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Optimising management practices to secure grassland agroecosystems' sustainability

Edited by
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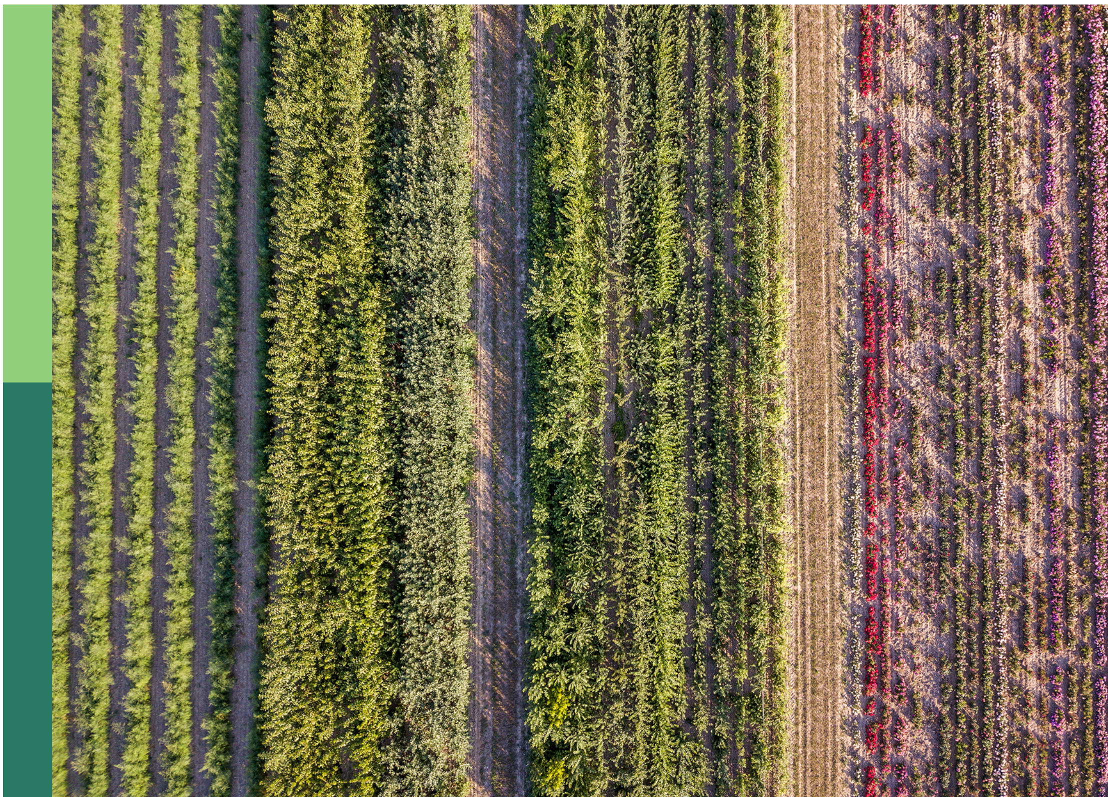

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Optimising management practices to secure grassland agroecosystems' sustainability

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Editorial: Optimising management practices to secure grassland agroecosystems' sustainability

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Editorial on the Research Topic
Optimising management practices to secure grassland agroecosystems' sustainability

Globally, the escalating and diverse pressures on natural and modified ecosystems urgently necessitate a paradigm shift in agricultural practices. In this new paradigm enhancing soil health and biodiversity is pivotal to guarantee global food security. Traditional agricultural systems, while effective historically, have shown limitations in sustaining productivity and ecological balance under contemporary societal challenges. This is especially true for grassland agroecosystems, which are vital for sustainable agriculture due to their ability to improve soil health, sequester carbon, and support biodiversity.

Grassland farming, integrating grasses and legumes, offers multifaceted benefits, such as reducing soil and water erosion, providing high-quality livestock feed, enhancing soil fertility, and facilitating carbon sequestration. These advantages are critical for ensuring the productivity, sustainability, and resilience of agroecosystems, particularly in the face of climate change.

This Research Topic aims to address the urgent need for optimized management practices in grassland agroecosystems to ensure sustainable agriculture and efficient resource use. The focus includes strategies like how management of grassland forage genetic diversity, grazing-density, and fertilizer application can help mitigate climate and environmental change and augment soilmicrobial diversity, tolerance, and soil function. This Research Topic seeks to answer critical questions on the adaptability and resilience of these systems.

The Research Topic comprises 17 articles, which explore several key areas:

1. Soil microbial communities and environmental stressors:

One study explores the effects of short-term drought and nitrogen application on soil microbial communities in alfalfa (*Medicago sativa L.*) grasslands of the Chinese Loess Plateau, one of the largest plateaus in the world. The findings revealing significant changes in soil organic carbon components, enzyme activity, and microbial diversity (Wang et al.). Another article investigates the influence of grazing densities and topographical positions on soil biochemical and microbial properties of mixed-grass prairie ecosystem in the North Dakota of the United States, highlighting the importance of intermediate grazing density for enhancing soil health and microbial activity (Bansal et al.). Another study examines the influence of nitrogen input forms and rates on phosphorus availability in karst grassland soils, underscoring how specific nitrogen forms impact soil phosphorus availability, plant root biomass, and enzyme activity, essential for sustainable nutrient management in these sensitive ecosystems (Zhou et al.).

2. Genetic diversity and grassland plant tolerance:

Research on the genetic diversity and salt tolerance of 51 alfalfa varieties under saline soil conditions in eastern China demonstrates significant genetic variation and identifies specific varieties with strong tolerance, providing a basis for improving saline-alkali land management (Fan et al.). In another study, the agronomical characters of alfalfa in acidic soils in southwest China identified varieties with excellent total dry weight and fresh weight, offering insights for developing alfalfa strains resistant to acidic soil conditions (Tian et al.). Additionally, an analysis on *Bacillus amyloliquefaciens* PG-4 inoculation in *Macrotyloma uniflorum* demonstrates improved salt stress tolerance, highlighting PGPR's role in enhancing plant resilience under abiotic stresses, crucial for fodder crops in marginal soils (Wu et al.).

3. Soil function and forage quality:

Studies on tropical range grasses in semiarid degraded lands and the effects of oat (*Avena sativa L.*) -pea (*Pisum sativum L.*) seeding ratios on the Qinghai-Tibetan Plateau highlight the potential of these practices to sustain soil functions, such as nutrient cycling and soil fertility, through improved nutrient availability, and improve silage quality, respectively (Liu et al.). An analysis of the productivity, water, and nitrogen utilization of intensified dryland farming with annual forages on the Chinese Loess Plateau suggests that integrating forage crops, such as forage rape and common vetch, into cropping systems can enhance soil fertility by increasing nitrogen retention and availability, and crop productivity (Deng et al.). Another study demonstrates how soil functionality is influenced by the plant maturity stage in a grass-legume system, noting significant nitrogen loss reduction measures for stages when nutrient cycling slows, which could inform better nitrogen management (Xie et al.). Research on a green manure-maize rotation on the Yunnan-Guizhou Plateau indicates that leguminous amendments like alfalfa and common vetch significantly boost maize yield, nitrogen use efficiency, and phosphorus use efficiency, underscoring the agronomic benefits of integrating legumes into maize systems (Gao et al.). Further research on tropical range grasses in semiarid degraded lands shows that certain species, such as Tri-Specific Hybrid (TSH) and Heteropogon contortus, exhibit resilience in sustaining soil functions despite nutrient depletion, emphasizing the role of unmanaged grass cover in maintaining carbon accumulation and nutrient cycling in degraded soils of the Bundelkhand area in India (Patidar et al.).

4. Carbon sequestration and climate mitigation:

The carbon balance of boreal legume grasslands in Finland under different management practices highlights the importance of organic fertilizers in enhancing carbon sequestration and sustaining ecosystem services, such as soil fertility, nutrient cycling, and climate regulation (Li et al.). Comparative analyses of lucerne termination methods using herbicides, rather than traditional tillage on the Loess Plateau demonstrate the effectiveness of herbicides in maintaining soil carbon and nitrogen contents, providing alternatives to conventional tillage (Zhao et al.). Additionally, Kentucky bluegrass studies in varying plantation ages in the Qinghai-Tibetan Plateau of China have revealed dynamic changes in plant and soil C:N:P stoichiometry, indicating that established pastures over 6 years can better support nutrient cycling and soil quality, critical in carbon sequestration efforts (Wei et al.).

5. Farmer behavior and risk management:

A study on the influence of risk preferences on forage planting behaviors among farmers in China's agro-pastoral zone highlights the role of risk management in promoting sustainable forage cultivation (Zhang et al.). Another article exploring crop-livestock integration demonstrates a system for resource efficiency and livelihood improvement, offering a model with greater nutrient cycling, reduced emissions, and enhanced food security for smallholders in vulnerable regions (Shanmugam et al.). Additionally, research on forage yield, competition, and economic indices of oat and common vetch intercrops in a semi-arid region provides insights into optimizing intercropping ratios for maximum productivity and economic return, supporting resource-efficient agricultural practices in water-scarce environments (Jiao et al.).

The findings in these articles highlighting the role of plant diversity in grassland agroecosystems, demonstrating that integrating legumes into grass-based systems can contribute to the development of sustainable agricultural practices that are resilient to environmental changes, improving soil function, and helping meet the growing demands for food security and soil health. We would like to extend our gratitude to all authors, reviewers, and editorial team members for their dedication and expertise, which have made this Research Topic possible.

Author contributions

ZL: Conceptualization, Writing – original draft. YL: Conceptualization, Writing – review & editing. BY: Writing – review & editing. CC: Writing – review & editing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Soil properties and silage quality in response to oat and pea seeding ratios and harvest stage on the Qinghai-Tibetan Plateau

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Livestock intensification improves production efficiency and enhances the demand for quality forage to feed ruminants. Novel combinations of forage plants, especially including Gramineae and Leguminous plants, benefit both ruminant animals and contribute to a sustainable environment. This study explored an oat-pea mixed seeding strategy as an approach to improving silage quality. Before ensialing, lactic acid bacteria (*Lactobacillus plantarum* and *Lactobacillus brucelli*) were added to forage from five different mixed seeding proportions of oats (O) and peas (P) (10:0, 8:2, 7:3, 5:5, and 0:10 oat to pea ratio) at two harvesting periods (the early flowering stage and the milk ripening stage for the oats). The results showed that mixed seeding changed the soil quality parameters. Moreover, the silage from the O5P5 (5:5 oat to pea ratio) group showed the lowest pH values (4.16) and highest LA contents (7.74% DM) after ensiling for 7d ($p < 0.05$) in early flowering stage. Also, the O5P5 group increased the number of tillers/branches and produced silage with the highest CP content (13.14–14.06) after ensiling for 7d in early flowering stage and both 7d and 30d in early flowering stage and milk ripening stage ($p < 0.05$). In conclusion, this study found that the selection of oat-pea mixed seeding as O5P5 and harvesting at the milk ripening stage of oat is recommended as a desirable oat-pea mixed seeding strategy for producing high quality silage.

KEYWORDS

oat, pea, mixed seeding, silage, harvest stage

1. Introduction

With increasing demands for livestock products, there is increasing emphasis on the need for good quality animal feeds and forages. This emphasis differs in different parts of the world and in the Qinghai-Tibetan Plateau, modern animal husbandry practices include an emphasis on the requirements for high quality seasonal and local forage products (Zhuang and Li, 2017) that are produced under conditions of the ecological benefit. Many different plant species are used to make forages for ruminants and an emerging area of potential is the silage production following mixed seeding of different plant species. The purpose of this approach is to (a) utilize the characteristics of stratification of different forage plants that may utilize water and nutrients separately and (b) to produce nutritious high-quality silage. Thus, the planting combination of *Gramineous* plants (such as cereal crops or grass) and *Leguminous* plants (such as peas or beans) shows great potential (Cui et al., 2014).

Oat (*Avena sativa*) is an annual forage crop and a main source of supplementary livestock feed in the cold season in alpine regions. It has strong cold resistance, high yield, and good quality (Dong et al., 2007). Peas (*Pisum sativum*) fix atmospheric nitrogen, are high-yielding and produce high-quality, high nutritional value, animal feeds, and human foods. Peas are extremely suitable for inclusion in ruminant diets in high-altitude plateau regions because of the excellent planting and growth conditions in the continental monsoon climate zone with long sunshine hours, high atmospheric transparency, strong solar radiation, and abundant light energy resources (Pflueger et al., 2020).

Oat and pea seeding mixtures have been shown to have high productivity (Pflueger et al., 2020) and produce silage, which is a usual low-cost forage processing technology (Muck, 2010), that shows great potential. However, to our knowledge, few studies have used oat-pea mixed forage with different seeding proportions to make silage in the Qinghai-Tibetan Plateau. Therefore, our study aimed to examine the effect of five sowing treatments differing in the seeding proportion of oats and peas and the two maturity stages at harvest, on soil properties and silage quality on the Qinghai-Tibetan plateau.

2. Material and methods

2.1. Site description, experimental design, and forage sampling

The study was conducted on 13.5 ha (each plot size was greater than 1 ha, see Figure 1A) of land on a farm in Qinghai Province, China, in the northwest of an area known as the Qinghai-Tibetan plateau. (36.65N, 101.20E, elevation 2,930 m, average temperature 1.0 °C, average annual precipitation 450 mm). The land area was divided into 10 plots, each receiving 150 kg/ha of diammonium phosphate and urea based fertilizer (diammonium phosphate: urea, 1:1). The based fertilizer was turned into the soil before sowing. Plots were randomly assigned to the five sowing treatments of different mixing ratios (10:0, 8:2, 7:3, 5:5, and 0:10 oat to pea ratio) according to the weight of expected forages wet yield of *Gramineae* and *Leguminous* plants (Oat, Qingtian No. 1 and Pea, Qingjian No. 1). Oats (O) and Peas (P) in different farm plot were seeded at the corresponding weight (Table 1) from May 12 to 13, 2021. Each plot of land was seeded with a seeder after the seeds were mixed according to the predetermined sowing plan (seed vigor above 95%), with the seeding depth of 6–10 cm, and a roller was used to flatten after sowing (Figure 1C). Five sowing proportions compound two forages harvest periods for oats (at the early flowering stage, from September 7 to 11, S1; the milk ripening stage, from October 11 to 13, S2), resulting in 10 different combinations (Table 1, Figure 1).

In each plot five 5 × 5 m large quadrats were chosen at the place with the same growth trend according to the "W" shape. Furthermore, five 0.5 × 0.5 m sampling quadrats were set in each large quadrat according to the 5-point sampling method (Figure 1D).

Twenty-five sampling quadrats (Figure 1D) were collected for each farm plot, yielding 125 quadrats for all five farm plots at each harvest time. The stubble on the ground was 15 cm, and all quadrats were harvested manually while the rest were with a combine harvester. Agronomic traits of oats and peas (wet weight yield, dry weight yield, the number of tillers/branches, stalk length of oat/stem length of pea, and natural height of pea) were determined immediately at the sampling time according to Guo et al. (2022).

2.2. Soil sampling and biochemical analysis

Before planting and at two harvest times in the autumn of 2021, soil samples from each large quadrat were collected from a depth of 0–30 cm, sieved (<2 mm) to remove visible fine roots and plant residues, stored at 4°C, and then analyzed according to the procedure described by (Jia Y. et al., 2022). Soil pH and Electro Conductibility (EC) were measured in water (1:2.5 w/v). The organic matter (OM) and cation exchange capacity (CEC) were measured according to Fan et al. (2020) and (Jia R. et al., 2022). Total nitrogen (TN) was measured by the Kjeldahl digestion (FOSS KjeltecTM 2300, FOSS NIR Systems Inc, Denmark). Total potassium (TK), total phosphorus (TP), and alkali-hydro nitrogen (AN), rapidly-available phosphorus (AP) and rapidly-available potassium (AK) were measured according to Shao et al. (2020). The concentrations of exchangeable Na (ANa), Ca (ACa), and Mg (AMg) were tested following the hot block acid digestion protocol (Huang and Schulte, 1985). NH_4^+-N and NO_3-N were determined according to Fan et al. (2020) using microplate spectrophotometer (Thermo1510, MULTISKAN GO, Thermo Fisher, USA). To calculate the soil quality index (SQI), each soil indicator was converted to a value between 0 and 1, following the procedure described by Zeraatpisheh et al. (2020), Zhou et al. (2020). The overall SQI score was estimated using an SQI-area approach according to the area of a radar diagram yielded by all standard soil indicators (Kuzyakov et al., 2020).

2.3. Silages preparation and analysis

Forages were harvested at S1 (at the early flowering stage of oats, from September 7 to 11) and S2 (the milk ripening stage of oats, from October 11 to 13) stage from each of the eight plots containing Oats, and were chopped by a combine harvester (GR80, LOVOL, China, Figure 1E) into pieces of about 2 cm immediately during harvesting. Lactic acid bacteria (LAB), commercial strains, were purchased from an animal husbandry market (Gansu Pro-Bicon Biotech. Co., Ltd., Lanzhou, Gansu, China) and the forage was added to the water containing LAB (1.0 × 10^5 colony forming unit/g on a fresh matter basis of forage) after chopping. Then the raw materials were mixed and packed as bales (38 × 40 × 68 cm) at a density of approximately 483.75 kg fresh weight (FW)/m^3 and put into the silage bags with the same specification. Wrapping and anaerobic sealing were carried out in the local common way shown in Figure 1F. Triplicate silage bags for each treatment were stored at ambient temperature (1–22°C) and opened for analysis after 7 and 30 days of ensiling.

All fresh silages from each bag were taken out by a forage sampler and then immediately mixed to obtain samples of about 500g by quartering. And among each silage sample, 20g subsamples

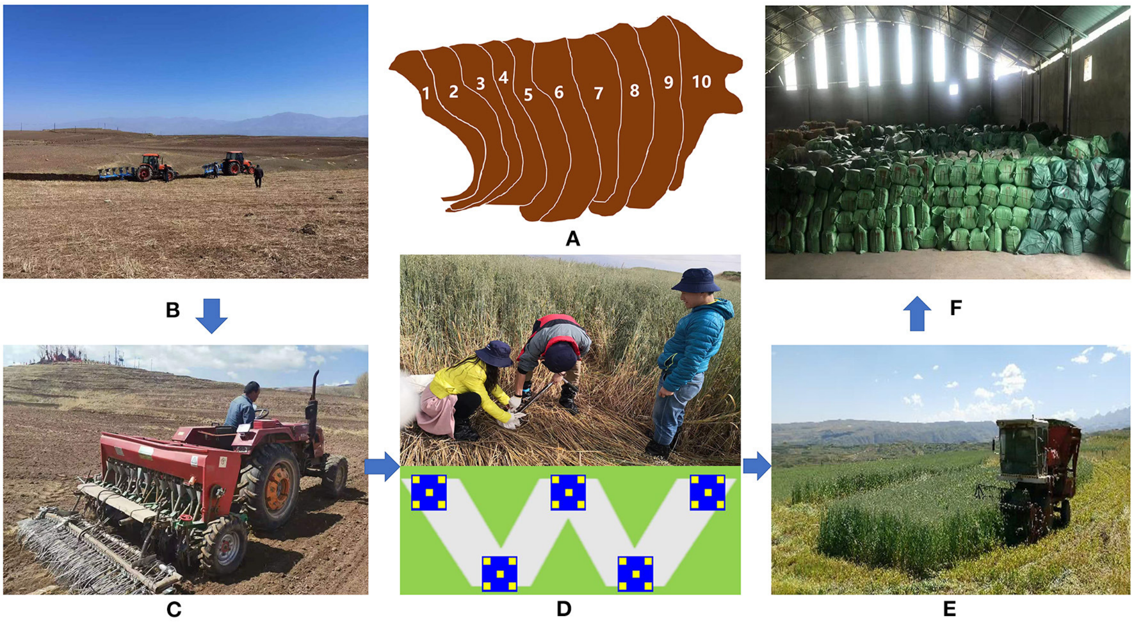

FIGURE 1
Explanations of the 10 plots and photos of the schematic diagram of the experiment. **(A)** Farm plot (Different number represents different treatment. 1. O0P10 at early flowering stage; 2. O10P0 at the early flowering stage; 3. O0P10 at the milk ripening stage; 4. O10P0 at the milk ripening stage; 5. O5P5 at the early flowering stage; 6. O8P2 at the early flowering stage; 7. O7P3 at the early flowering stage; 8. O5P5 at the milk ripening stage; 9. O8P2 at the milk ripening stage; 10. O7P3 at milk ripening stage); **(B)** ploughing the soil; **(C)** seeding; **(D)** sampling quadrats (large blue 5 × 5 m squares arranges across the plot in a W shape, and small yellow 0.5 × 0.5 m squares). **(E)** Harvesting; **(F)** bale silage.

TABLE 1 Overview of the experimental groups with seeding ratios and rates.

Farm plot Nos.	Treatments (Oat:Pea)	Harvest stage of oats	Oat sowing rate (kg/ha)	Pea sowing rate (kg/ha)	Total sowing rate (kg/ha)
1	O0P10	S1	0.0	120.0	120.0
2	O10P0	S1	165.0	0.0	165.0
3	O0P10	S2	0.0	120.0	120.0
4	O10P0	S2	165.0	0.0	165.0
5	O5P5	S1	82.5	60.0	142.5
6	O8P2	S1	132.0	24.0	156.0
7	O7P3	S1	115.5	36.0	151.5
8	O5P5	S2	82.5	60.0	142.5
9	O8P2	S2	132.0	24.0	156.0
10	O7P3	S2	115.5	36.0	151.5

Seeding ratios of Oats:Peas were 10:0, 8:2, 7:3, 5:5, and 0:10 giving groups denoted as O10P0, O8P2, O7P3, O5P5, and O0P10. Crops were harvested at the Early Flowering stage (S1) or the Milk Ripe stage (S2) of the oats.

were blended with sterilized distilled water (w:v = 1:9) for 1 min and filtered through three layers of qualitative filter paper. The filtrate was collected to measure pH. The NH_3-N level was measured using the phenol-hypochlorite method (Broderick and Kang, 1980) expressing in proportion to total nitrogen (%TN). Organic acids, such as lactic acid (LA), acetic acid (AA), propionic acid (PA), and butyric acid (BA), were evaluated using a high-performance liquid chromatography method, adjusted from those previously described (column, Shodex RS Pak KC-811; Showa Denko K.K., Kawasaki, Japan; detector, DAD, 210 nm, SPD-20A; Shimadzu Co., Ltd., Kyoto, Japan; eluent, 3 mmol L^{-1} $HClO_4$; flow speed, 1.0 ml min^{-1}; column oven temperature, 50°C) (Ohmomo, 1993). Dry matter (DM) was measured by oven-drying at 105°C for 3 h (Porter and Murray, 2010). All dried samples were ground in a hammer mill and filtered through a 1 mm screen for other analyses. Crude protein (CP) was measured according to the Association of Official Analytical Chemists (AOAC) International procedures (method 976.05; AOAC., 2000). Contents of neutral detergent fiber (NDF) and acid detergent fiber (ADF) (Van Soest et al., 1991) were measured using an Ankom 2000i fiber analyzer

(Ankom Technology Corp., Fairport, NY). Heat-stable α-amylase and sodium sulfite were used in the determination of NDF. Water soluble carbohydrate (WSC) content was determined using the improved anthrone-sulfuric acid assay (Webster, 1992) whereas Ether Extract (EE) was analyzed using an automatic Fat Analyzer (ANKOM XT15i, ANKOM Inc, USA) (AOAC., 2000).

2.4. Statistical analysis

The data were analyzed in a completely randomized 2x5 or 2×4 factorial design for Tables 2, 3, or for Table 4, using the Proc MIXED method of SAS (Statistical Analysis System, version 9.3) to determine significant differences among treatments. Mean values were compared using Tukey's test and the level of statistical significance was set to $P \leq 0.05$, a very significantly difference was set if $P \leq 0.01$. Soil physicochemical properties were ordinated by principal component analysis (PCA) using the R package FactoMineR v1.0.7 (Le et al., 2008).

3. Results

3.1. Soil properties and soil quality index

The soil physicochemical properties that were measured were not affected ($p > 0.05$) by the seeding ratio (Table 2) while harvesting stage significantly affected soil pH, EC, TP, TK, AN, and NO_3-N ($p < 0.05$). The overall soil pH and TP were higher at the early flowering harvest stage (S1) whereas EC, TK, AN, and NO_3-N were higher at the milk ripening harvest stage (S2). Seeding ratio by harvesting stage interaction effects were detected in exchangeable Na (Ana) and exchangeable Mg (Amg) with the highest and lowest Ana in O8P2 at S2 and O7P3 at S1, respectively ($p = 0.025$) and highest and lowest Amg in O5P5 at S2 and O8P2 at S1, respectively ($p = 0.036$). This is also shown in the PCA plots for the difference between harvest stages (Figure 2) and among seeding ratios within the harvest stage (Figure 3).

At the milk ripening stage, SQI comparison (Figure 4) between different mixed seeding ratios showed that the O5P5 group had higher soil quality parameters than others (although not significant).

3.2. Profiles of mixed seeding forage

Table 3 shows that the yield and chemical composition of mixed seeding forage was affected to varying degrees by different seeding ratios, harvesting stage, and seeding ratio by harvesting stage interaction. The highest wet weight yield was in O10P0 at S1 and the lowest was in O5P5 at S1 for Oats whereas O0P10 had the highest wet yield of Peas in S1 and S2. There was no significant difference in the dry weight yield of peas at S1 and oats at S2 of mixed seeding treatment groups. However, the highest dry weight yield for oats was shown at S2 in the single seeding group (O10P0). For the CP content at S2, the O7P3 group had the highest protein content in both oats and peas. In each planting pattern, the EE contents of both peas and oats were higher at S2 than at S1; the opposite was detected for NDF and ADF. The WSC content of oats decreased significantly ($p < 0.05$) with increasing ratio of pea seeding both at S1 and S2. In addition, the O5P5 group at S1 showed the highest ash content in all samples ($p < 0.05$).

The agronomic traits of mixed seeding forage are in Table 3. Except for single seeding (O10P0 and O0P10), the maximum number of tillers/branches of peas was in O5P5 at S1, while it was in O8P2 at S2 for oats. As far as the AH and NH were concerned, the O5P5 group also got the best results in mixed groups, and the S2 was better than S1. The seeding rate by stage of harvest interaction effects showed that O5P5 at S1 had the highest Ash contents of oats and peas and ADF content of peas, compared to all groups. O5P5 at S2 had the highest NDF and AH contents of peas. O7P3 at S1 had the highest NDF and ADF contents of oats and DM and NDF contents of peas ($p < 0.05$).

3.3. Fermentation quality and chemical composition of silages

After ensiling, all fermentation quality indicators (pH value, contents of LA, AA, PA, LA/AA ratio, and NH_3-N) and fermentation quality and chemical composition (DM, CP, NDF, ADF, WSC, and ash) were affected by varying degrees by different treatments (Table 4). After storage for 7 days (d), both mixed seeding ratio and harvest stage caused very significant differences ($p < 0.01$) in all the contents of LA, PA, DM, CP, NDF, WSC, ash, and LA/AA ratio, while very significant interaction effects occurred in the pH value, contents of LA, NDF, WSC, and ash ($p < 0.01$). However, for 30 d silage samples, only the AA and ash contents were very significantly affected ($p < 0.01$) by mixed seeding ratio and harvest stage among all fermentation quality and chemical composition parameters. For the four organic acids, PA was not detected in some treatment groups, but there were significant differences ($p < 0.05$) in the contents of LA, AA, and PA. Interestingly, O8P2 and O5P5 groups at S1 rather than O10P0 had the highest LA content in both storage times ($p < 0.05$). The O8P2 and O5P5 groups at S1 groups also resulted in higher AA content (especially greater than 3.6% DM at storing for 30 d). Overall, the LA/AA ratio of S2 samples was less than 2.00. At 7 and 30 d of ensiling, the O10P0 group at S1 showed the lowest NH_3-N content ($p < 0.05$), while the treatment at S2 after 7d was abnormally high.

The O10P0 group had the highest DM content in all treatments at 7 and 30 d, and O5P5 showed the highest CP content (except ensiling for 30 days in S1), which indicated the CP content of oat-pea silage generally increased with the proportion of peas in mixed seeding at the same harvest time. As far as cellulose was concerned, the NDF and ADF contents in silages seem mainly contributed by oats. Single seeding oats at S1 had higher NDF and ADF contents than at S2, which was also observed in most groups with the same mixed seeding ratio after storage for 7 or 30 d. There was lower WSC content after ensiling for 30 d than for 7 d (except O8P2 and O7P3 groups S1).

Seeding ratio by stage of harvest interaction effects were detected in all the fermentation quality and chemical composition indicators except in PA and DM after 7 days of ensiling, whereas

TABLE 2 Soil physicochemical properties in oat and pea mixed seeding plantations ($n = 5$).

Soil characteristics	Early flowering stage (S1)					Milk ripe stage (S2)					SEM	P-value		
	O10P0	O8P2	O7P3	O5P5	O0P10	O10P0	O8P2	O7P3	O5P5	O0P10		R	S	R × S
Soil pH	8.33	8.27	8.26	8.22	8.35	8.15	8.08	8.12	8.07	8.16	0.044	1.101	<0.001	0.968
EC (μS/cm)	161.70	182.89	179.11	172.65	164.28	236.95	214.80	238.44	233.92	208.55	7.911	0.069	<0.001	0.224
OM (g/kg)	35.78	26.72	35.26	36.36	29.06	23.02	34.32	27.32	35.84	26.36	5.178	0.570	0.325	0.366
CEC (cmol(+)/kg)	13.49	10.65	12.24	12.56	10.82	10.40	14.55	11.45	14.24	11.03	1.589	0.614	0.705	0.264
TN (g/kg)	1.99	1.35	1.83	1.97	1.45	1.23	2.07	1.55	2.10	1.51	0.265	0.324	0.871	0.097
TP (g/kg)	0.71	0.80	0.77	0.85	0.71	0.64	0.68	0.72	0.70	0.74	0.045	0.249	0.016	0.325
TK (g/kg)	18.98	16.66	17.04	17.78	18.31	18.76	19.57	18.28	19.81	17.91	0.652	0.315	0.010	0.067
AN (mg/kg)	74.99	55.67	64.95	76.88	56.44	60.52	101.85	80.50	95.24	81.21	11.289	0.467	0.015	0.136
AP (mg/kg)	30.99	41.47	38.59	45.30	40.18	23.18	46.34	32.2	32.58	34.66	5.431	0.053	0.116	0.595
AK (mg/kg)	114.88	120.65	108.70	130.12	97.15	110.68	107.48	95.58	106.15	106.14	8.408	0.202	0.095	0.384
Ana (mg/kg)	36.95	30.85	29.73	33.59	37.55	34.85	41.59	33.71	40.60	34.46	2.360	0.196	0.033	0.025
Aca (mg/kg)	5670	4857	5514	5580	5325	5184	5798	5367	59.01	5329	219.972	0.330	0.368	0.029
Amg (mg/kg)	192.30	138.17	154.36	162.52	175.18	190.63	179.16	191.94	216.87	165.64	11.786	0.041	0.002	0.036
NH_4^+-N (mg/kg)	3.66	1.98	2.29	4.15	2.97	2.47	2.06	1.25	1.49	3.40	0.736	0.220	0.067	0.259
NO_3-N (mg/kg)	2.03	1.39	1.06	1.61	0.54	2.49	2.62	2.89	2.15	2.56	0.457	0.443	0.000	0.427

O10P0, O8P2, O7P3, O5P5, and O0P10 mean the mixed seeding ratio of oat and pea was 10:0, 8:2, 7:3, 5:5, and 0:10; S, Harvest stage; R, Mixed seeding ratio; SEM, standard error of the mean; EC, electro conductibility; OM, organic matter; CEC, cation exchange capacity; TN, total nitrogen; TP, total phosphorus; TK, total potassium; AN, alkali-hydro nitrogen; AP, rapidly-available phosphorus; AK, rapidly-available potassium; Ana, exchangeable Na; Aca, exchangeable Ca; Amg, exchangeable Mg.

TABLE 3 The effect of seeding ratios and maturity stages at harvest on the chemical composition of oats and peas in mixed seeding plantations ($n = 5$).

Plant characteristics	Early flowering stage (S1)					Milk ripe stage (S2)					SEM	P-value		
	O10P0	O8P2	O7P3	O5P5	O0P10	O10P0	O8P2	O7P3	O5P5	O0P10		R	S	R × S
Oats														
WY (t/ha)	45.93^{a}	28.83^{b}	28.01^{b}	26.46^{b}	-	43.22	37.86	35.78	37.06	-	1.956	<0.001	<0.001	0.003
DY (t/ha)	11.88^{ab}	6.66^{b}	12.28^{a}	7.88^{ab}	-	14.62	12.49	11.42	11.49	-	1.206	0.006	0.001	0.052
DM (%)	25.82^{b}	24.44^{b}	31.97^{a}	31.37^{a}	-	34.1	34.08	31.8	31.05	-	0.970	0.325	<0.001	<0.001
CP (%DM)	4.58^{b}	5.75^{ab}	6.57^{a}	7.04^{a}	-	4.93^{b}	6.27^{ab}	7.66^{a}	7.61^{a}	-	0.349	<0.001	0.010	0.732
EE (%DM)	1.92	1.76	2	1.94	-	3.18	3.13	2.98	2.9	-	0.089	0.500	<0.001	0.056
NDF (%DM)	60.50^{b}	65.36^{a}	69.06^{a}	65.84^{a}	-	56.76	55.41	54.69	58.15	-	0.959	0.002	<0.001	<0.001
ADF (%DM)	33.75^{c}	37.89^{b}	42.17^{a}	41.64^{a}	-	32.44	32.09	30.62	32.87	-	0.607	<0.001	<0.001	<0.001
WSC (%DM)	21.95^{a}	15.31^{b}	9.10^{c}	8.02^{c}	-	19.16^{a}	12.16^{b}	12.65^{b}	13.73^{b}	-	0.800	<0.001	0.140	<0.001
Ash (%DM)	4.54^{c}	5.26^{ab}	5.84^{a}	6.38^{a}	-	4.14^{b}	5.42^{a}	5.09^{a}	5.49^{a}	-	0.137	<0.001	<0.001	0.001
NTB	4.10^{b}	5.78^{a}	3.34^{bc}	5.24^{a}	-	3.76^{b}	4.88^{a}	4.28^{ab}	4.72^{a}	-	0.222	<0.001	0.194	0.000
AH (cm)	133.85^{a}	123.70^{b}	125.01^{b}	132.98^{a}	-	147.91^{a}	137.20^{b}	139.95^{ab}	143.26^{ab}	-	1.805	<0.001	<0.001	0.589
Peas														
WY (t/ha)	-	17.61^{b}	18.78^{b}	26.27^{b}	63.40^{a}	-	6.28^{b}	11.69^{b}	16.72^{b}	88.00^{a}	2.531	<0.001	0.638	<0.001
DY (t/ha)	-	4.55	4.7	5.47	10.6	-	1.33^{b}	3.55^{b}	11.16^{ab}	16.77^{a}	2.618	0.000	0.311	0.175
DM (%)	-	26.69^{a}	27.43^{a}	23.14^{a}	16.70^{b}	-	19.34	20.33	24.03	19.15	1.269	<0.001	0.003	<0.001
CP (%DM)	-	21.95^{a}	18.54^{b}	21.67^{ab}	23.19^{a}	-	23.61^{a}	24.29^{a}	20.39^{b}	20.30^{b}	0.726	0.091	0.114	<0.001
EE (%DM)	-	2.01^{b}	2.27^{b}	2.22^{b}	3.19^{a}	-	1.85^{b}	2.26^{a}	2.21^{a}	2.44^{a}	0.076	<0.001	<0.001	<0.001
NDF (%DM)	-	42.67^{b}	48.77^{a}	46.16^{a}	44.12^{ab}	-	41.13^{b}	41.31^{b}	48.36^{a}	45.15^{ab}	1.217	0.000	0.096	0.001
ADF (%DM)	-	26.60^{b}	31.37^{a}	34.74^{a}	34.21^{a}	-	27.95	27.65	29.24	31.51	0.968	<0.001	0.000	0.005
WSC (%DM)	-	15.21^{a}	10.87^{b}	7.59^{c}	10.81^{b}	-	10.16	10.78	12.77	9.96	0.677	0.001	0.675	<0.001
Ash (%DM)	-	5.03^{b}	5.79^{a}	6.01^{a}	5.46^{ab}	-	4.76^{b}	4.60^{b}	4.84^{b}	5.74^{a}	0.162	0.000	<0.001	<0.001
NTB	-	1.78^{b}	1.40^{b}	19.32^{a}	19.7^{a}	-	1.23	1.17	1.2	1.24	0.162	<0.001	<0.001	<0.001
AH (cm)	-	130.04^{b}	137.40^{b}	138.14^{b}	148.76^{a}	-	133.82^{b}	148.41^{a}	157.86^{a}	150.20^{a}	2.305	<0.001	<0.001	0.000
NH (cm)	-	119.28^{a}	122.81^{a}	125.24^{a}	82.44^{b}	-	122.41^{b}	131.84^{a}	123.00^{ab}	96.32^{c}	2.065	<0.001	<0.001	0.001

$^{a-c}$Means in the same row with different superscripts differ significantly ($P < 0.05$); O10P0, O8P2, O7P3, O5P5, and O0P10 mean the mixed seeding ratio of oat and pea was 10:0, 8:2, 7:3, 5:5, and 0:10; S, Harvest stage; R, Mixed seeding ratio; SEM, standard error of the mean; WY, wet yield weight; DY, dry yield weight; WAT, water content; CP, crude protein; EE, ether extract; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, Acid detergent lignin; WSC, water-soluble carbohydrates; NTB, number of tillers/branches; AH, stalk length of oat/ stem length of pea; NH, natural height.

TABLE 4 The effect of ensiling time on fermentation quality and chemical composition of silage made from oats and peas planted in mixed seeding plantations ($n = 3$).

Items	Early flowering stage (S1)				Milk ripe stage (S2)				SEM	P-value		
	O10P0	O8P2	O7P3	O5P5	O10P0	O8P2	O7P3	O5P5		R	S	R × S
7d												
pH	4.12^{b}	4.39^{ab}	4.69^{a}	4.16^{b}	4.53	4.50	4.45	4.35	0.063	0.001	0.025	0.002
LA (%DM)	5.27^{b}	6.64^{a}	4.47^{b}	7.74^{a}	2.96	3.21	3.50	3.33	0.340	0.001	<0.001	0.001
AA (%DM)	3.13^{a}	2.84^{ab}	2.04^{b}	3.44^{a}	2.23	2.40	2.09	2.22	0.211	0.014	0.001	0.049
LA/AA	1.71^{b}	2.35^{a}	2.15^{ab}	2.25^{a}	1.33	1.34	1.70	1.50	0.100	0.008	<0.001	0.029
PA (%DM)	0.07^{b}	0.27^{b}	0.41^{a}	0.60^{a}	-	-	0.18	0.27	0.047	0.001	0.001	0.329
NH3-N (%TN)	2.84	6.64	5.21	6.79	10.65	4.87	5.84	4.84	1.427	0.866	0.275	0.021
DM (%)	34.27^{a}	31.84^{b}	31.74^{b}	27.17^{c}	35.62^{a}	32.59^{b}	33.54^{b}	28.49^{c}	0.381	<0.001	0.000	0.604
CP (%DM)	7.50^{c}	10.93^{b}	12.46^{a}	14.06^{a}	5.47^{c}	9.45^{b}	9.03^{b}	13.14^{a}	0.330	<0.001	<0.001	0.011
NDF (%DM)	69.12^{a}	66.97^{a}	69.46^{a}	61.58^{b}	59.30	60.22	58.02	58.78	0.767	0.000	<0.001	0.000
ADF (%DM)	41.94^{a}	39.43^{a}	42.43^{a}	37.77^{b}	35.20	34.27	34.96	35.62	0.669	0.014	<0.001	0.006
WSC (%DM)	7.06	5.25	5.16	6.17	17.30^{a}	9.02^{b}	14.07^{a}	9.57^{b}	0.963	0.001	<0.001	0.006
Ash (%DM)	8.86^{a}	6.30^{c}	7.88^{ab}	7.29^{b}	5.49	5.75	5.35	6.23	0.211	0.001	<0.001	<0.001
30d												
pH	4.10	4.16	4.76	4.20	4.82	4.51	5.09	4.38	0.278	0.126	0.061	0.788
LA (%DM)	6.02	7.31	4.88	7.90	3.11	4.62	2.86	5.59	0.863	0.017	0.001	0.955
AA (%DM)	3.06	3.66	3.36	3.63	1.88^{b}	2.98^{ab}	1.58^{b}	3.78^{a}	0.294	0.001	0.001	0.030
LA/AA	1.97	2.07	1.48	2.17	1.68	1.56	1.63	1.49	0.271	0.691	0.099	0.465
PA (%DM)	-	0.60	0.55	0.67	-	-	-	0.67	0.090	0.667	0.966	-
NH3-N (%TN)	3.49^{b}	8.97^{b}	8.82^{b}	19.60^{a}	4.35	9.38	5.45	10.27	2.875	0.011	0.179	0.295
DM (%)	32.70^{a}	31.03^{b}	29.89^{b}	28.21^{b}	34.07^{a}	29.49^{b}	29.71^{b}	27.44^{b}	0.792	<0.001	0.627	0.336
CP (%DM)	6.50	10.34	11.62	10.69	6.01^{b}	9.76^{b}	9.89^{b}	13.97^{a}	1.143	0.001	0.884	0.183
NDF (%DM)	67.81^{a}	62.30^{b}	65.20^{ab}	55.67^{c}	64.38^{a}	62.11^{a}	65.24^{a}	54.70^{b}	0.982	<0.001	0.120	0.305
ADF (%DM)	40.16^{a}	38.85^{a}	43.27^{a}	35.83^{b}	37.51	37.97	39.52	36.25	1.160	0.003	0.053	0.317
WSC (%DM)	5.32^{b}	6.81^{ab}	8.90^{a}	5.44^{b}	6.63	6.29	5.55	7.45	0.451	0.088	0.673	<0.001
Ash (%DM)	8.41^{a}	6.70^{b}	7.99^{a}	8.99^{a}	6.32	5.90	6.23	6.85	0.231	<0.001	<0.001	0.037

$^{a-c}$Means in the same row with different superscripts differ significantly ($P < 0.05$); O10P0, O8P2, O7P3, and O5P5 mean the mixed seeding ratio of oat and pea was 10:0, 8:2, 7:3, and 5:5; S, Harvest stage; R, Mixed seeding ratio; SEM, standard error of the mean; LA, lactic acid; AA, acetic acid; PA, propionic acid; NH_3-N, ammonia nitrogen; TN, total nitrogen; DM, dry matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; WSC, water-soluble carbohydrates. -, most of the initial data was lost so the samples were not analyzed.

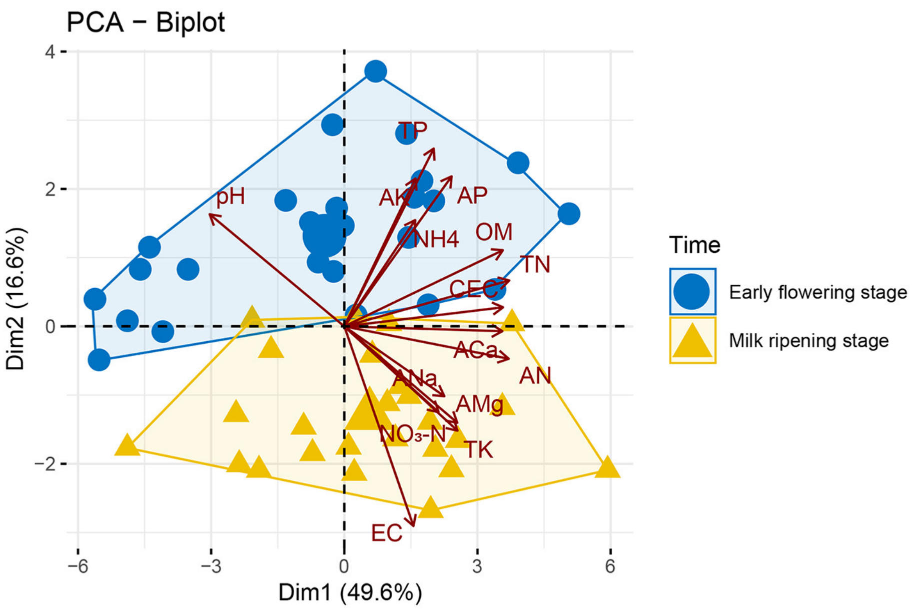

FIGURE 2
Principal component analysis (PCA) based on soil physicochemical properties as variables. The samples of the same harvest stage were outlined and grouped in different colors.

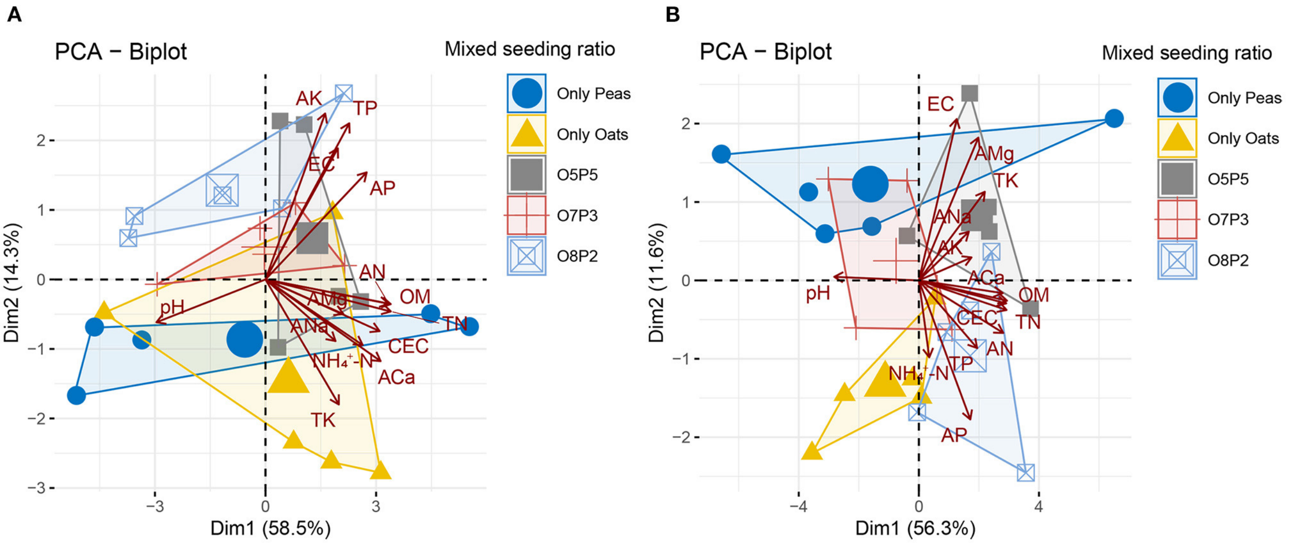

FIGURE 3
Principal component analysis (PCA) based on soil physicochemical properties as variables. The samples of the same mixed seeding ratio from the early flowering stage **(A)** and milk ripening stage **(B)** were outlined and grouped in different colors. Only Peas: O0P10; Only Oats: O10P0.

after 30 days of ensiling, interaction effects were detected only in AA, WSC, and Ash contents ($p < 0.05$). After 7 days of ensiling, O5P5 at S1 had the highest LA, AA, LA/AA, and CP contents compared to all groups. O10P0 at S1 had the highest contents of NDF, ADF, and Ash while O10P0 at S2 had the highest contents of NH_3-N and WSC. After 30 d of ensiling, O5P5 at S2 had the highest contents of AA whereas O5P5 at S1 and O7P3 at S1 had the highest Ash and WSC contents, respectively.

4. Discussion

The present study explored the silage production from mixed planting of two forage species, *Leguminous* and *Gramineous*, suitable for cultivation on the Qinghai-Tibetan Plateau. The results demonstrated that, using oats and peas, nutritional quality of silage and utilization potential can be improved by varying the mixed seeding ratio and the harvest time, and that they are suitable for producing high-quality silages for livestock.

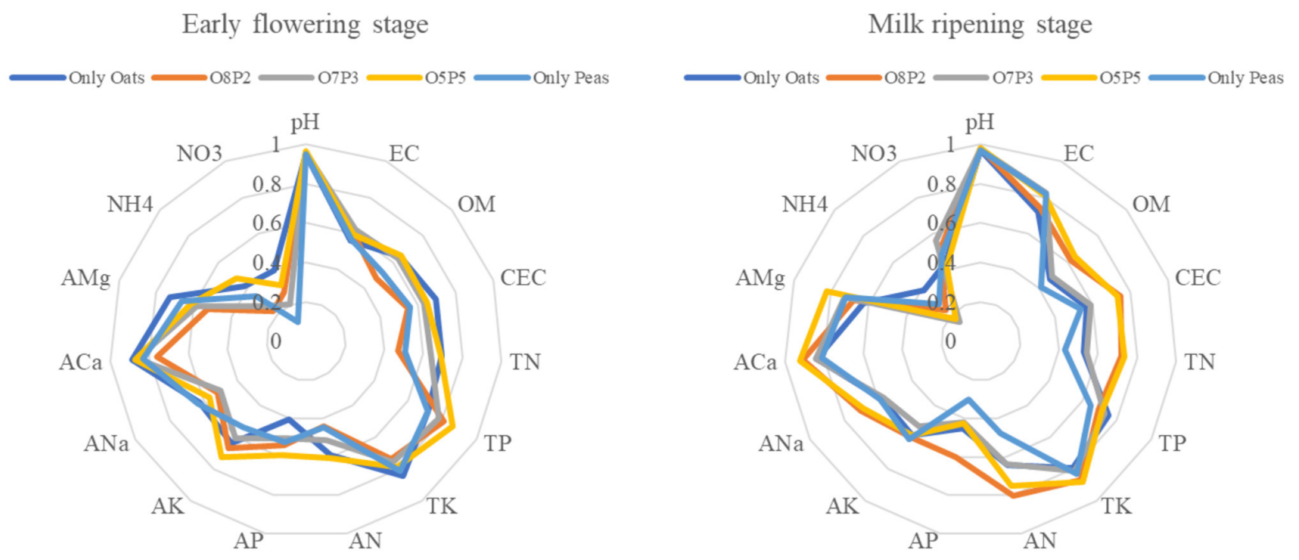

FIGURE 4
The radar graphs show the relative responses of soil properties to mixed seeding ratios at the early flowering stage and milk ripening stage. Only Peas: O0P10; Only Oats: O10P0.

Mixed sowing of *Leguminous* and *Gramineous* plants is considered an effective approach to improving total biomass and soil fertility (Berdahl et al., 2001; Tekeli and Ates, 2005; Salama et al., 2020). Our results indicated that although mixed seeding could alter the availability of some nutrients, it could not comprehensively change the soil quality. By scaling at the mixed seeding ratio, the O8P2 group obtained the highest total wet weight yield among the mixed seeding groups. Previous studies have found that the mixed sowing of peas and oats is a successful model (Lauk and Lauk, 2008). Similar to the results of our study, the maximum yield per unit area comes from pure crop (single seeding) plots but mixing with peas decreased the amount of additional nitrogen required. Specifically, they found that the addition of peas to oats resulted in a significant increase in protein content in cereals. Moreover, mixed seeding promoted tillering of oats, which may be due to reduced competition from grass weeds (Arlauskiene et al., 2021). Due to peas' chemical composition, the mixture was superior to single grain oat in protein yield (Soufan and Al-Suhaibani, 2021). Furthermore, the co-sowing of peas and oats has also been shown to have application potential in organic agriculture due to a relatively good harvest and high protein yield in the soil without fertilizer (Candelaria-Morales et al., 2022). This might provide a possible feed source for the organic production of dairy and meat products.

Several factors may be attributable to the characteristics of co-sowing pea-oat silages in our study. Nitrogen ammonification during silage due to high moisture content was of concern. Zhang et al. (2020) showed that the NH_3-N/TN (NH_3-N concentration expressed in TN%) of silage had a strong negative correlation with the DM intake of livestock. Thus, the content of ammonia nitrogen directly determined the feeding value of the silage. In the O5P5 group, regardless of harvesting and ensiling at S1 or S2, the DM content was always lower than in other treatments. At the same time, NH_3-N concentration was at a worrisome high level in these silages unexpectedly, even though the samples had low pH values and high LA content, that may explain the higher AA content of O8P2 and O5P5 groups at S1 groups. Silage with low moisture content delays the growth of anaerobic microorganisms (Mariotti et al., 2020), thus reducing the speed and ability of sugar to convert into organic acids (Zhang et al., 2014). When the moisture content of the silage was high, *Clostridium spp.* would multiply, and butyric acid, ammonia nitrogen, and other harmful substances would produce in large quantities, thus affecting the quality of silage fermentation (Zheng et al., 2018; Du et al., 2022). Therefore, successful ensiling fermentation by lactic acid bacteria can only be obtained if the water content is appropriate. Weinberg and Ashbell (2003) demonstrated that when the DM content of silage raw materials is high (the moisture content is low), the inhibitory effect on the activity of lactic acid bacteria and other microorganisms increases so that the silage cannot quickly reach a stable state.

Previous studies have attributed successful ensiling to the contribution of LAB (Muck, 2010). More studies have proved that LAB is the dominant fermentation in high-quality silage (Dunière et al., 2013). Compared with spontaneous fermentation, the exogenous addition of LAB has become a necessary part of silage production (Kung et al., 2018). In this study, all treatments were applied with the same LAB specie in equal loadings to accommodate subsequent animal production needs. Therefore, the difference in fermentation quality may be mainly due to the available substrates of LAB. Low pH and high LA content were key indexes indicating good silage quality. Results of our study showed that the ratio of forage materials seeding with O8P2 at S1 had higher WSC content than other mixing ratios, resulting in a lower pH value of silage samples in this treatment group. In addition, the lower WSC residuals and higher LA content after storage for 7 and 30 d also suggested that superior LAB had a better utilization effect on WSC during ensiling in this group (Zhang et al., 2017). In addition, the increasing ash content might be due to the chemical composition of raw material peas.

5. Conclusions

Our study found that the forages of the oat-pea mixed seeding altered the availability of soil nutrients and obtained the best fermentation quality at the O8P2 and O5P5 ratios on the Qinghai-Tibetan Plateau. The O5P5 ratio is further recommended as a desirable oat-pea mixed seeding silage production strategy giving the highest crude protein content.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Written informed consent was obtained from the individual(s) for the publication of any identifiable images or data included in this article.

Author contributions

Conceptualization: DB and AE. Methodology, formal analysis, and writing—original draft preparation: WL. Software and visualization: ZL. Validation: LZ and YakC. Investigation: YanC. Resources: LZ. Data curation: YS. Writing—review and editing, supervision, project administration, and funding acquisition: DB. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

AOAC. (2000). *Official Methods of Analysis*. Gaithersburg, MD, USA: The Association of Official Analytical Chemists.

Arlauskiene, A., Jablonskyte-Rašče, D., Šarunaite, L., Toleikiene, M., and Masilionyte, L., Gecaite, V., et al. (2021). Perennial forage legume cultivation and their above-ground mass management methods for weed suppression in arable organic cropping systems. *Chem. Biol. Technol. Agric.* 8, 34. doi: 10.1186/s40538-021-00228-5

Berdahl, J. D., Karn, J. F., and Hendrickson, J. R. (2001). Dry matter yields of cool-season grass monocultures and grass–alfalfa binary mixtures. *Agron. J.* 93, 463–467. doi: 10.2134/agronj2001.932463x

Broderick, G. A., and Kang, J. H. (1980). Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and in vitro media1. *J. Dairy Sci.* 63, 64–75. doi: 10.3168/jds.S0022-0302(80)82888-8

Candelaria-Morales, N. P., Grossman, J., Fernandez, A., and Rogers, M. (2022). Exploring multifunctionality of summer cover crops for organic vegetable farms in the upper midwest. *Renew. Agric. Food Syst.* 37, 198–205. doi: 10.1017/S1742170521000545

Cui, G. W., Li, H. Y., Sun, T., Wang, Z., and Xi, L. Q. (2014). An experimental study of variety screening, sequential cropping, compaction and mixed cropping techniques for the cultivation of annual forage crops in agro-pastoral area of tibet, china. *Int. J. Agric. Biol.* 16, 97–103. Available online at: https://www.fspublishers.org/

Dong, S. K., Kang, M. Y., Yun, X. J., Long, R. J., and Hu, Z. Z. (2007). Economic comparison of forage production from annual crops, perennial pasture and native grassland in the alpine region of the qinghai-tibetan plateau, china. *Grass Forage Sci.* 62, 405–415. doi: 10.1111/j.1365-2494.2007.00594.x

Du, Z., Lin, Y., Sun, L., Yang, F., and Cai, Y. (2022). Microbial community structure, co-occurrence network and fermentation characteristics of woody plant silage. *J. Sci. Food Agric.* 102, 1193–1204. doi: 10.1002/jsfa.11457

Dunière, L., Sindou, J., Chaucheyras-Durand, F., Chevallier, I., and Thévenot-Sergentet, D. (2013). Silage processing and strategies to prevent persistence of undesirable microorganisms. *Animal Feed Sci. Technol.* 182, 1–15. doi: 10.1016/j.anifeedsci.2013.04.006

Fan, X., Han, M., Wang, L., Li, X., and Zhou, J. (2020). Analysis of water quality change and its driving factors of the xiaoqing river estuary in recent ten rears. *Environ. Sci.* 41, 1619–1628. doi: 10.13227/j.hjkx.201909074

Guo, C., Wang, W., Pu, X., Duan, N., Wei, X., Peng, D., et al. (2022). The current situation and prospect of the integration of forage industry, agricultural machinery and agronomy in qinghai province. *Acta Agrestia Sinica* 30, 2483–2491. doi: 10.13594/j.cnki.mcjgjx.2020.01.005

Huang, C. Y. L., and Schulte, E. E. (1985). Digestion of plant tissue for analysis by icp emission spectroscopy. *Commun. Soil Sci Plant Analy.* 16, 943–958. doi: 10.1080/00103628509367657

Jia, R., Zhou, J., Chu, J., Shahbaz, M., Yang, Y., Davey, L., et al. (2022). Insights into the associations between soil quality and ecosystem multifunctionality driven by fertilization management: a case study from the north china plain. *J. Cleaner Prod.* 362, 132265. doi: 10.1016/j.jclepro.2022.132265

Jia, Y., Li, J., Zeng, X., Zhang, N., Wen, J., Liu, J., et al. (2022). The performance and mechanism of cadmium availability mitigation by biochars differ among soils with different ph: hints for the reasonable choice of passivators. *J. Environ. Manage.* 312, 114903. doi: 10.1016/j.jenvman.2022.114903

Kung, L., Shaver, R. D., Grant, R. J., and Schmidt, R. J. (2018). Silage review: interpretation of chemical, microbial, and organoleptic components of silages. *J. Dairy Sci.* 101, 4020–4033. doi: 10.3168/jds.2017-13909

Kuzyakov, Y., Gunina, A., Zamanian, K., Tian, J., Luo, Y., Xu, X., et al. (2020). New approaches for evaluation of soil health, sensitivity and resistance to degradation. *Front. Agric. Sci. Eng.* 7, 282–288. doi: 10.15302/J-FASE-2020338

Lauk, R., and Lauk, E. (2008). Pea-oat intercrops are superior to pea-wheat and pea-barley intercrops. *Acta agriculturae Scandinavica. Section B, Soil Plant Sci.* 58, 139–144. doi: 10.1080/09064710701412692

Le, S., Josse, J., and Husson, F. (2008). Factominer: an r package for multivariate analysis (article). *J. Stat. Softw.* 25, 1–18. doi: 10.18637/jss.v025.i01

Mariotti, M., Fratini, F., Cerri, D., Andreuccetti, V., Giglio, R., Angeletti, F. G. S., et al. (2020). Use of fresh scotta whey as an additive for alfalfa silage. *Agronomy* 10, 365. doi: 10.3390/agronomy10030365

Muck, R. E. (2010). Silage microbiology and its control through additives. *Rev. Brasileira De Zootecnia* 39, 183–191. doi: 10.1590/S1516-35982010001300021

Ohmomo, S. (1993). Analysis of organic acids in silage by high-performance liquid chromatography. *Bull. Natl. Grassl. Res. Inst.* 48, 51–56.

Pflueger, N. P., Redfearn, D. D., Volesky, J. D., Bolze, R., and Stephenson, M. B. (2020). Influence of oat and spring pea mixtures on forage characteristics in different environments. *Agronomy J.* 112, 1911–1920. doi: 10.1002/agj2.20144

Porter, M. G., and Murray, R. S. (2010). The volatility of components of grass silage on oven drying and the inter-relationship between dry-matter content estimated by different analytical methods. *Grass Forage Sci.* 56, 405–411. doi: 10.1046/j.1365-2494.2001.00292.x

Salama, H. S. A., Khalil, H. E., and Nawar, A. I. (2020). Utilization of thinned sunflower and soybean intercrops as forage: a useful strategy for small scale farms in intensive agricultural systems. *Int. J. Plant Prod.* 14, 487–499. doi: 10.1007/s42106-020-00099-0

Shao, T., Zhao, J., Liu, A., Long, X., and Rengel, Z. (2020). Effects of soil physicochemical properties on microbial communities in different ecological niches in coastal area. *Appl. Soil Ecol.* 150, 103486. doi: 10.1016/j.apsoil.2019.103486

Soufan, W., and Al-Suhaibani, N. A. (2021). Optimizing yield and quality of silage and hay for pea–barley mixtures ratio under irrigated arid environments. *Sustainability* 13, 13621. doi: 10.3390/su132413621

Tekeli, A. S., and Ates, E. (2005). Yield potential and mineral composition of white clover (trifolium repens l.)-Tall fescue (festuca arundinacea schreb.) Mixtures. *J. Central Eur. Agric.* 25, 27–34. doi: 10.5513/jcea.v6i1.243

Van Soest, P. J., Robertson, J. B., and Lewis, B. A. (1991). Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* 74, 3583–3597. doi: 10.3168/jds.S0022-0302(91)78551-2

Webster, J. (1992). "The biochemistry of silage (second edition)," in P. Mcdonald, A. R. Henderson and S. J. E. Heron (Marlow, Bucks, UK: Chalcombe Publications) (1991) 340. doi: 10.1017/S0014479700023115

Weinberg, Z. G., and Ashbell, G. (2003). Engineering aspects of ensiling. *Biochem. Eng. J.* 13, 181–188. doi: 10.1016/S1369-703X(02)00130-4

Zeraatpisheh, M., Bakhshandeh, E., Hosseini, M., and Alavi, S. M. (2020). Assessing the effects of deforestation and intensive agriculture on the soil quality through digital soil mapping. *Geoderma* 363, 114139. doi: 10.1016/j.geoderma.2019.114139

Zhang, G., Li, Y., Fang, X., Cai, Y., and Zhang, Y. (2020). Lactation performance, nitrogen utilization, and profitability in dairy cows fed fermented total mixed ration containing wet corn gluten feed and corn stover in combination replacing a portion of alfalfa hay. *Animal Feed Sci. Technol.* 269, 114687. doi: 10.1016/j.anifeedsci.2020.114687

Zhang, Q., Zhao, M., Wang, X., Yu, Z., and Na, R. (2017). Ensiling alfalfa with whole crop corn improves the silage quality and invitro digestibility of the silage mixtures. *Grassland Sci.* 63, 211–217. doi: 10.1111/grs.12168

Zhang, X. Q., Jin, Y. M., Zhang, Y. J., Yu, Z., and Yan, W. H. (2014). Silage quality and preservation ofurtica cannabina ensiled alone and with additive treatment. *Grass Forage Sci.* 69, 405–414. doi: 10.1111/gfs.12036

Zheng, M., Niu, D., Zuo, S., Mao, P., Meng, L., and Xu, C. (2018). The effect of cultivar, wilting and storage period on fermentation and the clostridial community of alfalfa silage. *Italian J. Animal Sci.* 17, 336–346. doi: 10.1080/1828051X.2017.1364984

Zhou, Y., Ma, H., Xie, Y., Jia, X., Su, T., Li, J., et al. (2020). Assessment of soil quality indexes for different land use types in typical steppe in the loess hilly area, china. *Ecol. Indic.* 118, 106743. doi: 10.1016/j.ecolind.2020.106743

Zhuang, M. H., and Li, W. J. (2017). Greenhouse gas emission of pastoralism is lower than combined extensive/intensive livestock husbandry: a case study on the qinghai-tibet plateau of china. *J. Cleaner Prod.* 147, 514–522. doi: 10.1016/j.jclepro.2017.01.126

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Effect of genotype and environment on agronomical characters of alfalfa (*Medicago sativa* L.) in a typical acidic soil environment in southwest China

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Alfalfa (*Medicago sativa* L.), an important perennial legume forage crop with high nutritional value and forage yield, is widely used in animal husbandry. However, it is very sensitive to aluminum, which severely limits its growth in acidic soils. In this study, we analyzed the genotype variation of each agronomic trait in 44 alfalfa varieties in two acidic soil environments. Then, analysis of variance (ANOVA) of the variance components was performed using the Residual Maximum Likelihood (REML). The best linear unbiased predictor analysis was used to obtain the mean trait of each variety, and the mean values were used to construct the mean matrix of varieties × traits and interaction analysis of varieties × years. The results showed that there was significant ($P < 0.05$) genotypic variation for each trait of the 44 varieties and the genetic diversity was abundant. The average repeatability (R value) of interannual plant height (PH), stem thickness (ST), number of branches (NS), fresh weight (FW), total fresh weight (TFW), and total dry weight (TDW) was high (0.21–0.34), whereas the genetics were relatively stable. PH, NS, FW, TFW, and dry weight (DW) were positively correlated ($P < 0.01$) with TDW. Six alfalfa varieties (Algonquin, Xinjiang daye, Trifecta, Vernal, WL354HQ, and Boja) with excellent TDW and TFW were identified in different years, environmental regions, and climatic altitudes. Our research results can provide suggestions and critical information regarding the future improvement and development of new alfalfa strains and varieties that are resistant to acidic soil conditions.

KEYWORDS

Medicago sativa, acidic soil, agronomic traits, genetic diversity, genotypic variation

1. Introduction

Alfalfa (*Medicago sativa* L.), which has a high protein content, high grass yield, and barren tolerance, is the most widely cultivated legume forage crop in the world (Avci et al., 2013; Arshad et al., 2017) and is often called the "King of Forages" (Feng et al., 2022). Worldwide, approximately 320 million hm^2 of land are used to cultivate alfalfa (Yuegao et al., 2010), which plays an important role in the development of animal husbandry. Alfalfa grows well in alkaline soils, but its yield decreases along with pH in acidic soils (Dogbatse et al., 2020). Approximately 50% of the world's potentially arable soils are acidic (Kochian et al., 2015), located predominantly in tropical, subtropical, and temperate regions (Ryan and Delhaize, 2010). China's acidic soil area is large, with a total area of $\sim 2.03 \times 10^8$ hm^2, spanning both tropical and subtropical regions in more than a dozen provinces and regions such as Guizhou, Sichuan, and Jiangxi south of the Yangtze River. Soil acidification is increasing with the use of fertilizers in agriculture and the emission of industrial waste and gases (Guo et al., 2010); in some areas, soil pH has reduced to <5.0 (Von Uexküll and Mutert, 1995; Zeng, 2000). At low pH, metallic aluminum in the soil dissolves in its toxic form and is absorbed by plants (Kinraide, 1991). The tips of plant roots are the most sensitive to aluminum toxicity, which inhibits cell division and elongation (Ciamporová, 2000, 2002). The subsoil may remain acidic despite raising the soil pH with lime, inhibiting root growth and leading to stunted plant growth and an increased susceptibility to drought (Sumner et al., 1986). This has severely limited the adoption of alfalfa in southern China.

Studying genetic diversity is crucial for the development of improved plant varieties, their selection, and exploration of species kinship. Studying plant genetic diversity based on the agronomic traits offers the advantage of being intuitive, simplicity, and practicality (Jia et al., 2019; Ambati et al., 2020). The identification, classification, and screening of plant germplasm resources by agronomic or phenotypic traits and the selection of breeding materials with excellent traits have become a common and effective research method (Kephart et al., 1990; Ta et al., 2020; Hakl et al., 2021). Many plants agronomic traits are quantitative, with a broad and complex genetic basis. Varieties with excellent traits can be screened for starting with their phenotype. However, if the relationship among traits is not clear, the selection of varieties tends to be speculative and ineffective (Yang et al., 2014). Understanding the correlations between traits and predicting them through arbitrary traits can save a lot of breeding resources and is necessary to select new varieties. Previous studies have used agronomic traits to analyze the genetic diversity of alfalfa (Ray et al., 1999; Ta et al., 2020; Hakl et al., 2021), among which stem weight, leaf dry weight, single plant dry weight, and single plant fresh weight have shown rich diversity and large coefficients of variation (Wu et al., 2018b). However, the research conclusions of these studies vary because of the differences in test materials, growth environment, and analysis methods. Talebi et al. (2011) conducted genetic diversity studies on alfalfa germplasm and found that with a wider source of materials, the genetic diversity and Shannon information index were greater and the results more meaningful.

Phenotypic expression and the observed variation in growth and development are influenced by the genotype (G) of a plant, the environment (E), and their interaction (G × E) (Zhu et al., 2009). Temporal adjustments include the effects of year-to-year and harvest time variations, where biotic and abiotic events among years or ripening times between harvests affect agricultural production and quality (Redpath et al., 2021). Dong et al. (2019) conducted a 2-year continuous evaluation of the genotypic variation, phenotype, and genotype correlations of 18 traits in 418 common vetch (*Vicia sativa* L.) germplasm resources and found that all traits had significant genotypic variation ($P < 0.05$) as well as genotype interaction ($P < 0.05$). Redpath et al. (2021) performed phenotypic analysis on various fruit quality-related traits in two consecutive harvests over 2 years at two sites and found that genotype is an important source of variation for most phenotypic characteristics. In addition, year × environment and genotype × year × environment interactions significantly influenced most of the phenotypic traits. Jia et al. (2022) analyzed the productivity of 10 alfalfa cultivars in the Hexi Corridor region for 3 years and found that there were yearly differences in agronomic traits and productivity between varieties. Genetic variation in key traits of other important forages and legumes has also been studied in detail, including that of ryegrass (*Lolium perenne* L.) (Humphreys, 1991), white clover (*Trifolium repens* L.) (Jahufer et al., 2002), and yellow sweet clover (*Melilotus officinalis* L.) (Luo et al., 2016). In recent years, alfalfa resources introduced in China have increased annually, but there are few studies on their agronomic traits and phenotypic genetic diversity in acidic soil areas (Dall'Agnol et al., 1996; Grewal and Williams, 2003; Lakić et al., 2019). Notably, these studies did not note the effects of genotype × year × environment interactions in acidic soil areas, so exploring the effects of alfalfa genotype × year × environment interactions in acidic soils is of great significance for the development of acid tolerant conditions and aluminum tolerant varieties in animal husbandry in southern China.

This study performed a genetic diversity analysis on 9 agronomic traits in 44 alfalfa varieties planted for 2 consecutive years at 2 sites. We pursued the following objectives: (1) estimating the genotype and environmental type variations of key traits, (2) clarifying the interrelationships among these traits, (3) screening for alfalfa varieties resistant to acidic soil conditions in China, and (4) provide reference data and breeding materials for the cultivation of new varieties of acid-resistant aluminum.

2. Materials and methods

2.1. Experimental sites

The experiments were performed at the experimental sites of Guizhou University in Huaxi District, Guiyang City (GY; 106° 39′ 28″ E, 26° 27′ 15″ N, 1,100 m.a.s.l.) and Beiping Village, Huaqiao Town, Shiqiao County, Tongren City (SQ; 108° 20′ 14″ E, 27° 32′ 03″ N, 614 m.a.s.l.), both in Guizhou Province. The monthly precipitation and temperature changes in the two study sites during the experimental period are shown in Figure 1. GY is located in the middle of Guizhou Province, which has a subtropical monsoon humid climate and clear plateau climate characteristics. The annual average temperature at this site is 14.80 °C, whereas the average annual precipitation is 1,347.30 mm (https://data.cma.cn/data/detail/dataCode/A.0012.0001.html, Figure 1). The vegetation primarily comprised natural weeds at an early stage of growth as no crops were planted. SQ is located in the eastern part of Guizhou Province and has a humid central subtropical monsoon climate, with sufficient sunshine and abundant rainfall, an average annual temperature of 17.24°C, and an average annual precipitation of 1,410.05 mm. Before the test, the SQ test point was corn field.

Previously, alfalfa had not been planted at either of the two test sites. Before planting, ∼20 cm of surface soil at the two test sites were collected, from which impurities were removed, air-dried, ground through a 2 mm sieve, and used to determine the basal nutrient composition and pH of the soil. Both places have yellow loam soil; SQ has a soil pH of 5.44 and GY has a soil pH of 6.14. The basic nutrients of both soils are detailed in Supplementary Table 1.

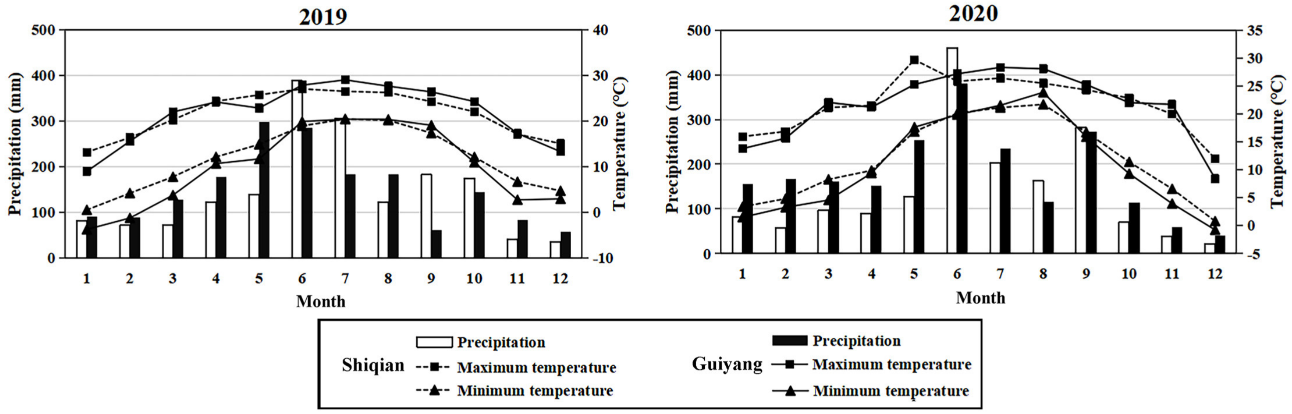

FIGURE 1
Monthly rainfall, minimum temperature, and maximum temperature in the study areas in 2019 and 2020.

TABLE 1 List of alfalfa traits 2019 and 2020.

Traits	Traits description and measurement
Stem biomass/leaf biomass ratio (RSL)	Description: The weight ratio of stems to leaves of the same alfalfa plant.
	Measurement: Each repeat randomly selects 10 alfalfa plants, weighs the stem and leaf weights separately, RSB = stem weight/leaf weight.
Stem thickness (ST, mm)	Description: The main stem of alfalfa plant is thick.
	Measurement: Select 10 plants randomly for each repetition and use vernier caliper to measure the diameter of the middle part of the second section of the main stem from the base upward (stubble height 5 cm).
Fresh weight (FW, g)	Description: The weight of a single alfalfa plant at the time of mowing.
	Measurement: Ten alfalfa plants (stubble height 5 cm) were selected at random for each repetition and weighed by electronic balance.
Total fresh weight (TFW, g)	Description: The fresh weight yield of alfalfa after cutting for 1 year.
Plant height (PH, cm)	Description: The natural height of the alfalfa plant.
	Measurement: Ten plants were randomly selected for listing in each repetition, and the height from the root neck to the top of the main stem was measured.
Number of stems (NS)	Description: The number of branches of alfalfa plants.
	Measurement: Ten plants were randomly selected for each repeat, and the number of branches was determined.
Dry weight/fresh weight ratio (RDF)	Description: The weight ratio of dry weight to fresh weight of alfalfa plant.
Dry weight (DW, g)	Description: The weight of a single alfalfa plant after drying.
	Measurement: Each repeat randomly selects 10 plants and fixation the trimmed alfalfa plants at 105°C, dried at 65°C To constant weight, and weighed on an electronic balance.
Total dry weight (TDW, g)	Description: The annual dry weight yield of alfalfa plants after cutting.

This study involved 44 alfalfa varieties, that is, 21 varieties from China; 17 from the United States; 2 each from France and Austria; and 1 each from Poland, the United Kingdom, Mexico, and Guatemala (Supplementary Table 2).

2.2. Experimental design

The experimental layout of the field trial was a randomized complete block design with three replicates, planted on September 27, 2018. The spacing between both plants and rows was 50 cm, and each replicate had 15 individual plants. During the test period, the plants grew naturally, without fertilization or artificial irrigation. When 50% of the plants of each variety in each test site flowered, they were harvested and data were recorded (harvest times are shown in Supplementary Table 3). Trait data for 10 individual plants of each variety were recorded in each replicate, presented in Table 1. Supplementary Table 1 lists the measurement methods used for each trait.

2.3. Data analysis

Using GenStat 21 (2021, VSN International Ltd.) data on the interactions of 9 agronomic traits, different years, and varieties × years of 44 alfalfa varieties cultivated in 2019 and 2020 were

TABLE 2 GGE yield rankings from 44 varieties of alfalfa in 2019 and 2020.

Rank (ID)	Variety	Rank (ID)	Variety	Rank (ID)	Variety	Rank (ID)	Variety
G1	Algonquin	G12	Gannong No. 1	G23	Gannong No. 4	G34	Gongnong No. 2
G2	Xinjiang daye	G13	Xinmu No. 2	G24	WL319HQ	G35	UC-1887
G3	Trifecta	G14	Ranger	G25	Gannong No. 6	G36	Gannong No. 5
G4	Vernal	G15	Derby	G26	Sanditi	G37	Saranac AR
G5	Zhongmu No. 1	G16	Zhonglan No. 1	G27	Arc	G38	Maverick
G6	Phabulous	G17	Archer	G28	Orca	G39	Zhongmu No. 2
G7	Sutter	G18	Gannong No. 2	G29	Gannong No. 7	G40	Longzhong
G8	WL354HQ	G19	Wudi	G30	Tianshui	G41	Dongmu No. 1
G9	Hunter River	G20	WL363HQ	G31	Gannong No. 3	G42	Longdong
G10	Boja	G21	Abi 700	G32	Aurora	G43	CUF 101
G11	Tumu No. 1	G22	WL343HQ	G33	WL168HQ	G44	UC-1465

The GGE yield ranking of each variety corresponds to its genotype ID.

analyzed. First, the genotypic variations of each agronomic trait in the 44 alfalfa varieties were analyzed (Dong et al., 2019). Then, analysis of variance (ANOVA) of the variance components was performed using the Residual Maximum Likelihood (REML) (White and Hodge, 1989). The best linear unbiased predictor analysis was used to obtain the mean trait of each variety, and the mean values were used to construct the mean matrix of varieties × traits and interaction analysis of varieties × years.

REML was used to estimate the average repeatability (R) of each trait in each variety using the following equation:

$$R = \frac{\sigma_g^2}{\sigma_g^2 + \frac{\sigma_{gy}^2}{n_l} + \frac{\sigma_\varepsilon^2}{n_l n_r}}, \tag{1}$$

where σ_g^2 is the genotype, σ_ε^2 is the experimental error, n_l is the year, and n_r is the number of replicates.

The coefficient of variation (CV) indicates the degree of dispersion of each trait and was calculated based on the following equation:

$$CV = S/X, \tag{2}$$

where S represents the standard deviation and X represents the mean.

Phenotypic correlation (rp) between traits was analyzed using GenStat 21 (2021, VSN International Ltd.). Stochastic linear models have been used to analyze inter-varietal variation in alfalfa traits (Nguyen et al., 2016). Pattern analysis combines principal component analysis (PCA) and cluster analysis (Gabriel, 1971; Kroonenberg, 1994) to provide a graphical overview of the data matrix for each trait between different varieties, where the direction vector represents the correlations between traits. A mixed linear model analysis with different varieties as fixed effects was also performed to study the differences in traits between different varieties of alfalfa (Watson et al., 1995).

The genotype main effects and genotype × environment interaction (GGE) model (Yan et al., 2000) was used to evaluate alfalfa yields in the two sites. Analyses were performed using GenStat 21 (2021, VSN International Ltd.).

3. Results

3.1. Yield evaluation of 44 alfalfa varieties

The total dry weight (TDW) of 44 alfalfa varieties was analyzed and ranked using GGE model analysis of G × E (Table 2). The varieties with the five highest yields were, in descending order, Algonquin, Xinjiang daye, Trifecta, Vernal, and Zhongmu No. 1. The varieties with the five lowest yields, in descending order, were Longzhong, Dongmu No. 1, Longdong, CUF 101, and UC-1465.

3.2. Diversity analysis of alfalfa agronomic traits

3.2.1. Genetic diversity of agronomic traits in a single year

Genotypic ANOVA of the agronomic traits of alfalfa varieties in 2019 and 2020 exhibited significant differences in yield between varieties ($P < 0.05$). Except for plant height (PH) in 2019, the results showed significant ($P < 0.05$) genotype differences among varieties (Tables 3, 4). Additionally, the average R values of each trait were significantly different. When comparing R values in 2019, those of the stem biomass/leaf biomass ratio (RSL), stem thickness (ST), number of branches (NS), and DW were the highest, at 0.45, 0.34, 0.32 and 0.32, respectively; PH, TDW and total fresh weight (TFW) followed with R values of 0.22, 0.23 and 0.24, respectively; whereas the fresh weight (FW) and dry weight/fresh weight ratio (RDF) R values were the lowest at 0.10 and 0.07, respectively. In 2020, DW and TDW R values were the highest at 0.33 and 0.61, respectively; followed by the FW and NS with R values of 0.2 and 0.22, respectively; TFW, RDF, RSL, ST and PH have lower R values of 0.12, 0.14, 0.14, 0.17 and 0.19, respectively (Table 4). The results showed that DW and TDW are hereditarily stable and less susceptible to environmental influence; thus, they could be used as important reference traits for variety selection. In 2019, FW, TFW, NS, DW, and TDW had high CVs of 29.91, 28.65, 23.40, 28.09, and 28.09%, respectively. The CVs of the RSL, ST, PH, and RDF were

TABLE 3 Average, maximum, minimum, least significant differences (*l.s.d.*$_{0.05}$), estimated genotypic (σ_g^2) and experimental error (σ_ε^2) variance components and associated standard errors (± SE), and mean repeatability (*R*) estimated from the 44 varieties of alfalfa evaluated in 2019.

Traits	RSL	ST (mm)	FW (g)	TFW (g)	PH (cm)	NS	RDF	DW (g)	TDW (g)
Average	1.04	2.81	44.26	135.32	54.33	36.56	0.31	13.10	39.29
Max	1.28	3.22	80.32	233.04	65.65	56.72	0.35	23.39	70.18
Min	0.91	2.43	19.57	66.63	44.01	21.06	0.26	5.82	17.44
l.s.d.$_{0.05}$	0.00	0.00	0.00	0.00	0.58	0.00	0.00	0.00	0.00
σ_g^2	0.006 ± 0.002	0.034 ± 0.026	6.800 ± 22.300	310.120 ± 420.002	7.630 ± 8.900	45.500 ± 19.100	0.001 ± 0.001	2.040 ± 1.440	26.800 ± 34.100
σ_ε^2	0.021 ± 0.003	0.243 ± 0.026	308.000 ± 46.400	2,358.201 ± 356.225	91.300 ± 13.760	446.300 ± 67.300	0.004 ± 0.001	22.180 ± 3.340	225.900 ± 34.100
σ_{gy}^2	0.008 ± 0.003	0.052 ± 0.030	20.700 ± 30.800	1,412.000 ± 489.084	16.840 ± 11.180	49.400 ± 31.000	0.001 ± 0.001	1.410 ± 1.710	99.800 ± 39.400
R	0.45	0.34	0.10	0.22	0.24	0.32	0.07	0.32	0.23
CV (%)	6.91	7.18	29.91	28.65	8.71	23.40	6.48	28.09	28.09

ST, stem thickness; PH, plant height; NS, number of branches; RSL, stem biomass/leaf biomass ratio; FW, fresh weight; DW, dry weight; RDF, dry weight/fresh weight ratio; TFW, total fresh weight; TDW, total dry weight.

TABLE 4 Average, maximum, minimum, least significant differences (*l.s.d.*$_{0.05}$), estimated genotypic (σ_g^2) and experimental error (σ_ε^2) variance components and associated standard errors (± SE), and mean repeatability (*R*) estimated from the 44 varieties of alfalfa evaluated in 2020.

Traits	RSL	ST (mm)	FW (g)	TFW (g)	PH (cm)	NS	RDF	DW (g)	TDW (g)
Average	1.20	2.76	182.03	985.35	69.79	54.31	0.33	58.27	291.17
Max	1.40	3.36	311.70	1,661.65	149.34	81.60	0.43	102.91	514.53
Min	1.05	2.32	64.50	387.55	60.12	27.28	0.30	23.53	117.64
l.s.d.$_{0.05}$	0.00	0.00	0.00	0.00	0.00	0.01	0.07	0.01	0.00
σ_g^2	0.001 ± 0.001	0.031 ± 0.045	1,343.100 ± 2,109.380	5,031.025 ± 1,023.001	13.000 ± 19.501	68.005 ± 94.014	0.001 ± 0.001	199.016 ± 155.104	4,421.340 ± 1,671.010
σ_ε^2	0.008 ± 0.001	0.484 ± 0.073	25,444.040 ± 3,836.840	108,531.108 ± 11,636.520	135.201 ± 14.507	1,399.050 ± 211.004	0.007 ± 0.001	2,149.504 ± 324.051	13,311.110 ± 14,19.090
σ_{gy}^2	0.002 ± 0.001	0.143 ± 0.070	2,163.250 ± 2,628.770	36,622.221 ± 16,172.010	69.100 ± 25.118	6.004 ± 124.601	0.001 ± 0.001	103.170 ± 171.220	1,169.720 ± 1,298.505
R	0.14	0.17	0.20	0.12	0.19	0.22	0.14	0.33	0.61
CV (%)	5.06	6.03	29.37	26.39	17.88	20.15	7.67	28.81	28.86

ST, stem thickness; PH, plant height; NS, number of branches; RSL, stem biomass/leaf biomass ratio; FW, fresh weight; DW, dry weight; RDF, dry weight/fresh weight ratio; TFW, total fresh weight; TDW, total dry weight.

relatively small at 6.91, 7.18, 8.71, and 6.48%, respectively (Table 3). In 2020, FW, TFW, NS, DW, and TDW had the highest CV values of 29.37, 26.39, 20.15, 28.81, and 28.86%, respectively. The CVs of PH, ST, RDF, and RSL were relatively low at 17.88, 6.03, 7.66, and 5.06%, respectively (Table 4).

3.2.2. Genetic diversity of interannual agronomic traits

The results of variance analysis of the mean value of agronomic traits under the interaction of variety × year show that there were extremely significant ($P < 0.01$) differences among the nine agronomic traits of 44 alfalfa varieties, excluding NS, indicating rich genotypic variations between them (Table 5). Comparing the *R* of each trait, ST and NS had high values of 0.31 and 0.34, respectively. RSL, FW, TFW, NS, and TDW had medium *R* values of 0.25, 0.22, 0.27, 0.28, and 0.21, respectively. RDF and DW had low *R* values of 0.03. The CVs of all agronomic traits except for RSL were high, ranging from 5.03 to 27.74%, indicating high agronomic trait diversity among alfalfa varieties. In terms of interannual performance, the highest values of DW and TDW were observed in Algonquin (61.45 and 340.82 g, respectively) and the highest values of NS, FW, and TFW were observed in WL354HQ (69.16, 189.72, and 946.60 g, respectively). The highest values of PH, ST, RSL, and RDF was observed in Derby (70.29 cm), Gannong No. 6 (3.06 mm), UC-1465 (1.26), and Xinmu No. 2 (0.37), respectively.

3.3. Pattern analysis and phenotypic correlation

3.3.1. Pattern analysis and phenotypic correlation in a single year

In 2019, the PCA showed that principal component 1 explained 50.49% of the total characteristic variation, and principal component 2 explained 16.45% of it (Figure 2A). According to PCA and correlation analyses (Table 6), both TFW and TDW were positively correlated with NS, DW, FW, PH, ST, and RSL (angle between direction vectors <90°, $P < 0.01$). Additionally, RDF was negatively correlated with DW, FW, TFW, TDW, and NS (angle between directional vectors >90°, $P < 0.01$). The smallest and largest positive *rp* were between RSL and DW (0.017) and FW and DW (0.969), respectively. Cluster analysis divided the 44 varieties into 2 groups (Figure 2A, Table 7). Group 1 contained 10 varieties, and group 2 contained 34 varieties. The mean values of RSL, ST, FW, TFW, PH, NS, DW, and TDW in the first group were greater than those in the second group.

In 2020, PCA showed that principal component 1 explained 42.96% of the total characteristic variation, and principal component 2 explained 20.18% of it (Figure 2B). According to PCA and correlation analyses (Table 8), among the traits, TFW and TDW were positively correlated with PH, DW, FW, and NS (angle between direction vectors <90°, $P < 0.01$), whereas they were negatively correlated with RSL and RDF (angle between direction vectors >90°, $P < 0.05$). ST, PH, FW, NS, and DW were significantly positively correlated ($P < 0.01$). Cluster analysis divided the 44 alfalfa varieties into three groups (Figure 2B, Table 7): group 1 contained 8 varieties, group 2 contained 23 varieties, and group 3 contained 13 varieties. Among them, group 1 had the highest values of TFW, PH, NS, and TDW, which were 1,421.05 g, 95.83 cm, 84, and 421.59 g, respectively. Group 3 had the lowest values for TFW, PH, NS, and TDW (707.81 g, 88.18 cm, 57, and 204.55 g, respectively).

3.3.2. Interannual agronomic trait pattern analysis and phenotypic correlation

Between 2019 and 2020, TFW and TDW were positively correlated with PH, DW, FW, NS, and ST (angle between direction vectors <90°) and negatively correlated with RSL and RDF (angle between direction vectors >90°) (Figure 2C). PCA showed that principal component 1 explained 47.87% of the total characteristic variation, and principal component 2 explained 19.07% of it. Cluster analysis divided the 44 alfalfa varieties into two groups (Figure 2C, Table 7), with group 1 containing 11 varieties and group 2 containing 33 varieties. The mean values of all traits in group 1 were greater than those of group 2, and the mean values of TFW, PH, NS, and TDW in group 1 were 764.26 g, 76.51 cm, 61, and 224.03 g, respectively. The same trend was observed in the agronomic traits across years (Figure 3), as varieties with high FW, DW, TFW, and TDW (Algonquin, Xinjiang daye, Trifecta, Vernal, WL354HQ, and Boja) also had relatively high ST, PH, and NS. This result was the same as that shown in Figure 2C.

The *rp* coefficients between the agronomic traits of the 44 alfalfa varieties, shown in Table 9, further demonstrate the correlation between the indicators in Figure 2C. TFW and TDW were positively correlated with PH, DW, FW, and NS ($P < 0.01$) and negatively correlated with RSL and RDF, reaching a significant correlation only with RDF. The smallest positive *rp* belonged to the PH and RSL correlation, whereas the largest belonged to the TDW and TFW correlation.

4. Discussion

Genetic variations in plants are formed by long-term natural selection and endows them with relatively stable and rich phenotypic and genotypic traits (Wu et al., 2018a). In this study, 5 alfalfa varieties (Algonquin, Xinjiang daye, Trifecta, Vernal, and Zhongmu No. 1) with strong adaptability and high grass yield were screened through GGE evaluation of the dry weights of 44 alfalfa varieties at multiple locations over 2 years. Jia et al. (2022) conducted a 3-year study on yield and agronomic traits of 10 alfalfa varieties in the Hexi Corridor region. Through the analysis of grass yield, they found that the yield of Xinjiang daye (11,416 kg/ha) was lower than that of Gannong No. 5 (13,923 kg/ha). However, in this study, Xinjiang daye showed good adaptability and high relative yields compared with Gannong No. 5. Zhao et al. (2022) conducted a 1-year adaptability study on 7 alfalfa varieties in Chifeng area, China, the total hay yield of each crop was in the order of Algonquin (12,859.40 kg/ha), Golden Empress (12,341.31 kg/ha), WL168(11,928.95 kg/ha), 55V12(10,258.90, Sanditi (9,394.60 kg/ha), Hunt River (8,782.25 kg/ha) and 54V09 (8,422.43 kg/ha). This was quite different from the performance of Algonquin, WL168 and Sanditi in this study, which may be due to a

TABLE 5 Average, maximum, minimum, least significant differences (*l.s.d.*$_{0.05}$), estimated genotypic (σ_g^2) and experimental error (σ_ϵ^2) variance components and associated standard errors (± SE), and mean repeatability (*R*) estimated from the 44 varieties of alfalfa evaluated in both 2019 and 2020.

ID	genotype	RSL	ST (mm)	FW (g)	TFW (g)	PH (cm)	NS	RDF	DW (g)	TDW (g)
G1	Algonquin	1.11	2.98	180.67	847.60	67.55	58.20	0.32	**61.45**	**340.82**
G2	Xinjiang daye	1.14	2.88	164.03	838.28	65.30	67.51	0.31	51.12	283.51
G3	Trifecta	1.13	3.04	183.13	856.54	67.42	56.43	0.30	55.36	305.22
G4	Vernal	1.15	2.58	167.26	773.02	64.99	61.06	0.29	48.32	268.83
G5	Zhongmu No. 1	1.14	2.97	126.89	658.33	63.99	49.69	0.30	38.63	225.82
G6	Phabulous	1.07	2.88	134.82	655.86	64.70	55.76	0.33	42.59	234.20
G7	Sutter	1.11	2.99	134.10	658.44	63.07	54.88	0.32	41.22	237.69
G8	WL354HQ	1.15	2.97	**189.72**	**946.60**	63.15	**69.16**	0.30	54.24	308.54
G9	Hunter River	1.19	2.88	122.27	524.13	67.29	44.79	0.31	37.59	211.01
G10	Boja	1.14	2.99	166.73	748.11	63.23	60.05	0.32	50.70	289.89
G11	Tumu No. 1	1.09	2.81	112.34	558.04	62.85	43.78	0.31	35.29	214.78
G12	Gannong No. 1	1.15	2.64	110.48	569.42	60.68	45.26	0.31	35.34	200.37
G13	Xinmu No. 2	1.09	2.70	102.76	578.03	64.26	48.88	**0.37**	35.35	206.11
G14	Ranger	1.18	2.96	145.49	745.55	65.93	50.14	0.33	44.88	274.01
G15	Derby	1.12	2.91	110.55	564.72	**70.29**	42.30	0.32	34.56	197.02
G16	Zhonglan No. 1	1.09	2.81	87.94	513.89	65.99	40.69	0.32	28.50	170.58
G17	Archer	1.21	2.88	140.06	678.56	66.23	50.23	0.31	42.86	258.88
G18	Gannong No. 2	1.09	2.66	86.25	426.60	59.74	41.63	0.31	26.53	166.02
G19	Wudi	1.03	2.82	88.08	441.52	59.70	40.19	0.32	28.58	173.98
G20	WL363HQ	1.09	2.72	117.36	506.92	58.22	45.28	0.36	51.92	267.99
G21	Abi 700	1.08	2.81	103.00	563.61	61.77	43.34	0.32	32.82	200.02
G22	WL343HQ	1.11	2.87	129.10	623.08	63.36	47.18	0.31	39.00	229.09
G23	Gannong No. 4	1.09	2.87	118.20	567.5	60.68	39.89	0.29	36.42	209.22
G24	WL319HQ	1.04	2.77	116.96	603.49	60.93	50.16	0.31	36.15	204.33
G25	Gannong No. 6	1.14	**3.06**	87.49	450.26	64.02	39.76	0.31	27.40	153.96
G26	Sanditi	1.05	2.55	86.09	427.99	59.48	32.69	0.32	28.08	157.81
G27	Arc	1.15	2.66	104.55	493.42	62.98	45.24	0.32	32.58	193.15

(Continued)

TABLE 5 (Continued)

ID	genotype	RSL	ST (mm)	FW (g)	TFW (g)	PH (cm)	NS	RDF	DW (g)	TDW (g)
G28	Orca	1.23	3.00	83.93	423.38	68.12	34.83	0.34	26.88	156.83
G29	Gannong No. 7	1.19	2.82	107.75	547.38	63.42	42.73	0.30	34.27	208.59
G30	Tianshui	1.14	2.82	90.11	500.82	62.54	44.39	0.33	29.89	176.67
G31	Gannong No. 3	1.17	2.78	126.86	598.97	60.61	38.46	0.30	38.95	229.98
G32	Aurora	1.10	2.77	105.06	509.28	59.37	39.24	0.33	34.93	203.36
G33	WL168HQ	1.11	2.62	99.20	534.60	58.42	45.08	0.32	30.35	176.97
G34	Gongnong No. 1	1.11	2.88	107.22	526.08	64.57	41.25	0.31	34.11	196.04
G35	UC-1887	**1.26**	2.80	95.69	498.42	68.06	40.38	0.30	29.12	176.26
G36	Gannong No. 5	1.08	2.71	98.33	474.54	65.89	42.63	0.31	30.00	164.59
G37	Saranac AR	1.13	2.76	86.92	448.75	62.44	35.96	0.34	28.10	169.29
G38	Maverick	1.11	2.43	82.80	400.58	55.85	43.17	0.30	24.94	148.46
G39	Zhongmu No. 2	1.08	2.91	84.86	393.19	61.89	38.13	0.30	26.19	162.63
G40	Longzhong	1.12	2.67	111.23	536.02	61.34	47.12	0.32	33.11	207.67
G41	Dongmu No. 1	1.09	2.63	79.50	402.28	54.94	37.41	0.31	23.95	147.91
G42	Longdong	1.12	2.63	74.60	398.26	60.52	44.17	0.32	23.78	144.21
G43	CUF 101	1.14	2.70	78.91	399.98	59.91	32.96	0.33	27.21	175.56
G44	UC-1465	1.19	2.52	49.22	242.66	58.44	27.26	0.34	16.78	104.74
Average		1.12	2.80	113.15	560.33	62.82	45.44	0.32	35.68	207.56
Max		1.26	3.06	189.72	946.60	70.29	69.16	0.37	61.45	340.82
Min		1.03	2.43	49.22	242.66	54.94	27.26	0.29	16.78	104.74
$l.s.d._{0.05}$		0.00	0.03	0.00	0.00	0.03	0.48	0.00	0.00	0.00
σ_g^2		0.001 ± 0.001	0.001 ± 0.001	2,288.052 ± 619.200	7,272.000 ± 5,505.001	16.700 ± 8.101	75.400 ± 29.400	0.001 ± 0.001	9.010 ± 41.000	570.003 ± 567.520
σ_ε^2		0.140 ± 0.010	0.052 ± 0.011	38,387.033 ± 2,893.086	73,529.001 ± 4,957.008	182.400 ± 12.300	998.000 ± 75.200	0.001 ± 0.001	1,791.000 ± 121.000	7,852.017 ± 529.610
σ_{gy}^2		0.020 ± 0.001	0.001 ± 0.001	3,100.100 ± 861.001	15,834.060 ± 6,114.140	3.200 ± 7.500	56.000 ± 26.900	0.001 ± 0.001	38.001 ± 60.000	1,796.204 ± 675.570
R		0.25	0.31	0.22	0.27	0.34	0.28	0.03	0.03	0.21
CV (%)		4.19	5.27	27.74	25.76	5.30	19.22	5.03	26.93	24.21

ST, stem thickness; PH, plant height; NS, number of branches; RSL, stem biomass/leaf biomass ratio; FW, fresh weight; DW, dry weight; RDF, dry weight/fresh weight ratio; TFW, total fresh weight; TDW, total dry weight.
The highest values in each column are shown in bold.

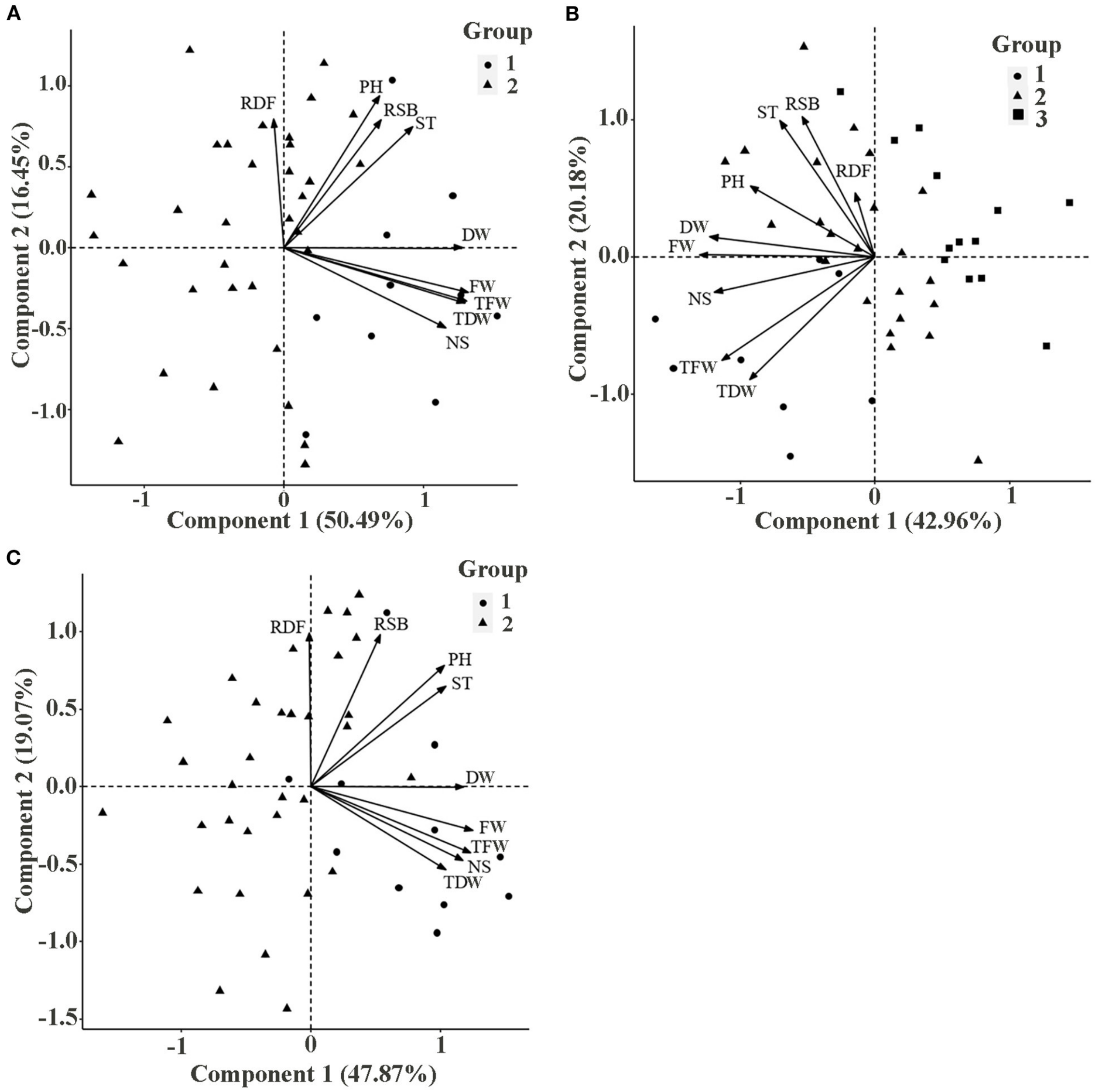

FIGURE 2
Principal component analysis of 9 agronomic traits of 44 alfalfa varieties in **(A)** 2019, **(B)** 2020, and **(C)** interannual. ST, stem thickness; PH, plant height; NS, number of branches; RSL, stem biomass/leaf biomass ratio; FW, fresh weight; DW, dry weight; RDF, dry weight/fresh weight ratio; TFW, total fresh weight; TDW, total dry weight.

combination of genes and the environment (Dungey et al., 2018) as the same variety will show significant phenotypic variation across different environments. Thus, the above discrepancies in results may be caused by differences among the varieties, test-site climate (especially temperature and precipitation), and soil physical and chemical properties.

Agronomic traits such as RSL, ST, FW, TFW, PH, NS, RDF, DW, and TDW are key indicators used to evaluate both the quality (Bhattarai et al., 2020; Jia et al., 2022; Sayed et al., 2022) and productivity of alfalfa varieties (Singer et al., 2017). However, previous studies on alfalfa have primarily focused on agronomic traits in a single place or year (Tucak et al., 2008; Inostroza et al., 2021) as well as the development of simple sequence repeat primers (Flajoulot et al., 2005; He et al., 2020) and transcriptome analysis (Chen et al., 2011; Liu et al., 2017). In this study, phenotypic and genotypic variation analyses of 9 agronomic traits in 44 varieties of alfalfa grown in acidic soil for 2 years showed that there was abundant genotypic variation in these agronomic traits. Overall, ST, PH, RSL, FW, NS, TFW, and TDW had relatively high *R* values (0.21–0.34, Tables 2–4), which indicate that the trait is mainly affected by genetic factors, is less susceptible to environmental factors, and has more stable genetics (Gonçalves et al., 2020). Generally, the genetic traits ST, PH, RSL, FW, NS, TFW, and TDW are genetically more stable than RDF and DW. Dong et al. (2019) found that the *R* values of ST, PH, and DW of 418 common vetch germplasms in 2015 and 2016 ranged 0.89–0.95, which is much larger than the *R* values of those traits seen in this study. This may be because vetch is a self-pollinating plant with more conserved and stable genetics, whereas alfalfa is a cross-pollinating plant with greater variation

TABLE 6 Correlation coefficients of phenotypes (*rp*) among 44 varieties of alfalfa in 2019.

Traits	RSL	ST	FW	TFW	PH	NS	RDF	DW	TDW
RSL	1								
ST	0.240**	1							
FW	−0.016	0.568**	1						
TFW	0.410**	0.492**	0.698**	1					
PH	0.196**	0.597**	0.464**	0.412**	1				
NS	0.078	0.300**	0.479**	0.409**	0.138*	1			
RDF	0.162**	0.152**	−0.246**	−0.240**	0.184**	−0.310**	1		
DW	0.017	0.544**	0.969**	0.647**	0.463**	0.455**	−0.080	1	
TDW	0.411**	0.468**	0.681**	0.934**	0.385**	0.403**	−0.230**	0.640**	1

ST, stem thickness; PH, plant height; NS, number of branches; RSL, stem biomass/leaf biomass ratio; FW, fresh weight; DW, dry weight; RDF, dry weight/fresh weight ratio; TFW, total fresh weight; TDW, total dry weight. *Denotes significant correlation of $P < 0.05$, **denotes extremely significant correlation of $P < 0.01$.

