Effects of Intraspecific Density on Garlic Mustard (*Alliaria Petiolata*) Sinigrin Concentration

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Abstract

The production of secondary defense chemicals in plants represents a trade-off between defense and the primary functions of growth and reproduction, but the relative allocation to growth versus defense varies across species, types of defenses, ontogeny, and environment. *Alliaria petiolata* (garlic mustard) is a brassica that produces glucosinolates, a class of constituent secondary metabolites that defend against herbivores and pathogens. Sinigrin, a hydrolyzed product of glucosinolate present in garlic mustard, may aid in its success as an invasive species by disrupting native plant–mycorrhizae mutualisms and decreasing forest species diversity in North America. Here, we measured sinigrin concentration in garlic mustard populations of different field densities and in greenhouse experiments to evaluate the relationship between sinigrin concentration and growth in response to density and varying environmental conditions. We found clear evidence for growth vs. defense tradeoffs in both experimental and field settings, as well as higher levels of defense in more densely growing, smaller individual plants. However, sinigrin levels and tradeoffs were not explained by soil fertility or light, allowing us to conclude that sinigrin expression is not controlled by limitations in the measured abiotic factors. Our findings suggest sinigrin leaf concentration increases at high densities despite the pressures of intraspecific competition that demand allocation to growth.

Introduction

It is generally accepted that plants balance allocation of resources between primary functions such as growth and reproduction, and secondary functions such as defense, due to limited resources (Herms and Mattson 1992). The devotion of resources to increased defense often comes with a reduction in growth or fecundity (Balhorn et al. 2014). The physiological tradeoff between these primary and secondary functions constrains the evolutionary responses of plants as they interact with their biotic and abiotic environments (Herms and Mattson 1992). However, the significance of this tradeoff varies across species, environments, and populations (Wallace and Eigenbrode, 2002; Siemens et al. 2002). While many hypotheses exist as frameworks to explain patterns of plant growth vs. defense, there is no general consensus that consistently accounts for the observed variation in plant defenses (Stamp 2003). To account for plasticity in defensive plant traits in response to biotic and abiotic environment, we need increased specificity and situational investigations of plant growth and defense.

Glucosinolates are the main secondary defense chemicals produced by the Brassicaceae mustard family, and are described as low weight compounds, highly toxic to unspecialized herbivores, and costly to produce (Herms and Mattson 1992; Feeny 1976; Stamp 2003). Garlic mustard (*Alliaria petiolata*), a widespread Eurasian biennial that is invasive in North America, predominantly produces the aliphatic glucosinolate, sinigrin (Renwick and Lopez 1999; Cipollini 2004). Previous research has classified sinigrin as defensive against herbivores in garlic mustard's native range (Lewis et al. 2006) and as allelopathic in the invaded range (i.e., disrupting native species growth through the release of phytochemicals), suppressing symbiotic fungi crucial to native plant growth (Roberts and Anderson 2001; Stinson et al. 2006; Wolfe et al. 2008; Poon et al. 2015; Wheeler et al. 2017). Sinigrin is also toxic to native pierid

butterfly larvae when females mistake invasive garlic mustard for their native mustard host plants (Chew and Courtney 1991; Cippolini 2002). While some theories on invasive plant chemical production posit that they will evolve increased competitive ability (EICA) overtime and reduce allocation to costly traits such as secondary defense chemicals, research specifically on the Brassicaceae suggest that glucosinolates may improve relative competition as well as defense– thus their allelopathic and anti-herbivory nature may reduce the evolutionary pressure to increase competitive ability in other ways (Siemens et al. 2002). Despite the importance to defense and potential to facilitate invasion, sinigrin production in garlic mustard is not well studied, and we lack an understanding of tradeoffs between sinigrin production and primary function in this and other Brassicaceae.

