CHEMOECOLOGY

Soil nitrogen availability and herbivore attack influence the chemical defenses of an invasive plant (*Linaria dalmatica*; Plantaginaceae)

Mary A. Jamieson · M. Deane Bowers

Abstract Chemical defenses are thought to contribute to the invasion success and impacts of many introduced plants; however, for most of these species, little is known about these compounds and how they vary in natural environments. Plant allelochemical concentrations may be affected by a variety of abiotic and biotic factors, including soil nutrients and herbivores. Moreover, such quantitative variation is likely to play an important role in species interactions involving these invasive plants. The purpose of this study was to examine patterns of variation in iridoid glycoside concentrations of the invasive plant *Linaria dalmatica* (Plantaginaceae). We conducted a greenhouse experiment to investigate the effect of soil nitrogen availability on iridoid glycoside concentrations. Results from this experiment showed that plant iridoid glycoside concentrations decreased with increased nitrogen availability. Additionally, plants were collected from multiple field sites in order to characterize the influence of population, soil nitrogen availability, and herbivore attack on iridoid glycoside variation. Results from field studies indicated that plants demonstrated considerable seasonal variation, as well as variation within and among populations, with iridoid glycoside concentrations ranging from approximately 1 to 15% dry weight. The relationship between soil nitrogen and plant iridoid glycosides varied among populations, with a strong negative correlation in one population, a marginally significant negative relationship in a second population, and no relationship in the remaining two populations. Additionally, we found a negative relationship between iridoid glycoside concentrations and plant injury by an introduced biocontrol agent, the stem-mining weevil *Mecinus janthinus* (Cucurlionidae). These results show that plant allelochemical concentrations can vary widely in natural environments and suggest that levels of plant defense may be reduced by increased soil nitrogen availability and herbivore attack in this invasive plant species.

Keywords *Linaria dalmatica* · Dalmatian toadflax · Iridoid glycosides · Biological control · *Mecinus janthinus* · Soil nitrogen availability · Herbivory · Allelochemical variation · Antirrhinoside · Linarioside

Introduction

Many plant species produce compounds that affect their interactions with other species (i.e. allelochemicals) and these compounds may be especially important for understanding ecological interactions involving invasive plants. Such compounds have been implicated in the invasion success and negative ecological effects of numerous invasive plant species (Blossey and Notzold 1995; Keane and Crawley 2002; Callaway and Ridenour 2004; Cappuccino and Arnason 2006; Inderjit et al. 2006). However, in most cases, there is a lack of basic knowledge about the allelochemistry of these species, in particular information on natural variation in levels of these compounds and factors that influence such variation.

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Plant allelochemical concentrations can demonstrate significant spatial and temporal variation in response to a number of abiotic and biotic factors (e.g., Bryant et al. 1983; Coley et al. 1985; Bazzaz et al. 1987; Chapin et al. 1987; Karban and Myers 1989; Herms and Mattson 1992; Bidart-Bouzat and Imeh-Nathaniel 2008). For invasive plants, such differences could have important consequences for ecological interactions in the introduced range, including interactions with native competitors or generalist enemies (e.g. herbivores and pathogens) as well as with specialist enemies (e.g. insect biological control agents). Thus, investigations examining allelochemical variation of invasive plants may help provide insight into their ecology and evolutionary biology as well as management (Callaway and Aschehoug 2000; Hierro and Callaway 2003; Callaway and Ridenour 2004; Cappuccino and Arnason 2006; Inderjit et al. 2006; Orians and Ward 2010).

The aim of this study was to investigate patterns of variation in allelochemical concentrations of Dalmatian toadflax, *Linaria dalmatica* (L.) P. Mill. (Plantaginaceae) and factors that influence these patterns. *Linaria dalmatica* is an invasive species found throughout the United States and Canada and the chemical defenses of this plant are thought to play a role in its success as an invader as well as its ecological and economic impacts (Vujnovic and Wein 1996; Wilson et al. 2005; Cappuccino and Arnason 2006). This species produces iridoid glycosides (Handjieva et al. 1993; Franzyk et al. 1999), which are a group of monoterpene-derived secondary metabolites found in more than 50 plant families (El-Naggar and Beal 1980; Boros and Stermitz 1990, 1991). Iridoid glycosides have been shown to act as chemical defenses against generalist insect herbivores and fungal pathogens as well as attractants and feeding stimulants for specialist herbivores (Bowers 1991; Marak et al. 2002; Biere et al. 2004; Beninger et al. 2008; Reudler Talsma et al. 2008). Moreover, quantitative variation in iridoid glycosides is known to influence plant interactions with generalist and specialist enemies; higher concentrations have been associated with greater negative effects on generalists and positive effects on specialists (e.g., Bowers and Puttick 1989; Biere et al. 2004; Harvey et al. 2005; Prudic et al. 2005; Reudler Talsma et al. 2008).

