

Interactive Effects of Simulated Nitrogen Deposition and Altered Precipitation Patterns on Plant Allelochemical Concentrations

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Abstract Global environmental change alters the supply of multiple limiting resources that regulate plant primary and secondary metabolism. Through modifications in resource availability, acquisition, and allocation, global change is likely to influence plant chemical defenses, and consequently species interactions that are mediated by these compounds. While many studies focus on individual global change factors, simultaneous changes in abiotic factors may interact to influence plant allelochemicals. In this study, we examined the individual and interactive effects of nitrogen enrichment and altered precipitation patterns on chemical defense compounds (iridoid glycosides) of an invasive plant, *Linaria dalmatica*. Plants were grown from seed in native mixed-grass prairie for 2 years. Nitrogen and water treatments were applied in each growing season over this period. Results indicate that soil water and nitrogen availability interact to shape plant chemical defense concentrations in *L. dalmatica*. Nitrogen addition decreased iridoid glycoside concentrations by approximately 25 % under reduced water availability, increased concentrations by 37 % in ambient water plots, and had no effect on these chemical defenses for plants growing under augmented water supply. Thus, results show differing patterns of allelochemical response to nitrogen enrichment, with

respect to both the magnitude and direction of change, depending on water availability. Our study demonstrates the importance of examining multiple environmental factors in order to predict potential changes in plant chemical defenses with climate change.

Keywords Invasive plant · Iridoid glycosides · Climate change · *Linaria dalmatica* (Dalmatian toadflax) · Nitrogen deposition · Water availability · Chemical defense · Antirrhinoid · Linarioside

Introduction

Global environmental change, including elevated atmospheric carbon dioxide and ozone, soil nitrogen enrichment, climate warming, and altered precipitation regimes can influence plant resource allocation and secondary metabolism, thereby modifying allelochemical concentrations and plant quality for herbivores (Throop and Lerdau 2004; Bidart-Bouzat and Imeh-Nathaniel 2008). Global change drivers do not occur in isolation, and may combine to result in additive, antagonistic, or synergistic effects. Understanding how these environmental changes interact to shape plant secondary metabolism and allelochemical concentrations is an important challenge for chemical ecologists, as these compounds play an essential role in plant defense and species interactions. Yet, our knowledge of interactive effects is limited.

Anthropogenic nitrogen inputs have increased substantially over the last several decades, resulting in ecological consequences for plant-insect interactions (Throop and Lerdau 2004). Nitrogen is a critical element regulating metabolic processes, growth, reproduction, and survival in plants and insects. Additionally, water is a key limiting resource for

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plants, in particular for dryland ecosystems, such as grassland habitats, which are predicted to experience greater desiccation and drought conditions with climate warming (e.g., Morgan et al. 2011). Plant water stress can alter plant-insect interactions, leading to both adverse and positive effects on insect herbivores, depending on the feeding guild or species under consideration (Huberty and Denno 2004). Global change may influence water availability in multiple ways, making it difficult to predict net effects on plants and insects. For example, while drought frequency and intensity are expected to increase with climate change, elevated atmospheric CO₂ can increase plant water use efficiency and therefore soil water availability (Morgan et al. 2011).

Biological invasions also represent an important driver of environmental change. For some introduced plants, including our study species, *Linaria dalmatica* (Plantaginaceae), chemical defenses are thought to be important traits contributing to their invasion success and ecological impact (Jamieson and Bowers 2010). Increases in resource availability, such as soil nitrogen, can facilitate plant invasions (Blumenthal 2009), and may alter the chemical defenses of these plants (Throop and Lerda 2004; Jamieson and Bowers 2012). Nitrogen enrichment can result in variable phytochemical responses, including increases, decreases, and no change in allelochemical concentrations (Koricheva et al. 1998; Throop and Lerda 2004).

Differential plant allelochemical response to nitrogen enrichment may be due to changes in other abiotic factors. In particular, water and nitrogen availability may interactively influence plant defensive chemistry, in turn modifying plant quality for and resistance to herbivores (Herms and Mattson 1992; Huberty and Denno 2004). These two plant resources are linked, as water availability can influence nitrogen supply; for example, low soil moisture can lead to poor nutrient uptake by roots. In this study, we examined the independent and interactive effects of water and nitrogen availability on iridoid glycoside (IG) content and concentration in *L. dalmatica*, which is a widespread invasive species in North America. Iridoid glycosides are a group of carbon-based terpenoid

compounds that act as defense compounds against insect herbivores and pathogens (Jamieson and Bowers 2010, references therein).

