

Allelopathic effects of invasive species (*Alliaria petiolata*, *Lonicera maackii*, *Ranunculus ficaria*) in the Midwestern United States

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ABSTRACT

Garlic mustard (*Alliaria petiolata*), Amur honeysuckle (*Lonicera maackii*) and lesser celandine (*Ranunculus ficaria*) are three invasive species in US Midwestern forests. The comparative allelopathic effects of leaf extracts of these species on germination and reproduction of *Arabidopsis thaliana* were investigated in a growth room. Highest extract concentrations (0.3 and 0.2 g fresh leaf tissue/mL distilled water) of *L. maackii* delayed germination in potting soil compared to the control. Extracts of *L. maackii* also decreased the number of siliques in potting soil compared to the control and to *A. petiolata* extracts, with extracts of *R. ficaria* having intermediate effects. In field soil, extracts of *L. maackii* and *R. ficaria* significantly decreased the number of siliques compared to extracts of *A. petiolata*. In a third experiment, effects on germination of three agricultural species (*Brassica oleracea*, *Lactuca sativa* and *Ocimum basilicum*), were studied. *Ranunculus ficaria* and *L. maackii* extracts were least harmful to germination, while *A. petiolata* extracts were most harmful. Germination of *L. sativa* and *O. basilicum* was more sensitive to *A. petiolata* and *R. ficaria* extracts, while germination of *B. oleracea* was more sensitive to *L. maackii* extracts. These results showed differential allelopathic effects of these invasive species, which varied with test species and experimental conditions.

Keywords: *Alliaria petiolata*, *Arabidopsis thaliana*, *Brassica oleracea*, exotic species, fig buttercup, germination inhibition, *Lactuca sativa*, leaf extracts, *Lonicera maackii*, *Ocimum basilicum*, phytotoxicity, *Ranunculus ficaria*.

INTRODUCTION

Invasive species pose a threat worldwide, negatively impacting biodiversity (34,49) and exerting significant economic costs (38). One focus in invasive species ecology is to determine the factors that contribute to their success (30,43), such as life history traits (2) and release from natural enemies (27). One hypothesis to explain invasive species success is the novel weapons hypothesis (2), whereby an invading species possesses a trait novel to the invaded ecosystem. The invasive species can then take advantage of this trait in its new ecosystem during interactions with native species that are evolutionarily-naïve to the trait (16). In plants, allelopathy can represent a novel weapon (6,8,24) and can have direct plant-to-plant effects, whereby allelochemicals directly impact

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other species (20). Alternatively, allelopathy may have indirect effects on other plants, through changes in soil ecology or mutualisms (7,44,50). Allelopathic effects may vary depending on target species (33) or conditions such as life stage (4) and nutrients (14).

Allelopathy can be studied using various experimental conditions (25), with differing degrees of realism and control. Simple, controllable, yet unrealistic, germination and growth experiments involve the application of specific chemicals or plant extracts with putative allelochemicals, usually in Petri dishes with a paper substrate (15,20,33). Other studies seek to increase the degree of realism at the expense of experimental control in greenhouse studies and field experiments, many times with the use of activated carbon as a manipulative tool (17,18,41). Generally, allelopathy studies start first with simple, controlled laboratory experiments before scaling up to field experiments.

Three important invasive species in forests and riparian areas in the Midwestern United States are garlic mustard (*Alliaria petiolata* (Bieb.) Cavara & Grand, Brassicaceae), Amur honeysuckle (*Lonicera maackii* (Rupr.) Maxim, Caprifoliaceae) and lesser celandine or fig buttercup (*Ranunculus ficaria* L., Ranunculaceae). *Lonicera maackii* (native to Asia) is found in 26 states in the eastern half of the United States (45) and negatively affects trees and understory plants (19,22,23). Its leaf extracts inhibit the germination of several test species in the laboratory (15,20) and adversely affect the growth of *Arabidopsis thaliana* in the greenhouse (14). Field soils collected from areas infested with *L. maackii* negatively impacted the growth of *A. thaliana* (11). Cipollini et al. (17) could not demonstrate any allelopathic effects of *L. maackii* on *Impatiens capensis* in a field study using activated carbon, though the sample size limited the conclusions of the study.