Introduced plants are most likely to invade environments where resource availability exceeds resource demand by resident species (e.g. nitrogen is not limiting) (Davis et al. 2000). Thus, soil nitrogen (N) availability may be an important factor influencing chemical defense and performance traits of invasive plants. Several plant defense hypotheses make specific predictions about the response of plant secondary compounds to nutrient availability (Stamp 2003). For example, the carbon-nutrient balance (Bryant et al. 1983) and growth-differentiation balance (Herms and Mattson 1992) hypotheses propose that production of

carbon-based secondary metabolites, such as iridoid glycosides, will be greatest when environmental conditions lead to photosynthates accumulating in excess of growth requirements, such as nitrogen-limited environments. Under these conditions, excess carbon can be allocated to secondary metabolism with little cost to plant growth. However, the predictions of these hypotheses have been difficult to adequately test and numerous studies attempting to do so have provided equivocal results (Koricheva et al. 1998; Hamilton et al. 2001; Stamp 2003; Stamp 2004). Nevertheless, for iridoid glycosides, research indicates that concentrations are often higher in plants grown under low-nutrient conditions compared to high nutrient conditions (Fajer et al. 1992; Darrow and Bowers 1999; Prudic et al. 2005).

Altered biotic interactions, in particular, escape from specialist pathogens and herbivores (Blumenthal 2005, 2006; Mitchell et al. 2006; Inderjit et al. 2006), may also affect plant defense and performance traits (Blossey and Notzold 1995; Keane and Crawley 2002; Bossdorf et al. 2005; Orians and Ward 2010). Likewise, re-introduction of co-evolved specialist herbivores can result in allelochemical shifts in introduced plant populations (e.g. Zangerl and Berenbaum 2005; Zangerl and Berenbaum 2008). In addition to such evolutionary changes, allelochemical concentrations may vary due to induced responses to plant damage, which may involve either active or passive responses and include both induced resistance and induced susceptibility (Karban and Myers 1989; Karban and Baldwin 1997; Karban 2011).

A previous study showed that iridoid glycoside concentrations of *L. dalmatica* plants demonstrated great variability within and among plant populations in the introduced range, over the course of the growing season, and among plant parts (Jamieson and Bowers 2010). In the present study, we conducted experimental greenhouse and observational field investigations to examine factors that may influence that variation. In the greenhouse study, we looked at the influence of soil nitrogen availability on plant iridoid glycoside concentrations. While plant allelochemical response to abiotic and biotic factors may be best studied in common garden and greenhouse experiments, it is also important to establish that these relationships exist under natural environmental conditions. Accordingly, we also collected plant and soil nitrogen samples from several populations in order to assess the relationship between soil nitrogen availability and plant iridoid glycoside concentrations in the field. Finding that the field data did not entirely support our experimental results, we then also examined the effect of herbivore attack on iridoid glycoside concentrations of field-collected plants. In particular, we investigated the relationship between plant injury caused by an abundant and widely distributed insect

biological control agent, the stem-mining weevil *Mecinus janthinus* Germar (Cucurlionidae) and plant iridoid glycoside concentrations.

Materials and methods

Study organisms

Linaria dalmatica is a perennial species that was originally introduced to North America (Canada and the US) as an ornamental plant from its native range in Eurasia (Alex 1962; Vujnovic and Wein 1996; Wilson et al. 2005). Synonyms of this species include *L. dalmatica* ssp. *dalmatica* (Griseb), *L. genistifolia* ssp. *dalmatica* (L.) Marie and Petitmengin, and *L. dalmatica* ssp. *macedonica* (Griseb) (Alex 1962; Vujnovic and Wein 1996; Weber and Wittmann 2001; USDA, NRCS 2009a). Due in part to substantial morphological and genetic variation, the taxonomy of *L. dalmatica* in the introduced and native ranges remains unclear (Alex 1962; Wilson et al. 2005; Gaskin personal communication). Adding to this confusion, *L. dalmatica* can hybridize with *L. vulgaris* (Ward et al. 2009). In our study populations, however, we did not observe any hybrids and *L. vulgaris* was not present at the field sites under investigation.

Mecinus janthinus is also native to Eurasia and was released in North America during the early 1990s as a biocontrol agent for invasive toadflax species (Wilson et al. 2005). This species has demonstrated the greatest promise as a biological control agent of *L. dalmatica* (Saner et al. 1994; Peterson et al. 2005; Wilson et al. 2005). A detailed description of *M. janthinus* biology and that of other toadflax biocontrol agents is presented in Wilson et al. (2005). Briefly, adults of *M. janthinus* emerge from natal stems in late spring and early summer, feed on leaf and stem tissues, reproduce, and lay eggs in stems. Females chew a hole into the stem, insert a single egg, and then cover the hole, leaving a distinctive oviposition scar for each oviposition event (Wilson et al. 2005). Complete metamorphosis occurs within toadflax stems during spring-summer months with larval feeding causing significant plant injury (Jeanneret and Schroeder 1992; Peterson et al. 2005; Wilson et al. 2005).

