ALLELOPATHIC INTERACTIONS AND EFFECTS OF *MICROSTEGIUM VIMINEUM* (JAPANESE STILT-GRASS) ON *ALLIARIA PETIOLATA* (GARLIC MUSTARD)

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ABSTRACT

The factors affecting the ability of some plant species to become invasive in non-native communities are the subject of active and important ecological study. One intriguing but disputed explanation for the success of invasive plants is the novel weapons hypothesis, which states that the allelopathic chemicals released by an invasive species more effectively inhibit plants outside of that species' native range than do those of its native competitors. The novel weapons hypothesis may play a role in the invasion of metropolitan forests of the northeastern US by the invasive herbs *Microstegium vimineum* (Japanese stilt-grass) and *Alliaria petiolata* (garlic mustard), given that evidence for allelopathy has been found for both species. However, the interactions between these species, as well as the allelopathic mechanisms of *M. vimineum*, have received only minimal study. Here I present my findings from a series of three experiments on the allelopathy of *M. vimineum* and its interactions with *A. petiolata*. First, I investigated the ideal solvent for extraction of *M. vimineum* allelochemicals. I found that extraction efficiency is correlated with solvent polarity, making water an effective solvent. Second, I performed a greenhouse study on the growth effects of exposure of both *A. petiolata* and *M. vimineum* to *M. vimineum* extract. I found no statistically significant effects, but subtle trends implied the possibility that *M. vimineum* may inhibit *A. petiolata* growth and/or facilitate its own growth. Third, I evaluated the overall effects of *M. vimineum* on *A. petiolata* establishment and growth through analysis of experimental field data. The results were ambiguous and often inconsistent, suggesting a complex relationship between the two species and other factors as yet unidentified. Overall, my findings contribute to the understanding of *M. vimineum*'s allelopathy, particularly in relation to *A. petiolata*.

INTRODUCTION: BACKGROUND

Problems of invasive species: Biotic invasions occur when species are transported to, and successfully proliferate within, environments other than their native range. Such species can displace native species and cause large-scale changes in ecological processes, with devastating results (Boppré 1991, Ehrenfeld et al. 2001, Levine et al. 2003, Mack et al. 2000, Pimentel et al. 2000, Turner et al. 1998, Vitousek et al. 1997, Whisenant 1990). Invasive species have become one of the greatest environmental problems of the natural world, on par with global warming; they cause over $138 billion of economic damage annually to the United States alone, even before considering their ecological impact (Crawley et al. 1996, D'Antonio and Vitousek 1992, Linz et al. 2007, Mack et al. 2000, Perrings 1996, Pimentel et al. 2000, Vitousek et al. 1997). Some invasive species may even directly threaten human health (Lakshmi and Srinivas 2007, Patel 2011, Pimentel et al. 2000, Vitousek et al. 1997)

Explaining invasions: Yet, not all species introduced outside of their native range become invasive; the vast majority of organisms transported to distant environments quickly die out, or establish only at innocuous levels. Only a small fraction of introduced species become problematic invaders (Crawley et al. 1996, Mack et al. 2000, Rejmánek 2000, Williamson and Fitter 1996b). A major focus of modern ecology is therefore identifying the factors that predict what species are likely to become invasive and/or influence the magnitude of invasive success, in order to enhance our ability to understand, prevent, and mitigate invasions. While there appears to be a random element to invasive success (Hamilton et al. 2005, Reichard and Hamilton 1997, Rejmánek 2000), there is abundant evidence that species characteristics also play a role, and can be predictive (Crawley et al. 1996, van Kleunen et al. 2010, Reichard and Hamilton 1997, Rejmánek 2000, Pyšek and Richardson 2007, Williamson and Fitter 1996a). Many traits, such as fast seedling growth (Grotkopp et al. 2002, Grotkopp and Rejmánek 2007), vegetative reproduction (Reichard and Hamilton 1997, Thompson et al. 1995), and specific leaf area (Grotkopp et al. 2002, Grotkopp and Rejmánek 2007, Lake and Leishman 2004), may contribute to invasiveness through increasing plant competitive ability (ability to outcompete competing species), which is in turn likely to predict invasive success (Keane and Crawley 2002, Mitchell et al. 2006, Pyšek and Richardson 2007, Thébaud et al. 1996). Other, less obvious traits, such as size (Crawley et al. 1996, Pyšek et al. 1995, Williamson and Fitter 1996a), insect pollination (Williamson and Fitter 1996a), and life cycle (Bradshaw et al. 2008, Sutherland 2004, Thompson et al. 1995), have been linked to invasiveness, sometimes inconsistently; some such traits may have environmentally-dependent relationships to invasiveness.

Predictions of invasiveness based on competitive ability suggest the existence of competitive hierarchies among plants, an unproven notion that nonetheless has some support (Bradshaw et al. 2008, Goldberg 1996, Keddy and Shipley 1989). It may be that a more competitive species tends to exclude a less competitive species where they co-occur (Armstrong and McGehee 1980, Crombie 1947). Considered further, this seems to suggest that the species of stable plant communities tend to have similar competitive ability, which would in turn predict a trend of invasive superiority between plant communities centered at different levels of competitive ability. The species of a more competitive community would be more likely both to successfully invade and to resist invasions from a less competitive community. There is currently insufficient data to test this hypothesis (Goldberg 1996), but it would fit well with certain findings (Keddy and Shipley 1989, Thébaud and Simberloff 2001) and may merit future investigation.

