AN EMPIRICAL TEST OF THE MUTUALISM DISRUPTION HYPOTHESIS: IMPACTS OF AN ALLELOPATHIC INVADER ON THE ECOPHYSIOLOGY OF A NATIVE FOREST HERB

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Alison N. Hale

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This dissertation was presented

by

Alison N. Hale

It was defended on

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and approved by

Dr. Jeffrey Lawrence, Dept. of Biological Sciences, University of Pittsburgh

Dr. David Mortensen, Dept. of Plant Science, Penn State University

Dr. Stephen Tonsor, Dept. of Biological Sciences, University of Pittsburgh

Dr. Brian Traw, Dept. of Biological Sciences, University of Pittsburgh

Dissertation Advisor: Dr. Susan Kalisz, Dept. of Biological Sciences, University of Pittsburgh

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Understanding how biotic and abiotic contexts modify the strength of species interactions is a key goal in ecology. Mutualism effectiveness is particularly sensitive to environmental conditions, and the invasion of non-native species is hypothesized to be one biotic factor that can drive mutualism disruption between native species and their partners. Using an ecophysiological approach, I tested this mutualism disruption hypothesis using the allelopathic invasive plant, *Alliaria petiolata*, and examined its impacts on the mutualism between symbiotic arbuscular mycorrhizal fungi (AMF) and *Maianthemum racemosum*, a common native herb in North America. To establish the potential for mutualism disruption in this system, I measured field concentrations of *A. petiolata*'s allelochemicals and tested the toxicity of these levels on AMF spore germination in a bioassay. I found that field-detected levels of allyl isothiocyanate, a key component of *A. petiolata*'s allelochemical profile, reduced spore germination by over 50% relative to controls. Additionally, by assessing fungal abundance in the field, I found that sites invaded by *A. petiolata* generally have reduced fungal hyphal lengths compared to uninvaded sites. In a separate common garden study, I demonstrated that *A. petiolata* allelochemicals significantly reduced soil respiration rates around *M. racemosum* plants, indicating active disruption of AMF associated with the plant roots. To investigate the impacts of mutualism disruption by *A. petiolata* on *M. racemosum*, I used a combination of field and greenhouse

studies. First, in a short, 2-week field study, I found that *A. petiolata* allelochemicals reduced physiological function and carbon acquisition in *M. racemosum*. Second, data from a season-long greenhouse study demonstrated that the physiological declines induced by *A. petiolata* allelochemicals were persistent and translated into reductions in allocation to key traits, including carbohydrate storage, root growth, and asexual reproduction. Together, these studies indicate that *A. petiolata* allelochemicals disrupted AMF function, resulting in water stress and altered source-sink dynamics for the native plant, and drove declines in both physiology and allocation to competing functions. Overall, my results suggest that allelopathic invasion is one critical, yet underexplored, biotic context that can dictate the outcome of plant-AMF mutualisms.

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PREFACE

I would like to begin by thanking the scientists who were critical in both the development of this dissertation and in my personal development as a researcher. First, I thank my advisor Susan Kalisz. Our weekly meetings often morphed into brain-storming sessions where her ideas and enthusiasm for my work injected me with confidence and a general excitement for science. She also devoted a tremendous amount of time and energy to my work, and for that I am truly grateful. Furthermore, she has shown me what it means to be a woman in science and that is invaluable. I would also like to thank the members of my committee. I give a big thanks to Stephen Tonsor for introducing me to plant physiology. He taught me how to use the LI-COR 6400 to measure leaf gas exchange rates and he was instrumental in retro-fitting this machine to measure soil respiration rates. He was also always ready to lend an ear and offer wisdom and advice, not only about experiments and analyses, but also about life. I thank Brian Traw for his assistance in measuring glucosinolates – his generosity with his time, supplies, and advice were critical in pinning down the ecological relevance of my work. I also thank Jeffrey Lawrence and David Mortensen for their constructive comments and experimental ideas. While she was not on my committee, I would also like to thank Line Lapointe from the Université Laval for her kindness in inviting me to Québec to learn how to measure carbohydrates. My month in her lab was one of the highlights of my time as a graduate student.

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Last, but certainly not the least, I thank my family. My husband, James Hale, is the best. Thanks for making me laugh when I needed it, for letting me cry when I needed it, and for giving me advice when I asked for it. I love you so much! I also thank my brother, Jeff Maye, for his hilariousness at the Pymatuning Laboratory of Ecology. This dissertation is dedicated to my parents, Richard and Monica Maye. I can never thank them enough for their love, prayers and support over these past five years. They have patiently listened to hours of mindless details about garlic mustard, mycorrhizae, and the inner-workings of the LI-COR 6400. For all your help drilling post holes and digging up plants – this Ph.D. belongs to you too.

1.0 INTRODUCTION

Mutualisms are interactions in which the exchange of resources and/or services between two species results in a net benefit for each of the partners. Such mutualistic interactions are ubiquitous – they can be found in nearly every ecosystem on earth and include a wide variety of organisms (Herre et al. 1999). While in the past, mutualisms were largely neglected by ecologists in favor of studying interactions such as competition and predation, the importance of mutualisms as a key species interaction is increasingly recognized (Bronstein 1994a). Indeed, every species on earth benefits either directly or indirectly from a mutualism (Kiers et al. 2010). Mutualisms are also hypothesized to act as foci around which biodiversity accumulates over ecological and evolutionary time (Bronstein et al. 2004).

Mutualisms are often studied in isolation, with little regard for the broader ecological context in which they occur (Bronstein et al. 2003). However, mutualism effectiveness is generally regarded as context-dependent (Heath and Tiffin 2007, Hoeksema et al. 2010, Morris et al. 2010, Schupp et al. 2010, Zwolak and Crone 2012, but see Chamberlain and Holland 2009), since both biotic and abiotic factors can strongly influence mutualism outcome (Bronstein 1994b, Kiers et al. 2010). Natural variation in the environment, including fluctuations in local partner density, resource availability, and overall community composition can impact the interaction strength between mutualistic partners (Bronstein 1994b). There is also increasing evidence that human-induced environmental changes, including climate change, habitat

loss/fragmentation, loss of large herbivore populations, and introduction of non-native species, can lead to mutualism disruption and eventually mutualism breakdown (Traveset and Richardson 2006, Memmot et al. 2007, Palmer et al. 2008, Kiers et al. 2010). To understand how mutualisms affect the physiology, abundance, and distribution of species, it is crucial that we move beyond investigations of two species isolated from biological reality and assess the impacts of outside factors on mutualism stability.

Plants are particularly reliant on mutualisms due to their sessile nature. The most widespread plant mutualism occurs with arbuscular mycorrhizal fungi (AMF). This relationship evolved over 460 million years ago (Redecker et al. 2000) and is based on a well-orchestrated exchange of resources between the two partners. The plant provides the obligately symbiotic AMF with carbohydrates produced via photosynthesis, and in return, the AMF provide mineral nutrients (mainly phosphorus and nitrogen) to the plant, along with a suite of other benefits, including enhanced water uptake (Augé 2001) and protection from pathogens (e.g. Newsham et al. 1995). While this mutualism is geographically widespread, it is especially common in forest ecosystems, where over 70% of herbaceous species associate with AMF (Hale et al. 2011).

One biotic factor that has the potential to disrupt the plant-AMF mutualism is the invasion of non-native species (Mitchell et al. 2006). Certainly, the effect of invaders on plant reproductive mutualisms, such as pollination and seed dispersal, is well-documented (reviewed in Traveset and Richardson 2006), but we know little about the effects of invaders on belowground mutualisms. The production of "novel weapons" – allelochemicals that native plants and their associated microbes have never experienced before – by invasive species is hypothesized to be an important mechanism underlying ecosystem invasion (Callaway and Ridenour 2004). Several invaders have been shown to produce allelochemicals that are toxic to

mycorrhizal fungi (e.g. Bainard et al. 2009, Sanon et al. 2009, Meinhardt and Gehring 2012, Urgenson et al. 2012), suggesting that mutualism disruption during invasion may be a general, but largely unrecognized, phenomenon.

In this dissertation, I test the mutualism disruption hypothesis for invasion using garlic mustard (*Alliaria petiolata*) and the mutualism between the native forest herb false Solomon's seal (*Maianthemum racemosum*) and AMF as a model system. I take a unique approach to test the mutualism disruption hypothesis, combining techniques from various fields, including analytical chemistry, plant physiology, and ecology. By evaluating the effect of an important biotic factor – a non-mutualistic, allelopathic invasive species – on a key plant mutualism, my research investigates mutualism function in a relevant ecological context.

In Chapter 2, I begin by testing the potential for mutualism disruption in this model system. In collaboration with two undergraduate students, Aaron Cantor and Justin Aaron, and Dr. Brian Traw and Dr. Susan Kalisz at the University of Pittsburgh, I assessed whether garlic mustard allelochemicals are released into field soils at concentrations that are toxic to AMF spore germination and hyphal growth. This work is published in *Biological Invasions* (Cantor et al. 2011).

In Chapter 3, I examine the results of two separate studies. First, I present findings from a common garden experiment that measured the impact of garlic mustard's allelochemicals on AMF associated with false Solomon's seal plants. Second, I introduce data from a short, 2-week field study that measured the physiological impacts of garlic mustard mediated-mutualism disruption on false Solomon's seal. This work was conducted with Dr. Stephen Tonsor and Dr. Susan Kalisz at the University of Pittsburgh and is published in *Ecosphere* (Hale et al. 2011).

In Chapter 4, I report the results from a season-long greenhouse experiment that assessed the physiological responses and changes in carbon allocation of false Solomon's seal plants experiencing persistent mutualism disruption by garlic mustard. Because garlic mustard's allelochemicals are likely present year-round on invaded sites, this study closely mimics the natural dynamics of mutualism disruption that native plants may face in the field. This work was also done in collaboration with Dr. Susan Kalisz.

In Chapter 5, I develop a framework of questions to guide future research on allelopathic disruption of plant mutualisms. In collaboration with Dr. Susan Kalisz, I explore the potential effects of allelopathy on the three major plant mutualisms - mycorrhizae, pollination and seed dispersal - and highlight areas of study that will be key in increasing the ecological significance of allelopathy research. This work is published in *Plant Ecology* (Hale and Kalisz 2012).

In Chapter 6, I conclude by summarizing my results and discussing their importance and relevance to plant physiological ecology and invasion biology. I also outline areas for future research on this topic.

2.0 LOW ALLELOCHEMICAL CONCENTRATIONS DETECTED IN GARLIC MUSTARD-INVADED FOREST SOILS INHIBIT FUNGAL GROWTH AND AMF SPORE GERMINATION

2.1 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 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 (*Alliaria petiolata*, Brassicaceae) 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 hours, 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.

2.2 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 0.42–0.93 $g \cdot (cm^3)^{-1}$. 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.

2.2.1 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 of soil on 12 June and 20 g of soil 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 hours 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 minutes, 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 de-sulfated 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 cm x 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 $ml \cdot min^{-1}$. 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 minutes. 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 minutes, 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 mg and 35.1 mg of the original 50 mg were recovered; Appendix A). Because we left our soil samples at 25°C for 24 hours 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.

2.2.2 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 x 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 x 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 $cm \cdot s^{-1}$ and the column flow was 1.7 $ml \cdot min^{-1}$. 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).

2.2.3 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 June 16 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 four 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 hours, mounted on glass slides, and examined at 20× magnification. Thirty areas of 0.375 mm × 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 mm × 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).

2.2.4 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 $\mu g \cdot g^{-1}$ 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 to 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 mM, 0.002 mM, 0.005 mM, 0.01 mM, 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 one 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.

2.3 RESULTS

2.3.1 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 (Appendix B). Only 1 of the 16 invaded soil samples collected during the early (growth) dates had detectable levels of sinigrin (Appendix B). Field concentrations of sinigrin ranged from 0.011 to 0.031 $\mu g \cdot g^{-1}$ dry soil (Figure 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 had an AITC concentration of 0.017 $\mu g \cdot g^{-1}$ soil, while a sample from 18 July had a lower concentration at 0.0042 $\mu g \cdot g^{-1}$ soil. Total and single ion chromatograms (m/z = 99.10) from our lowest AITC standard and the soil sample extracts were similar (Figure 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.

2.3.2 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 vs. control plots at the $P = 0.07$ level (Figure 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 length greater than the median value when compared to membranes from garlic mustard plots ($Z = -1.7884$; $P = 0.04$).

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

2.4 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. 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 et al. 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 eight 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 Appendix C), 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 at 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 (Figure 4). We found 57% inhibition of spore germination compared to controls with concentrations of AITC mimicking those detected in Trillium Trail field soil samples (~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 ~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 the early stages of 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.

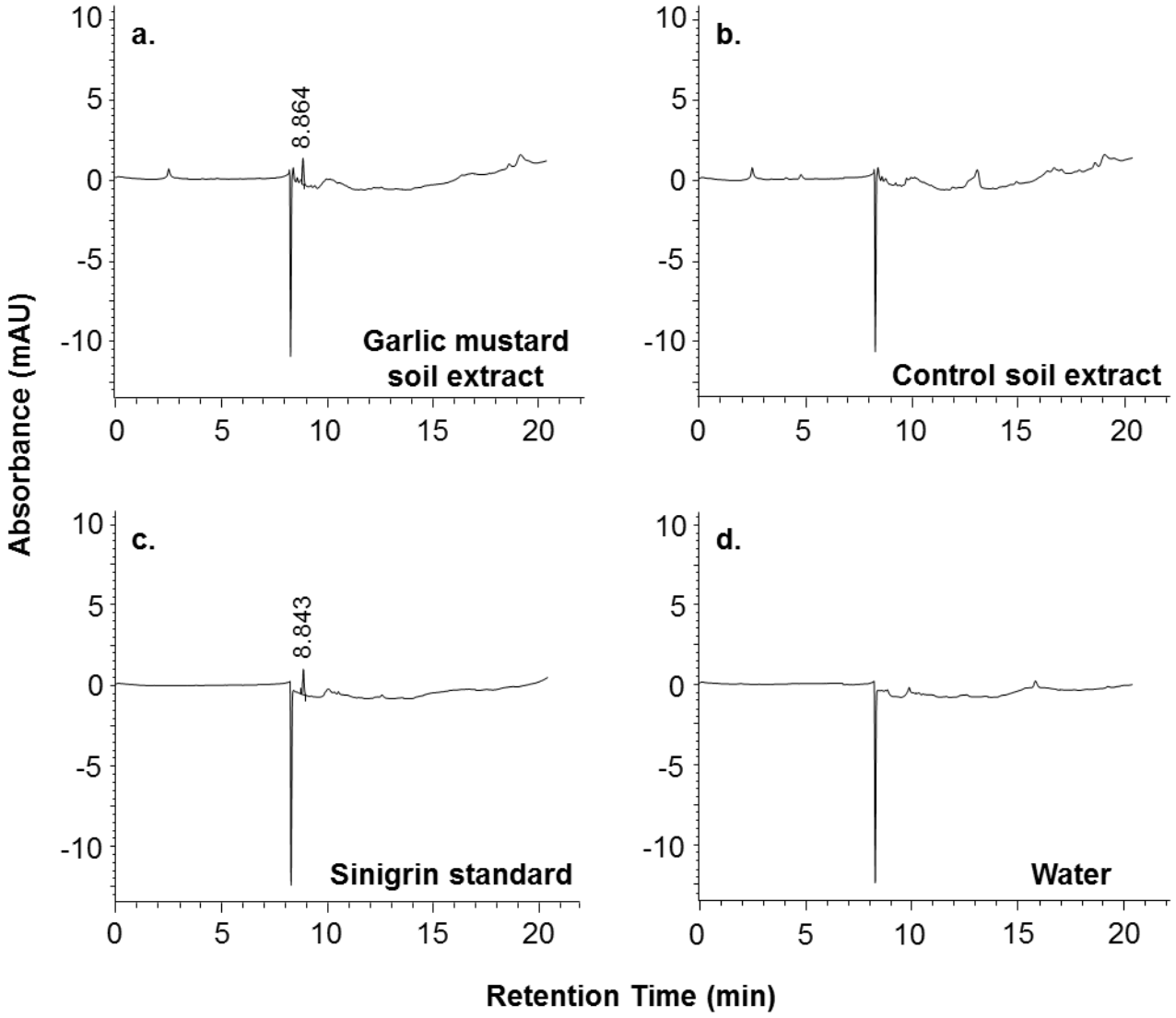


Figure 1. HPLC chromatograms of soil extracts collected from paired garlic mustard (a) and control plots (b), 0.1 µg sinigrin standard·ml^{-1} eluate (c), and water (d). The garlic mustard plot extract shows the detection of sinigrin, as a peak is detected at 8.8 minutes, as is seen in the sinigrin standard.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

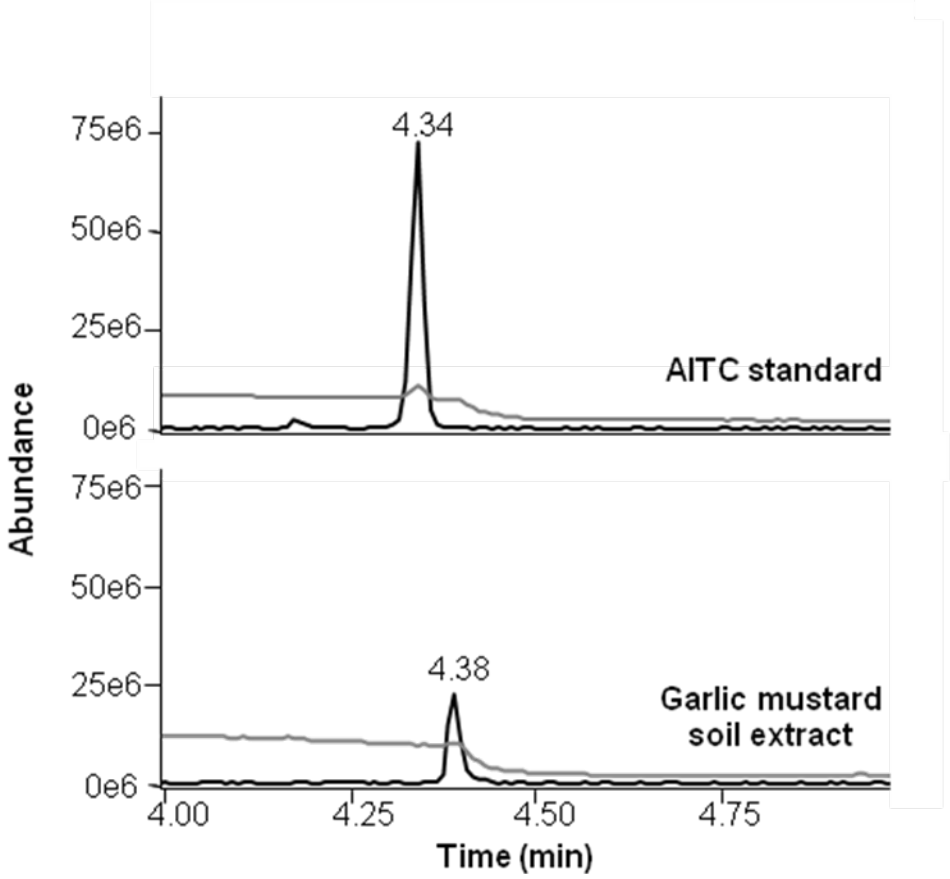


Figure 2. Total ion chromatograms (*gray line*) and selected ion chromatograms (*black line*) for an m/z of 99.10 of a 0.039 mM AITC standard (a) and a soil extract collected near garlic mustard adults at Trillium Trail (b). The garlic mustard plot extract shows the same peak as the AITC standard at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

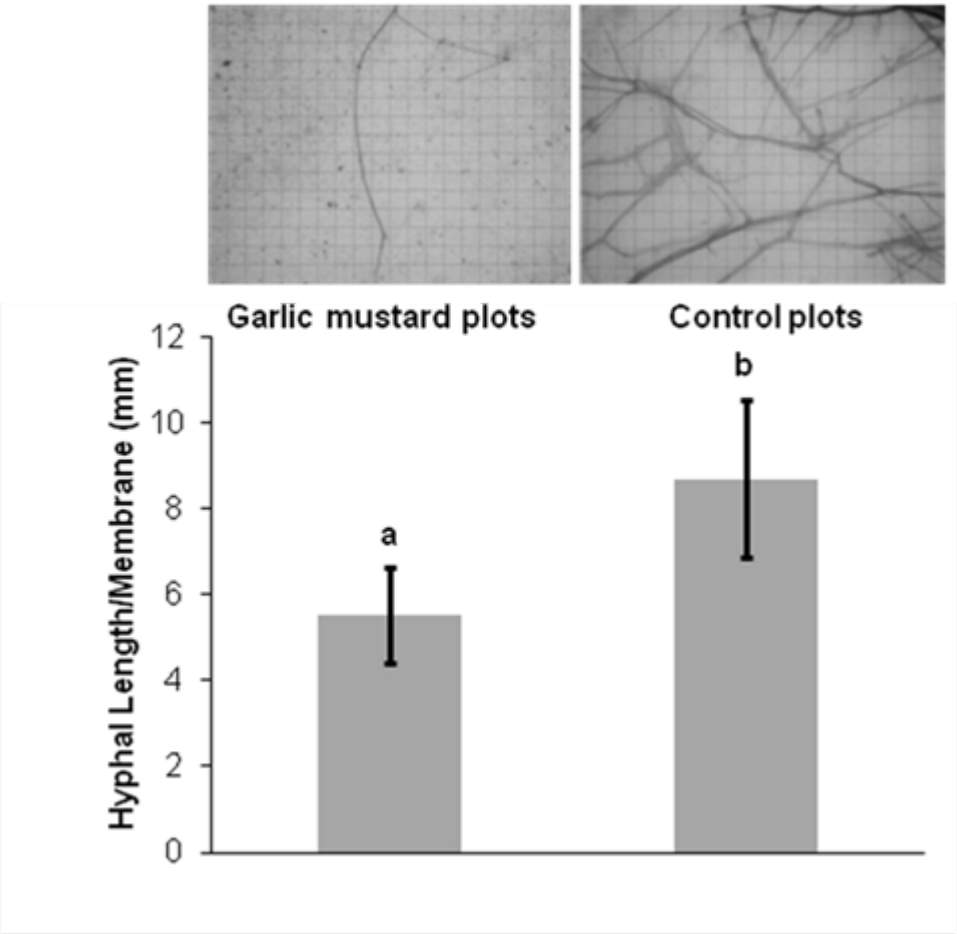


Figure 3. Mean fungal hyphal length (± one std. err.) on membranes placed in the field where garlic mustard is currently growing or is currently absent (*control*). Bars with the same letter are not significantly different from each other at the $P = 0.07$ level. Images of typical membranes from each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

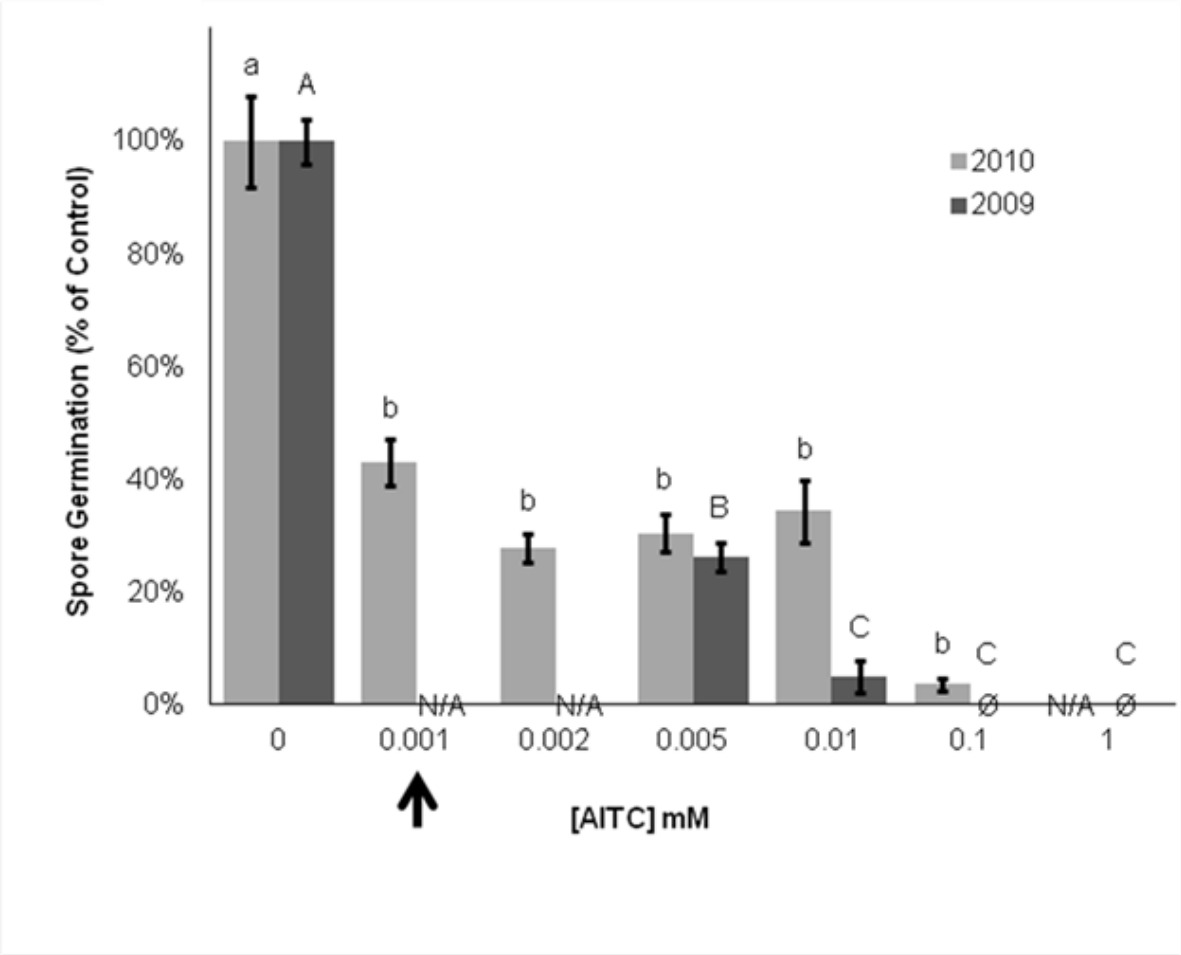


Figure 4. *In vitro* bioassay testing mean *Glomus clarum* spore germination (± one std. err.) across a range of AITC concentrations. Germination values are expressed as a % of total germination observed in the control (water only) treatment. N/A indicates that the concentration was not tested in a run 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

3.0 TESTING THE MUTUALISM DISRUPTION HYPOTHESIS: PHYSIOLOGICAL MECHANISMS FOR INVASION OF INTACT PERENNIAL PLANT COMMUNITIES

3.1 INTRODUCTION

Plants rely on mutualistic interactions for a number of services that are vital for reproduction, defense, dispersal, and nutrient acquisition. The most widespread plant mutualism is likely the interaction with arbuscular mycorrhizal fungi (AMF): it is estimated that up to 90% of all land plants participate in AMF mutualisms (Smith and Read 2008). The basis for this mutualism is a two-way exchange of resources. Up to 20% of a plant's carbon is shunted to the obligately symbiotic AMF, while AMF improve the supply of water, phosphorus and nitrogen to the plant (Smith and Read 2008). As a result of the numerous benefits that plants derive from AMF, these belowground mutualists can strongly influence the physiology (Figure 5), overall carbon gain, and likely the competitive ability of their host plants. Disruption of this key plant mutualism is hypothesized to facilitate invasion (Mitchell et al. 2006; Reinhart and Callaway 2006).

