Plant Soil (2024) 501:307–321
https://doi.org/10.1007/s11104-024-06517-w

RESEARCH ARTICLE

Nitrogen addition reduces the positive effect of *Ligularia virgaurea* on seed germination of alpine species on the Tibetan Plateau

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Received: 23 June 2023 / Accepted: 24 January 2024 / Published online: 13 February 2024

Abstract

Background and aims Nitrogen (N) deposition and native allelopathic plants may affect seed germination and growth of species through their effects on soil microbes and soil nutrient availability. However, our understanding of the interactions between N addition and allelopathic plants on the regeneration of alpine grasslands remains limited.

Methods Here, we investigated the effects of N addition and the presence of the allelopathic plant *Ligularia virgaurea* (Maxim.) Mattf. on seed germination, survival, and growth of native herbaceous species (*Elymus nutans*, *Delphinium kamaonense* and *Tibetia himalaica*) on the Qinghai-Tibet Plateau. We used piecewise structural equation modelling to assess both the direct effects of N addition, allelopathic plants, and their interactions and indirect effects mediated by soil properties, soil microbial richness and diversity, and soil enzyme activity.

Results We found that (1) *L. virgaurea* directly increased seed germination and early plant survival, and reduced plant root-to-shoot ratio; N addition directly increased early plant survival and biomass, (2) *L. virgaurea* indirectly increased plant biomass via bacterial richness, (3) N addition offsets the increase in seed germination promoted by the presence of *L. virgaurea* via soil acid phosphatase.

Conclusion Our study suggests the importance of direct and indirect roles of allelopathic plants, N addition and their interaction on seed germination, survival and plant growth. Our results highlight the need to consider the interactions between environmental and biological factors as well as their direct and indirect effects to obtain reliable predictions and mechanistic understanding of the response of alpine plants to future climate change.

Keywords Seed germination · Survival · Allelopathy · *Ligularia virgaurea* · Nitrogen addition · Qinghai-Tibet Plateau

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Responsible Editor: Roberta L C Dayrell.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11104-024-06517-w.

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Introduction

Ecosystems around the globe are experiencing environmental change (Midolo et al. 2019). Economic development and increasing human activities, including the widespread deposition of nitrogen (N), in many terrestrial ecosystems are major drivers of environmental change (Xia and Wan 2008; Humbert et al. 2016). Increasing levels of soil nitrate and ammonium can increase the growth and dominance of some N-limited species, at the expense of other species (Bobbink et al. 2010). Increasing soil N can increase seed germination and lead to the depletion of seed banks (Plassmann et al. 2008; Basto et al. 2015). Additionally, N enrichment can lead to the range expansion of allelopathic plants, which can decrease forage quality (Shi et al. 2018). Nitrogen deposition and the expansion of allelopathic plants has a major impact on plant community structure and composition (Ade et al. 2021; Huang et al. 2021). A better understanding of seed germination, survival, and plant growth responses to changes in N and the presence of allelopathic plants is critical for predicting vegetation dynamics under future global change scenarios.

Nitrogen deposition significantly affects the conditions that are suitable for plants (Clark et al. 2007; Bird and Choi 2017). For example, increasing soil NO_3^- and NH_4^+ can result in increased seedling growth and survival (Fenner and Thompson 2005; Onipchenko et al. 2012). Nitrogen deposition also alters enzyme activity in plant roots and soil, and can induce metabolic changes (Wang et al. 2022). Strategies for N use vary across species and functional groups, which may lead to subsequent changes in plant community structure and composition (Niu et al. 2008; Duprè et al. 2010; Zhong et al. 2019). For example, N addition can increase grass density (*Elymus nutans*) and decrease forb density (Zhang et al. 2016). Soil inorganic nitrogen can also have effects on seed germination and mortality which are species-specific (Davis 2007). Thus, we expect N addition to have a positive overall effect on seed germination and plant growth of herbaceous species, but with different taxa experiencing distinct effects.

Allelopathic plants have dramatically increased in a number of ecosystems, and these allelopathic plants have displaced high-quality forage and affected seed germination and plant establishment of surrounding plants (Shi et al. 2018). Allelopathy is defined as the effect of one plant on the growth of another plant through the release of secondary substances (e.g., volatile terpenoids, flavonoids, phenols) into the soil (Rice 1984). Allelopathic plants can inhibit seed germination and plant growth of surrounding species and may alter competitive interactions (Zhang et al. 2021). For example, the effects of allelochemicals on plant germination and growth may occur through a variety of mechanisms, including reduced mitotic activity in roots and hypocotyls, inhibition of hormonal and enzymatic activity, inhibition of photosynthesis and respiration etc. (Rice 1974). However, some studies suggest that allelopathic plants may benefit species in grassland ecosystems (Zhang et al. 2020). For example, the toxic odor of allelopathic plants can drive away livestock and insects, thereby hindering the soil seed bank and adjacent plants from being eaten and trampled (Oesterheld and Oyarzábal 2004). Plants can alter the rhizosphere microbial diversity and community composition by producing a range of root exudates and litter (Vieira et al. 2020), and below-ground factors such as soil microorganisms are important regulators of seed germination and plant productivity (Van Der Heijden et al. 2008). Previous research has shown that soil microbial biomass and soil enzyme activities are higher in allelopathic plant patches than in gap areas (Shi et al. 2011), which may in turn alter plant community structure (Sun et al. 2009; Mishra and Nautiyal 2012). Allelopathic plants can also promote soil nutrient and soil moisture retention through their well-developed root systems (Zhang et al. 2020). Thus, we hypothesized that the presence of allelopathic plants increased seed germination and plant growth of herbaceous species by altering soil microbes and enzyme activity.