Novel weapons: A major explanation for invasive plant success, central to my experiments, is the Novel Weapons Hypothesis (NWH), which is rooted in allelopathy. Allelopathy is a mechanism of inhibitory chemical interaction between plants; allelopathic species release chemicals (allelochemicals) which inhibit the growth and/or establishment of their competitors. Although some have questioned the prevalence of allelopathy (Blair et al. 2005, Hierro and Callaway 2003, Inderjit and Callaway 2003), there is substantial evidence that it is a significant interaction for many plants (Corbett and Morrison 2012, Hierro and Callaway 2003, Karachi and Pieper 1987, Kim and Lee 2011, Muller 1966, Prati and Bossdorf 2004, Ridenour and Callaway 2001, Wolfe et al. 2008). The NWH suggests that the allelopathy of non-native species may be more effective against the novel competitors of their invasive range than against their native competitors, because the novel competitors haven't had the opportunity to evolve resistance to their allelochemicals (Callaway and Ridenour 2004).

Although there are criticisms of the NWH (Hierro and Callaway 2003), such as the fact that its reasoning should also predict invasive species to be strongly affected by the allelopathy of their new competitors in their invasive range (Weidenhamer and Romeo 2005), there is evidence that it may contribute to the invasions of a number of allelopathic invasive species (Callaway and Aschehoug 2000, Callaway and Ridenour 2004, Cappuccino and Arnason 2006, Goslee et al. 2001, Hierro and Callaway 2003, Kim and Lee 2011, Prati and Bossdorf 2004). It may be most likely to aid species with both strong allelochemicals and strong resistance to enemy allelochemicals, or those that target mutualistic fungi on which their competitors, but not themselves, are dependent (Callaway et al. 2008, Callaway and Ridenour 2004, Roberts and Anderson 2001, Stinson et al. 2006). Invaders may also evolve to better exploit their allelopathic advantage, if they possess effective novel allelochemicals (Roberts and Anderson 2001, Stinson et al. 2006).

The NWH thus remains an area of active investigation in modern ecology. One notable gap in the literature is a lack of study on allelopathic interactions between simultaneously invading allelopathic species (Kuebbing et al. 2013). Invasive interactions can be significant, as many plant communities are experiencing more than one invasion at a time, and if they facilitate one another it can lead to an escalating series of invasions (Flory and Bauer 2014, Simberloff and Von Holle 1999). But invasive species should be non-native to one another's allelopathy by the NWH, as well, producing a complex situation ripe for study. Filling this gap was part of the purpose of my experiments.

Study system: I chose to study the allelopathy and interactions of *Microstegium vimineum* (Trin.) A. Camus (Poaceae, Japanese stilt-grass) and *Alliaria petiolata* (Bieb.) Cavara and Grande (Brassicaceae, garlic mustard), particularly in a metropolitan forest context. Metropolitan forests are fragmented forest environments heavily pressured by human activity, and increasingly important reservoirs of biodiversity as the primary forest environment in the urbanized American northeast (Corbett and Morrison 2012, Morrison et al. 2007, Vitousek et al. 1997). Therefore, understanding their ecology is important. *Alliaria petiolata* is a major and well-studied invasive herb of northeastern US forests. It is biennial and native to Europe, where it does not exhibit the problematic invasive spread that it does in the US (Callaway et al. 2008, Morrison et al. 2007). *Alliaria petiolata* has many competitive characteristics (Morrison et al. 2007, Myers et al. 2005), including well-studied allelopathy (based on release of glucosinolates that inhibit mutualistic fungi on which *A. petiolata* is not reliant) which has made it a frequent study species for the NWH (Callaway et al. 2008, Cipollini and Flint 2013, Prati and Bossdorf 2004, Roberts and Anderson 2001, Stinson et al. 2006, Vaughn and Berhow 1999, Wolfe et al. 2008). *Microstegium vimineum* is another major invasive species of the northeastern US. *Microstegium vimineum* is an annual, east Asian grass which is currently prolific across most of the eastern US (Corbett and Morrison 2012, Morrison et al. 2007). It has received less study than *A. petiolata*, and the factors influencing its dispersal are incompletely understood, but it is shade-tolerant and can modify soil pH and nutrient availability where it grows (Cole and Weltzin 2004, Ehrenfeld et al. 2001, McGrath and Binkley 2009, Morrison et al. 2007). There is evidence that *M. vimineum* is allelopathic, but it's allelopathy has received much less study to date than that of *A. petiolata* (Bauer and Flory 2011, Corbett and Morrison 2012, Pisula and Meiners 2010).

In addition to their individual significance, *A. petiolata* and *M. vimineum* are frequent co-invaders in metropolitan forests of the northeastern US, where they both occupy the herb layer. Thus, their interactions, which remain under-studied, are highly relevant to the context of metropolitan forest invasions (Flory and Bauer 2014, Kuebbing et al. 2013, Morrison et al. 2007). Therefore, I sought to explore their interactions, and the allelopathy of *M. vimineum*, in a series of three experiments: An extraction efficiency assessment, a greenhouse allelopathy assessment, and a forest growth comparison.

EXTRACTANT EFFICACY ASSESSMENT

Most previous extract-based studies of *M. vimineum* allelopathy, and of allelopathy in general, have utilized aqueous extraction techniques, but little serious evaluation has been given to the efficacy of water as the optimal extraction solvent (Blair et al. 2005, Corbett and Morrison 2012, Hierro and Callaway 2003, Kim and Lee 2011, Stinson et al. 2006). Therefore, the efficacy of water as an allelopathic extractant for *M. vimineum* merits examination in order to validate the usage of an extraction-application methodology for the study of this species' allelopathy. The main alternative to the use of allelopathic extracts in the study of allelopathy is the use of soils primed with allelopathic exudates by the growth of the allelopathic species under investigation. However, with that methodology, it is difficult to distinguish allelopathic effects from other effects, such as modification of nutrient availability or soil compaction, and so I focused on an extract-application methodology. There are criticisms of the validity of inferring an allelopathic contribution to plant competitiveness from such isolated laboratory studies (Hierro and Callaway 2003, Inderjit and Callaway 2003), but they can still serve as efficient early steps in the identification of potential allelopathic chemicals and mechanisms (Hierro and Callaway 2003, Pisula and Meiners 2010).

