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RESEARCH ARTICLE

Simulated nitrogen deposition increased herbivore resistance and competitive ability of an invasive plant species over a native co-occurring congeneric species

Tiantian Lin · Zening Wang · Man Ke · Wanci He · Guoqing Zhu · Abdul Manan · Klaas Vrieling · Bo Li

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Abstract

Background and Aims The deposition of atmospheric nitrogen has increased globally and interfered with plant growth and development. However, only a few studies have examined the influence of atmospheric nitrogen deposition on plant–herbivore interactions in the framework of plant invasion, and the underlying mechanisms remain unclear.

Methods In this study, we examined the changes in growth and herbivore resistance of the invasive weed *Alternanthera philoxeroides* and its native co-occurring congener *Alternanthera sessilis* in response to simulated nitrogen deposition. We further investigated the competitive ability of these two plant species grown in mono- and mix-culture conditions with or without herbivore pressures.

Results The results indicated that simulated nitrogen deposition largely increased the growth of both plant species, while *A. philoxeroides* produced more biomass than *A. sessilis*. The specialist and generalist herbivores showed contrasting preferences for the two plant species in the controls, whereas *A. philoxeroides* showed greater resistance to both herbivore species under simulated nitrogen deposition. Further investigation of host plant traits related to leaf structural and chemical defences suggested that such difference in herbivore resistance between the two plant species could be attributed to the increased levels of structural defense in *A. philoxeroides* and reduced levels of defensive metabolites in *A. sessilis*. The inter-specific competition test revealed that simulated nitrogen deposition largely increased the competitive advantage of *A. philoxeroides* over *A. sessilis*, regardless of the presence or absence of herbivores.

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Tiantian Lin and Zening Wang contributed equally to this work.

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T. Lin · M. Ke · G. Zhu · Bo. Li (✉)
Ministry of Education Key Laboratory for Transboundary Ecosecurity of Southwest China, Yunnan Key Laboratory of Plant Reproductive Adaptation and Evolutionary Ecology and Centre for Invasion Biology, Institute of Biodiversity, School of Ecology and Environmental Science, Yunnan University, Kunming 650504, Yunnan, China
e-mail: bool@ynu.edu.cn

T. Lin · Z. Wang · W. He · A. Manan
Key Laboratory of National Forestry & Grassland Administration On Forest Resources Conservation and Ecological Safety in the Upper Reaches of the Yangtze River, College of Forestry, Sichuan Agricultural University, Chengdu 611130, China

K. Vrieling
Above and Belowground Interactions, Institute of Biology, Leiden University, 2300 RA Leiden, The Netherlands

Conclusion Our study suggests that the invasiveness of *A. philoxeroides* may be enhanced by increased atmospheric nitrogen deposition.

Keywords Atmospheric nitrogen deposition · Chemical defense · Generalist herbivore · Interspecific competition · Specialist herbivore · Structural defense

Introduction

Atmospheric nitrogen deposition is a significant global change factor that involves the settling of atmospheric reactive nitrogen compounds from the atmosphere to the biosphere through gases, dry deposition, or precipitation (BassiriRad 2015). The increase in nitrogen deposition in global ecosystems, particularly in farming ecosystems, has been attributed to human activities such as the combustion of fossil fuels and the use of nitrogenous fertilizers (Ackerman et al. 2019; Galloway et al. 2004; Reay et al. 2008). Nitrogen is a crucial nutrient for the growth of plants, as its availability directly affects plant development, metabolism, and overall growth (Gaudinier et al. 2018; Lawlor et al. 2001). However, excessive nitrogen input in ecosystems can have detrimental effects on plants such as carbon limitation or photosynthesis inhibition (Galloway et al. 2004), and interfere with plant–herbivore interactions through altering the distribution of nutrients and defense substances in plant tissues (Chen and Ni 2011; Dietrich et al. 2004). For example, Throop and Lerdau (2004) have shown that nitrogen deposition promotes herbivore consumption on plant tissues through increased level of nitrogen content as well as reduced levels of defensive chemicals in plants. However, other studies have found that nitrogen addition could enhance plant resistance to herbivory due to induced levels of nitrogen-based defensive metabolites or increased leaf hairiness (Campbell and Vallano 2018; Zettlemoyer 2022). Thus, we argued that changes in plant–herbivore interactions mediated by nitrogen deposition might be species-specific both at plant and herbivore levels.

Herbivorous insects can be categorized into generalist and specialist herbivores based on their feeding habits (Lankau 2007). Generalist herbivores have a broad range of plant species that they can feed on, while specialist herbivores can feed only on specific plants. Consequently, plants have developed multiple defensive strategies that are in general all effective against generalist herbivores (Strauss et al. 2002); for example, mechanical defenses like leaf toughness and thickness, digestibility reducers like tannins and chemical defenses such as specialized metabolites like flavonoids or alkaloids (Kant et al. 2015). Mechanical defense is utilized by plants to form the first physical barrier to defend against both specialist and generalist herbivores (Lucas et al. 2000). Structural defense, as a type of mechanical defense, is derived from structural or anatomical characteristics such as leaf toughness, thickness, or trichomes (Lin et al. 2015a). Chemical defense, on the other hand, is used by plants to reduce herbivore feeding performance by increasing the amount of toxic chemicals in tissues (Lin et al. 2019). Specialist herbivores are often adapted to these defensive chemicals and often use them as an oviposition or feeding stimulants (Mithöfer and Boland 2012). Given the different sensitivities of specialist and generalist herbivores towards plant chemical and mechanical defenses, we proposed that the two types of herbivores may exhibit distinct responses to nitrogen-induced changes in plant defensive strategies. Therefore, we should incorporate both herbivore types and examine both defensive strategies in plants to gain a comprehensive understanding of the impact of increased atmospheric nitrogen deposition on the resource allocation to anti-herbivore defenses in plants.

Plant invasion refers to the range expansion of alien plant species into new geographic regions, typically resulting in significant economic and ecological losses for the ecosystems they have invaded (Sharma et al. 2023). As global environmental challenges become more intricate and severe, an increasing number of studies have explored the influence of various global change factors, such as climate change, drought, and elevated CO_2 levels, on the success of plant invasion (Liu et al. 2017; Vilà et al. 2007). Among these factors, only a limited number of studies have delved into the effects of simulated nitrogen deposition on the growth, development, and functional characteristics of invasive plant species (Guo et al. 2023; Li et al. 2014). These studies have revealed that invasive plants alter their adaptive strategies in terms of dry matter accumulation and resource allocation, resulting in increased biomass, competitiveness, and nitrogen use efficiency compared to co-occurring native plant species (Feng and

Fu 2008; Huangfu et al. 2016; Rickey and Anderson 2004; Vallano et al. 2012). However, only a few studies have examined the interactions between invasive plants and their associated herbivores in the context of atmospheric nitrogen deposition and the outcomes are mixed (Hu and Dong 2019; Lu et al. 2015). Besides that, these studies have rarely delved into the underlying mechanisms that drive changes in plant traits related to herbivore defense in invasive plants in response to nitrogen deposition. Furthermore, none of them have explored whether such changes could further impact the competitiveness of invasive plants.