Ligularia virgaurea (Maxim.) Mattf (hereafter *L. virgaurea*) is a perennial herb in the Asteraceae family. *L. virgaurea* can become dominant in grazed alpine communities due to its toxicity to grazers. The allelochemicals released by *L. virgaurea* (Wu et al. 2011) induce changes in alpine plant and soil microbial communities, affecting soil nutrients and forming positive feedback effects (Zhang et al. 2020; Ade et al. 2021). Seed germination and early seedling survival are considered to be two key stages of plant life history and may be impacted by factors associated with global climate change (An et al. 2020; Ganjurjav et al. 2020). Many studies have attempted to shed light on how N addition and allelopathic plants alter natural plant community composition and diversity (Broadbent et al. 2018; Midolo et al. 2019). However, few studies have examined the effects of N deposition and allelopathic plants and their interactions on seed germination, early plant survival, and growth (Cai et al. 2023). We aimed to explore how N addition and the presence of an allelopathic plant (*L. virgaurea*) would affect seed germination and growth of herbaceous species and to elucidate the potential mechanisms. In this study, we examined the following questions: (a) Do allelopathic plants and N addition (and the interaction between these factors) affect seed germination, early survival, and growth of plants on the Qinghai-Tibet Plateau? (b) What mechanisms drive the responses of seed germination, early survival, and growth to allelopathic plants and N addition?

Method

Study site

We performed our experiments at the Gannan Grassland Ecosystem National Observation and Research Station (101°51′ E, 33°40′ N) at 3550 m.a.s.l, Gansu Province, China. The average annual rainfall is 672 mm (mainly in summer and autumn), and the annual average temperature is 2.2 °C. The specific climate data during the experiment are shown in Table S1. The growing season generally begins in late April and ends in late October. The study site has alpine meadow soil. Total soil nitrogen is 5.28 g kg^{-1}, total soil phosphorus 1.31 g kg^{-1}, soil organic carbon 54.71 g kg^{-1} and soil pH 7.06. The grassland types are mainly alpine meadows, which are dominated by Cyperaceae, Fabaceae, Poacea and Ranunculaceae.

Experimental design

The experiment was conducted during the plant growth season of 2021 (June to September), we selected a flat and homogeneous ungrazed site (fenced with 60 × 60 m barbed wire). *L. virgaurea* was abundant in the site. We used two factorial designs with the presence/absence of the *L. virgaurea* fully crossed with N addition (with/without). We used polyvinyl chloride (PVC) pipes (20 cm diameter, 30 cm height) to create a microenvironment for all treatments. The PVC tubes were inserted in the soil by hammering into the soil and kept it level with the ground. There were eight replicates for each treatment. Thus, a total of 32 microenvironments were arranged in a completely randomized block containing all treatments (i.e., 8 blocks). A 5 m buffer zone was maintained between blocks, and the buffer zone was left untreated. The schematic diagram of our experimental site is shown in Fig. S1.

Nitrogen deposition is projected to increase to 40 g N m^{-2} yr^{-1} by 2050 (Zong et al. 2016), but 8 g N m^{-2} yr^{-1} has already led to soil nitrogen saturation in the current Qinghai-Tibet Plateau region (Xiao et al. 2020). In this experiment, the amount of nitrogen applied was 10 g N m^{-2} yr^{-1} and the N addition treatments were applied by dissolving 4 g of urea in 160 mL of water each time and applying it evenly to the microhabitat of each N treatment. Nitrogen was added once in June, July and early August, where each addition was one-third of the planned additive amount for the year. For treatments without added N, only equal amounts of water were added at the same time of N addition. For the *L. virgaurea* treatment, two evenly sized and well grown *L. virgaurea* seedlings were selected in the meadow, the PVC tubes were added surrounding these already established seedlings of *L. virgaurea*. We expect that the soil already contains allelochemicals. We did not choose to plant or transplant *L. virgaurea*, because we had a low survival after several attempts. For the without *L. virgaurea* treatment, we selected plots without the presence of *L. virgaurea* and framed gap soil where other native plants have been removed with PVC tubes embedded in the soil. In all treatments, we removed other native plants that were excluded from natural growth in the microenvironment (in the *L. virgaurea* treatment we only left *L. virgaurea*). A spade was used to obtain 0–5 cm of soil from each microenvironment and a sieve (0.2 cm pore size) was used to remove plant and fine root debris. To prevent the seed bank at the bottom of the microenvironment from interfering with our experiments, we laid down a layer of gauze. Finally, we filled the screened soil on top of the gauze. We sowed a one-time surface mixture of seeds from three species commonly found in alpine grasslands in the microenvironments, including *Elymus nutans* Griseb. (Poaceae), *Delphinium kamaonense* Huth var.glabrescens (Ranunculaceae), and *Tibetia himalaica* (Baker) H. P. Tsui (Fabaceae), thereafter referred to by their genus names. To prevent the seeds from being blown away by the wind, we cover the surface with a small amount of soil.

All seeds used in this study were collected in the autumn of 2020 in alpine meadows on the Tibetan Plateau and stored at room temperature. Before sowing, the seeds were cut to check their viability, which was between 95% and 100% depending on the species. From each species, 50 mature seeds were selected and sown in each microenvironment, and the number of seed germination was recorded every 15 days. At the start of September, above-biomass and below-biomass of plant were harvested. The biomass in each pot was separated by species, and all soil was washed from the roots and dried at 65 °C for 72 h. In each PVC tube (microenvironment), soil from the topsoil was collected with a soil auger (4 cm diameter). At each sampling, the shovel was cleaned, scrubbed with 75% alcohol wipes and air dried. Soil samples were packed in polyethylene bags and dispensed in 5 ml PE tubes, immediately stored in coolers with ice packs and transported to the laboratory. Soil samples were sieved (2 mm) and all visible roots, debris and stones were removed. Soil samples packed in polyethylene bags were stored at 4◦C for measuring the biogeochemical properties and enzymes of the soil, and soil dispensed in PE tubes stored at -80◦C for soil DNA extraction.