The assumption that water is the most effective solvent for experimental extraction of *M. petiolata* allelochemicals is a reasonable hypothesis, as aqueous transport would be a natural and effective means for *M. vimineum* to introduce competitor plants to its allelochemicals. However, non-water-soluble allelochemicals are not inconceivable; for instance, volatile transport of allelochemicals is a possibility, as has been found for the allelopathic terpenes of a variety of aromatic shrubs of Southern California (Muller 1966). Solid-phase transport of allelochemicals, such as through windborne or animal-carried particles, is also conceivable. While prior *M. vimineum* allelopathy research has indeed found a germination inhibition from the plant's aqueous extracts (Corbett and Morrison 2012, Pisula and Meiners 2010), this only proves that *M. vimineum* allelochemicals are not completely insoluble in water, not that water is their optimal solvent. The similarity in polarity of a solvent and a solute pair is typically the main indicator of how soluble the latter will be in that former (the "like dissolves like" paradigm). Therefore, extraction procedures of differing efficiency are likely to vary primarily based on the polarity of the solvent used.

This experiment was designed to establish the relative efficacy of various solvents at extracting *M. vimineum* allelochemicals, and in turn to characterize the nature of those allelochemicals insofar as can be inferred from their solubility. My prediction for this study was that I would observe extract-induced germination inhibition that decreased in inverse proportion to the polarity of the solvent, confirming the previously-assumed aqueous solubility of *M. vimineum* allelochemicals. This finding would also corroborate previous findings of an allelopathic inhibition of germination in lettuce by *M. vimineum* (Corbett and Morrison 2012), and lend credence to the use of aqueous solvents in experimental studies of *M. vimineum* allelopathy.

GREENHOUSE ALLELOPATHY ASSESSMENT

Prior research on the allelopathy of *Microstegium vimineum* has focused on the effects of its allelochemicals on the germination, establishment, and early growth of other plants (Pisula and Meiners 2010, Corbett and Morrison 2012). However, the species' allelopathic effects on later life stages of its competitors also merit investigation. Furthermore, the allelopathic interactions between *M. vimineum* and *A. petiolata* remain understudied, considering their potential importance to the frequent context in which they simultaneously invade the same environment. Therefore, this experiment was designed to assess the possible direct effect of *M. vimineum* allelochemicals on the experimental growth of *A. petiolata* beyond the germination stage. I grew *A. petiolata* under conditions of periodic exposure to either *M. vimineum* aqueous extract or control, then compared the final size and biomass of the two treatments. I expected to observe reduced growth in the plants receiving *M. vimineum* extract, due to a negative allelopathic effect.

Some researchers have raised concerns that allelopathic extractions may unintentionally extract phytochemicals other than those naturally exuded by the allelopathic species (Inderjit and Callaway 2003). In order to test the validity of my allelopathic extraction treatment against these concerns, I chose to also apply *M. vimineum* allelopathic extract to *M. vimineum* itself, as an expected negative control for allelopathic inhibition. Since the novel weapons hypothesis posits that native competitors are capable of evolving resistance to a species' allelochemicals, by extension a species should possess very strong resistance against its own allelochemicals (Callaway and Ridenour 2004). If impairment of growth with exposure to *M. vimineum* extract was observed in *M. vimineum* as well as *A. petiolata*, it would imply that the extract contained phytochemicals not released in the course of normal growth.

FOREST GROWTH COMPARISON

This portion of my study was based on analysis of the early results of a long-term, manipulative field study of the interactions between *A. petiolata* invasion, *M. vimineum* invasion, and deer herbivory, being conducted by the research group of Dr. Janet Morrison at The College of New Jersey. Field studies are capable of providing a more complete and representative picture of the interactions at play in a natural environment than the simplified circumstances of the greenhouse setting, although the added complexity does make it more difficult to isolate individual causes and effects. This study involved subjecting plots within six metropolitan forests of central New Jersey to fully-factorial, randomly-distributed treatments of *A. petiolata* introduction (by seed), *M. vimineum* introduction (by seed), and deer exclusion. The interaction of co-invasive species, particularly *A. petiolata* and *M. vimineum*, is an important yet under-studied topic (Kuebbing et al. 2013, Morrison et al. 2007) which this experimental design is well-poised to address. This analysis is based on the first year and a half of a planned five years of data collection following the establishment of the experimental conditions.

If the allelopathy of *M. vimineum* is a dominant factor in the interactions between these two species, then a negative correlation should be observed between *M. vimineum* abundance and *A. petiolata* abundance. I analyzed the data to determine if this was so, which would provide support for the hypothesis that an allelopathic effect operates during *M. vimineum* invasion and inhibits the co-invasion of *A. petiolata*.

METHODS: EXTRACTANT EFFICACY ASSESSMENT

I experimentally extracted potential *M. vimineum* allelochemicals with four different solvents of descending polarity: deionized (DI) water, ethanol, dichloromethane, and hexane. This procedure was a similar extraction methodology to that employed by Vaughn and Berhow (1999). I used these solvents to extract stem and leaf matter of different *M. vimineum* plants grown in a as described in the methodology for my greenhouse allelopathy assessment. This plant matter was dried at 60°C for three days before being extracted. I extracted with simple leaching for 24 hours in separate sealed glass flasks at room temperature for each solvent, at a ratio of 1 g plant meter per 20 mL solvent.