Garlic mustard occupies a variety of habitats from forest edge to understory (e.g., Byers and Quinn 1998) and in the invaded range. It can form dense monocultural stands in open areas as well as shaded forest habitats (Rogers et al. 2008). Both plant density and environmental parameters within a habitat may work in conjunction with plant development to affect production of defensive chemistry (Lewis et al. 2006). For example, high intraspecific density may result in density stress within a population, which is characterized by increased mortality, reduced plant size and reduced seed production by individual plants in a stand (Meekins and McCarthy 2002; Winterer et al. 2005). In addition, secondary compound concentration in herbaceous brassicas have been shown to vary in response to light, water, and surrounding native species (Kliebenstein et al. 2001; Brown et al. 2003; Cippolini 2004; Lankau et al. 2009; Lankau 2012; Frisch et al. 2014; Smith 2015). Light levels have been shown to impact rosette size of garlic mustard (Lewis et al. 2006), which in turn may affect sinigrin concentration either through physiological resource availability or by increasing the concentration in smaller plant tissues and diluting sinigrin in older, larger leaves (Lewis et al. 2006). However, it is not clear if or how allocation to secondary chemical production vs. primary function changes in response to the morphological and physiological effects of density stress and/or abiotic environmental conditions.

To investigate how allocation to garlic mustard defensive chemical production varies with intraspecific density and resource availability, we conducted field observations and a greenhouse experiment. To observe possible growth tradeoffs in response to sinigrin production, we compared correlations in growth metrics of garlic mustard in the field to sinigrin concentration in leaf tissue, recording variation in light levels and intraspecific density across a gradient. Plants in a greenhouse study were subject to experimental density and light treatments. We anticipated that density stress may impact defensive secondary compound production through impacts on morphology and plant size. We tested two alternative hypotheses: a) high population density leads to decreased sinigrin concentration in leaf tissue due to tradeoffs associated with competition, or b) high population density leads to increased sinigrin concentration in leaf tissue due to overall smaller plant size. We also predicted that high light conditions would favor higher sinigrin production than shade, as increased light resources should enhance a plant's overall primary function and reduce tradeoffs associated with defense. We found that plant size was negatively correlated with both density and sinigrin concentration. Higher density populations had smaller plants with higher sinigrin content, suggesting that large populations with suppressed plant sizes may be better defended. Plants also produced more sinigrin in high vs. low light conditions, but there was

an interaction effect between light level and density. Relative sinigrin production was similarly high across light levels at high densities, but lower among shaded populations at low densities. We conclude that sinigrin allocation may be driven by pressures of intraspecific competition that constrain plant size at high densities and, by contrast, dilution of sinigrin levels in larger leaves.

Methods

Garlic mustard is a self-compatible, biennial herb of Eurasian origin that first arrived in North America in 1868 in New York (Nuzzo 1993) and is now widespread in deciduous forest understories and along forest edges throughout the continent (Cavers et al. 1979; Myers and Anderson 2003). Garlic mustard can form dense, virtually monotypic stands that cover several hundred square meters and displace native species in the invaded habitats (Cavers et al. 1979; Nuzzo 1991; Yost et al. 1991; McCarthy 1997; Meekins and McCarthy 2002). It occurs in habitats with irradiance levels varying from deep shade— in forest understories, to nearly full sun— along forest edges, and varies in density across these different habitats (Cavers 1979; Myers and Anderson 2003). Its biennial life history consists of a basal rosette during the first year of growth, which over-winters, bolts primary stems and bears flowers and fruits in the spring of the second growth year (Nuzzo 1991). Siliques house the seeds that exhibit a dormancy period, which can last from 8–10 months or until cold stratification occurs, initiating germination from late February through April (Baskin and Baskin 1992; Anderson et al. 1996) followed by a release of seeds from July to October (Cavers 1979). The plant senesces in the late fall of the second growth year (Anderson et al. 1996).

In April 2017, we selected field sites with garlic mustard invasions in four secondary mixed deciduous hardwood forests in the adjacent towns of Northampton and Amherst, Massachusetts. Three sites were located in Amherst: Sylvan Woods, Brooks Wood, and Mill River Woods on the Northwest end of the University of Massachusetts Amherst (42 ° 24.036' N, 072 ° 31.355' W). The Northampton site consisted of a hardwood forest near the Mill River Reservoir that has a garlic mustard invasion extending along a trail (42 ° 18'46.37" N, 72 ° 39' 19.74" W). All sites had preexisting garlic mustard invasions at varying densities and were adjacent to agricultural land. Seedlings for the greenhouse experiments were collected in South Hadley, MA (42° 15' 16.79" N, 72 ° 35' 07.10" W) in an oak dominated hardwood forest adjacent to Stony Brook and east of the Connecticut River.