TABLE 7 Cluster analysis of grouping information for 44 varieties of alfalfa.

	Group	Variety numbers	RSL	ST (mm)	FW (g)	TFW (g)	PH (cm)	NS	RDF	DW (g)	TDW (g)
2019	1	10	0.99	2.98	39.14	193.49	58.70	39	0.29	10.06	55.02
	2	34	0.95	2.72	29.06	118.21	55.81	30	0.28	7.71	34.66
2020	1	8	0.85	3.36	358.83	1,421.05	95.83	84	0.31	91.50	421.59
	2	23	0.87	3.44	304.54	990.67	93.55	69	0.30	78.11	294.77
	3	13	0.86	3.33	238.89	707.81	88.18	57	0.31	60.27	204.55
2019–2020	1	11	0.93	3.28	196.14	764.26	76.52	61	0.29	49.23	224.03
	2	33	0.91	3.01	152.20	492.37	73.73	46	0.29	39.27	145.63

ST, stem thickness; PH, plant height; NS, number of branches; RSL, stem biomass/leaf biomass ratio; FW, fresh weight; DW, dry weight; RDF, dry weight/fresh weight ratio; TFW, total fresh weight; TDW, total dry weight.

TABLE 8 Correlation coefficients of phenotypes (*rp*) among 44 varieties of alfalfa in 2020.

Traits	RSL	ST	FW	TFW	PH	NS	RDF	DW	TDW
RSL	1								
ST	0.437**	1							
FW	0.745**	0.480**	1						
TFW	−0.208**	0.126**	0.198**	1					
PH	0.408**	0.331**	0.450**	0.189**	1				
NS	0.356**	0.475**	0.684**	0.414**	0.231**	1			
RDF	0.629**	0.249**	0.548**	−0.216**	0.219**	−0.074	1		
DW	0.609**	0.443**	0.850**	0.223**	0.439**	0.597**	−0.173**	1	
TDW	−0.207**	0.078	0.196**	0.813**	0.127*	0.324**	−0.182**	0.223**	1

ST, stem thickness; PH, plant height; NS, number of branches; RSL, stem biomass/leaf biomass ratio; FW, fresh weight; DW, dry weight; RDF, dry weight/fresh weight ratio; TFW, total fresh weight; TDW, total dry weight. *Denotes significant correlation of $P < 0.05$, **denotes extremely significant correlation of $P < 0.01$.

and susceptibility to environmental influence (Bagavathiannan and Van Acker, 2009).

Plant yield is an important indicator of forage production performance and is mainly affected by dry weight (Sayed et al., 2022). El-Hifny et al. (2019) found that alfalfa genotypes exhibited a high total variability in PH and pasture yield. The forage yield of alfalfa depends mainly on PH and tillering (Monirifar, 2011). This was also verified in this study, where visualization of interannual data showed that high FW and DW were usually accompanied by higher pH and NS (Tables 3–5, Figure 3). Studies have shown that PH is positively correlated with alfalfa yield; therefore, PH can be used as an indicator for selecting the best genotype and predicting high yield in hybridization and breeding programs (Davis and Buker, 1966; Tucak et al., 2008). The RSL, fresh yield, and dry yield are important indicators for evaluating the palatability of forage, which affects forage yield. However, when the RSL is too high, it indicates that the stem yield is too large and the leaf yield is relatively reduced. Although the overall yield increases with high RSL, the protein content is low and the palatability is poor. Wu et al. (2018a) conducted a genetic diversity analysis on seven agronomic traits, including PH, FW, and DW, and the results showed that the CV of each trait was >10%. The CV of each trait in this study was high (>5%, RSL CV <5% only for 2019–2020, Tables 3–5), which could be regarded as producing large variation (Bendjama and Ramdani, 2022), indicating that the agronomic traits in this study are rich in genetic diversity. The richer the genetic diversity of a species, the greater its ability to adapt to its environment and its evolutionary potential (Nevo et al., 2012). Under the interaction between varieties × environment, the traits showed large differences, which was also conducive to the study of alfalfa variety selection and introduction adaptability in the later stage.

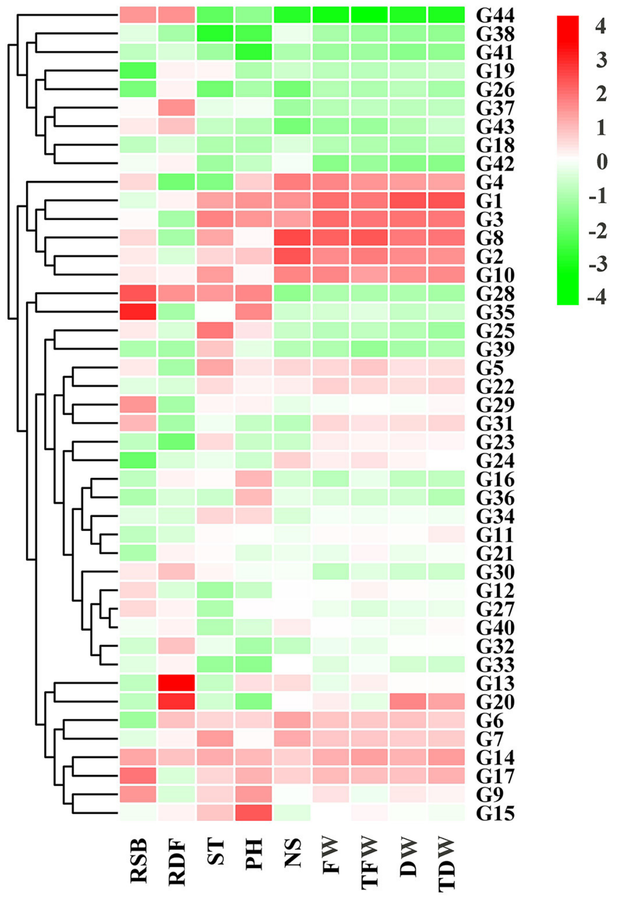

FIGURE 3
Heatmap showing 9 agronomic traits of 44 alfalfa varieties cultivated between 2019 and 2020. ST, stem thickness; PH, plant height; NS, number of branches; RSL, stem biomass/leaf biomass ratio; FW, fresh weight; DW, dry weight; RDF, dry weight/fresh weight ratio; TFW, total fresh weight; TDW, total dry weight.

Previously, the correlation between agronomic traits and yield as well as their contribution rate were used to determine whether agronomic traits could be used as reference indicators for germplasm selection (Monirifar, 2011; Jia et al., 2022). In this study, PCA and related analysis results showed that PH, NS, FW, TFW, DW, and TDW all had a significant positive correlation, and the higher the RSL, the better the yield performance. This may be a consequence of higher stem/leaf ratio being accompanied by a higher PH and NS. These results are consistent with those reported by other researchers (Kephart et al., 1990; Ray et al., 1999; Avci et al., 2018). The results showed that PH, DW, FW, and NS can be used as the main target traits for breeding, and it is necessary to increase the selection of target traits in the process of variety selection. However, the contribution of alfalfa agronomic traits varied across locations and years. In the first year of planting (2019), the ecological niche requirements of alfalfa were sufficiently met, and the available resources promoted vegetative growth (Weiner et al., 2009; Zhu et al., 2019); that is, agronomic traits were proportional to DW and FW. In the second year (2020), the competition between communities increased, the varieties obtained more resources by increasing PH, the proportion of stems increased, the resources were more inclined toward reproductive growth, RSL gradually increased, and RDF increased. Also taking into account the interannual data analysis, Algonquin, Xinjiang daye, Trifecta, Vernal, WL354HQ, and Boja varieties performed well in terms of TDW and TFW in different years, environmental

TABLE 9 Correlation coefficients of phenotypes (*rp*) among 44 varieties of alfalfa in both 2019 and 2020.

Traits	RSL	ST	FW	TFW	PH	NS	RDF	DW	TDW
RSL	1								
ST	0.287**	1							
FW	0.443**	0.537**	1						
TFW	−0.023	0.393**	0.580**	1					
PH	0.096*	0.549**	0.658**	0.703**	1				
NS	0.185*	0.529*	0.740**	0.611**	0.505**	1			
RDF	0.489**	0.180**	−0.294**	−0.335**	0.218**	−0.314**	1		
DW	0.331**	0.518**	0.906**	0.610**	0.671**	0.690**	−0.030	1	
TDW	−0.225**	0.361**	0.519**	0.931**	0.665**	0.565**	−0.391**	0.600**	1

ST, stem thickness; PH, plant height; NS, number of branches; RSL, stem biomass/leaf biomass ratio; FW, fresh weight; DW, dry weight; RDF, dry weight/fresh weight ratio; TFW, total fresh weight; TDW, total dry weight. *Denotes significant correlation of $P < 0.05$, **denotes extremely significant correlation of $P < 0.01$.

regions, and climatic altitudes (Tables 3–5). Thus, these varieties (genotypes) can be used in future projects to improve and develop new strains and varieties of alfalfa that can tolerate acidic soil conditions.

5. Conclusion

The agronomic traits of alfalfa are of great significance to the selection and preservation of excellent varieties, and dry weight plays a decisive role in yield. There were significant ($P < 0.05$) genotypic variations and genetic diversities in the traits of 44 alfalfa cultivars. Compared with other traits, PH, ST, NS, FW, TFW, and TDW *R* values were higher, and their genetics were relatively stable, suggesting they could be used as reference indicators for alfalfa variety selection. Genotypes Algonquin, Xinjiang daye, Trifecta, Vernal, WL354HQ, and Boja performed well under various climatic and environmental conditions. They can be used as commercial varieties as well as in projects to improve and develop new alfalfa strains that tolerate the acidic soil conditions in the south of China.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

RD and CC conceived the experiments. ZT, ZY, ZL, BL, YH, XW, FY, and SW performed the experiments. ZT, ZY, and RD analyzed the data. RD and ZT wrote this manuscript. All authors have contributed to the manuscript and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Ambati, D., Phuke, R. M., Vani, V., Sai Prasad, S. V., Singh, J. B., Patidar, C. P., et al. (2020). Assessment of genetic diversity and development of core germplasm in durum wheat using agronomic and grain quality traits. *Cereal Res. Commun.* 48, 375–382. doi: 10.1007/s42976-020-00050-z

Arshad, M., Feyissa, B. A., Amyot, L., Aung, B., and Hannoufa, A. (2017). MicroRNA156 improves drought stress tolerance in alfalfa (*Medicago sativa*) by silencing SPL13. *Plant Sci.* 258, 122–136. doi: 10.1016/j.plantsci.2017.01.018

Avci, M., Hatipoglu, R., Çinar, S., and Kiliçalp, N. (2018). Assessment of yield and quality characteristics of alfalfa (*Medicago sativa* L.) cultivars with different fall dormancy rating. *Legum. Res.* 41, 369–373. doi: 10.18805/LR-364

Avci, M. A., Ozkose, A., and Tamkoc, A. (2013). Determination of yield and quality characteristics of alfalfa (Medicago sativa L.) varieties grown in different locations. *J. Anim. Vet. Adv.* 12, 487–490. doi: 10.3923/javaa.2013.487.490

Bagavathiannan, M. V., and Van Acker, R. C. (2009). The biology and ecology of feral alfalfa (*Medicago sativa* L.) and its implications for novel trait confinement in North America. *Crit. Rev. Plant Sci.* 28, 69–87. doi: 10.1080/07352680902753613

Bendjama, A., and Ramdani, S. (2022). Genetic variability of some agronomic traits in a collection of wheat (Triticum turgidum L. sp. pl.) genotypes under South Mediterranean growth conditions. *Ital. J. Agron.* 17, 1976. doi: 10.4081/ija.2021.1976

Bhattarai, S., Biswas, D., Fu, Y. B., and Biligetu, B. (2020). Morphological, physiological, and genetic responses to salt stress in alfalfa: a review. *Agronomy* 10, 577. doi: 10.3390/agronomy10040577

Chen, Q., Zhang, X. D., Wang, S. S., Wang, Q. F., Wang, G. Q., Nian, H. J., et al. (2011). Transcriptional and physiological changes of alfalfa in response to aluminium stress. *J. Agric. Sci.* 149, 737–751. doi: 10.1017/S0021859611000256

Ciamporová, M. (2000). Diverse responses of root cell structure to aluminium stress. *Plant and Soil* 226, 113. doi: 10.1023/A:1026468403157

Ciamporová, M. (2002). Morphological and structural responses of plant roots to aluminium at organ, tissue, and cellular levels. *Biol. Plant.* 45, 161–171. doi: 10.1023/A:1015159601881

Dall'Agnol, M., Bouton, J. H., and Parrott, W. A. (1996). Screening methods to develop alfalfa germplasms tolerant of acid, aluminum toxic soils. *Crop Sci.* 36, 64–70. doi: 10.2135/cropsci1996.0011183X003600010011x

Davis, R. L., and Buker, R. J. (1966). Relationship among seedling height, greenhouse regrowth, fall height, and spring height in *Medicago sativa* L. 1. *Crop Sci.* 6, 201–204. doi: 10.2135/cropsci1966.0011183X000600020029x

Dogbatse, J. A., Arthur, A., Padi, F. K., Konlan, S., Quaye, A. K., Owusu-Ansah, F., et al. (2020). Influence of acidic soils on growth and nutrient uptake of cocoa (*Theobroma cacao* L.) varieties. *Commun. Soil Sci. Plant Anal.* 51, 2280–2296. doi: 10.1080/00103624.2020.1822384

Dong, R., Shen, S. H., Jahufer, M. Z., Dong, D. K., Luo, D., Zhou, Q., et al. (2019). Effect of genotype and environment on agronomical characters of common vetch (*Vicia sativa* L.). *Genet. Resour. Crop Evol.* 66, 1587–1599. doi: 10.1007/s10722-019-00789-3

Dungey, H. S., Dash, J. P., Pont, D., Clinton, P. W., Watt, M. S., and Telfer, E. J. (2018). Phenotyping whole forests will help to track genetic performance. *Trends Plant Sci.* 23, 854–864 doi: 10.1016/j.tplants.2018.08.005

El-Hifny, Z. M., Bakheit, B. R., Hassan, M. S., and El-Rady, A. (2019). Forage and seed yield variation of alfalfa cultivars in response to planting date. *SVU-Int. J. Agric. Sci.* 1, 21–33. doi: 10.21608/svuijas.2019.67079

Feng, Y., Shi, Y., Zhao, M., Shen, H., Xu, L., Luo, Y., et al. (2022). Yield and quality properties of alfalfa (*Medicago sativa* L.) and their influencing factors in China. *Eur. J. Agron.* 141, 126637. doi: 10.1016/j.eja.2022.126637

Flajoulot, S., Ronfort, J., Baudouin, P., Barre, P., Huguet, T., Huyghe, C., et al. (2005). Genetic diversity among alfalfa (*Medicago sativa*) cultivars coming from a breeding program, using SSR markers. *Theor. Appl. Genet.* 111, 1420–1429. doi: 10.1007/s00122-005-0074-4

Gabriel, K. R. (1971). The biplot graphic display of matrices with application to principal component analysis. *Biometrika* 58, 453–467. doi: 10.1093/biomet/58.3.453

Gonçalves, G., Ferreira-Gomes, R. L., Lopes, Â., and Vieira, P. (2020). Adaptability and yield stability of soybean genotypes by REML/BLUP and GGE biplot. *Crop Breed. Appl. Biot.* 20, e282920217. doi: 10.1590/1984-70332020v20n2a33

Grewal, H. S., and Williams, R. (2003). Liming and cultivars affect root growth, nodulation, leaf to stem ratio, herbage yield, and elemental composition of alfalfa on an acid soil. *J. Plant Nutr.* 26, 1683–1696. doi: 10.1081/PLN-120022381

Guo, J. H., Liu, X. J., Zhang, Y., Shen, J. L., Han, W. X., Zhang, W. F., et al. (2010). Significant acidification in major chinese croplands. *Science* 327, 1008–1010. doi: 10.1126/science.1182570

Hakl, J., Kunzová, E., Tocauerová, Š., Menšík, L., Mrázková, M., and Pozdíšek, J. (2021). Impact of long-term manure and mineral fertilization on yield and nutritive value of lucerne (*Medicago sativa*) in relation to changes in canopy structure. *Eur. J. Agron.* 123, 126219. doi: 10.1016/j.eja.2020.126219

He, F., Long, R., Zhang, T., Zhang, F., Wang, Z., Yang, X., et al. (2020). Quantitative trait locus mapping of yield and plant height in autotetraploid alfalfa (*Medicago sativa* L.). *Crop J.* 8, 812–818. doi: 10.1016/j.cj.2020.05.003

Humphreys, M. O. (1991). A genetic approach to the multivariate differentiation of perennial ryegrass (*Lolium perenne* L.) populations. *Heredity* 66, 437–443. doi: 10.1038/hdy.1991.53

Inostroza, L., Espinoza, S., Barahona, V., Gerding, M., Humphries, A., del Pozo, A., et al. (2021). Phenotypic diversity and productivity of *Medicago sativa* subspecies from drought-prone environments in mediterranean type climates. *Plants* 10, 862. doi: 10.3390/plants10050862

Jahufer, M. Z. Z., Cooper, M., Ayres, J. F., and Bray, R. A. (2002). Identification of research to improve the efficiency of breeding strategies for white clover in Australia-a review. *Aust. J. Agr. Res.* 53, 239–257. doi: 10.1071/AR01110

Jia, X., Zhang, Z., and Wang, Y. (2022). Forage yield, canopy characteristics, and radiation interception of ten alfalfa varieties in an arid environment. *Plants* 11, 1112. doi: 10.3390/plants11091112

Jin, L., Zhao, L., Wang, Y., Zhou, R., Song, L., Xu, L., et al. (2019). Genetic diversity of 324 cultivated tomato germplasm resources using agronomic traits and InDel markers. *Euphytica* 215, 69. doi: 10.1007/s10681-019-2391-8

Kephart, K. D., Buxton, D. R., and Hill, R. R. (1990). Digestibility and cell-wall components of alfalfa following selection for divergent herbage lignin concentration. *Crop Sci.* 30, 207. doi: 10.2135/cropsci1990.0011183X003000010045x

Kinraide, T. B. (1991). Identity of the rhizotoxic aluminium species. *PSILPH.* 717–728. doi: 10.1007/978-94-011-3438-5_80

Kochian, L. V., Piñeros, M. A., Liu, J., and Magalhaes, J. V. (2015). Plant adaptation to acid soils: the molecular basis for crop aluminum resistance. *Annu. Rev. Plant Biol.* 66, 571–598. doi: 10.1146/annurev-arplant-043014-114822

Kroonenberg, P. M. (1994). The TUCKALS line: a suite of programs for three-way data analysis. *Comput. Stat. Data Anal.* 18, 73–96. doi: 10.1016/0167-9473(94)90133-3

Lakić, Ž., Antić, M., Durdić, I., and Popović, V. (2019). Morphological characteristics of alfalfa genotypes tolerant to low soil pH. *Genetika-Belgrade* 51, 907–922. doi: 10.2298/GENSR1903907L

Liu, W., Xiong, C., Yan, L., Zhang, Z., Ma, L., Wang, Y., et al. (2017). Transcriptome analyses reveal candidate genes potentially involved in Al stress response in alfalfa. *Front. Plant Sci.* 8, 26. doi: 10.3389/fpls.2017.00026

Luo, K., Jahufer, M. Z. Z., Wu, F., Di, H., Zhang, D., Meng, X., et al. (2016). Genotypic variation in a breeding population of yellow sweet cover (*Melilotus officinalis*). *Front. Plant Sci.* 7, 972. doi: 10.3389/fpls.2016.00972

Monirifar, H. (2011). Path analysis of yield and quality traits in alfalfa. *Not. Bot. Horti Agrobo.* 39, 190–195. doi: 10.15835/nbha3926324

Nevo, E., Fu, Y. B., Pavlicek, T., Khalifa, S., Tavasi, M., and Beiles, A. (2012). Evolution of wild cereals during 28 years of global warming in Israel. *PNAS.* 109, 3412–3415. doi: 10.1073/pnas.1121411109

Nguyen, L., Lang, D., van Gessel, N., Beike, A. K., Menges, A., Reski, R., et al. (2016). Evolutionary processes as models for exploratory design. *In Bio. Res. Arch. Build. Construct.* 8, 295–318. doi: 10.1007/978-3-319-46374-2_15

Ray, I. M., Townsend, M. S., Muncy, C. M., and Henning, J. A. (1999). Heritabilities of water-use efficiency traits and correlations with agronomic traits in water-stressed alfalfa. *Crop Sci.* 39, 494–498. doi: 10.2135/cropsci1999.0011183X0039000200032x

Redpath, L. E., Gumpertz, M., Ballington, J. R., Bassil, N., and Ashrafi, H. (2021). Genotype, environment, year, and harvest effects on fruit quality traits of five blueberry (*Vaccinium corymbosum* L.) cultivars. *Agronomy* 11, 1788. doi: 10.3390/agronomy11091788

Rudgers, J. A., Hallmark, A., Baker, S. R., Baur, L., Hall, K. M., Litvak, M. E., et al. (2019). Sensitivity of dryland plant allometry to climate. *Funct. Ecol.* 33, 2290–2303. doi: 10.1111/1365-2435.13463

Ryan, P. R., and Delhaize, E. (2010). The convergent evolution of aluminium resistance in plants exploits a convenient currency. *Funct. Plant Biol.* 37, 275–284. doi: 10.1071/FP09261

Sayed, M. R., Alshallash, K. S., Safhi, F. A., Alatawi, A., ALshamrani, S. M., Dessoky, E. S., et al. (2022). Genetic diversity, analysis of some agro-morphological and quality traits and utilization of plant resources of alfalfa. *Genes* 13, 1521. doi: 10.3390/genes13091521

Singer, S. D., Hannoufa, A., and Acharya, S. (2017). Molecular improvement of alfalfa for enhanced productivity and adaptability in a changing environment. *Plant Cell Environ.* 41, 1955–1971. doi: 10.1111/pce.13090

Sumner, M. E., Shahandeh, H., Bouton, J., and Hammel, J. (1986). Amelioration of an acid soil profile through deep liming and surface application of gypsum. *Soil Sci. Soc. Am. J.* 50, 1254–1258. doi: 10.2136/sssaj1986.03615995005000050034x

Ta, H. T., Teixeira, E. I., Brown, H. E., and Moot, D. J. (2020). Yield and quality changes in lucerne of different fall dormancy ratings under three defoliation regimes. *Eur. J. Agron.* 115, 126012. doi: 10.1016/j.eja.2020.126012

Talebi, M., Hajiahmadi, Z., and Rahimmalek, M. (2011). Genetic diversity and population structure of four Iranian alfalfa populations revealed by sequence-related amplified polymorphism (SRAP) markers. *JCSB.* 14, 173–178. doi: 10.1007/s12892-011-0030-6

Tucak, M., Popovic, S., Bolaric, S., and Kozumplik, V. (2008). Agronomic evaluation of alfalfa genotypes under ecological conditions of eastern croatia. *Cereal Res. Commun.* 36, 651–654. Available online at: https://www.jstor.org/stable/90002788

Von Uexküll, H. R., and Mutert, E. (1995). Global extent, development and economic impact of acid soils. *Plant Soil* 171, 1–15. doi: 10.1007/BF00009558

Watson, S. L., DeLacy, I. H., Podlich, D. W., and Basford, K. E. (1995). GEBEI: an analysis package using agglomerative hierarchical classificatory and SVD ordination procedures for genotype × environment data. Centre for Statistics Research Report 57. Department of Agriculture, The University of Queensland, Australia.

Weiner, J., Campbell, L. G., Pino, J., and Echarte, L. (2009). The allometry of reproduction within plant populations. *J. Ecol.* 97, 1220–1233. doi: 10.1111/j.1365-2745.2009.01559.x

White, T. L., and Hodge, G. R. (1989). Predicting breeding values with applications in forest tree improvement. *Forest. Sci.* 33, 1. doi: 10.1007/978-94-015-7833-2

Wu, X. M., Guo, P., Chi, H. W., Fang, Z. H., Shi, Y. H., Wang, Y. Q., et al. (2018a). Diversity analysis of phenotypic traits and quality characteristics of alfalfa (Medicago sativa) introducted from abroad germplasm resources. *J. Plant Genet. Resour. C.* 19, 103–111. doi: 10.13430/j.cnki.jpgr.2018.01.012

Wu, Y. H., Fan, Z. L., Li, J., Guo, J. H., Guo, Y. K., and Wang, Y. L. (2018b). Phenotypic diversity of seeds and fruits in natural populations of Acer ginnala Maxim in China. *Guihaia* 38, 795–803. doi: 10.11931/guihaia.gxzw201711041

Yan, W., Hunt, L. A., Sheng, Q., and Szlavnics, Z. (2000). Cultivar evaluation and mega-environment investigation based on the GGE biplot. *Crop Sci.* 40, 597. doi: 10.2135/cropsci2000.403597x

Yang, K. M., Li, G. Q., Guo, S. J., and Qiao, L. (2014). Genetic diversity and association analysis of agronomic traits with SSR in a natura. *J. Nuclear Agric. Sci.* 9, 1576–1584. doi: 10.11869/j.issn.100-8551.2014.09.1576

Yuegao, H., Cash, D., Kechang, L., Suqin, W., Ping, Z., and Rong, G. (2010). Global status and development trends of alfalfa. Alfalfa management guide for Ningxia 1–14. United Nations Food Agriculture Organization. Rome.

Zeng, X. B. (2000). Acidification of red soils and control methods. Chin. *J. Soil Sci.* 31, 111–113. doi: 10.19336/j.cnki.trtb.2000.03.005

Zhao, M. Q. E., Wang, Z. J., Bao, J., Du, S., Hao, J. F., Yan, X., et al. (2022). Comprehensive performance evaluation of seven alfalfa varieties in Chifeng area. *Heilongjiang Anim. Sci. Vet. Med.* 652, 94–101+139. doi: 10.13881/j.cnki.hljxmsy.2021.09.0209

Zhu, M., Yu, M., and Zhao, S. (2009). Understanding quantitative genetics in the systems biology era. *Int. J. Biol. Sci.* 5, 161. doi: 10.7150/ijbs.5.161

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Soil bacterial community structure at different plant maturity stages in an annual grass–legume production system

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Introduction: To infer changes in soil function and thus determine appropriate agronomic management practices, this study evaluated the effects of plant maturity stage on root characteristics, soil chemical and enzymatic properties, and soil bacterial community composition in an annual grass–legume production system.

Methods: Annual ryegrass or rye was sown in combination with one of three legume species at a legume ratio of 50%. Eighteen plots (six plant combinations, three replicates per combination, 20m×25m plots) were tilled to a depth of 20cm after mowing. Soil samples from each plot were collected on four dates as the plants matured: January 4 (H1), March 14 (H2), April 21 (H3), and May 19 (H4). Bacterial community structures were characterized *via* 16S rRNA high-throught sequencing and the bio-informatics methods were used to evaluate the structural characteristics of soil bacteria.

Results: The most abundant root growth was observed at the H3 stage. No significant differences in organic matter, alkali-hydrolyzable nitrogen, available phosphorus, and available potassium contents ($p>0.05$) were observed on any sampling date. Soil collected at the H3 stage exhibited lower acid protease and urease activities ($p<0.05$) and higher nitrate reductase activity ($p<0.05$). The structure of the microbial community at stage H3 differed markedly from that at other stages, as evidenced by a higher abundance of Proteobacteria, Bacteroidetes, Acidobacteria, and Verrucomicrobia ($p<0.05$) and a lower abundance of Actinobacteria, Cyanobacteria, and Planctomycetes ($p<0.05$). At the class level, the relative abundances of Sphingobacteria, Betaproteobacteria, and Gammaproteobacteria in soil was higher at H3 stage than those of other stages ($p<0.05$). The order level for Sphingomonadales, Sphingobacteriales, and Burkholderiales and at the family level for Chitinophagaceae and Sphingomonadaceae showed the same trend toward higher.

Conclusion: Nutrient cycling in the soil was slowed at the H3 stage, and the loss of nitrogen would also be greater. Measures may need to be taken to improve the nitrogen fertilizer utilization efficiency to reduce denitrification and nitrous oxide production at this stage (the booting stage of grasses and budding stage of legumes).

KEYWORDS

soil bacterial community, maturity stage, grass–legume field, root characteristics, enzymatic properties

1. Introduction

In many agricultural systems, decades of intensive farming have reduced soil organic matter content, thereby reducing soil fertility and biodiversity (Gardi et al., 2013). As a consequence, important soil ecosystem services such as nutrient cycling are often impaired. Organic agriculture, including legume-based forage systems, is believed to be more environmentally sound than traditional agriculture and may enhance soil quality and plant diversity (Lu et al., 2017) through incorporation of large amounts of carbon into the soil *via* organic rather than mineral fertilizers (Lal, 2009).

There is a synergistic relationship between the microbial community and agricultural practices. For example, the inclusion of legumes has been shown to shape soil microbial community structure in the short term (Chávez-Romero et al., 2016; Lupwayi et al., 2018). The presence of legumes in the plant mixture enhanced soil bacterial biomass compared with grasses grown in monoculture, thereby changing the soil microbial community (Chen et al., 2008). Plant growth stage can also influence soil chemical and physical properties through its effects on root growth, which determines the quality and quantity of root exudates, thereby exerting selection on root-associated microorganisms (Baudoin et al., 2003; Ziegler et al., 2013; Li et al., 2014). The inclusion of legumes in organic agriculture can help to sustain soil fertility through biological nitrogen fixation, reducing greenhouse gas emissions from ruminants and soil in fertilized agricultural systems (Yadav et al., 2018).

In turn, microbial communities can enhance nutrient cycling, helping to maintain soil function (Lemanceau et al., 2016). Bacteria, the most abundant and diverse group of soil microbes, influence soil structure, organic matter decomposition, inorganic compound transformation, and nitrogen fixation in agricultural ecosystems (Gans et al., 2005; Sengupta and Dick, 2015). However, soil bacterial communities are particularly sensitive to changes in environmental factors such as soil status (Fierer et al., 2007), vegetation type (Leloup et al., 2018), and land use (Yergeau et al., 2006).

Recently, researchers have become interested in the effects of legume species on carbon and nitrogen cycling in grass production systems. Previous research has shown that seasonal variations in the activity and relative abundance of soil bacterial communities are plant dependent (Li et al., 2014). However, we still lack a comprehensive understanding of how soil microbial communities change during different stages of root growth. In addition, soil enzymes are also a small but vital component to shape soil organic matter since every biochemical action is dependent on or related to enzymes (Szajdak and Gaca, 2010). Since enzyme activity and nutrient characteristics of soils are closely related, it is critical to investigate the dynamics of enzyme activity in soil. In a recent study, we found that root characteristics of plants in an annual grass–legume field did not change linearly throughout plant growth and development. We inferred that the soil bacterial community structure and soil enzyme activity were also likely to evolve non-linearly as plants matured. By documenting key changes in soil bacterial community structure at different plant maturity stages, it may be possible to infer changes in soil function and thus determine appropriate agronomic management practices. In the present work, we collected soils from an annual grass–legume forage production system to determine the common features of the soil bacterial community at a site in southern China and analyze how these bacterial communities change at different stages of root growth.

2. Materials and methods

2.1. Site description and experimental design

The study was performed at the experimental station (E103°45′, N30°25′; 475 m altitude) of Sichuan Academy of Grassland Sciences, in Chengdu, P. R. China. The experimental site experiences a continental tropical humid monsoon climate with a mean annual temperature of 15°C and mean annual precipitation of about 1,300 mm. The soil type is a yellow clay soil (according to Chinese soil classification system) with a pH of 6.74 (Gong, 1992), organic matter content of 32.2 g kg^{-1}, alkali-hydrolyzable nitrogen (AN) of 185 mg kg^{-1}, available phosphorus (AP) of 41.8 mg kg^{-1}, and available potassium (AK) of 127.3 mg kg^{-1}. The annual sunshine duration is 1033.8 h, and the annual average frost-free period is 284 days.

The experiment was set up using a completely randomized block design. Annual ryegrass (*Lolium multiflorum* L.) or rye (*Secale cereale* L.) was sown together with one of the legume species milk vetch (*Astragalus sinicus* L.), bur clover (*Medicago polymorpha* L.), or common vetch (*Vicia sativa* L.) at a legume ratio of 50%. A total of 18 plots (six plant combinations, three replicates per combination, each 20 m × 25 m) were tilled to depth of 20 cm after mowing, and a pre-planting NPK fertilizer (1-1-1) was applied at a rate of 150 kg hm^{-2}. All mixtures were sown on 10–15 September, 2017. Seeding density was 18 kg hm^{-2} for the legume species and 22.5 kg hm^{-2} for the annual ryegrass and rye. To prevent weeds, atrazine was sprayed at 2.5 L hm^{-2} before tillage, and 2,4-dichlorophenoxyacetic acid was applied at 0.6 L hm^{-2} 1 week after germination.

2.2. Soil sampling and preparation

After each mowing in 2017, five 20-cm-deep soil cores were randomly collected using an 8 cm diameter soil auger from each plot on January 4 (H1, tiller stage of grasses and branching stage of legumes), March 14 (H2, jointing stage of grasses and branching stage of legumes), April 21 (H3, booting stage of grasses and budding stage of legumes), and May 19 (H4, milky stage of grasses and flowering stage of legumes). Roots were separated from the five soil cores and mixed as a composite sample. The root sample was washed with distilled water and characterized using a root analysis system (LC-4800, WinFOLIA, American). Sampling sites for subsequent samplings were selected near the previous locations to minimize the effects of spatial variation in soil properties on the bacterial community (Li et al., 2014). Adequate distance (> 0.5 m) should also be left for each sampling to avoid soil compaction. Samples from each plot were mixed and homogenized by passing through a 2-mm sieve to remove aboveground roots, visible residues, and stones, ultimately producing a composite soil sample. Each soil sample was divided into two subsamples: one was stored at 4°C for determination of soil physical and chemical properties, and the other was stored at −80°C for DNA extraction and microbial analysis.

2.3. Soil chemical and enzymatic properties

Soil acid protease activity (APA), urease activity (UA), and solid-nitrate reductase activity (NA) were measured by the method of Zhou

(1987). Soil pH was determined with a glass electrode. Soil AN was determined by the alkaline hydrolyzable diffusion method (Lu, 1999). Contents of organic matter (OM), AK, and AP were determined as described in Kong et al. (2006).

2.4. DNA extraction and PCR amplification

DNA extraction and PCR amplification were performed according to the method of Zeng et al. (2017). In brief, total genomic DNA was extracted from samples using the CTAB/SDS method. DNA concentration and purity were monitored on 1% agarose gels, and DNA was diluted to 1 ng µL^{-1} using sterile water. The primers 515F (5′-CCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) were used to amplify the V4 hypervariable region of the 16S rRNA gene with a barcode. All PCR reactions were carried out with Phusion High-Fidelity PCR Master Mix (New England Biolabs) and the PCR reaction volume was supplemented with distilled water to 20 µL. The PCR was performed using a thermal cycler Model (ABI GeneAmp 9700, Applied Biosystems Inc., San Diego, CA, United States) with the thermal cycling conditions: initial denaturation of 2 min at 95°C, 30 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 30 s, final extension at 72°C for 5 min and hold at 10°C. An equal volume of 1× loading buffer (containing SYB green) was mixed with the PCR products, and products were detected following electrophoresis on a 2% agarose gel. Samples with a bright main band between 400 and 450 bp were chosen for further experiments. PCR products were mixed in equidensity ratios and purified with the Qiagen Gel Extraction Kit (Qiagen, Germany).

2.5. Library preparation and sequencing

Sequencing libraries were generated using the TruSeq DNA PCR-Free Sample Preparation Kit (Illumina, USA) following the manufacturer's recommendations, and index codes were added. Library quality was assessed on a Qubit 2.0 Fluorometer (Thermo Scientific) and an Agilent Bioanalyzer 2100 system, and libraries were sequenced on the Illumina HiSeq 2500 platform to produce 250-bp paired-end reads.

Paired-end reads were merged using FLASH (V1.2.7; Magoč and Salzberg, 2011), then filtered to obtain high-quality clean tags according to the quality control process of QIIME (V1.7.0), which allowed for a two-nucleotide mismatch during primer matching (Bokulich et al., 2013). The tags were compared with the Gold database using the UCHIME algorithm to detect chimeric sequences, which were then removed.

Sequence analysis was performed with Uparse software (Uparse v7.0.1001; Edgar, 2013). Sequences with ≥97% similarity were assigned to the same OTU. Each representative sequence was annotated with taxonomic information using the GreenGene Database based on the RDP 3 classifier (Version 2.2) algorithm (DeSantis et al., 2006). To study phylogenetic relationships among different OTUs and characterize differences in the dominant species among different samples (groups), multiple sequence alignment was performed using MUSCLE software (Version 3.8.31; Edgar, 2004).

2.6. Data analysis

Before further analysis of alpha diversity, the sequences were normalized according to the lowest number of sequences for a single soil sample. Alpha diversity was calculated with QIIME (Version 1.7.0) and visualized using R software (Version 2.15.3). Principal coordinate analysis (PCoA) was performed and the results visualized using the R packages WGCNA, stat, and ggplot2 (R version 2.15.3) based on the Bray–Curtis dissimilarity. The non-strict version of linear discriminant analysis effect size (LEfSe; Segata et al., 2011) was applied to discover biomarkers for different root growth stages. The analysis was constructed in the Novogene platform using online interactive tools. The tools were used with default parameters.

ANOVA was performed to evaluate the effects of root growth stage on root characteristics, soil chemical properties, alpha diversity metrics, and relative abundances of dominant bacterial phyla. Duncan's multiple range test was used to identify statistically significant differences among the means. For all analyses, statistical significance was defined as $p < 0.05$.

3. Results

3.1. Root characteristics

Values of root characteristics did not increase linearly with growth time (Table 1). Although the differences were not statistically significant, the values of most root characteristics, including dry weight, length, surface area, volume, and number, initially tended to increase from H1 to H3 and then decrease at

TABLE 1 Root characteristics (mean/6.4cm^2) in an annual legume–grass forage production system.

	H1	H2	H3	H4	SEM	*p* value
Root dry weight (g)	1.53	2.01	2.91	2.40	0.20	0.098
Root length (cm)	669.0	889.3	1112.4	900.2	63.7	0.099
Root surface area (cm^2)	173.1	215.0	280.8	216.5	15.4	0.092
Average diameter (mm)	0.81	0.80	0.85	0.80	0.03	0.944
Root volume (cm^3)	3.66	4.39	6.07	4.39	0.38	0.136
Number of root tips	1885.0	2441.8	2946.0	2372.7	171.0	0.184

H1–4, root growth stages of mixed forage: January 4 (H1), March 14 (H2), April 21 (H3), and May 19 (H4). SEM, standard error of the mean.

H4. The most abundant root growth was observed at the H3 stage. However, the above-ground biomass production of the H1–H4 stages was 4,566, 5,512, 4,237, and 3,936 kg hm^{-2} (dry matter, DM), respectively. The maximum above-ground biomass production occurred in H2 stage.

3.2. Soil chemical and enzymatic properties

Soil APA decreased from 22.99 mg day^{-1} g^{-1} (DM) to 3.43 mg day^{-1} g^{-1} (DM) over the course of the season (Figure 1). Soil pH and NA reached their maximum values in the H2 and H3 phases,

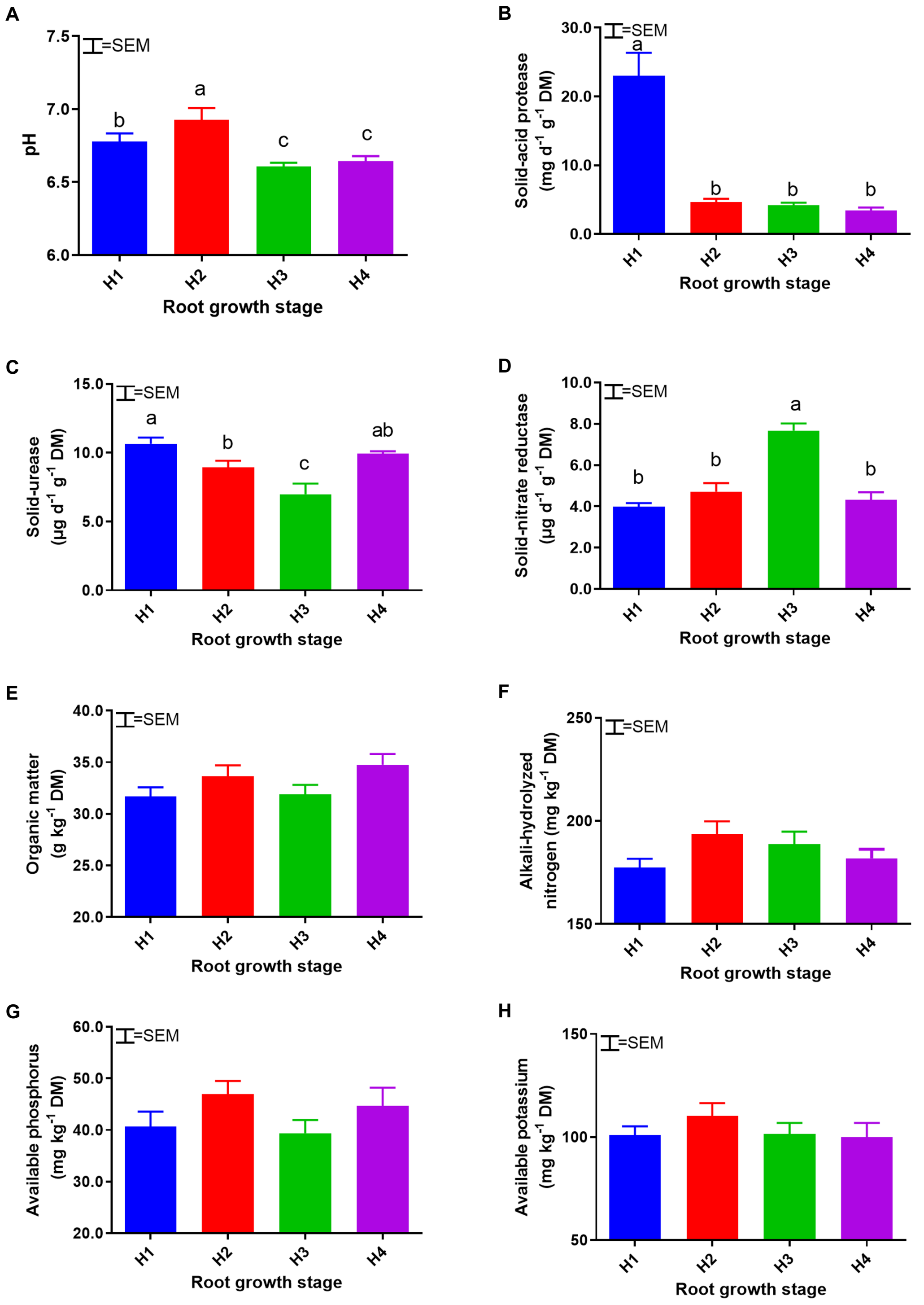

FIGURE 1
Soil chemical and enzymatic properties: pH **(A)**, solid-acid protease **(B)**, solid-urease **(C)**, solid-nitrate reductase **(D)**, organic matter **(E)**, alkali-hydrolyzable nitrogen **(F)**, available phosphorus **(G)**, and available potassium **(H)** at root growth stages H1–4. DM, dry matter. H1–4, root growth stages of mixed forage: January 4 (H1), March 14 (H2), April 21 (H3), and May 19 (H4). SEM, standard error of the mean (*n*=18). Means with different superscripts differ significantly ($p<0.05$).

respectively ($p < 0.05$). No significant differences ($p > 0.05$) were observed in the contents of OM, AN, AP, and AK between any of the plant maturity stages, H1–H4.

3.3. Soil bacterial community composition

Changes in bacterial alpha diversity during the season are shown in Figure 2. A total of 201,095 OTUs were obtained (on the basis of 97% sequence similarity), and the lowest number of OTUs (2594) occurred at H2. The Chao 1 and ACE diversity indices showed that bacterial community richness was highest at the H4 stage ($p < 0.05$). By contrast, whole-tree phylogenetic diversity (PD) decreased after H1 and then remained stable.

Bacterial community structure and relative abundance over the course of the experiment are shown in Figure 3. The dominant groups at the phylum level were Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes, Acidobacteria, Gemmatimonadetes, Cyanobacteria, Planctomycetes, Chloroflexi, and Verrucomicrobia, with a combined relative abundance of >93%. The identifiable dominant groups at the genus level were *Pseudomonas*, *Haliangium*, *Gemmatimonas*, and *Sphingomonas*, with a combined relative abundance of about 10%. Changes in the relative abundance of dominant bacterial phyla are shown in Figure 4. The relative abundances of Actinobacteria,

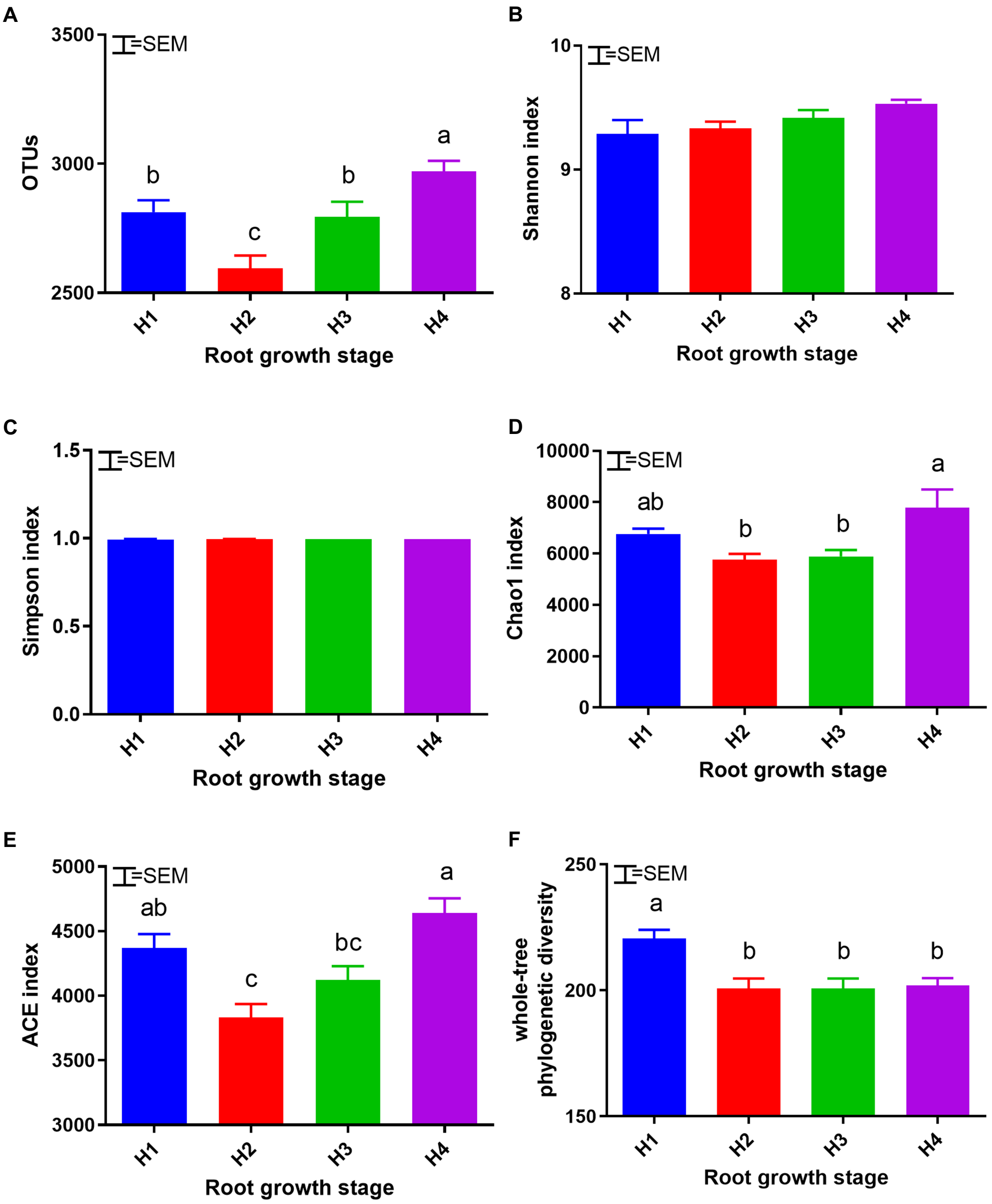

FIGURE 2
Soil bacterial alpha diversity: OTUs **(A)**, Shannon **(B)**, Simpson **(C)**, Chao 1 **(D)**, ACE **(E)**, and whole-tree PD **(F)** at root growth stages H1–4. H1–4, root growth stages of mixed forage: January 4 (H1), March 14 (H2), April 21 (H3), and May 19 (H4). SEM, standard error of the mean (n=18). Means with difference superscripts differ significantly (p<0.05).

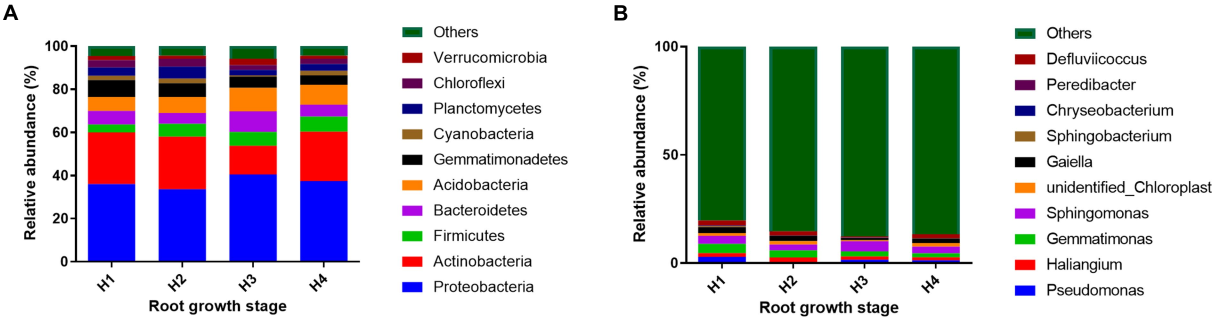

FIGURE 3
Soil bacterial community structure and relative abundance of taxa at the phylum **(A)** and genus **(B)** levels at root growth stages H1–4. H1–4, root growth stages of mixed forage: January 4 (H1), March 14 (H2), April 21 (H3), and May 19 (H4).

Cyanobacteria, and Planctomycetes were lower in H3 soil samples than in the others ($p < 0.05$), whereas relative abundances of Proteobacteria, Bacteroidetes, Acidobacteria, and Verrucomicrobia were higher than those of others ($p < 0.05$). The relative abundance of Firmicutes increased over the course of the season ($p < 0.05$), whereas that of Gemmatimonadetes decreased ($p < 0.05$).

Linear discriminant analysis effect size and PCoA analyses are shown in Figure 5. A total of 29 biomarkers were identified to distinguish the four root growth stages. The PCoA analysis showed that soil samples from H1, H2, and H4 were clustered together but were significantly separated from the H3 samples; the bacterial diversity of H3 therefore differed significantly from that at other stages. At the class level, the relative abundances of Sphingobacteria, Betaproteobacteria, and Gammaproteobacteria in soil was higher at H3 stage than those of other stages. The order level for Sphingomonadales, Sphingobacteriales, and Burkholderiales and at the family level for Chitinophagaceae and Sphingomonadaceae showed the same trend toward higher.

4. Discussion

Inclusion of legume forage is a potential strategy for enhancing carbon and nitrogen resources while sustaining soil health in the annual grass–legume forage systems of southern China (Lu et al., 2017, 2018). Here, we demonstrate that this practice can affect soil properties and the composition of the soil bacterial community. The two most abundant phyla detected in the present study were Proteobacteria (relative abundance 0.29–0.54) and Actinobacteria (0.08–0.36). Similar results were obtained by Ding et al. (2016), who reported that Proteobacteria (0.29–0.33) was the predominant phylum in agricultural soils of northeast China, followed by Acidobacteria (0.12–0.16) and Actinobacteria (0.09–0.11). Proteobacteria and Actinobacteria are considered to be the dominant bacterial taxa that influence grass–legume intercropping systems, and they have important roles in maintaining soil ecosystem function (Zhao, 2020). Many of these microbes have roles in nutrient cycling: Xanthobacteraceae in carbon cycling; Sphingomonadaceae, Rhizobiales, and Myxococcales in nitrogen cycling; and Actinomycetales in phosphorus utilization. Proteobacteria, including many nitrogen-fixing bacteria, can grow rapidly when labile substrates are available, and their abundance is generally positively correlated with soil carbon and nitrogen contents (Spain et al., 2009; Goldfarb et al., 2011; Zhang et al., 2016). Actinobacteria constitute the predominant phylum in many soil bacterial communities; they are generally considered to be remarkable decomposers of plant biomass and can survive under stress by secondary metabolism of filamentous mycelial growth (Priyadharsini and Dhanasekaran, 2015).

In the present study, changes in root characteristics as plants matured might have affected bacterial community composition and structure in the annual grass–legume production system. The Chao 1 and ACE richness indices were lower at the H2 and H3 stages, suggesting that an increase in harvesting frequency might disturb the evenness of the bacterial community. As expected, PCoA demonstrated that H3 samples tended to cluster together with high similarity, whereas samples from H1, H2, and H4 all grouped together. Correspondingly, more abundant root growth and marked changes in the dominant bacterial phyla were also observed at the H3 stage. These changes may be explained by changes in soil enzymatic properties and root characteristics over the course of the experimental period. Input of organic matter *via* roots and aboveground litter leads to accumulation of soil organic carbon and changes in available nitrogen and phosphorus contents that can shift aspects of the soil bacterial community (Mikkonen et al., 2011). In return, the soil bacterial community itself can serve as a dynamic indicator of soil quality, as it is sensitive to changes in soil function and aboveground plant composition (Hedlund, 2002).

The available soil nutrient contents did not vary significantly among different maturity stages in this study. However, it is not sufficient to assess soil fertility based on physical and chemical properties alone. Measurement of microbial and enzymatic indicators in the soil can provide a more complete understanding of the transformations that are taking place and affecting soil fertility and nutrient capacity (Wolna-Maruwka et al., 2018). In the present study, soil enzyme activities varied markedly among plant maturity stages, especially for enzymes associated with the nitrogen cycle. Protease and urease activities have been shown to be the most sensitive indicators of nitrogen transformation in soil (Wolna-Maruwka et al., 2018). Both urease and protease activities decreased at the H3 stage, implying that nutrient cycling in the soil was slowed. This may have been due to more vigorous root growth during the H3 stage and the increase in root nutrient uptake, as roots can compete with microorganisms for inorganic and small organic nitrogen forms, resulting in a decrease in soil urease and protease activity (Yang et al., 2021). At the same time, nitrate reductase activity was significantly higher at the H3 stage than at other stages, which accelerates the reduction of nitrate to nitrite. Nitrite ions are reduced to nitrous oxide by nitrite reductase, and this

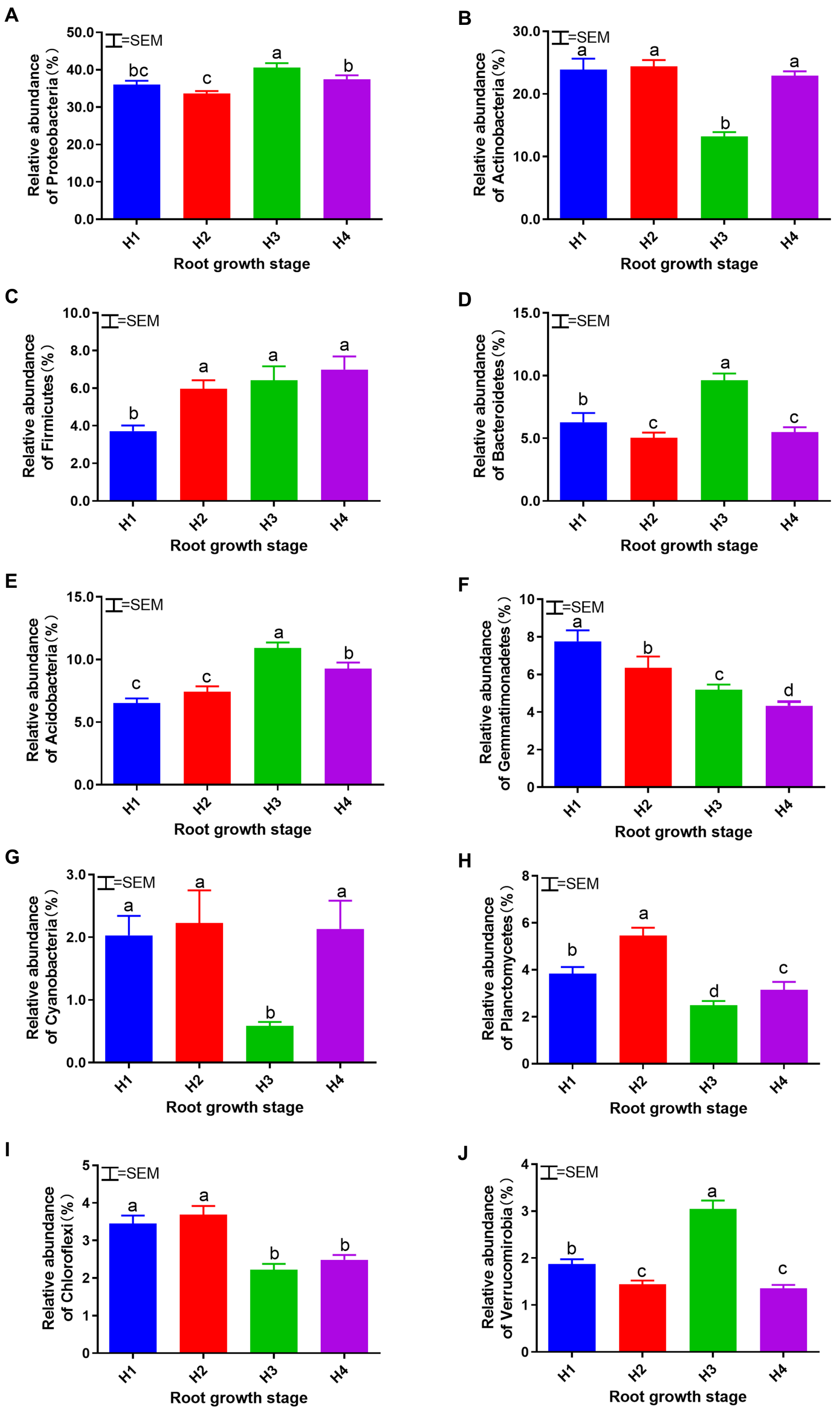

FIGURE 4
Relative abundance of dominant soil bacterial phyla: Proteobacteria **(A)**, Actinobacteria **(B)**, Firmicutes **(C)**, Bacteroidetes **(D)**, Acidobacteria **(E)**, Gemmatimonadetes **(F)**, Cyanobacteria **(G)**, Planctomycetes **(H)**, Chloroflexi **(I)**, and Verrucomicrobia **(J)** at root growth stages H1–4. H1–4, root growth stages of mixed forage: January 4 (H1), March 14 (H2), April 21 (H3), and May 19 (H4). SEM, standard error of the mean (*n*=18). Means with difference superscripts differ significantly (*p*<0.05).

process leads to loss of nitrogen from the soil. Zhao (2020) found that the abundance of Actinobacteria was positively correlated with soil health. By contrast, the greater the abundance of Acidobacteria and Verrucomicrobia, the more severe the soil nutrient loss. In our study, marked changes in microbial community structure and enzyme activity were observed together at the H3 stage, suggesting that the

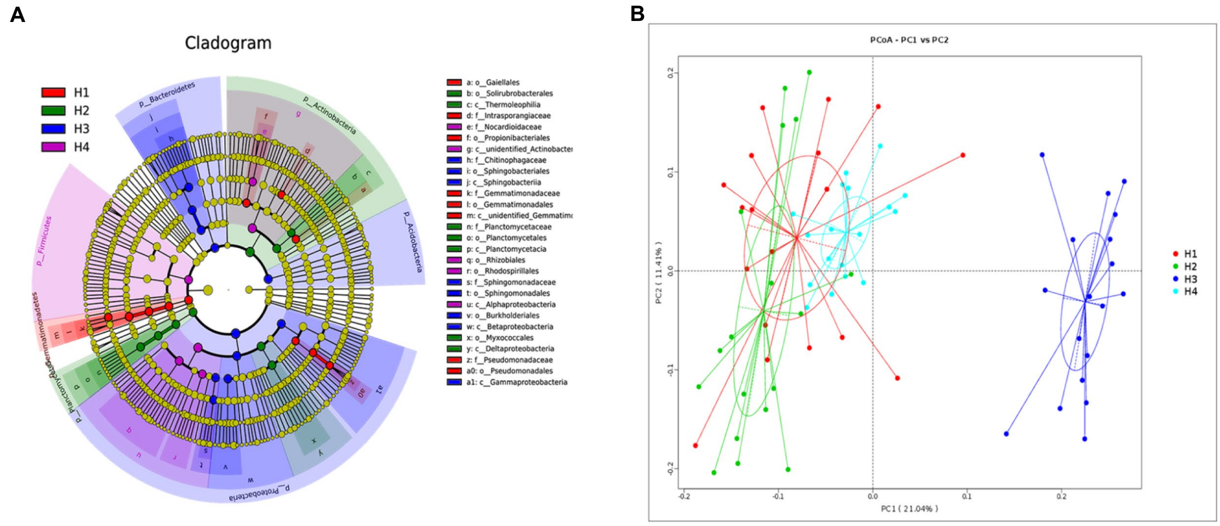

FIGURE 5
Evolutionary branch diagram of various soil bacterial species **(A)** and principal coordinate analysis (PCoA) of soil bacterial community structure **(B)**. H1–4, root growth stages of mixed forage: January 4 (H1), March 14 (H2), April 21 (H3), and May 19 (H4).

lower abundance of Actinobacteria, and higher abundance of Acidobacteria and Verrucomicrobia may be closely related to soil enzyme activity and nutrient conversion capacity. Furthermore, due to the higher abundance of Sphingobacteria, Betaproteobacteria, and Gammaproteobacteria at the class level, higher abundance of Sphingomonadales, Sphingobacteriales and Burkholderiales at the order level, and higher abundance of Chitinophagaceae and Sphingomonadaceae at the family level, the indicator species at H3 stage all have the potential to become the marker of changes in soil enzyme activity and nutrient conversion capacity.

Additional application of nitrogen fertilizer can be an effective means of providing nutrients for soil microorganisms and enhancing soil enzyme activity, microbial biomass and diversity (Yang et al., 2021), but it can also further aggravate nitrate pollution (Szajdak and Gaca, 2010). In terms of sustainable management and agroecology principles for grasslands, the application of nitrogen fertilizer will increase the accumulation of nitrogen in the soil, which might further increase nitrous oxide emissions and exacerbate the greenhouse effect. Therefore, to reduce soil nitrous oxide emissions, it is necessary to optimize fertilization practices and improve nitrogen fertilizer utilization efficiency (Canfield et al., 2010). Studies have shown that nitrous oxide emissions from ammonium sulfate applications are lower than those from urea (Bouwman et al., 2002). The application of polymer-coated nitrogen fertilizer could also significantly reduce soil nitrous oxide emissions (Hyatt et al., 2010). In addition, nitrification inhibitors such as dicyandiamide, 3,4-dimethylpyrazol phosphate and acetylene, which inhibit the production of nitrous oxide during nitrification and denitrification by soil microorganisms, can also be considered to be applied in the H3 stage (Menéndez et al., 2012; Akiyama et al., 2013).

5. Conclusion

The structure of the microbial community at stage H3 (booting stage of grasses and budding stage of legumes) was dramatically different from that at other stages. Nutrient cycling in the soil appeared to slow at the H3 stage, and increased nitrate reductase activity suggested that the loss of nitrogen would be greater. Measures need to be taken to improve the nitrogen fertilizer utilization efficiency to reduce denitrification and nitrous oxide production at this stage.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

WG and SB designed the study. YX, WL, and PL wrote and revised the manuscript. YX, PL, and ED carried out the data analysis. WG, DL, LZ, HS, YZ, QC, and CW performed the experiments. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Akiyama, H., Morimoto, S., Hayatsu, M., Hayakawa, A., Sudo, S., and Yagi, K. (2013). Nitrification, ammonia-oxidizing communities, and N_2O and CH_4 fluxes in an imperfectly drained agricultural field fertilized with coated urea with and without dicyandiamide. *Biol. Fertil. Soils* 49, 213–223. doi: 10.1007/s00374-012-0713-2

Baudoin, E., Benizri, E., and Guckert, A. (2003). Impact of artificial root exudates on the bacterial community structure in bulk soil and maize rhizosphere. *Soil Biol. Biochem.* 35, 1183–1192. doi: 10.1016/S0038-0717(03)00179-2

Bokulich, N. A., Subramanian, S., Faith, J. J., Gevers, D., Gordon, J. I., Knight, R., et al. (2013). Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nat. Methods* 10, 57–59. doi: 10.1038/nmeth.2276

Bouwman, A. F., Boumans, L. J. M., and Batjes, N. H. (2002). Emissions of N_2O and NO from fertilized fields: summary of available measurement data. *Glob. Biogeochem. Cycles* 16, 6–13. doi: 10.1029/2001gb001811

Canfield, D. E., Glazer, A. N., and Falkowski, P. G. (2010). The evolution and future of earth's nitrogen cycle. *Science* 330, 192–196. doi: 10.1126/science.1186120

Chávez-Romero, Y., Navarro-Noya, Y. E., Reynoso-Martínez, S. C., Sarria-Guzmán, Y., Govaerts, B., Verhulst, N., et al. (2016). 16S metagenomics reveals changes in the soil bacterial community driven by soil organic C, N-fertilizer and tillage-crop residue management. *Soil Tillage Res.* 159, 1–8. doi: 10.1016/j.still.2016.01.007

Chen, M., Chen, B., and Marschner, P. (2008). Plant growth and soil microbial community structure of legumes and grasses grown in monoculture or mixture. *J. Environ. Sci.* 20, 1231–1237. doi: 10.1016/S1001-0742(08)62214-7

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., et al. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environ. Microbiol.* 72, 5069–5072. doi: 10.1128/AEM.03006-05

Ding, J., Jiang, X., Ma, M., Zhou, B., Guan, D., Zhao, B., et al. (2016). Effect of 35 years inorganic fertilizer and manure amendment on structure of bacterial and archaeal communities in black soil of Northeast China. *Appl. Soil Ecol.* 105, 187–195. doi: 10.1016/j.apsoil.2016.04.010

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32, 1792–1797. doi: 10.1093/nar/gkh340

Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10, 996–998. doi: 10.1038/nmeth.2604

Fierer, N., Bradford, M. A., and Jackson, R. B. (2007). Toward an ecological classification of soil bacteria. *Ecology* 88, 1354–1364. doi: 10.1890/05-1839

Gans, J., Wolinsky, M., and Dunbar, J. (2005). Computational improvements reveal great bacterial diversity and high metal toxicity in soil. *Science* 309, 1387–1390. doi: 10.1126/science.1112665

Gardi, C., Jeffery, S., and Saltelli, A. (2013). An estimate of potential threats levels to soil biodiversity in EU. *Glob. Chang. Biol.* 19, 1538–1548. doi: 10.1111/gcb.12159

Goldfarb, K. C., Karaoz, U., Hanson, C. A., Santee, C. A., Bradford, M. A., Treseder, K. K., et al. (2011). Differential growth responses of soil bacterial taxa to carbon substrates of varying chemical recalcitrance. *Front. Microbiol.* 2:94. doi: 10.3389/fmicb.2011.00094

Gong, Z. (1992). *Classification of Soil Systems in China*. Beijing: Science Press.

Hedlund, K. (2002). Soil microbial community structure in relation to vegetation management on former agricultural land. *Soil Biol. Biochem.* 34, 1299–1307. doi: 10.1016/S0038-0717(02)00073-1

Hyatt, C. R., Venterea, R. T., Rosen, C. J., McNearney, M., Wilson, M. L., and Dolan, M. S. (2010). Polymer-coated urea maintains potato yields and reduces nitrous oxide emissions in a Minnesota loamy sand. *Soil Sci. Soc. Am. J.* 74, 419–428. doi: 10.2136/sssaj2009.0126

Kong, X., Zhang, F., Wei, Q., Xu, Y., and Hui, J. (2006). Influence of land use change on soil nutrients in an intensive agricultural region of North China. *Soil Tillage Res.* 88, 85–94. doi: 10.1016/j.still.2005.04.010

Lal, R. (2009). Soils and world food security. *Soil Tillage Res.* 102, 1–4. doi: 10.1016/j.still.2008.08.001

Leloup, J., Baude, M., Nunan, N., Meriguet, J., Dajoz, I., le Roux, X., et al. (2018). Unravelling the effects of plant species diversity and aboveground litter input on soil bacterial communities. *Geoderma* 317, 1–7. doi: 10.1016/j.geoderma.2017.12.018

Lemanceau, P., Creamer, R., and Griffiths, B. S. (2016). Soil biodiversity and ecosystem functions across Europe: a transect covering variations in bio-geographical zones, land use and soil properties. *Appl. Soil Ecol.* 97, 1–2. doi: 10.1016/j.apsoil.2015.07.017

Li, X., Rui, J., Mao, Y., Yannarell, A., and Mackie, R. (2014). Dynamics of the bacterial community structure in the rhizosphere of a maize cultivar. *Soil Biol. Biochem.* 68, 392–401. doi: 10.1016/j.soilbio.2013.10.017

Lu, R. (1999). *Soil Agricultural Chemical Analysis Method*. Beijing: Chinese Agriculture and Sciences Press.

Lu, X., Ji, S., Hou, C., Qu, H., Li, P., and Shen, Y. (2018). Impact of root C and N reserves on shoot regrowth of defoliated alfalfa cultivars differing in fall dormancy. *Grassl. Sci.* 64, 83–90. doi: 10.1111/grs.12190

Lu, X., Ji, S., Hou, C., and Shen, Y. (2017). Morphological development and dry weight distribution of alfalfa cultivars varying in fall dormancy under a short-term cultivation system. *Grassl. Sci.* 63, 23–28. doi: 10.1111/grs.12139

Lupwayi, N. Z., May, W. E., Kanashiro, D. A., and Petri, R. M. (2018). Soil bacterial community responses to black medic cover crop and fertilizer N under no-till. *Appl. Soil Ecol.* 124, 95–103. doi: 10.1016/j.apsoil.2017.11.003

Magoč, T., and Salzberg, S. L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27, 2957–2963. doi: 10.1093/bioinformatics/btr507

Menéndez, S., Barrena, I., Setien, I., González-Murua, C., and Estavillo, J. M. (2012). Efficiency of nitrification inhibitor DMPP to reduce nitrous oxide emissions under different temperature and moisture conditions. *Soil Biol. Biochem.* 53, 82–89. doi: 10.1016/j.soilbio.2012.04.026

Mikkonen, A., Kondo, E., Lappi, K., Wallenius, K., Lindström, K., Hartikainen, H., et al. (2011). Contaminant and plant-derived changes in soil chemical and microbiological indicators during fuel oil rhizoremediation with Galega orientalis. *Geoderma* 160, 336–346. doi: 10.1016/j.geoderma.2010.10.001

Priyadharsini, P., and Dhanasekaran, D. (2015). Diversity of soil Allelopathic Actinobacteria in Tiruchirappalli district, Tamilnadu. *J. Saudi Soc. Agric. Sci.* 14, 54–60. doi: 10.1016/j.jssas.2013.07.001

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12:R60. doi: 10.1186/gb-2011-12-6-r60

Sengupta, A., and Dick, W. A. (2015). Bacterial community diversity in soil under two tillage practices as determined by pyrosequencing. *Microb. Ecol.* 70, 853–859. doi: 10.1007/s00248-015-0609-4

Spain, A. M., Krumholz, L. R., and Elshahed, M. S. (2009). Abundance, composition, diversity and novelty of soil Proteobacteria. *ISME J.* 3, 992–1000. doi: 10.1038/ismej.2009.43

Szajdak, L. W., and Gaca, W. (2010). Nitrate reductase activity in soil under shelterbelt and an adjoining cultivated field. *Chem. Ecol.* 26, 123–134. doi: 10.1080/02757540.2010.501028

Wolna-Maruwka, A., Sulewska, H., Niewiadomska, A., Panasiewicz, K., Borowiak, K., and Ratajczak, K. (2018). The influence of sewage sludge and a consortium of aerobic microorganisms added to the soil under a willow plantation on the biological indicators of transformation of organic nitrogen compounds. *Pol. J. Environ. Stud.* 27, 403–412. doi: 10.15244/pjoes/74184

Yadav, G. S., Mitran, T., and Meena, B. L. (2018). *Legumes for Soil Health and Sustainable Management*. Singapore: Springer Singapore

Yang, J., Guo, W., Wang, F., Wang, F., Zhang, L., Zhou, B., et al. (2021). Dynamics and influencing factors of soluble organic nitrogen in paddy soil under different long-term fertilization treatments. *Soil Tillage Res.* 212:105077. doi: 10.1016/j.still.2021.105077

Yergeau, E., Bokhorst, S., Huiskes, A. H. L., Boschker, H. T. S., Aerts, R., and Kowalchuk, G. A. (2006). Size and structure of bacterial, fungal and nematode communities along an Antarctic environmental gradient. *FEMS Microbiol. Ecol.* 59, 436–451. doi: 10.1111/j.1574-6941.2006.00200.x

Zeng, Q., An, S., and Liu, Y. (2017). Soil bacterial community response to vegetation succession after fencing in the grassland of China. *Sci. Total Environ.* 609, 2–10. doi: 10.1016/j.scitotenv.2017.07.102

Zhang, C., Liu, G., Xue, S., and Wang, G. (2016). Soil bacterial community dynamics reflect changes in plant community and soil properties during the secondary succession of abandoned farmland in the loess plateau. *Soil Biol. Biochem.* 97, 40–49. doi: 10.1016/j.soilbio.2016.02.013

Zhao, Y. (2020). Study on advantage of alfalfa/ gramineae forage intercropping and mechanism of nitrogen efficiency and effect of soil microecological. Dissertation/Doctor's thesis. Gansu Agricultural University, Lanzhou.

Zhou, L. (1987). *Soil Enzymology*. Beijing: Science and Technology Press.

Ziegler, M., Engel, M., Welzl, G., and Schloter, M. (2013). Development of a simple root model to study the effects of single exudates on the development of bacterial community structure. *J. Microbiol. Methods* 94, 30–36. doi: 10.1016/j.mimet.2013.04.003

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Management practices during the renewal year affect the carbon balance of a boreal legume grassland

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Evaluating the net ecosystem carbon balance (NECB) of legume-based grasslands is crucial for optimizing grassland management and assessing the sustainability of the milk and beef industries. This study investigated the NECB of a boreal legume grassland in eastern Finland from May 2017 to May 2020, covering the entire three-year rotation cycle. We found that the grassland showed interannual variability in carbon sequestration, fixing 220g C m^{-2} in the first year, 334g C m^{-2} in the second year, and losing 146g C m^{-2} in the last year during the grassland renewal period. The study also examined the effects of mineral nitrogen fertilizer and digestate residue addition on the NECB of the grassland. No significant differences in net ecosystem carbon dioxide exchange were observed between the two treatments, but the application of digestate slurry increased the NECB, suggesting that organic fertilizers could potentially enhance carbon sequestration and sustain ecosystem services. In conclusion, our findings emphasize the importance of developing climate-friendly renovation management practices that maximize the photosynthetic period in boreal legume grasslands. These practices, combined with the use of organic fertilizers, can contribute to improved carbon sequestration and support the sustainability of milk and beef industries that rely on grasslands.

KEYWORDS

agricultural sustainability, boreal environment, climate change, GHG exchange, *Trifolium pratense*

1. Introduction

Grasslands are an essential component of European agriculture covering approximately 31% of the agricultural area (Olesen and Bindi, 2002; Eurostat, 2021). Grassland-based livestock production is the backbone of the Nordic socio-economy (Åby et al., 2014). It is an important source of livelihood and it maintains the population and vitality of rural areas. Production of milk and beef are interconnected in Finland, up to 80% of beef production is coupled with milk production (Åby et al., 2014). The milk and beef industry is currently being challenged for environmental reasons in light of the reduction in the carbon (C) footprint (Olesen and Bindi, 2002; Klumpp and Fornara, 2018). At the farm level, soil C storage enhancing measures that could be used cost-effectively, and without disrupting production, are lacking.

Incorporating legumes into grasslands through practices such as short rotation, intercropping, or mixed planting can potentially improve soil nutrient conditions (Suter et al., 2015), biomass yield (Finn et al., 2013), and ecosystem energy efficiency (Deng et al., 2021). Legumes can significantly affect the nitrogen (N) status through biological dinitrogen fixation (Gylfadóttir et al., 2007; Lüscher et al., 2014). N can be released from decaying biomass above and below ground, and the nodules and root exudates of legumes (Laidlaw et al., 1996; Suter et al., 2015). For example, using ^{15}N individual plant leaf labeling in a legume grassland in Iceland, a field study found that white clover (*Trifolium repens*) cultivated with smooth grass (*Poa pratensis*) provided about 2.5 g N m^{-2}, 50% of the total crop N requirement (Gylfadóttir et al., 2007). A coordinated continental-scale field experiment across 31 European sites reported that the yield of legume grasslands exceeded that of the average grass monoculture with low N fertilization by more than 97% over 3 years (Finn et al., 2013). In addition, a Finnish farm survey found that red clover (*Trifolium Pratense* L.) based grasslands yielded 7.5 ± 1.7 t dry matter (DM) ha^{-1} without N fertilization (Riesinger and Herzon, 2008).

Managed European grasslands are often fertilized with mineral and organic N fertilizers to further optimize the production and profitability of grasslands (Olesen and Bindi, 2002). The application of N fertilizers has been shown to affect soil C storage in grassland ecosystems (Conant et al., 2017). For example, a synthesis analysis comprising 50 studies from different parts of the world reported that fertilization with mineral or organic N fertilizers increased grassland soil C stock at an average rate of 0.57 t C ha^{-1} yr^{-1} (Conant et al., 2017). A study in a Scottish grassland showed that, compared to mineral fertilizers, organic treatment enhanced soil C storage after 6 years of the manure addition, despite increased rates of soil respiration (Jones et al., 2006). However, little is known about the potential impact of legumes on grassland C balance with the addition of mineral or organic fertilizer in the northern regions, where the long winter has a significant effect on C and N turnover in the plant–soil system.

The eddy covariance (EC) method allows continuous measurements of ecosystem C flows for periods of months to years (Baldocchi, 2020). EC-based measurements of CO_2 exchange from grasslands have the potential of providing valuable insights into the impact of management on the net ecosystem C balance (NECB, the net rate of C accumulation in or loss from ecosystems) and exploring opportunities for greenhouse gas mitigation (Chapin et al., 2006; Lind et al., 2016). Thus, as an alternative to monitoring soil organic C (SOC) content over time, changes in NECB can be determined from measured C imports and exports using the EC technique together with measured agronomic parameters, such as harvest and synthetic fertilizer or manure application (Lind et al., 2016; Rutledge et al., 2017; Ammann et al., 2020). Multi-year studies evaluating grassland NECB covering the entire rotation are crucial for understanding the C balance of rotational grasslands (Ammann et al., 2020). Such full-cycle experiments are especially important in the boreal region, characterized by cold climates and short growing seasons, where grasslands are renewed every three to 4 years (Virkajärvi et al., 2015) and wintertime has a significant effect on crop production and nutrient cycling (Maljanen et al., 2009). Currently, however, studies evaluating grassland NECB covering the entire rotation using EC techniques are lacking in the boreal region.

Here, we quantified the NECB of a legume grassland with the EC technique over a three-year rotation cycle in eastern Finland (Figure 1). Our objective in this study was to understand the impact of crop management practices (fertilization, harvesting, and grassland re-establishment) and interannual variability on ecosystem C flows and implications for ecosystem services (e.g., milk and beef production). In this study, we hypothesized that a legume grassland on mineral soil in a boreal environment is a sink for atmospheric C over a three-year rotation cycle and that the effect of fertilizer type on the NECB of legume grasslands varies over the rotation cycle.

2. Materials and methods

2.1. Site description and management

The study site is located in eastern Finland (63°09′ N, 27°140′ E, 89 m a.s.l.; Figure 1). The 30-year (1981–2010) mean annual temperature (MAT) and precipitation (MAP) in the region are 3.2°C and 612 mm, respectively. The soil at the study site is classified as a Haplic Cambisol/Regosol (Hypereutric, Siltic) (IUSS Working Group WRB, 2007) (silt loam; clay 25% ± 6%, silt 53% ± 9% and sand 22% ± 8%) based on the U.S. Department of Agriculture (USDA) textural classification system.

The study site, a 6.3-hectare agricultural field (280 m × 220 m) cultivated with timothy (*Phleum pratense* L. cv. Nuutti; seed rate 15 kg ha^{-1}) and red clover (cv. Ilte; 5 kg ha^{-1}), was established in 2015, reseeded in May 2017, and renewed in spring 2019 (Supplementary Table S1). In the autumn of 2018, glyphosate was applied to the field using a tractor-mounted sprayer to make the site devoid of any vegetation. Subsequently, the site was plowed using a tractor-mounted plow and left bare for the ensuing winter. In early June 2019, the site was renewed (Supplementary Table S1) with the seeding of a red clover and timothy seed mixture, along with barley (*Hordeum vulgare* L.) as a cover crop, using a tractor-mounted seed drill. Considering the frequency of prevailing wind directions (Supplementary Figure S1), the experimental site was divided into two plots that were treated with either mineral nitrogen (N_{min}) or digestate residue (N_{org}) over a rotation cycle: May 2017–May 2018, June 2018–May 2019, and June 2019–May 2020, hereafter referred to as R_1, R_2, and R_3, respectively. During both grass production years (R_1, R_2), the N_{min} plot was fertilized using a tractor-mounted fertilizer spreader at the start of each growing season (May) and after the 1st cut (mid to late June) with an average annual fertilization rate of 106 kg soluble N, 28 kg P, and 50 kg K ha^{-1}. In contrast, the N_{org} plot was fertilized once after the 1st cut with an average annual fertilization rate of 98 kg N total (of which 53 kg N was soluble), 13 kg P, and 83 kg K ha^{-1}, using a tractor-mounted slurry spreader. In the renovation year (R_3), the whole field received N_{min} plot fertilization with an annual rate of 45 kg N, 20 kg phosphorous, and 38 kg K ha^{-1} using a tractor-mounted fertilizer spreader, while the N_{org} plot did not receive any fertilizers (Supplementary Table S1). The grass was cut using a tractor-mounted mower, followed by a tractor-mounted rake and baler for forage harvesting.

Each treatment was further divided into two sub-plots to assess the grass growth patterns in different parts of the field. The physical and chemical properties of the topsoil (0–15 cm) are given in Supplementary Table S2. Each treatment was harvested typically two times per year, and once during the establishment year (2019) (Supplementary Tables S1, S2). The experimental field was harvested for pre-wilted silage using farm-scale machinery. The sward was cut to 8 cm with a conventional disk mower with conditioner, swathed and baled with farm machinery and the bales were individually weighed for each of the four subplots. Similarly, representative samples were taken from the swaths and oven-dried at 60°C for 40 h to determine DM and the chemical composition of the herbage. Soil and plant C content was analyzed using a Leco TruMac® CN analyzer.

2.2. Eddy covariance and environment measurements

2.2.1. Instrumentation

The EC tower was erected at the center of the study area on the boundary between N_{min} and N_{org} treatments (Figure 1). Measurements of CO_2 and H_2O fluxes were performed using a closed-path EC system with an adjacent weather station for supporting soil climate and meteorological data. The EC system consisted of a Li-7,000 infrared gas analyzer (IRGA, for CO_2 and H_2O mixing ratios, Li-COR Inc., Lincoln, NE, United States), and a sonic anemometer (for wind velocity components, sensible heat flux, and sonic temperature, R3-50, Gill Instruments Ltd., UK) mounted on an instrument tower at a height of 2.5 m above the soil surface. With a flow rate of 10 L min^{-1}, the air samples passed through a heated intake tube (inner diameter 6 mm, length 8 m, PTFE) with two filters (pore size 1.0 µm, PTFE, Gelman®). The IRGA was housed in a climate-controlled cabin and it was calibrated approximately every month during the growing season with a two-point calibration (0 and 399 µL L^{-1} of CO_2, AGA Oy, Finland) and additionally with a dew point generator (Li–610, LI–COR Inc.) for H_2O mixing ratio during conditions when the air temperature (T_a) was above 5°C.

Supporting climatic variables, i.e., net radiation (R_n, CNR1, Kipp & Zonen B.V.), T_a and relative humidity (RH, HMP45C, Vaisala Inc), photosynthetically active radiation (PAR, SKP215, Skye Instruments Ltd.), soil temperature (T_s, 107, Campbell Scientific Inc.), volumetric water content (θ_v, CS616, Campbell Scientific Inc.) at 5 and 20 cm depths, and air pressure (CS106 Vaisala PTB110 Barometer) were measured.

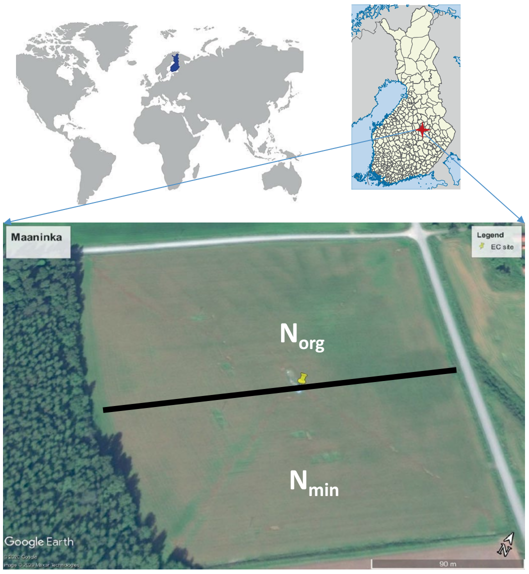

FIGURE 1
Site location at Maaninka (Kuopio, Finland) and experimental set-up.

Eddy covariance raw data were collected at 10 Hz using a data logger (CR3000, Campbell Scientific Inc.). All supporting meteorological and soil climate data were collected as 30 min mean values. Missing T_a, relative humidity, or precipitation data were filled using data from the Maaninka weather station operated by the Finnish Meteorological Institute (FMI), located about 6 km to the southeast of the site.

2.2.2. Processing of flux data

The 30 min EC flux values were calculated from the covariance of scalars and vertical wind velocity. Data processing was performed using EddyUH (Mammarella et al., 2016). Despiking limits were defined for CO_2 at 15 µmol mol^{-1}; 20 mmol mol^{-1} for H_2O; wind components ($u = 10$ m s $^{-1}$, $v = 10$ m s^{-1}, and $w = 5$ m s^{-1}) and temperature (5°C). Detected spikes were replaced by adjacent values or the average of previous values. Point-by-point dilution correction was applied after the despiking. The two-dimensional coordinate rotation was done on the sonic anemometer wind components. The angle of attack correction was not applied. Detrending was done using block averaging. Lag time due to the gas sampling line was calculated by maximizing the covariance. Low-frequency spectral corrections were implemented according to Rannik and Vesala (1999). For high-frequency spectral corrections, empirical transfer function calculations were done based on the procedure introduced by Aubinet et al. (1999). Humidity effects on sonic heat fluxes were corrected according to Schotanus et al. (1983). Additionally, flux values measured when winds were from behind the instrument cabin (85–130°), during rain, and during regular maintenance (e.g., calibration) were discarded.

Night-time NEE and u^* had no significant correlation, hence a default u^* filter of 0.1 m s^{-1} was used. Flux was considered non-stationary following Foken and Wichura (1996). The available flux data were further quality controlled. Both skewness and kurtosis of the data were checked, and the acceptable skewness range was set from −3 to 3 and −2 to 2, and kurtosis from 1 to 14 for CO_2/H_2O. Overall flags higher than 7 were removed (Foken et al., 2004). Finally, the data were visually inspected. From the available data, approximately 53% of the CO_2 and H_2O flux data were retained.

The gap-filling and flux partitioning of NEE were performed using the REddyProc Web online tool.[1] This tool considers both the co-variation of the fluxes with radiation, temperature, and vapor pressure deficit (VPD) and the temporal autocorrelation of the fluxes (Reichstein et al., 2005). The measured and quality-controlled flux data were used as inputs to the Flux partitioning tool. Total ecosystem respiration (R_E) was defined as the night-time measured net ecosystem CO_2 exchange (NEE). The regression between night-time NEE and T_a was calculated using an exponential regression model (Lloyd and Taylor, 1994). Using the model-estimated parameters, the missing half-hour R_E during night and daytime was estimated as a function of the continuous, measured dataset of T_a. Finally, gross photosynthesis

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[1] https://www.bgc-jena.mpg.de/bgi/index.php/Services/REddyProcWeb

(GPP) was calculated as a difference between NEE and R_E. In this paper, CO_2 released into the atmosphere is defined as a positive value and uptake from the atmosphere as negative.

2.3. Net ecosystem carbon balance

Annual and total net ecosystem C balances (NECB) were calculated for each treatment by adding all imports and exports of C to the calculated net ecosystem CO_2 exchange (NEE) (Chapin et al., 2006; Lind et al., 2016).

$$NECB_1 = NEE + C_{harvest} \text{ For the mineral } N \text{ treatment} \quad (1)$$

$$NECB_2 = NEE + C_{harvest} - C_{Norg}. \text{ For the organic } N \text{ treatment} \quad (2)$$

where C_{Norg} is the C added as organic fertilizer (digestate residue); $C_{harvest}$ is the dry biomass C in aboveground biomass removed by harvesting; Emissions of soil methane–C are ignored in this study as they are likely to be very small (Maljanen et al., 2009; Lind et al., 2016). $C_{harvest}$ was calculated as the total dry matter yield multiplied by the C content, $NECB_1$ is the total net ecosystem C balances accounting for harvested biomass and $NECB_2$ is the total C balances accounting also for C addition to the ecosystem in the form of the applied digested residue. Similar to NEE, a negative NECB indicates a (net) C retained in the ecosystem, and a positive sign indicates a (net) C emission or release to the atmosphere.

2.4. Controlling variable analyses

The relationship between daytime ($PAR > 20\,\mu mol\,m^{-2}\,s^{-1}$) NEE and PAR was examined during periods when the grass growth was at its peak (a week before each grass cutting event during the growing season each year). Prior to the analysis, PAR data were binned at an interval of $10\,\mu mol\,m^{-2}\,s^{-1}$. The values of NEE were plotted against PAR with a rectangular hyperbolic model.

$$NEE = \frac{P_{max} \times PAR \times \pm}{P_{max} + PAR \times \pm} + R_d \quad (3)$$

where P_{max} ($\mu mol\,m^{-2}\,s^{-1}$) is the theoretical maximum rate of photosynthesis at infinite PAR, ± is the apparent quantum yield, and R_d is the rate of dark respiration ($\mu mol\,m^{-2}\,s^{-1}$).

2.5. Statistical analyses

In this study, daily, monthly, seasonal, and annual NEE were calculated using gap-filled data in the R programming environment. Data quality control (see Section 2.2.2) was also conducted using R. The effects of PAR on NEE (Equation 3) were evaluated using the "nlme" package of R (Pinheiro et al., 2014). Multilevel correlations between climatic parameters and CO_2 fluxes were tested using the "correlation" package (Lüdecke et al., 2019). All figures were plotted using the "ggplot2" package (Wickham, 2016) in R.

3. Results

3.1. Climatic conditions during the study period

MAT during R_1, R_2, and R_3 was higher than the 30-year mean (3.2°C), with differences of 0.9°C, 1.6°C, and 1.8°C, respectively (Figure 2). The growing season duration varied across the rotations, with 136 days in R_1, 155 days in R_2, and 142 days in R_3. During R_1, the mean T_a from May to July was lower than the 30-year averages, while August and September values were similar (Figure 3). In contrast, R_2 exhibited a consistently higher mean T_a throughout the growing season compared to the 30-year average. The mean T_a during R_3's growing season was mostly in line with the 30-year averages, except for a higher value in June. The mean topsoil T_s during the growing seasons was 12.6°C for R_1, 14.1°C for R_2, and 12.9°C for R_3 (Figure 3). Corresponding subsoil temperatures were 12.1°C, 13.4°C, and 12.5°C, respectively.

MAP was lower than the 30-year mean (612 mm) during R_2 (542 mm) and R_3 (509 mm), while R_1 (624 mm) was wetter than normal (Figure 2). Precipitation during the growing season of R_3 (173 mm) was lower than that in R_1 (284 mm) and R_2 (252 mm), and precipitation values recorded during the growing seasons over the rotation cycle were all lower than that of the 30-year mean (318 mm, Figure 2). More precipitation was received outside the growing seasons over R_1, R_2, and R_3. Mean topsoil θ_v fluctuated with rain events during the growing season and was 34%, during R_1 and 27%, during R_2 and R_3, respectively, with the corresponding mean subsoil θ_v values of 29, 25, and 26% (Figure 3).

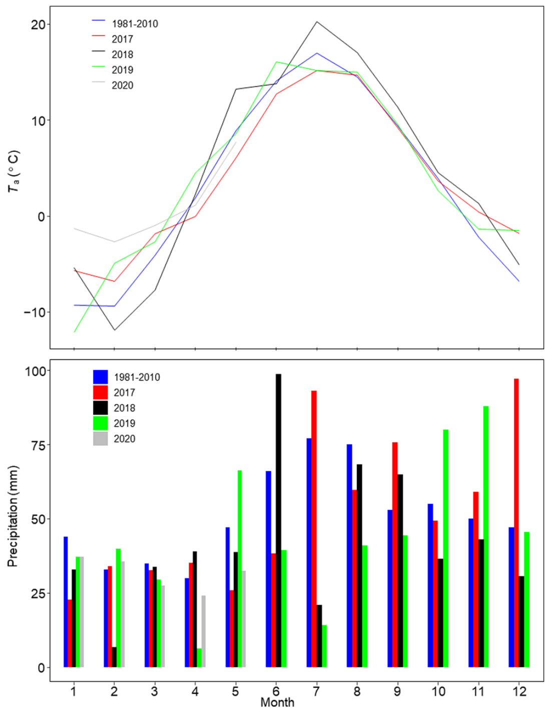

FIGURE 2
Monthly mean air temperature (T_a, °C), and precipitation sum (mm) during 2017–2020 compared with the 30-year average (1981–2010).

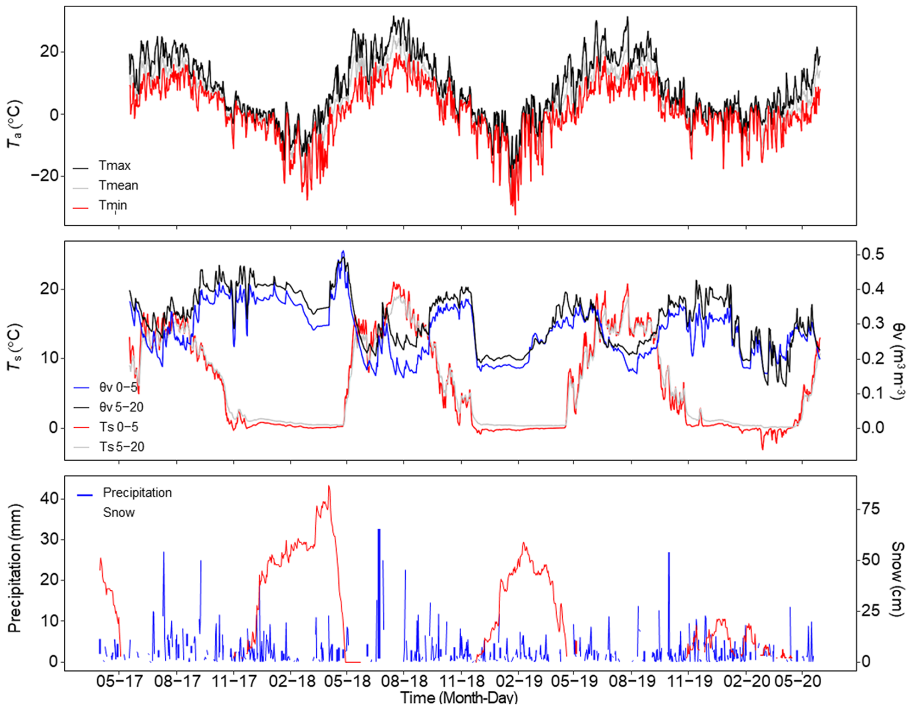

FIGURE 3
Climatic conditions at the study site from May 2017 to May 2020. Daily mean, maximum, and minimum air temperature (T_a, °C); daily averaged soil temperature (T_s, °C) and volumetric water content (θ_v, m^3 m^{-3}) at the top– (0–5cm) and subsoil (5–20cm); daily precipitation sum (mm) and snow depth (cm).

3.2. Biomass yields

The variability of biomass yield among years, treatments, and cuts is noteworthy (Table 1). The maximum annual biomass yield was reported in R_2. The yield from the first cut in R_1 was 25% lower than the second one under the N_{min} treatment, while the yields from the two cuts were about the same under N_{org}. During R_2, however, the yield from the first cut was 2.2 times higher than the second one under N_{min}, while under N_{org}, it was 2.5 times higher.

3.3. Daily net ecosystem CO_2 exchange

Daily NEE displayed distinct patterns reflecting the grass phenological development, harvesting impacts, and grassland renewal during the measurement period (Figure 4). In R_1 and R_2, two negative NEE peaks were observed before each grass cut in late June and early August. In contrast, R_3 had only one peak as the grass was cut once during the grassland renewal year (Figure 4).

In R_1, an uptake peak rate of 39 g CO_2 m^{-2} d^{-1} occurred in mid-June, followed by a post-cutting CO_2 source phase. A second CO_2 uptake peak of 22 g CO_2 m^{-2} d^{-1} was observed in late July. In R_2, the initial CO_2 uptake began 14 days earlier than in R_1, peaking at 48 g CO_2 m^{-2} d^{-1} by late May. After the first cut and second fertilizer application, a CO_2 source peak rate of 19 g CO_2 m^{-2} d^{-1} was observed, followed by a 38 g CO_2 m^{-2} d^{-1} uptake peak in late July. The third cut in R_2 was not performed due to low biomass accumulation rates.

TABLE 1 Harvest events and yield as dry matter (kg DM ha^{-1}) and the proportion of clover in grassland added with mineral nitrogen (N_{min}) or digestate residue (N_{org}) over the three-year rotation cycle (May 2017–May 2020).

Rotation cycle	Harvest date	Yield (kg DM ha^{-1})		Proportion of clover in DM (%)	
		N_{min}	N_{org}	N_{min}	N_{org}
R_1	June 29	2,500 ± 90	2,390 ± 296	50 ± 2	43 ± 5
	August 16	3,360 ± 33	2,490 ± 309	64 ± 5	65 ± 7
R_2	June 26	4,346 ± 1,131	4,860 ± 56	37 ± 1	44 ± 4
	August 7	1970 ± 71	1950 ± 6	57 ± 2	78 ± 0
R_3	August 6	3,410 ± 186	3,440 ± 149	na^a	na^a

R_1, R_2, and R_3 indicate three rotation cycles during May 2017–May 2018, June 2018–May 2019, and June 2019–May 2020, respectively. Data shown are mean ± standard error, $n = 3$.
^{a}The yield consisted mainly of whole-crop barley.

In R_3, following grassland re-establishment, the ecosystem reached a peak net uptake of 43 g CO_2 m^{-2} d^{-1} in mid-July. From the barley harvest in early August 2019 to the study's end in May 2020, the ecosystem remained a sustained CO_2 source (Figure 4).

3.4. Factors controlling CO_2 fluxes

A week before the first and second cuts under the N_{min} treatment in 2017 and 2018, and a week before the first cut under the N_{org} treatment in 2019 (Figure 5), high values of estimated P_{max} (potential photosynthetic

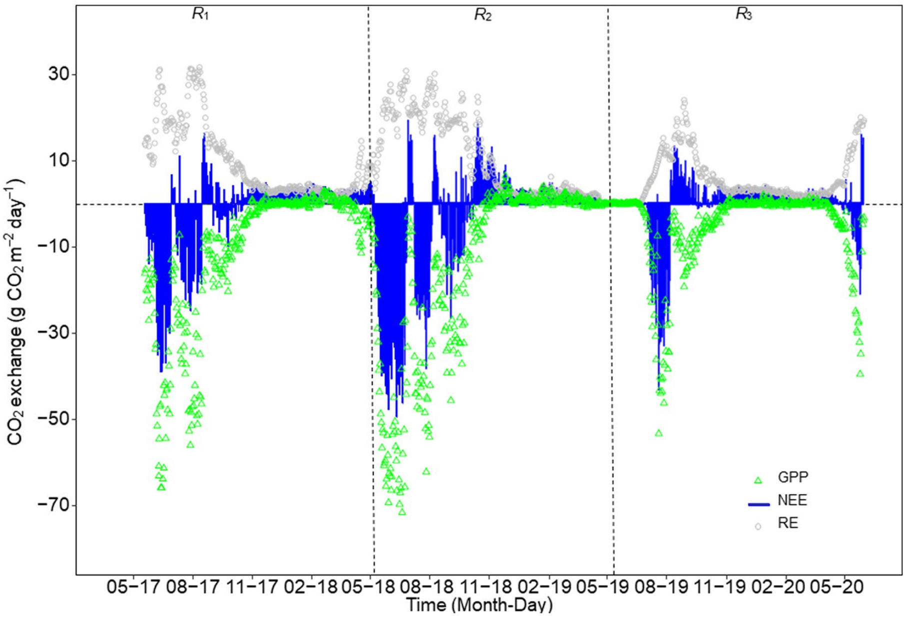

FIGURE 4
Daily CO_2 fluxes (g CO_2 m^{-2} day^{-1}) from grassland from May 2017 to May 2020; Gap-filled CO_2 fluxes were used. Net ecosystem CO_2 exchange (NEE), ecosystem respiration (R_E), and gross primary production (GPP). CO_2 released into the atmosphere is defined as a positive value.

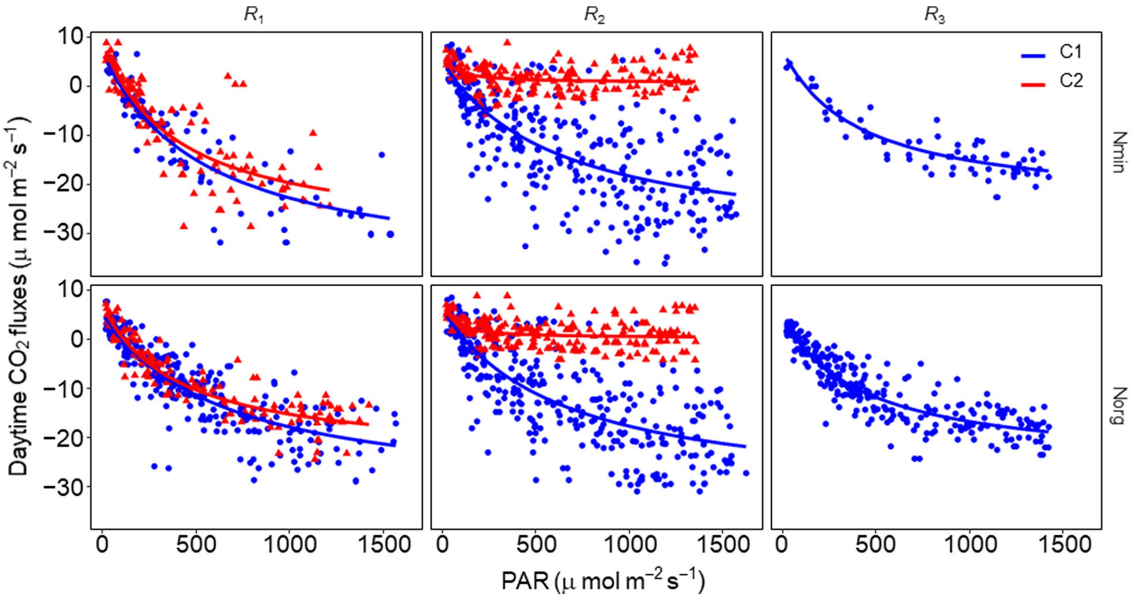

FIGURE 5
Relationship of day−time net ecosystem CO_2 exchange (NEE) with incident photosynthetically active radiation (PAR). Data of NEE were fitted with a nonlinear equation (the estimated parameters are presented in Table 3) with PAR (Section 2.3). R_1, R_2, and R_3 indicate three rotation cycles during May 2017–May 2018, June 2018–May 2019, and June 2019–May 2020, respectively. N_{min} and N_{org} indicate grassland added with mineral nitrogen or digestate residue, respectively. C1 and C2 indicate the first and second grass cuts, respectively. A week's worth of measured 30 min data pairs (NEE and PAR) available before each grass cut were used for the nonlinear regression analysis presented in this figure. Note that there was a single grass cut (C1) made in R3.

capacity) and α (light use efficiency) implied that the climatic conditions and increased leaf area during this time were favorable for high CO_2 uptake ($p < 0.05$, Table 3). The moderate, insignificant differences among light response curves suggested no major differences in NEE under the N_{min} and N_{org} treatments (Table 3, Figure 5).

3.5. Annual C balances

Cumulative R_E and GPP at the study site over the entire three-year study were 2,515 g and 3,061 g C m^{-2}, respectively (Table 2). Thus, based on NEE alone, the ecosystem sequestered 547 g C m^{-2} during

TABLE 2 The annual net ecosystem CO_2 exchange (NEE), ecosystem respiration (R_E), gross primary production (GPP), fertilizer C (C_{Norg}), and dry matter C ($C_{harvest}$) in g C m^{-2}.

	NEE	R_E	GPP	$C_{harvest}$	$NECB_1$	C_{Norg}	$NECB_2$
R_1	−220	965	1,185	257	37	−43	−6
R_2	−334	1,019	1,352	289	−45	−49	−95
R_3	7	531	524	139	146	0	146
Sum	−547	2,515	3,061	685	138	−92	45

Net ecosystem carbon balances (NECB) are presented here with the units of g C m^{-2}. R_1, R_2, and R_3 indicate three rotation cycles during May 2017–May 2018, June 2018–May 2019, and June 2019–May 2020, respectively. A negative NECB indicates C is retained in the ecosystem and while a positive value implies C is lost to the atmosphere. $NECB_1$ indicates the NECB accounting for *C* lost as harvested biomass under mineral *N* application, and the $NECB_2$ is the total net ecosystem *C* balances accounting for harvested biomass and *C* contained in the digested residue.