Determination of physical and chemical properties of soils

The soil water content (WC) used gravimetric analysis, i.e., 30 g of fresh soil was weighed in an oven at 105◦C for 48 h until a constant weight was reached. Soil pH was measured using a calibrated pH meter (PHS-3 C) with a fresh soil to water ratio of 1:5. The electrical conductance (EC) is determined by the electrode method using a conductivity meter. Soil organic carbon (SOC) was determined using the potassium dichromate volumetric method (Bao 2000). Soil total nitrogen (TN) content was determined by the semi-micro Kjeldahl method (Bao 2000). Soil total phosphorus (TP) was determined by the alkali fusion-Mo-Sb Anti spectrophotometric method (Bao 2000). The determination of soil fast-acting nitrogen (nitrate, NO_3^-; nitrate ammonium, NH_4^+) was performed by KCl leaching and indophenol blue colorimetric method. The content of fast total nitrogen, total phosphorus and fast-acting nitrogen was determined using a flow analyzer (Smartchem 200). Soil available phosphorus (AP) was determined using a 0.5 mol L^{-1} $NaHCO_3$ leaching-molybdenum antimony colorimetric method (Wang et al. 2022).

Determination of soil microorganisms and enzymes

Each soil sample was weighed at 0.25 g and soil DNA was extracted using the PowerSoil® DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA). Bacterial DNA was extracted using primers 515FB/806RB (GTG YCAGCMGCCGCGGTAA / GGACTACNVGGGTWT CTAAT) (Podell et al. 2019) and fungal DNA was amplified using primers ITS1F/ITS2 (CTTGGTCATTTAGAG GAAGTAA / GCTGCGTTCTTCATCGATGC) (Coince et al. 2013), respectively. The PCR products were then paired-end sequenced using the Illumina Novaseq PE250 platform. The double-end sequences were de-hybridized separately using Trimmomatic software: the sequences below 100 bp in length were finally removed. High-quality double-end sequences were concatenated using flash software with a minimum overlap region of 10 bp and a maximum mismatch rate of 0.2, and sequences containing ambiguous base N were removed. The quality-controlled double-end sequences were noise reduced using the dada2 plug-in of QIIME software, and then the valid sequences were clustered into Amplicon Sequence Variants (ASVs) for each sample based on the 97% sequence similarity. Finally, after sparsifying the rest of the samples based on the lowest ASV number among all samples, the resulting data tables can be used for subsequent analysis. The bacterial and fungal classifications were analyzed against SILVA (Pruesse et al. 2007) and UNITE (Kõljalg et al. 2005), respectively, and this process yielded a total of 27,354 bacterial OTUs and 3617 fungal OTUs.

In this study, three hydrolytic enzymes were measured, namely β-glucosidase related to carbon metabolism, N-acetyl-β-D-glucosaminidase related to N metabolism, and acid phosphatase related to phosphorus metabolism. The determination was performed by the methods supplied by the kit manufacturer (Suzhou Keming Biotechnology Co., Ltd., Suzhou, China). Then we measured the light density of the samples by colorimetric method. The enzyme activity was shown in µmol $day^{-1}g^{-1}$ dry soil.

Data analysis

The experiment used a block design, thus we specified block as a random effect in all models. Allelopathic plants, N addition and their interactions were considered as fixed factors. Seed germination, early plant survival, and relative abundance of soil microbes were analyzed using generalized linear mixed models with a binomial distribution. Microbial richness was analyzed using generalized linear mixed models with a negative binomial distribution. We also used linear mixed-effects models to assess effects of treatments on plant biomass, root-to-shoot ratio, soil properties, soil enzyme activity, bacterial and fungal Shannon diversity. In addition, for species-level analyses, we also considered the number of neighbouring species nested within block as a random effect. We used testUniformity function and testDispersion function to diagnose the model. For the residuals of the linear mixed models that did not satisfy the assumption of uniformity, we performed a log transformation of the response variable. For the residuals of generalized linear mixed models with over dispersion, we used the glmmTMB function with a beta binomial distribution. $P < 0.05$ was significant and $p < 0.1$ was marginally significant.

We conducted a categorical random forest analysis to determine the relative importance of soil physical variables, microbial community and enzyme activity in explaining seed germination, plant survival, biomass and root-shoot ratio. Prediction accuracy was averaged across all trees (10,000) to produce a final importance measure (Breiman 2001), and then we selected the most important attributes to construct piecewise structural equation modelling (piecewise SEM). Subsequently, piecewise SEM were used to synthesize the direct and indirect relationships of allelopathic plants (*L. virgaurea*), N addition and their interactions on seed germination, plant survival, biomass and root to shoot ratio via biotic and abiotic pathways. Adequacy of piecewise SEM was assessed by Shipley's d-separation test with Fisher's C-statistic (Shipley 2009).

All data were analyzed with R version 4.1.0 (R Core Team 2021). We used the freq.calc function in the R package 'spaa' to calculate relative abundance of microbes. We used the specnumber and diversity functions in the R package 'vegan' to calculate richness and Shannon diversity, respectively. The functions of the diagnostic model were derived from the 'DHARMa' package (Hartig 2019) and the 'glmmTMB' package was used to assess the significance of the model (Brooks et al. 2017). The 'lme4' package was used for conducting mixed-effects models. The 'emmeans' package was used to compute least squares means and standard errors (Lenth et al. 2020), the 'piecewiseSEM' package was used to compute piecewise SEM (Lefcheck 2016), and the 'ggplot2' package (Wickham 2008) was used to plot figures.