Alliaria petiolata (native to Europe) is found from coast-to-coast in 35 states (except the southern-most states) in the United States (45). It negatively affects understory plants (9,31,35) and exhibits direct allelopathic effects on germination of *Geum* species (40), though one study showed negligible effects (32). It has several candidate compounds that may be responsible for its allelopathic effects (12,13,46), but the exact compounds responsible have not been identified (3). Indirect allelopathic effects mediated through mycorrhizae have been demonstrated in the greenhouse (7,44), though the effect may vary with species or life stage (4). Its allelopathic effects have been shown in the field (17).

Ranunculus ficaria (native to Europe) is found in 21 states in the Northeast, Midwest, and Pacific Northwest regions of the United States (45). It is considered an invasive species (1), but only one paper confirms its negative impact (18). Because of its purported medicinal effects (10), it likely exhibits allelopathy (21). Its allelopathic effects on reproduction of *I. capensis* were shown in field (18), but the study could not differentiate direct and indirect effects. Hence more information is necessary to fully evaluate its impact as an invasive species, let alone the mechanism for its success.

While there is some evidence of allelopathy for all of these species, there is no comparative research on their allelopathic effects. Other studies have taken a comparative approach to study allelopathy and allelochemicals, either comparing a suite of invasive species (39) or comparing invasive species to co-occurring similar native species (5,33). As allelopathic effects can vary with the species on which they are tested (33), we tested multiple species [Broccoli (*Brassica oleracea*), Lettuce (*Lactuca sativa*) and Basil (*Ocimum basilicum*)] to get more generalizable results. A comparative approach using more than one test species and more than one invasive species is useful in restoration activities and the possible use of mitigation treatments [such as activated carbon (29)],

particularly in areas invaded by more than one species. This research aimed to compare the allelopathic effects of leaf extracts of three invasive species (*A. petiolata*, *L. maackii* and *R. ficaria*) on germination, growth, and reproduction of other test plant species.

MATERIALS AND METHODS

During spring, *L. maackii*, *A. petiolata* and *R. ficaria* leaves were collected from a natural area on the Wilmington College campus in Wilmington, Ohio. Leaves were then soaked for 48 h in distilled water and filtered. The extracts were then diluted to three different concentrations: 0.1, 0.2 and 0.3 g fresh leaf tissue/mL distilled water. The two low concentrations used were similar to previous studies (14,20). An additional higher concentration (0.3 g leaf/mL) was used in our current studies. While we have no information about natural concentrations of allelochemicals in the field, this high concentration represents approximately 30% of a mature *L. maackii* leaf in 1 mL of water (20), which is likely within field levels. Extracts were stored at 4°C until the start of the experiment. For all experiments, we used the fully factorial treatment combinations of extract type or species (*A. petiolata*, *L. maackii* or *R. ficaria*) and extract concentration (0.1, 0.2 or 0.3 g leaf/mL), for a total of 9 extract treatment combinations (3 extract species x 3 extract concentrations = 9 experimental treatment combinations), replicated 4 times. When a control solution was used, we used distilled water and therefore did not control for effects other than allelochemicals [such as altered pH, nutrients and osmality (25,47)], which limits our conclusions on the confirmation of allelopathy. As our goal is to compare the effects among invasive species, rather than simply compare our treatments to a control, a proper control becomes less necessary. Further, our controls served to validate the viability of our seeds in germination tests. The Columbia genotype of *Arabidopsis thaliana* (L.) Heynh (Brassicaceae) was chosen as a target test species in our first two experiments due to its sensitivity to allelochemicals (37) and its successful use in previous studies (11,14,20). We performed experiments in an air-conditioned growth room (23°C, 50 $\mu\text{mol}/\text{m}^2\cdot\text{s}$ PAR light and with 15 h days and 9 h nights).