The "mutualism disruption" hypothesis suggests that inhibition of native mutualists can provide invaders with a competitive advantage over mutualism-dependent native species. While this hypothesis has been tested extensively using reproductive mutualisms (reviewed in Traveset and Richardson 2006), it is increasingly recognized that AMF mutualism disruption may also play an important role in invasions. For example, plant invaders can reduce AMF density

(Roberts and Anderson 2001; Vogelsang and Bever 2009) and the diversity and abundance of AMF external hyphae (Mummey and Rillig 2006) in the soil. The adverse effects of invaders on AMF mutualists can result from either negative feedback (Vogelsang and Bever 2009) or novel chemical weapons (Callaway and Ridenour 2004). Many invaders are suspected of employing the latter, including *Alliaria petiolata* (garlic mustard, Brassicaceae), a rampant forest understory invader. If allelochemicals disrupt the function of AMF mutualists, physiological impairment of the plant host could provide a competitive edge for invaders.

Garlic mustard has become a model study system, and as a result, its allelopathic chemicals are increasingly understood. Garlic mustard produces numerous secondary compounds, including glucosinolates (Vaughn and Berhow 1999), which are unique to the Brassicaceae. Upon hydrolysis in the soil, glucosinolates are mainly converted to isothiocyanates, a class of compounds toxic to AMF and other soil organisms (Brown and Morra 1997). Greenhouse experiments demonstrate that soils from garlic mustard-invaded sites or soils pre-conditioned with garlic mustard reduce AMF colonization and biomass in native tree seedlings (Stinson et al. 2006) and increase mortality of herbaceous seedlings (Callaway et al. 2008). Recently, we showed that allyl isothiocyanate (AITC) – an abundant compound exclusive to garlic mustard (Vaughn and Berhow 1999; Barto et al. 2010a) – is present in garlic mustard-invaded soils (Cantor et al. 2011). Our companion bioassay revealed that even low AITC concentrations can reduce AMF spore germination by ~60%. Furthermore, garlic mustard-invaded areas at our study site showed reduced colonization by fungal hyphae compared to control areas (Cantor et al. 2011). These new results clearly demonstrate that garlic mustard's allelochemicals are present in field soil, and that even low AITC concentrations are capable of

reducing AMF growth and abundance. Thus, the forest invader, garlic mustard, is an ideal species for testing a "mutualism disruption" hypothesis.

The native understory perennial herbs of deciduous temperate forests are a phylogenetically diverse group of plants (Gilliam 2007) that are highly dependent on AMF (Brundrett and Kendrick 1988). The AMF-forest herb mutualism is unique: unlike AMF crop species whose arbuscules are short-lived (4-5 days), arbuscules within forest herbs' roots function for several months (Brundrett and Kendrick 1990). Furthermore, the coarse roots of many understory herbs lack fine root hairs, and AMF external hyphae may function as root hairs for these species (Brundrett 1991). Together these factors indicate high AMF dependence in forest herbs.

In this study we integrate the fields of plant ecophysiology and invasion biology to provide a first step in testing for physiological consequences of mutualism disruption. We propose that if an allelopathic invader depresses the function of AMF external hyphae, the influx of critical resources from the AMF to its host will be reduced, resulting in physiological stress of the host. We hypothesize that this stress could be manifested through several interacting physiological pathways (Figure 5). Decreases in nutrient supply rates can reduce photosynthetic capacity and demand for CO_2 through reductions in RuBisCO, indirectly causing partial stomatal closure (Figure 5, arrows 1&2). Limited water availability can directly cause partial stomatal closure (arrow 3). The interplay of the conflicting influences of water stress and CO_2 demand for photosynthesis largely determine stomatal conductance. A common physiological measurement - leaf internal CO_2 concentration - can be used to reveal the extent to which water and/or nutrient limitation is driving stomatal closure (Wong et al. 1979; Farquhar and Sharkey 1982). Under nutrient limitation, internal CO_2 concentrations remain unchanged as the partially

closed stomata provide sufficient CO_2 to the diminished photosynthetic machinery. In contrast, reduced internal CO_2 concentration occurs under water limitation because photosynthetic CO_2 demand outstrips its diffusion into the leaf. Finally, we expect that loss of sink strength via allelochemical-induced reductions in AMF hyphal function can further lower photosynthetic capacity (arrow 4). Here we use ecophysiological measurements in field and common garden experiments to determine which of these conflicting influences, water stress or nutrient driven reductions in CO_2 demand, impact physiological function. We show that a native perennial and its AMF exhibit reduced soil respiration rates with garlic mustard treatment and implicate water limitation in plant physiological declines.

3.2 METHODS

3.2.1 Focal species

Garlic mustard is a Eurasian biennial plant introduced to North America circa 1868 that has since spread throughout forest understory habitats (Rodgers et al. 2008). Garlic mustard releases powerful allelochemicals into the soil (Cantor et al. 2011) and can drive declines in native plant abundance and diversity in forests (reviewed in Rodgers et al. 2008).

We chose *Maianthemum racemosum* (false Solomon's seal; Liliaceae) as a focal understory species because it is common in both deciduous and coniferous forests throughout North America, often occurs in sites invaded by garlic mustard (Burke 2008; A. Hale, *personal observation*; Figure 6A), and is highly dependent on AMF. Like other understory perennial herbs, false Solomon's seal roots are highly colonized by AMF (76-94%; Brundrett and

Kendrick 1988; Burke 2008), and lack fine root hairs (Brundrett and Kendrick 1988; Figure 6B). These attributes led Brundrett and Kendrick (1988) to classify false Solomon's seal as obligately dependent on AMF for growth and survival. AMF external hyphae associated with understory herbs typically exhibit peak growth in mid to late summer (Brundrett and Kendrick 1990). This peak coincides with garlic mustard adults' senescence (Anderson et al. 1996) and the release of allelochemicals (Cantor et al. 2011), making the AMF mutualism in false Solomon's seal particularly susceptible to disruption by garlic mustard. Finally, false Solomon's seal's roots grow at shallow depths (2-4 cm below the soil surface; A. Hale, *personal observation*), which increases their probability of encountering allelochemicals leaching from garlic mustard leaf litter or root exudates.

3.2.2 Common garden experiment: garlic mustard allelochemicals' effect on belowground respiration

We established a 3 x 4 m plot at the University of Pittsburgh's Pymatuning Laboratory of Ecology (PLE) and created a grid of 30 cm deep holes using a post-hole digger. We collected false Solomon's seal plants (N = 34) on 4 June 2009 from Tryon-Weber Woods, a 34-hectare beech-maple forest in northwestern PA, USA that is not invaded by garlic mustard. To allow measurement of belowground respiration (described below), we employed a unique pot design. We built pots of 30 cm tall x 10 cm diameter PVC pipe with a mesh bottom (pore size: 1 mm) that allowed air and water to flow through the pot. We potted plants in a 50:50 mixture of Fafard:Turface (Conrad Fafard Inc., Agawam, MA, USA; Profile Products LLC, Buffalo Grove, IL, USA) with inoculum of local field soil collected around false Solomon's seal roots and 3.5 g Nutricote fertilizer (100 day release formula, Florikan E.S.A. Corporation, Sarasota, FL, USA).

We randomly assigned each plant to a grid location, making the top of each pot flush with the soil surface. Since AMF colonization begins in late spring for false Solomon's seal (Brundrett and Kendrick 1990), the AMF community was already established in the root systems at the time of collection. However, since we disturbed the external hyphae during transplanting, plants and AMF were allowed to re-establish for 6 weeks prior to treatment (Jakobsen et al. 1992) to allow hyphal regrowth. We enclosed the entire plot with a wire cage to exclude mammalian herbivores and attached a 60% shade cloth to the top of the cage to simulate forest understory light levels.

3.2.2.1 Treatments

We randomly applied one of three treatments to each pot: fresh garlic mustard tissue (N=11), fresh dame's rocket (*Hesperis matronalis*, Brassicaceae) tissue - another exotic mustard (N=11), or no plant tissue (N=12). We collected green adult garlic mustard leaves, stems and roots from Wallace Woods, a mature, second-growth forest owned by PLE that was invaded by garlic mustard within the last decade (T-L. Ashman, *personal communication*), and also collected green dame's rocket tissue on site at PLE. We placed 100 g of fresh garlic mustard or dame's rocket tissue into 20 x 20 x 1.5 cm fiberglass screen bags (pore size: 1 mm) and transported the bags to the common garden for application to the false Solomon's seal pots. We predicted that allelochemicals leaching from the garlic mustard treatment would kill AMF external hyphae. However, we predicted that the dame's rocket treatment would have a negligible effect on AMF as this species can sustain AMF colonization in its roots (Demars and Boerner 1995) despite its glucosinolate production (Larsen et al. 1992). For the no plant tissue treatment, we left the screen bags empty. Thus, the dame's rocket treatment allows us to separate the effects of garlic mustard glucosinolates from leaf tissue effects, while the empty screen bag treatment allowed us to assess background levels of soil respiration. We placed the screen bags at the base of the

plants and fastened them to the soil with stainless steel pins, ensuring that the bags were in direct contact with the soil surface. Treatments were imposed on 20 and 21 July 2010.

The application of garlic mustard tissue to the pots allowed us to closely simulate natural levels of allelochemicals, as decomposition is a major route of allelochemical release into the soil (Rice 1974), and removed the confounding factor of competition when garlic mustard and false Solomon's seal are grown together in pots. Because of the rapid decomposition of garlic mustard tissue (Rodgers et al. 2008) and sorption of isothiocyanates in soil (Matthiessen and Shackleton 2005), coupled with the quick turnover rate of AMF external hyphae (i.e. 5-6 days; Staddon et al. 2003), we determined that a one-week treatment would be sufficient for suppression of AMF external hyphal function.

3.2.2.2 Belowground respiration

We measured belowground respiration in each pot using a LI-COR 6400 infrared gas analyzer (IRGA; LI-COR Biosciences, Lincoln, NE, USA). We fabricated a sealed airflow path to pass CO_2-free air through the mesh bottom of each PVC pot, forcing the CO_2 in the soil matrix to flow out of the top of the pot into the IRGA (Appendix D). We recorded the ambient air temperature and the CO_2 concentration (our estimate of belowground respiration) of this air stream at time zero and every two minutes, for a total of 10 minutes. We chose this sampling interval because 1-hour trial runs revealed that >75% of available CO_2 in the soil was captured in the first 10 minutes. To separate the effect of garlic mustard on microbial vs. root respiration, at the end of the experiment we harvested each plant and recorded its fresh root mass. To assess the potential for direct effects of garlic mustard's allelochemicals on false Solomon's seal, we compared the fresh root mass across treatments.

3.2.2.3 Statistical analysis

We fit a curve ([CO_2] = time) to the data for each pot for a total of 34 regressions. The area under each curve, calculated using Mathematica 7.0 (Wolfram Research, Inc., Champaign, Illinois, USA), estimates the total CO_2 captured across the sampling duration. We compared belowground respiration in the garlic mustard vs. dame's rocket treatments using an analysis of covariance (ANCOVA) with root mass and ambient temperature as covariates. We corrected the final means from this analysis by subtracting the mean respiration value in the empty screen treatment, which represented background levels of soil respiration (all uncorrected values are shown in Appendix D). Since our a priori prediction was for lower respiration in the garlic mustard treatment relative to the dame's rocket treatment, we report one-tailed *P*-values. We compared root mass using a Kruskal-Wallis non-parametric test with the model: root mass = treatment.

3.2.3 Field experiment: garlic mustard allelochemicals' effect on native plant physiology

Our experimental site was the Trillium Trail Reserve, a 16-hectare mixed mesophytic forest that is owned and managed by the Fox Chapel Borough, PA, USA. We estimate that 73% of the 79 herbaceous species at Trillium Trail associate with AMF (Appendix E). Because garlic mustard invaded Trillium Trail in 1992 (L. Smith, *personal communication*), the garlic mustard plants in this young population are likely to have high glucosinolate concentrations (sensu Lankau et al. 2009). We have demonstrated that the detected AITC levels in invaded soil at this site can significantly reduce both AMF spore germination and fungal hyphal abundance (Cantor et al. 2011). Thus, the potential for AMF disruption at this site is high.

3.2.3.1 Experimental design

We paired false Solomon's seal plants (N = 18, 9 pairs) based on two factors that can influence physiology: individual plant size and microhabitat (Lambers et al. 2008). We used height as our proxy for plant size because it is highly correlated with total leaf area in false Solomon's seal at Trillium Trail (R^2 = 0.97, N = 23, S. Kalisz, *unpublished data*). In matching plants for microhabitat, we ensured that paired plants were no more than 1 m apart and experienced similar tree canopy cover and moisture regimes. We again prepared screen bags filled with garlic mustard tissue collected on site and an empty screen bag served as the control. We cleared the natural leaf litter from the immediate area surrounding each focal false Solomon's seal, and then randomly assigned one plant within each pair to the control, while the other received the garlic mustard treatment. We applied these treatments between 23 and 27 June 2008 and left them in place for two weeks.

3.2.3.2 Leaf gas exchange measures

We determined that 600 $\mu mol \cdot m^{-2} \cdot s^{-1}$ is a saturating irradiance level for false Solomon's seal (Appendix F) and used this light level for all subsequent physiological measurements because this maximizes our ability to detect water and/or nutrients, rather than light, as limiting resources. Between 15 and 21 June 2008, prior to imposing the treatments, we took pre-treatment physiological measures during which mean daily temperatures ranged from 15-22°C. For each plant we recorded net photosynthetic rate (A_n), stomatal conductance (g_s), transpiration rate (E), and leaf internal CO_2 concentration (C_i) every 15 seconds for 1 minute, yielding 5 measures for each physiological trait. Measurements were averaged to provide pre-treatment estimates for each plant. On 8 and 10 July 2008 mean daily temperatures ranged from 21-24°C and we repeated all physiological measurements to obtain post-treatment estimates.

3.2.3.3 Statistical Analysis

Because all of the physiological response variables are highly correlated, we first used a multivariate analysis of covariance (MANCOVA; physiology (conductance, transpiration, photosynthesis) = temperature + humidity + size + treatment) to determine the overall effect of garlic mustard on false Solomon's seal physiology. Temperature, relative humidity and plant size were covariates in the analyses, as all of these variables are known to affect one or more of the measured physiological traits (Lambers et al. 2008). All of the F-statistics reported for the MANCOVA were identical, therefore, here we report only the F-statistic for Roy's greatest root, as it leads the most naturally to post-hoc tests (Scheiner 2001). Upon obtaining a significant F-statistic, we conducted separate analysis of covariance (ANCOVA) tests for each physiological variable (conductance, transpiration, or photosynthesis = temperature + humidity + size + treatment). Because these were planned comparisons, Type I error correction is not necessary.

After obtaining the physiological response results from the ANCOVAs, we then performed a post-hoc analysis on the intercellular CO_2 concentration (C_i) data from both treatments with a Bonferroni correction to account for multiple comparisons ($P = 0.05/4 = 0.0125$; Scheiner 2001). Statistical analyses for the field and common garden experiments were run using SAS version 9.2, SAS Institute, Cary, North Carolina, USA.

3.3 RESULTS

3.3.1 Common garden experiment: belowground respiration

We found that belowground respiration was dramatically lower for garlic mustard-treated relative to dame's rocket-treated (control) pots (Figure 7A; $F_{3,18} = 2.42$, $P = 0.07$). Over the sampling period, CO_2 levels declined as the residual CO_2 in the soil matrix and current production of CO_2 was pushed through the pot (Figure 7B). At each time point, CO_2 levels from the garlic mustard-treated pots were significantly lower than those in dame's rocket-treated pots (Figure 7B). Ambient air temperature and root mass were not significant covariates in this analysis (temperature range = 21-35°C; root mass range = 2.1-8.2 g). Root mass did not differ across treatments (Kruskal-Wallis: df = 2; $P = 0.51$).

3.3.2 Field experiment: plant physiological responses

Prior to treatment, there was no significant difference in physiological rates among false Solomon's seal plants destined for control or garlic mustard treatments (MANOVA; Roy's greatest root, F = 0.46, $P = 0.72$). However, after two weeks of treatment, garlic mustard-treated plants displayed significantly lower physiological rates relative to control plants (MANOVA; Roy's greatest root, F = 3.70, $P = 0.05$; Figure 8A-C). Specifically, g_s had the strongest response to garlic mustard treatment: g_s in garlic mustard-treated plants was 36% lower than control plants (Figure 8B; $F_{4,13} = 13.11$, $P = 0.009$). Similarly, E and A_n were significantly reduced in garlic mustard-treated plants ($F_{4,13} = 8.73$, $P = 0.03$ and $F_{4,13} = 4.58$, $P = 0.05$, respectively). All

covariates in the g_s, E and A_n individual analyses were significant ($P < 0.05$), except for the ANCOVA for A_n, where plant size was not significant ($P = 0.47$).

C_i was significantly reduced in garlic mustard-treated plants compared to controls (Figure 8D; post-hoc ANCOVA $F_{4,13} = 10.21$, $P = 0.007$, well below the Bonferroni-corrected $P = 0.0125$). Here, only plant size was a significant covariate ($P = 0.003$).

3.4 DISCUSSION

Our field experimental data clearly demonstrate that short-term exposure to garlic mustard tissue, an allelopathic invasive species, can significantly reduce the physiological function of a native understory herb, false Solomon's seal. Stomatal conductance (g_s) in garlic mustard-treated false Solomon's seal adults was reduced by 36%, with concomitant reductions in transpiration (E; 25%) and photosynthesis (A_n; 17%) compared to controls (Figure 8A-C). This study is the first to reveal an explicit physiological mechanism underlying an allelopathic species' invasion of an established native plant community.

We attribute the aboveground physiological suppression (Figure 8A-C) by garlic mustard to allelopathic disruption of AMF external hyphal function. In our common garden experiment, total belowground respiration declined in garlic mustard-treated pots compared to dame's rocket control pots (Figure 7). Indeed, respiration in the garlic mustard-treated pots did not differ significantly from that measured in the no plant tissue treatment (Appendix D). Thus, while the flush of nutrients from the decomposing dame's rocket tissue stimulated microbial activity above background levels, the garlic mustard tissue did not. These findings are consistent with the prediction that any nutritional benefits of litter decomposition on microbial metabolism are offset

in the garlic mustard treatment by loss of AMF external hyphae. AMF are more sensitive to garlic mustard than other soil microbial groups (Lankau 2011a) and AMF hyphae can have a disproportionately large impact on overall soil respiration (Johnson et al. 2002). Additionally, our previous work has shown that even low levels of allelochemicals from garlic mustard disrupt AMF spore germination and depress fungal hyphal abundance in field soils (Cantor et al. 2011). Furthermore, we found no direct effects of garlic mustard on false Solomon's seal root mass. Together, our observed reductions in belowground respiration are consistent with a reduction in the function of AMF external hyphae in the garlic mustard treatment.

Our leaf internal CO_2 concentration (C_i) data provide important insight into which soil resource limits false Solomon's seal physiology. We observed significant reductions in both g_s and C_i in garlic mustard-treated plants (Figure 8B and 8D, respectively). This result implicates water limitation as the primary cause of the physiological suppression of false Solomon's seal. Together our data suggest a potential causal chain: garlic mustard inhibits external AMF hyphal function, which limits water availability to false Solomon's seal, which in turn reduces stomatal conductance and lowers photosynthetic rate.

Under natural conditions, native plants in the understory likely experience prolonged periods of exposure to garlic mustard's allelochemicals. In garlic mustard-invaded sites, the continuous presence of garlic mustard rosettes and/or adults, its high seedling and rosette mortality throughout the year (Davis et al. 2006), and a 2-month period of intense leaf litter input as adults senesce (Anderson et al. 1996, Cantor et al. 2011) likely contribute to the year-round release of allelochemicals into the soil. Given the significant physiological suppression shown by our short-term pulse experiments, we anticipate that longer-term exposure of AMF-dependent plants to garlic mustard allelochemicals could affect carbon storage and resource allocation.

Indeed, if carbohydrate storage of AMF-dependant species is impacted by season-long declines in physiology, then garlic mustard invasions could have long-term fitness effects on native forest perennial plants. Experiments currently under way in our lab are testing for long-term impacts of mutualism disruption.

Our results have broad implications for understanding how allelopathic invaders can lead to the collapse of established native plant communities in forest understories. Stressful environments diminish mutualism effectiveness (Bronstein 1994b; Kiers et al. 2010). Through secretion of allelochemicals in the soil, garlic mustard effectively creates a physiological stress that removes the native plants' mutualistic interactions with AMF. While physical disturbance often facilitates forest invasion (Luken 2003), our data provide a link between novel weapons and native plant declines, implicating physiological disturbance as the intermediate step underpinning invasion. Interference with nutritional mutualisms and subsequent physiological declines in natives may provide both the opportunity for invaders to establish and spread throughout previously stable ecosystems and increase the vulnerability of native species to other stressors that accompany global change.

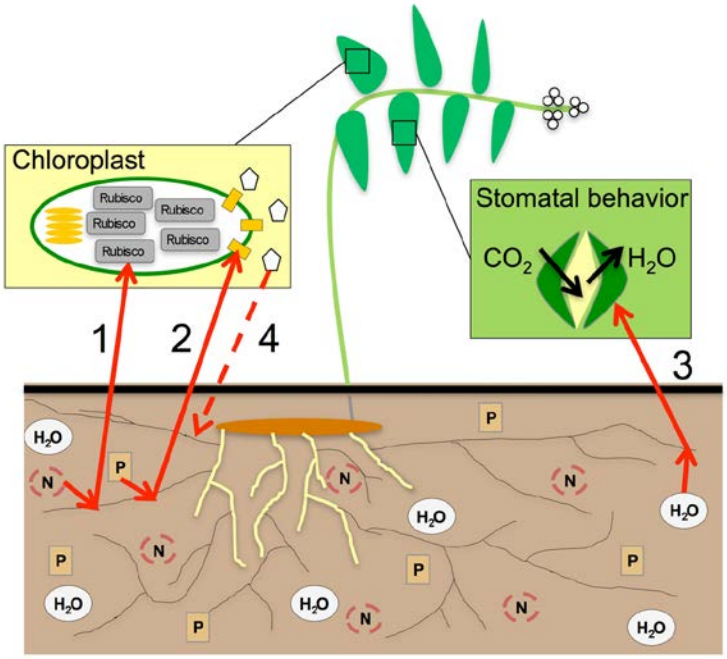


Figure 5. Plant physiology is dependent on both nutritional benefits (solid arrows) received from AMF and carbon costs (dashed arrows) delivered to AMF by the plant. In the soil, external hyphae of AMF (fine black lines) uptake soil nitrogen (N) and phosphorus (P) that is transported to the host plant. Arrow 1: Nitrogen is essential for the formation of chlorophyll and ribulose bisphosphate-carboxylase/oxygenase (RuBisCO), and as a result, photosynthetic rate (A_n) is highly correlated with total leaf nitrogen content (Evans 1989). Arrow 2: Phosphorus (P) is required to build ATP and other cofactors that play important roles in the Calvin cycle. P is also crucial for the transport of carbon assimilates (represented as pentagons) out of the chloroplast and P deficiencies can lead to a build-up of assimilates and down-regulation of A_n (Sivak & Walker 1986). Arrow 3: AMF enhance the plant's ability to capture water (H_2O) and increase water availability, which results in greater stomatal conductance (g_s) and transpiration (E; Augé 2001). Arrow 4: Maintenance costs and rapid turnover of AMF (Staddon *et al.* 2003) create an additional carbon sink and plants up-regulate photosynthetic rates in response. Studies in which non-mycorrhizal and mycorrhizal plants are matched for foliar [N] and [P] demonstrate that mycorrhizal plants have higher A_n than non-mycorrhizal plants (Wright *et al.* 1998; Miller *et al.* 2002)

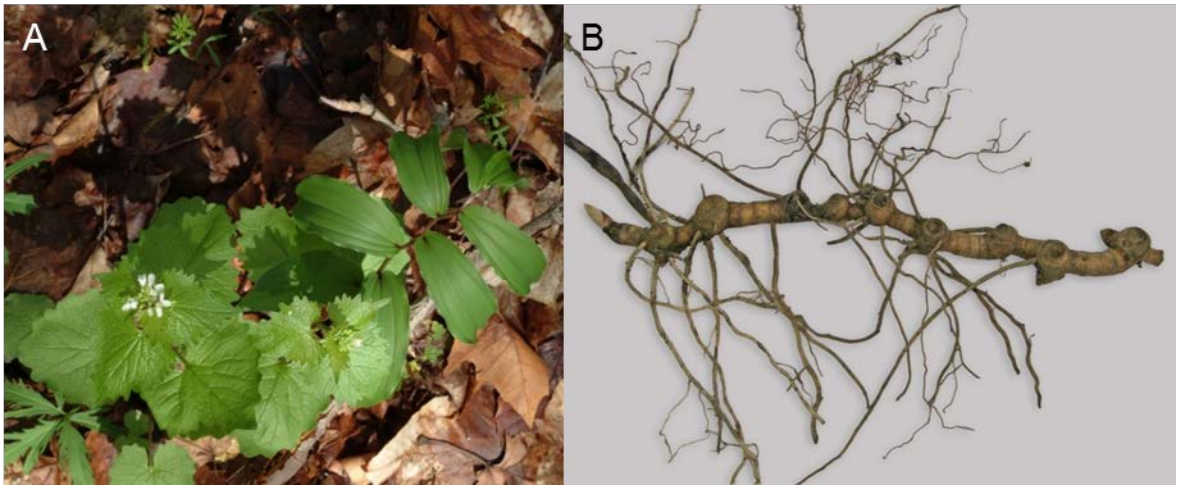


Figure 6. False Solomon's seal is an ideal native species for studying the impacts of mutualism disruption by garlic mustard because (A) it is commonly found in forests invaded by garlic mustard and (B) it also has very coarse roots that lack fine root hairs, suggesting a high degree of mycorrhizal dependency.