I set up five experimental blocks for each extract, as well as five control blocks for each pure solvent. Each block consisted of a plastic petri dish lined with two stacked circles of filter paper which I soaked with 2 mL of the appropriate extract or control shortly after the preparation of the extracts, then allowed to air dry for six hours. All four solvents were volatile, so that they could be evaporated to deposit dissolved allelochemicals onto the growth medium while preventing direct contact between solvent and seeds. Once the filter paper of each dish was dried, I re-moistened it with 2 mL of DI water, then evenly spread 20 lettuce seeds (*Lactuca sativa*) across the paper of each dish. Lettuce seeds have been frequently used in allelopathic germination assays, including those on the allelopathy of *M. vimineum*, owing to their speed and ease of germination and sensitivity to allelopathy (Corbett and Morrison 2012). I sealed each dish with parafilm and let them sit for 3 days in darkness at room temperature, then counted the number of germinated and ungerminated seeds in each dish. I analyzed the effects of solvent, of extract, and of their interaction on germination, by factorial analysis of variance (ANOVA) on the arcsine-transformed (to meet the normality assumption for ANOVA) percent germination data, performed through SAS.

GREENHOUSE ALLELOPATHY ASSESSMENT

In 2013, I collected ripe seeds of *A. petiolata* and *M. vimineum* from five wild populations of each plant. I supplemented these with leftover *M. vimineum* seeds previously collected for the forest growth comparison. The A. petiolata seeds were allowed to post-ripen at room temperature for 3 months after collection. I stratified the seeds of both species by refrigerating groups of seeds mixed into moist germination mix in sealed petri dishes, for three months for *A. petiolata* and for one month for *M. vimineum*.

Once the seeds were stratified, I planted seeds of both species in dense groups in germination mix within 7 cm x 7 cm x 6.5 cm plastic growing pots, and grew them in a heated greenhouse at ambient indoor humidity, from January through April of 2014. Once the planted seedlings of both species were thriving in their initial pots, they were transplanted to Pro-Mix potting mix within 10 cm x 10 cm x 8.5 cm plastic growing pots, either individually (for experimental plants) or in groups (for *M. vimineum* designated to produce allelopathic leachate). They were thereafter watered once every three. Shortly before the beginning of the experimental treatment, I selected the 48 individual plants of each species of the most median size (out of 66 *A. petiolata* and 108 *M. vimineum*) as the experimental plants.

I assigned each experimental plant to a random experimental treatment (*M. vimineum* aqueous extract or control DI water) and a random position within one of three experimental blocks in the greenhouse. At the start of the experimental treatment, I measured the initial above-ground size of each plant, in terms of total leaf area for *A. petiolata* and shoot length for *M. vimineum*. I prepared *M. vimineum* extract by separating the shoots (above-ground biomass) of greenhouse-grown *M. vimineum* from their roots, then briefly rinsing the shoots in water before drying them at 60°C for at least three days. Extracted *M. vimineum* was between one and three months old at the time of harvesting. After drying was complete, I soaked the whole dried shoot tissue in DI water at a ratio of 1 g plant matter to 50 mL of DI water for 24 hours in a sealed beaker at room temperature, then filtered the mixture through 1 mm wire mesh to exclude plant matter. I diluted this initial extract to 40% of its initial concentration with DI water, and used all extract within 12 hours of its production.

I administered 10 mL of this diluted extract to each plant assigned the experimental treatment once per week for three weeks, while the control plants received 10 mL of DI water on the same schedule. The watering schedule was adjusted to avoid watering on extract administration days. One week into the experimental treatment, all of the plants in the growth study received a one-time application of 75 mL of liquid fertilizer. After three weeks, I took final size measurements of each plant. Then I harvested all of the plants, separated the shoots from the roots, and rinsed both in tap water. I dried the roots and shoots of each plant in separate paper bags for three days at 60^{o} C, then recorded the mass of each.

I analyzed the effects of the allelopathic extract on the growth of *A. petiolata* and *M. vimineum* as separate experiments, through a series of analyses of covariance (ANCOVA), all run through SAS. Each ANCOVA was performed on the effects of extract treatment and experimental block on a different growth parameter, with starting size as a covariate. The growth parameters evaluated were final size, relative growth rate ((final size – starting size) / days of growth), final root, shoot, and total biomass, and ln-transformed final root to shoot biomass ratio.

FOREST GROWTH COMPARISON

Six suburban, forested sites in central New Jersey were selected for this experiment by the Morrison lab. These forests were designated Baldpate, Curlis, Eames, Herronton, Nayfield, and Rosedale, after the parks and preserves where they are located. Each forest was identified as either high deer pressure (Curlis, Eames, Rosedale) or low deer pressure (Baldpate, Herronton, Nayfield), based on deer management history and preliminary assessments of deer browse level.

Forty 4 m x 4 m plots were established 4 m apart from one another within each forest and randomly assigned an invasion treatment of either *A. petiolata*, *M. vimineum*, both species, or neither species, with 10 plots assigned each treatment. We seeded each plot with an equal quantity of seeds of the species it was assigned in November to December of 2012. These seeds were collected from 10 local populations of each species and mixed together before seeding. Additionally, half of the plots of each invasion treatment were randomly assigned a deer exclusion treatment, and surrounded by 2 m high deer exclusion fences in spring 2013. Such fences have been found to effectively exclude deer without significantly affecting levels of photosynthetically active radiation (Morrison and Brown 2004). As the study progressed, several plots had to be removed from the study due to storm damage.