Results

Soil properties, microbial community composition, and enzyme activity

The results of our linear mixed model showed that N addition increased soil NH_4^+ (Table 1; Table S2). While the pair-wise test showed no significant differences in soil EC and WC between different treatments, there was a trend for N addition decreased soil EC and *L. virgaurea* increased soil WC (Table 1; Table S2). The negative effects of the presence of *L. virgaurea* on soil pH and BD tended to be reduced by N addition, and the positive effects of the presence of *L. virgaurea* on the soil TP tended to be reduced by N addition (Table 1; Table S2). There was no significant effect of N addition and the presence of *L. virgaurea* on SOC, NO_3^-, TN and the AP (Table 1).

We found that the presence of *L. virgaurea* increased soil bacterial richness (Fig. 1a; Table 2). Bacterial Shannon diversity, soil fungal richness, and fungal Shannon diversity were not significantly affected by the treatments (Fig. 1b-d; Table 2). The relative abundance of bacteria belonged mostly to eight different phyla and the relative abundance of fungi belonged mostly to three different phyla (Table 1). We found that there was a trend for the presence of *L. virgaurea* increased the relative abundance of Actinobacteriota (Table 1; Table S2). Besides, there was an interactive effect between N addition and the presence of *L. virgaurea* on the relative abundance of Proteobacteria and Acidobacteriota (Table 1). Specifically, the negative effect of the presence of *L. virgaurea* on the relative abundance of Proteobacteria was reversed by N addition, and the positive effect of the presence of *L. virgaurea* on the

Table 1 Results from generalized linear mixed models on soil microbial community composition and from linear mixed model on soil properties at the end of the experiment

		Nitrogen (N)		*L. virgaurea* (L)		N×L	
Soil properties	df	F values	*P* values	F values	*P* values	F values	*P* values
WC	1	0.51	0.485	3.69	*0.069*	1.76	0.198
EC	1	4.56	**0.045**	0.79	0.382	0.79	0.386
pH	1	0.23	0.634	0.14	0.710	4.10	*0.056*
SOC(%)	1	1.97	0.175	0.17	0.840	0.01	0.940
TP(g kg^{-1})	1	1.03	0.322	1.82	0.191	4.16	*0.054*
TN(g kg^{-1})	1	0.03	0.861	0.04	0.839	0.00	0.995
NH_4^+	1	5.45	**0.030**	0.33	0.569	0.00	0.976
NO_3^-	1	0.01	0.915	0.66	0.426	0.58	0.455
BD	1	0.24	0.627	1.16	0.290	3.06	*0.091*
AP	1	0.20	0.658	0.65	0.429	1.63	0.216
Relative abundance of 11 most abundant lineages							
		Chisq	*P* values	Chisq	*P* values	Chisq	*P* values
Proteobacteria	1	0.00	0.946	2.49	0.114	18.62	**< 0.001**
Acidobacteriota	1	0.58	0.445	0.03	0.872	5.14	**0.023**
Actinobacteriota	1	0.64	0.422	3.21	*0.073*	2.02	0.156
Verrucomicrobiota	1	1.20	0.274	1.00	0.317	0.14	0.709
Gemmatimonadota	1	0.22	0.640	2.29	0.130	1.27	0.260
Bacteroidota	1	2.29	0.130	0.28	0.599	0.27	0.601
Firmicutes	1	0.00	0.984	0.00	0.980	0.00	0.997
Chloroflexi	1	1.08	0.300	0.08	0.772	0.05	0.828
Ascomycota	1	0.03	0.871	0.43	0.512	2.39	0.122
Basidiomycota	1	0.00	0.949	0.01	0.904	0.72	0.398
Mortierellomycota	1	0.11	0.745	0.53	0.466	0.52	0.471

Nitrogen addition (N), *L. virgaurea* (L) and their interactions were considered fixed factors. Differences shown in bold and italic are statistically significant at $P < 0.05$ and $P < 0.1$, respectively. *WC* water content, *EC* electrical conductivity, *SOC* soil organic carbon, *TP* total phosphorus, *TN* total nitrogen, NO_3^-: nitrate nitrogen; NH_4^+: ammonium nitrogen; *BD* bulk density, *AP* available phosphorus

Fig. 1 Effects of *L. virgaurea*, nitrogen addition and their interactions on (**a**) bacteria richness, (**b**) bacteria Shannon diversity, (**c**) fungal richness, (**d**) fungal Shannon diversity. The different lowercases show significant between treatments ($P < 0.05$). Error bars represent means ± standard error. Table 2 for details of models' outputs

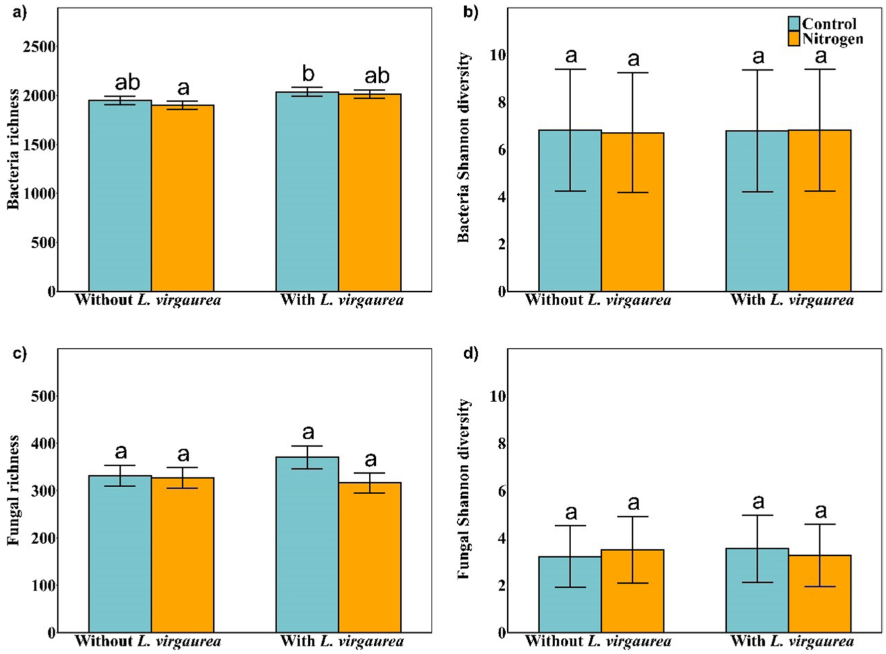

Table 2 Results from generalized linear mixed models on microbial richness and from linear mixed model on soil enzyme activity and microbial Shannon diversity at the end of the experiment