Methods and Materials

This study was conducted at the USDA-ARS High Plains Grasslands Research Station, west of Cheyenne, Wyoming, USA (41°N, 104°W) and was part of a larger global change experiment (see Blumenthal 2009 for further experimental design and study site details). For the work presented here, *L. dalmatica* plants were grown from seed (sown November 2003) in a native mixed-grass prairie under three levels of summer water availability (ambient, decreased, and increased) and two levels of nitrogen availability (ambient, increased). Ambient water and ambient nitrogen plots represent the experimental control group.

Treatments were arranged in a blocked (3, 2) split-plot design with two blocks, each containing one replicate of all factorial treatment combinations (a third block present within the larger experiment could not be used, due to the presence of older, pre-existing *L. dalmatica* plants within the harvest area). Nitrogen treatments were randomly assigned to subplots within water treatment whole plots. Subplots ($N=12$) measured approximately 3×1 m and nitrogen treatments were separated by at least 0.65 m. At the whole plot level, there was a 1 m buffer surrounding each plot. Data summarizing soil volumetric water content, *L. dalmatica* establishment success (seedling numbers over time), and plant productivity for the larger experimental project are presented in Blumenthal (2009) and references therein.

For the increased water treatment, plots were irrigated by hand to simulate a 50 % increase in precipitation compared to the local 30-year average between mid-May and mid-August. Specifically, we divided the 30-year average precipitation for each month by two, and applied ¼ of that amount 4 times per month. The decreased water treatment involved an approximate 50 % reduction in summer (mid-May through mid-August) precipitation using clear polyvinyl chloride (PVC)

Table 1 Mean (± 1 SE) plant number, aboveground biomass (mg), and carbon/nitrogen ratio (C:N) of *Linaria dalmatica* plants for each factorial combination of nitrogen treatment (ambient N, + N) and water treatment

	Ambient N			+ N			ANOVA		
	Ambient H ₂ O	- H ₂ O	+ H ₂ O	Ambient H ₂ O	- H ₂ O	+ H ₂ O	Nitrogen	Water	N × H ₂ O
Plant #	18.5±2.5	24±20	56±24	44.5±3.5	29±19	158±30	465.5 (1,3) ***	13.5 (2,2) *	205.6 (2,3) ***
Biomass	147.0±49.9	63.9±5.1	154.7±47.1	423.6±240.9	97.4±56.6	321.5±20.9	5.1 (1,3) NS	1.4 (2,2) NS	1.0 (2,3) NS
C:N	54.0±0.9	53.7±3.8	53.7±4.6	47.0±3.5	42.9±2.3	47.8±4.1	5.8 (1,3) *	0.9 (2,2) NS	0.21 (2,3) NS

(ambient H₂O, - H₂O, + H₂O) treatment in a blocked, split-plot experiment ($N=2$ subplots per N × H₂O treatment combination or 12 total subplots)

ANOVA results show F statistics with degrees of freedom in parentheses. Significant effects at $\alpha=0.1$ are in bold (* $P\leq 0.1$, ** $P\leq 0.01$, *** $P\leq 0.001$, NS non-significant)

rainout shelters (3.5×2.5 m), which covered entire plots and were angled to direct water into attached gutters that discharged water >1 m away from plots. This type of shelter can slightly reduce light availability (<10 %); however, in this ecosystem, belowground resources (i.e., water and nitrogen) rather than light availability are the key limiting resources for growth. Shelters had little effect on soil temperature, and effectively altered soil water content (Blumenthal 2009, references therein).

Increased nitrogen plots were treated with ammonium nitrate fertilizer twice per year (in April and August 2004 and May and July 2005), for a total of 2.63 g m⁻² N yr⁻¹, which simulated rates comparable to the highest levels of N deposition observed in mixed-grass prairies in the Northern Great Plains. After two growing seasons, plants were counted, and aboveground biomass was harvested (late July through early August 2005). Plant tissues (total aboveground biomass) from each subplot were dried at 60 °C for 3 days, weighed, and ground into a fine powder using a cyclone mill. That is, individual plants were pooled within each subplot ($N=2$ replicates per water × nitrogen treatment) prior to grinding. Across treatments, subplots ($N=12$ total) contained an average of 55 plants (SE=15.5; range=4–188; see Table 1 for mean number of plants and total biomass per treatment combination). To assess the efficacy of nitrogen treatments on plant nitrogen uptake, we examined the carbon-nitrogen ratio (C:N) of plant tissues. Carbon and nitrogen percent dry weights were determined using a Thermo Finnigan Flash EA 1112 elemental analyzer (ThermoFisher Scientific).

