Comparing Allelopathic Effects of Root and Leaf Extracts of Invasive *Alliaria petiolata, Lonicera maackii* and *Ranunculus ficaria* on Germination of Three Native Woodland Plants

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ABSTRACT. Invasive plant species can exhibit allelopathic effects on native plant species. The strength of this allelopathic effect can vary with invasive species, with target species and with type of plant tissue extract. The purpose of this study was to determine the direct effects of extracts from roots or leaves of three Midwestern US invasive plants (*Alliaria petiolata, Lonicera maackii* and *Ranunculus ficaria*) on the germination success of three native target species (*Anemone virginiana, Blephilia hirsuta* and *Elymus hystrix*) in a fully factorial experiment. Leaf extract treatments overall showed more germination inhibition compared to root extract treatments. As concentration increased, effects of extracts increased. Extracts of leaves of *A. petiolata* had the greatest inhibition of germination across all other treatments. Effects of root and leaf extracts of each invasive species varied with each target species. While *E. hystrix* showed little response to extracts of roots or leaves of *L. maackii* and *R. ficaria*, *B. hirsuta* and *A. virginiana* germination were reduced by leaf extracts of these two invasive species. This study confirms the strong direct allelopathic effects of *A. petiolata*, though the strength of the effect varies with target species and with type of tissue used to make extracts. This study is the first to directly compare the effects of these invasive species on a suite of native, ecologically-relevant target species.

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INTRODUCTION

Invasive species are a threat to biodiversity globally (McGeoch and others 2010) and have negative impacts on native species including reduced germination, growth, survival, and reproduction (e.g., Gould and Gorchov 2000; Miller and Gorchov 2004; Dorning and Cipollini 2006; Cipollini and others 2008a; Cipollini and others 2009). Understanding the mechanism by which invasive species negatively impact native species is an active area of invasive species research (Levine and others 2003). One important interaction between plants is allelopathy, which is suppression of one plant by substances produced by another plant. This mechanism may represent a novel weapon (Callaway and Ridenour 2004) and has been implicated in invasive species success (e.g., Callaway and Aschehoug 2000). Allelochemicals from invasive plants may affect other plants directly (Cipollini and others 2008b) or indirectly, such as through changing soil ecology and disrupting mutualisms (Cipollini and others 2012a).

Although field studies are necessary to see if allelopathic effects extend into complex field environments (Inderjit and Nielsen 2003), simple laboratory germination assays can be used as a first step to examine potential allelopathic effects of a plant species (Blum 1999; Inderjit and Callaway 2003). Laboratory studies also have the benefit of allowing the evaluation of direct impacts only (Cipollini and others 2012b). Laboratory bioassays further provide a simple tool to examine comparative allelopathic effects between several species (Pisula and Meiners 2010).

This study examined three common invasive species that are negatively impacting forests in Ohio and beyond. The first species, *Alliaria petiolata* (M. Bieb) Cavara & Grand [garlic mustard, Brassicaceae] is found in much of the US and Canada. It exhibits both direct (Prati and Bossdorf 2004; Cipollini and others 2012b) and indirect (Roberts and Anderson 2001; Stinson and others 2006; Callaway and others 2008) allelopathic effects on other plant species. Several possible allelochemicals have been described in *Alliaria petiolata* (Vaughn and Berhow 1999; Cipollini and Gruner 2007) but activity of putative compounds in the field needs to be better understood (Barto and Cipollini 2009). Probable allelopathy has been confirmed in the field (Cipollini and others 2008a). The second invasive species, *Lonicera maackii* (Rupr.) Maxim [Amur honeysuckle, Caprifoliaceae], is found throughout the eastern half of the US and Canada. Leaf extracts of *L. maackii* demonstrated direct allelopathic effects on seed germination of *Arabidopsis thaliana* and *Impatiens capensis* (Dorning and Cipollini 2006) and growth and reproduction of *A. thaliana* (Cipollini and others 2008b). Two flavones, apigenin and luteolin, were identified in *L. maackii* as possible allelochemicals