Greenhouse study

To examine the effects of soil nitrogen (N) availability on *L. dalmatica* iridoid glycosides, we conducted a greenhouse study in which seedlings were grown under one of four levels of N availability ($N = 25$ per group). Seedlings were collected on June 15, 2005 from a population located in Boulder County, Colorado, USA (Lefthand Canyon: N

40°7′14″; W 105°19′26″). These seedlings had germinated that year and were collected throughout the field site in an effort to select a random sample of offspring from many genetic families. Seedlings were transplanted into 4-L pots in a nutrient poor growing medium (Fafard Potting Mix #2, Conrad Fafard, Agawam, MA, USA) and were grown for 2 weeks prior to beginning experimental N treatments. Seedlings were between 8–12 cm in size and were randomly assigned to treatments.

Nitrogen treatments consisted of 100 ml of water with one of four levels of N availability added every one to two days (total rates equal to 0, 2, 4, and 8 g N/m²/yr). These rates were selected because they are within the range of current soil N availability in our study area. Burke et al. (1997) indicate that N mineralization rates in the Great Plains of the United States range from 1.5 to 10 g N/m²/yr. Plants were additionally watered twice during the course of the experiment with 100 ml of complete nutrient (minus N) solution (half-strength Hoagland's solution; described in Logan et al. 1999). All plants received equal amounts of water and nutrient solution, so that N availability was the only factor that varied among treatments. The placement of plants was re-randomized weekly to avoid bench effects. After 5 weeks, the aboveground biomass of plants (leaves and stems) was harvested and prepared for iridoid glycoside analysis. No plants showed any flower bud formation in this experiment.

Field studies

To investigate variation in the chemical defenses of plants growing under natural conditions, we collected plant samples from multiple populations during spring–summer of 2007. The sampling design included four field sites located in Boulder County, Colorado, USA: Rabbit Mountain (40°14′13″ N; 105°12′53″ W), Superior-Kelsall (N 39°55′5″; W 105°11′56″), Marshall Mesa (N 39°57′11″; W 105°13′35″), and Johnson (N 40°8′38″; W 105°15′55″). A fifth site, Lefthand Canyon, was not included as a study site in 2007 due to damage caused to the site by escaped cattle, although it was included in the 2009 study (see below). Field sites ranged from 1,650 to 2,000 m in elevation and were characterized as dry, rocky foothills grassland habitat with vegetation dominated by mixed grasses, forbs, and shrubs. Typical soil profiles at sites are described as clay loam, sandy loam, or a mixture of both (USDA, NRCS 2009b). The focus of this field study was to characterize patterns of variation in the iridoid glycoside concentrations of *L. dalmatica* plants growing in their introduced habitats. Additionally, this study focused on examining the relationship between soil nitrogen availability and plant iridoid glycoside concentrations in order to determine if plants growing under natural conditions

demonstrated similar responses as greenhouse-grown plants.

At each field site, we established 15 one-meter squared plots containing *L. dalmatica* and geo-referenced these plots using a global positioning system (GPS). We haphazardly selected plot locations to maximize distance between distinct patches of plants and in an attempt to sample broadly from multiple plant genotypes throughout each field site. However, *L. dalmatica* reproduces clonally as well as by seed, making it difficult to distinguish between ramets and genets without genetic analyses. To examine iridoid glycoside variation within and among populations as well as over the course of the growing season, we collected three ramets from each plot on three dates in 2007: May 25–30, July 10–13, and August 28–31 ($N = 15$ plant samples per site per date).

Soil nitrogen availability was measured during the peak growing period of *L. dalmatica*, from mid-May until the end of August, using ion exchange resin bags (methods described in Binkley and Vitousek 1987; Bowman et al. 2003). To prevent disturbance within plots, four resin bags were buried directly adjacent to each plot at a depth of 10 cm. Resin bags were collected at the end of July (2 bags/plot) and August (2 bags/plot) and taken to the laboratory for sample preparation and chemical analyses. The two resin bags from each plot and at each date were composited into a single sample, which was then extracted in 2N KCl. The extract was analyzed for NH_4+ and NO_3- using a Lachat colorimetric autoanalyzer (Milwaukee, Wisconsin, USA) in the Kiowa Lab at the University of Colorado, Boulder. Soil N availability was estimated as mean inorganic N flux per plot ($\mu g/bag/day$).

In 2009, we performed a second field study with the aim of identifying other potential factors that could contribute to variation in plant iridoid glycoside concentrations. In particular, we were interested in the effects of herbivore attack on *L. dalmatica* iridoid glycosides. In 2007 and 2008, we had noticed substantial plant damage caused by *Mecinus janthinus* and levels of weevil damage appeared to be quite variable within and among populations. Thus, in 2009, we investigated the relationship between *M. janthinus* attack and plant iridoid glycoside concentrations. In 2009, we surveyed the same four field sites as well as Lefthand Canyon, which had not been grazed since 2006. While other biocontrol agents (e.g., *Calophasia lunula*) were present at low levels at our field sites, herbivory and insect damage by these insects was minimal and substantially lower than that caused by *M. janthinus*. Additionally, we observed little to no evidence of damage by generalist herbivores, except for cattle herbivory late in the season, aphid damage on a few plants, and one instance of significant grasshopper herbivory. Plants with evidence of moderate to heavy herbivore and/or insect damage in

addition to that caused by *M. janthinus* were avoided during our sample collections.

