AN ASSESSMENT OF THE ALLELOPATHIC POTENTIAL
OF ALLIARIA PETIOLATA

A dissertation submitted in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

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ABSTRACT


Garlic mustard (GM), *Alliaria petiolata*, a European biennial herb invasive in North America, produces secondary metabolites that may contribute to its invasive success by allelopathically inhibiting growth of native plants. I tested this hypothesis by determining the levels, and stability, of these compounds in field soils. I also explored differences in the phytochemical profiles of GM and closely related North American species, and biogeographic differences in allelopathic effects on North American and European arbuscular mycorrhizal fungi (AMF). Furthermore, I exposed a North American annual herb, pale jewelweed (*Impatiens pallida*), and its AMF, to GM extracts in order to determine how GM metabolites interact to inhibit growth throughout the formation of mycorrhizal symbioses. Additionally, I determined whether or not allelopathic inhibition remained significant when resource competition was also occurring, by exposing jewelweed plants grown at various densities to GM extracts. Also, I assessed allelopathic effects on AMF diversity by performing terminal restriction fragment length polymorphism analysis on AMF DNA isolated from field soils collected
either in or outside of GM populations. Finally, I explored how three methods commonly used to kill GM influenced the health of jewelweed plants later planted in the same soil.

Secondary metabolites produced by GM decompose too quickly to account for long term inhibition, suggesting that degradates of these compounds are bioactive. None of the compounds produced by GM were found in four North American species, and North American AMF were more sensitive to allelopathic effects than European AMF. I found strong inhibition of seed germination and growth of uncolonized plants, but AMF appeared to protect colonized jewelweed plants from any allelopathic effects. Allelopathic effects were not as significant as effects of resource competition on jewelweed plant size. AMF diversity was lower in sites with GM than without. During simulated restorations, jewelweed plants were larger and more colonized by AMF in soils with the least amount of dead GM tissue remaining. GM can be allelopathic, but effects may not be strong enough to drive its invasive success. Instead, allelopathic effects may be more important in maintaining an invasion and must be addressed during restoration of invaded sites.
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ACKNOWLEDGMENTS

I would like to thank everyone in the Cipollini lab for helping with lab work over the years, especially Steph Enright. My committee members have provided much appreciated advice, and I sincerely thank them for their time and effort. Jeanette Frey, Gwyn Isenhower, Mill Miller, and Jerry Serviates provided technical assistance for fluorescence microscopy, chromatography, and molecular work. I was fortunate to collaborate with Ray Callaway (University of Montana), Kristina Stinson (Harvard University), and Pedro Antunes, Alex Koch, and John Klironomos (University of Guelph). I received funding from Wright State University and the Environmental Protection Agency’s Greater Research Opportunities Fellowship program (#91673701).
1 INTRODUCTION

1.1 ALLELOPATHY

Toxic effects of plant exudates were noticed as early as 1832 when DeCandolle hypothesized that secretions from rye roots were responsible for poor growth in nearby wheat plants. The next publication on the topic was not until 1881 when Stickney and Hoy suggested that sparse growth under black walnut trees was due to chemical secretions from the roots (Rice 1974). The term allelopathy was first used to describe the actions of plant exudates by Molisch in 1937 and comes from the Greek alleloi (each other) and pathy (to be affected by), giving “to be affected by each other”. Molisch intended the term to include both stimulatory and inhibitory interactions between plants and microorganisms (Gross 2003), but today the definition is typically restricted to inhibitory effects (direct or indirect) of one plant on another (Choesin and Boerner 1991, Barkosky et al. 1999). Allelopathic research flourished well into the 1960’s, and a review published in 1971 (Whittaker and Feeny 1971) described numerous plant interactions believed to be influenced by allelopathy. The authors optimistically proclaimed that “allelopathy may consequently be of widespread significance in plant communities”.

Allelopathy has been particularly well documented in crop species. Wheat produces phenolic compounds that increase cell permeability (Wu et al. 2002), and several mutagenic benzoxazinoids (Hashimoto and Shudo 1996) that accumulate in the
soil and inhibit growth of many plants, including wheat (Wu et al. 2000, Huang et al. 2003). Sorghum inhibits neighboring plants by exuding sorgoleone, a plastoquinone analog, which binds with photosystem II and blocks photosynthesis (Einhellig et al. 1993, Gonzalez et al. 1997).

Invasive species have received unfocused attention regarded allelopathy with the exception of several *Centaura* species that are invading the Western United States (Fletcher and Renney 1963, Muir and Majak 1983, Kelsey and Locken 1987, Callaway and Aschehoug 2000, Ridenour and Callaway 2001). *C. maculosa* exudes large enough quantities of (-)-catechin to inhibit germination and growth of native grasses, and actually triggers programmed cell death in the roots of neighboring plants (Bais et al. 2003b).

*Ailanthus altissima* inhibits seed germination and growth of many native North American plants (Lawrence et al. 1991). The native *Antennaria microphylla* produces hydroquinone which inhibits photosynthesis in the invading *Euphorbia esula*, thereby slowing the spread of this invasive plant (Barkosky et al. 1999).

The broad acceptance of allelopathy as an important ecosystem process was quickly replaced with skepticism of the methods used (mostly germination bioassays) to reach these conclusions (Harper 1975, Stowe 1979), and allelopathy was abandoned in favor of resource competition as an explanation for many plant interactions (Williamson 1990).

Although seed germination bioassays are still regularly published as evidence for allelopathy, more thorough experimental designs have also been called for (Weidenhamer et al. 1989, Williamson 1990, Choesin and Boerner 1991, Hierro and Callaway 2003,
In order to demonstrate that allelopathy is occurring one must show that resource competition alone cannot explain an observed interaction (Williamson 1990). One must identify the allelochemical involved (Williamson 1990), show that it is released into the environment by the donor plant, has a significant residence time in the environment, and has a deleterious effect on target plants at environmentally relevant concentrations (Choesin and Boerner 1991). Allelopathy is probably common in interactions involving invasive species simply because the plants involved are often being exposed to novel chemical compounds, and at least one of these conditions has been met for many invasive species. Seed germination bioassays have a place in this framework, but they are not sufficient to prove allelopathy.

*Alliaria petiolata* is an ideal species with which to apply these more rigorous experimental design guidelines for several reasons. This species produces many noxious compounds and is highly invasive in North America. There is some preliminary evidence for allelopathy in *A. petiolata* (Vaughn and Berhow 1999, Roberts and Anderson 2001, Prati and Bossdorf 2004, Stinson et al. 2006), but since most of the work has involved seeds or seedlings and unrealistically high exposure levels, there is still much work to be done. A more thorough understanding of the invasion mechanism will lead to more successful restoration efforts.

1.2 **GARLIC MUSTARD**

*Alliaria petiolata* (garlic mustard) is an herbaceous biennial plant that was introduced to North America from Europe in the 1860’s and has since become a problem across much of the Northeast and Midwest (Nuzzo 2002). This species is self-compatible
and produces large quantities of seed, meaning that only one seed is needed to begin an invasion. *Alliaria petiolata* suffers very little herbivory in North America (only 11 insect species are known to attack it, none of them specialists), while in Europe at least 70 insect species will eat *A. petiolata* and 5 of those specialize on the plant (Blossey et al. 2001). Insects native to North America (e.g. *Pieris napi oleracea* and *P. virginiensis*) will mistake *A. petiolata* for an acceptable host plant and oviposit, however the larvae do not develop as quickly on *A. petiolata* as on native Brassicaceae (Bowden 1971, Chew 1981, Keeler and Chew 2008). *Alliaria petiolata* does not form associations with symbiotic mycorrhizal fungi and rapidly invades disturbed woodland edges which are poor habitat for most plants partly because of low mycorrhizal inoculum potential. This plant can also form dense monocultures in the undisturbed forest understory, adversely impacting native plant abundance and biodiversity (McCarthy 1997), likely by inhibiting mycorrhizal growth and/or development (Stinson et al. 2006).

*Alliaria petiolata*, like other Brassicaceous plants, produces glucosinolates, which are β-thioglucoside N-hydroxysulfates with a sulfur-linked β-D-glucopyranose moiety and a side chain of varying composition (Figure 1.1) (Fahey et al. 2001). Glycosylation reduces the toxicity of the glucosinolates and makes them water soluble, allowing accumulation in vacuoles, sometimes to concentrations of 200 mM (Koroleva et al. 2000). The enzyme β-thioglucosidase, or myrosinase, that removes this sugar accumulates in specialized idioblasts called myrosin cells. These cells occur adjacent to cells containing high concentrations of glucosinolates, forming a “mustard oil bomb” that is activated after a cellular disturbance (e.g. herbivory, wounding). After myrosinase removes the sugar, the remaining aglycone spontaneously rearranges into
isothiocyanates, thiocyanates, nitriles, epithionitriles and/or oxazolidine-2-thiones (Figure 1.1). Each glucosinolate can form a range of degradates, influenced by cellular pH, iron concentration, and the presence of specifier proteins that encourage production of specific degradates. The glucosinolates produced by mustards degrade to mostly isothiocyanates and some epithionitriles, both of which are generally more volatile and less water soluble than their parent glucosinolates (Gardiner et al. 1999).

Glucotropaeolin (benzyl glucosinolate) is the most abundant glucosinolate produced by *A. petiolata*, followed by sinigrin (allyl glucosinolate). Glucotropaeolin is more abundant in roots than leaves and stems, and more abundant in autumn than spring. Degradation products produced after hydrolysis include benzyl isothiocyanate (BzITC), from glucotropaeolin (~36%), followed by allyl isothiocyanate (AITC), from sinigrin (~20%), and 2,3-epithiopropylnitrile, probably also from sinigrin, at (~8%) (Figure 1.1) (Vaughn and Berhow 1999).

Isothiocyanates have been implicated in the action of mustards against insects (Rask et al. 2000), arbuscular mycorrhizal fungi (AMF) (Schreiner and Koide 1993a, b), pathogenic fungi (Olivier et al. 1999), and other plants (Yamane et al. 1992). In contrast, a lack of effect against AMF (Glenn et al. 1988) and other plants (Choesin and Boerner 1991) has also been shown. Choesin and Boerner (1991) measured the amount of allyl isothiocyanate (AI) in the soil around *Brassica napus* plants, and then applied equivalent amounts to the soil around *Medicago sativa* plants and found no significant effect of AI on *M. sativa* growth. However, they also showed that at least 99% of AI either degrades or volatilizes within 12 hours of addition to soil. Therefore, the *M. sativa* plants in their experiment were exposed to much lower AI concentrations than the authors intended.
Glenn et al. (1988) used whole *Brassica campestris* or *B. napus* plants in their experiments, so any exuded allelochemicals would be continuously replenished. They added *Brassica* seedlings to agar plates containing germinated AM fungal spores, and then monitored fungal growth for two weeks. Mycorrhizal fungi are obligately symbiotic, meaning they cannot survive without a plant host, and can only grow for a limited time using the nutrients stored in the spore. Since the AM fungi had already germinated when *Brassica* seedlings were added it is entirely possible that the fungus had stopped growing before effects from plant exudates would be seen. Another explanation for the lack of effect is that young seedlings do not produce high enough concentrations of allelochemicals for an effect to be seen. Schreiner and Koide (1993b) were able to show effects of *B. napus* and *B. kaber* seedlings on AM fungal germination, so perhaps isothiocyanates function by inhibiting germination and not by slowing growth after germination.

The observed inhibitory effects of garlic mustard and other mustards have generally been assumed to be caused by glucosinolates and isothiocyanates, but allyl isothiocyanate and allylnitrile (the active forms of the glucosinolate sinigrin) have half-lives of about 40 and 100 hours respectively, meaning they actually spend very little time in the soil (Borek et al. 1994). Several glycosides produced by *A. petiolata* (alliarinoside and many glucopyranosides) are insecticidal, but neither the half-lives of these compounds nor their bioactivity against plants and fungi have been measured.

The cyanoallyl glucoside (2Z)-4-(β-D-glucopyranosyloxy)-2-butenenitrile (alliarinoside) deters feeding of first instars of *Pieris napi oleracea* apparently by inhibiting digestion, since larvae cease feeding about four hours after beginning
(Renwick et al. 2001). A flavonoid glycoside (isovitexin 6”-O-β-D-glucopyranoside, IVG) deters feeding of fourth instars by repelling larvae, which reject leaves without feeding, but after extensive palpation with mouthparts (Renwick et al. 2001) (Figure 1.1). Concentrations of alliarinoside peak in the plant throughout the fall and winter, while IVG levels peak in the spring (Haribal and Renwick 2001). This cycle is somewhat counterintuitive given that P. napi larvae typically emerge in early spring (when alliarinoside levels are lowest), and reach the fourth instar later in the season when IVG levels are lowest. This incongruity suggests that the insecticidal effects of these compounds may be incidental, with their primary effects perhaps lying elsewhere.

Alliarinoside has only been identified in A. petiolata and IVG only from A. petiolata and Gentiana arisannensis (Lin et al. 1997), suggesting that these compounds are likely to be more novel in invaded environments than the ubiquitous glucosinolates.

The backbones of IVG, apigenin and isovitexin, have been shown to have allelopathic, estrogentic, antioxidant, antibacterial, and respiration blocking properties. Apigenin inhibits seed germination of Raphanus sativus and Arabidopsis thaliana (Basile et al. 2000, Cipollini et al. 2008b), deters feeding by Spodoptera exigua (Cipollini et al. 2008b), and weakly induces nodulation in nitrogen fixing plants (Begum et al. 2001, Suominen et al. 2003). The antibacterial properties of apigenin remain unclear with strong activity against the gram-negative bacteria Proteus mirabilis, Pseudomonas aureginosa, Enterobacter aerogenes, and E. cloaceae, and no activity against the gram-positive Staphylococcus aureus reported in one case (Basile et al. 1999). In contrast, Sato et al. (2000) found strong activity against methicillin resistant S. aureus and no activity against P. mirabilis and E. cloaceae. The apigenin was isolated from different plant
species in each study and it is possible one or both also contained an antibacterial compound. Apigenin is weakly estrogenic, with the 4’-hydroxy being important for activity (Breinholt and Larsen 1998), and may actually serve as an antiestrogen by competitively inhibiting the binding of more active estrogens to receptor sites (Lee et al. 1998). The antioxidant properties of apigenin depend on the presence of 4’,5, and 7-hydroxyl groups, a keto at the 4 position, and a double bond between the second and third carbons. Multiple hydroxyls on the B-ring increase activity and the 7-hydroxy is particularly important (Križková et al. 1998, Miyazawa and Hisama 2003).

Isovitexin is generally less active than apigenin, probably since glycosylation increases water solubility and slows movement across membranes. Isovitexin inhibits mitochondrial respiration by blocking reoxidation of ubiquinone and serving as an uncoupler. Glycosylation decreases these effects but does not eliminate them (Wagner and van Brederode 1996). Isovitexin is not inhibitory to the plant pathogenic fungi Leptosphaeria maculans and Phoma lingam (Pedras et al. 2003), and actually decreased in susceptible maize lines after inoculation with Fusarium (Reid et al. 1992). Isovitexin has almost no antioxidant activity (Watanabe et al. 1997). Glycosylation may be a protective mechanism for the plant, minimizing its own exposure to the more active non-glycosylated flavonoids.

The glucosinolates produced by A. petiolata are produced by other species native to North America (Fahey et al. 2001), but only one of the flavonoid glycosides produced by A. petiolata (IVG), has been reported in another species (Gentiana arisanensis) (Lin et al. 1997). The presumed rarity, in North America, of many of the compounds produced by A. petiolata suggests that the Novel Weapons Hypothesis may explain the invasive
success of this species. The Novel Weapons Hypothesis posits that native organisms in invaded ranges will be naïve to secondary compounds produced by invading plants, making native organisms especially sensitive to any inhibitory effects (Bais et al. 2003a).

Plant, fungal, and bacterial populations native to North America experienced greater inhibition in soils where *A. petiolata* had grown than European populations, providing further support for the Novel Weapons Hypothesis and demonstrating that secondary compounds produced by *A. petiolata* can inhibit growth of a wide range of organisms (Callaway et al. 2008). In addition, *Geum lacinimum* and *G. urbanum* germinated poorly in Petri plates in soil where *A. petiolata* had grown (Prati and Bossdorf 2004). *Acer rubrum*, *A. saccharum*, and *Fraxinus americana* grown in soil where *A. petiolata* had grown were smaller with less AMF colonization than plants grown in soil uninvaded by *A. petiolata* (Stinson et al. 2006). Also, activated carbon improved growth of *Impatiens capensis* in the presence of live *A. petiolata* plants in field soils (Cipollini et al. 2008c).

Whole plant leachates in water had similar effects and also blocked AMF spore germination (Stinson et al. 2006). Using the same extraction procedure, Roberts and Anderson (2001) found that whole plant leachates in water inhibited germination of *Lycopersicum esculentum* and the mycorrhizal fungus *Gigaspora rosea*, and growth of *L. esculentum* and *Sorghum bicolor* on enriched agar in Petri plates. Colonization of *Lycopersicum esculentum* by *G. rosea* spores did not occur, but only ungerminated spores were used, which did not germinate when exposed to *A. petiolata* leachate (Roberts and Anderson 2001). It is possible that colonization could occur if mycorrhizal inoculum
containing germinated spores, hyphal fragments, and/or colonized root fragments was used instead of ungerminated spores.

Chloroform extracts of *A. petiolata* containing allyl and benzyl isothiocyanates inhibited growth of *Triticum aestivum* and *Lepidium sativum* on water agar in Petri plates, as did purified compounds applied individually (Vaughn and Berhow 1999). These compounds have been measured in *A. petiolata* tissues (Vaughn and Berhow 1999), but not in field soils; however, if transfer rates are similar to those of other mustards (Gardiner et al. 1999, Morra and Kirkegaard 2002) then glucosinolate concentrations in the soil can be expected to average $10^{-6}$M naturally and $10^{-4}$M after a freeze-thaw cycle. These values approach reported $I_{50}$ values for glucotropaeolin [$>1 \times 10^{-3}$M (wheat), $6.6 \times 10^{-4}$M (cress)] (Vaughn and Berhow 1999), so it is possible that the glucosinolates produced by *A. petiolata*, and their degradation products, reach bioactive concentrations in field soils.

Mycorrhizal inoculum potential was weakly negatively correlated ($r^2 = 0.2940$) with *A. petiolata* abundance in the field, however all sample plots were within established *A. petiolata* stands and the highest percent colonization found was less than 8% (Roberts and Anderson 2001). Mycorrhizal inoculum potential in healthy soils is typically 50 to 60%, so this evidence strongly supports the hypothesis that *A. petiolata* suppresses mycorrhizal fungi.

In contrast, McCarthy and Hanson (1998) found no evidence for allelopathy against several plant species when using water extracts of *A. petiolata* tissues. However, their extracts were made from macerated plant tissue, which would allow glucosinolates and myrosinases to mix, leading to conversion to isothiocyanates. The most abundant
glucosinolates in garlic mustard, glucotropaeolin and sinigrin, are converted to benzyl and allyl isothiocyanates, which are highly volatile and hydrophobic. It is therefore unlikely that McCarthy and Hanson exposed their target plants to appreciable amounts of either glucosinolates or isothiocyanates. Water extracts prepared from non-macerated tissue, which may release glucosinolates without exposing them to myrosinase for degradation, can inhibit germination and growth of plant seeds and AMF spores (Roberts and Anderson 2001, Stinson et al. 2006), but do not always (Cipollini et al. 2008a). However, in none of these studies were allelopathic compounds isolated from leachates and identified.

Most of these experiments measured only germination or growth and used artificial media, so even though there is evidence supporting allelopathy as an invasive mechanism in *A. petiolata*, more must be done. Specifically, it must be shown that resource competition alone cannot explain the success of *A. petiolata*. It must also be established that the previously observed effects remain if environmentally relevant concentrations are used. This will require some knowledge of field concentrations of these compounds, as well as their half-lives. Finally, the mechanism whereby *A. petiolata* avoids severe autotoxicity should be determined.

1.3 MYCORRHIZAL FUNGI

Non-mycorrhizal plant species, like *A. petiolata*, typically grow poorly in habitats with healthy AMF networks in the soil (Grime et al. 1987). The non-mycorrhizal plant *Salsola kali* defends against AMF colonization attempts by producing high enough levels of phenolics that the plant roots die (Allen et al. 1989). Therefore, a mechanism to limit
fungal growth would be all but necessary to ensure survival of *A. petiolata* in its native range. Since North American plants and mycorrhizal fungi are likely naïve to the secondary compounds produced by *A. petiolata*, any inhibitory effects would be even more noticeable in the invaded range.

Arbuscular mycorrhizal fungi (AMF) are aseptate and multinucleate obligately symbiotic fungi of the order Glomeromycota. These fungi associate with more than 80% of woodland plant species (Smith and Read 1997) and effectively increase the surface area of plant roots and provide increased water and nutrient (especially phosphorus) uptake to the plant in exchange for photoassimilates. Even though host plants can grow alone if supplied with necessary nutrients, growth is much improved by colonization, especially in soils deficient in one or more nutrients (Smith and Read 1997). AMF increase nutrient uptake of their host plants through several mechanisms. First, fungal hyphae are smaller than plant root hairs and can therefore explore smaller soil pores than are accessible to root hairs. Second, hyphae require less energy for development and growth than plant roots. Specifically referring to phosphorus uptake, the AMF P uptake enzyme has a lower $K_m$ than the plant equivalent, meaning it is more efficient at P uptake. Finally, AMF can solubilize inorganic and organic P sources not available to plants. It is not clear how this occurs but may involve the release of chelating agents and/or phosphatases by the fungus. AMF may be especially beneficial in areas with highly calcareous soils where most of the phosphorus is unavailable to plants as insoluble $\text{Ca}_3(\text{PO}_4)_2$, which AMF can utilize (Smith and Read 1997).
Four developmental stages occur in the formation and establishment of a mycorrhizae. First, the plant seed and AMF spore must germinate (GERMINATION). Second, the plant seedling and the AMF grow independently of each other before symbioses formation is initiated (PRESYMBIOSIS GROWTH). Third, the symbiosis is formed (SYMBIOSIS FORMATION), and fourth the symbiosis continues to grow and is maintained by both partners (SYMBIOSIS GROWTH).

Within the GERMINATION stage, germination of both plant seed and AMF spore must occur. Even though AMF are obligately symbiotic, they can germinate in the absence of a host plant. During the PRESYMBIOSIS GROWTH stage, the plant root and fungal hyphae lengthen without making contact with each other. In the absence of a host plant, hyphal networks totaling 20-30 mm of hyphal length have been observed (Smith and Read 1997). Spore germination and hyphal growth are enhanced in the presence of plant exudates, specifically those containing flavonoid compounds (Gianinazzi-Pearson et al. 1989, Tsai and Phillips 1991, Bécard et al. 1992).

As the hyphae near an acceptable host root, the SYMBIOSIS FORMATION stage is entered and hyphae branch extensively to initiate more than one contact point with the host. This step does require the presence of a host plant. At each contact point, the hyphae swell and form appresoria that adhere to the root, before narrowing into penetration pegs that actually enter the plant root. Intercellular hyphae extend throughout the plant root in extracellular spaces. The likely areas of nutrient exchange between the plant and AMF are either arbuscules or coils depending on the type of mycorrhizae, but both cause the plant cell plasma membrane to invaginate extensively, creating large amounts of surface area for nutrient exchange. Arbuscules and coils are transient.
structures, lasting about a week at the most. Vesicles are longer-lived structures formed mostly in extracellular spaces and likely function in nutrient storage. Auxiliary cells are similar to vesicles but are formed outside the root.

During the SYMBIOSIS GROWTH stage intercellular hyphae and arbuscules will continue to form, and extraradicle hyphae will grow back out of the plant root and perform various functions. Some extend much like plant roots and explore the soil for nutrient and water sources, and since mycorrhizal hyphae are so much smaller than plant roots they are more efficient at depleting these pools than their host plants (Smith and Read 1997). Some hyphae extend to other roots on the same plant, conspecifics, and even different species, and initiate new colonization points (Friese and Allen 1991). This results in a vast underground network that interconnects plants that appear discrete aboveground. Other hyphae develop to form asexual mycorrhizal spores, which resist desiccation and can be easily blown by the wind to new locations (Smith and Read 1997).

AMF have been shown to increase invasion success in some cases while protecting communities from invasion in others. *Centaurea maculosa* (spotted knapweed) releases allelopathic compounds that directly inhibit the growth of competing plants (Bais et al. 2003a), while maintaining a higher AMF colonization than those same plants (Marler et al. 1999), giving it a distinct competitive advantage. *Centaurea* species even draw photosynthate from other plants to themselves through the mycorrhizal network, further increasing their competitiveness (Carey et al. 2004). The invasive success of *Bidens pilosa*, an aster invading Hawaii, was slightly enhanced by two *Scutellospora* species, but significantly depressed by *Glomus sparcum* (Stampe and Daehler 2003). Growth of an invasive shrub in Florida, *Ardisia crenata*, was enhanced when colonized
by all of its associated AMF, but not when colonized with monospecific isolates (Bray et al. 2003), although this enhanced growth did not clearly enhance competitiveness.

Impacts of allelochemicals on AMF have rarely been demonstrated. Aqueous extracts of *Artemisia campestris*, an Asian native invading Great Lakes sand dunes, decreased AMF colonization in three native grasses (Yun and Maun 1997). In fact, no arbuscules were observed after exposure to the extracts, suggesting that the native grasses were receiving little if any benefit from the symbioses. No attempt was made to isolate specific compounds or to measure field concentrations, but other *Artemisia* species do produce allelopathic terpenoids (Yun and Maun 1997).

1.4 IMPLICATIONS FOR RESTORATION

Since Brassicaceous plants are non-mycorrhizal (Tester et al. 1987), allelopathy against mycorrhizae would be an effective invasion mechanism for *A. petiolata*, especially in phosphorus poor sites where many plants are particularly dependent on AMF (Hetrick et al. 1984). AMF are obligately symbiotic, and the spore bank in the soil is drastically reduced within two years in the absence of a host (Smith and Read 1997). Restoration of areas where *A. petiolata* has been established longer than two years may therefore need to include re-introduction of AMF. An understanding of the life stages affected by allelopathic compounds will better inform decisions about which life stages to reintroduce to a site, for example, when deciding whether spores or infected roots should be used for re-inoculation, or whether colonized plants are necessary.
1.5 OBJECTIVES

- Determine the field concentrations and half-lives of alliarinoside and the flavonoid glycosides produced by *A. petioalta*

- Test the Novel Weapons Hypothesis by comparing effects of *A. petiolata* on AMF from its native and invaded ranges, and by surveying native Brassicas for glucosinolate and flavonoid production

- Verify that the invasiveness of *A. petiolata* cannot be explained by resource competition through a density-dependent phytotoxicity experiment

- Compare AMF species diversity inside and outside of established *A. petiolata* patches

- Determine which plant and AMF life stages are least susceptible to the effects of *A. petiolata* using glass plate chambers

- Explore the effectiveness of various restoration protocols using a soil conditioning approach
1.6 REFERENCES


Figure 1.1. Putative allelochemicals produced by *Alliaria petiolata*.

Sinigrin and glucotraepolin are glucosinolates, which degrade to allyl isothiocyanate and benzyl isothiocyanate, respectively. IVG is the most abundantly produced flavonoid glycoside.
2 FIELD CONCENTRATIONS AND HALF-LIVES OF ALLIARINOSIDE AND THE FLAVONOID GLYCOSIDES PRODUCED BY ALLIARIA PETIOLATA

2.1 INTRODUCTION

Given the obvious and copious production of distasteful and malodorous compounds by many plant species, it is not surprising that so many plant species have been suggested to produce compounds that inhibit the growth of surrounding plants, a phenomenon known as allelopathy (Rice 1974). Allelopathy has been convincingly demonstrated for many species, including *Ailanthus altissima* and *Alliaria petiolata*, using a soil conditioning approach, where soil the allelopath has grown in inhibits growth of surrounding plants (Lawrence et al. 1991, Prati and Bossdorf 2004, Stinson et al. 2006). Another approach uses activated carbon, which adsorbs organic compounds, as a soil additive to demonstrate that organic compounds released into the environment by allelopaths, including *Centaurea* spp. and *A. petiolata*, limit growth of other plants (Mahall and Callaway 1992, Prati and Bossdorf 2004).

Despite the strengths of these two approaches, alone they cannot identify specific allelopathic compounds or show that the allelopathic compounds themselves are sufficient to inhibit growth of other plants. Bioassays monitoring seed germination rates after exposure to plant extracts have been used to demonstrate inhibition by specific
compounds or fractions, but these types of assays have been widely criticized for their lack of biological relevance (Harper 1975, Stowe 1979, Weidenhamer et al. 1989). A major criticism involves the concentrations used in bioassays, which are often assigned in an arbitrary fashion. For example, in bioassays testing allelopathic effects of extracts from garlic mustard [*Alliaria petiolata* (Bieb.) Cavara & Grande], doses have ranged from 1 to 100 mg tissue equivalents per g assay media (Table 2.1), with only one of the five papers cited providing a rationale for choosing the dose used (Callaway et al. 2008).