Starting in the spring of 2012, and repeating every fall and spring thereafter, we censused the herb layer of each experimental plot through a stratified pseudorandom sampling procedure. All species within the herb layer of each subsample were identified and assessed at a percent cover level in 10% intervals, from 1-10% up to 90-99% (in addition to 0% and 100%), based on visual estimation. Plot percent cover was the average of subsample percent coverage. In addition to these cover assessments, we performed a count of the number of individual *M. vimineum* and *A. petiolata* plants in each plot in July, 2013, and, for *A. petiolata*, again in April, 2014.

I analyzed the influence of *M. vimineum* on the establishment and growth of *A. petiolata* through a series of statistical analyses, all performed through SAS. An analysis of variance (ANOVA) was performed to assess the effects of forest, fencing, and *M. vimineum* invasive treatment on fall 2013 *A. petiolata* percent cover (arcsine-transformed to meet the normality assumption of ANOVA). I also performed a series of regression analyses on the relationship between arcsine-transformed fall 2013 *M. vimineum* percent cover and arcsine-transformed fall 2013 *A. petiolata* percent cover; both for all forests pooled together, and by individual forest. ANOVA was also performed on the effects of forest, fencing, and *M. vimineum* invasive treatment on summer 2013 *A. petiolata* counts, and on the effects of forest on *M. vimineum* arcsine-transformed percent cover. Finally, I performed a series of regression analyses on the relationship between *M. vimineum* abundance (assessed both in terms of arcsine-transformed fall 2013 percent cover and in terms of log-transformed summer 2013 plant count) and log-transformed summer 2013 *A. petiolata* counts, both for all forests pooled together and by individual forest data. All regression analyses examined only data points with nonzero values for *M. vimineum* cover/count, excluding plots in which *M. vimineum* was seeded but failed to establish.

RESULTS

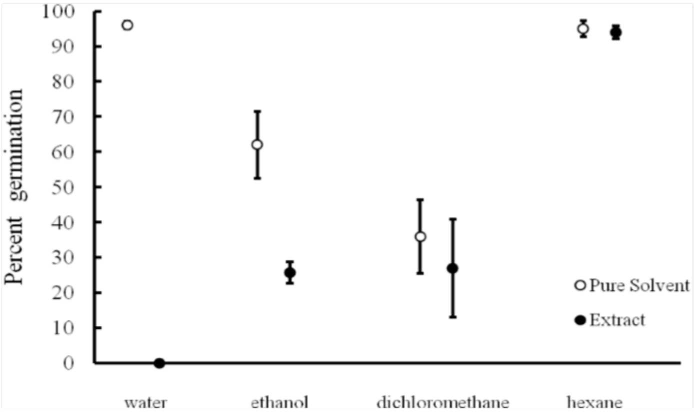

Figure 1. Germination (mean ± SE) of *L. sativa* seeds following three days of exposure to evaporated residue of either *M. vimineum* extract or pure solvent, for four different solvents (n = 5 for all). Solvents arranged in order of decreasing polarity from left to right.

EXTRACTANT EFFICACY ASSESSMENT

Highly significant effects were observed for solvent type ($F_{(3, 32)} = 27.44$, $P < 0.0001$), *M. vimineum* extraction ($F_{(1, 32)} = 46.75$, $P < 0.0001$), and solvent x extraction combination ($F_{(3, 32)} = 18.94$, $P < 0.0001$) on the percent germination of *L. sativa* when germinated on dried solvent-treated filter paper. Exposure to the evaporation residue of the different pure solvents had distinctly different impacts on *L. sativa* germination, with DI water and hexane yielding high germination success and dichloromethane and ethanol producing significantly lower germination (Figure 1). Exposure to the evaporation residue of *M. vimineum* extract was also clearly inhibitory, with the extract treatments for DI water and ethanol demonstrating clearly lower germination than their corresponding controls, and even the extract treatments for dichloromethane and hexane demonstrating a nonsignificant trend of lower germination than their corresponding controls.

Most importantly, examination of the means reveals the effect of extract x solvent interaction on germination success. There was a greater difference between extract and control germination for water and for ethanol than for dichloromethane or for hexane. Exposure to evaporated *M. vimineum* extract inhibited *L. sativa* germination relative to the corresponding control when the extract was prepared with DI water or ethanol, but not when the extract was prepared with dichloromethane or hexane. Furthermore, based on the relative magnitude of the differences between control and extract treatment, the allelopathic effectiveness of the extracts decreased as the solvent decreased in polarity, in the order water > ethanol > dichloromethane ≥ hexane.

GREENHOUSE ALLELOPATHY ASSESSMENT

For the *A. petiolata* growth experiment, the covariate, starting size of *A. petiolata,* significantly ($P < 0.05$) increased every growth parameter evaluated. Significant block effects were observed for final size and for relative growth rate, but no significant effects were observed from *M. vimineum* extract. However, while there was no