In this study, we utilized the invasive weed *Alternanthera philoxeroides* and its native congeneric species, *Alternanthera sessilis*, as experimental models to investigate their differences in herbivore resistance and competitive ability in response to simulated nitrogen deposition. This study aimed to address three specific questions: (1) Does simulated nitrogen deposition lead to differences in resistance to a specialist and a generalist herbivore species between the two plant species? (2) Are such differences in herbivore resistance between the two plant species due to the changes in host plant traits related to structural and chemical defense in response to simulated nitrogen deposition? (3) Do such differences in herbivore resistance between the two plant species further affect their competitive ability under simulated nitrogen deposition with different herbivore pressures? To further disentangle the mechanisms underlying the changes in herbivore resistance and competitive ability of *A. philoxeroides* and *A. sessilis* mediated by simulated nitrogen deposition, we examined plant growth performance and plant defensive traits (e.g. defensive chemicals, leaf toughness and thickness) in both plant species. The findings of this study have potential implications for understanding how increased atmospheric nitrogen deposition may facilitate plant invasions.

Materials and methods

Study species

Plant materials

Alternanthera philoxeroides (Amaranthaceae) is a herbaceous amphibious weed that is native to South America and has been introduced to many countries, including the United States, China, and Australia, since the late nineteenth century (Pan et al. 2007). This invasive species has successfully colonized both aquatic and terrestrial habitats, exerting strong suppressive effects on native plant species and ecosystems. It competes with local plants for nutrients and space, leading to a reduction in local biodiversity (Yan et al. 2020). *Alternanthera sessilis* (Amaranthaceae) is a native species that commonly co-occurs and competes with *A. philoxeroides* in wetland habitats in Asia and both species are highly similar to each other in morphology and phenotypic plasticity (Chen et al. 2013; Wang et al. 2021). Twenty individual plants of each species were collected from five populations in a forest park located in Chengdu, Southwestern China (30°41′ N, 103°51′ E). The distance between every two populations was over 200 m. Clonal fragments propagated from cuttings were used for experiments.

Herbivore species

The specialist herbivore species used in this study was *Agasicles hygrophila* (Coleoptera: Chrysomelidae), a specialized flea beetle that exclusively consumes the leaves of the *Alternanthera* genus (Zhao et al. 2015). This species originated from South America, but has been introduced to North America, China, and Australia as a biological control agent of *A. philoxeroides* (Sun et al. 2010). *Locusta migratoria* (Orthoptera: Oedipodidae) was employed as a generalist herbivore species. This pest species is highly polyphagous and has caused significant agricultural damage worldwide (Tanaka and Zhu 2005). Adults of *A. hygrophila* were collected from a natural population of *A. philoxeroides* in the same forest park where the plant material was collected. The 2nd-instar larvae of *L. migratoria* were provided by the College of Agriculture, Sichuan Agriculture University. Both herbivores were reared in a climate chamber under controlled conditions (25 °C, 70% RH, 16: 8 h L/D) with fresh *A. philoxeroides* leaves.

Experimental design

First experiment: single culture experiment

Plant cultivation Five clonal fragments of similar-height with 5 nodes of *A. philoxeroides* and *A. sessilis* from each population were planted individually in 1 L plastic pots (Ø 13.5 cm, height 11.5 cm). We ensured that each clonal fragment was selected from a different cutting. Each pot contained 30% potting soil and 70% sand. The potting soil had 4.58% organic matter, a pH of 7.08, nitrogen content of 0.72 $g \cdot kg^{-1}$, and phosphorus content of 0.77 $g \cdot kg^{-1}$. To simulate nitrogen deposition, we followed the method described by Chen et al. (2022). Based on observations of atmospheric nitrogen deposition in Southwestern China, the average annual nitrogen deposition was found to be 15 $g\ N \cdot m^{-2} \cdot yr^{-1}$ (Song et al. 2018). Considering that precipitation that fell during plant growth accounted for 60–70% of the total precipitation in the past five years, we set the level of soil nitrogen addition during the experiment to be 65% of the annual average nitrogen deposition, which is equivalent to 9.75 $g\ N \cdot m^{-2} \cdot yr^{-1}$. There were four experimental treatments: control + *A. philoxeroides*, control + *A. sessilis*, N + *A. philoxeroides* and N + *A. sessilis*. Each week a 20 mL solution containing 10.3 mM of NH_4NO_3 was added to each pot of the simulated nitrogen deposition treatment. The control group received 20 mL distilled water. In total, there were 2 plant species × 2 treatments × 5 populations × 5 clonal fragments = 100 plants. All plants were grown in a climate chamber for 8 weeks (25 °C, 70% RH, 16: 8 h L/D), with a photosynthetic photon flux density (PPFD) of 125 $\mu mol\ m^{-2}\ s^{-1}$.

Herbivore feeding bioassays A choice feeding bioassay was used to examine the feeding preferences of the specialist and generalist herbivores between *A. philoxeroides* and *A. sessilis* under control and simulated nitrogen deposition. As the distance between two plants was quite large for herbivores to move freely and make a choice, we used leaf-disc assay to test herbivore feeding preferences (Sanané et al. 2021). Five individual plants were selected from each treatment and we ensured that one individual plant from each population was selected. In total, there were 2 plant species × 2 treatments × 5 populations × 1 plant = 20 plants. Leaf discs (Ø 10 mm) were punched from the 1st to 10th fully expanded leaves, counted from the top, of each selected plant. Leaf discs of the five individual plants from each treatment were mixed together and paired with another treatment as the following pairs: control + *A. philoxeroides* vs. control + *A. sessilis*, control + *A. philoxeroides* vs. N + *A. philoxeroides*, control + *A. sessilis* vs. N + *A. sessilis*, N + *A. philoxeroides* vs. N + *A. sessilis*. In each bioassay, a leaf disc from one treatment was placed against a leaf disc from another treatment in a Petri dish (Ø 90 mm), on top of a moistened filter paper. One adult beetle of *A. hygrophila* or one larva of *L. migratoria* was individually introduced into the center of each Petri dish and was allowed to feed for 24 h. After 24 h the remaining area of each leaf disc was scanned by a flatbed scanner (Canon, Japan), and the amount of leaf area consumed was calculated. For each herbivore species, 20 individual insects were tested for each pair. In total, 4 plant pairs × 20 insects × 2 herbivore species = 160 insects were tested.

A no-choice feeding bioassay was conducted to investigate the feeding preference of the specialist and generalist on both plant species in the control and simulated nitrogen deposition treatment. Individual plants that had not been used in the choice feeding bioassay were used in this study. In total, there were 2 plant species × 2 treatments × 5 populations × 4 plants = 80 plants. One adult beetle of *A. hygrophila* or one larva of *L. migratoria* was placed on an expanded leaf and covered with a nylon net (5*5 cm). The opening of each net was closed with a rubber band to prevent the herbivores from escaping. After 24 h of feeding, the remaining leaves were scanned to measure the area, and the amount of leaf area consumed by herbivores was calculated. A total of 20 plants were used for each treatment and for each herbivore species, with each plant receiving one adult of *A. hygrophila* and one larva of *L. migratoria*. The leaf dry mass consumed by herbivores was determined by multiplying the herbivore-consumed leaf area by the leaf mass-area ratio measured as below.

Measurements of host plant traits The measurements of leaf, stem, root and total dry mass, number of leaves, and total leaf area were conducted on individuals of *A. philoxeroides* and *A. sessilis* used for the herbivore no-choice feeding bioassay (N = 20 for each treatment). Leaf chlorophyll content of each plant was measured from the 4th to 6th leaves, counted from the top of each plant, with a portable leaf chlorophyll meter (SPAD-502, Minolta, Japan). After that, the three leaves were harvested and dried in an oven at 60 °C for 3 days. Leaf nitrogen and carbon contents were analyzed using a CHN analyzer (Carlo Erba, Milan, Italy), following the method described by Lin et al. (2019).