TABLE 3 Relationship of day–time net ecosystem CO_2 exchange (NEE) with incident photosynthetically active radiation (PAR).

Year	Harvest	N_{min}				N_{org}			
	Time	P_{max}	α	R_d	R^2	P_{max}	α	R_d	R^2
R_1	C1	−46.10	−0.08	6.93	0.53	−37.90	−0.06	5.15	0.35
	C2	−40.30	−0.10	9.07	0.47	−31.20	−0.08	6.71	0.46
R_2	C1	−37.70	−0.06	4.75	0.62	−38.70	−0.06	5.87	0.59
	C2	−7.33	−0.16	6.76	0.77	−8.60	−0.20	8.55	0.68
R_3	C1	−31.50	−0.08	6.96	0.89	−30.70	−0.07	4.52	0.74

Data of NEE were fitted with a nonlinear equation (Equation 3). The NEE is the measured value of the half-hourly net ecosystem CO_2 exchange, PAR is the corresponding half-hourly value of photosynthetically active radiation, P_{max} is the potential net photosynthetic capacity of the vegetation (μmol CO_2 m^{-2} s^{-1}), R_d is the rate of dark respiration (μmol CO_2 m^{-2} s^{-1}), and α is the slope of the initial, linear increase in NEE with increasing PAR. R_1, R_2, and R_3 indicate three rotation years during May 2017–May 2018, June 2018–May 2019, and June 2019–May 2020, respectively. N_{min} and N_{org} indicate grassland added with mineral nitrogen or digestate residue, respectively. C1 and C2 indicate 1 week before the first or second harvest of aboveground biomass, respectively.

the 3-year measurement period. The three-year cumulative biomass yield was 685 g C m^{-2}. Accounting for C lost as harvested biomass, the NECB was 138 g C m^{-2} with synthetic N fertilization. Accounting for the 92 g C m^{-2} as an input of C to the ecosystem with the application of digestate residue, the NECB was reduced to 45 g C m^{-2} (Table 2).

The NECB of the ecosystem was 37, −45, and 146 g C m^{-2} over R_1, R_2, and R_3, respectively -under the mineral N fertilizer application and 6, −95, and 146 under the organic N application (Table 2). The ecosystem behaved as a source in R_1 and a sink in R_2 under the mineral N treatment, while as a small C sink in R_1, and a greater one in R_2 under organic N application, respectively. The ecosystem lost a large amount of C to the atmosphere in the renovation year under both treatments in R_3.

4. Discussion

Grasslands play a crucial role in agriculture by providing ecosystem services for the milk and beef industries in Nordic countries (Åby et al., 2014; Eurostat, 2021). Few studies have reported the CO_2 balance of grasslands in boreal environments, particularly for an entire rotation cycle including the renovation period. This information is vital for developing best management practices aimed at sustainable and climate-smart land use (Cowan et al., 2016; Li et al., 2021b). Our three-year study assessed the impact of various grassland management practices on the CO_2 balance of a legume grassland in eastern Finland.

The length of the growing season in 2018 was 20 days longer than in 2017, with an early start of 16 days in the spring (Figure 2). This study demonstrated high biomass accrual rates and an enhanced ability to sequester atmospheric CO_2 during early spring, indicative of the changes that can be expected in boreal environments under shifting climatic conditions (Ruosteenoja et al., 2011, 2016). In comparison to a 30-year (1981–2010) average climate, the growing season (May–September 2017) had a cooler mean temperature of 11.6°C, while the average temperature of the growing season in 2018 reached 15.1°C (Figure 2). Similarly, the mean soil temperatures at a 5 cm depth during the growing season in 2018 were 1.7°C higher than the seasonal average in 2017. This difference was mainly due to the warmer temperatures experienced during the 2018 spring. The average temperature difference between mid-April and early June (day 100–157) in the 2 years was 6.1°C, with a maximum difference of 17.7°C on day 136 (Figure 2). Precipitation sums from May to September in 2017 (287 mm) and 2018 (291 mm) were roughly equivalent. The vapor pressure deficit (VPD), an indicator of atmospheric dryness, was also elevated during the 2018 growing season (Figure 2). Higher VPD values under optimal soil moisture conditions promote increased photosynthetic uptake of atmospheric CO_2 by vegetation. The findings presented in this study have important implications for optimizing grassland management practices and identifying opportunities for soil C sequestration in response to the changing climate in boreal regions.

Given the interannual and within-season variability in climatic conditions, various management practices either decreased vegetative cover (due to biomass harvesting or grass cuts, senescence, land preparation—glyphosate application, plowing) or increased it (through phenological development and application of chemical or organic fertilizer). Depending on the management practice followed during a year, the NEE of the legume grassland ranged from being near neutral (R_1) to a large sink (R_2) and eventually to a large source in R_3. When the eddy covariance-based CO_2 exchange measurements were conducted in May 2017, the grassland was already in its second year of rotation, having been established in 2015 and reseeded in 2016. Consequently, September 2018 marked the end of the rotation cycle when the grassland was treated with glyphosate, plowed, and left bare during the subsequent winter. The period from the end of the growing season (late September) in 2018 to late May 2019 represented the typical phase of grassland rotation renewal. These practices led to a sustained soil C loss (Cowan et al., 2016; Li et al., 2021b), offsetting the soil C sequestered during previous seasons. In R_3, following regional practice, barley was cultivated as a cover crop alongside red clover and the grass mixture. The atmospheric CO_2 fixation during the short growth period from sprouting (June 4) to harvest (August 6) is primarily attributable to the vigorous growth of the cover crop (Figure 4). After the first cut, the grassland became a large source of C in R_3, likely due to dry climatic conditions in August and relatively low soil N concentration resulting from a small dose of 45 kg N ha^{-1} applied at planting time. Severe climatic stress and poor grass growth did not warrant a second cut in the season. Following the first cut, the ecosystem remained a sustained source of CO_2 to the atmosphere until the end of this study period (May 2020). Our observations, based on continuous CO_2 exchange measurements, reveal the impact of the renewal phase on the ecosystem balance of the entire 3-year rotation cycle in a boreal environment. These results strongly suggest that greater emphasis should be placed on developing climate-friendly renovation management under the Nordic climate (Klumpp and Fornara, 2018). Such management options could extend the length of production years between renovations, provided that grassland productivity can be maintained, re-seeding is performed rapidly in spring, and sufficient N fertilization is ensured for growth and photosynthesis.

Overall, based on the NECB, the legume grassland was a C source of 45 g C m^{-2} over a three-year rotation cycle (Table 2) considering the mineral N application. However, with additional C input to the ecosystem through the application of the organic fertilizer, the NECB values changed to a small C sink of 6 g C m^{-2} in R_1 and a greater sink of 95 g C m^{-2} in R_2. As no organic fertilizer was added in the last year, the NECB during R_3 remained a large C source. Applying organic N fertilizer has been shown to enhance NECB by indirectly enhancing photosynthesis and directly increasing the soil organic matter (Hirata et al., 2013; Conant et al., 2017; Miao et al., 2019; Zhu et al., 2021). A study at a cool temperate site in Japan reported that more than 80% of the C imported in applied manure remained in the grassland soil (Hirata et al., 2013). Previous studies on European grassland sites have also found that, in general, NECB at the grassland sites without organic matter input was a net loss of CO_2 to the atmosphere or neutral, while it was neutral or a net CO_2 sink at sites with organic matter application (Gilmanov et al., 2007). These observations from previous studies are in line with our results (Table 2), in which C incorporation in soil from the organic fertilizer resulted in the net accumulation of C in grassland soils. A study in a temperate grassland reported that the stimulation of C assimilation was greater than that of ecosystem respiration with the addition of organic N, and eventually increased the ecosystem C sequestration (Gilmanov et al., 2007; Luo et al., 2017). Thus, if NECB of the boreal legume grassland is not N limited, it might largely depend on the C balance between manure input and biomass output (Table 2), which warrants further investigation of the tradeoff between productivity and ecosystem benefits with a combination of mineral and organic N fertilizer. We also computed the hyperbolic light response of NEE to incident PAR from N_{min} and N_{org} treatments for a week before all cutting events over R_1, R_2, and R_3 (Table 3, Figure 5) because P_{max}, α, and R_d (Table 3, Figure 5) are important measures of the ecosystem's ability to exchange CO_2 with the atmosphere. The light response curves from the two treatments were similar in all years with minor differences during R_1 (Figure 5), which suggested that beyond the direct effect on photosynthesis, fertilizer type might affect NECB of boreal legume grassland in different manners, and further study is thus warranted. Additionally, the application of organic manure may enhance N_2O emissions (Jones et al., 2006; Li et al., 2021a), for a complete understanding of the sustainability of managed grasslands, N_2O emissions (CO_2-equivalent) need to be assessed in considering complete net GHG balance.

5. Conclusion

The net ecosystem CO_2 balance of a legume grassland over an entire rotation cycle in a boreal environment is critical for developing best management practices aimed at sustainable and climate-smart grassland management for the sustainability of dairy and beef farming industries. This study measured the NECB of a legume grassland in eastern Finland over a three-year (2017–2020) rotation cycle. Overall, the entire legume grassland was a carbon source of 45 g C m^{-2} over the rotation cycle. Specifically, it was a weak carbon sink during the first year of the rotation, a stronger carbon sink during the second year, and a large carbon source during the renovation year of the grassland. Management practices for grassland reestablishment, such as the application of glyphosate and plowing in the autumn of 2018, leaving the site bare during the following winter, and having a late and short growing season cover crop, resulted in significant soil carbon loss and offset soil carbon sequestration achieved in earlier grassland rotation years. Our results indicate that the ecosystem's carbon balance can be improved with the application of organic soil amendments. In conclusion, climate-friendly renovation management extending the length of the photosynthetic period under the Nordic climate can lead to higher carbon sequestration in boreal legume grasslands. The application of organic fertilizers can further enhance carbon sequestration, promoting more sustainable and climate-smart grassland management practices that support the sustainability of dairy and beef farming industries.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

YL: designed research, collected data, formal analysis, visualization, and writing—original draft. PK, SK, MM, and PV: designed research, data curation, and writing—review. NS: designed research, collected data, project administration, supervision, and funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2023.1158250/full#supplementary-material

References

Åby, B. A., Kantanen, J., Aass, L., and Meuwissen, T. (2014). Current status of livestock production in the Nordic countries and future challenges with a changing climate and human population growth. *Acta Agricult. Scand. Sect. A Anim. Sci.* 64, 73–97. doi: 10.1080/09064702.2014.950321

Ammann, C., Neftel, A., Jocher, M., Fuhrer, J., and Leifeld, J. (2020). Effect of management and weather variations on the greenhouse gas budget of two grasslands during a 10-year experiment. *Agric. Ecosyst. Environ.* 292:106814. doi: 10.1016/j.agee.2019.106814

Aubinet, M., Grelle, A., Ibrom, A., Rannik, Ü., Moncrieff, J., Foken, T., et al. (1999). "Estimates of the annual net carbon and water exchange of forests: the EUROFLUX methodology" in *Advances in ecological research*. eds. A. H. Fitter and D. G. Raffaelli (Academic Press), 113–175.

Baldocchi, D. D. (2020). How eddy covariance flux measurements have contributed to our understanding of global change biology. *Glob. Chang. Biol.* 26, 242–260. doi: 10.1111/gcb.14807

Chapin, F. S., Woodwell, G. M., Randerson, J. T., Rastetter, E. B., Lovett, G. M., Baldocchi, D. D., et al. (2006). Reconciling carbon-cycle concepts, terminology, and methods. *Ecosystems* 9, 1041–1050. doi: 10.1007/s10021-005-0105-7

Conant, R. T., Cerri, C. E. P., Osborne, B. B., and Paustian, K. (2017). Grassland management impacts on soil carbon stocks: a new synthesis. *Ecol. Appl.* 27, 662–668. doi: 10.1002/eap.1473

Cowan, N. J., Levy, P. E., Famulari, D., Anderson, M., Drewer, J., Carozzi, M., et al. (2016). The influence of tillage on N_2O fluxes from an intensively managed grazed grassland in Scotland. *Biogeosciences* 13, 4811–4821. doi: 10.5194/bg-13-4811-2016

Deng, J., Ni, H., Zhang, Z., Usman, S., Yang, X., Shen, Y., et al. (2021). Designing productive, energy-efficient, and environmentally friendly production systems by replacing fallow period with annual forage cultivation on the loess plateau of China. *J. Clean. Prod.* 320:128660. doi: 10.1016/j.jclepro.2021.128660

Eurostat (2021). *Milk and milk product statistics*.

Finn, J. A., Kirwan, L., Connolly, J., Sebastià, M. T., Helgadottir, A., Baadshaug, O. H., et al. (2013). Ecosystem function enhanced by combining four functional types of plant species in intensively managed grassland mixtures: a 3-year continental-scale field experiment. *J. Appl. Ecol.* 50, 365–375. doi: 10.1111/1365-2664.12041

Foken, T., Göockede, M., Mauder, M., Mahrt, L., Amiro, B., and Munger, W. (2004). "Post-field data quality control" in *Handbook of micrometeorology: A guide for surface flux measurement and analysis*. eds. X. Lee, W. J. Massman and B. E. Law (Dordrecht: Kluwer Academic Publishers), 181–208.

Foken, T., and Wichura, B. (1996). Tools for quality assessment of surface-based flux measurements. *Agric. For. Meteorol.* 78, 83–105. doi: 10.1016/0168-1923(95)02248-1

Gilmanov, T. G., Soussana, J. F., Aires, L., Allard, V., Ammann, C., Balzarolo, M., et al. (2007). Partitioning European grassland net ecosystem CO_2 exchange into gross primary productivity and ecosystem respiration using light response function analysis. *Agric. Ecosyst. Environ.* 121, 93–120. doi: 10.1016/j.agee.2006.12.008

Gylfadóttir, T., Helgadóttir, Á., and Høgh-Jensen, H. (2007). Consequences of including adapted white clover in northern European grassland: transfer and deposition of nitrogen. *Plant Soil* 297, 93–104. doi: 10.1007/s11104-007-9323-4

Hirata, R., Miyata, A., Mano, M., Shimizu, M., Arita, T., Kouda, Y., et al. (2013). Carbon dioxide exchange at four intensively managed grassland sites across different climate zones of Japan and the influence of manure application on ecosystem carbon and greenhouse gas budgets. *Agric. For. Meteorol.* 177, 57–68. doi: 10.1016/j.agrformet.2013.04.007

IUSS Working Group WRB, (2007). *World reference base for soil resources 2006, first update 2007*. FAO, Rome.

Jones, S. K., Rees, R. M., Kosmas, D., Ball, B. C., and Skiba, U. M. (2006). Carbon sequestration in a temperate grassland; management and climatic controls. *Soil Use Manag.* 22, 132–142. doi: 10.1111/j.1475-2743.2006.00036.x

Klumpp, K., and Fornara, D. A. (2018). "The carbon sequestration of grassland soils-climate change and mitigation strategies" in *27th general meeting of the European grassland federation* (Cork: Teagasc, Animal and Grassland Research and innovation Centre)

Laidlaw, A. S., Christie, P., and Lee, H. W. (1996). Effect of white clover cultivar on apparent transfer of nitrogen from clover to grass and estimation of relative turnover rates of nitrogen in roots. *Plant Soil* 179, 243–253. doi: 10.1007/BF00009334

Li, Y., Clough, T. J., Moinet, G. Y. K., and Whitehead, D. (2021a). Emissions of nitrous oxide, dinitrogen and carbon dioxide from three soils amended with carbon substrates under varying soil matric potentials. *Eur. J. Soil Sci.* 72, 2261–2275. doi: 10.1111/ejss.13124

Li, Y., Li, Z., Cui, S., Liang, G., and Zhang, Q. (2021b). Microbial-derived carbon components are critical for enhancing soil organic carbon in no-tillage croplands: a global perspective. *Soil Tillage Res.* 205:104758. doi: 10.1016/j.still.2020.104758

Lind, S. E., Shurpali, N. J., Peltola, O., Mammarella, I., Hyvönen, N., Maljanen, M., et al. (2016). Carbon dioxide exchange of a perennial bioenergy crop cultivation on a mineral soil. *Biogeosciences* 13, 1255–1268. doi: 10.5194/bg-13-1255-2016

Lloyd, J., and Taylor, J. A. (1994). On the temperature dependence of soil respiration. *Funct. Ecol.* 8, 315–323. doi: 10.2307/2389824

Lüdecke, D., Waggoner, P., and Makowski, D. (2019). Insight: a unified interface to access information from model objects in R. *J. Open Source Softw.* 4:1412. doi: 10.21105/joss.01412

Luo, Q., Gong, J., Yang, L., Li, X., Pan, Y., Liu, M., et al. (2017). Impacts of nitrogen addition on the carbon balance in a temperate semiarid grassland ecosystem. *Biol. Fertil. Soils* 53, 911–927. doi: 10.1007/s00374-017-1233-x

Lüscher, A., Mueller-Harvey, I., Soussana, J. F., Rees, R. M., and Peyraud, J. L. (2014). Potential of legume-based grassland–livestock systems in Europe: a review. *Grass Forage Sci.* 69, 206–228. doi: 10.1111/gfs.12124

Maljanen, M., Virkajärvi, P., Hytönen, J., Öquist, M., Sparrman, T., and Martikainen, P. J. (2009). Nitrous oxide production in boreal soils with variable organic matter content at low temperature – snow manipulation experiment. *Biogeosciences* 6, 2461–2473. doi: 10.5194/bg-6-2461-2009

Mammarella, I., Peltola, O., Nordbo, A., Järvi, L., and Rannik, Ü. (2016). Quantifying the uncertainty of eddy covariance fluxes due to the use of different software packages and combinations of processing steps in two contrasting ecosystems. *Atmos. Meas. Tech.* 9, 4915–4933. doi: 10.5194/amt-9-4915-2016

Miao, F., Li, Y., Cui, S., Jagadamma, S., Yang, G., and Zhang, Q. (2019). Soil extracellular enzyme activities under long-term fertilization management in the croplands of China: a meta-analysis. *Nutr. Cycl. Agroecosyst.* 114, 125–138. doi: 10.1007/s10705-019-09991-2

Olesen, J. E., and Bindi, M. (2002). Consequences of climate change for European agricultural productivity, land use and policy. *Eur. J. Agron.* 16, 239–262. doi: 10.1016/S1161-0301(02)00004-7

Pinheiro, J., Bates, D., DebRoy, S., and Sarkar, D., (2014). Nlme: linear and nonlinear mixed effects models. R package version 3.1–117.

Rannik, Ü., and Vesala, T. (1999). Autoregressive filtering versus linear detrending in estimation of fluxes by the eddy covariance method. *Bound.-Layer Meteorol.* 91, 259–280. doi: 10.1023/A:1001840416858

Reichstein, M., Falge, E., Baldocchi, D., Papale, D., Aubinet, M., Berbigier, P., et al. (2005). On the separation of net ecosystem exchange into assimilation and ecosystem respiration: review and improved algorithm. *Glob. Chang. Biol.* 11, 1424–1439. doi: 10.1111/j.1365-2486.2005.001002.x

Riesinger, P., and Herzon, I. (2008). Variability of herbage production in mixed leys as related to ley age and environmental factors: a farm survey. *Agric. Food Sci.* 17:394. doi: 10.2137/145960608787235522

Ruosteenoja, K., Jylhä, K., and Kämäräinen, M. (2016). Climate projections for Finland under the RCP forcing scenarios. *Geophysica* 51, 17–50.

Ruosteenoja, K., Räisänen, J., and Pirinen, P. (2011). Projected changes in thermal seasons and the growing season in Finland. *Int. J. Climatol.* 31, 1473–1487. doi: 10.1002/joc.2171

Rutledge, S., Wall, A. M., Mudge, P. L., Troughton, B., Campbell, D. I., Pronger, J., et al. (2017). The carbon balance of temperate grasslands part I: the impact of increased species diversity. *Agric. Ecosyst. Environ.* 239, 310–323. doi: 10.1016/j.agee.2017.01.039

Schotanus, P., Nieuwstadt, F. T. M., and De Bruin, H. A. R. (1983). Temperature measurement with a sonic anemometer and its application to heat and moisture fluxes. *Bound.-Layer Meteorol.* 26, 81–93. doi: 10.1007/BF00164332

Suter, M., Connolly, J., Finn, J. A., Loges, R., Kirwan, L., Sebastià, M. T., et al. (2015). Nitrogen yield advantage from grass–legume mixtures is robust over a wide range of legume proportions and environmental conditions. *Glob. Chang. Biol.* 21, 2424–2438. doi: 10.1111/gcb.12880

Virkajärvi, P., Rinne, M., Mononen, J., Niskanen, O., Järvenranta, K., and Sairanen, A. (2015). Dairy production systems in Finland. *Grassland Sci. in Eur.* 20, 51–66.

Wickham, H., (2016). *ggplot2: Elegant graphics for data analysis*. Springer, New York.

Zhu, Y., Merbold, L., Leitner, S., Wolf, B., Pelster, D., Goopy, J., et al. (2021). Interactive effects of dung deposited onto urine patches on greenhouse gas fluxes from tropical pastures in Kenya. *Sci. Total Environ.* 761:143184. doi: 10.1016/j.scitotenv.2020.143184

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Optimizing lucerne (*Medicago sativa*) termination on the Loess Plateau, China: a comparative analysis of conventional tillage and herbicide treatments

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Introduction: Lucerne (*Medicago sativa* L.) is a crucial component in agricultural rotation systems due to its ability to enhance soil carbon (C) and nitrogen (N) contents. In China, conventional tillage (CT) is commonly used for lucerne termination, leading to soil structure degradation and C and N losses. While herbicide application has been suggested for lucerne termination in the United States and EU, its impact on lucerne growth suppression and soil C and N contents in China remains uncertain.

Methods: In this study, we examined the effects of herbicide type [glyphosate and 2,4-D (G+2), glyphosate and dicamba (G+D), 2,4-D and dicamba (2+D), with water (W) and CT as controls] and concentration (100, 200, 300%) on various lucerne and soil properties.

Results and Discussion: Our findings revealed that G+2 and 2+D treatments significantly reduced the regreening rate, and the recommended herbicide concentration (100%) was sufficient for lucerne termination. CT and W treatments resulted in a higher soil pH compared to herbicide treatments. Moreover, herbicide treatments exhibited higher soil organic C (SOC) and total N (TN) levels than the CT treatment. The 2+D treatment demonstrated a higher SOC content compared to the G+2 treatment, while the 200 and 300% herbicide concentrations reduced SOC and TN levels. The G+2 treatment had the highest soil ammonium nitrogen content (7.94 ± 1.45 mg kg^{-1}), while the CT treatment showed the lowest (6.46 ± 1.54 mg kg^{-1}). In conclusion, our study suggests that applying the recommended herbicide dosage (100%) effectively terminates lucerne grassland on the Loess Plateau of China without negatively impacting soil C and N storage. Finally, it is important to acknowledge that one-year trials at a single site have inherent limitations, and the findings should be considered cautiously when informing policy decisions.

KEYWORDS

lucerne/alfalfa termination, herbicide, tillage, soil carbon, soil nitrogen

1. Introduction

Soil is the largest terrestrial carbon (C) pool (Scharlemann et al., 2014), with soil organic carbon (SOC) widely recognized as the core of soil quality, particularly in agricultural ecosystems due to its essential role in sustaining crop production and environmental resistance (Smith et al., 2016; Rumpel et al., 2018). Consequently, increasing SOC stocks could secure sustainable agricultural systems and food production and mitigate rising atmospheric carbon dioxide levels (Basile-Doelsch et al., 2020). Hence, optimizing agricultural management practices has been promoted to facilitate the SOC sequestration since agricultural management practices largely affect the turnover of SOC and relevant soil processes (Basile-Doelsch et al., 2020; Li et al., 2021).

Combined with minimum tillage, crop rotation increases agricultural SOC storage (Holland, 2004; Li et al., 2020). Studies have shown that legume-based rotations increase agricultural crop production (Zhao et al., 2022) and SOC content in global croplands (Liu et al., 2022). Incorporating legumes into cropping systems can improve system productivity under low fertilizer nitrogen (N) inputs and enhance soil C stabilization, particularly in N-limited deep subsoil (Peixoto et al., 2022). Deep-rooted perennial legumes can also improve subsoil C inputs and stabilization (Yuan et al., 2016).

Lucerne (*Medicago sativa* L.) is the most widely cultivated and used perennial leguminous forage worldwide (Wang et al., 2021). Lucerne's abundant underground biomass and root exudates promote soil aggregate formation and improve microbial C utilization efficiency, reducing soil heterotrophic respiration and system carbon dioxide emissions (Drinkwater, 1999). In addition, crop rotations with lucerne can improve soil quality, increase SOC and total nitrogen (TN) contents, and enhance agricultural SOC sequestration (Bell et al., 2012; Eerd et al., 2014), with significance for ecological security (Yuan et al., 2016).

Lucerne takes up plenty of water and has a high transpiration coefficient (Fourie, 2017). Therefore, lucerne growth over time inevitably leads to soil water deficit and even deep soil desiccation in water-limited areas (Shen et al., 2009; Ali et al., 2021). Thus, lucerne/annual crop rotations can maximize the benefits of lucerne in rotation systems and avoid the formation of dry soil layers so as not to impede subsequent crop growth (Davies and Peoples, 2003; Bell et al., 2012). However, lucerne termination in the rotation system frustrates producers. Conventional techniques, such as moldboard plowing, subsoiling, and disking, are commonly used to terminate lucerne; however, they increase soil erosion (Mohr et al., 1999), break down soil aggregates, and eventually decrease SOC (Entz et al., 1995), offsetting the sequestrated SOC from lucerne in the rotation system.

As an alternative to conventional techniques, herbicide application could terminate lucerne growth in the rotation system (Randall et al., 2001), with increasing interest in this technique among producers in the Canadian Prairies and northern US Great Plains (Entz et al., 1995). Herbicides that inhibit lucerne growth include glyphosate [N-(phosphonomethyl) glycine] (Davis et al., 1978; Clayton, 1982), dicamba (3,6-dichloro-2-methoxybenzoic acid) (Button, 1994), 2,4-D [(2,4-dichloro phenoxy) acetic acid] (Moomaw and Martin, 1976), and clopyralid (3,6-dichloropicolinic acid) (Clayton, 1982; Button, 1994). However, the effect of herbicides on suppressing lucerne growth and its impact on soil C and N contents in China remains unclear.

Lucerne is an important crop in the dryland farming systems of the Loess Plateau, China, occupying more than 70% of the farmland in north-western China (Han et al., 2010). Therefore, assessing herbicide efficiency in inhibiting lucerne growth would be meaningful for optimizing lucerne in crop rotation systems. Thus, our hypothesis was that different combinations of herbicides applied at various doses (100, 200, 300%) might result in different effects on lucerne termination and on C and N cycles.

2. Materials and methods

2.1. Site characterization

This study was performed at the Loess Plateau Research Station of Lanzhou University, Gansu, China (35°40′N, 107°51′ E, elevation 1,297 m), a typical rainfed agricultural area with a semiarid continental monsoon climate and more than 60% of the precipitation occurring from July to September. The mean annual temperature and precipitation (1970–2021) are 9°C and 561 mm, respectively (Li et al., 2022). The soil is classified as silty loam soil based on the FAO/UNESCO soil classification (Table 1). The soil pH is 8.4 (0–20 cm).

Lucerne grassland (*Medicago sativa* L. cv. Gannong No.3) was established in April 2016 at a seeding rate of 1.5 kg ha^{-1}, sowing depth of 3 cm, and row spacing of 30 cm (Pan et al., 2016). The experimental field received 140 kg ha^{-1} N and 70 kg ha^{-1} P, as urea and calcium superphosphate at sowing stage, and no irrigation. Lucerne was harvested twice a year.

2.2. Experimental design and setup

We conducted a preliminary study based on previous research to determine the types and combinations of herbicide (Davies and Peoples, 2003; Clark et al., 2021), from which three herbicides were selected: 41% glyphosate isopropylamine salt solution (Shandong Shengpeng Technology Co., Ltd., China, referred to herein as glyphosate), 90% 2,4-D isooctyl emulsifiable concentrate (Tianjin Boke Baisheng Technology Co., Ltd., China, 2,4-D), and 48% dicamba water agent (Zhejiang Baike Biotechnology Co., Ltd., China, dicamba).

The factorial experiment comprised two factors: (1) three herbicide type combinations [glyphosate and 2,4-D (G + 2), glyphosate and dicamba (G + D), 2,4-D and dicamba (2 + D)], with water (W) and conventional tillage (CT) used as controls; (2) three herbicide concentrations at 100, 200, and 300% of the recommended dose. The

TABLE 1 Measurements of soil nutrient content in the experimental field (mean±SD, n=4).

Soil depth (cm)	TN (gkg^{-1})	SOC (gkg^{-1})	NO_3^--N (mgkg^{-1})	NH_4^+-N (mgkg^{-1})
0–10	0.97 ± 0.17	9.22 ± 1.28	29.04 ± 9.92	7.95 ± 0.65
10–20	0.83 ± 0.05	7.96 ± 0.78	11.86 ± 2.85	7.94 ± 0.53
20–30	0.73 ± 0.07	6.89 ± 0.66	9.84 ± 1.86	7.00 ± 0.24
30–60	0.60 ± 0.15	6.75 ± 0.35	7.43 ± 0.83	6.97 ± 0.91
60–100	0.69 ± 0.06	6.94 ± 0.29	8.04 ± 0.99	6.71 ± 1.01

recommended doses for glyphosate, 2,4-D, and dicamba are $0.55\,\mathrm{mL\,m^{-2}}$, $0.07\,\mathrm{mL\,m^{-2}}$, and $0.09\,\mathrm{mL\,m^{-2}}$, respectively. The experimental plots were arranged randomly with four replications of each treatment and 1 m walkways between each plot.

The treatments were applied on September 16, 2021, when the average height of lucerne was about 15 cm. The CT treatment was spaded to a depth of 40 cm to remove lucerne roots. Herbicide treatments involved spraying a tank mixture of the herbicide combination on the lucerne. Soil samples were collected in December 2021, January 2022, and March 2022 to assess dynamic changes in SOC and TN contents.

2.3. NDVI, SPAD, plant height, and regreening rate

The normalized difference vegetation index (NDVI) was measured on September 20, September 30, and October 24, 2021, using GreenSeeker (RT100B, N-Tech, United States). The relative leaf chlorophyll content (characterized by SPAD) was measured with a portable SPAD chlorophyll meter (SPAD-502, Konica Minolta, Japan) on September 30, 2021. Five leaves in each plot were randomly selected to determine SPAD values, which were averaged.

The number of lucerne plants in a 1 m^2 quadrat was counted before the treatments were applied (September 2021) and after the regreening stage (April 2022) to calculate the regreening rate (Li, 2020):

$$\text{Regreening rate (\%)} = \frac{\text{Number of lucerne plants in the quadrat after greening}}{\text{Number of lucerne plants in the quadrat before winter}} \times 100.$$

Plant height was measured in May 2022 on four lucerne stems randomly selected in each plot, with the natural extension height from the root to the leaf tip measured with a ruler and the average value taken as plant height.

2.4. Soil sample collection and analysis

Soil samples at 0–10, 10–20, 20–30, 30–60, and 60–100 cm were collected with a soil drill (5 cm diameter). Part of the sample was air-dried and ground to pass through a 0.2 mm sieve to determine SOC and TN contents. Soil for ammonium-N (NH_4^+-N) and nitrate-N (NO_3^--N) analysis was stored at 4°C.

SOC content was determined using the potassium dichromate external heating method ($K_2Cr_2O_7$-H_2SO_4 oxidation method) (Chan et al., 2001) and subsequently titrated with o-phenanthroline indicator and $0.2\,\mathrm{mol\,L^{-1}}$ ferrous sulfate ($FeSO_4$) solution (Mccarty et al., 2010). TN content was determined using the Kjeldahl method (Kjeldahl, 1883). NO_3^--N and NH_4^+-N contents were determined with an automatic high flux intermittent chemical analyzer (smart 450 auto analyst, AMS, Italy) with 50 mL of $2\,\mathrm{mol\,L^{-1}}$ KCl extraction (Li et al., 2012). Soil pH was measured with an intelligent portable redox potentiometer (LD-QX6530, Laide-en, China).

2.5. Data analysis

NDVI, SPAD, and plant height at the regreening stage, regreening rate, and soil pH were analyzed by two-way ANOVA for herbicide type and concentration. SOC, TN, and NO_3^--N and NH_4^+-N contents were analyzed by three-way ANOVA for herbicide type, herbicide concentration, and sampling date. The data were tested for normality and homogeneity before performing the ANOVA. Significant results were compared using Tukey's HSD method with a significance level of 0.05. All data analyses were undertaken in the Agricola package (De Mendiburu, 2014) and plotted with the 'ggplot2' package (Wickham, 2016) in R (v4.1.3).

3. Results

3.1. Effect of herbicide type and concentration on lucerne growth

Herbicide type, herbicide concentration, and their interaction significantly affected the NDVI of lucerne grassland (Figure 1), which decreased over time ($p < 0.05$). The G + 2 treatment had a lower NDVI than the 2 + D treatment ($p < 0.05$). Regardless of herbicide type and concentration, the herbicide treatments had lower NDVI values than the W control (NDVI = 0.918 ± 0.010, $p < 0.05$).

Herbicide type, herbicide concentration, and their interaction significantly affected the SPAD value of lucerne (Figure 2). Except for the G + 2 (100%) treatment, the herbicide treatments had significantly lower SPAD values than the W control (49.91 ± 8.68). The G + 2 (200%) and G + 2 (300%) treatments had lower SPAD values than the G + D (200%) treatments ($p < 0.05$).

All herbicide types and concentrations significantly decreased the regreening rate of lucerne compared to the W control (regreening rate > 90%) (Table 2). There was no significant difference between herbicide types and concentration levels, and regreening rates under CT and herbicide treatments irrespective of types and concentrations were lower than 8%.

All herbicide types and concentrations significantly decreased lucerne height at the regreening stage compared to the W control (45.25 ± 1.13 cm) (Table 3). There was no significant difference between herbicide types and concentrations, and lucerne height under CT and herbicide treatments irrespective of types and concentrations were in the range of 10–25 cm, which was significantly lower than the W control.

3.2. Effect of herbicide type and concentration on soil pH and soil C and N contents

Herbicide type, herbicide concentration, and their interaction significantly affected soil pH from 0–100 cm (Figure 3). The CT and W controls had significantly higher soil pH values than the herbicide treatments. The G + D treatment had the lowest soil pH ($p < 0.05$). The herbicide treatments at 100% concentration had significantly higher soil pH values than the 200 and 300% concentrations, with no significant difference between the 200 and 300% concentrations. The 2 + D treatment (100, 200 and 300%) had higher soil pH values than the G + D treatment (100 and 300%, $p < 0.05$).

Herbicide type, sampling date, and their interaction significantly affected SOC content (Figure 4). The 2 + D treatment had higher SOC content than the G + 2 treatment and W control ($p < 0.05$). The 2 + D treatment had the highest SOC content from 30–100 cm ($p < 0.05$). December had higher SOC contents from 0–10 cm than January and March, while March had higher SOC contents from 20–100 cm than

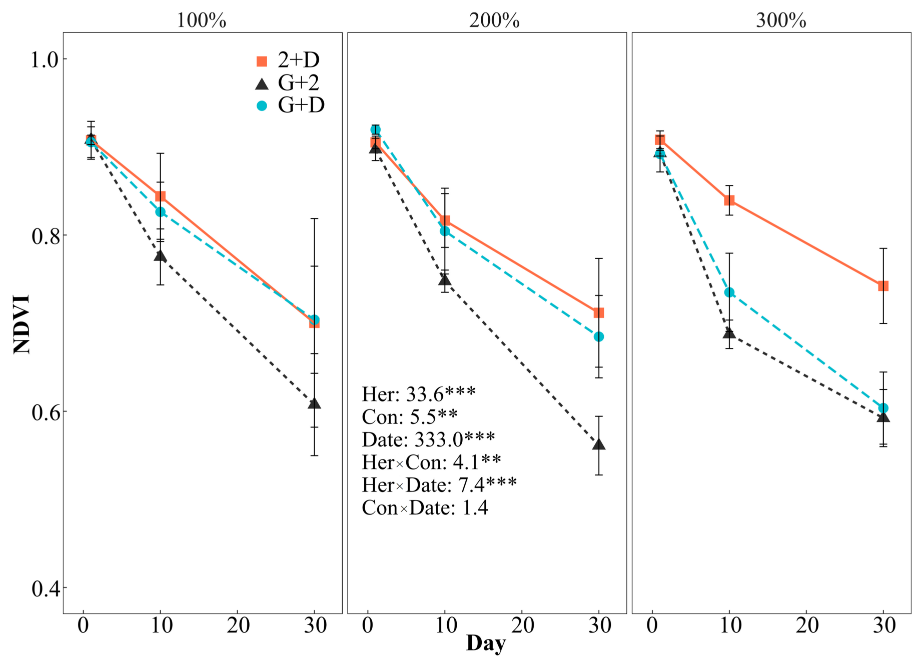

FIGURE 1
Effect of herbicide (2+D, G+D, G+2) and concentration (100%, 200%, 300%) on the NDVI of lucerne for 30days following herbicide application. Dots are means (bars represent standard deviation, *n*=4). *F* value is marked in the figure. *, **, and *** indicate significance levels at $p<0.05$, 0.01, and 0.001. Note the y-intercept is not 0. The three herbicide types were combined glyphosate and 2,4-D (G+2), glyphosate and dicamba (G+D), and 2,4-D and dicamba (2+D). Water (W) and conventional tillage (CT) was used as the control. The three herbicide concentrations were 100, 200, and 300% of the recommended dose.

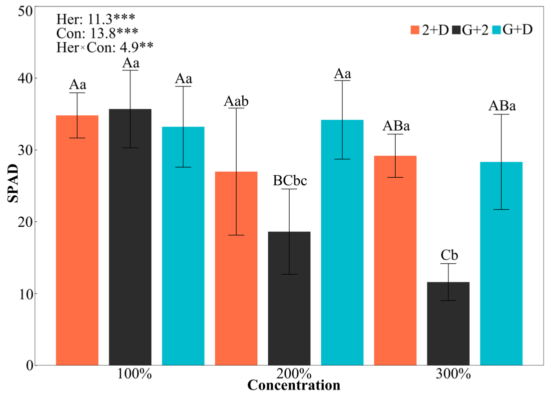

FIGURE 2
Effect of herbicide (2+D, G+D, G+2) and concentration (100, 200, 300%) on SPAD value of lucerne 14days after treatment application. Values are means of four replicates (± standard deviation). *F* value is marked in the figure. *, **, and *** indicate significance levels at $p<0.05$, 0.01, and 0.001. Different upper case letters indicate significant differences in SPAD values of lucerne under different concentrations of the same herbicide, and different lower case letters indicate significant differences in SPAD values of lucerne under different herbicide types with the same concentration ($p<0.05$). For a description of the abbreviations, refer to the Figure 1 caption.

TABLE 2 Effect of herbicide type and concentration on lucerne regreening rate (mean±SD, *n*=4).

Treatment	Regreening rate (%)		
	100%	200%	300%
G + 2	3.03 ± 1.24 b	3.03 ± 2.77 b	3.03 ± 1.24 b
G + D	3.03 ± 1.75 b	3.41 ± 2.59 b	3.03 ± 2.47 b
2 + D	4.17 ± 3.12 b	2.27 ± 2.90 b	0.76 ± 0.87 b
CT	3.21 ± 1.12 b		
W	95.37 ± 0.60 a		

For a description of the abbreviations, refer to the Figure 1 caption. Different lowercase letters indicate significant differences at $p < 0.05$ level among treatments of herbicide type and concentration.

January and December ($p < 0.05$). The 100% herbicide concentration had the highest SOC contents from 0–10 cm (10.65 ± 1.50 g kg^{-1}), even higher than the CT and W controls ($p < 0.05$).

Herbicide type, sampling date, and their interaction significantly affected soil TN content (Figure 5). The CT control and 2 + D and G + D treatments had higher TN contents than the W control ($p < 0.05$). Soil TN contents did not significantly differ in December and January but were higher than in March ($p < 0.05$). The herbicide types had similar soil TN contents in December, higher than the W control ($p < 0.05$). In January, the CT control had significantly higher TN content than the G + 2 treatment and W control, while the W control had significantly lower TN content than the G + D and 2 + D treatments. In March, no significant differences in TN content occurred between herbicide types.

Herbicide type, herbicide concentration, sampling date, and their interaction significantly affected soil NO_3^--N content (Figure 6). All herbicide treatments significantly increased soil NO_3^--N content compared to the W control but did not significantly differ from the CT control ($p > 0.05$). The 100% herbicide concentration increased soil

NO_3^--N content more than the 200 and 300% concentrations ($p < 0.05$). January had higher soil NO_3^--N contents than December ($p < 0.05$). Regardless of herbicide type, herbicide application produced higher soil NO_3^--N contents than the W control in January and March ($p < 0.05$). The G + D (100%) and G + 2 (300%) treatments had higher soil NO_3^--N contents than the W control ($p < 0.05$).

Herbicide type, herbicide concentration, sampling date, and their interactions significantly affected soil NH_4^+-N content (Figure 7). The G + 2 treatment had the highest soil NH_4^+-N contents ($7.94 \pm 1.45\,mg\,kg^{-1}$) (Figure 7, $p < 0.05$), while CT had the lowest ($6.46 \pm 1.54\,mg\,kg^{-1}$). Regardless of herbicide concentration, the herbicide treatments had higher soil NH_4^+-N contents than the CT and W controls ($p < 0.05$). December and March had lower soil NH_4^+-N contents than January ($p < 0.05$).

TABLE 3 Effect of herbicide type and concentration on lucerne height at regreening stage (mean±SD, *n*=4).

Treatment	Regreening height (cm)		
	100%	200%	300%
G + 2	15.92 ± 2.75 b	13.19 ± 2.19 b	11.17 ± 2.52 b
G + D	17.63 ± 1.65 b	14.56 ± 1.88 b	20.75 ± 5.15 b
2 + D	12.38 ± 8.76 b	12.5 ± 8.89 b	11.38 ± 10.03 b
W	45.25 ± 1.13 a		

For a description of the abbreviations, refer to the Figure 1 caption. Different lowercase letters indicate significant differences at $p < 0.05$ level among treatments of herbicide type and concentration.

4. Discussion

4.1. Herbicides inhibited lucerne growth

The NDVI and SPAD values indicated that the three herbicide combinations had a higher efficiency of terminating and inhibiting lucerne growth in a short time. While it is difficult to successfully terminate lucerne growth with a single herbicide, selecting an appropriate herbicide combination can inactivate lucerne roots (Buhler and Mercurio, 1988). Studies have reported a synergistic relationship between glyphosate and auxin herbicides, which may induce lucerne withering (Wehtje and Walker, 1997; Flint and Barrett, 1998).

All herbicide treatments significantly inhibited lucerne's regreening rate and plant height at the regreening stage, with the lowest NDVI and SPAD values in the G + 2 treatment (Figure 2), particularly at higher concentrations (200, 300%). The selected herbicides in this experiment are relatively safe. For instance, glyphosate has a half-life in soil and water that is relatively short (averaging about 30 d in temperate climates), and it does not bioaccumulate in organisms due to its high water solubility (Duke, 2020). Commercial 2,4-D is easily soluble in water and other solvents result in rapid penetration through the leaves/roots, and runoff of 2,4-D can be proper irrigation management (Islam et al., 2018). Dicamba is a chlorobenzene acid herbicide, and the half-life of dicamba in soil would be <12 weeks under typical conditions (Caux et al., 1993; Ochsner et al., 2006). Nonetheless, the recommended dosage, 100%, already

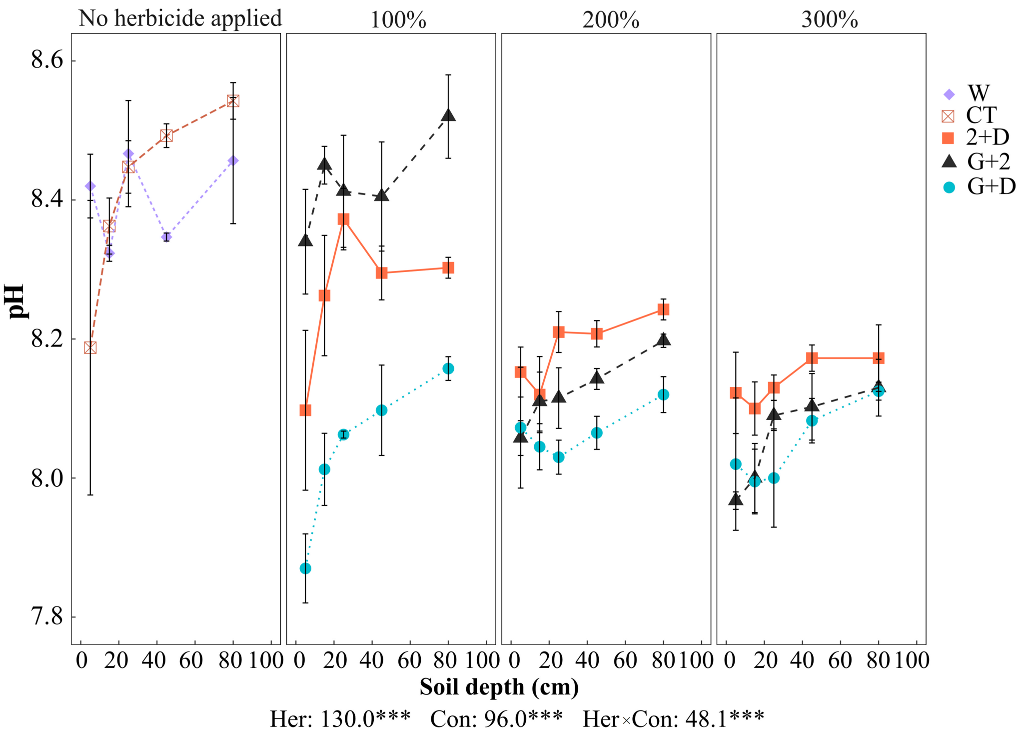

FIGURE 3
Soil pH at different depths (0–10, 10–20, 20–30, 30–60, 60–100cm) under different herbicides (2+D, G+D, G+2) and concentrations (100, 200, 300%) treatments at the end of this study in 2022. Values are means of four replicates (± standard deviation). *** indicate significance levels at $p < 0.001$. Note the y-intercept is not 0. For a description of the abbreviations, refer to the Figure 1 caption.

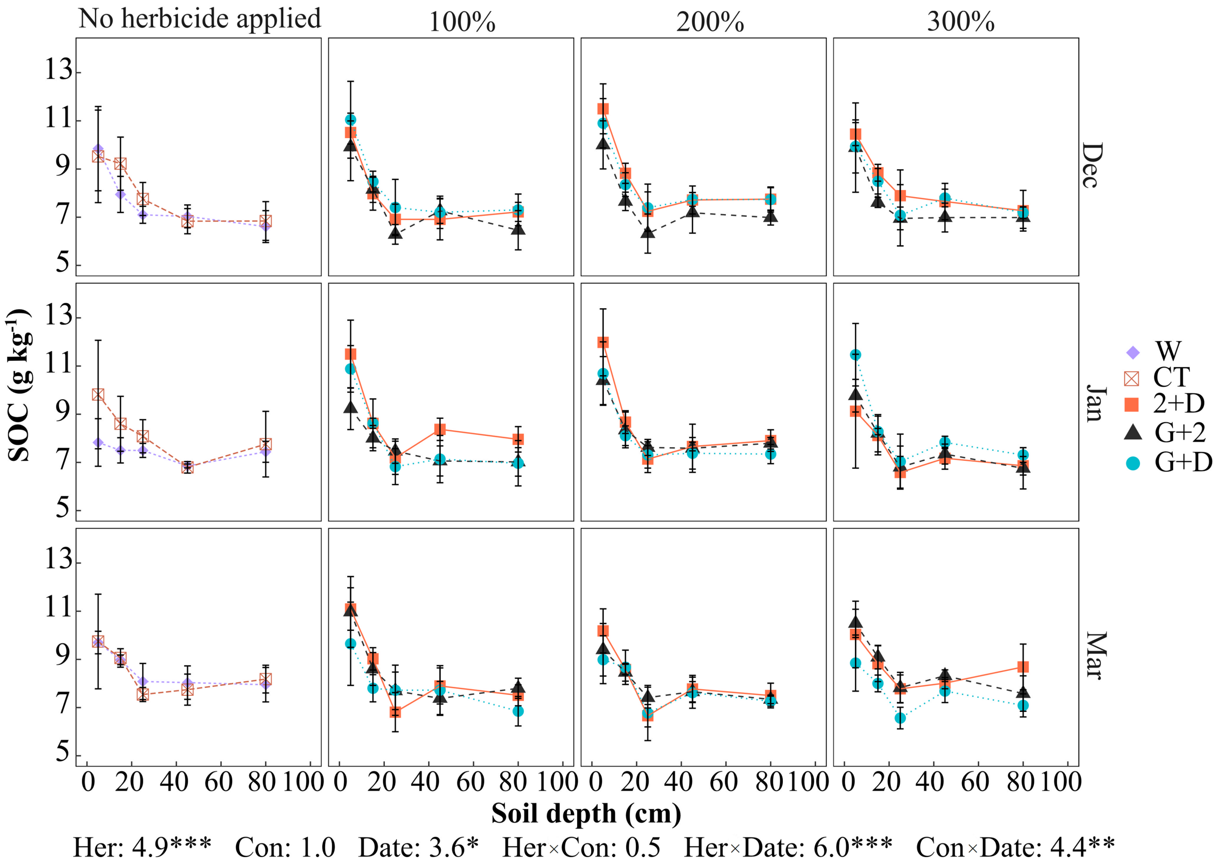

FIGURE 4
Effect of herbicide (2+D, G+D, G+2) and concentration (100%, 200%, 300%) on soil organic carbon (SOC) content at different depths (0–10, 10–20, 20–30, 30–60, 60–100cm) in December 2021, January 2022, and March 2022. *, **, and *** indicate significance levels at $p<0.05$, 0.01, and 0.001. Values are means of four replicates (± standard deviation). *F* value is marked in the figure. Note the y-intercept is not 0. For a description of the abbreviations, refer to the Figure 1 caption.

significantly decreased regreening rate (Table 2) and height at the regreening stage (Table 3). Thus, in light of environmental and economic costs, applying the recommended dosage of glyphosate and 2,4-D in autumn can mostly terminate lucerne growth on the Loess Plateau. However, the general outcome of this study may vary depending on the climate and the specific aspect being studied. Further research is warranted to validate the relevant findings across multiple sites and years. It is worth noting that one-year trials have limitations in terms of their ability to fully capture the long-term effects of treatments, and the results should be interpreted with caution when making policy decisions.

4.2. Effect of different treatments on soil pH and soil C and N contents

Soil C and N contents in the herbicide treatments significantly differed from the W and CT controls (Figures 4–7). For instance, the 2 + D treatment had significantly higher SOC contents from 30–100 cm than the CT and W controls (Figure 4), possibly because the tillage destroyed soil aggregate structure, releasing and mineralizing SOC. A study on lucerne showed that the herbicide treatment had higher SOC content than the tillage treatment (Malhi et al., 2010). In our study, the herbicide treatments had lower soil pH values than the CT and W controls, possibly due to the degradation of lucerne roots (killed by the herbicides) by microorganisms, subsequently releasing carbon dioxide from organic acids and reducing soil pH (Hinsinger et al., 2003). This suggested that lucerne roots are gradually apoptotic, releasing C and N substances in the herbicide treatments. However, soil C and N contents did not significantly differ between the different herbicide treatments (Figure 8). This may be because soil organic and labile compounds can persist not for weeks but years, with the initial stages of litter decomposition unable to explain the persistence of soil organic compounds in the long term (Schmidt et al., 2011). Therefore, treatment effects were not evident for organic C inputs or outputs during the seven-month experiment, possibly because significant changes in SOC take time. Additionally, this study did not monitor gaseous soil C and N losses that significantly contribute to soil C and N

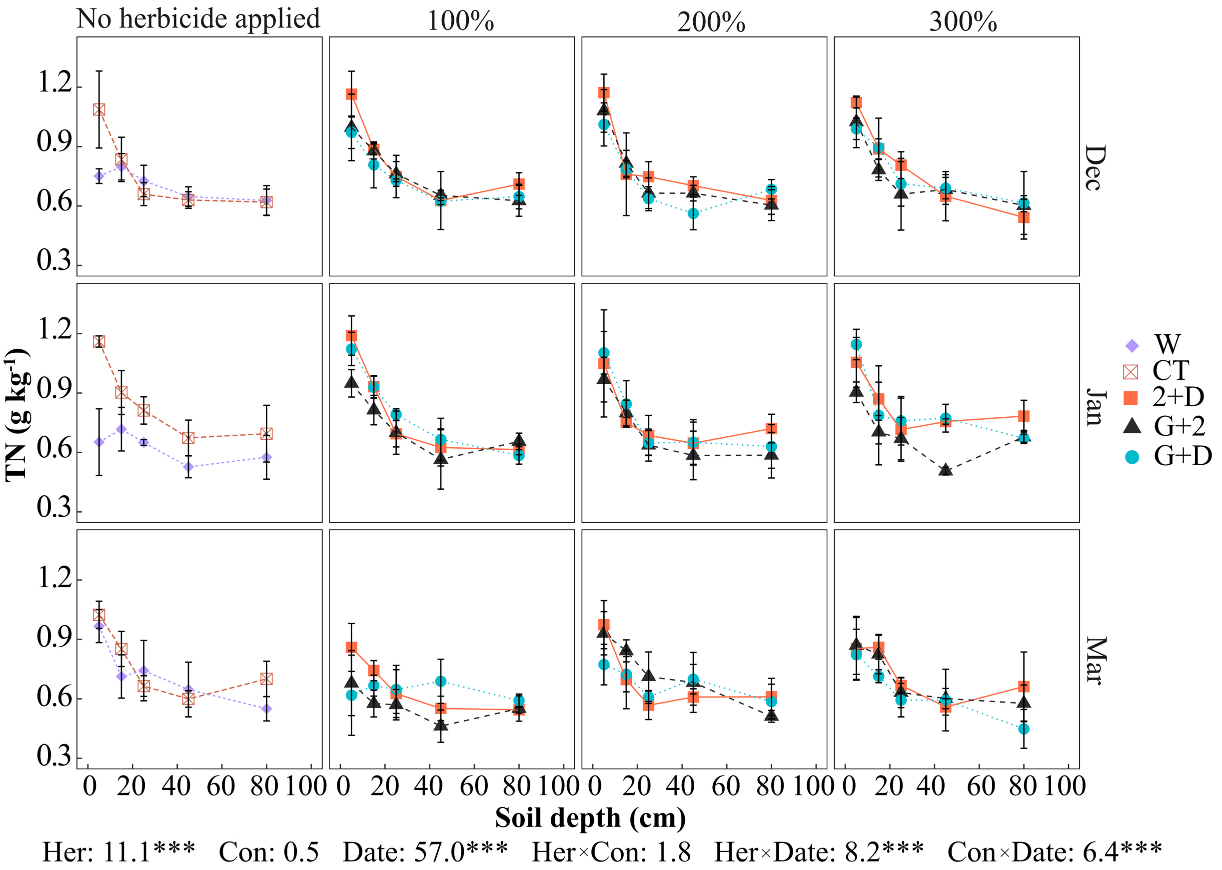

FIGURE 5
Effect of herbicide (2+D, G+D, G+2) and concentration (100%, 200%, 300%) on soil total N (TN) content at different depths (0–10, 10–20, 20–30, 30–60, 60–100cm) in December 2021, January 2022, and March 2022. Values are means of four replicates (± standard deviation). *F* value is marked in the figure.*** indicate significance levels at $p < 0.001$. Note the y-intercept is not 0. For a description of the abbreviations, refer to the Figure 1 caption.

dynamics. Thus, further study is warranted to assess gaseous and labile soil C and N dynamics in response to lucerne termination with herbicides.

The herbicide treatments and CT control increased soil TN and NO_3^--N contents in lucerne grassland relative to the W control, with significantly higher soil NO_3^--N content in the CT control than the herbicide treatments. This may be because CT adversely affects soil structure, disturbing aggregate formation (Six et al., 2002), increasing soil aeration, and releasing physically protected organic matter, thus promoting organic residue decomposition and mineralization (Jia, 2013). Nonetheless, lucerne termination with CT generally causes soil erosion, with large amounts of NO_3^--N runoff due to leaching and denitrification (Malhi and Nyborg, 1986; Nyborg et al., 1997). Herbicide-based lucerne termination can improve soil water conservation capacity, reduce water and soil erosion, and reduce carbon dioxide and nitrous oxide emissions relative to CT (Toderi et al., 2022). Although the termination effect of CT treatment was acceptable in this experiment, spading at 40 cm is a high energy consumption tillage operation, that is difficult to achieve in high clay soils.

4.3. Sampling date significantly affected soil C and N contents

March had significantly higher SOC contents from 20–100 cm under lucerne grassland than January and December. From December to March, the soils on the Loess Plateau experience severe freezing–thawing cycles (Li and Fan, 2014), which can increase SOC content by destroying soil aggregate structure and releasing physically protected SOC (Wang et al., 2012; Ma et al., 2021). Soil freezing also facilitates root death (Henry, 2007; Kreyling et al., 2012), increasing SOC content. The effect of soil frost on root mortality or function can also affect C dynamics because root respiration accounts for 40–60% of soil C losses (Hogberg et al., 2001).

Similarly, January had higher soil NO_3^--N and NH_4^+-N contents than December, with soil NH_4^+-N content decreasing in March relative to January, possibly due to the increasing temperature from January to March increasing nitrification (Hoyle et al., 2006; Wang et al., 2006), thus decreasing NH_4^+-N content (Nicolardot et al., 2001; Wang et al., 2006). Besides, the decrease in soil pH could convert NH_4^+ to NH_3, reducing soil N content. Changes in soil TN content depend on N accumulation and consumption. In this study, the change in soil

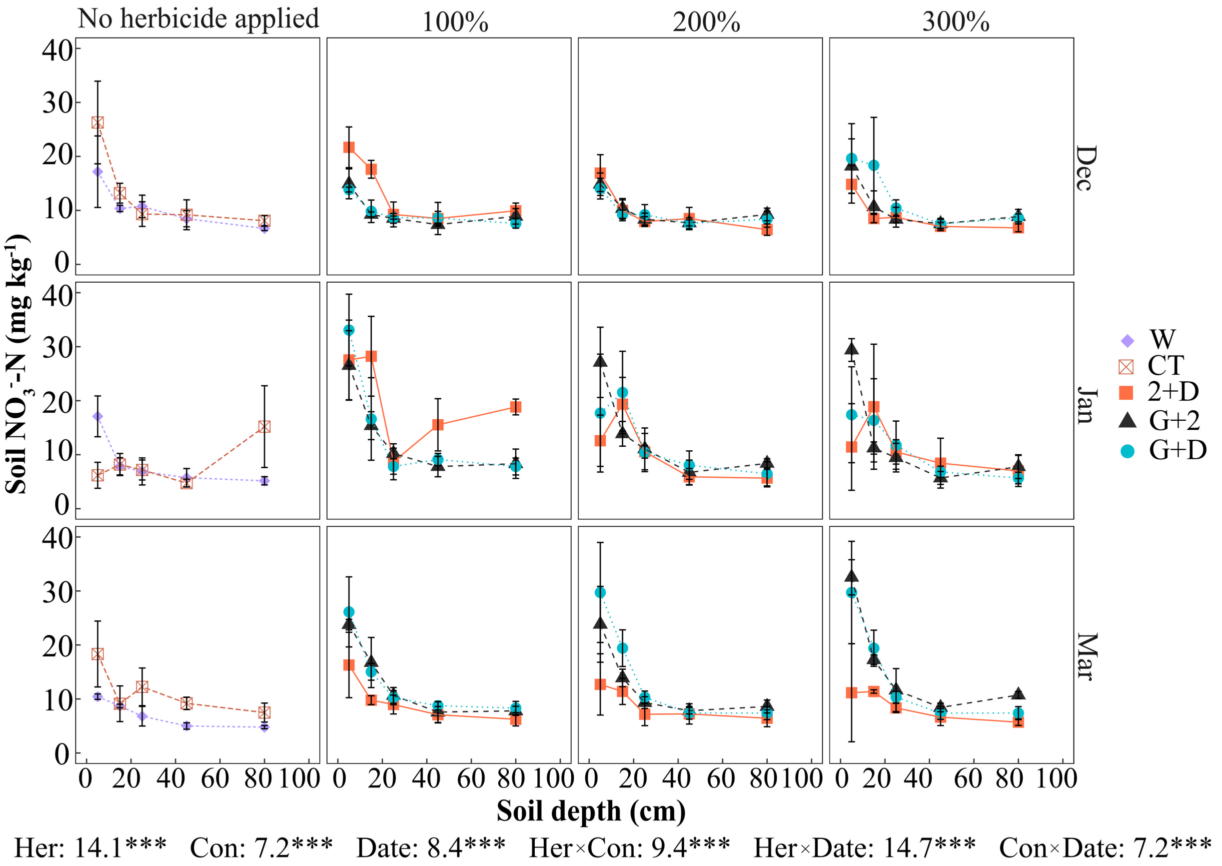

FIGURE 6
Effect of herbicide (2+D, G+D, G+2) and concentration (100%, 200%, 300%) on soil nitrate-N (NO_3^--N) content at different depths (0–10, 10–20, 20–30, 30–60, 60–100cm) in December 2021, January 2022, and March 2022. *** indicate significance levels at $p<0.001$. Values are means of four replicates (± standard deviation). *F* value is marked in the figure. For a description of the abbreviations, refer to the Figure 1 caption.

TN content lagged behind that of mineral N, reflecting a relatively stable soil TN content, but the change in soil N supply capacity is difficult to predict over a short period (Kaye et al., 2002).

5. Conclusion

Our findings indicate that the application of combined herbicides is effective for terminating lucerne growth on the Loess Plateau. We recommend using the suggested dosage (100%) of glyphosate and 2,4-D in autumn, considering both environmental and economic factors. Both conventional tillage and herbicide treatments contribute to increased SOC, soil TN, and NO_3^--N contents in lucerne grasslands. Herbicide treatments, compared to CT, further enhance SOC and soil NH_4^+-N content; however, higher herbicide concentrations (200 and 300%) may lead to decreased soil C and N levels. Future research should explore gaseous soil C and N losses, as well as the yield of subsequent crops such as maize (*Zea mays*) and spring wheat (*Triticum aestivum*), to assess the overall impact of herbicide application on lucerne termination. Additionally, it is worth noting that one-year trials have limitations in terms of their ability to fully capture the long-term effects of treatments, and the results should be interpreted with caution when making policy decisions. Therefore, further research, including multi-year trials, is needed to validate these findings and provide more robust evidence for policy stakeholders.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

YZ: conceptualization, methodology, investigation, writing–original draft, formal analysis, and visualization. JG: methodology, data curation, and visualization. SL: conceptualization, methodology, and writing–review. KS: writing–review & editing, validation, and visualization. YL and YS: conceptualization, writing–review &

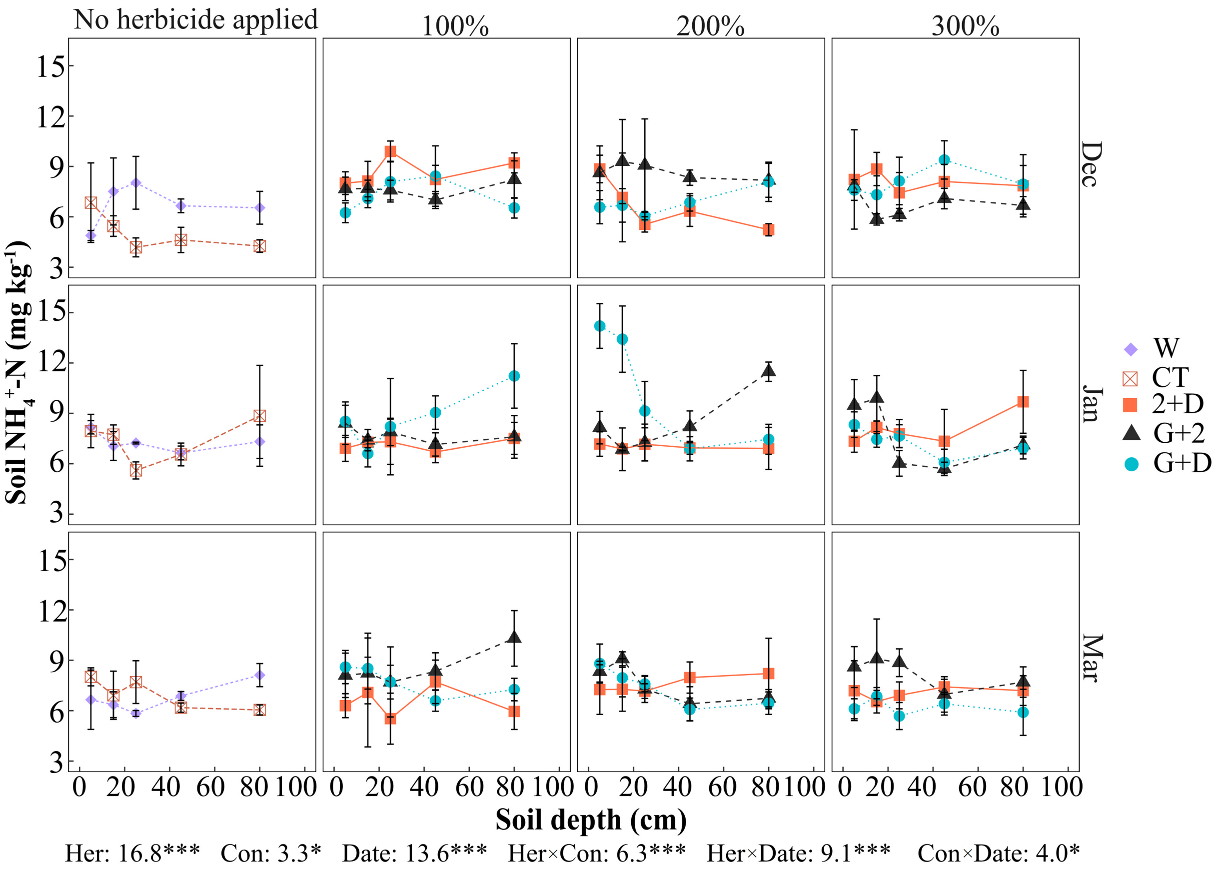

FIGURE 7
Effect of herbicide (2+D, G+D, G+2) and concentration (100%, 200%, 300%) on soil ammonium-N (NH_4^+-N) content at different depths (0–10, 10–20, 20–30, 30–60, 60–100cm) in December 2021, January 2022, and March 2022. * and *** indicate significance levels at $p < 0.05$ and 0.001. Values are means of four replicates (± standard deviation). *F* value is marked in the figure. Note the y-intercept is not 0. For a description of the abbreviations, refer to the Figure 1 caption.

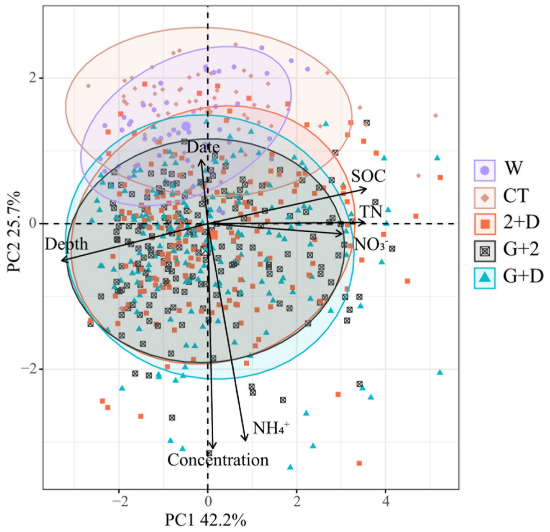

FIGURE 8
Principal component analysis of herbicide (2+D, G+D, G+2) and concentration (100%, 200%, 300%) effects on SOC, TN, NO_3^--N, NH_4^+-N content at different depths (0–10, 10–20, 20–30, 30–60, 60–100cm) in December 2021, January 2022, and March 2022.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Ali, G., Wang, Z., Li, X., Jin, N., Chu, H., and Jing, L. (2021). Deep soil water deficit and recovery in alfalfa fields of the loess plateau of China. *Field Crop Res.* 260:107990. doi: 10.1016/j.fcr.2020.107990

Basile-Doelsch, I., Balesdent, J., and Pellerin, S. (2020). Reviews and syntheses: the mechanisms underlying carbon storage in soil. *Biogeosciences* 17, 5223–5242. doi: 10.5194/bg-17-5223-2020

Bell, L. W., Sparling, B., Tenuta, M., and Entz, M. H. (2012). Soil profile carbon and nutrient stocks under long-term conventional and organic crop and alfalfa-crop rotations and re-established grassland. *Agric. Ecosyst. Environ.* 158, 156–163. doi: 10.1016/j.agee.2012.06.006

Buhler, D. D., and Mercurio, J. C. (1988). Vegetation management and corn growth and yield in untilled mixed-species perennial sod. *Agron. J.* 80, 454–462. doi: 10.2134/agronj1988.00021962008000030013x

Button, R., (1994). *Chemical control of alfalfa*, Soils and Crops Workshop.

Caux, P. Y., Kent, R. A., Tache, M., Grande, C., Fan, G. T., and MacDonald, D. D. (1993). Environmental fate and effects of dicamba: a Canadian perspective. *Rev. Environ. Contam. Toxicol.* 133, 1–58. doi: 10.1007/978-1-4613-9529-4_1

Chan, K. Y., Bowman, A., and Oates, A. (2001). Oxidizible organic carbon fractions and soil quality changes in an Oxic Paleustalf under different pasture leys. *Soil Sci.* 166, 61–67. doi: 10.1097/00010694-200101000-00009

Clark, J. D., Yost, M. A., Cardon, G. E., Ransom, C. V., and Creech, J. E. (2021). Tillage method and glyphosate-resistant alfalfa termination timing affect soil properties and subsequent corn yield. *Agron. J.* 113, 321–334. doi: 10.1002/agj2.20478

Clayton, G.W., (1982). *Zero tillage for cereal production on forage sods in Manitoba.*

Davies, S. L., and Peoples, M. B. (2003). Identifying potential approaches to improve the reliability of terminating a lucerne pasture before cropping: a review. *Aust. J. Exp. Agric.* 43, 429–447. doi: 10.1071/EA02042

Davis, H., Fawcett, R., and Harvey, R. (1978). Effect of fall frost on the activity of glyphosate on alfalfa (*Medicago sativa*) and quackgrass (*Agropyron repens*). *Weed Sci.* 26, 41–45. doi: 10.1017/S0043174500032665

De Mendiburu, F. (2014). Agricolae: statistical procedures for agricultural research. *R package version* 1, 1–4.

Drinkwater, L. (1999) in *Using plant species composition to restore soil quality and ecosystem function. Designing and testing crop rotations for organic farming.* eds. J. E. Olesen, R. Eltun, M. J. Gooding, E. S. Jensen and U. Kopke, 37–46.

Duke, S. O. (2020). Glyphosate: environmental fate and impact. *Weed Sci.* 68, 201–207. doi: 10.1017/wsc.2019.28

Eerd, L. L. V., Congreves, K. A., Hayes, A., Verhallen, A., and Hooker, D. C. (2014). Long-term tillage and crop rotation effects on soil quality, organic carbon, and total nitrogen. *Can. J. Soil Sci.* 94, 303–315. doi: 10.4141/cjss2013-093

Entz, M., Bullied, W., and Katepa-Mupondwa, F. (1995). Rotational benefits of forage crops in Canadian prairie cropping systems. *J. Prod. Agric.* 8, 521–529. doi: 10.2134/jpa1995.0521

Flint, J., and Barrett, M. (1998). Effects of glyphosate combinations with 2, 4-D or dicamba on field bindweed. *Weed Sci.* 37, 12–18. doi: 10.1017/S0043174500055776

Fourie, K.L., (2017). *Evaluation of salinity and irrigation guidelines for lucerne*, University of the Free State. Bloemfontein

Han, L., Tsunekawa, A., and Tsubo, M. (2010). Monitoring near-surface soil freeze–thaw cycles in northern China and Mongolia from 1998 to 2007. *Int. J. Appl. Earth Obs. Geoinf.* 12, 375–384. doi: 10.1016/j.jag.2010.04.009

Henry, H. A. L. (2007). Soil freeze-thaw cycle experiments: trends, methodological weaknesses and suggested improvements. *Soil Biol. Biochem.* 39, 977–986. doi: 10.1016/j.soilbio.2006.11.017

Hinsinger, P., Plassard, C., Tang, C. X., and Jaillard, B. (2003). Origins of root-mediated pH changes in the rhizosphere and their responses to environmental constraints: a review. *Plant Soil* 248, 43–59. doi: 10.1023/A:1022371130939

Hogberg, P., Nordgren, A., Buchmann, N., Taylor, A. F. S., Ekblad, A., Hogberg, M. N., et al. (2001). Large-scale forest girdling shows that current photosynthesis drives soil respiration. *Nature* 411, 789–792. doi: 10.1038/35081058

Holland, J. M. (2004). The environmental consequences of adopting conservation tillage in Europe: reviewing the evidence. *Agric. Ecosyst. Environ.* 103, 1–25. doi: 10.1016/j.agee.2003.12.018

Hoyle, F. C., Murphy, D. V., and Fillery, I. R. P. (2006). Temperature and stubble management influence microbial CO_2-C evolution and gross N transformation rates. *Soil Biol. Biochem.* 38, 71–80. doi: 10.1016/j.soilbio.2005.04.020

Islam, F., Wang, J., Farooq, M. A., Khan, M. S. S., Xu, L., Zhu, J., et al. (2018). Potential impact of the herbicide 2, 4-dichlorophenoxyacetic acid on human and ecosystems. *Environ. Int.* 111, 332–351. doi: 10.1016/j.envint.2017.10.020

Jia, S.W., (2013). *Effects of conventional tillage and conservation tillage on soil organic carbon, 2nd international conference on energy and environmental protection (ICEEP 2013).* Advanced Materials Research, Guilin, China, pp. 3832–3836.

Kaye, J., Barrett, J., and Burke, I. (2002). Stable nitrogen and carbon pools in grassland soils of variable texture and carbon content. *Ecosystems* 5, 461–471. doi: 10.1007/s10021-002-0142-4

Kjeldahl, J. (1883). Neue Methode zur Bestimmung des Stickstoffs in organischen Krpern. *Z. Anal. Chem.* 22, 366–382. doi: 10.1007/BF01338151

Kreyling, J., Persoh, D., Werner, S., Benzenberg, M., and Woellecke, J. (2012). Short-term impacts of soil freeze-thaw cycles on roots and root-associated fungi of Holcus lanatus and *Calluna vulgaris*. *Plant Soil* 353, 19–31. doi: 10.1007/s11104-011-0970-0

Li, M., (2020). *Evaluation on alfalfa resistance to diseases in Guyuan, Ningxia*. MA thesis Thesis, Lanzhou University. China

Li, G. Y., and Fan, H. M. (2014). Effect of freeze-thaw on water stability of aggregates in a black soil of Northeast China. *Pedosphere* 24, 285–290. doi: 10.1016/S1002-0160(14)60015-1

Li, Y., Li, Z., Chang, S. X., Cui, S., Jagadamma, S., Zhang, Q., et al. (2020). Residue retention promotes soil carbon accumulation in minimum tillage systems: implications for conservation agriculture. *Sci. Total Environ.* 740:140147. doi: 10.1016/j.scitotenv.2020.140147

Li, Y., Li, Z., Cui, S., Liang, G., and Zhang, Q. (2021). Microbial-derived carbon components are critical for enhancing soil organic carbon in no-tillage croplands: a global perspective. *Soil Tillage Res.* 205:104758. doi: 10.1016/j.still.2020.104758

Li, Y., Wang, G., Shurpali, N. J., and Shen, Y. (2022). Nitrogen addition affects nitrous oxide emissions of rainfed lucerne grassland. *Int. J. Environ. Res. Public Health* 19:7789. doi: 10.3390/ijerph19137789

Li, K. Y., Zhao, Y. Y., Yuan, X. L., Zhao, H. B., Wang, Z. H., Li, S. X., et al. (2012). Comparison of factors affecting soil nitrate nitrogen and ammonium nitrogen extraction. *Commun. Soil Sci. Plant Anal.* 43, 571–588. doi: 10.1080/00103624.2012.639108

Liu, X., Tan, S., Song, X., Wu, X., Zhao, G., Li, S., et al. (2022). Response of soil organic carbon content to crop rotation and its controls: a global synthesis. *Agric. Ecosyst. Environ.* 335:108017. doi: 10.1016/j.agee.2022.108017

Ma, R., Jiang, Y., Liu, B., and Fan, H. (2021). Effects of pore structure characterized by synchrotron-based micro-computed tomography on aggregate stability of black soil under freeze-thaw cycles. *Soil Tillage Res.* 207:104855. doi: 10.1016/j.still.2020.104855

Malhi, S. S., Lemke, R., and Schoenau, J. J. (2010). Influence of time and method of alfalfa stand termination on yield, seed quality, N uptake, soil properties and greenhouse gas emissions under different N fertility regimes. *Nutr. Cycl. Agroecosyst.* 86, 17–38. doi: 10.1007/s10705-009-9271-x

Malhi, S., and Nyborg, M. (1986). Increase in mineral N in soils during winter and loss of mineral N during early spring in north-Central Alberta. *Can. J. Soil Sci.* 66, 397–409. doi: 10.4141/cjss86-042

Mccarty, G.W., Iii, J., Yost, R., and Doraiswamy, P.C., (2010). *Evaluation of methods for measuring soil organic carbon in West African soils*. *Afr. J. of Agric. Res.* 5, 2169–2177.

Mohr, R. M., Entz, M. H., Janzen, H. H., and Bullied, W. J. (1999). Plant-available nitrogen supply as affected by method and timing of alfalfa termination. *Agron. J.* 91, 622–630. doi: 10.2134/agronj1999.914622x

Moomaw, R., and Martin, A. (1976). Herbicides for no-tillage corn in alfalfa sod. *Weed Sci.* 24, 449–453. doi: 10.1017/S0043174500066431

Nicolardot, B., Recous, S., and Mary, B. (2001). Simulation of C and N mineralisation during crop residue decomposition: a simple dynamic model based on the C: N ratio of the residues. *Plant Soil* 228, 83–103. doi: 10.1023/A:1004813801728

Nyborg, M., Laidlaw, J. W., Solberg, E. D., and Malhi, S. S. (1997). Denitrification and nitrous oxide emissions from a black chernozemic soil during spring thaw in Alberta. *Can. J. Soil Sci.* 77, 153–160. doi: 10.4141/S96-105

Ochsner, T. E., Stephens, B. M., Koskinen, W. C., and Kookana, R. S. (2006). Sorption of a hydrophilic pesticide: effects of soil water content. *Soil Sci. Soc. Am. J.* 70, 1991–1997. doi: 10.2136/sssaj2006.0091

Pan, J., Fan, Y., Li, R., Chen, L., and Hu, X. (2016). Screening of high efficient symbiotic rhizobium for *Medicago sativa* cv. Gannong no. 3 and *M. sativa* cv. Longdong. *Pratacultural Sci.* 33, 1536–1549. doi: 10.11829/j.issn.1001G0629.2016G0072

Peixoto, L., Olesen, J. E., Elsgaard, L., Enggrob, K. L., Banfield, C. C., Dippold, M. A., et al. (2022). Deep-rooted perennial crops differ in capacity to stabilize C inputs in deep soil layers. *Sci. Rep.* 12:10. doi: 10.1038/s41598-022-09737-1

Randall, G., Vetsch, J., and Murrell, T. (2001). Corn response to phosphorus placement under various tillage practices. *Better Crops* 85, 12–15.

Rumpel, C., Amiraslani, F., Koutika, L.-S., Smith, P., Whitehead, D., and Wollenberg, E., (2018). *Put more carbon in soils to meet Paris climate pledges*. Nature Publishing Group, 32–34, London.

Scharlemann, J. P. W., Tanner, E. V. J., Hiederer, R., and Kapos, V. (2014). Global soil carbon: understanding and managing the largest terrestrial carbon pool. *Carbon Manag.* 5, 81–91. doi: 10.4155/cmt.13.77

Schmidt, M. W. I., Torn, M. S., Abiven, S., Dittmar, T., Guggenberger, G., Janssens, I. A., et al. (2011). Persistence of soil organic matter as an ecosystem property. *Nature* 478, 49–56. doi: 10.1038/nature10386

Shen, Y., Li, L., Chen, W., Robertson, M., Unkovich, M., Bellotti, W., et al. (2009). Soil water, soil nitrogen and productivity of lucerne–wheat sequences on deep silt loams in a summer dominant rainfall environment. *Field Crop Res.* 111, 97–108. doi: 10.1016/j.fcr.2008.11.005

Six, J., Feller, C., Denef, K., Ogle, S. M., Sa, J. C. D., and Albrecht, A. (2002). Soil organic matter, biota and aggregation in temperate and tropical soils -effects of no-tillage. *Agronomie* 22, 755–775. doi: 10.1051/agro:2002043

Smith, P., House, J. I., Bustamante, M., Sobocká, J., Harper, R., Pan, G., et al. (2016). Global change pressures on soils from land use and management. *Glob. Chang. Biol.* 22, 1008–1028. doi: 10.1111/gcb.13068

Toderi, M., Dottavio, P., Francioni, M., Kishimoto-Mo, A. W., Santilocchi, R., and Trozzo, L. (2022). Short-term response of soil greenhouse gas fluxes to alfalfa termination methods in a Mediterranean cropping system. *Soil Sci. Plant Nutr.* 68, 124–132. doi: 10.1080/00380768.2021.1983869

Wang, E., Cruse, R. M., Chen, X., and Daigh, A. (2012). Effects of moisture condition and freeze/thaw cycles on surface soil aggregate size distribution and stability. *Can. J. Soil Sci.* 92, 529–536. doi: 10.4141/cjss2010-044

Wang, K., Liu, Y., Teng, F., Cen, H., Yan, J., Lin, S., et al. (2021). Heterogeneous expression of Osa-MIR156bc increases abiotic stress resistance and forage quality of alfalfa. *Crop J.* 9, 1135–1144. doi: 10.1016/j.cj.2020.11.009

Wang, C., Wan, S., Xing, X., Zhang, L., and Han, X. (2006). Temperature and soil moisture interactively affected soil net N mineralization in temperate grassland in northern China. *Soil Biol. Biochem.* 38, 1101–1110. doi: 10.1016/j.soilbio.2005.09.009

Wehtje, G., and Walker, R. H. (1997). Interaction of glyphosate and 2, 4-DB for the control of selected morningglory (Ipomoea spp.) species. *Weed Technol.* 11, 152–156. doi: 10.1017/S0890037X00041506

Wickham, H., (2016). *Data analysis, ggplot 2*. Springer, pp. 189–201, Germany.

Yuan, Z.-Q., Yu, K.-L., Guan, X.-K., Fang, C., Li, M., Shi, X.-Y., et al. (2016). *Medicago sativa* improves soil carbon sequestration following revegetation of degraded arable land in a semi-arid environment on the loess plateau, China. *Agric. Ecosyst. Environ.* 232, 93–100. doi: 10.1016/j.agee.2016.07.024

Zhao, J., Chen, J., Beillouin, D., Lambers, H., Yang, Y., Smith, P., et al. (2022). Global systematic review with meta-analysis reveals yield advantage of legume-based rotations and its drivers. *Nat. Commun.* 13:4926. doi: 10.1038/s41467-022-32464-0

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Productivity, water and nitrogen utilization of intensified dryland farming with annual forages on the Chinese Loess plateau

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Understanding the relationships of productivity performance and water utilization and soil nitrogen dynamics after annual forage planting during the fallow period (F) in winter wheat (*Triticum aestivum* L.; W) mono-cropping is critically important for maintaining sustainable livestock and grain production in semiarid regions. We used 2 years (2017–2019) of data to investigate soil nitrogen dynamics, production, water utilization, and fallow efficiency when forage rape (*Brassica campestris* L.; R) and common vetch (*Vicia sativa* L.; V) were planted in a 3-month summer fallow of the W-F-W-F cropping system. Three cropping systems were comprised of winter wheat-summer fallow-winter wheat-summer fallow (W-F-W-F), winter wheat-forage rape-winter wheat-forage rape (W-R-W-R), and winter wheat-forage rape-winter wheat-common vetch (W-R-W-V). The results showed that the annual forage planting decreased the average NO_3^--N content by 54.8% compared with the W-F-W-F cropping system. Compared with the W-F-W-F cropping system, planting annual forage in summer fallow increased the average system forage production by 4.93 t ha^{-1}. Local total annual precipitation can meet crop-water requirements, and the limiting factor for agricultural production was the drought due to the uneven seasonal distribution of precipitation. In comparison to the W-F-W-F cropping system, annual forage planting decreased the average available soil moisture storage by 50.3 mm above the 80 cm soil layer. Compared with that in the W-R-W-R (23.21 t ha^{-1}) and W-F-W-F (30.25 t ha^{-1}) cropping systems, the crop productivity in the W-R-W-V cropping system (33.23 t ha^{-1}) was relatively stable and high because the reduction in subsequent winter wheat yield (2.96 t ha^{-1}) was adequately offset by the forage yield (5.15 t ha^{-1}). Adding forage rape to the W-F-W-F cropping system decreased system crop-water productivity (CWP) by 40.9%. However, the CWP, precipitation use efficiency (PUE), and soil nitrate in the W-R-W-V cropping system increased by 30.4, 30.1, 110.9, and 82.0%, respectively, compared with those in the W-R-W-R cropping system. Therefore, the W-R-W-V cropping system is recommended for better water and fertility management as well as grain and forage production in semiarid regions. However, further study is required to involve drought years for better evaluation of the effect of long-term precipitation variability on the crop productivity.

KEYWORDS

dryland farming system, soil nitrogen, yield, water management, crop water productivity

1. Introduction

Given the increasing global population, the function of water and nitrogen fertilizer on agricultural production is irreplaceable (Li et al., 2019). In many arid and semiarid areas of the world, agricultural production activities will face severe challenges related to water and fertilizer shortages (Gan et al., 2015). Therefore, a more sustainable farming system for food production that could better meet the demand of society with a less detrimental impact on the environment is needed (Jensen et al., 2012).

In the region of Northwest China, winter wheat monoculture is the traditional crop production system, mainly practiced on the Chinese Loess Plateau. This cropping system involves a fallow of 3 months during the summer (July–September). Generally, because the availability of water and nitrogen are the main limiting factors for crop production in semiarid regions, summer fallow is considered a way to conserve soil moisture and soil nutrients, especially soil nitrate for subsequent crops (Grant et al., 2002; Campbell et al., 2008; Nielsen and Vigil, 2010). However, summer fallow may cause negative impacts on sustainable production (Unger et al., 1997; Farahani et al., 1998), such as nitrate leaching (Yang et al., 2017), loss of soil organic matter (Lemke et al., 2012), and low crop-water productivity (CWP) and precipitation use efficiency (PUE; Zhu et al., 1994). These negative impacts of summer fallow occurred because substantial rainfall happened during this period, which is accompanied by large amount of soil moisture evaporation because the soil is bare (Kang et al., 2001; Yang et al., 2018). In addition, there was an obvious enhancement in production systems in recent years due to the increased demand for food and animal products (Zhang et al., 2019a). However, meeting this demand poses a significant challenge in this region because of variable precipitation distribution, water scarcity and low quality and shortages of forage supply for animal feeding. These have been the typical constraints on crop production and farmer incomes, and these factors subsequently stall the regional livestock industry's economic development in semiarid regions (Hou et al., 2008; Komarek et al., 2015; Zhang et al., 2019b). Integrating forage crops to replace fallow is an effective way to alleviate erosion, improve PUE, and enhance forage yields (Aiken et al., 2013; Blanco-Canqui et al., 2015; Nielsen et al., 2015). Li et al. (2000) reported a 17% increase in precipitation utilization with a continuous wheat production system with a forage crop compared with continuous wheat production system on the Loess Plateau. In semiarid climates, Li et al. (2000) found that the success of integrating grain-forage production systems depends on the choice of forage crops. Cultivars with fast seedling emergence and short growth spans that can quickly achieve complete cover are considered most appropriate. Abate et al. (2003) reported a 2.0 t ha^{-1} increase in forage yield when forage vetch was planted during a short period of fallow. As an annual forage crop, forage rape resulted in a relatively high yield within a short period (Liu et al., 2014). However, Unger and Vigil (1998) reported that there is limited adoption of cover crops in dry regions, chiefly due to poor soil nutrients and low CWP. The availability of water strongly influences forage rape and common vetch production, as well as the yield stability of subsequent winter wheat in semiarid environments (Nielsen et al., 2016; Zhang et al., 2018). In the semiarid Loess Plateau of China, the annual precipitation varies considerably, from 300 to 600 mm (Ao et al., 2007). The precipitation ratio during summer fallow is as great as 62% compared to the annual value, although with a considerable variation from 35 to 88% (Wang et al., 2020). Therefore, it is necessary to investigate how forage production and soil moisture utilization are affected by precipitation.

Agriculture production is largely determined by the amount of soil moisture stored and residual nitrate in rainfed regions. When a single crop-fallow system is practiced, the amount of water lost from the soil due to evapotranspiration could be recouped by precipitation during the fallow season (Li et al., 2013). A modest reduction in yield (0–16%) could occur in normal precipitation years when a spring broadleaf crop replaces fallow. Non-summer fallowing practices also have an obvious impact on soil nitrogen, and the integration of pulse crops [dry pea (*Pisum sativum* L.) and lentil (*Lens culinaris* Medikus)] could enhance soil available nitrogen (Herridge et al., 2008). Yang et al. (2015) reported that nitrates were highly mobile. Therefore, due to the concentration of precipitation during the summer fallow period, the residual nitrates could be susceptible to leaching into the deeper layers of the soil. Hence, the availability of soil nitrate for crop production in rainfed regions primarily relies on the amount of available water (Gan et al., 2015). Therefore, the differences in the soil nitrate, water balance, and system CWP mainly rely on the precipitation amount and distribution, fallow period, cropping patterns, and evaporative demand (Lyon et al., 2004). However, little research has conducted the effects of integrating forage into winter wheat monocultures on evapotranspiration (ET). In addition, Deng et al. (2020) studied the crude protein productivity in relation to CWP and PUE and the economic return of replacing summer fallow with either forage rape and/or common vetch on the Chinese Loess Plateau. However, soil nitrate dynamics, soil moisture storage and water utilization during crop development in different cropping systems need to be explored.

Therefore, the main goal of the present study was to determine the effect of integrating forage rape and common vetch into the winter wheat monoculture as a replacement for summer fallow and specifically: (1) evaluate the crop yields as affected by the cropping system; (2) evaluate the effect of cropping system on soil nitrate dynamics, CWP, and PUE; (3) investigate the ET characteristics of the cropping system during crop development; and (4) assess soil moisture utilization sustainability under different cropping systems on the Loess Plateau.

2. Materials and methods

2.1. Experimental site and design description

The experimental site was located on the Chinese Loess Plateau at 1298 m above sea level. The area has a temperate, semiarid continental monsoon climate. The mean annual precipitation, solar radiation, and air temperature are 527.6 mm, 5543.0 MJ m^{-2}, and 9.2°C, respectively. The annual precipitation exhibits an uneven distribution, with 60–70% of it concentrated in July, August, and September. The field trial was conducted on level terrain, and the soil type is a typical Loess Plateau soil classified as the Heilu soil type (U.S. classification; Zhu et al., 1983). The properties of the soil and the nutrient profile at a depth of 0–160 cm in late September 2017 are shown in Supplementary Table 1. The soil from the 0–160 cm layer contained 4.1 g kg^{-1} organic matter, 31.1 mg kg^{-1} available nitrogen, 2.6 mg kg^{-1} available phosphorus, and 88.9 mg kg^{-1} available potassium. The field experiment was conducted in late September 2015 at the Lanzhou University Research Station located near Xifeng district, Qingyang, Gansu, China (35°39′N, 107°51′E) and was implemented for two cycles during the growing seasons of 2015–2019. Because the soil moisture measuring instrument was installed in 2017, the experimental data in this paper are from the two winter wheat growing seasons of 2017–2019. The field experiment contained three treatments: winter wheat-summer fallow-winter wheat-summer fallow (W-F-W-F), winter wheat-forage rape-winter wheat-forage rape (W-R-W-R), and winter wheat-forage rape-winter wheat-common vetch (W-R-W-V).

2.2. Experimental field management

A randomized complete block design was adopted in this study, and it was replicated three times, with each experimental plot measuring 4 m by 5 m. The seeding rate of the wheat was 225 kg ha^{-1}, while the spacing between rows was 15 cm. Furthermore, annual forage was sown in late June or early July immediately after the winter wheat harvest. The seeding rate for common vetch (Lanjian 3) was 120 kg ha^{-1}, and the inter-row spacing was 20 cm. Forage rape (Siyou 1) was surface-broadcasted at a rate of 15 kg ha^{-1}. All the plots were ploughed to a 30 cm depth using a chisel plough before planting. Nitrogen and phosphorus fertilizers were applied according to the local fertilization recommendations, which suggest an application of available N (70 kg ha^{-1}) and P (100 kg ha^{-1}) prior to planting. N and P fertilization was applied to the winter wheat and forage rape, but P fertilization was only applied to the common vetch. Manual hoeing was used to ensure all the experimental plots were weed free throughout the experiment and no irrigation was given in this study.

2.3. Measurements and methods

2.3.1. Weather conditions

During the experimental years, daily information on precipitation, radiation, and air temperature was recorded by an onsite weather station (Campbell Sci., Inc. Logan, UT, United States) located within the experimental station.

2.3.2. Yield measurements

The grain and dry matter yields of winter wheat were estimated at the physiological maturity of the crop. Samples were collected using three 0.3 m^2 (2 m × 0.15 m) quadrats that were randomly placed within each plot. The estimation of dry matter yield was carried out following the harvest of forage rape and common vetch at 50% flowering. Three quadrats (1 × 1 m) that were randomly placed in each plot were used for sampling. A distance of approximately 50 cm between sampling areas was used for all crops, and the sampling positions were away from the plot edges. The collected samples were oven dried at an initial temperature of 105°C for 30 min to kill the fresh tissues and subsequently dried to a constant weight at 80°C. The oven-dried samples were used to calculate grain and dry matter yield, which were extrapolated to tons per hectare (Deng et al., 2020).

2.3.3. Soil available nitrogen

When the winter wheat and forage crop harvest occurred, three soil samples were taken randomly at depths of 0–10, 10–20, 20–40, 40–60, 60–80, 80–100, and 100–120 cm with an auger in the middle of each plot. The soil samples were used for calorimetric determination of NO_3^-–N (soil nitrate-nitrogen) and NH_4^+–N (ammonium nitrogen) concentrations by using the salicylate-nitroprusside method with an automated continuous flow analyzer (AA3, Seal, Norderstedt, Germany; Luce et al., 2020).

2.3.4. Soil moisture

Soil moisture content was measured approximately every 7–13 days during the entire study. The soil moisture up to the 160 cm depth was measured at an interval of 10 cm using a Diviner-2000 device (Sentek Pty Ltd., Australia). PVC access tubes (internal diameter 40 mm) were installed in the center of each plot at the beginning of this experiment in late September 2015.

Soil water storage (SWS; mm) in the 0–160 cm soil layer was determined according to Zhang et al. (2017) as follows:

$$\mathrm{SWS} = \sum \mathrm{SWC}_i \times h_i, \tag{1}$$

where SWC_i is the volumetric soil water content of soil layer i and h_i is the height of soil layer i (mm), i (1–16).

Available soil water storage (ASWS) was calculated by multiplying the depth soil layer (10 cm) by the difference between the calculated volumetric water and the bottom limit of available water at each depth of water measurement (Ritchie, 1981; Ratliff et al., 1983). The bottom limit of available water at each of the 16 measurement intervals was initially determined in late September 2017 in the plot area as the lowest volumetric water value. The profile plant-available soil water storage was calculated by adding the individual values of available water at the 16 depths in each plot.

The ET (mm) from stage j (i.e., its starting time (d)) to stage $j+1$ (d) was calculated as follows (Ding et al., 2018):

$$\mathrm{ET}_{j-j+1} = P_{j-j+1} + \Delta\mathrm{SWS}, \tag{2}$$

where P_{j-j+1} is the precipitation from stage j to stage $j+1$ (mm) and ΔSWS is the change in SWS from stage j to stage $j+1$ (mm). The runoff and capillary rise of water were ignored because the topography and the underground water table of the experimental site were flat and approximately 50 m deep, respectively. Soil water percolation was also ignored based on the assumption that rainfall water rarely infiltrated to a 160 cm depth in the short term.

The evapotranspiration rate (ETR, mm d^{-1}) from stage i to stage $i+1$ was calculated as follows:

$$\mathrm{ETR}_{i-i+1} = \frac{\mathrm{ET}_{i-i+1}}{t_{i-i+1}}, \tag{3}$$

where t_{i-i+1} is the duration time from stage i to stage $i+1$ (d). Thus, CWP and PUE were calculated according to Li et al. (2018) as in the equations below:

$$\mathrm{CWP} = \mathrm{DM} / \mathrm{ET}, \tag{4}$$

$$\mathrm{PUE} = \mathrm{DM} / P_G, \tag{5}$$

where DM is dry matter yield of the total system. Fallow efficiency (FE) was calculated as:

$$\mathrm{FE} = \left(\mathrm{SWS}_a - \mathrm{SWS}_b\right) / P_F \times 100, \tag{6}$$

where $\mathrm{SWS_a}$ is the soil water storage at the initial stage of summer fallow, $\mathrm{SWS_b}$ is the soil water storage at the later stage of summer fallow, and P_F is the precipitation during the summer fallow period (mm).

2.4. Data analysis

The variables of soil nitrogen dynamics, production, water utilization, and fallow efficiency were subjected to analysis of variance using GenStat 17.0 (VSN International, Hemel Hempstead, United Kingdom). Significant differences between treatments were analyzed using Fisher's protected least significant difference test at a probability level of 5%. SigmaPlot 12.2 was used for drawing of figures.

3. Results

3.1. Precipitation and temperature

The precipitation in the 2017–2018 and 2018–2019 growing seasons, was 808.6 mm (398.5 mm for wheat and 410.1 mm for the summer fallow season) and 670.9 mm (278.3 mm for wheat and 392.6 mm for the summer fallow season), respectively (Figure 1; Supplementary Table 2). The long-term average (LTA) precipitation (2007–2017) was 557.8 mm (260.9 mm for wheat and 296.9 mm for the summer fallow season). The air temperature of LTA (2007–2017) was 10.0°C (7.0°C for winter wheat and 19.1°C for the summer fallow season). The air temperature was 10.1°C (6.8°C for winter wheat and 20.6°C for the summer fallow season) and 9.6°C (6.5°C for winter wheat and 19.9°C for the summer fallow season) for the growing seasons of 2017–2018 and 2018–2019, respectively.

3.2. Production

During the growing season of 2017–2018, the grain yield for winter wheat had no significant difference and the average was 5.2 t ha^{-1} across the cropping systems (Table 1). No significant difference was found in forage rape yield and the average was 4.0 t ha^{-1} across the W-R-W-R and W-R-W-V treatments. During the 2018–2019 growing season, the winter wheat grain yield in the W-R-W-R treatment had the lowest value, which was 82.6 and 58.7% lower than that in the W-F-W-F and W-R-W-V treatments, respectively ($p < 0.05$). The forage yield in the W-R-W-R treatment was 46.7% greater than that in the W-R-W-V treatment ($p < 0.05$). The system DM was significant ($p < 0.05$). The system DM yield in the W-R-W-V treatment was 30.2% greater than that in the W-R-W-R treatment ($p < 0.05$), but the difference with that in the W-F-W-F treatment was not statistically significant.

3.3. Soil available nitrogen in the 0–120 cm layer

The differences in the distribution of available soil nitrogen in the 0–120 cm layer among the cropping systems are presented in Figure 2. The NO_3^-–N content obtained among the different cropping systems after the wheat growing season in 2017–2018 was not significant ($p > 0.05$). After forage rape planting (24 September 2018), the NO_3^-–N content decreased by 54.8% ($p < 0.05$) compared to that in the W-F-W-F treatment. On 3 July 2019, the NO_3^-–N content in the W-R-W-R treatment had the highest average value (23.9 mg kg^{-1}) in the 0–60 cm soil layer, 50.0% higher than that in the W-F-W-F

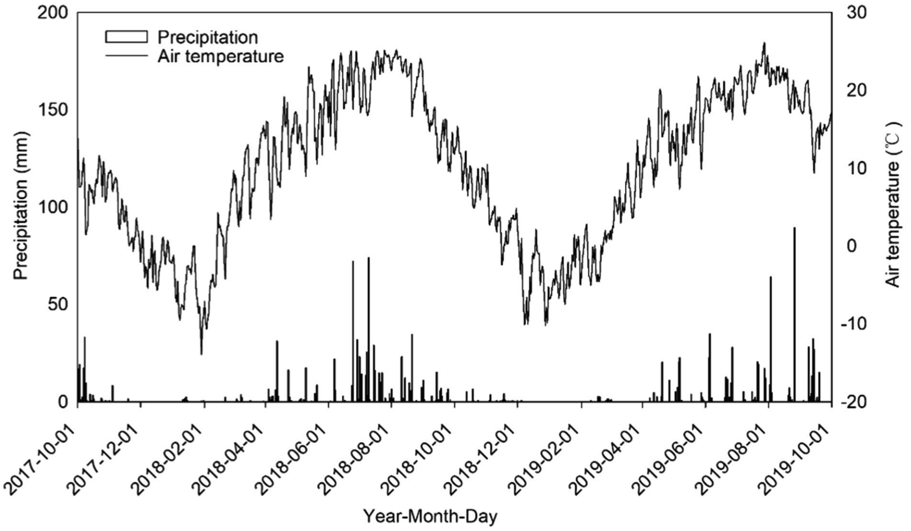

FIGURE 1
Daily precipitation distribution and air temperature during the growing seasons (October 2017–September 2019).

TABLE 1 Winter wheat grain yield, forage yield, and dry matter (DM) yield of the system under different cropping systems during the growing seasons (October 2017–September 2019) at Xifeng, Gansu, China.

Cropping systems[a]	2017–2018		2018–2019		System DM yield ($t\ ha^{-1}$)
	Winter wheat grain yield ($t\ ha^{-1}$)	Forage yield ($t\ ha^{-1}$)	Winter wheat grain yield ($t\ ha^{-1}$)	Forage yield ($t\ ha^{-1}$)	
W-F-W-F	5.32 ± 0.52 a	NA[b]	4.94 ± 0.95 a	NA	30.25 ± 4.92 ab
W-R-W-R	4.98 ± 1.23 a	3.85 ± 0.82 a	0.86 ± 0.08 c	1.80 ± 0.17 a	23.21 ± 3.34 b
W-R-W-V	5.22 ± 0.39 a	4.19 ± 0.83 a	2.08 ± 1.92 b	0.96 ± 0.13 b	33.23 ± 1.69 a

[a] W = winter wheat, F = summer fallow, R = forage rape, V = common vetch.

[b] NA, not applicable.

Different letters indicate significant differences among treatments ($p < 0.05$).

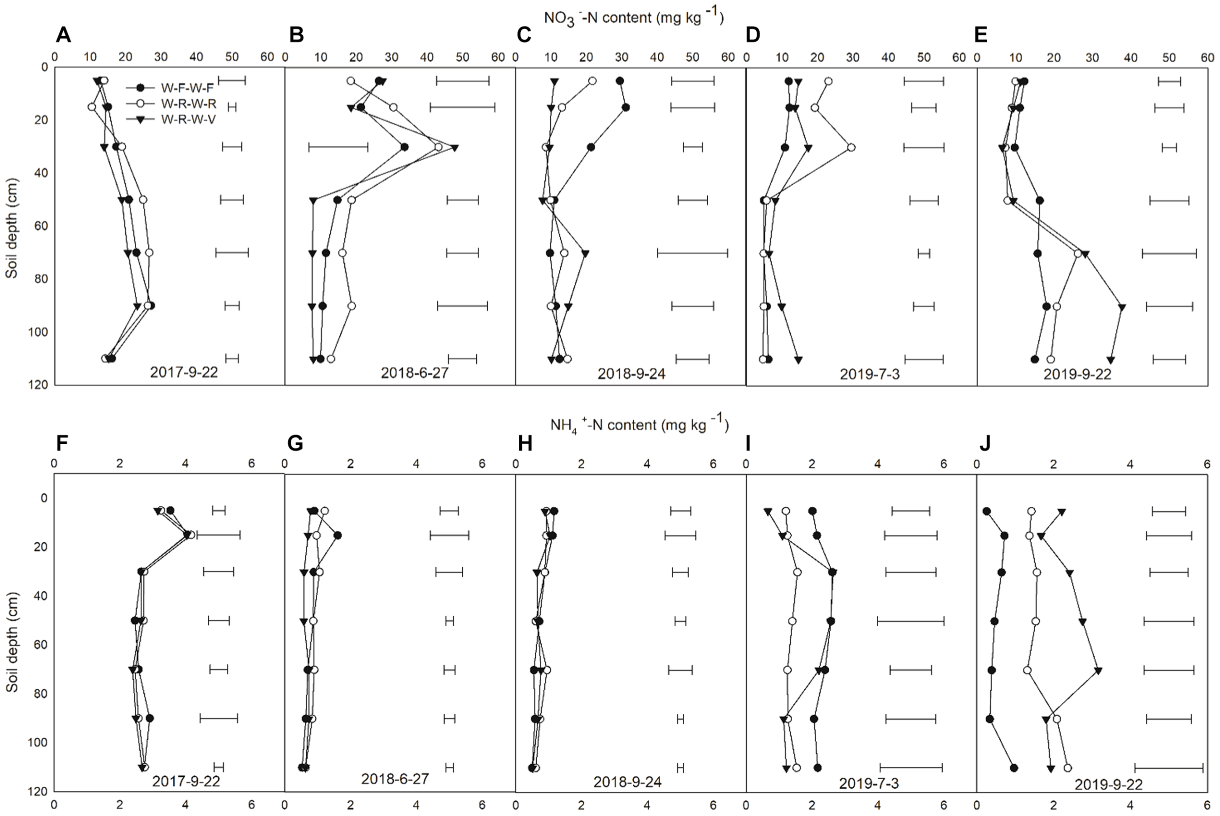

FIGURE 2
Vertical distribution of NO_3^-–N and NH_4^+–N content in the 0–120 cm soil layer for different sowing and harvesting dates of each crop among the cropping systems during the growing seasons (October 2017–September 2019). The bars adjacent to each soil layer represent the least significant difference ($p = 0.05$). W = winter wheat, F = summer fallow, R = forage rape, V = common vetch.

treatment ($p < 0.05$). At the forage harvesting stage (22 September 2019), no significant difference was found in the NO_3^--N content in the 0–70 cm soil layer. However, the NO_3^--N content in the W-R-W-V treatment was 51.0% greater than that in the W-F-W-F treatment in 70–120 cm soil layer ($p < 0.05$). There was no obvious difference in the NH_4^+-N content in the 0–120 cm soil layer among the treatments on 22 September 2017, 27 June 2018, 24 September 2018, and 3 July 2019. However, in the 30–70 cm soil layer, the NH_4^+-N content in the W-R-W-V treatment was 82.0% greater than that in the W-F-W-F treatment on 22 September 2019 ($p < 0.05$).

