

**NITROGEN ENRICHMENT DIFFERENTIALLY AFFECTS ABOVE- AND
 BELOWGROUND PLANT DEFENSE¹**

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- **Premise of the Study:** Human nitrogen (N) inputs to terrestrial ecosystems have greatly increased in recent years and may have important consequences for plant growth, reproduction, and defense. Although numerous studies have investigated the effects of nitrogen addition on plants, few have examined both above- and belowground responses within a range of predicted increase and apart from concomitant increases in other nutrients.
- **Methods:** We conducted a greenhouse experiment to study the consequences of increased nitrogen inputs, such as those from atmospheric N deposition, on plant performance, chemical defenses, and allocation tradeoffs for an invasive species, *Linaria dalmatica*. This plant produces iridoid glycosides, which are a group of terpenoid compounds.
- **Key Results:** Soil nitrogen enrichment increased growth, reproduction, and whole-plant iridoid glycosides while decreasing some costs of defense. Interestingly, nitrogen addition had varying effects on defense allocation to above- and belowground tissues. Specifically, there was no change in iridoid glycoside concentrations of shoots, whereas concentrations decreased in flowers by ~35% and increased in roots by >400%.
- **Conclusions:** Observed increases in plant performance and chemical defenses may have implications for the invasion potential of *L. dalmatica*. Moreover, our results highlight the importance of evaluating both above- and belowground plant defenses. In particular, findings presented here indicate that research focused on leaf-level defenses may not detect key allelochemical responses, including changes in plant resistance traits that could affect consumers (e.g., herbivores and pathogens) that specialize on different plant tissues as well as plant fitness and invasion success.

Key words: defense costs; growth differentiation balance hypothesis; invasive plant; iridoid glycosides; *Linaria dalmatica*; nitrogen deposition; optimal defense hypothesis; resource allocation; root defense; secondary metabolites.

Nitrogen (N) is the nutrient required in greatest quantity by plants (Chapin et al., 1987). Although most plants in temperate terrestrial ecosystems remain nitrogen-limited, nitrogen inputs from human activity have more than doubled since the early 1960s and continue to increase at a rapid rate (reviewed in Vitousek et al., 1997; Galloway et al., 2004). Atmospheric nitrogen deposition resulting from anthropogenic nitrogen oxide (NO_x) and ammonia (NH₃) emissions is leading to increased N availability at regional and global scales (Galloway et al., 2004). These N inputs are likely to have important effects on plant performance and resistance traits because soil nitrogen availability plays a key role in plant allocation to growth, reproduction, and defense (Coley et al., 1985; Bazzaz et al., 1987; Aerts and Chapin, 2000). In general, nitrogen fertilization increases plant growth and reproduction (Bazzaz et al., 1987; Aerts and Chapin, 2000), decreases concentrations of carbon-based secondary compounds (i.e., non-nitrogen-containing compounds such as phenolics and terpenoids), and increases nitrogenous compounds such as alkaloids (Koricheva et al., 1998).

However, compared with plant growth and reproduction, the effects of N fertilization on plant chemical defenses are less clear, with variable allelochemical responses within and among groups of compounds and plant species (reviewed in Koricheva et al., 1998; Throop and Lerda, 2004).

Although numerous studies have investigated the effects of nutrient fertilization on plant chemical defenses and allocation patterns, much less attention has been given to understanding the effects of nitrogen enrichment alone (i.e., without associated increases in phosphorus, potassium, or other nutrients) and within a range of increase that would be expected with current and projected nitrogen deposition (but see Throop and Lerda, 2004). Isolating the effects of nitrogen is especially important for predicting plant response to N deposition, because N enrichment can lead to other nutrient limitations (e.g., phosphorus) and ultimately affect plant allocation patterns (Gusewell, 2004; Vitousek et al., 2010). Additionally, many studies that address plant defenses focus on aboveground allocation patterns, often with little consideration of root defenses.

With increasing awareness of the effects of root-feeding herbivores and pathogens on plant fitness, there is growing interest in quantifying root defenses and understanding the relationship between above- and belowground defenses (reviewed in Van der Putten et al., 2001; Kaplan et al., 2008; Rasmann and Agrawal, 2008; van Dam, 2009). Several of these recent reviews have synthesized and compared above- and belowground defense allocation, but few consistent patterns have emerged. In general, constitutive defenses appear to be equally allocated between above- and belowground tissues (reviewed in Kaplan et al., 2008). Specifically, Kaplan et al. (2008) found that secondary metabolites were allocated proportionally to leaf and root tissues when examining data for all classes of chemical defenses

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(i.e., alkaloids, glucosinolates, phenolics, and terpenoids). However, when these classes were assessed individually, only phenolics and terpenoids were similarly distributed among leaves and roots. In addition to that study, other reviews indicate that there is considerable, unexplained variation in above- and belowground defense allocation (Rasmann and Agrawal, 2008; van Dam, 2009).