In the 2009 field season, plant samples were collected at random on one date (between June 18–25) from plots located at each of the five field sites ($N = 15$ samples per site). We quantified levels of attack by *M. janthinus* by counting the number of oviposition scars per cm stem. In another related study, 60% of dissected oviposition holes had developing larvae inside the stem cavity of ramets collected in July (Knochel et al., unpublished data). Moreover, in that study there was a strong correlation between the number of oviposition scars and the number of adult weevils from stems collected in September ($r = 0.86$) (Knochel et al., unpublished data). Other studies have demonstrated negative effects of *M. janthinus* larvae on plant physiology (Peterson et al. 2005) as well as the negative effects of increased weevil densities within stems on plant size (Van Hezewijk et al. 2010). Accordingly, the number of oviposition scars per cm can provide a good estimate of plant injury caused by this specialist herbivore.

Among the field sites under investigation, only Lefthand Canyon had detailed information on the history of the release of *M. janthinus*, which included location and date of release as well as the number of individuals released. Moreover, this site had a well-established and abundant *M. janthinus* population. Thus, we collected additional plant samples from this site in order to further evaluate the effects of *M. janthinus* on plant iridoid glycoside concentrations. Plant samples at Lefthand Canyon were collected on June 19 ($N = 15$), June 24 ($N = 15$), July 9 ($N = 60$).

Plant sample preparation and chemical analyses

Samples from greenhouse and field studies were oven-dried at 50°C to constant mass, weighed to the nearest 0.01 g, ground into a fine powder, and then 25–30 mg of each sample was prepared for chemical analysis. In the 2007 field study, samples for chemical analyses included combined leaf and stem tissue (3 ramets per sample). In the 2009 field study, samples for chemical analyses were leaf tissue from one ramet; stems were used to quantify plant injury. Methods for sample preparation and iridoid glycoside analysis by gas chromatography (GC) have been previously described (Gardner and Stermitz 1988; Bowers and Stamp 1993; Jamieson and Bowers 2010). Briefly, plant samples were extracted overnight in methanol; then, the filtered extract was evaporated to dryness, an internal standard (phenyl-β-D-glucopyranoside) was added, and the sample partitioned between water and ether. After removing hydrophobic compounds in the ether layer, the sample was evaporated again. An aliquot was derivatized with Tri-Sil-Z (Pierce Chemical Company, Rockford, Illinois) prior to analysis by gas chromatography (GC) on

a Hewlett-Packard (HP) 5890A system (Agilent Technologies). Data were processed with HP ChemStation software (version A.03.34).

Statistical analyses

We quantified amounts of the two primary iridoid glycosides found in *L. dalmatica*—antirrhinoside and linarioside. The concentrations of these compounds are correlated and demonstrate similar patterns of variation (Jamieson and Bowers 2010). In this study, we examined patterns of variation in antirrhinoside and linarioside combined (i.e. iridoid glycoside concentrations). Plant iridoid glycoside concentration data were arcsine square-root transformed in order to conform to assumptions of statistical analyses.

For the greenhouse study, analysis of variance (ANOVA) was used to explore the effects of soil N enrichment on (a) aboveground biomass and (b) iridoid glycoside concentrations. Changes in iridoid glycoside concentrations may be due to changes in resource allocation to defense, biomass (concentration-dilution effects), or a combination of both. In order to remove the effect of biomass, we additionally performed an analysis of covariance (ANCOVA) with ramet biomass as a covariate, which allowed us to isolate the effect of N treatment on iridoid glycoside concentrations.

For field-collected plant samples from 2007, the influence of site and seasonal variation on iridoid glycoside concentrations was examined by repeated-measures ANOVA with site as the between subjects factor and date as the within subject factor. Because 2009 samples were collected using a different sampling design (i.e. plant samples were collected on only one date), a separate ANOVA was conducted to examine variation in iridoid glycoside concentrations among field sites in this year. Tukey's HSD multiple comparisons tests were used to examine differences among groups for all significant ANOVAs.

We conducted correlation analyses using Pearson correlation coefficients to assess the relationship between iridoid glycoside concentrations and (1) soil N availability and (2) weevil attack (number of oviposition scars per ramet). In the first analysis, we pooled plant iridoid glycoside data as well as soil nitrogen data collected at the plot level over the course of the 2007 field season. Thus, this analysis revealed the overall relationship between soil N availability and plant iridoid glycosides concentrations at the plot level. To examine data collected in 2009, we first used a *t* test to compare iridoid glycoside concentrations of plants with and without *Mecinus janthinus* oviposition scars. Then, we performed a correlation analysis examining the relationship between the level of weevil attack (oviposition scars per ramet) and iridoid glycoside concentrations. In this analysis, we examined only plants with evidence of

M. janthinus oviposition scars and pooled data from all sites into a single correlation analysis. Soil N data were log $(x + 1)$ transformed and weevil oviposition scar counts were square-root transformed to meet assumptions of normality. All statistical analyses were conducted using SYSTAT (version 11).