In order to provide biologically relevant results, bioassays must use not only concentrations that mimic field levels, but doses must be reapplied as needed to maintain those levels (Williamson 1990). Unfortunately, for many species (including *A. petiolata*) nothing is known about field concentrations or half-lives of potential allelopathic compounds.

*Alliaria petiolata* produces a suite of secondary metabolites including glucosinolates, a cyanoallyl glycoside called alliarinoside, several flavonoid glycosides, and cyanide (Haribal and Renwick 1998, Vaughn and Berhow 1999, Haribal and Renwick 2001, Haribal et al. 2001, Renwick et al. 2001, Cipollini and Gruner 2007). The most abundantly produced glucosinolates, sinigrin and glucotropaeolin, are more abundant in belowground tissues than aboveground tissues, reaching concentrations of 3.5 and 50.2 mg per g freeze dried tissue, respectively (Vaughn and Berhow 1999). Sinigrin shows little seasonal variation, but glucotropaeolin is more than three times as abundant in the autumn than the spring (Vaughn and Berhow 1999). Isovitexin-6’’-O-β-D-glucopyranoside (IVG) is the most abundantly produced flavonoid glycoside and reaches levels of 12-15 mg per gram fresh tissue during the winter (Haribal and Renwick 2001).
Levels of IVG decline during the summer when levels of alliarinoside are highest; alliarinoside levels are lowest in the spring (Haribal and Renwick 2001). Cyanide reaches approximately ten times higher levels in aboveground tissues than belowground, with levels higher in younger leaves than old, and maximal levels of about 100 ppm fresh weight (Cipollini and Gruner 2007). Seasonal differences in cyanide production have not been investigated, but levels remained relatively constant over 8 weeks in a greenhouse (Cipollini and Gruner 2007).

Sinigrin is remarkably stable in non sterile soil water with a half life greater than 120 days (Tsao et al. 2000). Glucotropaeolin is much less stable with reported half lives in soil between 6 hours and 9 days (Gimsing et al. 2006, Gimsing et al. 2007). These glucosinolates degrade predominantly to allyl isothiocyanate and benzyl isothiocyanate, which have half-lives in soil of 1-40 days and less than 2 days, respectively (Tsao et al. 2000, Gimsing et al. 2007). Nothing is known about the stability of alliarinoside or the flavonoid glycosides produced by *A. petiolata*, or the concentrations in soil around *A. petiolata* of any of the compounds discussed here. The objectives of this experiment were to chronicle the levels of sinigrin, glucotropaeolin, alliarinoside and the flavonoid glycosides in the rhizosphere of *A. petiolata* throughout the plant’s life cycle, and to determine the half-lives of alliarinoside and the flavonoid glycosides in order to determine ecologically relevant concentrations for use in bioassays, that mimic field conditions.
2.2 METHODS

2.2.1 Bulk Soil Extractions

In September, 200 g soil were collected from the center of 30.5 cm square plots in the Wright State University Nature Preserve (39°47.0’N, 84°3.0’W), containing 1, 8, or 13 *A. petiolata* plants. Glucosinolates were extracted by boiling 100 g soil in 50 mL of 70% methanol for 10 minutes, then desulfating before HPLC analysis (Kiddle et al. 2001). Alliarinoside and flavonoids were extracted by boiling 100 g soil in 400 mL ethanol for 10 minutes, then drying and resuspending the extract in water before HPLC analysis (Callaway et al. 2008). All HPLC analyses were conducted using a Phenomenex Luna C18 (150 x 4.6 mm, 5µm) column and a Waters 2690 HPLC system equipped with a Waters 996 photodiode array detector. Identities of compounds were verified initially by electrospray ionization (ESI)/MS and MS/MS on a liquid chromatography quadrapole (LCQ) ion-mass spectrometer (ThermoQuest, USA), then by retention time and UV spectra.

2.2.2 Biomimetic Extractions

Field soil was monitored monthly for nine months using biomimetic extraction (Weidenhamer 2005). Ten *A. petiolata* patches, five containing only first year rosette stage plants and five containing only second year bolting plants were monitored and together provided a profile of chemicals found in the rhizosphere throughout the two-year life cycle of *A. petiolata*. Five sites with no *A. petiolata* were used as controls. Sites also contained *Acer saccharum*, *Anemonella thalictroides*, *Arisaema atrobrubens*, *Asarum*.
canadense, Cardamine concatenata, Galtum sp., Geum sp., Impatiens sp., Podophyllum peltatum, Smilacena racemosa, and Trilium sp. Alliaria petiolata density in one-meter square plots was recorded monthly and rosette plants were removed from bolting and control plots, while bolting plants were removed from rosette and control plots. A grid was laid out around a marked plant each sampling date and the corners of the plot may have rotated through time, leading to fluctuations in observed plant density.

A ten centimeter long piece of polydimethylsiloxane (PDMS) tubing (0.30 mm ID, 0.64 mm OD) was inserted in the center of each plot, and into the rhizosphere of an A. petiolata plant (except for control plots). The PDMS absorbs organic compounds from the soil in a manner analogous to live plant roots, and has been used to isolate analytes with a range of polarities (Tredoux et al. 2000, Weidenhamer 2005). Preliminary data showed that probes buried for less than 3 months did not absorb reliably detectable levels of compounds. Therefore, a new probe was buried each month, and collected after 3 months in the soil. For example, data reported for February represent probes buried in January and collected in March. PDMS tubing was desorbed in acetonitrile before HPLC analysis for alliarinoside and flavonoids (Haribal and Renwick 2001). This allowed the determination of compounds released by decomposition of, or leaching from, above ground plant parts as well as those exuded by living roots.

2.2.3 Half-Lives

Alliaria petiolata extracts were prepared by boiling leaves and roots in ethanol followed by a solvent change to water. Extracts were adjusted to a concentration of 2.69 gram leaf equivalents (gle) per mL (modified from Haribal and Renwick 2001). Extract
(9.45 mL) was mixed with 30g air-dried, sieved (<2mm) field soil to reach a final concentration of 0.85 gle per g. Preliminary experiments determined that this concentration was the lowest that would allow recovery of compounds from the soil that were above detection limits. Treatments included sterile soil at 25°C, non-sterile soil at 25°C, and non-sterile soil at 5°C to examine the influence of living microorganisms in the degradation of these compounds, and to inform planning for seed germination experiments that were conducted at cold temperatures. Soil was sterilized by autoclaving at 121°C for 20 minutes, followed by a 24 hour rest at 25°C, then autoclaving again at 121°C for 20 minutes (Trevors 1996). (Trevors 1996) Five flasks were prepared for each soil treatment, capped with foil and stored in the dark.

At each sampling date, soil in each flask was mixed and 2 g of soil from each flask was extracted as above to give a concentration of 5 ge/mL. Soil was sampled immediately after set up, then at 0.5, 1, 2, 4, 8, 12, 18, 24, and 36 hours after set up. Preliminary results suggested that compounds would not remain above detectable levels for more than 2 days. Glycosides were analyzed by HPLC (Haribal and Renwick 2001). Peak areas at 354 nm were used as a surrogate for concentration, declines in natural log of peak areas were modeled assuming first-order kinetics, the slopes of the best fit regression lines were defined as K (the degradation rate) and half-lives were calculated as \( \ln 2/K \) (Macías et al. 2004). The influence of soil treatment on the half-lives of each compound was compared individually using ANOVA with soil type as a fixed factor with SAS v9.1 (SAS Institute Inc. Cary, NC). Data for Compound 1 and IVG were log transformed. Data for Compound 2 did not need to be transformed to meet assumptions of ANOVA.
2.3 RESULTS

2.3.1 Bulk Extractions

Glucosinolates, alliarinoside, and the flavonoid glycosides produced by *A. petiolata* were undetectable in extracts of bulk field soil (Figure 2.1). This approach was therefore abandoned in favor of biomimetic extraction techniques.

2.3.2 Biomimetic Extractions

Abundance of rosette stage plants peaked in March at approximately 200 plants per square meter, then declined to less than 20 plants per square meter by August (Figure 2.2). Density of bolting stage plants was low and relatively constant throughout the experiment (Figure 2.2).

IVG was the only compound detected in the PDMS tubing, and was found in plots containing *A. petiolata* during January through March, coinciding with *A. petiolata* germination and the period of highest plant density (Figure 2.2). Very low levels of IVG were found in control plots in January (Figure 2.2).

2.3.3 Half-Lives

Three compounds were reliably extracted from spiked soils and demonstrated exponential decay, IVG and two unidentified flavonoid glycosides (Figure 2.3). Compound 1 had $\lambda_{\text{max}}$ values of 270 and 325 nm, while compound 2 had $\lambda_{\text{max}}$ values of
272 and 328 nm. Half-lives varied with compound, but were significantly longer in sterile soil than live soil at either temperature (Tables 2.2, 2.3). Temperature did not affect half-lives of any compound (Tables 2.2, 2.3).

Although the extract used to spike soils was prepared from plants collected in September when alliarinoside levels should have been high (Haribal and Renwick 2001), alliarinoside was not present in the extract and its degradation was therefore not assessed. Several other unidentified compounds sporadically appeared but did not follow exponential decay curves, so half-lives could not be calculated. These compounds also did not exist in large enough amounts to obtain UV spectra, so no identification was attempted.

2.4 DISCUSSION

The very short half-lives of the glucosinolates and flavonoid glycosides produced by *A. petiolata* likely account for the difficulties in extracting these compounds from bulk field soils. Biomimetic extraction was more successful, but compounds were still detected only in the spring when *A. petiolata* density peaked. Microbial degradation appears to be important for the flavonoid glycosides produced by *A. petiolata*, since half-lives were longer in sterile soils than in live soils. Interestingly, temperature had no effect on half-lives in live soils.

Compound abundance coincided with the period of peak plant density, but the precise mechanism of entry of allelopathic compounds into the soil was not determined. Live plants could release allelopathic compounds by root exudation or by leaching from leaves during rainfall (Rice 1974). Growth of *Impatiens pallida* was inversely correlated
with amount of dead *A. petiolata* root tissue in the soil, suggesting that allelopathic compounds also leach from decaying plant material (Barto and Cipollini 2008). Seedlings in the study site germinated in late March, and seedling mortality can be as high as 50% in early spring (Cavers et al. 1979), potentially leading to large inputs of dead plant tissue into the soil in early spring that could also contribute to high levels of IVG in early spring. Regardless of the mechanism of entry of allelopathic compounds into the soil, the highest levels were observed in probes that were buried during January through April, and suggest that allelopathic effects mediated by IVG will be most pronounced in early spring.

IVG and both flavonoid glycosides for which half-lives were calculated degrade so quickly (\(t_{1/2} = 3\) to 12 hours in live soil) that their functional viability as allelopathic compounds must be questioned. Although none of the glycosides produced by *A. petiolata* appear stable enough to accumulate in the soil, they may still be responsible for allelopathic effects if levels in the soil are continually replenished by living plants, or if the glycosides must be modified before becoming allelopathic. Toxic compounds produced by plants are often stored in plant tissues in their glycosylated forms, which serves to increase their water solubility and limit their toxicity. Both of these attributes are advantageous only before the compounds are released to the environment, and many glycosylated compounds quickly shed their sugars to become aglycones (Weidenhamer and Romeo 2004, Rasmussen et al. 2005, Johansen et al. 2007, Kong et al. 2007). In fact, Brassicaceous plants produce myrosinase, an enzyme that speeds up this process for glucosinolates (Bones and Rossiter 1996).
Allelopathic mechanisms where a plant produces a non-toxic compound that quickly degrades in the environment to produce a noxious compound appear to be widespread. *Oryza sativa* produces and exudes two flavone glycosides, which quickly degrades to produce allelopathic aglycones (Kong et al. 2007). *Sorghum bicolor* produces dhurrin, a glycosylated compound with a half-life of less than two hours in soil, which degrades to the toxic compound hydrogen cyanide (Johansen et al. 2007). *Polygonella myriophylla* produces quercetin and rhamnetin, glycosylated compounds which rapidly decompose in soil to the allelopathic gallic acid and hydroquinone (Weidenhamer and Romeo 2004). The production by plants of non-toxic compounds which rapidly decompose to toxic compounds extends beyond the simple mechanism of glycosylation. Ceratolin, which is not glycosylated, degrades to the allelopathic hydrocinnamic acid (Tanrisever et al. 1987).

The flavonoid glycosides produced by *A. petiolata* could degrade to isovitexin, which still retains one sugar, and finally the aglycone apigenin. These compounds are strongly bioactive, with apigenin inhibiting seed germination of *Raphanus sativus* and *Arabidopsis thaliana* (Basile et al. 2000, Cipollini et al. 2008) and mediating symbiotic interactions between plants and nodulating bacteria (Begum et al. 2001, Suominen et al. 2003). Isovitexin is generally less active than apigenin, but does inhibit mitochondrial respiration (Wagner and van Brederode 1996).

The mechanisms of allelopathy by *A. petiolata* are quite complex and more research is needed to precisely identify the bioactive compounds. Cyanide is produced by *A. petiolata* (Cipollini and Gruner 2007), and could be released by alliarinoside, although work is needed to verify this. Degradation of the flavonoid glycosides needs to be more
closely monitored and stability and bioactivity of each degradate determined. Until such data becomes available, soil conditioning approaches (Barto and Cipollini 2008) or bioassay doses estimated from known tissue and soil concentrations of similar compounds in related plants (Callaway et al. 2008) will continue to provide ecologically relevant models for assessing allelopathic effects.
2.5 REFERENCES


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Table 2.1. Doses used to test allelopathic potential of *Alliaria petiolata*, the justification (if given) for the dose used, and results of any bioassays.

<table>
<thead>
<tr>
<th>Dose&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Justification</th>
<th>No effect</th>
<th>Inhibition</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 none</td>
<td>No effect on germination or biomass of lettuce, rye, radish, and vetch.</td>
<td></td>
<td>McCarthy and Hanson 1998</td>
<td></td>
</tr>
<tr>
<td>3.3 Calculated from values of glucosinolates in tissue and soils near <em>Brassica</em> spp assuming similar transfer rates for garlic mustard.</td>
<td>Decreased spore viability of North American AM fungi.</td>
<td>Callaway et al. 2008</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.7&lt;sup&gt;b&lt;/sup&gt; none</td>
<td>Inhibited cress and wheat radicle elongation.</td>
<td>Vaughn and Berhow 1999</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Estimated field levels are approximately 3.3 mg tissue equivalents/g soil. Any doses higher than this are unlikely to be biologically relevant.*

Reduced growth and mycorrhizal colonization of sugar maple, red maple, and white ash.

Stinson et al. 2006

10.0  none

No effect on germination or biomass of lettuce, rye, radish, and vetch.

McCarthy and Hanson 1998

25.0  Cited Cote and Thibault (1988), who used a range of doses and gave no justification.

No effect on germination of sorghum.

Inhibited germination of Gigasporea rosea; reduced germination and root length in tomato; reduced root length in sorghum.

Roberts and Anderson 2001
25.0  Cited Roberts and Anderson (2001), who cited Cote and Thibault (1988), who used a range of doses and gave no justification.

28.5  none  Reduced germination of Glomus spp. and Acaulospora spp. Stinson et al. 2006

57.0  none  Inhibited cress and wheat radicle elongation. Vaughn and Berhow 1999

100.0 none  Inhibited cress and wheat radicle elongation. Vaughn and Berhow 1999

Inhibited cress and wheat radicle elongation. McCarthy and Hanson 1998

Doses given in mg tissue equivalents per g assay media. In order to calculate this dose we assumed that the lyophilized extract was pure benzyl glucosinolate, that the garlic mustard tissue used for the extract contained 53 mg glucosinolates per g freeze-dried tissue, and that 1 g dry garlic mustard tissue is equal to 3 g fresh weight. In order to calculate this dose we assumed that a 6-inch pot holds 750 g soil.
Table 2.2. Half-lives, in field soil, of flavonoid glycosides produced by *A. petiolata*.

Half-lives with different bold letters were significantly different at $\alpha = 0.05$. Data for each compound were analyzed individually.

<table>
<thead>
<tr>
<th>Retention Time (min)</th>
<th>Sterile Soil</th>
<th>Live Soil at $5^\circ$C</th>
<th>Live Soil at $25^\circ$C</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.6</td>
<td>$45.5 (\pm 6.3)_{\text{a}}$</td>
<td>$10.7 (\pm 1.2)_{\text{b}}$</td>
<td>$9.5 (\pm 2.0)_{\text{b}}$</td>
</tr>
<tr>
<td>13.4</td>
<td>$12.8 (\pm 1.0)_{\text{a}}$</td>
<td>$4.7 (\pm 0.4)_{\text{b}}$</td>
<td>$3.1 (\pm 0.6)_{\text{b}}$</td>
</tr>
<tr>
<td>14.1</td>
<td>$33.9 (\pm 6.2)_{\text{a}}$</td>
<td>$11.2 (\pm 0.7)_{\text{b}}$</td>
<td>$8.9 (\pm 1.0)_{\text{b}}$</td>
</tr>
</tbody>
</table>
Table 2.3. ANOVA table for half-life experiment.

<table>
<thead>
<tr>
<th>Retention Time (min)</th>
<th>df (model, error)</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.6</td>
<td>2,9</td>
<td>27.27</td>
<td>0.0002</td>
</tr>
<tr>
<td>13.4</td>
<td>2,11</td>
<td>52.45</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>14.1</td>
<td>2,11</td>
<td>34.21</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
Figure 2.1. HPLC chromatographs of bulk soil extracts from 0.09 square meter plots containing 1, 8, or 13 *A. petiolata* plants.
Figure 2.2. Seasonal abundance of *A. petiolata* plants and levels of isovitexin-6”-β-D-glycopyranoside (IVG) recovered from PDMS probes buried in the soil for three months.

A. Plots with rosette stage plants only. B. Plots with bolting stage plants only.
Figure 2.3. HPLC chromatograph of a spiked soil sample immediately after spiking. Compounds 1 and 2 are flavonoid glycosides, IVG - isovitexin-6"-β-D-glycopyranoside.
3 TESTING THE NOVEL WEAPONS HYPOTHESIS USING *ALLIARIA PETIOLATA*

3.1 INTRODUCTION

Success of invaders is a complicated, multifaceted issue and several theories have been advanced to explain the success of invasive plants. These theories typically include some facet of enemy release (Crawley 1997), since invaders usually lack specialized herbivores in their invaded ranges. This lack of herbivore pressure can lead to decreased production of costly defensive secondary metabolites and increased growth, as suggested by the Evolution of Increased Competitive Ability hypothesis (EICA) (Blossey and Nötzold 1995). While some support has been found for EICA (Blossey and Nötzold 1995, Siemann and Rogers 2001, Rogers and Siemann 2004), many invasive plants retain high levels of defensive compounds and/or protection from herbivory (Buschmann et al. 2005, Cipollini et al. 2005, Stastny et al. 2005). It is therefore likely that the compounds responsible for limiting herbivory in these plants are not being selected against and may actually contribute to the invasive success of the plant.

The Novel Weapons hypothesis (NWH) provides an explanation for the maintenance of defenses in invaders, suggesting that native organisms in invaded ranges will be naïve and therefore especially sensitive to the novel compounds produced by invasive plants (Bais et al. 2003). These novel weapons provide invaders with protection
from herbivory (Carpenter and Cappuccino 2005), and can also directly inhibit other plants and mycorrhizal fungi, a phenomenon known as allelopathy. Species that share a co-evolutionary history with the invader are expected to be more resistant to these allelopathic effects. The predictions of the NWH have been met for two Eurasian Centaurea species, which inhibit growth of North American grasses more than growth of European grasses (Callaway and Aschehoug 2000, Bais et al. 2003). Lepidium draba, Vaccinium myrtillus, and Alliaria petiolata also more strongly inhibit growth of plants from outside their native ranges (Mallik and Pellissier 2000, Prati and Bossdorf 2004, McKenney et al. 2007).

Alliaria petiolata (M.Bieb.) Cavara & Grande, Brassicaceae, (garlic mustard), an herbaceous biennial, was introduced to North America from Europe in the 1860’s and has since become invasive in much of North America (Nuzzo 2002). Alliaria petiolata can form dense monocultures in the undisturbed forest understory, adversely impacting native plant abundance and biodiversity (McCarthy 1997). Allelopathic effects of A. petiolata have been demonstrated against other plants and arbuscular mycorrhizal fungi (Vaughn and Berhow 1999, Roberts and Anderson 2001, Prati and Bossdorf 2004, Stinson et al. 2006).

Biologically active secondary compounds produced by A. petiolata include the glucosinolates glucotropaeolin and sinigrin and their degradates, a cyanoallyl glycoside called alliarinoside, several flavonoid glycosides, and cyanide (Haribal and Renwick 1998, Vaughn and Berhow 1999, Haribal and Renwick 2001, Haribal et al. 2001, Renwick et al. 2001, Cipollini and Gruner 2007). The evolutionary implications of the NWH suggest that other Brassicaceae closely related to A. petiolata will be most likely to
share similar phytochemical profiles. Species within the Brassicaceae can be divided into 25 tribes, with *A. petiolata* in the Thlaspi-deae (Figure 3.1). Of the 564 Brassicaceae species native to North America, none are in the Thlaspi-deae (USDA Plants Database, Al-Shehbaz et al. 2006). However, the closely related tribe Arabideae includes 183 species native to North America, 5 of which are found in the Midwest in forest understory habitats typically invaded by *A. petiolata* (Table 3.1) (USDA Plants Database, Al-Shehbaz et al. 2006). The presence of these compounds in Brassicaceous plants native to North America has not yet been fully assessed, and according to the NWH, they are most likely to occur in species closely related to *A. thaliana*, such as those in the Arabideae.

*Alliaria petiolata* does not form associations with symbiotic mycorrhizal fungi and rapidly invades disturbed woodland edges which are poor habitat for most plants partly because of low mycorrhizal inoculum potential. Arbuscular mycorrhizal fungi (AMF) are obligately symbiotic organisms that associate with plant roots and provide increased access to nutrients and water in exchange for fixed carbon from the plant. Although about 80% of woodland plants associate with AMF (Smith and Read 1997), *A. petiolata* does not (Tester et al. 1987), so inhibition of mycorrhizae would reduce interspecific competition with no risk of autotoxicity. A North American plant species naïve to *A. petiolata* suffered even greater inhibition than a plant species from *A. petiolata*’s native range (Prati and Bossdorf 2004), and AMF species are expected to follow the same patterns although this has not been tested yet.

The glucosinolates produced by *A. petiolata* are widely assumed to be responsible for its observed allelopathic effects, and while glucosinolates are definitely bioactive...
(Yamane et al. 1992, Schreiner and Koide 1993a, b, Vaughn and Berhow 1999), this assumption may be unfounded. The glucosinolates are ubiquitous among the Brassicaceae and therefore unlikely to be novel in North America. The glucosinolates produced by *A. petiolata*, glucotropaeolin and sinigrin, are also produced by species in eight other plant families (Fahey et al. 2001).

Several compounds produced by *A. petiolata* are arguably less commonly produced than the glucosinolates and are therefore more likely to be novel in North America. Alliarinoside, known only from *A. petiolata*; and isovitexin 6”-O-β-D-glucopyranoside (IVG), to date found only in *A. petiolata* and *Gentiana arisanensis*, inhibit feeding by the caterpillar *Pieris napi oleracea* (Lin et al. 1997, Renwick et al. 2001). Concentrations of alliarinoside peak in the plant throughout the fall and winter, while IVG levels peak in the spring (Haribal and Renwick 2001). This cycle is somewhat counterintuitive given that *P. napi* larvae typically emerge in early spring when alliarinoside levels are lowest, and reach the fourth instar later in the season when IVG levels are lowest. This incongruity suggests that the insecticidal effects of these compounds may be incidental, with their primary effects lying elsewhere.

The objectives of this experiment were to survey North American Brassicaceae for the presence of flavonoids and glucosinolates, compare AMF spore viability in North American and European soils exposed to *A. petiolata* extracts, and determine whether glucosinolate or flavonoid compounds were responsible for any observed effects. The Novel Weapons hypothesis predicts that compounds produced by *A. petiolata* will not be found in North American species, and that North American species will be more sensitive to these compounds than European species.
3.2 METHODS

3.2.1 Phytochemical Comparisons

Cauline leaves were collected from three plants each of *A. petiolata*, *Arabis laevigata* (rock cress), *Cardamine concatenata* (cutleaf toothwort), *Cardamine bulbosa* (spring cress), and *Cardamine douglassii* (purple cress) in early spring while the plants were flowering. *Arabis laevigata* is in a common tribe of Brassicaceae native to North America, and is also closely related to *A. petiolata* (USDA Plants Database, Al-Shehbaz et al. 2006), so it is likely to produce similar secondary compounds. The *Cardamine* species represent an abundant tribe in North America (Table 3.1) (USDA Plants Database, Al-Shehbaz et al. 2006), with *C. concatenata* being especially common in areas invaded by *A. petiolata* (D Cipollini, personal observation), and these species may therefore have driven strong co-evolutionary selection for resistance to compounds in their phytochemical profiles. *A. petiolata*, *C. concatenata*, and *C. douglassii* were collected at Wright State University, OH (39°48.0’N, 84°1.0’W). *A. laevigata* was collected in John Bryan State Park, OH (39°47.3’N, 83°51.1’W), and *C. bulbosa* was collected in Glen Helen Nature Preserve, OH (39°47.3’N, 83°52.8’W).

Flavonoids and alliarinoside were analyzed as in Cipollini *et al.* (2005), but with the flow conditions shown in Table 3.2. Glucosinolates were analyzed as described by Kiddle *et al.* (2001) using a Phenomenex Luna C18 (150 x 4.6 mm, 5µm) column and a Waters 2690 HPLC system equipped with a photodiode array detector (Waters 996), with a flow rate of 1 mL/min. Alliarinoside and the flavonoids produced by *A. petiolata* were
initially identified using electrospray ionization (ESI)/MS and MS/MS on a liquid chromatography quadrupole (LCQ) ion-trap mass spectrometer (ThermoQuest, USA). Subsequent identifications were based on retention time and UV spectra. The glucosinolates sinigrin and glucotropaeolin were identified by comparison with standards [sinigrin monohydrate from horseradish (Sigma-Aldrich, St. Louis, MO, USA), glucotropaeolin (POS Pilot Plant Corp, Saskatoon, SK, Canada]. Compounds that could not be identified were distinguished from each other by retention time and UV spectra.

3.2.2 Bioassay

Fractions enriched in flavonoids or glucosinolates were isolated from fresh leaf tissue of *A. petiolata* plants collected from a forest in southwestern Ohio (Wright State University, 39°48.0’N, 84°1.0’W). Production of glucosinolates in roots and leaves is well known in *A. petiolata* (Vaughn and Berhow 1999) and other plants in the Brassicaceae (Fahey et al. 2001). Flavonoids are also produced in both leaves and roots of *A. petiolata* plants (Figure 3.2). These extracts should therefore include compounds that enter the soil through exudation, as well as those which leach from leaves during rainfalls or from decomposing tissues.

Leaf tissue was boiled in ethanol, then filtered, dried, and defatted with hexane (modified from Haribal and Renwick 2001). The dried extract was dissolved in water, and then partitioned with *n*-butanol to isolate flavonoids and alliarinoside, while the charged glucosinolates remained in the water. Both extracts were dried and re-dissolved in water. Fractionated extracts were characterized using HPLC as described above to verify locations of compounds. The flavonoid fraction was enriched in glycosides,
including alliarnoside, isovitexin-6’-β-D-glucopyranoside, and other flavonoid glycosides. We also isolated a glucosinolate fraction devoid of these glycosides but enriched in glucosinolates, of which sinigrin and glucotropaeolin are known to predominate in *A. petiolata* (Vaughn and Berhow 1999) (Figure 3.3).

Ideally, dosages in allelopathy studies should be equivalent to levels found in field soil. However, there are no published reports of *A. petiolata* metabolite levels in soil. A related species, *Brassica napus*, has glucosinolate levels of 14-20 µmol/g leaf tissue (Gardiner et al. 1999, Morra and Kirkegaard 2002). Isothiocyanates, which are the breakdown products of glucosinolates, reach levels of 40 to 75 nmol per gram in soils following incorporation of *B. napus* plant material (Morra and Kirkegaard 2002). Assuming that a similar relationship exists for *A. petiolata*, soil levels should be about 300 times less than levels in the leaves, and extracts were diluted accordingly to give 0.0033 gram leaf equivalents (gle) per gram of soil. The lowest dose used to show allelopathic effects of *A. petiolata* in the past was 0.05 gle/mL extract (Table 2.1), which is an order of magnitude higher than the dose used here. McCarthy and Hanson (1998) used doses as low as 0.001 gle/mL extract, but only found inhibitory effects at 0.1 gle/mL extract. Although this dosage is based on data from a separate species, it is more ecologically relevant than the higher doses used in earlier studies.