For field observations at each forest, we placed six one m² quadrats systematically at least 10 m apart along multiple transects within a 200 m sample range. To assess garlic mustard's relative field density, we counted each stem of second year adults within the quadrat (Wilson 2007). High-density plots were characterized as having 29 or more adult garlic mustard stems per square meter and low-density plots were characterized as having 25 or fewer adult stems per square meter.

Within each quadrat, we measured soil temperature (Traceable® thermometer) and photosynthetic photon flux density (PPFD) (Li–Cor 250A Photometer) weekly from April –June. Soil temperatures were taken at a depth of ~7 cm and PPFD $\mu\text{mol}/\text{m}^2/\text{s}$ was measured at 60 cm above ground with a Li-190R

quantum sensor attached to the 250A Photometer $\pm$ 3h to solar noon at center of each quadrat. Four soil cores were systematically taken from each side around the center of the quadrat with an auger at the depth of 15 cm, homogenized, ground, and oven dried at 23 ° C. Soil samples were then processed at the University of Massachusetts Soil and Plant Nutrient Testing Laboratory Amherst, MA, for standard soil fertility tests measuring macro and micro nutrient availability, pH, extractable nutrients, cation exchange capacity and percent base saturation.

In each quadrat, the garlic mustard stem closest to the 50 cm intersect was tagged and sampled for growth metrics throughout the season. Leaf samples were taken from adult plants for glucosinolate measurements shortly after initial flowering, in June 2017. We sampled the second leaf node down from the tip of the flowering stem by taking a 1 cm hole punch sample from the leaf midrib and placing the leaf sample into a 2 mL vial of 99% menthol. After each leaf sample collection, the hole puncher was cleansed with 70% isopropanol. Leaf collection methods were adapted from Lankau 2001; Cipollini 2002; and Kliebenstein 2001. Growth metrics included the maximum height of the selected garlic mustard plant, relative chlorophyll content of the sampled leaf using SPAD-502 meter (Konica-Mintola, Japan), number of branches, and the number of leaf nodes to assess each plant's photosynthetic ability and vigor. We harvested focal plants at the end of June, just before senescence, and recorded the number of siliques, average seed weight per plant, above-ground and belowground biomass at the time of harvest.

Analyses were conducted in R Studio, software version 3.4.0 (R Development Core Team 2017) and JMP-Pro v15.1 (SAS Institute 2019). We constructed multiple linear regression models to assess relationships among garlic mustard density and soil nutrients, and among garlic mustard density, soil temperature, and PPFD. We also calculated and tabulated the means and standard errors for each environmental variable. We constructed linear regression models and plotted correlations between growth characteristics of adult plants as a function of sinigrin concentration to assess linear relationships and possible tradeoffs between defense and growth or reproductive traits. To examine the effect of density (adult and rosette stem count) on glucosinolate sinigrin concentration, we fit a multiple linear regression with log scaled sinigrin concentration as the dependent variable, and light, site, and garlic mustard stem density as factors. Variance inflation factors were calculated, and model selection was based on AIC comparison.

To experimentally test the effect of density on leaf sinigrin concentration, we established a randomized pot design at the College of Natural Science and Education Greenhouse at the University of Massachusetts, Amherst (Experiment 1: Density effect on plant traits and sinigrin production). We harvested cotyledon stage garlic mustard seedlings in mid-May from a hardwood dominated forest in South Hadley, MA and transplanted them into 10 cm plastic nursery pots at two density treatment levels. The low-density treatment consisted of one seedling per pot and the high-density treatment consisted of five seedlings per pot. Density treatment was replicated 61 times using individual pots as experimental units (N=122).

All pots were filled with ~3 L (0.14 cu ft.) of ProMix HP soil mixed with 1/3 of natural sand. Seedlings of the high-density 5 rosettes per pot treatment were planted equidistant of each other with one seedling in

the center of the pot. Seedlings grew on a bench in the greenhouse under controlled day lengths and temperatures programmed at 16 h, 21°C daylight; 8 h, 18°C night.

We randomly selected a subset of thirty-four of each high and low-density treatment pots (N=68) for sinigrin analysis using a random number generator. We took a holepunch sample and fresh leaf surface area measurements of the leaf of the tallest stem from the center rosette following the protocols outlined above after 10 weeks. We harvested all plants after 22 weeks in the greenhouse and assessed growth as specific leaf area, leaf mass ratio, and shoot, root, and total biomass at the time of harvest.