To examine the effects of water and nitrogen availability on *L. dalmatICA* chemical defenses, gas chromatography was used to quantify iridoid glycoside concentrations of homogenized plant tissues from each subplot. For each replicate (subplot), an approximate 25–30 mg sample was weighed to the nearest 0.01 mg, extracted overnight in methanol, filtered, and then partitioned between water and ether to remove hydrophobic compounds. Phenyl-β-D-glucose (PBG) was used as an internal standard. An aliquot of each sample was derivatized with Tri-Sil-Z™ (Pierce Chemical Company) and injected into a Hewlett Packard 5890 gas chromatograph (Agilent Technology) with an Agilent DB-1 column (30 m, 0.320 mm I.D., 0.25 μm film thickness). Further details for iridoid glycoside analyses by gas chromatography have been described previously (Jamieson and Bowers 2010, references therein). Amounts of iridoid glycosides (i.e., sum of two major compounds, antirrhinoside and linarioside, which represent >95 % of the total iridoid glycoside content) were quantified using ChemStation A.03.34 software. Purified standards of antirrhinoside and linarioside were provided by S.R. Jensen (Department of Chemistry, Technical University of Denmark, Lyngby, Denmark). Iridoid glycoside (IG) concentrations were calculated by dividing these amounts by the extracted tissue sample weights to yield proportions per mg of tissue.

For each subplot, we also estimated mean plant-level iridoid glycoside content (mg) by multiplying iridoid glycoside concentration by mean per plant biomass (mg).

Treatment effects on plant number, plant biomass, carbon/nitrogen ratio, IG content, and IG concentration were examined using general linear mixed models with restricted maximum likelihood (REML) methods in JMP version 9.2 (SAS Institute, Inc., Cary, NC, USA). Statistical models included water (whole-plot factor), nitrogen (subplot factor), and water × nitrogen as fixed effects in addition to block and block × water as random effects to account for the split-plot design. Iridoid glycoside concentrations (proportional data) were arcsine squareroot transformed to meet model assumptions of normality, however concentrations are presented as mean % dry weight in text and figures for illustrative purposes and ease of interpretation. *Post hoc* analyses were run for significant statistical models to examine pairwise mean differences. To balance between potential Type I and II errors, we evaluated our results at a significance level of $\alpha=0.10$, as recommended by Filion et al. (2000) for comparable global change

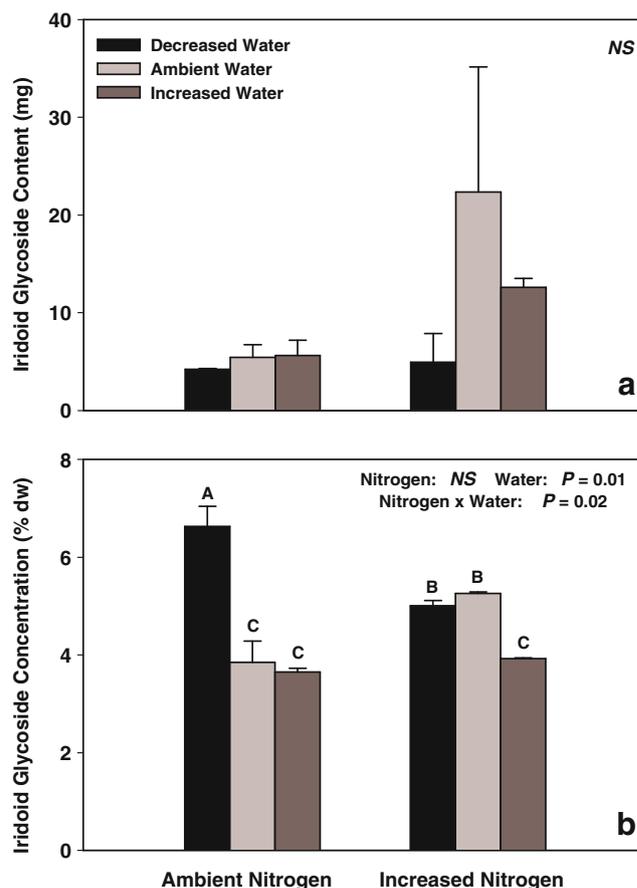


Fig. 1 Effects of water (H₂O) and nitrogen (N) availability on *Linaria dalmatICA* iridoid glycoside **a**) content and **b**) concentration. Bars are means ± 1 SE ($N=2$ subplots per N × H₂O treatment combination). Letters above bars indicate significant differences (*Post hoc* Tukey HSD contrasts). To balance between potential Type I and II errors, results were evaluated at $\alpha=0.10$

experiments, which have low replication and similar split-plot experimental designs.