Extracts were applied as either flavonoid, glucosinolate, or mixed fractions to soils by collaborators at The University of Montana. Soils were collected from six sites in North America [Oregon (45°30.3’N, 122°41.4’W), Vermont (42°52.3’N, 73°11.3’W), Massachusetts (42°31.4’N, 72°11.1’W), Minnesota (44°10.5’N, 93°58.4’W), Wisconsin (44°52.5’N, 91°41.2’W), and Indiana (39°11.4’N, 86°30.5’W)], and six sites in Europe
[Germany (51°28.3’N, 11°58.2’E), Spain (42°49.2’N, 1°17.3’W), Hungary (47°37.5’N, 18°19.2’E), Romania (47°10.2’N, 27°36.3’E), the Czech Republic (48°46.1’N, 14°18.1’E), and France (44°50.5’N, 0°28.0’W)]. These soils were dried slowly at room temperature and stored dry until use. On 2 February 2006, 3 gm of soil from each region was placed into each of 20 15 ml centrifuge tubes (total n=240). For each region, 5 samples each were treated with the flavonoid fraction, the glucosinolate fraction, and both glucosinolate and flavonoid fractions to give a final dose of 0.0033 gram leaf equivalents (gle) per gram of soil. Each treatment was applied in 1.5 mL of water and tubes were centrifuged for 15 seconds to spread the solution throughout the 3 gm of soil.

On 14 February, 2006 these soils were analyzed for AM spore viability by collaborators at the University of Guelph. AM fungal spores were extracted directly from the soil using a wet-sieving/centrifugation method (Klironomos et al. 1993), and counted under 20X magnification. Viability of those spores was assessed after placing them in iodonitrotetrazolium solution (1 mg ml⁻¹) for 48 hours (Carvalho et al. 2004).

3.2.3 Statistical analysis

There was very little overlap in chemical profiles between species, so phytochemical comparison data were not analyzed statistically.

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

3.3 RESULTS

3.3.1 Phytochemical Comparisons

Phytochemical profiles were consistent within a species, and showed very little overlap of compounds between species (Figure 3.4). None of the compounds produced by *A. petiolata* were also produced by any of the North American species surveyed. Among glucosinolates, compound 2 was present in two North American species, two of the three *Cardamine* surveyed. Four glucosinolates were detected in *C. douglassii*, while only one was found in each of the other species surveyed.

All the North American species produced more flavonoids than *A. petiolata*, and all produced compound a, which was not found in *A. petiolata*. *C. concatenata* and *C. douglassii* had very similar flavonoid profiles, but completely distinct glucosinolate profiles.

3.3.2 Bioassay

*A. petiolata* extracts were applied to soils from Europe and North America in order to compare geographic differences in effects on AMF spore viability. Effects differed significantly between sites within continents (region: $F_{10,192}=1.924$, $p=0.044$) (Figure 3.5), but patterns were largely similar within continents so pooled data are also
presented (Figure 3.6). Overall, the flavonoid glycoside enriched fraction was more inhibitory than the glucosinolate enriched fraction (treatment: F<sub>3,30</sub>=47.798, p=0.002), and effects were more pronounced in North American soils than in European soils (continent: F<sub>1,10</sub>=9.810, p=0.035). In European soils, only the flavonoid glycoside enriched fraction suppressed AMF spore viability (continent*treatment: F<sub>3,30</sub>=18.385, p=0.013). In North American soils, not only was suppression by the flavonoid glycoside enriched fraction greater than that seen in European soils, but the glucosinolate enriched fraction also suppressed AMF spore viability, leading to even greater suppression by a combined fraction (continent*treatment: F<sub>3,30</sub>=18.385, p=0.013) (Figure 3.6). The flavonoid glycoside enriched fraction and the combined extract reduced North American spore viability by an average of 53% and 74%, respectively.

### 3.4 DISCUSSION

All the data presented here are consistent with the Novel Weapons hypothesis, which predicts that invasive plants produce compounds that are novel in their invaded ranges, and that organisms in the invaded range will be more sensitive to those compounds than organisms in the native range. None of the glucosinolates, alliarinoside, or flavonoid glycosides produced by *A. petiolata* were found in four common North American Brassicaceae, although sinigrin has been reported in *A. laevigata* (Fahey et al. 2001). The only glucosinolate detected in cauline leaves of *A. petiolata* was sinigrin, although high levels of glucotropaeolin have been reported in roots (Vaughn and Berhow 1999). As seen here for *A. petiolata*, invasive plant species are generally more likely to have novel chemistry than non-invasive species (Cappuccino and Arnason 2006).
phytochemical profiles seen here may have been influenced by environmental variables, such as light and nutrient levels, which were not measured. However, qualitative patterns were nearly identical within a species, suggesting strong genetic control as well.

Seasonal differences in production of glucosinolates, alliarinoside, and flavonoids have also been reported (Vaughn and Berhow 1999, Haribal and Renwick 2001), emphasizing the importance of incorporating seasonal sampling of multiple tissue types into future surveys. Much lower doses were used in these bioassays than have typically been used in *A. petiolata* allelopathy research (Table 2.1), and the doses used here were chosen to mimic field levels, so effects seen here are likely to occur in the field as well. It is therefore especially alarming that these ecologically relevant doses of a complete *A. petiolata* extract reduced AMF spore viability by 75% (Figure 3.6).

This is the first report of allelopathic effects of the flavonoid glycosides produced by *A. petiolata*, although the backbone of these glycosides is apigenin, which is also allelopathic. Apigenin inhibits seed germination of *Raphanus sativus* and *Arabidopsis thaliana* (Basile et al. 2000, Cipollini et al. 2008), deters feeding by *Spodoptera exigua* (Cipollini et al. 2008). Studies using pure compounds may be necessary to understand the allelopathic mechanisms of *A. petiolata*, but we should remember that multiple compounds are necessary for the greatest inhibition, and that these compounds may also act synergistically.

In addition to the allelopathic effects of these novel weapons, they may also contribute to the low levels of insect herbivory on *A. petiolata* in North America (Lewis et al. 2006). Glucosinolates deter feeding by generalist herbivores, but attract specialist insects that are resistant to their effects (Renwick 2002). Alliarinoside and IVG, which
are produced by *A. petiolata*, inhibit the growth of one of these specialists, *Pieris napi oleracea*, in North America (Renwick et al. 2001), although these insects will feed on native Brassicaceae (Renwick 2002). Further support for the NWH comes from the observation that *P. oleracea* are adapting to *A. petiolata* in North America (Keeler and Chew 2008). Insects collected from sites containing *A. petiolata* oviposited more often on *A. petiolata* than insects collected from *A. petiolata* free sites. In addition, larvae from sites with *A. petiolata* survived to pupation more often on *A. petiolata* than did larvae from sites without *A. petiolata*, although pupation rates were still lower on *A. petiolata* than on native host plants.

As predicted by the Novel Weapons hypothesis, more pronounced inhibition was seen in North American soils than in European soils. Although glucosinolates suppressed AMF spore viability only slightly in this study, they have been implicated as novel weapons of another European Brassicaceae that is invasive in North America, *Lepidium draba* (McKenney et al. 2007). These differences may be due to the stability of the glucosinolates produced by each plant. Only two glucosinolates have been identified from *A. petiolata*, glucotropaeolin and sinigrin. Glucotropaeolin is unstable in soil with a half life between 6 hours and 9 days (Gimsing et al. 2006, Gimsing et al. 2007). Also in soil, the half life of benzyl isothiocyanate, a degradate of glucotropaeolin, is less than 2 days (Gimsing et al. 2007). However, sinigrin is remarkably stable in non sterile soil water with a half life greater than 120 days, and half lives of allyl isothiocyanate in soil are between 1 and 40 days (Tsao et al. 2000). Ten glucosinolates have been identified in *L. draba*, including glucotropaeolin (Fahey et al. 2001), and perhaps one or more of these
compounds are even more stable in soils (Kiemnec and McInnis 2002, McKenney et al. 2007).

Regardless of the stability of the allelopathic compounds produced, mixtures may be more inhibitory than single compounds, and may also delay development of resistance in native organisms (Wrubel and Gressel 1994). Resistance is expected to evolve quickly because the selection pressures exerted by allelopaths (e.g., mortality, reduced growth and fecundity) have direct impacts on population size (Jasieniuk et al. 1996). Herbicide resistance can appear in as little as 3 years, although mixtures of herbicides remain effective for longer periods (Wrubel and Gressel 1994). Resistance to allelopathic compounds, which have exposure rates much lower than herbicide applications, has also been observed. Native grass species surviving in sites invaded by *Centaurea maculosa* for 20-30 years were more resistant to allelopathic effects than native grass species from uninvaded sites (Callaway et al. 2005). Although the duration of sites sampled in this study was not noted, *Bromus erectus* collected near *Thymus vulgaris* plants producing predominantly phenolic allelopathic compounds were more resistant to those effects than when planted near *T. vulgvaris* producing predominantly non-phenolic allelopathic compounds, and vice versa (Ehlers and Thompson 2004). Insect resistance to allelopathic plants has been observed for *P. oleracea* on *A. petiolata*, in a site with a 35-55 year invasion history, which equates to 60-100 insect generations (Keeler and Chew 2008).

The results presented here provide some evidence for the Novel Weapons hypothesis, but further study is warranted. Additional experiments could include more
extensive phytochemical surveys of North American Brassicaceae, with sampling of multiple tissue types conducted throughout the plant’s life cycle. Any impact of environmental factors on production levels could be removed by using a common garden design. Comparisons of effects of the extracts used here on con-specific or con-generic plant populations from Europe and North America would also be informative, as would sampling across North America in sites with different invasion durations. Such studies would help to determine whether or not these mechanisms are important in the field and contribute to the invasive success of *A. petiolata.*
3.5 REFERENCES


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Tribe designations are from Al-Shehbaz et al. (2006).
Table 3.2. HPLC flow conditions for flavonoid and alliarinoside analysis.

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Figure 3.1. Phylogeny of the Brassicaceae, classified by tribe.

Lines indicate bootstrap distance, with smaller values indicating a higher degree of similarity. The number of species within each tribe that are native to North America is also indicated. Species collected in this study were sampled from tribes shown in bold. *Alliaria petiolata* - Thlaspideae; *Arabis laevigata* - Arabideae; *Cardamine cancatenata*, *C. bulbosa*, *C. douglassii* - Cardamineae. Modified from Al-Shebaz et al. (2006).
Figure 3.2. HPLC chromatographs from *A. petiolata* leaves (A), and roots (B). Alliarinoside (*), Isovitexin glucopyranoside (**).
Figure 3.3. Representative HPLC chromatographs of fractionated garlic mustard extracts.

A. Desulfated glucosinolate analysis of water fraction, asterisk indicates desulfated sinigrin. B. Flavonoid analysis of water fraction showing no flavonoid glycoside peaks. C. Desulfated glucosinolate analysis of butanol fraction, asterisk indicates a flavonoid glycoside. D. Flavonoid analysis of butanol fraction showing several flavonoid glycoside peaks.
Figure 3.4. Representative HPLC chromatographs showing glucosinolate and flavonoid profiles of five Brassicaceae.

Only peaks labeled with the same letter or number were present in more than one species. Compound 1 is sinigrin, no other labeled compounds were identified.
Figure 3.5. Effect of fractionated extracts from *Alliaria petiolata* on the viability of AM fungi in soils from 6 sites in Europe and 6 sites in North America.

Error bars represent 1 SE and ‘NS’ designates no significant differences among any treatments at a site. Different letters designate significant differences among treatments at a site determined by single ANOVA followed by Tukey tests. CZ: Czech Republic, FRA: France, GDR: Germany, HUN: Hungary, ROM: Romania, ESP: Spain, MA: Massachusetts, IN: Indiana, MN: Minnesota, OR: Oregon, VT: Vermont, WI: Wisconsin.
Figure 3.6. Effect of fractionated extracts from *Alliaria petiolata* on the viability of AMF in soils from 6 sites in Europe and 6 sites in North America, with regions combined.

Error bars represent 1 SE. Columns labeled with the same letter were not significantly different at $\alpha = 0.05$. 
4 DENSITY DEPENDENT PHYTOTOXICITY OF IMPATIENS PALLIDA
PLANTS EXPOSED TO EXTRACTS OF ALLIARIA PETIOLATA

4.1 INTRODUCTION

In natural communities, plants compete with each other in complex ways as they vie for the same territory and resources. These interactions can be classified as direct or indirect competition and are difficult to separate experimentally. Indirect competition is widely assumed to be the default natural condition, where two plants require access to a limited resource and the “winner” is more efficient at acquiring that resource (Connell 1990). Direct competition occurs through interference, with allelopathy being one of the most common mechanisms. Allelopathic plants release compounds into the environment that negatively impact surrounding plants, giving the allelopath a competitive advantage (Rice 1974).

Invasive plant species are by definition excellent competitors, whether by direct competition, indirect competition or both. The distinction is important for several reasons. Simply removing the invader may control an invasion spreading by indirect competition. However, in a site where direct competition is occurring, removal of the invader may not be enough. Allelopathic compounds released by the invader could remain in the soil and continue to inhibit growth of other plants, even after removal of the allelopathic invader. For this reason, considerable attention has been paid to exploring the

*Alliaria petiolata*, an herbaceous biennial, was introduced to North America from Europe in the 1860’s and has since become invasive in much of North America (Nuzzo 2002). The success of *A. petiolata* in North America has been attributed to its high propagule pressure (Cavers et al. 1979), escape from herbivores (Blossey et al. 2001), superior competitive ability (Meekins and McCarthy 1999), and allelopathic inhibition of surrounding plants (Vaughn and Berhow 1999, Roberts and Anderson 2001, Prati and Bossdorf 2004, Stinson et al. 2006). Given the broad range of bioactive secondary metabolites produced by garlic mustard (Haribal and Renwick 1998, Vaughn and Berhow 1999, Haribal and Renwick 2001, Haribal et al. 2001, Renwick et al. 2001, Cipollini and Gruner 2007), it is not surprising that a large part of the effort spent studying the invasive success of this species has focused on allelopathic effects. Crude extracts and purified compounds from *A. petiolata* inhibited germination and growth of other plants and their symbiotic arbuscular mycorrhizal fungi (Vaughn and Berhow 1999, Roberts and Anderson 2001, Stinson et al. 2006, Callaway et al. 2008). Seeds of *Geum* spp. sown in soil conditioned by *A. petiolata* germinated better if the soil also contained activated carbon, presumably indicating the presence of inhibitory organic compounds in the soil (Prati and Bossdorf 2004). Also, Cipollini et al. (2008b) found positive effects of adding carbon in the films on growth of *Impatiens capensis*. In contrast, *A. petiolata* extracts have also failed to inhibit growth of several plant species in some cases (McCarthy and Hanson 1998, Cipollini et al. 2008a)
The most common issues leading to equivocal results include the use of a broad range of doses in bioassays, only some of which are biologically relevant. For example, in bioassays for allelopathic effects of *A. petiolata*, doses of plant extracts ranging from 1 to 100 mg tissue equivalents per g bioassay media have been used (McCarthy and Hanson 1998, Vaughn and Berhow 1999, Roberts and Anderson 2001, Stinson et al. 2006, Callaway et al. 2008), while the highest biologically relevant dose is likely less than 4 mg tissue equivalents per g bioassay media (Table 2.1).

Also confusing the issue of allelopathy in *A. petiolata* is the wide range of secondary metabolites produced by this species and the range of extraction techniques used to isolate those compounds. Bioassays have been dosed with extracts prepared by soaking tissues in water (McCarthy and Hanson 1998, Roberts and Anderson 2001), ethanol (Callaway et al. 2008), and chloroform (Vaughn and Berhow 1999, Roberts and Anderson 2001). Polar solvents like water would extract a different suite of compounds than nonpolar solvents such as choloroform, and since only a few studies attempted to identify and/or quantify compounds in the extracts used (Vaughn and Berhow 1999, Callaway et al. 2008), it is impossible in many cases to know exactly which compounds, and at what levels, target plants were exposed to.

Activated carbon (AC) has been advocated for use as a soil amendment as a simple way to distinguish between allelopathy and resource competition, because it adsorbs organic compounds with little effect on inorganic nutrients (Inderjit and Callaway 2003). Theoretically, AC should remove allelopathic compounds from the environment, meaning any remaining inhibition is due to indirect competition for resources. Actual doses of allelopathic compounds in soils amended with AC are not
typically assessed or reported, so in most cases it is impossible to know if enough AC was incorporated into the soil to adsorb all organic compounds present. Even if enough AC is used, it may also adsorb inorganic compounds and could therefore alter indirect competition as well as removing effects of direct competition. Inderjit and Callaway (2003) have suggested fertilizing soils amended with AC to alleviate this concern, but the absorptive capacity of AC depends on soil type, plant species, and nutrient level (Lau et al. 2008), making it nearly impossible to include proper controls in any experimental design. Activated carbon is a valuable tool in allelopathy research, but multiple lines of evidence are required before claiming that allelopathic inhibition is occurring (Weidenhamer et al. 1989, Williamson 1990, Choesin and Boerner 1991, Hierro and Callaway 2003, Inderjit and Callaway 2003).

Allelopathic effects are highly dependent on densities of target species, and can be masked by resource competition at high densities (Weidenhamer et al. 1989, Weidenhamer 1996). In carefully designed experiments incorporating a range of plant densities, allelopathic effects can be distinguished from indirect competition for resources without the need to amend soils with AC. If resource competition is the dominating factor in an interaction then individual plant mass will decrease as density increases. However, if allelopathy is the dominant factor then individual plant mass will decrease more slowly, or even increase, as density increases, until a density is reached where resource competition becomes the dominant factor (Figure 4.1). This model assumes that all plants present must share the available dose, and as plant density increases the dose per plant decreases. At very high densities, each plant receives a dose so low that allelopathic inhibition disappears and resource competition becomes dominant (Weidenhamer et al. 1989, Weidenhamer 1996).
1989). This pattern has been observed for plants exposed to purified compounds (Hoffman and Lavy 1978, Andersen 1981, Weidenhamer et al. 1989), ground tissue from suspected allelopaths (Tseng et al. 2003), and soil conditioned by an allelopathic plant (Weidenhamer et al. 1989).

Allelopathic effects of *A. petiolata* may be masked by resource competition in some cases, and a better understanding of the dose-response relationship of secondary metabolites of *A. petiolata* would help clarify this issue. Synergism among allelopathic compounds may also be contributing to the inconsistency of results reported in the literature, since fractions of extracts and even purified compounds have been used (Vaughn and Berhow 1999, Roberts and Anderson 2001). The objective of this experiment was to use a density dependent phytotoxicity approach to determine the allelopathic potential of *A. petiolata* for a range of extract doses. We used fractionated extracts, enriched in either glucosinolates or flavonoid glycosides, to explore the contribution of different classes of compounds to observed allelopathic effects (see Chapter 3 for more information on fractionated extracts). I expect results to conform to predictions of the density dependent phytotoxicity model, with effects of a complete extract being more pronounced than effects of either fraction. The results of these experiments should help clarify many of the issues preventing a thorough understanding of the invasive mechanisms of *A. petiolata*.

### 4.2 METHODS

We chose *Impatiens pallida* (Nutt.) Balsaminaceae (pale jewelweed), as the target plant for this study because it grows in the same habitats invaded by *A. petiolata*, and has
been susceptible to allelopathic effects of garlic mustard in prior studies (Barto and Cipollini 2008). *I. pallida* seeds were collected from a population in Yellow Springs, OH (39°47.0’N, 83°52.5’W), and non-sterilized seeds were immediately stratified in sterile water at 5°C to stimulate germination, then seedlings were transferred to 6” pots containing sieved field soil mixed 1:1 with sand. The experiment was fully factorial with four densities (1, 2, 4, and 8 *I. pallida* plants), three *A. petiolata* fractions (glucosinolate enriched fraction, flavonoid enriched fraction, and a combined fraction), and six concentrations (0.25X, 0.5X, 1X, 2X, 4X, and 8X) with X equaling 3.3 mg tissue equivalents per gram soil. This dose was chosen to represent expected exposure levels in the field (Callaway et al. 2008). A set of controls receiving only water was planted at all four densities, and at least three replicate pots were planted for each treatment combination.

Extracts were prepared, and fractionated, as in Callaway et al. (2008). Pots were dosed weekly, and watered as needed. Plants were harvested after 6 weeks, air dried at 25°C for one week, and shoot and root dry masses were measured. Root to shoot ratios were calculated, and the log of root and shoot dry mass per plant, and log of root to shoot ratios, were regressed against the log of plant density per pot using PROC GLM with density and dose as factors, followed by Duncan’s Multiple Range Test with α=0.05 (Weidenhamer et al. 1989). SAS Version 9.1 (SAS Institute Inc., Cary, NC) was used for all analyses.
4.3 RESULTS

Across all other factors, mean shoot dry masses were higher in pots dosed with a glucosinolate enriched fraction than those receiving either a complete extract or a flavonoid enriched fraction ($F_{2,161}=3.55$, $p=0.0310$). Mean root dry masses showed a similar pattern, but the effect was only marginally significant ($F_{2,161}=2.88$, $p=0.0591$). Mean root and shoot mass per plant declined with increasing plant density and every dose.

Among pots dosed with a flavonoid enriched fraction, plant density was the most significant factor impacting shoot dry mass ($t=-19.07$, $p<0.0001$) (Figure 4.2A, Table 4.1). Dose was also significant ($t=-4.98$, $<0.0001$), but shoot mass after exposure to the 8X dose was lower than that of control plants only for the lowest density. This suggests that at densities greater than one plant per pot, resource competition among *I. pallida* plants was masking any allelopathic effects of *A. petiolata* extracts. The slopes of the regression lines were significantly different ($t=2.16$, $p=0.0336$), supporting predictions of the density dependent phytotoxicity model. However, the slope for the 1X dose could not be distinguished from that of the control, showing that at an ecologically relevant dose, allelopathic inhibition was not occurring. Plant density was also the most significant factor impacting root dry mass ($t=-12.81$, $p<0.0001$) of plants exposed to a flavonoid enriched fraction, but dose had no effect ($t=-1.90$, $p=0.068$) (Figure 4.2B, Table 4.1). Root dry mass was not affected allelopathically by a flavonoid enriched fraction, since the slopes of the regression lines were not significantly different ($t=0.26$, $p=0.7973$).

Effects were much less pronounced in pots dosed with a glucosinolate enriched fraction. Plant density was again the most significant factor affecting mean shoot and root
Dose significantly impacted mean shoot dry masses ($t=2.13$, $p=0.0363$), while mean root dry masses were unaffected ($t=-0.25$, $p=0.8025$). As before, the slopes of the regression lines were not significantly different in either case (shoot: $t=-0.17$, $p=0.8663$; root: $t=1.13$, $p=0.2640$), suggesting that resource competition among *I. pallida* plants is the dominant factor when they are exposed to glucosinolates alone.

Inhibitory effects were most pronounced in pots dosed with both flavonoid and glucosinolate enriched fractions, representing the complete phytochemical profile of *A. petiolata*. Plant density was again the most significant factor impacting mean shoot and root dry masses (shoot: $t=-15.34$, $p<0.0001$; root: $t=-10.91$, $p<0.0001$; Figure 4.4, Table 4.3). Extract dose significantly affected both mean shoot and root dry masses (shoot: $t=-3.76$, $p=0.0003$; root: $t=-3.35$, $p=0.0012$), with the strongest dose being the most inhibitory, but differences were significant only at the lowest density. Slopes of the regression lines for mean shoot mass and mean root mass against plant density were significantly different (shoot: $t=3.53$, $p=0.0007$; root: $t=2.03$, $p=0.0455$) when pots received a complete extract (Figure 4.4A). The highest dose led to the lowest slope, showing that at high doses allelopathic inhibition by *A. petiolata* is more important than resource competition between *I. pallida* plants. However, at the 1X dose expected to represent exposure levels in the field, the slope of the treatment regression lines could not be distinguished from that of control plants. Therefore, even though compounds produced by *A. petiolata* can inhibit *I. pallida* shoot and root growth, they did not do so at an ecologically relevant dose.
The root to shoot ratios of plants exposed to a flavonoid enriched fraction of an *A. petiolata* extract were significantly affected by both density and dose (density: \( t=2.97 \) \( p=0.0040 \); dose: \( t=2.16, p=0.0337 \)), although the slopes of the regression lines were not different \( t=-1.48, p=0.1430 \) (Figure 4.5A, Table 4.4). Root to shoot ratios were highest at a density of 4 plants per pot, indicating that *I. pallida* plants became more crowded, they invested resources preferentially in root growth, but these allocation patterns were not affected by exposure dose.

In *I. pallida* plants exposed to a glucosinolate enriched fraction of an *A. petiolata* extract, root to shoot ratios were generally higher in plants exposed to low doses than in plants exposed to high doses \( t=-2.54, p=0.0132 \) (Figure 4.5B, Table 4.5). Root to shoot ratios were not affected by plant density \( t=-0.21, p=0.8359 \), and the slopes of the regression lines were not different \( t=1.23, p=0.2209 \).

In contrast to the strong effects of a complete *A. petiolata* extracts and shoot and root dry mass of *I. pallida* plants, root to shoot ratios were not affected by density or dose (density: \( t=1.62, p=0.1083 \); dose: \( t=-0.33, p=0.7430 \)), and the slopes of the regression lines could not be distinguished \( t=-0.88, p=0.3796 \) (Figure 4.5C, Table 4.6).

### 4.4 DISCUSSION

*Alliaria petiolata* has the potential to exert strong allelopathic effects on surrounding plants, as shown by the reduced *I. pallida* growth observed in this study after exposure to high doses of either a flavonoid enriched fraction or a complete extract. In contrast to other reports of inhibition by glucosinolates at doses as low as 2 times field levels (Vaughn and Berhow 1999), I found no inhibition due to glucosinolates, even
using a dose 8 times higher than expected field levels. Studies using multiple fractions are rare, but the finding of much higher levels of inhibition when a complete extract was used support those seen other studies (Callaway et al. 2008). Despite the well-known potential for *A. petiolata* to be allelopathic (Vaughn and Berhow 1999, Roberts and Anderson 2001, Prati and Bossdorf 2004, Stinson et al. 2006, Callaway et al. 2008), intraspecific resource competition was the dominant process determining growth of the *I. pallida* plants used in this study. Allelopathic effects of *A. petiolata* seem unlikely to account for its ability to displace *I. pallida* populations (McCarthy 1997).

Invasive success could also be explained by superior resource competition by *A. petiolata*, however, seedlings of *Impatiens capensis*, a smaller congeneric of *I. pallida*, were actually more competitive than *A. petiolata* seedlings when both were planted at the same time (Meekins and McCarthy 1999). Although small *A. petiolata* seedlings did not out compete *I. capensis*, *A. petiolata* is a biennial, and bolts early in the spring of its second year before many other plants germinate. Therefore, second year *A. petiolata* plants are likely more competitive with an annual like *Impatiens* than *A. petiolata* seedlings, and may be able to out compete *I. pallida* seedlings.

Relative contributions of allelopathic inhibition and resource competition to the overall competitiveness of a plant can be determined using a series of density dependent phytotoxicity experiments coupled with replacement series designs (Liu et al. 2005). A more complex model could also incorporate environmental influences on resource competition and production of allelopathic compounds, which likely vary with the environmental characteristics of a site, time of year, and the identity of the target organisms. Environmental factors influencing the production of allelopathic compounds
include nutrient and water availability, soil texture, the presence of other soil contaminants, altitude, latitude, light levels, temperature, and pH of the soil (Blanco 2007). In *A. petiolata*, production of flavonoid glycosides varies seasonally (Haribal and Renwick 2001), and differences between populations may be due to environmental factors (Haribal and Renwick 2001) and/or be genetically controlled (Cipollini et al. 2005). Glucosinolate production also varies seasonally (Vaughn and Berhow 1999), and is influenced by environmental factors (Cipollini 2002). Further complicating matters are the differing sensitivities of different species of target plants. Only *I. pallida* plants were used in this study, but growth of several other herbaceous plants, as well as arbuscular mycorrhizal fungi and several tree species, is also inhibited by *A. petiolata* extracts (Vaughn and Berhow 1999, Roberts and Anderson 2001, Stinson et al. 2006, Callaway et al. 2008).

The importance of resource competition also likely varies with site and the identities of the species involved. Among the woodland plants compared by Meekins and McCarthy (1999), *I. capensis* was the most aggressive. Height and canopy diameter are among the most important factors influencing competitiveness among herbaceous plants (Keddy 1990), so *I. pallida*, which can reach 2.5 meters in height, are likely to be at least as competitive as *I. capensis*, which reaches only about 1 meter (USDA Plants Database, http://plants.usda.gov/, accessed on May 5, 2008). Allelopathic effects would likely be more pronounced on less competitive species than on more competitive species that could limit growth of the allelopathic plant through resource competition.