To examine the effect of density on plant traits and leaf chemistry, we constructed one-way ANOVA models with density category as the main effect and each plant trait and sinigrin concentration as the response variables. Post-hoc pairwise mean comparisons with 95% confidence levels were performed for significant ANOVA test variables.

To determine how light interacts with density to affect leaf sinigrin concentration, we conducted a second experiment at the greenhouse facility concurrently, with identical greenhouse conditions using seedlings sourced from the same field location as described above. In a 2x2 factorial randomized design, seventy-two pots were arranged on a single bench with low and high garlic mustard seedling density treatment levels. A light treatment of 50% aluminet knitted nylon shade cloth was draped over half of the bench shading thirty-six of the seventy-two pots. The density treatments were evenly distributed between the 50% shade and full sun. Thirty-six pots were randomly subsampled for sinigrin and fresh leaf surface area as above, with the two light treatments equally represented (Greenhouse Experiment 2: Density and light effects on plant traits and sinigrin production). The duration of the experiment and morphological traits measured match the density experiment as above.

To examine the interaction of light and density on sinigrin production, we constructed a two-way ANCOVA with density and light treatments as main effects and an interaction term. We used interaction plots with sinigrin concentration, specific leaf area, and total biomass as response variables to density and light treatments to determine effects of density treatment and light on growth and sinigrin production of the rosettes.

We measured sinigrin content using high performance liquid chromatography (HPLC). Leaf tissue samples were stored in the vials filled with methanol for a minimum of thirty days until extraction for sinigrin analysis. To prepare the plant material for HPLC analysis, leaf tissue samples were extracted through 96 well plate filter columns packed with QAE Sepahex A-25 (Sigma-Aldrich, St. Louis, MO, USA). The columns were pre-washed with sodium acetate buffer before the addition of 800µL of aqueous plant tissue sample extract. The columns were washed sequentially 2 x with 750 µL of 70% MeOH, 2 x with 750 µL of ddH₂O, 1 x with 750 µL of 20 mM NaOAc, and 2 x with ddH₂O to create optimal conditions for 30 µL of sulfatase from *Helix pomatia* type-H1 to cleave the sulfate bonds (adapted from Grosser and van Dam 2017). After 24 hours, desulphated glucosinolates were eluted with 150 µL of ultra-pure water (18.2 mΩ) purified with a Milli-Q water purification system (Millipore, Molsheim, France) into sample vials.

The samples were analyzed on an Alliance 2695 dual-wave UV HPLC instrument using a reserved-phased Symmetry C18 (150mm x 4.6mm i.d., 5 μ particle size) column at 40°C and a VanGuard precolumn and cartridge holder (Waters, Milford, MA). All sample extracts were injected at 20 μ L and spectra were generated using a diode-array detector at a UV wavelength of 229 nm. The linear gradient elution consisted of (A) HPLC grade water and (B) acetonitrile mobile phase at a flow rate of 1 mL/min with the following program set: 1.5% of B from 0 to 5 minutes 2.5% B 6 to 7 minutes; 5.0% B from 8 to 14 minutes; 18% B from 15 to 16 minutes; increased to 46% B from 17 to 23 minutes; then 92% B from 23–24 minutes, then re-equilibrated to initial conditions at 25 to 29 minutes. Standards of pure sinigrin monohydrate were run on a simplified gradient elution as described in Grosser and van Dam 2017. Sample peaks were compared to the calibration curve standard of sinigrin monohydrate, integrated using QuanLynx from MassLynx 4.1 Software (Waters, Milford, MA), and quantified using the linear calibration methods from Prasad 2015 and Grosser and van Dam 2017. The 2.11 mM sinigrin monohydrate stock concentration was used as a multiplier in the calibration QuanLynx methods. After glucosinolate extraction, leaf tissue samples were oven dried and weighed for dry mass. Sinigrin concentration was then calculated based on microMoles sinigrin per gram of dry leaf weight.

Results

Means and standard errors for garlic mustard density and the environmental variables at our field plots are given in Table 1. There were no correlations between garlic mustard density and any of the environmental variables we observed in the field (Table 2a-b).