The structural defense-related traits, including leaf mass-area ratio, leaf thickness, leaf tensile strength and leaf toughness, were measured on the 7th to 12th leaves of plants from the no-choice feeding bioassay ($N = 20$ plants for each treatment). The leaf mass-area ratio was calculated as dry mass of leaves (g)/total leaf area(m^2) (Poorter et al. 2009). Leaf thickness was measured on leaf sections from the middle of leaf images using a microscope (U-LHLEDC, Olympus, Tokyo, Japan) coupled with ImageJ 1.42q (Lin et al. 2015a). Three leaves were measured as technical replicates for each plant, and the average thickness was taken for statistical analysis. Leaf tensile strength and punch toughness were measured using a tensile strength testing machine (ZT969S, Xianglong, Guangzhou, China), following the method described by Graça and Zimmer (2020). Leaf tensile strength refers to the amount of force required to tear apart a leaf sample. Leaf punch toughness was estimated by measuring the force required to penetrate a leaf sample with a steel die. The die was installed in the same machine and positioned to pass through the leaf without any friction. When the leaf was fractured or pierced, a sharp increase in force was observed and the maximum force (N) was recorded. Three different leaves of each plant were measured as technical replicates for either leaf tensile strength or punch toughness, and the average was used for statistical analysis.

Metabolite extraction and profiling Individual plants from the no-choice feeding bioassay were used for leaf metabolome analysis. The 13th to 16th fully expanded leaves of the four plants from each population under the same treatment were harvested and mixed together as one sample. In total, there were 2 plant species × 2 treatments × 5 populations × 1 sample = 20 samples. Leaves from each sample were freeze-dried and ground into powder with liquid nitrogen. The extraction and analysis of metabolites of the leaf samples followed the protocol described by Su et al. (2021). Specifically, 50 mg ground powder was ultra-sonicated in 0.4 ml of methanol/water (4:1, v/v) for 30 min, followed by a 30-min incubation at -20 °C. An internal standard of 2-Chloro-L-phenylalanine (0.02 mg ml^{-1}) was added. The extracts were centrifuged at 13,000 rpm for 15 min at 4 °C and 200 µl of the supernatant was filtered through a 0.2 µm filter and analyzed using a UHPLC-Q Exactive HF-X Mass Spectrometer (ThermoFisher, Massachusetts, USA) equipped with an electrospray interface ACQUITY UPLC HSST3 (100 mm × 2.1 mm i.d., 1.8 µm, Waters, Milford, USA) platform.

Second experiment: competition experiment

The experiment consisted of two mono-cultures and three mixed-cultures, the latter without herbivore, with the specialist herbivore or with the generalist herbivore, according to Lin et al. (2015b) with a few modifications as shown in Fig. S1. For each pot, two similar-sized clonal fragment of *A. philoxeroides* or *A. sessilis* from different populations, with five nodes, were paired and planted in 1 L plastic pots (Ø 13.5 cm, height 11.5 cm). We ensured that the clonal fragments of the same plant species used in each paired treatment were equally selected from the five populations. Half of the pots were subjected to simulated nitrogen deposition. These pots received the same amount of nitrogen as described above in the single culture experiment. In total, there were 10 treatments × 10 replicates = 100 pots. The competition experiment lasted 8 weeks in a climate chamber (25 °C, 70% RH, 16: 8 h L/D), with a photosynthetic photon flux density (PPFD) of 125 µmol m^{-2} s^{-1}.

After 8 weeks, five specialist *A. hygrophila* adults or five generalist *L. migratoria* larvae were released in the pots subjected to herbivore treatment. Plastic cages with transparent nylon nets (Ø: 14–16 cm, height: 20 cm) were placed over all pots to prevent herbivores from escaping. After 7 days of feeding, the herbivores were removed from each cage and all plants were harvested. Leaf, stem, root, and total dry mass of each plant were then measured after being dried at 60 °C for 5 days. The proportional biomass of *A. philoxeroides* over *A. sessilis* in the mixed-cultures was calculated as the total dry mass of *A. philoxeroides* divided by the total dry mass of *A. sessilis* (Lin et al. 2015b).

Statistical analysis

We used Generalized Linear Mixed Models (GLMMs) with a Poisson error distribution, with plant status (invasive *A. philoxeroides* vs. native *A.*

sessilis) and nitrogen (control vs. simulated nitrogen deposition) as fixed factors and population as a random factor, to investigate the impact of plant species and simulated nitrogen deposition on host plant traits related to growth, leaf chemicals and structural defense in the single culture experiment. The same GLMMs were also used to analyze the leaf dry mass consumed by the two herbivores in the no-choice feeding bioassay between *A. philoxeroides* and *A. sessilis*. To further explore the significance of the observed differences among treatments, a Wald pairwise comparison was conducted. The herbivore preferences in the choice bioassay were analyzed with a Wilcoxon test. Spearman correlation two-tailed tests were employed to examine the correlations between leaf dry mass consumed by each herbivore in the no-choice bioassay and host plant traits related to leaf chemicals, leaf structural defense and defensive metabolites.

In the competition experiment, a comparison was first made between mixed-culture and mono-culture controls, with and without simulated nitrogen deposition (Fig. S1a-f). The effects of culture type (mono-/mixed-culture), plant status (invasive *A. philoxeroides* vs. native *A. sessilis*), and nitrogen (control / simulated nitrogen deposition) on leaf, stem, root, and total dry mass without herbivore feeding were examined using GLMMs with a Poisson error distribution, with culture type, plant status and nitrogen as fixed factors as well as population as random factor. Subsequently, the three mixed-cultures treatments of the control and simulated nitrogen deposition were analyzed (Fig. S1e-j). The same GLMMs were employed with plant status (invasive *A. philoxeroides* vs. native *A. sessilis*), nitrogen (control / simulated nitrogen deposition) and herbivory (specialist / generalist / no herbivore) as fixed factors as well as population as random factor, to assess their effects on dry mass of leaves, stems, roots, and the overall dry mass of individual plants. The total dry mass of *A. philoxeroides* and *A. sessilis* plants that were grown in the same pot was analyzed using a Wilcoxon test. Lastly, the effects of simulated nitrogen deposition and herbivore species on the proportional biomass of *A. philoxeroides* (the proportion of *A. philoxeroides* biomass) was tested using GLMMs with a Poisson error distribution, with nitrogen (control / simulated nitrogen deposition) and herbivory (specialist / generalist / no herbivore) as fixed factors and population as a random factor, followed by a Wald pair-wise comparison to test for differences among treatments. All of the aforementioned analyses were performed using SPSS 23.0 (IBM, Chicago, USA).

The metabolome data matrix obtained from the UPLC-MS analysis was analyzed using the freely available online platform provided by Majorbio (www.majorbio.com). Following the methodology described by Li et al. (2023), the peak areas obtained from LC/MS underwent preprocessing as follows: variables that exhibited a relative standard deviation (RSD) greater than 30% of the quality control (QC) samples were excluded from the dataset. Additionally, peak areas were log10 transformed and subsequently normalized by sum. A Hierarchical Cluster Analysis (HCA) was conducted to show the similarity in leaf metabolites among the four treatments. A principal Component Analysis (PCA) was conducted with unit variance scaling to classify all samples based on the metabolomic data. Next, a Partial Least Squares Discriminant Analysis (PLS-DA) was performed on the metabolomic data using pareto (Par) scaling. This analysis aimed to classify the observations into groups based on the largest predicted indicator variable. Heatmaps were generated that displayed the relative abundance of the top 20 Variable Importance in the Projection (VIP) as determined by the PLS-DA. Additionally, a PCA was conducted with unit variance scaling to classify individual plants from the four treatments of the no-choice herbivore bioassay based on a suite of host plant traits related to leaf structural and chemical defenses. Generalized Linear Models (GLMs) were employed with species and nitrogen as fixed factors to assess the effects of plant species and simulated nitrogen deposition on the six defensive metabolites from the VIP list.