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(Cipollini and others 2008c). Allelopathy of *L. maackii* on *A. thaliana* has been demonstrated in field soils using leaf extracts and conditioned soils, i.e., soils in which the invasive species had previously grown (Cipollini and others 2008b; Cipollini and Dorning 2008). The third invasive species, *Ranunculus ficaria* L. [lesser celandine, Ranunculaceae], is an emerging invasive species (Axtell and others 2010), with little published research from its invasive range in the Northeastern and Pacific Northwest regions of the US. Leaf extracts of *R. ficaria* negatively affected germination and growth of some model species in the laboratory (Cipollini and others 2102b), and probable allelopathic effects in the field have been revealed (Cipollini and Schradin 2011).

While each species of interest has demonstrated allelopathic effects, there is little information on comparative allelopathic effects of these species. Meiners and others (2012) call for more studies investigating the variation in strength of allelopathic interactions in order to understand these interactions in an ecological context. Further, understanding the difference in allelopathic effects may help in prioritizing and refining conservation activities related to prevention and mitigation of invasive species effects. Pisula and Meiners (2010) compared allelopathic effects of leaf extracts of 10 invasive species, finding *A. petiolata* as one of the top strongest germination inhibitors. Cipollini and others (2012b) found similar comparatively strong effects of *A. petiolata*, though effects varied with target species. Other studies have also found that effects of invasive species extracts can vary with target species (McEwan and others 2010; Shang and others 2011). Previous comparative studies have used agricultural or model laboratory organisms (Pisula and Meiners 2010; Cipollini and others 2012b) and there is a need for testing allelopathy with more ecologically relevant species. There may also be little allelopathic effect between plants within the same plant family; for example, there was no negative impact of *A. petiolata* on other members of the Brassicaceae (Cipollini and others 2008b; Cipollini and others 2012b). Additionally, the type of plant tissue used to make extracts may also have differential impacts (Dorning and Cipollini 2006; Cipollini and Dorning 2008; Moosavi and others 2011). The direct effect of root extracts has not been examined for *R. ficaria* or for *A. petiolata*, particularly in a comparative context.

This study aimed to investigate the effect of root and leaf extracts from three co-occurring invasive species on the germination of three native target species. We hypothesized that leaf extracts would have greater impacts than root extracts due to results from preliminary studies, and that inhibitory effects would increase with extract concentration. We hypothesized that *A. petiolata* would have the strongest impacts and that effects of invasive species would vary with target species. In particular, we hypothesized that extracts of *R. ficaria* would have negligible effects on a target species in its same plant family and have greater impacts on target species found in other plant families.

MATERIALS AND METHODS

Roots and leaves/shoots of *A. petiolata*, *L. maackii* and *R. ficaria* were collected in May 2010 in woodlots near Wilmington, OH. Three hundred grams of each tissue, leaf or root, were placed in 1 L of distilled water and allowed to soak at room temperature for 48 h to make a concentration of 0.3 g fresh tissue/mL distilled water. Solutions were then vacuum-filtered and the filtrate was stored frozen until use. The three native target species, *Blephilia hirsuta* (Pursh) Benth. [hairy pagoda plant, Lamiaceae], *Anemone virginiana* L. [tall thimbleweed, Ranunculaceae] and *Elymus hystrix* L. [bottlebrush grass, Poaceae], were chosen based on their availability, their ability to easily germinate and their native habitat overlap with all of the invasive species of interest. Seeds were obtained from Prairie Moon Nursery (Winona, MN). Two concentrations of root and leaf extract were used for the experiment, 0.3 and 0.15 g fresh tissue/mL distilled water; concentrations are similar to those used in previous experiments and thought be within field levels (Dorning and Cipollini 2006; Cipollini and others 2008b; Cipollini and others 2012b). The low concentration was created by diluting the high concentration by half with distilled water.