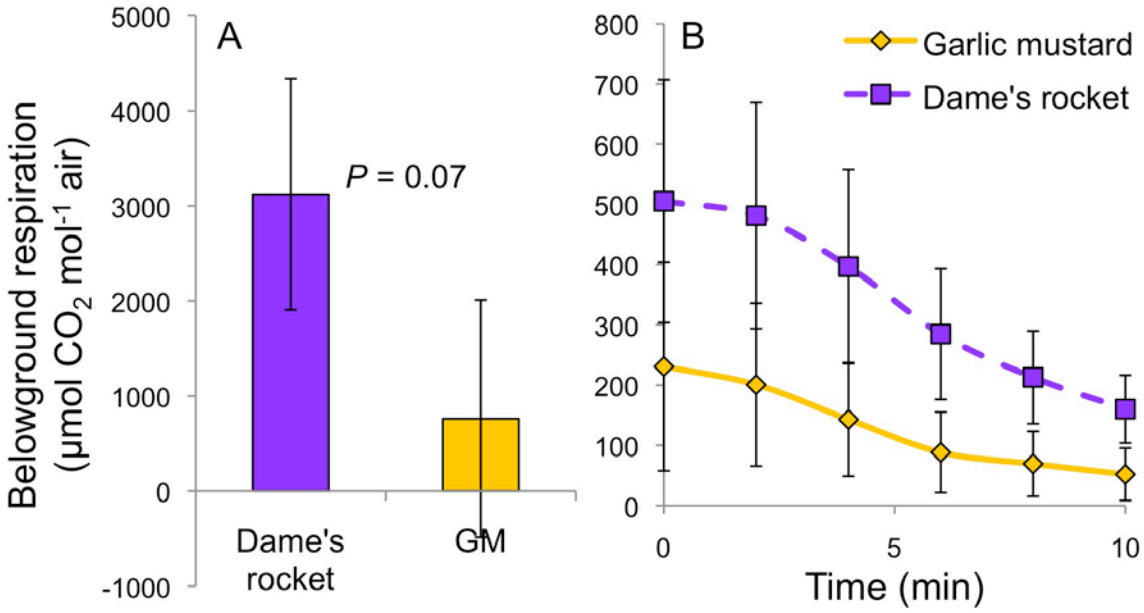


Figure 7. Belowground respiration from pots treated with garlic mustard (GM) is lower than in pots treated with dame's rocket tissue in our common garden experiment. (A) Total CO_2 captured in a ten-minute sampling period averaged across all plants within a treatment (least squares (LS) means ± 1SE). (B) The mean CO_2 captured per 2-minute sampling interval averaged across all plants within a treatment (least squares (LS) means ± 1SE).

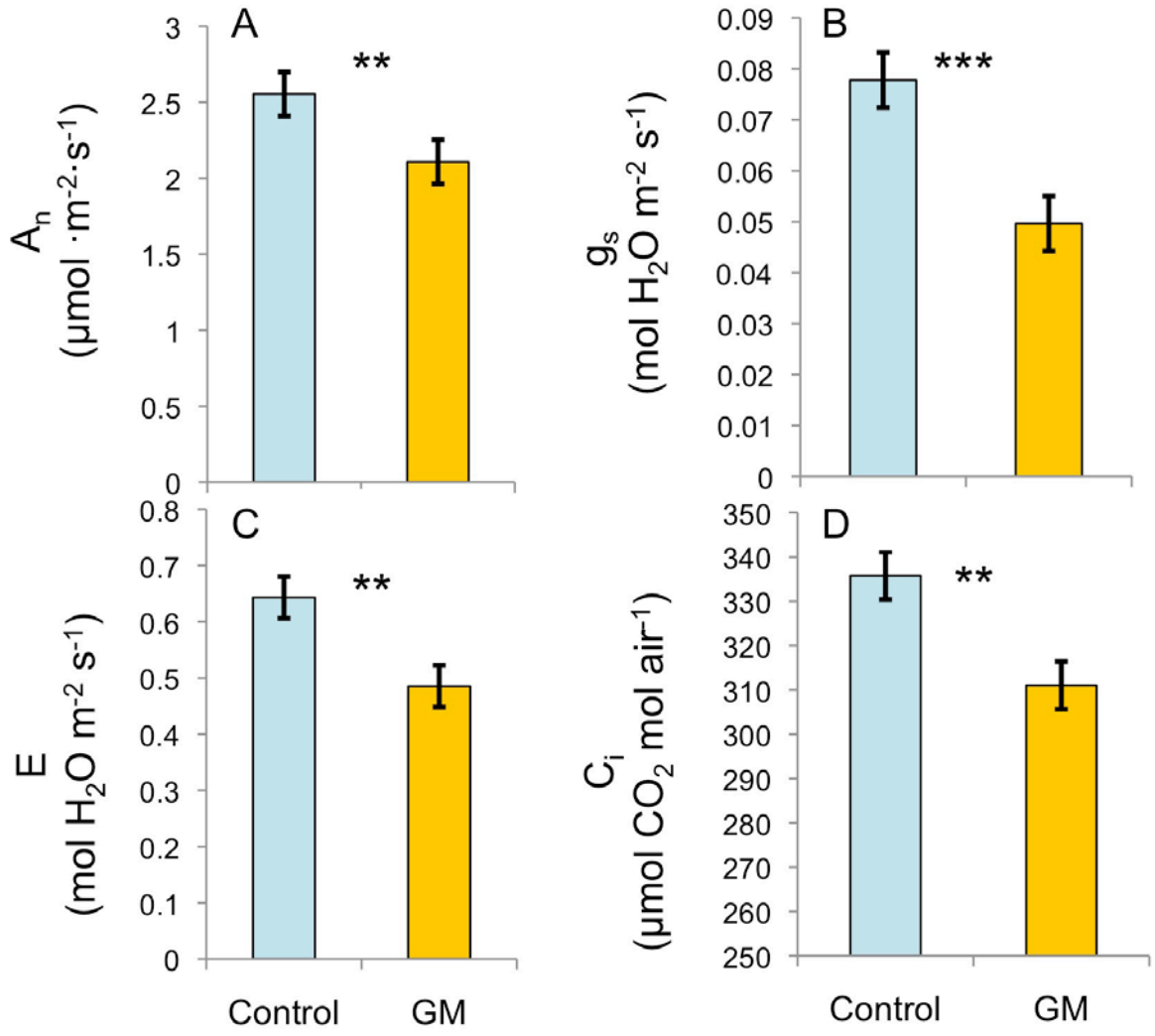


Figure 8. Garlic mustard tissue negatively affects false Solomon's seal's physiological function. All physiological parameters (A-C), photosynthetic rate (A_n), stomatal conductance (g_s), and transpiration (E) were significantly reduced (***, $P \leq 0.01$; **, $P \leq 0.05$) after two weeks of garlic mustard (GM) treatment relative to the controls. In a post-hoc analysis (D), intercellular CO_2 concentration (C_i) was also significantly lower (**, $P = 0.007$) for garlic mustard-treated plants compared to controls suggesting a potential role of stomatal limitation in the observed A_n declines (LS means ± 1SE).

4.0 MUTUALISM DISRUPTION AS A MECHANISM OF INVASION? AN ALLELOPATHIC INVADER DRIVES PERSISTENT DECLINES IN NATIVE PLANT PHYSIOLOGY AND ALTERS RESOURCE ALLOCATION

4.1 INTRODUCTION

The invasion of non-native species into novel habitats is considered one of the top threats to biodiversity (Salafsky 2008). The success of invaders is in part due to their ability to dramatically alter both the abiotic and biotic environment of native species. Invasive plants can influence soil nutrient inputs and cycling (reviewed by Ehrenfeld 2010 and Simberloff 2011), light availability (Asner et al. 2008), water availability (D'Antonio and Mahall 1991, Melgoza et al. 1990), and fire regimes (reviewed by Brooks et al. 2004). While these abiotic changes can directly influence invasive plant success, many of the hypothesized mechanisms for invasion invoke altered biotic interactions (Mitchell et al. 2006). The mutualism disruption hypothesis (Mitchell et al. 2006) states that invaders can impact interactions between native plants and their mutualistic partners. For example, invaders with attractive floral or fruit displays can effectively disrupt native pollination and seed dispersal mutualisms (reviewed by Traveset and Richardson 2006). However, the details of soil mutualism disruption during invasion remain poorly understood.

An important soil mutualism for a large percentage of plants is the interaction with arbuscular mycorrhizal fungi (AMF; Harley and Harley 1987, Brundrett and Kendrick 1988, Vogelsang and Bever 2009). In this mutualism, the plant provides obligately symbiotic AMF with carbon from photosynthesis, and in exchange, the AMF supply mineral nutrients and enhance water uptake to the plant (Parniske 2008). The mutualism disruption hypothesis predicts that if an invader can alter the effectiveness of this important plant mutualism, then it may gain a competitive advantage over native species.

There are two proposed mechanisms by which the native plant-AMF mutualism could be disrupted during invasion. First, if an invader associates less strongly with AMF than the native plants, then physical disturbances that disrupt the native plant-AMF mutualism could facilitate the establishment of invasives in the community. Over time, positive plant-soil feedbacks between the invader and the disturbed soil community could lead to long term declines in the mutualist population (i.e. "mutualist degradation" hypothesis; Vogelsang and Bever 2009). Second, toxic allelochemicals produced by an invasive species could kill AMF spores and active hyphae in the soil (i.e. "novel weapons" hypothesis; Callaway and Ridenour 2004). In either case, mutualism disruption is expected to result in significant short- and long-term consequences for native plants.

In the short-term, mutualism disruption could diminish the physiological performance of the native plant. Physiological function could be severely nutrient-limited by AMF disruption because AMF supply up to 80% and 25% of a plant's phosphorus and nitrogen, respectively (Marschner and Dell 1994). These nutrients are critical for the construction of photosynthetic enzymes, co-factors, and ATP, and reductions in the availability of these key nutrients are linked to declines in carbon acquisition (Sivak and Walker 1986, Evans 1989). Additionally, AMF

disruption could impose water limitations on plant physiology because AMF strongly influence plant water balance, transpiration, and stomatal conductance (Augé 2001). Lastly, because AMF sink activity influences plant photosynthetic rates (Wright et al. 1998, Miller et al. 2002, reviewed by Kaschuk et al. 2009), mutualism disruption could dramatically alter source-sink dynamics and carbon acquisition in the native plant. Clearly, the loss of AMF could compromise native plant function through a variety of mechanisms.

If AMF mutualism disruption persists, then declines in carbon assimilation could scale up to alter carbon allocation in the native plant. Carbon gained from photosynthesis is utilized for critical functions in plants, including storage, growth, and reproduction (Chapin et al. 1990). Plant must allocate among these competing functions to maximize lifetime fitness (i.e. optimal resource allocation theory; Bloom et al. 1985). AMF mutualism disruption may decrease the plant's ability to allocate sufficient resources to one or more of these functions. These impacts could affect the native plants' ability to effectively compete against an invader, persist in the population, and give rise to future generations.

In previous work, we have shown that the native plant-AMF mutualism is sensitive to disruption by an allelopathic invader. Garlic mustard (*Alliaria petiolata*), a widespread invasive species, releases glucosinolate-derived allelochemicals into the soil that are highly toxic to AMF spore germination and hyphal growth (Cantor et al. 2011). A two-week pulse experiment with garlic mustard tissue significantly reduced soil respiration rates and diminished physiological function of *Maianthemum racemosum* (Hale et al. 2011), a native forest understory herb. These results suggest that allelochemicals drive the concurrent loss of AMF activity and reductions in native plant physiology. However, it remains unclear if the observed physiological declines

would be persistent over an extended period of time or if they would scale up to impact seasonal carbon allocation patterns.

Here, we investigate the impacts of AMF mutualism disruption over a five-month period. Using data from this study, we address three major questions: 1) Are the physiological declines induced by AMF mutualism disruption (observed in Hale et al. 2011) persistent over time? 2) To what extent does water limitation, nutrient limitation, and/or altered sink activity underlie observed changes in physiological function? 3) Can the physiological declines drive changes in carbon storage and allocation to competing functions?

4.2 METHODS

4.2.1 Study species

Garlic mustard is an exotic biennial plant that was introduced to North America from Europe in the mid-1800s. Since its introduction, garlic mustard has become a prominent invader of forest understories throughout the United States and Canada (reviewed in Rodgers et al. 2008). Declines in perennial herb (Rodgers 2008) and tree seedling abundance (Stinson et al. 2007) have been documented on invaded sites, and at the community level, native plant species diversity is negatively correlated with garlic mustard density (Stinson et al. 2007). While numerous traits likely contribute to garlic mustard's invasiveness (reviewed in Rodgers et al. 2008), the success of this species has in large part been attributed to its allelopathic effects on mycorrhizal fungi (e.g. Roberts and Anderson 2001, Stinson et al. 2006, Callaway et al. 2008,

Cantor et al. 2011, Lankau 2011a). As a result, garlic mustard has rapidly emerged as a model system for the study of allelopathic mutualism disruption.

We chose *Maianthemum racemosum* (false Solomon's seal; Liliaceae) as our model native forest herb because it is often found on sites invaded by garlic mustard (Rodgers 2008, Burke 2008) and is hypothesized to have a high degree of mycorrhizal dependency (Brundrett and Kendrick 1988). The root system of *M. racemosum* is coarse, lacking fine root hairs, and highly mycorrhizal (colonization rates range from 76-94%; Brundrett and Kendrick 1988, Burke 2008), suggesting that this species may be particularly sensitive to AMF disruption. We also chose this species because its physiological phenology is understood. Like the majority of forest herbs, *M. racemosum* acquires most of its annual carbon early in the growing season (Neufeld and Young 2003) and allocates this carbon pool to various traits throughout the summer months (LaFrankie 1985). We used this information to select appropriate timings for our measurements during the experiment.

4.2.2 Experimental Design

The study was conducted at the University of Pittsburgh greenhouse facilities during the summer of 2010. We purchased bare-root, adult *M. racemosum* plants (N = 63; Prairie Moon Nursery, Winona, Minnesota, USA) on 13 May. Prior to treatment, we determined plant size by measuring the mass of each plant. We potted each plant (15.24 cm in diameter; Magnum pots) in a 3:1 mixture of autoclaved Fafard potting soil and Turface (Conrad Fafard Inc., Agawam, MA, USA; Profile Products LLC, Buffalo Grove, IL, USA). To ensure that all plants were colonized with AMF, we inoculated all plants by adding 150 g of field soil, which was collected by taking soil cores (10 cm diameter, 8 cm deep) $\leq$ 12 cm away from *M. racemosum* plants

growing in Trillium Trail Nature Preserve, Fox Chapel Borough, PA. After potting, the plants were watered every 2-3 days and allowed to grow, acclimate, and establish mycorrhizal colonization for one month. Because *M. racemosum* is a summer-green forest understory herb and accustomed to low light (Neufeld and Young 2003), the greenhouse was equipped with 2 layers of 65% shadecloth to maintain appropriate light conditions.

Prior to implementing our treatments, we assessed the effectiveness of the proposed allelochemical delivery system. We conducted a pilot study where we watered pots treated with "tea bags" full of garlic mustard leaves and collected the water as it leached out of the bottom of each pot. The water contained sinigrin (Appendix G), indicating that our garlic mustard treatment successfully delivers the toxic allelochemicals into the soil.

In June, we randomly assigned plants to one of three treatments: either 1 of 2 "tea bag" treatments or a drench with a non-systemic fungicide. For the tea bag treatments, we placed 25 g of fresh garlic mustard leaves or fresh dame's rocket (*Hesperis matronalis*) leaves on top of the pots. The dame's rocket tea bag serves as a negative control because while it produces glucosinolates (Larsen et al. 1992), it can sustain AMF colonization in its own root system (Demars and Boerner 1995). In contrast, as the garlic mustard leaves in the toxic teabag decompose, allelochemicals that should disrupt the function of AMF hyphae are delivered into the soil. The tea bags were first applied on 11 June and were re-applied every 2 weeks through 25 August.

The non-systemic fungicide drench served as a positive control, and should mimic the predicted negative effects of garlic mustard on the microbial community. For the drench, we used fungicides containing the active ingredient iprodione, either Chipco 26019 (containing 50% (w/w) iprodione) or OHP 26 GT-0 (containing 23.3% (w/w) iprodione). Treatments were

applied at monthly intervals (Gange and Nice 1997), beginning on 10 June and ending 10 August, at an application rate of ~0.1 g active ingredient per plant. After the treatments were applied, plants were watered every 2-4 weeks to maintain the allelochemicals and fungicide in the soil (Appendix G).

After two weeks of treatment (June 25), we inserted mixed cellulose ester membrane filters (Millipore; pore size = 45μm) into each pot to monitor AMF hyphal growth (Baláz and Vosátka 2001). Prior to insertion, we cut each membrane in half, moistened them with water, and inserted the halves in opposite corners of the pot. The small pore size of these membranes causes AMF hyphae to grow and adhere to the membrane surface. Total hyphal length on membranes can be readily estimated.

4.2.3 Physiology

We used a Li-Cor 6400 infrared gas analyzer (IRGA; Li-Cor Inc., Lincoln, NE, USA) to assess physiological performance on a weekly basis from 15 June to 14 July 2012. We focused on this time period because, as discussed above, forest understory herbs gain the majority of their seasonal carbon early in the growing season (Neufeld and Young 2003). To control abiotic factors that can influence leaf physiology, we set the block temperature to 25°C, maintained the relative humidity between 40-50% by manually adjusting air flow through the dessicant tube, held CO_2 levels constant at 400 μmol CO_2 mol^{-1} air by using an injector system, and set light levels to 600 $\mu mol \cdot m^{-2} \cdot s^{-1}$, which we have shown to be a saturating irradiance for *M. racemosum* (Hale et al. 2011). Plants were measured from 900 to 1600 hours, with the order being randomly determined each week. For each plant, the fourth leaf from the bottom of the stem was placed inside the leaf cuvette and allowed to acclimate to the conditions for 5 minutes. We

subsequently recorded photosynthetic rate (A_n), transpiration rate (E), stomatal conductance (g_s), and leaf intercellular CO_2 concentration (C_i) every 15 seconds for 1 minute. Overall, each plant was measured once prior to treatment to determine baseline physiological function, and then for four weeks following treatment to assess the impacts.

In addition to these four gas exchange traits, we also used the data to calculate the ratio of leaf intercellular CO_2 concentration to stomatal conductance (C_i/g_s ratio). We converted all stomatal conductance values to mmol $H_2O \cdot m^{-2} \cdot s^{-1}$ and divided a plant's leaf intercellular CO_2 concentration recorded in one week by its stomatal conductance observed in that week. The C_i/g_s ratio expresses the plant's demand for CO_2 independent of stomatal conductance, and thus serves as a strong indicator of leaf mesophyll efficiency (Sheshshayee et al. 1996).

The measurement of leaf CO_2 characteristics (C_i and the C_i/g_s ratio) is particularly important for this study, as these traits allow us to determine the limitations to net photosynthetic rate (Wong et al. 1979, Farquhar and Sharkey 1982) during mutualism disruption. If AMF disruption results in water stress for the native plant, then we would expect leaf C_i to be low. Because the stomata close to conserve water, CO_2 diffusion rates into the leaf decline, and the little CO_2 that is available in the mesophyll is rapidly utilized. In contrast, if the loss of AMF nutrients or sink strength is driving declines in photosynthesis, then leaf intercellular CO_2 concentrations would be high despite stomatal closure (large C_i/g_s ratio). The reductions in photosynthetic enzymes, co-factors, ATP, and general sink strength that accompany nutrient stress and sink loss would diminish the plant's overall capacity to utilize CO_2 (i.e. its mesophyll efficiency). Thus, leaf CO_2 traits can indicate how mutualism disruption affects native plant carbon acquisition.

4.2.4 Harvests

We harvested plants at four separate time points across the growing season – once prior to treatment (6 June) and three times post-treatment (9 July, 6 August, and at senescence) – to accomplish three major goals. First, we wanted to determine if our garlic mustard tea bag and fungicide treatments were effective in killing AMF hyphae across the growing season. Second, we measured foliar and rhizome nitrogen and phosphorus concentrations at each time point to determine if AMF disruption resulted in native plant nutrient-limitation. Third, we measured rhizome carbohydrate concentrations, below and aboveground growth, and asexual reproduction to determine if changes in carbon assimilation affect carbon allocation to storage, growth, and reproduction. As discussed above, forest herbs like *M. racemosum* continue to allocate carbon to traits throughout the growing season, so a multiple harvest approach captures the biological reality of this species. Throughout the experiment, we determined that plants were senesced when >40% of their leaf tissue was yellowed. To verify that plant activity was finished for the season, we also measured photosynthetic rates on the senesced plants and only harvested plants once photosynthetic rates were <1.0 μmol $CO_2 \cdot m^{-2} \cdot s^{-1}$.

4.2.4.1 AMF hyphal colonization

The membrane halves were collected (N = 6-8 plants/treatment/harvest), rinsed in DI water, and stained with trypan blue. We destained the membranes in glycerol for at least 24 hours, mounted the two halves together on a glass slide (5 × 7.5 cm), and examined them under a microscope at 20× magnification. We examined 30 areas on each membrane (15 areas/membrane half), and in areas where stained hyphae were observed we captured an image using a Nikon digital camera. We measured hyphal length in each image using the NeuronJ (Meijering et al. 2004) plugin for

ImageJ (Abramoff et al. 2004). For each membrane, we summed the measurements of hyphal length from all images to estimate hyphal length/membrane (mm).

4.2.4.2 Foliar and rhizome nutrient concentration and leaf mass per area

At the time of harvest, we separated the shoot from the roots and rhizome. The shoot was pressed and dried in an oven at 70°C for 5-6 days to a constant weight. The roots were clipped away from the rhizome and the rhizome was immediately flash frozen in liquid nitrogen. The rhizomes were then stored at -80°C and later dried in a lyophilizer for 2-3 days or to a constant weight.

After drying, we removed the leaves from the shoot, weighed them, and determined total leaf area per plant by scanning leaves in a flatbed scanner and analyzing the images in Adobe Photoshop CS3 (San Jose, California, USA). Using these data we calculated leaf mass per area (leaf dry mass/leaf area; lma). Higher values of lma indicate that plants have thicker leaves, denser leaf tissue or both (Wright et al. 2004). Dried rhizomes were also weighed, and we subsequently ground both the rhizomes and leaves into a fine powder using a Wiley mill. The samples were sent to the Penn State Agricultural Analytical Services Laboratory (University Park, PA, USA) where inductively coupled plasma (ICP) analysis determined foliar and rhizome nitrogen and phosphorus concentrations. Nitrogen and phosphorus concentrations are expressed as a % of the dry mass that was analyzed.

4.2.4.3 Allocation to storage carbohydrates and sucrose availability

We used HPLC to determine the inulin and sucrose content of the dried rhizome samples (Zuleta and Sambucetti 2001). For each sample, we treated 0.03 g of dried ground tissue with boiling water for 15 minutes while stirring continuously. We cooled and filtered the samples before

injection on the HPLC (Aminex HPX-87C anion-exchange column, deionized water at 85°C was set as the mobile phase with a flux rate of 0.6 mL/min). Inulin and sucrose concentrations are expressed as a % of the dry mass of the HPLC sample.

4.2.4.4 Allocation to above and belowground growth

Prior to drying, we recorded shoot, root and rhizome wet mass to assess allocation to above and belowground growth. Leaf area (methods described above) was also used to determine allocation to aboveground growth.

4.2.4.5 Allocation to reproduction

Asexual reproduction was scored as the number of new buds formed on the rhizome. This trait was only assessed for plants harvested during the late harvest because new bud formation is not complete until the end of the growing season (LaFrankie 1985). Because flowers are pre-formed 8-12 months before they open (LaFrankie 1985), treatments applied in one growing season cannot affect a plant's allocation to flower number within that same season. Therefore, we could not assess allocation to sexual reproduction in this study.

4.2.5 Statistical Analysis

4.2.5.1 Physiology

We used a linear mixed effects model in SAS version 9.2 (SAS Institute, Cary, North Carolina, USA) to analyze each physiological trait. The models included treatment and time main effects and a treatment*time interaction. While we attempted to control ambient temperature and humidity in the experiment, there was still significant variation in these variables over time.

Therefore, we included these factors as covariates in the analyses because they are known to strongly influence physiological performance (Lambers et al. 2008). We also included baseline physiology - recorded prior to treatment - as a covariate to account for inherent genetic differences among plants in physiological function. Lastly, we included plant size as a covariate to control for the possible influence of overall size on gas exchange. One or more of these covariates often accounted for a significant portion of the variation in the models (Table 1). Residuals for the C_i/g_s ratio data were highly skewed and non-normal, so the data were log-transformed prior to analysis. Data for all other variables met the assumptions of normality.