		Nitrogen (N)		*L. virgaurea* (L)		N×L	
Soil enzyme activity	df	F values	*P* values	F values	*P* values	F values	*P* values
N-acetyl-β-glucosaminidase	1	0.06	0.810	0.08	0.785	1.85	0.188
Soil β-glucosidase	1	0.26	0.612	0.21	0.655	0.87	0.360
Soil acid phosphatase	1	10.68	**0.004**	4.67	**0.042**	8.88	**0.007**
Soil microbial diversity							
Bacterial Shannon diversity	1	1.06	0.313	0.48	0.493	1.82	0.188
Fungal Shannon diversity	1	0.00	0.988	0.02	0.877	1.36	0.253
		Chisq	*P* values	Chisq	*P* values	Chisq	*P* values
Bacterial richness	1	0.67	0.412	5.37	**0.021**	0.11	0.740
Fungal richness	1	2.02	0.155	0.40	0.527	1.38	0.240

Nitrogen addition (N), *L. virgaurea* (L) and their interactions were considered fixed factors. Differences shown in bold and italic are statistically significant at $P < 0.05$ and $P < 0.1$, respectively relative abundance of Acidobacteriota was decreased by N addition (Table S2).

We found that the presence of *L. virgaurea* increased soil acid phosphatase by 4.7% (Fig. 2c; Table 2), while N addition reduced soil acid phosphatase by 6.8%. In addition, the interaction between N addition and *L. virgaurea* on soil acid phosphatase was significant (Fig. 2c; Table 2). Specifically, the increase in soil acid phosphatase occurred only in the presence of *L. virgaurea*, and the N addition was able to counterbalance this effect. There was no significant effect of treatments on soil N-acetyl-β-glucosaminidase and soil β-glucosidase (Fig. 2a, b; Table 2).

Seed germination, early plant survival, and growth

The presence of *L. virgaurea* increased the germination by 46.9%, plant survival by 36%, and biomass by 190.8%, respectively (Fig. 3a-c; Table 3). The presence of *L. virgaurea* decreased root–shoot ratio by 46.9% (Fig. 3d; Table 3). Nitrogen addition increased early plant survival by 30.7% and increased biomass by 143.4% (Fig. 3b-c; Table 3). Moreover, the interaction between N addition and *L. virgaurea* had a significant effect on seed germination: the positive effect of the presence of *L. virgaurea* on seed germination was offset by N addition (Fig. 3a; Table 3). At the species level, our results showed that the presence of *L. virgaurea* increased the germination of *Elymus, Delphinium*, and *Tibetia* (Fig. S2a-c). There was a marginally significant interaction effect on germination of *Delphinium* (Fig. S2b). The presence of *L. virgaurea* increased early survival of *Elymus* (Fig. S3a). There was a trend of increased early survival of *Delphinium* with N addition (Fig. S3b). The presence of *L. virgaurea* alone and the addition of N alone increased biomass of *Elymus* (Fig. S4a). Moreover, the presence of *L. virgaurea* alone and N addition alone positively affected the root-shoot ratio of *Tibetia* (Fig. S5c). We found that seed germination, biomass, and root–shoot ratio were all significantly affected by species identity, except for early survival (Table S3).

Our SEM explained 61%, 36%, 40%, 37% of the variation in the germination, survival, biomass, and root–shoot ratio, respectively (Fig. 4a-d). Specifically, the SEM analysis suggested that the presence of *L. virgaurea* had a direct positive effect on seed germination. In addition, the presence of *L. virgaurea* had an indirect positive effect on germination via acid phosphatases. Nitrogen addition had an indirect positive effect on germination via NH_4^+ and WC, but N addition also indirectly negatively affected germination via acid phosphatases (Fig. 4a). This may mean

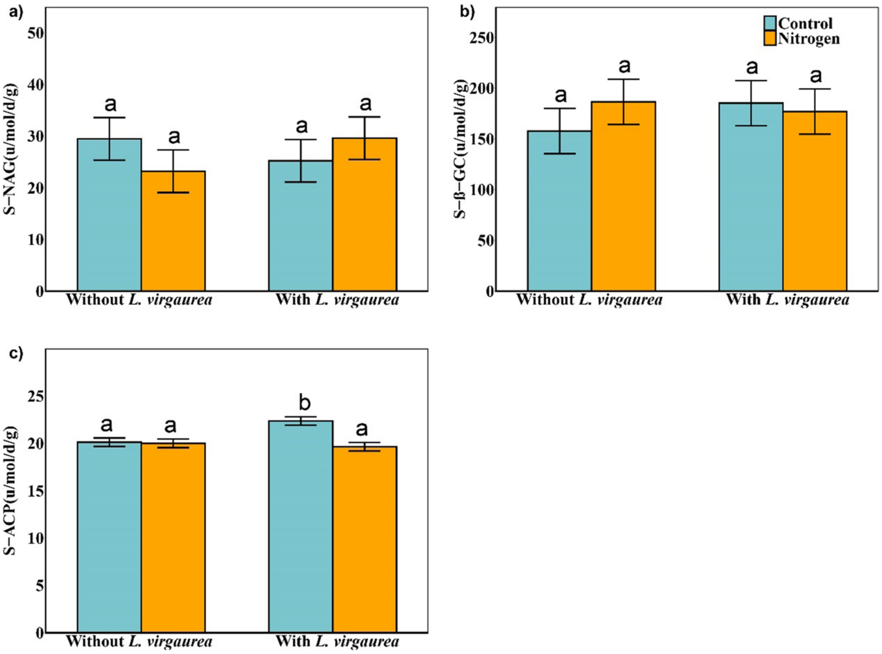

Fig. 2 Effects of *L. virgaurea*, nitrogen addition and their interactions on (**a**) N-acetyl-β-glucosaminidase (S-NAG), (**b**) soil β-glucosidase(S-β-GC), (**c**) soil acid phosphatase (S-ACP). The different lowercases show significant between treatments ($P < 0.05$). Error bars represent means ± standard error. Table 2 for details of models' outputs