Results

Greenhouse study

Soil nitrogen enrichment had a positive effect on plant growth ($F_{3,78} = 12.583$; $P < 0.001$; Fig. 1a). Mean plant biomass more than doubled from the zero N to the high N treatment (8 g N). Moreover, there was a significant decrease in mean iridoid glycoside concentrations with soil nitrogen enrichment ($F_{3,78} = 6.595$; $P < 0.001$; Fig. 1b), yielding a mean reduction of approximately 30% from the
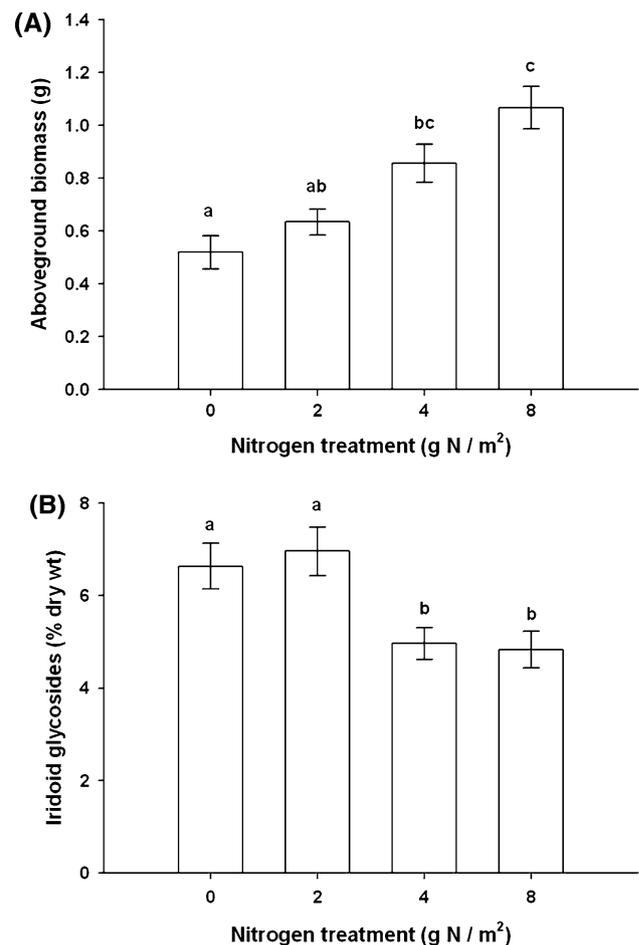

Fig. 1 Effects of soil nitrogen (N) enrichment on *Linaria dalmatica* **a** growth (aboveground biomass) and **b** chemical defenses (iridoid glycoside concentrations). Plants were grown in a greenhouse for five weeks under varying levels of nitrogen availability ($N = 82$)

two lower N treatments (0 and 2 g) to the two higher N treatments (4 and 8 g). ANCOVA results showed that the covariate of biomass had a significant effect on iridoid glycoside concentrations ($F_{1,77} = 5.516$, $P = 0.021$). After removing the effect of biomass, there was still a significant N treatment effect on plant iridoid glycosides ($F_{3,77} = 8.745$, $P < 0.0001$), indicating that changes in concentration were not due to plant size alone.

Field studies

Iridoid glycoside concentrations were highest early in the growing season and declined over time ($F_{2,112} = 17.70$, $P < 0.001$; Fig. 2). Additionally, there was a significant site effect on variation in iridoid glycoside concentrations ($F_{3,56} = 18.55$, $P < 0.001$; Fig. 2). Specifically, plants from Rabbit Mountain had significantly higher concentrations ($P < 0.05$) compared to plants collected from the other three sites in 2007. In 2009, there was also a significant effect of site on variation in iridoid glycoside concentrations ($F_{4,69} = 7.147$, $P < 0.001$); and again, plants from Rabbit Mountain showed the highest concentrations (Table 1). In addition to variation among sites, plants within populations demonstrated considerable variation in chemical defenses, with more than a 15-fold difference in iridoid glycoside concentrations measured in one population (i.e., Johnson) (Table 1).