Seeds (10 for *E. hystrix* and 25 for *B. hirsuta* and *A. virginiana*) were placed on paper towels, which were then saturated with 20 ml of designated extract or a control solution of distilled water, with four replicates for each treatment. There were a total of 156 experimental units (three invasive extract species x three native target species x two extract concentrations x two extract tissue types x four replicates + 12 controls). The seeds on the saturated paper towels were placed in plastic bags, which were haphazardly placed under lights with 12 h of full-spectrum light. Distilled water was added as needed throughout the experiment to keep seeds moist. The number of seeds that had germinated was recorded after eight weeks. Percent germination (out of average percent germination of control of each species) was analyzed using a four-way Analysis of Variance (ANOVA) with the fixed factors of extract species, extract type (root or leaf), extract concentration and target species using Minitab (Ryan and others 2005). Data did not meet the assumption of normality and were rank-transformed prior to analysis (e.g., Mitchell and others 1994, Mendes and Yigit 2013). Tukey's test was used to determine differences between treatments when significance was found in the ANOVA. The α-level for all tests was 0.05.

RESULTS

The mean (±SE) number of seeds germinated in the control treatments (distilled water only) was 7±0.7, 6±0.8 and 5±1.35 for *E. hystrix*, *B. hirsuta* and *A. virginiana*, respectively. There was a significant difference between extract species, with seeds treated with extracts of *A. petiolata* across all other treatments having lower percent germination (32.7±6.3%) than seeds treated with extracts of *R. ficaria* (66.9±7.8%) and *L. maackii* (67.9±7.6%) (Table 1). High extract concentrations had a larger inhibitory effect (50.8±6.1%) compared to low extract concentrations (61.0±6.3%) across all other treatments. Extracts of leaves inhibited germination (31.0±4.7%) more than extracts of roots (80.7±6.1%) across all other treatments. Germination of *E. hystrix*

TABLE 1
Results of Analysis of Variance (ANOVA) on final percent germination (out of number germinated in control treatment).

Source	df	F	p
Extract Species	2	29.9	<0.001
Extract Type (Root or Leaf)	1	140.3	<0.001
Extract Concentration	1	5.54	0.02
Target Species	2	97.9	<0.001
Extract Species*Extract Type	2	8.2	<0.001
Extract Species*Extract Concentration	2	0.5	0.61
Extract Species*Target Species	4	6.8	<0.001
Extract Type*Extract Concentration	1	1.9	0.17
Extract Type*Target Species	2	11.8	<0.001
Extract Concentration*Target Species	2	1.2	0.32
Extract Species*Extract Type*Extract Concentration	2	1.5	0.22
Extract Species*Extract Type*Target Species	4	2.9	0.02
Extract Species*Extract Concentration*Target Species	4	1.5	0.22
Extract Type*Extract Concentration*Target Species	2	0.1	0.90
Extract Species*Extract Type*Target Species*Extract Conc.	4	0.6	0.70
Error	108		

(81.8%±6.1%) and of *B. hirsuta* (70.3±8.3%) were higher than germination of *A. virginiana* (15.4±4.0%) across all other treatments.

The effect of extract type varied with extract species and with target species. There was a larger reduction between root and leaf extracts in *A. petiolata* extract treatments compared to *L. maackii* and *R. ficaria* extract treatments (Fig. 1A). *Anemone virginiana* was most sensitive to extracts of both *A. petiolata* and *R. ficaria*, while *E. hystrix* and *B. hirsuta* showed no response to extracts of *R. ficaria* (Fig. 1B). The interaction of extract type and target species was significant. The germination of *E. hystrix* was only ~40% less for leaf extracts than root extracts, while the germination of *A. virginiana* and *B. hirsuta* were ~80% less for leaf extracts than root extracts (Fig. 2). Each native target species responded significantly differently to root and leaf extracts of the three invasive extract species in different ways (Fig. 3). For example, all three target species generally strongly negatively responded to leaf extracts of *A. petiolata* compared with root extracts, with the largest differences seen in *E. hystrix* and *B. hirsuta*. *Anemone virginiana* had stronger responses to root extracts of *A. petiolata*, which attenuated the difference in response between root and leaf extracts. There was little difference between the effects of root and leaf extracts of *L. maackii* and *R. ficaria* on germination of *E. hystrix*, while *B. hirsuta* and *A. virginiana* responded more strongly to leaf extracts compared to root extracts of these two invasive species.