We incorporated a repeated measures approach to account for the covariance among measurements on the same plant across time (Littell et al. 2006). The best covariance structure was selected based on information criteria, including AIC, AICC, and BIC. For the models assessing photosynthesis and transpiration, a heterogeneous first order autoregressive covariance structure provided the best fit. However, a heterogeneous Toeplitz covariance structure provided a slightly better fit for the models assessing stomatal conductance, leaf intercellular CO_2 concentration, and the C_i/g_s ratio. Both of these covariance structures account for heterogeneous variance over time, which was a general trend across all physiological variables. These structures also assume that pairs of measurements separated by the same units of time have the same correlation. The only difference is that the Toeplitz model does not assume exponential decay of the correlations over time (Littell et al. 2006). To control for the type I error inflation that can accompany the use of these complex covariance models, we used the Kenward-Rogers degrees of freedom correction (Littell et al. 2006). Finally, if a significant treatment effect was found ($P < 0.05$), we specified least squares means be calculated and conducted pairwise comparisons to assess differences among the treatments.

4.2.5.2 AMF hyphal colonization

We used a two-way analysis of variance (ANOVA) to test the effect of our treatments on AMF hyphal length (model: AMF hyphal length = treatment + harvest date + treatment*harvest date). If the interaction term was non-significant, we dropped it from the model. To meet normality assumptions, data on AMF hyphal growth was log-transformed prior to analysis. Upon finding a significant treatment effect ($P < 0.05$), we then conducted pairwise comparisons to test for differences among treatments. Due to the presence of two influential outliers in the data (studentized residuals ≥ 3, Cook's D $\geq 4/n$), we present results from analyses both with and without the outliers.

4.2.5.3 Foliar and rhizome nutrient concentrations and leaf mass per area

We used two-way analysis of covariance tests to determine the impact of our treatments on foliar nitrogen concentration, rhizome nitrogen concentration, foliar phosphorus concentration, rhizome phosphorus concentration, and leaf mass per area (model: trait = treatment + harvest date + treatment* harvest date + initial plant size). If the interaction term was non-significant, we dropped it from the model. We used initial plant size as a covariate in each analysis because we expected size to influence to nutrient content and investment in tissues. As above, if a significant treatment effect was found ($P < 0.05$), we specified least squares means and conducted pairwise comparison tests.

4.2.5.4 Allocation

Because we expected allocation to certain traits to be correlated, prior to testing for treatment effects on allocation to each trait individually, we tested for significant correlations among the belowground traits (rhizome and root mass), aboveground traits (shoot mass and leaf area), and

carbohydrate traits (inulin and sucrose content). Depending on the normality of the variables, we calculated either Pearson or Spearman correlation coefficients (Rosner 2006). We then assessed overall treatments effects on allocation to these correlated traits using a multivariate analysis of covariance (MANCOVA; model: trait1, trait2 = treatment + harvest date + treatment*harvest date + initial plant size). If the interaction term was non-significant, we dropped it from the final model. Here, we report F-statistics for Roy's greatest root for all effects in the MANCOVA models, as Roy's greatest root has the greatest power among the MANCOVA test statistics (Scheiner 1993). We only proceeded with individual analysis of covariance tests (ANCOVAs) if a significant treatment effect was found in the MANCOVA (i.e. the "protected" ANOVA method). The ANCOVA models were of the form: trait = treatment + harvest date + treatment*harvest date + initial plant size. We included initial plant size as a covariate in all models (MANCOVAs and ANCOVAs), as it is well-established that biomass allocation scales with plant size (Niklas and Enquist 2001). Lastly, if a significant treatment effect was detected in the ANCOVA, we then requested pairwise comparisons among the least square means to determine how the treatments differed from each other. Since allocation to asexual reproduction was only assessed at the end of the growing season, we analyzed this trait using a simpler, one-way ANCOVA model (model: bud number = treatment + initial plant size) and subsequent protected pairwise comparison tests. All data were analyzed in SAS (v. 9.2 and v. 9.3, SAS Institute, Cary, North Carolina, USA).

4.3 RESULTS

4.3.1 AMF hyphal colonization

All membranes exhibited colonization. We detected only a weak effect of treatment on AMF hyphal length ($F_{2,59}$ = 1.40, P = 0.25 with two outliers; $F_{2,57}$ = 2.23, P = 0.12 excluding two outliers), although the response was in the predicted direction (Figure 9). Membranes from pots treated with garlic mustard exhibited a lower total hyphal length relative to membranes from the dame's rocket control. Furthermore, membranes from the fungicide treatment showed similar reductions in hyphal length to those in the garlic mustard treatment (Figure 9). Harvest date was highly significant indicating that in general, the membranes became progressively more colonized over time. ($F_{2,59}$ = 19.51, P < 0.0001 with two outliers; $F_{2,57}$ = 19.97, P = <0.001 excluding two outliers).

4.3.2 Physiology

Overall, *M. racemosum* plants treated with garlic mustard leaves or the fungicide had significantly reduced physiological function (Figure 10) over a 4-week period compared to plants treated with dame's rocket leaves. Stomatal conductance was the trait most strongly affected, with reductions of ~40% observed in plants treated with either garlic mustard leaves or a fungicide (P = 0.0002, P < 0.0001, respectively; Figure 10B). These treatments caused similarly dramatic reductions in net photosynthetic rate (A_n; Figure 10A) and transpiration rate (E; Figure 10C).

Average leaf intercellular CO_2 concentration (C_i) did not differ among the treatments (Table 1; F = 1.48; P = 0.24; Figure 11A). However, average C_i/g_s ratios were significantly higher in plants treated with garlic mustard or the fungicide compared to control plants (P = 0.0095, P < 0.0001, respectively; Figure 11B). This high C_i/g_s ratio indicates that CO_2 accumulates in the leaves of garlic mustard and fungicide treated plants relative to the leaves of control plants.

4.3.3 Foliar and rhizome nutrient concentrations and leaf mass per area

Treatment did not have a significant effect on foliar or rhizome nitrogen concentration (Table 2). Similarly, foliar and rhizome phosphorus concentrations were also not significantly affected by treatment (Table 2). Harvest date significantly affected each of these traits; foliar nutrients concentrations slowly declined across the growing season and rhizome nutrient concentrations increased (Figure 12).

In contrast, treatment had a highly significant effect on leaf mass per area (lma; $F_{2,57}$ = 6.46, P = 0.0031). On average, garlic mustard treated plants invested 51 grams of dry mass per m^2 leaf area, which was marginally significantly higher than the 49 grams of dry mass per m^2 leaf invested by plants in the dame's rocket control treatment (Figure 13, P = 0.07). In this case, fungicide treated plants did not respond similarly to plants in the garlic mustard treatment – in fact, the fungicide treated plants did not differ significantly in lma from the dame's rocket control treated plants (Figure 13).

4.3.4 Allocation

Treatment significantly affected rhizome carbohydrate content (Table 3). Upon examining each trait alone using an ANCOVA, we found that treatment had a significant effect on both inulin ($F_{2,60}$ = 5.42, P = 0.0071) and sucrose ($F_{2,60}$ = 7.52, P = 0.0013) concentrations. Because concentrations of these two major carbohydrates are negatively correlated (Figure 14A), plants in the garlic mustard treatment had 17% less inulin stored in their rhizomes (Figure 15A), but a significantly greater availability of sucrose (Figure 15B). While the fungicide treated plants do not show reduced rhizome inulin concentrations (Figure 15A), their sucrose content was intermediate between the dame's rocket control and garlic mustard treatments (Figure 15B).

Treatment had a significant effect on allocation to belowground growth (Table 4) and rhizome and root mass were positively correlated (Figure 14B). However, when we examined each trait individually, we found that the impact of treatment on root mass was highly significant ($F_{2,59}$ = 9.40, P = 0.0003) and dramatic, while treatment did not significantly affect rhizome mass ($F_{2,59}$ = 0.22, P = 0.8044). Thus, treatment effects on allocation to belowground growth appear to be driven largely by changes in allocation to root growth. Indeed, plants from both the garlic mustard and fungicide treatments produced ~25% less root mass than plants from the dame's rocket control (Figure 16A). Interestingly, while the two aboveground traits – shoot mass and leaf area – were highly positively correlated (r = 0.81, P < 0.0001) and the MANCOVA indicated a significant treatment effect on allocation to aboveground growth (Table 5), individual ANCOVAs showed no significant treatment effect on shoot mass ($F_{2,59}$ = 0.66, P = 0.5217) or leaf area ($F_{2,57}$ = 0.04, P = 0.9621).

Treatment had a marginally significant effect on asexual reproduction ($F_{2,19}$ = 3.01, P = 0.08). On average, plants in the dame's rocket control produced two more buds compared to

plants in the garlic mustard treatment (Figure 16B). Again, the fungicide treated plants produced an intermediate number of buds (Figure 16B).

4.4 DISCUSSION

Exposure to garlic mustard's allelochemicals suppresses physiological function and alters carbon allocation in *M. racemosum*. Stomatal conductance and photosynthetic rate were reduced by nearly 40% and 15%, respectively, relative to control plants (Figure 10). These persistent declines in carbon acquisition translated into reductions in allocation to a suite of ecologically important traits, including root growth, carbohydrate storage, and asexual reproduction. While it is known that invaders can alter native species' allocation to growth and reproduction (reviewed by Levine et al. 2003), our study is the first to link changes in both biomass and carbohydrate allocation to underlying physiological mechanisms.

Our data implicate AMF mutualism disruption as the driver of the observed declines in physiology and allocation. We base this conclusion on two lines of evidence. First, reductions in fungal hyphal length were nearly identical in the garlic mustard and fungicide treatments and both were lower than the dame's rocket controls (Figure 9). These results indicate that our garlic mustard treatment was as effective as a fungicide in reducing hyphal growth in the soil, and corroborate the findings of Hale et al. (2011), which showed that a garlic mustard "tea bag" treatment reduced soil respiration rates around *M. racemosum*. Second, *M. racemosum* plants treated with a non-systemic fungicide displayed strikingly similar changes in physiology and carbon allocation to those observed in the garlic mustard treated plants (Figure 10A-C; Figure 11A and B; Figure 15A and B; Figure 16A and B). Overall, our data are consistent with the

hypothesis that garlic mustard's negative impacts on native plants are the result of AMF mutualism disruption.

Mutualism disruption by garlic mustard allelopathy appears to drive changes in native plant physiology via a two-step process. Hale et al. (2011) found that a two-week garlic mustard pulse treatment reduced leaf intercellular CO_2 concentration in *M. racemosum*, implicating water limitation in their observed physiological declines. In contrast, here, we show that changes in leaf mesophyll efficiency were responsible for persistent reductions in carbon assimilation, as plants in the garlic mustard and fungicide treatments had significantly higher C_i/g_s ratios than control plants (Figure 11B). Thus, during the initial stages of mutualism disruption, decreases in water availability may cause stomatal closure and immediate reductions in physiology. Changes in the hydration of the root system can rapidly trigger stomatal closure via abscisic acid signaling (reviewed by Comstock 2002). However, in the long-term, reductions in mesophyll efficiency seem to limit physiological capacity.

Mesophyll efficiency is a complex trait that is a function of numerous factors, including mesophyll conductance (i.e. diffusion of gaseous CO_2 into intercellular spaces, dissolution of CO_2 into a liquid phase, conversion of CO_2 to HCO^{3-}, and diffusion across membranes; Lambers et al. 2008), the rate of the light reaction, inorganic phosphate (P_i) recycling, and ribulose bisphosphate-carboxylase/oxygenase (RuBisCO) content and efficiency (Krishna Prasad et al. 1996). Many of these aspects of mesophyll efficiency are tightly coupled with nutrient availability – for example, leaf nitrogen concentration is positively correlated with RuBisCO activity (Evans 1983). However, we found that foliar and rhizome nitrogen and phosphorus concentrations did not vary with treatment (Figure 12). When looking at leaf mass per area, garlic mustard treated plants appear to actually invest slightly more in their leaf tissue than

dame's rocket treated plants, although this comparison is marginally significant and fungicide treated plants do not respond in a similar manner (Figure 13). Thus, our data indicate that nutrient limitation is not responsible for the long term reductions in *M. racemosum* physiological function.

Rather, our data suggest that by killing AMF hyphae in the soil, garlic mustard alters source-sink dynamics in native plants. Photosynthetic processes are generally tightly linked to sink activity and can be up- or down-regulated depending on sink demand (Herold 1980, Paul and Foyer 2001). Due to the significant carbon costs of hyphal construction and turnover (Staddon et al. 2003), AMF typically exert tremendous sink strength on their plant partner and can consume up to 20% of a plant's photosynthate (Parniske 2008). Mutualism disruption and accompanying declines in AMF sink activity could affect two important aspects of mesophyll efficiency. Feedback inhibition can reduce both the rate at which P_i is recycled back to the Calvin cycle after sugar synthesis (Paul and Foyer 2001) and RuBisCO efficiency. Thus, the changes in mesophyll efficiency that we detected in the garlic mustard and fungicide treatments (Figure 11B) can be explained by the loss of AMF sink strength. In summary, our data indicate that mutualism disruption by garlic mustard knocks out the AMF sink and induces changes in mesophyll efficiency that drives physiological collapse in native forest herbs.

These physiological declines were followed by striking changes in carbon allocation. Plants treated with garlic mustard diminished allocation to carbohydrate storage (Figure 15A), root growth (Figure 16A), and asexual reproduction (Figure 16B). The declines in carbon assimilation likely led to decreases in the raw material available for allocation to these various functions. However, the sugar fixed via photosynthesis acts as both a substrate for construction and as a signal that modulates gene expression within the plant. Therefore, the reductions in

photosynthesis may have also affected the expression of genes that direct allocation to various traits. In general, when sugars fixed via photosynthesis are scarce, genes controlling storage and growth are repressed, while genes controlling photosynthesis and export are enhanced (Koch 1996). Low carbon fixation and subsequent repression of genes affecting inulin storage and root growth could explain the significant reductions in allocation to these traits for garlic mustard treated plants. Similarly, the slight increase in leaf mass per area in that we observed in the garlic mustard treated plants (Figure 13) and increased sucrose availability in the rhizome (Figure 15B) could be explained by this gene expression model. The possibility for invaders to alter gene expression in native plants by mutualism disruption is an intriguing possibility that, to our knowledge, is completely unexplored. This avenue of research warrants future study, as it could lead to a truly mechanistic understanding of the processes underlying the impacts of mutualism disruption.

Alternatively, the increased availability of sucrose in the rhizome could be an attempt by the plant to re-establish the AMF network in its root system. Because of its lack of fine root hairs and high degree of association with AMF in the field, some have classified *M. racemosum* as obligately dependent on AMF for growth and survival (Brundrett and Kendrick 1988). If AMF are critical to the survival of the plant, re-establishment of the fungal partners may be a priority after mutualism disruption. Sucrose is the pre-cursor to the hexose sugars that are ultimately exchanged with AMF arbuscules in the roots (Parniske 2008). Because AMF arbuscules inside the root system can remain intact after exposure to garlic mustard allelochemicals (Barto 2010), the sucrose we observed in the rhizome could be in transit to the roots to feed AMF arbuscules and re-establish the hyphal network in the soil. While this is a

difficult hypothesis to test empirically, we cannot rule it out as a possible explanation for the elevated sucrose levels.

The changes in allocation patterns could affect the persistence of native plants on invaded sites and their ability to cope with additional environmental stresses. First, declines in both AMF hyphal length (Figure 9) and root mass (Figure 16A) indicate that plants on invaded sites will be less able to scavenge the soil and effectively compete against native and exotic community members for soil resources. Second, since the invasion of garlic mustard and AMF disruption can diminish inulin storage by up to 17%, the ability of these forest herbs to tolerate high herbivory pressure may be compromised. Annually, herbivory by white-tailed deer (*Odocoileus virginianus*) in *M. racemosum* populations can consume up to 100% of flowering plants (N. Brouwer and S. Kalisz, unpublished data). High concentrations of storage carbohydrates are important for herbaceous perennials to survive repeated episodes of deer browse (Lapointe et al. 2010). Our data suggest that plants on invaded sites may be unable to store sufficiently large quantities of inulin to persist. Third, clonal reproduction is likely important in maintaining population growth in a species such as *M. racemosum*, which exhibits extremely slow growth and low germination rates (S. Kalisz, unpublished data). Overall, mutualism disruption by garlic mustard could lead to population decline and may explain the reduced abundance of forest herbs on invaded sites (Stinson et al. 2007).

In summary, we have shown that allelochemicals from a widespread invader reduce AMF hyphal growth in the soil and alter the sink capacity of native plants. Changes in sink strength lead to persistent declines in plant physiology that culminate in reduced allocation to functionally important traits, such as storage, growth, and reproduction. Ultimately, these impacts could facilitate further invasion of the ecosystem and compromise the ability of natives to respond to

other environmental stressors. While we used garlic mustard as a model system in this study, other exotic and invasive species have also been shown to produce allelochemicals that are toxic to mycorrhizal fungi (*Tamarix* sp., Meinhardt and Gehring 2012; *Amaranthus viridus*, Sanon et al. 2009; *Sisymbrium loeselii*; Bainard et al. 2009). Allelopathic mutualism disruption may be an important, but under-recognized, mechanism underlying ecosystem invasion. As the number of invasive species and their corresponding impacts continue to increase (Pimental 2005), linking studies of invasion with techniques from ecophysiology will be critical in revealing invasion mechanisms and informing management decisions.

Table 1. Summary of results from mixed model analyses of physiological traits (A_n = photosynthetic rate, g_s = stomatal conductance, E = transpiration rate, C_i = leaf intercellular [CO_2], and C_i/g_s = mesophyll efficiency). The factor plant size represents initial plant biomass, prior to the start of the experiment. Significant main effects and covariates are in bold print.

	A_n		**g_s**		**E**		**C_i**		**C_i/g_s**	
Factor	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*
Treatment	**10.47**	**0.0001**	**11.42**	**<.0001**	**12.44**	**<.0001**	1.48	0.237	**10.08**	**0.0002**
Week	**3.7**	**0.0138**	**9.11**	**<.0001**	**15.81**	**<.0001**	**43.75**	**<.0001**	**1.65**	**0.1841**
Treatment*Week	1.85	0.0968	**3.23**	**0.007**	1.44	0.206	0.82	0.556	1.95	0.0816
Leaf Temperature	**13.24**	**0.0004**	**39.34**	**<.0001**	1.28	0.259	**196.9**	**<.0001**	**31.8**	**<.0001**
Relative Humidity	**8.53**	**0.0041**	2.57	0.114	0.81	0.37	1.05	0.307	3.46	0.065
Baseline Trait Value	**35.41**	**<.0001**	**8.87**	**0.004**	**29.98**	**<.0001**	0.38	0.54	**42.96**	**<.0001**
Initial Plant Size	0.47	0.4943	**3.93**	**0.052**	3.82	0.055	1.56	0.217	0.1	0.7522

Table 2. Summary of results from ANCOVAs assessing the impacts of treatment and harvest date on foliar and rhizome nitrogen (N = 57, N = 61, respectively) and foliar and rhizome phosphorus (N = 59, N = 61, respectively) concentrations. The factor plant size represents initial plant biomass, prior to the start of the experiment. Significant main effects and covariates are in bold print.

	DF	**Foliar N**		**Rhizome N**		**Foliar P**		**Rhizome P**	
Factor		F	*P*	F	*P*	F	*P*	F	*P*
Treatment	2	2.26	0.1146	1	0.3743	1.49	0.2349	0.04	0.9578
Harvest	2	**178.44**	**<0.0001**	**12.49**	**<0.0001**	**11.35**	**<0.0001**	**6.7**	**0.0025**
Initial Plant Size	1	**5.98**	**0.0179**	0.78	0.3825	0.13	0.7243	2.54	0.117

Table 3. Summary of results from MANCOVA assessing the impacts of treatment and harvest date on rhizome carbohydrate content (inulin and sucrose (%)). The factor plant size represents initial plant biomass, prior to the start of the experiment. Significant main effects and covariates are in bold print.

Factor	**Roy's Greatest Root**	**Num DF**	**Den DF**	**F-value**	***P*-value**
Initial Plant Size	0.05	2	54	1.35	0.2690
Treatment	**0.32**	**2**	**55**	**8.80**	**0.0005**
Harvest Date	0.06	2	55	1.71	0.1909
Model: inulin + sucrose = initial plant size + treatment + harvest date					

Table 4. Summary of results from MANCOVA assessing the impacts of treatment and harvest date on allocation to belowground growth (root and rhizome wet mass). The factor plant size represents initial plant biomass, prior to the start of the experiment. Significant main effects and covariates are in bold print.

Factor	Roy's Greatest Root	Num DF	Den DF	F-value	*P*-value
Initial Plant Size	**10.44**	**2**	**49**	**255.78**	**<0.0001**
Treatment	**0.43**	**2**	**50**	**10.73**	**0.0001**
Harvest Date	**1.15**	**2**	**50**	**28.99**	**<0.0001**

Model: rhizome mass + root mass = initial plant size + treatment + harvest date

Table 5. Summary of results from MANCOVA assessing the impacts of treatment and harvest date on allocation to aboveground growth (shoot wet mass and leaf area). The factor plant size represents initial plant biomass, prior to the start of the experiment. Significant main effects and covariates are in bold print.

Factor	Roy's Greatest Root	Num DF	Den DF	F-value	*P*-value
Initial Plant Size	**1.59**	**2**	**51**	**40.52**	**<0.0001**
Treatment	**0.13**	**2**	**52**	**3.33**	**0.0434**
Harvest Date	**0.31**	**2**	**52**	**8.02**	**0.0009**

Model: shoot mass + leaf area = initial plant size + treatment + harvest date

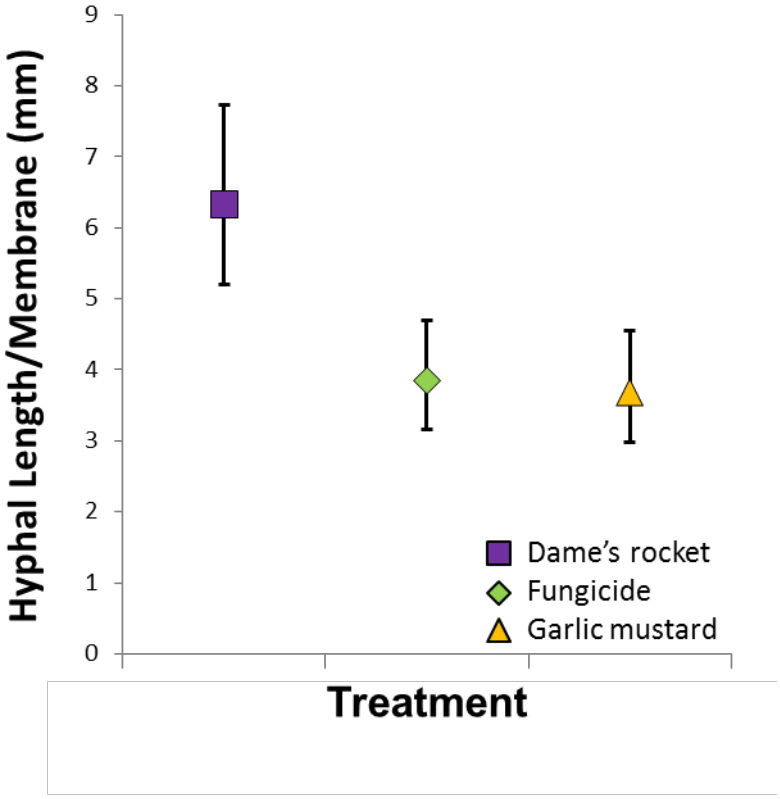


Figure 9. Average fungal hyphal length (mm) per membrane from soils exposed to different litter tea bag or fungicide treatments. Values are least squares means ± 1 standard error and from the model with two outliers dropped.

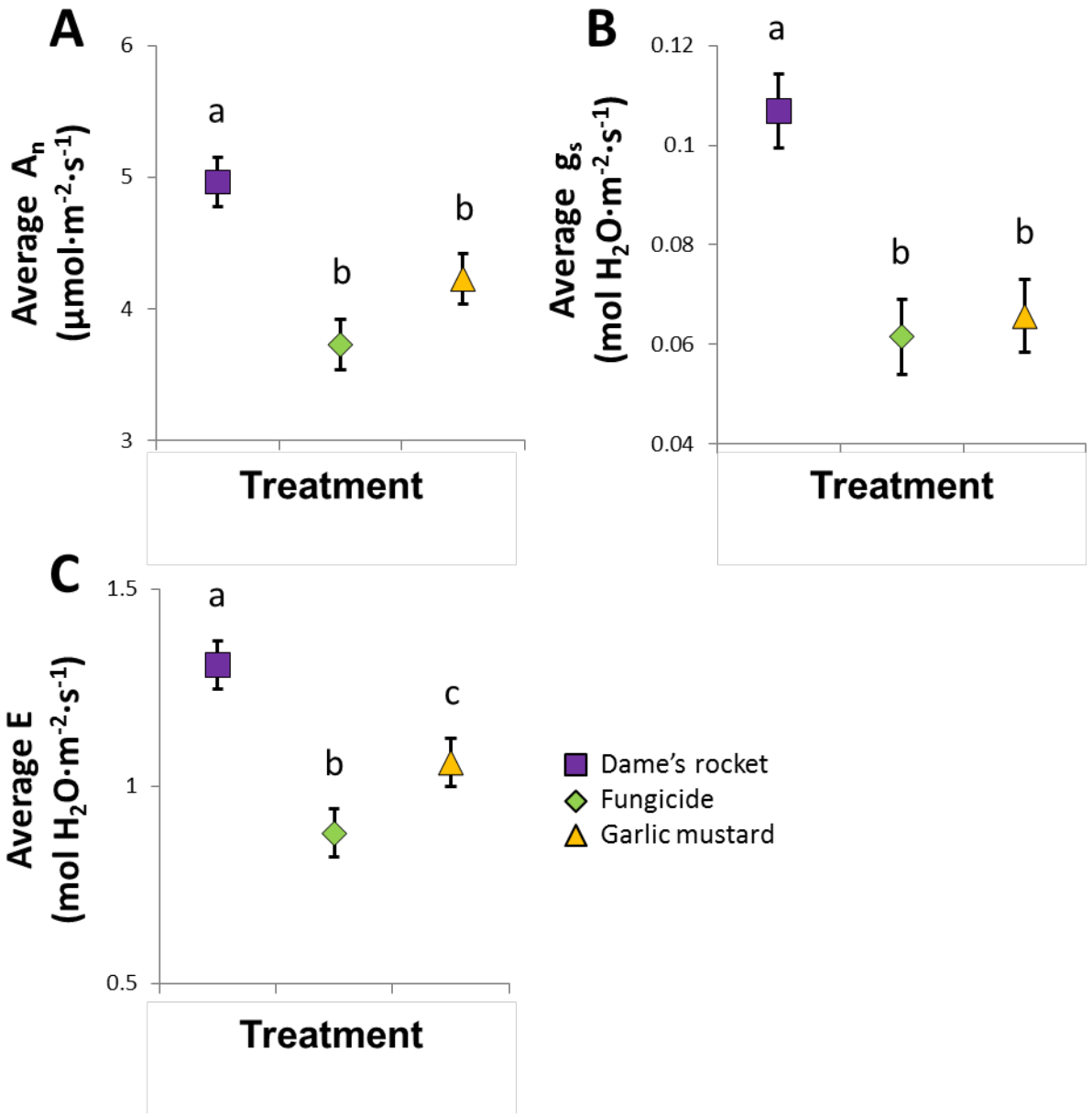


Figure 10. Average physiological performance of *M. racemosum* plants across a 4-week period exposed to different litter tea bag or fungicide treatments. Trends in A) photosynthetic rate (A_n), B) stomatal conductance (g_s), and C) transpiration rate (E) are shown. As determined by pairwise comparisons, treatments with different lowercase letters are significantly different from each other ($P < 0.05$; values are least squares means ± 1 standard error).