Results and Discussion

Relative to control plots (ambient N, ambient H₂O), increasing water and nitrogen availability positively influenced *L. dalmanica* establishment, whereas the number of plants decreased under reduced water and nitrogen treatments (Table 1). The significant N × H₂O treatment effect reflects an apparent synergistic effect of augmented water and nitrogen on plant establishment. Across water treatments, soil nitrogen enrichment led to greater plant nitrogen uptake, as indicated by the lower carbon-to-nitrogen (C:N) ratio of plant tissues (Table 1).

Water and nitrogen treatments had no significant effect on mean plant biomass or mean iridoid glycoside content (Table 1; Fig. 1a). However, there were significant water and water by nitrogen interaction effects on iridoid glycoside (IG) concentrations (Fig. 1b). In particular, increased water availability decreased IG concentrations on average, but this effect was less apparent with nitrogen addition. There was an approximate 35 % reduction in IG concentrations in plants from plots with added water compared to plants growing under decreased water availability. The effect of nitrogen on IG concentrations varied in both degree and direction of response with water level, leading to no main effect of nitrogen treatment.

Mean concentrations of iridoid glycosides were highest at 6.6 % (± 0.41 SE) in plants grown in decreased precipitation plots, without added nitrogen. This finding is consistent with predictions based on the growth-differentiation balance hypothesis, which suggests secondary metabolite concentrations may increase under resource limiting conditions, in particular, when growth processes are more strongly limited compared to photosynthesis (Herms and Mattson 1992). Under decreased water availability, simulated nitrogen deposition reduced IG concentrations by approximately 25 % ($F_{1,3}=19.9$, $P=0.02$). The reduction in iridoid glycoside concentrations with nitrogen enrichment mirrors the pattern of response found in a previous study examining *L. dalmanica* plants growing under field and greenhouse conditions (Jamieson and Bowers 2012). In general, nitrogen fertilization tends to reduce foliar concentrations of carbon-based secondary compounds as a group, although results are mixed when examining terpenoid compounds (Koricheva et al. 1998).

In contrast to plants growing under decreased water availability, iridoid glycoside concentrations increased by approximately 37 % in *L. dalmanica* plants when nitrogen was added to plots with ambient water availability (Fig. 1b; *Post hoc* contrast: $F_{1,3}=19.6$, $P=0.02$). These results demonstrate that variation in soil water availability can influence plant allelochemical response to nitrogen enrichment, affecting not

only the magnitude of change in concentrations of chemical defenses, but also the direction of response. Mean iridoid glycoside concentrations were lowest at 3.6 % (± 0.08 SE) in plants growing under increased water and control nitrogen treatments (Fig. 1b). Simulated nitrogen deposition had no effect on allelochemical concentrations of plants growing under increased water availability. Our results suggest that plants may be allocating resources, in particular nitrogen, equally to growth (i.e., biomass) and defense (i.e., iridoid glycoside content) when water availability is not limiting, thus resulting in steady state allelochemical concentrations with increased nitrogen availability. Further studies are necessary to elucidate the mechanism underlying plant allelochemical response to altered water and nitrogen supplies. Nonetheless, our study reveals that that altered precipitation patterns and nitrogen deposition will likely interact in complex ways, yielding varying effects on plant defensive chemistry, and potentially resistance to herbivory.

In summary, our study demonstrates that the effect of nitrogen enrichment on plant allelochemistry depends on water availability. Results presented here suggest that increased nitrogen may decrease chemical defenses of *L. dalmanica* if growing season water availability decreases with climate change. In contrast, iridoid glycoside concentrations may increase with nitrogen enrichment if growing season precipitation patterns remain stable or precipitation is comparable to mean levels observed during our study period. Such increases in iridoid glycoside concentrations could yield plants that are more resistant to herbivory and less susceptible to insect biocontrol efforts. The effects of nutrient-water interactions on plant traits, however, may not produce parallel responses in insect herbivores (Lower and Orians 2003). Moreover, the consequences of global environmental change on plant-insect interactions will depend on both modifications in plant defense traits and nutritional chemistry as well as the direct and indirect effects of global change drivers on insect herbivores and higher trophic level organisms.

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