Sinigrin concentration from the field density observations ranged between 0.184–5.99 μ M/g dry weight in high-density plots and from 0.3711–2.487 μ M/g dry weight in low-density plots. Sinigrin concentration was negatively correlated with adult stem height ($r = -0.65$, $p = 0.002$), shoot mass ($r = -0.63$, $p = 0.009$), and root mass ($r = -0.49$, $p = 0.052$) (Figure 1).

There was a positive relationship between second year stem density and sinigrin concentration ($r = 0.21$, $p = 0.37$) (Figure 2). Our models demonstrated an effect of adult stem density ($F_{(1,10)} = 7.34$, $P = 0.022$), but no effect of rosette density or PPFD on leaf sinigrin concentration in the field (Table 3).

All plant biomass traits and leaf mass ratios were consistently higher in the low-density treatment than in the high-density treatment in Experiment 1; however, SPAD readings and SLA did not differ significantly between the density treatments (Table 4). Sinigrin concentrations ranged from 0.011–0.786 μ M/g dry weight and was negatively correlated with shoot mass ($r = -0.34$, $p = 0.024$), root mass ($r = -0.38$, $p = 0.013$), and total biomass ($r = -0.41$, $p = 0.0054$) (Figure 3). Sinigrin concentration was higher in the high-density treatment than in the low-density treatment (Figure 4; Table 4).

In Experiment 2: Density and light treatments, sinigrin levels ranged from 0.0293–1.482 μ M/g dry weight. Similar to the density only treatment, sinigrin concentration was higher but plant size was smaller in the high-density treatments (Table 5; Figure 5a,c). In density and light treatments, specific Leaf Area was

higher in the high light treatment compared to the shade treatment (Figure 5b). No correlations between sinigrin concentration and the other plant traits (specific leaf area, total biomass or density) were observed in light and density treatments. There was a trend toward higher sinigrin concentration in high light but the model did not show significant effects of light or interactions between the light and density treatment effects on any of the measured plant traits (Table 5).

Discussion

This study investigated whether there is evidence of tradeoffs between chemical expression of sinigrin and primary plant function, and whether intraspecific density and light are external factors that alter the concentration of sinigrin in garlic mustard. Specifically, we investigated whether high population density leads to either decreased sinigrin concentration in leaf tissue due to tradeoffs associated with competition, or to increased sinigrin concentration due to a predicted reduction in overall plant size as a result of density-dependent resource limitation. We also predicted that high light conditions would favor higher sinigrin production than shade conditions, as increased light resources should enhance a plant's overall primary function and reduce tradeoffs associated with defense.

Reviewing tradeoffs in growth vs sinigrin concentration, the negative relationships between sinigrin concentration and growth metrics in both field and greenhouse populations of garlic mustard in our study demonstrate a direct cost to sinigrin production in this species. Glucosinolates are involved in resistance to generalist herbivores and pathogens as well as allelopathic suppression of competitors during invasion (e.g., Cipollini 2004; Stinson et al. 2006). As such, their production is of presumed benefit to the plant (Siemens et al. 2002) but may involve tradeoffs with primary growth and reproduction functions (Cipollini 2004). Such tradeoffs are common among plants in that resources are generally limiting for both primary function and production of secondary compounds (e.g., Herms and Mattson 1992; Donaldson et al. 2005).

The allocation of resources to the primary function of growth or the secondary function of defense often influence the success of invasive species (Mitchell-Olds et al. 1996; Keane and Crawley 2002; Cipollini 2004; Crawley 2007; Keeler and Chew 2008). The growth vs. defense framework suggests that defensive chemical production may lower competitive ability (Stamp 2003, but this relationship shifts within the context of range expansion, where allelopathy in particular can give invasive plants a competitive edge (Stinson et al. 2006). These "novel weapons" can be beneficial during the invasion process (Callaway et al. 2008; Siemens et al. 2002), as has been shown for garlic mustard in past studies where glucosinolates suppress native plants via disruption of mycorrhizal symbioses (Anthony et al. 2017). Chemical defense also allows garlic mustard to escape herbivory which increases its competitive abilities in the new range (Keeler and Chew 2008). In light of this past work, we can conclude from our findings that sinigrin production remains important as an allelopathic and defense compound during invasion, despite the direct costs to growth.