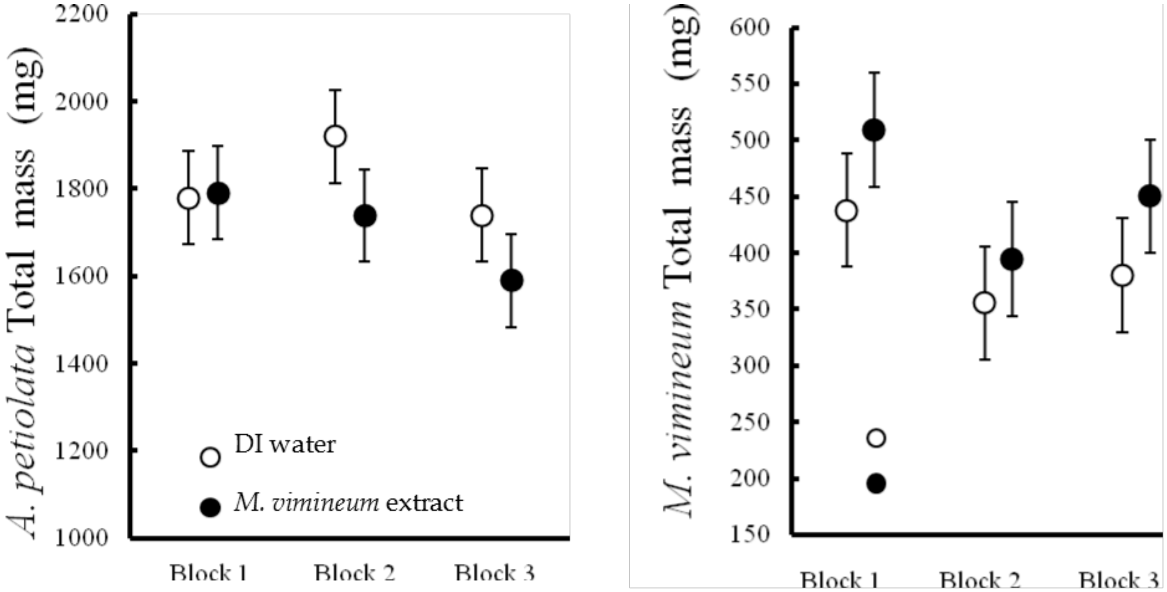

Figure 2. *Alliaria petiolata* (left) and *M. vimineum* (right) final dry total mass (mean ± SE) by block and allelopathic extract treatment (n = 8 for all).

consistent trend of a common difference in final size or relative growth rate between *A. petiolata* grown with and without exposure to *M. vimineum* extract, there was a consistent trend of reduced final biomass (for root, total, and to a lesser extent shoot biomass) in the experimental treatment relative to the control (Figure 2), accompanied by relatively close to significant P values for the effect of extract on root and total biomass ($P < 0.25$). This trend was most evident for blocks 2 and 3, and was reduced or reversed in block 1, which didn't appear to exhibit this trend as clearly (Figure 2). There was also a slight but consistent trend ($P = 0.41$) of decreased root:shoot ratio in the experimental treatment, which may indicate increased allocation of growth to shoots rather than roots.

For the *M. vimineum* growth experiment, no significant effects were observed upon any of the growth parameters assessed, even from experimental block or starting size. There is a consistent trend in the means of multiple growth parameters, however; all growth parameters (final size, relative growth rate, and root, shoot, and total biomass) tended to be at least slightly higher for plants receiving *M. vimineum* extract than for those receiving the control treatment (Figure 2 for total mass), although there was no consistent trend for root:shoot ratio.

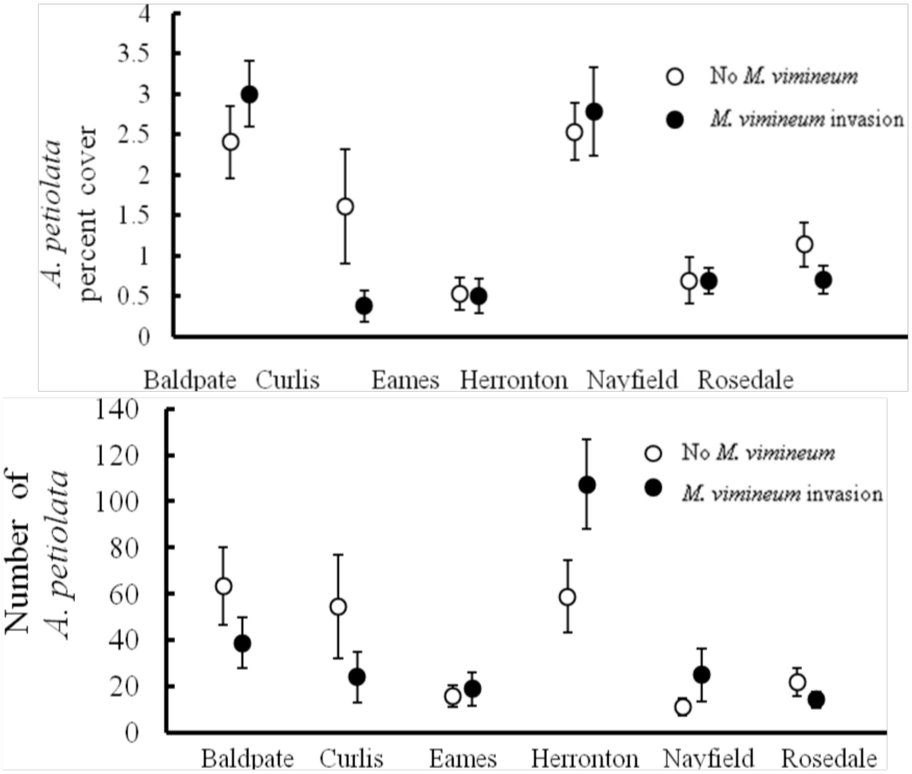

Figure 3. Fall 2013 *A. petiolata* percent cover (mean ± SE; above) and Summer 2013 *A. petiolata* counts (mean ± SE; below), grouped by forest and *M. vimineum* presence (n from left to right: Above, 10, 10, 8, 9, 10, 10, 9, 9, 10, 10, 8, 8; below, 8, 7, 8, 9, 10, 10, 9, 9, 10, 10, 8, 8 0).