Results

Effects of simulated nitrogen deposition on host plant traits

Growth-related traits

Although *A. philoxeroides* exhibited a relatively higher dry mass of leaves, stems and roots compared to *A. sessilis* in the control after 8 weeks of growth, these differences were not statistically significant

(Fig. S2). Simulated nitrogen deposition largely promoted the growth of leaves, stems and roots of both plant species, leading to a 38% greater total dry mass in *A. philoxeroides* compared to *A. sessilis* (Fig. 1a). On the other hand, simulated nitrogen deposition largely increased the number of leaves and total leaf area of the two plant species, with no significant differences observed between *A. sessilis* and *A. philoxeroides* (Fig. 1b and c). In addition, plant populations exerted no significant effect on the aforementioned growth-related traits.

Leaf chemical traits

The nitrogen and carbon contents in the leaves of *A. philoxeroides* and *A. sessilis* were similar in the control, while simulated nitrogen deposition led to a noticeable increase in leaf nitrogen and carbon levels in *A. philoxeroide* but not in *A. sessilis* (Fig. 2a and b). Moreover, *A. philoxeroides* had a 13% higher leaf chlorophyll content than *A. sessilis* in the control, whereas simulated nitrogen deposition increased the leaf chlorophyll content in both plant species (Fig. 2c). However, leaf water content was neither affected by plant status nor simulated nitrogen deposition (Fig. 2d). In addition, plant populations exerted no significant effect on the aforementioned leaf chemical traits.

Leaf structural defense

Both plant species exhibited similar levels of four leaf structural defense-related traits in the control (Fig. 3a-d). However, simulated nitrogen deposition led to a significant increase in these traits specifically in *A. philoxeroides* plants. As a result, *A. philoxeroides* had a 27%, 19%, 29%, and 44% higher leaf mass-area ratio, leaf thickness, leaf punch toughness, and leaf tensile strength, respectively, than *A. sessilis*. Furthermore, the plant populations did not have a notable impact on the four leaf traits.

Leaf metabolome

The non-targeted UPLC-QTOF/MSA annotated a total of 3140 compounds. Hierarchical cluster analysis (HCA) showed two main clusters, one for each species (Fig. S3a). The composition of leaf metabolites in the same plant species also differed between control and simulated nitrogen deposition. The PCA and PLS-DA plots both showed a clear separation in the leaf metabolites between the two plant species and between the two treatments (Fig. S3b and 4a). Specifically, the first component of the PLS-DA plot accounted for 64.3% of the variance, separating the species while the second component accounted for 8.1% separating the treatments.

From the top 20 metabolites with the highest VIP values from the PLS-DA, six compounds were identified as defensive metabolites belonging to the class of flavonoids or alkaloids (Fig. 4b). Among the six defensive metabolites, the relative abundances of all metabolites were affected by plant status (Fig. 4c). More specifically, Hydroquinidine, Kaempferol 3-(3R-glucosylrutinoside)

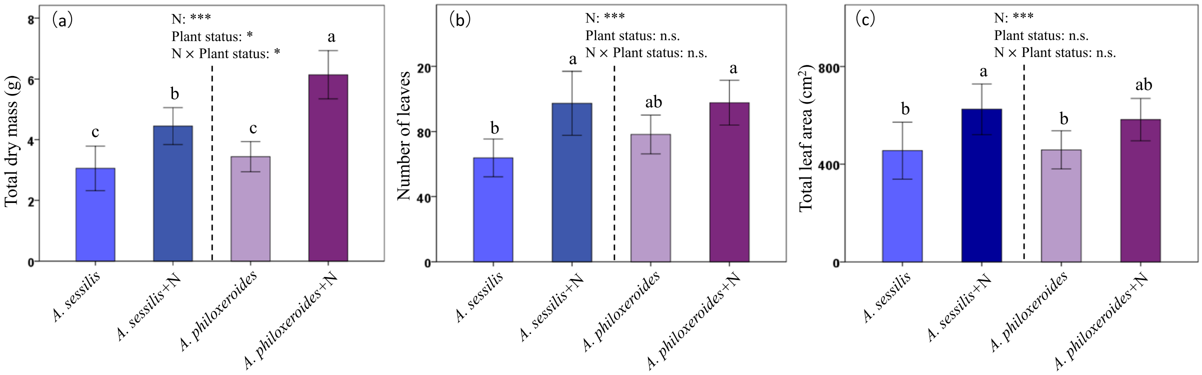

Fig. 1 Plant growth-related traits of *A.philoxeroides* and*A. sessilis* from control and simulated nitrogen deposition treatments.(**a**) total dry mass, (**b**) number of leaves, (**c**) total leaf area. N = 20 for each treatment. Values are means ± SE. Different letters indicate significant differences among treatments at $p < 0.05$ according to GLMMs followed by Waldpair - wise comparisons. The significance values of the GLMMs is indicated by n.s., not significant;*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$