Considering effects of abiotic environmental variation on sinigrin production, several studies have demonstrated that garlic mustard's growth and reproductive success vary with environmental conditions across continental (e.g., Lewis et al. 2006), regional (Blossey 2020), microsite (Hancock and Stinson 2016) and temporal (Lewis et al. 2006) scales. In particular, studies have observed physiological constraints to growth of garlic mustard because of light intensity (Smith and Reynolds 2014; Stinson and Seidler 2014; Myers and Anderson 2003; 2005). Light availability also influences garlic mustard's growth, reproduction, and long-term population growth (Meekins and McCarthy, 2000; Meekins and McCarthy 2002). Despite previous studies documenting variation in glucosinolate expression in the leaves of garlic mustard and related Brassica species in response to light levels (Kliebenstein 2001; Smith and Reynolds 2014), we found little evidence of this in our study. With the exception of increased SLA in high light, we did not see significant effects of soil temperature, pH, soil nutrients, or light on growth metrics, sinigrin production, or their interaction in garlic mustard. We did not measure soil moisture in this study, and it is possible that water limitation could affect our findings in the field (Byers and Quinn 1998). However, holding moisture conditions constant, as we did in our greenhouse experiments, we can conclude that soil conditions and light levels do not directly impact sinigrin production.

Exploring density effect on sinigrin production, garlic mustard has been observed growing across of range of field densities across North American forests (e.g., Pardini 2009; Lankau et al. 2009), as we have observed in the current study. We did see evidence that biotic conditions affect sinigrin, with consistently higher sinigrin levels in high-density conditions in both the greenhouse experiments and in the field. There are a number of possible explanations for this observation.

First is an extension of the apparency hypothesis, where plants with different life histories express different levels of chemical defense based on the species detectability and relative densities to surrounding plant species. Plants growing at low densities are considered non-apparent and should not have large investments in defense, while the opposite is expected for more apparent species (Coley 1987). Variation in sinigrin concentration across the density gradient in the field, as well as elevated sinigrin concentration differed at high experimental stem density suggests that garlic mustard may adjust sinigrin levels at high densities in order to escape herbivore pressure within the new range. Whether and how this phenomenon occurs in the home range, where garlic mustard densities are typically lower (Rodgers et al. 2008), requires further study.

A second possible explanation for higher levels of sinigrin at high densities is a simultaneous decline in density and defense allocation over time. The EICA hypothesis predicts that in the absence of adapted specialists and generalist herbivores in the new range, invasive plant populations should lose costly traits that aid in herbivory resistance and allocate resources to primary functions that provide competitive advantages such as increasing in size or fecundity (Callaway and Ridenour 2004; Rai 2015). For example, data from long-term monitoring of populations of garlic mustard in the invaded range suggests that glucosinolate concentration declines in garlic mustard populations over time (Lankau et al. 2009). Additional studies suggest there may be a drop in garlic mustard densities over time as well (Blossey et al. 2020). However, our sites were located in forests with similar land use histories and forest

age and were locally proximate to one another with likely similar invasion history, while studies on declines in age and density have been conducted at the regional–to–continental scale (Blossey et al. 2020; Winterer et al. 2005). Additionally, research with garlic mustard has demonstrated that populations in the invaded range experience less herbivory, but do not decrease defensive chemical production compared to plants in the home range (Lewis et al. 2006). Therefore, we think it is unlikely that the density and sinigrin gradient we observed reflects temporal variation in invasion history.

A third explanation for higher sinigrin levels at high densities is a developmental relationship between plant size and sinigrin expression (Herms and Mattson 1992). Meta-analyses of plant defense have shown that trends in defense chemical production also vary through ontogeny, depending on life history strategy and type of defense trait (Barton and Koricheva 2010). While studies focusing on secondary defense chemicals other than glucosinolates have found that older seedlings are better defended (Elger et al. 2009), experimental work monitoring secondary chemicals in seedlings of *Brassica juncea* reveals the opposite trend– defensive concentrations decrease with seedling age (Wallace and Eigenbrode 2002). Many studies have observed chemical concentration of secondary compounds to be at their highest during the early stages of seedling growth and/or at small size in other species as well (Herms and Mattson 1992; Kliebenstein et al. 2001; Myers and Anderson 2003). Garlic mustard shows significant investment in sinigrin production in early life stages, followed by dilution of secondary compounds as leaves grow (Lewis et al. 2006). Other work has noted a general decline of sinigrin concentration with maturity and plant size (Barton and Koricheva 2010), though the biennial life cycle requires the investment of resources for the primary functions of individual plant growth and survivorship during year one, followed by seed production at maturity in year two (Blossey and Notzold 1995). Here we found that plant size was negatively correlated with density, such that garlic mustard grown at higher density were smaller, but with higher sinigrin content. It is possible that developmental constraints on growth under high intraspecific densities explains the pattern we found, with smaller plants being better defended and larger plants demonstrating dilution of sinigrin (e.g., Lewis et al. 2006).