	Fall 2013 *A. petiolata* percent cover				**Summer 2013 *A petiolata* count**			
Forest	**N**	**P**	**Relationship slope**	**R2**	**N**	**P**	**Relationship slope**	**R2**
ALL	69	0.51	-0.03	0.006	65	0.47	1.83	0.008
Baldpate	12	0.001	**0.63 ***	0.68	8	0.40	-21.30	0.12
Herronton	10	0.51	-0.05	0.06	10	0.83	1.38	0.006
Nayfield	16	0.19	0.15	0.12	16	0.44	5.76	0.04
Rosedale	11	<0.01	**-0.11 ***	0.58	11	0.04	**-4.15 ***	0.40
Curlis	9	0.74	-0.06	0.02	9	0.57	6.95	0.05
Eames	11	<0.01	**0.28 ***	0.61	11	<0.01	**14.24 ***	0.60

Table 1. Regression test results on the relationships between arcsine-transformed fall 2013 *M. vimineum* percent cover and either arcsine-transformed fall 2013 *A. petiolata* percent cover or log-transformed summer 2013 *A. petiolata* count. * = significant relationship ($P < 0.05$).

FOREST GROWTH COMPARISON

Influences on fall 2013 *Alliaria petiolata* percent cover: I found no significant difference in fall 2013 *A. petiolata* percent cover based on the presence of *M. vimineum* co-invasion treatment ($F_{(1, 98)} = 0.68$, $P = 0.41$), the presence of deer exclusion fencing ($F_{(1, 98)} = 2.86$, $P = 0.09$), or the interaction of forest x co-invasion treatment ($F_{(5, 98)} = 1.15$, $P = 0.34$). A significant difference was observed based on the forest ($F_{(5, 98)} = 14.97$, $P < 0.0001$); post-hoc Tukey tests showed that *A. petiolata* cover was not significantly different between Herronton and Baldpate, but was significantly different between those two forests and the other four, which were not significantly different from one another (Figure 3). The only forest with a clear difference between the presence and absence of *M. vimineum* co-invasion treatment was Curlis, for which *A. petiolata* percent cover was significantly lower with *M. vimineum* treatment than without, but *M. vimineum* presence was not a significant factor among all forests. However, regression analysis revealed a positive relationship between fall 2013 *M. vimineum* percent cover and fall 2013 *A. petiolata* percent cover in Eames and Baldpate, a negative relationship in Rosedale, and no relationship at the other forests or across pooled data from all forests (Table 1).

Influences on summer 2013 *Alliaria petiolata* count: Although there was no significant relationship between *M. vimineum* co-invasion treatment and summer 2013 *A. petiolata* count ($F_{(1, 93)} = 0.08$, $P = 0.78$), the *A. petiolata* count did have a significant effect from forest ($F_{(5, 93)} = 8.08$, $P < 0.0001$) and a borderline-significant effect from the combination of forest and co-invasion treatment ($F_{(5, 93)} = 2.27$, $P = 0.05$). This latter relationship was unpredictable, as it varied in sign and magnitude between forests, but not according to any predictable pattern (Figure 3). The most significant effect was observed for Herronton, where *M. vimineum* treatment corresponded with higher counts of *A. petiolata*. Percent cover of *M. vimineum* varied significantly among the six forests ($F_{(5, 50)} = 4.48$, $P = 0.002$), but this pattern did not mirror that of the *A. petiolata* summer counts in the forests. Regression testing showed a positive relationship between fall 2013 *M. vimineum* percent cover and *A. petiolata* count in Eames, a negative relationship in Rosedale, and no relationship in any other forest (Table 1). Regression analysis showed no relationship between summer 2013 *M. vimineum* count and summer 2013 *A. petiolata* count in any forests.

Influences on spring 2014 *Alliaria petiolata* percent cover: The spring 2014 *A. petiolata* count results generally mirrored those found by the other analyses. There was no significant effect on spring 2014 *A. petiolata* count from *M. vimineum* invasion treatment ($F_{(1, 79)} = 0.03$, $P = 0.87$), deer exclusion treatment ($F_{(1, 79)} = 0.90$, $P = 0.35$), or the combination of forest and invasion treatment (ns), but there were significant effects from forest ($F_{(5, 79)} = 8.22$, $P < 0.0001$) and from the combination of forest and deer exclusion treatment ($F_{(5, 79)} = 2.66$, $P = 0.03$). The only potentially significant effect of *M. vimineum* invasion treatment was seen for Curlis, where *A. petiolata* count was lower with *M. vimineum* treatment than without. Regression analysis showed a positive relationship between fall 2013 *M. vimineum* percent cover and spring 2014 *A. petiolata* count in Baldpate ($R^2 = 0.41$, $P < 0.05$) and in Eames ($R^2 = 0.76$, $P < 0.05$), and no significant relationship in any other forest.

DISCUSSION: EXTRACTANT EFFICACY ASSESSMENT

Allelochemicals capable of inhibiting the germination of *L. sativa* were extracted and transported in allelopathically-active quantity and form by soaking *M. vimineum* shoots and leaves in water and in ethanol, but not in dichloromethane or hexane. There was also direct solvent inhibition on germination from ethanol and dichloromethane treatment. This direct effect was unexpected; however, it is possible that non-volatile contaminants present to some small level in the pure solvents (possibly by-products from their synthesis) were deposited on the filter paper by the ethanol and dichloromethane control treatments, and inhibited germination in those cases. Therefore, the results clearly indicated that the above-ground tissues (shoots) of *M. vimineum* possess allelochemicals that are capable of inhibiting the germination of *L. sativa*, and that these allelochemicals are appreciably soluble in water and ethanol without being destroyed, whereas, in dichloromethane and hexane, these allelochemicals are either insoluble or inactivated by the solvent.

The results confirm the expected importance of polarity to allelochemical solvent efficacy. The most obvious trend in these results with regard to solubility is that water and ethanol are the more polar two of the four solvents, while dichloromethane and hexane are the less polar two. Furthermore, inspection of the means reveals that the magnitude of the difference between control and extract treatments decreased as the polarity of the solvent decreased in the order water > ethanol > dichloromethane ≥ hexane. This result suggests that the solubility of *M. vimineum*'s allelochemicals is proportional to the polarity of the solvent, which in turn suggests that they are substantially polar compounds themselves.