3.4. Evapotranspiration characteristics and soil water storage

During the experimental period of 2017–2018, there was no significant difference in winter wheat ET among the different cropping systems at the early stage (Figure 3). However, in April 2018, the average ET in the W-F-W-F treatment was 7.8% higher than that in the W-R-W-R and W-R-W-V treatments ($p < 0.05$), and the average ET in the W-F-W-F treatment was 10.2% lower than that in the W-R-W-R and W-R-W-V treatments in June 2018

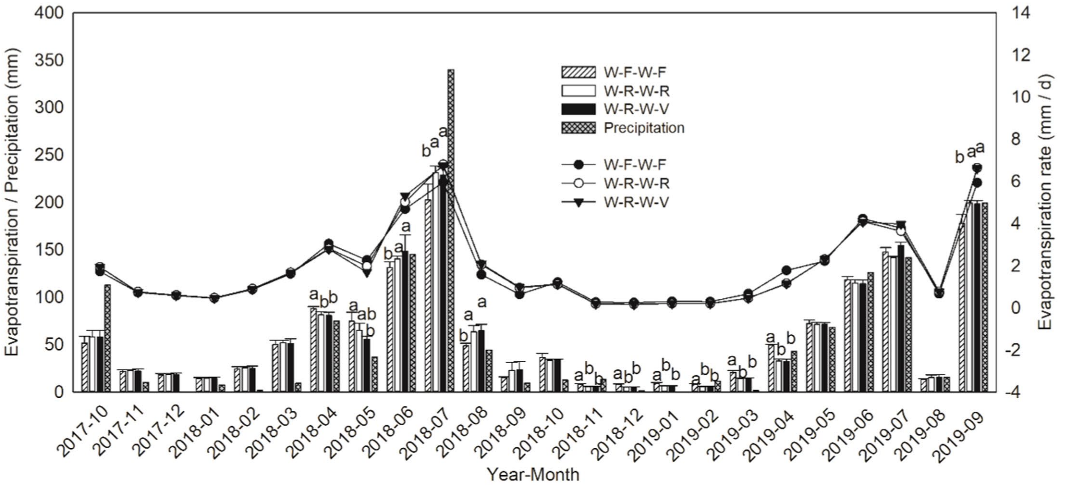

FIGURE 3
Effects of different cropping systems on evapotranspiration and the corresponding evapotranspiration rate during the growing seasons (October 2017–September 2019). The vertical bars represent the least significant difference ($p = 0.05$). Different letters indicate significant differences among treatments ($p < 0.05$). W = winter wheat, F = summer fallow, R = forage rape, V = common vetch.

($p < 0.05$). During the summer fallow period in 2018, the average ET in the W-F-W-F treatment was lowest in July, which was 13.5% lower than that in the W-R-W-R and W-R-W-V treatments ($p < 0.05$). In August 2018, the average ET in the W-F-W-F treatment was 24.0% less than that in the W-R-W-R and W-R-W-V treatments ($p < 0.05$). There was no cropping system effect on ET in September (average 18.6 mm). During the experimental period of 2018–2019, the average ET in the W-F-W-F treatment had the highest value, 29.7, 60.1, 21.9, 10.4, and 61.1% greater than that in the other treatments in November, December, January, February, March, and April, respectively ($p < 0.05$). The cropping system had no significant effect on the ET of winter wheat at the emergence and harvesting stages. During the summer fallow period in 2019, there was no significant difference in July and August. In September, ET in the W-F-W-F treatment was lowest, which was 10.7 and 10.3% lower than that in the W-R-W-R and W-R-W-V treatments, respectively ($p < 0.05$).

Table 2 shows the soil water supply and demand (SWSD) of the system under different cropping systems across the experimental period from 2017 to 2019. The system SWSD in the W-F-W-F treatment had the greatest value, followed by that in W-R-W-V and finally that in W-R-W-R.

The ASWS in the 0–80 cm soil layer declined gradually, and then increased rapidly but without a cropping system effect in the wheat-growing season of 2017–2018 (Figure 4A). After 1 August 2018, the ASWS in the W-F-W-F treatment in the 0–80 cm soil layer was an average 40.5% higher than that in the W-R-W-R treatment ($p < 0.05$). A similar ASWS trend was found in the 80–160 cm soil layer during the growing season but without a cropping system effect among the W-F-W-F, W-R-W-R, and W-R-W-V treatments. At the regreening stage of winter wheat (2 April 2019), the ASWS in the W-F-W-F treatment in the 0–80 cm soil layer was 36.2 and 29.8% greater than that in the W-R-W-R and W-R-W-V treatments, respectively ($p < 0.05$; Figure 4B). After May, there was no significant difference in the ASWS among the cropping systems (average = 56.1 mm). A similar ASWS trend was found in the 80–160 cm soil layer during the growing season but without a cropping system effect among the W-F-W-F, W-R-W-R, and W-R-W-V treatments.

3.5. Crop water productivity and fallow efficiency

The system CWP in the W-R-W-V treatment was 30.4% greater than that in the W-R-W-R treatment ($p < 0.05$) but statistically similar to that in the W-F-W-F treatment (Figure 5A). The system PUE in the W-R-W-V treatment had the highest value, which was 8.9 and 30.1% greater than that in the W-F-W-F and W-R-W-R treatments, respectively ($p < 0.05$; Figure 5B). The FE in the W-F-W-F treatment was 53.1 and 21.7% greater than that in the W-R-W-R and W-R-W-V treatments, respectively ($p < 0.05$; Figure 5C).

4. Discussion

4.1. Relationship of production performance with soil moisture and nitrogen

In the Chinese Loess Plateau region, Li et al. (2000) reported that the rainy season occurs around the forage growing periods (July to September), and the growth of such crops relies on growing-period precipitation. Therefore, forages, such as soybean [*Glycine max* L. (Merr.)] and common vetch, has relatively less influence on soil moisture storage. However, our results showed that forage in the W-R-W-R and W-R-W-V treatments sharply decreased the ASWS in 2017–2018 (Figure 4). Water depletion obviously occurred in the 0–80 cm soil layer (Figure 4; Supplementary Figure 1), which hindered the subsequent wheat from completing the regreening stage in the next year, thereby resulting in a significant reduction in winter wheat grain yield compared to that in the W-F-W-F treatment (Table 1; Figure 5). Similarly, Nielsen et al. (2017) also found up to a 17%

TABLE 2 System soil water supply and demand (SWSD) under different cropping systems during the experimental period of 2017–2019 during the growing seasons (October 2017–September 2019) in Xifeng, Gansu, China.

Cropping system[a]	Precipitation (mm)			ET (mm)			SWSD (mm)
	Growing season	Fallow period	Total precipitation	Growing season	Fallow period	Total ET	
W-F-W-F	644.6	791.6	1436.5	1059.2	305.1	1364.3	72.2
W-R-W-R	1436.5	NA[b]	1436.5	1377.4	NA	1377.4	59.1
W-R-W-V	1436.5	NA	1436.5	1372.9	NA	1372.9	63.6

[a] W = winter wheat, F = summer fallow, R = forage rape, V = common vetch.

[b] NA, not applicable.

reduction in the yield of subsequent winter wheat due to the depletion of soil moisture storage to 47 mm by planting the forage triticale (*X Triticosecale rimpaui* Wittm.) in a wheat-maize (*Zea mays* L.)—fallow rotation system. Precipitation during the summer fallow period in 2018 was 23.2% higher than that in the LTA; the precipitation variability degree was approximately 38.1% (Supplementary Table 2), and 85.8% of the precipitation was mainly concentrated in the late growing season of winter wheat and the early forage rape growing season (Figure 1). Hence, forage rape grew fast, and the strong root system had a negative impact on the 0–80 cm soil moisture content in the late forage rape growing season. Moreover, the soil moisture consumption by forage rape might not be offset by precipitation (snow and rain) from November to April, which could result in the failure of the subsequent crop. Therefore, it is necessary to implement supplementary irrigation to ensure successful crop development and ultimately achieve optimum yield. In addition, the wheat grain yield in the W-R-W-V treatment were better than those in the W-R-W-R treatment when drought occurred (Table 1; Figure 5); this result was likely due to the impact of the different preceding forage crops between W-R-W-V and W-R-W-R treatments in 2017. Our results showed that eliminating summer fallow with common vetch had a relatively small impact on subsequent wheat production and obtained a relatively high market price compared to that for forage rape. Parihar et al. (1999) analyzed the net return among different crop rotations and found a similar result: the net return in a rice (*Oryza sativa* L.)-sunflower (*Helianthus annuus* L.) system was 16.2% lower than that in a rice-peanut (*Arachis hypogaea* L.) system. Compared to that of forage rape, the shallow root system of common vetch (Gan et al., 2011) can preserve soil moisture for subsequent deeper-rooted crops in the rotation, decreasing the risk of reducing yield (Wang et al., 2012). Figure 4 also shows that the ASWS in the W-R-W-V treatment was greater than that in the W-R-W-R treatment, although no significant difference was found. In addition, in comparison to when wheat monoculture is continuous, when forage legumes are integrated into wheat monoculture, there was a significant increase in N content through biological N fixation (Li et al., 2002; Luce et al., 2016). A similar result was found in the current study, where enhanced NO_3^--N and NH_4^+-N contents were obtained when common vetch was planted in the fallow period. However, planting forage rape had an obvious negative impact on the NO_3^--N content in the 0–30 cm soil layer (Figure 2). Of the treatment, the W-F-W-F cropping system had the lowest NO_3^--N content, which also explained the reduction in wheat production in the 2018–2019 growing season.

4.2. Evapotranspiration characteristics

In the rainfed regions on the Loess Plateau, precipitation is sufficient but uneven, resulting in frequent drought (Ding et al., 2016). To mitigate the effect of extreme weather and environmental conditions on soil moisture utilization and crop-forage production, ET characteristics of the crop growing season under different cropping systems need to be explored. Our study showed that ET among the cropping systems had no obvious difference during early winter wheat growth, but there was a significant difference in ET during late wheat growth (Figure 3). This difference was mainly related to the precipitation distribution and soil moisture storage. Ding et al. (2018) showed that the amount of local precipitation and its spatiotemporal

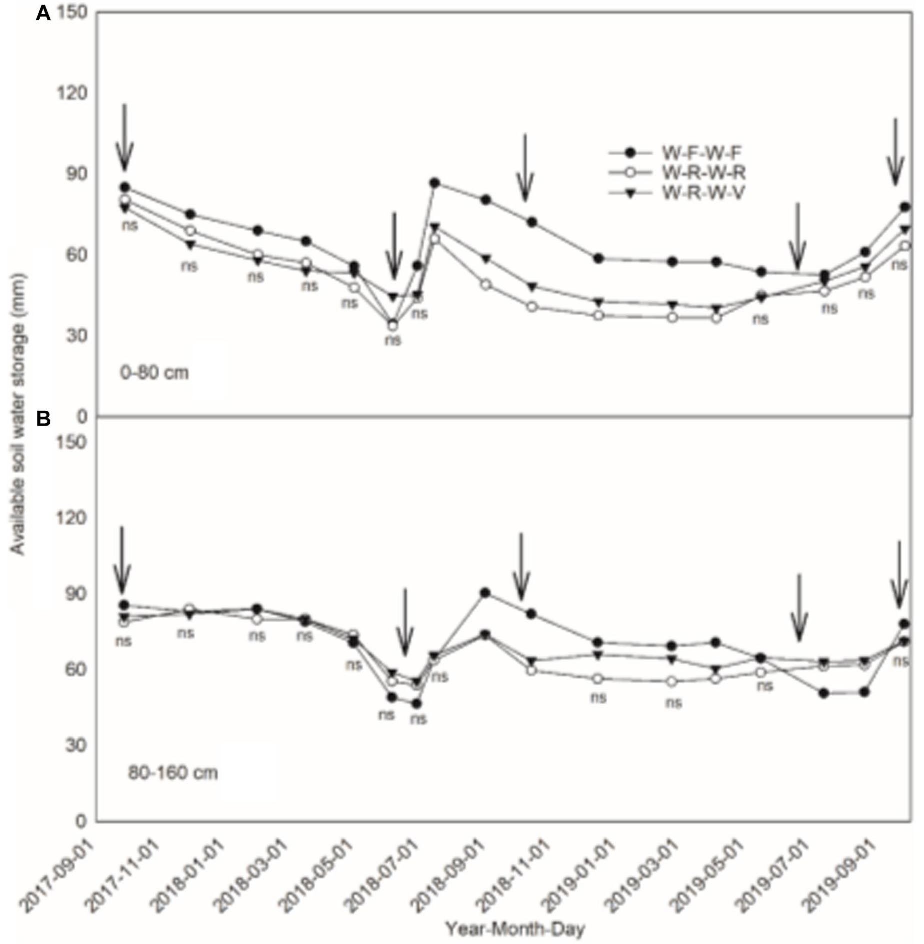

FIGURE 4
Variation in available soil water storage in the 0–80 cm **(A)** and 80–160 cm **(B)** soil layers under different cropping systems during the growing seasons (October 2017–September 2019). Vertical bars represent the least significant difference ($p = 0.05$). W = winter wheat, F = summer fallow, R = forage rape, V = common vetch.

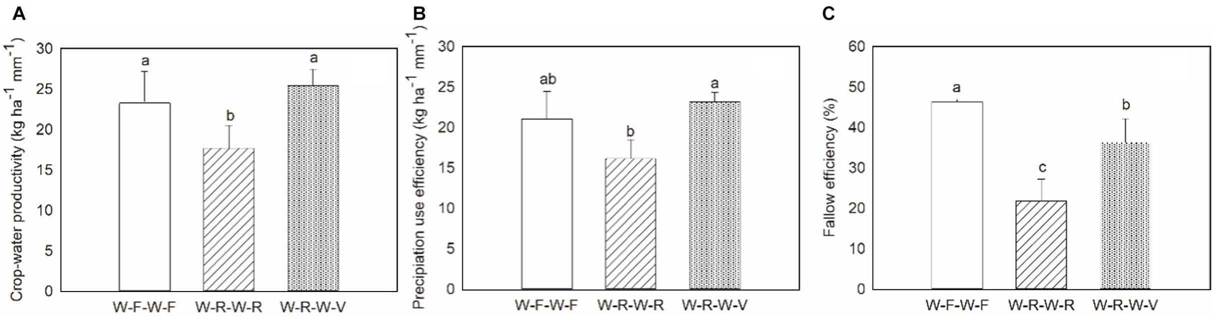

FIGURE 5
System crop-water productivity **(A)**, precipitation use efficiency **(B)**, and fallow efficiency **(C)** among the different cropping systems. Different letters indicate significant differences between treatments ($p < 0.05$). W = winter wheat, F = summer fallow, R = forage rape, V = common vetch.

distribution greatly affect the ET and soil moisture content during winter wheat growing seasons. Fallow practices result in substantial soil evaporation (267–397 mm) because 55.3% of annual precipitation was concentrated in the summer fallow period during the experimental period and the air temperatures in the summer fallow period were relatively high (Figure 1; Supplementary Table 2). However, there was no significant difference in ET between the W-R-W-R and W-R-W-V treatments, indicating that in comparison to the cropping system, precipitation had a much larger impact on ET when the rainfall amount was relatively high on the Chinese Loess Plateau. Ding et al. (2018) reported that relatively low air temperatures in the wheat-growing season result in a decline in soil evaporation, thereby explaining the present study results.

4.3. Crop-water productivity and sustainability

Water is an important driving factor in agriculture, and the efficient utilization of precipitation determines agriculture sustainability (Peterson et al., 1996). Therefore, a system that enhances water storage efficiency and CWP must be developed to ensure agricultural sustainability on the Chinese Loess Plateau. In comparison with the W-F-W-F treatment, all other rotation treatments significantly increased the period of crop coverage in the rainy season and the PUE of the system (Huang et al., 2003). Our results showed that the system PUE in the W-R-W-V treatment presented a greater value than that in the W-R-W-R treatment but no significant difference from that in the W-F-W-F treatment (Figure 5B). This result was primarily because the W-R-W-R treatment had the lowest system DM yield among the treatments, and no significant difference in system DM yield was found between the W-F-W-F and W-R-W-V treatments (Table 1). Evans (1993) also reported that a reduction in the individual crop yield in alternative cropping system intensification system with at least two crops could be offset by a cumulative increase in the annual yield. Our study revealed that the decline in the subsequent winter wheat grain yield in 2018–2019 was adequately offset by the common vetch yield in 2019. The result suggests that compared to the W-R-W-R system, the W-R-W-V system is a plausible alternative cropping system that can ensure Loess Plateau farming system sustainability. Similarly, in an environment with water limitations, efficiency and high production in relation to water use are important to select cropping systems that could be sustainable (Neal et al., 2011). In addition, the SWSD averaged 64.9 mm across the cropping system (Table 2). Although planting forage crops in the summer fallow decreased the FE (Figure 5C), precipitation during the summer fallow period still restored the soil moisture content well. Because precipitation was generally sufficient during the experimental years in this semiarid region of the Loess Plateau compared with the long-term average (Figure 1; Supplementary Table 2), losses in agricultural production do not solely depend on rainfall scarcity but also the seasonal variability in rainfall distribution (Li, 1998). Furthermore, the difference in soil moisture was significant among the cropping systems in 2018 when the forage crop was harvested, but no significant difference was found in 2019 (Figure 4; Supplementary Figure 1), indicating that the effect of substituting summer fallow with forage crops mainly depends on the precipitation distribution on the Loess Plateau. However, Wang et al. (2020) reported 135.9 mm of evaporated water from bare soil for the corresponding period from planting to late harvesting. This value is almost half of the water used by synchronous cover crops in a dry year. Therefore, common vetch should be planted solely or intercropped in a greater proportion when there is limited rainfall during the fallow period. In addition, our study showed that the W-R-W-R cropping system posed a threat to subsequent winter wheat production based on soil moisture consumption. Hence, in comparison to the other treatments, the W-R-W-V system was had a better performance for maintaining the sustainable development of agricultural production. However, since the experimental years in the present study fell above the long term precipitation mean, quantification of soil moisture should be conducted prior to planting fallow crops so as to prevent moisture shortage during the subsequent winter wheat production in the event of low precipitation year (below the long time average). In addition, further study with a longer experimental period that could capture the long term rainfall variability in the Loess Plateau is required.

5. Conclusion

Based on this two-year field study, we found that the challenge to sustainable agricultural production is not necessarily the absolute scarcity of precipitation but rather its uneven seasonal distribution on the Chinese Loess Plateau. In comparison to the W-F-W-F cropping system, planting forage rape and common vetch in summer fallow could increase the average system forage production by 4.93 t ha^{-1}. However, planting these crops decreased the available soil moisture storage and NO_3^-–N content by an average of 50.3 mm and 54.8%, respectively, resulting in a decline in subsequent winter wheat yield due to the drought resulting from the erratic monthly precipitation distribution in the 2018–2019, especially in the W-R-W-R cropping system. The reduced yield of winter wheat in the system had been offset by the forage yield in the W-R-W-V cropping system which helped to maintain the productivity of the system. Adding forage rape into the W-F-W-F cropping system decreased the CWP of the total system by 40.9%, without a significant difference in PUE. However, the PUE of the W-R-W-V system were 30.1% higher than those in the W-R-W-R system. Therefore, the W-R-W-V cropping system has shown a decreased soil evaporation and could be a stable pattern for grain and forage production that will significantly contribute to the sustainability of soil moisture and fertility on the Chinese Loess Plateau. Further study with a longer experimental period that could capture the long-term rainfall variability in the Loess Plateau is required.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2023.1139453/full#supplementary-material

References

Abate, T., Berhanu, T., Bogale, S., and Worku, D. (2003). Potential of forages legumes to replace the traditional fallow-barley rotation system in the cool-high land of bale. *Challenges and opportunities of livestock marketing in Ethiopia proceedings of the 10th annual conference of the Ethiopian Society of Animal Production*. Addis Ababa, Ethiopia, 21–23 August 2003.

Aiken, R. M., O'Brien, D. M., Olson, B. L., and Murray, L. (2013). Replacing fallow with continuous cropping reduces crop water productivity of semiarid wheat. *Agron. J.* 105, 199–207. doi: 10.2134/agronj2012.0165

Ao, Y. H., Wen, J., and Lü, S. H. (2007). A study of the water and energy transfer at the soil surface in cropped field on the loess plateau of northwestern China. *Environ. Earth Sci.* 52, 595–603. doi: 10.1007/s00254-006-0490-z

Blanco-Canqui, H., Shaver, T. M., Lindquist, J. L., Shapiro, C. A., Elmore, R. W., Francis, C. A., et al. (2015). Cover crops and ecosystem services: insights from studies in temperate soils. *Agron. J.* 107, 2449–2474. doi: 10.2134/agronj15.0086

Campbell, C. A., Zentner, R. P., Basnyat, P., Jong, R. D., Lemke, R., and Desjardins, R. L. (2008). Nitrogen mineralization under summer fallow and continuous wheat in the semiarid Canadian prairie. *Can. J. of Soil Sci.* 88, 681–696. doi: 10.4141/CJSS07115

Deng, J. Q., Zhang, Z. X., Liang, Z. T., Li, Z., Yang, X. L., Wang, Z. K., et al. (2020). Replacing summer fallow with annual forage improves crude protein productivity and water use efficiency of the summer fallow-winter wheat cropping system. *Agric. Water Manag.* 230:105890. doi: 10.1016/j.agwat.2019.105980

Ding, D. Y., Feng, H., Zhao, Y., Liu, W. Z., Chen, H. X., and He, J. Q. (2016). Impact assessment of climate change and later-maturing cultivars on winter wheat growth and soil water deficit on the loess plateau of China. *Clim. Chang.* 138, 157–171. doi: 10.1007/s10584-016-1714-1

Ding, D. Y., Zhao, Y., Feng, H., Hill, R. L., Chu, X. S., Zhang, T. B., et al. (2018). Soil water utilization with plastic mulching for a winter wheat-summer maize rotation system on the loess plateau of China. *Agric. Water Manag.* 201, 246–257. doi: 10.1016/j.agwat.2017.12.029

Evans, L. T. (1993). *Crop Evolution, Adaptation and Yield*. Cambridge University Press, Cambridge.

Farahani, H. J., Peterson, G. A., and Westfall, D. G. (1998). Dryland cropping intensification: a fundamental solution to efficient use of precipitation. *Adv. Agron.* 64, 197–223. doi: 10.1016/S0065-2113(08)60505-2

Gan, Y. T., Hamel, C., O'Donovan, J. T., Cutforth, H., Zentner, R. P., Campbell, C. A., et al. (2015). Diversifying crop rotations with pulses enhances system productivity. *Sci. Rep.* 5:14625. doi: 10.1038/srep14625

Gan, Y. T., Liu, L. P., Cutforth, H., Wang, X. Y., and Ford, G. (2011). Vertical distribution profiles and temporal growth patterns of roots in selected oilseeds, pulses and spring wheat. *Crop Pasture Science* 62, 457–466. doi: 10.1071/CP10406

Grant, C. A., Peterson, G. A., and Campbell, C. A. (2002). Nutrient considerations for diversified cropping systems in the northern Great Plains. *Agron. J.* 94, 186–198. doi: 10.2134/agronj2002.1860

Herridge, D. F., Peoples, M. B., and Boddey, R. M. (2008). Global inputs of biological nitrogen fixation in agricultural systems. *Plant Soil* 311, 1–18. doi: 10.1007/s11104-008-9668-3

Hou, F. J., Nan, Z. B., Xie, Y. Z., Li, X. L., Lin, H. L., and Ren, J. Z. (2008). Integrated crop-livestock production systems in China. *Rangel. J.* 30, 221–231. doi: 10.1071/RJ08018

Huang, M. B., Shao, M. A., Zhang, L., and Li, Y. S. (2003). Water use efficiency and sustainability of different long-term crop rotation systems in the loess plateau of China. *Soil Till. Res.* 72, 95–104. doi: 10.1016/S0167-1987(03)00065-5

Jensen, E. S., Peoples, M. B., Boddey, R. M., Gresshoff, P. M., Hauggaard-Nielsen, H., Alves, B. J. R., et al. (2012). Legumes for mitigation of climate change and the provision of feedstock for biofuels and biorefineries. *A review. Agron. Sustain. Dev.* 32, 329–364. doi: 10.1007/s13593-011-0056-7

Kang, S. Z., Zhang, L., Song, X. Y., Zhang, S. H., Liu, X. Z., Liang, Y. L., et al. (2001). Runoff and sediment loss responses to rainfall and land use in two agricultural catchments on the loess plateau. *Hydrol. Process.* 15, 977–988. doi: 10.1002/hyp.191

Komarek, A. M., Bell, L. W., Whish, J. P. M., Robertson, M. J., and Bellotti, W. D. (2015). Whole-farm economic, risk and resource-use trade-offs associated with integrating forages into crop–livestock systems in western China. *Agric. Syst.* 133, 63–72. doi: 10.1016/j.agsy.2014.10.008

Lemke, R. L., Vandenbygaart, A. J., Campbell, C. A., Lafond, G. P., McConkey, B. G., and Grant, B. (2012). Long-term effects of crop rotations and fertilization on soil C and N in a thin black Chernozem in southeastern Saskatchewan. *Can. J. Soil Sci.* 92, 449–461. doi: 10.4141/cjss2010-047

Li, F. R. (1998). *Studies on arid agricultural ecosystems (In Chinese)*. Shanxi Science and Technology Press, Xian.

Li, F. R., Gao, C. Y., Zhao, H. L., and Li, X. Y. (2002). Soil conservation effectiveness and energy efficiency of alternative rotations and continuous wheat cropping in the loess plateau of Northwest China. *Agric. Ecosyst. Environ.* 91, 101–111. doi: 10.1016/S0167-8809(01)00265-1

Li, Y., Li, Z., Cui, S., Chang, S. X., Jia, C. L., and Zhang, Q. P. (2019). A global synthesis of the effect of water and nitrogen input on maize (*Zea mays*) yield, water productivity and nitrogen use efficiency. *Agric. For. Meteorol.* 268, 136–145. doi: 10.1016/j.agrformet.2019.01.018

Li, S. X., Wang, Z. H., Li, S. Q., Gao, Y. J., and Tian, X. H. (2013). Effect of plastic sheet mulch, wheat straw mulch, and maize growth on water loss by evaporation in dryland areas of China. *Agric. Water Manag.* 116, 39–49. doi: 10.1016/j.agwat.2012.10.004

Li, Z., Yang, X., Cui, S., Yang, Q., Yang, X. L., Li, J. C., et al. (2018). Developing sustainable cropping systems by integrating crop rotation with conservation tillage practices on the loess plateau, a long-term imperative. *Field Crops Res.* 222, 164–179. doi: 10.1016/j.fcr.2018.03.027

Li, F. R., Zhao, S. L., and Geballe, G. T. (2000). Water use patterns and agronomic performance for some cropping systems with and without fallow crops in a semi-arid environment of Northwest China. *Agric. Ecosyst. Environ.* 79, 129–142. doi: 10.1016/S0167-8809(99)00149-8

Liu, Y. B., Tian, L. H., Zhang, Q. P., and Shen, Y. Y. (2014). Grain and forage performances of winter wheat in a rape and wheat rotation system. *Pratacultural Sci.* 31, 1336–1342.

Luce, M. S., Grant, C. A., Ziadi, N., Zebarth, B. J., O'Donovan, J. T., Blackshaw, R. E., et al. (2016). Preceding crops and nitrogen fertilization influence soil nitrogen cycling in no-till canola and wheat cropping systems. *Field Crops Res.* 191, 20–32. doi: 10.1016/j.fcr.2016.02.014

Luce, M. S., Lemke, R., Gan, Y. T., Mcconkey, B., May, W., Campbell, C., et al. (2020). Diversifying cropping systems enhances productivity, stability, and nitrogen use efficiency. *Agron. J.* 112, 1517–1536. doi: 10.1002/agj2.20162

Lyon, D. J., Baltensperger, D. D., Blümenthal, J. M., Burgener, P. A., and Harveson, R. M. (2004). Eliminating summer fallow reduces winter wheat yields but not necessarily system proftability. *Crop Sci.* 44, 855–860. doi: 10.2135/cropsci2004.8550

Neal, J. S., Fulkerson, W. J., and Hacker, R. B. (2011). Differences in water use efficiency among annual forages used by the dairy industry under optimum and deficit irrigation. *Agric. Water Manag.* 98, 759–774. doi: 10.1016/j.agwat.2010.11.011

Nielsen, D. C., Lyon, D. J., Hergert, G. W., Higgins, R. K., and Holman, J. D. (2015). Cover crop biomass production and water use in the central Great Plains. *Agron. J.* 107, 2047–2058. doi: 10.2134/agronj15.0186

Nielsen, D. C., Lyon, D. J., Higgins, R. K., Hergert, G. W., Holman, J. D., and Vigil, M. F. (2016). Cover crop effect on subsequent wheat yield in the central Great Plains. *Agron. J.* 108, 243–256. doi: 10.2134/agronj2015.0372

Nielsen, D. C., Lyon, D. J., and Miceli-Garcia, J. J. (2017). Replacing fallow with forage triticale in a dryland wheat-corn-fallow rotation may increase profitability. *Field Crops Res.* 203, 227–237. doi: 10.1016/j.fcr.2016.12.005

Nielsen, D. C., and Vigil, M. F. (2010). Precipitation storage efficiency during fallow in wheat-fallow systems. *Agron. J.* 102, 537–543. doi: 10.2134/agronj2009.0348

Parihar, S. S., Pandey, D., Shukla, R. K., Verma, V. K., Chaure, N. K., Choudhary, K. K., et al. (1999). Energetics, yield, water use and economics of rice-based cropping system[J]. *Indian J. Agron.* 44, 205–209.

Peterson, G. A., Schlegel, A. J., Tanaka, D. L., and Jones, O. R. (1996). Precipitation use efficiency as affected by cropping and tillage systems. *J. Prod. Agric.* 9, 180–186. doi: 10.2134/jpa1996.0180

Ratliff, L. F., Ritchie, J. T., and Cassel, D. K. (1983). A survey of field-measured limits of soil water availability as related to laboratory-measured properties. *Soil Sci. Soc. Am. J.* 47, 770–775. doi: 10.2136/sssaj1983.03615995004700040032x

Ritchie, J. T. (1981). Soil water availability. *Plant Soil* 58, 327–338. doi: 10.1007/BF02180061

Unger, P. W., Schomberg, H., Dao, T. H., and Jones, O. R. (1997). Tillage and crop residue management practices for sustainable dryland farming systems. *Ann. Arid Zone* 36, 209–232.

Unger, P. W., and Vigil, M. F. (1998). Cover crops effects on soil water relationships. *J. Soil Water Conserv.* 53, 200–207.

Wang, X. Y., Gan, Y. T., Hamel, C., Lemke, R., and McDonald, C. (2012). Water use profiles across the rooting zones of various pulse crops. *Field Crops Res.* 134, 130–137. doi: 10.1016/j.fcr.2012.06.002

Wang, Z. K., Jiang, H. L., and Shen, Y. Y. (2020). Forage production and soil water balance in oat and common vetch sole crops and intercrops cultivated in the summer-autumn fallow season on the Chinese loess plateau. *Eur. J. Agron.* 115:126042. doi: 10.1016/j.eja.2020.126042

Yang, X. L., Lu, Y. L., Tong, Y. A., and Yin, X. F. (2015). A 5-year lysimeter monitoring of nitrate leaching from wheat–maize rotation system: comparison between optimum N fertilization and conventional farmer N fertilization. *Agric. Ecosyst. Environ.* 199, 34–42. doi: 10.1016/j.agee.2014.08.019

Yang, X. L., Lu, Y. L., Yan, D., Yin, X. F., Raza, S., and Tong, Y. A. (2017). Optimising nitrogen fertilisation: a key to improving nitrogen-use efficiency and minimising nitrate leaching losses in an intensive wheat/maize rotation (2008-2014). *Field Crops Res.* 206, 1–10. doi: 10.1016/j.fcr.2017.02.016

Yang, X., Zheng, L. N., Yang, Q., Wang, Z. K., Cui, S., and Shen, Y. Y. (2018). Modelling the effects of conservation tillage on crop water productivity, soil water dynamics and evapotranspiration of a maize-winter wheat-soybean rotation system on the loess plateau of China using APSIM. *Agric. Syst.* 166, 111–123. doi: 10.1016/j.agsy.2018.08.005

Zhang, Q. P., Bell, L. W., Shen, Y. Y., and Whish, J. P. M. (2018). Indices of forage nutritional yield and water use efficiency amongst spring-sown annual forage crops in north-West China. *Eur. J. Agron.* 93, 1–10. doi: 10.1016/j.eja.2017.11.003

Zhang, Z. X., Christensen, M., Nan, Z. B., Whish, J. P. M., Lindsay, W. B., Wang, J. F., et al. (2019b). Plant development and solar radiation interception of four annual forage plants in response to sowing date in a semi-arid environment. *Ind. Crop. Prod.* 131, 41–53. doi: 10.1016/j.indcrop.2019.01.028

Zhang, Z. X., Whish, J. P. M., Bell, L. W., and Nan, Z. B. (2017). Forage production, quality and water-use-efficiency of four warm-season annual crops at three sowing times in the loess plateau region of China. *Eur. J. Agron.* 84, 84–94. doi: 10.1016/j.eja.2016.12.008

Zhang, Z. X., Yu, K. L., Jin, X. L., Nan, Z. B., Wang, J. F., Niu, Y. N., et al. (2019a). Above- and below-ground dry matter partitioning of four warm-season annual crops sown on different dates in a semiarid region. *Eur. J. Agron.* 109:125918. doi: 10.1016/j.eja.2019.125918

Zhu, X. M., Li, Y. S., Peng, X. L., and Zhang, S. G. (1983). Soils of the loess region in China. *Geoderma* 29, 237–255. doi: 10.1016/0016-7061(83)90090-3

Zhu, Z. X., Stewart, B. A., and Fu, X. J. (1994). Double cropping wheat and corn in a subhumid region of China. *Field Crops Res.* 36, 175–183. doi: 10.1016/0378-4290(94)90109-0

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How do risk preferences influence forage planting behaviors among farmers in the agro-pastoral ecotone of China?

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Introduction: This study analyzes the influence of risk preference on the forage planting behavior of farmers in the agro-pastoral zone from three aspects-whether or not to plant forage, the scale of forage planting, and the duration of forage planting—and pays attention to the indirect effect of credit on the farmers' forage planting behavior, as well as the heterogeneity of the influence of risk preference on the forage planting behavior of farmers from the perspectives of different farming scales, types of farmers, and differences between generations.

Methods: Experimental economics, a two-stage model, is used to analyze this problem.

Results and conclusion: The results show that, first, risk preference can significantly promote farmers' forage planting probability, expand forage planting scale, and increase forage planting duration. Specifically, when risk preference increases by one unit, the probability of farmers choosing to plant forage increases by 7.8%, the planting scale increases by 0.205 hm^2, and the planting duration increases by 0.519 years. This conclusion remained robust after changing the explanatory variables. Second, risk preference not only directly affects farmers' forage planting behavior but also indirectly affects farmers' forage planting behavior by influencing farmers' participation in credit. Third, heterogeneity analysis shows that risk preference has a significant effect on forage planting behavior among farmers with a medium breeding degree, among pure farmers and concurrent farmers, as well as middle-aged and elderly farmers.

KEYWORDS

risk preference, farmers' forage planting behavior, experimental economics, agro-pastoral ecotone, farmer heterogeneity

1. Introduction

In response to the upgrading of the food consumption structure of the people and the prominent structural contradictions in agriculture, the Chinese government put forward the concept of establishing large grain in 2015 to ensure food security. The big food concept requires not only ensuring the safety of rations but also ensuring the safety of non-rations, such as meat, eggs, and milk (Dong et al., 2015). Under the premise of absolute food safety, how to guarantee the adequate supply of livestock products and quality safety are important problems that need to be solved urgently. Being influenced by traditional farming culture, the feeding mode of "straw + concentrate" is extended in our animal husbandry. Due to the long-term lack of high-quality forage as "staple food" to develop herbivore animal husbandry, resulting in low production efficiency of animal husbandry, livestock product quality and safety are not large issues (Chen et al., 2012; Zhang et al., 2013). Related research shows that, if the feeding method of herbivorous livestock is changed, the bottleneck of animal husbandry development will be alleviated (Ren, 2013). In this way, the development of high-quality forage has become a necessary way to speed up the transformation of herbivorous animal husbandry and ensure an adequate supply of livestock products.

In 2015, the Chinese government vigorously implemented the policy of "food-fodder change". This policy is aimed at the development of herbivore animal husbandry, encouraging and guiding farmers to plant high-quality forage grass such as whole-plant silage corn (Shi and Hu, 2022). These silages are harvested, processed, and converted into herbivore livestock such as cattle and sheep in the form of silage, forming a development model of circular agriculture combining planting and feeding (Guo, 2019). As a circular agricultural production and management mode, it can not only promote China's agricultural structure adjustment but also increase the supply of high-quality forage and ensure the quantity and quality safety of livestock products. In 2022, the 14th Five-Year Plan for the Development of the National Forage Industry further proposed to actively promote grain conversion in the agro-pastoral ecotone, and by 2025, the national yield of high-quality forage grass is expected to reach 98 million tons. This means that the forage planting degree in China will continue to expand in the future, and the farmers involved will also be further expanded. However, the problem is that the policy of "food-fodder change" has been implemented for 8 years. Also, the forage planting rate of farmers is still low, the forage planting is not continuous, and the high-quality forage supply is at a low level (Guo et al., 2020; Yang et al., 2023). In this context, it is an important issue that government departments and academia need to explore the factors that affect forage planting behavior decision-making of farmers. This is of great practical significance for promoting the sustainable development of "food-fodder change" policy, comprehensively consolidating the foundation of food security and practicing the concept of big food.

A review of the existing literature revealed that scholars investigated the important factors affecting farmers' behavioral decisions on forage planting from the aspects of farmer characteristics, policy incentives, and crop prices. For example, Wang et al. (2018) based on the micro-survey data of two provinces found that household characteristics (age of household head, number of livestock, and amount of forage) and regional characteristics all had a positive impact on the operation behavior of combined planting and breeding. Zhang et al. (2018), based on the survey data of farmers in the agro-pastoral intercropping zone in Ningxia, found that policy support (participation in the feeding-shed project) could significantly increase the planted area of artificial forage land. Wang (2021), based on the data from 19 major alfalfa-producing provinces in China from 2001 to 2017, found that the high transportation cost of alfalfa has an inhibitory effect on the expansion of alfalfa production scale, and increasing the per capita arable land area has an incentive effect on promoting alfalfa planting. Irungu (1998), in a study of smallholder dairy farmers in the Kiambu region, found that the head of household's farming years, horticultural/dairy area affiliation, off-farm employment, and dairy cooperative/farmer organization affiliation had a positive impact on the probability of adopting Napier grass. Milk price has a negative impact on the probability of adopting Napier forage among sample farmers. Javeed et al. (2020) conducted personal interviews with dairy farmers in the Northeast Transition Zone of Karnataka, India, and found that group size, land holdings, annual income, distance to veterinary institutions, extension participation, decision-making ability, scientific orientation, and economic variables, such as orientation and risk orientation, were significantly associated with attitudes toward green forage crop cultivation. Age and education level were negatively correlated with attitudes toward green forage crop cultivation. Wang et al. (2015) analyzed the influencing factors of farmers' alfalfa planting behavior in the Huaihai area by using statistical data and found that corn prices had a significant positive effect on the alfalfa planting area. However, as a forage with relatively high risk, especially after converting grain corn into whole silage corn, it may face risks such as inadequate technical suitability, uncertain market conditions, and natural disasters. Therefore, the influence of risk preference on the forage planting behavior of farmers cannot be ignored.

In terms of risk appetite perspective, existing studies have also shown that risk preference plays an important role in farmers' behavioral decision-making (Wossen et al., 2015; Xu et al., 2020). Chavas and Holt (1996) found that farmers' risk preferences will have an impact on land allocation decisions in the process of agricultural production through the study of corn and soybean planting areas. Using data from a preliminary survey of onion growers in India, Khanal et al. (2019) explored the relationship between risk appetite and farm diversification strategies. Hasibuan et al. (2022) conducted a study on citrus growers and considered the role of risk in household fertilizer and pesticide expenditure decisions. Salimonu and Falusi (2007) measured the risk preference of food crop farmers and found that risk preference will have an impact on resource use and allocation patterns in agricultural production. Vollenweider et al. (2011) focused on the adoption of pro-environmental behaviors in pasture planting and studied the impact of risk preference on the probability of farmers participating in the Rural Environmental Protection Program (REPS). However, at present, only Gao (2020) studied farmers' forage planting behavior decisions based on risk management strategies and confirmed the role of risk management strategies. The conclusion of this study has a certain reference value, but there are still some shortcomings: in terms of research content, on the one hand, this study only focused on the impact of risk management strategies on farmers' forage planting area but did not involve farmers' forage planting decisions and forage planting duration and other issues, so the dynamic change description and investigation of farmers' forage planting decision-making behavior were not comprehensive and in-depth. On the other hand, only the influence of risk management strategies on the forage planting decisions of farmers was discussed, but the mechanism of such influence was not deeply analyzed. From the perspective of research, it focuses on the discussion of risk management strategies but lacks the analysis of how risk preference affects farmers' forage planting behavior. This provides a space for further study in this article.

Therefore, based on the 1,479 survey data of the agro-pastoral ecotone, this study intends to analyze how risk preference affects farmers' forage planting behavior. The reason why this area is chosen as the research object is because the agro-pastoral ecotone is the edge intersection zone, the key area where the terrestrial ecosystem responds to global environmental changes and human disturbance, which is also the transition zone between grassland and cultivated land and between animal husbandry and planting (Li et al., 2018). Specifically speaking, this region has a fragile resource background, dry, windy, and heavy rainfall climate, loose soil texture, high content of sand and silty sand, sparse and low vegetation, and is an area where water erosion, desertification, and sandstorms occur and develop strongly (Li et al., 2015). These factors lead to the disruption of grain production and yield loss in the study area. It also threatens local and global food security (Shi et al., 2021). Considering that this area is an advantageous area for animal husbandry development, to improve the efficiency of land use, the Chinese government encourages local farmers to plant high-quality forage, especially whole silage corn. Many studies have shown that, under the same conditions, they have more advantages than other crops and the returns of forage cultivation are higher than that of food crops (Du and Han, 2020). Meanwhile, planting forage can reduce feed costs, help farmers manage costs during market fluctuations, and reduce financial risks associated with livestock rearing (Nong et al., 2021). However, we believe that there is still a great risk for farmers to plant forage. Such as, first, in the policy of promoting forage planting, a complete policy system has not been formed, and the specific implementation and subsidy methods are not perfect. Second, in the production of farmers, the technology of planting forage is not skilled, and the problem of insufficient planting equipment hinders farmers from planting forage. Third, in the sales process, there are contradictions such as a poor connection between forage production and sales and lagging cultivation in the order market. This means that farmers' forage planting decisions will be influenced by their risk preferences.

Compared with the existing literature, the marginal contribution of this study is mainly reflected in the following two aspects: First, analyzing farmers' forage planting behavior and exploring its mechanism from the perspective of risk preference is helpful to enrich the lack of research on farmers' forage planting behavior in related fields and provide a new direction for the study of key factors of farmers' forage planting behavior. Second, the experimental economics method is adopted to measure the risk preference of farmers in the farming-pastoral ecotone, to make the measurement results more scientific and accurate, and to enrich the relevant research on the influence of risk preference on the decision-making behavior of farmers.

2. Material and methods

2.1. Study area and data collection

The data used in this study are from the micro survey of farmers in Shaanxi and Gansu provinces in August 2022. The choice of this study area mainly considers the following three factors: First, from the perspective of geographical location, the Shaanxi and Gansu provinces cover the different agricultural ecosystems of the desertification degradation area along the Great Wall of China and the loess hilly-gully region. Second, from the perspective of agricultural conditions, the corn planting area in this region is large but the yield is low and unstable. It is the dominant area of animal husbandry, but the production efficiency of animal husbandry is low. Third, from the perspective of natural conditions, under the influence of continental climate, agricultural production in the research area faces great risks.

After confirming the sample provinces, based on the comprehensive consideration of regional agricultural and animal husbandry development scale, economic development level, and other factors, stratified sampling and random sampling methods were adopted to finally select Huan County, Zhenyuan County, Zhengning County, Yuyang District, and Dingbian County, a total of 5 sample counties (Figure 1). Then, approximately 4–5 sample towns were selected in each sample county. Later, approximately 4–5 sample villages were selected in each sample town. Finally, approximately 14–17 grain farmers were randomly selected in each sample village for one-to-one questionnaire interviews. The survey involved 96 administrative villages in 24 towns and villages in 2 provinces, 2 cities, and 5 counties. A total of 1,600 questionnaires were issued to farmers in total. After eliminating the questionnaires of key information mismatch, serious missing variables, and extreme values, 1,479 effective questionnaires were obtained from farmers, with an effective rate of 92.43%. The contents of the questionnaire mainly include the basic characteristics of farmers and families, input and output information of farmers planting whole silage corn, input and output information of livestock breeding, farmers' risk perception, and social capital.

2.2. Theoretical analysis

2.2.1. Effect of risk preference on farmers' forage planting behavior

Risk preference refers to a subjective psychological attitude of farmers in the face of risks associated with agricultural production and operation, which can be divided into risk preference, risk neutral, and risk aversion. According to prospect theory (Kahnemand and Amos, 1984), farmers' risk preferences affect their risk decision-making behavior, and there are significant differences between different farmers' risk preferences under the situation of uncontrollable production risks and asymmetric information. For example, risk-averse farmers will inhibit agricultural technology adoption (Visser et al., 2020; Yu et al., 2021), as well as fertilizer and pesticide reduction (Pan et al., 2020; Qiao and Huang, 2021). However, farmers with strong risk preferences are more inclined to adopt technology or produce risky behaviors (Liu and Huang, 2013).

The agro-pastoral ecotone is a temperate continental climate with obvious climate change, especially heavy rain and drought. In terms of farmers in the region planting whole silage maize, when it comes to dry seasons, using corn as silage can reduce yield losses and protect farmers' incomes. However, when the silage harvest season meets the rainy season, the silage machinery cannot enter the cultivated land and miss the best harvest time, which not only leads to the decrease of forage yield but also decreases the nutritional value of forage silage. Meanwhile, planting whole silage corn also has the risk of insufficient technical suitability and lag in order market cultivation, which means that, when farmers make forage planting decisions, in addition to considering cost

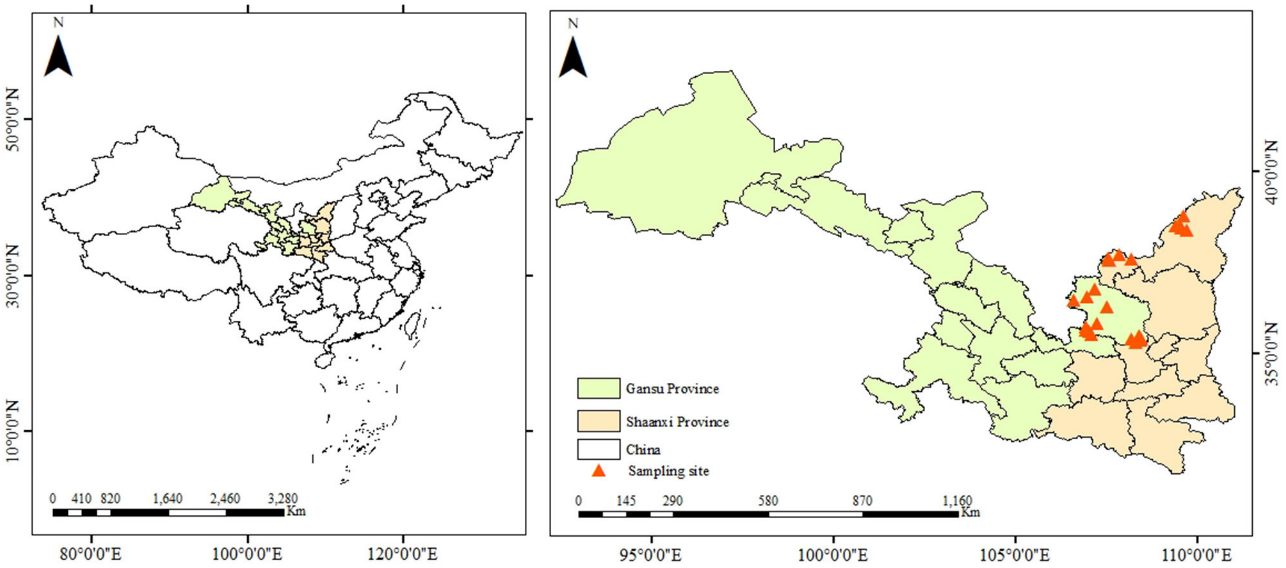

FIGURE 1
Location of sampling sites.

and benefit factors, they also need to comprehensively consider the risk factors they face. Therefore, when farmers have a high-risk preference, they pay less attention to technical and market risks brought by forage planting and more attention to economic benefits brought by forage planting, promoting the forage planting behavior. Relevant studies have shown that risk preference has a significant positive impact on farmers' behavior decision-making, decision-making scale, and duration (Mao et al., 2022). Based on the above analysis, the following assumptions are proposed:

> Hypothesis 1: Risk preference may affect the forage planting behavior of farmers in the agro-pastoral.
> Hypothesis 1a: The higher the risk preference of farmers, the greater the possibility of forage planting behavior, the larger the forage planting scale, and the longer the forage planting duration.

2.2.2. The mediating effect credits

In terms of planting whole silage corn, on the one hand, a large amount of productive financing is needed in the early stage, such as the construction of silage cellars, the purchase of silage packages, and the leasing of silage machinery. Therefore, the shortage of funds is an important factor restricting farmers' forage planting (Imail et al., 2012). By participating in credit, farmers can obtain financial credit support, relieve the financial pressure in the process of farming and animal husbandry operation, and promote the degree of agricultural investment, which can solve the transformation of agricultural production mode and stimulate farmers to plant forage (Fu et al., 2022). However, it is a fact that agricultural production is greatly affected by natural conditions, and farmers' credit is risky, so whether farmers participate in credit is affected by their risk preferences. In other words, although farmers' participation in credit can alleviate the constraints of fund shortages in farming and animal husbandry production when forage grass cultivation suffers losses, they must repay the loan within the stipulated loan period; otherwise, they will bear huge compensation for breach of contract (Gine and Yang, 2009). According to this logic, risk preference farmers pay more attention to the substantial increase in economic benefits brought by planting whole corn, have higher enthusiasm to participate in credit, and are more likely to choose forage planting. However, risk-averse farmers are less likely to participate in forage planting through loans. Based on the above analysis, the following assumptions are proposed:

> Hypothesis 2: Credit has a mediating effect on the influence of risk preference on the forage planting behavior of farmers.
> Hypothesis 2a: The higher the probability of farmers participating in credit, the greater the possibility of planting forage, the larger the planting forage scale, and the longer the planting forage duration.

2.2.3. Effects of risk preference on forage planting behavior of heterogeneity farmers

Under the background of an incomplete factor market, farmers cannot rely on the market to alleviate the problem of insufficient initial capital endowment, which makes farmers with different breeding scales have relatively different demands for forage, and thus produce different forage supply behaviors, that is, risk preference has different effects on the forage planting behavior of farmers with different breeding scales. Needless to say, small-scale farmers and medium-sized and above farmers have completely different production goals and production capacities (Tan et al., 2022). Compared with small-scale households, medium-scale and above farmers have a stronger demand for forage grass and a stronger ability to regulate production factors. At the same time, with the development of the social economy, the deepening division of labor makes the transfer of labor in agricultural and non-agricultural sectors an inevitable choice. In terms of peasant households, the division of labor leads to the division of part-time and non-part-time labor resources in the household, which then leads to changes in the input mix of production factors, such as changes in land management decisions, including land management area decisions and crop planting decisions (Luo, 2020). For farmers with different degrees of differentiation, their resource endowment and dependence on agriculture differ in land management decisions. Therefore, the influence of risk preference on farmers' forage planting behavior also varies. Compared with other farmers, pure farmers and I-concurrent farmers are more dependent on agricultural production, and they prefer to plant forage grass to meet the food nutrition of livestock and increase the income of animal husbandry. Relevant studies have shown that the same social events have different influences on different age groups, which leads to different group characteristics in preferences, attitudes, and behaviors among different generation groups (Joshi et al., 2010). As far as the current situation of China's rural areas is concerned, it is common for the young generation to go out for work. The rural labor population force is mostly middle-aged and elderly. This group is mainly engaged in agriculture and hopes to improve their agricultural income by changing the existing agricultural management model. Based on the above analysis, the following assumptions are proposed:

> Hypothesis 3: The effects of risk preference on the forage planting behavior of farmers are different in terms of breeding scale, differentiation type, and intergenerational issues.
> Hypothesis 3a: Compared with small-scale farmers, risk preference has a more obvious influence on the forage planting behavior of farmers with medium-scale and above farming.
> Hypothesis 3b: Compared with other types of farmers, risk preference has a more obvious influence on the forage planting behavior of pure farmers and I-concurrent farmers.
> Hypothesis 3c: Compared with the young generation, the influence of risk preference on forage planting behavior of the middle-aged and elderly generations is more obvious.

2.3. Variable selection

2.3.1. Explained variable

The explained variable of this study is the farmers' forage planting behavior, which includes farmers' forage planting decision, forage planting scale, and forage planting duration. In this study, farmers' forage planting decision is defined by the question: "Whether farmers will plant whole silage corn in 2021?" Values are 1–0. According to statistics, there were 664 whole silage corn growers and 815 non-growers in the survey area. Forage planting scale refers to the area of whole silage corn planted by farmers in 2021. Forage planting duration refers to the number of years for farmers to plant whole silage corn by 2022.

TABLE 1 Design of risk preference game scheme for farmers.

Game coding	A: Win a lottery ticket	B: Get a fixed amount
01	50% chance of getting 100 RMB	1
02	50% chance of getting 100 RMB	5
03	50% chance of getting 100 RMB	10
04	50% chance of getting 100 RMB	15
05	50% chance of getting 100 RMB	20
06	50% chance of getting 100 RMB	25
07	50% chance of getting 100 RMB	30
08	50% chance of getting 100 RMB	40
09	50% chance of getting 100 RMB	50
10	50% chance of getting 100 RMB	60
11	50% chance of getting 100 RMB	70
12	50% chance of getting 100 RMB	80
13	50% chance of getting 100 RMB	90
14	50% chance of getting 100 RMB	99

2.3.2. Explanatory variable

Drawing on relevant research (Holt and Laury, 2002), experimental economics is used to measure farmers' risk preferences. Specifically speaking, the experimental game consists of 14 questions, each containing two options A and B (Table 1). Option A of the 14 questions is set up the same way, that is, if the farmer buys a lottery ticket, they have a 50% chance of winning 100 RMB. Option B is that farmers will definitely receive a certain amount of reward, increasing gradually from question 1 to question 14. For each question, farmers have to choose between option A and option B. Rules of the game: researchers are asked to start with the first question and ask farmers for answers one by one. If the farmer chooses A from the first question, then the researchers begin to ask the second question, if the farmer continues to choose A, then the researchers continue to ask the third question, and so on, until the end of the 14th question. However, as long as the farmer's answer is directed from option A to option B, the game is over, and the answers to the remaining questions should all be defined as B. Of course, there are also farmers who choose option B in the first set of games, then the game is over, and the farmer's choice in the subsequent 13 sets of games is defined as B. Through 14 groups of game schemes, we can more accurately measure and analyze farmers' risk preferences. The calculation formula for farmers' risk preference degree is as follows:

$$RP = 1 - \frac{\textit{choose the number of } B}{14} \tag{1}$$

In Formula (1), *RP* is the value of farmers' risk preference degree, if farmers choose option B in all 14 groups of game schemes, then the *RP* value indicates that farmers have extreme risk aversion. On the contrary, if farmers choose option A in all 14 groups of game schemes, then the *RP* value is 1, indicating that farmers have extreme risk preferences.

TABLE 2 The meaning of variables and descriptive statistics.

Variable name	Variable assignment and its meaning	Mean	Standard deviation
Explained variable			
Forage planting decisions	Plant whole silage corn = 1;unplanted whole silage corn = 0	0.449	0.497
Forage planting scale	Area of farmers planting whole silage corn in 2021 (hm^2)	0.643	2.691
Forage planting duration	By 2021, the number of years that farmers have planted whole silage corn (year)	2.090	3.935
Explanatory variable			
Farmers' risk preference	Expressed as an index of risk preference	0.303	0.348
Mediating variable			
Credit	Farmers loans amount in 2021(RMB¥ 10,000)	5.793	14.933
Control variable			
Age	Respondent age	53.832	10.534
Education	Length of schooling	6.864	3.647
Breeding duration	Breeding duration of respondent (year)	16.564	14.709
Agricultural labor force	Household labor force size (person)	1.766	0.876
Operating area of cultivated land	Operating area of household cultivated land in 2021 (hm^2)	1.319	13.718
Number of livestock raised	Number of Livestock in 2021 (Standard sheep Units)	78.632	137.419
Government training	Participated in whole silage corn training: 1 = yes; 0 = no	0.125	0.332
Government propaganda effort	Government publicity for whole silage corn: 1 = never; 2 = very small; 3 = general; 4 = greater; 5 = large	2.536	1.499
Trust degree of farmers	Do you agree or disagree that most people in this society can be trusted: 1 = disagree; 2 = less agree; 3 = general; 4 = more agree; 5 = strongly agree	4.060	0.966
The frequency of communication	Frequency of communication with agricultural technology extension personnel: 1 = no contact; 2 = less frequent; 3 = general; 4 = more frequently; 5 = very often	2.221	1.200
Village status	When there are important things in the village, will you consult your opinion: 1 = no; 2 = Occasionally; 3 = general; 4 = yes; 5 = often	3.663	1.184
Whole silage corn sales cognition	Cognition of marketing difficulty of whole silage corn: 1 = very easy; 2 = easier; 3 = general; 4 = more difficult; 5 = very difficult	2.535	1.014
Price cognition of whole silage corn	Cognition of whole silage corn price: 1 = very low; 2 = lower; 3 = general; 4 = higher; 5 = very high	3.308	1.109

2.3.3. Control variable

There are abundant research results on the analysis of factors affecting farmers' decision-making behavior (e.g., Meng et al., 2019; Feng et al., 2020; Andries et al., 2023). With reference to existing studies, this study selected control variables that may affect farmers' forage planting behavior, including five aspects: farmers' personal characteristics, family management characteristics, external environment characteristics, social capital, and farmers' cognition of forage grass. The meanings and descriptive statistics of variables are shown in Table 2.

2.4. Model construction

2.4.1. Based model setting

This article constructed an econometric model to analyze the influence of risk preference on forage planting decision, planting degree, and planting duration of farmers. The benchmark measurement model is set as follows:

$$Y_1 = \beta_0 + \beta_1 Risk + \beta_2 X + \varepsilon_1 \tag{2}$$

$$Y_2 = \gamma_0 + \gamma_1 Risk + \gamma_2 X + \varepsilon_2 \tag{3}$$

$$Y_3 = \delta_0 + \delta_1 Risk + \delta_2 X + \varepsilon_3 \tag{4}$$

Y_1, the forage planting decision of farmers, that is, whether to plant whole silage corn. Y_2, the forage planting degree, namely the planting area of whole silage corn. Y_3, forage planting duration, that is, planting time of whole silage corn until 2022; *Risk*, the degree of farmers' risk preference; X, control variable; β_0, γ_0, and δ_0, the constant term; β_1, γ_1, δ_1, β_2, γ_2, and δ_2 are the coefficients to be estimated; ε_1, ε_2, and ε_3 are the random perturbation term.

It should be noted that, when the explained variable is whether farmers plant forage grass, it belongs to the binary variable, and the Probit model is selected for estimation. When the explained variable is forage planting degree and planting duration, it belongs to the blocking variable, including the 0 value. If the model is set as a linear equation and the least square method is used to estimate the regression, biased estimation results will be obtained (Sun and Guo, 2016). Therefore, the Tobit model is used for estimation in this study.

2.4.2. Mediation effect test

To test the mediating role of farmers' credit, this study draws on relevant literature on the mediating effect test method (Wen and Ye, 2014) and constructs the following measurement model:

$$Y_i = \alpha_0 + \alpha_1 Risk + \alpha_2 X + \varepsilon_3 \tag{5}$$

$$M_i = \delta_0 + \alpha_1 Risk + \alpha_2 X + \varepsilon_4 \tag{6}$$

$$Y_i = \zeta_0 + \alpha_1 Risk + \alpha_3 M + \alpha_4 X + \varepsilon_5 \tag{7}$$

Y_i, the farmers' forage planting behavior; M_i, intermediate variable, that is, credit; α_1, α_2, α_3, and α_4 are parameters or parameter matrices to be estimated; α_0, δ_0, and ζ_0 are constant terms; and ε_3, ε_4, and ε_5 are random disturbance terms.

3. Results and discussion

3.1. Results of hypothesis 1

Table 3 reports the baseline estimation results of the influence of risk preference on the forage planting behavior of farmers. Among them, columns (1), (3), and (5) only consider the influence of risk preference on the forage planting behavior of farmers when no control variables are added. To avoid the endogeneity problem caused by missing variables, control variables are added in columns (2), (4), and (6). The estimated results showed that the marginal coefficient and significance level of risk preference did not exhibit significant changes, indicating that risk preference had a significant promoting effect on farmers' forage planting behavior after controlling other factors. Specifically:

The regression columns (1)–(2) are the estimation results of the influence of risk preference on farmers' forage planting decisions. According to the results, after the introduction of control variables, risk preference has a significant positive impact on farmers planting forage decisions at the statistical level of 5%, indicating that the stronger risk preference farmers have, the greater likely they are to plant forage. The marginal effect estimation coefficient of the model further indicates that when other characteristics remain unchanged, the possibility of farmers choosing forage planting increases by 7.8% for each unit increase in the risk preference index of farmers.

The regression columns (3)–(4) are the estimated results of the influence of farmers' risk preference on their forage planting scale. The results showed that, after controlling the influence of other variables, risk preference significantly positively affected farmers' forage planting scale at the statistical level of 1%. The marginal effect coefficient of the model indicated that the forage planting scale of farmers would increase by 0.205 hm^2 with each increase of one unit of the risk preference index.

The regression columns (5)–(6) are the regression results of the influence of farmers' risk preference on their forage planting duration. It can be observed from the results that farmers' risk preference positively affects the forage planting duration at the significance level of 1%. The marginal effect coefficient indicates that the forage planting duration increases by 0.519 years for every unit increase in the risk preference coefficient of farmers.

In summary, the higher the risk preference of farmers, the higher the probability of participating in forage planting, the larger the planting scale, and the longer the planting time. Therefore, the results support Hypothesis 1 and Hypothesis 1a. These results are similar to those of Wang and Zhao (2023). The study area's economic structure was single, with traditional animal husbandry as the main industry. Compared with traditional corn, high-quality whole silage corn can significantly improve livestock production characteristics such as lactation and daily gain (Cheng et al., 2022; Huo et al., 2022) and thus increase the economic benefits for farmers (Chen et al., 2018). Planting whole silage corn will also face some risks such as technology, market, and so on, which cannot be ignored. Therefore, for risk-seeking farmers, their sensitivity to benefits is higher than their sensitivity to risks, and they are more willing to try the new farming mode of "forage + concentrate" to improve their income.

3.2. Robustness test

To verify the robustness of the baseline regression results, this study replaces "farmer household risk preference index" with "farmer household stock investment preference" and adopted the same econometric model to re-estimate the relationship between risk preference and farmers' forage planting behavior. It is worth explaining that the specific measure of farmers' preference for stock investment is "Suppose 10% loss occurs in the stock you invested in, how will you deal with the stock? 1 = sell everything; 2 = sell 50%; 3 = Hold, not sell." If the farmer answers 1, the farmer is identified as risk averse. If the farmer answers 2, it is risk neutral. If the farmer answers 3, it is defined as risk preference. The estimated results are shown in Table 4. The results showed that the coefficient and significance of stock investment preference had no substantial change and had a significant positive effect on forage planting behavior, indicating that risk preference significantly promoted the forage planting behavior of farmers.

3.3. Results of hypothesis 2

According to the process of mediating effect test, Table 5 reports the results of the mechanism test of credit. For the convenience of comparison, the regression results of column (2) in Table 3 are added to column (1) in Table 5. Table 5 (2) shows the regression

TABLE 3 Estimation results of the effect of risk preference on farmers' forage planting behavior.

	Forage planting decisions		Forage planting scale		Forage planting duration	
	(1)	(2)	(3)	(4)	(5)	(6)
Risk preference	0.150*** (0.036)	0.078** (0.037)	0.591*** (0.108)	0.205*** (0.064)	0.825*** (0.194)	0.519*** (0.198)
Age		−0.004*** (0.001)		−0.007*** (0.002)		−0.024*** (0.007)
Education		−0.010*** (0.004)		−0.016*** (0.006)		−0.046** (0.019)
Breeding duration		−0.004*** (0.001)		−0.004** (0.002)		0.005 (0.005)
Agricultural labor force		0.027* (0.015)		0.067*** (0.024)		0.129* (0.076)
Land area		0.000 (0.000)		0.004*** (0.000)		0.001* (0.000)
Number of livestock		0.000 (0.000)		0.000 (0.000)		0.000 (0.000)
Government training		0.174*** (0.039)		0.254*** (0.068)		1.891*** (0.649)
Government propaganda effort		0.004 (0.009)		−0.012 (0.015)		−0.019 (0.150)
Trust degree of farmers		0.009 (0.013)		−0.002 (0.024)		−0.004 (0.073)
The frequency of communication		0.006 (0.011)		0.019 (0.019)		−0.005 (0.060)
Village status		0.007 (0.011)		0.046** (0.020)		0.082 (0.062)
Whole silage corn sales cognition		−0.075*** (0.014)		−0.144*** (0.022)		−0.372** (0.069)
Price cognition of whole silage corn		0.079*** (0.012)		0.103*** (0.020)		0.371*** (0.063)
Constant term	−0.389 (0.070)	0.153 (0.512)	−34.605 (2.938)	−0.267 (0.606)	−2.074*** (0.311)	0.335 (1.815)
Sample number	1,479	1,479	1,479	1,479	1,479	1,479
Prob>chi2	0.000	0.000	0.000	0.000	0.000	0.000
Pseudo R^2	0.008	0.077	0.004	0.189	0.003	0.018

Robust standard error in brackets. ***, **, and * are statistically significant at 1, 5, and 10%, respectively. The parameter in the model is a conditional marginal effect.

TABLE 4 Robustness test.

	Change the core explanatory variable					
	Farmers' Stock investment preference	Constant term	Control variable	Sample number	Prob>chi2	Pseudo R^2
Forage planting decisions	0.025* (0.014)	−0.411	Yes	1,479	0.076	0.002
Forage planting area	0.101** (0.044)	−2.390	Yes	1,479	0.020	0.001
Forage planting duration	0.180*** (0.067)	−0.317	Yes	1,479	0.000	0.020

Control variables are the same as Table 3; Robust standard error in brackets; ***, **, and * are statistically significant at the level of 1, 5, and 10%, respectively. The parameter in the model is the conditional marginal effect.

results of credit on farmers' plant forage decisions. The results show that farmers' participation in credit significantly positively influences farmers' forage planting decisions. Column (3) is the estimation result of risk preference on farmers' participation in credit. The results show that risk preference has a significant positive effect on farmers' participation in credit. Column (4) is the estimated result of introducing both risk preference and credit. It can be observed from the estimation results that both risk preference and farmers' credit passed the significance test at the 1% statistical level, and compared with column (1), the coefficient value of risk preference decreased, indicating that credit played a partial intermediary role in the process of risk preference affecting farmers' forage planting decision. Furthermore, through the value of the intermediary effect, it can be found that the intermediary effect value of farmer's credit is 0.004, and its proportion in the total effect is $ab/c = 1.403 \times 0.003/0.078 = 0.054$. To some extent, approximately 5.4% of the effect of risk preference on whether farmers choose forage planting is realized through the intermediary role of credit, which shows the transmission mechanism of risk preference-credit-farmers' forage planting decision. According to this logic, this study also tested the mediating effect of credit in the influence of risk preference on farmers' forage planting scale and duration. The regression results were shown in Tables 6, 7, respectively. As can be observed from column (4) of Tables 6, 7, both risk preference and credit coefficients were positive and were statistically significant at least by 10%, indicating that credit is part of the intermediary variable that risk preference affects the forage planting scale and planting duration of farmers, that is,

TABLE 5 Mechanism test of the influence of risk preference on forage planting decisions of farmers.

Variable name	Forage planting decision (1)	Forage planting decision (2)	Credit (3)	Forage planting decision (4)
Risk preference	0.078** (0.037)		1.403** (0.683)	0.072*** (0.037)
Credit		0.003** (0.001)		0.003*** (0.001)
Control variable	Under control	Under control	Under control	Under control
Constant term	−4.001(9.092)	0.215 (0.493)	20.858 (6.675)	0.027 (0.503)
Observed value	1,479	1,479	1,479	1,479
Prob>F	0.000	0.000	0.000	0.000

Values in brackets are robust standard errors. ***, **, and * shows significance level at 1%, 5% and 10%. The parameter in the model is a conditional marginal effect.

TABLE 6 Mechanism test of the influence of risk preference on forage planting degree of farmers.

Variable name	Forage planting scale (1)	Forage planting scale (2)	Credit (3)	Forage planting scale (4)
Risk preference	0.205*** (0.064)		1.403** (0.683)	0.194*** (0.063)
Credit		0.005*** (0.001)		0.005*** (0.001)
Control variable	Under control	Under control	Under control	Under control
Constant term	−0.267(0.606)	−0.037 (0.593)	20.858 (6.675)	−0.402 (0.604)
Observed value	1,479	1,479	1,479	1,479
Prob>F	0.000	0.000	0.000	0.000

Values in brackets are robust standard errors. ***, **, and * shows significance level at 1%, 5% and 10%. The parameter in the model is a conditional marginal effect.

risk preference will promote farmers' participation in credit and further promote the forage planting scale and planting duration of farmers. In conclusion, farmers' credit plays a partial mediating role in the influence of risk preference on farmers' forage planting behavior. Therefore, the results support hypotheses 2 and 2a. The results are similar to the study conducted by Mao et al. (2022). Based on the methodology of experimental economics, this paper explores the mediating role of credit in relation to risk aversion and farmers' adoption of climate adaptation technology. The results indicate that farmers who are more risk-averse are less likely to participate in credit and adopt climate adaptation technology. This leads to a lower degree of adoption as well as a shorter duration of adoption. We believe the possible reason is that, in the early stage of whole silage corn planting, large capital needs to be invested, and farmers' participation in credit can alleviate financial constraints and promote their forage planting behavior. However, due to climate, technology, market, and other reasons, agricultural investment cost is difficult to recover and there is the possibility of an increase in the cost of credit default (Gine and Yang, 2009). Therefore, risk-averse farmers will reduce the possibility of credit, and the shortage of funds further inhibits farmers' enthusiasm in forage planting.

3.4. Results of hypothesis 3

3.4.1. Cultivation scale heterogeneity

According to the standards of breeding scale of <30, between 30 and 100, and ≥100, the farming scale of farmers was divided into three groups of "small," "medium," and "large," and control variables were included in each group for grouping regression. Table 8 shows the estimated results of the marginal effect of the model (similarly below). The results showed that risk preference significantly promoted forage planting behavior (decision, scale, and duration) of medium-scale farmers, and the effect of promoting forage planting of farmers is the largest among all groups. Specifically, when farmers have the medium cultivation scale, the forage planting probability can be increased by 14.2%, the planting scale can be increased by 0.418 hm^2, and the forage planting duration can be increased by 0.752 years. However, the findings in the current study are inconsistent with the study of Tan et al. (2022), who concluded that farmers with smaller farming scales are more likely to produce green production behaviors. Although forage planting behavior is also a kind of green production, in our study region, the main livelihood of medium-scale farming households comes from agricultural income, and the main goal of agricultural production is to save costs and increase efficiency. The studies of Wang (2015) showed that, if 3 kg of alfalfa was added to the livestock diet, the daily concentrate could be reduced by 1–1.5 kg, and the cost of disease control in the whole feeding process could be reduced by approximately RMB¥ 1,000. However, the influence of risk preference on forage planting decisions of large-scale farmers is not significant. We believe that the possible reason is that large-scale farmers themselves have good bargaining power and can meet the forage needs of livestock by themselves. Therefore, risk preference has a limited promotion effect on the forage planting of these farmers. Therefore, hypothesis 3a is partially verified.

3.4.2. Household differentiation heterogeneity

According to the definition of standard of household types based on China's national statistical data, farmers were divided into pure households, I-concurrent households (agricultural concurrent

TABLE 7 Test of the mechanism of influence of risk preference on forage planting duration of farmers.

Variable name	Forage planting duration (1)	Forage planting duration (2)	Credit (3)	Forage planting duration (4)
Risk preference	0.519*** (0.198)		1.403** (0.683)	0.503** (0.198)
Credit		0.008* (0.004)		0.007* (0.004)
Control variable	Under control	Under control	Under control	Under control
Constant term	0.335 (1.815)	1.025 (1.786)	20.858 (6.675)	0.115 (1.819)
Observed value	1,479	1,479	1,479	1,479
Prob>F	0.000	0.000	0.000	0.000

Values in brackets are robust standard errors. ***, **, and * shows significance level at 1%, 5% and 10%. The parameter in the model is a conditional marginal effect.

TABLE 8 Grouping regression of farmers' breeding scale.

	Degree of cultivation	Risk preference	Control variable	Prob>chi2	Sample number
Forage planting decision	Small-scale	0.048 (0.057)	Under control	0.000	573
	Medium-scale	0.142** (0.058)	Under control	0.000	562
	Large-scale	0.026 (0.252)	Under control	0.000	344
Forage planting area	Small-scale	0.066 (0.080)	Under control	0.000	573
	Medium-scale	0.418*** (0.126)	Under control	0.000	562
	Large-scale	0.086 (0.105)	Under control	0.000	344
Forage planting duration	Small-scale	0.496 (0.316)	Under control	0.000	573
	Medium-scale	0.752** (0.311)	Under control	0.000	562
	Large-scale	0.210 (0.437)	Under control	0.000	344

***, **, and * shows significance level at 1%, 5% and 10%.

households), and II-concurrent households (non-agricultural concurrent households) according to the proportion of household agricultural income ≥80%, between 50 and 80% (including 50%), and between 0 and 50% (excluding 0%). Group regression was carried out. The estimated results are shown in Table 9. According to the results, risk preference can significantly increase the forage planting probability of pure farmers by 16.9%; increase the forage planting scale of pure farmers and I-concurrent farmers by 0.383 hm^2 and 0.226 hm^2, respectively, and increase the forage planting duration of pure farmers by 1.114 years. In general, risk preference mainly promoted the forage planting behavior of pure farmers, followed by I-concurrent farmers, but had a small promoting effect on II-concurrent farmers, and both failed the significance test. This finding was supported by Cheng et al. (2021). This is mainly because pure farmers and I-concurrent farmers are more dependent on agriculture and want to stimulate the increase in income by changing their production and management mode. According to the author's investigation, 15 hectares of medium farmland planting grain corn yield is basically approximately 400–750 kg, and planting whole silage corn per mu yield can reach approximately 2,000–4,000 kg and can replace approximately 400–800 kg concentrate. At the same time, relevant studies showed that if 15 Kg silage corn was added to the dairy cow diet, the milk yield could be increased by 2.3 kg/day compared with free feeding of hay (Li and Liu, 2010). This means that forage planting can save the purchase cost of forage, increase the output of agriculture and animal husbandry, and thus increase the income of farmers. Hypothesis 3b is tested.

3.4.3. Household differentiation heterogeneity

Referring to relevant literature, we define the farmers born before 1982 as the middle-aged and elderly generation and those born after 1982 as the young generation. Grouping regression is carried out to analyze the possible heterogeneity of risk preference affecting farmers' forage planting behaviors (Table 10). According to the estimation results, the influence coefficients of risk preference on forage planting decision, planting scale, and planting duration of the middle-aged and elderly generation were higher than those of the young generation, and all passed the significance test. Specifically, risk preference can increase the forage planting probability of the middle-aged and elderly generation by 9%, expand the forage planting scale of the middle-aged and elderly generation by 0.23 hm^2, and increase the planting time of the middle-aged and elderly generation by 0.65 years. However, the findings in the current study are inconsistent with the study of Belay et al. (2017) and Wu and Li (2023), who concluded that the middle and older generation has a stronger tendency to leave farming. The main source of these differences is that the region they studied is the North China Plain, where agricultural and non-agricultural employment is common. However, our study region is in the agro-pastoral ecotone, where farmers' livelihoods are based on agriculture, and the middle-aged and elderly generations became the main force of agricultural production, so risk preference is more likely to affect the forage planting behavior of these groups. Hypothesis 3c is tested.

To sum up, hypothesis 3 has been verified.

TABLE 9 Group regression of farmer types.

	Farmers type	Risk preference	Control variable	Prob>chi2	Sample number
Forage planting decisions	Pure farmer	0.169*** (0.064)	Under control	0.000	415
	I farmer	0.046 (0.078)	Under control	0.000	313
	II farmer	0.012 (0.252)	Under control	0.000	751
Forage planting area	Pure farmer	0.383** (0.150)	Under control	0.000	415
	I farmer	0.226* (0.137)	Under control	0.000	313
	II farmer	0.071 (0.057)	Under control	0.000	751
Forage planting duration	Pure farmer	1.114*** (0.421)	Under control	0.000	415
	I farmer	0.491 (0.378)	Under control	0.000	313
	II farmer	0.041 (0.270)	Under control	0.000	751

***, **, and * shows significance level at 1%, 5% and 10%.

TABLE 10 Grouping regression of intergenerational perspective.

	Intergenerational classification	Risk preference	Control variable	Prob>chi2	Sample number
Forage planting decisions	The middle-aged and elderly generation	0.090** (0.040)	Under control	0.000	1,293
	Young generation	0.061 (0.097)	Under control	0.000	186
Forage planting area	The middle-aged and elderly generation	0.233*** (0.071)	Under control	0.000	1,293
	Young generation	0.136 (0.144)	Under control	0.021	186
Forage planting duration	The middle-aged and elderly generation	0.648*** (0.221)	Under control	0.000	1,293
	Young generation	0.164 (0.450)	Under control	0.009	186

***, **, and * shows significance level at 1%, 5% and 10%.

4. Conclusion and implications

4.1. Conclusion

Based on the micro-survey data of 1,479 farmers in the farming-pastoral ecstatic zone, this study used the two-stage econometric model to comprehensively and systematically analyze the influence of risk preference on farmers' forage planting behavior and its mechanism from three aspects: forage planting decision, forage planting scale, and planting duration. The heterogeneity of the effects was further investigated from three dimensions: cultivation scale, household differentiation, and intergenerational difference.

The study found the following: First, risk preference has a significant positive effect on farmers' forage planting behavior, that is, risk preference can significantly improve farmers' forage planting probability and increase forage planting scale and duration. This means that farmers who tend to have risk preferences have a higher possibility of forage planting, a higher scale of planting, and a longer planting duration. In the risk-aversion farmers, the forage planting possibility is smaller, the planting scale is smaller, and the planting duration is shorter.

Second, risk preference can affect forage planting behavior through farmers' credit. The more risk preferences farmers have, the more likely they are to participate in credit, the more likely they are to plant forage, the greater the forage planting scale, and the longer planting duration.

Third, the influence of risk preference on the forage planting behavior of farmers was significantly heterogeneous in terms of cultivation scale, household differentiation, and intergenerational difference. Specifically, risk preference significantly promoted the forage planting behavior of farmers with medium cultivation levels but had no significant effect on small and large farmers. Risk preference mainly promoted the forage planting behavior of pure farmers and I-concurrent farmers but had no significant effect on II-concurrent farmers. Risk preference significantly increased the forage planting probability, the forage planting degree, and the planting time of the middle-aged and elderly generation.

4.2. Policy implications

The research conclusion of the article has certain policy significance. First, risk preference can significantly promote the forage planting behavior of farmers. Since most farmers in China are risk-averse farmers and there is a lack of formal risk avoidance mechanisms in rural China, farmers tend to deviate from the profit maximization goal in their production decisions to avoid risks, and they are very cautious when making forage planting decisions. Therefore, to reduce the risk of forage planting for farmers and improve the avoidance mechanism of forage planting, an insurance system suitable for forage planting in China should be constructed.

Second, increase financial support, relax credit amount and credit time constraints on farmers, relieve credit rationing pressure on farmers, promote agricultural insurance and loan linkage mechanism, improve farmers' risk coping ability, promote farmers' participation in credit, and improve their enthusiasm in forage planting. Third, in view of the fact that farmers with a medium breeding degree, pure farmers, I-concurrent, and the elderly generation have a strong dependence on agriculture, the sunk cost of forage planting is high, but the promoting effect of risk preference is relatively strong. This study argues that, on the one hand, more attention and financial support should be given to these groups to ensure that forage planting subsidy funds are timely and fully issued, to reduce the worries of farmers in forage planting. On the other hand, the forage planting publicity and education should be focused on this part of the population to improve their forage planting intention. At the same time, relevant training work should be done to solve the technical bottleneck of forage planting and reduce potential technical risks by innovating training forms and enriching training content.

There are also some limitations in this study. First of all, this study only considers the situation of farmers planting whole silage corn and needs to carry out research on how risk preference affects farmers' participation behavior for different high-quality forage. Second is the limitation of space. The research carried out in this study is based on the micro-survey data of farmers in the agro-pastoral ecotone. So, this study has not observed the participation of farmers' forage behavior in other regions and whether the study conclusion is applicable to the whole of China needs to be further discussed in the follow-up study.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent from the participants was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

WZ: conceptualization, investigation, methodology, data curation, writing—original draft, writing—review, and editing. AK: writing—review and editing. YL and TQ: investigation. MZ: project administration, resources, supervision, writing—review, and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Andries, A. M., Plopeanu, A. P., and Sprincean, N. (2023). Institutional determinants of households' financial investment behaviour across European countries. *Econ. Anal. Policy* 77, 300–325. doi: 10.1016/j.eap.2022.11.015

Belay, A., Recha, J. W., Woldeamanuel, T., and Morton, J. F. (2017). Smallholder farmers' adaptation to climate change and determinants of their adaptation decisions in the Central Rift Valley of Ethiopia. *Agric. Food Secur.* 6, 1–13. doi: 10.1186/s40066-017-0100-1

Chavas, J. P., and Holt, M. T. (1996). Economic behavior under uncertainty: a joint analysis of risk preferences and technology. *Rev. Econ. Stat.* 78, 329–335. doi: 10.2307/2109935

Chen, L. K., Du, L. X., Wang, Y. C., Ren, Q. Z. X., Bian, Z. G., and Li, H. Q. (2012). Current situation analysis and industrialization development of herbage seed. *J. Grassland Forage Sci.* 7, 46–49.

Chen, Y. P., Zheng, A. R., Sun, X., Lin, J. X., Xiao, J. N., Liu, B. S., et al. (2018). Effects of whole corn silage and corn stalk silage treated by different methods on growth performance and economic benefits of beef cattle. *Chinese J. Animal Nutr.* 30, 2571–2580. doi: 10.3969/j.issn.1006-267x.2018.07.017

Cheng, G. L., Qiu, J., Wang, X. G., Xu, T. J., Chen, C. J., Zhang, C. Y., et al. (2022). Changes of agronomic characters, biological yield and quality of silage maize combination (variety). *J. Agr. Sci. Tech-Iran.* 24, 30–37. doi: 10.13304/j.nykjdb.2021.0453

Cheng, Q. W., Yu, R., Yu, W. F., and Chen, Q. (2021). Research on the impact of different types of livelihood strategies on farmers' willingness to choose forest species: based on the survey data of 477 Farmers in Fujian Province. *Eco. Econ.* 37, 119–124.

Dong, W. L., Wang, X. B., and Yang, J. (2015). Future perspective of China's feed demand and supply during its fast transition period of food consumption. *J. Integr. Agr.* 14, 1092–1100. doi: 10.1016/S2095-3119(14)60992-8

Du, Z. X., and Han, L. (2020). Research on the impact of supply-side production changes on China's food security. *China Rural Econ.* 10, 2–14.

Feng, L., Zhang, M. H., Li, Y. X., and Jiang, Y. (2020). Satisfaction principle or efficiency principle? Decision-making behavior of peasant households in China's rural land market. *Land. Use Policy* 99, 104943. doi: 10.1016/j.landusepol.2020.104943

Fu, L. S., Wang, S. G., and Qin, T. (2022). Income-increasing effect of agricultural insurance and its mechanism. *China Popul. Resour. Environ.* 32, 153–165.

Gao, H. X. (2020). *Empirical study on planting decision behavior of forage grass producers in China*. Chinese academy of agricultural sciences thesis. Dissertation. Beijing: Chinese academy of agricultural sciences.

Gine, X., and Yang, D. (2009). Insurance, credit, and technology adoption: field experimental evidence from Malawi. *J. Dev. Econ.* 89, 1–11. doi: 10.1016/j.jdeveco.2008.09.007

Guo, Q. H. (2019). Ecological care under the action of "grain to feed"——Based on the discussion of the quality of cultivated land in the main grain producing areas of Northeast China. *Agric. Econ. Issues.* 21, 89–99. doi: 10.13246/j.cnki.iae.2019.10.008

Guo, S. J., Hu, T. H., Hu, X. D., and, S. U., Y. (2020). Where should the "food-fodder change" subsidy policy go? Based on the simulation of beef cattle farmers in the pilot area. *Issues Agri. Econ.* 14, 101–110. doi: 10.13246/j.cnki.iae.2020.09.010

Hasibuan, A. M., Gregg, D., and Stringer, R. (2022). Risk preferences, intra-household dynamics and spatial effects on chemical inputs use: Case of small-scale citrus farmers in Indonesia. *Land. Use Policy* 122: 106323. doi: 10.1016/j.landusepol.2022.106323

Holt, C. A., and Laury, S. K. (2002). Risk aversion and incentive effects. *Am. Econ. Rev.* 92, 1644–1655. doi: 10.1257/000282802762024700

Huo, L. M., Li, Y., Dong, L. X., Zheng, B. Q., Li, A. J., and Tang, S. N. (2022). Effects of three treatments on silage corn feeding beef cattle. *China Cattle Sci.* 48, 14–16.

Imail, K. S., Gaiha, R., Thapa, G., and Annim, S., k. (2012). Microfinance and poverty- a macro perspective. *World Dev.* 40, 1675–1689. doi: 10.1016/j.worlddev.2012.04.013

Irungu, P. M. (1998). *An economic analysis of factors affecting the adoption of Napier grass in smallholder dairying in Kiambu district, Kenya.* (Doctoral dissertation).

Javeed, M. A., Veeranna, K. C., Thirumalesh, T., Rathod, P., Gopala, G. T., and Biradar, C. (2020). Attitude of dairy farmers towards cultivation of green fodder crops in north eastern transition zone of Karnataka, India. *J. Livestock Res.* 10, 169–173. doi: 10.5455/ijlr.20200305080442

Joshi, A., Dencher, J. C., Franz, G., and Martocchio, J. J. (2010). Unpacking generational identities in organizations. *Acad. Manage. Rev.* 35, 392–414. doi: 10.5465/AMR.2010.51141800

Kahnemand, D., and Amos, T. (1984). Choice, values and frames. *Am. Psychol.* 39, 341–350. doi: 10.1037/0003-066X.39.4.341

Khanal, A. R., Mishra, A. K., and Kumar, A. (2019). Risk preference and adoption of risk management strategies: evidence from high-value crop production in emerging economy. *Agric. Appl. Econ. Assoc.* 25, 17. doi: 10.22004/ag.econ.291285

Li, S., An, P., Pan, Z., Wang, F., Li, X., and Liu, Y. (2015). Farmers' initiative on adaptation to climate change in the northern agro-pastoral ecotone. *Int. J. Disast. Risk Res.* 12, 278–284. doi: 10.1016/j.ijdrr.2015.02.002

Li, X. L., Yang, L. X., Tian, W., Xu, X. F., and He, C. S. (2018). Land use and land cover change in agro-pastoral ecotone in Northern China: A review. *Chinese J. Appl. Ecol.* 29, 3487–3495. doi: 10.13287/j.1001-9332.201810.020

Li, Z. Q., and Liu, C. L. (2010). Nutritional value of silage and its application in ruminant production. *J. Domest. Animal Ecol.* 31, 95–98. doi: 10.19556/j.0258-7033.20230424-01

Liu, E. M., and Huang, J., k. (2013). Risk preferences and pesticide use by cotton farmers in China. *J. Dev. Econ.* 103, 202–215. doi: 10.1016/j.jdeveco.2012.12.005

Luo, B. L. (2020). Small household operation, function transformation, strategy options: how can small household incorporate into the modern agricultural development pattern? *Issues Agri. Econ.* 12, 29–47. doi: 10.13246/j.cnki.iae.2020.01.004

Mao, H., Fu, Y., Peng, P., and Chai, Y. J. (2022). Farmers' risk aversion and adoption behavior of climate adaptation technology: evidence from cotton farmers in Xinjiang, China. *China Rural Surv.* 3, 126–145.

Meng, X. Y., Tan, X. C., Wang, Y., Wen, Z. G., Tao, Y.uan., and Qian, Y. (2019). Investigation on decision-making mechanism of residents' household solid waste classification and recycling behaviors. *Resour. Conser. Recy.* 140, 224–234. doi: 10.1016/j.resconrec.2018.09.021

Nong, Y. X., Qian, X. P., Yin, C. B., Wang, Y. Q., and Fu, L. B. (2021). Analysis of the effect of the cycle mode of grass-growing and planting in orchards—Based on a comparative study of orchards in Luxi County, Yunnan. *China Agric. Resour. Reg. Plann.* 42, 7–14. doi: 10.7621/cjarrp.1005-9121.20211102

Pan, D., He, M. M., and Kong, F. B. (2020). Risk attitude, risk perception, and farmers' pesticide application behavior in China: A moderation and mediation model. *J. Clean. Prod.* 276, 124241. doi: 10.1016/j.jclepro.2020.124241

Qiao, F. B., and Huang, J. K. (2021). Farmers' risk preference and fertilizer use. *J. Integr. Agr.* 20, 1987–1995. doi: 10.1016/S2095-3119(20)63450-5

Ren, J. Z. (2013). China's traditional agricultural structure does not change not to die - nine consecutive increases of grain after the hidden concern. *Acta Pratac. Sinica* 22, 1–5. doi: 10.11686/cyxb20130301

Salimonu, K. K., and Falusi, A. O. (2007). Risk preferences and resource allocation differentials of food crop farmers. *J. Rural Econ. Dev.* 16, 1–12. doi: 10.22004/ag.econ.147513

Shi, W. J., Wang, M. L., and Liu, Y. T. (2021). Crop yield and production responses to climate disasters in China. *Sci. Total Environ.* 750, 141147. doi: 10.1016/j.scitotenv.2020.141147

Shi, Z. Z., and Hu, X. D. (2022). The impact of planting structure adjustment on China's feed grain supply and demand and livestock and poultry breeding. *Resour. Sci.* 44, 2567–2579. doi: 10.18402/resci.2022.12.14

Sun, X. L., and Guo, P. (2016). The impact of risk aversion on farmer's behavior of land transfer: evidence from Jilin Province, Shandong Province, Shaanxi Province and Hunan Province. *China Land Sci.* 30, 35–44.

Tan, Y. F., Lu, Q., and Zhang, S. X. (2022). Can contract farming promote farmers' green production transition? *J. Agrotech. Econ.* 2, 16–33. doi: 10.13246/j.cnki.jae.20220428.001

Visser, M., Jumare, H., and Brick, k. (2020). Risk preferences and poverty traps in the uptake of credit and insurance amongst small-scale farmers in South Africa. *J. Econ. Behav. Organ.* 180, 826–836. doi: 10.1016/j.jebo.2019.05.007

Vollenweider, X., Di, F. S., and O'Donoghue, C. (2011). *Risk preferences and voluntary agri-environmental schemes: does risk aversion explain the uptake of the rural environment protection scheme?* Working paper. 726-2016-49886. doi: 10.1115/IMECE2011-64074

Wang, M. L. (2015). New ideas to solve the problem of food security effectively: Focus on the development of forage industry. *Chinese Rural Econ.* 5, 63–74.

Wang, W. (2021). Improving China's alfalfa industry development: an economic analysis. *China Agr Econ. Rev.* 13, 211–228. doi: 10.1108/CAER-07-2019-0128

Wang, W. X., Cai, S. P., and Wang, G. (2015). Empirical analysis of factors affecting alfalfa plantation behavior of farmers in Huang-huai-hai region. *Trans. Chinese Soc. Agric. Eng.* 31, 284–290. doi: 10.3969/j.issn.1002-6819.2015.zl.034

Wang, X. Q., and Zhao, X. Y. (2023). Farmers' perception and choice preference of grassland ecosystem services: Evidence from the northeastern region of the Qinghai-Tibet Plateau. *Land Use Policy* 132, 1006768. doi: 10.1016/j.landusepol.2023.106768

Wang, Y. R., Sun, F., and Zhang, H. X. (2018). An empirical analysis of the combination of crop farming and livestock breeding of farmers in the background of food reform. *J. Hebei North Univ.* 34, 76–81. doi: 10.3969/j.issn,2095-462X.2018.06.018

Wen, Z. L., and Ye, B. J. (2014). Mediation effect analysis: Methodology and model development. *Adv. Psychol. Sci.* 22, 731–745. doi: 10.3724/SP.J.1042.2014.00731

Wossen, T., Berger, T., and Di Falco, S. (2015). Social capital, risk preference and adoption of improved farm land management practices in Ethiopia. *Agr. Econ.* 46, 81–97. doi: 10.1111/agec.12142

Wu, H., and Li, J. (2023). Risk preference, interlinked credit, and insurance contracts, and agricultural innovative technology adoption. *J. Innov. Knowl.* 8, 100282. doi: 10.1016/j.jik.2022.100282

Xu, L. F., Jin, W. D., and Chen, K. (2020). Risk preference, information acquisition and pig-breeding pollution exposure behaviors: an empirical analysis based on 1489 pig breeders survey data. *China Rural Surv.* 4, 129–144.

Yang, T., Dong, J. W., Huang, L., Li, Y. Z., Yan, H. M., Zhai, J., et al. (2023). A large forage gap in forage availability in traditional pastoral regions in China. *Fundam. Res.* 3, 188–200. doi: 10.1016/j.fmre.2023.01.003

Yu, L. L., Chen, C., Niu, Z. H., Gao, Y., Yang, H. R., and Xue, Z. H. (2021). Risk aversion, cooperative membership and the adoption of green control techniques: Evidence from China. *J. Clean Prod.* 279, 123288. doi: 10.1016/j.jclepro.2020.123288

Zhang, H. P., Wang, D. X., and Yang, Y. F. (2018). Ecological reward and compensation for returning grazing land to grassland and famers' alternative behavior within planting and breeding. *Issues Agri. Econ.* 23, 118–128. doi: 10.13246/j.cnki.iae.2018.07.008

Zhang, Y. J., Reng, J. Z., Wang, M. L., and Yang, G. W. (2013). Discussion on the Position and Development Distribution of Forage Industry in China's Agricultural Industry Structure. *J. Agric. Sci. Technol.* 15, 61–71. doi: 10.3969/j.issn.1008-0864.2013.04.11

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Tropical range grasses can sustain soil functions despite nutrient depletion in semiarid degraded land

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Soil function encompasses numerous functions and services the soil provides and can be measured using physical, chemical, and biological characteristics. In any event, research on fertility and biological activity is increasingly being stressed as indices of ecosystem services. Rangeland degradation is one of the world's most widespread land use changes, with serious consequences for soil processes and ecosystem services. The influence of uncontrolled grass cover on soil functioning in semiarid environments is little understood. In the current study, the following seven varieties of grass coverings were studied in a semiarid area of central India: *Cenchrus ciliaris* (CC), *Megathyrsus maximus* (MM), *Chrysopogon fulvus* (CF), *Heteropogon contortus* (HC), *Sehima nervosum* (SN), *Vetiveria zizanioides* (VZ), and Tri-Specific Hybrid (TSH). The carbon accumulation proficiency of TSH, MM, and HC was ∼2.23, 2.17, and 2.07 times greater than fallow. The nutrient cycling capacity of grasses was ∼1.1 to 1.6 times greater than that of fallow, despite the grasses' depleted macronutrients in the soil. The biological activity under MM and HC was ∼1.23 and 1.67 times higher than fallow. Overall soil functionality under TSH and HC was 83 and 25% greater than MM, respectively. These soil functions augment ecosystem services like climate regulation, biomass production, nutrient recycling, water, and airflow. However, more attention should be paid to the management of inputs for greater ecosystem services from this grass cover in semiarid, degraded land.

KEYWORDS

grass cover, biomass productivity, nutrient recycling, biological activity, climate regulation

Highlights

- The nutrient cycling capacity of grasses was ∼1.1 to 1.6 times greater than that of fallow.
- The carbon accumulation proficiency of grasses improved by ∼2 times over fallow.
- Soil functionality under TSH was 83% greater than that under *Megathyrus*.
- Grass-augmented services such as climate regulation, biomass production, and nutrient recycling.

1. Introduction

Soils, also designated as "reserves of natural capital", serve many ecosystem services. To verify the role of soil in the sustainability of ecosystems, researchers have developed the idea of soil function (Pulleman et al., 2012; Baradwal et al., 2022). The capacity of soil to support microbial activity, the delivery of nutrient elements, structural stability, and support for biomass production are the prime qualities of soil functioning. As most ecosystem services could be linked to soils, restoring degraded land's potential to support soil functions and ecosystems should be a conservation goal (Perring et al., 2015).

Low rainfall, inefficient crop water and nutrient usage, and poor soil fertility are key challenges in semiarid regions (Wani et al., 2007), leading to below-average yields, little agricultural revenue, little organic carbon, and insufficient nutritional inputs in the region (Nosetto et al., 2006; Wani et al., 2007). Growing range grasses with a high capacity for sequestering carbon might therefore be a solution for semiarid areas (Li et al., 2017; Notenbaert et al., 2021). The capacity of grasses to bind soil particles, reduce erosion, establish themselves quickly, and create humus makes them more remarkable (Ola et al., 2015). The influence of different grasses on certain soil parameters was described by earlier workers (Wilekson et al., 2010). As a result, grasses can be paired with mechanical techniques to provide soil protection (Prajapati et al., 1973). Water cycling, carbon sequestration, nitrogen cycling, gas exchange with the environment, climate change mitigation, and aesthetic enjoyment are all critical activities of rangeland grasses in dry and semiarid environments (Franzluebbers, 2012; Fuglie et al., 2021). In semiarid regions, the primary stable food source for the majority of ruminants worldwide is grasses; they contain higher carbohydrate content but lower levels of protein, minerals, vitamins, and lipids than legumes (Tambara et al., 2017; Waliszewska et al., 2021). Despite this, grasses are the most frequently used fodder.

The primary ecosystem services of soil are carbon sequestration, food or biomass production, provision of microbial habitat, nutrient recycling, etc. (Tellen and Yerima, 2018). However, the actual magnitude of soil functions provided by range grasses has never been quantified. Moreover, there is little data on the impact of range grasses on soil functional indicators in semiarid regions. The goal of this study was to assess the impact of range grasses on soil functionality and establish a strong linkage with ecosystem services in semiarid ecosystems in central India. Three research issues are addressed in this study: (1) Do the soil fertility levels and nutrient supply capacity of the soil change differently for various grasses? (2) Under which grasses are microbial communities and soil biota most active? (3) How does the range of grasses impact the soil functionality in various soil layers? In response to these inquiries, we postulated that range grasses would boost carbon sequestration, food or biomass production, provision of microbial habitat, and nutrient recycling at various soil depths (i.e., 0–15 and 16–30 cm). Our findings are anticipated to help us boost ecosystem services, optimize land cover plans, and better understand how soil operates under various range grasses.

2. Materials and methods

2.1. Experimental site description

The study was carried out in India's Jhansi District (Figure 1). The study site is located in the Bundelkhand area of India. The soil type at the experimental site was sandy loam. They are shallow, well-drained, and vary in color from yellowish red to dark brown. A field experiment was carried out between 2015 and 2022 at the ICAR-Indian Grassland and Fodder Research Institute's Technology Demonstration Unit (25° 31′ 36.33′′ N lat, 78° 32′ 54.18′′ E long, and altitude 177 m above mean sea level). The research location is located in a semiarid environment with high temperatures, irregular rainfall, a high aridity index, and inadequate soil moisture. The study area's long-term average annual rainfall (1939–2015) is 908 mm, with most of it falling between June and August (Rai et al., 2018). The climate here is characterized by dry air, an excessively hot summer, and a chilly winter (late November to the middle of March). From July to September, the southwest monsoon occurs. The average annual rainfall (2015–2023) in the district is 840 mm. Approximately 90% of it occurs during the southwest monsoon, and the rest occurs throughout the year. Rainfall patterns in the Bundelkhand area are variable, resulting in cyclic droughts. The average daily maximum and lowest temperatures in January were 21.34 and 6.84°C, respectively (the coldest month). The average daily maximum temperature in May from 2015 to 2020 was 41.48°C (the warmest month). Peak temperatures in May and June can reach 48°C. The month of June had the highest mean daily evaporation (12.80 mm day^{-1}).

2.2. Factors and level of land degradation

Wind erosion and a lack of fertile soil are the reasons that make the land susceptible to land degradation. All characteristics were compared to a fallow land to remove any room for doubt (located on a comparable slope, topography, soil texture, and parent material). In terms of soil nutrients and biological characteristics, we considered that fallow land had experienced minimal changes. We also assumed that climate had a comparable impact on the soils of alternate land use systems (ALUS) and fallow land, respectively. The soil texture at the study site comprised sandy loam and was a member of the Typic Haplustepts hypothermic family. They range from pale yellowish-red to dark brown. The soil lacked mineral N (183 kg ha^{-1}), low SOC (0.35%), low plant-available P (9 kg ha^{-1}), and medium K contents (250 kg ha^{-1}). These soils have a modest ability to retain nutrients and water. The soil's ability to store water at saturation level was 32.5% (v/v). Throughout May and July, the average wind speed is >8 km/h, which results in soil erosion of between 35 and 50 Mg ha^{-1} yr^{-1}.

2.3. Experimental details

Seven perennial tropical range grasses, namely *Cenchrusciliaris* (CC), *Megathyrsus maximus* (MM), *Chrysopogon fulvus* (CF), *Heteropogon contortus* (HC), *Sehima nervosum* (SN), *Vetiveria*

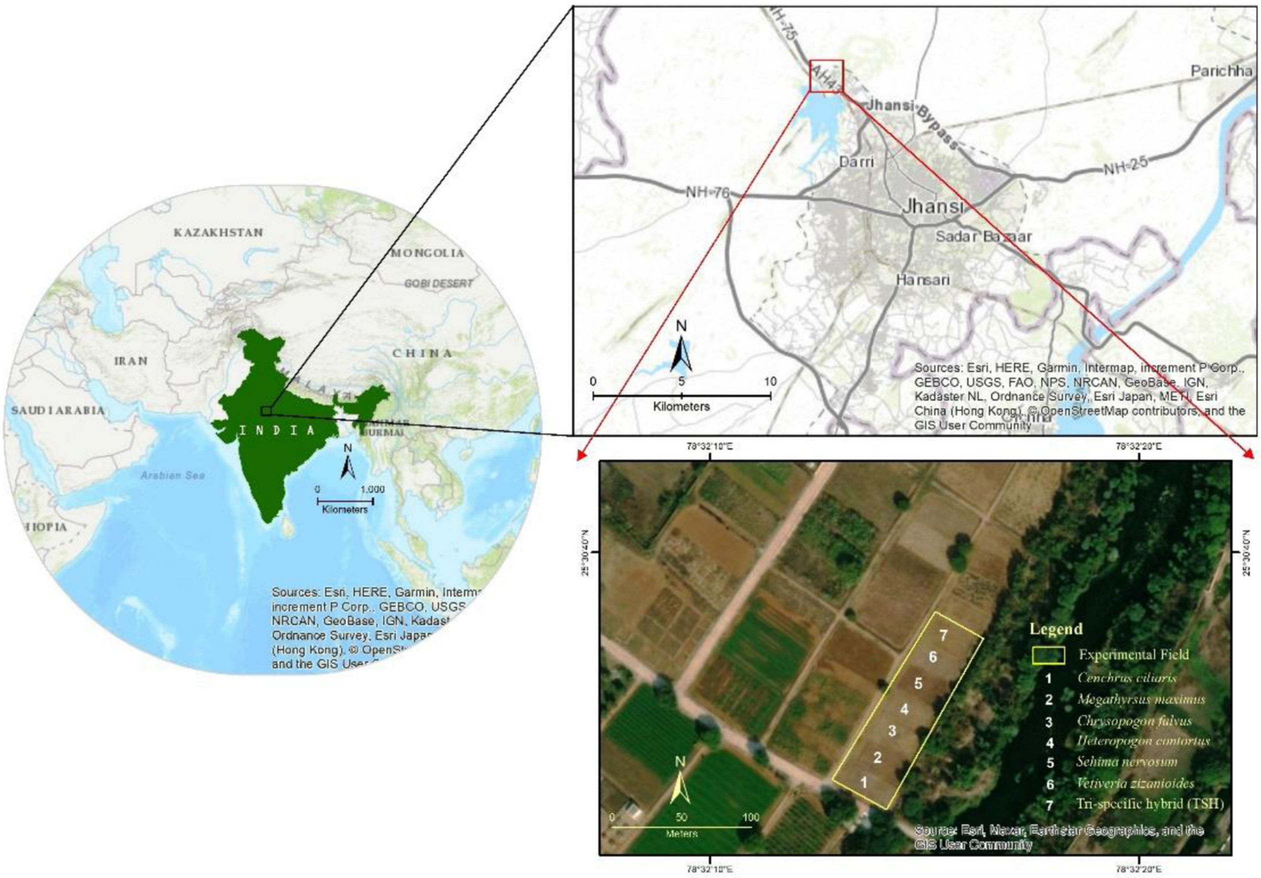

FIGURE 1
Geographic location of the experimental site.

zizanioides (VZ), and Tri-Specific Hybrid (TSH: *Pennisetum glaucum x Pennisetum purpureum x Pennisetum squamulatum*) were evaluated in this study. The grass seedlings were transplanted to the field on 15th July 2015. At the establishment stage, 2–3 irrigations were permitted to ensure uniformity and were afterward maintained in rainfed circumstances. Every year on 15th November, the grasses were trimmed 5 cm from the root and left to grow during the wet season (June–July). The biomass production of grasses was estimated using a uniform plot size of 10 × 15 m.