Pot culture (Potting soil)

In May 2008, we planted 10 seeds of *Arabidopsis thaliana* in 100 mL pots containing potting soil (Pro-Mix BX, Premier Horticulture, Inc., Quakertown, PA) and 1 mL of slow release fertilizer (Osmocote, The Scotts Company, Marysville, OH). In earlier studies, we have grown *A. thaliana* in pots of this size (14) and did not notice any evidence of space limitations. Pots with seeds were irrigated with 10 mL of their specified extract (or control). The number of seedlings emerged in each pot were recorded daily for 2 weeks, at which time they were thinned to one plant per pot. No plants germinated after 7 days. Each pot was irrigated with 10 mL of extract on alternate weeks and plants were watered as needed. We recorded the date of first flowering. After 13 weeks, using a blinded technique, we counted the number of siliques per plant and randomly collected 10 siliques per plant to determine mass of seeds per silique.

For the effect of extract concentration on germination over 7 days, we performed a MANOVA for each extract species, using each date as a separate variable (47). When significance was found in the MANOVA, we ran separate ANOVAs for each date,

followed by Tukey's test. For the final response variables, due to design constraints, we could not use a fully-crossed two-way ANOVA with control treatments in the model. We first performed three two-way ANOVAs with the factors of extract species and extract concentration and their interaction on the response variables of days to flowering, silique number and seed mass. There was a significant effect of extract species for the response variables of silique number and days to flowering ($F_{2,25} = 3.98$, $p = 0.031$ and $F_{2,25} = 3.42$, $p = 0.049$, respectively). There were no significant differences for the factor of extract concentration or the interaction term for any response variable.

This study aimed to statistically compare the differences between the invasive species and the control. Since the effect of concentration was not significant for any response variable, we made a *post hoc* decision to remove concentration from the model. We then performed a MANOVA with the response variables of days to flowering, silique number and seed mass with the factor of extract species, either control or one of the three invasive species. When significance was found in the MANOVA, we ran separate univariate ANOVAs for each response variable, followed by Tukey's test. We set α at 0.05 for all tests and used Type III sums of squares in this unbalanced design. Minitab was used for all statistical analyses (42).

Pot culture (Field soil)

To better simulate field conditions, we used field soil. In August 2009, we planted *Arabidopsis thaliana* in 100 mL pots containing field soil (collected from a local woodlot free of invasive species). Because we had found with previous treatments that *A. petiolata* extracts served as a negative control for *A. thaliana* in both field soil and potting soil (see 14 and results above, respectively), we did not use a control of distilled water for this study. Pots with seeds were irrigated with 10 mL of their specified extract. Plants were thinned to one plant per pot one week later. Each pot was treated with 10 mL of extract on alternate weeks and water was given to the plants as needed. Ten mL of 0.4 g/L fertilizer (Peters 20-20-20 N-P-K plus micronutrients; Grace-Sierra, Milpitas, CA) dissolved in distilled water was added when extracts were added, as this level of fertilizer allows allelopathic effects to occur (14). After 10 weeks, we counted the number of siliques per plant. We performed an ANOVA with the response variable of silique number, with the fully-crossed factors of species and extract concentration, followed by Tukey's test. In all tests, we set α at 0.05.

Petri plate bioassay

In April 2009, the direct allelopathic potential on germination, removing any soil effects, was further explored by applying extracts to three agricultural species [*Brassica oleracea* 'Copenhagen Early Market' (Brassicaceae), *Lactuca sativa* 'Grand Rapids, Tipburn Resistant' (Asteraceae) and *Ocimum basilicum* (Lamiaceae)]. We chose these species as they were readily available, germinate easily and represent different plant families. Additionally, agricultural species such as lettuce and radish are frequently used in allelopathy studies (32,39). Four replicates were used for each treatment combination (3 extract species x 3 extract concentrations x 3 test species x 4 replicates). Four replicates of distilled water served as a control. Ten seeds of each test species were placed on folded paper towels and watered with 10 ml of extract solution or of distilled water (control). Paper towels were placed in plastic sandwich bags and placed under lights. Germination

(measured as emergence of the radicle) was recorded for 28 days; however, no seeds germinated after 14 days.