In addition to the complex factors influencing resource competition between donor and target plants, and production of allelopathic compounds by donor plants, there
may also be interactions between the two processes. Resource competition can cause shifts in root to shoot ratios, which could alter levels of allelopathic compounds produced preferentially in roots or shoots (Goldberg 1990). Flavonoid glycosides are more abundant in the shoots of *A. petiolata* (Figure 3.2), while glucosinolates are more abundant in the roots (Vaughn and Berhow 1999).

Goslee et al. (2001) used data from lab bioassays to truth a computer model predicting plant abundance at varying levels of resources and allelopathy for the potentially allelopathic plant *Acroptilon repens*. Model predictions matched observed plant densities in the field only when allelopathic inhibition was included in the model. Direct competition through allelopathic inhibition and indirect competition for resources could both play a role in the invasion success of *A. petiolata*. Modeling approaches incorporating both processes may ultimately prove more successful in explaining the invasion success of *A. petiolata* than approaches focusing on either process alone.
4.5 REFERENCES


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Table 4.1. Shoot and root dry masses of *I. pallida* plants exposed to a flavonoid enriched fraction of an *A. petiolata* extract.

In dose column X represents expected field levels of 3.3 mg *A. petiolata* tissue equivalents per g soil. Means with the same letter are not significantly different at $\alpha = 0.05$ using Duncan's Multiple Range Test.

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<td>115 ± 8 ghi</td>
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Mean root dry mass per plant (mg ± 1 SE)

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</thead>
<tbody>
<tr>
<td>0X</td>
<td>1557 ± 320 a</td>
<td>808 ± 188 bcdefg</td>
<td>527 ± 90 fghijkl</td>
<td>404 ± 27 ijkln</td>
</tr>
<tr>
<td>0.25X</td>
<td>980 ± 8 abcd</td>
<td>675 ± 80 cdefghi</td>
<td>490 ± 49 ghijkl</td>
<td>381 ± 36 jkllm</td>
</tr>
<tr>
<td>0.5X</td>
<td>1524 ± 318 a</td>
<td>595 ± 110 efghij</td>
<td>490 ± 15 ghijkl</td>
<td>288 ± 50 mn</td>
</tr>
<tr>
<td>1X</td>
<td>1236 ± 165 ab</td>
<td>871 ± 173 bcdefg</td>
<td>641 ± 167 defghij</td>
<td>329 ± 20 klnn</td>
</tr>
<tr>
<td>2X</td>
<td>1193 ± 290 ab</td>
<td>697 ± 62 cdefgh</td>
<td>592 ± 67 defghij</td>
<td>287 ± 42 mn</td>
</tr>
<tr>
<td>4X</td>
<td>1088 ± 77 abc</td>
<td>623 ± 28 defghij</td>
<td>475 ± 27 ghijkl</td>
<td>248 ± 54 n</td>
</tr>
<tr>
<td>8X</td>
<td>999 ± 239 abcd</td>
<td>546 ± 29 fghijk</td>
<td>422 ± 39 hijklm</td>
<td>317 ± 23 lmn</td>
</tr>
</tbody>
</table>
Table 4.2. Shoot and root dry masses of *I. pallida* plants exposed to a glucosinolate enriched fraction of an *A. petiolata* extract.

In dose column X represents expected field levels of 3.3 mg *A. petiolata* tissue equivalents per g soil. Means with the same letter are not significantly different at $\alpha = 0.05$ using Duncan's Multiple Range Test.

<table>
<thead>
<tr>
<th>Dose</th>
<th>Mean shoot dry mass per plant (mg ± 1 SE)</th>
<th>Mean root dry mass per plant (mg ± 1 SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 plant per pot</td>
<td>2 plants per pot</td>
</tr>
<tr>
<td></td>
<td>2 plants per pot</td>
<td>4 plants per pot</td>
</tr>
<tr>
<td>0X</td>
<td>394 ± 93 abc</td>
<td>221 ± 11 bcdefgh</td>
</tr>
<tr>
<td></td>
<td></td>
<td>189 ± 27 defghi</td>
</tr>
<tr>
<td>0.25X</td>
<td>291 ± 106 bcdefg</td>
<td>302 ± 43 abcde</td>
</tr>
<tr>
<td></td>
<td></td>
<td>118 ± 14 hijklm</td>
</tr>
<tr>
<td>0.5X</td>
<td>302 ± 43 abcde</td>
<td>211 ± 10 bcdefgh</td>
</tr>
<tr>
<td></td>
<td></td>
<td>118 ± 14 hijklm</td>
</tr>
<tr>
<td>1X</td>
<td>564 ± 85 a</td>
<td>258 ± 21 bcdef</td>
</tr>
<tr>
<td></td>
<td></td>
<td>130 ± 4 ghijkl</td>
</tr>
<tr>
<td>2X</td>
<td>409 ± 18 ab</td>
<td>211 ± 10 bcdefgh</td>
</tr>
<tr>
<td></td>
<td></td>
<td>118 ± 14 hijklm</td>
</tr>
<tr>
<td>4X</td>
<td>426 ± 139 abcd</td>
<td>258 ± 21 bcdef</td>
</tr>
<tr>
<td></td>
<td></td>
<td>118 ± 14 hijklm</td>
</tr>
<tr>
<td>8X</td>
<td>615 ± 216 a</td>
<td>229 ± 45 bcdefgh</td>
</tr>
<tr>
<td></td>
<td></td>
<td>229 ± 45 bcdefgh</td>
</tr>
<tr>
<td></td>
<td></td>
<td>229 ± 45 bcdefgh</td>
</tr>
</tbody>
</table>
Table 4.3. Shoot and root dry masses of *I. pallida* plants exposed to a complete *A. petiolata* extract.

In dose column X represents expected field levels of 3.3 mg *A. petiolata* tissue equivalents per g soil. Means with the same letter are not significantly different at $\alpha = 0.05$ using Duncan's Multiple Range Test.

<table>
<thead>
<tr>
<th>Dose</th>
<th>1 plant per pot</th>
<th>2 plants per pot</th>
<th>4 plants per pot</th>
<th>8 plants per pot</th>
</tr>
</thead>
<tbody>
<tr>
<td>0X</td>
<td>394 ± 93 ab</td>
<td>221 ± 11 bcd</td>
<td>115 ± 8 fghi</td>
<td>89 ± 5 hi</td>
</tr>
<tr>
<td>0.25X</td>
<td>371 ± 50 ab</td>
<td>180 ± 6 cdef</td>
<td>161 ± 25 cdefg</td>
<td>75 ± 2 i</td>
</tr>
<tr>
<td>0.5X</td>
<td>378 ± 62 ab</td>
<td>221 ± 44 bcde</td>
<td>112 ± 10 fghi</td>
<td>78 ± 3 hi</td>
</tr>
<tr>
<td>1X</td>
<td>377 ± 130 ab</td>
<td>185 ± 18 cdef</td>
<td>122 ± 6 efghi</td>
<td>69 ±2 i</td>
</tr>
<tr>
<td>2X</td>
<td>446 ± 33 a</td>
<td>289 ± 109 bc</td>
<td>110 ± 7 fghi</td>
<td>76 ± 4 i</td>
</tr>
<tr>
<td>4X</td>
<td>279 ± 76 abc</td>
<td>221 ± 16 bcde</td>
<td>115 ± 11 fghi</td>
<td>84 ± 3 hi</td>
</tr>
<tr>
<td>8X</td>
<td>196 ± 48 cdef</td>
<td>204 ± 71 cdef</td>
<td>144 ± 33 defgh</td>
<td>95 ± 12 ghi</td>
</tr>
</tbody>
</table>

Mean root dry mass per plant (mg ± 1 SE)

<table>
<thead>
<tr>
<th>Dose</th>
<th>1 plant per pot</th>
<th>2 plants per pot</th>
<th>4 plants per pot</th>
<th>8 plants per pot</th>
</tr>
</thead>
<tbody>
<tr>
<td>0X</td>
<td>1557 ± 320 ab</td>
<td>808 ± 188 bcdefg</td>
<td>527 ± 90 efghi</td>
<td>404 ± 27 ghij</td>
</tr>
<tr>
<td>0.25X</td>
<td>1257 ± 180 abc</td>
<td>765 ± 136 cdefg</td>
<td>772 ± 127 bcdefg</td>
<td>253 ± 16 j</td>
</tr>
<tr>
<td>0.5X</td>
<td>1224 ± 122 abc</td>
<td>852 ± 95 bcdef</td>
<td>474 ± 11 efghij</td>
<td>394 ± 24 ghij</td>
</tr>
<tr>
<td>1X</td>
<td>1500 ± 77 ab</td>
<td>974 ± 197 abcde</td>
<td>452 ± 88 fghij</td>
<td>424 ± 106 ghij</td>
</tr>
<tr>
<td>2X</td>
<td>1707 ± 142 a</td>
<td>1273 ± 454 abc</td>
<td>437 ± 40 fghij</td>
<td>341 ± 19 ij</td>
</tr>
<tr>
<td>4X</td>
<td>1162 ± 291 abcd</td>
<td>659 ± 127 cdefgi</td>
<td>483 ± 126 fghij</td>
<td>390 ± 96 hij</td>
</tr>
<tr>
<td>8X</td>
<td>730 ± 108 cdefg</td>
<td>600 ± 194 defghi</td>
<td>535 ± 126 efghij</td>
<td>336 ± 77 ij</td>
</tr>
</tbody>
</table>
Table 4.4. Root to shoot ratios of dry masses of *I. pallida* plants exposed to a flavonoid enriched fraction of an *A. petiolata* extract.

Means with the same letter are not significantly different at $\alpha = 0.05$ using Duncan's Multiple Range Test.

<table>
<thead>
<tr>
<th>Dose</th>
<th>1 plant per pot</th>
<th>2 plants per pot</th>
<th>4 plants per pot</th>
<th>8 plants per pot</th>
</tr>
</thead>
<tbody>
<tr>
<td>0X</td>
<td>$4.05 \pm 0.23$ ab</td>
<td>$3.61 \pm 0.67$ abc</td>
<td>$4.51 \pm 0.52$ ab</td>
<td>$4.60 \pm 0.54$ ab</td>
</tr>
<tr>
<td>0.25X</td>
<td>$2.86 \pm 0.36$ bc</td>
<td>$3.71 \pm 0.71$ abc</td>
<td>$4.55 \pm 0.79$ ab</td>
<td>$4.69 \pm 0.68$ ab</td>
</tr>
<tr>
<td>0.5X</td>
<td>$3.52 \pm 0.60$ abc</td>
<td>$2.96 \pm 0.29$ bc</td>
<td>$4.28 \pm 0.13$ ab</td>
<td>$3.54 \pm 0.49$ abc</td>
</tr>
<tr>
<td>1X</td>
<td>$2.97 \pm 0.55$ bc</td>
<td>$4.03 \pm 0.38$ ab</td>
<td>$4.94 \pm 1.31$ ab</td>
<td>$4.18 \pm 0.36$ ab</td>
</tr>
<tr>
<td>2X</td>
<td>$2.25 \pm 0.34$ c</td>
<td>$3.41 \pm 0.18$ abc</td>
<td>$5.36 \pm 0.58$ a</td>
<td>$3.53 \pm 0.73$ abc</td>
</tr>
<tr>
<td>4X</td>
<td>$4.22 \pm 0.65$ ab</td>
<td>$3.92 \pm 0.53$ ab</td>
<td>$4.78 \pm 0.35$ ab</td>
<td>$3.67 \pm 0.99$ abc</td>
</tr>
<tr>
<td>8X</td>
<td>$5.05 \pm 1.13$ ab</td>
<td>$3.44 \pm 0.23$ abc</td>
<td>$4.59 \pm 0.48$ ab</td>
<td>$4.77 \pm 0.40$ ab</td>
</tr>
</tbody>
</table>
Table 4.5. Root to shoot ratios of dry masses of *I. pallida* plants exposed to a glucosinolate enriched fraction of an *A. petiolata* extract.

Means with the same letter are not significantly different at $\alpha = 0.05$ using Duncan's Multiple Range Test.

<table>
<thead>
<tr>
<th>Dose</th>
<th>1 plant per pot</th>
<th>2 plants per pot</th>
<th>4 plants per pot</th>
<th>8 plants per pot</th>
</tr>
</thead>
<tbody>
<tr>
<td>0X</td>
<td>4.05 ± 0.23 ab</td>
<td>3.61 ± 0.67 ab</td>
<td>4.51 ± 0.52 ab</td>
<td>4.60 ± 0.54 ab</td>
</tr>
<tr>
<td>0.25X</td>
<td>5.39 ± 1.25 a</td>
<td>4.58 ± 0.79 ab</td>
<td>3.90 ± 0.23 ab</td>
<td>4.33 ± 0.27 ab</td>
</tr>
<tr>
<td>0.5X</td>
<td>5.20 ± 0.60 a</td>
<td>4.65 ± 0.45 ab</td>
<td>4.16 ± 0.96 ab</td>
<td>4.10 ± 0.90 ab</td>
</tr>
<tr>
<td>1X</td>
<td>2.62 ± 0.49 b</td>
<td>3.13 ± 0.76 ab</td>
<td>4.52 ± 1.00 ab</td>
<td>4.76 ± 0.38 ab</td>
</tr>
<tr>
<td>2X</td>
<td>3.99 ± 1.14 ab</td>
<td>5.31 ± 1.12 a</td>
<td>3.86 ± 0.36 ab</td>
<td>2.93 ± 0.34 ab</td>
</tr>
<tr>
<td>4X</td>
<td>3.81 ± 0.80 ab</td>
<td>3.68 ± 0.58 ab</td>
<td>2.95 ± 0.51 ab</td>
<td>3.65 ± 0.58 ab</td>
</tr>
<tr>
<td>8X</td>
<td>2.63 ± 0.34 b</td>
<td>3.28 ± 0.27 ab</td>
<td>4.01 ± 0.72 ab</td>
<td>3.98 ± 0.34 ab</td>
</tr>
</tbody>
</table>

(mean ± 1 SE)
Table 4.6. Root to shoot ratios of dry masses of *I. pallida* plants exposed to a complete *A. petiolata* extract.

There were no significant differences among means using Duncan's Multiple Range Test ($\alpha = 0.05$).

<table>
<thead>
<tr>
<th>Dose</th>
<th>1 plant per pot $\text{ (mean } \pm 1 \text{ SE)}$</th>
<th>2 plants per pot $\text{ (mean } \pm 1 \text{ SE)}$</th>
<th>4 plants per pot $\text{ (mean } \pm 1 \text{ SE)}$</th>
<th>8 plants per pot $\text{ (mean } \pm 1 \text{ SE)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0X</td>
<td>$4.05 \pm 0.23$</td>
<td>$3.61 \pm 0.67$</td>
<td>$4.51 \pm 0.52$</td>
<td>$4.60 \pm 0.54$</td>
</tr>
<tr>
<td>0.25X</td>
<td>$3.47 \pm 0.52$</td>
<td>$4.24 \pm 0.75$</td>
<td>$4.98 \pm 0.98$</td>
<td>$3.38 \pm 0.32$</td>
</tr>
<tr>
<td>0.5X</td>
<td>$3.31 \pm 0.23$</td>
<td>$4.21 \pm 0.96$</td>
<td>$4.28 \pm 0.30$</td>
<td>$5.06 \pm 0.36$</td>
</tr>
<tr>
<td>1X</td>
<td>$4.75 \pm 1.13$</td>
<td>$5.56 \pm 1.69$</td>
<td>$3.63 \pm 0.56$</td>
<td>$6.21 \pm 1.66$</td>
</tr>
<tr>
<td>2X</td>
<td>$3.82 \pm 0.03$</td>
<td>$4.64 \pm 1.28$</td>
<td>$3.96 \pm 0.18$</td>
<td>$4.50 \pm 0.41$</td>
</tr>
<tr>
<td>4X</td>
<td>$4.39 \pm 1.00$</td>
<td>$2.93 \pm 0.35$</td>
<td>$4.15 \pm 1.02$</td>
<td>$4.75 \pm 1.29$</td>
</tr>
<tr>
<td>8X</td>
<td>$4.27 \pm 1.16$</td>
<td>$3.18 \pm 0.72$</td>
<td>$3.78 \pm 0.64$</td>
<td>$3.45 \pm 0.47$</td>
</tr>
</tbody>
</table>
Figure 4.1. Predictions of the density-dependent phytotoxicity model.

The bold line represents expectations when resource competition is dominant, and the dashed line represents expectations when allelopathy is dominant. Dotted lines represent expected patterns at intermediate chemical doses.
Figure 4.2. Response of *I. pallida* plants exposed to a flavonoid enriched fraction of an *A. petiolata* extract (N=2-3).

A. Relationship of log mean shoot mass per plant and log plant density. B. Relationship of log mean root mass per plant and log plant density.
Figure 4.3. Response of *I. pallida* plants exposed to a glucosinolate enriched fraction of an *A. petiolata* extract (N=2-4).

A. Relationship of log mean shoot mass per plant and log plant density. B. Relationship of log mean root mass per plant and log plant density.
Figure 4.4. Response of *I. pallida* plants exposed to a complete *A. petiolata* extract (N=2-3).

**A.** Relationship of log mean shoot mass per plant and log plant density.  **B.** Relationship of log mean root mass per plant and log plant density.
Figure 4.5. Root to shoot ratios of *I. pallida* plants exposed to *A. petiolata* extracts.

A. Response to a flavonoid enriched fraction of an *A. petiolata* extract (N=2-3). B. Response to a glucosinolate enriched fraction of an *A. petiolata* extract (N=2-4). C. Response to a complete *A. petiolata* extract (N=2-3).
5 DIVERSITY OF ARBUSCULAR MYCORHIZAL FUNGI IN AND OUTSIDE OF ESTABLISHED ALLIARIA PETIOLATA PATCHES

5.1 INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) are obligately symbiotic fungi that plants in 90% of families surveyed associate with and depend on to some extent for normal growth and development (Smith and Read 1997). In exchange for photosynthate from their host plants, these fungi provide nutrients and water to their host plants, and enhance resistance to pathogenic organisms (Smith and Read 1997). Although AMF are widely assumed to benefit their plant hosts, they can also have detrimental effects on growth (Wilson 1984, Johnson 1993, Klironomos 2003). AMF are not host specific and any particular fungus can colonize more than one host plant simultaneously, meaning that the majority of plants in an area are connected by a vast underground hyphal network nicknamed the “wood wide web” (Helgason et al. 1998). This interconnectedness allows for broad ecosystem level effects of AMF as well, including both increases and decreases in plant diversity (van der Heijden et al. 1998b, Hartnett and Wilson 1999) and increases in community productivity (van der Heijden 2002). These effects depend not only on the abundance of AMF, but also the diversity of the AMF community and even the specific AMF species present (van der Heijden et al. 1998b, Bray et al. 2003).
This is not surprising given that AMF species develop internal and external hyphal structures and spores at different rates (Wilson and Trinick 1983, Jakobsen et al. 1992, Bever et al. 1996), and have differing capacities for nutrient uptake (Arines et al. 1989, Raju et al. 1990, Kothari et al. 1991, Jakobsen et al. 1992, Bürkert and Robson 1994). These differences also depend, at least to some extent, on the identity of the host plants as well. In a fully factorial experiment with five plant species and four AMF species, Helgason et al. (2002) found that phosphorus concentrations in leaves and roots, and plant biomass, depended on the identities of plant and fungus. In similar factorial experiments, van der Heijden et al. (1998a, 1998b) also found that plant and fungal identities determined intensity of effects on plant size and phosphorus concentrations of plant tissues.

Any change in the mycorrhizal community can be expected to dramatically affect the plant community. In addition to directly impacting growth of their plant hosts, AMF also mediate interactions between plants, and can enhance competitiveness of invasive plants. *Centaurea maculosa*, an invasive knapweed in the western United States, maintains a higher mycorrhizal colonization with greater numbers of extraradical hyphae than surrounding plants (Marler et al. 1999, Walling and Zabinski 2004), giving it a distinct competitive advantage. *Centaurea* species even draw photosynthate from other plants to themselves through the mycorrhizal network, further increasing their competitiveness (Carey et al. 2004). The strength of interactions also depends on the identities of the colonizing AMF species. The invasive success of *Bidens pilosa*, an aster invading Hawaii, was slightly enhanced by two *Scutellospora* species, but significantly depressed by *Glomus sparcum* (Stampe and Daehler 2003). Growth of an invasive shrub
in Florida, *Ardisia crenata*, was enhanced when colonized by its associated mycorrhizae, but not when colonized with monospecific isolates (Bray et al. 2003), although this enhanced growth did not clearly enhance competitiveness.

Some invasive plants are also allelopathic, meaning they release noxious natural products that inhibit the growth of surrounding plants (Rice 1974). This inhibition can be direct, as is the case for *Centaurea maculosa* (Ridenour and Callaway 2001, Bais et al. 2003), or indirect by limiting the growth of AMF (Yun and Choi 2002, Stinson et al. 2006, Callaway et al. 2008).

Garlic mustard [*Alliraia petiolata* (M. Bieb.) Cavara and Grande, Brassicaceae] is a Eurasian native that was introduced to North America in the late 1800’s and has become widespread throughout midwestern and northeastern North America and southern Canada (Nuzzo 2002). *Alliaria petiolata* forms dense monocultures in the undisturbed forest understory, territory typically inaccessible to invaders, adversely impacting native plant abundance and biodiversity (McCarthy 1997). *Alliaria petiolata* can directly inhibit growth of surrounding plants (Vaughn and Berhow 1999, Roberts and Anderson 2001, Prati and Bossdorf 2004), and recent research has focused on indirect mechanisms of inhibition. *Alliaria petiolata* cannot associate with mycorrhizal fungi so any allelopathic effects targeted towards AMF will not affect *A. petiolata*, thereby minimizing autotoxicity. Woody species are thought to be more dependent on AMF, especially during seedling stages, than many herbaceous species, and growth of *Acer saccharum*, *Acer rubrum*, and *Fraxinus americana* were suppressed when grown in soil in which *A. petiolata* had previously grown (Stinson et al. 2006).
Although *A. petiolata* extracts have been demonstrated to reduce abundance of AMF (Roberts and Anderson 2001, Stinson et al. 2006), we know nothing about allelopathic effects on diversity of the AMF community. The objectives of this experiment were to explore the effects of *A. petiolata* on the abundance and diversity of AMF communities associated with *Acer saccharum* in two regions of North America.

### 5.2 METHODS

#### 5.2.1 Site Selection and Sampling

*Acer saccharum* Marsh. (sugar maple) associates with AMF as seedlings and its growth is suppressed by *A. petiolata* indirectly through suppression of AMF associating with *A. saccharum* (Stinson et al. 2006). Eight sites were selected (4 in Massachusetts and 4 in Ohio), each encompassing an established *A. petiolata* population and an adjacent uninvaded area separated by approximately 30 m, to give 16 sampling sites. Five *A. saccharum* seedlings (less than 30 cm tall) were collected from each site, and all *A. saccharum* seedlings collected in invaded sites were growing within 15 cm of an *A. petiolata* plant. Approximately 100 mg of root tissue was freeze dried for subsequent DNA extraction and terminal restriction fragment length polymorphism (T-RFLP) analysis to assess AM fungal diversity (Gollotte et al. 2004, Johnson et al. 2004).

#### 5.2.2 T-RFLP Analysis

DNA was extracted from freeze dried plant roots using a DNeasy 96 Plant Kit (Qiagen Inc., Mississauga, ON, Canada), then amplified using a nested PCR approach.
Fungal DNA was amplified in the first PCR with LR1/FLR2 primers (Trouvelot et al. 1999), followed by a second PCR with 5’-labelled primers FLR3-FAM/FLR4-VIC (Applied Biosystems, Foster City, CA, USA) to amplify AMF DNA (Gollotte et al. 2004). Both PCR reactions consisted of 1 x Green GoTaq® Reaction Buffer (Promega, Madison, WI, USA), 1.7 mM MgCl$_2$, 0.13 mM each dNTP, 0.33 mM of each primer, 1.25 u GoTaq® DNA Polymerase, and 1 µl of template DNA in a final volume of 15 µL. The PCR conditions were 93°C for 2 min; then 35 cycles of 93°C for 1 min, 55°C for 1 min, 72°C for 1 min; then 72°C for 10 min in a Mastercycler® ep thermocycler (Eppendorf, Hamburg Germany). We tested the first PCR step using three concentrations of purified DNA; the original concentration, a 1:50 dilution of purified DNA, and a 1:100 dilution of purified DNA. No dilution successfully amplified DNA from every sample, so we combined the PCR product from all three attempts, and used a 1:100 dilution of the combined PCR product as the template for the second PCR. The final PCR product was purified with a Qiaquick cleanup kit (Qiagen Inc.) before quantification of DNA in each sample with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Two restriction enzymes, AluI and MboI (Invitrogen Inc., Burlington, ON, Canada) were used in digests consisting of 50 ng DNA, 1X REact® 1 buffer (for AluI) or REact® 2 buffer (for MboI), and 1 unit of enzyme in 20 µL total volume. Digestions were incubated at 37°C for 6 hours before analysis on an ABI 3730 DNA Analyzer (Applied Biosystems) with LIZ-500 as the size standard.
5.2.3 Data Analysis

Terminal restriction fragment (T-RF) sizes and peak heights were determined using Peak Scanner software v. 1.0 (Applied Biosystems) with a threshold of 50 AU for \textit{MboI}, and 100 AU for \textit{AluI}, which had more background noise. Stutter peaks are PCR artifacts that appear on profiles near real peaks with up to 20\% the peak area of real peaks, and a fragment length one or two base pairs less than the real peak. Each profile was scanned and stutter peaks were manually deleted, then total fluorescence of each profile was standardized (Dunbar et al. 2001). TR-Fs were aligned with T-Align (http://inismor.ucd.ie/~talign/) using a confidence interval of 1.0 to combine fragment lengths differing by less than one base pair into the same TRF (Smith et al. 2005). This ensures that a peak appearing with different fragment lengths in more than one profile, for example at 147.4 bp in one profile and 147.8 in another profile, will be identified as the same TRF. TRFs occurring in only one sample were excluded from further analysis.

Since only the terminal restriction fragment is labeled in T-RFLP analysis, each T-RF theoretically represents one DNA sequence, or one species. In reality, there may be variation within a species leading to multiple T-RFs, and/or overlap of T-RFs between species, but the total number of T-RFs per sample correlates with species richness. Total T-RFs per sample were analyzed using a nested ANOVA with state, site nested with state, and invasion status as factors. Using two labeled primers for each of two restriction enzymes provides four ways to quantify species richness in each sample. Forward and reverse sequences for each restriction enzyme were analyzed individually, and are expected to produce similar results. All data for \textit{MboI} and VIC-labeled T-RFs for \textit{AluI} were log transformed to meet assumptions of ANOVA. Data for FAM-labeled T-RFs for
AluI needed no transformation. Analyses were conducted with SAS v9.1 (SAS Institute Inc., Cary, NC).

5.3 RESULTS

When using MboI, 23 FAM-labeled T-RFs and 15 VIC-labeled T-RFs were identified across all samples. Patterns were similar for AluI, with 20 FAM-labeled T-RFs and 16 VIC-labeled T-RFs identified across all samples.

More T-RFs were detected in sites not invaded by A. petiolata than in invaded sites, but only for FAM-labeled primers, and the pattern was only marginally significant with MboI (Figure 5.1A) (FAM-MboI: $F_{1,64}=3.58$, $p=0.0631$; FAM-AluI: $F_{1,60}=4.33$, $p=0.0417$). Species richness was consistently higher in Ohio than in Massachusetts, with approximately twice as many T-RFs detected per site, on average. More T-RFs were detected per site in Ohio than Massachusetts for all primers and both restriction enzymes (Figure 5.1B) (FAM-MboI: $F_{1,64}=12.08$, $p=0.0009$; VIC-MboI: $F_{1,64}=27.96$, $p<0.0001$; FAM-AluI: $F_{1,60}=11.60$, $p=0.0012$; VIC-AluI: $F_{1,60}=9.03$, $p=0.0039$). No other factors or combination of factors significantly impacted total number of T-RFs per site.