2.4. Collection of soil and plant samples

Areas with comparable physiography, geology, and climatic conditions were chosen to create a homogenous sampling arrangement. In each of the grasses, three locations were selected. During winter, after cutting grass in October 2021, five soil samples (from the four corners and the center of the plots) were collected from two layers of 0–15 and 16–30 cm at each location. Soil samples were also collected from fallow land. Soil samples were immediately taken to the laboratory and divided into two halves: one half was air-dried and utilized for physical and chemical analyses, while the other half was maintained at 4°C for microbiological property evaluation. The plants were harvested, kept in perforated brown paper bags, suitably labeled, and dried to attain constant weight in a thermostatically controlled oven at $70 \pm 2^\circ$C (Ghosh et al., 2019). The dry weight was recorded and expressed in terms of dry matter accumulation by plants (g m^{-2}). Root morphology was studied using a root analyzer coupled with Win-Rhizo.

2.5. Analysis of soil physical, chemical, and biological parameters

Soil particle size distribution (soil texture), bulk density, pH, and electrical conductivity were measured (Jackson, 1973). Soil organic carbon (SOC) and nitrogen were determined using chromic acid oxidation and permanganate oxidation. Available phosphorus (P) was determined by $NaHCO_3$ extraction (Olsen, 1954), followed by spectrophotometric estimation. Available potassium (K) was extracted using the acetate salt of ammonium (Hanway and Heidel, 1952). The availability of micronutrients (iron, manganese, zinc, and copper) was determined using diethylene triaminepenta acetic acid. Microbial biomass C was measured with 45% extraction efficiency (Jenkinson and Powlson, 1976). Important nutrient-cycling enzymes, such as the activities

TABLE 1 Relative evaluation of ecosystem services from different grass covers in this study.

Type of ecosystem service	Ecosystem services	Soil functions	Indicators
Provisioning	Biomass production	Grass productivity	Dry matter yield
Regulating	Climate regulation	Carbon storage	SOC concentration and carbon accumulation proficiency
	Purification of soil air	Aeration	Porosity
Supporting	Nutrient cycling	Nutrient supply capacity	The concentration of available nutrients
	Microbial habitat provision	Biological activity	Soil enzyme activity

of β-D-glucosidase, urease, and alkali phosphatase, were assessed (Dick, 2020).

2.6. Computation of indices of soil functionality for understanding ecosystem services

The pertinent ecosystem services and their indicator soil parameters have been listed in Table 1. Soil functionality was thought to be a function of nutrient supply capacity, carbon accumulation proficiency, biomass output, and microbial activity. It was calculated to determine the total potential of range grasses to boost soil functions.

A biological activity index was computed by using a formula (Ghosh et al., 2021).

$$\mathrm{BAI} = \frac{1}{4}\sum_{\mathrm{i}=1}^{4} \mathrm{Pi}\ldots, \tag{1}$$

where Pi is the ratio of the activity of the i^{th} enzyme in experimental soils to that in fallow land. BAI is typically calculated as the average of all enzyme activity responses in cultivated land over fallow ground.

Nutrient supply capacity (NSC), a measure of nutrient dynamics, was calculated (Baradwal et al., 2022).

$$\mathrm{NSC} = \frac{1}{7}\sum_{\mathrm{i}=1}^{7} \mathrm{Ni}\ldots \tag{2}$$

Ni is the proportion of the availability of ith nutrient in ALUS to that in fallow.

Carbon accumulation proficiency (CAP), a measure of ecosystem carbon sequestration, was calculated (Baradwal et al., 2022).

$$\mathrm{CAP} = \frac{\mathrm{Cr}}{\mathrm{Cf}}\ldots \tag{3}$$

Cr is SOC accumulation in restored land, and Cf is SOC accumulation in fallow land. The biomass yield of grasses was normalized using a Z-score.

$$\mathrm{Z} = \frac{\mathrm{x}-\mathrm{u}}{€}\ldots \tag{4}$$

x is the biomass yield of grass, μ is the mean yield of grasses, and € is the standard deviation of grass yield.

Soil functionality (SF) was estimated as (Baradwal et al., 2022).

$$SF = 10^{\log\,(NSC\times BAI\times CAP\times Z)}\ldots \tag{5}$$

2.7. Statistical analysis

The collected data were processed for variance analysis (ANOVA) as applicable to one-way ANOVA to examine for differences among the range grasses specified by Gomez and Gomez (1984). Tukey's honest significant difference test ($P < 0.05$) was employed as a mean separation test. Node analysis was performed to understand the contribution of each grass to specific ecosystem services. The figures were created using MS Office Excel 2020–2021.

3. Results

3.1. Biomass productivity and root growth of grasses

The TSH produced the highest fodder yield. It had ∼138, 87, and 64% greater yields than SN, VZ, and HC. The yield of CC and CF was similar. However, the yield of MM was ∼101, 58, and 39% greater than that of SN, VZ, and HC, respectively. The biomass productivity of SN was the lowest (Table 2). Important qualities such as root length density (RLD) and root surface density (RSD) were highest for *Vetiveria zizanoides* grass (64.71 cm cm^{-3} and 8.59 cm^2 cm^{-3}, respectively), followed by *Hetropogon contortus* and *Chrysopogon fulvus* grass. However, the root weight density (RWD) was higher under TSH grass (117.01 mg cm^{-3}), followed by *Megathyrsus maximus* grass (110.07 mg cm^{-3}), and the lowest was observed in *Cenchrus ciliaris* (79.53 mg cm^{-3}), whereas *Megathyrsus maximus* had the lowest RLD (39.73 cm cm^{-3}), and RSD (6.07 cm^2 cm^{-3}). Similarly, the highest number of root tips was recorded for *Megathyrsus maximus* grass (9,115.25), followed by *Vetiveria zizanoides* grass (6,944.50). The average root diameter of different grasses varied from 0.30 to 0.46 mm. The maximum root diameter was recorded for *Sehima nervosum* grass (0.46 mm), followed by TSH grass (0.38 mm) and *Vetiveria zizanoides* grass (0.38 mm) (Table 2). In the case of root volume, the highest root volume (157.49 cm^3) and root dry weight (23.07 g plant^{-1}) were observed for *Megathyrsus maximus* grass, while the lowest values were with *Heteropogon contortus* and *Cenchrus ciliaris* grass.

TABLE 2 Root morphological traits [root length density (RLD), root weight density (RWD), and root surface density (RSD)] and biomass productivity of different tropical perennial range grasses in semiarid India.

Grasses	RLD (cm cm^{-3})	Root dry weight (g plant^{-1})	RWD (mg cm^{-3})	RSD (cm^2 cm^{-3})	Estimated volume (cm^3)	Dry matter production (Mg ha^{-1})
#CC	45.23bc	10.55e	79.53d	6.67d	118.13e	7.76b
MM	39.73c	23.07a	110.07b	6.07e	157.49a	9.83ab
CF	47.52b	11.68d	83.72c	8.06b	124.91d	7.83b
HC	48.37b	9.47f	84.51c	7.40c	110.72f	7.09b
SN	41.88bc	11.57d	86.21c	7.32c	122.62d	4.88c
VZ	64.71a	19.43b	80.90d	8.59a	141.30b	6.21bc
TSH	45.50bc	15.46c	117.01a	6.70d	128.50c	11.65a

This means that similar lower-case letters within a column are not significantly different according to Tukey's HSD test ($p < 0.05$).

#CC, *Cenchrus ciliaris*; MM, *Megathyrsus maximus*; CF, *Chrysopogon fulvus*; HC, *Heteropogon contortus*; SN, *Sehima nervosum*; VZ, *Vetiveria zizanioides*; TSH, tri-specific hybrid.

TABLE 3 Bulk density, porosity, soil moisture content, and penetration resistance of soil under different tropical perennial range grasses at the surface (SL; 0–15 cm) and subsurface (SSL; 16–30 cm) soil layers in semiarid India.

Grass	Bulk density (Mg m^{-3})		Porosity (%)		Soil moisture (%)		Penetration resistance (kPa)
	SL	SSL	SL	SSL	SL	SSL	0–30 cm
#CC	1.66cd	1.76c	37.44ab	33.40b	8.44b	7.83c	1004cd
MM	1.65d	1.87b	37.71ab	29.47bc	6.38c	6.37c	1102bc
CF	1.70c	1.81c	35.75b	31.75bc	11.51a	8.89b	1137b
HC	1.58e	1.63d	40.28a	38.54a	11.92a	10.78a	1132b
SN	1.66cd	1.80c	37.47ab	32.04ab	8.93b	8.15b	1072c
VZ	1.75b	1.79c	34.07bc	32.55ab	12.9a	11.86a	935d
TSH	1.55e	1.54e	30.13c	30.66bc	11.91a	11.65a	840d
F	1.88a	1.92a	28.87c	27.69c	5.51d	5.62d	1386a

This means that similar lower-case letters within a column are not significantly different according to Tukey's HSD test ($p < 0.05$).

#CC, *Cenchrus ciliaris*; MM, *Megathyrsus maximus*; CF, *Chrysopogon fulvus*; HC, *Heteropogon contortus*; SN, *Sehima nervosum*; VZ, *Vetiveria zizanioides*; TSH, tri-specific hybrid; F, fallow.

3.2. Soil bulk density, porosity, moisture content, and penetration resistance

The plots under VZ, HC, TSH, CF, SN, CC, and MM had ∼ 134, 116, 116, 108, 62, 53, and 15.78% higher soil moisture than fallow land in the 0–15 cm soil surface. However, the VZ, HC, TSH, CF, SN, CC, and MM had ∼ 111, 107, 91, 58, 45, 39, and 13%, respectively, superior soil moisture as compared to fallow land at the 16–30 cm soil layer, while the moisture of the MM plot was comparable to the moisture of fallow land in both soil layers (Table 3).

At the surface and subsurface soil, porosity increased by nearly 4–40% under grass cover. At the 0–15 cm layer, porosity under MM and HC was ∼27 and 37% higher than TSH, whereas, at the 16–30 cm layer, HC and TSH had ∼40 and 11% greater porosity than fallow. At the 0–15 cm soil surface, the soil under HC had a 15% lower bulk density than the fallow land (Table 3). However, at the 16–30 cm soil layer, HC, MM, SN, CC, and CF had ∼16, 13, 12, 12, and 10% lower soil bulk density than fallow land (Figure 2). The plots under TSH, VZ, SN, CC, HC, MM, and CF had ∼39, 33, 23, 13, 11, 6, and 4% higher soil penetration resistance than fallow land (Table 3).

3.3. Soil organic C and nutrient availability

Range grasses significantly improved the SOC status at both soil layers over fallow land. The soil under TSH had ∼35, 19, and 17% greater SOC than VZ, SN, and CF at the soil surface. However, the soil under HC had ∼26, 10, and 8% greater SOC than VZ, SN, and CF, respectively (Figure 2). At the 16–30 cm soil layer, soils under all grasses had similar SOC, except MM. The range grasses either depleted or improved the soil mineral N in the surface layer. However, N under VZ and SN was depleted by ∼11 and 6%, respectively, compared to fallow land. However, the N status under TSH, HC, and MM was maintained at the soil surface. Interestingly, at the subsurface, TSH and SN improved mineral N status by ∼33 and 24% over fallow. All other range grasses except CC and VZ significantly improved the mineral N status at the soil subsurface (Figure 2). Range grasses significantly reduced the available P at the soil surface. The CF, HC, SN, VZ, and TSH depleted P by ∼24, 42, 20, 60, and 26% over fallow. At the 16–30 cm soil layer, CC, MM, CF, and TSH improved soil P status by 73, 37, 11, and 9% over fallow. Soil P availability declined by ∼35 and 16% under HC and SN over fallow. All the range grasses depleted soil K remarkably over the fallow

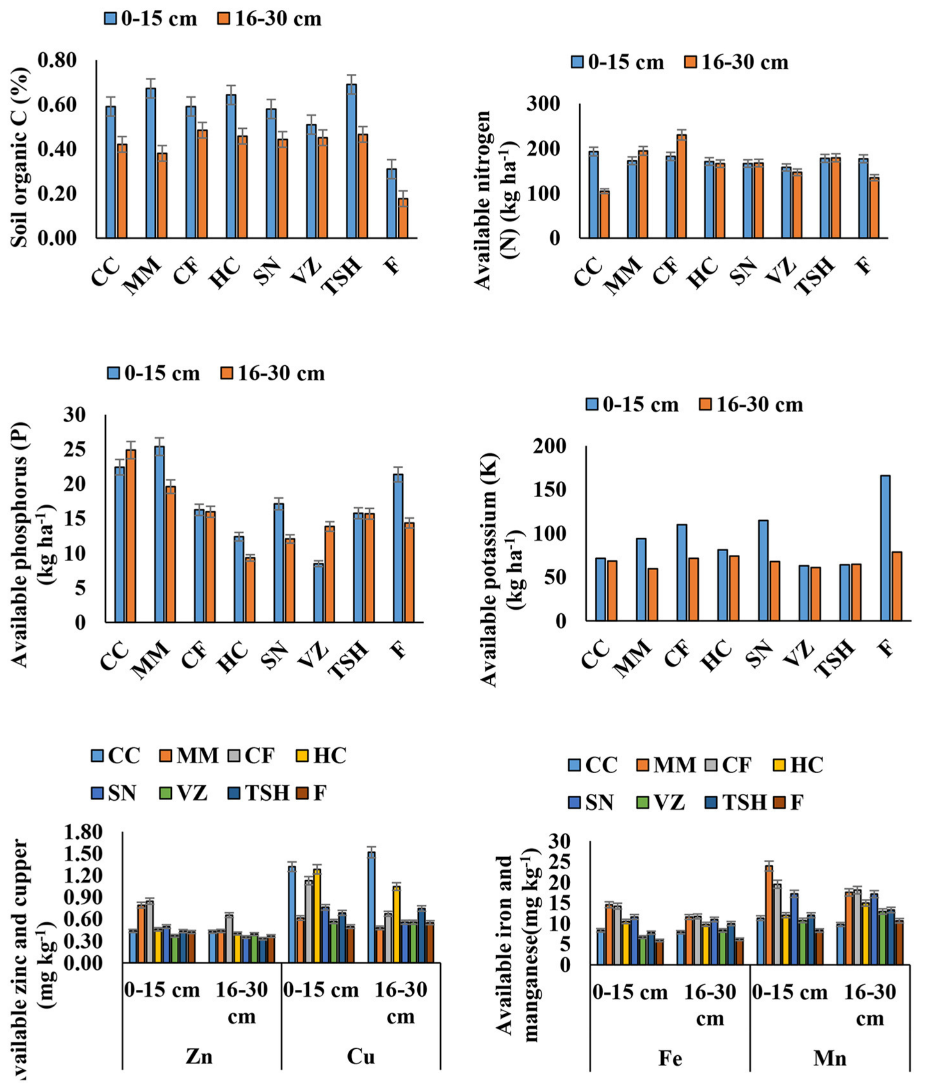

FIGURE 2
Nutrient availability in soil under different tropical perennial range grasses in semiarid India. The error bars indicate the LSD value according to Tukey's HSD test ($p < 0.05$). CC, *Cenchrus ciliaris*; MM, *Megathyrsus maximus*; CF, *Chrysopogon fulvus*; HC, *Heteropogon contortus*; SN, *Sehima nervosum*; VZ, *Vetiveria zizanioides*; TSH, tri-specific hybrid; F, fallow.

period. The VZ and TSH contributed to the highest depletion of K at the surface and subsurface layers. The concentrations of micronutrients (Fe, Mn, Zn, and Cu) were mostly improved by range grasses at both soil layers, although a few trend breakers were present (Figure 2).

3.4. Soil microbial activity

Soils under TSH, HC, VZ, MM, and CF had ∼130, 119, 90, 70, and 50% higher urease enzyme activity than fallow land in a 0–15 cm soil layer. However, CC and SN had ∼30 and 29% lower urease enzyme activity than fallow land in a 0–15 cm soil layer (Figure 3). At the 16–30 cm soil layer, TSH, HC, and SN had ∼93, 66, and 31% higher urease enzyme activity than fallow land. However, CF, MM, CC, and VZ had ∼57, 50, 50, and 49% lower urease enzyme activity than fallow land. Soils under VZ had ∼15% lower alkaline phosphatase enzyme activity than fallow land in a 0–15 cm soil layer. However, MM had ∼22% lower alkaline phosphatase activity than fallow land. The soils under HC, CF, and MM had ∼192, 58, and 45%, respectively, more DHA enzyme activity than fallow land in 0–15 cm soil layers. However, SN, VZ, CC, and TSH had ∼80, 70, 44, and 42%, respectively, less DHA enzyme activity than fallow land in a 0–15 cm soil layer. At a 16–30 cm soil layer, CF had ∼45% higher DHA enzyme activity than fallow land (Figure 3). However, CC, TSH, MM, SN, VZ, and HC had ∼93, 91, 90, 60, 55, and 15%, respectively, lower DHA enzyme activity than fallow land. In the 16–30 cm soil layer, TSH, SN, and CF had ∼68, 57, and 34% higher β- glucosidase enzyme activity than fallow land. However, MM, VZ, CC, and HC had ∼74, 58, 34, and 11% lower β-glucosidase enzyme activity than fallow land, respectively. The soils under CF, HC, SN, MM, TSH, and CC had ∼173, 135, 63, 58, 22, and 5% higher β-glucosidase enzyme activity than fallow land in 0–15 cm soil layer. However, VZ had ∼34%

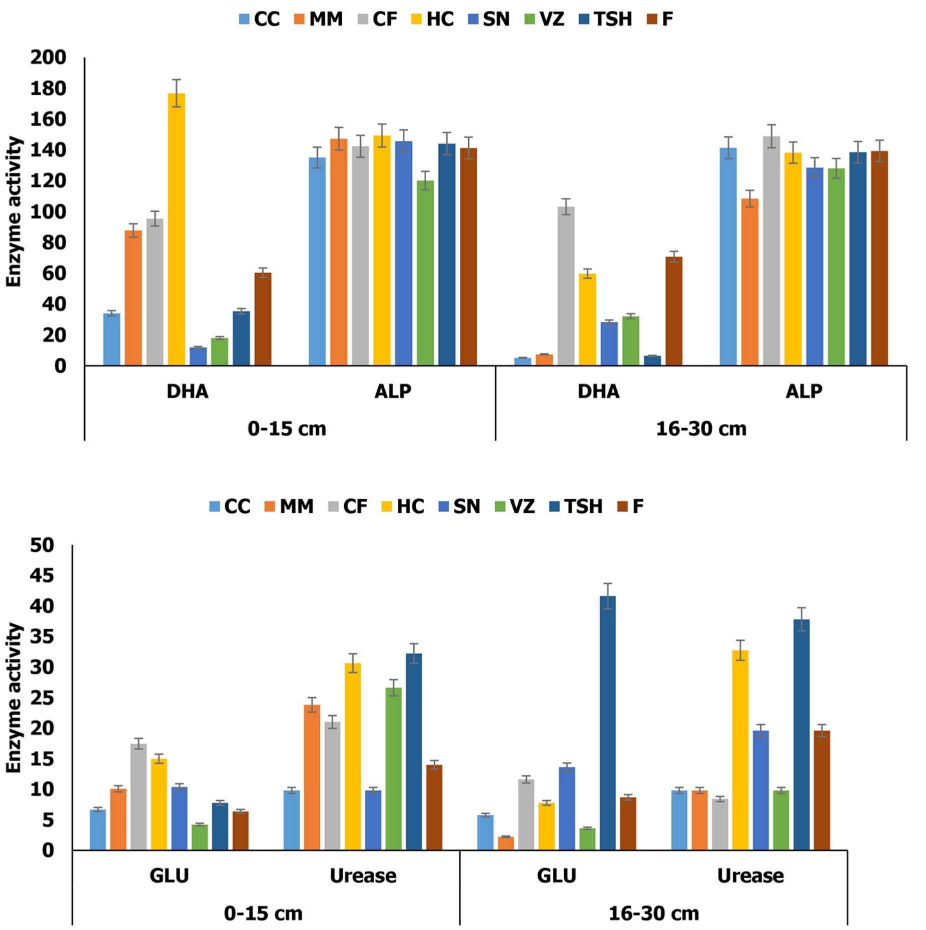

FIGURE 3
The activity of dehydrogenase (DHA; μg TPF g soil^{-1} 24 h^{-1}) alkaline phosphatase (ALP; μg PNP g soil^{-1} 2 h^{-1}), glucosidase (GLU; μg PNP g soil^{-1} h^{-1}), and urease (μg NH$_4^+$g soil^{-1} 2 h^{-1}) in soil under different tropical perennial range grasses in semiarid India. The error bars indicate the LSD value according to Tukey's HSD test ($p < 0.05$). CC, *Cenchrus ciliaris*; MM, *Megathyrsus maximus*; CF, *Chrysopogon fulvus*; HC, *Heteropogon contortus*; SN, *Sehima nervosum*; VZ, *Vetiveria zizanioides*; TSH, tri-specific hybrid; F, fallow.

minimum β-glucosidase enzyme activity than fallow land in a 0–15 cm soil layer. At the 16–30 cm soil layer, TSH, SN, and CF had ~68, 57, and 34% higher β- glucosidase enzyme activity than fallow land. However, MM, VZ, CC, and HC had ~74, 58, 34, and 11% lower β-glucosidase enzyme activity than fallow land (Figure 3).

3.5. Soil functions and their indices

In the surface soil (0–15 cm), carbon accumulation proficiency (CAP) was found to be highest under the SN grass system, which was statistically at par with the MM and HC grass systems. The lowest CAP was found in the VZ-based system. CAP in the SN, MM, and HC systems was ~122, 117, and 107% higher than the fallow (Table 4). In sub-surface soil (16–30 cm), CAP was found to be highest under the CF-based system, which was statistically at par with the HC, VZ, and SN-based systems. The lowest CAP was found in the MM-based system. CAP in the CF, TSH, HC, VZ, and SN-based systems was ~91, 87, 107, 65, and 122%, significantly higher than fallow (Table 4). In the surface soil (0–15 cm), nutrient supply capacity (NSC) was found to be highest in the VZ-based system. The lowest NSC was found in the CF-based system, which was significantly NSC under VZ based system was ~39% significantly higher than the CF-based system. TSH and HC-based systems also showed ~24 and 19% higher NSC than CF-based systems, respectively (Table 4). NSC was found highest on the CC-based system, followed by the VZ-based system in sub-surface soil (16–30 cm). The lowest NSC was found in the CF-based system. CC and VZ-based systems show ~43 and 27% higher NSC than the CF-based system, respectively (Table 4). The biological activity index (BAI) in surface soil (0–15 cm) was found to be highest in the HC-based system, followed by the CF-based system, which was statistically at par with the MM-based system. The VZ-based system found the lowest BAI, which was statistically at par with the CC and SN-based systems. The HC-based system showed ~120% higher BAI than the VZ-based system (Table 4).

In sub-surface soil (16–30 cm), BAI was found highest in the TSH-based system, followed by the CF-based system. CC-based systems showed the lowest BAI, which was statistically at par with the MM-based systems. The TSH-based system showed ~100% higher BAI than the CC-based system. In the surface soil (0–15 cm), soil functionality (SF) was found to be highest in the HC-based

TABLE 4 Carbon accumulation proficiency (CAP), nutrient supply capacity (NSC), biological activity index (BAI), and soil functionality (SF) of soil under different tropical perennial range grasses at the surface (SL; 0–15 cm) and subsurface (SSL; 16–30 cm) soil layers in semiarid India.

	SL				SSL			
	CAP	NSC	BAI	SF	CAP	NSC	BAI	SF
#CC	1.91bc	1.23cd	0.84d	15.19cd	2.37b	1.13a	0.68e	14.12b
MM	2.17ab	1.14d	1.21b	29.49ab	1.58c	0.81c	0.54e	6.74c
CF	1.91bc	1.10de	1.24b	20.47bc	2.73a	0.70d	1.14b	17.18b
HC	2.07ab	1.31bc	1.67a	32.29a	2.58ab	0.89c	1.00c	16.31b
SN	2.22a	1.15d	0.80d	9.99d	2.50ab	0.89c	0.80d	8.63c
VZ	1.65d	1.53a	0.76d	11.89d	2.54a	1.01b	0.73d	11.58bc
TSH	1.87c	1.36b	0.99c	29.32a	2.63a	0.86c	1.36a	36.00a
F	1.00e	1.00e	1.00c	1.00e	1.00d	1.00b	1.00c	1.00d

This means that similar lower-case letters within a column are not significantly different according to Tukey's HSD test ($p < 0.05$).

#CC, *Cenchrus ciliaris*; MM, *Megathyrsus maximus*; CF, *Chrysopogon fulvus*; HC, *Heteropogon contortus*; SN, *Sehima nervosum*; VZ, *Vetiveria zizanioides*; TSH, tri-specific hybrid; F, fallow.

system. SF was found in the order: HC > TSH > MM > CF > CC > VZ > SN > F. HC and TSH-based systems showed ∼ 223 and 193% higher SF over SN-based systems, respectively. SF was found highest on the TSH-based system, followed by the CF-based system in sub-surface soil (16–30 cm). The lowest SF was found in the MM-based system. SF was found in the order TSH > CF > HC > CC > VZ > SN > MM > F (Table 4).

4. Discussion

4.1. Tropical grasses could deplete macronutrient status

The chemical and physical properties of the soil can be significantly impacted by grass cover. The amount and type of crop debris and organic materials that reach the soil also affect the possible outcomes (Bakhshandeh et al., 2019; Paramesh et al., 2022). The emphasis in this situation is on studying and evaluating the condition of C and soil nutrients (as indicators of soil function) in addition to the numerous physical and chemical features of the soil (Kooch et al., 2019; Fan and Han, 2020). In actuality, changes in the spatial distribution of environmental factors, such as land use and management, microbial activity, and soil organic matter, affect soil fertility conditions (Basso et al., 2000). Our findings demonstrated that grass cover dramatically changed the nutritional condition of the soil. Since grasses absorb nitrogen, grass cover did not increase the N content, even though the C input from straw and grassroots considerably enhanced SOC (Chamberlain Lucas et al., 2022). The results of this study are consistent with those of de Oliveira et al. (2015), who discovered that land use clearly affects the quality and quantity of litter input, litter decomposition rates, and organic matter stabilization processes in soils. As a result, land use and the corresponding plant cover significantly alter the organic matter content. However, there are a number of other potential causes for the fall in soil P and K content under grass cover, including soil erosion, nitrogen absorption by these grasses, nutrient leaching during the wet season, etc. Increased micronutrient status under grass cover may be caused by weak absorption and the creation of strong bonds with organic materials.

To assess the state of ecosystems, one factor that may be taken into account is soil nutrients (Kooch et al., 2019). The C-P and C-N ratios in the current investigation revealed statistically superior variations among the grasses, and it appears that their absolute values were better able to capture the deterioration in soil quality. Significant data indicates that soil enzymatic activity can be utilized to indicate soil fertility and soil microbial activities. Soil enzyme activities are particularly responsive to grass cover (Wang et al., 2012; Parsapour et al., 2018; Ram et al., 2022). Wider C-N and C-P ratios suggest that the decreased BAI values under CC, SN, and VZ may result from severe food restriction. Several soil function indicators in the current study showed considerable improvements, whereas others showed significant declines. To sustain the soil nutrient strategy on semiarid, degraded land, greater focus should be placed on managing grasses. Although grass covers typically receive favorable responses, range grasses appear to require a fertilizing approach to enhance soil conditions.

4.2. Grasses strengthen the linkage between soil functions and ecosystem services

4.2.1. Climate regulation through carbon preservation

The CAP parameter investigates how grass cover may affect carbon flows, retention, and sequestration. The CAP provides a signal for C cycles, flows, and the systems' relative operational supremacy (Ghosh et al., 2021). The capacity of the grass cover to store and transport atmospheric carbon and produce biomass, among other things, enhanced CAP (Sainepo et al., 2018). The technology of grass cover had a more protected SOC than fallow land because of the comparatively high CAP ratings. In semiarid India, this research discovered that TSH and MM are viable choices for conserving or recovering SOC contents (Figure 4).

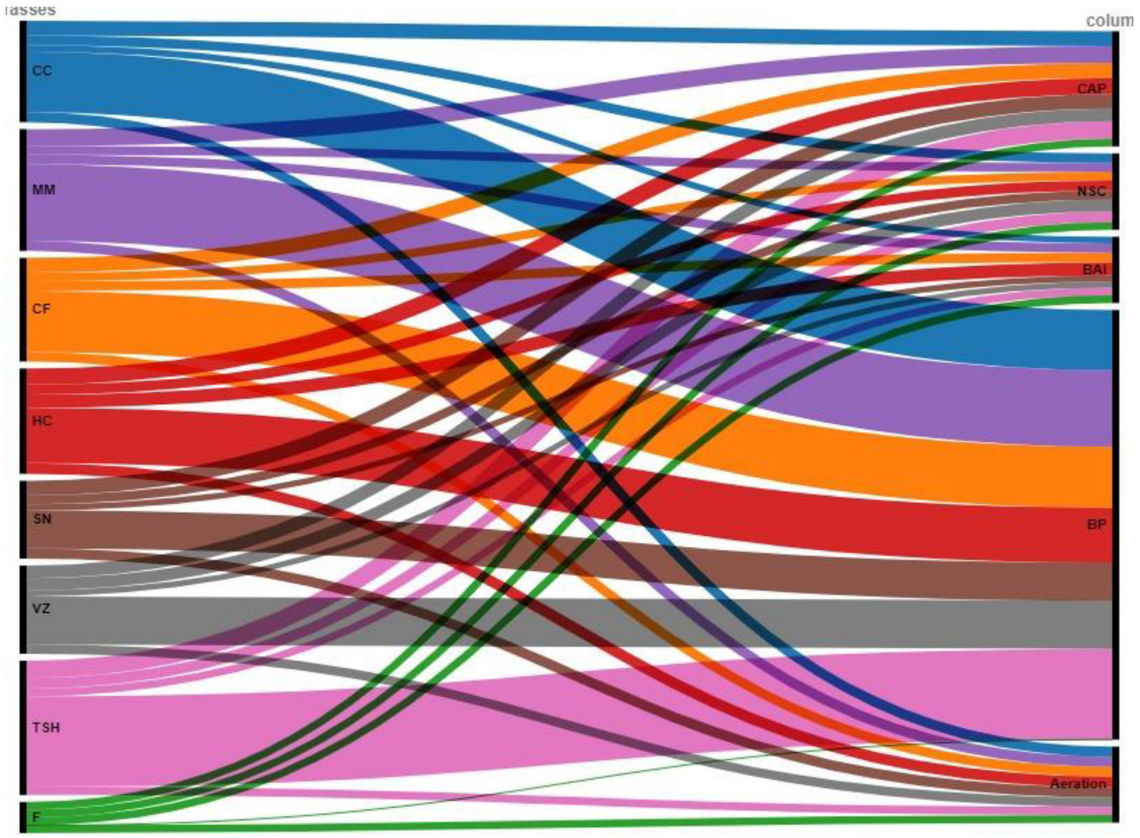

FIGURE 4
Node analysis plot depicting the contribution of different grasses toward soil functional indicators and their linkage to ecosystem services. The width of the lines indicates the strength of the linkage. CAP, Carbon accumulation proficiency; NSC, nutrient supply capacity; BAI, biological activity index; SF, soil functionality; BP, biomass productivity. CC, *Cenchrus ciliaris*; MM, *Megathyrsus maximus*; CF, *Chrysopogon fulvus*; HC, *Heteropogon contortus*; SN, *Sehima nervosum*; VZ, *Vetiveria zizanioides*; TSH, tri-specific hybrid; F, fallow.

4.2.2. Microbial activity and nutrient supply capacity

The ongoing supply of nutrients provided by live roots is beneficial to microbial cells. In contrast to fallow, CC, VZ, and SN, this may have increased BAI in MM and HC and maintained BAI under TSH (Guyonnet et al., 2018). The capacity to thrive in poor soil conditions, thick, deep networks of fine roots, and faster biomass development are just a few of the traits that assist in boosting biological activity under MM, HC, and TSH (Hu et al., 2021). By possibly increasing biomass productivity, these grasses help maintain soil biological activity, biodiversity, and ecosystem processes (Ghosh et al., 2021). The NSC parameter also evaluates the dynamics of nutrients in agroecosystems. Crop yields are increased due to the mineralization and decomposition of litter under grass cover (Figure 4). Nutrient deposition from leaves and rain may supplement litter decomposition, which is the main source of nutrients.

Moreover, the observed increases in NSC levels may result from a decrease in the distribution of nutrients out of the systems due to limited leaching in tree rows brought on by the impact of sheltering (Alva et al., 1999). Furthermore, the observed increase in SOC beneath the grass cover may result in a greater CEC and a consequent rise in the aptitude to retain nutrients (Lehmann, 2007; Bambrick et al., 2010). The nutrient outflow has been seen to have an impact on cations, particularly K+, in soil profiles (Jobbágy and Jackson, 2004). Litter deposit and breakdown are crucial to preserving NSC, particularly in less naturally fertile soils used for raising grasses. These processes are regulated by fauna, soil temperature, and management options (de Freitas et al., 2018; Ghosh et al., 2020).

4.2.3. Biomass productivity and soil airflow

The higher SOC content and root development may be related to the higher soil porosity under all grass cover compared to fallow. As a result, during the crucial low flow phase, soil water status and the ongoing river water flow to the soil profile are maintained. In the end, the deteriorated soil produced a sizable quantity of biomass due to the NSC and CAP (Figure 4). The production of plant biomass (fodder) serves as the foundation for ecosystem services connected to animal production. The productivity of grasses was lower than the stated yield, even though fodder production fluctuates with soil nutrients and moisture.

5. Conclusions

Soil functionality indicators, which frequently highlight the traits of soil ecology and fertility, offer important insights into ecosystem services. Functionality indicators in the soil can be considerably impacted by the type of grass. The habitats of semiarid areas, which are frequently situated in vulnerable locations, are particularly significant in this regard. Based on this, the current study examined how seven grasses affected the physical, chemical, and biological characteristics of the soil. According to our research, unmanaged grass coverings decreased soil nutrients, including N, P, and K, even if they increased SOC.

Nonetheless, regardless of the grass type, they could maintain their micronutrient status. Long-term grass growth led to the emergence of soil organisms that are better suited to cycling nutrients. In addition, more plant residuals entered the soil in the long term. Accordingly, grasses boosted soil functions such as green forage productivity, nutrient cycling capacity, carbon accumulation, and water and airflow compared to fallow. These functions of soil augment ecosystem services like climate regulation, biomass production, nutrient recycling, and water and airflow. In the long run, soil functionality under TSH and *Heteropogon* increased significantly in semiarid regions. In general, more attention should be paid to the management of inputs, such as nutrients, water, and manure, for greater ecosystem services.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

PP: data analysis and investigation. AS: formal analysis. AG: conceptualization, methodology, writing—original draft preparation, and software. MS: conceptualization, methodology, and supervision. SM and VK: validation. DO and AC: project administration. RC: software, validation, and editing revised manuscript. HE: data analysis, validation, and editing revised manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Alva, A. K., Prakash, O., Fares, A., and Hornsby, A. G. (1999). Distribution of rainfall and soil moisture content in the soil profile under citrus tree canopy and at the dripline. *Irrigat. Sci.* 18, 109–115. doi: 10.1007/s002710050051

Bakhshandeh, E., Hossieni, M., Zeraatpisheh, M., and Francaviglia, R. (2019). Land use change effects on soil quality and biological fertility: a case study in northern Iran. *Eur. J. Soil Biol.* 95, 103119. doi: 10.1016/j.ejsobi.2019.103119

Bambrick, A. D., Whalen, J. K., Bradley, R. L., Cogliastro, A., Gordon, A. M., Olivier, A., et al. (2010). Spatial heterogeneity of soil organic carbon in tree-based intercropping systems in Quebec and Ontario, Canada. *Agroforest. Syst.* 79, 343–353. doi: 10.1007/s10457-010-9305-z

Baradwal, H., Ghosh, A., Kumar, A., Singh, P. D., Sannagoudar, M. S., Ahamad, S., et al. (2022). Ecological restoration of degraded lands with alternate land use systems improves soil functionality in semiarid tropical India. *Land Degrad. Dev.* 33, 1076–1087. doi: 10.1002/ldr.4225

Basso, F., Bove, E., Dumontet, S., Ferrara, A., Pisante, M., Quaranta, G., et al. (2000). Evaluating environmental sensitivity at the basin scale through the use of geographic information systems and remotely sensed data: an example covering the Agri basin (Southern Italy). *Catena* 40, 19–35. doi: 10.1016/S0341-8162(99)00062-4

Chamberlain Lucas, A., Aguayo, T., Zerega, N. J. C., Dybzinski, R., and Egerton-Warburton, L. M. (2022). Rapid improvement in soil health following the conversion of abandoned farm fields to annual or perennial agroecosystems. *Front. Sustain. Food Syst.* 6, 1010298. doi: 10.3389/fsufs.2022.1010298

de Freitas, W. K., Portz, A., de Carvalho Peres, A. A., Tarré, R. M., and de Melo Campos, M. (2018). Soil nutrient content and plant phytosociology in agroforestry systems of the Rio de Janeiro State highlands, Brazil. *Acta Sci. Biol. Sci.* 40, 1–8. doi: 10.4025/actascibiolsci.v40i1.35368

de Oliveira, S. P., de Lacerda, N. B., Blum, S. C., Escobar, M. E. O., and de Oliveira, T. S. (2015). Organic carbon and nitrogen stocks in soils of northeastern Brazil converted to irrigated agriculture. *Land Degrad. Dev.* 26, 9–21. doi: 10.1002/ldr.2264

Dick, R. P. ed. (2020). *Methods of Soil Enzymology*, Vol. 26. Madison, WI: John Wiley & Sons.

Fan, L., and Han, W. (2020). Soil respiration after forest conversion to tea gardens: a chronosequence study. *Catena* 190, 104532. doi: 10.1016/j.catena.2020.104532

Franzluebbers, A. J. (2012). Grass roots of soil carbon sequestration. *Carbon Manag.* 3, 9–11. doi: 10.4155/cmt.11.73

Fuglie, K., Peters, M., and Burkart, S. (2021). The extent and economic significance of cultivated forage crops in developing countries. *Front. Sustain. Food Syst.* 5, 712136. doi: 10.3389/fsufs.2021.712136

Ghosh, A., Das, A., Das, D., Ray, P., Bhattacharyya, R., Biswas, D. R., et al. (2020). Contrasting land use systems and soil organic matter quality and temperature sensitivity in North Eastern India. *Soil Till. Res.* 199, 104573. doi: 10.1016/j.still.2020.104573

Ghosh, A., Kumar, R. V., Manna, M. C., Singh, A. K., Parihar, C. M., Kumar, S., et al. (2021). Eco-restoration of degraded lands through trees and grasses improves soil carbon sequestration and biological activity in tropical climates. *Ecol. Eng.* 162, 106176. doi: 10.1016/j.ecoleng.2021.106176

Ghosh, A., Kumar, S., Manna, M. C., Singh, A. K., Sharma, P., Sarkar, A., et al. (2019). Long-term in situ moisture conservation in horti-pasture system improves biological health of degraded land. *J. Environ. Manag.* 248, 109339. doi: 10.1016/j.jenvman.2019.109339

Gomez, K. A., and Gomez, A. A. (1984). *Statistical Procedures for Agricultural Research*. New York, NY: John Wiley & Sons.

Guyonnet, J. P., Cantarel, A. A., Simon, L., and Haichar, F. E. Z. (2018). Root exudation rate as functional trait involved in plant nutrient-use strategy classification. *Ecol. Evol.* 8, 8573–8581. doi: 10.1002/ece3.4383

Hanway, J. J., and Heidel, H. (1952). *Soil Analysis Methods as Used in Iowa State College Soil Testing Laboratory, Bulletin 57*. Ames, IA: Iowa State College of Agriculture. p. 131.

Hu, H., Umbreen, S., Zhang, Y., Bao, M., Huang, C., and Zhou, C. (2021). Significant association between soil dissolved organic matter and soil microbial communities following vegetation restoration in the Loess Plateau. *Ecol. Eng.* 169, 106305. doi: 10.1016/j.ecoleng.2021.106305

Jackson, M. L. (1973). *Soil Chemical Analysis*. New Delhi: Prentice Hall of India Pvt. Ltd.

Jenkinson, D. S., and Powlson, D. S. (1976). The effects of biocidal treatments on metabolism in soil—V: A method for measuring soil biomass. *Soil Biol. Biochem.* 3, 209–213.

Jobbágy, E. G., and Jackson, R. B. (2004). The uplift of soil nutrients by plants: biogeochemical consequences across scales. *Ecology* 85, 2380–2389. doi: 10.1890/03-0245

Kooch, Y., Sanji, R., and Tabari, M. (2019). The effect of vegetation change in C and N contents in litter and soil organic fractions of a Northern Iran temperate forest. *Catena* 178, 32–39. doi: 10.1016/j.catena.2019.03.009

Lehmann, J. (2007). Bio-energy in the black. *Front. Ecol. Environ.* 5, 381–387. doi: 10.1890/1540-9295(2007)5381:BITB2.0.CO;2

Li, C., Fultz, L. M., Moore-Kucera, J., Acosta-Martínez, V., Horita, J., Strauss, R., et al. (2017). Soil carbon sequestration potential in semiarid grass- lands in the Conservation Reserve Program. *Geoderma* 294, 80–90. doi: 10.1016/j.geoderma.2017.01.032

Nosetto, M. D., Jobbágy, E. G., and Paruelo, J. M. (2006). Carbon sequestration in semiarid rangelands: comparison of *Pinus ponderosa* plantations and grazing exclusion in NW Patagonia. *J. Arid Environ.* 67, 142–156. doi: 10.1016/j.jaridenv.2005.12.008

Notenbaert, A. M., Douxchamps, S., Villegas, D. M., Arango, J., Paul, B. K., Burkart, S., et al. (2021). Tapping into the environmental Co-benefits of improved tropical forages for an agroecological transformation of livestock production systems. *Front. Sustain. Food Syst.* 5, 742842. doi: 10.3389/fsufs.2021.742842

Ola, A., Dodd, I. C., and Quinton, J. N. (2015). Can we manipulate root system architecture to control soil erosion? *Soil* 1, 603–612. doi: 10.5194/soil-1-603-2015

Olsen, S. R. (1954). *Estimation of available phosphorus in soils by extraction with sodium bicarbonate (No. 939)*. US Department of Agriculture.

Paramesh, V., Singh, S. K., Mohekar, D. S., Arunachalam, V., Misra, S. D., Jat, S. L., et al. (2022). Impact of sustainable land-use management practices on soil carbon storage and soil quality in Goa State, India. *Land Degrad. Dev.* 33, 28–40. doi: 10.1002/ldr.4124

Parsapour, M. K., Kooch, Y., Hosseini, S. M., and Alavi, S. J. (2018). Litter and topsoil in *Alnus subcordata* plantation on former degraded natural forest land: a synthesis of age-sequence. *Soil Till. Res.* 179, 1–10. doi: 10.1016/j.still.2018.01.008

Perring, M. P., Standish, R. J., and Price, J. N. (2015). Advances in restoration ecology: rising to the challenges of the coming decades. *ESA J. 2* 6, 1–25. doi: 10.1890/ES15-00121.1

Prajapati, M. C., Phadake, A. B., and Agrawal, M. C. (1973). Studies on suitability of grasses for protection of field earthen structures in Agra region of Yamuna ravines. *Ind. For.* 99, 193–204.

Pulleman, M., Creamer, R., Hamer, U., Helder, J., Pelosi, C., Peres, G., et al. (2012). Soil biodiversity, biological indicators and soil ecosystem services—an overview of European approaches. *Curr. Opin. Environ. Sustain.* 4, 529–538. doi: 10.1016/j.cosust.2012.10.009

Rai, S. K., Dixit, A. K., Choudhary, M., and Kumar, S. (2018). Climatic variability and prediction of annual rainfall using stochastic time series model at Jhansi in central India. *Mausam* 69, 73–80. doi: 10.54302/mausam.v69i1.232

Ram, B., Singh, A. P., Singh, V. K., Durgude, S. A., and Nath, A. (2022). Effect of different land-use systems on microbial population and urease enzyme activity in a mollisol. *Biol. For.* 14, 455–459.

Sainepo, B. M., Gachene, C. K., and Karuma, A. (2018). Assessment of soil organic carbon fractions and carbon management index under different land use types in Olesharo Catchment, Narok County, Kenya. *Carbon Bal. Manag.* 13, 1–9. doi: 10.1186/s13021-018-0091-7

Tambara, A. A. C., Sippert, M. R., Jauris, G. C., Flores, J. L. C., Henz, É. L., and Velho, J. P. (2017). Production and chemical composition of grasses and legumes cultivated in pure form, mixed or in consortium. *Acta Sci. Anim. Sci.* 39, 235–241. doi: 10.4025/actascianimsci.v39i3.34661

Tellen, V. A., and Yerima, B. P. (2018). Effects of land use change on soil physicochemical properties in selected areas in the North West region of Cameroon. *Environ. Syst. Res.* 7, 1–29. doi: 10.1186/s40068-018-0106-0

Waliszewska, B., Grzelak, M., Gawe,ł, E., Spek-Dzwigała, A., Sieradzka, A., and Czekała, W. (2021). Chemical characteristics of selected grass species from polish meadows and their potential utilization for energy generation purposes. *Energies* 14, 1669. doi: 10.3390/en14061669

Wang, B., Xue, S., Liu, G. B., Zhang, G. H., Li, G., and Ren, Z. P. (2012). Changes in soil nutrient and enzyme activities under different vegetations in the Loess Plateau area, Northwest China. *Catena* 92, 186–195. doi: 10.1016/j.catena.2011.12.004

Wani, P. A., Khan, M. S., and Zaidi, A. (2007). Synergistic effects of the inoculation with nitrogen-fixing and phosphate-solubilizing rhizobacteria on the performance of field-grown chickpea. *J. Plant Nutr. Soil Sci.* 170, 283–287. doi: 10.1002/jpln.200620602

Wilekson, W. M. C. J., Hewage, R. S., Gajaweera, C. J., and Seresinhe, T. (2010). “Effect of Guinea grass (*Panicum maximum*) on selected properties of reddish-brown earths soils,” in *Conference Paper, International Symposium, Faculty of Agriculture* (Mapalan, NJ).

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Genetic diversity and salt tolerance assessment of 51 alfalfa (*Medicago sativa*) varieties under saline soil conditions

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Introduction: Salt stress significantly impacts plant growth worldwide. Although alfalfa exhibits some tolerance to salt-alkali soils, the specific salt tolerance levels across different alfalfa varieties remain inadequately understood. For this purpose, we conducted a study to investigate the diversity of agronomic traits of alfalfa (*Medicago sativa*) under different soil environments in order to elucidate the salt tolerance of 51 alfalfa varieties.

Methods: We chose three different soil conditions for our study: normal conditions in Yantai as control, saline conditions in Yantai with soil salt concentrations ranging from 0.25 to 0.33%, and saline-alkali conditions in Dongying with soil salt concentrations ranging from 0.28 to 0.32%. We chose 51 alfalfa varieties from different sources as our experimental materials. Planting was conducted following a completely randomized block design with three replicates for each variety. Harvesting was performed when the plants reached 50% flowering, cutting them at a height of 5cm above the ground. Comprehensive assessments encompassed 10 parameters, namely fresh weight, dry weight, stem-leaf ratio, branch count, plant height, crude protein content, crude fat content, acid detergent fiber, neutral detergent fiber, and ash content.

Results: Our findings revealed that the 51 studied alfalfa varieties displayed significant generalized heritability and coefficient of variation, indicating a high level of genetic diversity. Using principal component and cluster analyses, we categorized the varieties into three distinct clusters based on their agronomic traits. Grey correlation degree analysis revealed the commendable performance of Ying st, PI 672734, and Mei zuo across diverse environments. Genotype emerged as a predominant determinant of all parameters, except crude fat, acid detergent fiber, and neutral detergent fiber. Through the application of membership function analysis, PI 672768 emerged as a variety that demonstrated strong tolerance in both saline soil contexts.

Discussion: Despite extensive previous studies indicating Zhong Mu No. 1 as a salt-tolerant alfalfa variety, its performance in this experiment did not distinguish itself. The findings of this study provide a fundamental basis for improving the management of saline-alkali lands and advancing alfalfa cultivation practices.

KEYWORDS

agronomic characters, varietal assessment, forage breeding, salt stress, genotype and environment interaction

1. Introduction

Soil salinity is an abiotic stress that significantly affects plant growth. In recent decades, climate change and concurrent water scarcity have contributed to a gradual increase in soil salinization. This phenomenon has spread to over 100 countries worldwide, causing a growing global concern. According to UNESCO and FAO, the extent of saline soil across the globe has expanded to cover an area totaling 932.2 million hectares (Rengasamy, 2006; Mbarki et al., 2020). The widespread occurrence of soil salinization has resulted in a decrease in available arable land, leading to a decline in both food production and pasture areas. Consequently, lower pasture yields have occurred, leading to increased competition for sustenance between humans and animals. This prevalent predicament of limited land use has prompted the cultivation and careful screening of crop varieties with enhanced salt tolerance. This is particularly significant in the context of shrinking land availability, necessitating the selection of plant varieties capable of thriving in saline-alkali environments to improve land utilization efficiency (Zhang et al., 2008; Lu et al., 2021; Zhang et al., 2023).

Given these challenges, there has been a notable increase in global research focused on the cultivation of alfalfa (*Medicago sativa*). Despite its moderate salt tolerance, alfalfa has shown notable effectiveness in improving saline-alkali soils. Alfalfa, widely distributed in China, has earned the reputation of being the “queen of forage” due to its high protein content, nutritional richness, and prolific yield. However, indigenous alfalfa varieties within China are limited, with a significant proportion being imported from foreign sources. These imported varieties exhibit notable variations in their salt tolerance capabilities. Therefore, it is imperative to investigate the diversity of agronomic traits in the context of saline-alkali soil environments and to identify alfalfa varieties suitable for cultivation under these conditions. These efforts hold the potential to improve the utilization of saline-alkali soils and provide a theoretical foundation for enhancing alfalfa varieties.

Researchers worldwide have made significant progress in unraveling the intricacies of alfalfa’s salt tolerance. For instance, Yu et al. (2021) investigated 20 alfalfa varieties subjected to salt stress during the seedling stage and categorized them based on their response: a high-salt tolerance variety, salt-tolerant, medium salt-tolerant, and salt-sensitive. Similarly, Liang et al. (2017) studied the performance of seven traits across 10 alfalfa varieties under salt stress, pinpointing four varieties distinguished by commendable salt tolerance. Wang et al. (2023) examined germination potential and root length diversities among 29 alfalfa varieties during seed germination to discern five varieties endowed with robust salt tolerance. These studies highlight the recognition of salt tolerance variations among alfalfa varieties and hold substantial potential for refining breeding strategies (Al-Khatib et al., 1992). The results of previous studies provide great inspiration and reference for this experiment (Tavakoli et al., 2019; Benabderrahim et al., 2020; Yu et al., 2021).

Building upon the existing knowledge base, this study delves into the phenotypic and quality attributes of alfalfa cultivated under salt stress. It aspires to ascertain the agronomic trait diversity among 51 alfalfa species across three distinct environments. In this study, we investigated the diversity of agronomic traits in 51 alfalfa species using a gray correlation method with the aim of identifying superior varieties, exploring the relationship between traits, genotypes, and the environment through gene–environment interactions, and finally screening for high-quality alfalfa varieties with pronounced salt tolerance using an affiliation function. The outcomes of this investigation hold the promise of furnishing a theoretical foundation for the selection of salt-tolerant alfalfa varieties, thus advancing the field of alfalfa breeding and variety improvement.

2. Materials and methods

2.1. Test site and test material

The test site for both the Salt Pond and the control group was situated in Zhifu District, Yantai City. The salt pond was irrigated with seawater, resulting in a salt content ranging from 0.25 to 0.33%. In Yantai, the soil’s organic matter content accounted for 7.51% of dry weight, with total nitrogen at 1.187 mg/kg, ammonia nitrogen at 6.39 mg/kg, nitrate nitrogen at 4.89 mg/kg, and total phosphorus at 0.25 mg/kg. The saline-alkali land in Dongying is situated in Kenli District, Dongying City. In the Dongying soil, organic matter content accounted for 5.73% of dry weight, with total nitrogen at 1.04 mg/kg, ammonia nitrogen at 5.38 mg/kg, nitrate nitrogen at 5.87 mg/kg, total phosphorus at 0.29 mg/kg, and a salt content ranging from 0.28 to 0.32%. In this study, “control” refers to the natural environment in Yantai, “Yantai” designates the salt field environment in Yantai, and “Dongying” signifies the saline-alkali land environment in Dongying. Supplementary Table S1 provides a summary of the rainfall, minimum temperature, and maximum temperature data during the experimental period.

2.2. Experimental design

In April 2020, we initiated the germination process by sowing three alfalfa seeds in greenhouse pots. After emergence, we carefully selected and retained a single robust alfalfa seedling to ensure consistent growth conditions. Subsequently, in mid-May 2020, we transplanted 51 distinct alfalfa varieties into three different locations: Yantai, the Yantai salt pond, and Dongying’s saline-alkali land. The details regarding the varieties can be found in Supplementary Table S2. We planted the alfalfa varieties with a spacing of 30 cm between individual plants. The experimental setup included three replicates for each variety, following a randomized complete block design. After 3 months, we scheduled the harvest when the alfalfa plants reached 50% flowering, maintaining a 5 cm elevation above ground level. The experimental setup did not involve fertilization or artificial irrigation practices. Measurement indices and methodologies are detailed in Table 1.

2.3. Statistical analysis

Statistical analysis of the amassed data was conducted using Excel 2022. For correlation analysis and cluster analysis, SPSS 16 was

TABLE 1 Determination indexes and methods.

Traits	Method
Fresh weight (FW, g)	The cutting height of alfalfa was 5 cm. The fresh weight of alfalfa was measured by electronic balance after cutting.
Dry weight (DW, g)	The cut alfalfa was put into an envelope and baked at 105°C for 30 min, then baked at 75°C to constant weight and weighed to get dry weight.
Fresh/Dry ratio	Fresh weight/Dry weight
Stem/leaf ratio (S/L R)	Stem/leaf dry weight
Plant height (PH, cm)	The straightening length from the base of the plant to the tip of the main stem or inflorescence.
Branch number (BN)	The number of branches of alfalfa per plant was determined.
Crude protein (CP, %)	Kjeldahl method (Han et al., 2022)
Ether extract (EE, %)	Soxhlet extraction method (Han et al., 2022)
Acid detergent fiber (ADF, %)	Van Soest method (Han et al., 2022)
Neutral detergent fiber (NDF, %)	Van Soest method (Han et al., 2022)
Ash (%)	Complete combustion method (Han et al., 2022)

employed. Graphical representation was executed using Origin 9 software.

2.3.1. Genetic diversity analysis

Genetic diversity indices were organized into 10 distinct grades. The initial grade, denoted as Xi > (X + 2σ), was juxtaposed with the tenth grade, Xi > (X + 2σ). The computation of the genetic diversity index, commonly recognized as the Shannon-Weaver diversity index (H′), adhered to the formula H′ = -ΣPilnPi, accommodating a 0.5σ variation between each grade.

Where: X represents the average value of each index, σ signifies the standard deviation of the index, and Pi represents the proportion of specimens within the total cohort displaying grade I characteristics.

2.3.2. Grey relation analysis

Building upon the foundations of grey correlation theory, the study treated the 10 alfalfa traits as a grey system. The construction of the ideal variety, denoted as X_0, relied on the optimal values encompassing each individual trait. The ensuing correlation coefficient and correlation degree calculations adhered to established equations (Zeng et al., 2020).

$$\varepsilon_i(k) = \frac{\min_i \min_k \left|X_0(k) - X_i(k)\right| + \rho \max_i \max_k \left|X_0(k) - X_i(k)\right|}{\left|X_0(k) - X_i(k)\right| + \rho \max_i \max_k \left|X_0(k) - X_i(k)\right|}$$

$$R_i = \frac{1}{n}\sum_{k=1}^{n} \varepsilon_i(k)$$

$$w_k = \frac{R_i}{\sum R_j}$$

$$R_i' = \sum_{k=1}^{n} W_k \varepsilon_i(k)$$

where: $|X_0(k)\text{-}X_i(k)|$ was the absolute difference between X_0 series and X_i series at k, minimink$|X_0(k)\text{-}X_i(k)|$ was the second level minimum difference, maximaxk$|X_0(k)\text{-}X_i(k)|$ was the second level maximum difference, ρ was the distinguishing coefficient, the value was considered to be 0.5, W_k was weight, R'_i was weighted relevance.

2.3.3. Heritability calculation

The generalized heritability is calculated according to the following formula (Tian et al., 2023).

$$R = \frac{\sigma_g^2}{\sigma_g^2 + \sigma_{gy}^2 + \frac{\sigma_\varepsilon^2}{n_l n_r}}$$

where σ_g^2 is the genotype, σ_{gy}^2 is the interaction variance, $\frac{\sigma_\varepsilon^2}{n_l n_r}$ is the error, n_l is the environment, and n_r is the number of replicates.

2.3.4. Salt tolerance index and membership function value

The assessment of salt tolerance was determined through the utilization of the Salt Tolerance Index, calculated as the ratio of the performance of the tested sample (T test) to that of the control sample (Roshdy et al., 2011). To further refine the analysis, the principles of membership function within the realm of fuzzy mathematics were incorporated. This entailed the standardization of the salt tolerance coefficient, enabling a more comprehensive and nuanced evaluation of the salt tolerance characteristics of the examined varieties (Jin et al., 2018).

$$U(X_j) = \frac{X_j - X_{min}}{X_{max} - X_{min}}$$

Where X_j represents the salt tolerance index of j index of the i variety, and X_{max} and X_{min}, respectively, represent the maximum and minimum values of the index.

The weight of each comprehensive index:

$$W_j = \frac{P_j}{\sum P_j}$$

Comprehensive salt tolerance:

$$D = \sum_{j=1}^{n}\left[U(X_j) \times W_j\right]$$

where, W_j represents the importance or weight of the j-th comprehensive indicator among all indicators, P_j represents the contribution rate of the j-th comprehensive indicator for each material, and D represents the comprehensive evaluation value of heat tolerance obtained by evaluating each material under high temperature stress using comprehensive indicators.

3. Results

3.1. Analysis of agronomic trait diversity of alfalfa in different environments

Under controlled conditions, the coefficient of variation for the 51 studied alfalfa varieties ranged from 3.78 to 70.47% (Table 2). The highest coefficient of variation was observed in dry weight (DW) at 70.47%, while the lowest was recorded in CP at 3.78%. Assessing the genetic diversity index, all 10 physiological indices showed values exceeding 1, indicating significant genetic diversity among the alfalfa varieties. Notably, the highest genetic diversity was observed in CP and ADF at 1.98.

Within the Yantai environment, the coefficient of variation across agronomic traits within the 51 alfalfa specimens ranged from 3.91 to 69.92% (Table 3). The maximal coefficient of variation was noted in DW at 69.92%, while the minimum coefficient was witnessed in CP at 3.91%. Genetic diversity exhibited alterations, with increments observed in FW, DW, PH, ADF and ash content. Conversely, reductions were noted in BN, S/L R, CP, and EE. Notably, the genetic diversity of NDF remained constant, with the highest genetic diversity observed in ash content at 2.02.

In Dongying, the coefficient of variation for the 51 agronomic traits of alfalfa ranged from 3.81 to 74.09% (Table 4). The lowest coefficient of variation was found in CP at 3.81%, while the highest was observed in DW at 74.09%. In comparison to the Yantai environment, the genetic diversity index in Dongying showed increased genetic diversity in FW, DW, EE, NDF, and ADF. However, genetic diversity trends diverged, with declines observed in BN, S/L R, CP, and ash content.

In contrast to the control conditions, exposure to the saline environment resulted in significant changes. Specifically, DW, FW, PH, and S/L R exhibited reductions in the salt-affected environment. Conversely, BN, CP, and ash content displayed increases. Conversely, limited fluctuations were observed in EE, ADF, and NDF. Furthermore, compared to the control, the mean values of DW, FW, PH, and S/L R declined by 24.68, 24.45, 19.15, and 15.18%, respectively, in Yantai. At the same time, BN, CP, and ash content exhibited increases of 10.81, 10.04, and 19.94%, respectively. Analogous trends were observed in the Dongying environment, where DW, FW, PH, and S/L R underwent reductions of 24.45, 24.68, 19.15, and 15.18% respectively, while BN, CP, and ash content experienced increments of 13.51, 10.04, and 19.94%, respectively.

3.2. Correlation analysis of agronomic traits in diverse alfalfa environments

We conducted a comprehensive correlation analysis involving the diverse set of 51 alfalfa species under controlled conditions, revealing intricate relationships among the evaluated traits (Figure 1A). The results revealed significant correlations with FW showing positive associations with BN, S/L R, EE, and ADF. Meanwhile, DW showed positive correlations with FW, BN, S/L R, and ADF, while EE was positively correlated with ADF. Furthermore, CP displayed positive correlations with EE and was as significantly positively correlated with ash content. Notably, a negative correlation was observed between CP and NDF, while EE revealed a notable negative correlation with NDF and positive correlation with Ash content. The ADF content exhibited a positive correlation with NDF, while a significant negative correlation was observed between NDF and ash content.

In the Yantai environment, we presented a thorough correlation analysis among the 51 agronomic traits, providing insights into trait interactions (Figures 1B,C and Figures 2). Importantly, differences from the control environment become apparent, with DW showed a significant positive correlation with EE and ADF content. Conversely, in the saline soil environment of Dongying, BN displayed a positive correlation with ADF. Notably, the content of CP and NDF demonstrated significant negative correlations when contrasted with the control environment, diverging from other correlations which largely mirrored those of the control environment. Additionally, it is worth noting that PH did not show significant correlation with the other measured traits in this context.

3.3. Principal component analysis and cluster analysis

Utilizing advanced analytical techniques, we employed principal component analysis (PCA) and cluster analysis to gain deeper insights into the complex interrelationships among the agronomic traits of diverse alfalfa species across different environments.

In controlled conditions, we enhanced the cluster analysis by converting FW into the Fresh-to-Dry Ratio (F/D R). We then applied the Ward method to cluster the standardized results of the 51 agronomic traits of alfalfa. As a result, the alfalfa varieties coalesced into three distinct groups (Table 5). The primary group comprised 25

TABLE 2 Summary of mean, standard deviation, coefficient of variation, and genetic diversity for 10 agronomic traits of 51 alfalfa species in Yantai's control environment.

Item	FW (g)	DW (g)	PH (cm)	S/L R	BN	CP (%)	EE (%)	ADF (%)	NDF (%)	Ash (%)
Mean	139.92	40.93	95.02	31.02	0.74	19.33	2.35	31.13	40.84	9.33
SD	73.43	28.84	14.10	9.98	0.13	0.73	0.13	2.34	1.87	0.52
CV (%)	52.48	70.47	14.83	32.19	17.81	3.78	5.39	7.52	4.58	5.60
H′	1.71	1.40	1.93	1.89	1.93	1.98	1.90	1.98	1.95	1.96

FW, fresh weight; DW, dry weight; PH, plant height; S/L R, stem/leaf ratio; BN, branching number; CP, crude protein; EE, ether extract; ADF, acid detergent fiber; NDF, neutral detergent fiber; SD, standard deviation; CV, coefficient of variation; H′, genetic diversity index.

TABLE 3 Summary of mean, standard deviation, coefficient of variation, and genetic diversity for 10 agronomic traits of 51 alfalfa species in Yantai's salt pond environment.

Item	FW (g)	DW (g)	PH (cm)	S/L R	BN	CP (%)	EE (%)	ADF (%)	NDF (%)	Ash (%)
Mean	105.71	30.83	76.82	26.31	0.82	21.27	2.35	31.14	40.77	11.19
SD	53.40	21.56	13.36	8.37	0.14	0.83	0.13	2.33	1.89	0.64
CV (%)	50.51	69.92	17.39	31.79	17.47	3.91	5.45	7.48	4.63	5.70%
H′	1.79	1.43	1.96	1.81	1.90	1.94	1.88	1.99	1.95	2.02

FW, fresh weight; DW, dry weight; PH, plant height; S/L R, stem/leaf ratio; BN, branching number; CP, crude protein; EE, ether extract; ADF, acid detergent fiber; NDF, neutral detergent fiber; SD, standard deviation; CV, coefficient of variation; H′, genetic diversity index.

TABLE 4 Summary of mean, standard deviation, coefficient of variation, and genetic diversity for 10 agronomic traits of 51 alfalfa species in Dongying's saline-alkali land.

Item	FW (g)	DW (g)	PH (cm)	S/L R	BN	CP (%)	EE (%)	ADF (%)	NDF (%)	Ash (%)
Mean	106.56	31.53	78.09	26.32	0.84	21.27	2.35	31.14	40.77	11.19
SD	57.98	23.36	12.57	8.56	0.16	0.81	0.13	2.37	1.91	0.63
CV (%)	54.41	74.09	16.10	32.51	19.33	3.81	5.38	7.62	4.68	5.61
H′	1.80	1.45	1.96	1.90	1.80	1.86	1.95	2.00	1.99	1.98

FW, fresh weight; DW, dry weight, PH; plant height, S/L R, stem/leaf ratio; BN, branching number; CP, crude protein; EE, ether extract; ADF, acid detergent fiber; NDF, neutral detergent fiber; SD, standard deviation; CV, coefficient of variation; H′, genetic diversity index.

varieties, characterized by elevated F/D R, PH, Ash content, and NDF. The secondary group consisted of 10 varieties, distinguished by the highest average PH and Ash content. Meanwhile, the tertiary group, encompassing 16 varieties, showcased heightened average values for DW, BN, S/L R, EE, and ADF. The insights obtained from principal component analysis supported these findings. Principal component 1 explained 25.6% of the total trait variation, and principal component 2 accounted for 24.7% of the overall variation in the controlled environment.

In the Yantai environment, similar procedures led to the clustering of the 51 alfalfa species into three distinct groups (Table 6). The primary cluster encompassed 19 varieties distinguished by elevated PH, ADF, and NDF. The secondary cluster, containing 20 varieties, exhibited the highest Fresh-to-Dry Ratio (F/D R) and average CP. Conversely, the tertiary cluster, composed of 12 varieties, exhibited superior average values for DW, BN, S/L R, EE, and Ash content. Principal component analysis reaffirmed these findings, with principal component 1 and 2 explained 25.3 and 24.1% of the total trait variation, respectively.

In the Dongying environment, principal component analysis also revealed intricate patterns. Principal component 1 and 2 collectively expounded 25.1 and 23.9% of the total trait variation, respectively. Cluster analysis led to the categorization of the 51 alfalfa species into three discernible groups (Table 7). The primary group, constituted of 17 varieties, exhibited the highest average values for CP, ADF, NDF, and Ash content. Meanwhile, the secondary group, featuring 21 varieties, was distinguished by superior average F/D R and PH. The tertiary group, housing 13 varieties, displayed heightened average values for DW, BN, S/L R, and EE.

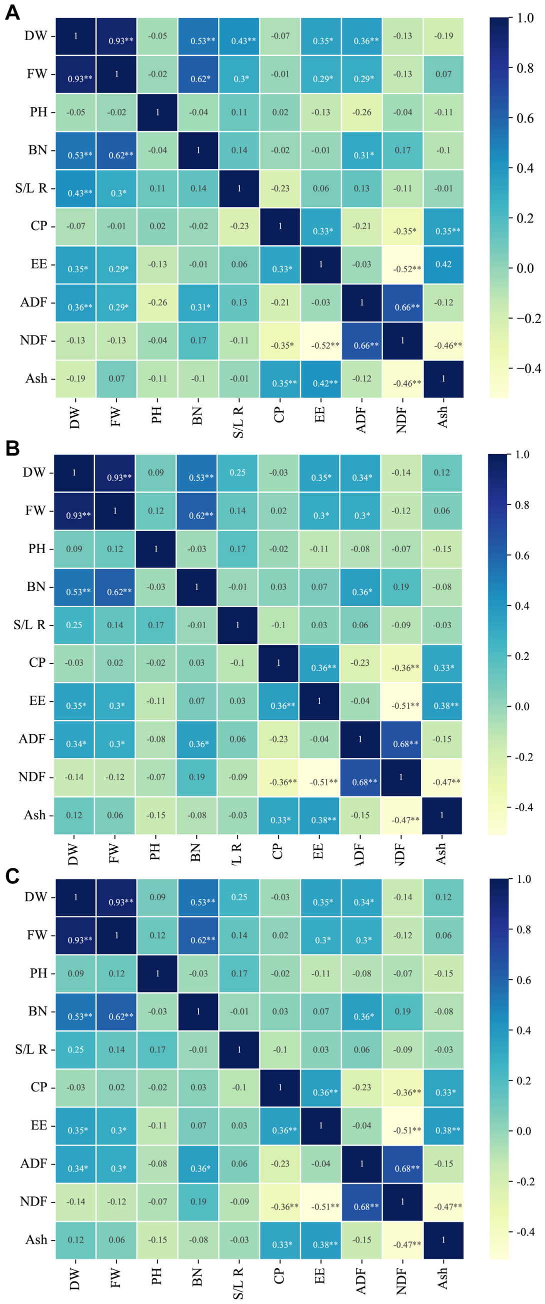
The integration of principal component analysis and cluster analysis elucidated the intricate trait associations and groupings across distinct environments, thereby affording valuable insights into the varietal behavior of alfalfa under differing conditions.

3.4. Screening of excellent forage performance varieties

The process of identifying superior alfalfa varieties, enriched by rigorous analytical methods, aimed to reveal strains that exhibited exceptional performance across various traits and environments. To create a representative ideal variety designated as X_0, each attribute was optimized to maximize values, except for ADF, NDF, and Ash, where the goal was to minimize values. This approach facilitated the normalization of data through the initial value method, enabling the computation of both minimink$|X_0(k)$-$X_i(k)|$ and maximaxk$|X_0(k)$-$X_i(k)|$.

Subsequently, the weighted correlation degree was employed as a discerning criterion for the stratification of the alfalfa varieties (Table 8). The outcomes of this process revealed the top five ranked varieties across the entire spectrum. In a broader context, the Ying st, PI 672734, Mei zuo, Gan nong NO.6 and Nan mu 501 emerged as prominently ranked, indicating their superior quality across diverse traits. This trend persisted within the Yantai environment, where Ying st, PI 672734, Mei zuo, Gan nong No.6 and Xiang yang No.6 garnered the highest acclaim. Likewise, within the Dongying environment, the elite rankings included PI 672734, Ying st, Nan mu 501, Gan Nong 6, and Mei zuo. It is worth noting the consistent excellence exhibited by Ying st, PI 672734, Gan nong No.6, and Mei zuo, confirming their exceptional qualities across all three distinct environments, firmly establishing them as high-caliber alfalfa varieties.

The meticulous process of strain selection, supported by comprehensive analytical techniques, serves to underscore the significance of Yingst, PI 672734, Gan nong No.6 and Mei zuo as robust candidates for continued agricultural propagation and advancement. This discerning approach aligns with the pursuit of enhancing alfalfa cultivation while attaining optimal yields and performance across varying conditions.



A

	DW	FW	PH	BN	S/L R	CP	EE	ADF	NDF	Ash
DW	1	0.93**	-0.05	0.53**	0.43**	-0.07	0.35*	0.36**	-0.13	-0.19
FW	0.93**	1	-0.02	0.62*	0.3*	-0.01	0.29*	0.29*	-0.13	0.07
PH	-0.05	-0.02	1	-0.04	0.11	0.02	-0.13	-0.26	-0.04	-0.11
BN	0.53**	0.62**	-0.04	1	0.14	-0.02	-0.01	0.31*	0.17	-0.1
S/L R	0.43**	0.3*	0.11	0.14	1	-0.23	0.06	0.13	-0.11	-0.01
CP	-0.07	-0.01	0.02	-0.02	-0.23	1	0.33*	-0.21	-0.35*	0.35**
EE	0.35*	0.29*	-0.13	-0.01	0.06	0.33*	1	-0.03	-0.52**	0.42
ADF	0.36**	0.29*	-0.26	0.31*	0.13	-0.21	-0.03	1	0.66**	-0.12
NDF	-0.13	-0.13	-0.04	0.17	-0.11	-0.35*	-0.52**	0.66**	1	-0.46**
Ash	-0.19	0.07	-0.11	-0.1	-0.01	0.35**	0.42**	-0.12	-0.46**	1

B

	DW	FW	PH	BN	S/L R	CP	EE	ADF	NDF	Ash
DW	1	0.93**	0.09	0.53**	0.25	-0.03	0.35*	0.34*	-0.14	0.12
FW	0.93**	1	0.12	0.62**	0.14	0.02	0.3*	0.3*	-0.12	0.06
PH	0.09	0.12	1	-0.03	0.17	-0.02	-0.11	-0.08	-0.07	-0.15
BN	0.53**	0.62**	-0.03	1	-0.01	0.03	0.07	0.36*	0.19	-0.08
S/L R	0.25	0.14	0.17	-0.01	1	-0.1	0.03	0.06	-0.09	-0.03
CP	-0.03	0.02	-0.02	0.03	-0.1	1	0.36**	-0.23	-0.36**	0.33*
EE	0.35*	0.3*	-0.11	0.07	0.03	0.36**	1	-0.04	-0.51**	0.38**
ADF	0.34*	0.3*	-0.08	0.36*	0.06	-0.23	-0.04	1	0.68**	-0.15
NDF	-0.14	-0.12	-0.07	0.19	-0.09	-0.36**	-0.51**	0.68**	1	-0.47**
Ash	0.12	0.06	-0.15	-0.08	-0.03	0.33*	0.38**	-0.15	-0.47**	1

C

	DW	FW	PH	BN	S/L R	CP	EE	ADF	NDF	Ash
DW	1	0.93**	0.09	0.53**	0.25	-0.03	0.35*	0.34*	-0.14	0.12
FW	0.93**	1	0.12	0.62**	0.14	0.02	0.3*	0.3*	-0.12	0.06
PH	0.09	0.12	1	-0.03	0.17	-0.02	-0.11	-0.08	-0.07	-0.15
BN	0.53**	0.62**	-0.03	1	-0.01	0.03	0.07	0.36*	0.19	-0.08
S/L R	0.25	0.14	0.17	-0.01	1	-0.1	0.03	0.06	-0.09	-0.03
CP	-0.03	0.02	-0.02	0.03	-0.1	1	0.36**	-0.23	-0.36**	0.33*
EE	0.35*	0.3*	-0.11	0.07	0.03	0.36**	1	-0.04	-0.51**	0.38**
ADF	0.34*	0.3*	-0.08	0.36*	0.06	-0.23	-0.04	1	0.68**	-0.15
NDF	-0.14	-0.12	-0.07	0.19	-0.09	-0.36**	-0.51**	0.68**	1	-0.47**
Ash	0.12	0.06	-0.15	-0.08	-0.03	0.33*	0.38**	-0.15	-0.47**	1

FIGURE 1
Correlation analysis of 10 agronomic traits of 51 alfalfa species under **(A)** normal environment in Yantai, **(B)** salt pond in Yantai, and **(C)** saline soil in Dongying.

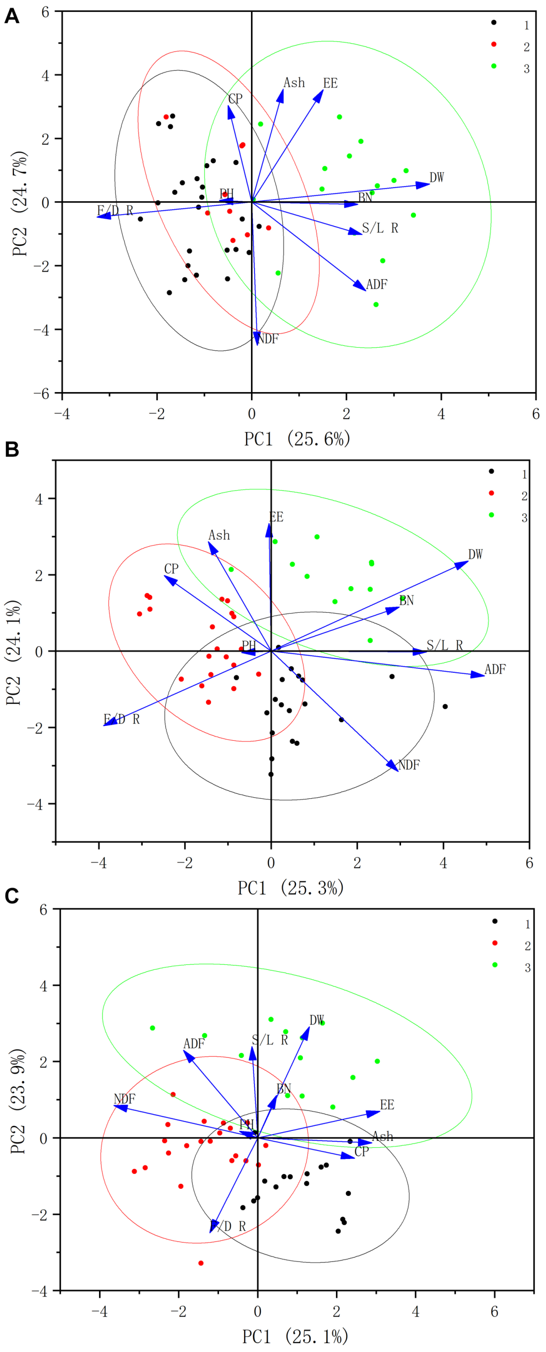


FIGURE 2
Results of cluster analysis and principal component analysis of agronomic traits of alfalfa under **(A)** control environment, **(B)** salt pond in Yantai, and **(C)** saline soil in Dongying.

3.5. Genotype-by-environment (G × E) analysis and identification of salt-tolerant varieties

A thorough investigation of G × E interactions, supported by meticulous data analysis (Tables 9, 10), formed the basis for assessing 10 distinct agronomic traits in alfalfa. The findings underscored the undeniable influence of genotype on the majority of traits ($p < 0.01$). Nevertheless, traits such as F/D R, EE, ADF, and NDF remained unaffected by the environment, thus indicating the inherent genetic stability of these traits in the face of environmental variability. Conversely, crucial traits including DW, PH, BN, S/L R, CP and ash content were significantly impacted by environmental conditions. This substantiated that the genetic integrity of F/D R, EE, ADF, and NDF endured regardless of external environmental factors. Interestingly, an interaction between genetic makeup and environmental factors was observed, especially concerning DW and ash content. Notably, the relative significance ranking of the six traits significantly influenced by the genotypic environment emerged as

TABLE 5 Average values of 10 agronomic traits of 51 alfalfa species across three groups in Yantai's normal environment.

Group	DW (g)	Fresh/Dry ratio	PH (cm)	S/L R	BN	CP (%)	EE (%)	ADF (%)	NDF (%)	Ash (%)
1	24.04b	4.38a	99.58a	29.04b	0.71b	19.37a	2.29b	29.86b	41.05a	9.14b
2	25.33b	4.16a	83.67b	27.20b	0.66b	19.45a	2.38a	31.94a	40.69a	9.60a
3	77.06a	2.95b	95.00a	36.50a	0.84a	19.20a	2.43a	32.63a	40.59a	9.46ab

DW, dry weight; PH, plant height; S/L R, stem/leaf ratio; BN, branching number; CP, crude protein; EE, ether extract; ADF, acid detergent fiber; NDF, neutral detergent fiber; SD, standard deviation; CV, coefficient of variation; H′, genetic diversity index. Different lower-case letters indicate a significant difference among treatments.

TABLE 6 Average values of 10 agronomic traits of 51 alfalfa species across three groups in Yantai's salt pond environment.

Group	DW (g)	Fresh/Dry ratio	PH (cm)	S/L R	BN	CP (%)	EE (%)	ADF (%)	NDF (%)	Ash (%)
1	25.43b	4.08a	80.63a	29.65a	0.80b	20.89b	2.26c	32.28a	42.54a	10.74b
2	16.16c	4.43a	71.98a	20.82b	0.77b	21.54a	2.37b	29.45b	39.69b	11.42a
3	63.83a	2.86b	78.88a	30.19a	0.92a	21.41ab	2.47a	32.13a	39.78b	11.52a

DW, dry weight; PH, plant height; S/L R, stem/leaf ratio; BN, branching number; CP, crude protein; EE, ether extract; ADF, acid detergent fiber; NDF, neutral detergent fiber; SD, standard deviation; CV, coefficient of variation; H′, genetic diversity index. Different lower-case letters indicate a significant difference among treatments.

TABLE 7 Average values of 10 agronomic traits of 51 alfalfa species across three groups in Dongying's saline-alkali land.

Group	DW (g)	Fresh/Dry ratio	PH (cm)	S/L R	BN	CP (%)	EE (%)	ADF (%)	NDF (%)	Ash (%)
1	17.18b	4.17a	74.61a	22.53b	0.81b	21.60a	2.39a	28.85b	39.14c	11.47a
2	20.74b	4.50a	81.24a	26.02b	0.81b	21.11a	2.27b	32.02a	42.26a	10.87b
3	67.72a	2.63b	77.54a	31.77a	0.94a	21.09a	2.43a	32.71a	40.50b	11.35a

DW, dry weight; PH, plant height; S/L R, stem/leaf ratio; BN, branching number; CP, crude protein; EE, ether extract; ADF, acid detergent fiber; NDF, neutral detergent fiber; SD, standard deviation; CV, coefficient of variation; H′, genetic diversity index. Different lower-case letters indicate a significant difference among treatments.

follows: Ash > CP > DW > S/L R > PH > BN in Yantai; Ash > DM > CP > S/L R > PH > BN in Dongying.

To holistically evaluate the salt tolerance of alfalfa, the six agronomic traits concurrently influenced by both genotype and environment were harnessed as evaluative indices: DW, PH, BN, S/L R, CP, and Ash. Transmuting these attributes into a salt tolerance index, a membership function was invoked for comparison. The resulting rankings were presented in Table 11, revealing the relative standing of each trait. The hierarchy of traits significantly influenced by the genotypic environment mirrored the following sequence: Ash > DW > CP > S/L R > PH > BN.

Within the Yantai environment, excellent salt-tolerant varieties were Gu yuan zi hua, Ao han, Huang guan, PI 672768, and WL366. Similarly, the ranking of salt-tolerant varieties in the Dongying environment were lead PI 672734, Qian jing, Xiang yang No. 6, PI 672768, and PI 672763. This discerning analysis substantiates the multifaceted nature of salt tolerance within the gamut of alfalfa species, offering invaluable insights into the adaptation and performance of these varieties across contrasting environments.

4. Discussion

4.1. Genetic diversity and environmental adaptation

The phenomenon of long-term natural selection has endowed plants with a relatively stable phenotype and a rich reservoir of genetic diversity (Wu et al., 2018). As evidenced by the substantial divergence in growth parameters, different varieties of the same species exhibit distinctive responses to varied environments (An et al., 2021). This divergence becomes notably pronounced under the stress of salt-induced conditions, where the growth of salt-sensitive varieties is impeded, ultimately posing a threat of plant mortality (Li et al., 2010). Central to assessing alfalfa quality are agronomic traits encompassing DW, PH, and nutrient composition (Akdeniz et al., 2019). The statistical analysis of 51 alfalfa traits across three distinct environments underscores the elevated genetic diversity and coefficient of variation inherent to alfalfa's adaptive repertoire across these varied settings.

4.2. Salt stress and genetic diversity

Salt stress stands as a formidable abiotic factor that profoundly influences alfalfa's growth and development. Prior studies, predominantly conducted under simulated salt stress conditions, offer a myriad of insights into alfalfa's salt tolerance (Annicchiarico, 1992; Tucak et al., 2008). However, a divergence often exists between simulated stress and actual saline-alkali conditions (Qados, 2011; Mann et al., 2019). Correspondingly, Al-Khatib et al. (1992) observed a heightened genetic diversity in alfalfa under salt stress. In congruence with these findings, our experiment illuminates that the genetic diversity of more than half the traits underwent augmentation in response to salt stress. Generally, the imposition of salt stress leads to escalated osmotic pressure in the soil, impairing water absorption and exerting a detrimental impact on alfalfa growth. Consequently, a

TABLE 8 Ranking of 51 alfalfa species in three environments based on grey correlation degree analysis results.

Variety	Rank			Variety	Rank		
	Control	Yantai	Dongying		Control	Yantai	Dongying
A er gang jin	41	40	39	Qian jing	37	34	31
Biao ba	34	41	32	Qing chun	44	44	34
Ao han	35	29	26	Sa lan na si	23	23	18
Bo la tu	29	33	23	San de li	50	48	46
Bei ni dong	22	24	24	Su lian No.36	42	42	35
Gan nong No.1	24	25	27	Lei ting	32	35	33
Gan nong No.3	15	13	7	Long mu 801	49	49	49
Gan nong No.6	4	3	4	Tiao zhan zhe	38	32	41
Fa duo	43	43	45	PI 672763	27	28	28
Mu xu wang	28	19	30	PI 672767	45	46	48
WL366	26	20	25	PI 672768	12	14	8
WL440HO	11	10	11	Zhong mu No.1	18	18	16
WL-SQT	21	21	14	PI 672734	2	2	1
Gong nong No.1	30	39	38	Kang bao	13	17	19
Gu yuan zi hua	40	27	47	PI 672764	36	38	37
De guo da ye	20	26	17	PI 631976	16	22	29
Huang guan	7	5	12	PI 631975	31	36	36
Jin huang hou	14	7	13	Xi mo	9	12	20
Jin neng	17	16	15	Xiang yang No.6	6	6	6
Kai en	25	30	21	Yan shi	19	11	9
Nan mu 501	5	8	3	Ying st	1	1	2
PI 672741	48	50	44	PI 672759	8	9	10
PI 672755	33	31	42	Zhong mu No.3	10	15	22
Liang mu No.2	51	51	50	Tu ku man	46	45	51
Mei zuo	3	4	5	Wei shen	39	37	40
Long mu No.1	47	47	43				

TABLE 9 Two-factor analysis of variance of 51 alfalfa species in Yantai's normal environment and salt pond environment based on 10 agronomic traits.

Character	F (G)	F (E)	F (G × E)	R
DW(g)	82.69**	170.30**	2.24**	0.96
Fresh/Dry ratio	6.68**	0.16	0.23	
PH(cm)	7.5**	175.20**	0.32	0.87
S/L R	14.00**	48.14**	0.47	0.93
BN	6.49**	24.54**	0.02	0.85
CP (%)	28.60**	2258.21**	0.27	0.97
EE (%)	66.46**	0.60	0.15	
ADF (%)	598.22**	0.001	0.93	
NDF (%)	198.57**	3.18	0.71	
Ash (%)	239.12**	31470.23**	2.85**	0.98

DW, dry weight; PH, plant height; S/L R, stem/leaf ratio; BN, branching number; CP, crude protein; EE, ether extract; ADF, acid detergent fiber; NDF, neutral detergent fiber.

**Indicates very significant correlation.

TABLE 10 Two-factor analysis of variance of 51 alfalfa species in Yantai's normal environment and Dongying's saline soil environment based on 10 agronomic traits.

Character	F (G)	F (E)	F (G × E)	R
DW (g)	93.45**	155.60**	1.62*	0.98
Fresh/Dry ratio	5.99**	0.05	0.27	
PH (cm)	7.08**	153.33**	0.40	0.86
S/L R	13.34**	44.79**	0.41	0.93
BN	5.55**	33.15**	0.45	0.82
CP (%)	29.17**	2363.62**	0.24	0.97
EE (%)	58.70**	0.52	0.12	
ADF (%)	582.11**	0.03	0.91	
NDF (%)	207.08**	3.18	0.72	
Ash (%)	316.73**	42387.25**	3.32**	0.99

DW, dry weight; PH, plant height; S/L R, stem/leaf ratio; BN, branching number; CP, crude protein; EE, ether extract; ADF, acid detergent fiber. NDF: neutral detergent fiber.

*Indicates correlation.

**Indicates very significant correlation.

TABLE 11 Ranking of 51 alfalfa species in two salt environments based on two-factor analysis of variance results and membership function theory.

Variety	Rank		Variety	Rank	
	Yantai	Dongying		Yantai	Dongying
A er gang jin	21	42	Qian jing	37	2
Biao ba	26	46	Qing chun	8	27
Ao han	2	41	Sa lan na si	27	17
Bo la tu	41	44	San de li	31	7
Bei ni dong	33	48	Su lian No.36	19	39
Gan nong No.1	24	16	Lei ting	25	9
Gan nong No.3	6	22	Long mu 801	7	26
Gan nong No.6	10	32	Tiao zhan zhe	38	45
Fa duo	18	24	PI 672763	40	5
Mu xu wang	17	36	PI 672767	34	31
WL366	5	40	PI 672768	4	4
WL440HO	15	23	Zhong mu No.1	36	18
WL-SQT	46	20	PI 672734	43	1
Gong nong No.1	29	47	Kang bao	11	30
Gu yuan zi hua	1	33	PI 672764	20	15
De guo da ye	49	10	PI 631976	51	51
Huang guan	3	49	PI 631975	47	6
Jin huang hou	48	34	Xi mo	35	11
Jin neng	22	25	Xiang yang No.6	13	3
Kai en	50	50	Yan shi	12	13
Nan mu 501	14	14	Ying st	39	12
PI 672741	16	38	PI 672759	30	19
PI 672755	45	43	Zhong mu No.3	42	29
Liang mu No.2	28	8	Tu ku man	32	21
Mei zuo	9	37	Wei shen	23	35
Long mu No.1	44	28			

decrease in FW, DW, and PH is an anticipated outcome. These outcomes are in line with previous research that posits salt stress amplifies the S/L R of alfalfa, thereby enhancing overall quality a trend corroborated by our experiment's results.

4.3. Nutrient content and salt stress

Reinforcing the intricate relationship between nutrient content and salt stress, Akdeniz et al. (2019) establish a direct correlation between high CP levels and elevated Ash content. Additionally, Ferreira et al. (2015) and Wan et al. (2023) unveil salt stress's role in augmenting CP content while diminishing ADF and NDF concentrations. Nonetheless, discrepancies persist due to variable factors including alfalfa variety and experimental locale.

4.4. High quality alfalfa varieties

Grey system theory can standardize all kinds of indicators and assign weight coefficients to them, which can not only effectively overcome the limitations caused by single indicators, but also objectively and accurately conduct comprehensive evaluation of alfalfa varieties (lines), and has been widely used in comprehensive evaluation of forage. Wang et al. (2021) According to the study of 8 kinds of alfalfa quality traits by grey correlation degree, WL358, WL440HQ, WL656HQ, and WL366HQ are excellent alfalfa varieties. According to the study of 8 kinds of alfalfa quality traits by grey correlation degree, WL358, WL440HQ, WL656HQ, and WL366HQ are excellent alfalfa varieties. Lu et al. (2021) also evaluated 12 kinds of alfalfa by grey correlation degree and found that WL656HQ, WL525, and WL440HQ were relatively excellent varieties, indicating that grey correlation degree analysis is a commonly used and reliable evaluation method for alfalfa evaluation. Li et al. (2021) found that Ying st performed better in the study on the introduction of alfalfa in Yunnan province, and Ying st performed better in the three environments in this experiment, while Quan et al. (2023) found that Ying st was not prominent in the variety comparison test in Gansu province. This is different from the results of this study, and the reason for this result is the different environment of the planting site.

4.5. G × E interaction and salt tolerance evaluation

A pivotal facet in plant agronomy lies in comprehending the intricate interplay between genotype and environment. Such interactions significantly impact agronomic traits across varied plant species, including wheat, oats, and alfalfa (Purchase, 1997; Peterson et al., 2005; Qiu et al., 2023; Tian et al., 2023). Distinct acidic soils further underscore genotype's pivotal role in shaping alfalfa traits, as elucidated by Achir et al. (2020), who deem environmental influence on alfalfa yield as paramount. In our study, genotype emerged as the principal driver of diverse agronomic traits in control and saline-alkali soil environments. Conversely, the environment significantly affected multiple traits, excluding F/D R, EE, Acid detergent Fiber, and Neutral detergent Fiber. Interestingly, the traits influenced by genotype and environment generally exhibited higher average generalized heritability, reflecting predominant genetic influence. However, as a comprehensive quantitative trait, salt tolerance is a complex trait influenced by many genetic and non-genetic factors (Ashraf and Foolad, 2013), and it is difficult for a single trait to reflect the salt tolerance of plants, comprehensive salt tolerance evaluation demands the amalgamation of morphological, physiological, and biochemical indices (El-Hendawy et al., 2005; Badran et al., 2015; Guo et al., 2022). In the past, Al-Khatib et al. (1994) believed that branch length could be used as an evaluation index of salt-tolerance of alfalfa, while Tavakoli et al. (2019) and Benabderrahim et al. (2020) believed that reliable results could be obtained only when the results of multiple indexes were comprehensively considered. In this experiment, G × E analysis found that only part of the traits studied were affected by environmental factors. Therefore, we used the membership function method to comprehensively evaluate these traits and finally screened out Gu yuan zi hua, Ao han, Huang guan, PI 672768, WL366, PI 672734, Qian jing, Xiang yang No. 6, and PI 672763 excellent salt-tolerant varieties.

In essence, this study contributes to our understanding of alfalfa's genetic diversity, environmental responsiveness, and salt tolerance. The interplay between genotype and environment profoundly shapes the alfalfa's performance, offering insights into suitable varieties for saline-alkali land improvement. By effectively marrying analytical techniques, this research aligns with the overarching goal of optimizing alfalfa cultivation and bolstering agricultural productivity. The screened high-quality purple alfalfa varieties can provide materials for the breeding of purple alfalfa varieties, and at the same time, high-quality salt-tolerant varieties have value in future saline-alkali land improvement.

5. Conclusion

In this study, an exhaustive exploration of agronomic traits across 51 alfalfa varieties under three distinct environments was conducted, yielding valuable insights into the plant's genetic diversity and adaptive capabilities. The observed richness in genetic diversity across the diverse array of agronomic traits underscores the inherent flexibility of alfalfa to respond to varying conditions. Among the plethora of varieties assessed, four standouts emerged: Yingst, PI 672734, Gan nong No.6 and Mei zuo, all manifesting exceptional traits across the different environments studied.

Furthermore, the assessment of salt tolerance in alfalfa under diverse saline and alkaline conditions revealed intriguing nuances. Contrary to conventional assumptions, the highest-performing alfalfa varieties did not universally exhibit superior salt tolerance. An exemplary case is PI 672768, which demonstrated remarkable salt tolerance despite not being classified among the highest-performing varieties under normal conditions. This finding underscores the complexity of salt tolerance mechanisms within alfalfa varieties.

The outcomes of this study hold significant implications for the selection and breeding of alfalfa varieties optimized for diverse environments. The elucidation of genetic diversity, coupled with a nuanced understanding of salt tolerance, lays the foundation for more effective alfalfa variety selection and improvement strategies. Ultimately, these findings contribute to the advancement of sustainable agriculture practices by harnessing the genetic potential of alfalfa to thrive in challenging and diverse conditions.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

SF: Investigation, Resources, Writing – review & editing. JC: Data curation, Writing – original draft. JM: Resources, Writing – original draft. MZ: Funding acquisition, Project administration, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

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References

Achir, C., Annicchiarico, P., Pecetti, L., Khelifi, H. E., and Laouar, M. (2020). Adaptation patterns of sixteen alfalfa (*Medicago sativa* L.) cultivars across contrasting environments of Algeria and implications for the crop improvement. *Ital. J. Agron.* 15, 57–62. doi: 10.4081/ija.2020.1578

Akdeniz, H., Hosaflioglu, I., Koç, A., Hossain, A., Islam, M. S., Iqbal, M. A., et al. (2019). Evaluation of herbage yield and nutritive value of eight forage crop species. *Appl. Ecol. Environ. Res.* 17, 5571–5581. doi: 10.15666/aeer/1703_55715581

Al-Khatib, M., Mcneilly, T., and Collins, J. (1992). The potential of selection and breeding for improved salt tolerance in lucerne (*Medicago sativa* L.). *Euphytica* 65, 43–51. doi: 10.1007/BF00022198

Al-Khatib, M. M., McNeilly, T., and Collins, J. C. (1994). Between and within cultivar variability in salt tolerance in lucerne, (*Medicago sativa* L.). *Genet. Resour. Crop. Evol.* 41, 159–164. doi: 10.1007/BF00051632

An, Y., Gao, Y., Tong, S., and Liu, B. (2021). Morphological and physiological traits related to the response and adaption of Bolboschoenus Planiculmis seedlings grown under salt-alkaline stress conditions. *Front. Plant Sci.* 12:567782. doi: 10.3389/fpls.2021.567782

Annicchiarico, P. (1992). Cultivar adaptation and recommendation from alfalfa trials in Northern Italy. *J. Genet. Breed.* 46:269.

Ashraf, M., and Foolad, M. R. (2013). Crop breeding for salt tolerance in the era of molecular markers and marker-assisted selection. *Plant Breed.* 132, 10–20. doi: 10.1111/pbr.12000

Badran, A. E., ElSherebeny, E. A. M., and Salama, Y. A. (2015). Performance of some alfalfa cultivars under salinity stress conditions. *J. Agric. Sci.* 7:281. doi: 10.5539/jas.v7n10p281

Benabderrahim, M. A., Guiza, M., and Haddad, M. (2020). Genetic diversity of salt tolerance in tetraploid alfalfa (*Medicago sativa* L.). *Acta Physiol. Plant.* 42, 1–11. doi: 10.1007/s11738-019-2993-8

el-Hendawy, S. E., Hu, Y., Yakout, G. M., Awad, A. M., Hafiz, S. E., and Schmidhalter, U. (2005). Evaluating salt tolerance of wheat genotypes using multiple parameters. *Eur. J. Agron.* 22, 243–253. doi: 10.1016/j.eja.2004.03.002

Ferreira, J., Cornacchione, M., Liu, X., and Suarez, D. (2015). Nutrient composition, forage parameters, and antioxidant capacity of alfalfa (*Medicago sativa* L.) in response to saline irrigation water. *Agriculture* 5, 577–597. doi: 10.3390/agriculture5030577

Guo, S., Ma, X., Cai, W., Wang, Y., Gao, X., Fu, B., et al. (2022). Exogenous proline improves salt tolerance of alfalfa through modulation of antioxidant capacity, ion homeostasis, and proline metabolism. *Plants (Basel)* 11:2994. doi: 10.3390/plants11212994

Han, F., Fu, G., Yu, C., and Wang, S. (2022). Modeling nutrition quality and storage of forage using climate data and normalized-difference vegetation index in alpine grasslands. *Remote Sens.* 14:3410. doi: 10.3390/rs14143410

Jin, Y., Wang, Y., Chen, J., Guo, Q., and Xu, B. (2018). Comparison of salt tolerance of 14 alfalfa hybrid combinations. *Pratacult. Sci.* 35, 2931–2939. doi: 10.11829/j.issn.1001-0629.2017-0497

Li, R., Shi, F., Fukuda, K., and Yang, Y. (2010). Effects of salt and alkali stresses on germination, growth, photosynthesis and ion accumulation in alfalfa (*Medicago sativa* L.). *Soil Sci. Plant Nutr.* 56, 725–733. doi: 10.1111/j.1747-0765.2010.00506.x

Li, Y., Zhang, M., Ren, J., Yi, J., Li, X., Yang, Y., et al. (2021). New variety comparison experiment *medicago sativa* l. in subtropical zone of Yunnan province. *J. West China For. Sci.* 50, 82–86. doi: 10.16473/j.cnki.xblykx1972.2021.03.011

Liang, X., Qi, Y., Li, Y., Shi, Y., Cao, W., Li, W., et al. (2017). Comprehensive evaluation on salinity performance of 10 alfalfa varieties by comprehensive index and gray correlation methods. *Crops* 2017:6. doi: 10.16035/j.issn.1001-7283.2017.04.008

Lu, X., Wang, X., Yang, W., Wang, L., Zhang, X., and Zhao, Y. (2021). Production performance and quality evaluation of 12 alfalfa varieties (lines) in Tai 'an, Shandong Province. *Shandong J. An. Sci. Vet. Med.* 42, 1–6. doi: 10.3969/j.issn.1007-1733.2021.01.001

Mann, A., Kaur, G., and Kumar, A., Sanwal, SK., Singh, J., and Sharma, P. (2019). Physiological response of chickpea (*Cicer arietinum* L.) at early seedling stage under salt stress conditions. *Legume Res. Int. J.*, 42, 625–632. doi: 10.18805/LR-4059

Mbarki, S., Skalicky, M., Talbi, O., Chakraborty, A., Hnilicka, F., Hejnak, V., et al. (2020). Performance of *Medicago sativa* grown in clay soil favored by compost or farmyard manure to mitigate salt stress. *Agronomy* 10:94. doi: 10.3390/agronomy10010094

Peterson, D. M., Wesenberg, D. M., Burrup, D. E., and Erickson, C. A. (2005). Relationships among agronomic traits and grain composition in oat genotypes grown in different environments. *Crop Sci.* 45, 1249–1255. doi: 10.2135/cropsci2004.0063

Purchase, J. L. (1997). Parametric analysis to describe genotype x environment interaction and yield stability in winter wheat. [Doctoral dissertation]. University of the Free State.

Qados, A. (2011). Effect of salt stress on plant growth and metabolism of bean plant *Vicia faba* (L.). *J. Saudi Soc. Agric. Sci.* 10, 7–15. doi: 10.1016/j.jssas.2010.06.002

Qiu, Y., Wang, Y., Fan, Y., Hao, X., Li, S., and Kang, S. (2023). Root, yield, and quality of alfalfa affected by soil salinity in Northwest China. *Agriculture* 13:750. doi: 10.3390/agriculture13040750

Quan, J., Li, X., Gan, H., Ma, Y., and Wang, P. (2023). Adaptability of 43 *medicago sativa* varieties in the irrigated agricultural area of Zhangye, Gansu province. *Pratacult. Sci.* 40, 1888–1901. doi: 10.11829/j.issn.1001-0629.2022-0893

Rengasamy, P. (2006). World salinization with emphasis on Australia. *J. Exp. Bot.* 57, 1017–1023. doi: 10.1093/jxb/erj108

Roshdy, A. E. D., Alebidi, A., Almutairi, K., Al-Obeed, R., and Elsabagh, A. (2011). The effect of salicylic acid on the performances of salt stressed strawberry plants, enzymes activity, and salt tolerance index. *Agronomy* 11:775. doi: 10.3390/agronomy11040775

Tavakoli, M., Poustini, K., Besharati, H., and Ali, S. (2019). Variable salinity responses of 25 alfalfa genotypes and comparative salt-response ion distribution. *Russ. J. Plant Physiol.* 66, 231–239. doi: 10.1134/S1021443719020158

Tian, Z., Yang, Z., Lu, Z., Luo, B., Hao, Y., Wang, X., et al. (2023). Effect of genotype and environment on agronomical characters of alfalfa (*Medicago sativa* L.) in a typical acidic soil environment in Southwest China. *Front. Sust. Food Syst.* 7:1144061. doi: 10.3389/fsufs.2023.1144061

Tucak, M., Popović, S., Čupić, T., Grljušić, S., Bolarić, S., and Kozumplik, V. (2008). Genetic diversity of alfalfa (*Medicago* spp.) estimated by molecular markers and morphological characters. *Period. Biol.* 110, 243–249.

Wan, W., Liu, Q., Zhang, C., Li, K., Sun, Z., Li, Y., et al. (2023). Alfalfa growth and nitrogen fixation constraints in salt-affected soils are in part offset by increased nitrogen supply. *Front. Plant Sci.* 14:358. doi: 10.3389/fpls.2023.1126017

Wang, X., Wang, L., Yang, W., Pan, X., Zhao, Y., Jiang, H., et al. (2021). Comprehensive evaluation of alfalfa quality based on near infrared spectroscopy methods. *Chin. J. Grassland* 43, 38–44. doi: 10.16742/j.zgcdxb.20190253

Wang, M., Zhao, Z., Hu, Z., and Wu, X., Gong, M., He, Z., et al. (2023). Evaluation of heat tolerance of local germplasm resources of leek in Guangxi based on membership function and cluster analysis. *Southw. China J. Agric. Sci.*, 36, 541–549. doi: 10.16213/j.cnki.scjas.2023.3.012

Wu, X. M., Guo, P., Chi, H. W., Fang, Z. H., Shi, Y. H., Wang, Y. Q., et al. (2018). Diversity analysis of phenotypic traits and quality characteristics of alfalfa (*Medicago sativa*) introducted from abroad germplasm resources. *J. Plant Gen. Res.* 19, 103–111. doi: 10.13430/j.cnki.jpgr.2018.01.012

Yu, R., Wang, G., Yu, X., Li, L., Li, C., Song, Y., et al. (2021). Assessing alfalfa (*Medicago sativa* L.) tolerance to salinity at seedling stage and screening of the salinity tolerance traits. *Plant Biol.* 23, 664–674. doi: 10.1111/plb.13271

Zeng, L. S., Li, P. Y., Sun, X. F., and Sun, Z. J. (2020). A multi-trait evaluation of drought resistance of bermudagrass (*Cynodon dactylon*) germplasm from different habitats in Xinjiang province. *Acta Pratacul. Sin.* 29, 155–169. doi: 10.11686/cyxb2019486

Zhang, C., Chen, Y., Xu, F., Song, S., and Zhao, N. (2008). Study on the alfalfa cultivation in the young poplar growing *Medicago sativa*. *Grassland Turf* 128, 63–65. doi: 10.13817/j.cnki.cyycp.2008.03.017

Zhang, L., Song, Q., Ma, S., Yu, X., Wang, C., and Yang, X. (2023). Applying grey correlation degree analysis to evaluate nutritional value of 12 *Medicago sativa* varieties. *China Feed* 725, 136–140+161. doi: 10.15906/j.cnki.cn11-2975/s.2022080032-02

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Effects of short-term drought, nitrogen application and their interactions on the composition and functional genes of soil microbial communities in alfalfa grassland on the Loess Plateau

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Drought and nitrogen addition are important factors influencing soil microorganisms and changes in the soil environment. In the future, droughts will become more frequent, shorter, and more severe. However, little is known about the soil organic carbon components, enzyme activity, and composition, structure, and functional genes of soil microbial communities under short-term drought and nitrogen application conditions. In this study, we used metagenomics sequencing technology to explore the changes in the composition and functional genes of soil microbial communities under short-term drought, nitrogen application, and their interactions in the artificial grasslands of the Loess Plateau. The results indicated that (I) short-term drought, nitrogen application, and their interactions all increased the particulate organic carbon (POC) content. (II) Short-term drought increased the contents of soil microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN), as well as soil enzyme activity. (III) Short-term drought significantly increased the bacterial alpha diversity, whereas the interactions of short-term drought and nitrogen application enhanced the fungal alpha diversity. (IV) The interactions of short-term drought and nitrogen application inhibited *Fusarium* to prevent plant diseases. (V) Short-term drought enriched the relative abundance of genes related to carbon cycling and amino acid metabolism, while nitrogen application reduced genes related to carbon cycling but enriched genes related to glycan biosynthesis and metabolism. These results clearly showed that, short-term drought altered the composition and functional genes of soil microbial communities. Our research suggests that in the event of frequent short-term droughts in the future, nitrogen addition can be considered to maintain the diversity of soil microbial communities and sustain soil carbon and nitrogen cycling.