Several hypotheses have been proposed to explain patterns of variation in plant secondary metabolites, including the “growth–differentiation balance” (Loomis, 1932; Herms and Mattson, 1992) and “optimal defense” (McKey, 1974, 1979; Rhoades, 1979) hypotheses (reviewed in Stamp, 2003). A common assumption of these hypotheses is that resource allocation to defense imposes a fitness cost to plants and that the availability of limiting resources (e.g., nitrogen) influences these allocation costs, which can be demonstrated as tradeoffs (or negative correlations) between growth or reproduction and defense (Koricheva, 2002). However, the predicted relationship between resource availability and allocation costs as well as the predicted response of secondary metabolism to increased nutrient availability differ among these hypotheses. At the whole-plant level, the growth–differentiation balance (GDB) hypothesis predicts that concentrations of secondary metabolites will be greatest at intermediate levels of resource availability, based on the supposition that resources are preferentially allocated to growth under low- and high-resource conditions (Herms and Mattson, 1992). Additionally, this hypothesis presumes that costs of defense are reduced under moderate nutrient limitation because growth is more strongly limited than photosynthesis, resulting in excess carbon available for allocation to secondary metabolism. By contrast, the optimal defense (OD) hypothesis predicts that allocation to defense will increase, linearly, with increasing resource availability, because defense costs are greatest when nutrients are limiting (Rhoades, 1979). For within-plant defense allocation, the GDB hypothesis does not explicitly address variation in above- and belowground defense allocation, and the OD hypothesis proposes that tissues with higher fitness value and greater risk of attack will have the highest levels of defense.

We examined the effects of soil nitrogen enrichment on above- and belowground plant allocation patterns and allocation tradeoffs in the invasive species Dalmatian toadflax, *Linararia dalmatica* (L.) P. Mill. (Plantaginaceae). This species is a perennial plant native to Eurasia, and its success as an invader has been attributed to several traits, including high seed production, rhizomatous growth habit, and chemical defenses (Vujnovic and Wein, 1997; Wilson et al., 2005; Cappuccino and Arnason, 2006; Jamieson and Bowers, 2010). Given that *L. dalmatica* is a perennial species that reproduces sexually and vegetatively, both flower and root tissues should have high fitness values and levels of chemical defense. However, the relative importance of sexual and vegetative reproduction in the population dynamics and spread of this species has not been investigated. Moreover, resource availability may alter the fitness value of these tissues. For example, Kaplan et al. (2008) suggested that roots might become more valuable when water and nutrients are limiting. We conducted a greenhouse experiment to address two primary questions. (1) How does soil nitrogen enrichment influence whole-plant allocation to, and tradeoffs among, growth, sexual reproductive potential, and constitutive chemical defenses? (2) What are the effects of increased nitrogen availability on within-plant biomass and defense allocation patterns? Finally, we evaluated general predictions (discussed above) of the GDB and OD hypotheses.

MATERIALS AND METHODS

Study system—*Linararia dalmatica* is invasive in the United States and Canada, demonstrating negative ecological and economic effects in its introduced habitats (Vujnovic and Wein, 1997; Duncan et al., 2004; Wilson et al., 2005). This species produces iridoid glycosides (Handjieva et al., 1993; Franzyk et al., 1999; Jamieson and Bowers, 2010), a group of terpenoid compounds found in >50 plant families (Boros and Stermitz, 1990; Boros and Stermitz, 1991). These secondary metabolites act as plant defenses (feeding deterrents and toxins) against various natural enemies, including herbivores and pathogens (Bowers, 1991; Marak et al., 2002; Biere et al., 2004; Beninger et al., 2008). In the United States, there are eight known species of intentionally or accidentally introduced specialist insects that use *L. dalmatica* as a host plant. These exotic insects include species that specialize on different plant parts, including flowers, seed capsules, leaves, roots, and stems (for species descriptions, see Wilson et al., 2005).

Iridoid glycosides, in particular antirrhinoside and linarioside, comprise a significant portion of *L. dalmatica* biomass, averaging 6–12% dry mass early in the growing season, with concentrations exceeding 15–20% in some plant tissues (Jamieson and Bowers, 2010). Antirrhinoside is phloem mobile and can be transported from source tissues, such as leaves, to other plant tissues (Gowan et al., 1995; Voitsekhojskaja et al., 2006; Beninger et al., 2007). This compound has been shown to reduce the growth of gypsy moth larvae (*Lycmantria dispar* L.) at concentrations as low as 3.3% (Beninger et al., 2008). Iridoid glycosides can be induced in response to above- and belowground herbivore and pathogen damage (Darrow and Bowers, 1999; Marak et al., 2002; Wurst et al., 2008), and the potential for chemical induction adds a layer of complexity in understanding defense allocation (Bezemer and van Dam, 2005). However, induction of foliar iridoid glycosides is not always observed after plant damage (e.g., Jarzowski et al., 2000; Fontana et al., 2009), and it is unknown whether antirrhinoside and linarioside are inducible. In the present experiment, plants were not subjected to herbivore or pathogen damage, and the focus of the study is on constitutive levels of antirrhinoside and linarioside combined.