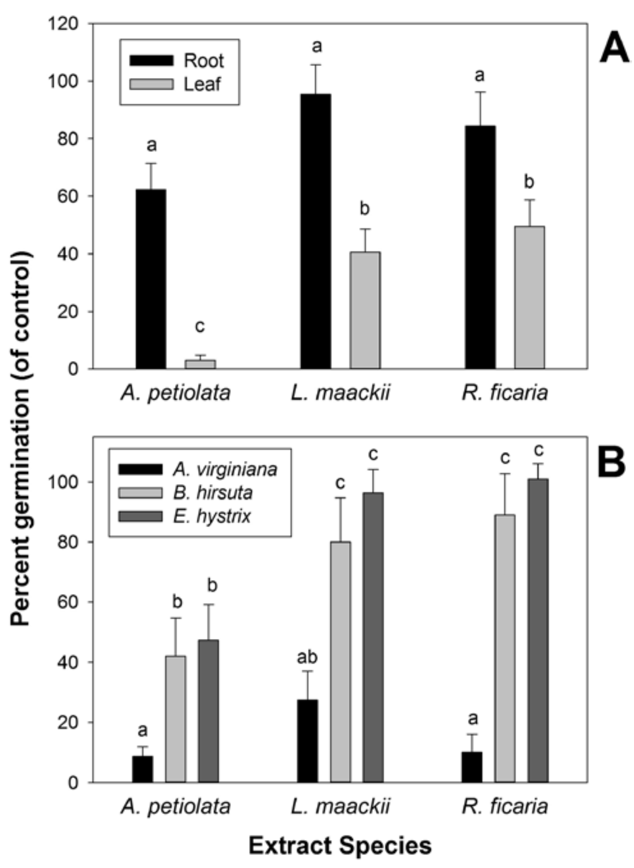

FIGURE 1. Mean (±SE) percent germination (out of average germinated in distilled water control) for seeds of three native species (*A. virginiana, B. hirsuta*, and *E. hystrix*) treated with root and leaf extracts of three invasive species (*A. petiolata, L. maackii*, and *R. ficaria*) at two concentrations. A) Percent germination treated with root and leaf extracts of three invasive species, across all concentration and target species treatments. B) Percent germination of three target native species (*A. virginiana, B. hirsuta*, and *E. hystrix*) treated with extracts of three invasive species (*A. petiolata, L. maackii,* and *R. ficaria*), across all extract tissue type and concentration treatments. Letters indicate significant differences using Tukey's test at α = 0.05.

DISCUSSION

In this study, *A. petiolata* had the strongest overall direct inhibitory effects on germination, confirming results from studies using a comparative approach (Pisula and Meiners 2010; Cipollini and others 2012b). This study extends previous comparative work; for example, Pisula and Meiners (2010) did not include *L. maackii* and *R. ficaria* in their study. Additionally, this study used native species while previous studies used agricultural or model laboratory species (Pisula and Meiners 2010; Cipollini and others 2012b). Using multiple native species as target species provided more insight into interactions that could occur in the field, an identified need in allelopathic studies (Meiners and others 2012).

Invasive species effects varied with target species (McEwan and others 2012; Cipollini and others 2012b). *Blephilia hirsuta* was more sensitive to extracts of *L. maackii* than *E. hystrix*, a pattern that was also found