KEYWORDS

short-term drought, nitrogen application, soil enzyme activity, alpha diversity, bacterial and fungal communities, functional genes

1 Introduction

Drought is one of the most important factors affecting soil microorganisms, plants, and the soil environment (Bogati and Walczak, 2022). The emergence of extreme weather events, especially drought, puts pressure on the function and composition of soil microorganisms, reducing their activity and abundance and affecting enzyme production and nutrient cycling, thereby leading to a decrease in soil fertility and inhibition of plant growth (Geisen et al., 2019; Jansson and Hofmockel, 2020; Bogati and Walczak, 2022). Climate models predict an increase in the frequency and intensity of extreme drought weather events (Iglesias and Garrote, 2015; Spinoni et al., 2015). In arid and semi-arid areas, extreme drought events may become shorter but more severe and frequent (Ochoa-Hueso et al., 2018). Such frequent short-term drought may increase the risk of dryness and high temperatures in specific regions or seasons. Plants need sufficient water during the growing season, resulting in plant damage and reduced yields. Moreover, short-term drought may lead to changes in soil organic carbon composition and extracellular enzyme activity related to soil organic matter decomposition (Barnard et al., 2013), as well as changes in microbial communities and functions (Hueso et al., 2012; Ochoa-Hueso et al., 2018). Therefore, it is necessary to explore the impact of short-term drought on soil microbial communities and functions for agricultural development.

The impacts of drought on microorganisms are very complex and may be influenced by the frequency, intensity, and duration of drought (Kundel et al., 2020). Short-term drought is not a "weakened version" of long-term drought; it has its own unique features (Yakushev et al., 2023). At present, the assembly of microbial communities is easily affected by short-term climate change (especially drought), which may affect the participation of microorganisms in nutrient decomposition and cycling (Ochoa-Hueso et al., 2018). In particular, the structure of bacterial communities is more easily affected by short-term environmental changes (Yuste et al., 2014). Although the changes caused by extreme drought have negative impacts, they may induce the adaptation of microorganisms and plants to the environment by altering related functional genes, allowing them to continue to survive and reproduce (Sayer et al., 2021). In addition, the impacts of extreme drought on microorganisms can lead to changes in soil extracellular enzyme activity and redistribute resources through feedback mechanisms that affect carbon and nitrogen cycling in the soil (Austin et al., 2004; Barnard et al., 2013; Schimel, 2018). At present, it is not yet clear how short-term drought affects soil carbon cycling.

Nitrogen addition also has a major impact on soil properties and soil microorganisms. The application of nitrogen can be used to improve soil fertility and plant productivity and further reduce the negative effects of extreme weather events on soil fertility (Lu et al., 2015; Nkebiwe et al., 2016). However, the impacts of nitrogen addition on soil organic carbon components and extracellular enzyme activity are contradictory, and some studies have shown that soil organic carbon components and extracellular enzyme activity increase (Maaroufi et al., 2015; Jian et al., 2016), decrease (Ochoa-Hueso et al., 2018), or stay the mostly the same (Chen et al., 2015). These differences may be due to differences in ecosystem types, nitrogen fertilizer methods, and experimental times (Lu et al., 2011). Moreover, the responses of the composition and structure of soil microbial communities to nitrogen addition are inconsistent (Roberts et al., 2011). For example, the composition of microbial communities did not respond to nitrogen addition in soil (Roberts et al., 2011), or nitrogen addition led to a decrease in bacterial diversity in grassland soil (Zeng et al., 2016). Therefore, further exploration of the effects of nitrogen application on soil organic carbon components, soil enzyme activity, and soil microorganisms based on specific circumstances is necessary.

Surprisingly, the negative impact of drought on the soil can be reduced by adding nutrients. The addition of nitrogen generally improves soil fertility, which may alleviate the negative effects of drought on soil function and plant productivity. The addition of nitrogen alleviates but cannot fully offset the legacy effects of extreme weather events (Nguyena et al., 2018). The functions of soil microbial communities, as well as changes in the composition and structure of communities, importantly influence soil function (Sayer et al., 2021). However, there is currently little understanding of the effects of extreme drought events and nitrogen addition on soil microbial communities and the processes involved in carbon cycling (Nguyena et al., 2018). It is still unknown whether the impacts of short-term drought on microbial communities and carbon cycling can be regulated by adding appropriate amounts of nitrogen. Therefore, continuing to explore the interaction between drought and nitrogen application is crucial for understanding carbon cycling in agricultural systems under extreme climate conditions.

Due to limited knowledge of how short-term drought, nitrogen addition, and their interactions affect soil carbon components, soil enzyme activity, the composition of soil microbial communities and their functional genes, we designed an experiment involving short-term drought and nitrogen application on artificial alfalfa grasslands in the Loess Plateau of China. In addition, we used a metagenomic sequencing strategy to explore the changes in functional genes involved in carbon and nitrogen cycling. Therefore, in our study, we aim to address the following questions: (I) How do short-term drought and nitrogen application and their interactions affect changes in soil organic carbon components, MBC, MBN, and soil enzyme activity? (II) How do short-term drought and nitrogen application and their interactions change the composition and structure of soil microbial communities? (III) Do the relative abundances of functional genes related to carbon and nitrogen cycling change under short-term drought, nitrogen application and their interactions? (IV) Is it possible to mitigate the effects of drought on both soil microbial communities and soil functions by adding a suitable amount of nitrogen over a short period of time?

2 Materials and methods

2.1 Experimental location and design

This experiment was conducted at the National Field Scientific Observation and Research Station of Grassland Agricultural Ecosystem in Qingyang, Gansu Province, China (35°40'N, 107°51′E, altitude 1,297 m), which belongs to the hinterland on the Longdong Loess Plateau, with a warm temperate semi-arid continental monsoon climate. The average temperature is 9.4°C, and the average potential evaporation is 1,500 mm. The average annual rainfall is 527.6 mm, mainly concentrated during the growth period, with July to September accounting for approximately 60% of the annual rainfall. The research object of the experiment is the artificial grassland dominated by alfalfa, which exhibits strong regeneration and ecological adaptability. It can be harvested three times a year, every time during the early flowering period.

To explore the effects of short-term drought, nitrogen application and their interactions on soil organic carbon components, soil enzyme activity, and composition and structure of soil microbial communities, the study adopted a completely randomized block design with two factors (short-term drought and nitrogen application) and four treatments (C: blank control, P: short-term drought, N: nitrogen application, NP: nitrogen application × short-term drought), with four zones per treatment, totaling 16 experimental plots, each with an area of 4 m × 4 m (16 m^2). A distance of 1 m was reserved between the plots to facilitate experimental sampling and avoid interference from human activities on adjacent experimental plots (Figure 1).

The experiment included two rainfall scenarios, natural rainfall and drought (reducing rainfall by 50%), and used canopies to achieve the drought treatment. The height of canopies from the ground was 1.30–1.60 m. Tile-shaped transparent groove plates measuring 20 cm in width were used to intercept rainwater and channel it into the rainwater collection device through PVC sinks and conduits. To eliminate the differences between the treatments caused by the light transmittance of PVC pipes in the canopies, the canopies were also placed in the area without short-term drought treatment, and the pipes were inverted to achieve the effect of natural precipitation. Trenches were dug around the sampling area and PVC boards were buried at a depth of 60 cm, exposing the surface for 10 cm to prevent soil moisture from migrating between the samples. There were two levels of nitrogen addition, namely, nitrogen application and no nitrogen application. Based on the soil nitrogen content in the Loess Plateau region, development trends, and relevant research cases, the nitrogen application rate was set at 10 g N m^{-2} $yr.^{-1}$. Based on the nitrogen content of urea, the needed amount of urea for each experimental plot was calculated and applied evenly in the treatment area using the dry method before rainfall each year (divided into three separate times).

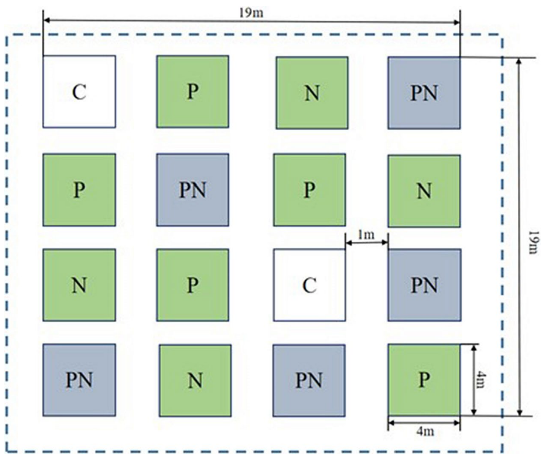

FIGURE 1
Design drawing of the sample plot for this experiment. (C: blank control, N: nitrogen application, P: short-term drought, NP: short-term drought × nitrogen application).

2.2 Soil sampling

This experiment was conducted in April 2022. The soil was sampled on 4 September 2022. After harvesting alfalfa, we randomly selected four sampling locations within each plot and collected soil cores with a 38 mm inner diameter at depths of 0–10, 10–20, 20–30, and 30–50 cm. Soil samples from the same soil layer were mixed. Four biological replicates were performed per group.

2.3 Methods for measuring soil carbon components

Method for determining total soil organic carbon (TOC): soil samples from each soil layer were ground through a 0.25 mm soil sieve under external heating (oil bath temperature, 180°C; boiling time, 5 min). Soil organic carbon was oxidized with a certain concentration of potassium dichromate and sulfuric acid solutions, the remaining potassium dichromate was titrated with ferrous sulfate, and the content of organic carbon was calculated from the amount of potassium dichromate consumed. Compared with the dry burning method, only 90% of organic carbon is oxidize using this method. Therefore, the measured organic carbon was multiplied by a correction factor of 1.1 to calculate the amount of organic carbon. This experiment used a ferrous solution of o-phenanthroline as an indicator.

Easily oxidized organic carbon (EOC) refers to the organic carbon in soil that is easily oxidized and unstable after interacting with oxidants. Based on the total organic carbon content of the soil, the amount of soil sample containing 15 mg of carbon was calculated as the weight of the sample to be tested (approximately 2.0–2.5 g of soil), and then the sample was transferred to 50 ml plastic centrifuge tubes with covers, with no soil sample added to the blank sample (three biological replicates). Then, 25 mL of potassium permanganate solution with a concentration of 333 mmol/L was added to the centrifuge tubes, the centrifuge tubes were shaken for 1 h (normal shaking was sufficient), and the tubes were centrifuged at a speed of 4,000 r/min for 5 min. The supernatant was diluted with deionized water (1:250), and the absorbance at 565 nm was measured by a spectrophotometer. The change in potassium permanganate concentration was calculated from the difference between the absorbance of the blank without soil and that of the soil sample, and then the amount of oxidized carbon could be calculated.

Particulate organic carbon (POC) refers to organic particulate matter insoluble in water, which plays an important role in the carbon cycle. Air-dried soil was passed through a 2 mm sieve to remove visible plant roots and other debris. The samples then dried overnight and stored at 4°C. Ten grams of pre-treated soil sample was weighed, put into reagent bottles, 30 mL of 5 g/L sodium hexametaphosphate solution was added, and the sample was shaken on a reciprocating oscillator for 15 h to disperse it. The dispersed solution was then passed through a 53 μm sieve and rinsed with clean water until the leachate clarified. The separated components were dried at 60°C and weighed, and their percentage in the soil was calculated.

Dissolved organic carbon (DOC) refers to the organic carbon extracted with water, dilute salt, and other extractants, followed by passage through a 0.45 μm pore filter membrane. Each layer of soil sample was ground through a 2 mm sieve, 5 g of soil sample was weighed, and the sample was transferred to 100-mL plastic bottles with covers. K_2SO_4 solution (0.5 mol/L) was added to complete 25 mL (soil: water ratio 1:5), and the mixture was shaken for 30 min. The mixture was left to settle for 30 min after the end of the shock. A pipette gun was then used to accurately transfer 2.5 ml of the supernatant of the original solution to 25 ml volumetric flasks (diluted to 5 ~ 15 mg/L, depending on the concentration of the original solution and diluted approximately 10 times in the Loess Plateau area). Then, the constant-volume solution was drawn into a 30 ml syringe and passed through a 0.45 μm pore filter membrane, and the absorbance of the filtered solution was measured by a UV spectrophotometer at a wavelength of 254 nm. Finally, a regression equation was established with the concentration and absorbance value to calculate the results.

2.4 Determination of MBC and MBN

The MBC was determined by weighing six fresh soil samples (passed through a 2 mm soil sieve) each to 12.5 g and placing them in small beakers. The three small beakers were then placed into a vacuum dryer, with three beakers placed at the bottom of the dryer, each containing chloroform, water, and low-concentration sodium hydroxide solution (with a small amount of glass beads in the beakers). When vacuuming, chloroform was allowed to boil vigorously for 3–5 min, the vacuum dryer valve was then closed, and the sample was placed in a dark room for 24 h. After the fumigation was completed, the dryer valve was opened, and the chloroform was removed (and placed in a fume hood). The other three portions of soil were put into another dryer, and the above operation was repeated, but chloroform was not added. All the fumigated soil samples were then transferred to 150 ml triangular flasks, 50 mL of 0.5 mol/L K_2SO_4 (K_2SO_4: water ratio 1:4) was added, the samples were shaken for 30 min and filtered. The operation for non-fumigated soil samples was the same, and blanks were made at the same time. The determination of MBN required repeating the above steps to obtain the extraction solution and then using a Kjeldahl azotometer for determination.

2.5 Enzyme activity

This experiment used the fluorescence microplate assay to measure the activity of soil enzymes (Qi et al., 2016). The enzymes selected for measurement in this experiment were *β*-glucosidase (Glu), *β*-cellobiosidase (β-CB), *β*-1,4-xylosidase (Xyl), leucine aminopeptidase (LAP), peroxidase (POD), and N-acetylamino glucosidase (NAG).

2.6 Experimental procedures of metagenomic sequencing

DNA was extracted from soil samples using the CTAB method. The DNA degradation degree, potential contamination and DNA concentration were measured using the Agilent 5,400 system. The sequencing library was generated using the NEBNext® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA, Catalogue#: E7370L) following the manufacturer's recommendations. Briefly, genomic DNA samples were fragmented by sonication to a size of 350 bp. Then, DNA fragments were end-polished, A-tailed, and ligated with the full-length adapter for Illumina sequencing, followed by further PCR amplification. After PCR, the products were purified by the AMPure XP system (Beckman Coulter, USA). Subsequently, library quality was assessed on the Agilent 5,400 system (Agilent, USA) and quantified by qPCR (1.5 nM). The qualified libraries were pooled and sequenced on Illumina platforms with the PE150 strategy, according to the effective library concentration and data amount needed. The raw data of bacteria, fungi, and viruses in soil samples were obtained by metagenomic sequencing using the Illumina NovaSeq high-throughput sequencing platform.

2.7 Statistical analysis

First, the experimental data were processed in Excel 2013, which included data import, cleaning, conversion and formatting to ensure the accuracy and consistency of the data. Then, statistical tests were conducted using SPSS 25.0 software, and soil carbon components, soil enzyme activity, MBC, and MBN data were analyzed using *t*-tests, analysis of variance, and other functions (statistical significance was determined at a level of $p \leq 0.05$). Microbial data were analyzed using R software (version 3.1.1). We used the "vegdist," "adonis" and "capscale" functions in the vegan package and used the ggplot2 package for data visualization[1].

3 Results

3.1 Changes in soil organic carbon components, MBC, MBN, and pH under short-term drought and nitrogen application

The differences in soil organic carbon components among the four treatments are shown in Figure 2. There was no significant change in TOC or DOC among the four treatments. Compared with that in the C treatment, the POC in all treatments increased, with the increase in the surface layer being the most significant ($p < 0.05$). The NP treatment had the highest POC content. In the four treatments, the contents of EOC in the 0–10 and 10–20 cm soil layers showed significant changes ($p < 0.05$). Compared with that in the C treatment, the content of EOC in the N treatment increased.

Compared with that in the C treatment, the contents of MBC and MBN in the N treatment, P treatment and NP treatment were slightly increased. Moreover, the increase in MBC and MBN in the P treatment was higher than that in the other treatments (Figure 3). The minimum MBC and MBN values were 5.80 and 2.19 mg/kg, respectively, and the maximum MBC and MBN values were 139.78 and 20.67 mg/kg, respectively.

The pH values of the four treatments were similar, but compared with the C treatment, the N treatment showed a downwards trend, the

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[1] https://bioinfogp.cnb.csic.es/tools/venny/index.html

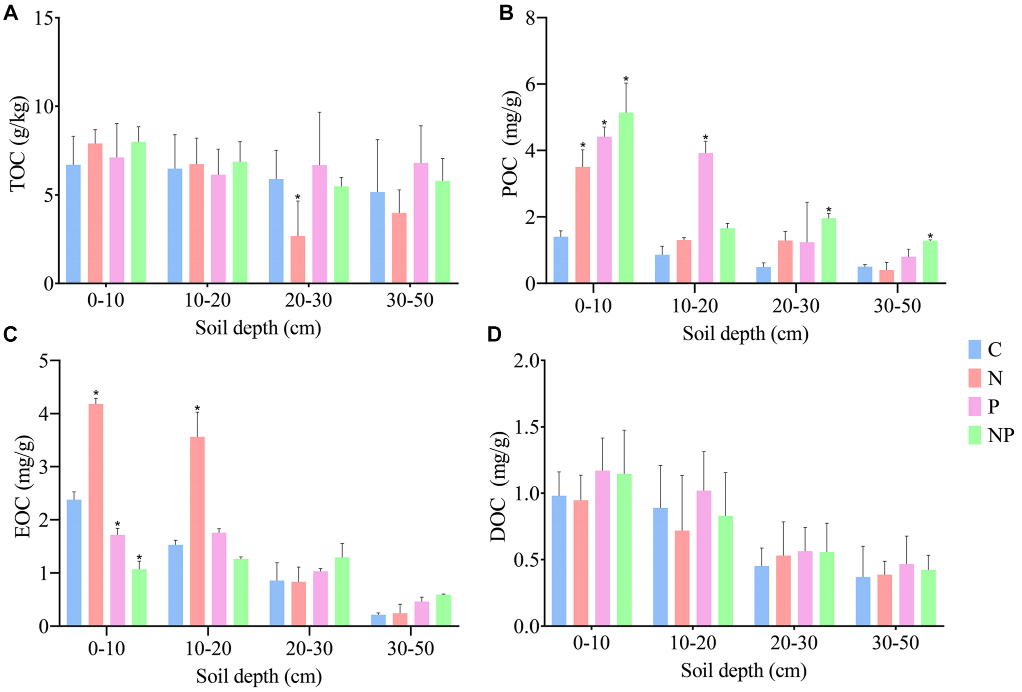

FIGURE 2
Differences in the contents of soil organic carbon components in C (blank control), N (nitrogen application), P (short-term drought), NP (short-term drought × nitrogen application), **(A)** TOC content, **(B)** POC content, **(C)** EOC content, and **(D)** DOC content. (*) Represents significant differences between treatments ($p < 0.05$).

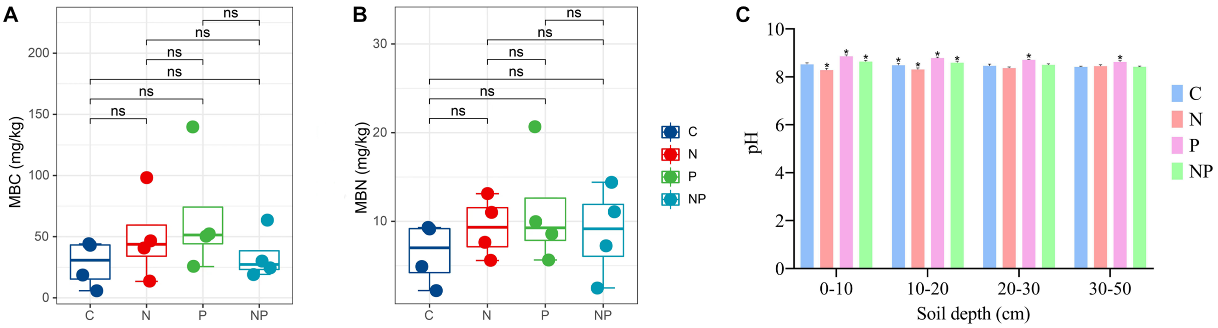

FIGURE 3
The contents of MBC, MBN, and soil pH in C (blank control), N (nitrogen application), P (short-term drought), and NP (short-term drought × nitrogen application), **(A)** MBC content, **(B)** MBN content, **(C)** pH value. (*) Represents significant differences between treatments ($p < 0.05$).

P treatment showed an upwards trend, and the NP treatment was similar to the C treatment (Figure 3).

3.2 Short-term drought stimulates enzyme activity

The soil enzyme activities in the four treatments are shown in Figure 4. Compared with those in the C treatment, the activities of Xyl, Glu, and LAP increased in the P and NP treatments but were not different in the N treatment. The activities of POD, NAG and β-CB did not change significantly among the four treatments.

3.3 Changes in the diversity of soil microbial communities under short-term drought and nitrogen application

We used Venn diagrams (Figure 5) to show the numbers of common and unique species in the four treatments. Compared to the C treatment, the numbers of operational taxonomic units (OTUs) of fungi and bacteria were increased in the other three treatments. Bacterial OTU analysis showed that all treatments shared 1,399 OTUs, among which the P treatment had the highest number of bacterial OTUs (2,485). Fungal OTU analysis showed that all treatments shared 88 OTUs, among which the NP treatment had the highest number of

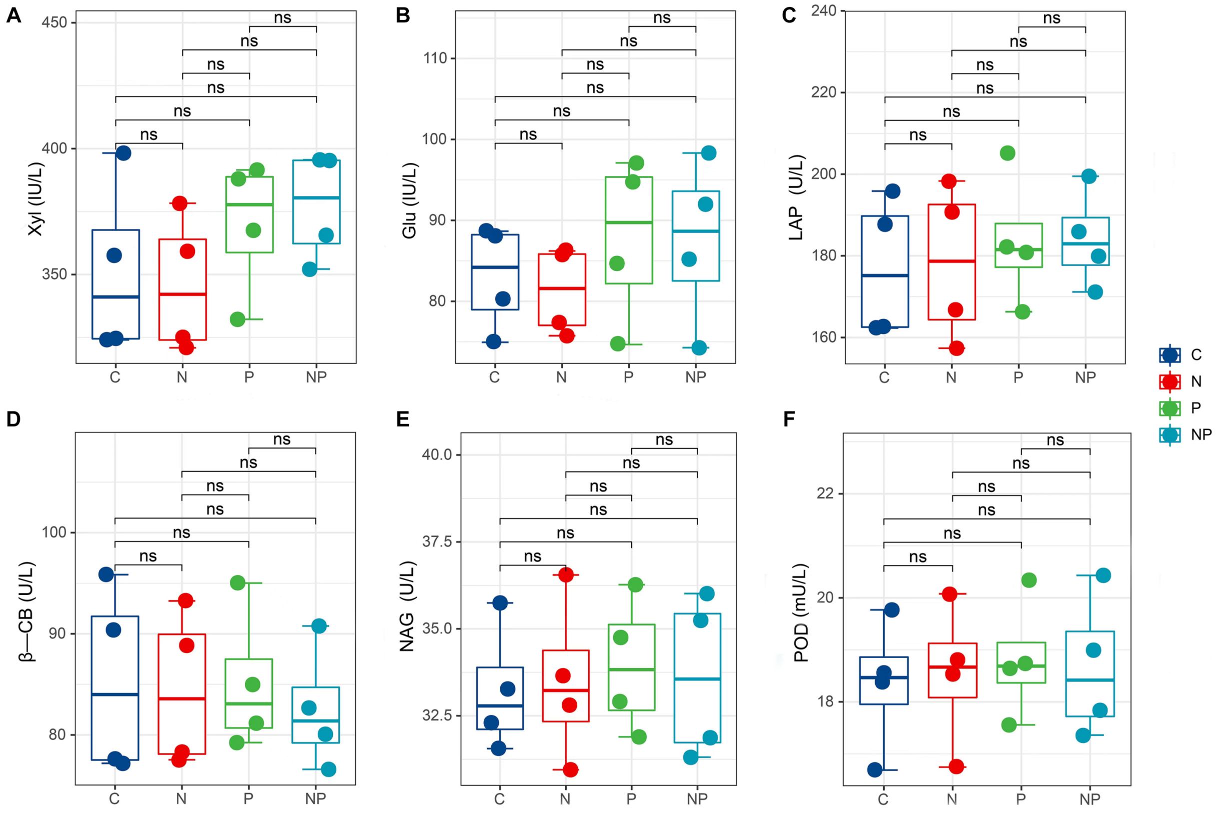

FIGURE 4
Box plots show the activities of soil enzymes among C (blank control), N (nitrogen application), P (short-term drought), NP (short-term drought × nitrogen application). **(A)** Xyl activity, **(B)** Glu activity, **(C)** LAP activity, **(D)** β-CB activity, **(E)** NAG activity, **(F)** POD activity.

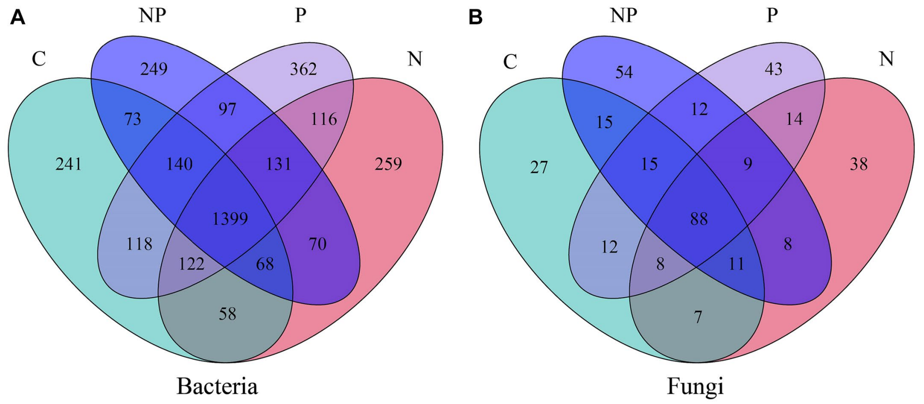

FIGURE 5
Venn diagrams of the unique and shared operational taxonomic units (OTUs) between C (blank control), N (nitrogen application), P (short-term drought), NP (short-term drought × nitrogen application). **(A)** Venn diagrams of bacterial OTUs. **(B)** Venn diagrams of fungal OTUs.

fungal OTUs (212). This indicated that the fungal community diversity of the NP treatment was higher, while the bacterial community diversity of the P treatment was higher. As shown in Figure 6, the P treatment significantly enhanced bacterial alpha diversity (Chao1, Ace, and Shannon) ($p < 0.05$). However, the NP treatment increased fungal alpha diversity (Ace and Shannon), while the N treatment had almost no effect on bacterial or fungal alpha diversity.

3.4 Changes in the composition and structure of soil microbial communities under short-term drought and nitrogen application

The relative abundances of the top 10 bacterial and fungal phyla and genera among the four treatments are shown in Figure 7. At the

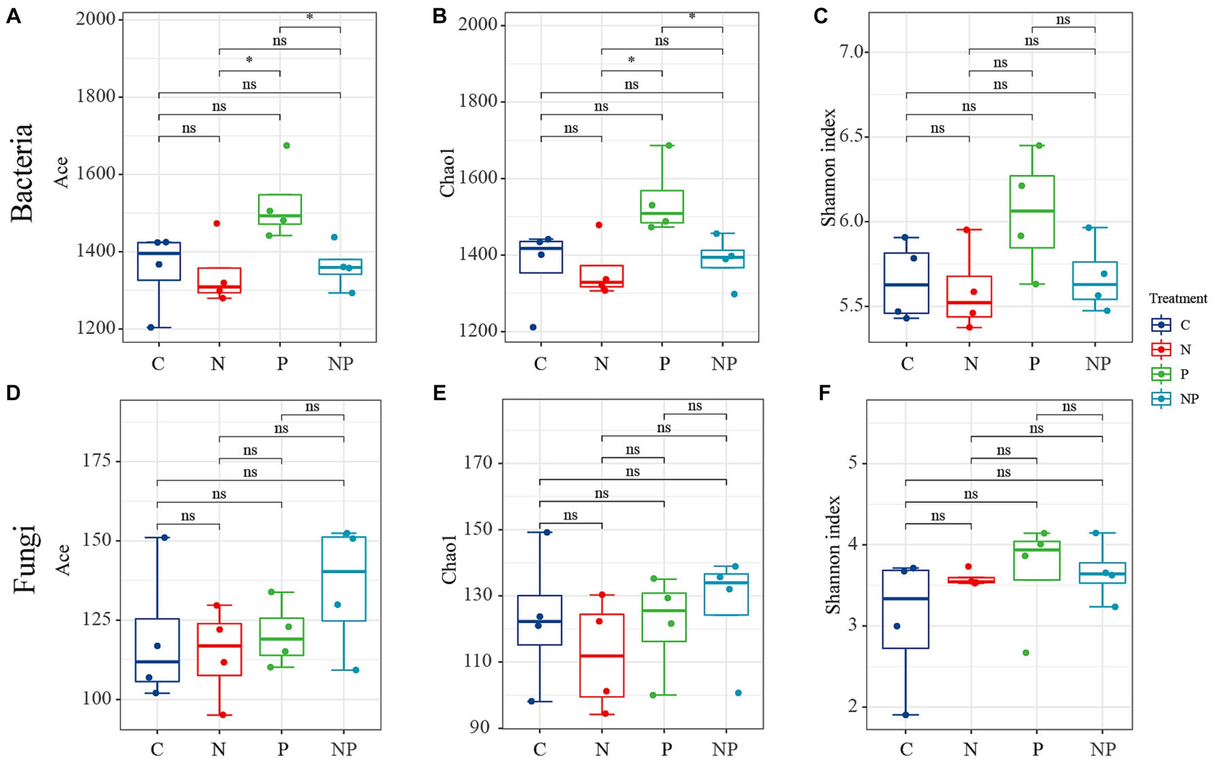

FIGURE 6
Box plots of bacteria and fungi alpha diversities among the four treatments, (*) represents significant differences between treatments ($p < 0.05$). (C: blank control, N: nitrogen application, P: short-term drought, NP: short-term drought x nitrogen application.) **(A)** ACE index in bacterial alpha diversity, **(B)** Chao1 index in bacterial alpha diversity, **(C)** Shannon index in bacterial alpha diversity, **(D)** ACE index in fungi alpha diversity, **(E)** Chao1 index in fungi alpha diversity, **(F)** Shannon index in fungi alpha diversity.

bacterial phylum level, *Proteobacteria* accounted for the highest proportion of bacterial communities in all treatments, but the proportion of *Proteobacteria* remained almost unchanged among the four treatments. The other two dominant bacterial phyla among the four treatments were *Actinobacteria* and *Firmicutes*. The proportions of *Actinobacteria* in the C, N, P, and NP treatments were 24.8, 24.18, 29.99, and 27.38%, respectively. The proportions of *Firmicutes* in the C, N, P, and NP treatments were 1.32, 1.51, 2.05, and 1.73%, respectively. Compared with those in the C treatment, the proportions of *Actinobacteria* and *Firmicutes* in the P and NP treatments increased, while there was no significant change in the N treatment. In addition, the proportion of *Cyanobacteria* in the NP treatment was higher than that in the other three treatments. At the fungal phylum level, the top three dominant fungal communities among the four treatments were *Ascomycota*, *Mucoromycota*, and *Basidiomycota*. Their proportions were 52.95, 9.9, 2.07% in the C treatment, 49.4, 6.38, 4.6% in the N treatment, 50.87, 19.39, 3.76% in the P treatment, and 53.86, 13.08, 2.35% in the NP treatment, respectively. Compared with that in the C treatment, the proportions of *Mucoromycota* in the P and NP treatments increased, while those in the N treatment decreased, and the proportions of *Basidiomycota* in the N, P, and NP treatments increased.

At the genus level, in terms of the composition and structure of bacterial communities, there was no significant change in the relative abundance of the top 10 species under the N, P, and NP treatments compared to the C treatment. At the fungal genus level, the top four dominant fungal communities in the four treatments were *Metarhizium*, *Fusarium*, *Rhizophagus*, and *Cladosporium*, and their proportions were 24.57, 13.45, 8.6, and 0.99% in the C treatment, 8.86, 10.26, 5.29, and 6.91% in the N treatment, 34.5, 17.74, 18.44, and 3.96% in the P treatment, and 17.7, 13.44, 11.16, and 3.28% in the NP treatment, respectively. Compared with that in the C treatment, the proportion of *Metarhizium* in the P treatment increased, while that in the N and NP treatments decreased. The proportion of *Rhizophagus* in the P and NP treatments increased, while that in the N treatments decreased. The proportions of *Cladosporium* in the N, P, and NP treatments increased. Furthermore, it is worth noting that *Purpureocillium* accounted for 0.58% of the community in the C treatment and 2.94% in the P treatment.

3.5 Changes in functional genes under short-term drought and nitrogen application

The metagenomic reads were annotated based on the KEGG database (Figure 8). The P treatment increased the relative abundances of pathway genes related to the carbon cycle and amino acid metabolism. The N treatment decreased the abundances of pathway genes related to the carbon cycle, such as starch and sucrose

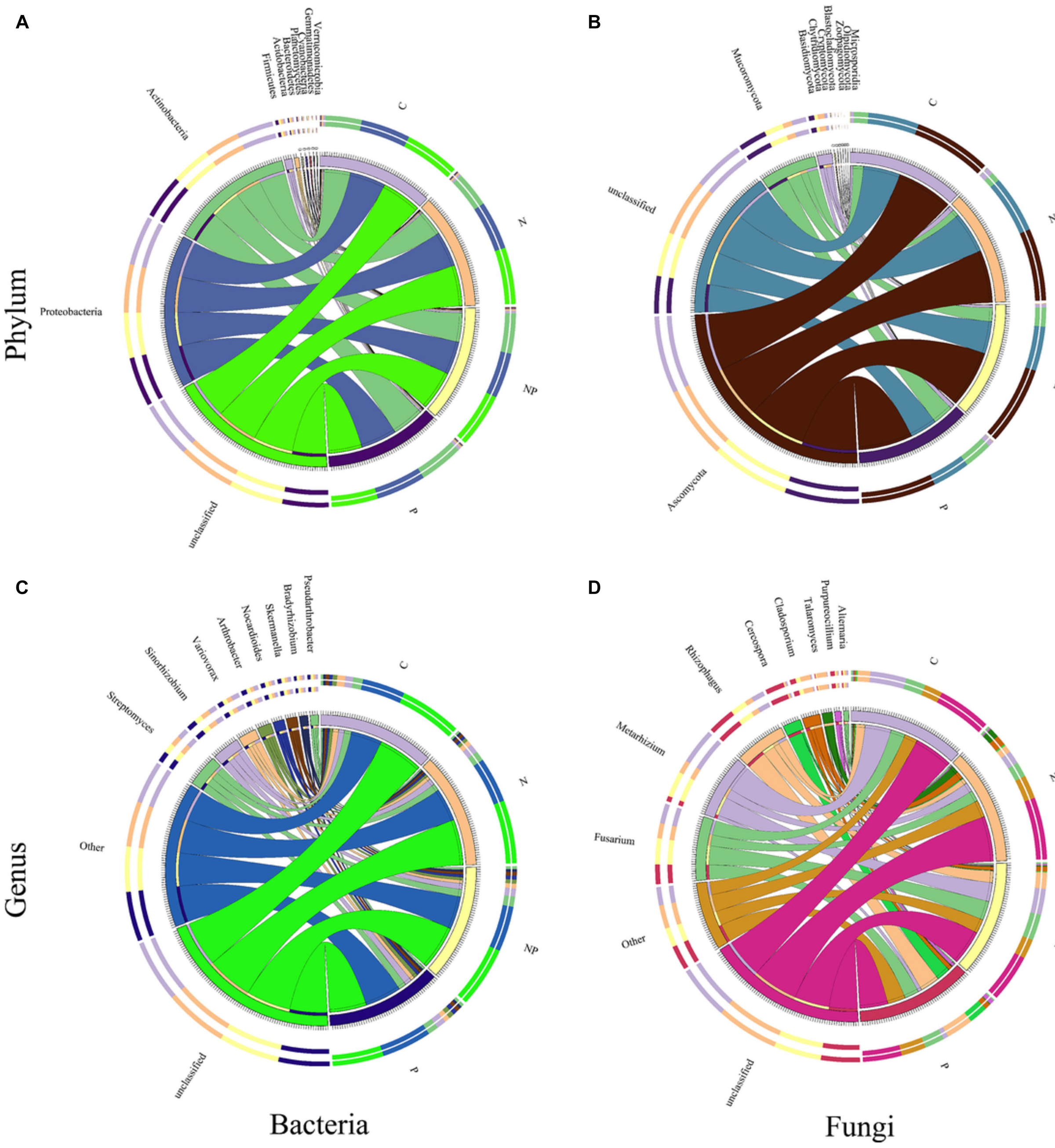

FIGURE 7
The relative abundances of dominant bacterial and fungal communities at the phylum and genus levels in C: blank control, N: nitrogen application, P: short-term drought and NP: short-term drought × nitrogen application. **(A,C)** The relative abundances of the top 10 bacterial phyla and genera among the four treatments. **(B,D)** The relative abundances of the top 10 fungal phyla and genera among the four treatments.

metabolism, inositol phosphate metabolism, pentose phosphate pathway, pentose and glucuronic acid transformation, ascorbate and aldarate metabolism, but increased the relative abundances of genes related to glycan biosynthesis and metabolism, such as glycosphingolipid biosynthesis-globo and isoglobo series, other glycan degradation, lipopolysaccharide biosynthesis, glycosaminoglycan degradation, and various types of N-glycan biosynthesis. Moreover, N treatment increased the abundances of genes related to the nitrogen cycle. There were no significant changes in the NP treatment.

4 Discussion

4.1 Effects on soil physicochemical properties and soil enzyme activities

Soil is a large carbon pool and its carbon content is three times that of the atmosphere (Lal, 2004). The amount of soil organic carbon directly affects soil quality and productivity (Liu et al., 2014) and reflects the relationship between input and decomposition rates, but it is difficult to detect changes under short-term climate change

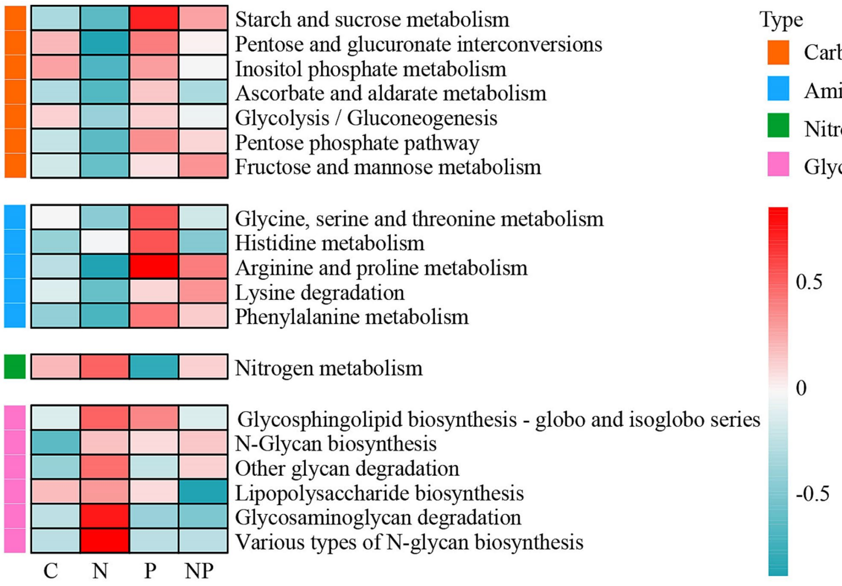

FIGURE 8
Heatmaps of the relative abundances of functional genes related to the carbon and nitrogen cycles for all treatments based on KEGG database. (C: blank control, N: nitrogen application, P: short-term drought, NP: short-term drought × nitrogen application). The blue and red colors indicate abundances from low to high, respectively.

(Yang et al., 2005). Recently, many studies have found that unstable organic carbon, such as POC, DOC, EOC, and MBC, is a more sensitive and useful indicator (Plaza-Bonilla et al., 2014). In this study, we found that the N, P and NP treatments increased POC (Figure 2). The short-term droughts led to an increase in POC, which may be due to POC being mainly imported from fresh plant stubble and the return of litter and dead fine roots. Nitrogen application also increased POC, possibly due to nitrogen enrichment promoting the accumulation of soil POC by increasing large aggregate carbon and causing acidification of the soil (Wang et al., 2020).

Both the N and P treatments increased the soil MBC and MBN (Figure 3). Nitrogen application increased soil MBC and MBN, which may be because appropriate nitrogen application can meet the nitrogen demand of soil animal, plant and microbial life activities and stimulate soil microbial activities (Zhu et al., 2021). At the same time, the sufficient supply of soil nutrients after nitrogen application alleviates the competition between plants and soil microorganisms, thus promoting the accumulation of MBC and MBN (Zhu et al., 2021). We also found that short-term drought increased soil microbial biomass carbon and nitrogen, which may be caused by the fact that under drought conditions, plant growth is inhibited, and a large number of plants dry and fall to the ground. The nutrient competition between plants and soil microorganisms decreases, and the litter input into the soil is decomposed by microorganisms, which further promotes the increase in microbial biomass (Zhang et al., 2017; Li et al., 2024).

In our study, soil enzyme activity was increased under short-term drought treatment (Figure 4), which may be due to the accumulation of enzymes and their reaction substrates under low moisture conditions, thus driving enzyme reactions under more arid conditions (Austin et al., 2004). The increase in soil enzyme activity may also be related to reduced competition between plants and microorganisms for soil resources and nutrients under drought conditions (Schwinning and Sala, 2004). Usually, the water limitation threshold of microbes is much lower than that of plants (Schwinning and Sala, 2004; Delgado-Baquerizo et al., 2013). Higher enzyme activity may be associated with higher organic matter input and rhizosphere activity, while drought can lead to root death and leaf shedding, resulting in more input of additional organic matter into the soil (Ogaya and Peñuelas, 2004; Sinsabaugh et al., 2008) and thus enhancing soil enzyme activity. The activity of Glu and Xyl increased under short-term drought conditions because cellulose accounts for 40% of plant tissue, and drought may increase the input of cellulose to soil, ultimately increasing Glu activity (Toberman et al., 2010). The LAP activity in the P treatment also increased, which may be due to the short-term drought increasing the nitrogen requirements of soil microorganisms and limiting their nutrient metabolism (Xiao et al., 2022).

4.2 Short-term drought increases bacterial diversity, while its interaction with nitrogen application increases fungal diversity

Under short-term drought conditions, more dead plants drop their branches, resulting in higher soil organic matter (Ogaya and Peñuelas, 2004). In this study, alfalfa, a leguminous plant rich in nutrients such as amino acids, sugars, and flavonoids, was more conducive to bacterial growth and development, resulting in the increased species diversity of bacteria in the P treatment (Isobe et al., 2001; Figures 5, 6). Moreover, the input of organic matter also provides more carbon for bacterial growth and promotes an increase in bacterial diversity (Liao et al., 2021). Similarly, we found that short-term drought resulted in an increase in the alpha diversity of soil bacteria, possibly due to the decrease in pore connectivity caused by low water content, and dry pore spaces further created isolated habitats and niches that could accommodate less competitive bacteria.

Compared with bacteria, fungi are less restricted under short-term drought, which may be related to their mycelium system connecting through dry pore spaces (Tecon and Or, 2017). Compared with the C treatment, the change in microbial species diversity in the N treatment was minimal (Figures 5, 6), revealing that the overall response of the microbial communities was not sensitive to nitrogen addition (McHugh et al., 2017). Generally, nitrogen-induced soil acidification is an important mechanism for altering the composition of bacterial communities (Zhang et al., 2017). However, our experimental site was located on the Loess Plateau, and as nitrogen application can only cause a slight decrease in soil pH, the soil remained alkaline (Liu et al., 2015; Figure 3C). In addition, we also found that the fungal species diversity increased under the NP treatment (Figures 5, 6), indicating that under short-term drought, nitrogen application may increase fungal diversity. This is mainly because, the fungal communities were more abundant and diverse in the area with less rainfall, and adding an appropriate amount of nitrogen fertilizer can further provide nutrients for fungi (Classen et al., 2015; Kaisermann et al., 2015).

4.3 Impact on the composition and structure of soil microbial communities

Drought is a major climatic driver of soil microbial community composition and structure (Maestre et al., 2015). Our study showed that the microbial community composition and structure were highly susceptible to short-term drought, which was consistent with a previous study (Ochoa-Hueso et al., 2018). This may affect microbiome-mediated soil nutrient cycling, with implications for ecosystem function. The common explanation for changes in microbial composition is niche selection, where the relative abundance of drought-tolerant taxa increases compared to drought-sensitive taxa (Figure 7). Over time, the entire soil microbial community can develop drought resistance, which may lead to an increase in microbial activity rates at low soil moisture (Goransson et al., 2013). In the P treatments, the relative abundance of *Actinobacteria* and *Firmicutes* increased, and *Actinobacteria* typically exhibited drought resistance. *Actinobacteria* decompose organic matter in soil, such as chitin and cellulose, and have strong resistance to dry and low-resource conditions, which may explain why they outperform other microorganisms under drought conditions. The majority of *Firmicutes* are gram-positive bacteria, which possess thick cell walls (Aislabie et al., 2009) and are able to form spores under extreme conditions (Singh et al., 2007), allowing them to survive under drought conditions. Compared to that in the other treatments, the relative abundance of *Cyanobacteria* increased in the NP treatment, this is mainly because *Cyanobacteria* had strong stress resistance and drought tolerance, when nitrogen was added it increased the response ratio of the relative abundance of blue–green algae (She et al., 2018).

Compared to Bacteria, at the phylum level, the number of *Mucoromycota* treated with P and NP increased, while the number of *Mucoromycota* treated with N decreased (Figure 7). This may be due to the evolution of three life forms in *Mucoromycota*: saprotrophic, mycorrhizal, and parasitic. The saprophytic form survives by obtaining nutrients via the breakdown of dead and decaying organic matter. Short-term drought increased the number of dead plants in the soil, providing more organic matter for mainly saprophytic microbes. The addition of nitrogen may inhibit the activity of saprophytic fungi, which also causes the relative abundance of *Mucoromycota* treated with NP to be lower than that treated with P (Wen et al., 2018). *Basidiomycetes* can tolerate drought and may be superior to other taxa in water-scarce environments due to their advanced osmoregulatory functions and strong cell walls that allow them to resist limiting conditions. Under nitrogen application conditions, the relative abundance of *Basidiomycetes* increased, which may be due to the provision of nutrients for *Basidiomycetes* (Bogati and Walczak, 2022).

In this study, changes at the fungal genus level were also observed. The relative abundance of *Fusarium* decreased under nitrogen application but increased under drought treatment. *Fusarium* includes plant pathogens of many important crops, many of them are soil-borne, and their accumulation in the soil may exacerbate crop diseases (Li et al., 2022). Drought is one of the factors that increases the incidence rate and severity of *Fusarium*-induced diseases. The relative abundance of *Purpureocillium* in the P treatment also increased compared with that in the other three treatments. *Purpureocillium* has an inhibitory effect on a variety of plant pathogenic bacteria and viruses and can reduce *Fusarium* abundance. The increase in the relative abundance of *Fusarium* under drought conditions also promotes the increase in *Purpureocillium*, which may be due to the self-regulation of the microbial community (Hao et al., 2018). However, nitrogen application reduced the relative abundance of *Fusarium* in soil. According to the theory of hypotrophic replicative nutrition, the tendency of *Fusarium* to be hypotrophic is negatively correlated with the content of inorganic nitrogen (Yang et al., 2019). In our study, we found no change in the relative abundance of *Fusarium* in the NP treatment compared to the C treatment (Figure 7). This suggests a potential strategy: appropriate nitrogen application in times of drought may inhibit *Fusarium* and contribute to plant protection.

The relative abundance of *Metarhizium* increased under drought treatment, decreased under nitrogen treatment, and remained unchanged under NP treatment. *Metarhizium* is a broad-spectrum insect pathogen. According to statistics, *Metarhizium* is the host of more than 200 insects, can cause insect disease, and can cause repeated infections within populations. At present, *Metarhizium* has been developed into a fungal insecticide second only to *Beauveria bassiana* (Zimmermann, 2007). Drought, pests and diseases, and pathogen-related damage often occur at the same time, and additional stress from climate change and drought may make plants more vulnerable to pests and pathogens. The increase in *Metarhizium* content under drought conditions may indicate an increase in the soil pest population, and the decrease in *Metarhizium* content under nitrogen application conditions may indicate a decrease in the soil pest population. Therefore, appropriate nitrogen application during drought may inhibit the occurrence of pests.

4.4 Short-term drought enriches pathway genes related to the carbon cycle and amino acid metabolism

The crop planted in the experimental site was alfalfa, which contains a large amount of protein and amino acids and is a high-quality forage. Short-term drought caused alfalfa litter to enter the soil, and the litter was decomposed by microorganisms, which promoted the increase in genes related to amino acid metabolism. At this time, microorganisms can easily obtain nutrients, further promoting the soil carbon cycle (Figure 8). Moreover, plant litter is the main source of soil organic carbon and has a positive effect on microbial carbon cycling genes (Zhong et al., 2018). The input of plant litter to soil stimulated the enrichment of soil microbial carbon cycling genes. The decrease in the abundance of pathway genes associated with the carbon cycle after nitrogen treatment may be due to the addition of nitrogen inhibiting the activity of saprophytic fungi, which produce enzymes to decompose organic matter that is difficult to degrade, which may not be conducive to carbon decomposition, resulting in a decrease in the abundance of pathway genes associated with the carbon cycle. However, the addition of nitrogen reduced the decomposition rate of plant litter, and the microbial communities under nitrogen treatment utilized sugar as a carbon source to maintain activity (Zhang et al., 2016; Wen et al., 2018; Wang et al., 2020). Thus, nitrogen fertilization enriched the abundance of genes associated with glycan biosynthesis and metabolism. In addition, appropriate nitrogen application may also promote an increase in the abundance of nitrogen cycling-related genes in soil, which is conducive to the nitrogen cycle (Figure 8).

5 Conclusion

The soil organic carbon components, soil enzyme activity, and composition and functional genes of the soil microbial community of alfalfa artificial grassland on the Loess Plateau show different responses to short-term drought, nitrogen application and their interactions. Short-term drought, nitrogen application and their interactions all increased soil POC, while only nitrogen application increased the soil EOC content. Short-term drought increased soil MBC and MBN and promoted an increase in soil enzyme activity. Additionally, short-term drought increased the alpha diversity of soil bacteria, and the interaction between short-term drought and nitrogen application increased the alpha diversity of soil fungi. Short-term drought stress also increased the relative abundance of drought-resistant bacteria such as *Actinobacteria* and *Firmicutes*. During short-term drought, moderate nitrogen application reduced the relative abundance of the *Fusarium* and *Metarhizium* genera, which may prevent plant diseases during droughts and pest outbreaks. Short-term drought enriched the abundance of genes related to the carbon cycle and amino acid metabolism, while nitrogen application decreased the abundance of genes related to the carbon cycle and enriched the abundance of genes related to glycan biosynthesis and metabolism and the nitrogen cycle. Overall, short-term drought altered the composition and functional genes of soil microbial communities. Moreover, in the case of frequent short-term drought in the future, nitrogen addition can be considered to maintain the diversity of the soil microbial community and sustain soil carbon cycling.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: NCBI BioProject, accession number PRJNA1046644, http://www.ncbi.nlm.nih.gov/bioproject/1046644.

Author contributions

RW: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Writing – original draft, Writing – review & editing. JGZ: Data curation, Software, Writing – review & editing. TM: Resources, Supervision, Writing – review & editing. WL: Funding acquisition, Supervision, Writing – review & editing. YS: Project administration, Visualization, Writing – review & editing. QY: Methodology, Resources, Writing – review & editing. XW: Project administration, Writing – review & editing. JL: Formal analysis, Methodology, Writing – review & editing. QX: Formal analysis, Methodology, Writing – review & editing. LL: Formal analysis, Methodology, Writing – review & editing. JJZ: Data curation, Formal analysis, Writing – review & editing. JM: Conceptualization, Funding acquisition, Investigation, Writing – review & editing. ZZ: Analyzed data and created figures.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Aislabie, J., Jordan, S., Ayton, J., Klassen, J. L., Barker, G. M., and Turner, S. (2009). Bacterial diversity associated with ornithogenic soil of the Ross Sea region, Antarctica. *Can. J. Microbiol.* 55, 21–36. doi: 10.1139/W08-126

Austin, A. T., Yahdjian, L., Stark, J. M., Belnap, J., Porporato, A., Norton, U., et al. (2004). Water pulses and biogeochemical cycles in arid and semiarid ecosystems. *Oecologia* 141, 221–235. doi: 10.1007/s00442-004-1519-1

Barnard, R. L., Osborne, C. A., and Firestone, M. K. (2013). Responses of soil bacterial and fungal communities to extreme desiccation and rewetting. *ISME J.* 7, 2229–2241. doi: 10.1038/ismej.2013.104

Bogati, K., and Walczak, M. (2022). The impact of drought stress on soil microbial community, enzyme activities and plants. *Agronomy* 12:189. doi: 10.3390/agronomy12010189

Chen, H., Li, D. J., Gurmesa, G. A., Yu, G. R., Li, L. H., Zhang, W., et al. (2015). Effects of nitrogen deposition on carbon cycle in terrestrial ecosystems of China: a meta-analysis. *Environ. Pollut.* 206, 352–360. doi: 10.1016/j.envpol.2015.07.033

Classen, A. T., Sundqvist, M. K., Henning, J. A., Newman, G. S., Moore, J. A. M., Cregger, M. A., et al. (2015). Direct and indirect effects of climate change on soil microbial and soil microbial-plant interactions: what lies ahead? *Ecosphere* 6, 1–21. doi: 10.1890/ES15-00217.1

Delgado-Baquerizo, M., Maestre, F. T., Rodrıguez, J. G. P., and Gallardo, A. (2013). Biological soil crusts promote N accumulation in response to dew events in dryland soils. *Soil Biol. Biochem.* 62, 22–27. doi: 10.1016/j.soilbio.2013.02.015

Geisen, S., Wall, D. H., and van der Putten, W. H. (2019). Challenges and opportunities for soil biodiversity in the Anthropocene. *Curr. Biol.* 29, R1036–R1044. doi: 10.1016/j.cub.2019.08.007

Goransson, H., Godbold, D. L., Jones, D. L., and Rousk, J. (2013). Bacterial growth and respiration responses upon rewetting dry forest soils: impact of drought-legacy. *Soil Biol. Biochem.* 57, 477–486. doi: 10.1016/j.soilbio.2012.08.031

Hao, Z. T., Hao, X. H., Yan, J., Xie, B. Y., and Liang, L. Q. (2018). Research progress on the secondary metabolite of *Purpureocillium lilacinum*. *Biol. Bull.* 34, 11–17. doi: 10.13560/j.cnki.biotech.bull.1985.2018-0049

Hueso, S., García, C., and Hernández, T. (2012). Severe drought conditions modify the microbial community structure, size and activity in amended and unamended soils. *Soil Biol. Biochem.* 50, 167–173. doi: 10.1016/j.soilbio.2012.03.026

Iglesias, A., and Garrote, L. (2015). Adaptation strategies for agricultural water management under climate change in Europe. *Agric. Water Manage.* 155, 113–124. doi: 10.1016/j.agwat.2015.03.014

Isobe, K., Tateishi, A., Nomura, K., Inoue, H., and Tsuboki, Y. (2001). Flavonoids in the extract and exudate of the roots of leguminous crops. *Plant Prod. Sci.* 4, 278–279. doi: 10.1626/pps.4.278

Jansson, J. K., and Hofmockel, K. S. (2020). Soil microbiomes and climate change. *Nat. Rev. Microbiol.* 18, 35–46. doi: 10.1038/s41579-019-0265-7

Jian, S. Y., Li, J. W., Chen, J., Wang, G. S., Mayes, M. A., Dzantor, K. E., et al. (2016). Soil extracellular enzyme activities, soil carbon and nitrogen storage under nitrogen fertilization: a meta-analysis. *Soil Biol. Biochem.* 101, 32–43. doi: 10.1016/j.soilbio.2016.07.003

Kaisermann, A., Maron, P. A., Beaumelle, L., and Lata, J. C. (2015). Fungal communities are more sensitive indicators to non-extreme soil moisture variations than bacterial communities. *Appl. Soil Ecol.* 86, 158–164. doi: 10.1016/j.apsoil.2014.10.009

Kundel, D., Bodenhausen, N., Jørgensen, H. B., Truu, J., Birkhofer, K., Hedlund, K., et al. (2020). Effects of simulated drought on biological soil quality, microbial diversity and yields under long-term conventional and organic agriculture. *FEMS Microbiol. Ecol.* 96, fiaa205. doi: 10.1093/femsec/fiaa205

Lal, R. (2004). Soil carbon sequestration impacts on global climate change and food security. *Science* 304, 1623–1627. doi: 10.1126/science.1097396

Li, Y., Li, Y., Zhang, Q. P., Xu, G., Liang, G. P., Kim, D., et al. (2024). Enhancing soil carbon and nitrogen through grassland conversion from degraded croplands in China: assessing magnitudes and identifying key drivers of phosphorus reduction. *Soil Tillage Res.* 236:105943. doi: 10.1016/j.still.2023.105943

Li, Q., Wu, Q., Zhang, T., Xiang, P., Bao, Z. J., Tu, W. Y., et al. (2022). Phosphate mining activities affect crop rhizosphere fungal communities. *Sci. Total Environ.* 838:156196. doi: 10.1016/j.scitotenv.2022.156196

Liao, L. R., Wang, X. T., Wang, J., Liu, G. B., and Zhang, C. (2021). Nitrogen fertilization increases fungal diversity and abundance of saprotrophs while reducing nitrogen fixation potential in a semiarid grassland. *Plant Soil* 465, 515–532. doi: 10.1007/s11104-021-05012-w

Liu, J. B., Fa, K. Y., Zhang, Y. Q., Wu, B., Qin, S. G., and Jia, X. (2015). Abiotic CO2 uptake from the atmosphere by semiarid desert soil and its partitioning into soil phases. *Geophys. Res. Lett.* 42, 5779–5785. doi: 10.1002/2015GL064689

Liu, E., Teclemariam, S. G., Yan, C., Yu, J., Gu, R., Liu, S., et al. (2014). Long-term effects of no-tillage management practice on soil organic carbon and its fractions in the northern China. *Geoderma* 213, 379–384. doi: 10.1016/j.geoderma.2013.08.021

Lu, Y. H., Sun, Y. T., Liao, Y. L., Nie, J., Xie, J., Yang, Z., et al. (2015). Effects of the application of controlled release nitrogen fertilizer on rapeseed yield, agronomic characters and soil fertility. *J. Agric. Sci. Technol.* 16, 1216–1221. doi: 10.16175/j.cnki.1009-4229.2015.06.031

Lu, M., Zhou, X. H., Luo, Y. Q., Yang, Y. H., Fang, C. M., Chen, J. K., et al. (2011). Minor stimulation of soil carbon storage by nitrogen addition: a meta-analysis. *Agric. Ecosyst. Environ.* 140, 234–244. doi: 10.1016/j.agee.2010.12.010

Maaroufi, N. I., Nordin, A., Hasselquist, N. J., Bach, L. H., Palmqvist, K., and Gundale, M. J. (2015). Anthropogenic nitrogen deposition enhances carbon sequestration in boreal soils. *Glob. Chang. Biol.* 21, 3169–3180. doi: 10.1111/gcb.12904

Maestre, F. T., Delgado-Baquerizo, M., Jeffries, T. C., Eldridge, D. J., Ochoa, V., Gozalo, B., et al. (2015). Increasing aridity reduces soil microbial diversity and abundance in global drylands. *Proc. Natl. Acad. Sci. U. S. A.* 112, 15684–15689. doi: 10.1073/pnas.1516684112

McHugh, T. A., Morrissey, E. M., Mueller, R. C., Gallegos-Graves, V., Kuske, C. R., and Reed, S. C. (2017). Bacterial, fungal, and plant communities exhibit no biomass or compositional response to two years of simulated nitrogen deposition in a semiarid grassland. *Environ. Microbiol.* 19, 1600–1611. doi: 10.1111/1462-2920.13678

Nguyena, L. T. T., Osanaia, Y., Laia, K., Andersona, I. C., Bangeb, M. P., Tissuea, D. T., et al. (2018). Responses of the soil microbial community to nitrogen fertilizer regimes and historical exposure to extreme weather events: flooding or prolongeddrought. *Soil Biol. Biochem.* 118, 227–236. doi: 10.1016/j.soilbio.2017.12.016

Nkebiwe, P. M., Weinmann, M., Bar-Tal, A., and Müller, T. (2016). Fertilizer placement to improve crop nutrient acquisition and yield: a review and meta-analysis. *Field Crop Res.* 196, 389–401. doi: 10.1016/j.fcr.2016.07.018

Ochoa-Hueso, R., Collins, S. L., Delgado-Baquerizo, M., Hamonts, K., Pockman, W. T., Sinsabaugh, R. L., et al. (2018). Drought consistently alters the composition of soil fungal and bacterial communities in grasslands from two continents. *Glob. Chang. Biol.* 24, 2818–2827. doi: 10.1111/gcb.14113

Ogaya, R., and Peñuelas, J. (2004). Phenological patterns of *Quercus ilex*, Phyllerea latifolia and *Arbutus unedo* growing under a field experimental drought. *Ecoscience* 11, 263–270. doi: 10.1080/11956860.2004.11682831

Plaza-Bonilla, D., Álvaro-Fuentes, J., and Cantero-Martínez, C. (2014). Identifying soil organic carbon fractions sensitive to agricultural management practices. *Soil Tillage Res.* 139, 19–22. doi: 10.1016/j.still.2014.01.006

Qi, R., Li, J., Lin, Z., Li, Z., Li, Y., and Yang, X. (2016). Temperature effects on soil organic carbon, soil labile organic carbon fractions, and soil enzyme activities under long-term fertilization regimes. *Appl. Soil Ecol.* 102, 36–45. doi: 10.1016/j.apsoil.2016.02.004

Roberts, B. A., Fritschi, F. B., Horwath, W. R., Scow, K. M., Rains, W. D., and Travis, L. R. (2011). Comparisons of soil microbial communities influenced by soil texture, nitrogen fertility, and rotations. *Soil Sci.* 176, 487–494. doi: 10.1097/SS.0b013e31822769d3

Sayer, E. J., Crawford, J. A., Edgerley, J., Askew, A. P., Hahn, C. Z., Whitlock, R., et al. (2021). Adaptation to chronic drought modifies soil microbial community responses to phytohormones. *Commun. Biol.* 4:516. doi: 10.1038/s42003-021-02037-w

Schimel, J. P. (2018). Life in dry soils: effects of drought on soil microbial communities and processes. *Annu. Rev. Ecol. Evol. Syst.* 49, 409–432. doi: 10.1146/annurev-ecolsys-110617-062614

Schwinning, S., and Sala, O. E. (2004). Hierarchy of responses to resource pulses in arid and semi-arid ecosystems. *Oecologia* 141, 211–220. doi: 10.1007/s00442-004-1520-8

She, W. W., Bai, Y. X., Zhang, Y. Q., Qin, S. G., Feng, W., Sun, Y. F., et al. (2018). Resource availability drives responses of soil microbial communities to short-term precipitation and nitrogen addition in a desert Shrubland. *Front. Microbiol.* 9:186. doi: 10.3389/fmicb.2018.00186

Singh, B. L., Munro, S., Potts, J. M., and Millard, P. (2007). Influence of grass species and soil type on rhizosphere microbial community structure in grassland soils. *Appl. Soil Ecol.* 36, 147–155. doi: 10.1016/j.apsoil.2007.01.004

Sinsabaugh, R. L., Lauber, C. L., Weintraub, M. N., Ahmed, B., Allison, S. D., Crenshaw, C., et al. (2008). Stoichiometry of soil enzyme activity at global scale. *Ecol. Lett.* 11, 1252–1264. doi: 10.1111/j.1461-0248.2008.01245.x

Spinoni, J., Naumann, G., and Vogt, J. (2015). Spatial patterns of European droughts under a moderate emission scenario. *Adv. Sci. Res.* 12, 179–186. doi: 10.5194/asr-12-179-2015

Tecon, R., and Or, D. (2017). Biophysical processes supporting the diversity of microbial life in soil. *FEMS Microbiol. Rev.* 41, 599–623. doi: 10.1093/femsre/fux039

Toberman, H., Laiho, R., Evans, C. D., Artz, R. R. E., Fenner, N., Straková, P., et al. (2010). Long-term drainage for forestry inhibits extracellular phenol oxidase activity in Finnish boreal mire peat. *Eur. J. Soil Sci.* 61, 950–957. doi: 10.1111/j.1365-2389.2010.01292.x

Wang, Y. J., Liu, L., Yang, J. F., Duan, Y. M., Luo, Y., Taherzadeh, M. J., et al. (2020). The diversity of microbial community and function varied in response to different agricultural residues composting. *Sci. Total Environ.* 715:136983. doi: 10.1016/j.scitotenv.2020.136983

Wen, X., Xiao, C., Xin, J., and Biao, Z. A. (2018). Meta-analysis of soil extracellular enzyme activities in response to global change. *Soil Biol. Biochem.* 123, 21–32. doi: 10.1016/j.soilbio.2018.05.001

Xiao, L., Zhao, M., Liu, G. B., Li, P., Liu, F. Y., and Xue, S. (2022). Soil biochemical index-based assessment of the effect of drought stress on the rhizosphere soil quality in three typical grass species in the loess plateau, China. *J. Soils Sediments* 22, 2982–2994. doi: 10.1007/s11368-022-03291-z

Yakushev, A. V., Zhuravleva, A. I., and Kuznetsova, I. N. (2023). Effects of long-term and short-term droughts on the hydrolytic enzymes in haplic Luvisol. *Eurasian Soil Sci.* 56, 782–792. doi: 10.1134/S1064229323600380

Yang, X. D., Ma, L. F., Ji, L. F., Shi, Y. Z., Yi, X. Y., Yang, Q. L., et al. (2019). Long-term nitrogen fertilization indirectly affects soil fungi community structure by changing soil and pruned litter in a subtropical tea (*Camellia sinensis* L.) plantation in China. *Plant Soil* 444, 409–426. doi: 10.1007/s11104-019-04291-8

Yang, C. M., Yang, L. Z., and Ouyang, Z. (2005). Organic carbon and its fractions in paddy soil as affected by different nutrient and water regimes. *Geoderma* 124, 133–142. doi: 10.1016/j.geoderma.2004.04.008

Yuste, G. C., Fernandez-Gonzalez, A. J., Fernandez-Lopez, M., Ogaya, R., Penuelas, J., Sardans, J., et al. (2014). Strong functional stability of soil microbial communities under semiarid Mediterranean conditions and subjected to long-term shifts in baseline precipitation. *Soil Biol. Biochem.* 69, 223–233. doi: 10.1016/J.SOILBIO.2013.10.045

Zeng, J., Liu, X., Song, L., Lin, X., Zhang, H., Shen, C., et al. (2016). Nitrogen fertilization directly affects bacterial community composition. *Soil Biol. Biochem.* 92, 41–49. doi: 10.1016/j.soilbio.2015.09.018

Zhang, W. D., Chao, L., Yang, Q. P., Wang, Q. K., Fang, Y. T., and Wang, S. L. (2016). Litter quality mediated nitrogen effect on plant litter decomposition regardless of soil fauna presence. *ESA* 97, 2834–2843. doi: 10.1002/ecy.1515

Zhang, Y., Shen, H., He, X., Thomas, B. W., Lupwayi, N. Z., and Hao, X. (2017). Fertilization shapes bacterial community structure by alteration of soil pH. *Front. Microbiol.* 8:1325. doi: 10.3389/fmicb.2017.01325

Zhong, Y., Yan, W., Wang, R., Wen, W., and Shangguan, Z. (2018). Decreased occurrence of carbon cycle functions in microbial communities along with long-term secondary succession. *Soil Biol. Biochem.* 123, 207–217. doi: 10.1016/j.soilbio.2018.05.017

Zhu, L., Li, Y. X., Wang, Y., Wang, Y. Y., Zhang, X. Y., Chen, A. J., et al. (2021). Effects of nitrogen application on maize nitrogen uptake and soil biological and chemical properties under drought stresses at seedling stage. *J. Soil Water Conserv.* 35, 267–274. doi: 10.13870/j.cnki.stbcxb.2021.04.037

Zimmermann, G. (2007). Review on safety of the entomopathogenic fungus *Metarhizium anisopliae*. *Biocontrol Sci. Tech.* 17, 879–920. doi: 10.1080/09583150701593963

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Influences of nitrogen input forms and levels on phosphorus availability in karst grassland soils

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The availability of soil phosphorus (P), a crucial nutrient influencing plant productivity and ecosystem function, is impacted by continuously increasing nitrogen (N) enrichment, which changes the soil P cycle. The effect of varying forms of N input on soil P dynamics in P-limited karst grassland ecosystems remains unclear. To address this knowledge gap, we conducted a greenhouse experiment to explore the effects of various forms of N addition [$Ca(NO_3)_2$, NH_4Cl, NH_4NO_3, Urea] on soil P fractions in these ecosystems, applying two levels (N1: 50 mg N kg^{-1}soil, N2: 100 mg N kg^{-1}soil) of N input in two soils (yellow soil, limestone soil). Results indicated that P fractions in both soil types were significantly affected by N additions, with yellow soil demonstrating a higher sensitivity to these additions, and this effect was strongly modulated by the form and level of N added. High N addition, rather than low N, significantly affect the P fractions in both soil types. Specially, except for $Ca(NO_3)_2$, high N addition significantly increased the available P in both soils, following the order: Urea and $NH_4NO_3 > NH_4Cl > Ca(NO_3)_2$, and decreased $NaHCO_3$-Pi in both soils. High N addition also significantly reduced NaOH-Po and C.HCl-Po fractions in yellow soil. Additionally, the response of root biomass and alkaline phosphatase activity in both soils to N input paralleled the trends observed in the available P fractions. Notably, changes in soil available P were strongly correlated with plant root biomass and soil alkaline phosphatase activity. Our study highlights that the N addition form significantly influences soil P availability, which is closely tied to plant root biomass and alkaline phosphatase activity. This finding underscores the importance of considering N input form to boost soil fertility and promote sustainable agriculture.

KEYWORDS

phosphorus fractions, nitrogen input, nitrogen form, phosphatase activity, phoC, phoD

1 Introduction

Phosphorus (P) is a crucial nutrient required for plant growth and the integrity of terrestrial ecosystem function, playing a pivotal role in biodiversity conservation, global sustainability, and biogeochemical cycling (Sattari et al., 2016; Hou et al., 2021; Bai and Cotrufo, 2022). A significant amount of P in soil is bound by insoluble compounds, leaving only a limited amount of inorganic P accessible to plants and microbes, leading to widespread P limitation across terrestrial ecosystems (Ashley et al., 2011; Ahmad et al., 2018). Concurrently, nitrogen (N) enrichment from atmospheric deposition, biological fixation, and over-fertilization has more than doubled the input of reactive N in various forms, such as ammonium, nitrate, and organic N, including urea and amino acids (Penuelas et al., 2020). This increase in N input not only alters P cycling in the soil but also exacerbates P scarcity in ecosystems (Hou et al., 2021). Understanding the effects of N input on soil P cycling is therefore crucial for predicting the future functioning of terrestrial ecosystems.

P in soil exists in multiple complex chemical forms, and its availability, behavior, and mobility change with varying soil conditions (Wang et al., 2022). Previous studies have developed a framework categorizing inorganic (Pi) and organic P (Po) into different labile and non-labile pools via a sequential fractionation procedure (Hedley et al., 1982; Tiessen and Moir, 1993). N input can affect these P fractions by altering factors such as soil pH, microbial activity, content of alkaline cations, cation exchange capacity, and plant biomass (Mahmood et al., 2021; Cui et al., 2022). Despite extensive research, findings on the effects of N on soil P fractions are inconsistent. For instance, some studies have found that N input increases $NaHCO_3$-Pi and decreases or does not affect moderate-labile P fractions (Liu et al., 2022; Guan et al., 2023). Conversely, other studies indicate that N addition reduces labile P (Resin-Pi, $NaHCO_3$-Pi and $NaHCO_3$-Po) while increasing moderately labile P (NaOH-Pi, NaOH-Po) (Kai et al., 2014; Chen et al., 2018; Zhang and Shenglei, 2020). At the same time, compared to labile and moderately labile P, non-labile P fractions are less affected by N input (Chen et al., 2018; Liu et al., 2022; Guan et al., 2023). Additionally, while some studies suggest that N input generally increases soil inorganic P and reduces soil organic P fractions (Jing et al., 2021), there are also reports to the contrary (Zhang et al., 2022). Such variation could be attributed to the different N forms used in these studies (Hu et al., 2022). As terrestrial ecosystems receive a variety of N inputs, it's important to understand how these different N forms affect soil P fractions.

Phosphatases, particularly acid phosphatase, and alkaline phosphatase, secreted by plant roots and soil microorganisms, are crucial in the transformation and cycling of soil P (Fan et al., 2019; Wang et al., 2023). N input is known to alter the activity of these enzymes, which catalyze the conversion of organically bound P to inorganic P (Yokoyama et al., 2017; Chen et al., 2020). While acid phosphatase is primarily produced by plants, bacteria, and fungi, alkaline phosphatase is predominantly secreted by soil microorganisms, especially bacteria (Nannipieri et al., 2011; Cao et al., 2022). The *phoC* gene and *phoD* gene, encoding soil acid and alkaline phosphatase respectively, play a significant role in the soil P cycle (Tan et al., 2013; Wang et al., 2021). However, the specific effects of different N forms, such as ammonium, nitrate, and organic N, on phosphatase activity and the abundance of these genes are not well understood.

Karst ecosystems are widely distributed around the world, and the subtropical karst area located in southwest China is one of the largest carbonate bedrock development areas in the world (Li et al., 2018; Wu et al., 2020). In the past 20 years, most degraded land in the karst region has been gradually transformed into woodland and grassland (Wen et al., 2016). During this succession process, the soil N content has increased, primarily due to atmospheric N deposition, fertilizer application, and biological N fixation. However, due to the shallow, uneven and fragile characteristics, the soil in this area is susceptible to leaching, resulting in the loss of N and P (Wen et al., 2016; Fenton et al., 2017; Xiao et al., 2020). Moreover, in karst areas, the high calcium ion content in the soil leads to a serious lack of P, which makes P a key nutrient limiting secondary succession and ecological restoration in the southwest karst area (Liu et al., 2018). Despite the substantial N input from land use activities, few studies have focused on the impact of different N forms on soil P fractions in karst areas. This study aims to explore these effects through greenhouse experiments, examining changes in soil P fractions, phosphatase activity, and the abundance of *phoC* and *phoD* genes under various N additions. We hypothesize that: (1) different N forms have distinct impacts on soil P fractions, considering that different forms of N have different effects on factors that control soil P cycling; (2) alterations in soil P fractions due to N input are predominantly influenced by biotic factors, considering that abiotic factors typically exhibit less variability in the short term.

2 Materials and methods

2.1 Experimental design and treatments

We conducted a greenhouse experiment to explore the effect of N input on soil P fractions at Guizhou University (106° 39′ 29″ E, 26° 26′ 59″ N), Guiyang, Guizhou. The experiment comprised three N addition levels (N0: $0\,mg\,N\,kg^{-1}$ soil，N1: $50\,mg\,N\,kg^{-1}$ soil, N2: $100\,mg\,N\,kg^{-1}$ soil) combined with four forms of N [$Ca(NO_3)_2$, NH_4Cl, NH_4NO_3 and Urea] and two soil types (yellow soil and limestone soil). This resulted in a total of 72 experimental pots, with each treatment replicated four times. Topsoil samples (0–15 cm) of two types of soils were collected from Guizhou, located in the southwestern karst area's core area, which are the primary soil types in the karst region (Piao et al., 2000; Zhang et al., 2021). The limestone soil was collected from Dafang County grasslands and yellow soil from Huaxi County grasslands (Table 1). Based on the FAO-UNESCO soil classification framework, the limestone soil utilized in our research is classified as Calcic Cambisol, and the yellow soil is designated as Ferralsol. The soils were air-dried at room temperature, homogenized and placed into pots (16 cm in diameter, 12 cm in depth), each containing 1,500 g of soil with an average particle size of <2 mm. The greenhouse was maintained under a 20°C/15°C day (16 h)/night (8 h) temperature cycle.

White clover (*Trifolium repens* L.) and perennial ryegrass (*Lolium perenne* L.) were selected as their prevalence in the area's mixed plantings. Each pot was planted with eight seedlings (four perennial ryegrass and four white clovers). The experiment was carried out in a greenhouse during the autumn and winter (August to December), with plants watered 1–2 times weekly to maintain soil water holding capacity at about 60%. Seedling management and pest control were also conducted (Van Lenteren, 1988). Specifically, clear weeds in time during seedling stage, spray 50% carbendazim and 50% mancozeb 500 times solution to control pests and diseases after thin out seedling (Zhang et al., 2004).

2.2 Sampling and chemical analysis

After 4 months, the plants were harvested, divided into above and below-ground parts, and the roots were dried at 65°C for 48 h to measure dry weight. Soil from each pot was sieved (2 mm mesh) and divided into three subsamples for different analyses. The first subsample was stored at −80°C for *phoC* and *phoD* gene abundance

TABLE 1 Basic physicochemical properties of the soil.

Characteristic	BD	pH	SOM	NH_4^+-N	NO_3^--N	AP	Exchangeable Ca^{2+}	Exchangeable Mg^{2+}
	($g\,cm^{-3}$)		($g\,kg^{-1}$)	($mg\,kg^{-1}$)	($mg\,kg^{-1}$)	($mg\,kg^{-1}$)	($mg\,kg^{-1}$)	($mg\,kg^{-1}$)
Limestone soil	1.04	6.01	31.05	31.57	4.75	3.02	4050.32	258.73
Yellow soil	1.08	5.46	9.54	19.67	1.41	7.99	3375.67	582.81

analysis. The second subsample was kept at 4°C for phosphatase activity determination, and the third subsample was air-dried for assessing soil physical and chemical properties and P fractions.

2.3 Soil chemical and biological properties

Soil samples were dried at 105°C for soil moisture content (SM) determination (Geng et al., 2022). Soil pH was measured in triplicate at a 1: 5 soil to deionized water (w/v) using digital PHS-3E/pH meter (Leici Instrument Branch of Shanghai Yidian Scientific Instrument Co., Ltd., Shanghai, China); NH_4^+-N and NO_3^--N concentrations were assessed with a continuous flow analyzer (Vario EL III, Elementar, Germany) after 2 mol L^{-1} KCl solution leaching (Ding et al., 2023). Soil phosphatase activities (S-ACP and S-ALP) were measured using commercially available kits (Beijing Solarbio Technology Co., Ltd.), with the activity determined by the phenol produced at 660 nm after hydrolyzing benzene disodium phosphate (Ding et al., 2023; Yang et al., 2023).

2.4 Soil P fractions

The soil P fractions were determined using the Hedley continuous extraction method (Hedley et al., 1982). The procedure is detailed below: A 0.5 g sample of air-dried soil, sieved through a 2 mm mesh, was placed in a 50 mL centrifuge tube. Sequential extractions were conducted using 30 mL of distilled water, 0.5 mol L^{-1} $NaHCO_3$, 0.1 mol L^{-1} NaOH solution, 0.5 mol L^{-1} HCl solutions, and 1 mol L^{-1} HCl solutions. Each extraction involved shaking the sample for 4 h using an overhead shaker. After shaking, the soil suspension from each step was centrifuged for 10 min to separate the supernatant. The final extract was then digested using concentrated H_2SO_4 and H_2O_2. The P content in 0.5 mol L^{-1} $NaHCO_3$, 0.1 mol L^{-1} NaOH, and concentrated HCl extracts was determined using the molybdate-ascorbic acid method, post-digestion with H_2SO_4 and potassium persulfate. Total P in the extracts was quantified, and the concentration of organic P (Po) was calculated by subtracting inorganic P (Pi) from the total P in each extract (Tiessen and Moir, 1993).

2.5 DNA extraction and real-time PCR

Soil microbial genomic DNA was extracted from 0.5 g of fresh soil using the Omega soil DNA isolation kit (Omega Biotek Inc., Georgia, USA), following the manufacturer's instructions. The concentration of extracted DNA was evaluated through agarose gel electrophoresis and quantification with a NanoDrop ND-2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Quantitative PCR (qPCR) was performed using the ABI 7500 Real-Time PCR System (Applied Biosystems, Germany) to amplify the *phoC* and *phoD* genes (Walker, 2001). The primer pairs for amplifying the *phoC* and *phoD* genes were *phoC*-A-F1 and *phoC*-A-R1, and *phoD*-ALPS-F730 and *phoD*-ALPS-1101 (Sakurai et al., 2007).

The PCR mixture consisted of 10 μL of Power SYBR® Green PCR Master Mix (Applied Biosystems™, Thermo Fisher Scientific Inc., MA, USA), 0.4 μL of each primer, and 1 μL of the extracted DNA template. The PCR conditions were as follows: an initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 15 s, and annealing at 60°C for 30 s. The specificity of the amplification was verified by analyzing the melting curve. To quantify the gene copies, a standard curve was constructed by serial tenfold dilution of a plasmid containing the target gene. The copy numbers of the *phoC* and *phoD* genes were calculated based on this standard curve and expressed in units per gram of dry soil. These copy numbers represent the abundance of the *phoC* and *phoD* genes.

2.6 Statistical analyses

Statistical analyses were performed using SPSS 20.0 (SPSS, Inc., Chicago, IL, USA). A three-way ANOVA was conducted to assess the effects of N addition level (NL), N addition form (NF), and soil type (SF) on soil physical and chemical properties, plant root biomass, soil phosphatase activity, and the abundance of *phoC* and *phoD* genes, as well as soil P fractions. Additionally, an independent samples T-test was utilized to compare differences between the various N treatments and the control group. Pearson correlation analysis was used to explore the relationships between soil P fractions and other variables, including soil physiochemical properties, plant root biomass, phosphatase activity, and the abundance of *phoC* and *phoD* genes. All statistical tests were performed with a significance level set at 0.05.

3 Results

3.1 Effects of N addition on soil properties and root biomass

Soil properties responded variably to N addition, dependent on the level and form applied (Table 2 and Figure 1). Low-N treatment generally did not significantly affect soil properties ($p > 0.05$; Figure 1), whereas high-N treatment significantly altered these characteristics ($p < 0.05$; Figure 1). Specifically, under high N levels, all four forms of N input [NH_4Cl, $Ca(NO_3)_2$, NH_4NO_3, and Urea] significantly reduced the NH_4^+ and AN content in yellow soil. However, in limestone soil, this significant reduction in NH_4^+ and AN content was only observed with the addition of NH_4NO_3 and Urea. There were significant

TABLE 2 **Effects of soil type (ST), N addition levels (NL), N addition forms (NF) and their interaction on soil properties (NH_4^+, NO_3^-, AN, pH, and SM), phosphatase activity (ACP, ALP), phoC and phoD gene abundance, root biomass (RB) and soil P fractions.**

ResponseVar	ST	NL	NF	ST × NL	ST × NF	NL × NF	ST × NL × NF
NH_4^+	2.11	118.28***	7.93***	0.20	4.72**	10.75***	7.78***
NO_3^-	440.32***	12.82***	8.35***	1.06	2.15	1.25	3.75*
AN	0.03	100.43***	8.08***	0.03	5.75**	12.8***	9.10***
pH	3165.01***	11.46**	198.13***	3.18	17.48***	25.06***	5.67**
SM	268.30***	0.07	6.22**	5.33*	1.42	8.31***	4.70**
RB	338.40***	54.36***	10.39***	15.36***	1.39	9.50***	4.26*
ACP	15.95***	0.03	3.23*	0.29	3.12*	3.03*	1.90
ALP	63.61***	0.56	58.08***	3.82	1.71	8.27***	3.38*
phoC	997.82***	27.15***	24.78***	6.02*	7.51***	22.13***	6.56***
phoD	775.24***	0.58	1.66	0.92	1.34	1.47	1.49
H_2O-Pi	137.60***	166.28***	29.91***	26.46***	9.43***	19.62***	6.74***
$NaHCO_3$-Pi	367.35***	44.99***	1.60	17.15***	1.27	1.12	2.10
$NaHCO_3$-Po	237.31***	6.50*	1.25	0.01	1.16	2.97*	4.18*
NaOH-Pi	1268.89***	9.12**	22.75***	11.21**	23.35***	38.38***	37.81***
NaOH-Po	78.29***	1.23	3.94*	10.22**	5.44**	2.43	8.31***
DHCl-Pi	232.70***	6.52*	6.69***	0.56	8.44***	0.88	1.28
CHCl-Pi	761.04***	29.21***	6.08**	2.23	0.97	0.66	3.21*
CHCl-Po	4.54*	0.11	2.50	0.67	2.71	3.40*	1.38
Residue-Pt	333.13***	102.32***	0.10	0.50	2.53	4.60**	2.02

Asterisks denote significance: $*P < 0.05$; $**P < 0.01$, $***P < 0.001$.

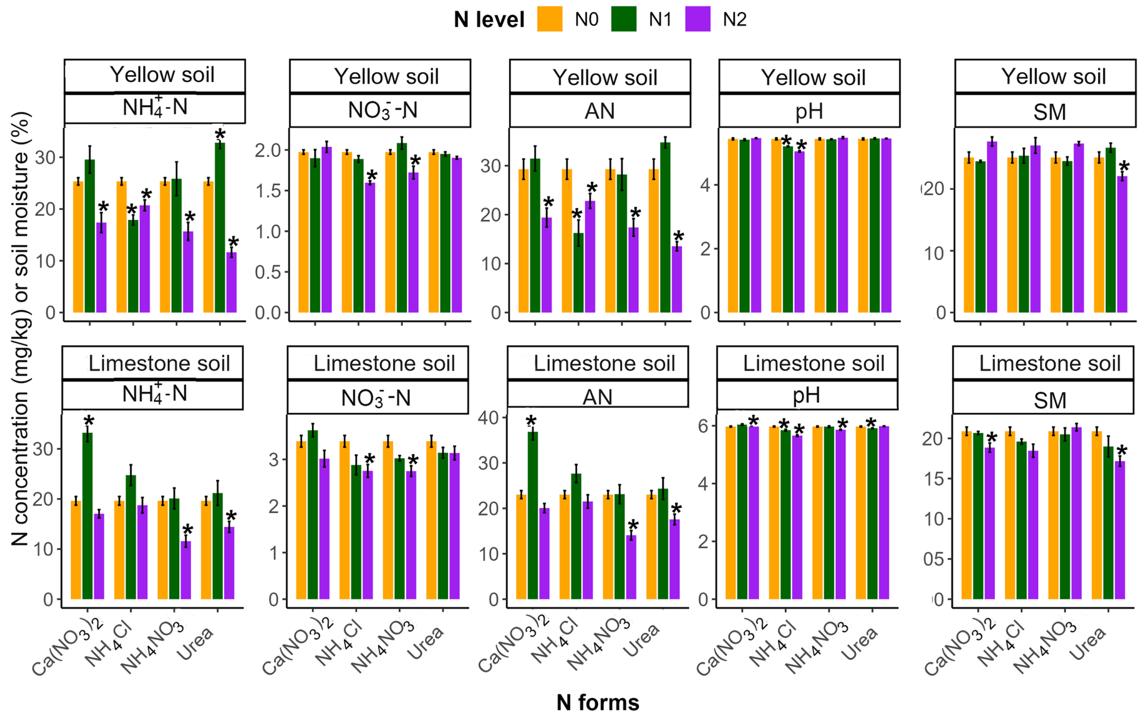

FIGURE 1
The effects of different N addition levels (N0, 0 mg N kg⁻¹ soil; N1, 50 mg N kg⁻¹ soil; N2, 100 mg N kg⁻¹ soil) and N addition forms [$Ca(NO_3)_2$, NH_4Cl, NH_4NO_3 and Urea] on the properties of yellow soil and limestone soil. The error bars represent the standard error (*n* = 4). "*" Indicates significant differences between the N addition treatment and the control group ($p < 0.05$).

differences in NH_4^+ and AN contents across different N forms treatments in both soil types, following a consistent pattern: $NH_4Cl > Ca(NO_3)_2 > NH_4NO_3 >$ Urea. Regarding NO3 content, high NH_4Cl and NH_4NO_3 had a negative effect in both soils. Additionally, soil pH significantly decreased under high NH_4Cl, more so than with other N forms. Although N treatments generally had less effect on soil moisture (SM), high Urea addition significantly reduced SM in both soils.

All N treatments significantly increased plant root biomass (RB) in both soil types ($p < 0.05$; Figure 2). In our study, Urea resulted in the greatest increase in RB, whereas $Ca(NO_3)_2$ contributed to the least increase in this parameter ($p < 0.05$; Supplementary Table S2). In addition, limestone soil exhibited a higher RB compared to yellow soil, with increased N levels contributing to more significant enhancements in RB (Figure 2 and Supplementary Table S2).

3.2 Effect of N addition on soil P fractions

In both yellow and limestone soils, inorganic P was the predominant form, accounting for 66.55 and 56.78% of total P, respectively (Supplementary Table S1). Additionally, yellow soil exhibited higher contents and proportions of moderately labile P fractions (NaOH-Pi, NaOH-Po) and non-labile P fractions (C.HCl-Pi, C.HCl-Po, and Residual-Pt) compared to limestone soil. However, the content and proportion of $NaHCO_3$-Po and H_2O-Pi were lower in yellow soil than in limestone soil (Table S1).

The influence of N addition on soil P fractions varied based on soil type, N level, and form (Table 2 and Figures 3, 4). Generally, high-N additions significantly affected the P fraction in both soils, unlike low-N additions (Figures 3, 4). With the exception of $Ca(NO_3)_2$, high N addition significantly increased the H_2O-Pi fraction and decreased the $NaHCO_3$-Pi fraction, without affecting the $NaHCO_3$-Po fraction ($p < 0.05$; Figures 3, 4). Significant differences in the soil H_2O-Pi fraction were found among various high-N treatments, with NH_4NO_3 and Urea showing higher levels than $Ca(NO_3)_2$ (Supplementary Table S3). Other P fractions in the two soils, however, exhibited divergent responses to N inputs (Figures 3, 4). In yellow soil, high N negatively affected the C.HCl-Pi, C.HCl-Po, and NaOH-Po fractions, except for C.HCl-Pi fractions with Urea and NaOH-Po fractions with $Ca(NO_3)_2$ (Figure 3). Conversely, these P fractions in limestone soil were not significantly impacted. Moreover, at high N levels, there were significant differences between various N forms in C.HCl-Pi and NaOH-Po fractions ($p < 0.05$; Figure 3). Specifically, the NaOH-Po fraction in $Ca(NO_3)_2$ was significantly higher than in other N forms, and the C.HCl-Pi fraction was significantly higher with NH_4NO_3 and Urea compared to other N forms (Supplementary Table S3).

3.3 Effect of N addition on soil phosphatase activity and *phoC* and *phoD* gene abundance

In both yellow and limestone soils, the activity of acid phosphatase (ACP) exceeded that of alkaline phosphatase (ALP). While ACP activity generally remained unaffected by N addition, ALP activity was significantly influenced by various N treatments ($p < 0.05$; Figure 5). The impact of N treatments varied based on soil type, N level, and form (Table 2). The $Ca(NO_3)_2$ treatment significantly reduced ALP activity in both soils, regardless of the addition level. Conversely, high levels of the other three N forms significantly increased ALP activity in yellow soil but decreased it in limestone soil, with the exception of NH_4Cl ($p < 0.05$; Figure 5). Notably, the ALP activity with $Ca(NO_3)_2$ treatment was significantly lower than that with the other three N forms in both soils ($p < 0.05$, Figure 5). Moreover, low-level additions of Urea and NH_4NO_3 significantly increased ALP activity in yellow soil ($p < 0.05$), but did not significantly affect limestone soil ($p > 0.05$; Figure 5).

The abundance of *phoC* and *phoD*, in response to N input, varies based on N level, and N form (Figure 5 and Table 2). Notably, high Urea addition significantly increased the *phoC* abundance in both soils

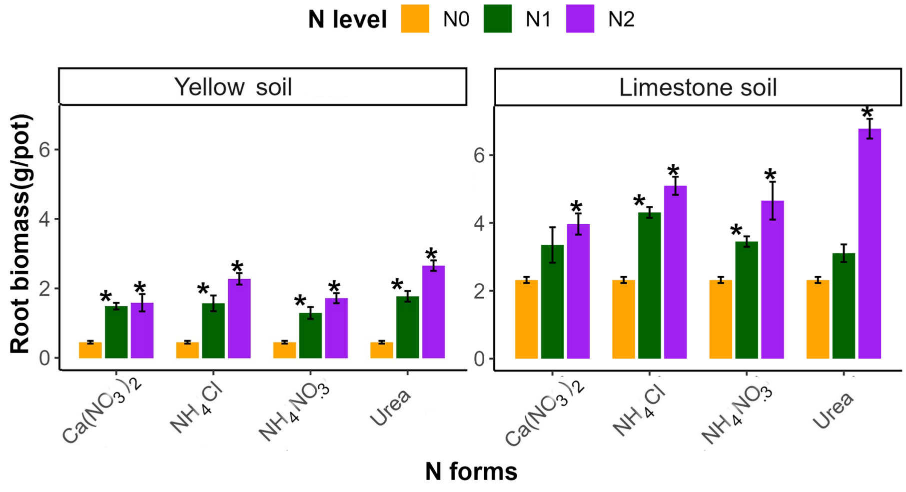

FIGURE 2
The effects of different N addition levels (N0, 0 mg N kg^{-1} soil; N1, 50 mg N kg^{-1} soil; N2, 100 mg N kg^{-1} soil) and N addition forms ($Ca(NO_3)_2$, NH_4Cl, NH_4NO_3 and Urea) on the root biomass of yellow soil and limestone soil. The error bars represent the standard error ($n = 4$). "*" Indicates significant differences between the N addition treatment and the control group ($p < 0.05$).

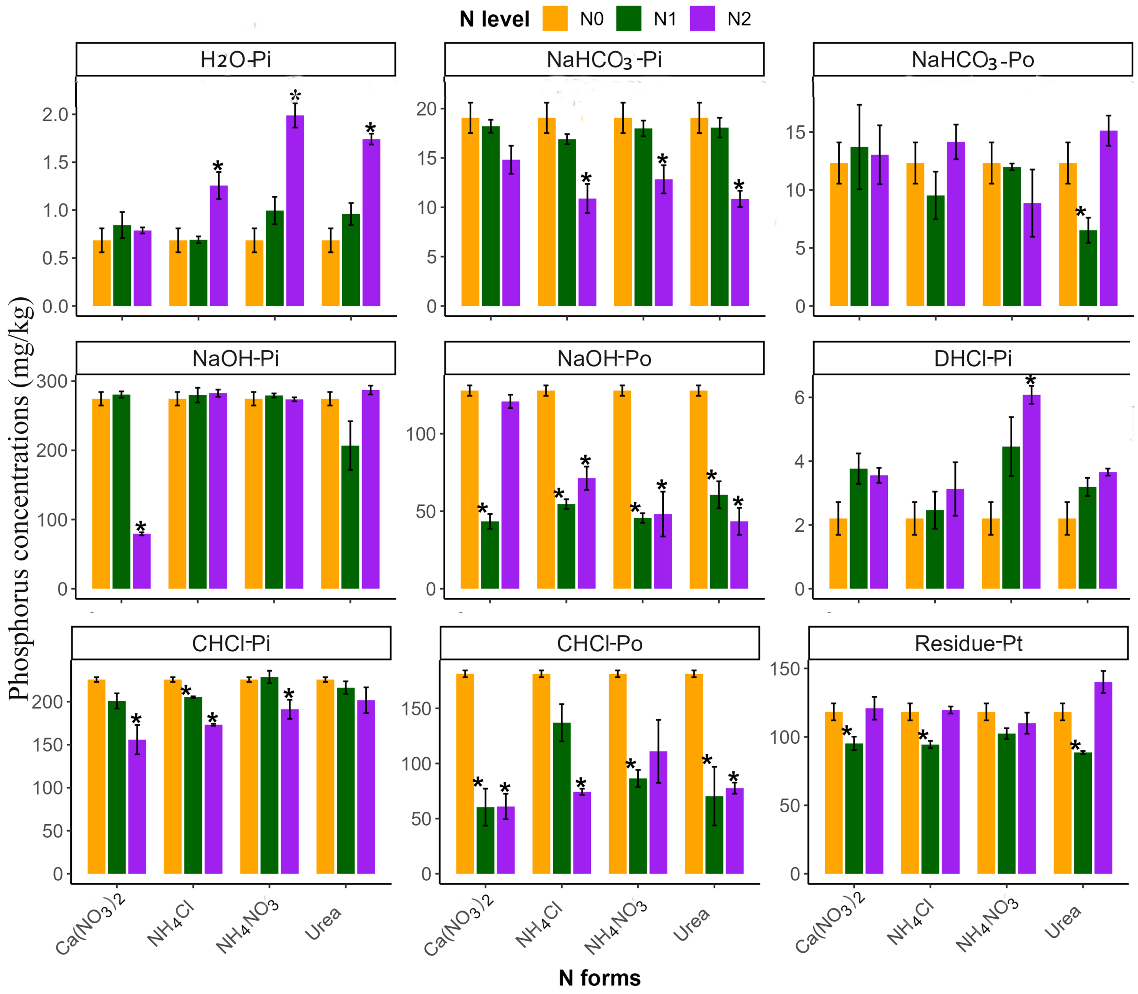

FIGURE 3
The effects of different N addition levels (N0, 0 mg N kg^{-1} soil; N1, 50 mg N kg^{-1} soil; N2, 100 mg N kg^{-1} soil) and N addition forms ($Ca(NO_3)_2$, NH_4Cl, NH_4NO_3 and Urea) on the P fractions concentrations (mg kg^{-1}) in yellow soil. The error bars represent the standard error ($n = 4$). "*" Indicates significant differences between the N addition treatment and the control group ($p < 0.05$).

($p < 0.05$; Figure 5). On the other hand, $Ca(NO_3)_2$ addition only decreased *phoC* abundance in limestone soil, while other N forms did not notably affect *phoC* abundance in either soil type ($p < 0.05$; Figure 5). Furthermore, in both soil types, the *phoC* abundance resulting from high Urea addition was significantly higher compared to other N additions (Supplementary Table S2). As for the *phoD* gene abundance in yellow soil, all forms of N addition generally had a positive effect, showing the same order as above ($p < 0.05$; Figure 5). In contrast, in limestone soil, high NH_4Cl and $Ca(NO_3)_2$ addition significantly reduced *phoD* gene abundance, while other N treatments had no significant effect. Additionally, compared to yellow soil, limestone soil exhibited higher *phoC* and *phoD* gene abundance (Figure 5 and Table 2).

3.4 Correlation between soil properties and P fractions

Correlation analysis revealed distinct relationships between soil properties and P fractions in yellow and limestone soils. In yellow soil, H_2O-Pi exhibited a significant negative correlation with NH_4^+-N, NO_3^--N and AN ($p < 0.05$; Figure 6A), while it showed a positive correlation with ALP activity, *phoC* gene abundance, *phoD* gene abundance, and RB ($p < 0.05$; Figure 6A). Conversely, $NaHCO_3$-Pi displayed an opposite trend in its correlations with these factors. Additionally, both NaOH-Po and C.HCl-Po were significantly negatively correlated with RB in yellow soil ($p < 0.05$; Figure 6A). In limestone soil, H_2O-Pi was significantly negatively correlated with NH_4^+-N and AN, and positively correlated with *phoC* gene abundance and RB ($p < 0.05$; Figure 6B). Furthermore, NaOH-Pi, D.HCl-Pi, and C.HCl-Po in limestone soil showed significant positive correlations with RB ($p < 0.05$; Figure 6B).

4 Discussion

4.1 The impact of N input on soil P availability depends on the input level and form of N

N input modifies P fractions by influencing biotic and abiotic factors in the soil P cycle (Sales et al., 2017; Hou et al., 2018; Fan et al.,

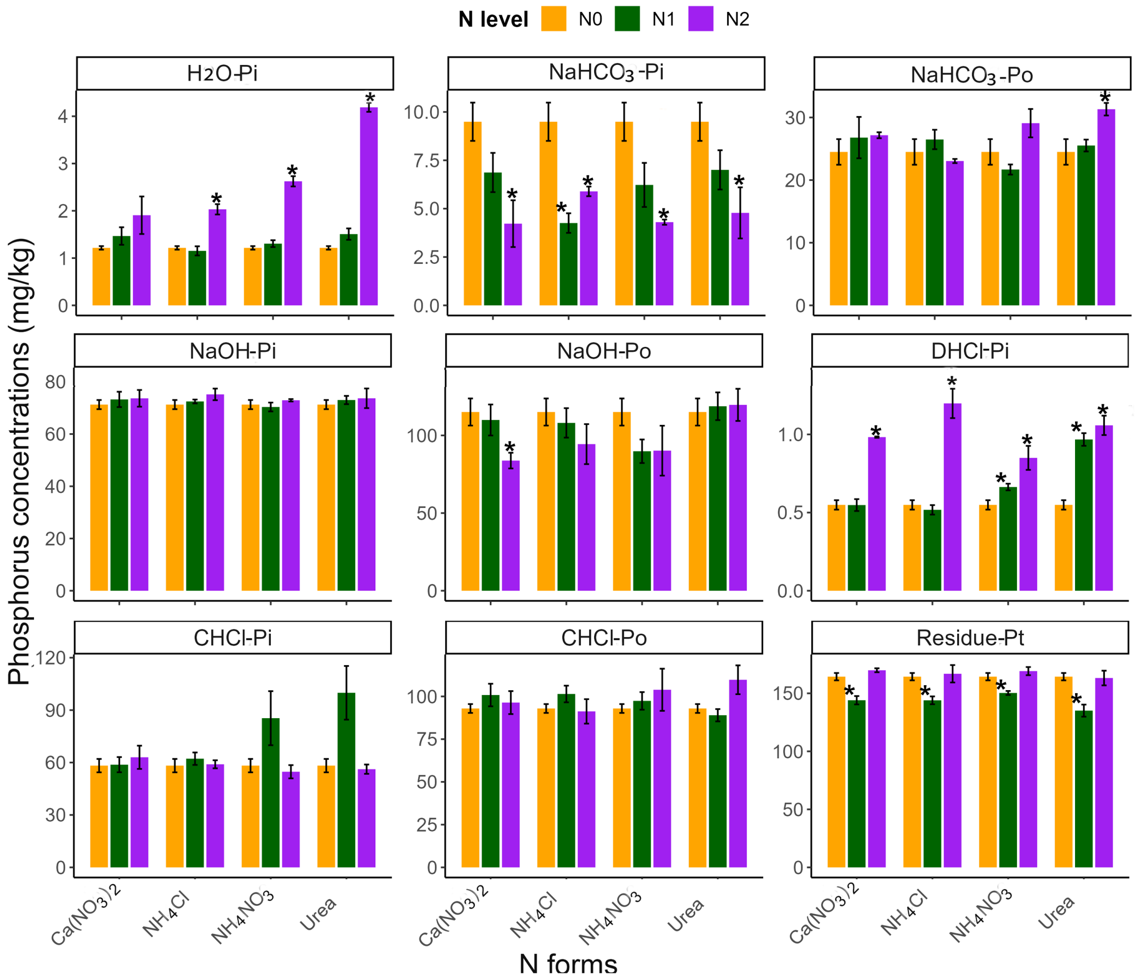

FIGURE 4
The effects of different N addition levels (N0, 0 mg N kg^{-1} soil; N1, 50 mg N kg^{-1} soil; N2, 100 mg N kg^{-1} soil) and N addition forms [$Ca(NO_3)_2$, NH_4Cl, NH_4NO_3 and Urea] on the P fractions concentrations (mg kg^{-1}) in limestone soil. The error bars represent the standard error (*n* = 4). "*" Indicates significant differences between the N addition treatment and the control group ($p < 0.05$).