Additional considerations:

As mentioned above, other unmeasured environmental variables such as soil moisture could have affected sinigrin concentrations in the plants from our field observations (Lakshmi et al. 2003; Cantor 2011), although we observed the same patterns of high sinigrin at high experimental densities under constant water conditions. In Cipollini 2002, total glucosinolate levels did not vary significantly across garlic mustard field populations but soil characteristics did. Whether and how soil moisture and other abiotic conditions determine production of sinigrin remains an open question. Glucosinolate concentration excreted into field soil was not measured because of their little to no detectability (Cipollini 2004; Cantor et al. 2011). Due to their volatile nature and short half-lives, the presence of glucosinolates can dissipate in as little as three to twelve hours once excreted into the soil (Barto and Cipollini 2009; Cantor et al. 2011). In addition, in our study we only measured the constitutive aliphatic secondary compound sinigrin, but inducible indole compounds and flavonoids likely play a role in defense and have been shown to vary by external stressors in other garlic mustard plant tissues such as roots and seeds

(Cipollini 2002; Brown et al. 2003; van Geem et al. 2016). Further research on the total phytochemistry of garlic mustard could provide insight into the broader question of resource allocation to growth vs. defense and relationships between defense and intraspecific density in garlic mustard.

Reconsidering management, garlic mustard can grow at a range of densities, from a few individuals to vast monocultures, in its new range (Nuzzo 1999). There is some evidence that populations differ in their production of the phytochemical sinigrin, which functions as an allelopathic “novel weapon” during the invasion process (Rai 2015) as well as an anti-herbivore defense (Frisch et al. 2014). However, few studies have tested whether sinigrin production represents a tradeoff between growth and secondary chemistry, the specific abiotic under which sinigrin varies, or the effects of intraspecific densities on sinigrin production. Here, we measured sinigrin levels in garlic mustard at different field densities and in greenhouse experiments to test the relationship between sinigrin concentration and growth in response to density and varying environmental conditions.

We found clear evidence for growth vs. defense tradeoffs in both experimental and field settings, as well as higher levels of defense in more densely growing, smaller individual plants. However, sinigrin levels and tradeoffs were not explained by soil fertility or light, allowing us to conclude that sinigrin expression is not controlled by limitations in the measured abiotic factors. Our findings suggest sinigrin leaf concentration increases at high densities despite the pressures of intraspecific competition that demand allocation to growth. Possible mechanisms for the patterns we observed might include increased apparency of high-density populations in that plants at high densities were better defended (Feeny 1976), a decline in defense over time as posited by the EICA hypothesis (Callaway and Ridenour 2004; Rai 2015), growth constraints at high densities accompanied by dilution of glucosinolates in larger leaves or glucosinolate variation due to developmental constraints (Brown et al. 2003, Stamp 2003). We conclude that tradeoffs in growth and expression of sinigrin may represent a competitive constraint on plant size in our study populations.

Regardless of the mechanisms involved, our data demonstrate variation in sinigrin levels across garlic mustard populations, suggesting that there should also be fine-scaled spatial variation in its effects on native species. By examining intrapopulation chemical expression within the framework of invasion mechanism tradeoffs, it may be possible to predict future behaviors of populations in particular sites given the habitat characteristics and target crucial growth stages with effective management techniques (Meekins and McCarthy 2002; Pardini et al. 2009). Specifically, our findings suggest that high-density patches of garlic mustard are more toxic to fungi and native caterpillar herbivores (Stinson et al. 2007, van Geem et al. 2016) than low-density patches, making them a more important target for management.

Declarations

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Code availability: Analyses were conducted in R Studio, software version 3.4.0 (R Development Core Team 2017) and JMP–Pro v15.1 (SAS Institute 2019).

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Tables

Table 1: Means and standard errors for measured field environmental variables and garlic mustard density.