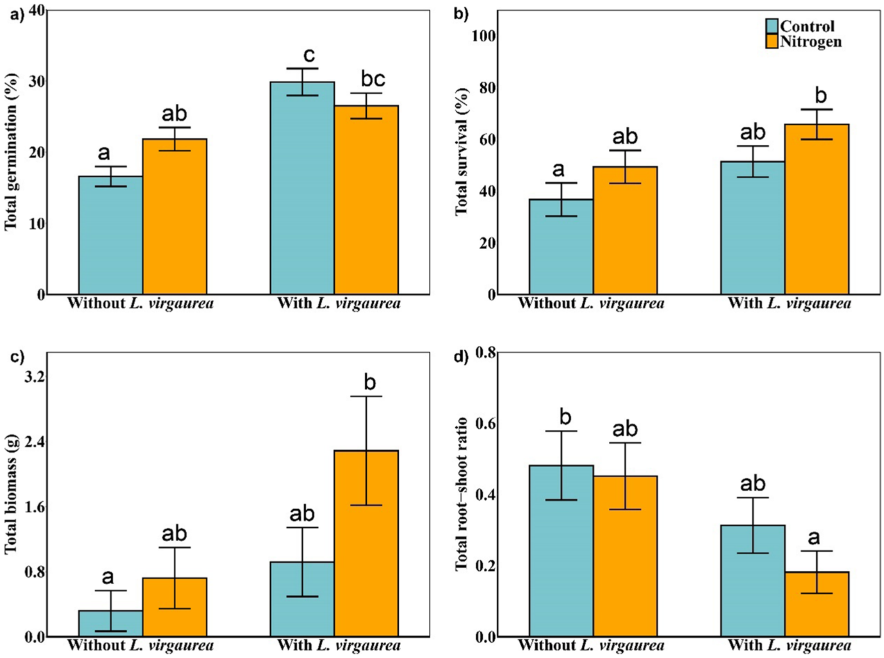

Fig. 3 Effects of *L. virgaurea*, nitrogen addition and their interactions on (**a**) total seed germination, (**b**) total plant early survival, (**c**) total plant biomass, and (**d**) total root-shoot ratio for all species (*Elymus, Delphinium*, and *Tibetia*). The different lowercases show significant between treatments ($P < 0.05$). Error bars represent means ± standard error. Table 3 details of models' outputs

Table 3 Results from generalized linear mixed models on seed germination and plant survival and from linear mixed model on biomass and root-shoot ratio at the end of the experiment

Fixed-factors		Seed germination		Plant survival		Plant biomass		Root-shoot ratio	
	df	Chisq	*P* values	Chisq	*P* values	F values	*P* values	F values	*P* values
Nitrogen (N)	1	1.58	0.208	4.69	**0.030**	3.60	*0.068*	1.41	0.248
L. virgaurea (L)	1	53.89	**< 0.001**	6.16	**0.013**	5.70	**0.024**	8.45	**0.008**
N×L	1	13.31	**< 0.001**	0.02	0.877	0.37	0.549	0.73	0.403

Nitrogen addition (N), *L. virgaurea* (L) and their interactions were considered fixed factors. Differences shown in bold and italic are statistically significant at $P < 0.05$ and $P < 0.1$, respectively

that there was no significant effect of N addition on seed germination by positive and negative offsetting each other (Fig. S6a). The interaction between N addition and the presence of *L. virgaurea* negatively affected germination via acid phosphatases (Fig. 4a). The presence of *L. virgaurea* alone or N addition alone had a direct positive effect on survival (Fig. 4b). Nitrogen addition had a direct positive effect on biomass; the presence of *L. virgaurea* had an indirect positive effect on biomass via bacterial richness (Fig. 4c). Moreover, there was a direct negative effect of *L. virgaurea* on root-shoot ratio; *L. virgaurea* also positively affected the root-shoot ratio indirectly through bacterial richness and Shannon diversity (Fig. 4d). However, the total effect of *L. virgaurea* was a negative effect on the root-shoot ratio (Fig. S6d).

Discussion

Positive effects of *L. virgaurea* on seed germination, early survival, and biomass, and negative effects on plant root-to-shoot ratio

We found that the presence of *L. virgaurea* significantly increased seed germination, early survival and biomass of alpine plants. Several previous studies found that some plants secrete allelochemicals that exert negative effects on neighboring plants and exhibit negative