Greenhouse experimental design—Plants were grown from seeds collected from three populations in Boulder County, Colorado, USA: Lefthand Canyon (40°7'14"N, 105°19'26"W), Hall Ranch (40°12'42"N, 105°17'20"W), and Rabbit Mountain (40°14'13"N, 105°12'53"W). Seeds were collected from many plants (>15 per population) and were mixed together in an effort to select a random sampling of offspring from many genetic families. On 15 May 2007, seeds were sown in flats filled with potting soil (Fafard custom mix; peat with vermiculite and no added nutrients; manufactured by Conrad Fafard, Agawam, Massachusetts, USA). Seedlings were transplanted in mid-June into 15 × 40 cm pots containing a sterile soil medium composed of equal parts Fafard custom mix, triple-washed play sand, and calcined clay (Turface MVP, Turface Athletics, Buffalo Grove, Illinois, USA).

These seedlings were then randomly assigned to one of three N treatments: 2, 6, and 12 g N/m²/yr ($n = 20$ plants per treatment). Nitrogen treatments were added weekly in a 200-mL aqueous NH₄NO₃ solution and were continued for 10 wk to yield the total above rates. Additionally, we provided plants with 200 mL of a complete (excluding N) nutrient solution (quarter-strength Hoagland's minus N; for description, see Logan et al., 1999) every 2 wk to avoid other nutrient limitations, to focus on the effects of N addition alone. Burke et al. (1997) estimated N mineralization rates of 1.5–10 g N/m²/yr in the central United States, and 4–6 g N/m²/yr along the Colorado Front Range. In the western United States, the highest known deposition levels in wildland areas are 2.5–4.5 g N/m²/yr, and deposition rates downwind of urban and agricultural areas can be as high as 3–9 g N/m²/yr (Fenn et al., 2003). Thus, our midlevel N treatment (6 g) represents the approximate nitrogen level that plants would experience growing in the field. The low-N treatment (2 g) represents an N-limited condition, and the high-N treatment (12 g) simulates an elevated N-deposition environment.

After 10 wk of treatments, we counted the number of flowers (to serve as an estimate of sexual reproductive potential) and then harvested above- and belowground biomass. We did not evaluate asexual reproduction because no new ramets were observed developing from lateral roots or vegetative propagation, nor did we observe adventitious buds on roots that would be indicative of asexual reproduction. Roots were gently washed with water to separate them from the potting medium. Immediately after harvest, plant tissues were oven dried at 50°C for 36–48 h and weighed by plant part (flowers, shoots, roots).

Chemical analyses—We quantified iridoid glycosides in flower, shoot (leaves + stems), and root tissues for each experimental plant. Plant tissues

were ground separately into a fine powder. A 25–30 mg sample was weighed to the nearest 0.01 mg and then extracted in 5 mL of methanol for ~24 h. We filtered the extract, evaporated to dryness, added 1 mL of an internal standard (phenyl- β -D-glucopyranoside), and then partitioned the sample between water and ether to remove hydrophobic compounds (from the ether layer). An aliquot of the prepared sample 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, Santa Clara, California, USA). Iridoid glycoside (antirrhinose + linarioside) data were processed with HP ChemStation software (version A.03.34). Additional details for iridoid glycoside extraction and analysis by GC are provided elsewhere (Gardner and Stermitz, 1988; Bowers and Stamp, 1993; Jamieson and Bowers, 2010). For each plant sample, we first calculated iridoid glycoside (IG) concentration (% dry mass for each tissue type by taking the GC-quantified IG amount (mg) divided by the extracted tissue sample mass (mg). Within-plant IG content was calculated by taking IG concentration and multiplying that by the biomass of respective plant tissues. Whole-plant IG content represents the sum of these values, and whole-plant IG concentration was calculated by dividing whole-plant content by total plant biomass.

Statistical analyses—All statistical analyses were performed in JMP Pro version 9.0.2 (SAS Institute, Cary, North Carolina, USA). Data were transformed as indicated below to satisfy model assumptions of normality. To assess whole-plant allocation patterns, we used analysis of variance (ANOVA) to examine effects of N treatment on overall plant growth (total biomass), sexual reproductive potential (square-root transformed flower number), and constitutive defenses (arcsine square-root transformed iridoid glycoside concentrations). To test for whole-plant allocation tradeoffs, we conducted nonparametric correlation analyses (using Spearman's correlation coefficient: ρ or r_s) on untransformed data to evaluate relationships among plant biomass, flower production, and iridoid glycoside content within each nitrogen treatment level. We used nonparametric correlation analyses because they are robust to outliers (and violations of normality) and are advantageous over corresponding parametric correlation measures (Pearson product-moment correlation coefficient), which only describe the linear part of the relationship between two variables (Potvin and Roff, 1993). Additionally, for these whole-plant allocation tradeoff analyses, we used iridoid glycoside content because plants produce total quantities of compounds, and concentration data may lead to spurious negative correlations given that biomass is the denominator of this ratio value (Koricheva, 1999).