2019). Based on the availability of plants and organisms, soil P can be categorized into available P (H_2O-Pi), labile P fraction ($NaHCO_3$-Pi and $NaHCO_3$-Po), moderately labile P fraction (NaOH-Pi, NaOH-Po and D.HCl-Pi), non-labile P fraction (C.HCl-Pi, C.HCl-Po and residual-Pt), respectively (Hedley et al., 1982; Redel et al., 2019; Mahmood et al., 2021). Notably, available P and labile P are more influenced by human activities than moderately and non-labile P fractions (Chen et al., 2018; Guan et al., 2023). In this study, two distinct soil types were examined: limestone soil and yellow soil. In yellow soil, the fractions of H_2O-Pi, $NaHCO_3$-Pi, NaOH-Po, C.HCl-Pi, and C.HCl-Po were significantly affected by N input, while in limestone soil, only H_2O-Pi and $NaHCO_3$-Pi fractions were significantly influenced, suggesting a higher sensitivity of yellow soil to N inputs (Figures 3, 4). The variation in soil available P can be attributed to the differences in initial available P concentrations and soil pH between these soil types. A recent meta-analysis reported that pH plays an important role in change of soil P contents (Li et al., 2023). In comparison to limestone soil (available P: 1.22 mg kg^{-1}, pH: 6.01), yellow soil has lower available P and soil pH (available P: 0.68 mg kg^{-1}; pH: 5.46). Prior research has highlighted that soil P dynamics are highly responsive to N inputs in P-limited environments, particularly in acidic soils ($pH < 5.5$) (Deng et al., 2017). Addition of N significantly affects soil properties, primarily by lowering soil pH and leading to soil acidification (Fan et al., 2019). This change can substantially alter soil phosphorus availability by influencing the chemical and biological reactions that control P migration (Li et al., 2019).

Furthermore, our findings support the first hypothesis that N input significantly alters soil P fractions, with the effect depending on both the N level and form. This aligns with Liu et al. (2021), who found that low-level N inputs in the short term do not significantly affect soil P fractions, while high-level inputs do. This difference might be attributed to the lesser influence of low-level N on biotic and abiotic factors controlling soil P fractions in the short term (Figures 1, 5). Fertilization and land use mainly influence H_2O-Pi and $NaHCO_3$-Pi fractions (Tian et al., 2020). Our findings indicated that high N inputs, except for $Ca(NO_3)_2$, significantly increased available P and decreased the $NaHCO_3$-Pi fraction in both soils (Figures 3, 4). This result is similar to previous findings in subtropical forests and temperate larch plantations (Fan et al., 2019; Huang et al., 2021). Additionally, in

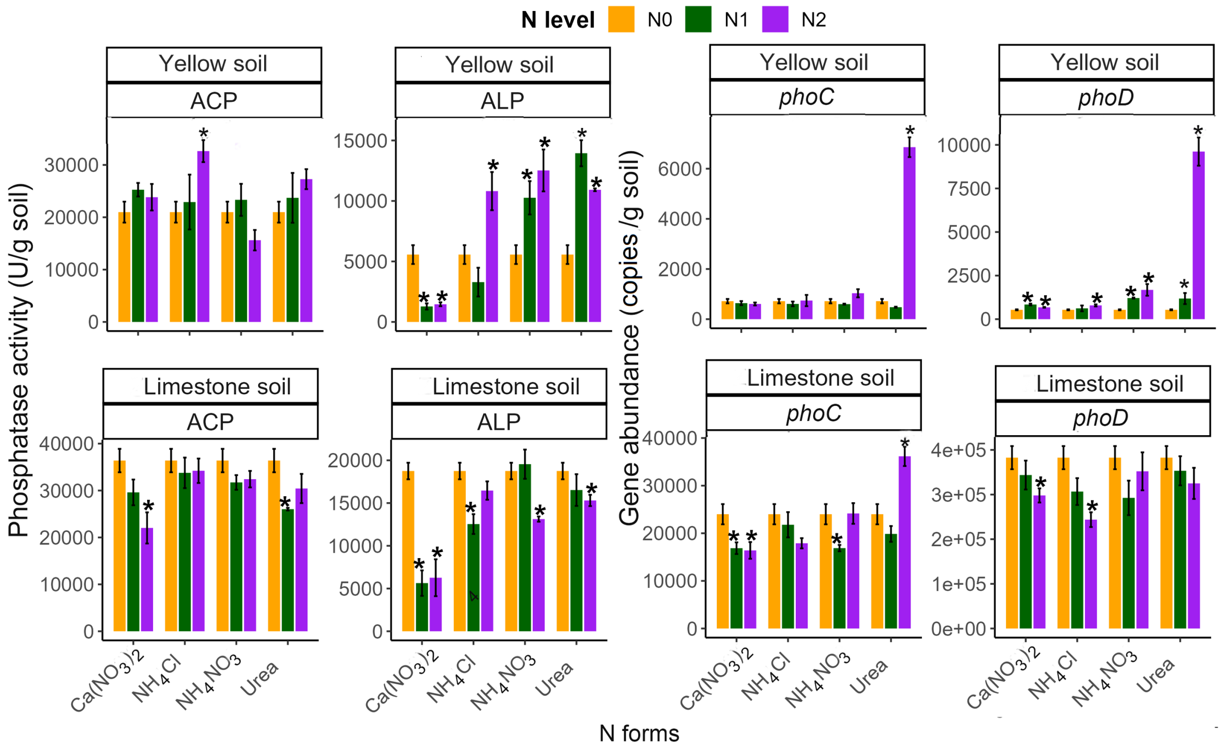

FIGURE 5

The effects of different N addition levels (N0, 0 mg N kg^{-1} soil; N1, 50 mg N kg^{-1} soil; N2, 100 mg N kg^{-1} soil) and N addition forms [Ca (NO$_3$)$_2$, NH$_4$Cl, NH$_4$NO$_3$ and Urea] on the activities of acid phosphatase (ACP) and alkaline phosphatase (ALP), the abundance of *phoC* and *phoD* genes in yellow soil and limestone soil. The error bars represent the standard error ($n = 4$). "*" Indicates significant differences between the N addition treatment and the control group ($p < 0.05$).

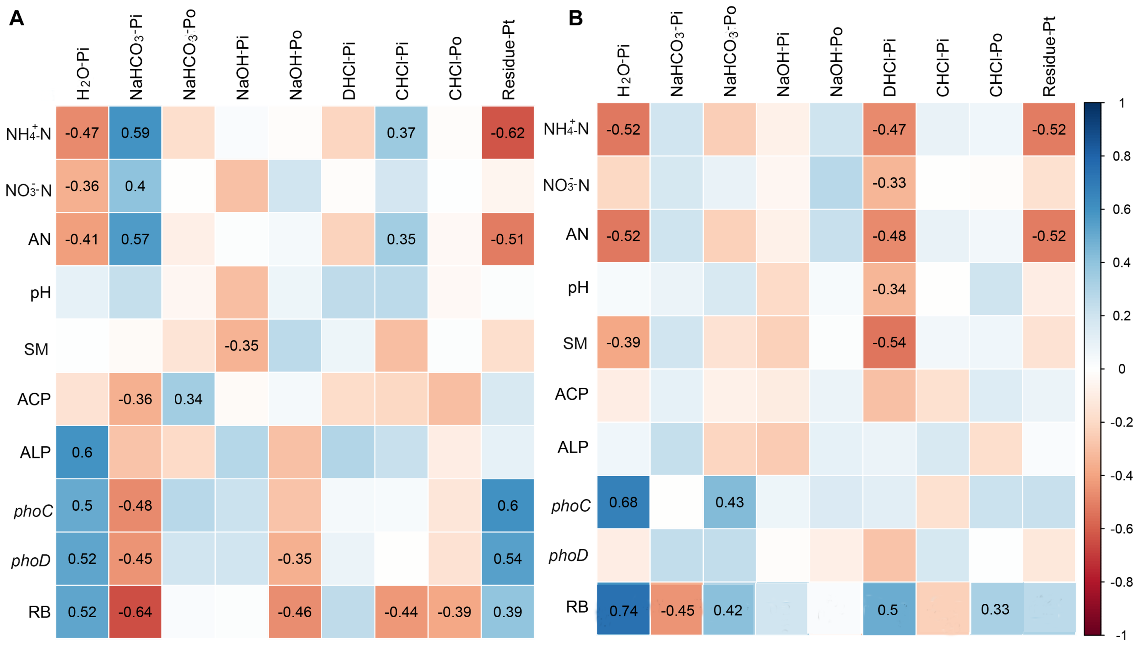

FIGURE 6

Relationship (Pearson's) between P fractions of yellow soil **(A)** and limestone soil **(B)** and biotic and abiotic factors. Only significant correlation coefficients are marked in the figure ($p < 0.05$). AN, available nitrogen; ACP, acid phosphatase; ALP, alkaline phosphatase; RB, root biomass; SM, soil water contents.

yellow soil, but not limestone soil, N input also reduced the NaOH-Po and C.HCl-Po fractions. The dynamic behavior of soil P, which involves processes like dissolution, adsorption, desorption, precipitation, and transformation (Rawat et al., 2020), implies that N additions could increase the availability of soil P. This potential increase may result from the enhanced desorption of inorganic P and the accelerated mineralization of organic P. This interpretation was supported by the significant correlation between soil available P and ALP activity, and plant root biomass (Figure 6A).

In our study, different forms of nitrogen exhibited varied effects on the available phosphorus, consistently following the order in both yellow soil and limestone soil: NH_4NO_3 and Urea $>NH_4Cl > Ca(NO_3)_2$ (Figures 3, 4). This pattern was consistent with Amin's (2023) research on the efficiency of different N fertilizers in increasing P availability. The differential effects of N forms on soil available P can be attributed to several factors. Firstly, Ca^{2+}, as an alkaline cation, can form low-soluble calcium phosphate with phosphate anions in soil, potentially reducing available P, especially when adding $Ca(NO_3)_2$ (Jalali and Jalali, 2016; Deng et al., 2017; Xu et al., 2020). Furthermore, the higher soil pH following $Ca(NO_3)_2$ addition could contribute to this phenomenon (Penn and Camberato, 2019). Conversely, NH_4Cl application leads to more severe soil acidification due to nitrification, promoting inorganic P desorption (Raza et al., 2019; Mahmood et al., 2021). Differently, in contrast to $Ca(NO_3)_2$ and NH_4Cl, the addition of Urea, particularly in limestone soil, results in a greater increase in root biomass and phosphatase activity, thereby enhancing the availability of soil P. Similarly, high NH_4NO_3 treatment significantly increased soil available phosphorus compared to the addition of $Ca(NO_3)_2$ and NH_4Cl, yet this trend was not mirrored in the root biomass (Figure 2). Considering that this study included only white clover and perennial ryegrass, we speculate that the enhanced soil available phosphorus due to NH_4NO_3 treatment may be associated with a higher proportion of white clover in the community (unpublished data). Previous research shows that white clover is more efficient than perennial ryegrass at releasing organic ions, which aids in the desorption, chelation, and complexation of iron and aluminum oxides, ultimately improving phosphorus release for plant uptake (Touhami et al., 2020). Considering urea's affordability and accessibility (Apthorp et al., 1987), using it as the primary N source can improve soil P availability, reduce fertilizer costs in the studied grasslands. Additionally, the role of different N forms should be considered in future research on N deposition, given the global changes in N deposition components, to accurately evaluate their impact on grassland soil P cycling.

4.2 Relationship between changes in soil available P and plant root biomass and phosphatase activity

Our research revealed a clear connection between alterations in soil available P, plant root biomass, and phosphatase activity, aligning with our second hypothesis. These findings illuminate the dynamic interplay among these factors in soil P cycling, particularly under N input. In both yellow and limestone soils, we observed a close link between available P and plant root biomass. This finding is consistent with previous studies in various ecosystems, which highlight the pivotal role of plant roots in soil P dynamics (Fan et al., 2019).

Plant roots are critical in mediating soil P availability, especially following N input. Increased N leads to a greater P demand, consequently stimulating root growth and enhancing carbon allocation towards P acquisition (Zhang et al., 2016; Fan et al., 2018). The resulting increase in root biomass facilitates the release of root exudates, including organic acids and phosphatases, which are instrumental in mobilizing mineral P and promoting phosphate dissolution (Kai et al., 2014; Yang et al., 2019). Our study observed a significant rise in soil available P, particularly H_2O-Pi, under N input, which is likely a direct consequence of enhanced P desorption and bioavailability. This observation parallels findings by Yang et al. (2019), who noted similar effects in short-term N addition studies.

Soil phosphatase, pivotal in mineralizing soil organic P, is also influenced by N input (George et al., 2018; Cao et al., 2022; Ma et al., 2023). We found that N input significantly increased ALP activity and the abundance of the *phoD* gene in yellow soil, while the response was less pronounced for acid phosphatase (ACP) and *phoC* abundance (Figure 5). This variation might be due to the distinct origins of these enzymes. Microorganisms that harbor the *phoD* gene tend to increase alkaline phosphatase production under soil P scarcity, a response that is likely exacerbated by N input (Marklein and Houlton, 2012; Nasto et al., 2015; Wang et al., 2021). Conversely, the response of these enzymes to N input in limestone soil differed (Figure 5). This may be related to the fact that the pH in limestone soil is more sensitive to N input, N-induced decreases in soil pH are positively correlated with N-induced changes in soil phosphatase activity (Chen et al., 2020). The significant increase in alkaline phosphatase activity under N input, particularly with Urea and NH_4NO_3 treatments, underscores the role of these enzymes in mitigating P limitation. This is further supported by the observation that N-induced changes in soil phosphatase activity are positively correlated with alterations in soil P fractions (Figure 6A). This may be due to the Urea input providing microbially available carbon, enhancing microbial activity (Zhang et al., 2020). However, $Ca(NO_3)_2$ appeared to inhibit phosphatase activity, likely due to Ca^{2+} inhibition (Halstead, 1964), suggesting it may not be the optimal N source for enhancing P availability in these soils. The discrepancy in *phoD* gene abundance and alkaline phosphatase activity trends under different N inputs (Figure 5) could stem from the fact that alkaline phosphatase is encoded not only by *phoD* but also by *phoA* and *phoX* genes (Hu et al., 2018).

Our study highlights the importance of considering the form of N input when assessing its impact on soil P dynamics. While all forms of N input altered alkaline phosphatase activity to some degree, the distinct patterns observed suggest varying effects on the microbial processes involved in P mineralization, necessitating further exploration to fully grasp the implications of different N forms on soil P cycling and plant-microbe interactions.

5 Conclusion

This study investigated the effects of N addition on two typical soil P fractions in the karst grasslands of southwestern China and found that this effect was significantly and strongly modulated by both the form and amount of N added. In particular, compared with $Ca(NO_3)_2$ addition, other high-level N sources (especially Urea and NH_4NO_3) significantly increased available P in both soils, with the order of effects being Urea and $NH_4NO_3 > NH_4Cl > Ca(NO_3)_2$. This highlights the considerable role of N form in determining soil P availability. Further analysis revealed a significant correlation between changes in soil available P and factors such as plant root biomass and alkaline phosphatase activity, underscoring the importance of biological factors in the dynamics of soil P in the context of N input. In terms of management implications, we recommend that high levels of Urea and NH_4NO_3 be prioritized over $Ca(NO_3)_2$ in management practices to increase soil P availability and plant utilization. This has important implications for improving soil fertility in the region and supporting sustainable agricultural practices.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

JZ: Data curation, Writing – original draft. FY: Data curation, Writing – review & editing. XZ: Writing – review & editing. XG: Writing – review & editing. CC: Writing – review & editing. JC: Funding acquisition, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2024.1343283/full#supplementary-material

References

Ahmad, M., El-Naggar, A. H., Usman, A. R. A., Abduljabbar, A., Vithanage, M., Elfaki, J., et al. (2018). Aging effects of organic and inorganic fertilizers on phosphorus fractionation in a calcareous Sandy loam soil. *Pedosphere* 28, 873–883. doi: 10.1016/S1002-0160(17)60363-1

Amin, A. E.-E. A. Z. (2023). Effect of co-applying different nitrogen fertilizers with bone char on enhancing phosphorus release in calcium carbonate-rich soil: an incubation study. *J. Soil Sci. Plant Nutr.* 23, 1565–1575. doi: 10.1007/s42729-023-01217-3

Apthorp, J., Hedley, M., and Tillman, R. (1987). The effects of nitrogen fertilizer form on the plant availability of phosphate from soil, phosphate rock and mono-calcium phosphate. *Fertilizer Res.* 12, 269–283. doi: 10.1007/BF01315111

Ashley, K., Cordell, D., and Mavinic, D. (2011). A brief history of phosphorus: from the philosopher's stone to nutrient recovery and reuse. *Chemosphere* 84, 737–746. doi: 10.1016/J.CHEMOSPHERE.2011.03.001

Bai, Y., and Cotrufo, M. F. (2022). Grassland soil carbon sequestration: current understanding, challenges, and solutions. *Science* 377, 603–608. doi: 10.1126/science.abo2380

Cao, N., Zhi, M., Zhao, W., Pang, J., Hu, W., Zhou, Z., et al. (2022). Straw retention combined with phosphorus fertilizer promotes soil phosphorus availability by enhancing soil P-related enzymes and the abundance of phoC and phoD genes. *Soil Tillage Res.* 220:105390. doi: 10.1016/j.still.2022.105390

Chen, H., Chen, M., Li, D., Mao, Q., Zhang, W., and Mo, J. (2018). Responses of soil phosphorus availability to nitrogen addition in a legume and a non-legume plantation. *Geoderma* 322, 12–18. doi: 10.1016/j.geoderma.2018.02.017

Chen, J., van Groenigen, K. J., Hungate, B. A., Terrer, C., van Groenigen, J. W., Maestre, F. T., et al. (2020). Long-term nitrogen loading alleviates phosphorus limitation in terrestrial ecosystems. *Glob. Chang. Biol.* 26, 5077–5086. doi: 10.1111/gcb.15218

Cui, H., Fan, M., Wang, Y., Zhang, X., Xu, W., Li, Y., et al. (2022). Impacts of mowing and N addition on soil organic phosphorus mineralization rates in a semi-natural grassland in Northeast China. *Plant Soil* 482, 7–23. doi: 10.1007/s11104-022-05670-4

Deng, Q., Hui, D., Dennis, S., Reddy, K. C., and Xu, X. (2017). Responses of terrestrial ecosystem phosphorus cycling to nitrogen addition: a meta-analysis. *Glob. Ecol. Biogeogr.* 26, 713–728. doi: 10.1111/geb.12576

Ding, Y., Geng, Y., Zhou, W., and Li, D. (2023). Habitat-specific environmental factors regulate the spatial variability of biological soil crust microbial communities on the Qinghai-Tibet Plateau. *Sci. Total Environ.* 901:165937. doi: 10.1016/j.scitotenv.2023.165937

Fan, Y., Lin, F., Yang, L., Zhong, X., and Yang, Y. (2018). Decreased soil organic P fraction associated with ectomycorrhizal fungal activity to meet increased P demand under N application in a subtropical forest ecosystem. *Biol. Fertil. Soils* 54, 149–161. doi: 10.1007/s00374-017-1251-8

Fan, Y., Zhong, X., Lin, F., Liu, C., Yang, L., Wang, M., et al. (2019). Responses of soil phosphorus fractions after nitrogen addition in a subtropical forest ecosystem: insights from decreased Fe and Al oxides and increased plant roots. *Geoderma* 337, 246–255. doi: 10.1016/j.geoderma.2018.09.028

Fenton, O., Mellander, P.-E., Daly, K., Wall, D., Jahangir, M., Jordan, P., et al. (2017). Integrated assessment of agricultural nutrient pressures and legacies in karst landscapes. *Agric. Ecosyst. Environ.* 239, 246–256. doi: 10.1016/j.agee.2017.01.014

Geng, Y., Peng, C., Wang, Z., Huang, S., Zhou, P., and Li, D. (2022). Insights into the spatiotemporal differences in tailings seepage pollution by assessing the diversity and metabolic functions of the soil microbial community. *Environ. Pollut.* 306:119408. doi: 10.1016/j.envpol.2022.119408

George, T. S., Giles, C. D., Menezes-Blackburn, D., Condron, L. M., and Haygarth, P. M. (2018). Organic phosphorus in the terrestrial environment: a perspective on the state of the art and future priorities. *Plant Soil* 427, 191–208. doi: 10.1007/s11104-017-3488-2

Guan, Z.-H., Cao, Z., Li, X. G., Kühn, P., Hu, G., Scholten, T., et al. (2023). Effects of winter grazing and N addition on soil phosphorus fractions in an alpine grassland on the Qinghai-Tibet Plateau. *Agric. Ecosyst. Environ.* 357:108700. doi: 10.1016/j.agee.2023.108700

Halstead, R. L. (1964). Phosphatase activity of soils as influenced by lime and other treatments. *Can. J. Soil Sci.* 44, 137–144. doi: 10.4141/cjss64-017

Hedley, M. J., Stewart, J. W. B., and Chauhan, B. S. (1982). Changes in inorganic and organic soil phosphorus fractions induced by cultivation practices and by laboratory incubations. *Soil Sci. Soc. Am. J.* 46, 970–976. doi: 10.2136/sssaj1982.03615995004600050017x

Hou, E., Wen, D., Jiang, L., Luo, X., Kuang, Y., Lu, X., et al. (2021). Latitudinal patterns of terrestrial phosphorus limitation over the globe. *Ecol. Lett.* 24, 1420–1431. doi: 10.1111/ele.13761

Hou, E., Wen, D., Kuang, Y., Cong, J., Chen, C., He, X., et al. (2018). Soil pH predominantly controls the forms of organic phosphorus in topsoils under natural broadleaved forests along a 2500 km latitudinal gradient. *Geoderma* 315, 65–74. doi: 10.1016/j.geoderma.2017.11.041

Hu, W., Tan, J., Shi, X., Lock, T. R., Kallenbach, R. L., and Yuan, Z. (2022). Nutrient addition and warming alter the soil phosphorus cycle in grasslands: a global meta-analysis. *J. Soils Sediments* 22, 2608–2619. doi: 10.1007/s11368-022-03276-y

Hu, Y., Xia, Y., Sun, Q., Liu, K., Chen, X., Ge, T., et al. (2018). Effects of long-term fertilization on phoD-harboring bacterial community in karst soils. *Sci. Total Environ.* 628-629, 53–63. doi: 10.1016/j.scitotenv.2018.01.314

Huang, J., Zhou, L. H., Liu, S. J., Han, T. F., Hayatu, N. G., Li, D. C., et al. (2021). Vertical distribution of phosphorus fractions and the environmental critical phosphorus level in acidic red soil under long-term fertilizer and lime application in southern China. *J. Plant Nutr. Soil Sci.* 184, 585–595. doi: 10.1002/jpln.202100098

Jalali, M., and Jalali, M. (2016). Relation between various soil phosphorus extraction methods and sorption parameters in calcareous soils with different texture. *Sci. Total Environ.* 566-567, 1080–1093. doi: 10.1016/j.scitotenv.2016.05.133

Jing, D., Yan, Y., Ren, T., Lu, J., Wang, X., Chen, J., et al. (2021). Effects of nitrogen application rate on phosphorus transformation in an Alfisol: results from phosphate-oxygen isotope ratios. *Appl. Geochem.* 134:105094. doi: 10.1016/j.apgeochem.2021.105094

Kai, Y., Jiaojun, Z., Jiacun, G., Lizhong, Y., and Wang, Z. (2014). Changes in soil phosphorus fractions after 9 years of continuous nitrogen addition in a *Larix gmelinii* plantation. *Ann. For. Sci.* 72, 435–442. doi: 10.1007/s13595-014-0444-7

Li, Y., Cui, S., Chang, S. X., and Zhang, Q. (2019). Liming effects on soil pH and crop yield depend on lime material type, application method and rate, and crop species: a global meta-analysis. *J. Soils Sediments* 19, 1393–1406. doi: 10.1007/s11368-018-2120-2

Li, Y., Li, Y., Zhang, Q., Xu, G., Liang, G., Kim, D., et al. (2023). Enhancing soil carbon and nitrogen through grassland conversion from degraded croplands in China: assessing magnitudes and identifying key drivers of phosphorus reduction. *Soil Tillage Res.* 236:105943. doi: 10.1016/j.still.2023.105943

Li, D., Liu, J., Chen, H., Zheng, L., and Wang, K. (2018). Soil gross nitrogen transformations in responses to land use conversion in a subtropical karst region. *J. Environ. Manag.* 212, 1–7. doi: 10.1016/j.jenvman.2018.01.084

Liu, Y., Bing, H., Wu, Y., Zhu, H., Tian, X., Wang, Z., et al. (2022). Nitrogen addition promotes soil phosphorus availability in the subalpine forest of eastern Tibetan plateau. *J. Soils Sediments* 22, 1–11. doi: 10.1007/s11368-021-03064-0

Liu, C., Li, W., Xu, J., Wei, W., and Yan, H. (2021). Response of soil nutrients and stoichiometry to grazing management in alpine grassland on the Qinghai-Tibet plateau. *Soil Tillage Res.* 206:104822. doi: 10.1016/j.still.2020.104822

Liu, C., Liu, Y., Guo, K., Qiao, X., Zhao, H., Wang, S., et al. (2018). Effects of nitrogen, phosphorus and potassium addition on the productivity of a karst grassland: plant functional group and community perspectives. *Ecol. Eng.* 117, 84–95. doi: 10.1016/j.ecoleng.2018.04.008

Ma, X., Zhou, Z., Chen, J., Xu, H., Ma, S., Dippold, M. A., et al. (2023). Long-term nitrogen and phosphorus fertilization reveals that phosphorus limitation shapes the microbial community composition and functions in tropical montane forest soil. *Sci. Total Environ.* 854:158709. doi: 10.1016/j.scitotenv.2022.158709

Mahmood, M., Tian, Y., Ma, Q., Hui, X., Elrys, A. S., Ahmed, W., et al. (2021). Changes in phosphorus fractions in response to long-term nitrogen fertilization in loess plateau of China. *Field Crop Res.* 270:108207. doi: 10.1016/j.fcr.2021.108207

Marklein, A. R., and Houlton, B. Z. (2012). Nitrogen inputs accelerate phosphorus cycling rates across a wide variety of terrestrial ecosystems. *New Phytol.* 193, 696–704. doi: 10.1111/j.1469-8137.2011.03967.x

Nannipieri, P., Giagnoni, L., Landi, L., and Renella, G. (2011). "Role of phosphatase enzymes in soil" in *Phosphorus in action*. eds. E. Bunemann, A. Oberson and E. Frossard (Berlin, Heidelberg: Springer), 215–243.

Nasto, M. K., Alvarez-Clare, S., Lekberg, Y., Sullivan, B. W., Townsend, A. R., Cleveland, C. C., et al. (2015). Interactions among nitrogen fixation and soil phosphorus acquisition strategies in lowland tropical rain forests. *Ecol. Lett.* 17, 1282–1289. doi: 10.1111/ele.12335

Penn, C. J., and Camberato, J. J. (2019). A critical review on soil chemical processes that control how soil pH affects phosphorus availability to plants. *Agriculture* 9:120. doi: 10.3390/agriculture9060120

Penuelas, J., Janssens, I. A., Ciais, P., Obersteiner, M., and Sardans, J. (2020). Anthropogenic global shifts in biospheric N and P concentrations and ratios and their impacts on biodiversity, ecosystem productivity, food security, and human health. *Glob. Chang. Biol.* 26, 1962–1985. doi: 10.1111/gcb.14981

Piao, H., Wu, Y., Hong, Y., and Yuan, Z. (2000). Soil-released carbon dioxide from microbial biomass carbon in the cultivated soils of karst areas of Southwest China. *Biol. Fertil. Soils* 31, 422–426. doi: 10.1007/s003749900189

Rawat, P., Das, S., Shankhdhar, D., and Shankhdhar, S. C. (2020). Phosphate-solubilizing microorganisms: mechanism and their role in phosphate Solubilization and uptake. *J. Soil Sci. Plant Nutr.* 21, 49–68. doi: 10.1007/s42729-020-00342-7

Raza, S., Chen, Z., Ahmed, M., Afzal, M. R., Aziz, T., and Zhou, J. (2019). Dicyandiamide application improved nitrogen use efficiency and decreased nitrogen losses in wheat-maize crop rotation in loess plateau. *Arch. Agron. Soil Sci.* 65, 450–464. doi: 10.1080/03650340.2018.1506584

Redel, Y., Staunton, S., Durán, P., Gianfreda, L., Rumpel, C., and de La Luz Mora, M. (2019). Fertilizer P uptake determined by soil P fractionation and phosphatase activity. *J. Soil Sci. Plant Nutr.* 19, 166–174. doi: 10.1007/s42729-019-00024-z

Sakurai, M., Wasaki, J., Tomizawa, Y., Shinano, T., and Osaki, M. (2007). Analysis of bacterial communities on alkaline phosphatase genes in soil supplied with organic matter. *Soil Sci. Plant Nutr.* 54, 62–71. doi: 10.1111/j.1747-0765.2007.00210.x

Sales, M. V., Aleixo, S., Gama-Rodrigues, A. C., and Gama-Rodrigues, E. F. (2017). Structural equation modeling for the estimation of interconnections between the P cycle and soil properties. *Nutr. Cycl. Agroecosyst.* 109, 193–207. doi: 10.1007/s10705-017-9879-1

Sattari, S., Bouwman, A., Martinez Rodríguez, R., Beusen, A., and Van Ittersum, M. (2016). Negative global phosphorus budgets challenge sustainable intensification of grasslands. *Nat. Commun.* 7:10696. doi: 10.1038/ncomms10696

Tan, H., Barret, M., Mooij, M. J., Rice, O., Morrissey, J. P., Dobson, A., et al. (2013). Long-term phosphorus fertilisation increased the diversity of the total bacterial community and the phoD phosphorus mineraliser group in pasture soils. *Biol. Fertil. Soils* 49, 661–672. doi: 10.1007/s00374-012-0755-5

Tian, H., Xu, R., Pan, S., Yao, Y., Bian, Z., Cai, W. J., et al. (2020). Long-term trajectory of nitrogen loading and delivery from Mississippi River basin to the Gulf of Mexico. *Glob. Biogeochem. Cycles* 34:e2019GB006475. doi: 10.1029/2019GB006475

Tiessen, H., and Moir, J. O. (1993). Characterization of available P by sequential extraction. *Soil sampling and methods of analysis*. 7, 225–229.

Touhami, D., McDowell, R. W., and Condron, L. M. (2020). Role of organic anions and phosphatase enzymes in phosphorus acquisition in the rhizosphres of legumes and grasses grown in a low phosphorus pasture soil. *Plan. Theory* 9:1185. doi: 10.3390/plants9091185

Van Lenteren, J. E., and Woets, J. V. (1988). Biological and integrated pest control in greenhouses. *Annu. Rev. Entomol.* 33, 239–269. doi: 10.1007/0-306-47585-5_22

Walker, N. J. (2001). Real-time and quantitative PCR: applications to mechanism-based toxicology. *J. Biochem. Mol. Toxicol.* 15, 121–127. doi: 10.1002/jbt.8

Wang, Z., Tao, T., Wang, H., Chen, J., Small, G. E., Johnson, D., et al. (2023). Forms of nitrogen inputs regulate the intensity of soil acidification. *Glob. Chang. Biol.* 29, 4044–4055. doi: 10.1111/gcb.16746

Wang, C., Xue, L., and Jiao, R. (2021). Soil phosphorus fractions, phosphatase activity, and the abundance of phoC and phoD genes vary with planting density in subtropical Chinese fir plantations. *Soil Tillage Res.* 209:104946. doi: 10.1016/j.still.2021.104946

Wang, R., Yang, J., Liu, H., Sardans, J., Zhang, Y., Wang, X., et al. (2022). Nitrogen enrichment buffers phosphorus limitation by mobilizing mineral-bound soil phosphorus in grasslands. *Ecology* 103:e3616. doi: 10.1002/ecy.3616

Wen, L., Li, D., Yang, L., Luo, P., Chen, H., Xiao, K., et al. (2016). Rapid recuperation of soil nitrogen following agricultural abandonment in a karst area, Southwest China. *Biogeochemistry* 129, 341–354. doi: 10.1007/s10533-016-0235-3

Wu, L., Wang, S., Bai, X., Tian, Y., Luo, G., Wang, J., et al. (2020). Climate change weakens the positive effect of human activities on karst vegetation productivity restoration in southern China. *Ecol. Indic.* 115:106392. doi: 10.1016/j.ecolind.2020.106392

Xiao, D., Xiao, L., Che, R., Tan, Y., Liu, X., Yang, R., et al. (2020). Phosphorus but not nitrogen addition significantly changes diazotroph diversity and community composition in typical karst grassland soil. *Agric. Ecosyst. Environ.* 301:106987. doi: 10.1016/j.agee.2020.106987

Xu, X., Mao, X., Zwieten, L. V., Niazi, N. K., and Wang, H. (2020). Wetting-drying cycles during a rice-wheat crop rotation rapidly (im) mobilize recalcitrant soil phosphorus. *J. Soils Sediments* 20, 3921–3930. doi: 10.1007/s11368-020-02712-1

Yang, C., Xia, L., Zeng, Y., Chen, Y., and Zhang, S. (2023). Hexaploid Salix rehderiana is more suitable for remediating lead contamination than diploids, especially male plants. *Chemosphere* 333:138902. doi: 10.1016/j.chemosphere.2023.138902

Yang, L., Zhijie, P., Yuanzhen, L., Yanyu, X., Decheng, L., and Yusheng, Y. Y. (2019). Evaluating P availability influenced by warming and N deposition in a subtropical forest soil: a bioassay mesocosm experiment. *Plant Soil* 444, 87–99. doi: 10.1007/s11104-019-04246-z

Yokoyama, D., Imai, N., and Kitayama, K. (2017). Effects of nitrogen and phosphorus fertilization on the activities of four different classes of fine-root and soil phosphatases in Bornean tropical rain forests. *Plant Soil* 416, 463–476. doi: 10.1007/s11104-017-3225-x

Zhang, T., Qin, M., Wei, C., Li, D., Lu, X., and Zhang, L. (2020). Suspended particles phoD alkaline phosphatase gene diversity in large shallow eutrophic Lake Taihu. *Sci. Total Environ.* 728:138615. doi: 10.1016/j.scitotenv.2020.138615

Zhang, L. F., and Shenglei. (2020). Effects of nitrogen deposition and increased precipitation on soil phosphorus dynamics in a temperate forest. *Geoderma* 380:114650. doi: 10.1016/j.geoderma.2020.114650

Zhang, H., Shi, L., Wen, D., and Yu, K. (2016). Soil potential labile but not occluded phosphorus forms increase with forest succession. *Biol. Fertil. Soils* 52, 41–51. doi: 10.1007/s00374-015-1053-9

Zhang, Y., Wang, H., Zhao, Y., Wang, J., and Chen, S. (2004). Causes and control countermeasures of main pests and diseases in artificial grassland in Moqi, Inner Mongolia. *Grassland Res.* 21:3. doi: 10.3969/j.issn.1001-0629.2004.07.015

Zhang, W., Zhang, L., and Deng, J. (2021). Comparison of retention and output of sulfur in limestone soil and yellow soil and their responses to acid deposition in a small karst catchment of Guizhou Province, Southwest China. *Environ. Sci. Pollut. Res.* 28, 60993–61007. doi: 10.1007/s11356-021-15039-2

Zhang, X., Zhao, Q., Sun, Q. Y., Mao, B., and Zeng, D. H. (2022). Understory vegetation interacts with nitrogen addition to affect soil phosphorus transformations in a nutrient-poor *Pinus sylvestris* var. mongolica plantation. *For. Ecol. Manag.* 507:120026. doi: 10.1016/j.foreco.2022.120026

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Impacts of stocking densities on soil biochemical and microbial properties in a mixed-grass prairie ecosystem at two landscape positions

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Grazing management is a critical land-use requirement that facilitates the preservation of plant community composition, soil properties and environmental quality. Grazing density of livestock has a significant impact on soil health, and there is a need to study the interactions of grazing densities and topographical positions influencing soil biochemical and microbial properties. This study was conducted at Cottonwood Field Station in Philip, South Dakota to assess the influence of more than 7 years of low, medium, and high grazing stocking densities (0.33, 0.41, 0.72 animal units/ac, respectively) at summit and footslope landscape positions on soil carbon (C) and nitrogen (N) fractions, microbial community composition, and enzymatic activities in a mixed-grass prairie ecosystem. Medium grazing density showed a 16% increase in soil N at the footslope compared with summit. Low grazing density significantly reduced microbial biomass C (~269 $\mu g\ g^{-1}$ soil) and N (~26 $\mu g\ g^{-1}$ soil) at summit compared with other grazing densities and landscape positions, except, the summit at high grazing density. Medium grazing density significantly enhanced hot-water extractable N by 21–23% at footslope compared with low grazing density at the footslope and high grazing density at the summit. Low grazing density increased urease (3.64 $\mu g\ NH_4^+\ g^{-1}$ soil h^{-1}) at footslope than all other grazing densities and landscape positions. Low grazing density enhanced β-glucosidase by 75% than high grazing density; alkaline phosphatase was significantly greater by 60% at footslope than summit. High grazing density at the summit decreased total PLFA (mean 56.53 nmol g^{-1} soil) due to lower AM fungi, G (+), G (−) and actinomycetes biomass. Microbial stress indicators such as G (+)/G (−), saturated/unsaturated, monosaturated/polysaturated, GNeg stress revealed that high grazing density especially at summit position posed elevated physiological stressed conditions to the microbial community. Overall, long-term medium grazing density of 0.41 animal units/ac may enhance soil N, microbial composition, microbial biomass C and N, hot-water extractable C and N fractions, and reduce stress conditions for microbial community at both footslope as well as summit landscape positions. Moreover, long-term overgrazing of pastures, particularly at summit slopes, appears to inhibit microbial populations and degrade overall soil health.

KEYWORDS

stocking density, mixed-grass prairie, soil health, carbon, nitrogen, phospholipid fatty acid, soil enzymes

1 Introduction

Grazing management is critical to maintain ecological sustainability and environmental quality, along with providing ecosystem services, especially after long-term grazing. Grazing can impact overall soil properties such as pH, soil organic matter, and soil nutrient concentrations (Stumpp et al., 2005; Paz-Kagan et al., 2016). The impact of grazing on soil quality is dependent on the selection of appropriate stocking density, which is the most important decision a producer can make (Gillen and Sims, 2002; Smart et al., 2010). Stocking density exceeding the "carrying capacity" can adversely impact the long-term sustainability of a grassland system (Dunn et al., 2010). Additionally, different stocking rates can enhance species diversity in grasslands with short- and long-term biological implications (Dunn et al., 2010). The purpose of using variable stocking densities in this experiment was to determine the stocking density essential to enhance the soil biochemical properties and microbial activity while maintaining the plant community composition. Apart from stocking density, landscape position can strongly influence soil health by causing changes in microclimate based on runoff, drainage, soil temperature variation, wind and water erosion, nutrient redistribution and deposition processes (Mwanjalolo Jackson-Gilbert et al., 2015). Moreover, landscape position can provide different substrates for microbes, and nutrients that can ultimately influence soil microbial biomass and communities (Sekaran et al., 2019). Hence, topography should be accounted for while assessing the influence of grazing on soil C and N cycling (Frank et al., 2011; Zhang et al., 2018).

Previous studies on lipids demonstrated that nutritional status and environmental conditions can affect phospholipid fatty acid (PLFA) markers (Findlay and White, 1983; Guckert et al., 1986), therefore fatty acid composition can subsequently be used to indicate stress in the soil microbial community (Smith et al., 2000; Willers et al., 2015). For instance, relative dominance of a bacterial group can be assessed through the ratio of gram-positive to gram-negative bacterial lipids in a particular ecosystem and several studies associate an increase in gram-negative PLFAs with stress conditions (Willers et al., 2015). The composition of microbial communities can also determine the resistance and resilience to abiotic and biotic stresses (Griffiths et al., 2000; Patra et al., 2005). Additionally, animals return part of their ingests in the form of dung and urine, thereby increasing N inputs and labile organic matter fraction in soil (Bardgett and Wardle, 2003; Prieto et al., 2011). It is evidenced that grazing can influence aboveground plant community composition over time, not only affecting the size of the organic matter input but also the soil organic matter quality (Bardgett and Wardle, 2003; Prieto et al., 2011). Soil microbial biomass and enzymes are important parts of biochemical functioning and are strongly linked to soil organic matter and nutrient cycling (Saviozzi et al., 1999; Dodor and Ali Tabatabai, 2005; Patra et al., 2005; Makoi and Ndakidemi, 2008; Bottomley et al., 2020).

Soil microbial biomass and enzyme activities have been considered as sensitive indicators of microbial activity due to their rapid response to shifts in environmental conditions and disturbances (Anderson and Domsch, 1989). Soil enzymes are involved in C (e.g., β-glucosidase), N (e.g., urease), phosphorus (P) (e.g., phosphatases), and sulfur (S) (e.g., arylsulfatase) cycling and can be influenced by landscape position (Sekaran et al., 2019). Some studies suggest that grazing may increase or decrease biochemical activity depending on the presence of animal excreta or deteriorated soil structure due to trampling (Manzano and Návar, 2000; Yates et al., 2000; Conant et al., 2001; Steffens et al., 2008). Enzyme activities and microbial composition related to nutrient composition have previously been evaluated in agricultural practices (Bending et al., 2004; Moscatelli et al., 2007), however, knowledge of similar research is scarce in terms of grassland ecosystem with existing work reporting conflicting results on microbial biomass C (Banerjee et al., 2000; Craine et al., 2009; Katsalirou et al., 2010).

Great Plains rangelands serve as vital ecological components, offering significant contributions to wildlife habitat preservation, watershed protection, recreation, and conservation of genetic diversity. During the early twentieth century, the overall condition of these rangelands declined, attributable to livestock mismanagement, extensive cultivation and abandonment and persistent drought (Schacht et al., 2011). These issues resulted in reduced vegetation cover, increased wind and water erosion, and the widespread growth of invasive plant species. Efficacious management of grazed rangelands required the strategic deployment of tools, with particular emphasis on optimal stocking rates, which play a pivotal role in maintaining or enhancing range conditions (Holechek et al., 1989). The research gap has been identified due to a complex interaction of grazing density affecting soil properties, and microbial activities. There is need to assess the impact of different grazing densities on belowground components such as soil nutrient cycling and microbial activities which are still not fully understood (Bardgett and Wardle, 2003). Additionally, there is a lack of long-term rangeland studies focusing on topographical variability and patterns of grazing stocking density on soil health. Addressing these knowledge gaps may improve grazing management and allow the validity of stocking density recommendations based on assessed biochemical and microbial parameters. These knowledge gaps restrict the potential to theorize the effect of grazing animals, grazing intensity or management practices on grassland functioning. The objectives of this study were

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Abbreviations: C, carbon; N, nitrogen; HWC, hot-water extractable carbon; HWN, hot-water extractable nitrogen; CWC, cold-water extractable carbon; CWN, cold-water extractable nitrogen; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen; AM fungi, arbuscular mycorrhizal fungi; G (–), gram-negative bacteria; G (+), gram-positive bacteria; TBB, total bacterial biomass; TF, total fungi; F/B, fungi/bacteria; TPLFA, total phospholipid fatty acid; AP, alkaline phosphatase; AS, arylsulfatase.

to understand the soil C and N fractions, enzymatic activities, soil microbial community composition and microbial stress indicators as influenced by three different grazing stocking densities at two landscape positions in a long-term mixed-grass prairie system.

2 Materials and methods

The experiment was established at the Cottonwood Field Station in Philip, South Dakota (43°57′41″N, 101°51′36″W) and this site has primarily been used for livestock grazing research for 70 years. It is one of five long-term rangeland study sites in the US. The Cottonwood Field Station is situated in the Northern Great Plains mixed grass prairie ecosystem in west-central South Dakota. The soil type at the study site is Kyle clay (very fine, montmorillonitic, mesic Aridic Haplusterts) and Pierre clay (fine, montmorillonitic, mesic Aridic Haplusterts) developed over a Pierre shale formation (US Department of Agriculture, Soil Conservation Service, 1987; Dunn et al., 2010). The pH of the study site ranged between 6.9 and 7.4. The mixed grass prairie ecosystem at the study site is composed primarily of C3 green needlegrass (*Nassella viridula*) and western wheatgrass (*Pascopyrum smithii*) and C4 blue grama (*Bouteloua gracilis*) and buffalograss (*Buchloe dactyloides*). Topography is slightly sloping with hills and relatively flat-topped ridges. The study site has a Köppen climate classification of continental and semiarid with hot summers and cold winters. Mean annual precipitation is approximately 440 mm. In the 1940s, six pastures at the station were assigned to 3 levels of grazing intensity (low, medium, and high), with yearling steers (*Bos taurus* L.) on 100-acre fields to create pastures in low, good, and excellent range condition. The study consists of three grazing stocking densities of low, 0.33; medium, 0.41; and high, 0.72 animal units/acre and two landscape positions, i.e., summit and footslope. The study design is split-plot with six replications.

2.1 Soil sampling

Following cool-season plant dormancy, soil samples were collected randomly from 0 to 6 cm depth in Fall, 2020 using a push probe. Within each replication, 10 cores were composited from either summit or footslope positions for further analyses and immediately stored in a refrigerator at either 4°C or −20°C for microbial analysis. The moisture content of soil was determined gravimetrically after drying for 48 h at 60°C. Air-dried soil samples were passed through 2-mm sieve for C and N fractions analyses and soil pH was determined using 1:1 soil/water suspension with a pH meter (Thermo Fisher Scientific, United States).

2.2 Soil organic carbon and nitrogen

Air-dried soil samples (<2 mm) were ground and weighed ~0.250-g in a tin foil cup for analyzing SOC and N with a LECO TruSpec Analyzer (LECO Corporation, St. Joseph, MI) using dry combustion method.

2.3 Hot- and cold-water extractable carbon and nitrogen

Hot and cold water extractable C and N were analyzed using the methodology of Ghani et al. (2003) and Sekaran et al. (2019). Soil samples were weighed (3-g oven dry equivalent) into 50 mL centrifuge tube and 30 mL distilled water was added for extraction of easily soluble C and N. Samples were placed on a shaker for 30 min at 30 rpm and centrifuged at 3,000 rpm for 25 min. Supernatant was filtered using 0.45 μm filter into glass scintillation vials and analyzed for cold-water extractable C and N. For hot-water extractable C and N, 30 mL of distilled water was added to the same centrifuge tubes containing 3-g soil samples. These tubes were kept in hot water bath at 80°C for ~15 h. Each tube was shaken on a vortex for about 10 s to ensure that hot-water C and N released from the soil organic matter was fully suspended in the extraction medium. The tubes were further centrifuged at 3,000 rpm for 25 min. Supernatant was filtered through 0.45 μm filter into glass scintillation vials. The cold water and hot water extractable C and N fractions were determined using the TOC-L analyzer (Shimadzu Corporation, Kyoto, Japan).

2.4 Microbial biomass carbon and nitrogen

Microbial biomass C and N were assessed using the chloroform-fumigation and extraction method (Vance et al., 1987; Singh et al., 2021). Ten grams of fresh soil samples were weighed for each fumigated and non-fumigated analysis. The non-fumigated subsample was extracted immediately using 0.5 M K_2SO_4. However, the samples subjected to fumigation were placed in a desiccator with suspended alcohol-free chloroform and extracted with 0.5 M K_2SO_4 after 24 h period in the dark. These samples were shaken for 1 h on an oscillating shaker at 30 rpm and centrifuged for 3 min. Supernatant was filtered through 0.45 μm filter paper and the extractant was analyzed for C and N using TOC analyzer (Shimadzu Corporation, Kyoto, Japan). Microbial biomass C and N were calculated as the difference between non-fumigated and fumigated sample C and N concentrations and divided by a factor of 0.45 as extraction efficiency (Beck et al., 1997).

2.5 Enzymatic analysis

Enzymatic analysis was determined to gain insights into soil enzyme dynamics as reflected by microbial activity and nutrient cycling in the studied environment. Urease (EC 3.5.1.5) was analyzed using colorimetric determination of ammonium method by Kandeler and Gerber (1988), with results expressed as μg NH_4-N g^{-1} soil h^{-1}. *β*-glucosidase (EC 3.2.1.21) activity was assessed following the approach of Eivazi and Tabatabai (1988), and results were expressed as μmol *p*NP g^{-1} soil h^{-1}. Alkaline Phosphatase activity (EC 3.1.3.1) was determined using the method of Tabatabai and Bremner (1969) and Eivazi and Tabatabai (1977), with results expressed as μg *p*NP g^{-1} soil h^{-1}. Arylsulfatase activity assessment followed the procedure of Tabatabai and Bremner (1970), utilized *p*-nitrophenol solution as the substrate, and results were expressed as μg *p*NP g^{-1} soil h^{-1}.

2.6 Phospholipid fatty acid analysis

2.6.1 Extraction

Microbial community composition was assessed by extracting total soil lipids by shaking ~1–2 g of soil in 4 mL of Blight & Dyer reagent (200 mL 50 mM K_2HPO_4 buffer in deionized H_2O, 500 mL methanol, 250 mL chloroform) and 19:1 phosphatidylcholine (Avanti Polar Lipids, United States) internal PLFA standard followed by sonication at room temperature. Solid and liquid phases were separated by centrifuging the samples in a 5,804 R centrifuge, (Eppendorf, United States) at 4,000 rpm for 15 min. Supernatant was added with of 1 mL of each deionized water and chloroform and centrifuged again at 4,000 rpm for 15 min. Separated liquid phase was placed in a SpeedVac™ vaccum concentrator (Thermo Scientific, United States) for drying at low/ambient temperature for 1 h.

2.6.2 Lipid separation

The samples were dissolved with 1 mL chloroform and transferred to conditioned HyperSep™ solid-phase extraction (SPE) columns (Thermo Scientific, United States), containing 50 mg silica per 1 mL column, and allowed to gravity drain. A 1.5 mL clean glass catch vial was placed below each column and phospholipids were eluted using 0.5 mL of the 5:5:1 chromatography eluent solution (methanol: chloroform: deionized water) to the SPE columns. The collected solution was dried in a SpeedVac™ vaccum concentrator for ~1 h at ambient temperature.

2.6.3 Trans-esterification

A 0.2 mL of trans-esterification reagent was added to the dried samples followed by incubation at 37°C for 15 min. A 0.4 mL of 0.075 M acetic acid and 0.5 mL of chloroform was added to each tube and bottom phase after vortex was transferred to a GC vial followed by drying in SpeedVac™ vaccum concentrator for 20–30 min at ambient temperature. The samples were further resuspended using 100 μL of hexane and analyzed using an Agilent 2030-GC equipped with a CP-7693 auto-sampler and a flame ionization detector (FID). Fatty acid peaks were identified by comparing the retention times to MIDI PLFAD2 calibration mix using SHERLOCK software v.6.2 (MIDI Inc., United States). Fatty acids were used as functional group signatures for various microorganisms and each PLFA was expressed as nmol g^{-1} soil.

2.7 Microbial stress factors

PLFA analysis can be used to indicate the microbial community stress where the obtained signature fatty acid biomarkers allow assessment and differentiation of various microbial groups including fungi and bacteria (Frostegård and Bååth, 1996; Bertram et al., 2012). For instance, since the cell structure of gram (−) bacteria benefits in stress resistance than gram (+), an increase in gram (+)/gram (−) ratio has been used as an indicator to recognize whether a given microbial community is stressed (Kaur et al., 2005; Bertram et al., 2012). The specific microbial stress indicators included in this study were gram (+)/gram (−), monounsaturated/polysaturated, saturated/unsaturated fatty acids, and GNeg stress. The GNeg stress indicator is based on the observations of increased gram (−) stress PLFAs with stress conditions. Increased ratios of saturated to monounsaturated fatty acids, trans- to cis monoenoic fatty acids and cyclopropyl fatty acids to their monoenoic precursors are used as stress signatures (Willers et al., 2015).

3 Statistical analyses

The analysis was conducted using SAS 9.4 (SAS Institute Inc., Cary, United States) with mixed model ANOVA procedure. The dependent variables included soil organic C and N, hot and cold-water extractable C and N, microbial biomass C and N, PLFA, enzymatic activities and microbial stress factors. Grazing densities and landscape positions were treated as fixed effects and replicates as random effects. Data were analyzed for normality using Shapiro–Wilk's test. Mean separation for treatments was performed using Fisher's protected least significant difference (LSD) at a significance level of 0.05. Principal component analysis (PCA) and correlation were performed using multivariate methods in JMP® Pro 16 (SAS Institute Inc., Cary, United States) for the measured parameters. Principal component analysis was conducted to simplify the data and observe the response variables which followed a similar trend and explained greatest variation in the data. Correlation was performed to see the relationship between different C and N fractions, and microbial communities.

4 Results

4.1 Soil organic C and N, MBC, and MBN

The summary of statistical significance (*p*-values) of measured soil-related parameters as affected by grazing density and landscape position is presented in Table 1. The interaction between grazing densities and landscape positions was significant ($p \leq 0.05$) for most of the parameters. Although SOC did not vary under different grazing densities, it was enhanced significantly (+12%) at the footslope compared with summit position. Within medium grazing density, soil N was reduced by 25% at summit when compared to the footslope position.; however, it did not vary significantly from the low and high grazing densities at either landscape position (Figure 1).

In terms of C and N related to microbial biomass, the majority of the variation was observed under low and high grazing densities at both landscape positions (Figure 1). Microbial biomass C was significantly greater for medium and high grazing densities at footslope as well as summit, with a mean value between 403 and 510 μg g^{-1} soil. These did not vary significantly from the footslope position (445 μg g^{-1} soil) at low grazing density. Microbial biomass C was significantly lowered by 39–47% under low grazing density at summit position, compared to other grazing densities/landscape positions, except it did not differ from the summit position at high grazing density. Under medium grazing density, MBN was enhanced under footslope as well as summit position, when compared to low and high grazing densities (Figure 1). Microbial biomass N was reduced at summit positions under low and high grazing density by 25 and 22 μg g^{-1} soil, respectively, compared to the footslope. The ratio of MBC:MBN was comparatively ($p < 0.05$) greater at summit position (mean = 11.8) than the footslope position (mean = 9.11). At summit position, low grazing density showed significantly greater HWC:HWN ratio than the medium grazing density (Data not presented).

TABLE 1 Statistical significance (*p*-values) of the influence of grazing density and landscape position on soil C and N fractions and microbial stress indicators.

Effect	SOC	Soil N	MBC	MBN	HWC	HWN	CWC	CWN	G(+)/G(−)	Sat/unsat	Mono/poly	GNeg stress
Grazing density	NS	NS	NS	NS	NS	NS	NS	NS	**	**	NS	**
Landscape position	***	**	***	***	**	**	NS	***	NS	*	***	NS
Grazing*Landscape	NS	**	**	**	**	*	NS	**	**	**	**	NS

SOC, soil organic carbon; soil N, soil nitrogen; HWC, hot water extractable carbon; HWN, hot water extractable nitrogen; CWC, cold water extractable carbon; CWN, cool water extractable nitrogen; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen; G(+)/G(−), gram positive/gram negative; sat/unsat, saturated/unsaturated; mono/poly, monosaturated/polysaturated; GNeg stress, gram-negative stress. ***Significantly different at $p \leq 0.0001$. **Significantly different at $p \leq 0.05$. *Significantly different at $p \leq 0.1$. NS, not significant at $p \leq 0.05$.

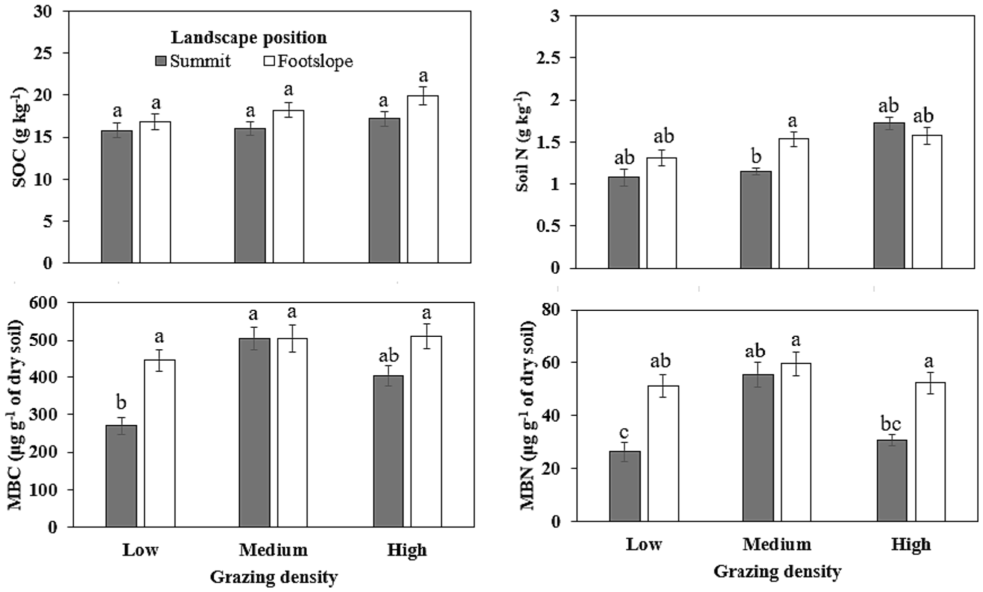

FIGURE 1
Soil organic carbon and nitrogen fractions as influenced under different grazing densities at two landscape positions. Bars with different lowercase letters represent significant differences between grazing densities and landscape positions at $p \leq 0.05$. SOC, soil organic carbon; soil N, soil nitrogen; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen.

4.2 Hot-water and cold-water extractable C and N

Medium grazing density increased HWC by ~22% at the footslope position compared with summit position (Figure 2). In terms of HWN, 21–23% increase was noted for medium grazing density at footslope position when compared with the low grazing density at footslope and high grazing density at summit position. Cold-water extractable C was not influenced by either grazing density or landscape position. Cold-water extractable N was enhanced under high grazing density at footslope (mean 11.2 mg kg^{-1}), and it reduced significantly to 7.3, 4.9, and 4.0 mg kg^{-1} under low, medium, and high grazing density, respectively, at summit position.

4.3 Enzymatic activities

Urease and arylsulfatase activities were significantly greater under low grazing density at footslope position and reduced by 25–79% under other grazing density/landscape position interactions (Table 2). However, arylsulfatase activity did not vary between medium and low grazing densities and footslope position. Averaging over landscape positions, β-glucosidase activity was enhanced significantly by 75% under low grazing density compared with the high grazing density. Moreover, alkaline phosphatase activity was higher under low grazing density by 62% than medium grazing density. Compared to footslope position, alkaline phosphatase activity decreased under summit by ~8 µg *p*-NP g^{-1} soil h^{-1}.

4.4 Soil microbial community composition

Total PLFA was reduced significantly by 38–48% under high grazing density at summit position compared with the other grazing densities and landscape positions. In particular, high grazing density reduced the total PLFA by 43% at summit when compared with the footslope position (Table 3).

Soil microbial lipid composition was significantly influenced by the interaction of grazing densities and landscape positions, except fungal population. High grazing density reduced the AM fungi population by 1.59–2.87 nmol g^{-1} soil at summit over the other grazing densities and landscape positions (Table 3). Medium and high grazing

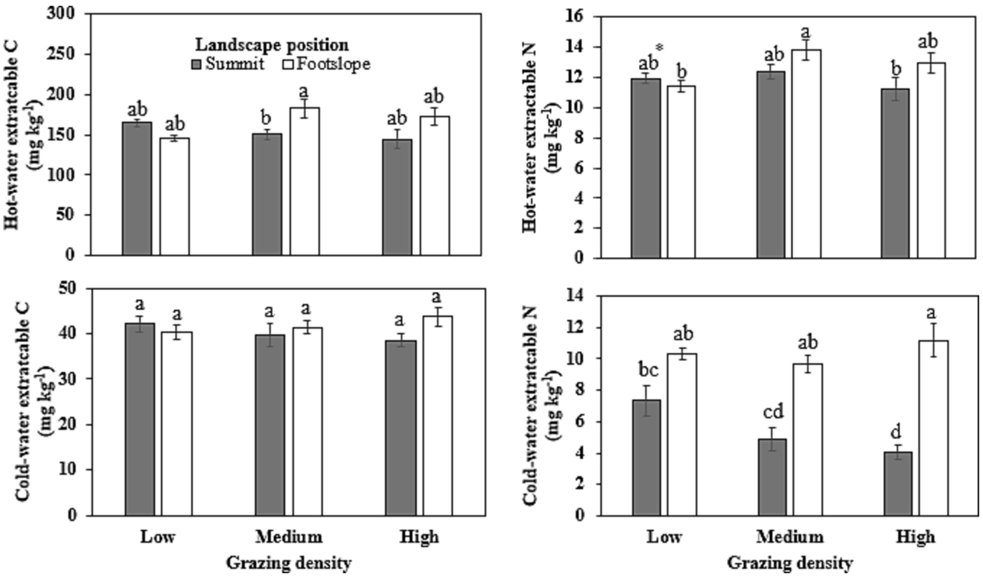

FIGURE 2
Hot-water and cold-water extractable carbon and nitrogen fractions as influenced under different grazing densities at two landscape positions. Bars with different lowercase letters represent significant differences between grazing densities and landscape positions at $p \leq 0.05$. *Represents significant interaction at $p \leq 0.10$.

TABLE 2 Influence of different grazing densities at two landscape positions on soil enzyme activity.

Treatments	Urease (μg NH_4^+ g^{-1} soil h^{-1})	*β*-glucosidase (μmol *p*-NP g^{-1} soil h^{-1})	Alkaline phosphatase (μg *p*-NP g^{-1} soil h^{-1})	Arylsulfatase (μg *p*-NP g^{-1} soil h^{-1})
Grazing density (GD)				
Low	2.59^{a†}	101.92^{a}	20.87^{a}	27.19
Medium	1.28^{b}	98.43^{ab}	12.92^{b}	24.53
High	1.29^{b}	58.11^{b}	19.20^{ab}	23.15
Landscape position (LP)				
Summit	1.05^{b}	75.57	13.57^{b}	20.76^{b}
Footslope	2.39^{a}	96.73	21.76^{a}	29.15^{a}
GD—LP				
Low—summit	1.54^{b}	97.83	16.71	19.97^{b}
Low—footslope	3.64^{a}	106.01	25.03	34.40^{a}
Medium—summit	0.76^{b}	75.42	8.49	21.75^{b}
Medium—footslope	1.81^{b}	121.44	17.34	27.30^{ab}
High—summit	0.85^{b}	53.48	15.50	20.56^{b}
High—footslope	1.73^{b}	62.74	22.90	25.74^{b}
ANOVA ($P>F$)				
GD	<0.0001	0.0536*	0.0406	0.1999
LP	<0.0001	0.1493	0.0028	<0.0001
GD—LP	0.0757*	0.4818	0.9744	0.0141

†Means followed by similar lowercase letters are not significantly different at $P \leq 0.05$. *Represents significance at $P \leq 0.10$.

densities at summit position reduced the G (−) bacteria by 26 and 44%, respectively, compared with the medium grazing at footslope position. High grazing density also lowered the G (+) bacteria (20.87 nmol g^{-1} soil) at summit position compared with the medium grazing density at summit and footslope and high grazing density at footslope. Furthermore, the actinomycetes population was reduced under high grazing density at the summit position (8.71 nmol g^{-1} soil) than the other grazing densities and landscape positions. Overall, the

TABLE 3 Influence of different grazing densities at two landscape positions on soil microbial structure as identified by phospholipid fatty acid biomarkers.

Treatments	AM fungi	G (−)	G (+)	Fungi	Actinomycetes	Total bacterial biomass	Fungi/bacteria	Total fungi	Total PLFA
nmol g^{-1} soil									
Grazing density (GD)									
Low	4.04^{ab†}	30.95^{ab}	33.83^{ab}	4.10^{ab}	15.79^{ab}	64.78^{ab}	0.13	8.14^{a}	88.71^{ab}
Medium	4.71^{a}	33.16^{a}	41.26^{a}	4.56^{a}	17.57^{a}	74.42^{a}	0.13	9.28^{a}	101.27^{a}
High	3.24^{b}	27.63^{b}	30.66^{b}	2.80^{b}	13.33^{b}	58.29^{b}	0.11	6.04^{b}	77.66^{b}
Landscape position (LP)									
Summit	3.53^{b}	26.91^{b}	31.72^{b}	4.29^{a}	13.62^{b}	58.63^{b}	0.14^{a}	7.82	80.07^{b}
Footslope	4.47^{a}	34.25^{a}	38.78^{a}	3.35^{b}	17.51^{a}	73.03^{a}	0.11^{b}	7.82	98.36^{a}
GD—LP									
Low—summit	4.01^{a}	31.22^{ab}	35.19^{ab}	4.34	16.15^{a}	66.42^{a}	0.13^{a}	8.35	90.92^{a}
Low—footslope	4.07^{a}	30.68^{ab}	32.47^{ab}	3.87	15.42^{a}	63.14^{ab}	0.13^{a}	7.94	86.50^{ab}
Medium—summit	4.13^{a}	28.12^{bc}	39.10^{a}	5.30	15.99^{a}	67.23^{a}	0.14^{a}	9.43	92.65^{a}
Medium—footslope	5.29^{a}	38.20^{a}	43.41^{a}	3.82	19.16^{a}	81.61^{a}	0.12^{a}	9.12	109.89^{a}
High—summit	2.43^{b}	21.37^{c}	20.87^{b}	3.25	8.71^{b}	42.24^{b}	0.14^{a}	5.68	56.63^{b}
High—footslope	4.05^{a}	33.88^{ab}	40.45^{a}	2.35	17.96^{a}	74.33^{a}	0.09^{b}	6.40	98.69^{a}
ANOVA ($P > F$)									
GD	0.0005	0.0360	0.0155	0.0060	0.0297	0.0143	0.1002	0.0008	0.0101
LP	0.0018	<0.0001	0.0208	0.0384	0.0033	0.0017	<0.0001	0.9966	0.0041
GD—LP	0.0841*	0.0066	0.0105	0.6569	0.0082	0.0068	0.0015	0.7498	0.0115

†Means followed by similar lowercase letters are not significantly different at $p \leq 0.05$. *Represents significance at $p \leq 0.10$. AM fungi, arbuscular mycorrhizal fungi; G (−), gram-negative bacteria; G (+), gram-positive; PLFA phospholipid fatty acid.

high grazing density reduced total bacterial biomass 37% at the summit position compared to the other grazing densities, which corresponds with the observed decreases in gram (+), gram (−) and actinomycetes population. At the footslope, the fungal/bacterial ratio was significantly lower with a mean value 0.09 than the other grazing densities.

4.5 Microbial stress indicators

Gram (+)/gram (−) ratio did not vary between the two landscape positions under low and medium grazing densities, except at high grazing density where the summit position showed a lower ratio (0.10) than the footslope position (0.13) (Figure 3). Similar results were observed for saturated/unsaturated fatty acids, where reduced ratio was observed under high grazing density at the summit position (0.09) when compared with the other grazing densities and landscape positions, except it did not vary significantly with low grazing density at summit position (0.12). Monosaturated/ polysaturated fatty acids were significantly higher under high grazing density at the footslope position (1.76) than the other grazing densities and landscape positions, however, it did not differ significantly than the medium grazing density at footslope position (1.11). GNeg stress or gram (−) stress varied significantly only among different grazing densities; and medium grazing density showed 13 and 20% higher GNeg stress, respectively, than the low and high grazing densities.

4.6 Principal component analysis

The PCA results demonstrated that principal component 1 (PC1) and PC2 explained 36 and 15% of variation, respectively. It suggests that medium and high grazing densities influenced C and N fractions (e.g., SOC, N, HWC, HWN, CWN) and enzymes (urease, arylsulfatase, alkaline phosphatase) at the footslope position (Figure 4). However, the microbial community composition was mainly influenced by medium grazing density at both footslope and summit positions.

4.7 Pearson's correlation coefficient

Pearson's correlation revealed that total PLFA or microbial community composition was positively correlated with most of the C and N fractions mainly SOC, HWC, HWN, CWN, MBC, and MBN (Figure 5). The microbial community had strong positive correlation with each other at $p \leq 0.05$, e.g., AMF, G (−), G (+), fungi, actinomycetes.

5 Discussion

Long-term grazing can directly or indirectly influence the soil physicochemical and biological properties (Yates et al., 2000; Prieto

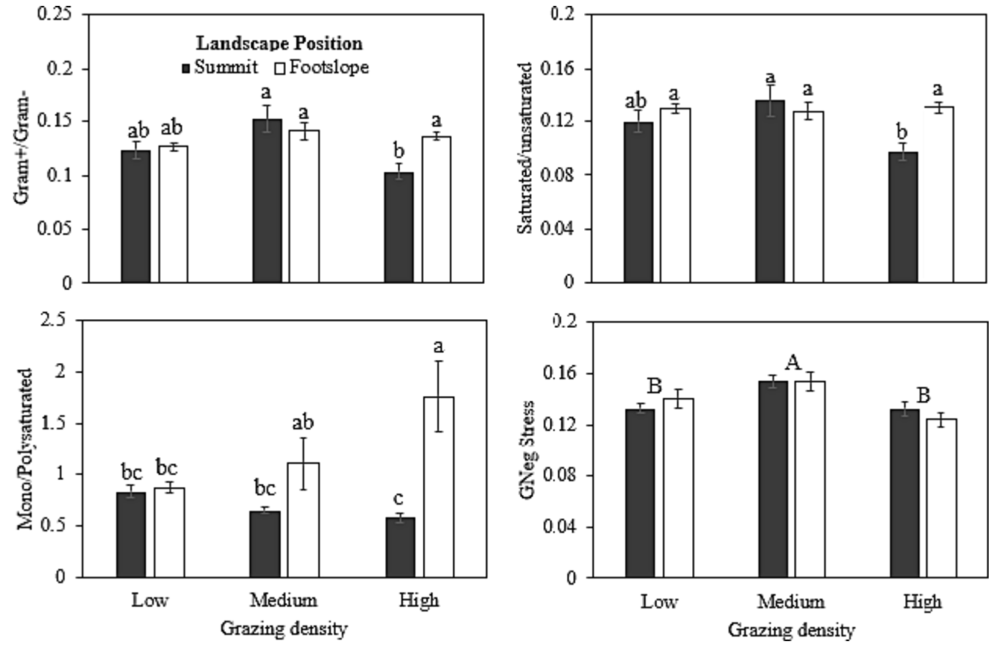

FIGURE 3
Stress indicators ratio calculated from PLFA analyses as influenced under different grazing densities at two landscape positions. Bars with different lowercase letters represent significant differences between grazing densities and landscape positions are significantly different at $p \leq 0.05$. Capital letters in GNeg stress represent significant differences among three grazing densities.

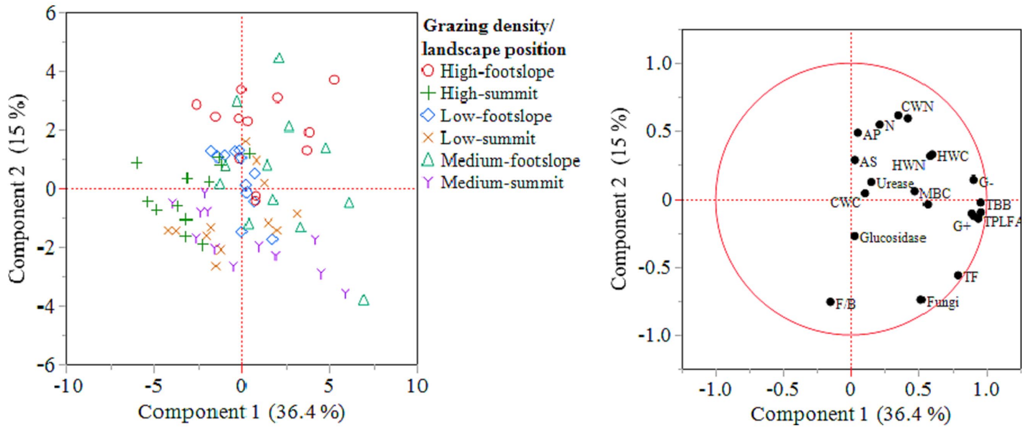

FIGURE 4
Score plot (left) and eigenvectors (right) of the soil physicochemical and biological parameters with principal component analysis (PCA) showing PC1 and PC2 (left) and eigenvectors (right). C, carbon; N, nitrogen; HWC, hot-water extractable carbon; HWN, hot-water extractable nitrogen; CWC, cold-water extractable carbon; CWN, cold-water extractable nitrogen; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen; AM fungi, arbuscular mycorrhizal fungi; G (–), gram-negative bacteria; G (+), gram-positive bacteria; TBB, total bacterial biomass; TF, total fungi; F/B, fungi/bacteria; TPLFA, total phospholipid fatty acid; AP, alkaline phosphatase; AS, arylsulfatase.

et al., 2011). Our results suggest that labile fractions of C (e.g., HWC, MBC) can be considered as sensitive indicators while determining the subtle variations within a mixed-grass system. However, there were no discernable trends across all treatments and indicators, aligning with previous findings by Ghani et al. (2003). The effects of grazing densities were more pronounced on HWC compared to CWC at different landscape positions. The higher HWC under medium grazing density at the footslope position suggests the presence of carbohydrates, phenols, and lignin monomers compared to the summit position (Landgraf et al., 2006). Hot-water extractable C usually represents 3–6% of total organic C in soil and can serve as an early indicator of organic matter loss (Ghani et al., 2003). Our data indicate that the rate of decomposition was favored over accumulation of carbon under medium grazing density at summit position. While the lowest MBC and N were observed under low grazing density at the summit, they did not significantly differ from high grazing density at the same position. Contrary to soil C, this study showed changes in the total soil N as well as labile fraction of soil N (HWN, CWN, and

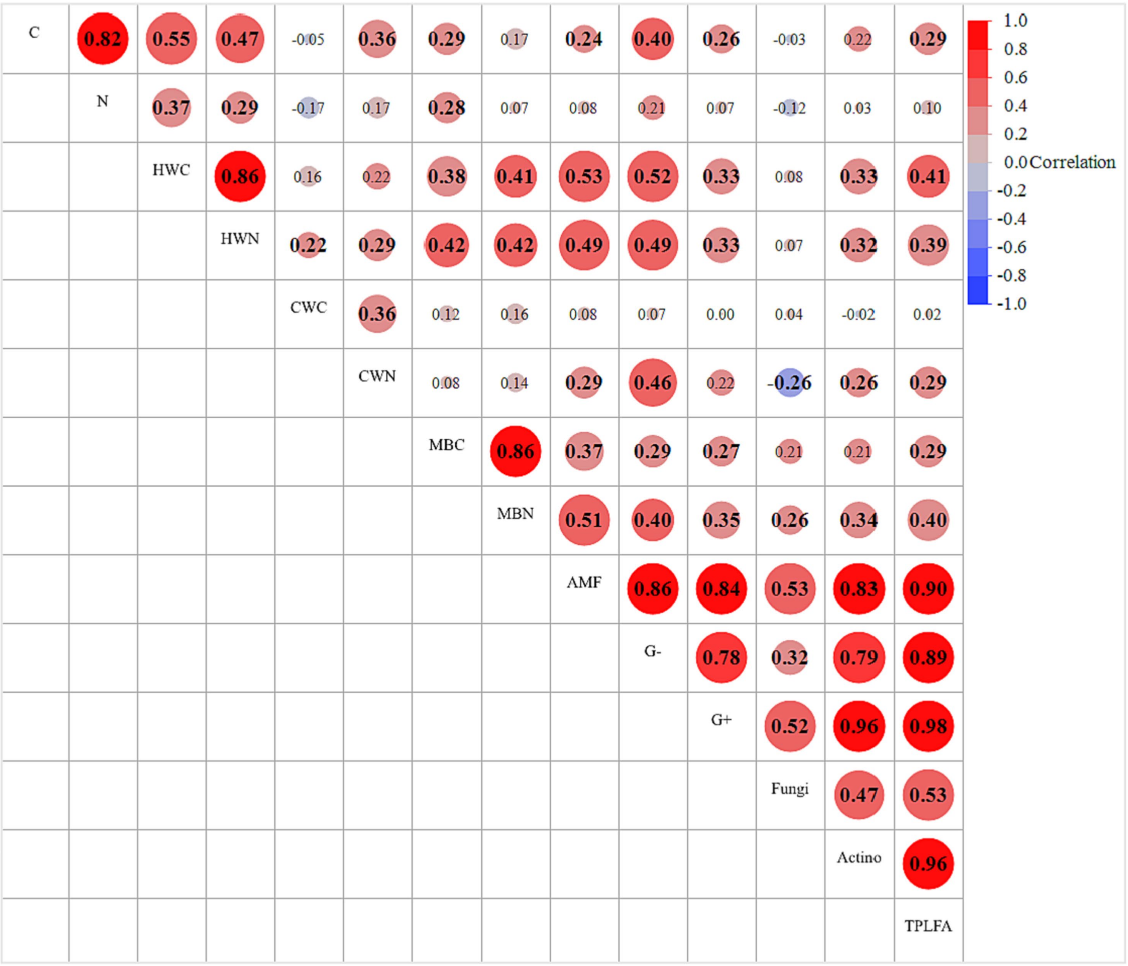

FIGURE 5
Pearson's correlation coefficient (r) between soil physicochemical and biological properties observed under different grazing densities and landscape positions. C, carbon; N, nitrogen; HWC, hot-water extractable carbon; HWN, hot-water extractable nitrogen; CWC, cold-water extractable carbon; CWN, cold-water extractable nitrogen; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen; AMF, arbuscular mycorrhizal fungi; G (–), gram-negative bacteria; G (+), gram-positive; TPLFA, total phospholipid fatty acid. Bold numeric values represent significance at $p \leq 0.05$.

MBN) as affected under different grazing densities at two landscape positions. Moreover, rhizodeposition and root exudates can greatly influence C and N turnover in soils, affecting their accrual or decomposition in rhizosphere (Kuzyakov et al., 2001).

The observed lack of variation in the SOC based on grazing density can also lead to insignificant changes in the β-glucosidase and phosphatase activities, consistent with prior studies associating reduced SOC with decreased β-glucosidase and phosphatase activities under intensive grazing (Prieto et al., 2011). Averaging over landscape positions, β-glucosidase activity was 1.8 times higher under low grazing density than the high grazing density, which suggested that the primary source of energy for the living soil microbial communities of low grazing system is attained from disaccharides such as cellobiose, a derivative of cellulose (Burns and Dick, 2002; Hewins et al., 2015). This study found no interaction effect between grazing densities and landscape positions on C cycling enzyme β-glucosidase, indicating that much of the existing soil organic matter may already be relatively stable following microbial decomposition (Fierer et al., 2009; Moore et al., 2011; Hewins et al., 2015). Urease and arylsulfatase enzymes were usually higher under low grazing density and at footslope positions, indicating a shared pattern of nitrogen and sulfur decomposition within SOM, potentially fulfilling the growth and cellular functions of microbial communities (Hewins et al., 2015). Independent of the landscape position, greater urease and alkaline phosphatase under low grazing density suggested greater mineralization with greater microbial community supported by organic nitrogen and phosphorus sources in ecosystems rich in grass production and associated litter (Mitchell et al., 2015). Lower enzymatic activities under high grazing density can also be associated with the reduced microbial abundance, especially AM fungi, G (–), G (+), fungi, actinomycetes. It is postulated that under high grazing density, microbial communities may not be supported by enzymes involved in nitrogen, carbon, and phosphorus cycling, experiencing adverse impacts compared to low or medium grazing densities.

The fungal to bacterial ratio was reduced by 26–40% under high grazing density at footslope position compared to other grazing densities and landscape positions. The non-AM fungal population was reduced significantly by 39% at the high grazing density compared to medium grazing density. Previous studies linked increased intensity of physical disturbance and decrease in the fungal biomarker (by 29.5–43.3%) and fungal:bacterial ratio in response to grazing (Kaur et al., 2005). Moreover, the AM fungi population was significantly declined under high grazing density at summit position compared to other grazing densities and landscape positions, likely due to the sensitivity of filamentous fungi to physical disturbances under high grazing pressure over low and medium grazing (Bardgett et al., 2001). In addition to fungal biomarkers, single-celled organisms showed lower abundance, particularly under high grazing density at the summit landscape position.

A physiological stress in certain bacterial species can be derived from the PLFA analysis, serving as an assessment tool for evaluating microbial community function under various treatments (Kaur et al., 2005). Microbial stress indicators revealed that gram (+)/gram (−) and saturated/unsaturated fatty acids were significantly lower under high grazing density at the footslope position. This reduction in the gram (+)/gram (−) bacterial ratio represented an increase in abundance of gram (−) bacterial PLFA, with the concomitant decrease in gram (+) bacterial PLFA, suggesting stressful conditions under high grazing density at summit. The higher saturated fatty acids reflected a well-adapted microbial community to environmental conditions in an ecosystem. However, communities experiencing stressed conditions tend to increase the proportion of unsaturated fatty acids, as noted under high grazing density at summit. The decreased ratio of sat/unsaturated fatty acids under high grazing density at summit position may indicate slightly weakened and unstable microbial community. Similarly, the observed lower mono/polysaturated ratio, especially under high grazing density at summit position, depicted prolonged stress likely due to environmental conditions such as animal trampling, intense grazing, pH, nutrient starvation, low moisture conditions. GNeg stress was calculated as the ratio of the sum of two monosaturated fatty acids (16:1ω7c and 18:1ω7c) and two cyclopropanoic fatty acids, i.e., 17:0 cyclo ω7c and 19:0 cyclo ω7c. Gram (−) bacteria generate cyclopropanoic fatty acids under stressed conditions, therefore, the higher the GNeg ratio, the less stressed the soil is. Medium grazing density represented the least stress for microbial communities among different grazing densities. Overall, these microbial stress ratios suggested that low and medium grazing densities at summit and footslope positions can provide sufficient nutrients for balanced bacterial growth through root exudates in rhizosphere. However, high grazing density, especially at summit position, might indicate elevated physiological stress for the soil microbial community, possibly due to higher exposure to radiation and evaporation rates, compared to footslope position (Saul-Tcherkas and Steinberger, 2009; Ben-David et al., 2011; Frostegård et al., 2011). While PLFA primarily focuses on changes in microbial community structure and biomass as indicators of stress, it captures diverse markers present across multiple microbial groups. Based on the PCA results, it was summarized that relatively stable and unstressed conditions for C and N fractions, enzymes related to nitrogen, phosphorus, and sulfur nutrients and microbial community might be present under medium grazing density at both summit as well as footslope positions. Positive correlations among C and N fractions and microbial communities indicate their capacity to influence each other synergistically.

6 Conclusion

High grazing density at summit landscape position mostly affected soil physicochemical and microbial composition, likely attributed to direct factors such as of trampling by hoof action and environmental exposure of the landscape position to radiation, wind erosion and evaporation. Increased grazing density of 0.72 animal units/ac at summit position declined N, C and S cycling by reducing urease, β-glucosidase, and arylsulfatase. Furthermore, microbial biomass nitrogen, hot-water extractable carbon and nitrogen, cold-water extractable nitrogen were also notably affected by high grazing density. High grazing density led to reductions in AM fungi, G (−), G (+), non-AM fungi, actinomycetes, fungi-to-bacteria ratio and total PLFA of the soil, along with an increase in microbial stress indicators. However, high grazing density, if practiced at footslope position, can partially mitigate some negative impacts on soil health parameters such as labile C and N fractions, as well as total PLFA. In contrast, medium grazing density of 0.41 animal units/ac at both summit and footslope position showed relatively stable and less stressed conditions with greater C and N fractions, enzymes associated with N, P and S nutrients, and the microbial community. Therefore, considering these positive outcomes, medium grazing density emerges as a more conducive alternative compared to high grazing density, particularly to ameliorate the potential adverse effects of overgrazing on soil health of mixed-grass prairie ecosystem. These results are important in terms of developing grazing regimes that balance livestock forage needs while maintaining a resilient and healthy ecosystem. Ongoing research on this rangeland study site is targeting to investigate microbiome diversity, abundance, and their relationship with microbial functions across distinct grazing densities. Moreover, further exploration into rhizosphere dynamics, particularly focusing on root interactions and nutrient cycling processes is needed.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

SB: Data curation, Formal analysis, Methodology, Software, Writing – original draft. DS: Methodology, Writing – review & editing. CG: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Writing – review & editing. JG: Methodology, Resources, Supervision, Validation, Writing – review & editing. SK: Investigation, Resources, Supervision, Writing – review & editing. HM: Methodology, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Anderson, T.-H., and Domsch, K. H. (1989). Ratios of microbial biomass carbon to total organic carbon in arable soils. *Soil Biol. Biochem.* 21, 471–479. doi: 10.1016/0038-0717(89)90117-X

Banerjee, M. R., Burton, D. L., McCaughey, W., and Grant, C. (2000). Influence of pasture management on soil biological quality. *J. Range Manag.* 53, 127–133. doi: 10.2307/4003403

Bardgett, R. D., Jones, A. C., Jones, D. L., Kemmitt, S. J., Cook, R., and Hobbs, P. J. (2001). Soil microbial community patterns related to the history and intensity of grazing in sub-montane ecosystems. *Soil Biol. Biochem.* 33, 1653–1664. doi: 10.1016/S0038-0717(01)00086-4

Bardgett, R. D., and Wardle, D. A. (2003). Herbivore-mediated linkages between aboveground and belowground communities. *Ecology* 84, 2258–2268. doi: 10.1890/02-0274

Beck, T., Joergensen, R., Kandeler, E., Makeschin, F., Nuss, E., Oberholzer, H., et al. (1997). An inter-laboratory comparison of ten different ways of measuring soil microbial biomass C. *Soil Biol. Biochem.* 29, 1023–1032. doi: 10.1016/S0038-0717(97)00030-8

Ben-David, E. A., Zaady, E., Sher, Y., and Nejidat, A. (2011). Assessment of the spatial distribution of soil microbial communities in patchy arid and semi-arid landscapes of the Negev Desert using combined PLFA and DGGE analyses. *FEMS Microbiol. Ecol.* 76, 492–503. doi: 10.1111/j.1574-6941.2011.01075.x

Bending, G. D., Turner, M. K., Rayns, F., Marx, M.-C., and Wood, M. (2004). Microbial and biochemical soil quality indicators and their potential for differentiating areas under contrasting agricultural management regimes. *Soil Biol. Biochem.* 36, 1785–1792. doi: 10.1016/j.soilbio.2004.04.035

Bertram, J. E., Orwin, K. H., Clough, T. J., Condron, L. M., Sherlock, R. R., and O'Callaghan, M. (2012). Effect of soil moisture and bovine urine on microbial stress. *Pedobiologia* 55, 211–218. doi: 10.1016/j.pedobi.2012.03.004

Bottomley, P. J., Angle, J. S., and Weaver, R. (2020). *Methods of soil analysis, part 2: microbiological and biochemical properties* John Wiley & Sons, Wisconsin, Madison.

Burns, R. G., and Dick, R. P. (2002). *Enzymes in the environment: activity, ecology, and applications* Marcel Dekker, New York: CRC Press.

Conant, R. T., Paustian, K., and Elliott, E. T. (2001). Grassland management and conversion into grassland: effects on soil carbon. *Ecol. Appl.* 11, 343–355. doi: 10.1890/1051-0761(2001)011[0343:GMACIG]2.0.CO;2

Craine, J. M., Ballantyne, F., Peel, M., Zambatis, N., Morrow, C., and Stock, W. D. (2009). Grazing and landscape controls on nitrogen availability across 330 south African savanna sites. *Austral Ecol.* 34, 731–740. doi: 10.1111/j.1442-9993.2009.01978.x

Dodor, D. E., and Ali Tabatabai, M. (2005). Glycosidases in soils as affected by cropping systems. *J. Plant Nutr. Soil Sci.* 168, 749–758. doi: 10.1002/jpln.200521761

Dunn, B. H., Smart, A. J., Gates, R. N., Johnson, P. S., Beutler, M. K., Diersen, M. A., et al. (2010). Long-term production and profitability from grazing cattle in the northern mixed grass prairie. *Rangel. Ecol. Manag.* 63, 233–242. doi: 10.2111/REM-D-09-00042.1

Eivazi, F., and Tabatabai, M. (1977). Phosphatases in soils. *Soil Biol. Biochem.* 9, 167–172. doi: 10.1016/0038-0717(77)90070-0

Eivazi, F., and Tabatabai, M. (1988). Glucosidases and galactosidases in soils. *Soil Biol. Biochem.* 20, 601–606. doi: 10.1016/0038-0717(88)90141-1

Fierer, N., Grandy, A. S., Six, J., and Paul, E. A. (2009). Searching for unifying principles in soil ecology. *Soil Biol. Biochem.* 41, 2249–2256. doi: 10.1016/j.soilbio.2009.06.009

Findlay, R. H., and White, D. C. (1983). Polymeric beta-hydroxyalkanoates from environmental samples and *Bacillus megaterium*. *Appl. Environ. Microbiol.* 45, 71–78. doi: 10.1128/aem.45.1.71-78.1983

Frank, D. A., Depriest, T., McLauchlan, K., and Risch, A. C. (2011). Topographic and ungulate regulation of soil C turnover in a temperate grassland ecosystem. *Glob. Chang. Biol.* 17, 495–504. doi: 10.1111/j.1365-2486.2010.02219.x

Frostegård, A., and Bååth, E. (1996). The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. *Biol. Fertil. Soils* 22, 59–65. doi: 10.1007/BF00384433

Frostegård, Å., Tunlid, A., and Bååth, E. (2011). Use and misuse of PLFA measurements in soils. *Soil Biol. Biochem.* 43, 1621–1625. doi: 10.1016/j.soilbio.2010.11.021

Ghani, A., Dexter, M., and Perrott, K. (2003). Hot-water extractable carbon in soils: a sensitive measurement for determining impacts of fertilisation, grazing and cultivation. *Soil Biol. Biochem.* 35, 1231–1243. doi: 10.1016/S0038-0717(03)00186-X

Gillen, R. L., and Sims, P. L. (2002). Stocking rate and cow-calf production on sand sagebrush rangeland. *J. Range Manag.* 55, 542–550.

Griffiths, B., Ritz, K., Bardgett, R. D., Cook, R., Christensen, S., Ekelund, F., et al. (2000). Ecosystem response of pasture soil communities to fumigation-induced microbial diversity reductions: an examination of the biodiversity–ecosystem function relationship. *Oikos* 90, 279–294. doi: 10.1034/j.1600-0706.2000.900208.x

Guckert, J., Hood, M., and White, D. (1986). Phospholipid ester-linked fatty acid profile changes during nutrient deprivation of *Vibrio cholerae*: increases in the trans/cis ratio and proportions of cyclopropyl fatty acids. *Appl. Environ. Microbiol.* 52, 794–801. doi: 10.1128/aem.52.4.794-801.1986

Hewins, D. B., Fatemi, F., Adams, B., Carlyle, C. N., Chang, S. X., and Bork, E. W. (2015). Grazing, regional climate and soil biophysical impacts on microbial enzyme activity in grassland soil of western Canada. *Pedobiologia* 58, 201–209. doi: 10.1016/j.pedobi.2015.10.003

Holechek, J. L., Pieper, R. D., and Herbel, C. H. (1989). *Range management*. Principles and practices. Prentice-Hall.

Kandeler, E., and Gerber, H. (1988). Short-term assay of soil urease activity using colorimetric determination of ammonium. *Biol. Fertil. Soils* 6, 68–72. doi: 10.1007/BF00257924

Katsalirou, E., Deng, S., Nofziger, D. L., and Gerakis, A. (2010). Long-term management effects on organic C and N pools and activities of C-transforming enzymes in prairie soils. *Eur. J. Soil Biol.* 46, 335–341. doi: 10.1016/j.ejsobi.2010.06.004

Kaur, A., Chaudhary, A., Kaur, A., Choudhary, R., and Kaushik, R. (2005). Phospholipid fatty acid–a bioindicator of environment monitoring and assessment in soil ecosystem. *Curr. Sci.*:89, 1103–1112.

Kuzyakov, Y., Ehrensberger, H., and Stahr, K. (2001). Carbon partitioning and below-ground translocation by *Lolium perenne*. *Soil Biol. Biochem.* 33, 61–74. doi: 10.1016/S0038-0717(00)00115-2

Landgraf, D., Leinweber, P., and Makeschin, F. (2006). Cold and hot water–extractable organic matter as indicators of litter decomposition in forest soils. *J. Plant Nutr. Soil Sci.* 169, 76–82. doi: 10.1002/jpln.200521711

Makoi, J. H., and Ndakidemi, P. A. (2008). Selected soil enzymes: examples of their potential roles in the ecosystem. *Afr. J. Biotechnol.* 7, 181–191.

Manzano, M. G., and Návar, J. (2000). Processes of desertification by goats overgrazing in the Tamaulipan thornscrub (matorral) in North-Eastern Mexico. *J. Arid Environ.* 44, 1–17. doi: 10.1006/jare.1999.0577

Mitchell, J. P., Shrestha, A., Horwath, W. R., Southard, R. J., Madden, N., Veenstra, J., et al. (2015). Tillage and cover cropping affect crop yields and soil carbon in the San Joaquin Valley, California. *Agron. J.* 107, 588–596. doi: 10.2134/agronj14.0415

Moore, T. R., Trofymow, J., Prescott, C. E., and Titus, B. (2011). Nature and nurture in the dynamics of C, N and P during litter decomposition in Canadian forests. *Plant Soil* 339, 163–175. doi: 10.1007/s11104-010-0563-3

Moscatelli, M. C., Di Tizio, A., Marinari, S., and Grego, S. (2007). Microbial indicators related to soil carbon in Mediterranean land use systems. *Soil Tillage Res.* 97, 51–59. doi: 10.1016/j.still.2007.08.007

Mwanjalolo Jackson-Gilbert, M., Makooma Moses, T., Rao, K. P., Musana, B., Bernard, F., Leblanc, B., et al. (2015). Soil fertility in relation to landscape position and land use/cover types: a case study of the Lake Kivu pilot learning site. *Adv. Agric.* 2015, 1–8. doi: 10.1155/2015/752936

Patra, A. K., Abbadie, L., Clays-Josserand, A., Degrange, V., Grayston, S. J., Loiseau, P., et al. (2005). Effects of grazing on microbial functional groups involved in soil N dynamics. *Ecol. Monogr.* 75, 65–80. doi: 10.1890/03-0837

Paz-Kagan, T., Ohana-Levi, N., Herrmann, I., Zaady, E., Henkin, Z., and Karnieli, A. (2016). Grazing intensity effects on soil quality: a spatial analysis of a Mediterranean grassland. *Catena* 146, 100–110. doi: 10.1016/j.catena.2016.04.020

Prieto, L. H., Bertiller, M. B., Carrera, A. L., and Olivera, N. L. (2011). Soil enzyme and microbial activities in a grazing ecosystem of Patagonian Monte, Argentina. *Geoderma* 162, 281–287. doi: 10.1016/j.geoderma.2011.02.011

Saul-Tcherkas, V., and Steinberger, Y. (2009). Temporal and shrub adaptation effect on soil microbial functional diversity in a desert system. *Eur. J. Soil Sci.* 60, 871–882. doi: 10.1111/j.1365-2389.2009.01165.x

Saviozzi, A., Biasci, A., Riffaldi, R., and Levi-Minzi, R. (1999). Long-term effects of farmyard manure and sewage sludge on some soil biochemical characteristics. *Biol. Fertil. Soils* 30, 100–106. doi: 10.1007/s003740050594

Schacht, W. H., Volesky, J. D., Bauer, D. E., and Stephenson, M. B. (2011). *Grazing systems for Nebraska Sandhills rangeland*. Lincoln: University of Nebraska.

Sekaran, U., McCoy, C., Kumar, S., and Subramanian, S. (2019). Soil microbial community structure and enzymatic activity responses to nitrogen management and landscape positions in switchgrass (*Panicum virgatum* L.). *Glob. Change Biol. Bioenergy.* 11, 836–851. doi: 10.1111/gcbb.12591

Singh, S., Mayes, M. A., Shekoofa, A., Kivlin, S. N., Bansal, S., and Jagadamma, S. (2021). Soil organic carbon cycling in response to simulated soil moisture variation under field conditions. *Sci. Rep.* 11, 1–13. doi: 10.1038/s41598-021-90359-4

Smart, A., Derner, J., Hendrickson, J., Gillen, R., Dunn, B., Mousel, E., et al. (2010). Effects of grazing pressure on efficiency of grazing on north American Great Plains rangelands. *Rangel. Ecol. Manag.* 63, 397–406. doi: 10.2111/REM-D-09-00046.1

Smith, C. A., Phiefer, C. B., Macnaughton, S. J., Peacock, A., Burkhalter, R. S., Kirkegaard, R., et al. (2000). Quantitative lipid biomarker detection of unculturable microbes and chlorine exposure in water distribution system biofilms. *Water Res.* 34, 2683–2688. doi: 10.1016/S0043-1354(00)00028-2

Steffens, M., Kölbl, A., Totsche, K. U., and Kögel-Knabner, I. (2008). Grazing effects on soil chemical and physical properties in a semiarid steppe of Inner Mongolia (PR China). *Geoderma* 143, 63–72. doi: 10.1016/j.geoderma.2007.09.004

Stumpp, M., Wesche, K., Retzer, V., and Miehe, G. (2005). Impact of grazing livestock and distance from water source on soil fertility in southern Mongolia. *Mt. Res. Dev.* 25, 244–251. doi: 10.1659/0276-4741(2005)025[0244:IOGLAD]2.0.CO;2

Tabatabai, M. A., and Bremner, J. M. (1969). Use of p-nitrophenyl phosphate for assay of soil phosphatase activity. *Soil Biol. Biochem.* 1, 301–307. doi: 10.1016/0038-0717(69)90012-1

Tabatabai, M., and Bremner, J. (1970). Arylsulfatase activity of soils. *Soil Sci. Soc. Am. J.* 34, 225–229. doi: 10.2136/sssaj1970.03615995003400020016x

US Department of Agriculture, Soil Conservation Service. (1987). *Jackson County soil survey*. Washington, DC: Government Printing Office.

Vance, E. D., Brookes, P. C., and Jenkinson, D. S. (1987). An extraction method for measuring soil microbial biomass C. *Soil Biol. Biochem.* 19, 703–707. doi: 10.1016/0038-0717(87)90052-6

Willers, C., Jansen van Rensburg, P., and Claassens, S. (2015). Phospholipid fatty acid profiling of microbial communities–a review of interpretations and recent applications. *J. Appl. Microbiol.* 119, 1207–1218. doi: 10.1111/jam.12902

Yates, C. J., Norton, D. A., and Hobbs, R. J. (2000). Grazing effects on plant cover, soil and microclimate in fragmented woodlands in South-Western Australia: implications for restoration. *Austral Ecol.* 25, 36–47. doi: 10.1046/j.1442-9993.2000.01030.x

Zhang, B., Thomas, B. W., Beck, R., Liu, K., Zhao, M., and Hao, X. (2018). Labile soil organic matter in response to long-term cattle grazing on sloped rough fescue grassland in the foothills of the Rocky Mountains, Alberta. *Geoderma* 318, 9–15. doi: 10.1016/j.geoderma.2017.12.019

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Leguminous green manure amendments improve maize yield by increasing N and P fertilizer use efficiency in yellow soil of the Yunnan-Guizhou Plateau

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The low utilization efficiencies of chemical N and P fertilizers largely threat the sustainability of agriculture. Green manure is conducive to increasing crop yield. This study aimed to explore the effects of leguminous and non-leguminous green manures on the maize production, and N and P fertilizers use efficiency. A green manure-maize rotation experiment was conducted in the Karst region of the Yunnan-Guizhou Plateau. The responses of maize yield, N (NUE) and P (PUE) fertilizer use efficiency to winter fallow fields treated with no fertilizer (CK) and traditional chemical fertilizers (WF), WF with alfalfa (AL), common vetch (CV) and oilseed rape (OR) green manures in 2020 and 2021. The results showed that compared with WF, the maize yield was significantly increased on average by 22% and 15% in AL and CV, respectively, while it was hardly affected in OR. AL significantly increased NUE and PUE by an average of 103% and 66%, and CV increased NUE by an average of 74% and 41%, respectively, while RA had no significant effects on PUE, and decreased NUE by 39% in the second year. Structural equation modeling (SEM) showed that green manures indirectly affected NUE and PUE due to soil available N and P, which directly and indirectly influenced maize N and P uptake, and then enhanced NUE and PUE. Linear regression results showed that maize production had positive relationships with NUE and PUE. Our findings highlight that non-legume green manure would hardly influence grain yield, while legume green manure could be effective for increasing maize production by increasing NUE and PUE, especially for alfalfa in yellow soil of the Yunnan-Guizhou Plateau.

KEYWORDS

alfalfa, legume green manure, crop rotation, NUE, PUE

1 Introduction

Nitrogen (N) and phosphorus (P) are the main factors affecting crop growth (Umar et al., 2020). Crops absorb a large amount of N from soils, and a shortage of N leads to low crop yield by limiting crop photosynthesis (Mu and Chen, 2021). In addition, soil P deficiency and its low availability may constrain crop productivity (Katsalirou et al., 2016), contributing 35%–40% to the restriction of maize yield in China (Cao et al., 2021). Chemical N and P fertilizers are used worldwide to increase crop production and maintain soil fertility; however, only 30%–35% and 18%–20% of mineral N and P fertilizers can be used in the current season for crop growth (Balemi and Negisho, 2012), while the rest are fixed or lost through leaching, runoff and volatilization, leading to the risk of soil degradation and environmental pollution problems (Karamesouti and Gasparatos, 2017). Moreover, chemical P fertilizer is produced from nonrenewable natural phosphate rock, but the input of P fertilizer is increasing to maximize crop production due to the large demand for food (Balemi and Negisho, 2012). Thus, improving N and P fertilizer use efficiency is critical for easing environmental problems and the lack of phosphate ore and developing sustainable agriculture in the long term. The management of organic fertilizers in combination with chemical fertilizers shows positive effects on nutrient use efficiency and environment (Zhang et al., 2023).

Globally, green manuring can reduce soil erosion and promote soil hydraulic properties, which can reduce the loss of chemical fertilizer through runoff and leaching (Lei et al., 2022). Additionally, green manuring has positive effects on soil physicochemical properties and nutrient transformation, such as soil available nutrients, microbial quantity and enzymatic activity (He et al., 2020; Khan et al., 2020; Gao et al., 2021), which further enhance N and P use efficiency and crop yield, especially for legume green manuring (Xie et al., 2016; Yang et al., 2019). For example, common vetch (*Vicia sativa* L.), lupin (*Lupinus*) and lablab (*Lablab purpureus* L.) green manures can increase soil available N (AN) and P (AP), and production in wheat and rice systems (He et al., 2020; Amede et al., 2021). Previous studies have indicated that the combined application of milk vetch (*Astragalus sinicus* L.) green manure and chemical N fertilizer increased NUE by 182%−203% compared with chemical N fertilizer alone (Meng et al., 2019), and the P fertilizer use efficiency increased by 10%−14% with the application of legume green manure in paddy fields (Gao et al., 2022).

There are several mechanisms for enhancing soil nutrients and N and P use efficiency by incorporation of green manure. First, legume plants can fix atmospheric N to improve soil available N, meanwhile arbuscular mycorrhizal fungi and rhizobia in the rhizosphere of legume plants can enhance the N and P uptake of the following crops (Meng et al., 2015; Allito et al., 2020). Second, green manures that exude high rates of organic acids are effective for dissolving soil Fe-P and Al-P to increase the labile P content (Haynes and Mokolobate, 2001). At the same time, organic forms of P released during green manure decomposition are less susceptible to strong adsorption on functional groups of oxides and hydroxides of Fe and Al than inorganic forms (Pavinato et al., 2017). Third, green manure incorporation can improve soil aeration and physical properties (Meena et al., 2018), which is conducive to nutrient retention and water storage in soil. Furthermore, green manure incorporation increases soil enzyme activity and microorganisms, further enhancing nutrient cycling (Zhou et al., 2020). Although many studies have addressed the effect of green manure on crop nutrient cycling, it is as yet not well understood how green manure improves N and P use efficiency through soil available nutrients and whether there is an interaction between N and P use efficiency.

The Karst area in southwest China covers an area of 55 × 10^6 ha, which is one of the largest continuous Karst region in the world (Li et al., 2017). It is particularly susceptible to severe soil degradation due to a complex network of soil pockets, rock matrices, and flow paths with variable hydraulic conductivity (Fu et al., 2015; Li A. et al., 2016). In addition, cultivated land in Karst landforms is highly sensitive and vulnerable due to shallow and thin topsoil (Li S. L. et al., 2020). Yellow clayed soil accounts for 46.2% of soil in this region (Li et al., 2016), which characterized by weathering, erosion and a lack of available N and available P contents (Liu et al., 2017). The yellow soil in Karst areas has been seriously degraded owing to intensive nutrient leaching, which can risk low fertilizer use efficiency, crop productivity and soil N and P losses in agroecosystems (Wang et al., 2019; Li S. L. et al., 2020). The application of sweet pea (*Lathyrus odoratus* L.) green manure led to significantly increased soil available P content and maize yield in northwest China (Ablimit et al., 2022). The application of February Orchid (*Orychophragmus violaceus*) as green manure improved maize grain yield and nitrogen use efficiency and reduced nitrogen losses in northern China (Bai et al., 2015). Therefore, it is essential to reduce N and P losses by increasing chemical fertilizer use efficiency for green manuring management in Karst landforms. Although many studies have been conducted on the effects of green manure incorporation on crop yields and soil fertility around the world (He et al., 2020; Amede et al., 2021), there are still very few available reports regarding the coupling responses of NUE and PUE to legume and non-legume green manure incorporation in Karst maize cultivation systems.

To characterize the combined nutrient use efficiencies and maize yield feedback attributable to different green manures in combination with chemical fertilizers and chemical fertilizers alone, we used alfalfa, common vetch and soilseed rape as green manures in a Karst maize ecosystem in Southwest China. The objectives were (1) to determine the difference in nutrient use efficiency and grain yield between legume and non-legume green manure and (2) to assess the interaction of N and P in a green manure-maize rotation system. We hypothesized that the application of green manures would improve soil fertility and maize yield by increasing N and P use efficiency in karst landforms.

2 Materials and methods

2.1 Experimental site

The experiment was conducted in a typical Karst area in Machang town (26°25′N, 106°27′E), Guiyang, China. The region possesses a subtropical humid monsoon climate. The previous crop was maize, which is fallow in winter. The maize growing season is from April to September, with precipitation of 932.60 and 784.00 mm and mean temperatures of 20.35 and 21.09°C in 2020 and 2021, respectively. The soil type is yellow loam. The basic soil pH was 5.28, and the organic matter, total N and P were 34.94, 1.30, and 1.24 g kg^{-1}, respectively.

2.2 Field treatment and management

In order to choose the suitable green manure to improve the maize yield, NUE, and PUE, different green manure treatments, including (1) winter fallow field + no fertilization (CK); (2) winter fallow field + traditional chemical fertilizer (WF); (3) alfalfa + traditional chemical fertilizer (AL); (4) common vetch + traditional chemical fertilizer (CV); and (5) oilseed rape + traditional chemical fertilizer (OR), were established in randomized plots (3 m × 6 m) with a plot spacing of 0.3 m. There were three replicates (plots) for each treatment. The traditional chemical fertilizer application rate was N 244 kg ha^{-1} and P_2O_5 145 kg ha^{-1}.