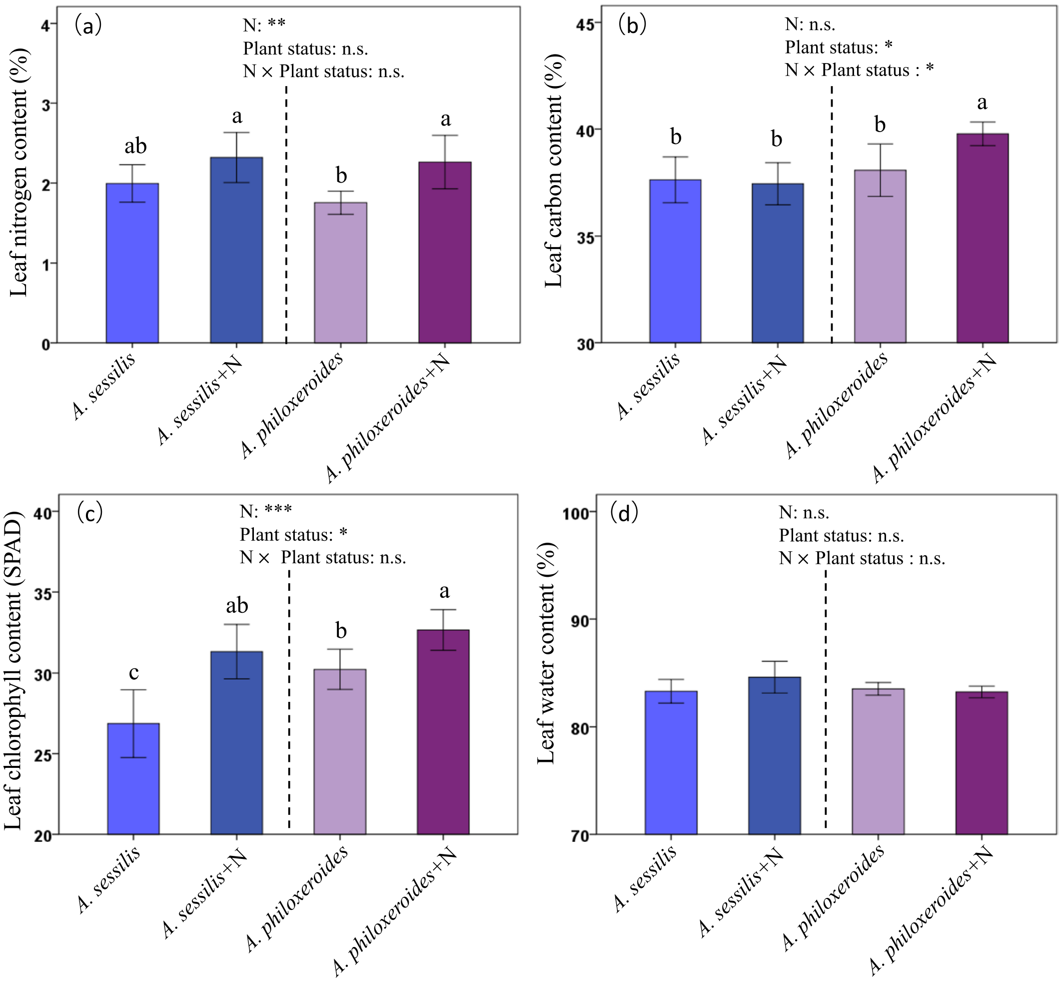

Fig. 2 Leaf chemical traits of *A.philoxeroides* and *A.sessilis*from control and simulated nitrogen deposition treatments. (**a**) Leaf nitrogen content, (**b**) leaf carbon content, (**c**) leaf chlorophyll content and (**d**) leaf water content (N = 20 for each treatment). Values are means ± SE. Different letters indicate significant differences among treatments at $p< 0.05$ according to GLMMs followed by Waldpair - wise comparisons. The significance values of the GLMMs is indicated by n.s., not significant; *, $P \leq 0.05$; **, $P \leq 0.01$; ***,$P \leq 0.001$

and (2R)-6,8-Diglucopyranosyl-4',5,7-trihydroxyflavanone were found in higher concentrations in *A. sessilis* leaves compared to *A. philoxeroides* leaves, regardless of simulated nitrogen deposition treatments. Simulated nitrogen deposition significantly reduced the relative amounts of Apigenin 6-C-arabinosyl-8-C-glucoside and Diosmin in *A. sessilis* leaves, but had no effect on *A. philoxeroides* leaves. However, the levels of P-chlorophenylalanine increased in the leaves of both plant species, whereas those in *A. philoxeroides* leaves were significantly higher than in *A. sessilis* leaves.

Effects of simulated nitrogen deposition on herbivore preferences

In the choice feeding bioassays, the specialist and generalist herbivores showed contrasting feeding preferences for the leaf discs of both plant species in the control (Fig. 5a and b). Given a choice between leaves from the same plant species of the control or simulated nitrogen deposition, the specialist herbivores preferred the leaves from the control for both plant species, while the generalist did prefer the control leaf discs of *A. philoxeroides* and had a significant

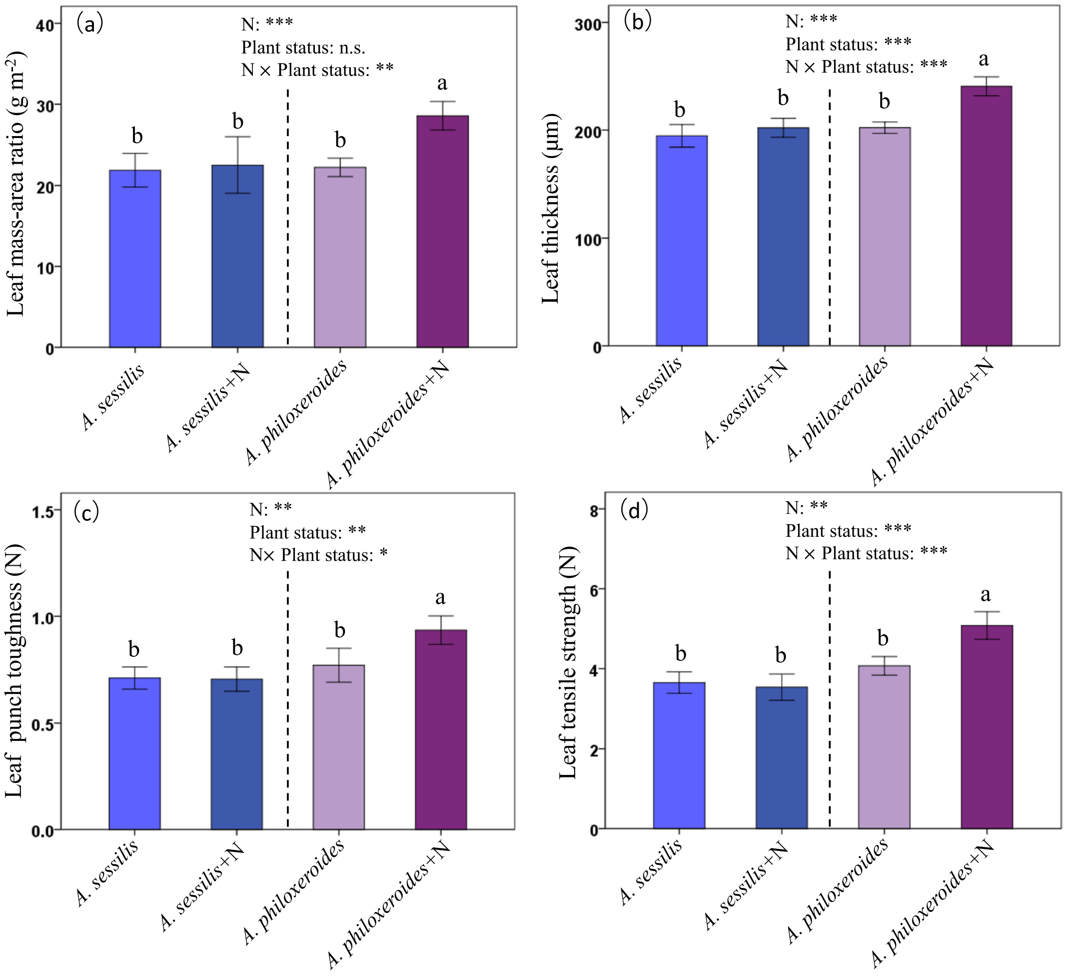

Fig. 3 Host plant traits related to structural defense of *A.philoxeroides*and *A.sessilis* from control and simulated nitrogen deposition treatments. (**a**) leaf mass-area ratio, (**b**) leafthickness, (**c**) leaf punch toughness and (**d**) leaf tensile strength (N = 20 plants fo reach treatment). Values are means ± SE. Different letters indicate significant differences among treatments at $p < 0.05$ according to GLMMs followed by Waldpair - wise comparisons. The significance values of the GLMMs is indicated by n.s., not significant; *, $P \leq 0.05$; **, $P \leq 0.01$; ***,$P \leq 0.001$

preference for leaf discs of *A. sessilis* from the simulated nitrogen deposition treatment. Given a choice between leaf discs of *A. philoxeroides* and *A. sessilis* from the simulated nitrogen deposition treatment, *A. philoxeroides* received 65% and 75% less consumption by *A. hygrophila* and *L. migratoria*, respectively, compared to *A. sessilis*.

In addition, the no-choice feeding bioassays perfectly reflected the outcome of the choice leaf disc assay. The specialist herbivore *A. hygrophila* consumed 42% more leaf dry mass from *A. philoxeroides* than from *A. sessilis* in the control (Fig. 5c). This was reversed for the simulated nitrogen deposition treatment, resulting in 51% more feeding on the leaves of *A. sessilis* than on *A. philoxeroides*. The generalist *L. migratoria* consumed on average 31% more leaf dry mass of *A. sessilis* than *A. philoxeroides* in the control, with this difference increasing to 138% under simulated nitrogen deposition (Fig. 5d). In addition, the leaf dry mass consumed by both herbivores was not significantly influenced by plant population.