5.4 DISCUSSION

Sites uninvaded by A. petiolata tended to have more T-RFs than invaded sites, but the pattern was only significant for FAM-AluI fragments, and marginally significant for FAM-MboI fragments. Since all four primer:restriction enzyme combinations should produce similar results and invasion status was significant for only one of the four, the
biological relevance of this result must be questioned. This suggests that observed reductions in AMF colonization after exposure to *A. petiolata* (Chapter 7, Stinson et al. 2006) are due to reduced AMF growth, rather than a reduction in the number of AMF species present. There were significant regional differences in AMF species richness, with more species found in Ohio than in Massachusetts. *Alliaria petiolata* was first noted in North America in New York (Nuzzo 2002), and likely reached Massachusetts before Ohio. According to the novel weapons hypothesis, species naïve to the allelopathic compounds produced by *A. petiolata* should be more sensitive than species with a longer exposure history. Therefore, plant populations growing in Massachusetts, where *A. petiolata* has likely been invasive for longer than in Ohio, would be expected to be more resistant to allelopathic effects than plant populations of the same species growing in Ohio. However, more species were found in Ohio, suggesting that the differences may be due to other environmental factors, such as climatic conditions, soil type, and plant diversity, instead of *A. petiolata* invasion history. A more thorough sampling of sites around the Northeast and Midwest encompassing a range of invasion histories and soil types would help resolve this issue.

Although AMF species richness was not affected by *A. petiolata* presence, AMF species diversity may have been. Theoretically, individual species can be identified from a T-RFLP profile based on the fragment lengths of each T-RF, since each unique DNA sequence will produce one T-RF per labeled primer. In practice, there can be variation within a species leading to more than one T-RF per species, and/or overlap when two species produce the same T-RF. By using two labeled primers for each of two restriction enzymes, we have four independent assessments of the AMF community in each sample
that will be compared in order to determine which AMF species were present in each sample. Using a library of AMF species and their expected T-RFs, created from published DNA sequences or T-RFLP analysis of monospecific AMF cultures, the T-RFs found in this study will be identified to species by Pedro Antunes (Freie Universität, Berlin, Germany). Then canonical correspondence analysis will be done to determine how AMF species diversity is affected by *A. petiolata* invasion. This technique is viable for AMF because less than 200 species have been identified worldwide (Smith and Read 1997) and species richness within a site is usually low enough that overlap of T-RFs among species is rare. Mummey and Rillig (2006) used this technique to show that fewer AMF species were found in sites dominated by the invasive plant *Centaurea maculosa* than in sites dominated by native grasses, and that different AMF species were abundant in each site.

AMF diversity could be affected by *A. petiolata* presence, with no changes in species richness, if sensitive species are replaced by resistant species. AMF species resistant to allelopathic effects of *A. petiolata* appear to protect their host plants from those allelopathic effects (Chapter 6), so the presence of sensitive and resistant AMF species at a particular site could be used to predict the risk of *A. petiolata* invasion at that site. Sites with only sensitive AMF species would be more at risk for invasion than sites with resistant AMF species. Plant and AMF species diversity are tightly linked, with particular plant species promoting growth of specific AMF species (van der Heijden et al. 1998a, van der Heijden et al. 1998b, Helgason et al. 2002). Planting native plants that promote the growth of AMF species resistant to allelopathic effects of *A. petiolata* could therefore be a cost effective means of promoting natural restoration of invaded sites.
REFERENCES


Figure 5.1. Mean number of terminal restriction fragments (T-RFs) per site (± 1 SE), by fluorescent label and restriction enzyme.

Each of the four sets of T-RFs was analyzed individually, and bars within each primer:restriction enzyme category that are labeled with different letters were
significantly different after Bonferroni comparisons at $\alpha = 0.05$. A. Differences between sites invaded and uninvaded by *A. petiolata*. B. Differences between sites in Ohio and Massachusetts.
6 EFFECTS OF EXTRACTS OF *ALLIARIA PETIOLATA* ON *IMPATIENS PALLIDA* AND ITS ASSOCIATED ARBUSCULAR MYCORRHIZAL FUNGI ACROSS MULTIPLE LIFE STAGES

6.1 INTRODUCTION

Allelopathy has historically been defined as direct inhibition of plant growth by organic compounds released by a donor plant (Choesin and Boerner 1991, Barkosky et al. 1999). These compounds act through several mechanisms, including causing DNA mutations (Hashimoto et al. 1984, Hashimoto and Shudo 1996, Wu et al. 2000), blocking photosynthesis (Einhellig et al. 1993, Gonzalez and Estevez-Braun 1997), and/or triggering programmed cell death (Bais et al. 2003). While these mechanisms clearly act directly on the target plants, many potential mechanisms of indirect inhibition exist as well (Inderjit and Weiner 2001). For example, compounds released by a donor plant can influence nutrient availability in a way that is more beneficial for the donor plant (Inderjit and Mallik 1999). Also, microbial populations supported by a donor plant can selectively inhibit growth of surrounding plants (Kaminsky 1981), or microbes which benefit surrounding plants could be inhibited by a donor plant (Stinson et al. 2006).

Such beneficial microbes include the arbuscular mycorrhizal fungi (AMF), with which more than 80% of plants associate (Smith and Read 1997). AMF are obligately symbiotic and provide their host plants with mineral nutrients and water in exchange for
photosynthate (Smith and Read 1997). Many plants depend on AMF for normal growth, and any inhibition of AMF would therefore also indirectly slow plant growth. Experimental designs to assess such indirect effects are necessarily more complex than the simple germination bioassays often used to demonstrate direct inhibition. However, indirect effects may be more common in the field than direct effects (Inderjit and Weiner 2001).

Many invasive plants are allelopathic (including *Ailanthus altissima*, *Centaurea maculosa*, and *Alliaria petiolata*) and in many cases their allelopathic effects are believed to enhance invasion success (Lawrence et al. 1991, Ridenour and Callaway 2001, Roberts and Anderson 2001). However, the germination bioassays used to demonstrate these effects have been called into question, and are not easily relatable to effects in the field (Harper 1975, Stowe 1979). Determination of the environmentally relevant levels and stabilities of the allelopathic compounds is also necessary (Choesin and Boerner 1991), but often overlooked (but see Chapter 2).

flavonoid glycosides on plant growth have not been determined. Effects of glucosinolates on established symbioses are also not clear.

I explored the effects of *A. petiolata* extracts on the four major developmental stages of mycorrhizae (1. germination, 2. presymbiosis growth, 3. symbiosis formation, and 4. symbiosis growth), each in a separate experiment. The germination phase includes any stratification requirement up to and including emergence of the radicle from the seed, or hypha from the spore. Seeds and spores are the most cost effective way to reintroduce species to an invaded site during a restoration program, but any inhibition of germination would directly limit further population size. Therefore, if seeds and spores are sensitive to allelopathic compounds produced by *A. petiolata*, they will need to be applied in even greater numbers to achieve desired plant densities during restorations.

Growth of the plant before colonization by AMF is included in the presymbiosis growth phase. Although the non-mycorrhizal condition is usually rare in the field, sites with a long invasion history are likely to have low AMF colonization potential (Roberts and Anderson 2001), and this experiment will demonstrate how well a re-introduced native grows when exposed to *A. petiolata* extracts without the benefit of AMF.

During the symbiosis formation phase, contact between AMF and the plant is initiated and fungal structures begin to form inside the plant root. Allelopathic compounds could interfere with signaling between host plants and AMF, thereby limiting the formation of the symbiosis.

Finally, the symbiosis continues to operate throughout the remainder of the plants lifetime in the symbiosis growth phase. Once a symbiosis is formed, fungal structures inside the root are likely to be somewhat insulated from allelopathic effects. However,
fungal hyphae in the soil will still be exposed, and limited growth of fungal hyphae would compromise the ability of the fungus to absorb water and nutrients for its plant host. Plants can reject such parasitic associations (Smith and Read 1997), killing the fungus, but also slowing plant growth to less than that found in a mutualistic association.

The objectives of this experiment were to assess the effects of glucosinolate and flavonoid glycoside enriched fractions of *A. petiolata*, alone and in concert, on growth of a North American native plant and its associated AMF. These effects were assessed across multiple life stages (germination, presymbiosis growth, symbiosis formation, symbiosis growth) in order to determine the importance of AMF in mediating any observed allelopathic effects. In addition, these results will better inform restoration efforts by identifying which developmental stages are most susceptible to the allelopathic effects of *A. petiolata*, so those life stages can be avoided in restoration plantings. I expect germination to be inhibited, as has been shown previously (Roberts and Anderson 2001, Stinson et al. 2006), but not as severely since lower, more realistic, doses will be used. Stinson et al. (2006) suggested that inhibitory effects were mediated my mycorrhizae, so I expect the symbiosis formation and symbiosis growth phases to be more sensitive than the presymbiosis growth phase.

### 6.2 METHODS

#### 6.2.1 Extract Preparation

Extracts were prepared from *A. petiolata* plants randomly collected from the Wright State University Nature Preserve during late Spring, when the establishment of mycorrhizal symbioses with host plants is actively occurring. Plants were either extracted
immediately after collection, or flash frozen and stored, to maintain a standard lot of material for extract preparation. Glucosinolates were separated from the flavonoid glycosides using a butanol/water fractionation as described in Callaway et al. (2008). All experiments were dosed at a rate equivalent to 3.3 mg *A. petiolata* tissue equivalents per g assay media, a dose that has been estimated to represent expected does in the field (Callaway et al. 2008).

**6.2.2 Target Plant**

*Impatiens pallida* Nutt. (pale jewelweed) was chosen as the target plant because it grows in the same woodland habitats invaded by *A. petiolata* and is dependent on AMF for normal growth (K Barto, personal observation). While AMF are not generally thought to be host specific, certain plant:fungus associations are more effective than others (Stampe and Daehler 2003, Johnson et al. 2004), so I used naturally associated AMF of *I. pallida*. AMF spores were isolated from field soil collected near *I. pallida* plants using a wet sieving technique (Klironomos et al. 1993). Mycorrhizal inoculum consisted of finely chopped *I. pallida* roots collected from a population in Yellow Springs, Ohio (39°47.0’N, 83°52.5’W). *Impatiens pallida* seed were collected from the same population for all experiments. Inoculum was prepared in the fall and stored at 5°C until needed. The rhizosphere of *I. pallida* in southwestern Ohio is dominated by many small (60-110 µm diameter) white and yellow *Glomus* and a larger spored (170-240 µm diameter) *Glomus*, possibly *G. constrictum* (K. Barto, unpublished data).
6.2.3 *Growth Chambers*

The seed germination experiment was conducted in small glass dishes and the spore germination experiment used 96-well plates. The presymbiosis plant growth experiment and the symbiosis formation and symbiosis growth experiments occurred in chambers made of 13 cm x 30 cm x 0.15 cm glass plates held ½ cm apart by silicon on 3 sides (Figure 6.1). With the exception of the spore germination experiment, all other experimental chambers were filled with a 1:1 mix of field soil and sterile coarse sand or the same mix with activated carbon added at a rate of 20 mL/L soil, as has been used in other experiments with *A. petiolata* (Prati and Bossdorf 2004). Local soil has a high proportion of clay and forms impenetrable bricks in the chambers without the addition of sand (K. Barto, personal observation). Field soil was collected from an *A. petiolata* free area of the Wright State University Nature Preserve. Root viewing chambers were covered with foil and stored at a slight angle to encourage root and hyphal growth along one glass plate (Friese and Allen 1991). Chambers were placed under fluorescent grow-lights (75 µmol PAR/m²/sec) at ~22°C in the laboratory. Four separate experiments were conducted using these chambers, examining one developmental stage per experiment.

6.2.4 *Germination*

This experiment examined the effects of treatments on the germination stage of seeds or spores, determined by concurrently adding test fractions and ungerminated seeds or spores to chambers. Seed germination was assessed by placing five *I. pallida* seeds on the soil surface and storing plates at 5°C. Doses were applied every other week. Plates
were covered but not sealed to allow oxygen to circulate. Germination was scored weekly for six months. There were five replicates for each treatment combination.

Spore germination was assessed by placing thirty randomly selected spores in a well of a 96-well plate. Doses were applied once at the beginning of the experiment. Plates were covered and stored at 25°C for two weeks, at which time spores were removed and mounted in PVLG (INVAM). All spores were then identified to morphotype and scored for germination. There were ten replicates in each treatment group.

For both plant and AMF germination experiments, there were four treatments (glucosinolate fraction, glycoside fraction, combined fraction, and water control). The seed germination experiment also included two activated carbon treatments (with or without).

6.2.5 Presymbiosis Plant Growth

This experiment examined the presymbiosis plant growth stage by simultaneously adding test fractions and germinated seeds to root-viewing chambers. *Impatiens pallida* seeds were germinated in sterile water at 5°C (Leck 1979). Preliminary experiments showed that seedlings quickly became colonized with AMF, so chambers were treated with 190 mg/L chlorothalonil (Daconil) every other day to maintain the uncolonized status of seedlings in this experiment. Preliminary experiments determined that this dosage schedule would block mycorrhizal colonization of seedlings without directly impacting *I. pallida* growth (K. Barto, personal observation). Presymbiosis seedling growth was assessed by placing one newly germinated *I. pallida* seed on the soil surface
while adding test compounds. Doses were applied once a week throughout the experiment. The roots were traced unto transparency film and digitized every three days until a root reached the bottom of the chamber.

Fractal techniques can be used to quantify exploration efficiency, depletion zone volume, and homogeneity of branching patterns of roots and hyphae (Walk et al. 2004). These traits correlate well with P uptake in *Phaseolus vulgaris* (Nielsen et al. 1999).

Total root length, area of the root system, and density of roots in the root system (pixels/area) were quantified using ImageJ (NIH: [http://rsb.info.nih.gov/ij/](http://rsb.info.nih.gov/ij/)). Rhizosphere density can be artificially inflated in small root systems because the rhizosphere area is small, so I also measured the box-counting fractal dimension (FD), which measures exploration efficiency of the system independently of rhizosphere size. Fractal dimension and lacunarity, which quantifies homogeneity of the system, were calculated using the FracLac plugin for ImageJ ([http://rsb.info.nih.gov/ij/plugins/frac-lac.html](http://rsb.info.nih.gov/ij/plugins/frac-lac.html)). This FD depends on both the spatial arrangement and branching extent of the root system. In order to calculate the FD a grid is drawn over the root system and the number of boxes needed to completely cover the root system is counted. I repeated this process for 10 box sizes and calculated FD as the negative slope of the linear regression of log(box size (length of side)) on log(number of boxes required to cover the root system). Larger FDs indicate more efficient exploration of the soil, with a maximum of 2 indicating complete coverage (Bernston 1994). Since planar box counting fractal dimensions represent a slice of the root system, they are typically fragmented, with FDs less than 1, and reported values range from 0 to 1.75 (Fitter and Stickland 1992, Nielsen et al. 1997, Eshel 1998, Nielsen et al. 1999, Walk et al. 2004, Dannowski and Block 2005).
portion of *I. pallida* was also recorded once a week. Plants were harvested at the end of the experiment, dried at 30°C to constant mass, and root and shoot dry mass were measured.

### 6.2.6 Symbiosis Formation

This experiment examined the symbiosis formation stage by injecting compounds into inoculated chambers with one growing *I. pallida* seedling. *Impatiens pallida* seeds were stratified in water at 5°C to stimulate germination, then planted in chambers. Mycorrhizal inocula, which consisted of chopped roots of field collected *I. pallida*, was mixed with the soil at a rate of 1g inocula/100 g soil before filling chambers. Injections began as soon as seeds were added, and doses were applied once a week throughout the experiment. Mycorrhizal structures were identified non-destructively in the chambers by their fluorescence after excitation with 460 nm light (Friese and Allen 1991) (Figure 6.2). The thickness of the chambers limits magnification to 100x, so fine mycorrhizal structures were not visible. However, it was possible to identify “landmarks” in the soil and to therefore repeatedly visit the same area.

Chambers were observed along horizontal 6 cm transect lines centered at the middle of the chamber and spaced vertically 5 cm apart. (Figure 6.1A). The first transect line was 2.5 cm below the top of the chamber and additional lines were observed as the roots grew down through the chamber. Chambers were monitored weekly and root colonization and soil colonization indices were calculated as described in Chapter 7 (Barto and Cipollini 2008). Height of the above ground portion of *I. pallida* was also
recorded each week. Plants were harvested at the end of the experiment, dried at 30°C to constant mass, and root and shoot dry mass were measured.

There were four treatments (glucosinolate enriched fraction, flavonoid glycoside enriched fraction, combined fraction, and water control) for each of two carbon amendments (with or without). There were ten replicate chambers per treatment combination yielding 80 chambers total.

6.2.7 Symbiosis Growth

This experiment examined the symbiosis growth stage by injecting compounds into chambers with already established symbioses between *I. pallida* and its associated mycorrhizae. Established symbioses were generated by growing *I. pallida* seedlings in glass plate chambers in field soil containing mycorrhizal inoculum. When seedlings were four weeks old, colonization was verified by fluorescence microscopy before beginning injections of compounds. Injections were repeated weekly thereafter until the end of the experiment. Fungal development characteristics were monitored as in the symbiosis formation experiment. Plates were monitored weekly with observations beginning when plants were three weeks old. Plants were harvested at the end of the experiment, dried at 30°C to constant mass, and root and shoot dry mass were measured. There were four treatments (glucosinolate enriched fraction, flavonoid glycoside enriched fraction, combined fraction, and water control) for each of two carbon amendments (with or without). There were ten replicate chambers per treatment combination yielding 80 chambers total.
6.2.8 Statistical Analyses

For the seed germination experiment, percent germination at each time point was analyzed individually using PROC GLM and ANOVA with activated carbon and extract as fixed factors. Since data collected at each time point were not independent of other time points, I calculated Bonferroni adjustments (Gotelli and Ellison 2004). However, this adjustment is extremely conservative and greatly increases the Type II error rate (Perneger 1998, Gotelli and Ellison 2004), so I do not advocate its use. Adjusted and unadjusted p-values are provided throughout and I leave it up to the reader to decide which significance level they deem appropriate. I also plotted percent germination versus time and calculated the slope of the best-fit regression line, which was then analyzed using ANOVA with activated carbon, and extract as fixed factors. Data were not transformed.

For the spore germination experiment, percent germination was analyzed using PROC GLM and ANOVA with extract and spore morphotype as fixed factors. Data were not transformed.

For the presymbiosis growth experiment, plant height, root length, rhizosphere area, box-counting fractal dimension of the root system, rhizosphere density, and root system lacunarity at each time point were analyzed individually using PROC GLM and ANOVA with activated carbon and extract as fixed factors. I calculated Bonferroni adjusted p-values as described above. To capture the effect of time, slopes of best-fit regression lines through the data over time were calculated for plant height, root length, rhizosphere area, and box-counting fractal dimension and analyzed by ANOVA using the same factors used to analyze individual time points. Rhizosphere density and lacunarity did not demonstrate linear growth patterns, so slopes were not analyzed. Life spans of
plants were analyzed using PROC GLM and ANOVA with activated carbon and extract as fixed factors. Life span was chosen instead of dry masses since so many plants died before the end of the experiment that dry masses were not available for plants in all treatment groups. All data were transformed as necessary to meet assumptions of normality.

For the symbiosis formation experiment, data were analyzed as described for the presymbiosis growth experiment. In addition, the RCI, SCI, and RCI/SCI at each time point, and the slopes of their best-fit regression lines were analyzed individually using PROC GLM and ANOVA with activated carbon, extract, and depth in the chamber as fixed factors. Correlation analyses using PROC CORR were used to compare RCI, SCI, and RCI/SCI values over time with final plant height, the growth rate of plant height, shoot and root dry weights, the root to shoot ratio, final total root length, the growth rate of plant roots, final rhizosphere area, the growth rate of rhizosphere area, the final box-counting fractal dimension, the lacunarity of the root system, and the density of the root system.

For the symbiosis growth experiment, plant height, shoot and root dry masses, root to shoot ratios, RCI, SCI, RCI/SCI were analyzed as for the symbiosis formation experiment. Correlation analysis using PROC CORR were used to compare RCI, SCI and RCI/SCI values over time with final plant height, the growth rate of plant height, shoot and root dry weights, and the root to shoot ratio. SAS Version 9.1 (SAS Institute Inc.) was used for all analyses.
6.3 RESULTS

Activated carbon generally acted as expected, ameliorating effects of *A. petiolata* extracts. Therefore, I will focus on effects of extracts in soil without activated carbon, still noting overall effects of activated carbon.

6.3.1 Germination

*I. pallida* seeds began to germinate after 20 weeks of stratification and no additional seed germinated after 24 weeks (Figure 6.3). Activated carbon did not affect germination rates (Table 6.1). Among treatments without activated carbon, seeds treated with the flavonoid enriched fraction had the highest final germination rates (76%), followed by seeds given water only (68%), then glucosinolate treated (52%) and finally seeds exposed to a combined fraction (28%) ($F_{3,16}=6.22$, $p=0.0053$).

Overall, AMF spore germination was not affected independently by any *A. petiolata* extracts (Figure 6.4). However, three distinct morphotypes of spores were used in this experiment, and each responded differently to *A. petiolata* fractions (Figure 6.4, treatment*morphotype: $F_{6,98}=2.49$, $p=0.0276$). The brown and black spore morphotypes were unaffected by *A. petiolata* fractions. The flavonoid enriched fraction tended to stimulate germination of the white spore morphotype, while no spore germination occurred in control or glucosinolate enriched fractions. Overall, significantly more spores of the brown morphotype germinated than either the black or white morphotype (morphotype: $F_{2,98}=5.78$, $p=0.0042$).
6.3.2 Presymbiosis Growth

*I. pallida* plants in chambers with activated carbon grew faster than plants in chambers without activated carbon and were also taller throughout the course of the experiment (Figure 6.5, Table 6.2). *Alliaria petiolata* extracts had no effect on *I. pallida* height (Figure 6.5, Table 6.2).

All of the *I. pallida* plants grown without AC, and exposed to a glucosinolate containing fraction or a combined fraction, died before the end of the experiment and dry weights were therefore not measured for these plants. Dry weight data were not statistically analyzed since two complete treatment groups would have been omitted from the analysis.

Total root length and rhizosphere area of *I. pallida* plants increased more quickly in plants grown in soil containing AC than in plants grown without AC (Figure 6.6, Tables 6.3, 6.4). Plants grown in soil containing activated carbon completely filled the growth chambers by the end of the experiment, which had a soil area of about 290 cm$^2$. Among the plants grown without AC, control plants and those exposed to a flavonoid enriched fraction had the highest final total root length. Plants exposed to a combined fraction grew to only about 14% of those levels (Figure 6.6A). Rhizosphere areas of plants exposed to a combined fraction reached only about 25% of those reached by control plants (Figure 6.6B). Since these plants were not yet colonized by AMF, these impacts represent direct effects of *A. petiolata* extracts on root growth of *I. pallida* plants.

Root structure of *I. pallida* plants was also affected by *A. petiolata* extracts. The planar box-counting fractal dimension, which quantifies the exploration efficiency of the root system, increased more quickly in chambers containing AC than in chambers
without AC (Figure 6.7A, Table 6.5). After Day 9, the fractal dimension remained significantly higher in chambers with AC than in chambers without (Figure 6.7A, Table 6.5). Among chambers without AC, the fractal dimensions of all plants were initially very similar. The fractal dimensions of roots of control plants or those exposed to a flavonoid enriched fraction increased throughout the experiment, while the fractal dimension of roots of plants exposed to a combined extract did not change over time (slope: $F_{3,33}=4.17$, $p=0.0131$). A less sophisticated measure of exploration efficiency, rhizosphere density, generally followed the same patterns as the fractal dimension, although results were not as pronounced (Figure 6.7B, Table 6.6). In contrast to the fractal dimension, rhizosphere density is easily influenced by rhizosphere area, leading to high values initially when the root system is small. Although rhizosphere density was low in plants exposed to a combined fraction between Days 9 and 18, the high final values are due to the fact that so many of these plants remained small throughout the experiment. Heterogeneity of *I. pallida* root systems, measured as lacunarity, increased over time, but was unaffected by activated carbon or *A. petiolata* extract (Figure 6.7C, Table 6.7).

*Impatiens pallida* plants grown in soil with AC lived longer than plants in soil without AC (Figure 6.8, AC: $F_{1,68}=43.43$, $p<0.0001$). The glucosinolate enriched fraction and a complete *A. petiolata* extract significantly shortened *I. pallida* life span, while a flavonoid enriched fraction had no effect (Figure 6.8, extract: $F_{3,68}=7.64$, $p=0.0002$). This inhibition was only seen in chambers without AC (Figure 6.8, AC*extract: $F_{3,68}=12.35$, $p<0.0001$).
6.3.3 Symbiosis Formation

During the symbiosis formation phase of growth, *I. pallida* height was unaffected by AC or *A. petiolata* extract (Figure 6.9, Table 6.8). Shoot and root dry weights of plants at the end of the experiment were also unaffected by AC or *A. petiolata* extracts.

*Impatiens pallida* total root length and rhizosphere area increased through time for all treatments, but values were unaffected by AC or *A. petiolata* extracts (Figure 6.10, Tables 6.9, 6.10).

Root structure of *I. pallida* plants was unaffected by AC, and heterogeneity of root systems was also unaffected by *A. petiolata* extracts (Figure 6.11, Table 6.11). However, regardless of AC treatment, root systems of plants exposed to a glucosinolate enriched fraction had significantly higher fractal dimensions than those exposed to a combined fraction (Table 6.12). Root systems of control plants or those exposed to a flavonoid enriched fraction had fractal dimensions indistinguishable from either extreme. Also regardless of AC treatment, root systems of plants exposed to a glucosinolate enriched fraction had significantly higher fractal dimensions that those of plants exposed to either a combined fraction or a flavonoid enriched fraction (Table 6.13). Root systems of control plants had fractal dimensions indistinguishable from either extreme.

The root colonization index (RCI) declined over time in all treatments, but was higher in plants growing in soil containing AC than in soil without AC for the first two weeks of the experiment (Figure 6.12A, Table 6.14). RCI values were unaffected by *A. petiolata* extracts (Table 6.14). RCI values were higher in roots near the bottom of the chamber than near the top of the chamber throughout the experiment (Table 6.14). This corresponds to higher colonization of young roots than old roots. The RCI declined
fastest over time in roots near the top of the chamber and slowest in roots near the bottom of the chamber (Table 6.14).

The soil colonization index (SCI) remained relatively constant over time, but was lower in chambers with AC than without AC during the first week of the experiment (Figure 6.12B, Table 6.15). Among chambers without AC, SCI values were significantly higher in those receiving a flavonoid enriched fraction than in those receiving a combined fraction, during the final week of the experiment, meaning that AMF hyphae were less abundant in the later chambers (Figure 6.12B). As for RCI, SCI values varied with the location in the chamber, and were also higher near the base of the chamber than near the top of the chamber (Table 6.15).

The ratio of root to soil colonization (RCI/SCI), was calculated to quantify the degree to which AMF parasitize their plant hosts. This ratio was higher in chambers with activated carbon than without during the first two weeks of the experiment, and declined fastest in chambers with AC (Figure 6.12C, Table 6.16). The parasitism ratio was unaffected by A. petiolata extracts (Table 6.16). As for RCI and SCI, the parasitism ratio was highest near the base of the chambers and lowest near the top (Table 6.16). This ratio remained constant throughout the experiment near the top of the chambers, but declined rapidly near the base of the chambers (Table 6.16).

Correlation analysis were performed to determine how mycorrhizal abundance related to I. pallida size and root morphology. RCI and SCI were not correlated with any measure of root size or structure (Table 6.17). The RCI/SCI values during Weeks 1 and 2 were negatively correlated with final rhizosphere area, meaning that as the degree of parasitism increased, the area of the rhizosphere declined (Table 6.17). RCI and RCI/SCI
values during Week 2 were positively correlated with final plant height (Table 6.18). SCI values during Week 2 were negatively correlated with root and shoot dry weight, while SCI values during Week 3 were negatively correlated with root dry weight and height growth rate (Table 6.18).

### 6.3.4 Symbiosis Growth

During the symbiosis growth phase, plant height increased more quickly in chambers containing AC than in chambers without (Figure 6.13, Table 6.19). *Impatiens pallida* height was unaffected by *A. petiolata* extracts (Table 6.19). Root and shoot dry weights were higher in chambers without AC than in chambers with AC, but were unaffected by *A. petiolata* extracts (Table 6.20).

Since *I. pallida* were given a month in the chambers before doses began, roots had filled the chambers to such an extent that it would have been extremely difficult to track further development. Root morphology data was therefore not collected.

RCI values were higher in plants grown in AC than in plants grown without AC during Week 1 (Figure 6.14A, Table 6.21). RCI values were unaffected by *A. petiolata* extracts (Figure 6.14A, Table 6.21). As during the symbiosis formation phase, RCI values varied with root position in the chambers. Patterns were identical to those seen during the symbiosis formation phase, with higher RCI values near the base of the chamber than at the top of the chamber (Table 6.21). RCI values increased over time at the top of the chambers, but decreased quickly near the base of the chambers.

SCI values were higher in chambers without AC than in chambers with AC throughout the experiment (Figure 6.14B, Table 6.22). Among chambers without AC,
RCI values were highest in control chambers and those receiving a combined fraction, and lowest in chambers receiving a flavonoid enriched fraction (Figure 6.14B). SCI values varied with position in the chamber, but followed different patterns than those observed during the symbiosis formation growth phase. During Week 1, SCI values were lowest at the base of the chamber and highest near the top. During Week 3, values were highest at either end of the chamber, and lowest in the middle.