Association between soil nitrogen availability and iridoid glycosides

The relationship between soil nitrogen availability and iridoid glycoside concentrations varied among populations
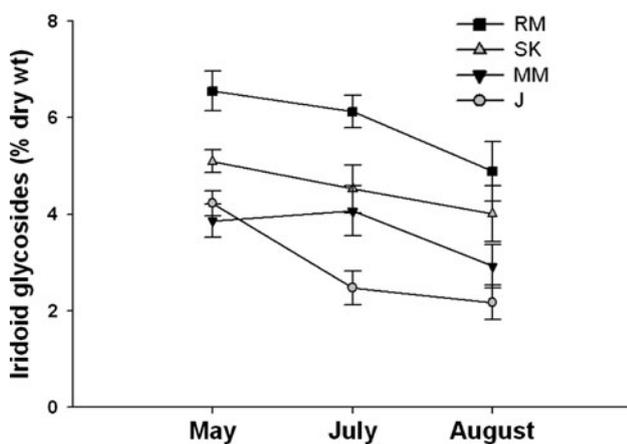

Fig. 2 Pattern of variation in *Linaria dalmatica* iridoid glycoside concentrations (mean ± SE). Plant samples (3 ramets per sample) were collected in 2007 at field sites located in Boulder County, Colorado, USA ($N = 180$): Rabbit Mountain (*RM*), Superior-Kelsall (*SK*), Marshall Mesa (*MM*), and Johnson (*J*)

(Fig. 3). Specifically, there was a strong negative correlation between soil N flux and iridoid glycoside concentrations of plants found at Rabbit Mountain ($r = -0.73$, $P = 0.002$) and a marginally significant negative correlation at Superior-Kelsall ($r = -0.51$, $P = 0.068$). However, there was no relationship ($P \gg 0.05$) between soil N flux and plant iridoid glycosides in the remaining two populations (Johnson and Marshall Mesa).

Association between weevil attack and iridoid glycosides

There was substantial variation in oviposition activity by *M. janthinus* within and among field sites, with the number of oviposition scars greatest on plants from Lefthand Canyon (Table 1). Across field sites, the number of oviposition scars ranged from 0 to 295 per ramet (mean = 48.3 ± 5.8 SE) and plants with *M. janthinus* had lower iridoid glycoside concentrations compared to plants that were not attacked by this biocontrol agent ($t = 2.126$; df = 130; $P = 0.035$; Fig. 4). Moreover, there was a significant negative correlation between weevil attack levels (measured as the number of oviposition scars per ramet) and iridoid glycoside concentrations for plants showing evidence of weevil attack ($r = -0.476$; $P < 0.001$; Fig. 5).

Discussion

In this study, we documented natural variation in iridoid glycoside concentrations of *L. dalmatica*, an ecologically and economically important invasive plant, as well as factors that may influence quantitative variation in these compounds. Our study revealed substantial temporal and spatial variation in *L. dalmatica* chemical defenses. Specifically, results showed a significant decline in iridoid glycoside concentrations of approximately 40% from May to August. In addition to seasonal variation, we found great variation in iridoid glycoside concentrations both within and among populations—ranging from less than 1 up to 15% dry weight. These patterns are similar to those found in a previous study (Jamieson and Bowers 2010) and may have important implications for ecological interactions involving *L. dalmatica*.

Quantitative variation in allelochemicals is likely to influence plant interactions with both generalist and specialist herbivores. High iridoid glycosides concentrations can have a greater negative effect on the growth and performance of generalist insect herbivores compared to low concentrations (e.g., Bowers and Puttick 1989; Biere et al. 2004; Beninger et al. 2008). In contrast, iridoid glycosides are often positively associated with the preference and performance of specialist insect herbivores (e.g., Bowers 1984; Pereyra and Bowers 1988; Bowers and Puttick 1989;

Table 1 Variation in *Linaria dalmatica* chemical defenses and *Mecinus janthinus* attack levels

Field site	Iridoid glycoside concentrations (mean ± SE)	Maximum	Minimum	No. oviposition scars (mean ± SE)
Rabbit Mountain				
June 25 (N = 15)	8.46 ± 0.6**a**	14.56	4.96	7.2 ± 2.4
Superior-Kelsall				
June 22 (N = 15)	4.49 ± 0.5**b**	8.70	1.45	8.2 ± 4.3
Marshall Mesa				
June 18 (N = 15)	4.84 ± 0.5**b**	9.01	2.29	2.0 ± 1.1
Johnson				
June 23 (N = 15)	5.07 ± 0.6**b**	9.54	0.63	0.6 ± 0.4
Lefthand Canyon				
All dates (N = 97)	4.00 ± 0.3**b**			75.0 ± 8.1
June 19 (N = 15)	5.91 ± 0.6	10.49	2.40	94.8 ± 18.7
June 24 (N = 52)	3.95 ± 0.4	11.90	0.75	51.0 ± 7.2
July 9 (N = 30)	3.12 ± 0.5	11.75	0.10	106.7 ± 19.8

Plant samples were collected in 2009 at field sites located in Boulder County, Colorado, USA

Bold letters indicate significant mean differences. Lefthand Canyon data from all dates were analyzed together