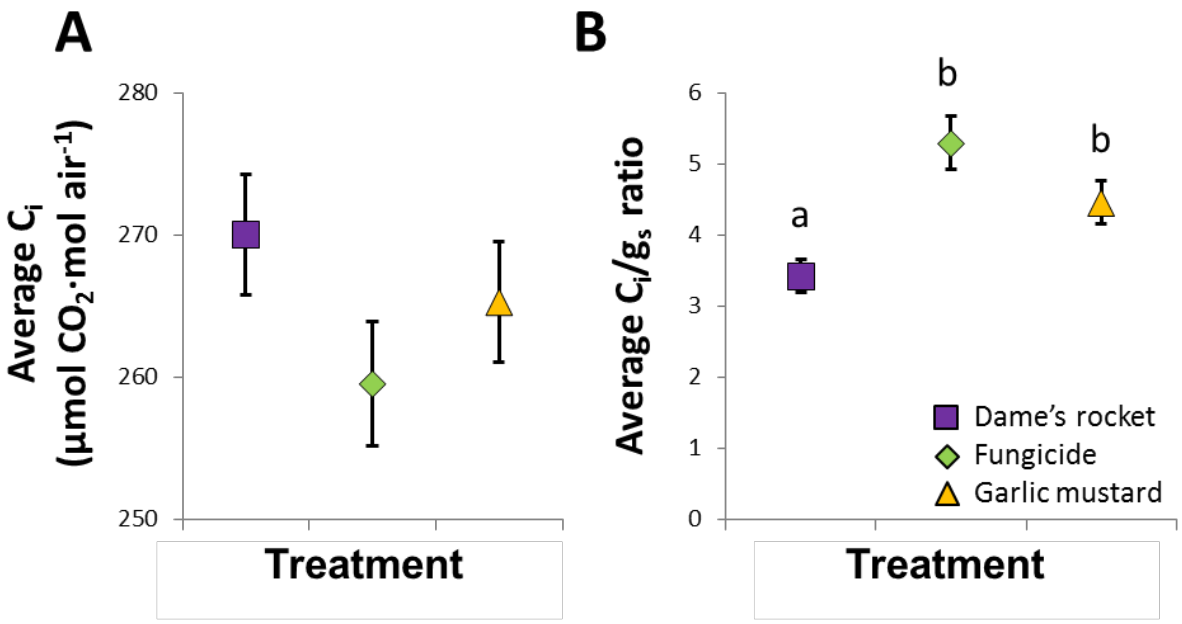


Figure 11. Average leaf CO_2 characteristics of *M. racemosum* plants across a 4-week period exposed to different litter tea bag or fungicide treatments. Trends in A) leaf intercellular CO_2 concentration (C_i) and B) mesophyll efficiency (C_i/g_s ratio) are shown. As determined by pairwise comparisons, treatments with different lowercase letters are significantly different from each other ($P < 0.05$; values are least squares means ± 1 standard error).

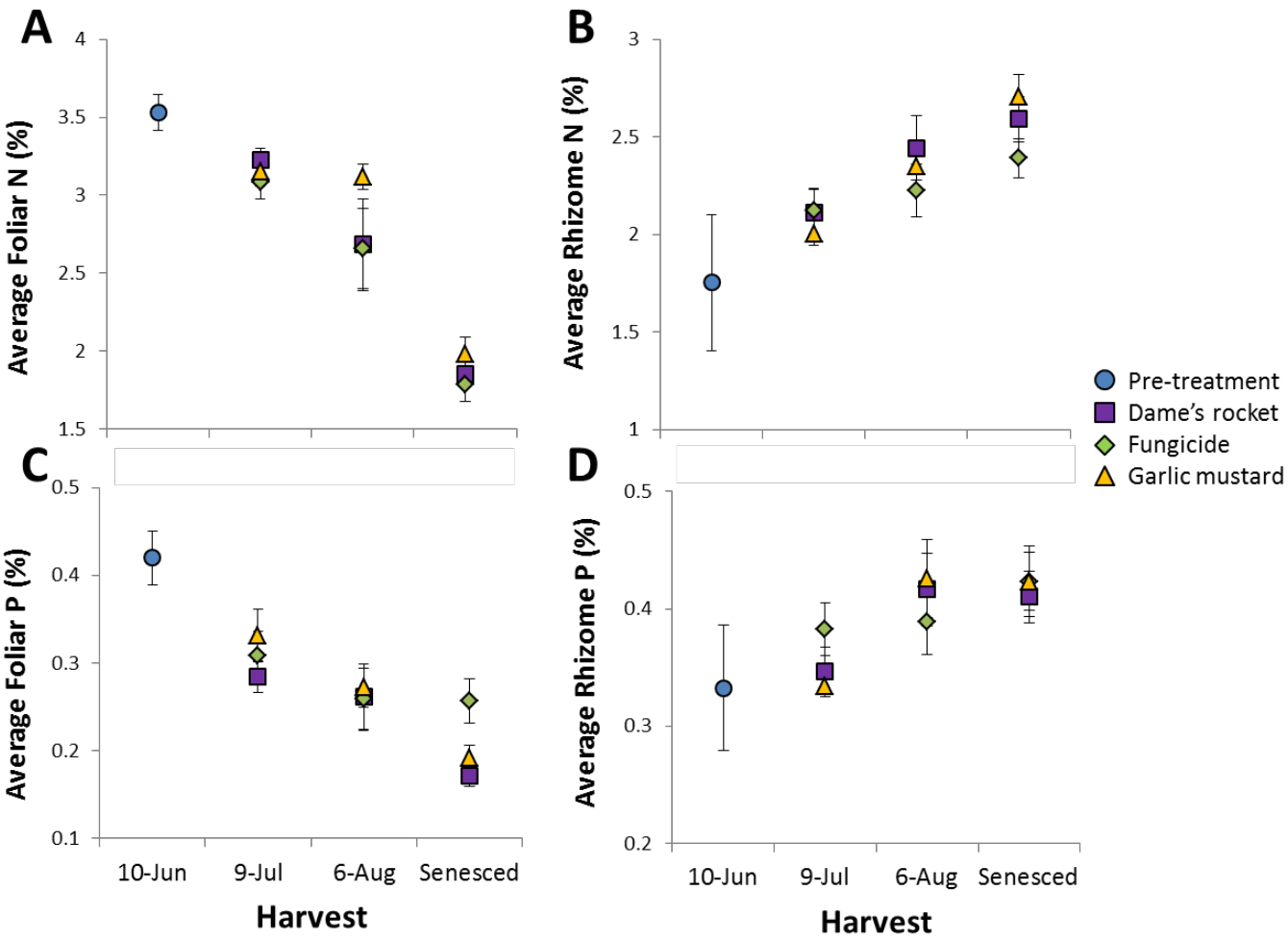


Figure 12. Average nutrient concentrations of different *M. racemosum* tissues harvested at four time points across the growing season from plants that were exposed to different litter tea bag or fungicide treatments. Trends in A) average foliar nitrogen, B) average rhizome nitrogen, C) average foliar phosphorus, and D) average rhizome phosphorus are shown. Values are means ± 1 standard error.

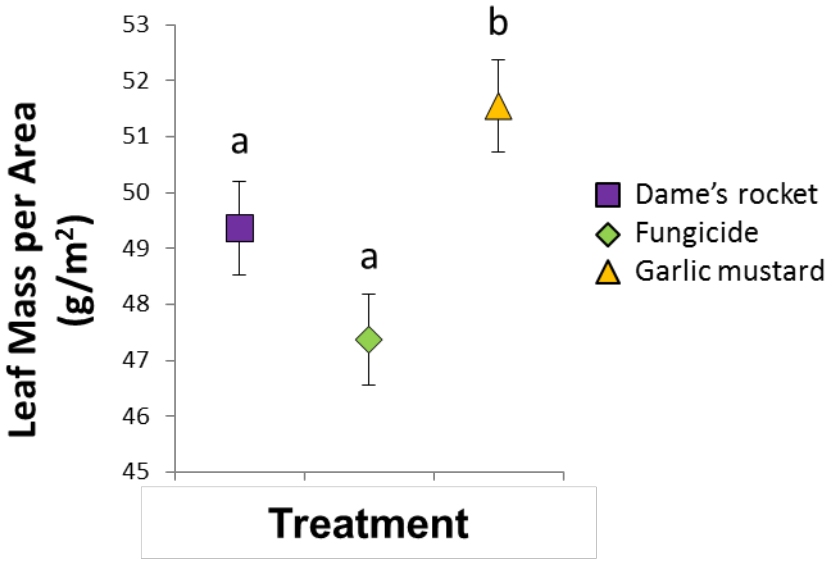


Figure 13. Average leaf mass per area for *M. racemosum* plants exposed to different litter tea bag or fungicide treatments. As determined by pairwise comparisons, treatments with different lowercase letters are significantly different from each other ($P < 0.07$; values are least squares means ± 1 standard error).

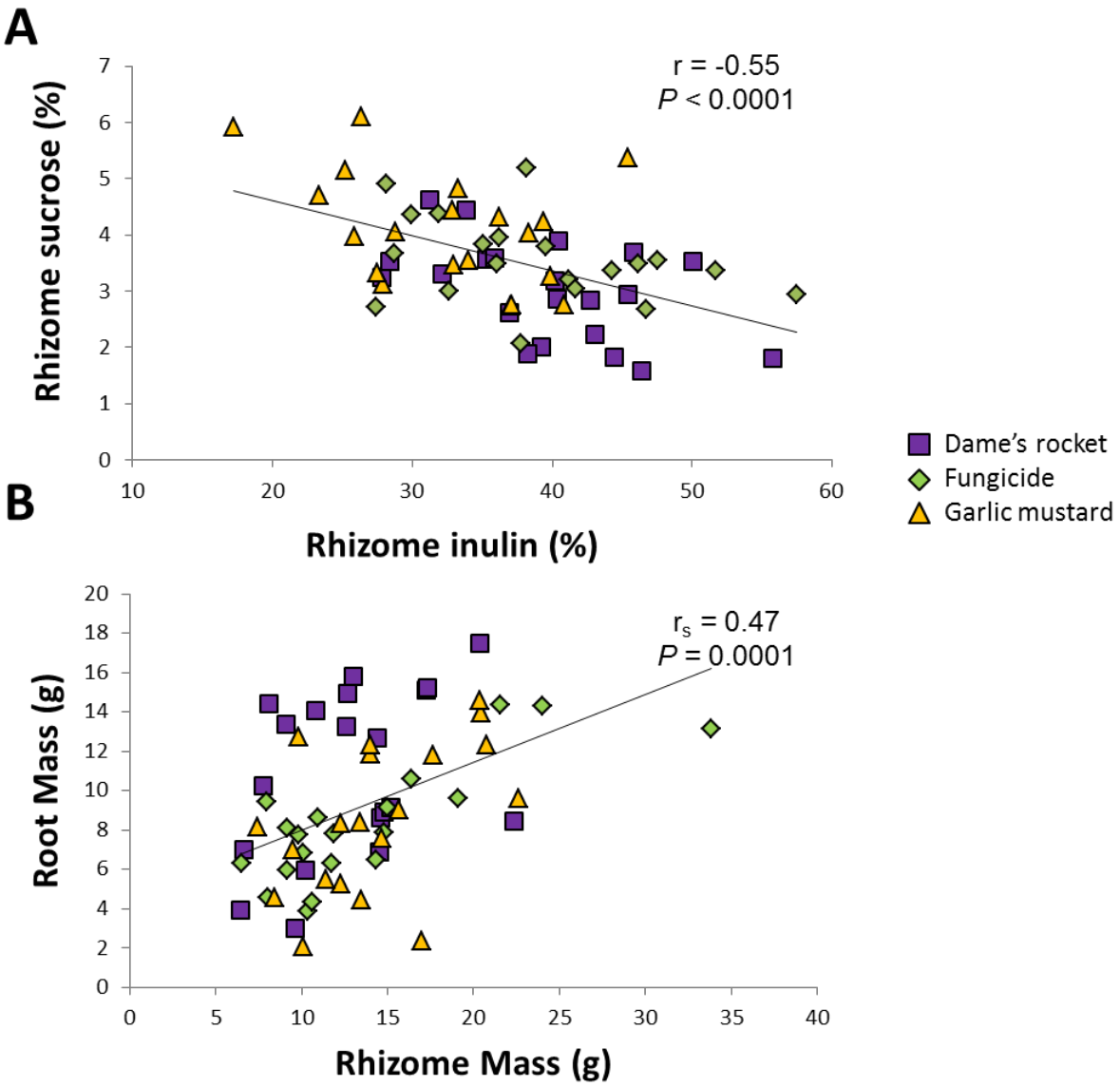


Figure 14. Correlation between A) rhizome inulin and sucrose content and B) root and rhizome mass for *M. racemosum* plants exposed to different litter tea bag or fungicide treatments. r_s = Spearmann correlation coefficient; r = Pearson correlation coefficient.

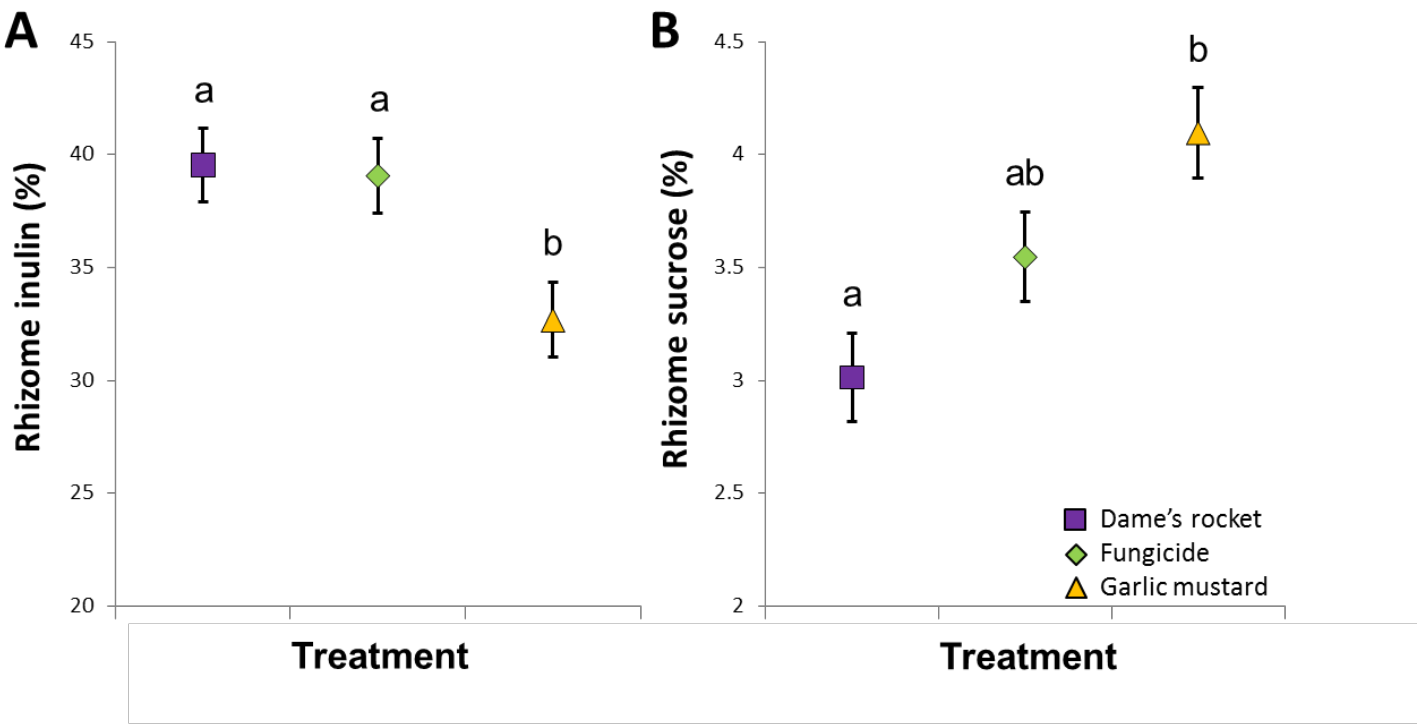


Figure 15. Rhizome carbohydrate content for *M. racemosum* plants exposed to different litter tea bag or fungicide treatments. Impacts on A) allocation to carbohydrate storage (inulin) and B) mobile sugar (sucrose) availability are shown. As determined by pairwise comparisons, treatments with different lowercase letters are significantly different from each other ($P < 0.05$; values are least squares means ± 1 standard error).

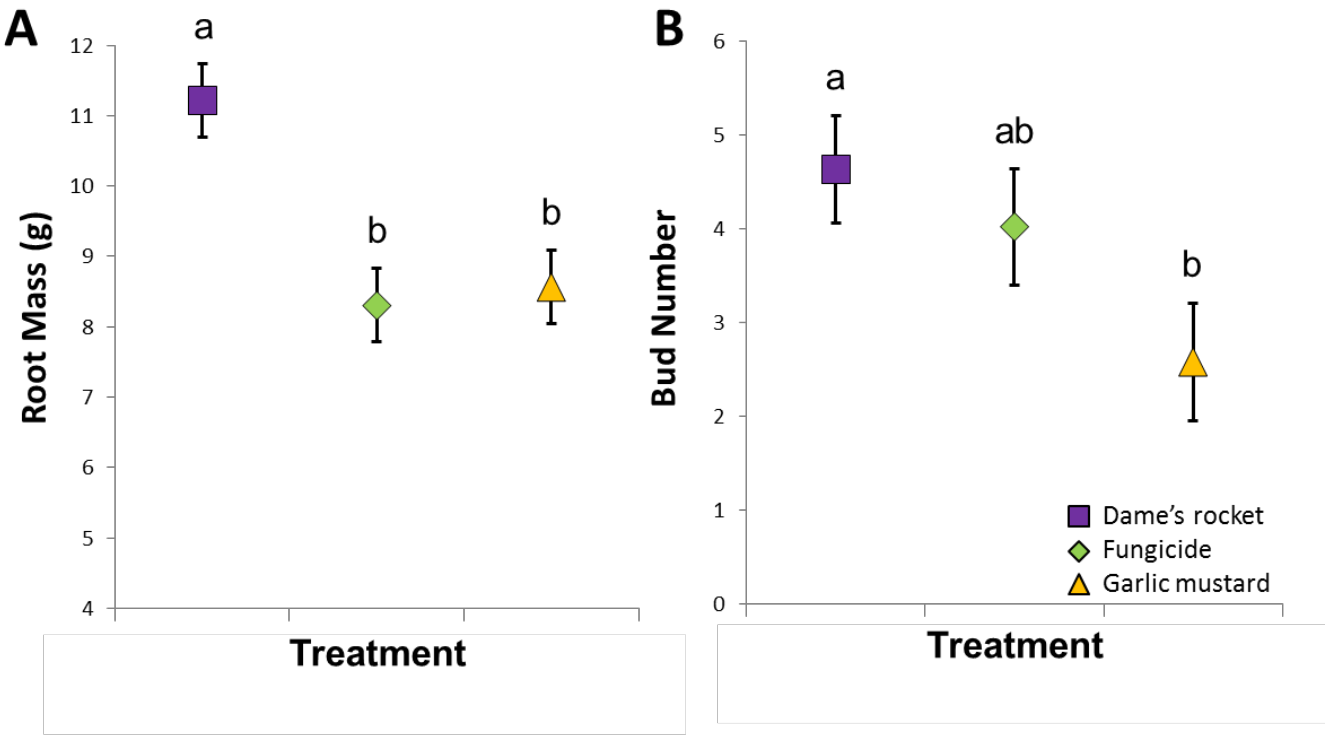


Figure 16. Allocation to A) root growth and B) asexual reproduction for *M. racemosum* plants exposed to different litter tea bag or fungicide treatments. Asexual reproduction was measured as new bud production along the rhizome at the last harvest. As determined by pairwise comparisons, treatments with different lowercase letters are significantly different from each other ($P < 0.05$; values are least squares means ± 1 standard error).

5.0 PERSPECTIVES ON ALLELOPATHIC DISRUPTION OF PLANT MUTUALISMS: EXPLORING POTENTIAL CONSEQUENCES ON INDIVIDUAL- AND POPULATION-LEVEL FITNESS

5.1 INTRODUCTION

Mutualisms involve the exchange of resources and/or services between two partner species. For each species, the benefits received from the interaction outweigh the costs, resulting in a net fitness gain. The overwhelming majority of plant species participate in one or more mutualistic interactions with microorganisms and animals. While the symbiosis with mycorrhizal fungi is likely the most widespread mutualism (up to 90% of terrestrial plants participate in mycorrhizal interactions; Smith and Read 2008), ~ 75% of all flowering plant species take part in pollination mutualisms (National Research Council 2007) and 60-80% have evolved seed dispersal mutualisms (Jordano et al. 2011). Dependence on these key mutualisms varies widely among plant species and local habitat conditions, ranging from facultative to obligate (Bronstein 1994b). However, for the majority of plants, fitness (i.e. germination, establishment, survival and reproductive success) is intricately linked to the benefits derived from mutualistic interactions.

Abiotic and biotic conditions dictate the effectiveness and stability of plant mutualisms (reviewed by Bronstein 1994b, Kiers et al. 2010). Allelopathy (i.e. inhibition of the establishment and/or growth of neighboring plants by plant-produced chemicals; Inderjit et al.

2011a) is increasingly recognized as a widespread ecological phenomenon (see articles in this special issue). Surprisingly, the influence of this environmental stressor on plant mutualisms has been almost completely overlooked. While allelopathy can directly inhibit plant functioning (reviewed by Duke and Dayan 2006), allelopathy could also impact plant fitness by weakening the effectiveness of key plant mutualisms. We suggest that allelopathy could negatively affect plant mutualisms through two distinct, but not mutually exclusive, pathways: 1) direct interference with the plant's ability to produce resources and rewards for its mutualistic partners, and 2) direct or indirect alteration in the behavior of a plant's mutualists. Allelopathy can have dramatic effects in both agro-ecosystems and natural habitats, by influencing the success of crop and weed species' (reviewed in Weston and Duke 2003) and exotic species' invasion (i.e. "novel weapons"; Callaway and Ridenour 2004). Yet, in most cases it remains undetermined if the observed impacts result from direct allelopathy on the plant, allelopathic disruption of plant mutualisms, or a combination of these factors.

Here, we explore the general consequences of allelopathy for plant-mutualist interactions. First, we discuss two classes of allelochemicals, phytotoxins and antimicrobials, and briefly describe their effects, highlighting what is known about how they can alter plant mutualisms. We then develop a framework of key questions focused on the three most common plant mutualisms - mycorrhizae, pollination, and seed dispersal. In assessing each question, we draw upon the agricultural and ecological literature to highlight current research results and the potential fitness effects of allelopathic mutualism disruption. We then flip these questions around to ask under what conditions plant-produced chemicals could exert positive effects on plants and their mutualists. Lastly, we identify gaps and opportunities in allelopathy and plant mutualism research.

5.2 CLASSES OF ALLELOCHEMICALS

Allelochemicals are plant-derived secondary compounds with negative effects on other plants (Inderjit et al. 2011a) that enter the environment through root exudation, litter decomposition, foliar leaching, and/or volatilization (Inderjit and Duke 2003). Two broad classes of allelochemicals - phytotoxins and antimicrobials - can impact plants and their mutualistic partners. Phytotoxins directly inhibit the plant, while antimicrobials can inhibit bacteria and fungi.

Phytotoxic allelochemicals can be released as exudates (e.g. Czarnota et al. 2001, Kong et al. 2006) or volatiles (e.g. Kong et al. 2002, Jassbi et al. 2010, Inderjit et al. 2011b) and can target a wide range of molecular sites, including plant photosynthetic machinery and/or mitochondrial respiration enzymes (reviewed by Duke and Dayan 2006). One well-known phytotoxic exudate is sorgoleone, a chemical produced by roots of the crop species *Sorghum bicolor* that inhibits photosystem II electron transport (Czarnota et al. 2001). Some allelochemicals are such potent phytotoxins that the active ingredients of several agricultural herbicides are derived from their chemical structures (Vyvan 2002). Because phytotoxic allelochemicals can cause reductions in carbon acquisition and plant growth (e.g. Patterson 1981, Helj and Koster 2004, Hussain and Reigosa 2011) these chemicals undoubtedly alter the quantity of resources and quality of reproductive structures that a plant has available for mutualist interactions.