These findings are in keeping with the hypothesis that *M. vimineum* allelochemicals would be predominantly soluble in aqueous medium, as an effective natural mode of transport to competitor species for inhibition. My results support prior findings of an inhibitory effect of *M. vimineum* aqueous extracts on germination, and validate the aqueous extraction techniques that such studies have generally employed as the most effective means of experimentally extracting and transporting *M. vimineum* allelochemicals (Corbett and Morrison 2012, Pisula and Meiners 2010). Furthermore, the finding that the most effective extraction was the aqueous extraction, which most closely mimics the theoretical natural process of allelochemical release through leaching by rainwater, supports the theory that the germination-inhibiting allelochemicals of aqueous *M. vimineum* extract are indeed naturally exuded by *M. vimineum* in the field.

GREENHOUSE ALLELOPATHY ASSESSMENT

Although no effects on the growth of *A. petiolata* from extract treatment were formally significant ($P = 0.05$), inspection of the means does reveal a consistent trend of reduced biomass of *A. petiolata* treated with *M. vimineum* extract relative to those treated with DI water, and the P values for the relationship between extract treatment and root and total biomass are conspicuously close to. Given that this experiment only consisted of three weeks of periodic, post-germination exposure to the experimental treatment, this result may indeed indicate a subtle allelopathic influence of *M. vimineum* extract on the growth of *A. petiolata*. Repeating this experiment over a longer period of time, on a younger stage of target plant growth, and/or using higher quantities of *M. vimineum* extract might reveal an allelopathic effect that was masked by the limitations of this experiment.

As was expected, *M. vimineum* did not experience any significant effects from exposure to *M. vimineum* extract. It is interesting to note, however, that inspection of the means reveals a consistent trend of greater size and biomass of extract treatments relative to control treatments. Furthermore, except for root:shoot ratio, every growth parameter had a conspicuously low P value ($P < 0.30$) for its relationship with extract treatment. This suggests that there may actually be a slight positive effect of *M. vimineum* extract on *M. vimineum* growth that, as reasoned for *A. petiolata* above, might not have had time to evidence itself in the context of this experiment but might appear in a broader one. Such an effect would suggest the existence of a cooperative phytochemical in *M. vimineum*, some released substance that is water-soluble and which facilitates the growth of other *M. vimineum* nearby. Such a substance could be an unexplored aspect of the invasive success of *M. vimineum*, and part of its ability to rapidly spread and to form dense, monospecific stands. Evidence for similar chemical autofacilication has been found for both *Anthroxanthum odoratum L.* (sweet vernalgrass) (Hierro and Callaway 2003) and *Sapium sebiferum* L. Roxb. (Tallow) (Conway et al. 2002).

This potential facilitative exudate of *M. vimineum* therefore merits further study.

FOREST GROWTH COMPARISON

The large variety of analyses of the field data did not reveal any consistent relationships between *M. vimineum* co-invasion and *A. petiolata* establishment and/or growth. More consistent and significant effects on *A. petiolata* abundance were observed when regression analysis was used to consider *M. vimineum* abundance as a continuous variable, suggesting that differences in between-plot recruitment and growth of *M. vimineum* have a significant effect on these results. However, no analysis showed a consistent effect of *M. vimineum* across forests, nor did any forest show a consistent effect of *M. vimineum* across analyses. I could identify no underlying factor which might explain why *M. vimineum* would have a positive effect on *A. petiolata* in some forests, a negative effect in others, and no effect in still others. Positive effects in particular are contrary to the expectations of an allelopathic effect, and suggest the presence of one or more other, positive interactions at play. There is no trend in deer pressure or in overall *M. vimineum* cover by forest that matches the changes in the direction and magnitude of the apparent relationship between *M. vimineum* presence and *A. petiolata* success. Cole and Weltzin (2004) previously found indications that *M. vimineum* has complex interactions with environmental factors, as the relationship they observed between *M. vimineum* abundance and pH varied between forests with no clear explanation, and canopy openness and biomass of other species were the only other significant environmental predictors they could establish for *M. vimineum* abundance; it is likely that allelopathy is another quality of *M. vimineum* that co-varies with biotic or abiotic factors yet to be determined. Such factors might include, for instance, nutrient availability (Isaac 1992), environmental pH levels (Ehrenfeld et al. 2001), or the set of native competitors present. It may be possible to reach more confident conclusions about the interactions between *A. petiolata* and *M. vimineum* when the forest study has completed its five-year course of data collection.

CONCLUSIONS

This series of experiments has made some significant strides towards understanding the allelopathy of *Microstegium vimineum*, although many questions remain. The extraction efficacy assessment corroborated prior findings of an inhibitory effect of *M. vimineum* extracts on germination, and characterized the responsible extracted allelochemicals as soluble primarily in water or other aqueous solvents. Although the greenhouse allelopathy assessment found no statistically significant effects, consistent trends constituted noteworthy indications of both inhibition of *A. petiolata* and facilitation of *M. vimineum* by *M. vimineum* extract, suggesting merit to further study. The forest growth comparison did not identify a consistent effect of the *M. vimineum* on *A. petiolata*. Instead, we found effects that varied unpredictably between forests and methods of analysis, suggesting that the effect of *M. vimineum* on *A. petiolata* is dependent on other, underlying factors which are not yet clear but which vary between the forests of the field study.

The ambiguity of these collective findings highlights the complexities of ecological systems and the need to continue studying their unknowns. If these findings are built upon, they may enhance our ability to understand and respond to two of the most dangerous invaders of the metropolitan forests that struggle to preserve the natural world in the face of human development.

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