Fig. 3 Relationship between soil nitrogen (N) availability and *Linaria dalmatica* iridoid glycoside concentrations at four field sites located in Colorado, USA. Soil N data are inorganic N (NH_4+ and NO_3-) flux measurements, which are presented as log (μg N/bag/day)
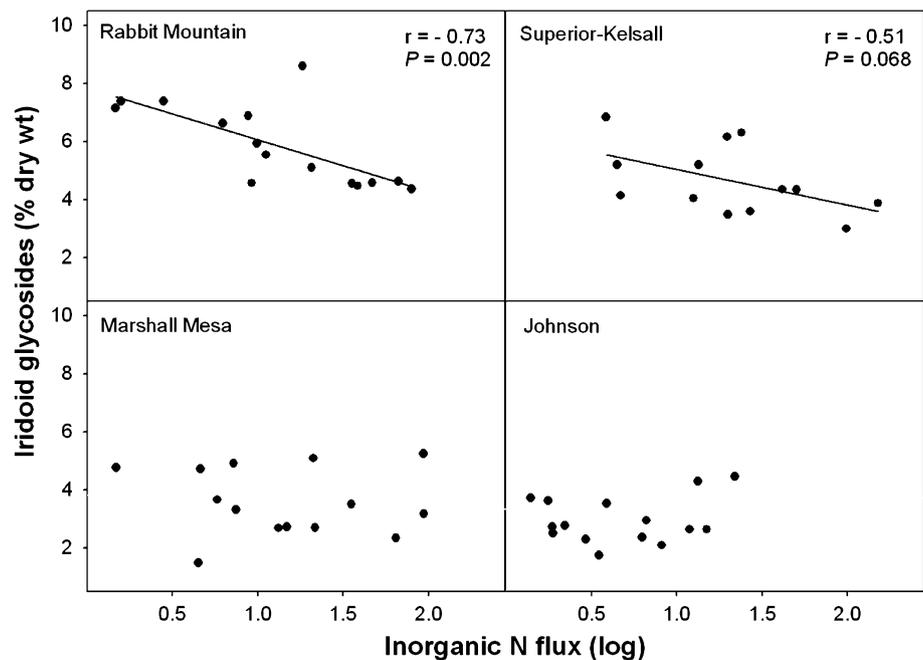
Klockars et al. 1993; Nieminen et al. 2003; Harvey et al. 2005; Prudic et al. 2005; Saastamoinen et al. 2007; Reudler Talsma et al. 2008). For *L. dalmatica*, the large range of variation in iridoid glycosides that we observed indicates that individual plants within populations, as well as different populations over time will provide a chemically variable landscape for both specialist and generalist herbivores.

Our research suggests that natural variation in soil nitrogen availability may influence *L. dalmatica* iridoid glycosides. In this study, we found a strong negative relationship between soil N availability and iridoid glycoside concentrations in at least one population and a marginally

significant negative relationship in a second population. Although our field results are correlative, they support the findings of our greenhouse experiment, which showed a 30% decrease in iridoid glycosides from the two lower to two higher N treatments, as well as other studies that have also demonstrated that iridoid glycoside concentrations are higher under low-nutrient conditions compared to high nutrient conditions (Fajer et al. 1992; Darrow and Bowers 1999; Prudic et al. 2005). Moreover, our results are congruent with predictions of prominent plant defense hypotheses, such as the growth rate, carbon-nutrient balance, and growth-differentiation balance hypotheses (Coley
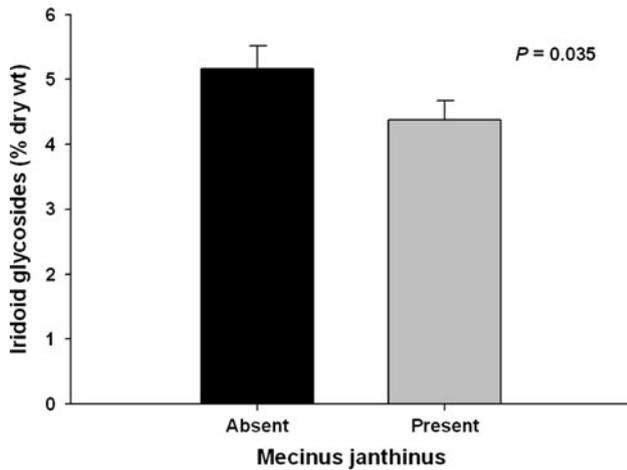
Fig. 4 Comparison of mean (± SE) iridoid glycoside concentrations for plants with no evidence of *Mecinus janthinus* attack (*N* = 101) and plants with oviposition scarring (*N* = 55)
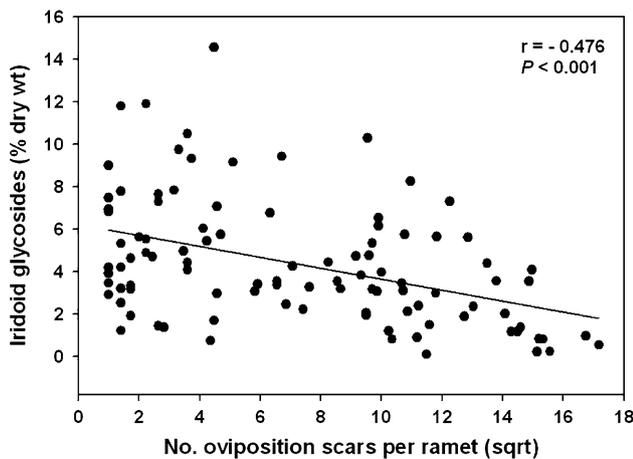

Fig. 5 Relationship between *Mecinus janthinus* attack levels and *Linaria dalmatica* iridoid glycoside concentrations (*N* = 101). Weevil attack data are square-root transformed counts of oviposition scars per ramet

et al. 1985; Bryant et al. 1983; Herms and Mattson 1992). Nevertheless, our research also suggests that plant allelochemistry may be influenced by other important ecological and/or evolutionary factors such as herbivory.