Antimicrobial allelochemicals commonly inhibit the growth of soil microbes (reviewed by Cipollini et al. 2012). Clear evidence for direct antimicrobial allelopathy comes from the glucosinolate-producing members of the mustard family, Brassicaceae. Upon hydrolysis, glucosinolates in the soil are converted into various biologically active compounds (e.g. Brown

et al. 1991, Morra and Kirkegaard 2002, Gimsing and Kirkegaard 2006), which are highly toxic to a wide range of soil bacterial and fungal pathogens, nematodes, and other invertebrates (reviewed by Brown and Morra 1997). As a result, many mustard species are actively planted as cover crops in agricultural fields, where they are later plowed under to control disease and pest species in the soil (Gimsing and Kirkegaard 2009). However, these allelochemicals can also directly inhibit beneficial microbes involved in plant mutualisms, including mycorrhizal fungi (Schreiner and Koide 1993, Vierheilig et al. 2000). A direct allelopathic effect on a microbial partner will decrease its ability to supply resources to its plant partner.

5.3 ALLELOPATHIC DISRUPTION OF PLANT MUTUALISMS

In this section, we briefly describe three key plant mutualisms associated with roots, flowers and fruits and develop a framework of specific questions regarding the impact of allelochemicals on these mutualisms. These questions, the predicted effects of allelopathy, and the fitness consequences for each partner are summarized in Table 6.

5.3.1 Allelopathy and plant-mycorrhizal mutualisms

Mycorrhizal fungi are a unique fungal group that associate with plant roots and provide plants with numerous benefits including soil-derived resources, protection from pathogens, enhanced drought resistance, and increased sink strength (Smith and Read 2008). In return, the plant supplies these fungal symbionts with carbon from photosynthesis (Smith and Read 2008). Thus, each partner in the symbiosis incurs costs and benefits, and the mutualism is only maintained

when the benefits outweigh the costs for both partners (Johnson et al. 1997). Recent research demonstrates that a reciprocal reward system is responsible for stabilizing cooperation in the mycorrhizal mutualism; the more carbohydrates the plant provides, the more nutrients the mycorrhizal fungi provides, and *vice versa* (Kiers et al. 2011). Thus, in mycorrhizal symbioses, mutualism effectiveness is largely dictated by the interaction of the plant's ability to supply carbon and the fungal partner's ability to acquire soil nutrients. We pose questions regarding the impact of allelochemicals on both components of this interaction (Table 6A) and discuss supporting evidence below.

5.3.1.1 Can direct anti-fungal effects of allelochemicals reduce mycorrhizal fungi spore germination and/or the abundance of functional hyphae in the soil, thereby reducing nutrient flow to the plant partner?

While many studies have demonstrated evidence of direct allelopathic toxicity on mycorrhizal fungi (summarized in Table 7), there are few complete stories. One species that has emerged as a model system for studying allelopathic impacts on mutualistic soil microbes is *Alliaria petiolata* (garlic mustard; Brassicaceae), a widespread invader in North America. Identifying and demonstrating the potential for allelopathic impacts in an ecological setting is a critical first step in research on suspected allelopathic species (Blair et al. 2009). *Alliaria petiolata*'s allelochemicals are well characterized; chemicals identified to date include alliarinoside (Haribal et al. 2001), cyanide (Cipollini and Gruner 2007), flavonoid glycosides (Haribal and Renwick 1998), and glucosinolates (Vaughn and Berhow 1999). This suite of chemicals completely inhibits mycorrhizal spore germination when applied in bioassays using whole plant extracts (Roberts and Anderson 2001, Stinson et al. 2006). *Alliaria petiolata*'s glucosinolates and flavonoids are each inhibitory to spore germination, but it is their combined effect that makes *A.*

petiolata extraordinarily toxic (Callaway et al. 2008). The above bioassay results were recently corroborated for the first time under field conditions. Cantor et al. (2011) detected the glucosinolate, sinigrin, and its breakdown product, allyl isothiocyanate (AITC), in soils invaded by *A. petiolata*. In a companion laboratory bioassay, the field-detected concentrations of AITC reduced spore germination by 57% relative to the control (Cantor et al. 2011), establishing that *A. petiolata*'s anti-mycorrhizal allelochemicals are present in field soils at bioactive concentrations.

Multiple studies have demonstrated the effectiveness of *A. petiolata* in disrupting mycorrhizal fungi. *Alliaria petiolata* density is negatively correlated with soil mycorrhizal inoculum potential (Roberts and Anderson 2001) and fungal hyphal abundance is reduced in invaded sites (Cantor et al. 2011). Tree seedlings collected from invaded sites show reduced colonization by arbuscular mycorrhizal fungi (AMF; Barto et al. 2011) and ectomycorrhizal fungi (EMF; Wolfe et al. 2008). Tree seedlings grown in soils experimentally invaded or "conditioned" by *A. petiolata* also experience little to no AMF (Stinson et al. 2006) or EMF colonization (Wolfe et al. 2008).

Interestingly, *A. petiolata*'s allelopathic effects on mycorrhizal fungi can differ among sites or studies (e.g. Burke 2008, Barto et al. 2010b). Variation in *A. petiolata*'s allelochemical production across populations likely contributes to these differences. Per capita glucosinolate production is highest in newly invading *A. petiolata* populations (Lankau et al. 2009) and in sites where it is in low abundance (Lankau 2012), resulting in significant variation among sites in allelochemical production. AMF community composition and richness also vary dynamically with age since *A. petiolata* invasion (Lankau 2011a, Lankau 2011b). These changes could result in shifts in the identity of the fungal partners involved in plant mutualisms (Barto et al. 2011,

Lankau 2011b), evolved changes in the mycorrhizal community (Lankau 2011a), or extinction of mycorrhizal partners.

Direct allelopathic effects of *A. petiolata* on mycorrhizal fungi can result in significant negative consequences for the plant partner across multiple plant life stages and life histories, particularly for species that are heavily dependent on the mutualism. For example, the loss of mycorrhizal spore viability, infectivity, and the altered mycorrhizal community composition in soils treated with *A. petiolata* depressed growth in seedlings of six native tree species (Stinson et al. 2006, Lankau 2011b) and reduced emergence and increased post-recruitment mortality in native herbaceous seedlings (Callaway et al. 2008). Similarly, significant reductions in photosynthetic rate (Hale et al. 2011), growth, asexual reproduction, and carbohydrate storage (Hale and Kalisz, in prep) of adult *Maianthemum racemosum* plants are linked to diminished AMF function following *A. petiolata* treatment. Like most temperate forest understory perennial herbs, *M. racemosum* is highly dependent on mycorrhizal fungi (Brundrett and Kendrick 1988). Because the negative consequences of mutualism disruption likely scale with a plant's reliance on its partner (Kiers et al. 2010), allelopathic disruption of the AMF mutualism could be devastating for all *M. racemosum* life stages, with effects on seedlings and adults. In general, the loss of mycorrhizal fungi could diminish native plants' population stability and potentially drive diversity loss on invaded sites (Stinson et al. 2007).

There is also preliminary evidence of direct allelopathic effects on mycorrhizal fungi in other species. Two studies on another exotic invasive, *Amaranthus viridus*, demonstrate that invaded soils have reduced AMF spore abundance and hyphal length and that tree seedlings grown in invaded soils show reduced mycorrhizal colonization and growth (Sanon et al. 2009, Sanon et al. 2012, Table 7). In other systems, such as rice (*Oryza sativa*), it is clear that

allelochemicals have a general inhibitory effect on the soil microbial community, but specific impacts on mycorrhizal fungi remain unknown (Kong et al. 2008). Given the large number of species with direct allelopathic toxicity on beneficial mycorrhizal fungi (Table 7) and those that remain to be tested, we anticipate that antimicrobial allelopathic species will play a powerful role in structuring diversity and composition of plant soil communities through mutualism disruption.

5.3.1.2 Can phytotoxic chemicals adversely impact plant-mycorrhizal mutualisms by reducing photosynthesis and lowering carbon availability/allocation to the mycorrhizal partner?

Several studies on allelopathy demonstrate that allelochemicals can cause significant reductions in photosynthesis in target plants (e.g. Patterson 1981, Helj et al. 1993, Jose and Gillespie 1998, Hussain and Reigosa 2011). Because mycorrhizal plants can expend up to 20% of their carbon resources to support the fungi (Bago et al. 2000), a reduction in carbon availability within the plant will likely decrease its ability to provide resources to mycorrhizal fungi. These effects would be analogous to the negative impacts of herbivory on mycorrhizal colonization, which are proposed to occur as a result of reduced carbon allocation to the mycorrhizal fungi (reviewed by Gehring and Whitham 1994). At the very least, under allelopathy the carbon cost of supporting the mycorrhizal fungi would significantly increase, as the support of any mycorrhizal partners detracts from current carbon demands within the plant. Although a recent meta-analysis (Corrêa et al. 2012) suggests that plants only use excess carbon for "luxury resource exchange" (*sensu* Kiers and van der Heijden 2006, page 1630) with their mycorrhizal partners, others have stated that resources are never truly in excess when they can be stored for reproduction or future growth (e.g. Chapin et al. 1990). Even if a plant continues to provide ample carbon to its mycorrhizal partners despite allelopathy-driven photosynthetic declines, this continued cost could reduce

carbon reserves and constrain future growth and reproduction. Thus, phytotoxic allelochemicals that diminish photosynthetic capacity will, in theory, alter a plant's ability to participate in mycorrhizal mutualisms and cause either immediate or long-term fitness declines for the plant. Immediate impacts will arise as a result of the reciprocal nature of the mutualism; reduced carbon flow to the mycorrhizal partner results in reduced nutrient flow to the plant. Long term impacts will arise because as the plant continues to provide carbon to the mycorrhizal fungi, it will have less for itself to use or store.

5.3.2 Allelopathy and plant-pollinator and plant-seed disperser mutualisms

Numerous animal species provide pollination and seed dispersal services to plants, including a diversity of birds, mammals, insects, and lizards (National Research Council 2007, Valido and Olesen 2007). To attract these mutualists, plants expend resources to create a variety of showy structures and signals. For pollinators, floral traits such as individual flower size and shape (Darwin 1859; Bradshaw et al. 1998), color (Grotewold 2006), scent (Pichersky and Gershenzon 2002; Raguso 2008), longevity (Ashman and Schoen 1994) and overall floral display size (Wyatt 1982) act as advertisements. For seed dispersers, plants produce conspicuous, colorful fruits to stimulate visitation (reviewed in Schaefer and Schaefer 2007). These signals and rewards are energetically expensive. Nectar is typically composed of a mixture of sugars including glucose, fructose, and sucrose, amino acids (Baker and Baker 1986) and sometimes lipids. Likewise, pollen is a costly reward that can contain proteins, starch, lipids, and vitamins (Roulston and Cane 2000). Fruit production represents an even greater cost; in one perennial plant, the production of a single fruit can reduce leaf area in the following year by up to 2% (Snow and Whigham 1989). Despite these costs, participation in pollination and seed disperser mutualisms

is critical for the growth and persistence of plant populations (e.g. avoidance of inbreeding and inbreeding depression, reviewed by Charlesworth and Charlesworth 1987, and Janzen-Connell effects, Wills et al. 1997, Harms et al. 2000).

Like the mycorrhizal symbiosis, pollinator and seed disperser mutualism effectiveness involves both the ability of the plant to provide sufficient signals and rewards, and the behavior of the animal mutualist. We propose that allelopathy could diminish the effectiveness of these reproductive mutualisms largely through the plant's side of the interaction. Below, we outline three questions that address potential impacts that allelopathic disruption of reproductive could have at the individual and population levels (Table 6B).

5.3.2.1 Can phytotoxic allelochemicals adversely affect plant-pollinator or seed disperser mutualisms through declines in plant photosynthesis and/or vigor, ultimately resulting in smaller displays with fewer, poorer rewards?

Because phytotoxic allelochemical exposure can cause significant reductions in photosynthesis and plant function (see above), affected plants will have fewer resources available for allocation to energy-intensive displays and rewards. The impacts of phytotoxins on plant reproduction can be seen in the allelopathic suppression of an African legume, *Vigna subterranea*, by the sunflower (*Helianthus annuus*). This legume produced no flowers when grown in soils mixed with either fresh or decomposing sunflower litter, while control plants produced an average of 6-7 flowers (Batlang and Shushu 2007). Further, sunflower litter also completely suppressed root nodulation by nitrogen-fixing bacteria (Batlang and Shushu 2007). An interesting aspect of this study is that *V. subterranea* produces only small, cleistogamous, self-pollinating flowers (Onwubiko et al. 2011). These results indicate that allelopathy can reduce plant resources and drive total reproductive failure even for species that invest little in attractive floral structures or

pollinator rewards. Since the resource cost of chasmogamous flower production is significantly higher than that of cleistogamous flower production (e.g. Schemske 1978), we expect that phytotoxic allelochemicals would have an even greater negative impact on the reproductive allocation of chasmogamous species and could reduce pollinator visitation by diminishing floral display size. Finally, although the results in this case are clear, the chemicals and mechanisms responsible are not. The phytotoxic effects of sunflower's sesquiterpene lactones (Macías et al. 1996, Macías et al. 2006) could diminish carbon availability for reproduction and exchange with nodulating bacteria. In addition, *Helianthus annuus* produces other compounds with anti-fungal effects (Giudici et al. 2000, Prats et al. 2007) that could be acting in this study, but their effects on bacteria are unknown. Thus, the results of Batlang and Shushu (2007) do not rule out the possibility that the decline in flower production is the result of sunflower allelochemicals directly inhibiting N-fixing bacteria associated with the legume.

In addition to flower or fruit number, the quantity of the reward and/or the reward's nutritional quality is expected to diminish upon exposure to phytotoxic allelochemicals. Nectar production can demand up to 37% of a plant's daily carbon assimilate during flowering (Southwick 1984). Given that phytotoxins such as juglone can cause a 3-fold reduction in photosynthetic rate in target plants (Jose and Gillespie 1998), phytotoxic depression of a plant's resource status of could have a significant impact on its ability to produce high-quality or sufficient quantities of nectar. Similarly, pollen production is sensitive to the daily capture of photosynthate: plants in high light environments produce significantly more pollen than plants in low light environments (Etterson and Galloway 2002). Reductions in pollen production as a result of allelopathy could alter pollinator visitation rates, the duration of pollinator visits and their general effectiveness in pollen export. In a similar fashion, early fruit growth can be

strongly limited by a decreased supply of carbon assimilates (Zhang et al. 2005). Reductions in fruit size can depress seed disperser visitation rates (reviewed in Jordano 2000). Allelopathy could therefore likewise disrupt the efficiency of seed dispersal mutualisms.

5.3.2.2 Can the uptake of allelochemicals into plant tissues alter the attractiveness and nutrition of rewards?

Quantifying the uptake and translocation of allelochemicals is a major methodological challenge in the field of allelopathy (Inderjit and Duke 2003). The most widely used approach is radioactive isotopic labeling and tracking of the putative allelochemicals (Chiapusio and Pellissier 2001, Chiapusio et al. 2004, Hachinohe et al. 2004, Dayan et al. 2009, but see Sánchez-Moreiras et al. 2010). One such study revealed that radish seedlings readily take up 2-benzoxazolinone, an allelochemical commonly found in cultivated grain crops (Chiapuso et al. 2004). Because radiolabeled allelochemicals are often not commercially available (Loi et al. 2008), increasingly sophisticated tools that allow for direct monitoring of allelochemical uptake are being applied. Using solid-phase microextraction, Loi et al. (2008) demonstrated that the soil application of just 50μM 1,8-cineole, an allelochemical produced by *Artemisia* sp. (Barney et al. 2005, Jassbi et al. 2010), resulted in the uptake and translocation of this allelochemical 30cm up the stem of tomato plants. While such studies demonstrate that allelochemical uptake and translocation can occur, the uptake rate varies widely across species, further complicating one's ability to detect a general pattern. Variation has been ascribed to differences in the target plant's ability to detoxify the allelochemical after uptake (Inderjit and Duke 2003, Hachinohe et al. 2004), the life history stages of target plants tested (Dayan et al. 2009), and the presence of microbes (Chiapusio and Pellissier 2001).

Work to date has demonstrated allelochemical uptake and translocation to stem and foliar tissue of target plants, but it remains unknown if allelochemicals are conducted into floral tissues. Evidence from serpentine endemic plants suggests that uptake of toxic materials is a distinct possibility. It is well known that some serpentine endemics hyperaccumulate and translocate heavy metals to stem and leaf tissue, which increase herbivore defense, sequestration, and/or elemental allelopathy (Boyd 2004). High levels of nickel have also been detected in flowers (Jaffré et al. 1976) and fruits (Boyd et al. 2006) of serpentine plants. Insects that feed on the pollen and fruits of these nickel hyperaccumulators exhibit elevated nickel levels in their bodies (Boyd et al. 2006), implicating heavy metals in the nutritional rewards for seed dispersers and pollinators. While we acknowledge that plant-produced allelochemicals differ significantly in their chemical properties from heavy metals, these data suggest the possibility for allelochemical incorporation into floral rewards and fruits.

We expect that if allelochemical accumulation is high in pollinator and seed-disperser rewards, the palatability and attractiveness of these rewards could be diminished. Work by Strauss et al. (1999) illustrates that the presence of glucosinolates in *Brassica nigra* can make pollen and nectar less palatable to its pollinators. When pollinators were allowed to choose among lines of *B. nigra* that varied in their myrosinase production from low to high, pollinators spent significantly more time per flower and visited more flowers per plant on low-myrosinase lines relative to the high-myrosinase line plants (Strauss et al. 1999). Because the total number of visitors did not differ between the lines - indicating no difference in the attractiveness of the floral display - it is hypothesized that quality or quantity of the reward drove the changes in pollinator behavior (Strauss et al. 1999). Similarly, a recent comparative study across multiple taxa of *Nicotiana* demonstrated that taxa that produce lower levels of the anti-herbivore

compound nicotine in their nectar tend to be more outcrossing while taxa that produce nectar with high levels of nicotine are highly selfing (Adler et al. 2012). Although Adler et al. (2012) did not directly measure pollinator preference, the implications are similar to those of Strauss et al. (1999) and illustrate that the chemical traits of reward tissues can potentially play a role in mutualist attraction or repulsion. The influence of allelochemicals on the palatability of these rewards is an interesting, albeit, completely unexplored potential side effect of allelopathy.

5.3.2.3 Can the release of volatile allelochemicals mask attractive scents of neighboring plants or directly repel pollinators and seed dispersers?

Volatile allelochemicals could reduce pollen and seed dispersal of surrounding plants by masking their scent signal to pollinators and seed dispersers. The ability of floral scent to successfully communicate information to pollinators is dependent on the local context (Raguso 2008). Background odors can influence the effectiveness of floral scents as attractants to distant pollinators, particularly when the plant producing the scent is patchily distributed among other scent-producing plants (Raguso 2008). Thus, volatile allelochemicals could diminish pollinator visitation through dilution of an attractive scent or repel pollinators by overpowering the attractive scent. These ideas are strongly supported by studies testing the effects of background volatiles on insect herbivores that also utilize volatiles scents as cues. When barley plants were caged with *Cirsium arvense* for five days and then exposed to aphids, more aphids settled on the control plants than on the plants that had been exposed to *C. arvense* volatiles (Glinwood et al. 2004). The authors suggest that *C. arvense* volatiles may have directly adhered to the barley and repelled the herbivores or altered barley phytochemistry in some way (Glinwood et al. 2004). Another study found that volatiles from *Chenopodium album* deterred aphids from settling on barley in the lab and the field (Ninkovic et al. 2009). Together, these studies suggest that

volatiles can strongly interfere with insect preference for a host plant. While these ideas have not yet been tested on plant-pollinator or plant-seed disperser mutualisms, it is plausible that other animal mutualists would respond in a similar ways.

5.4 FEEDBACK BETWEEN MUTUALISMS

The three major plant mutualisms discussed above are not independent, and the disruption of any one could have cascading effects on another. For instance, experimental studies demonstrate that the presence of mycorrhizal fungi can enhance floral traits that are important for pollinator attraction. AMF have been shown to increase overall inflorescence size (Wolfe et al. 2005), individual flower size (Gange et al. 2005, Varga et al. 2010 Aguilar-Chama and Guevara 2012), number of functional stamens (Varga et al. 2010), flower number, nectar sugar content, and nectar secretion rate (Gange et al. 2005) in their plant partners relative to non-mycorrhizal controls. In two studies, the enhancement of floral traits by AMF was linked to increases in pollinator visitation (Gange et al. 2005, Wolfe et al. 2005). These species-level effects are mirrored at the community level: plots treated with benomyl to suppress AMF experienced a 67% decrease in pollinator visitation as a result of significantly decreased floral displays at the plot level relative to control plots (Cahill et al. 2008). However, the effects of mycorrhizal fungi on floral traits can be context-dependent and variable. AMF only increased individual flower size for *Datura stramonium* under high light environments, where carbon was not limiting (Aguilar-Chama and Guevara 2012) and the mutualism less costly. Similarly, the presence of AMF decreased the production of floral scent (Becklin et al. 2011), demonstrating a cost of the AMF mutualism to the plant at the expense of the pollinator mutualism. Despite the complexity of

these tri-trophic interactions, the loss of AMF through antimicrobial allelopathy could interrupt aboveground pollination mutualisms, and ultimately have downstream effects on seed dispersers.

5.5 POTENTIAL FOR POSITIVE INFLUENCES OF PLANT-PRODUCED CHEMICALS ON PLANT MUTUALISMS?

To this point, we have focused our discussion on the negative fitness impacts that allelopathy may have on plant mutualisms. However, there may be conditions where these plant-produced chemicals could have positive effects on mutualism function. Here we consider the flip side of this coin - the potential for these chemicals to facilitate mutualism effectiveness.

5.5.1.1 To what extent do antimicrobial chemicals eradicate soil pathogens, providing a net positive effect to the plant and its mutualistic partners?

Although we know of no data addressing this question for wild populations, allelopathic cover crops or mulch are used to suppress parasitic nematodes (Halbrendt 1996) and soil bacterial and fungal pathogens (see above; Gimsing and Kirkegaard 2009). These farming practices suggest that anti-microbial chemicals could have similar positive effects in wild plant populations, particularly if they target only pathogenic microbial strains and leave beneficial microbes unaffected.

5.5.1.2 Can the uptake of antimicrobial chemicals increase the attractiveness of floral and fruit rewards to pollinators and seed dispersers?

We envision that the uptake of antimicrobial chemicals could increase the attractiveness or palatability of floral or fruit rewards in two ways. First, chemicals could prevent microbial degradation of the rewards. For example, yeast that grow in floral nectar can actively degrade sucrose into fructose and glucose (Herrera et al. 2008, de Vega and Herrera 2012), making the nectar less tasty to the bees, who prefer nectar with high sucrose concentrations (e.g. Baker and Baker 1982; Cnaani et al. 2006). Yeast subsequently consume these simple sugars, which reduces total nectar sugar content (Herrera et al. 2008, de Vega and Herrera 2012), produces ethanol (Ehlers and Olesen 1997, Wiens et al. 2008), and further degrades nectar quality. Likewise, dispersers generally prefer ripe fruits over rotted, fermenting fruits (Levey 2004), and thus rapid microbial colonization of fruits can diminish their attractiveness (e.g. Buchholz and Levey 1990). While ethanol-rich nectar does not affect mammalian pollinators (Wiens et al. 2008), insect pollinators and mammalian seed dispersers can exhibit sluggish or drunken behavior after feeding at such nectaries (Kevan et al. 1988, Ehlers and Olesen 1997, Adler 2000) or on fermented fruits (reviewed by Levey 2004). Secondary compounds in toxic nectar and fruits suggest that some plants have evolved chemical defenses against microbial degradation of rewards (Adler 2000, Herrera 1982, Cipollini and Levey 1997, Levey et al. 2007). Capsaicin, the chemical responsible for the heat in chilies, has strong antifungal properties. In the field fruits with higher levels of capsaicin showed no signs of fungal infection after 45 days, while 12% of fruits with low capsaicin levels had fungal infections (Levey et al. 2007). In a similar fashion, uptake of antimicrobial chemicals and translocation into nectar, pollen or fruits could accomplish

essentially the same functions as toxic nectar or spicy chilies: the prevention of reward degradation, and increasing palatability to visitors and visitation rates.

The second way in which the uptake of antimicrobial chemicals could increase the function of floral or fruit rewards is through their antibiotic function. Self-medicating behavior is increasingly recognized in the animal kingdom (Clayton and Wolfe 1993) and has been well studied in insects ranging from monarch butterflies to honeybees (reviewed in Parker et al. 2011). Insects can rid themselves of parasites or microbial pathogens by ingesting food plants that contain specific chemical compounds (e.g. Singer et al. 2009). Likewise, alkaloid-containing nectar has been shown to significantly reduce pathogen infection in bumblebees suggesting the potential for self-medication of pollinators through floral rewards (Manson et al. 2010). The potential for uptake and translocation of antimicrobial chemicals could increase the attraction and function of floral and fruit rewards and in some cases help to maintain healthy and effective mutualist populations. This research area clearly warrants further attention.

5.6 SYNTHESIS AND FUTURE DIRECTIONS

Our framework of questions focused on the ecological consequences of allelopathic disruption of mycorrhizal, pollination, and seed dispersal mutualism. We found general support for the idea that allelopathy is a stressor that can disrupt each of these critical plant mutualisms (Table 6) and provide evidence of how the direct allelopathic effects on one mutualistic partner can scale up and impact the other partner (e.g. Stinson et al. 2006, Callaway et al. 2008, Hale et al. 2011, Lankau 2011b). However, the bulk of the evidence for direct effects comes from studies of a single species (*Alliaria petiolata*) on a single mutualism, (plant-mycorrhizal fungi), at a single

target stage (seedling, but see Hale et al. 2011; Hale and Kalisz, in prep). The many partial and suggestive answers to our questions indicate that numerous research opportunities exist in the context of allelopathy and plant mutualisms. Three areas ripe for future investigations are: 1) allelochemicals' indirect effects on the behavior and function of a plant's mutualist partners, 2) the cascading effects of allelopathy across multiple mutualisms, and 3) scaling up to population-level consequences of allelopathic mutualism disruption. Novel approaches and technologies are needed to expand into these areas, which we briefly discuss below.