The parasitism ratio was highest in chambers with AC during Week 1, but declined quickly in those chambers (Figure 6.14C, Table 6.23). Among chambers without AC, RCI/SCI values were highest in chambers receiving a flavonoid enriched fraction and lowest in control chambers and those receiving a combined fraction (Figure 6.14C). As during the symbiosis formation growth phase, the parasitism ratio was highest near the base of the chambers and lowest near the top of the chambers throughout the experiment. Values remained constant throughout the experiment at the top of the chambers, but declined quickly near the base of the chambers.

RCI values during Week 2 were negatively correlated with *I. pallida* plant height (Table 6.24). SCI and the parasitism ratio were not correlated with any measure of plant size (Table 6.24).

### 6.4 DISCUSSION

Glucosinolate and flavonoid enriched fractions from *A. petiolata* extracts influenced the growth of *I. pallida* and its associated AMF. Activated carbon ameliorated those effects for the most part, as has been seen in other allelopathy studies (Ridenour and Callaway 2001, Prati and Bossdorf 2004).
Although total AMF spore germination was not affected by *A. petiolata* extracts, effects varied by spore morphotype. AMF and their plant hosts do not appear to be obligately species specific, but certain plant-fungus associations are more beneficial than others (van der Heijden et al. 1998b, Bray et al. 2003). The differential effects on AMF morphotypes seen here could lead to changes in AMF species diversity in the field, which could then impact plant species diversity (van der Heijden et al. 1998a, van der Heijden et al. 1998b). Additional work on AMF diversity in field sites impacted by *A. petiolata* will help clarify the importance of this phenomenon in the field, and is presented as a separate chapter in this dissertation.

Synergistic effects of the flavonoid and glucosinolate enriched fractions were observed in the germination and presymbiosis growth phases, but not in either symbiosis phase. The flavonoid enriched fraction had no effect alone, but appeared to enhance the slight toxicity of the glucosinolate enriched fraction, leading to the very low germination or growth rates observed after exposure to a combined fraction. Synergistic interactions among allelopathic compounds also occur in *Triticum*, where phenolic compounds increase cell permeability in root tips, allowing greater uptake of mutagenic benzoxazinoids (Blum et al. 1992, Hashimoto and Shudo 1996). Synergistic mechanisms also operate in *Desmodium* and *Sorghum*, which block parasitism by *Striga* plants by concurrently stimulating germination of *Striga* seeds while inhibiting further root growth (Chang et al. 1986, Weston et al. 1989, Yoder 2001, Tsanuo et al. 2003). While the specific mechanism of action remains to be elucidated, synergistic effects among compounds produced by *A. petiolata* are clearly limiting plant and AMF growth during the early stages of development.
All measures of AMF health, RCI, SCI, and RCI/SCI, were higher near the base of the chambers throughout the symbiosis formation and growth phases, suggesting that young roots support higher colonization loads than old roots. This pattern has been demonstrated previously in *I. pallida* (Barto and Cipollini 2008), although it is not a universal phenomenon since colonization in *Trifolium repens* and *Allium porrum* is not related to root age (Smith et al. 1992).

The RCI declined over time in both the symbiosis formation and symbiosis growth phases of the experiment, although values were highest during the formation of the symbiosis. The continual decline of RCI could be an artifact of the small size of the growth chambers. As the plants aged and their roots filled up the soil volume, assistance from AMF would become less necessary. Plants have some control over intraradical fungal growth (Smith and Read 1997) and could have been limiting fungal development as the plants became root bound. This is supported by the decline in RCI values in chambers containing AC, as well as control chambers receiving only water.

SCI values remained relatively constant throughout the symbiosis formation and growth stages, suggesting that extraradical fungal structures are less dependent on the age or status of the symbiosis than intraradical fungal structures. The plant likely has more control over intraradical fungal structures than extraradical, and the continued growth of extraradical structures may represent a last ditch effort by the fungus to find a new plant host in the face of decreasing support, via intraradical structures, by its current host. Clearly, knowledge of the abundance and longevity of intraradical and extraradical fungal structures is necessary in order to fully understand mycorrhizal symbioses.
Correlation analyses were conducted to assess the relationship between AMF and plant health. As expected, during the symbiosis formation phase, RCI values were positively correlated with plant height and negatively correlated with rhizosphere area. This pattern has been observed previously (Barto and Cipollini 2008), presumably because a high AMF colonization allows the plant to invest fewer resources in root biomass, leaving more for aboveground biomass. However, SCI values were negatively correlated with plant height growth rates and root and shoot dry weights, suggesting that other factors also mediate this interaction. The only correlation observed during the symbiosis growth phase was a negative correlation between RCI and plant height, suggesting that AMF benefit plant growth primarily early in development.

A more thorough test of the validity of this index for quantifying the degree to which AMF parasitize their plant hosts is necessary. Such a test should control for a wide range of RCI/SCI values, since despite the range of extracts applied to AMF during these experiments, only a narrow range of RCI/SCI values were produced. Maintaining a range of fertilization levels should be an effective strategy.

In contrast to prior work demonstrating inhibition of AMF by *A. petiolata* (Stinson et al. 2006, Callaway et al. 2008), I found little evidence for impacts on AMF, and no evidence that those impacts translated to reduced plant growth. Stinson et al. (2006) used doses of *A. petiolata* extracts twice as high as those used here, which could account for the inhibition observed in that study. The doses used here are more likely to be ecologically relevant (Callaway et al. 2008), and any impacts not seen at these doses
are unlikely to be important in the field. Callaway et al. (2008) measured AMF spore viability instead of germination, and viability is usually higher and less variable than germination (Walley and Germida 1995). Germination requirements for AMF are not well understood, and the poor germination in this study may therefore have been due to culture conditions instead of *A. petiolata* extracts. The approach used by Callaway et al. (2008), which showed reduced viability of AMF spores after exposure to the same glucosinolate and flavonoid enriched fractions used here, was therefore a more robust approach than that used here.

I found extensive evidence of inhibition of seed germination and presymbiosis plant growth in the absence of AMF. The lack of these non-AMF mediated inhibitory effects during the symbiosis formation and growth phases suggests that the AMF may actually be protecting the plant from the allelopathic compounds. Non-mycorrhizal species are typically easily outcompeted by mycorrhizal species (Grimet et al. 1987). The non-mycorrhizal species *Salsola kali* produced high enough levels of phenolics as a defense response to AMF colonization attempts to kill its own plant roots (Allen et al. 1989). Amelioration of allelopathic effects by AMF (Yun and Choi 2002). and ectomycorrhizal fungi has also been demonstrated (Nilsson et al. 1993, Souto et al. 2000).

In summary, allelopathic effects of *A. petiolata* on *I. pallida* were more consistent and more severe during early life stages when the plants were not colonized by AMF. Despite the apparent resistance of colonized plants to these inhibitory effects, *I. pallida* populations in the field could still be severely impacted. When exposed to a complete *A. petiolata* extract, *I. pallida* seed germination was less than 41% that of seeds dosed with
water. In the next growth stage, uncolonized *I. pallida* plants treated with a complete *A. petiolata* extract grew to less than 18% of the total root length of plants treated with water. Although colonized plants in the final growth stages were more resistant to allelopathic effects, it is unclear whether severely inhibited uncolonized plants would ever be able to recover and initiate mutualistic mycorrhizal associations.

Regardless of effects on older plants, the 41% reduction in seed germination suggests that *I. pallida* populations invaded by *A. petiolata* would have significantly fewer adult *I. pallida* plants than uninvaded populations. These differences would be magnified each year as fewer and fewer seed were produced, leading to dramatic reductions in *I. pallida* individuals over time. Although fieldwork is needed to verify this, restoration plantings using colonized *I. pallida* plants seem more likely to succeed than either uncolonized plants or seed.
6.5 REFERENCES


Inderjit, and J. Weiner. 2001. Plant allelochemical interference or soil chemical ecology? Perspectives in Plant Ecology, Evolution and Systematics 4:3-12.


Table 6.1. Results of ANOVA for percent germination of *I. pallida* seeds exposed to *A. petiolata* extracts over time, with AC (activated carbon) and extract as factors.

Bold p-values indicate significance at $\alpha = 0.05$, and italic p-values indicate significance at the Bonferroni adjusted level of $\alpha = (0.05/5) = 0.01$ where appropriate.

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Table 6.2. Results of ANOVA for height of *I. pallida* plants exposed to *A. petiolata* extracts during the presymbiosis phase over time, with AC (activated carbon) and extract as factors.

Bold p-values indicate significance at $\alpha = 0.05$, and italic p-values indicate significant at the Bonferroni adjusted level of $\alpha = (0.05/5) = 0.01$ where appropriate.

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<td>7.86</td>
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Table 6.3. Results of ANOVA for total root length of *I. pallida* plants exposed to *A. petiolata* extracts during the presymbiosis phase over time, with AC (activated carbon) and extract as factors.

Bold p-values indicate significance at $\alpha = 0.05$, and italic p-values indicate significance at the Bonferroni adjusted level of $\alpha = (0.05/7) = 0.007$ where appropriate.

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Table 6.4. Results of ANOVA for rhizosphere area of *I. pallida* plants exposed to *A. petiolata* extracts during the presymbiosis phase over time, with AC (activated carbon) and extract as factors.

Bold p-values indicate significance at $\alpha = 0.05$, and italic p-values indicate significance at the Bonferroni adjusted level of $\alpha = (0.05/7) = 0.007$ where appropriate.

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<td><strong>0.0021</strong></td>
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161
Table 6.5. Results of ANOVA for the box-counting fractal dimension of root systems of *I. pallida* plants exposed to *A. petiolata* extracts during the presymbiosis phase over time, with AC (activated carbon) and extract as factors.

Bold p-values indicate significance at $\alpha = 0.05$, and italic p-values indicate significance at the Bonferroni adjusted level of $\alpha = (0.05/7) = 0.007$ where appropriate.

| Source      | df | F    | p    | F    | p    | F    | p    | F    | p    | F    | p    | F    | p    | F    | p    | F    | p    |
|-------------|----|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
| AC          | 1  | 3.45 | 0.0675 | 7.38 | 0.0085 | 19.18 | <0.0001 | 11.08 | 0.0015 | 8.43 | 0.0053 | 20.26 | <0.0001 | 54.51 | <0.0001 | 21.20 | <0.0001 |
| extract     | 3  | 0.70 | 0.5550 | 2.87 | 0.0431 | 4.65 | 0.0056 | 2.92 | 0.0421 | 3.49 | 0.0216 | 2.77 | 0.0521 | 6.35 | 0.0007 | 5.87 | 0.0013 |
| AC*extract  | 3  | 0.19 | 0.9032 | 0.62 | 0.6065 | 0.90 | 0.4475 | 0.50 | 0.6862 | 1.17 | 0.3279 | 1.30 | 0.2855 | 2.79 | 0.0471 | 1.45 | 0.2356 |
| Error df: 68 |    |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| Error df: 64 |    |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| Error df: 58 |    |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| Error df: 56 |    |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| Error df: 54 |    |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| Error df: 46 |    |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| Error df: 68 |    |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| Error df: 68 |    |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
Table 6.6. Results of ANOVA for rhizosphere density of *I. pallida* plants exposed to *A. petiolata* extracts during the presymbiosis phase over time, with AC (activated carbon) and extract as factors.

Bold p-values indicate significance at $\alpha = 0.05$, and italic p-values indicate significance at the Bonferroni adjusted level of $\alpha = (0.05/7) = 0.007$ where appropriate.

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</table>
Table 6.7. Results of ANOVA for root system lacunarity of *I. pallida* plants exposed to *A. petiolata* extracts during the presymbiosis phase over time, with AC (activated carbon) and extract as factors.

Bold p-values indicate significance at $\alpha = 0.05$. No results were significant at the Bonferroni adjusted level of $\alpha = (0.05/7) = 0.007$.

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<td>0.76</td>
<td>0.5205</td>
<td>0.37</td>
<td>0.7785</td>
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<td>0.6496</td>
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<td>0.0237</td>
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<td>0.33</td>
<td>0.8040</td>
<td>0.83</td>
<td>0.4834</td>
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Table 6.8. Results of ANOVA for height of *I. pallida* plants exposed to *A. petiolata* extracts during the symbiosis formation phase over time, with AC (activated carbon) and extract as factors.

No factors were significant at $\alpha = 0.05$.

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<td>0.0511</td>
<td>1.14</td>
<td>0.2886</td>
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<td>0.4171</td>
<td>1.03</td>
<td>0.3832</td>
<td>0.51</td>
<td>0.6763</td>
<td>0.22</td>
<td>0.8813</td>
<td>0.17</td>
<td>0.9152</td>
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<td>0.7063</td>
<td>0.68</td>
<td>0.5651</td>
<td>0.53</td>
<td>0.6606</td>
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<td>0.8062</td>
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</tbody>
</table>
Table 6.9. Results of ANOVA for total root length of *I. pallida* plants exposed to *A. petiolata* extracts during the symbiosis formation phase over time, with AC (activated carbon) and extract as factors.

Bold p-values indicate significance at \( \alpha = 0.05 \). No factors were significant at the Bonferroni adjusted \( \alpha = (0.05/7) = 0.007 \).

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<th>Day 9</th>
<th>Day 12</th>
<th>Day 15</th>
<th>Day 18</th>
<th>Final</th>
<th>Slope</th>
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<tbody>
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<td>0.3243</td>
<td>0.85</td>
<td>0.3599</td>
<td>0.02</td>
<td>0.8863</td>
<td>0.55</td>
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<td>0.4831</td>
<td>0.78</td>
<td>0.5106</td>
<td>0.39</td>
<td>0.7600</td>
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<td>AC*extract</td>
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<td>0.62</td>
<td>0.6047</td>
<td>0.22</td>
<td>0.8811</td>
<td>0.23</td>
<td>0.8721</td>
<td>0.11</td>
<td>0.9518</td>
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</tbody>
</table>

| Error df        | 72 | 70     | 70    | 72     | 69     | 57     | 68     | 72    |       |

166
Table 6.10. Results of ANOVA for rhizosphere area of *I. pallida* plants exposed to *A. petiolata* extracts during the symbiosis formation phase over time, with AC (activated carbon) and extract as factors.

No factors were significant at $\alpha = 0.05$.

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<td>0.7049</td>
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<td>0.6735</td>
<td>0.42</td>
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<td>1.05</td>
<td>0.3784</td>
<td>0.66</td>
<td>0.5795</td>
<td>0.82</td>
<td>0.4891</td>
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Error df: 72 | Error df: 70 | Error df: 71 | Error df: 72 | Error df: 69 | Error df: 57 | Error df: 71 | Error df: 72
Table 6.11. Results of ANOVA for rhizosphere lacunarity of *I. pallida* plants exposed to *A. petiolata* extracts during the symbiosis formation phase over time, with AC (activated carbon) and extract as factors.

No factors were significant at the Bonferroni adjusted $\alpha = (0.05/7) = 0.007$.

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Error df: 72 | Error df: 70 | Error df: 71 | Error df: 72 | Error df: 69 | Error df: 57 | Error df: 72
Table 6.12. Results of ANOVA for the box-counting fractal dimension of the root systems of *I. pallida* plants exposed to *A. petiolata* extracts during the symbiosis formation phase over time, with AC (activated carbon) and extract as factors.

Bold p-values indicate significance at $\alpha = 0.05$. No factors were significant at the Bonferroni adjusted $\alpha = (0.05/7) = 0.007$.

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<td>0.7435</td>
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Error df: 72  | Error df: 70 | Error df: 71 | Error df: 72 | Error df: 69 | Error df: 57 | Error df: 72 | Error df: 71
Table 6.13. Results of ANOVA for rhizosphere density of *I. pallida* plants exposed to *A. petiolata* extracts during the symbiosis formation phase over time, with AC (activated carbon) and extract as factors.

Italic p-values indicate significance at the Bonferroni adjusted $\alpha = (0.05/7) = 0.007$.

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</table>

Error df: 72  | Error df: 70 | Error df: 71 | Error df: 72 | Error df: 68 | Error df: 57 | Error df: 72
Table 6.14. Results of ANOVA for root colonization index (RCI) of *I. pallida* plants exposed to *A. petiolata* extracts during the symbiosis formation phase over time, with AC (activated carbon), extract, and line (depth in chamber) as factors.

Bold p-values indicate significance at $\alpha = 0.05$ for Slope, and at the Bonferroni adjusted $\alpha = (0.05/3) = 0.017$ for all other data.

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<th>p</th>
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Table 6.15. Results of ANOVA for soil colonization index (SCI) of *I. pallida* plants exposed to *A. petiolata* extracts during the symbiosis formation phase over time, with AC (activated carbon), extract, and line (depth in chamber) as factors.

Bold p-values indicate significance at $\alpha = 0.05$, and italic p-values indicate significance at the Bonferroni adjusted level of $\alpha = (0.05/3) = 0.017$ where appropriate.

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Bold p-values indicate significance at $\alpha = 0.05$, and italic p-values indicate significance at the Bonferroni adjusted level of $\alpha = (0.05/5) = 0.01$ where appropriate.

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Table 6.17. Results of correlation analysis between Root Colonization Index (RCI), Soil Colonization Index (SCI), and Parasitism Index (RCI/SCI), and root morphology of *I. pallida* plants exposed to *A. petiolata* extracts during the symbiosis formation phase.

Pearson Correlation Coefficient (p value). Length – final total root length, Length Growth – growth rate of root length, Area – final rhizosphere area, Area Growth – growth rate of rhizosphere area, FD – final box-counting fractal dimension of root system, Heterogeneity – final lacunarity of root system, Density – final density of root system. N=79-80. Italic p-values indicate significance at the Bonferroni adjusted $\alpha = (0.05/3) = 0.017$.

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Table 6.18. Results of correlation analysis between Root Colonization Index (RCI), Soil Colonization Index (SCI), and Parasitism Index (RCI/SCI), and size of *I. pallida* plants exposed to *A. petiolata* extracts during the symbiosis formation phase.

Pearson Correlation Coefficient (p value). Height – final plant height, Height Growth – growth rate of plant height, Shoot DW – shoot dry weight, Root DW – root dry weight, RS Ratio – ratio of root to shoot dry weight. N=79-80. Bold p-values indicate significance at $\alpha = 0.05$, and italic p-values indicate significance at the Bonferroni adjusted $\alpha = (0.05/3) = 0.017$.

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Table 6.19. Results of ANOVA for the height of \textit{I. pallida} plants exposed to \textit{A. petiolata} extracts during the symbiosis growth phase over time, with AC (activated carbon), and extract as factors.

Bold p-values indicate significance at $\alpha = 0.05$. No factors were significant at the Bonferroni adjusted $\alpha = (0.05/5) = 0.01$.

<table>
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<th>Week 1 F</th>
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<th>Week 1 F</th>
<th>p</th>
<th>Week 1 F</th>
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<th>Week 1 F</th>
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<td>0.4892</td>
<td>&lt;0.01</td>
<td>0.9685</td>
<td>0.30</td>
<td>0.5850</td>
<td>0.34</td>
<td>0.5593</td>
<td>0.09</td>
<td>0.7609</td>
<td>5.41</td>
<td>bold0229</td>
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<td>Error df: 72</td>
<td>Error df: 72</td>
<td>Error df: 72</td>
</tr>
<tr>
<td>extract</td>
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<td>1.98</td>
<td>0.1240</td>
<td>2.37</td>
<td>0.0797</td>
<td>2.42</td>
<td>0.0729</td>
<td>1.87</td>
<td>0.1431</td>
<td>0.61</td>
<td>0.6112</td>
<td>1.89</td>
<td>0.1386</td>
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<td>Error df: 72</td>
<td>Error df: 72</td>
<td>Error df: 72</td>
</tr>
<tr>
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<td>0.06</td>
<td>0.9814</td>
<td>0.35</td>
<td>0.7926</td>
<td>0.15</td>
<td>0.9264</td>
<td>0.45</td>
<td>0.7188</td>
<td>0.13</td>
<td>0.9400</td>
<td>0.30</td>
<td>0.8223</td>
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Table 6.20. Results of ANOVA for dry-weights of *I. pallida* plants exposed to *A. petiolata* extracts during the symbiosis growth phase, with AC (activated carbon), and extract as factors.

Bold p-values indicate significance at $\alpha = 0.05$.

<table>
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<th>p</th>
<th>Root F</th>
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<th>Root:Shoot F</th>
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</thead>
<tbody>
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<td>AC</td>
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<td>5.83</td>
<td>0.0183</td>
<td>4.42</td>
<td>0.0391</td>
<td>2.87</td>
<td>0.0949</td>
</tr>
<tr>
<td>extract</td>
<td>3</td>
<td>1.80</td>
<td>0.1546</td>
<td>0.73</td>
<td>0.5362</td>
<td>1.44</td>
<td>0.2386</td>
</tr>
<tr>
<td>AC*extract</td>
<td>3</td>
<td>0.35</td>
<td>0.7863</td>
<td>1.02</td>
<td>0.3904</td>
<td>0.84</td>
<td>0.5922</td>
</tr>
</tbody>
</table>

Error df: 71  Error df: 71  Error df: 70
Table 6.21. Results of ANOVA for the root colonization index (RCI) of *I. pallida* plants exposed to *A. petiolata* extracts during the symbiosis growth phase over time, with AC (activated carbon), extract, and line (depth in chamber) as factors.

Bold p-values indicate significance at $\alpha = 0.05$, and italic p-values indicate significance at the Bonferroni adjusted level of $\alpha = \frac{0.05}{3} = 0.017$ where appropriate.

<table>
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<th></th>
<th>Week 3</th>
<th></th>
<th>Slope</th>
<th></th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
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<td>p</td>
<td>F</td>
<td>p</td>
<td>F</td>
<td>p</td>
<td>F</td>
<td>p</td>
</tr>
<tr>
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<td>6.29</td>
<td><strong>0.0127</strong></td>
<td>1.06</td>
<td>0.3050</td>
<td>0.01</td>
<td>0.9423</td>
<td>0.38</td>
<td>0.5368</td>
</tr>
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<td>extract</td>
<td>3</td>
<td>1.68</td>
<td>0.1704</td>
<td>1.58</td>
<td>0.1943</td>
<td>0.19</td>
<td>0.9014</td>
<td>1.04</td>
<td>0.3766</td>
</tr>
<tr>
<td>AC*extract</td>
<td>3</td>
<td>1.53</td>
<td>0.2063</td>
<td>5.47</td>
<td><strong>0.0011</strong></td>
<td>0.18</td>
<td>0.9103</td>
<td>0.51</td>
<td>0.6790</td>
</tr>
<tr>
<td>line</td>
<td>4</td>
<td>14.17</td>
<td><strong>&lt;0.0001</strong></td>
<td>14.10</td>
<td><strong>&lt;0.0001</strong></td>
<td>7.34</td>
<td><strong>&lt;0.0001</strong></td>
<td>2.94</td>
<td><strong>0.0223</strong></td>
</tr>
<tr>
<td>AC*line</td>
<td>4</td>
<td>1.60</td>
<td>0.1740</td>
<td>0.58</td>
<td>0.6802</td>
<td>0.61</td>
<td>0.6561</td>
<td>0.86</td>
<td>0.4874</td>
</tr>
<tr>
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<td>0.3802</td>
<td>1.73</td>
<td>0.0595</td>
<td>0.55</td>
<td>0.8807</td>
<td>0.84</td>
<td>0.6079</td>
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<td>AC<em>extract</em>line</td>
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<td>1.10</td>
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<td>0.84</td>
<td>0.6048</td>
<td>0.53</td>
<td>0.8922</td>
<td>0.35</td>
<td>0.9646</td>
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</table>

Error df: 293 Error df: 302 Error df: 223 Error df: 160
Table 6.22. Results of ANOVA for the soil colonization index (SCI) of *I. pallida* plants exposed to *A. petiolata* extracts during the symbiosis growth phase over time, with AC (activated carbon), extract, and line (depth in chamber) as factors.

Bold p-values indicate significance at $\alpha = 0.05$, and italic p-values indicate significance at the Bonferroni adjusted level of $\alpha = (0.05/3) = 0.017$ where appropriate.

<table>
<thead>
<tr>
<th>Source</th>
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<th>p</th>
<th>Week 2 F</th>
<th>p</th>
<th>Week 3 F</th>
<th>p</th>
<th>Slope F</th>
<th>p</th>
</tr>
</thead>
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<tr>
<td>AC</td>
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<td>12.49</td>
<td><strong>0.0005</strong></td>
<td>5.81</td>
<td><strong>0.0164</strong></td>
<td>3.99</td>
<td>0.0467</td>
<td>2.39</td>
<td>0.1230</td>
</tr>
<tr>
<td>extract</td>
<td>3</td>
<td>1.04</td>
<td>0.3747</td>
<td>4.67</td>
<td><strong>0.0033</strong></td>
<td>1.34</td>
<td>0.2629</td>
<td>0.73</td>
<td>0.5375</td>
</tr>
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<td>AC*extract</td>
<td>3</td>
<td>1.25</td>
<td>0.2924</td>
<td>1.91</td>
<td>0.1276</td>
<td>0.96</td>
<td>0.4115</td>
<td>2.31</td>
<td>0.0770</td>
</tr>
<tr>
<td>line</td>
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<td>3.38</td>
<td><strong>0.0098</strong></td>
<td>1.41</td>
<td>0.2285</td>
<td>5.94</td>
<td><strong>0.0001</strong></td>
<td>0.84</td>
<td>0.4992</td>
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<td>1.52</td>
<td>0.1958</td>
<td>1.94</td>
<td>0.1028</td>
<td>0.56</td>
<td>0.6908</td>
<td>1.50</td>
<td>0.2028</td>
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<td>0.80</td>
<td>0.6491</td>
<td>0.29</td>
<td>0.9912</td>
<td>1.12</td>
<td>0.3419</td>
<td>0.62</td>
<td>0.8258</td>
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<td>0.83</td>
<td>0.6234</td>
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<td>0.8574</td>
<td>0.85</td>
<td>0.5965</td>
<td>1.31</td>
<td>0.2149</td>
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Table 6.23. Results of ANOVA for the parasitism index (RCI/SCI) of *I. pallida* plants exposed to *A. petiolata* extracts during the symbiosis growth phase over time, with AC (activated carbon), extract, and line (depth in chamber) as factors.

Bold p-values indicate significance at \( \alpha = 0.05 \), and italic p-values indicate significance at the Bonferroni adjusted level of \( \alpha = (0.05/3) = 0.017 \) where appropriate.

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<th>Week 2</th>
<th></th>
<th>Week 3</th>
<th></th>
<th>Slope</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
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<td>p</td>
<td>F</td>
<td>p</td>
<td>F</td>
<td>p</td>
<td>F</td>
<td>p</td>
</tr>
<tr>
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<td>\textless 0.0001</td>
<td>3.70</td>
<td>0.0552</td>
<td>0.48</td>
<td>0.4910</td>
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<td>\textbf{0.0418}</td>
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<td>0.0653</td>
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<td>\textbf{0.0031}</td>
<td>0.58</td>
<td>0.6292</td>
<td>1.68</td>
<td>0.1710</td>
</tr>
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<td>0.5051</td>
<td>2.22</td>
<td>0.0857</td>
<td>0.29</td>
<td>0.8332</td>
<td>0.28</td>
<td>0.8426</td>
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<tr>
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<td>4</td>
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<td>\textless 0.0001</td>
<td>13.96</td>
<td>\textless 0.0001</td>
<td>8.49</td>
<td>\textless 0.0001</td>
<td>3.63</td>
<td>\textbf{0.0065}</td>
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<td>0.57</td>
<td>0.8617</td>
<td>0.75</td>
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<td>12</td>
<td>0.64</td>
<td>0.8090</td>
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<td>0.9969</td>
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Error df: 293   Error df: 302   Error df: 223   Error df: 331
Table 6.24. Results of correlation analysis between Root Colonization Index (RCI), Soil Colonization Index (SCI), and Parasitism Index (RCI/SCI), and size of *I. pallida* plants exposed to *A. petiolata* extracts during the symbiosis growth phase.

Pearson Correlation Coefficient (p value). Height – final plant height, Height Growth – growth rate of plant height, Shoot DW – shoot dry weight, Root DW – root dry weight, RS Ratio – ratio of root to shoot dry weight. N=64-79. Bold p-values indicate significance at $\alpha = 0.05$. No correlations were significant at the Bonferroni adjusted $\alpha = (0.05/3) = 0.017$.