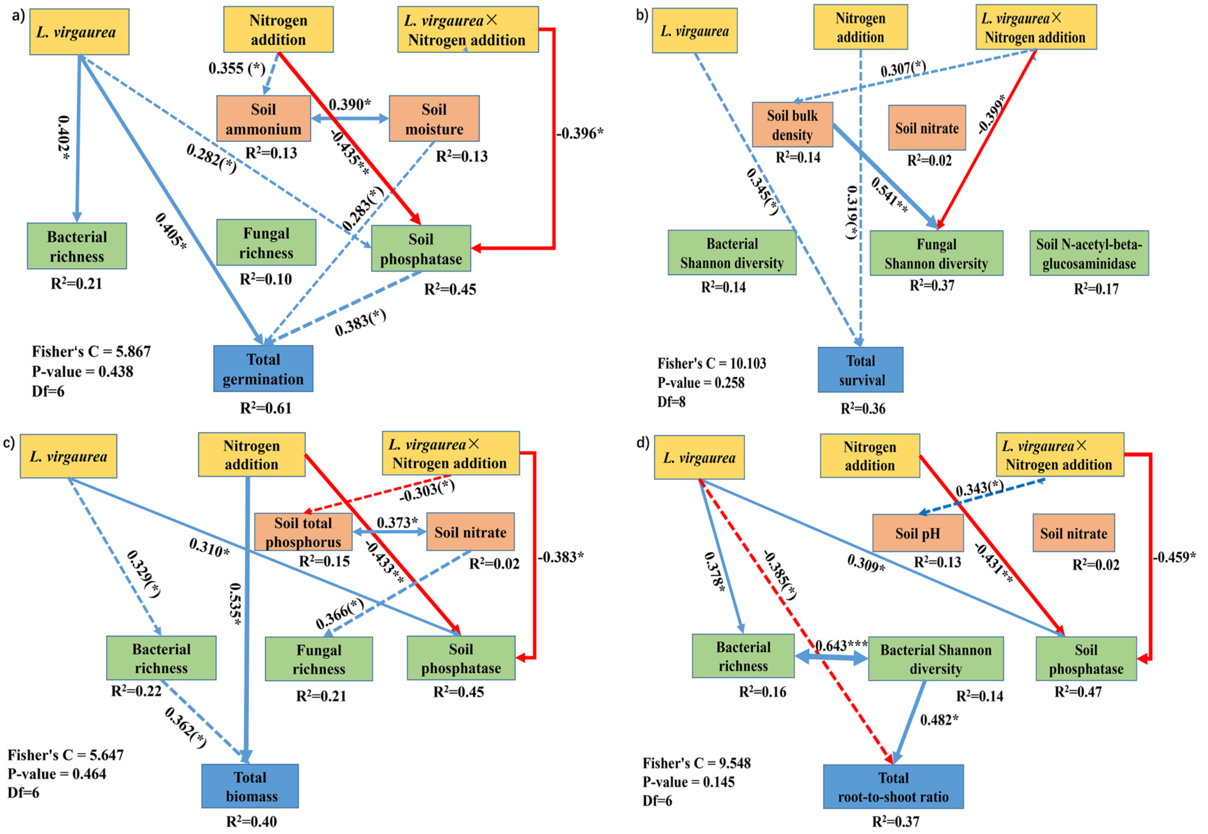

Fig. 4 Results of the SEM analyses indicating effects of *L. virgaurea*, nitrogen addition and their interactions on (**a**) seeds germination (**b**) plant early survival, (**c**) plant biomass, and (**d**) root-shoot ratio. Only marginally significant and significant pathways were shown. Blue and red solid arrows indicate significant positive and negative effects (at the level $P < 0.05$), respectively. While the dashed arrows indicate marginally significant effect (at level $P < 0.1$). Arrow width corresponds directly to the standardized path coefficient. R^2 values associated with response variables indicate the proportion of explained variation by relationships with other variables. Values associated with arrows represent standardized path coefficients. (*): $P < 0.1$, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$

effects on the seed germination and plant performance (Callaway and Ridenour 2004; Inderjit et al. 2011; Kim and Lee 2011). In our study, the observed effects may be because *L. virgaurea* densities are not high enough to have a negative impact. It has been found that at low densities, *L. virgaurea* has a positive effect on the maintenance of the productivity and diversity of grasslands (Wang et al. 2008). We found that aboveground biomass was higher than belowground biomass in the presence of *L. virgaurea*, which may be related to the shade of *L. virgaurea*. For example, Reich (2014) proposed that leaf traits are closely related to plant ecological processes, and in alpine meadow plant communities, *L. virgaurea* has a relatively large leaf area that favors high light interception, can lead to the growth of above-ground parts of plants in its lower layers to compete for light resources. In the absence of light, plants allocate more biomass to aboveground parts to compete for space and light for their own growth and reproduction (Mokany et al. 2006). However, light interception data was not measured in this study to reflect the shading effect of *L. virgaurea*. Therefore, future studies need to be refined in order to obtain a more comprehensive and reasonable explanation.

Our piecewise SEM results showed that *L. virgaurea* increased seed germination both directly, and indirectly through increasing soil acid phosphatase. Shi et al. (2011) had previously showed an increased phosphatase activity under *L. virgaurea* patches compared with normal grassland, which was associated with increased soil microbial biomass through the quality and quantity of litter and exudates. Soil acid phosphatase has a crucial catalytic function in promoting seed germination (Seneviratne et al. 2019) and we were able to detect a trend confirming this effect. Moreover, we found that *L. virgaurea* increased plant biomass via soil bacterial community richness. It has been previously shown that an allelopathic plant (*Acacia dealbata*) alters soil microbial community structure and increases bacterial richness (Lorenzo et al. 2010). Our results suggest that the increase in bacterial richness promoted by the presence of *L. virgaurea* influenced nutrient cycling in the soil and provided an enhanced soil environment for plant growth. In addition, *L. virgaurea* had no significant effect on fungal communities. This result may be due to the fact that soil fungi are more stable than bacteria and less affected by allelochemicals, litter and other mechanisms (Agnelli et al. 2004; Lorenzo et al. 2013). We found that *L. virgaurea* was associated with an increase in bacterial richness, which in turn affected Shannon diversity indirectly affecting the root-to-shoot ratio. A recent study showed significant changes in the structure and diversity of soil microbial communities in plant communities dominated by *L. virgaurea* and *L. sagitta* (Ade et al. 2021). In our study, *L. virgaurea* had a tendency to increase the relative abundance of Actinomycetes. Actinomycetes can protect seeds and plants using enzymes and anti-fungus compounds to inhibit the growth of potential pathogens (Jones et al. 2017).