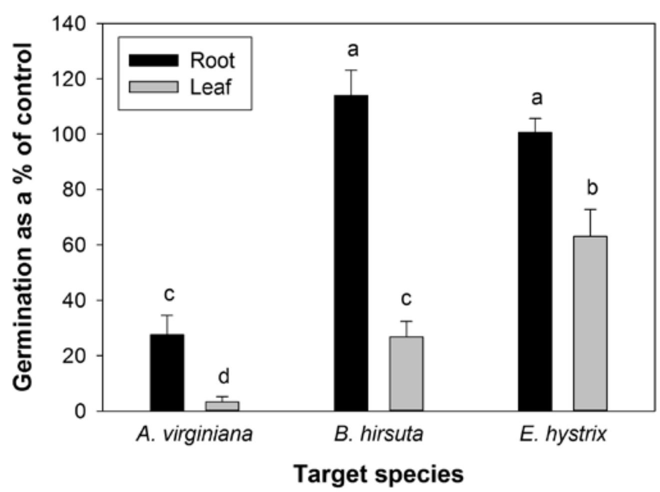

FIGURE 2. Mean (±SE) percent germination (out of average germinated in distilled water control) for seeds of three native target species (*A. virginiana, B. hirsuta,* and *E. hystrix*) treated with root and leaf extracts, across all invasive species and concentration treatments. Letters indicate significant differences using Tukey's test at α = 0.05.

by Bauer and others (2012). However, contrary to previous work (Cipollini and others 2008b; Cipollini at al. 2012b), there was no evidence of a family-specific resistance to allelochemicals. Germination of *A. virginiana* was inhibited by leaf extracts of *R. ficaria*, which are both in the plant family Ranunculaceae. These results provide preliminary evidence that the possible allelochemical may not be ubiquitous in the Ranunculaceae. The study of more species within this family is needed however in order to make any definitive conclusions about the effect of phylogenetic relationships and allelopathy.

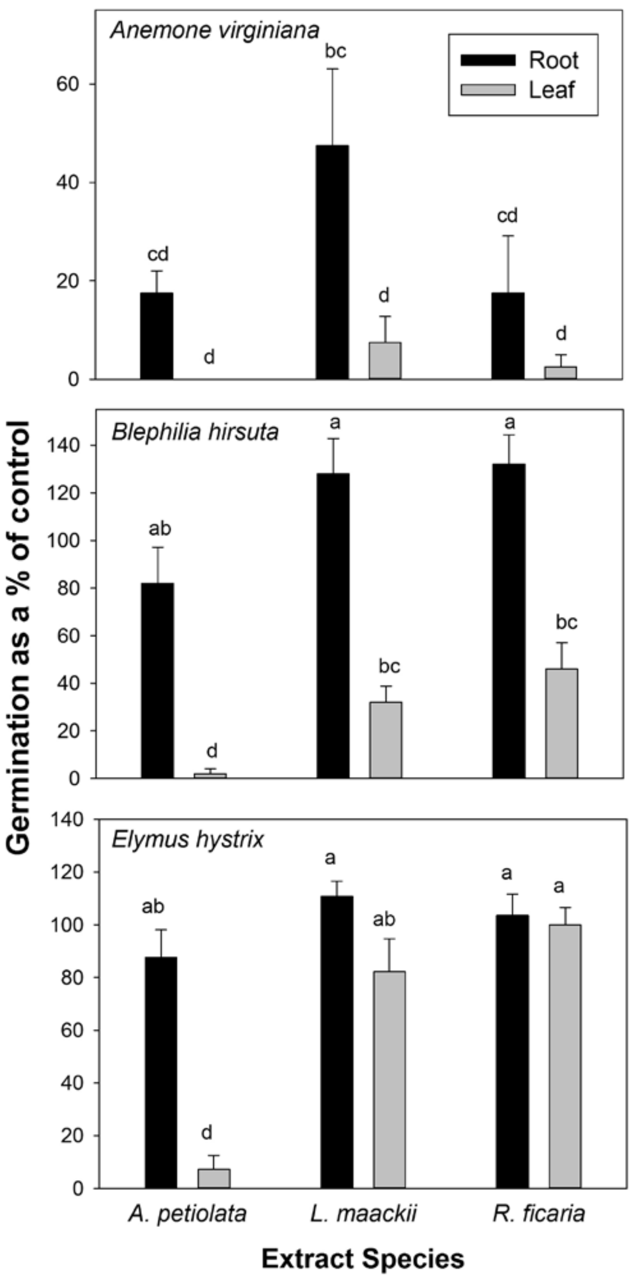

FIGURE 3. Mean (±SE) percent germination (out of average germinated in distilled water control) for seeds of three native target species (*A. virginiana, B. hirsuta,* and *E. hystrix)* treated with root and leaf extracts of three invasive species (*A. petiolata, L. maackii,* and *R. ficaria*), across concentration treatments. Letters indicate significant differences using Tukey's test at α = 0.05.