Further tests showed strong negative correlations between the leaf dry mass consumed by both herbivore species and the four leaf traits related to structural defense in *A. philoxeroides* (Table S1, Fig. S4). Moreover, the leaf dry mass consumed

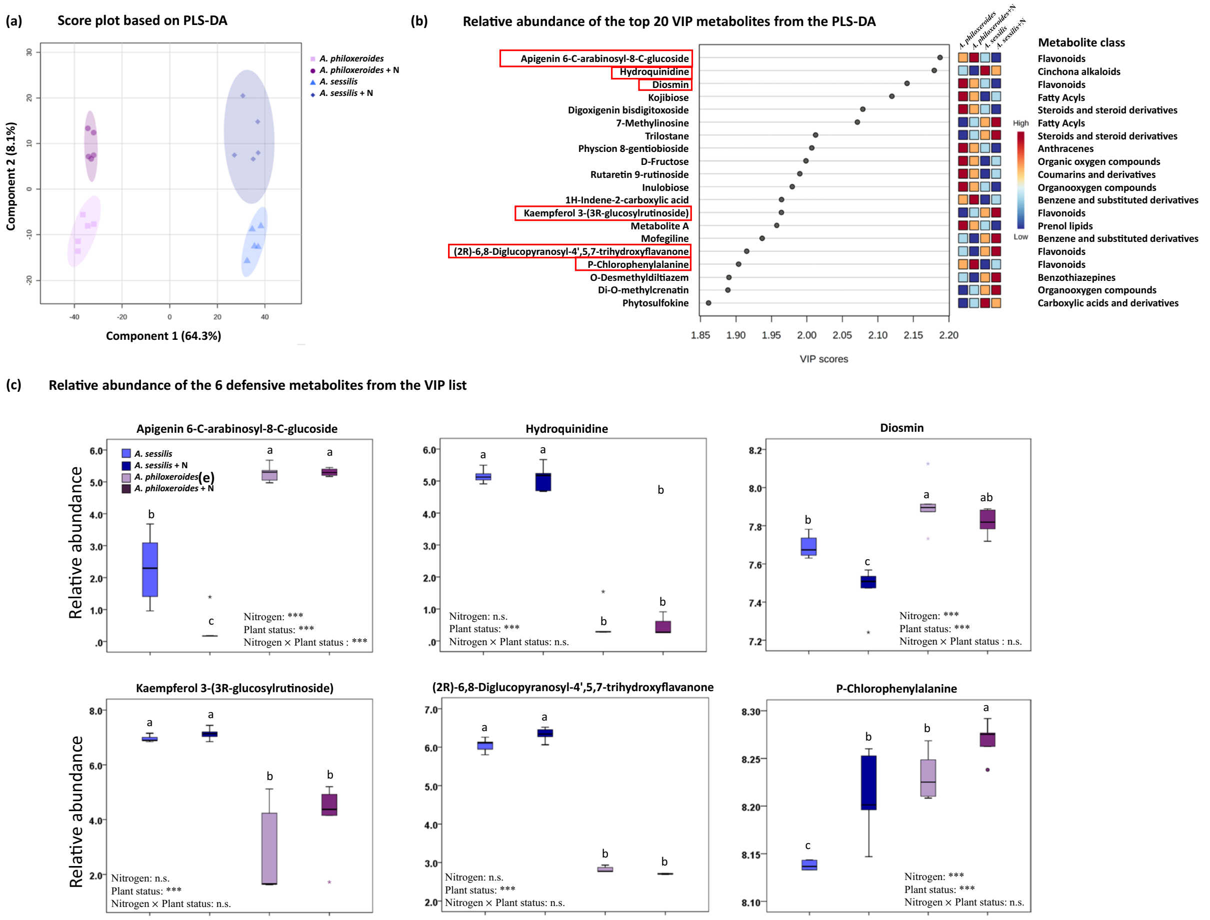

Fig. 4 Overview of leaf metabolome differences of *A.philoxeroides*and *A.sessilis* from control and simulated nitrogen deposition treatments.(**a**) Partial Least Squares Discriminant Analysis (PLS - DA) plot of all detected leaf metabolites in *A.philoxeroides* and *A.sessilis* for all treatments. (**b**) Relative of metabolites with top 20 variable importance in the projection (VIP) based on the PLS -DA. Red boxes indicate metabolites potentially involved in defense against herbivores. (**c**) Differences in the relative abundance of the 6 defensive metabolites from the VIP list among plants from different treatments. The abundance of each compound was log transformed and values are means ± SD. Different letters indicate significant differences among treatments at $p < 0.05$ according to GLMs followed by Waldpair – wise comparisons. The significance values of the GLMs is indicated by n.s., not significant;***, $P \leq 0.001$

by the specialist *A. hygrophila* was negatively correlated with leaf nitrogen, carbon and chlorophyll contents in *A. philoxeroides* leaves, while the leaf dry mass consumed by the generalist *L. migratoria* was positively correlated with leaf nitrogen content in both plant species. In addition, the leaf dry mass consumed by the generalist *L. migratoria* was also negatively correlated with leaf carbon content in *A. philoxeroides* leaves but positively correlated with leaf chlorophyll content in *A. sessilis* leaves. Furthermore, we tested the correlations between the three defensive metabolites from the VIP list that were strongly affected by simulated nitrogen deposition (Fig. 4c), and the leaf dry mass consumed by both herbivore species. The result showed that only the relative abundance of Apigenin 6-C-arabinosyl-8-C-glucoside in *A. sessilis* leaves was positively correlated with the leaf dry mass consumed by specialist *A. hygrophila*, while it showed a negative correlation that was nearly significant with the leaf dry mass consumed by the generalist *L. migratoria* (Table S2, Fig. S5).