We analyzed the germination percentage (of control treatment average) at 14 days, using a fully-crossed three-way ANOVA with the factors of extract species (*A. petiolata*, *L. maackii* or *R. ficaria*), extract concentration (0.1, 0.2 or 0.3 g/mL) and test species (*B. oleracea*, *L. sativa* or *O. basilicum*). Data were arcsine-square root transformed prior to analysis to meet model assumptions. We used Tukey's test to determine significance between means. We set α at 0.05 for all tests.

RESULTS

Pot culture (Potting soil)

For seed germination over 7 days, there was a significant difference for *L. maackii* extracts in the MANOVA ($p = 0.007$). In the univariate ANOVA, there was significant inhibition of germination in the first 2 days ($p = 0.002$, $p < 0.001$). On the first day of germination, there was greater inhibition in all extract treatments than the control (Fig. 1). On the second day of germination, there was greater inhibition in the 0.2 g/mL and 0.3 g/mL concentrations, compared to the control and the 0.1 g/mL concentration (Figure 1). For the final response variables, there was a significant effect of extract species in the MANOVA ($p = 0.046$). In the ANOVA, there was a significant effect of extract species for silique number ($p = 0.049$) and a near significant effect of extract species for flowering ($p = 0.071$). There was significantly greater inhibition of silique production in the *L. maackii* extract treatment than the control and *A. petiolata* extract treatments, with the *R. ficaria* extract treatment having intermediate effects (Figure 2). Because the effect of extract species on days to flowering was significant in the first full ANOVA model, we present here the means for each extract treatment to investigate the nature of the effect (Figure 3). Flowering in plants treated with *R. ficaria* extracts was slightly inhibited compared to *A. petiolata* extract treatments at $p = 0.10$.

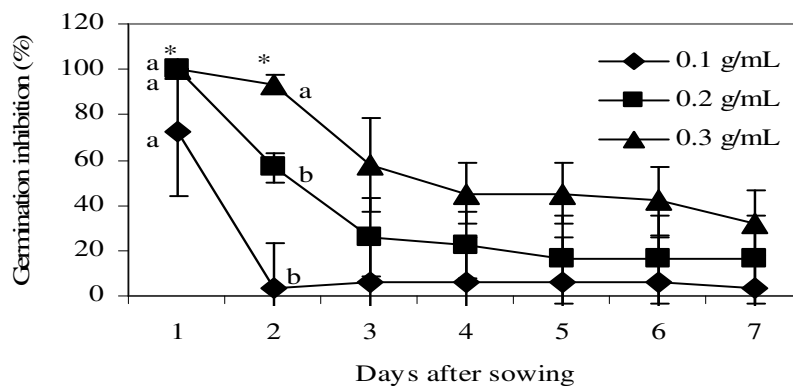


Figure 1. Effects of *Lonicera maackii* leaf extracts on seed germination of *Arabidopsis thaliana*. Asterisks indicate dates for which there were significant differences between treatments. Letters indicate significant differences within each date using Tukey's test at $\alpha = 0.05$.