As expected, germination inhibition increased with increasing concentrations of extracts (Dorning and Cipollini 2006; Cipollini and others 2008b; Cipollini and others 2012b). Although *L. maackii* leaf and root extracts have been compared (Dorning and Cipollini 2006; Cipollini and Dorning 2008), this study is the first to compare direct effects of root and leaf extracts of *A. petiolata* and *R. ficaria*. Root extracts had less impacts than leaf extracts as has been seen previously (Dorning and Cipollini 2006; Cipollini and Dorning 2008; Shang and others 2011), though in some cases roots can have greater impacts (Moosavi and others 2011).

A strength of this study is that it examines direct effects on germination in a highly controlled manner in a single experiment, which allows direct comparative effects. Some weaknesses do exist with this simple controlled approach. Because change in pH may in fact be the mechanism by which one species affects another, we did not control for pH and other water chemistry differences between control and treatment solutions as suggested by Inderjit and Nielsen (2003). Bauer and others (2012) found that pH of *L. maackii* extracts differed from other treatments, yet still found effects on germination when pH was adjusted to account for these differences. In fact, in this study *A. virginiana* was negatively affected by any treatment solution compared to the control, as evidenced by its overall low germination percentage across all treatments, suggesting that conditions in extracts were different enough from the control to have significant effects on germination. The low number of seeds that germinated in each treatment and the low number of replications limited the ability of this study to detect subtle differences; this study therefore reveals only the largest differences in treatments and is fairly conservative in nature.

Ideally, future studies will include more realistic conditions, as allelopathic effects can vary with experimental conditions (Cipollini and others 2012b; Bauer and others 2012). Indirect effects mediated through soil microbes were not considered in this study and can be important in the field (Cipollini and others 2012a). Recent research has focused on indirect effects of *A. petiolata* (e.g., Stinson and others 2006) on other species. This study illustrates that direct impacts are likely also important as well and should be considered when trying to understand the total impact and mechanism of impact of an invasive species. Direct impacts such as those simulated here could occur if seeds are found within the leaf litter layer, with direct leaching of allelochemicals from leaves. Successful establishment of seed is critical in the successful establishment of plant populations. For example, Louda and others (1990) found that a "3-fold reduction in seeds…led to a 6 to 37 reduction in the eventual number of new adults." Therefore, even if an invasive species is only affecting germination, it could have large impacts on native plant populations. Invasive species may have different impacts on different life stages; for example, *A. petiolata* has been shown to have largest allelopathic effects on very young seedlings, prior to mycorrhizal inoculation (Barto and others 2010). In contrast, Parepa and others (2012) found little effects on germination by invasive knotweed compared to larger negative effects on later life stages. Further studies should seek to evaluate impacts on other life stages beyond germination.

Identical concentrations of each extract on a per unit mass basis were used to compare allelopathic strength, similar to other comparative approaches (Szabo 2000; Pisula and Meiners 2010; Cipollini and others 2012b). Each of these invasive species is expected to make differing amounts of leaf litter; therefore, while *A. petiolata* has higher levels of allelopathy at a given concentration level, another species such as *L. maackii* may have larger impacts through higher in situ concentrations generated by higher leaf litter mass. Additionally, factors such as leaf decomposition, which can be rapid in *L. maackii*, should be considered (Blair and Stowasser 2009). This study provides information about the relative strength of allelopathic effects of each of these species and the possibility that they may have allelopathic effects in the field during the key germination period. This information can be used to aid in deciding which invasive species to focus additional research and to focus control efforts, as well as what type of restoration, such as carbon application to mitigate allelopathy (e.g., Kulmatiski and Beard 2006), might be appropriate. Additionally, in the case of active re-seeding after invasive species removal, the information can be used to aid in the selection of seed species that may be resistant to the effects of allelochemicals or that may need a greater seed application rate to ensure restoration success.

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