	Mean	StdErr
Number of Adult stems	62.900	27.676
pH	5.476	0.126
P	6.725	1.952
K	114.667	11.587
CA	1011.425	130.755
MG	127.595	22.388
Soil temp. °C	16.071	0.196
PAR $\mu\text{mol}/\text{m}^2/\text{s}$	195.060	36.480

Table 2a: Correlation Probabilities from multiple regression of soil nutrients and garlic mustard density in the field.

	pH	P	K	CA	MG	Adult stems
pH						
P	0.125					
K	0.750	0.337				
CA	0.025	0.908	0.419			
MG	0.020	0.545	0.844	0.004		
GM						
density	0.553	0.375	0.427	0.611	0.526	

Table 2b: Correlation probabilities from multiple regression of soil temperature °C, PAR $\mu\text{mol}/\text{m}^2/\text{s}$, and garlic mustard density in the field.

	MEAN		
	gm stems	PAR	TEMP
gm stems			
MEAN			
PAR60	0.418		
MEAN TEMP	0.397	0.172	

Table 3: Analysis of variance for effects of density and light on sinigrin concentration in field populations of garlic mustard.

	df	SS	F	p
adult density	1,10	2.667	7.342	0.022
rosette density	1,10	0.156	0.429	0.527
light	1,10	0.601	1.678	0.224

Table 4: Analysis of variance results for effects of density treatment on plant traits and sinigrin concentration in Greenhouse Experiment 1.

<i>Effect</i>	<i>Density Treatment</i>			
<i><u>Plant trait</u></i>	<i><u>df</u></i>	<i><u>mean ss</u></i>	<i><u>F</u></i>	<i><u>p</u></i>
SPAD	51	34.42	0.92	0.34
LMR	51	0.01	13.33	0.00
shoot mass	51	113.85	32.39	<0.0001
root mass	51	2100.10	27.65	<0.0001
total biomass	51	3306.96	32.11	<0.0001
SLA	51	4385283.00	0.34	0.56
[sinigrin]	64	10.50	6.90	0.01

Table 5: Analysis of variance for effects of Density, Light, and Density * Light Interaction terms on plant traits and sinigrin concentration in garlic mustard plants from Greenhouse Experiment 2.

<i>Effect</i>		<i>Density Treatment</i>			<i>Light Treatment</i>			<i>Density x Light</i>		
<i>Plant trait</i>	<i>df</i>	<i>mean ss</i>	<i>F</i>	<i>p</i>	<i>mean ss</i>	<i>F</i>	<i>p</i>	<i>mean ss</i>	<i>F</i>	<i>p</i>
SPAD	27	5.26	0.12	0.73	211.64	4.95	0.03	46.44	1.09	0.31
LMR	30	0.01	7.55	0.01	0.00	0.00	0.95	0.00	0.06	0.80
shoot mass	31	8.34	25.38	<0.0001	0.56	1.17	0.20	0.09	0.28	0.60
root mass	31	23.34	4.34	0.05	3.99	0.74	0.40	5.22	0.97	0.33
total biomass	31	66.88	9.25	0.00	1.06	0.15	0.70	8.68	1.20	0.28
[sinigrin]	28	0.63	6.14	0.02	0.01	0.14	0.71	0.00	0.05	0.84

Figures

Figure 1

Correlations between growth metrics and sinigrin concentration of adult garlic mustard plants in the field observation study. Relationship strength is indicated by Spearman coefficient with 95% confidence intervals of significance for (A) second year adult stem height, (B) shoot mass, and (C) root mass.

Figure 2

Correlation between sinigrin concentration of garlic mustard plants and adult garlic mustard stem count (density) in field populations of garlic mustard. Spearman coefficient and p value indicate relationship strength and significance.

Figure 3

Correlations between sinigrin concentration and final (A) shoot mass (B) root mass (C) total biomass of harvested garlic mustard rosettes in Greenhouse Experiment 1. Relationship strength and significance are indicated by Spearman coefficient and p value.

Figure 4

Mean sinigrin concentration and standard errors by density treatment of garlic mustard rosettes in Greenhouse Experiment 1.

Figure 5

Means and standard deviations for (A) sinigrin concentration, (B) specific leaf area, and (C) total biomass by density category and light treatment in Greenhouse Experiment 2.