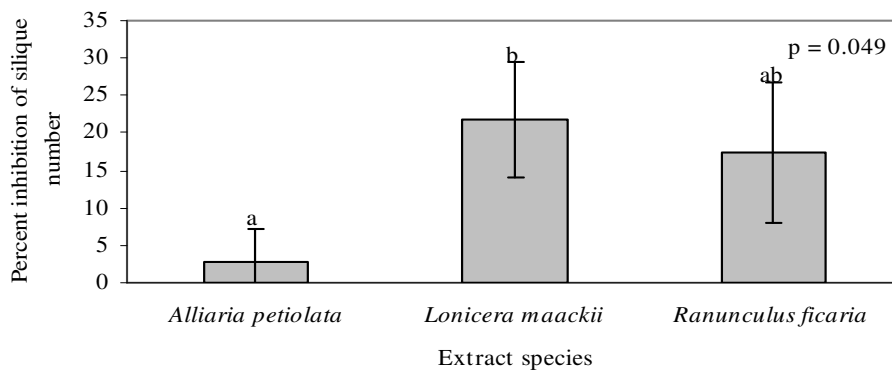


Figure 2. Mean (\pm SE) percent inhibition of silique production (versus control treatment) of *Arabidopsis thaliana* in potting soil, treated with extracts of invasive *Alliaria petiolata*, *Lonicera maackii* or *Ranunculus ficaria*. Mean silique production for the control treatment was 743 (\pm 66) siliques. Treatments with different letters were significantly different from each other using Tukey's test at $\alpha = 0.05$.

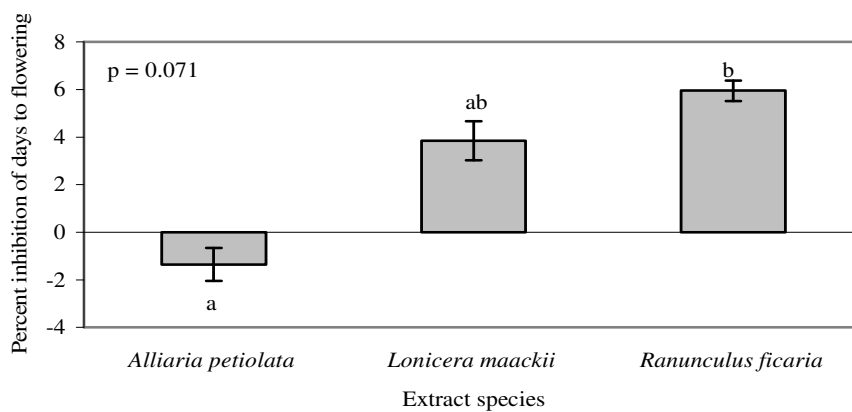


Figure 3. Mean (\pm SE) percent inhibition (versus control) of days to flowering of *Arabidopsis thaliana* in potting soil for treatments containing leaf extracts of invasive *Alliaria petiolata*, *Lonicera maackii* or *Ranunculus ficaria*. Mean days to flowering for the control treatment was 25 (\pm 1.5) days. Treatments with different letters were significantly different from each other using Tukey's test at $\alpha = 0.10$. These data are presented to show the nature of this near-significant trend.