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<th>RS Ratio</th>
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<td>0.0216</td>
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<tr>
<td></td>
<td>(0.4887)</td>
<td>(0.7336)</td>
<td>(0.8705)</td>
<td>(0.9605)</td>
<td>(0.8495)</td>
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<td>Week 2</td>
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<td>-0.1254</td>
<td>-0.0197</td>
<td>-0.0564</td>
<td>-0.0627</td>
</tr>
<tr>
<td></td>
<td><em>(0.0457)</em></td>
<td><em>(0.2705)</em></td>
<td><em>(0.8626)</em></td>
<td>*(0.6212)</td>
<td><em>(0.5826)</em></td>
</tr>
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<tr>
<td></td>
<td>(0.1701)</td>
<td>(0.2422)</td>
<td>(0.9040)</td>
<td>(0.5209)</td>
<td>(0.2930)</td>
</tr>
<tr>
<td></td>
<td>Week 1</td>
<td>Week 2</td>
<td>Week 3</td>
<td>RCI/SCI</td>
<td></td>
</tr>
<tr>
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<td>--------------</td>
<td>--------------</td>
<td>--------------</td>
<td>--------------</td>
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<tr>
<td></td>
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<td>(0.7650)</td>
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<td>-0.0341</td>
<td>-0.0002</td>
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<td>(0.9984)</td>
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<td>-0.1792</td>
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<td>(0.1670)</td>
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<td>0.0349</td>
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<td>(0.7843)</td>
<td>(0.7843)</td>
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<td>0.0353</td>
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<td>(0.7817)</td>
<td></td>
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</table>
Figure 6.1. Root-viewing chambers.

A. Diagram of root-viewing chamber, showing plant roots visible against the upper glass plate. Bold lines represent locations of transect lines. B. Arrangement of root-viewing chambers in growth chambers.
Figure 6.2. *Impatiens pallida* roots observed with epifluorescence microscopy.

Figure 6.3. Percent germination of *I. pallida* seeds exposed to *A. petiolata* extracts during stratification, means ± 1 SE.

AC - Mean of all chambers with activated carbon. Control - Chambers without activated carbon treated with water only. Flav - Chambers without activated carbon treated with a flavonoid enriched fraction. Gluc - Chambers without activated carbon treated with the glucosinolate enriched fraction. Combined - Chambers without activated carbon treated with both flavonoid and glucosinolate enriched fractions. Different letters indicate differences among treatments without activated carbon significant at $\alpha = 0.05$, different bold letters indicate differences significant at the Bonferroni adjusted $\alpha = (0.05/5) = 0.01$. With activated carbon (N=20), without activated carbon (N=5).
Figure 6.4. Percent germination of AMF spores exposed to *A. petiolata* extracts, means ± 1 SE (N=7-10).

Control - Treated with water only. Flav - Treated with a flavonoid enriched fraction. Gluc - Treated with the glucosinolate enriched fraction. Combined - Treated with both flavonoid and glucosinolate enriched fractions.
Figure 6.5. Heights of *I. pallida* plants exposed to *A. petiolata* extracts during the presymbiosis growth phase, means ± 1 SE.

Figure 6.6. Root growth parameters of *I. pallida* plants exposed to *A. petiolata* extracts during the presymbiosis growth phase, means ± 1 SE.

AC - Mean of all chambers with activated carbon. Control - Chambers without activated carbon treated with water only. Flav – Chambers without activated carbon treated with a flavonoid enriched fraction. Gluc - Chambers without activated carbon treated with the glucosinolate enriched fraction. Combined - Chambers without activated carbon treated with both flavonoid and glucosinolate enriched fractions. Different letters indicate differences among treatments without activated carbon significant at $\alpha = 0.05$.

A. Total root length, with activated carbon (N=32-39), without activated carbon (N=4-10). B. Rhizosphere area, with activated carbon (N=32-39), without activated carbon (N=4-10).
Figure 6.7. Root structural parameters of *I. pallida* plants exposed to *A. petiolata*
extracts during the presymbiosis growth phase, means ± 1 SE.

AC - Mean of all chambers with activated carbon. Control - Chambers without activated carbon treated with water only. Flav - Chambers without activated carbon treated with a flavonoid enriched fraction. Gluc - Chambers without activated carbon treated with the glucosinolate enriched fraction. Combined - Chambers without activated carbon treated with both flavonoid and glucosinolate enriched fractions. Different letters indicate differences among treatments without activated carbon significant at $\alpha = 0.05$, different bold letters indicate differences significant at the Bonferroni adjusted $\alpha = (0.05/7) = 0.007$

A. Planar box-counting fractal dimension, with activated carbon (N=32-39), without activated carbon (N=4-10). B. Rhizosphere density (length pixels/area pixels), with activated carbon (N=32-39), without activated carbon (N=2-10). The inset more clearly shows differences after Day 9. C. Heterogeneity of root system, measured as lacunarity, with activated carbon (N=33-39), without activated carbon (N=3-10).
Figure 6.8. Life span of *I. pallida* plants exposed to *A. petiolata* extracts during the presymbiosis growth phase, means ± 1 SE (N=9-10).

AC - activated carbon, Control - Chambers treated with water only, Flav - Chambers treated with a flavonoid enriched fraction, Gluc - Chambers treated with a glucosinolate enriched fraction, Combined - Chambers treated with both flavonoid and glucosinolate enriched fractions. Different letters indicate differences among treatments significant at α = 0.05,
Figure 6.9. Heights of *I. pallida* plants exposed to *A. petiolata* extracts during the symbiosis formation phase, means ± 1 SE.

Figure 6.10. Root growth parameters of *I. pallida* plants exposed to *A. petiolata* extracts during the symbiosis formation phase, means ± 1 SE.

AC - Mean of all chambers with activated carbon. Control – Chambers without activated carbon treated with water only. Flav - Chambers without activated carbon treated with a flavonoid enriched fraction. Gluc - Chambers without activated carbon treated with the glucosinolate enriched fraction. Combined - Chambers without activated carbon treated with both flavonoid and glucosinolate enriched fractions. **A.** Total root length (cm), with activated carbon (N=32-40), without activated carbon (N=7-10). **B.** Rhizosphere area (cm²), with activated carbon (N=32-40), without activated carbon (N=7-10).
Figure 6.11. Root structural parameters of *I. pallida* plants exposed to *A. petiolata*
extracts during the symbiosis formation phase, means ± 1 SE.

Figure 6.12. Mycorrhizal health in *I. pallida* plants exposed to *A. petiolata* extracts.
during the symbiosis formation phase, means ± 1 SE.

AC - Mean of all chambers with activated carbon. Control - Chambers without activated carbon treated with water only. Flav - Chambers without activated carbon treated with a flavonoid enriched fraction. Gluc - Chambers without activated carbon treated with the glucosinolate enriched fraction. Combined - Chambers without activated carbon treated with both flavonoid and glucosinolate enriched fractions. Asterisks indicate differences among treatments without activated carbon significant at the Bonferroni adjusted $\alpha = (0.05/3) = 0.017$. A. Root Colonization Index (RCI), with activated carbon (N=166-184), without activated carbon (N=38-46). B. Soil Colonization Index (SCI), with activated carbon (N=200), without activated carbon (N=45-50). C. Parasitism Index (RCI/SCI) with activated carbon (N=166-184), without activated carbon (N=38-46).
Figure 6.13. Heights of *I. pallida* plants exposed to *A. petiolata* extracts during the symbiosis growth phase, means ± 1 SE.

Figure 6.14. Mycorrhizal health in *I. pallida* plants exposed to *A. petiolata* extracts
during the symbiosis growth phase, means ± 1 SE.

AC - Mean of all chambers with activated carbon. Control - Chambers without activated carbon treated with water only. Flav - Chambers without activated carbon treated with a flavonoid enriched fraction. Gluc - Chambers without activated carbon treated with the glucosinolate enriched fraction. Combined - Chambers without activated carbon treated with both flavonoid and glucosinolate enriched fractions. Different letters indicate differences among treatments without activated carbon significant at \( \alpha = 0.05 \), different bold letters indicate differences significant at the Bonferroni adjusted \( \alpha = (0.05/3) = 0.017 \). A. Root Colonization Index (RCI), with activated carbon (N=130-171), without activated carbon (N=25-46). B. Soil Colonization Index (SCI), with activated carbon (N=160-200), without activated carbon (N=30-50). C. Parasitism Index (RCI/SCI) with activated carbon (N=130-171), without activated carbon (N=25-46).
7 METHOD USED TO KILL *ALLIARIA PETIOLATA* PLANTS AFFECTS THE ESTABLISHMENT OF A NATIVE PLANT AND ITS MYCORRHIZAE: IMPLICATIONS FOR RESTORATION

7.1 INTRODUCTION

Invasive plant species are a major economic and ecological problem in the United States, with 5,000 introduced species costing about $34 billion in damage and control efforts annually (Morse et al. 1995, Pimentel 2002). A large percentage of management budgets are typically spent on controlling invasive plants, and removal efforts must be repeated regularly, often annually, to maintain any level of control (D'Antonio and Meyerson 2002).

Even if removal efforts are successful, many invasive plants modify their environments in ways that make it difficult for native plants to reestablish. Nitrogen fixing invasive plants can leave abnormally high levels of soil N (Vitousek and Walker 1989, Witkowski 1991), which may give other exotics an advantage over native plants. Other plants concentrate minerals from the soil and redeposit them on the soil surface as they die, preventing new plants from growing (Vivrette and Muller 1977). Many invasive species [e.g., *Ailanthus altissima* (tree-of-heaven), *Lonicera maackii* (bush honeysuckle), *Centaurea maculosa* (spotted knapweed), and *Alliaria petiolata* (garlic mustard)] are allelopathic, meaning they produce compounds that can inhibit the growth of surrounding

*Alliaria petiolata* excels at invading previously undisturbed forest understories, territory typically inaccessible by invaders (Nuzzo 2002). *Alliaria petiolata* does not seem to inhibit other plants directly (McCarthy and Hanson 1998); instead, it inhibits growth of symbiotic mycorrhizal fungi that other plants depend on for normal growth (Stinson et al. 2006).

Arbuscular mycorrhizal fungi (AMF) are obligately symbiotic, meaning they cannot survive unless associated with a host plant that provides them with fixed carbon. In exchange for this “food”, AMF serve as an extended root system and provide their hosts with water and nutrients. Over 90% of plant families surveyed can form associations with AMF, and many depend on AMF for maximal growth (Smith and Read 1997). *Alliaria petiolata*, as a member of the Brassicaceae, does not establish symbioses with AMF (Tester et al. 1987), so by blocking AMF it could limit growth of surrounding plants without negatively impacting its own growth. This could have implications for restoration of sites invaded by *A. petiolata* because AMF populations in the soil can be drastically reduced within one year in the absence of a suitable host (Thompson 1987, 1994).

Several removal methods have proven effective for controlling *A. petiolata*. Glyphosate is effective in a 1-3% solution (Nuzzo 1996, Carlson and Gorchov 2004, Slaughter et al. 2007). Clipping just above the ground may not kill *A. petiolata* plants, but does reduce plant size and seed production (Nuzzo 1991, Rebek and O'Neil 2005). Dormant season burning reduces *A. petiolata* abundance, but must be repeated annually.
to maintain control (Nuzzo et al. 1996). All of these methods leave the *A. petiolata* root system in the ground, which can release allelopathic compounds into the soil as the root system dies and decays. Most of the root system is removed if the plant is pulled out of the ground, but the subsequent soil disturbance may facilitate increased *A. petiolata* germination (Nuzzo 2002). Soil disturbance also disrupts AMF hyphal networks in the soil, thereby making it easier for non-mycorrhizal plant species to colonize a site (Grime et al. 1987, Allen et al. 1989). Also, the time required to pull plants by hand makes this technique the last choice of land managers. No removal method is likely to be effective unless applied over a broad area to minimize seed rain from untreated plants, and repeated annually for at least five years, in order to deplete the seed bank (Nuzzo 1991, Baskin and Baskin 1992, Slaughter et al. 2007).

The objective of our study was to determine whether the method used to remove *A. petiolata* plants (glyphosate treatment, clipping off the shoot, pulling out the entire plant) during restoration efforts influenced health of native plants introduced later. These removal techniques lead to different amounts of dead *A. petiolata* root tissue and/or different rates of death and therefore different rates of degradation of the *A. petiolata* root tissue that remains. We also explored the effectiveness of two soil amendments, activated carbon and AMF inocula. Since *A. petiolata* inhibits AMF, we expected additional AMF inocula to augment any residual AMF remaining in the soil and enhance plant growth. We expected activated carbon to adsorb allelopathic compounds and therefore ameliorate inhibitory effects on plants and/or AMF. Thus, overall, we hypothesized that native plants would be healthiest when grown after hand pulling of *A. petiolata* in soil amended with activated carbon and AMF inocula.
7.2 METHODS

7.2.1 Study Species

*Alliaria petiolata* seeds were collected from the Wright State University campus woods (lat 39°47’N, long 84°3’W) and stratified in sterile water at 5 °C for 3 months to stimulate germination. Seedlings were maintained at 5 °C and planted within 1 month of germination.

We used *Impatiens pallida* (pale jewelweed) as the target species because it is dependent on mycorrhizae [non-mycorrhizal *I. pallida* plants grow to only half the size of AMF colonized plants (K. Barto, unpublished data)], grows in the same habitats that *A. petiolata* invades, and its congener, *I. capensis*, recovers quickly after *A. petiolata* removal, suggesting that inhibition is occurring (McCarthy 1997). We collected *I. pallida* seeds from a large population in Yellow Springs, OH (lat 39°80’N, long 83°89’W). Seeds were immediately separated from seed capsules and stored in sterile water at 3 °C to stimulate germination. Water was replaced weekly and seeds began to germinate after 3.5 months of stratification. Seedlings were maintained at 3 °C after germination and were planted within a week of germination.

7.2.2 Experimental Set-Up

We made root-viewing chambers by sandwiching two 13 x 30.5 x 0.15 cm glass plates 0.5 cm apart with silicone on three sides, leaving drainage holes along the 13 cm bottom edge (Friese and Allen 1991). Each chamber held about 180 mL of field soil
containing natural AMF inocula that was collected from an *A. petiolata*-free area of the Wright State University woods, sieved to 2 mm, then combined with sand in a 1:1 ratio. We added finely ground activated carbon (Black Magic Professional Grade; Aquarium Pharmaceuticals, Inc., Chalfont, PA) to the soil in half the root-viewing chambers (20 mL AC/L soil) (Prati and Bossdorf 2004). We then planted one newly germinated *A. petiolata* seed in each chamber, and wrapped chambers in foil to prevent exposure of roots to light. Chambers were placed in a growth room at 20-25 °C under fluorescent lights providing approximately 135 µmol/m²/sec PAR with 14 hours of light per day. Chamber positions were randomized weekly. We observed sporadic germination of unidentified seeds present in the field soil, and these seedlings were immediately removed from chambers. All *A. petiolata* plants grew at similar rates, and were approximately the same size when removed from chambers after 4 months. We removed *A. petiolata* plants by one of three methods: 1.) painting leaves with a 2 % glyphosate solution [RoundUp™ (Monsanto)], which left the entire root system to die quickly, 2.) clipping and removing the shoot only which left the entire root system to die slowly, or 3.) pulling out as much of the plant as possible, which left only small amounts of root tissue to die quickly. In chambers where *A. petiolata* was clipped, several plants did resprout and were reclipped as needed until they died. Even though clipped *A. petiolata* plants were not permitted to reach more than 1 cm in height there was potential for direct resource competition between *A. petiolata* and *I. pallida* in these chambers. In order to minimize any effects due to resource competition, we periodically fertilized the chambers throughout the experiment with a low phosphorus fertilizer (N:P:K, 75:1:30) to encourage AMF colonization.
The field soil used contained natural AMF inocula, but we expected *A. petiolata* to kill much of this inocula. Therefore, one week after *A. petiolata* removal, we added fresh inocula consisting of finely chopped colonized *I. pallida* roots to the soil surface of half the chambers in each treatment group to ensure that AMF were present in the chambers. We then planted one newly germinated *I. pallida* plant in each chamber. Chambers were returned to the growth rooms described above for an additional six weeks. The experiment was a fully factorial combination of activated carbon (2 levels), *A. petiolata* removal (3 levels), and AMF inocula (2 levels) treatments. Each of our twelve treatment combinations was replicated in five root-viewing chambers, for a total of sixty experimental units.

### 7.2.3 Monitoring

Every week we measured plant height to the nearest mm from the soil surface to the tip of the shoot. Every three days we traced plant roots visible against the upper glass plate, digitized and skeletonized the tracings, and determined total root length, area of the root system, and density of roots in the root system (length pixels/area pixels) using ImageJ (NIH: [http://rsb.info.nih.gov/ij/](http://rsb.info.nih.gov/ij/)). We also calculated the planar box-counting fractal dimension (FD) using the FracLac plugin for ImageJ ([http://rsb.info.nih.gov/ij/plugins/frac-lac.html](http://rsb.info.nih.gov/ij/plugins/frac-lac.html)). This FD quantifies exploration efficiency of systems and depends on both the spatial arrangement and branching extent of the system. In order to calculate the FD a grid is drawn over the system and the number of boxes needed to completely cover the system is counted. We repeated this process for 10 box sizes and calculated FD as the negative slope of the linear regression of log(box size...
(defined as length of side)) on log(number of boxes required to cover the system). Larger FDs indicate more efficient exploration of the soil, with a maximum of 2 indicating complete coverage (Bernston 1994). Reported values for root systems range from 0 to 1.75 (Fitter and Stickland 1992, Nielsen et al. 1997, Eshel 1998, Nielsen et al. 1999, Walk et al. 2004, Dannowski and Block 2005).

Weekly, we monitored extra- and intraradical AMF using epifluorescence microscopy along five 6-cm horizontal transect lines centered in the middle of the chambers and spaced 5 cm apart. The first transect line was 2.5 cm below the top of the chamber. In order to view mycorrhizae, chambers were examined at 400x using a Nikon Eclipse TE 2000-S microscope with a B-2E/C filter cube, which excites with blue light, causing AMF to fluoresce green (Friese and Allen 1991).

AMF health is usually assessed by quantifying only intraradical fungal structures (Giovannetti and Mosse 1980, McGonigle et al. 1990). However, intraradical structures may actually benefit the fungus more than the plant since fixed carbon is exchanged inside the root. Although mineral nutrients and water are also exchanged inside the root, the fungus cannot obtain these without healthy extraradical hyphae, which are rarely quantified.

Methods used to measure extraradical hyphal abundance include directly weighing hyphae after picking them out of the soil (eg., Graham et al. 1982). Hyphae can also be collected on membrane filters and total length estimated after staining (eg., Hamel et al. 1990). Both of these methods are tedious and most likely underestimate fungal hyphae biomass. Methods providing more precise quantification include immunofluorescence staining (eg., Wright and Morton 1989), phospholipid fatty acid
analysis (Grigera et al. 2007), or using stable isotopes to detect active hyphae (Francis and Read 1984). All these methods require destructive sampling and therefore only allow one snapshot of the system. They also have limited spatial resolution, with most homogenizing a soil sample as the first step in the procedure. Epifluorescence microscopy, coupled with the root-viewing chambers used here, addresses both of these concerns and allows for repeated quantification of AMF structures inside and outside of the same plant roots, while preserving the spatial arrangement of all structures.

Traditional methods for quantifying AMF colonization assume that roots are randomly arranged (Giovannetti and Mosse 1980, McGonigle et al. 1990). However, we monitored AMF in situ, so our roots were not randomly arranged and traditional methods were not appropriate. Intensity of colonization varies widely between microscopic fields of view, so we calculated Root and Soil Colonization Indices (RCI, SCI) for each transect line that gave more weight to heavily colonized areas of roots and soil than to minimally colonized areas. Weighted indices have been used in medical research to emphasize longer durations of exposure when calculating exposure risks, and to more heavily weight factors deemed important by expert opinion when describing patient behavior (Pekovic et al. 1998, Villeneuve et al. 2002). We believe this is the first use of a weighted index to assess mycorrhizal abundance.

In order to calculate the RCI, we scanned along each transect line, and estimated the percent colonization of all the roots crossing that transect line as 0%, 1-25%, 26-50%, 51-75%, or 76-100%. We then calculated the percentage of roots observed in each category as P0, P25, P50, P75 and P100, respectively.

$$\text{RCI} = 4(P100) + 3(P75) + 2(P50) + (P25)$$
In order to calculate the SCI, we also scanned along each transect line, but due to time constraints we could not quantify hyphal abundance along the entire transect line. Instead, in every third field of view that did not contain a root, we classified hyphal abundance as 0, 1-3, 4-6, 7-9, or more than 9 hyphae. We then calculated the percentage of hyphae observed in each category as P0, P3, P6, P9 and P10, respectively.

\[ SCI = 4(P10) + 3(P9) + 2(P6) + (P3) \]

We calculated a ratio of RCI to SCI to quantify the extent to which AMF were parasitizing the plant host. A plant with a high RCI and a low SCI would be supporting a large amount of mycorrhizal tissue inside the plant root, while the fungus would only be allocating a small portion of this investment to growth of extraradical structures. Since the fungus cannot absorb water and nutrients without extraradical hyphae, this association is presumably more beneficial for the fungus than the plant, and the fungus is acting as a parasite in this case. At the other extreme, a plant with a low RCI and a high SCI still has intraradical fungal structures, so the fungus can still exchange nutrients with the plant. In addition, this fungus has allocated resources to extraradical hyphae growth and therefore has a large uptake capacity, making this association beneficial for the plant. We therefore expect RCI/SCI to be low in beneficial associations and high in parasitic associations. Johnson (1993) suggested comparing numbers of hyphae and arbuscules to numbers of vesicles in order to quantify the effectiveness of a mycorrhizal symbiosis, but we believe this is the first use of ratios of intra- to extraradical structures to estimate the degree of mycorrhizal parasitism.
7.2.4 Statistical Analyses

Although we made multiple observations on replicates over time, we did not use repeated measures ANOVA to analyze our response variables because there were unavoidable gaps in the data. These gaps arose because we monitored plants until 3 weeks after the roots reached the bottom of the glass chambers. Some plants grew slower than others (but treatments did not affect weeks to completion) and were therefore monitored longer, providing more data points. Therefore, repeated measures ANOVAs could only be run on the first three weeks of collected data. Gotelli and Ellison (2004) advocate collapsing the repeated measures into a single variable for each individual, such as the slope of the regression line through the repeated measure data. Thus, we plotted each endpoint versus time and calculated the slope of the regression line, which we then analyzed using ANOVA with activated carbon, inocula, removal method, and transect line as fixed factors.

The regression slopes analysis method captures effects of treatments on changes through time. We also analyzed the final time point for all response variables to capture effects of treatment on absolute values of response variables. We used PROC GLM and ANOVA with activated carbon, inocula, removal method, and transect line as fixed factors. Data were transformed as necessary to meet assumptions of normality and constant variance, assessed by Shapiro-Wilk and Levene’s tests, respectively. SAS Version 9.1 (SAS Institute Inc.) was used for all analyses.
7.3 RESULTS

Although all treatment groups contained five replicates at the start of the experiment, due to mortality during the experiment some treatment groups contained two replicates at the end of the experiment. Plants appeared healthy and free of insect damage, so mortality may have been due to the small size of the chambers. Mortality rates were not affected by treatments.

Impatiens pallida plants grew taller faster in chambers with activated carbon than in chambers without (Figure 7.1A; height slope, AC: $F_{1,46}=8.18$, $p=0.0064$). Removal method was also significant, and plants in chambers where A. petiolata were pulled grew taller faster than plants in chambers where A. petiolata were either clipped or sprayed (Figure 7.1B; height slope, removal method: $F_{2,46}=11.49$, $p<0.0001$). At the end of the experiment, I. pallida plants in chambers where A. petiolata were pulled were taller than I. pallida plants in chambers where A. petiolata plants were either clipped or sprayed (Figure 7.1B; height, removal method: $F_{2,41}=10.11$, $p = 0.0003$). No other factors or interactions between factors significantly impacted plant height or changes in plant height.

At the end of the experiment, total root lengths were longer in chambers containing activated carbon than in those without (Figure 7.2A; root length, AC: $F_{1,26}=4.80$, $p=0.0375$). Root system length increased fastest in chambers where A. petiolata were clipped, and slowest in chambers where A. petiolata were pulled (Figure 7.2B; root length slope, removal method: $F_{2,46}=4.46$, $p=0.0169$). No other factors or interactions of factors impacted root length.
Root systems were denser in chambers containing activated carbon than in those without at the end of the experiment (Figure 7.2C; density, AC: $F_{1,26}=5.99$, $p=0.0215$). Root system density increased most quickly in chambers where *A. petiolata* was clipped, and most slowly in chambers where *A. petiolata* was pulled (Figure 7.2D; density slope, removal method: $F_{2,46}=6.22$, $p=0.0041$). No other factors or interactions of factors impacted root system density.

The rate of change of the FD, which measures exploration efficiency of the root system, was not affected by any factor or any interactions between factors. However, the final FD was higher in chambers containing activated carbon than in chambers without activated carbon (Figure 7.2E; FD, AC: $F_{1,26}=6.81$, $p=0.0148$). The interaction between AMF inocula and *A. petiolata* removal method was also significant (Figure 7.2F; FD, inocula*removal method: $F_{2,46}=3.73$, $p=0.0377$), with values in chambers without AMF inocula being similar, regardless of removal method. In chambers with AMF inocula, clipping or spraying *A. petiolata* plants led to higher FD values than pulling *A. petiolata* plants. No other factors or interactions between factors affected FD.

At the end of the experiment, RCI was higher in chambers where *A. petiolata* was pulled than in chambers where *A. petiolata* was either clipped or sprayed (Figure 7.3A; RCI, removal method: $F_{2,154}=3.16$, $p=0.0452$). Also at the end of the experiment, RCI values were higher deeper in the chambers, and therefore highest in young roots (Figure 7.3B; RCI, line: $F_{4,154}=2.55$, $p=0.0416$). RCI values declined fastest at the base of the chambers in young roots, and slowest in older roots near the top of the chambers (RCI slope, line: $F_{4,176}=2.94$, $p=0.0220$). The interaction between AMF inocula and *A. petiolata* removal method was significant with regard to slope of RCI, with AMF inocula
decreasing the rate of decline of RCI in chambers where *A. petiolata* was clipped, and increasing the rate of decline in other chambers (Figure 7.4A; RCI slope, inocula*removal method: $F_{2,176}=5.20, p=0.0064$). The interaction between AMF inocula, *A. petiolata* removal method, and activated carbon was also significant with regard to slope of RCI, with chambers containing AMF inocula but not activated carbon encompassing both extremes. Slopes declined fastest in chambers where *A. petiolata* was sprayed, and slowest in chambers where *A. petiolata* was clipped, both also containing AMF inocula but not activated carbon (Figure 7.4B; RCI slope, inocula*removal method*AC: $F_{2,126}=5.79, p=0.0037$). No other factors or interactions between factors significantly impacted RCI.

We calculated a soil colonization index (SCI) to quantify the abundance of AMF hyphae in the soil. The interaction between activated carbon and removal method was significant at the end of the experiment (Figure 7.4C; SCI, AC*removal method: $F_{2,195}=4.48, p = 0.0125$) with activated carbon only affecting SCI in chambers treated with glyphosate, where values declined. Rates of change in SCI varied significantly among removal treatments (Figure 7.3C; SCI slope: removal method: $F_{2,175} = 4.78, p = 0.0096$). SCI declined only slightly over time in chambers treated with glyphosate, while the decline was significantly more rapid in chambers where *A. petiolata* was clipped. In chambers where *A. petiolata* was pulled SCI also declined with time, but the rate was not significantly different from any other treatment (Figure 7.3C). No other factors or interactions between factors significantly impacted SCI.

We calculated a parasitism index (RCI/SCI) to quantify the degree to which AMF parasitize their plant hosts. At the end of the experiment, the three way interaction
between activated carbon, AMF inocula and removal method was significant (Figure 7.4D; RCI/SCI, inocula*removal method*AC: $F_{2,154} = 3.14, p = 0.0460$), with values in chambers with AC being similar within removal treatments regardless of presence of AMF inocula. In chambers without AC, RCI/SCI values were unaffected by AMF inocula only if *A. petiolata* plants were sprayed. In chambers where *A. petiolata* were clipped, RCI/SCI values were higher in chambers with inocula than those without, while in chambers where *A. petiolata* were pulled, RCI/SCI values were lower in chambers with inocula than in chambers without. After an early spike in values, RCI/SCI values declined over time in chambers containing activated carbon, and increased over time in chambers without activated carbon (Figure 7.3D; RCI/SCI slope, AC: $F_{1,217} = 7.12, p = 0.0082$). No other factors or interactions between factors significantly impacted RCI/SCI.