For within-plant allocation patterns, we used multivariate analyses of variance (MANOVA) to examine the effects of N treatment on above- and below-ground (1) biomass partitioning and (2) iridoid glycoside concentrations (arcsine square-root transformed). These analyses allowed us to examine the effects of N treatment on overall responses of flowers, shoots, and roots. The statistical models included N treatment, plant part, and a treatment \times plant part interaction term (F statistics are reported for Wilks's λ values). After determining whether overall responses were significant, we followed up with univariate ANOVAs to individually assess N treatment effects for flowers, shoots, and roots. For significant ANOVAs, post hoc Tukey's HSD tests were used for pairwise mean comparisons. Type III sums of squares were used for interpreting results of all MANOVA and ANOVA models. Finally, we also used nonparametric correlation analyses to evaluate relationships among iridoid glycoside concentrations of different plant tissues.

Graphical vector analysis—Because iridoid glycoside concentration and content are influenced by plant biomass, it is difficult to disentangle the effect of changing biomass on plant defenses. For example, changes in iridoid glycoside concentration may be due to altered allelochemical content (increased or decreased defense allocation) or altered growth and biomass (concentration or dilution effects). Such causal relationships are impossible to detect with phenotypic correlations alone. However, graphical vector analysis (GVA) has been recommended as a method for interpreting phenotypic variation in plant allelochemical concentrations and content (Koricheva, 1999). GVA allows for comparison of relative changes (which are calculated on the basis of a control group or reference treatment) in allelochemical content (x -axis), concentration (y -axis), and biomass (z -axis), where concentration = content/biomass. For our analyses, values for the midlevel N treatment (6 g N) were used as the reference point for calculating relative changes (biomass, iridoid concentration, and content) in the low- and high-N treatment groups.

In GVA, allelochemical and biomass changes are interpreted by examining the direction and length of vectors that extend beyond the reference point. Simultaneous examination of all three parameters allows for an interpretation of response. For example, if the relative change in biomass is greater than the rela-

tive change in allelochemical content, variation in allelochemical concentration is likely due to a concentration or dilution effect resulting from decreased or increased plant growth. By contrast, if relative changes in content (and concentration) are stronger than relative changes in biomass, we can assume that allelochemical variation is due to a change in the production or translocation of these secondary metabolites. Vertical and horizontal shifts indicate no change in content or concentration, respectively. We used GVA to help understand mechanisms that underlie shifts in iridoid glycoside concentrations. It is important to note that these analyses do not include an estimate of error, and they are illustrative rather than quantitative. Thus, we focused on interpreting vector shifts for allelochemical changes that were statistically significant, as determined by MANOVA and ANOVA. Methods for the application and interpretation of GVA in plant nutrient and allelochemical analyses are described in Haase and Rose (1995) and Koricheva (1999).

RESULTS

Whole-plant allocation patterns—Soil nitrogen enrichment had an overall positive effect on plant biomass ($F_{2,56} = 21.368$, $P < 0.0001$), flower number ($F_{2,56} = 11.077$, $P < 0.0001$), iridoid glycoside concentration ($F_{2,56} = 16.531$, $P < 0.0001$), and iridoid glycoside content ($F_{2,56} = 18.846$; $P < 0.0001$) (Table 1). Evidence of tradeoffs between resource allocation to growth and defense was mixed (Table 2A). Specifically, across nitrogen treatments, total biomass (growth) and iridoid glycoside content (defense) were positively correlated, demonstrating that larger plants produce higher overall levels of iridoid glycosides, regardless of nitrogen availability (Table 2A). However, analyses examining tradeoffs between defense and reproduction revealed evidence of fitness costs of defense (Table 2A) and showed a significant negative correlation between iridoid glycoside content (defense) and flower number (reproduction) for plants in the low-N treatment. Finally, although there was no evidence of tradeoffs between growth and reproduction, nitrogen availability affected the relationship between these response variables. Notably, for plants growing under high-N conditions, there was a significant positive correlation between biomass and flower number, but no significant relationship in the low- and midlevel-N treatments.

Within-plant allocation patterns—MANOVA of biomass allocation patterns demonstrated significant overall effects of N treatment ($F_{2,56} = 21.37$, $P < 0.0001$), plant part ($F_{2,55} = 419.44$, $P < 0.0001$), and a treatment \times plant part interaction ($F_{4,110} = 20.37$, $P < 0.0001$), indicating that the plant tissues (flowers, shoots, and roots) responded differently to the nitrogen treatments. Subsequent univariate ANOVA results indicated that N enrichment significantly increased biomass of flowers ($F_{2,56} = 13.15$, $P < 0.0001$), shoots ($F_{2,56} = 50.87$, $P < 0.0001$), and roots ($F_{2,56} = 7.24$, $P = 0.0016$) (Fig. 1A). For flowers and shoots, there were significant increases in mean biomass with each level of N enrichment. For roots, there was an increasing trend across treatments; however, mean root biomass values were significantly different only between the low- and high-N treatment groups.