5.6.1 Beyond the seedling stage

The vast majority of allelopathy studies use the seed or seedling stages of target species (e.g. Kong et al. 2002, Chiapusio et al. 2004, Helj and Koster 2004, Stinson et al. 2006, Callaway et al. 2008, Dayan et al. 2009, Hussain and Reigosa 2011, Lankau 2011b). Focus on this early stage makes many studies feasible, is practical for establishing general allelopathic effects, and lends insight into allelopathic effects on plant recruitment. However, to understand the extent to which allelopathy disrupts pollination and seed dispersal mutualisms or how disruption of any mutualism alters plant fitness and population dynamics will require an expanded focus that includes survival to flowering and adult plant reproduction.

5.6.2 Application of biochemical and analytical chemistry methods

Expanded use of analytical methods (Blair et al. 2009) and technologies to track the fate of allelochemicals within the plant and its partner, such as the solid-phase microextraction (Loi et al. 2008), will expand our ability to follow allelochemicals, assess their modes of action and

allow us to answer critical questions: Are allelochemicals readily taken up by neighboring plants? If so, are they broken down, or do they remain intact? To which plant organs are the chemicals translocated? Do these allelochemicals then affect pollinator or disperser behavior? Data on allelochemical uptake into flowers or fruits coupled with data on plant reproductive traits (i.e. quantity and quality of flowers, nectar and seeds or fruits), pollinator visitation, pollen removal, pollen and seed dispersal would provide unique insights into allelopathy's role in individual plant fitness and population-level processes.

5.6.3 Conduct manipulative experiments

While exploring the linkages between multiple trophic levels is inherently complex, manipulative experiments can be used to simplify the study system and target ecologically relevant questions. For example, recent studies used fungicides to suppress AMF and then examined the effect of reduced colonization on plant-pollinator interactions (Cahill et al. 2008, Becklin et al. 2011). Future studies could likewise apply antimicrobial allelochemicals at field-detected concentrations (i.e. Blair et al. 2009, e.g. Cantor et al. 2011) to the soil around target plants to examine the link between allelopathic reduction in mycorrhizal function and plant performance or pollinator/disperser preferences. Similarly, experimental addition of allelochemicals to nectar could be used to assess positive or negative effects on pollinator preferences.

5.6.4 Scale up from individual- to population-level processes

Quantification of target plant lifetime fitness enables us to understand and predict how mutualism disruption scales up from individual effects to their consequences for plant population performance. The use of demographic tools, such as matrix models, can improve our understanding of the population-level impacts for target plants and the sensitivity of population growth rate (de Kroon et al. 1986) to allelopathic mutualism disruption. Life table response experiment analyses (Caswell 2001) of data from manipulative experiments (e.g. Cahill et al. 2008, Becklin et al. 2011) would allow dissection of the stage specific effects of allelochemicals on the population growth rate of natural populations.

Understanding the factors that can modify species interaction strength is a key goal of ecology (Agrawal et al. 2007). The evidence presented here supports the idea that allelopathy is likely one important context that can alter the function of plant mutualisms by shifting the cost:benefit ratio of the interaction for one or both organisms involved. The ubiquity of mutualisms and the sweeping effects that their disruption can have on individual plant fitness are predicted to scale up and suppress plant population growth and diminish overall community diversity (Kiers et al. 2010). This ultimate scaling from the individual to community level highlights the need to understand allelopathy as one proximate driver of mutualism disruption. Future research in these areas will help to cement the broader ecological relevance of allelopathy as a field of study.

Table 6. Hypothesized effects and predictive framework for allelopathic impacts on plant mutualisms.

Mutualism	Potential effect of allelochemical	Predicted consequence for mutualistic partner[a]
A. Plant-mycorrhizae		
Phytotoxic	↓ plant vigor	↓ carbon available for mycorrhizae
Antimicrobial	↓ growth of active mycorrhizal fungi ↓ germination and density of mycorrhizal spores	↓ plant mineral nutrient content ↓ plant water balance ↓ plant physiological function ↓ plant growth ↓ plant protection from soil pathogens
B. Plant-pollinator and plant seed-disperser		
Phytotoxic	↓ plant vigor Volatile allelochemicals mask attractive scents	↓ inflorescence and infructescence size ↓ flower and fruit number ↓ pollen quantity and quality ↓ nectar quantity and quality ↓ attractiveness of floral and fruit scents ↓ visitation of plant by pollinators and seed dispersers ↓ outcrossing rates for plant ↓ pollen and seed dispersal distance ↓ population stability for plant and specialists
Antimicrobial	Uptake into nectar/pollen or fruits and seeds	↓ palatability of reward ↓ visitation of plant by pollinators and seed dispersers ↓ outcrossing rates for plant ↓ pollen and seed dispersal distance ↓ population stability for plant and specialists

[a]The primary predicted consequences for the mutualistic partner and secondary consequences (indented) that result from the primary consequences.

Table 7. Summary of the evidence for plant species producing allelochemicals that are toxic to mycorrhizae (arbuscular mycorrhizal fungi = AMF; ectomycorrhizal fungi = EMF).

Species	Family	Type of mycorrhizae studied	Type of study	Reference
Amaranthus viridis	Amaranthaceae	AMF	Field observation, Greenhouse - Type 1, Greenhouse - Type 2	Sanon et al. 2009, Sanon et al. 2012
Asparagus officinalis	Asparagaceae	AMF	Greenhouse - Type 1	Pedersen et al. 1991
Asparagus officinalis	Asparagaceae	AMF	Lab Bioassay, Greenhouse - Type 1	Wacker et al. 1990a
Asparagus officinalis	Asparagaceae	AMF	Field observation	Wacker et al. 1990b
Asparagus officinalis	Asparagaceae	AMF	Greenhouse - Type 1	Elmer and Pignatello 2011
Artemisia campestris ssp. *caudata*	Asteraceae	AMF	Greenhouse - Type 1	Yun et al. 2007
Artemisia princeps var. *orientalis*	Asteraceae	AMF	Greenhouse - Type 1	Yun and Choi 2002
Centaurea maculosa	Asteraceae	AMF	Field observation	Mummey et al. 2005
Centaurea maculosa	Asteraceae	AMF	Field observation	Mummey and Rillig 2006
Solidago candensis	Asteraceae	AMF	Greenhouse - Type 1, Greenhouse - Type 2, Greenhouse - Type 4	Zhang et al. 2007
Alliaria petiolata	Brassicaceae	AMF	Field observation	Barto et al. 2011
Alliaria petiolata	Brassicaceae	AMF	Lab bioassay, Field observation	Cantor et al. 2011

Alliaria petiolata	Brassicaceae	EMF	Field observation, Greenhouse - Type 3, Lab bioassay	Wolfe et al. 2008
Alliaria petiolata	Brassicaceae	AMF	Greenhouse - Type 3, Lab Bioassay	Callaway et al. 2008
Alliaria petiolata	Brassicaceae	AMF	Greenhouse - Type 1, Greenhouse - Type 2, Greenhouse - Type 3, Lab bioassay	Stinson et al. 2006
Alliaria petiolata	Brassicaceae	AMF	Field observation, Lab bioassay, Greenhouse - Type 1	Roberts and Anderson 2001
Brassica kaber	Brassicaceae	AMF	Lab bioassay	Schreiner and Koide 1993
Brassica nigra	Brassicaceae	AMF	Greenhouse - Type 2	Lankau and Strauss 2007
Brassica nigra	Brassicaceae	AMF	Field observation	Lankau et al. 2011c
Brassica oleraceae var. capita	Brassicaceae	AMF	Field experiment, Greenhouse - Type 4	Kluson 1995
Sisymbrium loeselii	Brassicaceae	AMF	Lab Bioassay, Greenhouse - Type 2	Bainard et al. 2009
Athyrium filix-femina	Dryopteridaceae	EMF	Lab Bioassay	Pellisier 1993
Calluna vulgaris	Ericaceae	EMF	Lab Bioassay	Robinson 1972
Empetrum hermaphroditum	Ericaceae	EMF	Lab Bioassay, Greenhouse - Type 1	Nilsson et al. 1993
Kalmia angustifolia	Ericaceae	EMF	Field observation	Yamasaki et al. 1998
Kalmia angustifolia	Ericaceae	EMF	Lab Bioassay	Mallik and Zhu 1995
Kalmia angustifolia	Ericaceae	EMF	Field observation	Hong and Mallik, unpub. data
Vaccinium myrtillus	Ericaceae	EMF	Lab Bioassay	Pellisier 1993
Laurel nobilis	Lauraceae	AMF	Greenhouse - Type 1	Hassiotis and Dina 2011
Abutilon theophrasti	Malvaceae	AMF	Greenhouse - Type 1	Koide and Li 1991

Picea abies	Pinaceae	EMF	Lab Bioassay	Pellisier 1993
Dicanthium annulatum	Poaceae	AMF	Greenhouse - Type 1	Javaid 2008
Imperata cylindrica	Poaceae	AMF	Greenhouse - Type 1	Afzal et al. 2000
Molinia caerulea	Poaceae	EMF	Greenhouse - Type 4	Timbal et al. 1990
Setaria lutescens	Poaceae	AMF	Greenhouse - Type 1	Koide and Li 1991
Polygonum × *bohemicum*	Polygonaceae	EMF	Field observation	Urgenson et al. 2012
Rubus idaeus	Rosaceae	EMF	Field observation, Lab bioassay	Coté and Thibault 1988
Populus trichocarpa	Salicaceae	AMF	Field observation, Greenhouse - Type 1	Piotrowski et al. 2008
Tamarix sp.	Tamaricaceae	AMF, EMF	Field observation, Greenhouse - Type 4	Meinhardt and Gehring 2012

Notes: Data were compiled through a literature search in ISI Web of Science using the search terms "allelo* mycorrhiza*". We also added references from our personal archives that have been collected over a period of several years. We used all studies that conducted at least one of the following tests and found evidence for allelopathy:

1. Lab bioassay: mycorrhizal spore germination/hyphal growth was measured on filter paper soaked with the proposed allelochemical or a water control

2. Greenhouse experiment - Type 1: mycorrhizal plants were treated with chemical extracts and water controls

3. Greenhouse experiment - Type 2: non-mycorrhizal plants were grown in soils from sites with/without the proposed allelopathic plant

4. Greenhouse experiment - Type 3: non-mycorrhizal plants were potted in field soils “conditioned” with/without the proposed allelopathic plant

5. Greenhouse experiment - Type 4: mycorrhizal plants were grown with/without the proposed allelopathic plant

6. Field observation: comparing mycorrhizal abundance in sites with/without the proposed allelopathic plant

6.0 CONCLUSIONS

This dissertation provides an empirical test of the mutualism disruption hypothesis for invasion. I demonstrate that an allelopathic invasive species can effectively disrupt the mycorrhizal mutualism of a native plant. Furthermore, I show that mycorrhizal disruption by allelopathy can drive physiological declines and reductions in allocation to competitive traits in a native plant. In conclusion, I will discuss the novelty and implications of some key findings, synthesize my results, and address areas of future research.

An immediate challenge I faced in my dissertation was determining whether garlic mustard allelochemicals can kill AMF under natural conditions and cause mutualism disruption in the field. Detecting putative allelochemicals in field soils is a major challenge in allelopathy research (Inderjit and Duke 2003), yet this is a critical step in validating the ecological relevance of any laboratory bioassay or greenhouse study (Blair et al. 2009). For garlic mustard, previous work had identified allyl isothiocyanate (AITC), the breakdown product of sinigrin, as a likely AMF-toxic compound (Vaughn and Berhow 1999). However, both of these compounds eluded detection in field soils (Barto and Cipollini 2009b). In Chapter 2, I present results that show the first successful detection of sinigrin and AITC in field soils. Using these data, I was able to calculate field concentrations of these allelochemicals and with the help of my collaborators, test these concentrations against AMF spore germination in the lab. We found that field detected concentrations of AITC reduced spore germination by 57% relative to a control, and

furthermore, that the growth of fungal hyphae was generally reduced on sites invaded by garlic mustard (Cantor et al. 2011). In addition to measuring allelochemical concentrations in the field, I also collected water and soil samples from garlic mustard treated pots to determine the effectiveness of my greenhouse allelochemical treatments (Appendix G). My work answers the call for increased incorporation of analytical chemistry techniques in allelopathy research (Blair et al. 2009) and, by doing so, highlights the ecological importance of allelopathy in dictating the outcome of species interactions.

In Chapters 3 and 4, I assess the allelopathic impacts of garlic mustard on the function of two mutualistic partners – the common perennial forest herb, false Solomon's seal, and its AMF. Because perennial plants grow and reproduce slowly, it can be difficult to rapidly assess impacts on their population growth. However, physiological tools can reveal subtle alterations in organism function that over time could scale up to impact fitness (Cooke and Suski 2008). The application of physiological tools to conservation issues is gaining in popularity and has emerged as its own field, now known as conservation physiology (Wikelski and Cooke 2006). I found that garlic mustard treatment reduced soil respiration rates (my proxy for AMF physiological function), as well as a suite of leaf gas exchange traits in native false Solomon's seal plants (Hale et al. 2011). Furthermore, I demonstrate that the garlic mustard-mediated physiological declines in false Solomon's seal are persistent and translate into declines in allocation to key fitness traits, such as root growth, asexual reproduction, and carbohydrate storage. While multiple studies have assessed plant physiological responses to climate change (see meta-analyses by Curtis 1996, Ainsworth et al. 2002), to my knowledge, the studies presented in Chapters 3 and 4 are the first to explicitly test the physiological response of a native plant species and its AMF to invasion. In general, studies of long-lived taxa could benefit from the use of physiological

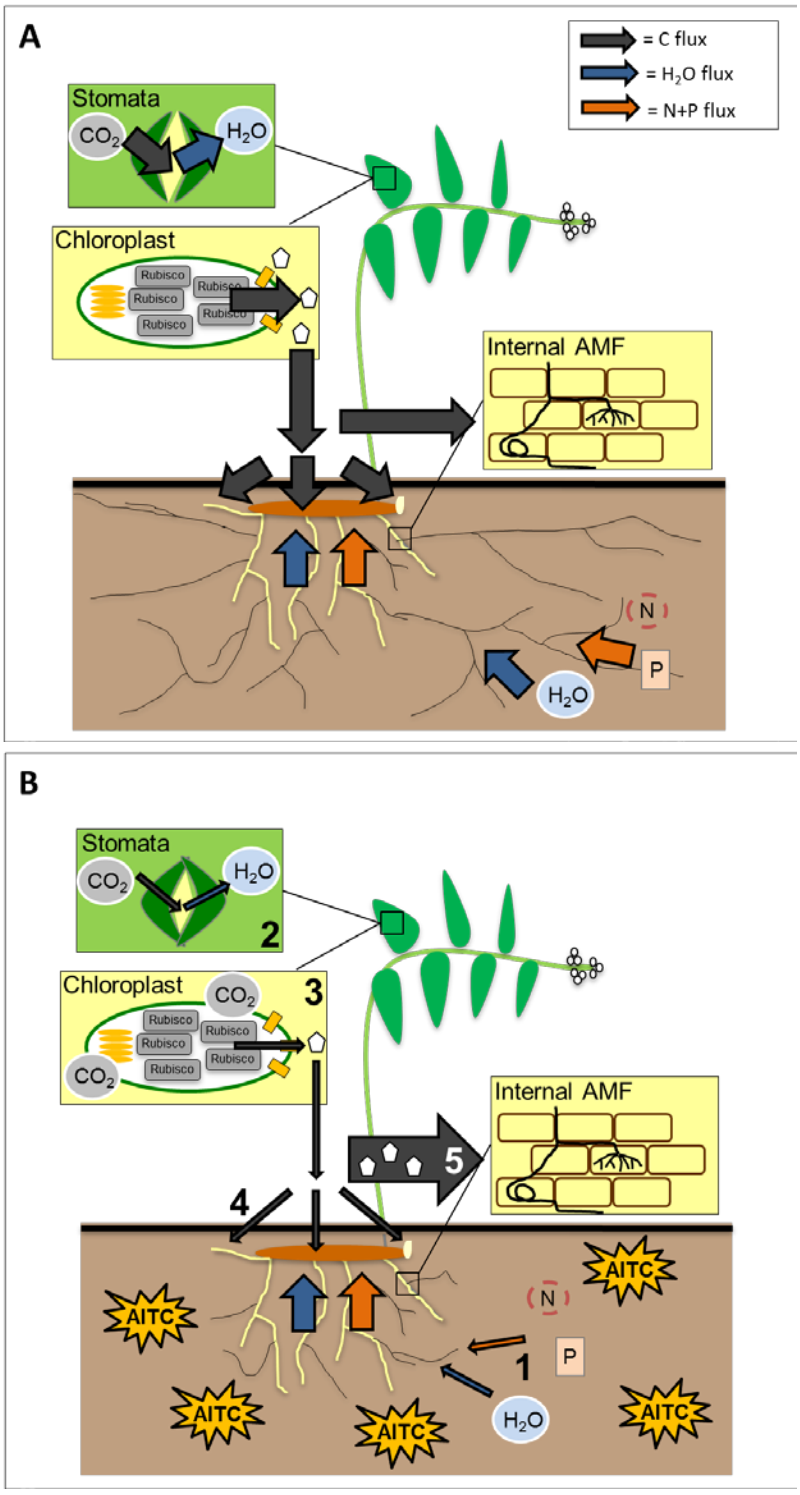
metrics to reveal impacts of anthropogenic environmental change that may otherwise go undetected. Additionally, future research that couples measures of individual physiology with long-term population demographics could truly link physiological responses with changes in population growth (see Ricklefs and Wikelski 2002).

Using the data presented in Chapters 2, 3, and 4, I have developed an integrative model that outlines the changes in carbon, water, and nutrient fluxes that occur during allelopathic mutualism disruption (Figure 17). In brief, allelochemicals from the invader garlic mustard kill AMF hyphae in soil (Ch. 2, 3, 4). In the short term, this AMF disruption results in water stress for the native plant (Ch. 3). Native plant stomata close to limit water loss, but this also diminishes intercellular CO_2 available for photosynthesis (Ch. 3). In the long term, the AMF disruption results in altered sink dynamics within the native plant (Ch. 4). The altered sink activity causes inefficient use of CO_2 by the mesophyll and photosynthetic rates remain low (Ch. 4). Rather than putting the limited amounts of sucrose into storage, growth, and reproduction, sucrose remains in a mobile form (Ch. 4). I predict that sucrose remains mobilized to support the internal AMF and re-establish the vast hyphal network (Future work). Overall, this model represents a complete synthesis of my dissertation and will be useful in guiding my future research on mutualism disruption.

While this dissertation focuses on the impact of allelopathic invasion on the plant-AMF mutualism, the effects of allelopathy on pollination and seed dispersal mutualisms remain largely unknown. In Chapter 5, I present a predictive framework to guide future research in this area (Hale and Kalisz 2012). I propose that allelopathy could diminish a plant's ability to produce flower, pollen, and nectar rewards for pollinators and/or fruit for seed dispersers. Volatile allelochemicals could mask attractive floral scents and further disrupt pollination mutualisms.

Additionally, because AMF enhance flower size (Gange and Smith 2005, Varga and Kytöviita 2010, Aguilar-Chama and Guevara 2012), flower number, nectar sugar content, and nectar secretion (Gange and Smith 2005), allelopathic disruption of mycorrhizal mutualisms could have cascading effects on other mutualisms. These ideas are intriguing, but unexplored, and would be interesting avenues for future research.

Understanding the influence of biotic and abiotic factors on species interactions is critical in understanding how these interactions shape species' abundance and distributions (Agrawal et al. 2007). In this dissertation, I have shown that the presence of an allelopathic invasive species can influence the effectiveness of plant-AMF mutualisms. The mutualism disruption that I observed was followed by physiological declines in the plant partner and reduced carbon acquisition and allocation to fitness traits. Ultimately, mutualism disruption may, in part, explain the disappearance of native species from invaded sites. In the future, I hope to apply the toolset developed in this dissertation to other urgent conservation concerns and continue to explore the dynamics underpinning species' declines.



In contrast to optimal mutualism functioning (A), during mutualism disruption (B):

1. Garlic mustard allelochemicals kill AMF hyphae (Ch. 2, 3, 4).
2. Resulting water stress causes stomatal closure, decreasing intercellular CO_2 available for photosynthesis (Ch. 3).
3. Over time, altered sink activity also reduces mesophyll efficiency in using CO_2 (Ch. 4).
4. Allocation to storage, growth, and reproduction is reduced (Ch. 4).
5. *Hypothesis*: Sucrose (⬠) remains mobilized to support the internal AMF and re-establish the vast hyphal network (Future work).

Figure 17. An integrative model outlining the impacts of allelopathic mutualism disruption in the garlic mustard-false Solomon's seal-AMF model system. A) Carbon, water, and mineral nutrient fluxes when the mutualism is well-established and functioning optimally. B) Altered fluxes after allelopathic mutualism disruption.

APPENDIX A

TEST OF SINIGRIN EXTRACTION EFFICIENCY

One pair of soil samples from the 20 June 2007 collection date was spiked with 50 mg of a commercial sinigrin standard to determine the efficiency of our sinigrin extraction method. After measuring the area below each peak (Figure 18) and relating it to a standard sinigrin curve, we found that we successfully recovered 65% (32.6 mg) and 70% (35.1 mg) of the sinigrin from the garlic mustard spiked soils and the control soils, respectively. This test demonstrates that our extraction methods are adequate for detecting sinigrin in garlic mustard-invaded soils.

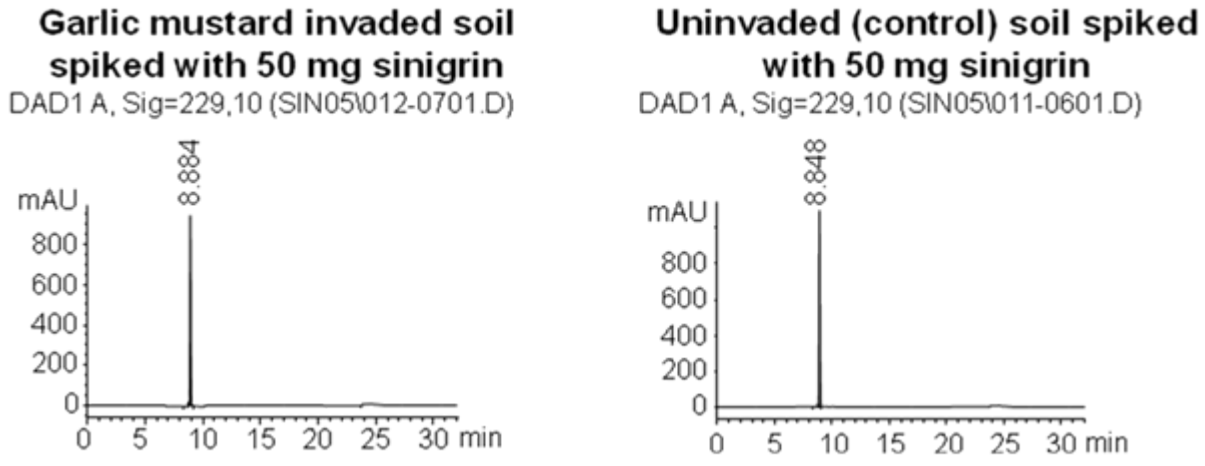


Figure 18. The resulting chromatograms from sinigrin spiked soil samples.

APPENDIX B

CHROMATOGRAMS FROM INVADED SAMPLES WHERE SINIGRIN WAS DETECTED AND PAIRED CONTROL SAMPLES

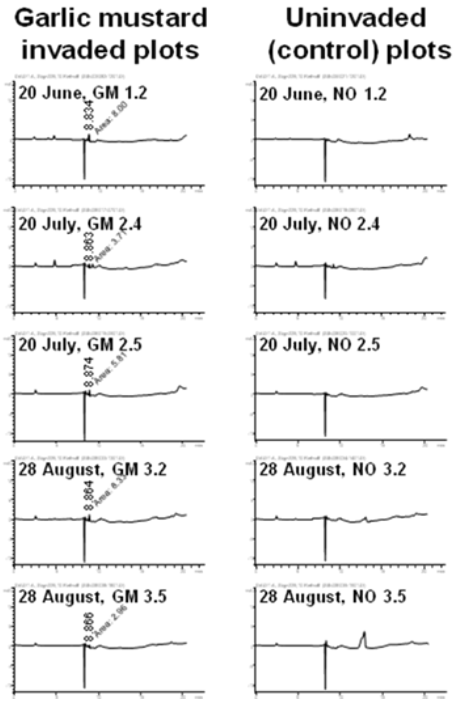


Figure 19. Chromatograms from the 5 pairs of soil samples in which sinigrin was detected in soils from the garlic mustard invaded site. Note that peaks meet both of our criteria for identification as sinigrin: peaks fall between 8.83 and 8.87 minutes and have an area greater than 2.33 mAU. None of the paired control samples contained sinigrin. 80% (4 out of 5) of our detections occurred during the period of garlic mustard senescence, suggesting that the decomposition of adult garlic mustard tissue releases allelochemicals into the soil.

APPENDIX C

ATTEMPTS TO DETECT AITC VIA UV-SPECTROSCOPY AND HPLC

The short half-life and volatility of AITC (Borek et al. 1995) prompted us to try two additional detection methods. First, we attempted UV spectroscopy of non-volatile cyclocondensation precipitate products formed between AITC and toluene dithiol (Zhang et al 1992). This option worked exceptionally well for standards from which we created a calibration curve for determining AITC concentration of unknown soil samples. However, when we tried this method for soil samples, impurities from the soil, including heavy metals, catalyzed the cyclocondensation reaction and produced precipitates that correlated with very high molar concentrations of AITC. We knew that these were false positive results and tried several chelating solutions, including ethylenediaminetetraacetic acid, to remove the heavy metals. Despite our efforts, the recommended purifications and the chelating solutions failed to prevent the intense precipitates observed during the cyclocondensation reaction between soil samples and toluene dithiol.