7.4 DISCUSSION

The method used to remove an invasive plant significantly impacted size of a native plant later planted in the same soil. *Impatiens pallida* plants in chambers where *A. petiolata* was pulled were significantly taller by the end of the experiment than plants in chambers where other removal techniques were used. These tall plants also had the smallest root systems, and their root systems were least efficient at exploring the soil. While small and inefficient root systems might seem to be detrimental to plant health, low root:shoot ratios are commonly seen in plants heavily colonized by beneficial AMF (Oliver et al. 1983). Indeed, by the end of the experiment, RCI values were highest in chambers where *A. petiolata* had been pulled, indicating higher levels of intraradical colonization in chambers with the least amount of dead *A. petiolata* tissue.
Although SCI values in all chambers were low by the end of the experiment, the rates of decline varied with *A. petiolata* removal method. SCI values in chambers sprayed with glyphosate remained low throughout the experiment, as would be expected if a large pulse of allelopathic compounds were released from a complete *A. petiolata* root system that had been killed suddenly. The slow decline in SCI values in chambers where *A. petiolata* was clipped may have been due to the slow deaths of the *A. petiolata* plants in these chambers, which survived several weeks after clipping in some cases. Since allelochemicals likely leach from decomposing tissues (Rice 1974), allelochemical levels in these chambers would have started low and gradually increased as the *A. petiolata* plants died. Over time, allelochemical loads should have approached those in chambers treated with glyphosate, which also retained a complete *A. petiolata* root system. As expected, by the end of the experiment SCI values in chambers where *A. petiolata* was clipped were indistinguishable from those where *A. petiolata* was sprayed. In chambers where *A. petiolata* plants were pulled, some fine roots remained which would have decomposed quickly and released a small dose of allelopathic compounds into the soil.

The low SCI values in chambers where *A. petiolata* was pulled could indicate that AMF are especially sensitive to the allelopathic compounds produced by *A. petiolata* (Schreiner and Koide 1993a, b, Callaway et al. 2008). Alternatively, low SCI values could be due to the small size of the root viewing chambers, which allowed the plants to completely explore the soil volume by week 4, thereby removing their dependence on mycorrhizae. For this experiment, we were only interested in recovery of sites after *A. petiolata* removal, so we did not include a treatment without exposure to *A. petiolata*. However, in separate experiments using *I. pallida* in these same root-viewing chambers,
SCI values in control chambers never exposed to *A. petiolata* remained relatively constant for seven weeks (K Barto, unpublished data), suggesting that SCI values were not limited by chamber size.

The method used to remove *A. petiolata* plants did not impact the parasitism ratio we calculated. However, the ratio declined over time in chambers containing activated carbon and increased over time in chambers without activated carbon. This is the first report of an index comparing intra- and extraradical fungal abundance and it is not yet clear how well the ratio correlates with degree of fungal parasitism. While extremely low levels of extraradical hyphae will not be beneficial, low levels of intraradical structures may not allow enough nutrient exchange to be beneficial either. The relationship between RCI/SCI and fungal parasitism may therefore be nonlinear, with optimum levels somewhere between both extremes. More research is needed to further clarify this important question.

This experiment was conducted over six weeks, which is much shorter than the annual life span of *I. pallida*, so we cannot conclude that these effects would persist without further experimentation. For logistical reasons we used first-year rosette stage *A. petiolata* plants in these experiments even though mature second-year plants are usually targeted during restoration efforts. While levels of natural products produced by *A. petiolata* do vary, the phytochemical profile remains very similar throughout the plant’s life cycle, (Vaughn and Berhow 1999, Haribal and Renwick 2001), Therefore, we expect relative effects of conditioning by mature plants to be very similar to the effects we observed using first-year rosette plants, although the size of second-year plants may magnify the treatment effects.
We have not tested the mechanism proposed here in the field, but it could explain the lack of effect on *I. pallida* size or fruit production a year after spraying 1% glyphosate on *A. petiolata* patches (Carlson and Gorchov 2004). Since the entire *A. petiolata* plant was left in the soil to decay over the winter in both the sprayed and unsprayed plots, *Impatiens pallida* plants the following year were exposed to equivalent amounts of dead *A. petiolata* tissue in sprayed and unsprayed plots. Abundance of rosette stage plants the year after spraying was high (~100 individuals/m²), and unaffected by treatment, so resource competition or allelopathy from living *A. petiolata* may also explain the results observed by Carlson and Gorchov (2004).

While it is possible that any inhibition of growth of *I. pallida* plants in chambers treated with glyphosate was due to direct effects of the herbicide, this is unlikely for several reasons. First, we painted a solution of glyphosate directly onto *A. petiolata* leaves, which were removed before *I. pallida* seeds were planted. Any glyphosate reaching the soil would therefore have been translocated by *A. petiolata* to the roots and leached out of degrading roots. While glyphosate is readily transported through the phloem, it follows source to sink flow patterns (Bromilow and Chamberlain 2000). When *A. petiolata* plants were killed, they were still producing new leaves which would have been acting as the major photosynthate sinks in the plant, so glyphosate would have accumulated in the leaves and therefore been removed from the chambers. Any glyphosate reaching the soil would likely have degraded quickly or adsorbed onto soil particles (Vereecken 2005), making it unavailable for later absorption by other plants. These factors combine to make it unlikely that large enough amounts of glyphosate were
available to significantly inhibit growth of *I. pallida* plants added to the chambers after *A. petiolata* removal.

### 7.5 CONCLUSIONS

The method used to kill *A. petiolata* plants was more important for the health of *I. pallida* plants than soil amendment with either activated carbon or AMF inocula. Pulling *A. petiolata* plants, which left the smallest amount of dead root tissue remaining, was the most beneficial removal method. Clipping the shoots of *A. petiolata* or spraying with glyphosate, which both left the entire *A. petiolata* root system in the soil, led to smaller *I. pallida* plants.

Total plant removal is generally frowned upon during restoration because of the soil disturbance it causes. While this is a valid concern, we wish to emphasize that dead root tissue, especially of allelopathic species, can have significant impacts on the health of native plants, even if they are introduced after the allelopathic plant has been killed.
7.6 REFERENCES


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Figure 7.1. Height of *I. pallida* plants over time, means ± 1 SE.

A. Response to activated carbon amendment. (N=25-30) B. Response to *A. petiolata* removal method. Clipped: *A. petiolata* shoots were removed, Sprayed: *A. petiolata* leaves were painted with glyphosate, Pulled: the entire *A. petiolata* plant was removed. Each week was analyzed individually. Within each week, data points with different letters were significantly different at p=0.05. (N=15-20).
Figure 7.2. Root morphology of *I. pallida* plants over time, means ± 1 SE.

Clipped: *A. petiolata* shoots were removed, Sprayed: *A. petiolata* leaves were painted with glyphosate, Pulled: the entire *A. petiolata* plant was removed. Within each day, data points with different letters were significantly different at p=0.05. A. Total root length response to activated carbon amendment (N=15-30). B. Total root length response to *A. petiolata* removal method (N=12-20). C. Root system density (length pixels/area pixels) response to activated carbon amendment (N=16-30). D. Root system density (length pixels/area pixels) response to *A. petiolata* removal method (N=12-20). E. Response of box-counting fractal dimension of the root system to activated carbon amendment (N=15-30). F. Response of box-counting fractal dimension of the root system to *A. petiolata* removal method and AMF inocula. (N=5-8).
Figure 7.3. AMF development over time, means ± 1 SE.

Clipped: *A. petiolata* shoots were removed, Sprayed: *A. petiolata* leaves were painted with glyphosate, Pulled: the entire *A. petiolata* plant was removed. Within each week, data points with different letters were significantly different at p=0.05. A. Response of root colonization index, RCI, to *A. petiolata* removal method (N=25-83). B. Response of root colonization index, RCI, to chamber depth. 1: shallowest transect line in chamber through 5: deepest transect line in chamber (N=3-47). C. Response of soil colonization index, SCI, to *A. petiolata* removal method (N=34-95). D. Response of parasitism ratio, RCI/SCI, to activated carbon amendment (N=34-117).
Figure 7.4. AMF abundance, means ± 1 SE.

Over 5,000 introduced plant species are established and/or invasive in the United States of America, meaning their populations have stabilized or are increasing in size, with detrimental impacts on the native ecosystem (Morse et al. 1995, Hallett 2006). Invasive plants reduce native species diversity and abundance through resource competition, competition for pollinators, and direct allelopathic inhibition (Levine et al. 2003, Henderson et al. 2006). They also modify ecosystem functioning by altering resource availability and fire and flood regimes (Levine et al. 2003, Henderson et al. 2006). Efforts to control plant invasions and restore invaded areas cost about $34 billion annually (Pimentel 2002), spurring much research on mechanisms of invasion.

*Alliaria petiolata* (M.Bieb.) Cavara & Grande, Brassicaceae (garlic mustard) is a biennial herb that was introduced to North America from Western Europe in the 1860’s (Nuzzo 2002). In its native range, which includes northern Europe and the British Isles, *A. petiolata* grows in disturbed habitats such as hedgerows and shaded waysides, and is occasionally found in open woods (Pursey 1978). In North America, *A. petiolata* is most abundant in the Northeast and Midwest and grows well on dry or moist soils in partially shaded areas such as forests and forest edges (Nuzzo 2002). *Alliaria petiolata* was
harvested as a potherb in Europe and may have been intentionally transported to the New World to provide flavor for cooking throughout the winter when most other plants are not green. After over-wintering as basal rosettes, *A. petiolata* plants bolt in April and May and can reach heights of almost two meters and densities of about 100 plants/m² (Nuzzo 2002).

### 8.1 BEFORE AN INVASION

Theories proposed to explain the invasive success of *A. petiolata*, and other invasive plants, fall into three categories, those attempting to identify traits common to invaded communities, those attempting to identify traits common to invasive plants, and those focused on the evolutionary history of the invader. Theories attempting to identify traits common to invaded communities include the Biotic Resistance and Empty Niche hypotheses, which state that pristine communities with high species diversity will be resistant to invasion (Elton 2000, Rejmánek 2000). *Alliaria petiolata* can form dense monocultures in both undisturbed forest understories and along disturbed edges (McCarthy 1997, Nuzzo 2002), suggesting that community structure in the invaded range is not controlling invasion success.

Numerous attempts have been made to correlate biotic characteristics such as plant size and habitat preference with invasibility in order to identify traits common to invasive plants (eg., Williamson and Fitter 1996, Goodwin et al. 1999, Milbau and Stout 2008). Such attempts have failed to identify universal predictors (Hayes and Barry 2008), but characteristics of the invader may still be important in specific cases. *Alliaria petiolata* is highly competitive (Meekins and McCarthy 1999), produces large quantities
of seed, and is self-compatible (Nuzzo 2002), meaning that only one seed is needed to begin an invasion.

The final group of invasion theories emphasize how interactions between organisms with a co-evolutionary history differ from interactions between organisms without such a history. The Novel Weapons Hypothesis (NWH) suggests that allelopathic inhibition is common in plant communities and that species sharing co-evolutionary histories are at least partially adapted to the allelopathic compounds produced by other species in the community. When an invasive plant colonizes an area outside its native range, other species in the invaded range will be naïve to the allelopathic compounds produced and therefore more sensitive to any inhibitory effects than plants in the native range of the invader (Callaway and Aschehoug 2000, Bais et al. 2003). *Alliaria petiolata* produces glucosinolates and flavonoid glycosides not found in four common North American Brassicaceae (Chapter 3), providing some support for the NWH. Further support comes from the observation that North American species are more sensitive to inhibition by *A. petiolata* than European species (Chapter 3, Prati and Bossdorf 2004, Callaway et al. 2008).

The Enemy Release Hypothesis (ERH) has a similar foundation, but the focus is on plant insect interactions instead of on plant plant interactions. Herbivory in the invaded range is assumed to be more strongly inhibited by the invader than herbivory in the native range, due to the production of novel weapons (Keane and Crawley 2002). Any reduction in herbivory in the invaded range is assumed to allow for increased growth of the invasive plant. *Alliaria petiolata* has certainly escaped herbivory in North America,
where only 11 insect species feed on it, compared to 70 in its native Europe (Blossey et al. 2001, Evans and Landis 2007).

The Evolution of Increased Competitive Ability (EICA) hypothesis elaborates on the ERH by suggesting that release from herbivory selects for reduced production of the costly secondary metabolites that limit herbivory, allowing even greater allocation of resources to growth by invasive plants (Blossey and Nötzold 1995). Contrary to predictions of the EICA hypothesis, *A. petiolata* in North America are not producing lower levels of secondary metabolites than *A. petiolata* from Europe (Cipollini et al. 2005). The EICA hypothesis predicts that these compounds will be unnecessary in North America, but the evidence supporting the NWH and the ERH suggest that high levels of secondary metabolites are enhancing invasion success, and would therefore not be selected against.

In summary, evidence suggesting that *A. petiolata* would be invasive in North America includes its ability to grow in disturbed and undisturbed habitats (McCarthy 1997, Nuzzo 2002), and various biotic characteristics such as self-fertilization and copious seed production (Nuzzo 2002). The invasiveness of *A. petiolata* is also expected based on the production of secondary metabolites novel in North America (Chapter 3), the increased sensitivity of North American species to effects of those compounds (Chapter 3, Prati and Bossdorf 2004, Callaway et al. 2008) and the low numbers of potential insect herbivores in North America (Blossey et al. 2001). The remainder of this review will focus on the allelopathic potential of *A. petiolata*, and how allelopathic effects could enhance invasion success.
8.2 DURING INVASION

The production of novel weapons by *A. petiolata* suggests allelopathy may be important for this species (Chapter 3), but bioassays must be conducted to verify that these compounds do in fact inhibit the growth of other organisms. In order to provide ecological relevance, target organisms used during bioassays should come from communities where the presumed allelopath also grows. Organisms should be chosen to maximize the likelihood of inhibitory effects appearing, so that if no inhibition is observed, similar results can be expected to occur in less sensitive target organisms as well. For example, *A. petiolata* may inhibit growth of other plants indirectly, by limiting growth of AMF (Chapter 3, Roberts and Anderson 2001, Stinson et al. 2006). Target organisms should therefore include AMF (Chapters 3, 5, 6, 7), and plant species dependent on AMF for normal growth. *Impatiens pallida* (pale jewelweed) is an herbaceous annual common in moist forested areas in North America invaded by *A. petiolata*. *Impatiens pallida* plants not colonized by AMF reach only about half the size of colonized plants (K Barto, unpublished data), so this species is highly dependent on AMF, increasing the chances of observing indirect inhibition of growth. *Acer saccharum* (sugar maple) is also common in forested areas in North America invaded by *A. petiolata*, and depends on AMF as seedlings (Stinson et al. 2006). Both *I. pallida* and *A. saccharum* are therefore appropriate target species for bioassays involving *A. petiolata* (Chapters 4-7).

In order for results from bioassays to be meaningful, ecologically relevant exposure levels must be used. This can be achieved by several complementary approaches; soil conditioning by live plants, the use of activated carbon soil amendments,
and extracts prepared from plant tissues. In a soil conditioning approach, the suspected allelopath is grown in soil for a period of time to “contaminate” it, then removed and replaced with a target plant. Major strengths of this approach are the use of soil instead of artificial media and the use of live plants to ensure that exposure levels are not unrealistically high. The allelopathic plant must be removed before planting the target plant in order to eliminate effects of resource competition, so exposure levels may be unrealistically low if allelopathic compounds degrade quickly. Soil conditioning approaches have been used to demonstrate inhibition of seed germination of an herbaceous plant up to eight weeks after *A. petiolata* removal (Prati and Bossdorf 2004), decreased AMF colonization of woody and herbaceous plants four months after *A. petiolata* removal (Stinson et al. 2006), and decreased AMF colonization and plant size of an herbaceous annual six weeks after *A. petiolata* removal (Chapter 7).

These long-term effects could be caused by unstable allelopathic compounds if growth suppression immediately after removal of the allelopath persisted throughout the experiment without further suppression. This seems unlikely for *A. petiolata* since AMF hyphal abundance declined throughout four weeks following *A. petiolata* removal (Chapter 7). Instead, the evidence suggests that inhibitory compounds remain in the soil for at least 4 weeks. It is then somewhat surprising that the glucosinolates and flavonoid glycosides produced by *A. petiolata* have half-lives in soil ranging from 3 hours to 9 days (Chapter 2, Gimsing et al. 2006, Gimsing et al. 2007), suggesting that they are not responsible for persistent inhibitory effects.

This apparent conundrum can be reconciled by the observation that many allelopathic species produce unstable non-toxic compounds that quickly degrade to
strongly bioactive compounds (Tanrisever et al. 1987, Kong et al. 1999, Weidenhamer and Romeo 2004, Johansen et al. 2007). Glucosinolates decompose primarily to isothiocyanates, which are bioactive (Yamane et al. 1992, Schreiner and Koide 1993a, b, Olivier et al. 1999), but are even less stable than their parent compounds (Gimsing et al. 2007), making them unlikely to be responsible for long-term inhibitory effects. The flavonoid glycosides produced by *A. petiolata* could decompose to isovitexin and apigenin after one and two deglycosylation events, respectively (Figure 8.1). Both of these compounds are bioactive (Wagner and van Brederode 1996, Basile et al. 2000, Begum et al. 2001, Suominen et al. 2003, Cipollini et al. 2008), and further research elucidating the degradation pathways of the flavonoid glycosides, and the stability of their degradates is warranted. No degradate peaks were observed in these studies, but the HPLC conditions used here were optimized to separate alliarinoside and the flavonoid glycosides, and smaller degradates may have come off the column too early to be distinguished from other small compounds appearing as junk peaks very early in the chromatographs.

When using a soil conditioning approach, suspected allelopaths and target plants are not grown simultaneously in order to eliminate resource competition as a factor. This means that any allelopathic compounds that must be continually replenished by the allelopath will not be present. Activated carbon has been used as a soil amendment to address this problem. Theoretically, activated carbon adsorbs organic compounds, making them unavailable for uptake by plants or microorganisms, without affecting inorganic nutrients (Inderjit and Callaway 2003). A simple experimental design would
include simultaneous plantings of allelopaths and target plants, in soils with and without activated carbon. Both resource competition and allelopathy will be operating in the soil without activated carbon, but only resource competition will be occurring in the soil with activated carbon. The contributions of resource competition and allelopathy can therefore be easily separated, but only if inorganic nutrients are unaffected by activated carbon. Inderjit and Callaway (2003) suggested fertilizing plants grown during this type of experiment in order to ensure that equal amounts of inorganic nutrients are available in both types of soils. However, the absorptive capacity of activated carbon depends on soil type, nutrient level, and the particular species being grown (Lau et al. 2008), making it nearly impossible to include proper controls in any experimental design. Despite these shortcomings, activated carbon certainly adsorbs organic compounds more strongly than inorganic compounds, and remains a valuable tool in allelopathy research, especially when integrated with other approaches.

Another valuable approach exposes target organisms to extracts from tissues of the suspected allelopath. Effects of resource competition are completely removed in this approach, without the use of activated carbon, since live allelopathic plants are not used. Further benefits include the ability to split extracts into various fractions in order to determine which compounds are allelopathic and how those compounds interact with each other (Chapter 6). It is imperative that an ecologically relevant dose, one which target organisms are likely to be exposed to in the field, be used in bioassays. The major drawback with this type of experiment relates to the difficulty in determining ecologically relevant doses.
Although a wide range of compounds, with two glucosinolates and several flavonoid glycosides being the most abundant, are detectable in *A. petiolata* tissues (Haribal and Renwick 1998, Vaughn and Berhow 1999, Haribal et al. 2001, Cipollini and Gruner 2007), I was unable to isolate any glucosinolates or flavonoid glycosides from bulk soils collected under *A. petiolata* plants (Chapter 2). Biomimetic extraction was more successful, but only one compound (IVG) was detected, and only in early spring during peak *A. petiolata* densities (Chapter 2). Glucosinolates are charged and therefore more likely to remain in soil water than be adsorbed by PDMS, and isothiocyanates are extremely volatile and unlikely to be detected by liquid chromatography, so it is not surprising that no glucosinolates were detected using biomimetic extraction. The difficulty in detecting *A. petiolata* metabolites in soils may also be related to the short half-lives of these compounds, which may indicate that they become bioactive only after being chemically modified. The glucosinolates, flavonoid glycosides, and alliarinoside produced by *A. petiolata* are glycosylated, or have at least one sugar attached to the compound (Figure 1.1, Figure 8.1). Glycosylated compounds are often unstable, with removal of the sugar being the first step in many degradation pathways (Weidenhamer and Romeo 2004, Johansen et al. 2007, Kong et al. 2007). Aglycones are typically easier to detect in soils than their glycosylated parent compounds (Weidenhamer and Romeo 2004, Kong et al. 2007), and attempts to isolate the aglycones derived from *A. petiolata* metabolites may therefore prove more fruitful than attempts to isolate their parent compounds. Once the degradation pathways of the compounds produced by *A. petiolata* are understood, levels of those degradates in field soils can be determined.
Until such data becomes available, ecologically relevant doses can be estimated using data from closely related species with similar biochemistry. *Brassica napus* produces glucosinolate at levels of 14-20 µmol/g leaf tissue (Gardiner et al. 1999, Morra and Kirkegaard 2002). Isothiocyanates, which are the breakdown products of glucosinolates, reach levels of 40 to 75 nmol per gram in soils following incorporation of *B. napus* plant material (Morra and Kirkegaard 2002). Assuming that a similar relationship exists for *A. petiolata*, soil levels should be about 300 times less than levels in the leaves, or 0.0033 gram leaf equivalents (gle) per gram of soil.

Using this dose, I exposed *Impatiens pallida* plants and their associated AMF fungi to *A. petiolata* extracts throughout development to determine which life stages were most sensitive. I found strong direct inhibition of germination of seeds and reduction in viability of spores (Figure 8.2). Seed germination was reduced by almost 60% after exposure to a complete *A. petiolata* extract (Chapter 6). Spore viability was reduced by 74% after exposure to the same extract (Chapter 3). Growth of uncolonized plants was also strongly inhibited by *A. petiolata* extracts, with root lengths and rhizosphere areas of exposed plants reaching only 14% and 25% of those of control plants, respectively (Chapter 6). Uncolonized *I. pallida* plants exposed to either a glucosinolate enriched fraction or a complete *A. petiolata* extract survived less than half as long as plants exposed to either a flavonoid enriched fraction or water alone (Chapter 6). *Impatiens pallida* plants exposed to extracts while initiating an association with AMF, or after establishing a symbiosis, were largely unaffected (Chapter 6). This suggests that AMF can protect *I. pallida* from the allelopathic effects of *A. petiolata*, rather than intensifying them, as has been shown for several woody species (Stinson et al. 2006). An additional
experiment exposing uncolonized plants to *A. petiolata* extracts for a period of time, then adding inocula, would determine whether or not AMF can rescue already damaged plants.

In summary, extracts from *A. petiolata* directly inhibited plant and AMF growth, but only when each partner was grown alone (Figure 2). This may be related to the synergistic effects observed between the flavonoid enriched and glucosinolate enriched fractions. Seed germination and plant growth before colonization were more strongly inhibited by a glucosinolate enriched fraction than a flavonoid enriched fraction (Chapter 6), while spore viability tended to be more strongly depressed by a flavonoid enriched fraction than a glucosinolate enriched fraction (Chapter 3). In both cases, a complete extract tended to cause more inhibition than either fraction alone (Chapters 3, 6). It is of note that the sensitivity of the plant to a glucosinolate enriched fraction does not seem to combine with the sensitivity of AMF to a flavonoid enriched fraction, in order to severely limit growth of the symbiosis. Instead, it appears that the ability of the plant to grow normally when exposed to a flavonoid enriched fraction and the ability of AMF to somewhat resist effects of a glucosinolate enriched fraction combine to benefit both partners during a symbiosis. This pattern is contradictory to results obtained using woody species, where AMF colonization was greatly reduced in soils conditioned by *A. petiolata* (Stinson et al. 2006). Further research is needed to determine whether herbaceous plants are consistently affected differently by *A. petiolata* than woody plants.

After establishing that the potential for allelopathy exists by using laboratory bioassays, it must be determined whether or not allelopathic inhibition also occurs in more natural settings. Among *I. pallida* plants grown in a greenhouse in field collected
soils, plant size was more strongly affected by interspecific resource competition than any inhibition due to *A. petiolata* extracts, although doses 4 times higher than estimated field levels did inhibit growth at the lowest plant density (Chapter 4). Such experiments emphasize that community structure is influenced by many forms of competition, including competition for resources and more direct mechanisms such as limiting the growth of surrounding plants through allelopathic inhibition. Both of these mechanisms likely operate simultaneously in the field, and future research should focus on determining the contributions of each mechanism to community structure. Computer models have proved valuable for predicting plant abundance at varying levels of resources and allelopathy for the potentially allelopathic plant *Acroptilon repens*. (Goslee et al. 2001), and such models may also be useful in determining the contributions of resource competition and allelopathy to the invasive success of *A. petiolata*.

**8.3 AFTER INVASION**

Although allelopathy may not be the driving force behind the invasive success of *A. petiolata*, the densities reached in some invaded sites (100 plants/m²) (Nuzzo 2002) suggest that allelopathic compounds may reach high enough levels in soils to be important in maintaining an invasion. The presence of allelopathic compounds in the soil will certainly need to be addressed during restoration efforts. Such efforts can be informed by an understanding of the mechanism of invasion. For example, colonized *I. pallida* plants are much more resistant to allelopathic effects of *A. petiolata* than seeds or uncolonized plants (Chapter 6). It therefore appears that transplanting colonized plants into a site after *A. petiolata* removal may be more effective that sowing seed.
Alternatively, one could sow enough seed to compensate for the expected decline in germination due to allelopathic effects (Chapter 6), and to keep densities of native plants high enough to limit effects of allelopathic inhibition through density dependent phytotoxicity (Chapter 4). This method may prove especially cost effective if the effects of resource competition among native plants are limited to growth reduction (Chapter 4), while allelopathic effects include high mortality rates (Chapter 6).

In addition to the methods used to re-introduce native plants to a restored area, the method used to remove the allelopathic plant can also influence the success of the restoration project. Allelopathic plants, by definition, produce compounds that limit the growth of surrounding plants, so any tissue from the allelopathic plant remaining in a site can continue to exert allelopathic effects. This also applies to dead tissues, which are often left on site after treatments to kill invading plants. Herbicide sprays leave the entire plant, while clipping or mowing can be used to remove above ground tissues. Hand-pulling can be used to remove the entire plant, but only if dead plants are disposed of off site. In a laboratory experiment simulating these removal methods, I. pallida plants added to chambers after A. petiolata removal were larger, with higher AMF colonization, in chambers where A. petiolata plants were hand-pulled (Chapter 7). Further research is needed to verify that these effects are also apparent in the field, and persist for longer than the 6-week duration of the lab experiment.

A final method of invasive species control, requiring no human intervention, relies on the natural adaptation of organisms in the native range to the novel weapons produced by the invader. North American populations of plants and insects have begun to adapt to the novel weapons produced by invaders from Europe in only 50 years since
many invasions began (Callaway et al. 2005, Keeler and Chew 2008). Studies exploring
the sensitivities of North American plants and insect populations invaded by *A. petiolata*
for different lengths of time would help to gauge the rate of evolution of resistance in
those populations.

### 8.4 CONCLUSIONS

Following an early over-reliance on seed germination bioassays (Harper 1975,
Stowe 1979), allelopathy research has since been held to extremely rigorous standards
2003, Inderjit and Callaway 2003). These standards demanded that one show that
resource competition alone could not account for observed effects (Williamson 1990). In
addition, inhibitory compounds had to be identified in the allelopathic plant and in the
environment, and be bioactive at ecologically relevant doses (Williamson 1990, Choesin
and Boerner 1991). Furthermore, the methods of release of inhibitory compounds from
the allelopathic plant and uptake by target plants were also seen as necessary pieces of
information (Willis 1985). A wide variety of approaches, such as those described here,
will be more beneficial in achieving these goals than reliance on one or two simple
techniques.

The complementary mechanisms of resource competition and herbivory have
never been held to such high standards, and Inderjit et al. (2005) argue that it is
unreasonable to continue to do so for allelopathy research. Instead, they argue that an
understanding of the mechanisms involved, and how they operate in the field, are more
meaningful goals (Inderjit et al. 2005). In light of these new guidelines, the case for
allelopathy in *A. petiolata* is quite strong. Further research focused on effects in natural environments will continue to deepen our understanding of how allelopathy, enemy release, and resource competition interact to create invasive plants.
8.5 REFERENCES


Figure 8.1. Chemical structures of IVG – isovitexin 6‴-O-β-D-glucopyranoside, isovitexin, and apigenin.
Figure 8.2. Life cycles of two *Impatiens pallida* populations, the one on the right also containing *Alliaria petiolata*.

① Germination – Seed germination is inhibited by *A. petiolata*. ② Presymbiosis Growth – Presymbiosis growth is inhibited by *A. petiolata*, and mortality rates are high after exposure to either a glucosinolate enriched fraction or a complete extract. ③ Symbiosis Formation – Symbioses formed normally when exposed to *A. petiolata* extracts. ④ Symbiosis Growth – AMF colonized *I. petiolata* plants were not affected by *A. petiolata* extracts. Even though AMF appear to protect *I. petiolata* plants from allelopathic effects, inhibition of seed germination will limit *I. pallida* population size.