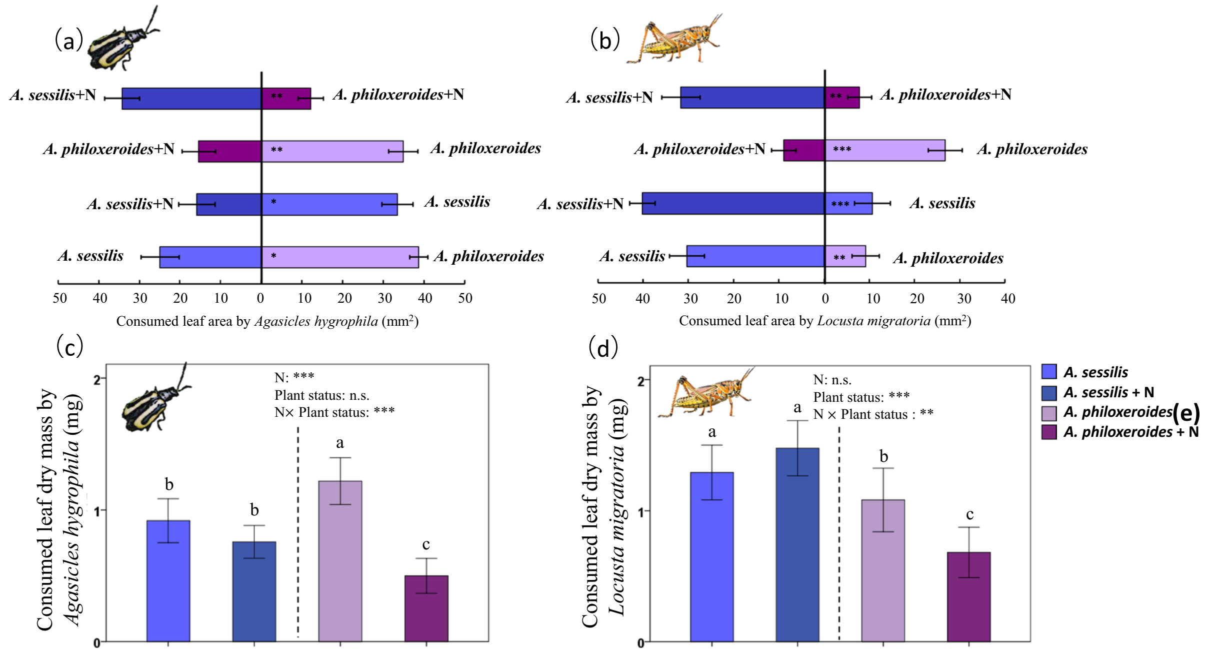

Fig. 5 Herbivore feeding preference in the choice feeding bioassays on leaf discs and no – choice feeding bioassays on whole plants of *A.philoxeroides*and *A.sessilis* grow non control and simulated nitrogen deposition soils.Leaf area Consumed by *Agasicleshygrophila* (**a**) and *Locustamigratoria* (**b**) adults in choice feeding bioassays. N= 20 replicates for each treatments and for each herbivore species. Values are means± SE. Asterisks the bars indicate significant differences between the two choices according to a Wilcoxon test: *, 0.01 < $P \leq$ 0.05; **, 0.001 <$P \leq$ 0.01; ***, $P \leq$ 0.001. area consumed by *Agasicleshygrophila* (c) and *Locustamigratoria*adults (d) in the no – choice feeding bioassay. N=20 replicates for each treatment both herbivore species. Values are means ± SE. Different letters indicate significant differences among treatments at $p<$ 0.05 according to GLMMs followed by Waldpair – wised comparisons. The significance values of the GLMMs is indicated by n.s., not significant; **, 0.001 < $P \leq$ 0.01; ***, $P \leq 0.001$

Effects of simulated nitrogen deposition on competitive ability

We first compared the mono-cultures and mixed-cultures without herbivore pressures, to interpret the effects of culture type, plant status and nitrogen on the growth of *A. philoxeroides* and *A. sessili* in the absence of herbivore pressure (Fig. S1a-f). Leaf, stem, root and total dry mass of individual plants were significantly affected by the three factors (Table 1). In addition, all four interactions significantly influenced leaf and total dry mass, while plant status × nitrogen and culture type × plant status × nitrogen had a strong effect on root dry mass. In addition, simulated nitrogen deposition largely increased the total dry mass of *A. philoxeroides* and *A. sessili*, compared to the control in the mono-cultures (Fig. 6a).

We then compared plant dry mass of *A. philoxeroides* and *A. sessili* in the mixed cultures with and without the two herbivores and under control or simulated nitrogen deposition (Fig. S1e-j). The results showed that all factors significantly affected the leaf, stem, root and total dry mass of individual plants, except for nitrogen × herbivore, which had no strong effect on stem and root dry mass (Table 1). Moreover, the feeding of both herbivore species resulted in reduced growth of the two plant species grown in mixed cultures, regardless of whether or not they received simulated nitrogen deposition treatment (Fig. 6b). We further examined the effect of herbivore pressures and simulated nitrogen deposition on the competitive ability of *A. philoxeroides* by calculating the proportional biomass of *A. philoxeroides* over its neighboring *A. sessilis* in terms of total dry mass. In all cases, *A. philoxeroides* outcompeted *A. sessilis* except in the control when consumed by the specialist herbivore *A. hygrophila* (Fig. 6c). In addition, plant populations

Table 1 χ^2 values of GLMMs with plant status (invasive *A. philoxeroides* / native *A. sessilis*), culture (mono- / mixed-culture), N (control / simulated nitrogen deposition) as fixed factors, leaf, stem, root and total dry mass as variables without herbivores. The second part of the table shows χ^2 with plant status (invasive *A. philoxeroides* / native *A. sessilis*), soil treatment (control / simulated nitrogen deposition) and herbivory (specialist / generalist / no herbivore) as fixed factors, while leaf, stem, root and total dry mass as variables for mixed-cultures only. Plant population was used as random factors

Mode	Df	Leaf dry mass	Stem dry mass	Root dry mass	Total dry mass
Without herbivore feeding					
Culture	112	1.417	5.918*	11.365***	12.158***
Plant status	112	136.729***	40.027***	61.131***	136.588***
N	112	324.142***	31.099***	89.495***	210.322***
Culture × Plant status	112	85.934***	24.899***	44.986***	92.978***
Culture × N	112	7.047**	0.154	3.287	4.146*
Plant status × N	112	36.451***	1.968	10.005**	20.807***
Culture type × Plant status × N	112	28.549***	1.461	7.562**	15.919***
Population		0.360	0.276	0.352	0.082
With herbivore feeding and this is only for the mixed cultures					
Plant status	108	341.048***	160.436***	190.871***	265.695***
N	108	175.354***	9.788**	134.331***	218.805***
Herbivory	108	36.700***	3.668*	13.580***	36.255***
Plant status × N	108	220.614***	67.653***	104.083***	245.204***
Plant status × Herbivory	108	38.282***	18.317***	20.532***	49.540***
N × Herbivory	108	21.999***	1.028	1.352	9.792***
Plant status × N × Herbivory	108	41.154***	26.770***	26.765***	63.091***
Population		1.108	1.263	0.778	0.814

Values are χ^2 from the GLMMs. The significances are shown as follows: *, $0.01 < P \leq 0.05$; **, $0.001 < P \leq 0.01$; ***, $P \leq 0.001$

exerted no significant effect on the proportional biomass of *A. philoxeroides*.

Discussion

In this study, we found that simulated nitrogen deposition in the soil strongly promoted the growth of both plant species, though the invasive weed *A. philoxeroides* exhibited a better growth performance than its native congener *A. sessilis*. In addition, the herbivore bioassays indicated that simulated nitrogen deposition largely strengthened the resistance of *A. philoxeroides* against both herbivore species. Such enhanced herbivore resistance further contributed to a greater competitive ability of *A. philoxeroides* over *A. sessilis*. The results suggested that increased atmospheric nitrogen deposition might facilitate the invasion of *A. philoxeroides*.

Similarly, several invasive plant species have been documented to have an enhanced growth performance compared to native co-occurring plant species under nitrogen treatment (Li et al. 2014; Rickey and Anderson 2004). It has been suggested that these invasive plant species could alter their adaptive strategies in terms of dry mass accumulation and resource allocation in response to high nitrogen availability (Huangfu et al. 2016; Vallano et al. 2012).

The herbivore choice feeding bioassay revealed that the specialist *A. hygrophila* and the generalist *L. migratoria* displayed contrasting feeding preferences when they were offered with the leaf discs from *A. philoxeroides* and *A. sessilis* in the control treatment. This result is consisting with the enemy release hypothesis, which proposes that the invasive plants can leave their natural specialist enemies behind when introduced to a new habitat and consequently contribute to increased growth performance (Keane and Crawley 2002). However, simulated nitrogen deposition strongly decreased the feeding preference of both herbivore species towards *A. philoxeroides*, resulting in an increased consumption of *A. sessilis* leaf discs by the herbivores. The herbivore no-choice feeding bioassay provided additional