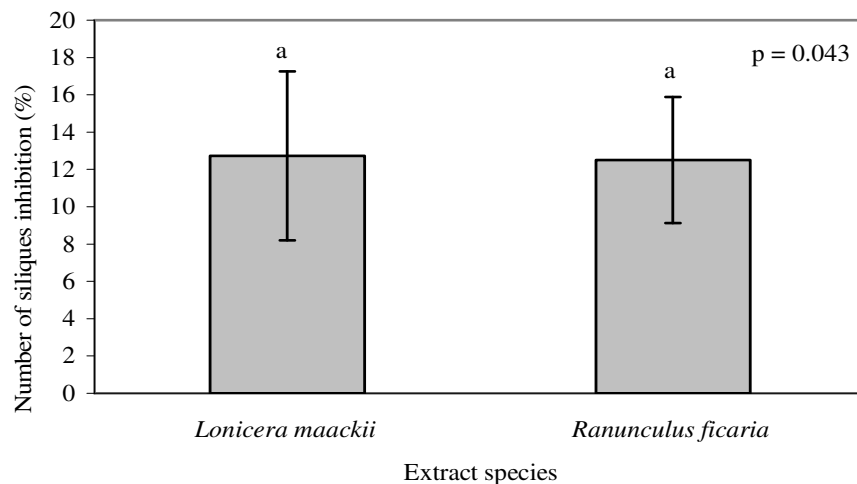


Figure 4. Mean (\pm SE) percent inhibition of siliques production (versus control treatment) of *Arabidopsis thaliana* in field soil, treated with extracts of invasive *Lonicera maackii* or *Ranunculus ficaria*. Mean siliques production for the *Alliaria petiolata* treatment, which served as a control treatment, was 298 (\pm 13) siliques. Treatments with the same letters were not significantly different from each other using Tukey's test at $\alpha = 0.05$. Both treatments significantly differed from the *A. petiolata* control treatment.

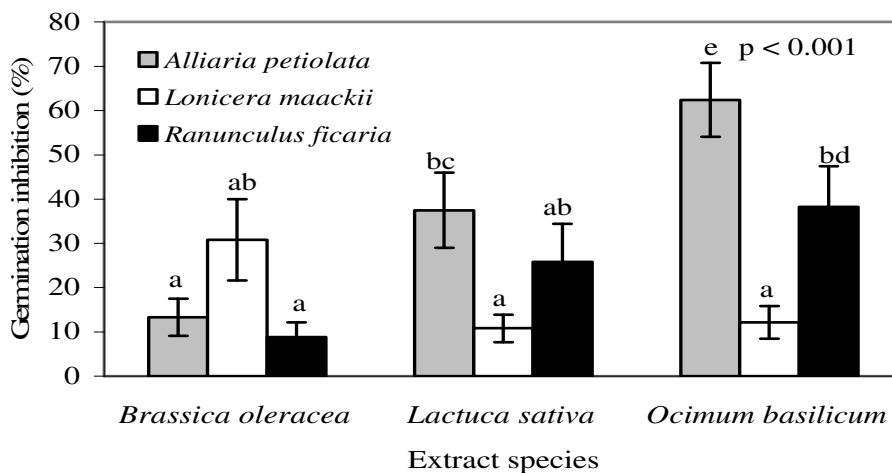


Figure 5. Influence of leaf extracts of invasive *Alliaria petiolata*, *Lonicera maackii* and *Ranunculus ficaria* on seed germination of *Brassica oleracea*, *Lactuca sativa* and *Ocimum basilicum*. Different letters indicate significant differences using Tukey's test at $\alpha = 0.05$.