In two of our study populations, there was no relationship between soil nitrogen availability and iridoid glycoside concentrations, suggesting that other variables, such as herbivory, may have an important effect on *L. dalmatica* allelochemicals. Research by Zangerl and Berenbaum (2005, 2008) demonstrated evolutionary shifts in levels of furanocoumarins found in the introduced plant *Pastinaca sativa* (Apiaceae) after re-introduction of a specialist herbivore from its native range. Compared to that system, the re-introduction of *M. janthinus* with *L. dalmatica* is a more recent event and the role of this specialist herbivore in the

evolution of *L. dalmatica* defense traits remains to be seen. There are a number of factors, both ecological (e.g. herbivory, water availability) and evolutionary (e.g. genetic variation due to varying selective pressures), that may be important drivers of variation in *L. dalmatica* allelochemistry. Other studies investigating plants containing iridoid glycosides have demonstrated that genetic, ontogenetic, biotic, and abiotic environmental variation can all influence these defense compounds (Bowers et al. 1992; Bowers and Stamp 1993; Adler et al. 1995; Darrow and Bowers 1999; Marak et al. 2002; Fuchs and Bowers 2004; Barton and Bowers 2006; Barton 2007; Wurst et al. 2008).

Like many invasive plant species, little is known about the chemical ecology of *L. dalmatica,* even though the defensive chemistry of this plant has been implicated in its ecological and economic impacts, as well as its success as an invader. Further research is necessary to assess the role of iridoid glycosides in mediating species interactions between this plant and its natural enemies, in particular its biological control agents. We found no evidence that *M. janthinus* induces the production of iridoid glycosides, as found in some other studies examining plant species containing iridoid glycosides (e.g. Darrow and Bowers 1999; Fuchs and Bowers 2004). Rather, our study suggests that weevil attack may decrease *L. dalmatica* iridoid glycosides. We found that iridoid glycoside concentrations of plants with damage were significantly lower than those without damage. Moreover, for plants with damage, there was a negative relationship between the amount of damage and iridoid glycoside concentrations.

While these patterns could also reflect the influence of plant allelochemicals on insect preference and performance, our results suggest plants may be experiencing induced susceptibility resulting from *M. janthinus* damage. As discussed earlier, other studies indicate that specialist insects often respond positively to higher iridoid concentrations rather than lower concentrations, suggesting that these specialist weevils would be attracted to plants with higher levels of iridoid glycosides, not lower. Additionally, damage by stem-mining *M. janthinus* larvae has been shown to cause deleterious effects to the primary physiology of *L. dalmatica,* leading to decreased photosynthesis (Peterson et al. 2005). These deleterious effects could also reduce available photosynthates and thus resource allocation to the production of iridoid glycosides. Moreover, plant injury caused by larvae may disrupt the flow of phloem and consequently antirrhinoside that is transported in phloem (Gowan et al. 1995).

Still, we cannot eliminate the possibility that iridoid glycosides confer resistance to *M. janthinus* or the possibility that insects are demonstrating preferences for plant nutritional characteristics that may be correlated with iridoid glycoside concentrations. For example, weevils

may show preferences for higher plant nitrogen content, which could be associated with lower iridoid glycoside concentrations. Further experimental research is necessary to test for induced susceptibility and/or resistance. Although induced resistance has historically received greater attention, a meta-analysis by Nykänen and Koricheva (2004) indicates that induced susceptibility is also a common plant response to herbivory. In particular, that study revealed that terpenoids tend to decrease in the foliage of herbivore-damaged evergreens. Similar to those results as well as to our research, Barton (2008) found that herbivory reduced iridoid glycoside concentrations by 65% in *Plantago lanceolata*.

Our research shows the importance of environmental variation, both abiotic and biotic, in understanding quantitative variation in plant allelochemicals. The data from this study provide a better understanding of *L. dalmatica* allelochemistry and clearly show that iridoid glycoside concentrations can vary considerably among individual plants in their introduced habitats. Thus, specialist and generalist enemies of *L. dalmatica* will experience spatially and temporally variable chemical defenses. Moreover, our results indicate that the relationship between environmental variables and plant allelochemicals can be context-dependent—for example, as shown in the relationship between soil nitrogen and plant iridoid glycosides at our study sites. Such field observations can provide a critical perspective for past and future experimental studies that directly test the influence of soil N and herbivory on plant allelochemistry.

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