For iridoid glycoside concentrations, MANOVA revealed significant effects of N treatment ($F_{2,56} = 8.60$, $P = 0.0006$), plant part ($F_{2,55} = 7.40$, $P = 0.0014$), and, perhaps most interesting, a treatment \times plant part interaction ($F_{4,110} = 10.78$, $P < 0.0001$), indicating variable allelochemical responses to N enrichment. In the low-N treatment, flower iridoid glycoside concentrations were ~3 times higher than those of shoots and ~9 times higher than those of roots (Fig. 1B), and there were no significant correlations among these defenses (Table 2B). In the

TABLE 1. Effects of soil nitrogen (N) enrichment on *Linaria dalmatica* growth, flower production, and whole-plant iridoid glycoside (IG) levels. Mean differences identified by Tukey's HSD post hoc analyses are indicated by different letters ($P < 0.05$).

| Response variable | N treatment (means ± SE) | | |
|-------------------------------|------------------------------------|------------------------------------|-------------------------------------|
| | 2 g/m ² /yr (n = 19) | 6 g/m ² /yr (n = 20) | 12 g/m ² /yr (n = 20) |
| Total biomass (g) | 15.0 ± 1.7 a | 26.1 ± 2.0 b | 35.9 ± 2.9 c |
| Flower number | 24.4 ± 5.3 a | 43.3 ± 7.9 a | 96.2 ± 16.6 b |
| IG concentration (% dry mass) | 2.3 ± 0.3 a | 5.8 ± 0.5 b | 4.9 ± 0.6 b |
| IG content (g) | 0.34 ± 0.05 a | 1.63 ± 0.21 b | 1.92 ± 0.25 b |

midlevel-N treatment, mean flower iridoid glycosides were nearly twice those of shoot and root defenses (Fig. 1B). At this level of N availability, shoot iridoid glycoside concentrations were positively related to concentrations found in roots and flowers (Table 2B). In the high-N treatment, mean flower, shoot, and root iridoid glycoside concentrations were similar (Fig. 1B). Yet only root and flower iridoid glycoside concentrations demonstrated a significant positive relationship (Table 2B).

Across all experimental treatments, overall (mean ± SE) iridoid glycoside concentrations were 8.1 ± 0.5% for flowers, 4.1 ± 0.4% for shoots, and 4.5 ± 0.5% for roots. Univariate ANOVAs revealed that nitrogen treatment had significant effects on iridoid glycoside concentrations for flowers ($F_{2,50} = 6.33, P = 0.0035$) and roots ($F_{2,56} = 31.69, P < 0.0001$), but not for shoots ($F_{2,56} = 1.58, P = 0.2140$; Fig. 1B). For flowers, there was a significant decrease in iridoid glycoside concentrations from the low- and midlevel-N treatments to the high-N treatment (Fig. 1B). For roots, there was a significant increase in iridoid glycoside concentrations from the low-N treatment to the midlevel- and high-N treatments (Fig. 1B).

A conceptual model for interpreting GVA results is shown in Figure 2A. Our results (Fig. 2B) indicated that the effects of N treatment on iridoid glycoside concentrations were likely caused by changes in secondary metabolite production and allocation as well as biomass concentration–dilution effects. Specifically, nitrogen limitation, as seen in the low-N (2 g) treatment, had a strong negative effect on root and whole-plant iridoid glycosides but not on flower or shoot iridoid glycosides. Furthermore, relative changes in iridoid glycoside concentrations and content were greater than changes in biomass for roots and at the whole-plant level. Thus, N enrichment from the 2-g to 6-g treatments apparently increased iridoid glycoside concentrations, primarily because of changes in allocation to

defense rather than biomass. In contrast, the change in flower iridoid glycoside content was small in relation to change in biomass, which suggests that the observed significant decrease in flower defense concentrations under high N availability was most likely caused by dilution effects (biomass accumulation—that is, the production of larger and/or more flowers without corresponding increases in allocation to floral iridoid glycosides).

DISCUSSION

Whole-plant allocation patterns—We found that N enrichment had a positive effect on both plant performance and defense traits—in particular, growth, flower production, and whole-plant iridoid glycosides (although iridoid glycoside responses varied among plant parts; see below). Flower production and iridoid glycoside content demonstrated especially strong responses, with a fourfold increase in flower number and nearly sixfold increase in total iridoid glycoside content from the low- to high-N treatment. For both total biomass and whole-plant iridoid glycoside concentrations, there was an approximate twofold increase. A prior field experiment showed that N addition at a rate of 15 g N/m²/yr doubled the biomass and seed capsule number of *L. dalmatica* plants, whereas biomass production of the surrounding vegetation increased by only 20% in fertilized plots (Jamieson et al., 2012). In combination with those findings, results demonstrated here suggest that nitrogen enrichment could influence the invasiveness of *L. dalmatica* by enhancing both plant performance (growth and reproduction) and chemical defenses (iridoid glycoside content and concentrations), which provides support for the link between increased nitrogen availability and plant invasions that has been made in

TABLE 2. Summary of correlation analyses examining (A) whole-plant allocation tradeoffs and (B) within-plant iridoid glycoside concentrations (IG) for *Linaria dalmatica* plants growing under three levels of nitrogen availability. Whole-plant allocation tradeoffs were examined by testing relationships among total biomass (growth), number of flowers produced (reproduction), and total iridoid glycoside content (defense). Reported values are nonparametric Spearman correlation coefficients (r_s) and statistical significance (P , bold = significant).