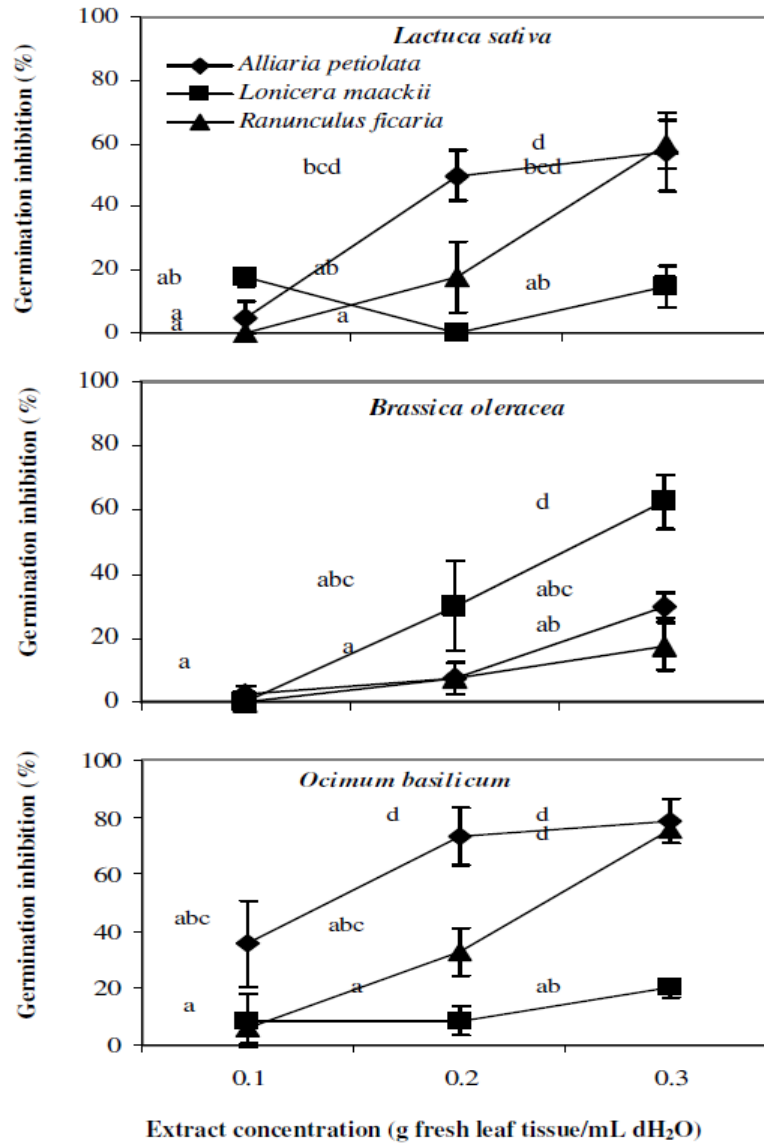


Figure 7. Influence of three concentrations of leaf extracts of *Alliaria petiolata*, *Lonicera maackii* and *Ranunculus ficaria* on seed germination of *Brassica oleracea*, *Lactuca sativa* and *Ocimum basilicum*. Different letters indicate significant differences using Tukey's test at $\alpha = 0.05$.

Pot culture (Field soil)

In the ANOVA, there was a significant effect of extract species on silique number ($p = 0.043$) and a near-significant effect of extract concentration on silique number ($p = 0.052$). There was significantly more inhibition of silique production in plants treated with extracts of *L. maackii* or *R. ficaria* compared to plants treated with *A. petiolata* (Figure 4).

Petri plate bioassay

All seeds of *L. sativa* and *B. oleracea* germinated in the control treatments. In the control for *O. basilicum*, nearly all seeds germinated (mean \pm SE = 93 ± 5). In the ANOVA, there was a significant effect of test species, extract species and extract concentration on germination ($p < 0.001$ for all three factors). Across all other treatments, *B. oleracea* (17.5 ± 3.8) and *L. sativa* (24.7 ± 4.4) had less inhibition than *O. basilicum* (35.8 ± 5.4). Across all other treatments, there was significantly more inhibition with *A. petiolata* extract (37.3 ± 5.2) than with *L. maackii* and *R. ficaria* extracts (18.0 ± 3.7 and 24.1 ± 4.7 , respectively). Across all other treatments, with each increase in concentration, there was an increase in germination inhibition (8.4 ± 2.7 , 25.5 ± 4.5 , 46.2 ± 4.6 for 0.1 g/mL, 0.2 g/mL and 0.3 g/mL, respectively). There was a significant effect of the interaction of extract species with test species and with extract concentration ($p < 0.001$, $p = 0.006$, respectively). The effect of extract species varied with test species, with *A. petiolata* extracts had the strongest inhibitory effects on germination of *L. sativa*, and *O. basilicum* and *L. maackii* extracts having the strongest inhibitory effects on germination of *B. oleracea* (Figure 5). Extracts of *R. ficaria* had stronger inhibitory effects than extracts of *L. maackii* on the germination of *O. basilicum*. The effect of extract concentration varied with extract species, with greater inhibition of germination with increasing concentration in extracts of *A. petiolata* and *R. ficaria* compared to extracts of *L. maackii*, which had smaller changes with increasing extract concentration (Figure 6). Additionally, there was a significant 3-way interaction of test species, extract concentration and extract species ($p < 0.001$). Essentially, each test species responded to increasing concentration of extracts of each invasive species in different ways. For example, while increasing concentrations of *L. maackii* extract had strong inhibitory effects on germination of *B. oleracea*, increasing concentrations of *L. maackii* had little effects on *L. sativa* and *O. basilicum* (Figure 7). Increasing concentrations of *R. ficaria* inhibited germination of *O. basilicum* and *L. sativa*, with little effect on *B. oleracea*.