As a second option, we mixed garlic mustard soil samples in the field with a solution of N-acetylcysteine. As described by Zhang et al. (1996), this conjugation step creates a non-volatile compound comprised of an N-acetylcysteine-AITC complex. This non-volatile complex

still reacts with toluene dithiol, and it also prevents the loss of AITC in the soil sample between the steps of soil collection and reaction with toluene dithiol. Our final cyclocondensation product that we created from standards did have a unique signal on the HPLC chromatogram like that found by Zhang et al. (1996) but, again, we were not able to reproduce this result for any of our field soil samples due to soil impurities that buried relevant signals.

APPENDIX D

RAW DATA FROM COMMON GARDEN EXPERIMENT MEASURING SOIL RESPIRATION RATES

Table 8. Total belowground respiration (TBR) data (μmol CO2 mol-1 air over 10 minutes; least square means) for three treatments in common garden experiment at the Pymatuning Laboratory of Ecology.

Treatment	TBR	SE	Sample Size
No tissue	10354.57	870.20	12
Dame's rocket	13553.77	869.25	11
Garlic mustard	11162.48	914.78	11

Table 9. Mean belowground respiration (μmol CO2 mol-1 air) at each 2-minute sampling interval for three treatments in common garden experiment at the Pymatuning Laboratory of Ecology.

	Mean belowground respiration at each 2-minute sampling interval											
Treatment	0 min	SE	2 min	SE	4 min	SE	6 min	SE	8 min	SE	10 min	SE
No tissue	1745.28	120.75	1440.50	93.58	1100.71	59.80	815.28	41.18	609.10	29.07	467.21	21.90
Dame's rocket	2250.68	161.51	1921.82	162.59	1497.50	149.53	1100.00	100.41	822.04	71.21	627.25	51.87
Garlic mustard	1976.18	124.01	1641.23	97.54	1243.78	72.87	903.57	52.71	678.46	45.54	519.12	37.65

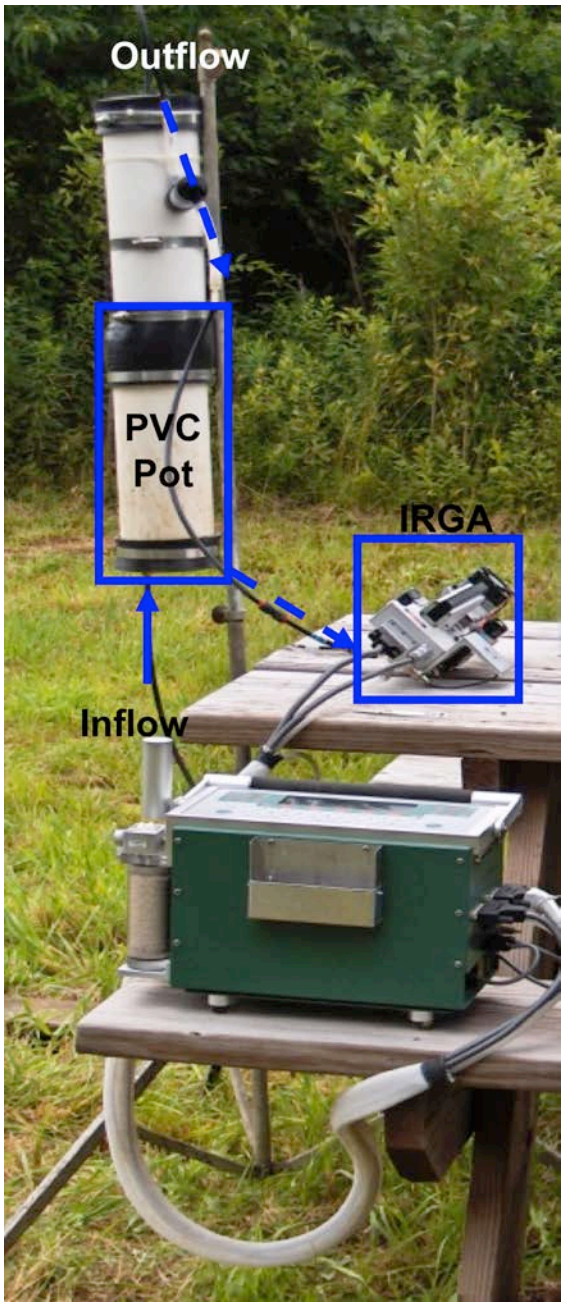


Figure 20. The sealed air-flow path through the PVC pot and into the IRGA of the LI-COR 6400. CO2-free air was passed through the bottom of the PVC pot (inflow, solid arrow), forcing the CO2 in the soil matrix to flow out of the top of the pot into the IRGA (outflow, dashed arrows).

APPENDIX E

MYCORRHIZAL STATUS OF FOREST HERBS AT TRILLIUM TRAIL NATURE RESERVE, FOX CHAPEL, PA

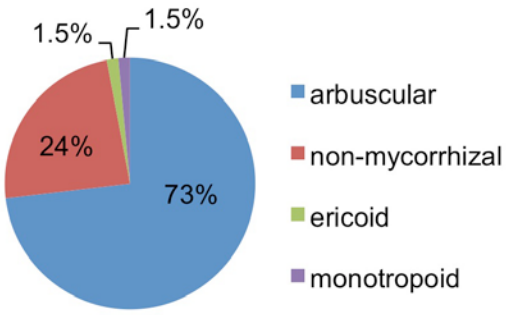


Figure 21. Pie chart shows the percentage of forest understory herbs at Trillium Trail Reserve that associate with each type of mycorrhizae.

Table 10. Mycorrhizal status of 79 herbaceous species at Trillium Trail in Fox Chapel Borough, PA, USA.

Scientific Name	Family	AMF status	Native status	Source
Actea pachipoda	Ranunculaceae	AM	Native	1
Alliaria petiolata	Brassicaeae	NM	Invasive	4
Allium tricoccum	Liliaceae	AM	Native	1
Anemone quinquefolia	Ranunculaceae	Unknown	Native	
Aquilegia canadensis	Ranunculaceae	AM	Native	1
Aralia nudicaulis	Araliaceae	AM	Native	1
Arasum canadense	Aristolochiaceae	AM	Native	1
Arisaema triphyllum	Araceae	AM	Native	1
Aster divaricatus	Asteraceae	AM, NM	Native	3
Aster pilosus	Asteraceae	AM, NM	Native	3
Campanula americana	Campanulaceae	AM	Native	3
Cardamine concatenata	Brassicaeae	NM	Native	1
Cardamine diphylla	Brassicaceae	NM	Native	3
Cimicifuga racemosa	Ranunculaceae	AM, NM	Native	3
Circaea quadrisulcata	Onagraceae	AM	Native	1
Claytonia virginica	Portulaceae	NM	Native	1
Clintonia umbellata	Liliaceae	Unknown	Native	
Corylis sempervirens	Papaveraceae	Unknown	Native	
Dicentra canadensis	Papaveraceae	NM	Native	1
Dicentra cucullaria	Papaveraceae	NM	Native	1
Epifagus virginiana	Orobanchaceae	NM	Native	1
Erigeron annuus	Asteraceae	AM	Native	4
Erythronium americanum	Liliaceae	AM	Native	1
Eupatorium purpureum	Asteraceae	AM, NM	Native	3
Eupatorium rugosum	Asteraceae	AM, NM	Native	3
Floerkea proserpinacoides	Limnanthaceaea	NM	Native	2
Galium odoratum	Rubiaceae	AM, NM	Non-native	4
Galium spp.	Rubiaceae	AM	Native	3
Gaultheria procumbens	Ericaeae	Ericoid	Native	4
Geranium maculatum	Geranaceae	AM	Native	3
Glechoma hederacea	Lamiaceae	AM, NM	Invasive	4
Helianthus divaricatus	Asteraceae	Unknown	Native	
Hepatica nobilis	Ranunculaceae	AM	Native	3
Houstonia caerulea	Rubiaceae	Unknown	Native	
Hydrophyllum virginianum	Hydrophyllaceae	NM	Native	1
Impatiens capensis	Balsaminaceae	AM	Native	1
Impatiens pallida	Balsaminaceae	AM, NM	Native	3
Laportea canadensis	Urticaceae	AM	Native	1
Maianthemum canadensis	Liliaceae	AM	Native	1
Maianthemum racemosa	Liliaceae	AM	Native	1
Medeola virginiana	Liliaceae	AM	Native	3
Mertensia virginica	Boraginaceae	NM	Native	3
Microstegium vimenium	Poaceae	AM, NM	Invasive	3

Mitella diphylla	Saxifragaceae	NM	Native	3
Monotropa uniflora	Monotropaceae	Monotropoid	Native	4
Osmorhiza claytonii	Apiaceae	NM	Native	3
Osmorhiza longistylus	Apiaceae	AM	Native	3
Panax trifolius	Araliaceae	Unknown	Native	
Phlox divaricata	Polemoniaceae	AM	Native	1
Phlox stolonifera	Polemoniaceae	Unknown	Native	
Phytolacca americana	Phytolaccaeae	NM	Native	3
Pilea pumila	Urticaceae	AM	Native	1
Podophyllum peltatum	Berberidaceae	AM	Native	1
Polygala paucifolia	Polygalaceae	Unknown	Native	
Polygonatum biflorum	Liliaceae	AM	Native	1
Polygonum cuspidatum	Polygonaceae	AM, NM	Invasive	4
Polygonum persicarium	Polygonaceae	AM, NM	Invasive	4
Ranunculus arbortivus	Ranunculaceae	AM	Native	1
Ranunculus ficaria	Ranunculaceae	AM, NM	Invasive	4
Sanguinaria canadensis	Papaveraceae	AM	Native	1
Sanicula marilandica	Apiaceae	Unknown	Native	
Saxifraga virginiensis	Saxifragaceae	Unknown	Native	
Sedum ternatum	Crassulaceae	NM	Native	3
Silene virginica	Caryophyllaceae	NM	Native	3
Silene vulgaris	Caryophyllaceae	NM	Invasive	4
Stellaria media	Caryophyllaceae	AM, NM	Invasive	4
Symplocarpus foetidus	Araceae	Unknown	Native	
Thalictrum dioicum	Ranunculaceae	AM	Native	1
Thalictrum thalictroides	Ranunculaceae	AM	Native	2
Tiarella cordifolia	Saxifragaceae	AM	Native	3
Trillium erectum	Liliaceae	AM	Native	1
Trillium grandiflorum	Liliaceae	AM	Native	1
Trillium sessile	Liliaceae	AM	Native	2
Tussilago farfara	Asteraceae	AM, NM	Invasive	4
Urtica dioica	Urticaceae	AM, NM	Native	4
Uvularia perfoliata	Liliaceae	AM, NM	Native	3
Viola blanda	Violaceae	Unknown	Native	
Viola canadensis	Violaceae	AM	Native	1
Viola eriocarpa	Violaceae	AM	Native	1

Notes: Mycorrhizal status was determined through a literature search. Blank cells in the Source column indicate that no articles were found describing the mycorrhizal status of that particular species. Sources are: 1, Brundrett and Kendrick (1988); 2, Demars (1996); 3, J.M. Trappe, *unpublished data*; 4; Wang and Qiu (2006).

APPENDIX F

LIGHT SATURATION CURVES FOR FALSE SOLOMON'S SEAL

To determine the appropriate quantum flux density for leaf gas exchange measurements on false Solomon's seal, we calculated light saturation curves in the field on 15-June-2008 at Trillium Trail and 24-June-2009 and 14-July-2009 at PLE between 1000 and 1200 hours using an IRGA. Measurements were made at ambient temperature and humidity, while CO_2 levels were held constant at 400 µmol CO_2 mol^{-1} air using an injector system. We measured photosynthetic rate, A_n at 1500, 1000, 700, 600, 500, 300, 100, 50, 25, 10, and 0 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at PLE, and 10 through 700 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at Trillium Trail. On each plant, the second distal leaf was placed in the leaf cuvette and allowed to acclimate for 5 minutes before A_n was recorded. This was repeated for each irradiance level. We combined our data with published data for false Solomon's seal (Hull 2002). A non-rectangular hyperbola, which accurately models the shape of light saturation curves (Ögren 1993), was fit to all data points (PROC NLIN, SAS v. 9.2): 600 $\mu mol \cdot m^{-2} \cdot s^{-1}$ is a saturating irradiance level for false Solomon's seal and was used for all subsequent physiological measurements (Figure 22).

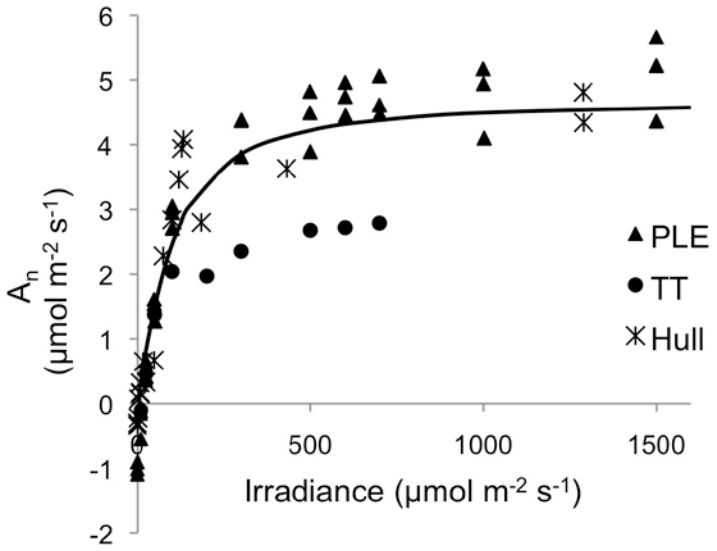


Figure 22. Light saturation curves for false Solomon's seal. Low observed maximum photosynthetic rates (An) and light saturation at low irradiance levels for false Solomon's seal are typical of forest understory herbs. Data from Trillium Trail (TT; 1 plant), Pymatuning Laboratory of Ecology (PLE; 3 plants), and from Hull (2002; 2 plants; used with permission).

APPENDIX G

TEST OF ALLELOCHEMICAL DELIVERY TO SOIL BY GARLIC MUSTARD "TOXIC" TEA BAGS AND POTENTIAL FOR WATER TO LEACH ALLELOCHEMICALS FROM SOIL

To investigate 1) the extent to which our garlic mustard toxic "tea bag" treatment effectively delivered allelochemicals into the soil and 2) the potential for water to leach allelochemicals out of the soil, I conducted a growth chamber study during December 2011. I prepared pots (15 cm diameter, Magnum) containing a 3:1 (w/w) mixture of autoclaved Fafard and Turface. To prevent garlic mustard leaves from becoming mixed into the soil matrix, I covered the surface of each pot with a 15 x 15 cm square of window screen. I collected garlic mustard rosettes from Trillium Trail Nature Reserve on 2 December, and immediately upon returning to the lab, I placed 25 g of leaves on top of each pot (N = 40). I left three pots bare to serve as controls. Pots were then placed in individual trays (Figure 23) and randomized among three Percival chambers in which temperature and light conditions were set to mimic an average day during the summer growing season (daytime temperature: 27°C, nighttime temperature: 20°C with a 13-hour daylight cycle).

After applying the garlic mustard leaves, I immediately watered all pots to saturation. From eight of the garlic mustard pots and one control pot, I collected 15 mL of water that leached into the trays. The water leachate samples were microwaved for 3 minutes to denature myrosinase and prevent the enzymatic conversion of sinigrin to allyl isothiocyanate, a compound which is not detectable from soil samples via HPLC (Cantor et al. 2011, Appendix C) and is considerably more difficult to capture due to its volatility (Borek et al. 1995). Water leachate samples were stored in the refrigerator until filtration and HPLC analysis (see below). Additionally, to assess sinigrin concentration in the soil, I removed the garlic mustard leaf litter from the surface of each pot by lifting out the window screen and then collected ≤ 400 g of soil from the top 5 cm of each pot. The soil samples were air dried and stored at room temperature. Every three days, I sampled an additional eight garlic mustard treated pots and one control pot. At each of these sampling time points, I only watered four of the pots prior to collecting the water and soil samples (water samples were only collected from the pots that were watered). My goal was to create "well-watered" vs. "drought" soil treatments to determine if constant watering diluted the concentration of allelochemicals in the soil.

To date, I have analyzed only the water leachate samples. Following the methods described in Cantor et al. (2011), I captured and washed the glucosinolates from the samples in open columns containing 0.1 g DEAE Sephadex A-25 (Amersham Biosciences, Uppsala, Sweden). I de-sulfated the glucosinolates by adding 1 mL of a 2 mg/mL solution of sulfatase (Sigma-Aldrich, St. Louis, MO, USA; Agerbirk et al. 2001) to the open columns. I added 5 mL of water to elute the desulfoglucosinolates and analyzed the resulting samples on HPLC using the same model, detector, and solvent program described in Cantor et al. (2011). To verify the identity of my glucosinolates and determine their concentrations, I created pure sinigrin

standards (Sigma-Aldrich, St. Louis, MO, USA). My standards had retention times between 8.48 and 8.54 minutes. I qualitatively analyzed the chromatograms from my water leachate samples and determined that only peaks between 8.48 and 8.54 minutes were indicative of sinigrin (Figure 24). For any sample with a peak falling within this range, I measured the integrated peak area and used my standard curve (Figure 25) to determine sinigrin concentration.

All water leachate samples collected immediately after the application of garlic mustard leaves to the pot (2 December) contained sinigrin (Table 11). The concentrations of sinigrin in these samples ranged from 0.09 to 0.22 µg (Table 11), which are at the lower end of the field concentrations of sinigrin detected by Cantor et al. (2011) (0.22 – 0.62 µg). Additionally, I also detected sinigrin in one out of the four water leachate samples collected on the second watering date (5 December; Table 11) at a concentration of 0.1 µg. Sinigrin was never detected in water leachate samples from the control pots (Figure 24).

These data indicate that sinigrin is highly water soluble and can be washed out of soil as a result of its polar nature. This result likely explains why native potted plants that are watered every 2-3 days do not respond to a garlic mustard treatment, while those that are watered less frequently respond strongly (Chapter 4). While plants that are watered infrequently may be more susceptible to garlic mustard's allelopathic effects because they are water stressed (Tang et al. 1995), the data from this current experiment also suggest that these plants may be exposed to higher, continuous concentrations of the allelochemical. Plants that are watered every 2-3 days likely only experience a brief pulse of the allelochemicals before they are washed out of the pot. Future analyses of the soil samples from this experiment will further clarify the role of water in diluting allelochemical concentration.

These findings have broad implications for the ecology of garlic mustard's allelopathic interactions. The effects of garlic mustard allelopathy on native plants are known to vary across sites and studies, with some authors finding little to no effect of garlic mustard on native plants and their AMF (e.g. Burke 2008; Barto et al. 2010b). This variation can in part be explained by the variation in glucosinolate production among garlic mustard populations – individuals in younger, recently invaded populations tend to produce greater concentrations of glucosinolates than individuals in older populations (Lankau et al. 2009). However, my data also suggest that local precipitation patterns at a site could influence the strength of allelopathic interactions. Indeed, the inhibitory effects of allelochemicals have been shown to be correlated with precipitation in at least one study (Richardson and Williamson 1988). Thus, precipitation may underlie variation in allelopathic interactions across a variety of habitats and could influence the survival of natives on sites invaded by allelopathic species.

Table 11. Water leachate samples in which sinigrin was detected.

Pot #	Harvest Date	Peak Area	Sinigin Concentration (µg)
38	Dec. 2, 2011	2.76	0.22
31	Dec. 2, 2011	1.29	0.11
15	Dec. 2, 2011	1.29	0.11
35	Dec. 2, 2011	1.65	0.14
24	Dec. 2, 2011	2.50	0.20
9	Dec. 2, 2011	1.04	0.10
14	Dec. 2, 2011	0.97	0.09
8	Dec. 2, 2011	1.67	0.14
13	Dec. 5, 2011	1.05	0.10

Figure 23. Photo showing a garlic mustard treated pot with tray from which water leachate samples were collected. Photo was taken seven days after the experiment began, so garlic mustard leaves are beginning to decompose.

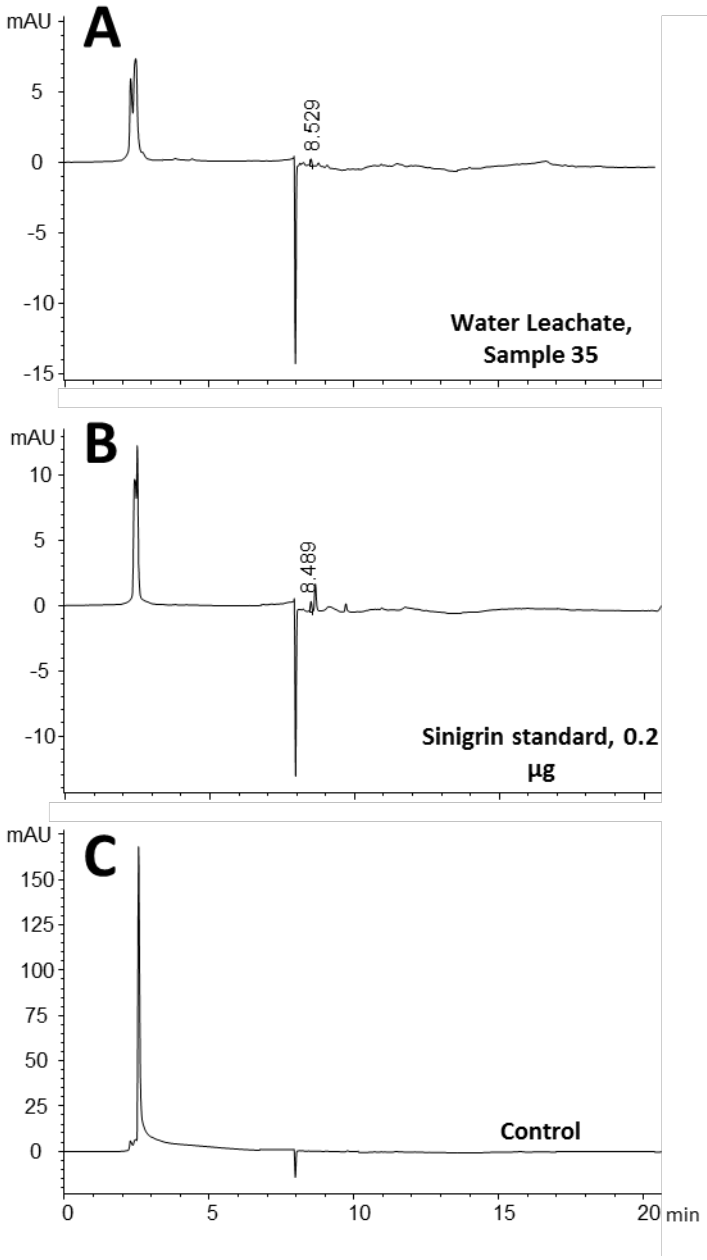


Figure 24. HPLC chromatograms from a A) a water leachate sample, B) the 0.2 µg sinigrin standard, and C) a control sample. The water leachate sample shows the detection of sinigrin with a peak at 8.52, as does the sinigrin standard with a peak at 8.48. Sinigrin was not detected in the control sample – note the absence of a peak between 8.48-8.54 minutes.

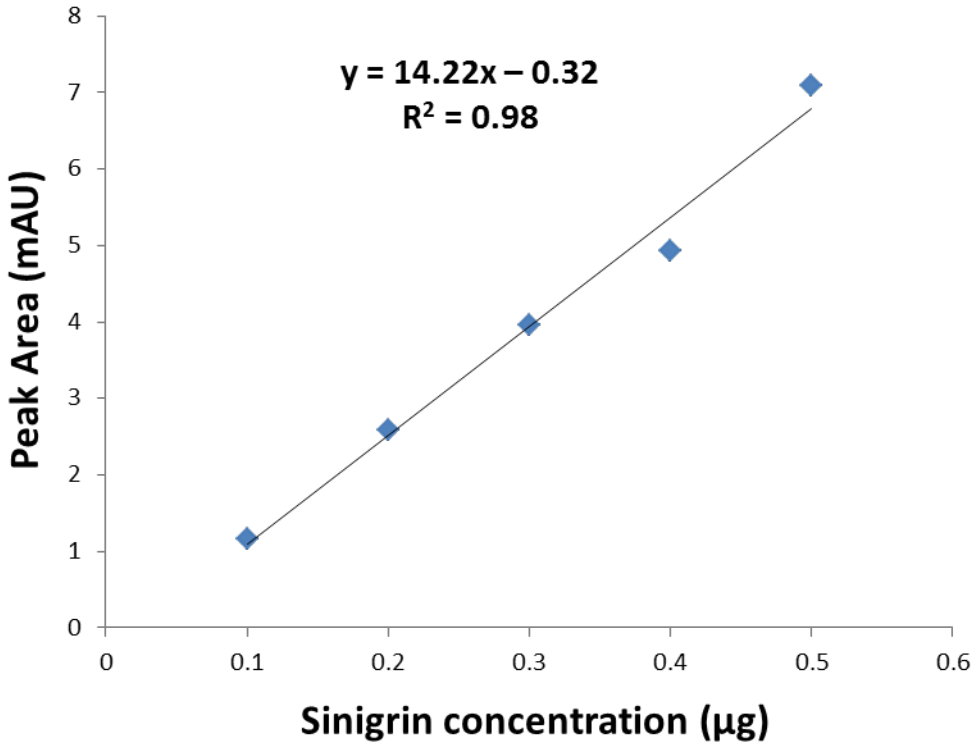


Figure 25. Sinigrin standard curve created from five pure sinigrin standards. The detector signal is given in absorbance units (mAU).

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