| | Nitrogen treatment | | | | | |
|--|--------------------|------------------|----------------|------------------|-----------------|------------------|
| | 2 g N (n = 19) | | 6 g N (n = 20) | | 12 g N (n = 20) | |
| | r_s | P | r_s | P | r_s | P |
| (A) Whole-plant allocation | | | | | | |
| Growth vs. defense | 0.711 | <0.001 | 0.878 | <0.001 | 0.735 | <0.001 |
| Reproduction vs. defense | -0.494 | 0.032 | -0.313 | 0.179 | 0.405 | 0.076 |
| Growth vs. reproduction | -0.274 | 0.257 | -0.209 | 0.376 | 0.443 | 0.050 |
| (B) Within-plant defense allocation | | | | | | |
| Shoot [IG] vs. root [IG] | 0.084 | 0.550 | 0.474 | 0.035 | 0.229 | 0.332 |
| Shoot [IG] vs. flower [IG] | 0.168 | 0.732 | 0.513 | 0.030 | 0.373 | 0.105 |
| Root [IG] vs. flower [IG] | -0.400 | 0.140 | 0.416 | 0.086 | 0.632 | 0.003 |

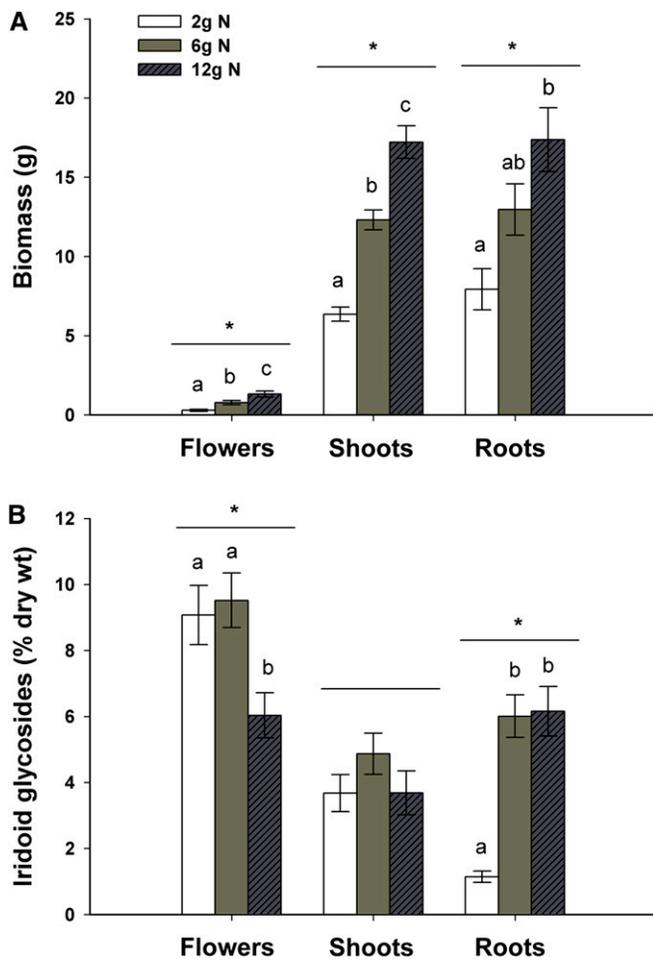


Fig. 1. Effects of soil nitrogen (N) enrichment on *Linaria dalmatICA* within-plant allocation patterns to (A) growth and reproduction (flower, shoot, and root biomass) and (B) chemical defenses (iridoid glycoside concentrations). Data are means \pm SE. An asterisk indicates significant within-plant part differences ($P < 0.05$), with letters used to distinguish pairwise mean differences as determined by post hoc Tukey's HSD tests. These analyses were performed separately for plant parts (flowers, shoots, roots), and lines above bars representing means are used to illustrate groupings for post hoc statistical tests.

other studies (e.g., Daehler, 2003; Blumenthal, 2006). However, further research is necessary to characterize the role of quantitative variation in chemical defenses and plant resistance to herbivores and pathogens for this invasive species.

Our finding of increased iridoid glycoside concentrations with elevated soil nitrogen is consistent with predictions of the OD hypothesis but not with those of the GDB hypothesis, which predicts that allocation to defense will decrease under high resource availability, because growth processes are favored under these conditions (reviewed in Stamp, 2003). However, four to five resource levels are recommended for adequately testing predictions of the growth differentiation balance hypothesis (Stamp, 2003, and references therein). Moreover, results presented here are in contrast to those of a previous greenhouse experiment, which showed a negative relationship between nitrogen availability and iridoid glycoside concentrations of *L. dalmatICA* growing under four N levels, ranging from zero to 8 g/m² (Jamieson and Bowers, 2012b), but those results did not fully support predictions of the