DISCUSSION

In our experiments, we provided additional information about the allelopathic potential of leaves of three invasive Midwestern species and, more importantly, provided information on the comparative effect of each. Pisula and Meiners (39) similarly used standardized methods to compare a suite of 10 invasive species, but they did not use either *L. maackii* or *R. ficaria* in their study. Pisula and Meiners (39) found *A. petiolata* to be one of the four highest inhibitory invasive species, though only one test species, radish, was used. Our comparative approach was enhanced by the use of multiple test species, as previous work shows that allelopathic effects vary with test species (33,36,40).

Additionally, our use of germination assays allowed the assessment of direct impacts of each invasive species.

Allelopathic effects of each invasive species varied with test species. Generally, effects of extracts of *L. maackii* were greatest on test species from the Brassicaceae, while extracts of *A. petiolata* and *R. ficaria* had the highest inhibitory effect on test species in other families (Asteraceae and Laminaceae). Extracts of *A. petiolata* did not strongly affect the two test species in the Brassicaceae, as was found in previous work (14). This is most likely caused by the similar chemical composition of plants in the same family, which makes *A. thaliana* and *B. oleracea* more resistant to the effects of these chemicals. Effects of extracts of *R. ficaria* were generally weaker though still had allelopathic effects, particularly at the highest concentration. *Ranunculus ficaria* had strongest effects on germination of *L. sativa* and *O. basilicum*.

Allelopathic effects of each invasive species also varied by experimental conditions. Extracts of *R. ficaria* showed a trend to reduce reproduction and to delay flowering in *A. thaliana* in potting soil, while extracts of *R. ficaria* significantly inhibited silique production of *A. thaliana* in field soil. There was also higher seed production in potting soil compared to field soils, suggesting differing growing conditions, which may have influenced the differential response to allelopathy (11,14). Interestingly, we found little long-term effect of extract of *L. maackii* on germination in *A. thaliana* in potting soil, as germination was only delayed by 2 days. This contrasts previous work, which showed 50% reduction of germination of *A. thaliana* on filter paper after one week (20), though an increasing sample of seeds in each treatment might have revealed a significant difference. There was no significant effect of extract concentration on response variables in potting soil and only a near-significant effect in field soil, in comparison to previous work that found strong effects of concentration in similar experimental conditions (14). In comparison, differing concentrations did affect germination on paper. Further, the concentration effect varied with extract species and with test species, increasing the difficulty in finding a simple, generalizable result from this study.

While our study provides some interesting insights into the comparative effects of allelopathy for these three invasive species, there is still much research to be done to fully evaluate the allelopathic potential of these species in the field. First, a proper control, which accounts for differences in such factors as pH and nutrients between extract solutions and control solutions must be used (26). Indeed, previous studies have not adequately controlled for these effects (e.g., 3, 14,20,32). In order to evaluate whether the allelopathic effects truly represent novel weapons to native plants, a comparative approach using co-occurring native species should be used (5, 33). Additionally, a combination of field and laboratory experiments should seek to identify allelopathic compounds and determine their bioactivity and persistence *in situ* (3,25). Finally, the ability of our study to find subtle effects was limited by the small number of seeds used in germination tests; therefore, our results are fairly conservative in nature. Nevertheless, our study provides important information on the relative allelopathic impact of each invasive species, as well as illustrates the importance of using multiple test species and experimental conditions to incorporate consideration of differing sensitivities to and conditions for allelopathic effects. Finally, our study also importantly provides additional information about the direct and the possibility of indirect allelopathic potential of *R. ficaria*, a species for which

there is little published information (18), despite increasing interest in its role as an invasive species (1).

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