Positive effects of N addition on early survival and plant biomass

Our results showed that N addition increased seedling survival and biomass. Alpine meadows are distributed in high altitude areas all over the world, and are a common ecosystem in the Qinghai-Tibet Plateau. Despite high soil N stores, N limitation is frequent because of slow mineralization of soil organic matter under low temperature and water deficiency in alpine meadow ecosystems (Baumann et al. 2009; Chen et al. 2013). Nitrogen addition can increase the photosynthetic efficiency of plants, thereby increasing plant survival. In addition, plants can accumulate more above-ground biomass when soil N resources are unrestricted (Wang et al. 2022). Therefore, increasing N inputs can increase the amount of available N in the soil, eliminate or alleviate N limitation, improve seedling survival, and promote plant growth (Meng et al. 2021; Zhao et al. 2021; Cai et al. 2023).

Different functional groups respond differently to N addition: we found a significant effect of N addition only on the biomass of *Elymus* (grass), but not on that of the other two species, *Delphinium* (forb) and *Tibetia* (legume). Grasses are tall plants in alpine herb communities and have fibrous root systems. Thus, *Elymus* (a dominant grass in these communities) may compete rapidly and effectively for water, nutrients and light in alpine communities (Hautier et al. 2009), while *Delphinium* and *Tibetia* are mainly found in the understory. Although N addition can alleviate the nutrient limitation of herbaceous growth (Alvarez-Clare et al. 2013), the lack of effect on *Delphinium* and *Tibetia* could be due to the enhanced shading effect of the tall and fast growing *Elymus* forming the upper canopy .Based on the SEM results, we found that ammonia nitrogen and water content associated with N addition had an indirect positive effect on germination, but N addition had a negative effect by affecting acid phosphatase. These effects may counteract each other, resulting in no significant effect of N addition on germination.

Negative effects of interaction between *L. virgaurea* and N addition on seed germination

We found that N addition counterbalanced the increase in seed germination in the presence of *L. virgaurea* by exerting an opposing negative effect on acid phosphatase activity. Acid phosphatase is mainly produced by plant roots (Speir and Cowling 1991; Susanne KraÈmer and Green 2000). We observed that N addition inhibited the positive effect of *L. virgaurea* on acid phosphatase. We speculated that with increased N, plants may reduce the allocation of belowground resources by reducing root yield and using more nutrients for aboveground growth and reproduction (Wallenstein et al. 2006; Kiær et al. 2013). That altered allocation strategies of allelopathic plants have potential negative effects on the synthesis and activity of soil acid phosphatase. Thus, the simultaneous presence of allelopathic plants and N addition is unfavourable for seed germination. In addition, our results showed that the coverage of *L. virgaurea* was significantly higher with the N addition. This was perhaps due to the fact that N addition increases N availability, which stimulates *L. virgaurea* growth and canopy cover, which can increase its capacity to intercept light. Therefore, with

N addition, light availability may be lower under canopy of *L. virgaurea*. Light is one of the key factors for seed germination, light can also regulate the levels of many enzymes and hormones that affect seed respiration and metabolism, as well as the transport of substances (Li et al. 2020). Thus, the interaction between N addition and *L. virgaurea* reduces seed germination, suggesting that it is necessary to consider the potential interaction of multiple factors for seed germination and early seedling establishment.

Our results showed that there was an interaction between N addition and *L. virgaurea* on the relative abundance of Proteobacteria. In particular, nitrogen addition counteracted the negative effect of *L. virgaurea* on the relative abundance of Proteobacteria, possibly because Proteobacteria are eutrophic bacteria that thrive in nutrient-rich environments (Dai et al. 2018). Consequently, N addition may be beneficial for Proteobacteria colonization. Previous studies have shown a positive correlation between Proteobacteria abundance and plant growth under shrubs in soil ecosystems (Lozano et al. 2017), however, we did not observe such an association in our study. Perhaps the duration of our experiment was relatively short, and future studies should take into account whether temporal changes in microbial abundance can exert significant impacts on plant growth.

Conclusion

Our results indicate that the presence of allelopathic plants and N addition may not only individually affect the regenerative stages of alpine plant communities on the Tibetan Plateau but also mutually modulate the effects of each other. We found a positive effect of allelopathic plants alone and/or N addition alone on seed germination, seedling survival and growth. However, the positive effect of allelopathic plants on seed germination can be cancelled out by N addition. Such phenomena may be attributed to the decrease of soil acid phosphatase associated with allelopathic plants by N addition. Overall, our study highlights the need to understand the interactions between factors associated with climate change (e.g., N deposition) and the presence of allelopathic plants to better predict plant community dynamics and whether management and conservation measures succeed or fail in the alpine grasslands of the Tibetan Plateau.

Acknowledgements This work was supported by the National Natural Science Foundation of China (41830321, 32071532, 31870412), Joint Funds of National Natural Science Foundation of China (U21A20186), the “111 Project” (BP0719040), the Natural Science Foundation of Gansu Province (22JR5RA402), the Second Tibetan Plateau Scientific Expedition and Research:Program (2019QZKK0302) and Core Facility of School of Life Sciences, Lanzhou University. We thank Yanhu Li and Yaya Chen for helpful comments on this work. We are also grateful to the editors and anonymous reviewers for many valuable comments on our manuscript.

Author contributions S.X. and J.W. designed the experiments; K.L., Z.L., X.J., J.L., J.C., H.C., L.A., H.S., Z.Y., J.W. and Y.W. collected field and laboratorial data. J.W. and S.X. performed statistical analysis and made the figures. J.W. drafted the manuscript. S.C., S.P.B. and S.X. revised the manuscript. All authors contributed to the manuscript.

Data availability The datasets are available from corresponding authors upon reasonable request.

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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