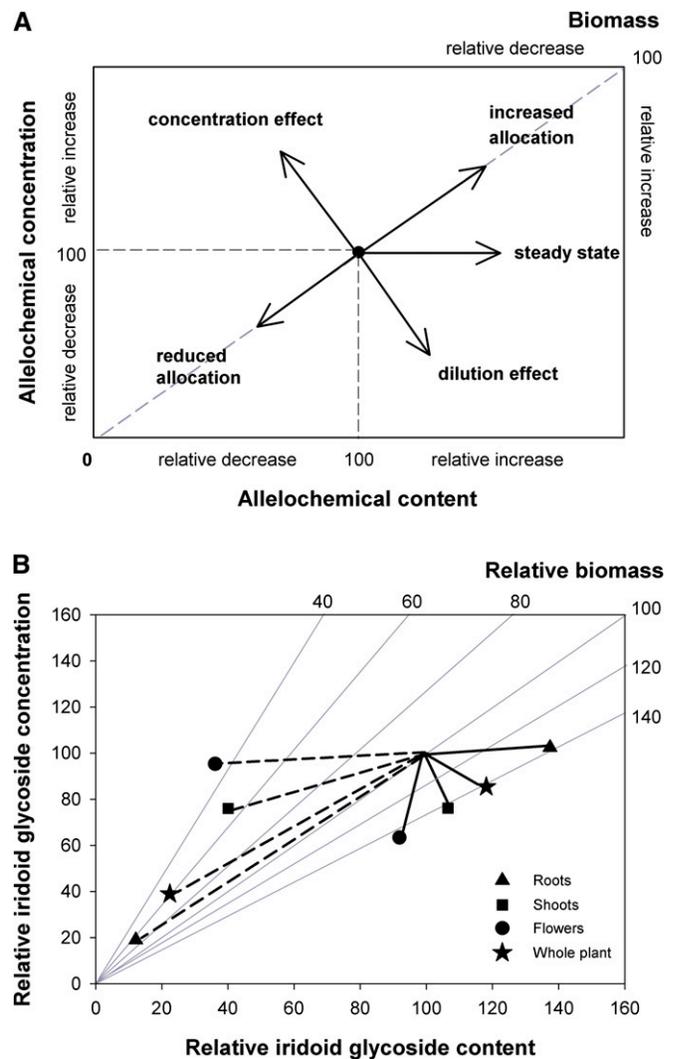


Fig. 2. Graphical vector analysis (GVA). (A) Conceptual model illustrating the use of GVA to interpret changes in plant chemical defenses. Changes in allelochemical content and concentration (x- and y-axes, respectively) and biomass (z-axis) are compared on a relative basis to a standardized reference point (with mean values for the control group normalized to 100). Model adapted from Koricheva (1999). (B) Experimental results demonstrating relative changes in *Linaria dalmatICA* biomass and chemical defenses in response to soil nitrogen (N) availability. Plants were grown under one of three N treatments: 2, 6, or 12 g N/m²/yr. Treatment mean values were compared on a relative basis with those of plants grown under the middle N-supply level (6 g). Dashed lines are relative responses for the low-N (2 g) treatment, and solid lines are relative responses for the high-N (12 g) treatment. Symbols are vector endpoints, where values >100 indicate a positive change and values <100 indicate a negative change.

OD or GDB hypotheses, either. We propose that variation in plant ontogeny may be one factor contributing to such observed differences in the relationship between nitrogen availability and chemical defense concentrations. Plant ontogeny plays an important role in defense allocation and allocation tradeoffs (Boege and Marquis, 2005; Barton and Koricheva, 2010) and may lead to nonlinear allelochemical changes as well as transient costs of defense (e.g., Orians et al., 2010; Quintero and Bowers, 2012). However, predictions arising from the OD or GDB hypotheses do not adequately address the influence of ontogeny.

In general, negative phenotypic correlations between growth and defense are as common as negative correlations between reproduction and defense (Koricheva, 2002). However, in the present study, we found no evidence of tradeoffs between growth and defense, but some support for tradeoffs between reproduction and defense. Similarly, a study by Marak et al. (2003) showed no evidence of a growth-versus-defense tradeoff in *Plantago lanceolata* (Plantaginaceae), but plants selected for high iridoid glycoside levels produced fewer inflorescences with lower reproductive-tissue mass, indicating a tradeoff between reproduction and defense. A meta-analysis examining fitness costs of defense at low and high levels of nutrient availability indicated that costs were more likely to be detected under high-nutrient conditions (Koricheva, 2002). However, when comparisons were restricted to studies that simultaneously examined both low- and high-nutrient conditions, there were no differences between the two conditions. Our study demonstrates that the level of nutrient addition can greatly influence interpretation of observed results. For example, our conclusions about changes in whole-plant iridoid glycoside concentrations and fitness costs of defense would be entirely different if we had examined only two levels of N addition (e.g., 6 g and 12 g/m²). Variation in nutrient treatments, including differences in the types of nutrients altered, total amount of nutrients added, and rates of addition pose challenges for cross-study comparisons.

In addition to variation in nutrient availability, several factors, including the type of plant defense under consideration and how costs are measured, can affect the ability to detect fitness costs of defense (Koricheva, 2002; Strauss et al., 2002). Additionally, plasticity in reproductive strategies, in particular vegetative propagation, may influence allocation tradeoffs (Van Der Putten, 2003). In the present study, we are limited in our understanding of how N enrichment may influence the overall reproductive potential of *L. dalmatica*, because vegetative propagation could not be examined in our experiment. Finally, allocation tradeoffs are only one measure of fitness costs of defense, and plants can also experience other types of defense costs, such as indirect ecological costs (e.g., reduced tolerance or reduced competitive ability), which are expressed when plants interact with other species (Koricheva, 2002; Strauss et al., 2002). Ongoing research with the *L. dalmatica* study system will allow us to incorporate asexual reproduction into allocation tradeoff analyses and further explore other types of defense costs.