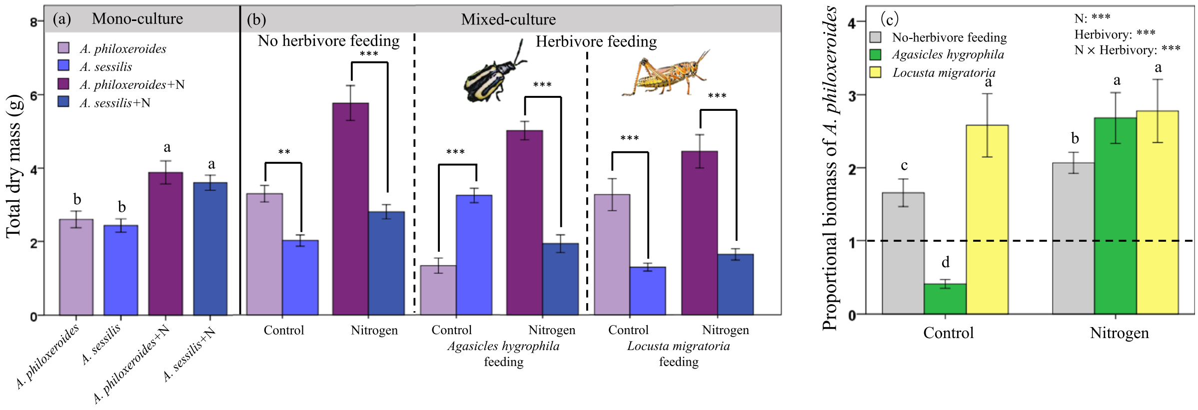

Fig. 6 The effect of herbivore pressure and competition on the growth of *A. philoxeroides* and *A. sessilis* from control and simulated nitrogen deposition treatments. (**a**) Total dry mass of *A. philoxeroides*and *A. sessilis* in the mono-culture grown on non control and nitrogen deposition soil. Values are means ± SE. Different letters indicate significant differences among treatments at $p < 0.05$ according to GLMMs followed by Wald-pair-wise comparisons. (**b**) Total dry mass of *A.philoxeroides* and *A.sessilis* in the mixed-culture grown on controls oil and nitrogen deposition soil with and without herbivores. N = 10 replicates for each plant species in the same treatment. Asterisks above the bars indicate significant differences between *A.philoxeroides* and *A.sessilis*grown in pairs according to a Wilcoxon test: **, $P \le 0.01$; ***, $P \le 0.001$. (**c**) Proportional biomass of the *A.philoxeroides* (= total dry mass of *A. philoxeroides*/ total dry mass of *A. sessilis* per pot). The dashed line indicates the proportional biomass of the *A. philoxeroides* = 1. The proportional biomass of the *A. philoxeroides* > 1 indicates that the *A. philoxeroides* out competes *A. sessilis*. The proportional biomass of the *A. philoxeroides*< 1 indicated that *A. sessilis* out competes *A. philoxeroides*. Values are means ± SE. Different letters indicate significant differences among treatments at $p < 0.05$ according to GLMMs followed by Waldpair – wise comparisons. The significance values of the GLMMs is indicated by **, $0.001 < P \le 0.01$; ***, $P \le 0.001$

support by demonstrating that both herbivore species exhibited reduced feeding on the leaves of *A. philoxeroides* in the simulated nitrogen deposition treatment. In contrast, a field experiment demonstrated that the addition of soil nitrogen (2 g N L^{-1}) led to an increase in herbivore defoliation on both *A. philoxeroides* and *A. sessilis*, while no significant difference was observed between these two species (Lu et al. 2015). Furthermore, Hu and Dong (2019) observed that *A. philoxeroides* experienced greater leaf damage than *A. sessilis* when it was attacked by the specialist *A. hygrophila* under high level of nitrogen availability (60 mg N L^{-1}). As the nitrogen concentration we applied in this study was 288.45 mg N L^{-1}, we suggested that the effect of nitrogen addition on plant–herbivore interactions might be dose-dependent.

We further found a negative correlation between the leaf dry mass consumed by herbivores and host plant traits associated with structural defense, including leaf mass-area ratio, leaf thickness, leaf tensile strength and leaf punch toughness for both herbivore species. These four traits are widely recognized as being closely linked to plant resistance against leaf herbivores (Lin et al. 2015a). As our findings indicated, simulated nitrogen deposition could drastically increase the levels of the four leaf structural defense-related traits in *A. philoxeroides*, but not in *A. sessilis*. This helped to partially explain why the two herbivore species showed a reduced preference for feeding on *A. philoxeroides* compared to *A. sessilis*.

Subsequent analysis of the leaf metabolome revealed that *A. sessilis* exhibited lower levels of leaf defensive metabolites compared to *A. philoxeroides* under simulated nitrogen deposition. Given that generalist herbivores are frequently adversely affected by plant defensive compounds, whereas many specialists have developed counter-defense mechanisms against these chemicals, it has been suggested that generalist herbivores may exhibit a stronger preference compared to specialists when these chemical defenses are reduced (Haber et al. 2018). We indeed found a positive correlation between the level of Apigenin 6-C-arabinosyl-8-C-glucosidea and the leaf dry mass consumed by the specialist herbivore, while a negative correlation was observed with that of the generalist herbivore. However, such correlations were only apparent in *A. sessilis* leaves but not in *A. philoxeroides*, suggesting that these two herbivores were more sensitive to the changes in defensive metabolites of *A. sessilis*.

When the two plant species were cultivated together in a mixed-culture, *A. philoxeroides* exhibited a greater competitive ability than its local congener *A. sessilis*. The superior competitive ability of invasive plant species can not only be attributed to the absence of natural enemies in the introduced ranges but also to the reallocation of their resources and energy from herbivore defense towards growth, as proposed by the evolution of increased competitive ability hypothesis and shifting defense hypothesis (Blossey and Nötzold 1995; Joshi and Vrieling 2005). Interestingly, the growth of *A. philoxeroides* individuals in a mixed-culture setting under simulated nitrogen deposition consistently exceeded that of neighboring *A. sessilis* plants, regardless of whether or not they were subjected to specialist or generalist herbivores. This suggests that the two species have different nitrogen acquisition and allocation strategies. In general, previous studies have shown that invasive plant species exhibit a greater capacity for resource acquisition and absorption in response to soil nitrogen availability, which contributes to their superior growth potential and higher competitive ability compared to co-occurring native plant species (Guo et al. 2023; Huangfu et al. 2016; Vallano et al. 2012). In conjunction with the findings of enhanced levels of structural defenses in *A. philoxeroides* and diminished levels of defensive chemicals in *A. sessilis* under simulated nitrogen deposition, we suggested that *A. philoxeroides* might invest greater resources towards growth and herbivore resistance in response to elevated atmospheric nitrogen deposition, leading to the failure of biological control on *A. philoxeroides* and the displacement of *A. sessilis* in nitrogen-enriched habitats.

Conclusions

In this study, we observed that simulated nitrogen deposition significantly enhanced the growth performance of the invasive species *A. philoxeroides* compared to its native congener *A. sessilis*. Herbivore feeding bioassays revealed that the specialist herbivore preferred *A. philoxeroides*, whereas the generalist herbivore preferred *A. sessilis* from the control treatment. However, simulated nitrogen deposition enhanced the resistance of *A. philoxeroides* to both herbivores. This can be attributed to the nitrogen-enhanced structural defenses in *A. philoxeroides* and reduced levels of leaf defensive compounds in *A. sessilis*. The inter-specific competition tests provided further evidence that simulated nitrogen deposition significantly increased the competitive advantage of *A. philoxeroides* over *A. sessilis*, irrespective of the type of herbivores present. Collectively the data suggest that the invasiveness of non-indigenous plant species may be promoted by increased atmosphere nitrogen deposition due to enhanced herbivore resistance and competitive ability.

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Author contributions TL and BL planned and designed the research; ZW and MK performed experiments and analyzed data; GZ and AM contributed to material collection and method development; TL, ZW, KV and BL wrote the manuscript. All authors contributed to the corrections of the manuscript.

Data availability The data that support the findings of this study are openly available in Dryad at https://datadryad.org/stash/share/EcctktVqEYY11f9089JBTk8g3zt7Tm_SidsFRtvremw.

Declarations

Conflict of Interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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