Within-plant defense allocation—Although whole-plant iridoid glycoside concentrations increased with N enrichment, our study demonstrated that within-plant defense allocation patterns differed across N treatments. Higher concentrations of flower iridoid glycosides compared with those found in shoot and root tissues is consistent with predictions of the OD hypothesis only if flowers are assumed to have a greater fitness value under low to moderate nutrient conditions. However, as originally proposed by Rhoades (1979), roots should have the highest fitness value, then shoots, and finally flowers, because the development of each successive tissue type depends on the previous one. In the present study, in particular in the midlevel- and high-N treatments, the distribution of shoot and root iridoid glycosides was consistent with the general pattern of equal allocation to above- and belowground defenses found by Kaplan et al. (2008), but these compounds were not equally distributed to shoots and roots in the low-N treatment. Correlation analyses revealed that nitrogen availability influenced relationships among flower, shoot, and root iridoid glycoside concentrations, and positive correlations

for tissue-specific defenses were observed only in the midlevel- and high-N treatment groups. Hence, differences in resource availability may influence some unexplained variation in above- and belowground defense allocation patterns found in cross-study comparisons (e.g., Kaplan et al., 2008). Furthermore, our results suggest that defense allocation tradeoffs among tissue types (e.g., flowers vs. roots) may be more common under nutrient-stress or nutrient-limiting conditions compared with moderate and high levels of resource availability.

The effects of N enrichment on iridoid glycoside concentrations evidently resulted from changes in allelochemical synthesis and partitioning as well as biomass concentration–dilution effects. The apparent dilution effect associated with increased biomass allocation to flowers and reduced iridoid glycoside concentrations (~35% decrease in the high-N treatment) provides some evidence that plants may allocate more resources to growth and reproduction over chemical defense under high resource availability, as suggested by the GDB hypothesis. In contrast to floral defenses, root iridoid glycoside concentrations increased by >400% from the low-N treatment to the midlevel- and high-N treatments, likely because of decreased allocation to root iridoid glycosides under low N availability. This pattern could reflect changing fitness values of root tissues related to shifts in reproductive strategy under different resource environments. For example, if clonal reproduction is favored when nutrients are abundant, root tissues may have a higher fitness value and, thus, greater level of defense, which would correspond with optimal defense. However, one problem with testing the OD hypothesis is that fitness values of tissues are rarely measured in plant chemical-defense studies (including the present study), which results in much speculation about which tissue types (flowers vs. roots, etc.) are most valuable for plant fitness. Alternatively, the observed changes in root defenses could be a consequence of allocation tradeoffs among plant parts (higher defense allocation to flowers at a cost to roots under low N availability) or potential tradeoffs and interactive effects with other *L. dalmatica* defenses that we did not examine, such as the alkaloid peganine, which is also found in root and shoot tissues (Gröger and John, 1965).

In other species that produce iridoid glycoside, root iridoid glycoside concentrations are often substantially lower than those found in shoot tissues (e.g., Quintero and Bowers, 2012). A comparative study by Beninger et al. (2009) indicated that plant reproductive strategy may influence defense allocation to roots. Results of that study showed high concentrations of antirrhinoside in roots of *L. vulgaris*, which also reproduces sexually and vegetatively, and much lower levels in *Antirrhinum majus*, which reproduces only through flower/seed production. The functional role of root iridoid glycosides requires further investigation, but research on *P. lanceolata* suggests that these compounds also play a defensive role belowground (Wurst et al., 2008; De Deyn et al., 2009). Belowground defenses may be especially important for understanding the invasion potential of some introduced plants (Van Der Putten, 2003, and references therein). Thus, additional research examining vegetative propagation, root defenses, and belowground herbivore and pathogen attack is needed for a full understanding of *L. dalmatica* invasion dynamics.

In conclusion, our results demonstrate that nitrogen enrichment can increase plant performance, reduce costs of defense, and differentially affect above- and belowground plant defenses. Such changes may have important consequences for ecological interactions between plants and their natural enemies

(e.g., see Throop, 2005; Zehnder and Hunter, 2008; Jamieson and Bowers, 2012a). Results presented here suggest that *L. dalmatica* root tissues may be more susceptible to herbivore and pathogen attack under N limitation and better defended under high-N conditions, and that the opposite would be true for flower tissues. Although the effects of nitrogen enrichment on growth and reproduction are relatively straightforward (Bazzaz et al., 1987; Vitousek et al., 1997), predicting the influence of resource availability on plant chemical defenses has proved a difficult challenge (Herms and Mattson, 1992; Koricheva et al., 1998; Hamilton et al., 2001). We propose that current plant-defense hypotheses and future studies examining the role of resource availability on plant chemical defenses would be improved by greater consideration of life-history strategies, both whole-plant and within-plant allocation tradeoffs, and relationships between above- and belowground defenses.

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