Alfalfa, common vetch and oilseed rape were sown after maize harvest in 2019 and 2020 by broadcast seeding, with seeding amounts of 27, 45, and 45 kg ha^{-1}, respectively. No fertilizers were applied during the green manure growing season. All green manures were cut into 5–10 cm pieces and incorporated into the tillage layer (20 cm) 1 week before planting maize. The properties of green manures are shown in Table 1.

The maize varieties were starnuo41 and Yingtai863A in 2020 and 2021, respectively. All the seeds were planted in hill-holes, with three–four seeds per hill-hole, with a distance between the holes of ~60 cm. After the emergence of seedlings, each hole contained two plants. Monoammonium phosphate fertilizer (including N 11%, P_2O_5 44%) was applied as a base fertilizer on the day of sowing maize, and urea (N 46%) as the top fertilizer was applied at the seedling stage (June 15, 2020, June 2, 2021) and before plucking and silking (July 10, 2020, July 6, 2021), accounting for 35 and 55% of the total N amount, respectively. Other management practices were the same as local conventional field practices.

2.3 Sampling and analyses

Three topsoil (0–20 cm) samples were collected simultaneously with the maize harvest period in each plot, mixed evenly using the quartic method, then divided into two subsamples. One subsample were sieved through 2 mm sieves and stored at −20°C, and another subsample was air dried and sieved through 0.15 and 0.25 mm sieves when relevant soil properties were measured. At the maize harvest stage, ten maize plants were randomly harvested in each plot to measure grain yield and stem and leaf biomasses. The stem and leaf samples were dried for 30 min at 105°C and then dried for 48 h at 60°C. The dried grain, stem, and leaf were sieved (0.15 mm) to determine their N and P contents.

NH_4^--N and NO_3^+-N were extracted with 1 M KCl (1:5 w/v) and measured with a flow analyzer (Cleverchem 380, Germany). Soil AN content contained NH_4^--N and NO_3^+-N contents. The total N content was measured by the Kjeldahl digestion procedure (Bremner and Tabatabai, 1972). The soil total P content was determined by the molybdenum blue method after digestion with concentrated $HClO_4$-H_2SO_4 (1:10 v/v), and the soil AP was determined via the molybdenum blue method after extraction with 0.5 M $NaHCO_3$ at pH 8.5.

2.4 Calculations of nutrient use efficiency

The maize N or P uptakes of grain (Eq. 1), stem (Eq. 2), leaf (Eq. 3), and straw (Eq. 4) were calculated as follows:

$$\text{Grain N or P uptake } \left(\text{kg ha}^{-1}\right) = \text{grain N or P content } \left(\text{g kg}^{-1}\right) \times \text{grain yield } \left(\text{kg ha}^{-1}\right)/1{,}000 \quad (1)$$

$$\text{Stem N or P uptake } \left(\text{kg ha}^{-1}\right) = \text{stem N or P content } \left(\text{g kg}^{-1}\right) \times \text{stem yield } \left(\text{kg ha}^{-1}\right)/1{,}000 \quad (2)$$

$$\text{Leaf N or P uptake } \left(\text{kg ha}^{-1}\right) = \text{leaf N or P content } \left(\text{g kg}^{-1}\right) \times \text{leaf yield } \left(\text{kg ha}^{-1}\right)/1{,}000 \quad (3)$$

$$\text{Straw N or P uptake } = \text{stem N or P uptake } + \text{leaf N or P uptake} \quad (4)$$

NUE (Eq. 5) and PUE (Eq. 6) were determined as follows (Cao et al., 2021):

$$\text{N or P uptake efficiency } = U \times 100/F \quad (5)$$

$$\text{N or P use efficiency } = \left(U_{+\text{N or P}} - U_{-\text{N or P}}\right) \times 100/F \quad (6)$$

where F denotes the amount of N and P applied (kg ha^{-1}), U_{+NorP} and U_{-NorP} are the total N and P uptake by grain and straw in the chemical fertilizer treatment and CK (kg ha^{-1}), respectively.

2.5 Statistical analysis

The LSD test was used to test the differences between the yields, N and P concentrations in maize plants, N and P uptake, soil available N and soil available P content among treatments at $P < 0.05$, and a t-test was used to test the differences in NUE and PUE at $P < 0.05$. Two-way ANOVAs were used to determine the effects of green manure type, year, and their interaction on maize yield and nutrient use efficiency at $P < 0.05$. The "piecewise SEM" package in R (v4.1.1) was used for structural equation modeling (SEM). The a priori model included all possible pathways among these factors. To reduce the complexity of SEM, the representing indices of green manure N and P concentrations were calculated using PCA with the "FactoMineR" package in R (v4.1.1). All relationships were fitted using "lm" linear regression (Xiao et al., 2021).

3 Results

3.1 Grain yield and straw biomass

All the green manure treatments improved maize grain yield, straw and total biomass compared to CK in both years ($P < 0.05$,

TABLE 1 The nutrient contents and uptake efficiency of green manure in 2020 and 2021.

Items		Alfalfa		Common vetch		Oilseed rape	
		2020	2021	2020	2021	2020	2021
Nutrient contents (g kg^{-1})	C	446.7	482.4	427.4	462.1	422.4	429.4
	N	31.4	36.4	23.2	32.0	14.3	20.8
	P	3.0	2.5	2.2	3.0	3.1	3.1
Nutrient ratios	C/N	14.2	13.2	18.4	14.5	29.6	20.7
	C/P	148.9	178.5	198.3	144.0	138.3	137.2
Nutrient accumulations (kg ha^{-1})	C	616.4	1,413.4	1,767.7	1,693.6	1,573.0	1,514.9
	N	43.3	106.7	96.0	117.3	53.3	73.4
	P	4.1	7.3	9.1	11.0	11.5	10.9
Nutrient uptake efficiency (%)	N	17.8	43.7	39.3	48.1	21.8	30.1
	P	6.5	11.6	14.4	17.4	18.2	17.3

Figure 1), with the highest yields observed in AL. Compared with WF, AL and CV significantly increased maize grain yield by 15 and 21% in 2021 and straw biomass by 44 and 51% in 2020 ($P < 0.05$), and OR decreased grain yield by 9% in 2021 ($P > 0.05$). Compared with WF, AL and CV increased total biomass by 24 and 21% on average over the 2 years ($P < 0.05$), respectively. Compared with the OR treatment, the AL and CV treatments had higher grain yields, straw yields and total yields, which increased by 7%—32%, 21%—27% and 13%—27% in 2020 and 2021, respectively.

Two-way ANOVA showed that year and green manure application had significant effects on grain yield, straw and total yield ($P < 0.05$), but there was no significant interaction effect between them ($P > 0.05$, Table 2).

3.2 Maize N and P concentration and uptake

Green manure incorporation increased the N concentration of maize grains, stems and leaves in 2020 and leaves in 2021 compared to CK ($P < 0.05$, Figure 2A). Both AL and CV increased the grain N concentration compared to WF, but the mean increase intensity differed between AL (23%, $P < 0.05$) and CV (5%, $P > 0.05$). OR decreased the grain N concentration in 2020 ($P > 0.05$) and 2021 ($P < 0.05$, Figure 2A). The P concentration in maize plants was lowest in CK and highest in AL (Figure 2B). The grain, stem and leaf P concentrations in the AL treatment increased by 3%, 20% and 9% on average, respectively, compared with those in the WF treatment.

Both total maize N and P uptake showed consistent changes in 2020 and 2021, with the highest uptake in AL, followed by CV and OR, and the lowest uptake in CK (Figure 3). All fertilizer treatments significantly increased the maize grain, straw, and total N and P uptake compared to CK in 2020 and 2021 ($P < 0.05$, Figure 3). Compared with WF, the maize grain, straw and total uptake in AL and CV increased by 49 and 21%, 87 and 88%, and 63 and 45% on average, while the RA decreased by 20%, 9%, and 11%. The AL treatment significantly increased the maize grain, straw and total N uptake compared with the WF treatment ($P < 0.05$, Figure 3A). AL had the highest grain, straw and total P uptake, and the straw and total P uptake was significantly higher than WF ($P < 0.05$, Figure 3B). Compared with CK, all the treatments significantly increased the maize grain N/P ratio except OR; AL and CV increased the leaf N/P ratio in 2020 and 2021, and OR increased the stem N/P ratio in 2021 ($P < 0.05$, Table 3).

3.3 Maize N and P use efficiency

Legume green manure incorporation showed an overall trend of improving N and P fertilizer use efficiencies, while non-legume green manure (OR) application showed an opposite trend in the second year (Table 4). Compared with the WF treatment, the UEN and NUE increased by an average of 63 and 103% in the AL treatment and by an average of 45 and 74% in the CV treatment, respectively; the UEP and PUE increased by an average of 35 and 66% in the AL treatment and by 22 and 41% in the CV treatment, respectively. AL significantly increased UEP and PUE compared to WF in both years ($P < 0.05$). In addition, the UEP and PUE in AL and CV were higher than those in OR. Two-way ANOVA showed that both N and P fertilizer use efficiencies were significantly affected by green manure type ($P < 0.05$), and there were no significant interactions between green manure type and year ($P > 0.05$, Table 4).

3.4 Soil N and P contents

AL significantly increased the soil available and total N contents compared with the other treatments in 2020 and 2021 (Figures 4A, B, $P < 0.05$). The soil available N of CV and OR was significantly higher than that of CK and WF in 2021 ($P < 0.05$, Figure 4A), and there were no significant differences in total N among CK, WF, CV and OR in both years ($P > 0.05$, Figure 4B). The soil available P in AL and CV was significantly higher than that in CK and WF in 2020 and 2021 ($P < 0.05$, Figure 4C). The soil total P content had no significant changes among fertilizer treatments ($P > 0.05$, Figure 4D).

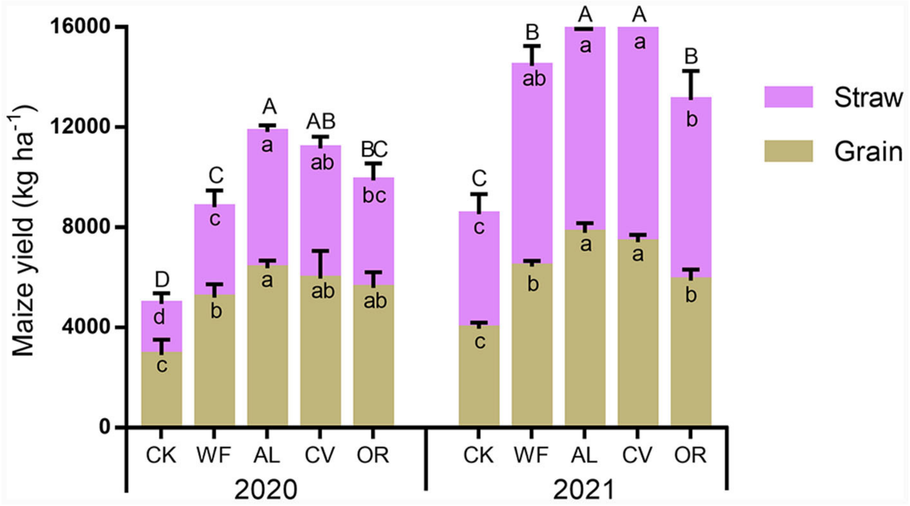

FIGURE 1
Maize grain yield, straw and total biomass in 2020 and 2021 (means ± SEs). Straw biomass including stem and leaf biomass. Different letters in the same item represent a significance level of 0.05 for grain, straw (lowercase) and total biomass (capital).

TABLE 2 The effects of green manure type and year on maize yields.

Treatment	df	Grain		Straw		Total biomass	
		F	*P*-value	*F*	*P*-value	*F*	*P*-value
Green manure (G)	4	30.90	<0.01	182.89	<0.01	150.84	<0.01
Year (Y)	1	42.00	<0.01	30.50	<0.01	52.42	<0.01
G × Y	4	1.16	0.36	1.80	0.17	1.74	0.18

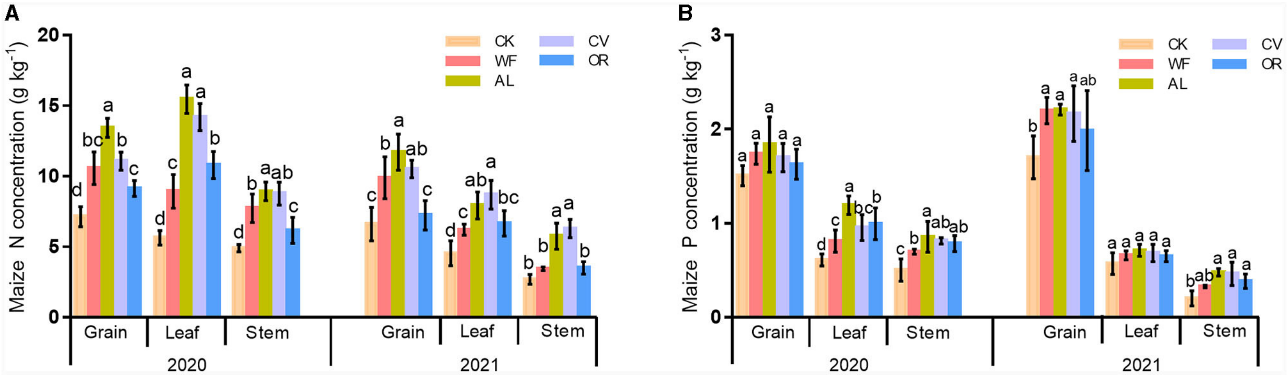

FIGURE 2
The N **(A)** and P **(B)** concentrations in grain, stem and leaf under different treatments in 2020 and 2021 (means ± SEs). Different letters represent a significance level of 0.05 among treatments.

3.5 Controlling factors for NUE, PUE and grain yields

The SEM explained 99 and 96% of the variations in NUE and PUE, respectively (Figure 5). NUE was directly affected by grain and straw N uptake, and PUE was directly affected by grain and straw P uptake. Moreover, green manure indirectly affected NUE due to soil available N and P contents and N uptake in grain and straw, while green manure indirectly affected PUE due to soil available N and P contents and P uptake in grain and straw (Figure 5). Maize total biomass exhibited a significantly positive linear relationship to NUE and PUE (Figures 6A, B), and there were significant positive linear relationships between NUE and PUE, grain yield and grain N/P ratio in 2020 and 2021 ($P < 0.05$, Figures 6C–E).

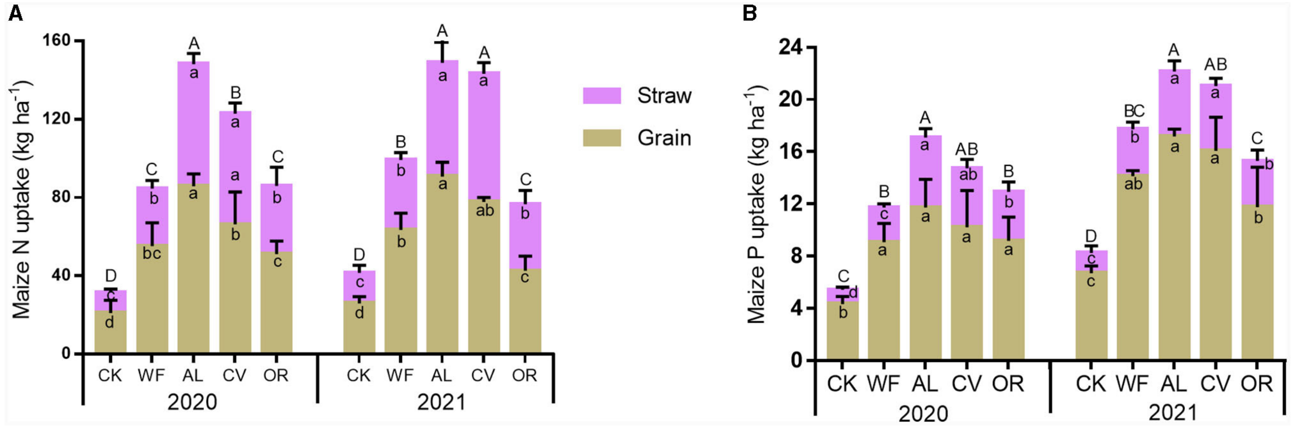

FIGURE 3
The maize N uptake **(A)** and P uptake **(B)** of grain and straw under different treatments (means ± SEs). Different letters in the same item represent a significance level of 0.05 for straw, grain (lowercase) and total uptake (capital).

TABLE 3 The maize N:P ratio in different treatments.

Treatments	Grain		Leaf		Stem	
	2020	2021	2020	2021	2020	2021
CK	4.78 ± 1.37c	3.87 ± 1.99b	9.24 ± 0.51c	7.98 ± 0.27c	10.08 ± 1.37ab	14.22 ± 1.99a
WF	6.07 ± 0.63b	4.50 ± 0.38ab	11.12 ± 0.97bc	9.48 ± 0.76bc	11.00 ± 0.63a	10.53 ± 0.38ab
AL	7.41 ± 1.18a	5.29 ± 0.65a	13.02 ± 0.47ab	11.12 ± 0.2ab	10.68 ± 1.18ab	11.96 ± 0.65ab
CV	6.53 ± 0.84ab	4.92 ± 1.48a	15.03 ± 0.91a	12.81 ± 1.01a	10.85 ± 0.84ab	14.03 ± 1.48a
OR	5.64 ± 0.48bc	3.69 ± 0.75b	11.05 ± 1.00bc	10.26 ± 0.71b	7.88 ± 0.48b	9.25 ± 0.75b

Different letters in the same column represent a significance level of 0.05.

4 Discussion

4.1 Effects of green manures on maize yield

In the present study, alfalfa green manure incorporation significantly increased maize grain and straw yields in 2020 and 2021, and common vetch had a positive effect on grain yield in the second year, while rape green manure reduced grain yield in 2021 (Figure 1). Previous studies showed that the application of milk vetch green manure significantly increased maize yield by 31% in a 2-year study (Tao et al., 2017), while legume hairy vetch application had no significant effect on maize yield, and non-legume annual ryegrass and cereal rye green manure application decreased maize grain yield by 4% in a 5-year study (Qin et al., 2021). A meta-analysis showed that legume and non-legume green manures increased maize yield by 12 and 9% in northern China, respectively (Ma et al., 2021), while non-legume cover crops resulted in a 4% yield decrease (Abdalla et al., 2019). These results are similar to our findings, suggesting that the effects of green manure incorporation on crop yields are closely associated with green manure type and application year.

We found that legume green manures were more effective in promoting maize growth and grain yield than no legume (Figure 1). This may be due to the following mechanisms. First, the soil N content and legume green manure N content were higher than those in the non-legume treatment (Table 1 and Figure 4A). The accumulation of N in leguminous plants is normally higher than that in non-leguminous plants because legumes can fix N_2 from the atmosphere into soils through rhizobia (Zandvakili et al., 2017), which leads to an increase in soil N supplementation and high N concentrations in leguminous plants (Dovrat et al., 2020). Second, the C/N ratio is one of the determining constraints for effective decomposition of green manure. Plant residues contain a lesser proportion of carbon to nitrogen than the 24 perfectly balanced diet soil microorganisms need, the microbes consumed the plant residues and leave the excess nitrogen in the soil. This surplus nitrogen in the soil is available for growing plants. Plant residues with a C/N ratio >24 result in a temporary nitrogen deficit (immobilization), and those with a C/N ratiol <24 result in a temporary nitrogen surplus (mineralization) (United States Department of Agriculture, 2011). The C/N ratios of alfalfa and common vetch were much lower than that of oilseed rape in the present study (Table 1). The decomposition and mineralization rates of legume residues are faster than those of non-legume residues due to the narrow C/N ratio of legume green manures (Calegari et al., 2013; Toom et al., 2019), which release N and P faster, thus improving maize growth and yield. However, non-legume green manure with a high C/N ratio would first stabilize soil available N, which limited the N applied for crop growth (Li et al., 2020).

TABLE 4 **Effects of green manure on N and P use efficiency.**

Treatments	Uptake efficiency (%)		Nutrient use efficiency (%)	
	N	P	N	P
2020				
WF	34.69 ± 3.73b	18.51 ± 1.48b	21.77 ± 3.73b	9.95 ± 1.48b
AL	60.73 ± 0.32a	26.98 ± 1.67a	47.81 ± 0.32a	18.42 ± 1.67a
CV	50.34 ± 5.18a	23.26 ± 3.18ab	37.42 ± 5.18a	14.70 ± 3.18ab
OR	35.20 ± 1.62b	20.41 ± 1.01b	22.28 ± 1.62b	11.85 ± 1.01b
2021				
WF	40.69 ± 2.86b	28.02 ± 0.48b	23.64 ± 2.86b	14.97 ± 0.48b
AL	61.00 ± 3.33a	35.00 ± 1.23a	43.96 ± 3.33a	21.95 ± 1.23a
CV	58.69 ± 1.48a	33.22 ± 2.78ab	41.65 ± 1.48a	20.17 ± 2.78ab
OR	31.40 ± 3.08c	24.15 ± 3.50b	14.35 ± 3.08c	11.10 ± 3.50b
Green manure (G)	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$
Year (Y)	0.24	$P < 0.01$	0.55	0.04
G × Y	0.20	0.43	0.20	0.48

Different letters indicate a significance level of 0.05 in the same column.

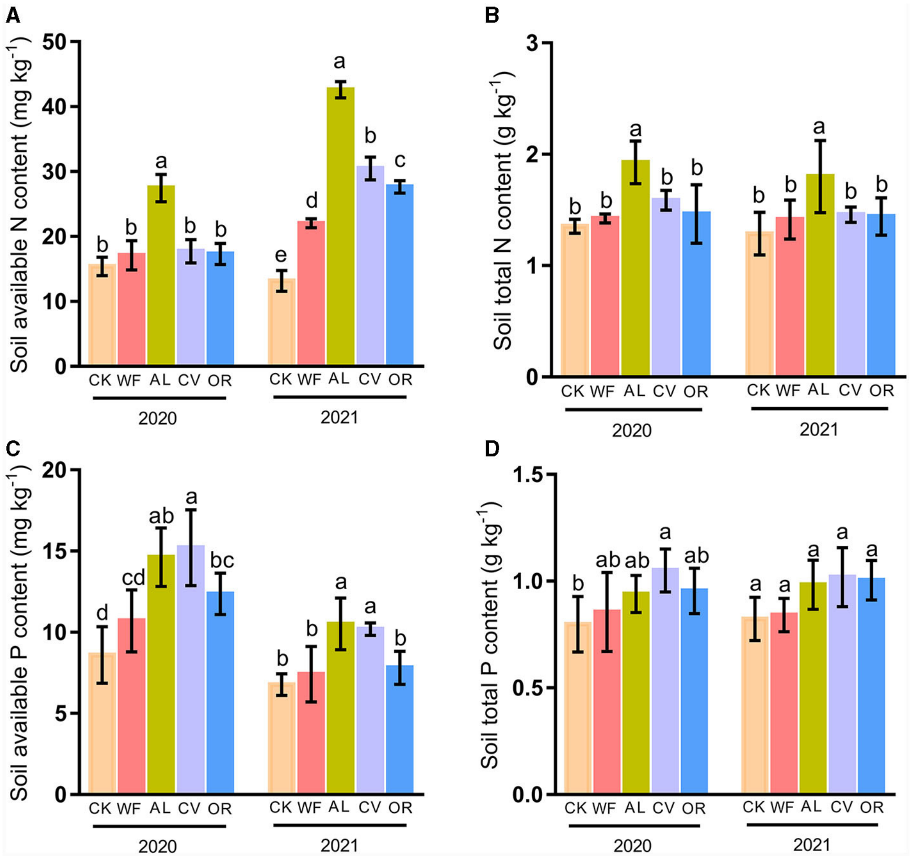

FIGURE 4
Variations in soil available N **(A)**, total N **(B)**, available P **(C)**, and total P **(D)** contents **(D)** among treatments. Different letters in the same item are significantly different at $P < 0.05$.

Furthermore, green manure application may increase available P for succeeding crops (Haynes and Mokolobate, 2001), and net P mineralization from legume green manure is commonly positively correlated with the P concentration in green manure (Pypers et al., 2005). Legume green manure application could increase grain yield by improving a large amount of soil active

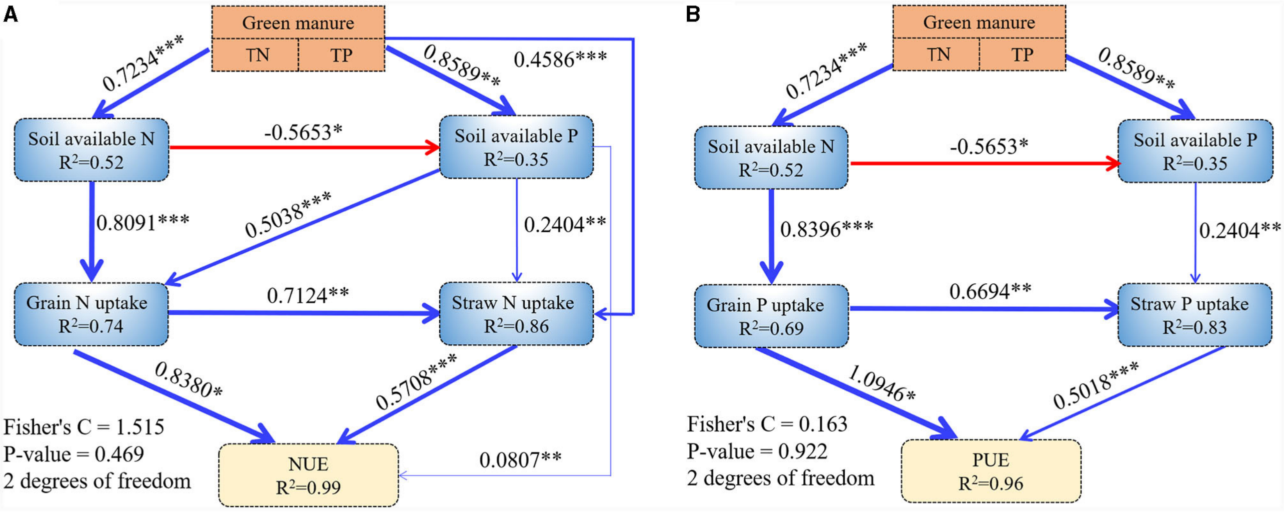

FIGURE 5
The SEM to evaluate the direct and indirect effects of green manure, soil available N content (AN), soil available P content (AP), and N and P uptake on maize NUE **(A)** and PUE **(B)** from 2020 to 2021. Soil available N content including NH_4^+-N and NO_3^--N contents. Continuous arrows represent the significant relationships. Blue and red lines represent positive and negative correlations, respectively. The adjacent number in the same direction as the arrow represents the path coefficients, and the width of the arrow is proportional to the degree of the path coefficients. R^2 is the variance explained by each response variable. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

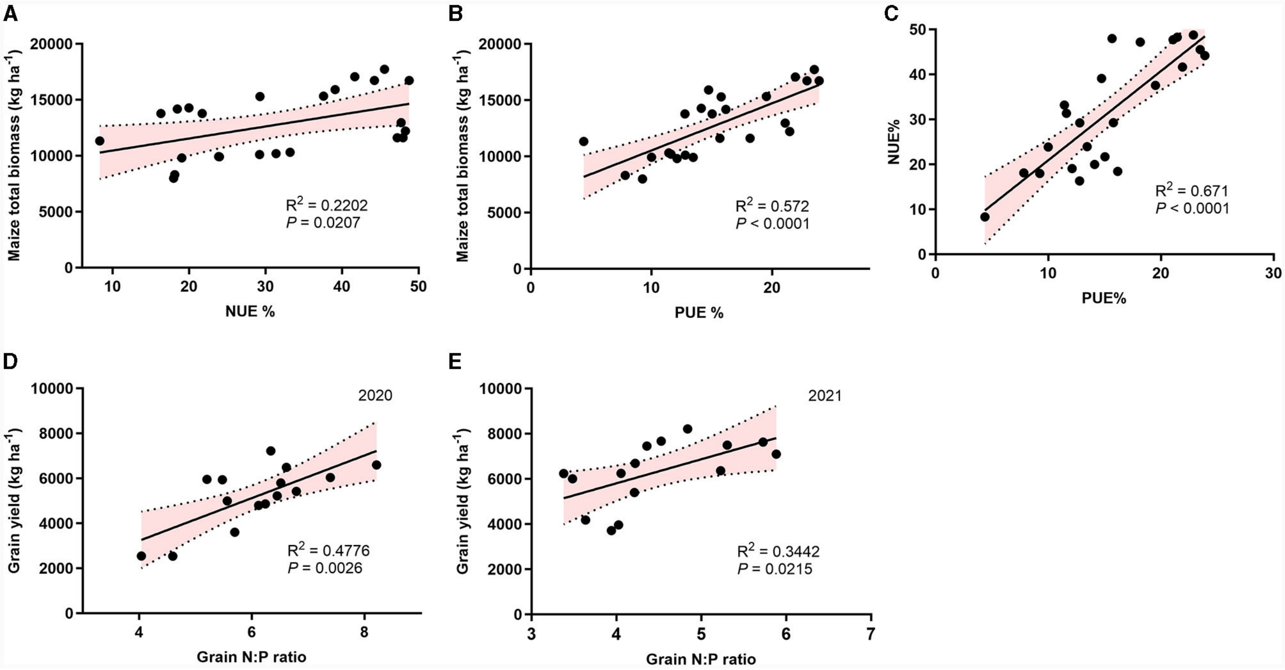

FIGURE 6
Linear relationships between maize above-ground biomass and NUE, and PUE **(A–C)** and between maize grain yield and the grain N:P ratio in 2020 and 2021 **(D, E)**.

P (Gao et al., 2016). Although the P concentration in oilseed rape was the highest in the present study, the soil available P content was lower than those in AL and CV (Figure 4) because the low N concentration and large C/N ratio of oilseed rape limit the decomposition of rape residues and further affect available P release.

4.2 Effects of green manures on maize NUE

Maize has a relatively high nutrient requirement for N, while the average NUE of maize is low (Abbasi et al., 2013). In this study, the NUE ranged from 14 to 48%, and the application of alfalfa and common vetch increased the NUE by 103 and 74% and decreased it by 19% under oilseed rape green manure (Table 4), which coincided with the trends in maize biomass, N uptake, and available N content between fertilization treatments (Figures 1, 3A). Similarly, Murungu et al. (2011) found that the contributions of grazing vetch (*Vicia darsycarpa*) and forage pea (*Pisum sativum*) green manures to maize N uptake were higher than that of oat green manure. Red clover significantly improved maize N availability, but oilseed radish (*Raphanus sativus*), oat and rye green manures had no significant effect on maize N availability (Vyn et al., 2000). Chinese milk vetch increased N uptake and use efficiency by 39%—51% (Zhu et al., 2014). Increasing crop biomass and N concentration are effective ways to increase NUE (Sinclair and Vadez, 2002). The N contributions of alfalfa and common vetch green manures to maize plants were higher than those of rape green manure, causing higher NUE in the AL and CV treatments than in the OR treatment (Figure 2A and Table 4). Legume green manure is not only used as a direct source of available N for plants but also has great potential in increasing the availability of soil N to crops and preserving N (Ashraf et al., 2004). Legume green manure increased NUE mainly by directly increasing N uptake and indirectly improving soil available N and P contents (Figure 5A), suggesting that the improvement in soil available N content may be responsible for the increased crop yields and N uptake and further enhances NUE (Zhang et al., 2020). Therefore, it is suitable for enhancing N supply and NUE in alfalfa green manure-maize rotation systems.

There are several factors affecting soil available N content, which in turn affect crop N uptake and N use efficiency after green manure return to the field. First, organic N in the soil began a rapid initial fixation or mineralization, followed by a slow linear mineralization, and the C/N ratio of green manure determined the decomposition of green manure and N mineralization in soil (Cambardella et al., 2010). In general, green manure with a small C/N ratio mineralizes faster, increasing the availability of mineral N in the soil (Figure 4A), which is the major N source for absorption by crops (Radicetti et al., 2017). Second, the lower C:N ratio of green manure resulted in more rapid N mineralization, and a higher C/N ratio of green manure can prolong the microbial fixation of N available in the intensive cropping system, and its effect is more significant in the early stage of maize growth, thus limiting the N uptake and potential yield in the short term (Radicetti et al., 2017). Moreover, legume and non-legume green manure incorporation exhibited differential responses to the soil microbial community (Khan et al., 2020). Leguminous green manure rich in N easily decomposes following application, probably increasing the soil microbial functional community and soil enzyme activities (Chavarría et al., 2016), which might be attributable to carbon availability in the early stages of maize growth, further enhancing crop nutrient absorption. This was supported by our findings that AL and CV significantly increased soil urease activity and the relative abundance of Proteobacteria (He, 2022). In addition, lower N uptake in maize directly caused the soil available N content to increase with oilseed rape application in 2021. In our study, oilseed rape green manure incorporation reduced the N uptake and NUE in the second year, mainly attributable to maize grain yield reduction (Figures 1A, 3A and Table 4), which may be due to the large C/N ratio of oilseed rape and slow decomposition rate, resulting in the fixation of organic N and little contribution to maize N supplied (Radicetti et al., 2017; Carciochi et al., 2021).

4.3 Effects of green manures on maize PUE

We found that alfalfa and common vetch green manure incorporation increased the P uptake of maize straw, while the effects on maize grain P uptake were insignificant (Figure 3B). Previous studies have found that the P uptake in rice straw with alfalfa and broad bean green manure application was higher than that in chemical fertilizer alone (Gao et al., 2016), and maize P uptake in cobs had no significant changes under the *O. violaceus* green manure-maize system compared with the continuous maize system (Zhang et al., 2022). The PUE of AL and CV increased by 35%—85% in the present study (Table 3), suggesting that short-term application of legume green manure has the potential to improve the maize PUE. Sesbania green manure application increased P uptake and the recovery efficiency of P and PUE in a rice–wheat double system (Mitran and Mani, 2017). Alfalfa and broad bean green manure in combination with chemical fertilizer significantly increased rice PUE by 10%—14% compared with the application of chemical fertilizer alone (Gao et al., 2022). Green manure incorporation can increase soil available P content and crop P uptake and then improve PUE (Pavinato et al., 2017; Gao et al., 2022). Wang et al. (2021) reported that soil properties such as total N, available N, microbial biomass C and N were closely related to PUE. In the present study, SEM analysis showed that the improvement in PUE was attributed to soil available N and P contents and maize P uptake (Figure 6B).

Phosphorus cycling-related microbial and enzyme activities transform soil P through dissolution, mineralization, and absorption, converting soil insoluble P into inorganic P, which is more easily absorbed by plants (Gao et al., 2019). Specifically, the organic instability pool in surface soil was increased through mycorrhizal colonization of green manure, and the arbuscular mycorrhizal fungal abundance of subsequent crops was increased (Arruda et al., 2021). By coincidence, maize plants preferred the mycorrhizal pathway at suboptimal soil available P (Zhang et al., 2021). In addition, organic acids released during the decomposition of green manure can enhance P availability by chelating with aluminum and iron oxides, reducing the number of binding sites and reducing soil P adsorption strength by dissolving soil mineral P (Haynes and Mokolobate, 2001). Moreover, green manure effectively increased the abundances of P-solubilizing bacteria and enhanced phosphatase activity (Wang et al., 2021).

4.4 Implications for green manure maize cropping systems

Balanced nutrient supply is the key factor in nutrient use efficiency and increased crop production (Janssen, 1998; Dash et al., 2015). There was a positive linear correlation between NUE and PUE in our study (Figure 6C). The crop N/P ratio is a direct function of N uptake and an inverse function of P uptake, and its efficiency depends on the range of the N/P ratio. Alfalfa and common vetch green manures increased the maize grain N/P ratio, while oilseed rape had no significant effect on it compared with the winter fallow, and there was a positive relationship between grain yield and grain N/P ratio (Figure 6D), suggesting that lower C/N ratio of legume green manure could enhance maize N and P uptake and consequently increase maize yield owning to the mineralization of green manure (United States Department of Agriculture, 2011). Previous studies showed that crop P uptake and PUE were positively correlated with P concentration in green manure and negatively correlated with the C/P ratio of green manure (Garg and Bahl, 2008; Gao et al., 2022). This study showed the different result that the applicaiton of oilseed rape with the highest P concentration and the lowest C/P ratio had the lowest P uptake and PUE, as well as NUE. The probably reason is that the higher C/N ratio of oilseed rape limited the N supplied and P releasing from oilseed rape residues (Calegari et al., 2013; Toom et al., 2019). Legume green manures rich in N and P contents, in particular N content, may enhance NUE and PUE by improving soil available N and P. In general, legume green manure incorporation generally has positive contributions to N and P in cropping systems.

5 Conclusion

In the 2-year green manure-maize rotation system, alfalfa and common vetch green manure incorporation increased maize yield by 22 and 15%, soil available N content by 77 and 42%, soil available N content by 39 and 40%, NUE by 103 and 74%, and PUE by 66 and 41%, respectively. The improvement in NUE and PUE was attributed to soil available N and P contents, N uptake, and P uptake. Our findings suggest that alfalfa green manure is beneficial for promoting maize NUE and PUE, which will promote the green and sustainable development of agricultural Karst landforms, and that replacing partial chemical N and P fertilizers with alfalfa green manure may be an effective alternative for reducing the application rate of chemical fertilizers and for enhancing maize yields.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

XG: Conceptualization, Writing – review & editing, Writing – original draft. YH: Investigation, Writing – original draft. YC: Investigation, Writing – review & editing. MW: Investigation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Abbasi, M. K., Tahir, M. M., and Rahim, N. (2013). Effect of N fertilizer source and timing on yield and N use efficiency of rainfed maize (*Zea mays* L.) in Kashmir–Pakistan. *Geoderma* 195–196, 87–93. doi: 10.1016/j.geoderma.2012.11.013

Abdalla, M., Hastings, A., Cheng, K., Yue, Q., Chadwick, D., Espenberg, M., et al. (2019). A critical review of the impacts of cover crops on nitrogen leaching, net greenhouse gas balance and crop productivity. *Glob. Change Biol.* 25, 2530–2543. doi: 10.1111/gcb.14644

Ablimit, R., Li, W., Zhang, J., Gao, H., Zhao, Y., and Cheng, M. (2022). Altering microbial community for improving soil properties and agricultural sustainability during a 10-year maize-green manure intercropping in Northwest China. *J. Environ. Manage.* 321, 115859–115859. doi: 10.1016/j.jenvman.2022.115859

Allito, B. B., Ewusi-Mensah. N., and Logah, V. (2020). Legume-rhizobium strain specificity enhances nutrition and nitrogen fixation in faba bean (*Vicia faba* L.). *Agronomy* 10:826. doi: 10.3390/agronomy10060826

Amede, T., Legesse, G., Agegnehu, G., Gashaw, T., Degefu, T., Desta, G., et al. (2021). Short term fallow and partitioning effects of green manures on wheat systems in East African highlands. *Field Crops Res.* 269:108175. doi: 10.1016/j.fcr.2021.108175

Arruda, B., Herrera, W. F. B., Rojas-García, J. C., Turner, C., and Pavinato, P. S. (2021). Cover crop species and mycorrhizal colonization on soil phosphorus dynamics. *Rhizosphere* 19:100396. doi: 10.1016/j.rhisph.2021.100396

Ashraf, M., Mahmood, T., Azam, F., and Qureshi, R. M. (2004). Comparative effects of applying leguminous and non-leguminous green manures and inorganic N on biomass yield and nitrogen uptake in flooded rice (*Oryza sativa* L.). *Biol Fertil. Soils* 40, 147–152. doi: 10.1007/s00374-004-0756-0

Bai, J., Cao, W., Xiong, J., Zeng, N., Gao, S., and Katsuyoshi, S. (2015). Intergrated application of February Orchid (*Orychophragmus violaceus*) as green manure with chemical fertilizer for improving grain yield and reducing introgen losses in improving grain yield and reducing nitrogen losses in spring maize system in norther China. *J. Inter. Agr.* 14, 2490–2499.

Balemi, T., and Negisho, K. (2012). Management of soil phosphorus and plant adaptation mechanisms to phosphorus stress for sustainable crop production: a review. *J. Soil Sci. Plan. Nutr.* 12, 547–562. doi: 10.4067/S0718-95162012005000015

Bremner, J., and Tabatabai, M. (1972). Use of an ammonia electrode for determination of ammonium in Kjeldahl analysis of soils. *Commu. Soil Sci. Plant* 3, 159–165. doi: 10.1080/00103627209366361

Calegari, A., Tiecher, T., Hargrove, W. L., Ralisch, R., Tessier, D., Tourdonnet, S., et al. (2013). Long-term effect of different soil management systems and winter crops on soil acidity and vertical distribution of nutrients in a Brazilian Oxisol. *Soil Till. Res.* 133, 32–39. doi: 10.1016/j.still.2013.05.009

Cambardella, C. A., Moorman, T. B., and Singer, J. W. (2010). Soil nitrogen response to coupling cover crops with manure injection. *Nutr. Cycl. Agroecosyst.* 87, 383–393. doi: 10.1007/s10705-010-9345-9

Cao, D., Lan, Y., Chen, W., Yang, X., Wang, D., Ge, S., et al. (2021). Successive applications of fertilizers blended with biochar in the soil improve the availability of phosphorus and productivity of maize (*Zea mays* L.). *Eur. J. Agron.* 130, 126344. doi: 10.1016/j.eja.2021.126344

Carciochi, W. D., Crespo, C., Eliceche, M., and Barbieri, P. A. (2021). Nitrogen and sulfur recycling and diagnostic in cover crop-maize systems. *J. Soil Sci Plant Nutr.* 21, 801–812. doi: 10.1007/s42729-020-00402-y

Chavarría, D. N., Verdenelli, R. A., Serri, D. L., Restovich, S. B., Andriulo, A. E., Meriles, J. M., et al. (2016). Effect of cover crops on microbial community structure and related enzyme activities and macronutrient availability. *Eur. J. Soil Biol.* 76, 74–82. doi: 10.1016/j.ejsobi.2016.07.002

Dash, A., Singh, H., Mahakud, T., Pradhan, K., and Jena, D. (2015). Interaction effect of nitrogen, phosphorus, potassium with sulphur, boron and zinc on yield and nutrient uptake by rice under rice-rice cropping system in inceptisol of coastal Odisha. *Inter. Res. J. Agri. Sci. Soil Sci.* 5, 14–21. doi: 10.14303/irjas.2014.080

Dovrat, G., Bakhshian, H., Masci, T., and Sheffer, E. (2020). The nitrogen economic spectrum of legume stoichiometry and fixation strategy. *New Phytol.* 227, 365–375. doi: 10.1111/nph.16543

Fu, Z., Chen, H., Zhang, W., Xu, Q., Wang, S., Wang, K., et al. (2015). Subsurface flow in a soil-mantled subtropical dolomite karst slope: a field rainfall simulation study. *Geomorphology* 250, 1–14. doi: 10.1016/j.geomorph.2015.08.012

Gao, S., Cao, W., Zhou, G., and Rees, R. M. (2021). Bacterial communities in paddy soils changed by milk vetch as green manure: a study conducted across six provinces in South China. *Pedosphere* 31, 521–530. doi: 10.1016/S1002-0160(21)60002-4

Gao, X., He, Y., Zhang, T., An, Y., Sun, C., Xu, H., et al. (2022). Alfalfa green manure amendment improved P use efficiency and reduced P losses from paddy fields. *Nutr. Cycl. Agroecosyst.* 123, 35–47. doi: 10.1007/s10705-022-10195-4

Gao, X., Shi, D., Lv, A., Wang, S., Yuan, S., Peng, Z., et al. (2016). Increase phosphorus availability from the use of alfalfa (*Medicago sativa* L) green manure in rice (*Oryza sativa* L.) agroecosystem. *Sci Rep.* 6:36981. doi: 10.1038/srep 36981

Gao, X. L., Li, X. G., Zhao, L., and Kuzyakov, Y. (2019). Regulation of soil phosphorus cycling in grasslands by shrubs. *Soil Biol. Biochem.* 133, 1–11. doi: 10.1016/j.soilbio.2019.02.012

Garg, S., and Bahl G. (2008). Phosphorus availability to maize as influenced by organic manures and fertilizer P associated phosphatase activity in soils. *Bioresource Technol.* 99, 5773–5777. doi: 10.1016/j.biortech.2007.10.063

Haynes, R., and Mokolobate, M. (2001). Amelioration of Al toxicity and P deficiency in acid soils by additions of organic residues: a critical review of the phenomenon and the mechanisms involved. *Nutr. Cycl. Agroecosyst.* 59, 47–63. doi: 10.1023/A:1009823600950

He, H. B., Li, W. X., Zhang, Y. W., Cheng, J. K., Jia, X. Y., Li, S., et al. (2020). Effects of Italian ryegrass residues as green manure on soil properties and bacterial communities under an Italian ryegrass (*Lolium multiflorum* L.)-rice (*Oryza sativa* L.) rotation. *Soil Till. Res.* 196:104487. doi: 10.1016/j.still.2019.104487

He, Y. (2022). Effects of *Green Manure Inc*orporation on *Soil Nutrients, Bacterial Community Structure and Maize N and P use Efficiency in Yellow Soil of Guizhou*. Guiyang: Guizhou Minzu University.

Janssen, B. H. (1998). Efficient use of nutrients: an art of balancing. *Field Crop. Res.* 56, 197–201. doi: 10.1016/S0378-4290(97)00130-5

Karamesouti, M., and Gasparatos, D. (2017). *Adaptive Soil Management: From Theory to Practices*. Singapore: Springer Press, 189–214. doi: 10.1007/978-981-10-3638-5_9

Katsalirou, E., Deng, S., Gerakis, A., and Nofziger, D. L. (2016). Long-term management effects on soil P, microbial biomass P, and phosphatase activities in prairie soils. *Eur. J. Soil Biol.* 76, 61–69. doi: 10.1016/j.ejsobi.2016.07.001

Khan, M. I., Gwon, H. S., Alam, M. A., Song, H. J., Das, S., and Kim, P. J. (2020). Short term effects of different green manure amendments on the composition of main microbial groups and microbial activity of a submerged rice cropping system. *Appl. Soil Ecol.* 147, 103400. doi: 10.1016/j.apsoil.2019.103400

Lei, B., Wang, J., and Yao, H. (2022). Ecological and environmental benefits of planting green manure in paddy fields. *Agriculture* 12:223. doi: 10.3390/agriculture12020223

Li, F., Srensen, P., Li, X., and Olesen, J. E. (2020). Carbon and nitrogen mineralization differ between incorporated shoots and roots of legume versus non-legume based cover crops. *Plant Soil* 446, 243–257. doi: 10.1007/s11104-019-04358-6

Li, S. L., Xu, S., Wang, T. J., Yue, F. J., Peng, T., Zhong, J., et al. (2020). Effects of agricultural activities coupled with Karst structures on riverine biogeochemical cycles and environmental quality in the karst region. *Agr. Ecosyst. Environ.* 303:107120. doi: 10.1016/j.agee.2020.107120

Li, Y., Li, X., and Wang, B. (2016). Effects of four soil amendments on improving soil quality and acidity of yellow soils. *Earth Environ.* 44, 683–690. doi: 10.14050/j.cnki.1672-9250.2016.06.013

Li, Z., Xu, X., Liu, M., Li, X., Zhang, R., Wang, K., et al. (2017). State-space prediction of spring discharge in a karst catchment in Southwest China. *J. Hydrol.* 549, 264–275. doi: 10.1016/j.jhydrol.2017.04.001

Li, Z., Xu, X., Yu, B., Xu, C., Liu, M., Wang, K., et al. (2016). Quantifying the impacts of climate and human activities on water and sediment discharge in a karst region of southwest China. *J. Hydrol.* 542, 836–849. doi: 10.1016/j.jhydrol.2016.09.049

Liu, Z., Rong, Q., Zhou, W., and Liang, G. (2017). Effects of inorganic and organic amendment on soil chemical properties, enzyme activities, microbial community and soil quality in yellow clayey soil. *PLoS ONE* 12:e0172767. doi: 10.1371/journal.pone.0172767

Ma, D., Yin, L., Ju, W., Li, X., Liu, X., Deng, X., et al. (2021). Meta-analysis of green manure effects on soil properties and crop yield in northern China. *Field Crop. Res.* 266:108146. doi: 10.1016/j.fcr.2021.108146

Meena, B., Fagodiya, R., Prajapat, K., Dotaniya, M., Kaledhonkar, M., Sharma, P., et al. (2018). "Legume green manuring: an option for soil sustainability," in *Legumes for Soil Health and Sustainable Management*, eds R. S. Meena, A. Das, and G. S. Yadav (Berlin: Springer Press), 387–408. doi: 10.1007/978-981-13-0253-4_12

Meng, L., Zhang, A., Wang, F., Han, X., Wang, D., Li, S., et al. (2015). Arbuscular mycorrhizal fungi and rhizobium facilitate nitrogen uptake and transfer in soybean/maize intercropping system. *Front. Plant Sci.* 6:339. doi: 10.3389/fpls.2015.00339

Meng, X., Li, Y., Zhang, Y., and Yao, H. (2019). Green manure application improves rice growth and urea nitrogen use efficiency assessed using ^{15}N labeling. *Soil Sci. Plant Nutr.* 65, 511–518. doi: 10.1080/00380768.2019.1635872

Mitran, T., and Mani, P. K. (2017). Effect of organic amendments on rice yield trend, phosphorus use efficiency, uptake, and apparent balance in soil under long-term rice-wheat rotation. *J. Plant Nutr.* 40, 1312–1322. doi: 10.1080/01904167.2016.1267205

Mu, X., and Chen, Y. (2021). The physiological response of photosynthesis to nitrogen deficiency. *Plant Physiol. Bioch.* 158, 76–82. doi: 10.1016/j.plaphy.2020.11.019

Murungu, F. S., Chiduza, C., Muchaonyerwa, P., and Mnkeni, P. N. S. (2011). Decomposition, nitrogen and phosphorus mineralization from winter-grown cover crop residues and suitability for a smallholder farming system in South Africa. *Nutr. Cycl. Agroecosyst.* 89, 115–123. doi: 10.1007/s10705-010-9381-5

Pavinato, P. S., Rodrigues, M., Soltangheisi, A., Sartor, L. R., and Withers, P. J. A. (2017). Effects of cover crops and phosphorus sources on maize yield, phosphorus uptake, and phosphorus use efficiency. *Agron. J.* 109, 1039–1047. doi: 10.2134/agronj2016.06.0323

Pypers, P., Verstraete, S., Thi, C. P., and Merckx, R. (2005). Changes in mineral nitrogen, phosphorus availability and salt-extractable aluminium following the application of green manure residues in two weathered soils of South Vietnam. *Soil Biol. Biochem* 37, 163–172. doi: 10.1016/j.soilbio.2004.06.018

Qin, Z., Guan, K., Zhou, W., Peng, B., Villamil, M. B., Jin, Z., et al. (2021). Assessing the impacts of cover crops on maize and soybean yield in the U.S. *Midwestern agroecosystems. Field Crop. Res.* 273:108264. doi: 10.1016/j.fcr.2021.108264

Radicetti, E., Campiglia, E., Marucci, A., and Mancinelli, R. (2017). How winter cover crops and tillage intensities affect nitrogen availability in eggplant. *Nutr. Cycl. Agroecosyst.* 108, 177–194. doi: 10.1007/s10705-017-9849-7

Sinclair, T. R., and Vadez, V. (2002). Physiological traits for crop yield improvement in low N and P environments. *Plant Soil* 245, 1–15. doi: 10.1023/A:1020624015351

Tao, J., Liu, X., Liang, Y., Niu, J., Xiao, Y., Gu, Y., et al. (2017). Maize growth responses to soil microbes and soil properties after fertilization with different green manures. *Appl. Microbiol. Biot.* 101, 1289–1299. doi: 10.1007/s00253-016-7938-1

Toom, M., Tamm, S., Talgre, L., Tamm, I., Tamm, Ü., Narits, L., et al. (2019). The effect of cover crops on the yield of spring barley in Estonia. *Agriculture* 9:172. doi: 10.3390/agriculture9080172

Umar, W., Ayub, M. A., Zia-ur-Rehman, M., Ahmad, H. R., Farooqi, Z. U. R., Shahzad, A., et al. (2020). "Nitrogen and phosphorus use efficiency in agroecosystems," in *Resources use efficiency in agriculture*, eds S. Kumar, R. S. Meena, and M. K. Jhariya (Singapore: Springer press), 213–257. doi: 10.1007/978-981-15-6953-1_7

United States Department of Agriculture (2011). *Carbon to Nitrogen Ratios in Cropping* Systems. Washington, DC: United States Department of Agriculture.

Vyn, T. J., Faber, J. G., Janovicek, K. J., and Beauchamp, E. G. (2000). Cover crop effects on nitrogen availability to corn following wheat. *Agron. J.* 92, 915–924. doi: 10.2134/agronj2000.925915x

Wang, K., Zhang, C., Chen, H., Yue, Y., Zhang, W., Zhang, M., et al. (2019). Karst landscapes of China: patterns, ecosystem processes and services. *Landsc. Ecol.* 34, 2743–2763. doi: 10.1007/s10980-019-00912-w

Wang, Y., Huang, Q., Gao, H., Zhang, R., Yang, L., Guo, Y., et al. (2021). Long-term cover crops improved soil phosphorus availability in a rain-fed apple orchard. *Chemosphere* 275:130093. doi: 10.1016/j.chemosphere.2021.130093

Xiao, Y., Liu, X., Zhang, L., Song, Z., and Zhou, S. (2021). The allometry of plant height explains species loss under nitrogen addition. *Ecol. Lett.* 24, 553–562. doi: 10.1111/ele.13673

Xie, Z., Tu, S., Shah, F., Xu, C., Chen, J., Han, D., et al. (2016). Substitution of fertilizer-N by green manure improves the sustainability of yield in double-rice cropping system in south China. *Field Crop. Res.* 188, 142–149. doi: 10.1016/j.fcr.2016.01.006

Yang, L., Zhou, X., Liao, Y., Lu, Y., Nie, J., Cao, W., et al. (2019). Co-incorporation of rice straw and green manure benefits rice yield and nutrient uptake. *Crop Sci.* 59, 749–759. doi: 10.2135/cropsci2018.07.0427

Zandvakili, O. R., Ebrahimi, E., Hashemi, M., Barker, A. V., and Akbari, P. (2017). The potential of green manure mixtures to provide nutrients to a subsequent lettuce crop. *Commun. Soil Sci. Plant Anal.* 48, 2246–2255. doi: 10.1080/00103624.2017.1408819

Zhang, J., Nie, J., Cao, W., Gao, Y., Lu Y., and Liao Y. (2023). Long-term green manuring to substitute partial chemical fertilizer simultaneously improving crop productivity and soil quality in a double-rice cropping system. *Eur. J. Agron.* 142:126641. doi: 10.1016/j.eja.2022.126641

Zhang, L., Chu, Q., Zhou, J., Rengel, Z., and Feng, G. (2021). Soil phosphorus availability determines the preference for direct or mycorrhizal phosphorus uptake pathway in maize. *Geoderma* 403:15261. doi: 10.1016/j.geoderma.2021.115261

Zhang, Q., Song, Y., Wu, Z., Yan, X., Gunina, A., Kuzyakov, Y., et al. (2020). Effects of six-year biochar amendment on soil aggregation, crop growth, and nitrogen and phosphorus use efficiencies in a rice-wheat rotation. *J. Clean. Prod.* 242:118435. doi: 10.1016/j.jclepro.2019.118435

Zhang, Z., An, J., Xiong, S., Li, X., Xin, M., Wang, J., et al. (2022). *Orychophragmus violaceus*-maize rotation increases maize productivity by improving soil chemical properties and plant nutrient uptake. *Field Crop. Res.* 279:108470. doi: 10.1016/j.fcr.2022.108470

Zhou, G., Gao, S., Lu, Y., Liao, Y., Nie, J., Cao, W., et al. (2020). Co-incorporation of green manure and rice straw improves rice production, soil chemical, biochemical and microbiological properties in a typical paddy field in southern China. *Soil Till. Res.* 197:104499. doi: 10.1016/j.still.2019.104499

Zhu, B., Yi, L., Hu, Y., Zeng, Z., Lin, C., Tang, H., et al. (2014). Nitrogen release from incorporated ^{15}N-labelled Chinese milk vetch (*Astragalus sinicus* L.) residue and its dynamics in a double rice cropping system. *Plant Soil* 374, 331–344. doi: 10.1007/s11104-013-1808-8

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Crop–livestock-integrated farming system: a strategy to achieve synergy between agricultural production, nutritional security, and environmental sustainability

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Introduction: Climate change, nutritional security, land shrinkage, and an increasing human population are the most concerning factors in agriculture, which are further complicated by deteriorating soil health. Among several ways to address these issues, the most prominent and cost-effective means is to adopt an integrated farming system (IFS). Integrating farming systems with livestock enables a way to increase economic yield per unit area per unit of time for farmers in small and marginal categories. This system effectively utilizes the waste materials by recycling them via linking appropriate components, thereby minimizing the pollution caused to the environment. Further integrating livestock components with crops and the production of eggs, meat, and milk leads to nutritional security and stable farmer's income generation. So, there is a dire need to develop an eco-friendly, ecologically safe, and economically profitable IFS model.

Methods: An experiment was conducted to develop a crop–livestock-based integrated farming system model for the benefit of irrigated upland farmers in the semi-arid tropics for increasing productivity, farm income, employment generation, and food and nutritional security through efficient utilization of resources in the farming system.

Results and discussion: The IFS model has components, viz., crop (0.85 ha) + horticulture (0.10 ha) + 2 cattles along with 2 calves in dairy (50 m^2) + 12 female goats and 1 male goat (50 m^2) + 150 numbers of poultry birds (50 m^2) + vermicompost (50 m^2) + kitchen garden (0.02 ha) + boundary planting + supporting activities (0.01 ha) in a one-hectare area. The model recorded a higher total MEY (162.31 t), gross return (689,773), net return (317,765), and employment generation (475 mandays). Further negative emissions of −15,118 CO_2-e (kg) greenhouse gases were recorded under this model. The study conclusively reveals that integration of crop, horticulture, dairy, goat, poultry, vermicompost production, kitchen garden, and boundary planting models increases the net returns, B:C ratio, employment generation, nutritional security, and livelihoods of small and marginal farmers.

KEYWORDS

employment generation, greenhouse gas emission, nutritional security, profitability, sustainability

1 Introduction

Agriculture and livestock are the key contributors to a country's economy and agricultural occupation. However, changing climate and erratic distribution of rainfall negatively affect the agriculture and livestock sectors. Since the 1980s, climate change has already reduced crop yields by about 5%—10% in notable areas (Iizumi and Ramankutty, 2016). The overall production of maize, rice, wheat, and soya is projected to fall to 9% by 2030 and 23% by 2050 worldwide (Haile et al., 2017). Major coastal areas with their high levels of agriculture are currently prone to be mainly affected; this includes Bangladesh and Vietnam, and drought-prone locations such as large parts of East and West Africa, Morocco, and parts of South Asia and China. This not only damages agriculture but also has a huge economic impact since climate change leads to fluctuating food prices, which have an indirect negative effect on production as these fluctuations signal risk and reduce incentives to invest in agriculture. At present, employment in agriculture has been drastically reduced, and there is practically no scope for horizontal growth of land for food production; which is mostly owing to the ever-increasing human population and diminishing land resources. To ensure that farm households receive a sufficient amount of money on a regular basis, only vertical expansion is feasible by integrating appropriate farming components that demand less area and time. Further global warming is a major threat to humans for sustainable food production. Approximately 20% of the yearly contribution of carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O) gas emissions is from the agriculture sector. So, it is necessary to develop alternative strategies to mitigate GHG emissions as well as to improve the production and productivity of crops. Studies conducted in Indonesia on reducing greenhouse gas emissions by adopting IFS practices also show promising results (Munandar et al., 2015).

Integrated farming system (IFS) with livestock is one of the traditional practices in rural India among farmers of small and medium categories. It is very important for farmers to diversify their crops with dairy, goatery, fishery, poultry, duckery, and so on to increase income (Ray et al., 2012). The holdings should be evenly distributed compared to land holdings among the farmers (Kochewad et al., 2017). An IFS places an emphasis on recycling farm wastes and reducing the hurdles of their management in an effective and useful way. This can be achieved by making various components of the farming system work together for higher total productivity than individual productions. The wastes/and by-products from one enterprise are used as inputs for another to enhance productivity and lower the cost of production. With India's shifting agrarian situation, the IFS appears to be a viable option. Overall, an IFS achieves numerous goals, including helping farmers become self-sufficient by ensuring that family members have balanced food, raising living standards by increasing employment and total net income, reducing risk and uncertainty, and maintaining environmental harmony (Mali et al., 2014). Furthermore, IFS is considered to be the most viable and efficient option in Southeast Asian regions, especially for enhancing the productivity of small and marginal farmers; similarly, IFS experiments conducted throughout various countries also showed a positive impact on farm productivity. In Indonesia, it has been found that the IFS integration of corn/horticulture-cow, supported by irrigation ponds, can increase income from IDR 4,094,000 in 2005 to IDR 9,696,300 in 2008, an increase of 136.84 percent (Adijaya et al., 2009). Further experiments with integrated farming systems in the Philippines and Thailand also registered higher yields with improved net returns. In Bangladesh, the integration of different components such as crops (0.65 ha), vegetable farms (0.12 ha), other crops fields (0.09 ha), and a homestead (0.08 ha) with 2.17 numbers of cattle and 11.69 numbers of poultry birds recorded higher profitability (Uddin and Takeya, 2006).

In African countries, integrated farming systems can contribute positively to increasing the productivity of agricultural systems and improving the overall biomass and ecosystem (Duncan et al., 2013). Erick et al. (2013) concluded in their research that livestock-based integration not only helps agriculture but also helps mitigate the most important problems in Africa, such as food security and malnutrition in both animals and humans. This was supported by the findings of Ezeaku et al. (2015). Similarly, results were also observed by Ugwumba et al. (2010) in crop + livestock IFS in the Awka South Agricultural Zone of Anambra State, Nigeria. Integrated crop and livestock systems in Ghana, West Africa, improve overall farm productivity and income through effective recycling of available resources on the farm (Asante et al., 2020). Othman (2006) revealed that adopting IFS increased food production and enhanced economic profitability through the integration of different allied enterprises into the ecosystem. Hence, IFS experiments were conducted to improve productivity, farm income, employment generation, nutritional food security, and reduce GHG emissions for the livelihood improvement of small and marginal farmers in irrigated upland.

2 Materials and methods

2.1 Site description and prevailing weather conditions

The experiments were conducted on the irrigated upland of Tamil Nadu Agricultural University, Coimbatore, from 2017 to 2022. The experimental site was located at 10°12′ and 11°24′ N latitude and between 76°39′ and 77°30′ E longitude of 426.7 m above MSL. The experimental site has a tropical wet and dry

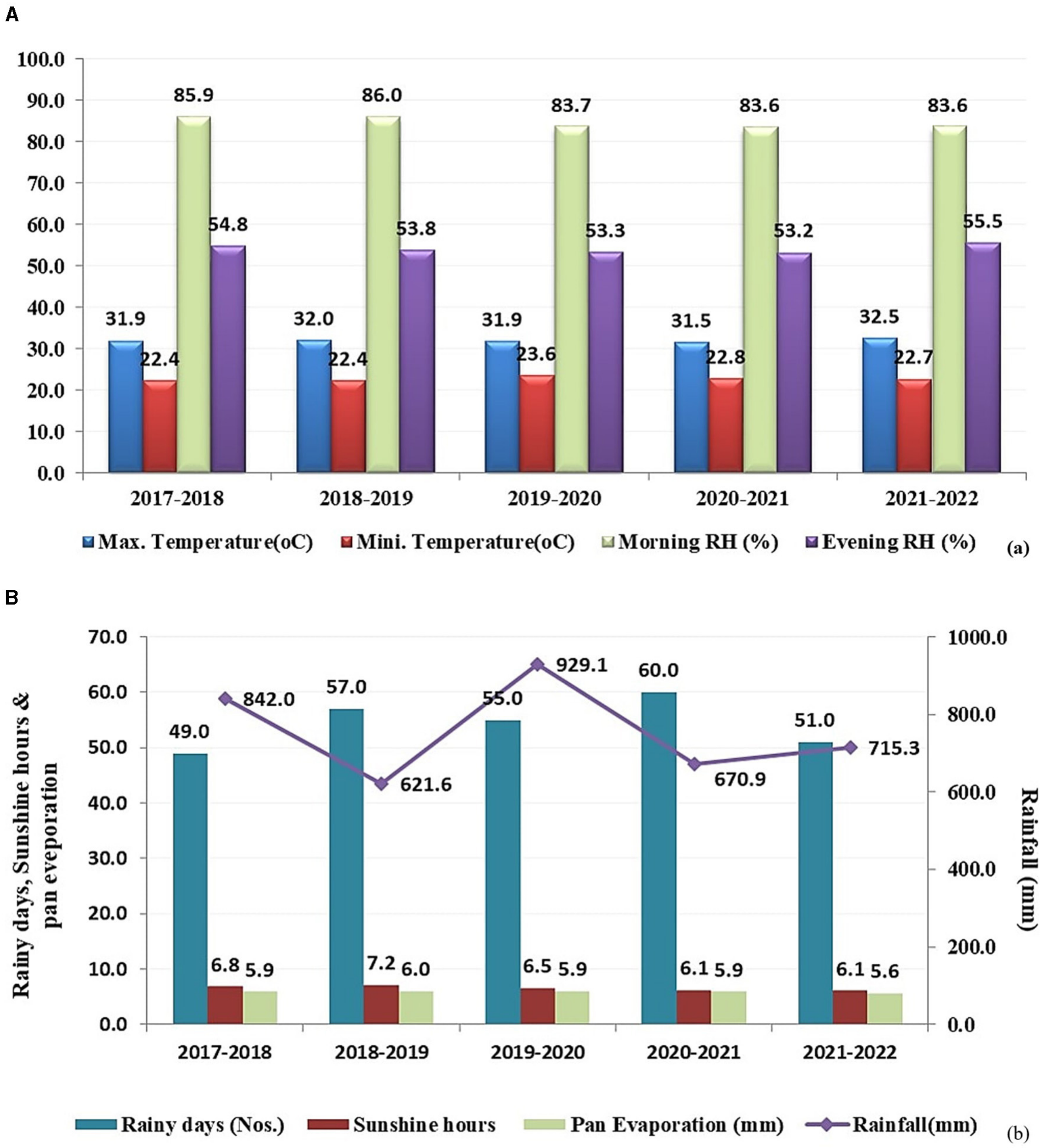

FIGURE 1
(A) Mean meteorological weather data (maximum, maximum temperature and morning, evening relative humidity) for the crop season from 2017 to 2022. **(B)** Mean meteorological weather data (rainy days, sunshine hours, pan evaporation, and rainfall) for the crop season from 2017 to 2022.

climate. During the study, the mean maximum and minimum temperatures were 32.0°C and 22.8°C, respectively. Similarly, the mean morning and evening relative humidity were 84.6% and 54.1%, respectively. A total rainfall of 3,778.9 mm was recorded with 272 rainy days (Figures 1A, B). A mean sunshine hour of 6.5 and a mean evaporation of 5.9 mm were also registered during the cropping period.

2.2 Soil characteristics

The soil of the experimental field was clay loam in texture, with the underlying Periyanaikenpalayam soil series having a bulk density (mg m^{-2}) of 1.24. The soil was alkaline (8.49), medium in organic carbon (0.60), medium in available N (250 kg/ha), medium in P_2O_5 (21.9 kg/ha), and high in K_2O (624 kg/ha).

2.3 Enterprises details

The IFS model was established during 2017–2018 in a 1.0 ha area. Six components were included, viz., crop (0.85 ha) + horticulture (0.10 ha) + 2 cattles along with 2 calves in dairy (50 m^2) + 12 female goats and one male goat (50 m^2) + 150 numbers of poultry birds (50 m^2) + kitchen garden (200 m^2) + boundary planting + vermicompost (50 m^2) + value addition. In addition to

TABLE 1 Details of the crops and components integrated into the model.

Components	Area (m^2)	Details				
Crop	8,500		*Kharif*	*Rabi*	*Summer*	*Area* (m^2)
		CS I	Cowpea (VBN 3)	Ragi (CO 15)	Dhaincha	1,500
		CS II	Maize (COH(M) 6)	Sunflower (SF hybrid CO 2)	Dhaincha	1,500
		CS III	Prosomillet CO (PV) 5	Chillies (Samba)	Dhaincha	1,500
		CS IV	Pearl millet CO (Cu) 10	Cotton (TCH1819)	Dhaincha	1,500
		Fodder unit	Bajra Napier grass (CO BN 5) and *Desmanthus* (CO 1)			1,500
			Grazing unit: *Cenchrusciliaris* (CO 2) and fodder trees			1,000
Horticulture	1,000	Fruit trees: Sapota PKM 2 and PKM 1 (3:1), Guava (Lucknow 49), Amla NA7 and BSR1 (4:1), Pomegranate (Bhaguva)				
Dairy (2 + 2)	50	Native breeds (Gir and Kankrej)				
Goat rearing (12 + 1)	50	Native breed (Salem black)				
Poultry (150 birds)	50	Desi poultry birds (Aseel) for meat purposes (50 per batch; 3 batches per year)				
Vermicompost	50					
Kitchen garden	200	Vegetables and greens				
Border planting		Annual moringa (PKM 1), curry leaf (Senkambu), agathi, *Gliricidia sepium*				
Areas for supporting activities	100	Manure pit, fodder chopping unit, bakery unit, *etc.*				
Total	10,000					

this, a 100 m^2 area was allotted for supporting activities (100 m^2) (Table 1). A benchmark survey of 150 farmers from three districts (Erode, Salem, and Coimbatore) in the western zone of Tamil Nadu has been taken as the data for conventional farming systems.

2.3.1 Cropping system, horticultural, kitchen garden, and border planting

In the cropping component, five cropping systems were included in a total area of 0.85 ha. After assessing the fodder needs of the dairy and goat components in terms of green fodder, dry fodder, and concentrated feed requirements, the promising fodder components were identified and included in the cropping system. Considering the annual green fodder requirement of the dairy and goat units, an area of 0.25 ha was allotted for the Bajra Napier grass (CO (BN) 5) and leguminous *Desmanthus virgatus* (CO1) to get a year-round green fodder supply. To supplement the fodder requirement during lean summer months, *Sesbania grandiflora* (Agathi) and *Gliricidia sepium* were raised along the field boundary. To effectively utilize the field boundaries, annual moringa, curry leaf, and agathi were planted. In the horticultural component fruit trees, viz., sapota, guava, amla, and pomegranate, were planted (Table 2). Intercropping with vegetables like bottle guard, pumpkin, snake guard, and bitter guard was raised in between the space of fruit trees to reduce evaporation losses, weed population, and gain additional income. In the kitchen garden, vegetables (cluster beans, brinjal, radish, lablab, bhindi, and tomato) and greens (fenugreek, coriander, amaranthus, and palak) were raised to meet the nutritional balance of members. A recommended package of practices was followed for agricultural, horticultural crops, and border planting. The surface irrigation system was used to irrigate both horticultural and agricultural crops. Weeds were managed by spraying pre-emergence herbicides. Pests and diseases were controlled by spraying insecticides and seed treatments with biocontrol agents.

2.3.2 Dairy

The dairy unit was maintained with two desi-breed cows (Gir and Kankrej) and two calves. For the feeding schedule, 25 kg of green fodder, 7 kg of dry fodder, and 3.5 kg of concentrate feed were fed daily. FMD vaccinations were administered every 6 months, and artificial insemination was used to reproduce. Deworming was done at a 2-month interval. Milk obtained from the dairy animals was sold regularly. Cow dung obtained from the unit is weighed regularly and used for vermicompost production.

2.3.3 Goat

In the goat unit, Salem black (12 female and 1 male) was maintained on an elevated platform raised 7–8 feet above ground level with good ventilation. In the feeding schedule, 3.5 kg of grass fodder, 2.0 kg of legume fodder, and 1.5 kg of tree fodder were fed. The green grass and dry fodder obtained from crop components and border planting were recycled and utilized as feed for animals. The feed was given at 1.0%—1.5% of their body weight. In addition to this, to maintain health and improve the digestive system, the goats were allowed to graze for 2–3 h in *Cenchrus ciliaris* grazing

TABLE 2 Adopted crops, varieties, and fertilizer schedule for cropping system.

Cropping system	*Kharif*			*Rabi*			Summer	
	Crop and variety	Seed rate (kg/ha)	Fertilizers (N, P_2O_5 and K_2O kg/ha)	Crop and variety	Seed rate (kg/ha)	Fertilizers (N, P_2O_5 and K_2O kg/ha)	Crop and variety	Seed rate (kg/ha)
Cowpea (G)-ragi-G.Manure (*Dhaincha*)	Cowpea VBN 3	25	25:50:25	Ragi CO 15	20	40:20:20	Dhaincha	40
Maize-sunflower-dhaincha	Maize COH (M) 6	20	250:75:75	Sunflower SF hybrid CO 2	5	60:90:60	Dhaincha	40
Prosomillet-chillies-dhaincha	Prosomillet CO (PV) 5	8–12	60:30:20	Chillies samba	41,667 Seedlings	120:60:30	Dhaincha	40
Pearlmillet-cotton-dhaincha	Peralmillet CO (Cu) 10	5	70:35:35	Cotton TCH 1819	10	80:40:40	Dhaincha	40
Perennial fodder grass and leguminous fodder: Grazing area-Cenchrus	Bajra Napier Grass (CO 5), *Desmanthus* (CO 1), Cenchrus (CO 1), fodder trees				Staggered daily harvest depending on requirement			
Border planting	Curry leaf (Senkambu), annual moringa (PKM 1), Agathi (Local), Gliricidia				Periodical			

area. Vaccination for FMD was done once every 6 months and for enterotoxemia once a year, respectively. Furthermore, deworming was given at the 1st month of age and then once a month up to 6 months of age. After attaining a body weight of 15–20 kg, goats were sold periodically.

2.3.4 Poultry

In poultry rearing, 150 Assel desi birds were reared with a deep litter system. The litter was spread to a depth of 5 cm on the floor before introducing chicks. The litter was stirred once a week. The vaccination was done as per the standard schedule. The poultry bird was fed with a combination of nutrients of carbohydrate, protein, fiber, and mineral salts. Grains such as maize, ragi, prosomillet, cumbu, and cowpea obtained from the IFS are grained in the required proportion and used as feed. After attaining a body weight of 2 kg, the birds were sold.

2.3.5 Vermicompost production

A vermicompost shed was constructed in a 50-m^2 area, and earthworms were released at 1 kg/m^2. For effective utilization of resources, the animal waste from the dairy and goat units, along with the unutilized feed wastes and crop residues or by-products, were collected for preparing vermicompost. Periodical harvesting of vermicompost was used for agricultural, horticultural, and kitchen gardens.

2.4 Observations recording

2.4.1 Productivity

The system was analyzed by quantifying productivity and income to what extent. The productivity of various allied enterprises was converted to maize grain equivalent yield (MEY) based on farm price.

$$\text{Maize equivalent yield (MEY)} = \frac{\text{Yield of a crop} \times \text{Market value of the crop}}{\text{—Market value of maize}}$$

2.4.2 Economics

The cost of inputs and the price of produce at prevailing market rates were considered for working out the cost of cultivation, returns, and B:C ratio as below.

2.4.2.1 Net return

Net returns were obtained by subtracting the cost of cultivation from the gross return for each component.

$$\text{Net return} = \text{Gross return } - \text{Cost of cultivation}$$

2.4.2.2 Benefit-cost ratio (BCR)

The benefit-cost ratio was worked out using the following formula.

$$\text{Benefit-cost ratio (BCR)} = \frac{\text{Total cost of cultivation}}{\text{Gross return}}$$

TABLE 3 Productivity of different cropping systems in IFS.

Cropping system	Land allocation for cropping sequences (m^2)	MEY (t./ha)					Mean
		2017–2018	2018–2019	2019–2020	2020–2021	2021–2022	
Cowpea-ragi-dhaincha	1,500 (17.65%)	1.67	1.74	1.65	1.57	1.37	1.60
Maize-sunflower-dhaincha	1,500 (17.65%)	2.04	1.9	1.81	1.97	1.52	1.85
Proso millet-chillies-dhaincha	1,500 (17.65%)	1.94	2.08	1.83	1.85	1.59	1.86
Pearl millet-cotton-dhaincha	1,500 (17.65%)	1.96	1.83	1.67	1.64	1.43	1.71
BN hybrid grass and desmanthus	1,000 (29.41%)	4.19	5.4	5.05	2.54	1.68	3.77
Total	8,500 (100%)	11.8	12.95	12.01	9.57	7.59	10.78

MEY, Maize equivalent yield.

2.4.3 Greenhouse gas emissions

The information was calculated using previously forecasted values, fertilizer, machinery, and chemical usage for various crops. For easier comparison, the greenhouse gas emissions from various cropping sequences and other components were converted to carbon dioxide equivalents between components.

$$\text{Emission} = \text{A} \times \text{EF}$$

were,

Emission = Annual emissions in units of kg of CO_2 eq. per farm.

A = Activity data (kg of N used, liters of fuel used, etc.).

EF-Emission factor = IPCC default emission factors or country-specific emission factors.

The IPCC uses CO_2 as a reference gas; hence, all other GHGs are converted into carbon dioxide equivalents to ensure uniformity.

2.4.4 Data interpretation

The production data of different enterprises for five consecutive years were presented year-wise and average under respective parameters. The conventional farming system (crop + horticulture + livestock) was considered as a control to compare with the integrated farming system.

$$(\text{Crop} \leftrightarrow \text{Horticulture} \leftrightarrow \text{Dairy} \leftrightarrow \text{Poultry} \leftrightarrow \text{Vermicomposting})$$

The difference between the two farming systems was expressed as a fold change (conventional farming systems were considered as 1).

3 Results and discussion

3.1 Productivity and economics of crop components

The ever-increasing demand for food grain production requires crop diversification, which is essential to increasing productivity, profitability, and sustainability. In this study, a higher maize equivalent yield of 1.86 t in a 1,500 m^2 area was obtained from prosomillet-chillies-dhaincha (Tables 3, 4). This might be due to the rooting systems of the crops; the shallow and fibrous root systems of the prosomillet and deep root systems of the chillies enable for better uptake of nutrients from the top and bottom layers of the soil in their consecutive seasons of cropping. Prosomillet is included in the cropping system for its nutritional content and significance. At present, millet is considered an important source of energy and protein for African and Asian countries (Amadou et al., 2013), and considering its nutritional superiority and climate-resilient features of prosomillet, it can be a better climate-smart alternative to the predominant cereals (Rajasekaran et al., 2023).

Similarly, vegetable-based cropping systems like prosomillet-chillies-dhaincha will provide year-round production with additional revenue, thereby guaranteeing nutritional security (Arti et al., 2019). This result was followed by maize-sunflower-dhaincha (1.85 t.). Integration of maize-sunflower cropping in

TABLE 4 **Economics of different cropping systems in IFS.**

Cropping system	Mean over 5 years (2017–2022)			
	Gross return ₹	Cost of Production ₹	Net return ₹	B:C ratio
Cowpea-ragi-dhaincha	34,554	17,085	17,469	2.02
Maize-sunflower-dhaincha	39,451	19,895	19,556	1.98
Proso millet-chillies-dhaincha	51,768	23,619	28,149	2.19
Pearl millet-cotton-dhaincha	39,384	17,528	21,842	2.25
BN hybrid grass and desmanthus	77,976	41,791	36,185	1.87
Total	243,133	119,918	123,215	2.03

TABLE 5 **Productivity of different farm enterprises in the IFS model.**

Components	MEY (t./ha)					
	2017–2018	2018–2019	2019–2020	2020–2021	2021–2022	Mean
Cropping	11.8	12.95	12.01	9.57	7.59	10.78
Horticulture	0.53	0.71	1.01	1.8	1.21	1.05
Dairy	12.55	13.07	3.44	3.81	2.96	7.17
Goat	6.36	7.58	4.3	3.81	3.06	5.02
Poultry	2.56	1.88	2.59	2.78	2.15	2.39
Vermicompost	3.06	6.71	2.56	4.61	3.78	4.14
Kitchen garden	0.83	0.87	0.57	0.7	0.62	0.72
Boundary planting	1.07	1.22	1.1	1.37	1.16	1.18
Total	38.76	44.99	27.58	28.45	22.53	32.46
Conventional (crop + horticulture + livestock)	19.04	19.12	19.87	20.05	20.19	19.7
Fold change (times)	+2.04	+2.35	+1.39	+1.42	+1.12	1.65

the IFS system plays a pivotal role in animal nutrition. The maize grain and sunflower oil cake produced from the cropping system can be utilized as concentrate feed for livestock and feed for poultry, thus reducing the overall cost of production via better recycling. Experiments conducted in southern Africa revealed that maize-sunflower rotation produced significantly higher yields (Thierfelder et al., 2013). Furthermore, Shanmugam and Ramamoorthy (2014) in their study concluded that the maize-sunflower cropping system in a sericulture-based integrated farming system increased the productivity of maize as well as that of sunflowers with enhanced net return and B:C ratio. Similarly, crop rotation with dhaincha significantly improved the yield. This can be attributed to the efficient and adequate nutrients supply from dhaincha biomass decomposition and the released nutrients for the crop (Sarwar et al., 2017).

The overall crop productivity of 10.78 t. recorded in 8,500 m^2. Similarly, Meena et al. (2022) revealed that crop diversification with multiple crops like sugarcane-ratoon and sugarcane-wheat registered a higher sugarcane equivalent yield of 26.83 t./year. Further conventional farming systems, or monocropping, resulted in lower productivity and economic returns. Monocropping with rice is less economical when compared with a diversified cropping system. This is mainly due to the fact that the cultivation of a larger number of crops on the same piece of land has a synergistic impact on each other and compensates for nutrients requirements with minimal environmental risk (Mahapatra and Behera, 2011). Based on the economics of the cropping component, the highest net return of/28,149/ha was recorded with prosomillet-chillies-dhaincha with a B:C ratio of 2.19; however, pearlmillet-cotton-dhaincha recorded a higher B:C ratio of 2.25 with a net return of/21,842/ha. In the fodder system, Bajra Napier hybrid grass, Desmanthus, and Cenchrus recorded a net return of/36,185/ha with a B:C ratio of 1.87.

3.2 Productivity

The productivity of the various components in the integrated farming system was converted into maize equivalent yield (Table 5). Among the different enterprises, crop components alone recorded a higher maize equivalent yield (10.78 t./ha), followed by dairy (7.17 t./ha), goat (5.02 t./ha), vermicompost (4.14 t./ha), poultry (2.39 t./ha), boundary planting (1.18 t./ha), horticulture (1.05 t./ha), and kitchen garden (0.72 t./ha). Higher productivity was due to residue recycling, increased nutrient addition from vermicompost, and the incorporation of green manure in the

TABLE 6 **Profitability of different farm enterprises in the IFS model.**

Components	Total cost/	Gross return/	Net return/	B:C	Net return contribution (%)
Cropping	103,337	209,435	106,098	2.03	33.39
Horticulture	7,309	22,815	15,506	3.12	4.88
Dairy	125,261	155,501	30,240	1.24	9.52
Goat	49,208	122,687	73,479	2.49	23.12
Poultry	26,600	49,598	22,998	1.86	7.24
Vermicompost	49,964	89,880	39,916	1.80	12.56
Kitchen garden	5,196	14,801	9,605	2.85	3.02
Boundary planting	5,133	25,056	19,923	4.88	6.27
Total	372,008	689,773	317,765		100
Conventional (crop + horticulture + livestock)	164,231	266,236	102,005	1.62	
Fold change (times)	+2.27	+2.59	+3.12		

B:C, Benefit-cost ratio.

summer, which improved soil fertility and subsequently increased crop productivity. A higher total rice equivalent yield of 20.83 t. was recorded due to the application of organic manures, viz., farmyard manure, vermicompost, and *azolla* by recycling, which might have contributed to better crop productivity in IFS (Porpavai and Marimuthu, 2018). The total productivity of the developed IFS was 162.31 t./ha, which was higher when compared with the conventional farming system (crop+horticulture+livestock) (98.27 t./ha). This result is in agreement with the findings of Goverdhan et al. (2020), who opined that integration of crop+horticulture+dairy+goat+vermicompost+backyard poultry fowl recorded higher productivity (26.58 t. of total maize equivalent yield), which was higher than conventional farming systems.

3.3 Economics

Gross return, net return, production cost, and B:C ratio were worked out as individual components in the IFS model. A total gross return of ₹689,773/- and a net return of ₹317,765/- were recorded with the IFS model. The integrated farming system, which includes crops, livestock, poultry, and fisheries, provided a net return of ₹189,069/ha/year as opposed to ₹74,552/ha/year in conventional cotton, which was 2.5 times less than the IFS system (Vinodakumar et al., 2017; Paramesh et al., 2022). This might be due to the inclusion of livestock in the system, which produced higher returns for the farmer.

Among the different components, cropping recorded a higher net return of ₹106,098/- (33.39%) (Table 6). This was followed by a goat, which recorded a net return of ₹73,479/- (23.12%), and the cost of production was lower when compared with the dairy unit. Dairy recorded a net return of ₹30,240/-. This was mainly due to reduced production costs. Expenditure was reduced in the farming system mainly due to the reduction of fodder costs since fodder was grown on the farm itself and fed to animals and birds. Besides, vermicompost produced from the waste was well utilized for the production of fodder. Maintaining goats as an allied enterprise generated year-round income for the farmers with less investment. The goat component contributed to 33.1% of the total gross income. Next to goat, vermicompost production recorded a net return of ₹39,916/-, which contributed about 12.56%. For effective utilization of resources, the animal waste from dairy and goat units, along with the unutilized feed and crop residues/and by-products was used for the production of vermicompost. This provided an excellent opportunity for organic recycling and reduced farmers' dependency on externally purchased inputs.

The IFS models with coconut + goat + cow + turkey + *azolla* + vermicompost recorded a higher gross return, net return, and B:C ratio compared to conventional cultivation (Thavaprakaash and Premavathi, 2019). Similarly, in the crop + dairy + poultry + vermicompost + *azolla* IFS model with a net return of ₹10,525/-, the dairy unit contributed a higher net income of 9.52% due to the supply of balanced nutrition by green and dry fodder produced in the system (Shankar et al., 2017). The dairy component produced a considerably higher net return of ₹79,476/- in the IFS model with crop + dairy + poultry + fishery (Singh et al., 2007). Kumara et al. (2017) and Vinodakumar and Desai (2017) reported that the IFS model in a 1-ha area with crop + horticulture + dairy (one buffalo + two HF cows) + sheep (10 + 1) + vermicompost + *azolla* produced a higher net return from crops (₹80,795/-), followed by horticulture (₹38,526/-), dairy (₹47,278/-), and sheep unit (₹17,876/-).

The poultry component also recorded a considerable net return of ₹22,998/-, with a share of 7.24%. This was mainly due to reduced production costs from reduced purchases of feed from outside markets. The grains such as maize, cumbu, and prosomillet obtained from the farming system are used as feed for poultry. This will create interest among the farmers in rearing poultry as a component of the IFS model. The meat and eggs obtained from the poultry unit generate additional income for the farmers. Similarly, Kumar et al. (2017) reported that the integration of crops with poultry, goats, and mushrooms was more suitable for achieving better income for the farmers.

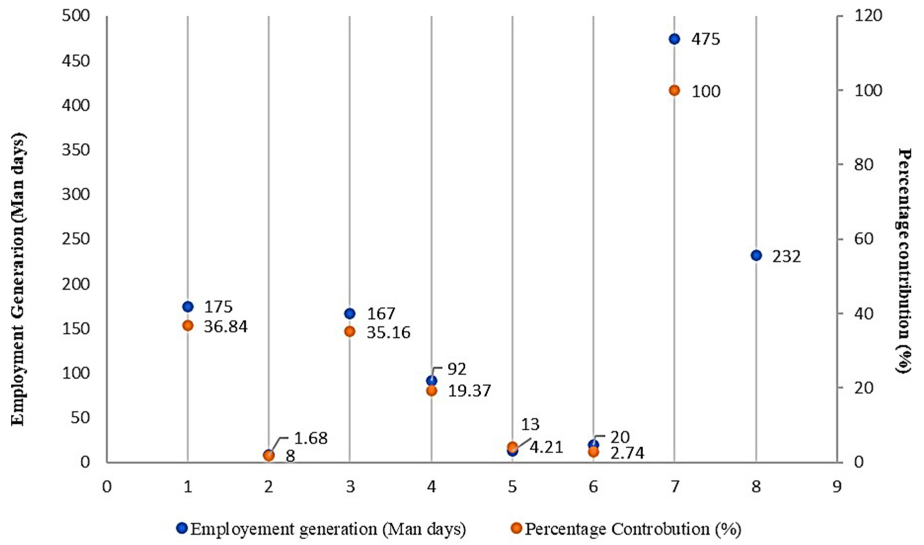

FIGURE 2
Employment generation and percentage contribution in different enterprises of IFS model.

Boundary planting, horticulture, and the kitchen garden generated a net return of ₹19,923/-, 15,506/-, and 9,605/-, respectively. Integrating horticulture, the kitchen garden, and boundary planting are the sources for increasing family income and the nutritive values of foods and fodder.

The conventional farming system with crop + horticulture + livestock in 1-ha area of the farm recorded a lower gross return (₹266,236/-), a lower net return (₹102,005/-), and a lower B:C ratio (1.62). Lower economic returns in the conventional farming system are mainly due to minimum component integration. Dey et al. (2010) and Sachinkumar et al. (2012) have reported similar findings.

3.4 Employment generation and percentage contribution

On employment generation, cropping activity in the IFS model generates 175 mandays of employment, and multi-enterprise included in the farming system generates 300 mandays of employment with even distribution over the course of the year (Figure 2). Total employment generation from the IFS model was 475 mandays, which was higher when compared with the conventional farming system (232 mandays). This was primarily due to the diversified nature of the numerous activities related to various allied enterprises included in integrated farming systems, which offer a lot of employment opportunities, keep farmers and their families engaged for a longer period of time, and help to improve employment for rural poor people. Integration of cropping with different enterprises such as dairy, mushroom, and biogas recorded higher employment generation (875 mandays) (Sivamurugan, 2001). Combining crops with other enterprises such as horticulture + goat + poultry increases labor requirements, which provide scope to employ more labor throughout the year without giving breaks in the lean period as in conventional agriculture (Ravisankar et al., 2007; Kumar et al., 2012). Sharma et al. (2017) observed that crop + horticulture + kitchen garden + dairy + goat + poultry + duck + vermicompost in irrigated conditions of Chhattisgarh recorded higher employment generation for 1,033 mandays for 1.5 ha area and also in gardenland (Siddeswaran et al., 2012). This is mainly due to the diversified nature of multifarious activities related to different enterprises included in the integrated farming system.

3.5 Food and nutritional security

The integrated farming system fulfills the basic family needs of cereals, pulses, oil, fruits, vegetables, and proteins through milk and meat, which improves the family income status and maintains balanced nutrition for the farm family members. It also alleviates malnutrition deficiencies in rural areas. In this study, adopting an integrated farming system (Table 7) with different components produced 1,679 kg of cereals, 1,918 kg of vegetables, 1,497 L of milk, and 300 kg of meat, which is more than sufficient for an average farm family of six members. Further production of green fodder (46,624 kg) and dry fodder (2,434 kg) was also used as feed for livestock units such as cattle and goats, which reduced the production cost considerably in livestock rearing. IFS strengthens food and nutritional security more than conventional systems. A field experiment conducted in Modipuram reported that integrating crops with dairy, fish, and boundary planting produced 3,040 kg of cereals, 289.9 kg of pulses, 4,228 L of milk, 5,560 kg of fruits, and 2,960 kg of vegetables (Panwar et al., 2019). Higher nutritional security was achieved by producing 1,738 kg of cereals, 239 kg of pulses, 283 kg of oilseeds, 5,902 L of milk, 162 kg of fruits, 1,581 kg of vegetables, 675 kg of eggs, 26,560 kg of green fodder, 3,761 kg of dry fodder, and 810 kg of fuel wood by adopting crops with dairy, horticulture, goat, poultry, and biocompost in a 1.45-ha area (Ravisankar et al., 2019).

TABLE 7 **Livelihood assessment of the integrated farming system model.**

Farm enterprises	Production (year-wise)					Average (5 years)	Requirement/family (6 members)	Surplus for market sale
	2017–2018	2018–2019	2019–2020	2020–2021	2021–2022			
Crop								
Cereals (kg)	2,030	1,704	1,618	1,573	1,470	1,679	1,550	129 (+)
Pulses (kg)	221	205	208	195	185	202.8	200	2.80 (+)
Oilseeds (kg)	275	282	376	226	206	273	130	143 (+)
Green fodder (kg)	51,500	67,866	65,301	27,428	21,024	46,624	-	46,624 (+)
Dry fodder (kg)	1,124	3,222	2,367	2,385	3,072	2,434	-	2,434 (+)
Horticulture								
Vegetables (kg) (intercrop)	150	126	289	654	548	353.4	-	
Fruits (kg)	532	275	593	443	890	546.6	200	346.6
Livestock								
Milk (L)	3,845	2,485	265	458	434	1,497	1,120	377 (+)
Goat meat (kg)	94	198	196	114	116	144	-	144 (+)
Poultry								
Poultry meat (kg)	192	183	145	136	125	156	-	156 (+)
Boundary planting								
Vegetables (kg)	560	668	600	716	857	680.2	-	
Green fodder (kg)	1,110	1,165	1,087	1,327	1,101	1,158	-	1,158 (+)
Kitchen garden								
Vegetables (kg)	1,110	1,213	657	734	709	884.6	-	
Total vegetables (kg)						1,918.2	900	1,018.2 (+)
Vermicompost (kg)	1,050	10,844	9,047	9,215	9,456	7,922	-	7,922 (+)

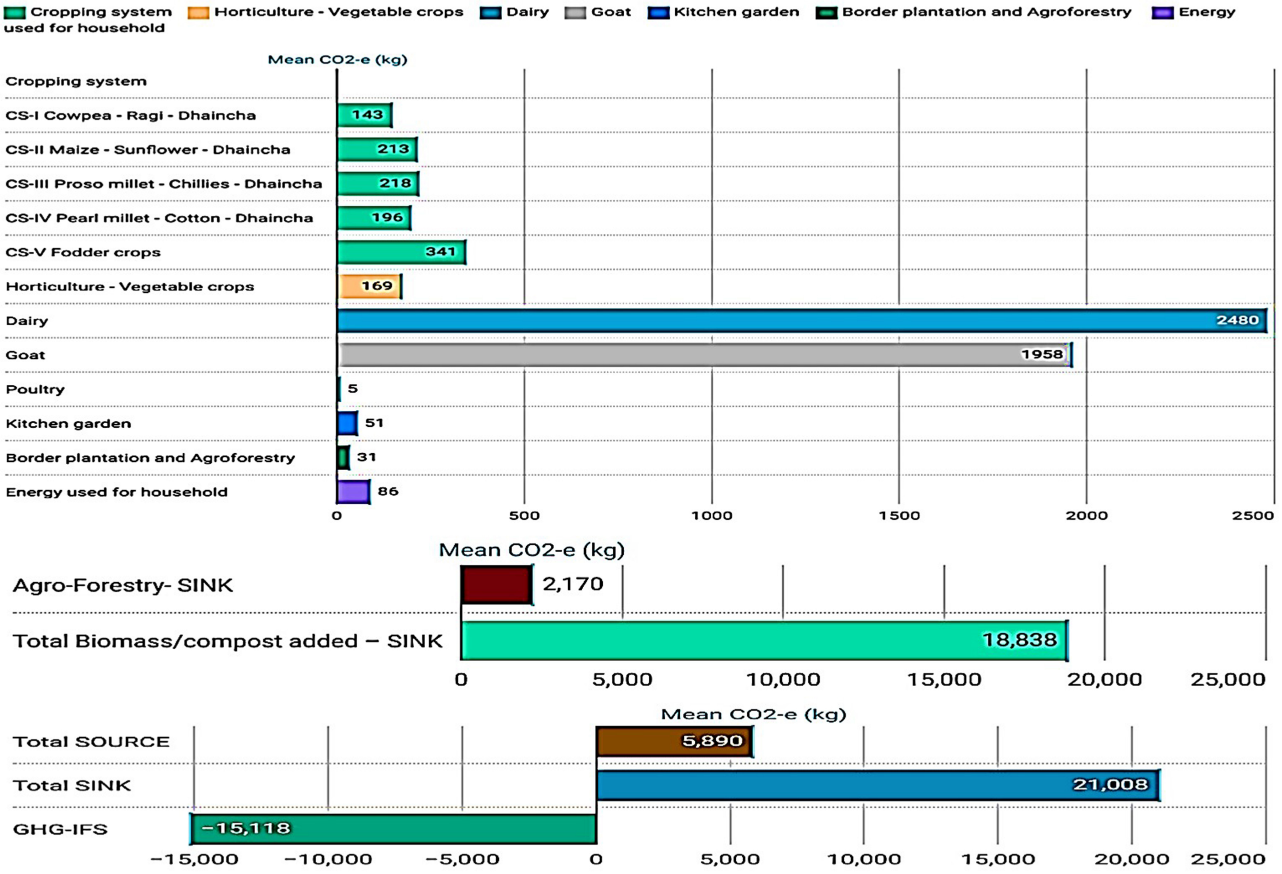

FIGURE 3
GHG emissions in different enterprises of IFS model.

3.6 GHG emissions

The high utilization of agricultural chemicals in crop production produced higher GHG emissions from crop fields and other allied enterprises (Figure 3). It was demonstrated that in the integrated farming system, the highest GHG was emitted by livestock components measured at 2,480 CO_2-e (kg), followed by goats at 1,958 CO_2-e (kg) and horticultural vegetable crops and cropping systems. Among the different cropping systems, cowpea-ragi-dhaincha released a minimum of 143 CO_2-e (kg) into the atmosphere. On the contrary, prosomillet-chillies-dhaincha released a maximum of 218 CO_2-e (kg), followed by maize-sunflower-dhanicha, which released 213 CO_2-e (kg), and pearl millet-cotton-dhaincha, which released 196 CO_2-e (kg). The total sink in the IFS unit was 21,008 CO_2-e (kg), and the total GHG emission from the IFS unit was −15,118 CO_2-e (kg). Fruit trees and boundary plantations, which resulted in a negative GHG emission and a greater carbon sink in the IFS model, allow for further agricultural or enterprise intensification. The integrated farming system is considered a viable strategy to reduce greenhouse gas emissions through the adoption of suitable nutrient recycling (Barbosa et al., 2015; Sharma and Sharma, 2018). Considerably, IFS produced low greenhouse gas emissions. These results are in close conformity with the findings of Meena et al. (2022), who opined that crop + dairy + horticulture + fish pond + mushroom + poultry + kitchen garden + vermicompost + boundary planting registered negative GHG emissions. Negative (−3,175 kg CO_2 eq./ha) GHG emissions were recorded in crops with dairy, horticulture, fishery, poultry, duckery, goatery, apiary, vermicompost, biogas, liquid manure, and FYM production integration (Ravisankar et al., 2019). Perennial forages combined with cropping techniques including agroforestry, alley cropping, and intercropping provide a variety of choices for mitigating the effects of climate change by enhancing carbon sequestration and nutrient availability with better grazing management (Bell et al., 2014; Ericksson and Crane, 2018). Further border planting of trees reduces CH and CO emissions by sequestering carbon as biomass and modifying the soil environment, and the roots act as sinks of carbon (Franzluebbers et al., 2016; MacCarthy et al., 2018; Shyam et al., 2023). Therefore, it can be said that IFS is a prominent means to reduce the effects of climate change.

3.7 Limitations and Barriers

Although the IFS system overall is efficient, it also has certain limitations in terms of implementation and practicality. The major limitation has to be the high initial cost associated with various components of the system as well as the requirement of manpower. Furthermore, the system also needs a farm with a sizable area to accommodate all the components, which is a major barrier among small- and medium-scale farmers. The system also needs its initial operational time to become efficient and effective, and the economic stability of the farmers during this time period is also a major concern, especially for small farmers. This was further supported by the findings of Pandey et al. (2019). The availability of suitable planting materials and their combination of plants, animals, and inputs, as well as the complexities of managing, are also constraints, as mentioned in the findings of Nurcholis and Supangkat (2011) and Pushpa (2010). During the course of this study, it was understood that the limitations of the integrated farming system, although prominent, outweigh the advantages, and as the system progresses in the long run, it becomes more efficient and effective than the conventional farming system, thereby providing a stable and sustainable income for the farmers.

4 Conclusion

The developed IFS integrated farming system in 1.0-ha integrating agriculture crops, horticulture, dairy, goat, poultry, vermicompost, kitchen garden, and boundary planting registered higher productivity with generating more mandays employment and fetched a higher gross return and net return. In addition, it also provides nutritional security to the farm household by producing a high amount of cereals, vegetables, milk, and meat, which alleviates nutritional deficiency. This IFS model also helps to reduce the impact of global warming by reducing negative GHG, thus becoming eco-friendly. Thereby, it can be considered the economically feasible, ecologically sustainable, and efficient IFS model for the resource-poor farmers of irrigated upland. This model is developed under on-farm conditions in the western zone of Tamil Nadu, and the results thus obtained are based on the regional-specific conditions. Therefore, in future studies, more emphasis needs to be provided on understanding the variabilities associated with the combinations of the integrated farming system under different climatic and regional conditions, as well as identifying the possibilities and potentials of recycling and optimizing the available resources in a wider national-specific context.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

Author contributions

PS: Conceptualization, Formal analysis, Funding acquisition, Methodology, Validation, Writing – original draft, Writing – review & editing. SS: Validation, Conceptualization, Formal analysis, Funding acquisition, Methodology, Writing – original draft. PCP: Conceptualization, Funding acquisition, Methodology, Writing – review & editing. SV: Writing – original draft, Writing – review & editing. AR: Formal analysis, Validation, Writing – review & editing. NR: Conceptualization, Funding acquisition, Writing – review & editing. PP: Funding acquisition, Writing – review & editing. TP: Funding acquisition, Writing – review & editing. NS: Funding acquisition, Writing – review & editing. SN: Funding acquisition, Writing – review & editing. MG: Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Adijaya, N., Trisnawati, W., Mahaputra, K., and Agus,. K. (2009). *Laporan Akhir Prima Tani Lahan Kering Dataran Rendah Iklim Kering di Kabupaten Buleleng.* Balai Pengkajian Teknologi Pertanian, Bali: Denpasar.

Amadou, I., Gounga, M. E., and Le, G. W. (2013). Millets: nutritional composition, some health benefits and processing-A review. *Emirates J. Food Agric.* 25, 501–508. doi: 10.9755/ejfa.v25i7.12045

Arti, B. E., Jha, K. K., Lily, M., and Kispotta, M. (2019). Identification of suitable vegetable based cropping sequences in relation to economic yield and profitability. *J. Pharmacogn. Phytochem.* 5, 37–41.

Asante, B. O., Villano, R. A., and Battese, G. E. (2020). Evaluating complementary synergies in integrated crop-livestock systems in Ghana. *Int. J. Soc. Econ.* 47, 72–85. doi: 10.1108/IJSE-04-2019-0274

Barbosa, F. A., SoaresFilho, B. S., Merry, F. D., de Oliveira Azevedo, H., Costa, W. L. S., Coe, M. T., et al. (2015). *Cenários para a pecuária de corteamazônica.* Universidade Federal de Minas Gerais: Belo Horizonte, Brazil, 154.

Bell, L. W., Moore, A. D., and Kirkegaard, J. A. (2014). Evolution in crop– livestock integration systems that improve farm productivity and environmental performance in Australia. *Eur. J. Agron.* 57, 10–20. doi: 10.1016/j.eja.2013.04.007

Dey, M. M., Paraguas, F. J., Kambewa, P., and Pemsl, D. E. (2010) The impact of integrated aquaculture–agriculture on small-scale farms in Southern Malawi. *Agric. Econ.* 41, 67–79. doi: 10.1111/j.1574-0862.2009.00426.x

Duncan, J., Tarawali, S. A., Thorne, P. J., Valbuena, D., Descheemaeker, K., and Tut, S. H. K. (2013). Integrated Crop-Livestock Systems-a Key to Sustainable Intensification in Africa, Zimbabwe. *Trop. Grassl.- Forrajes Trop.* 1, 202–206. doi: 10.17138/TGFT(1)202-206

Erick, O. O., Mlingi, F. T., Nyonje, B. M., Charo-Karisa, H., and Munguti, J. M. (2013). Can integrated livestock-fish culture be a solution to East Africa's food insecurity? A review. *African J. Food, Agric. Nutr. Dev.* 13, 8058–8076. doi: 10.18697/ajfand.59.12920

Ericksson, P., and Crane, T. (2018). *The feasibility of low emissions development interventions for the East African livestock sector: Lessons from Kenya and Ethiopia.* International Livestock Research Institute, Research Report 46. Nairobi, Kenya. Available online at: https://cgspace.cgiar.org/bitstream/handle/10568/91527/RR46.pdf (accessed October 15, 2023).

Ezeaku, I. E., Mbah, B. N., Baiyeri, K. P., and Okechukwu, E. C. (2015). Integrated crop livestock farming system for sustainable agricultural production in Nigeria. *Afr. J. Agric. Res.* 10, 4268–4274. doi: 10.5897/AJAR2015.9948

Franzluebbers, A. J., Chappell, J. C., Shi, W., and Cubbage, F. W. (2016).Greenhouse gas emissions in an agroforestry system of the Southeastern USA. *Nutr. Cycl. Agroecosyst.* 108, 85–100. doi: 10.1007/s10705-016-9809-7

Goverdhan, M., Kumari, C. P., Sridevi, S., Ramana, M. V., and Suresh, K. (2020). Evaluation of integrated farming system model for small and marginal farmers of Telangana State. *Curr. Appl. Sci.* 39, 26–134. doi: 10.9734/cjast/2020/v39i1030636

Haile, M. G., Wossen, T., Tesfaye, K., and von Braun, J. (2017). Impact of climate change, weather extremes, and price risk on global food supply. *Econ. Disaster Clim. Chang.* 1, 55–75. doi: 10.1007/s41885-017-0005-2

Iizumi, T., and Ramankutty, N. (2016). Changes in yield variability of major crops for 1981–2010 explained by climate change. *Environ. Res. Lett.* 11:034003. doi: 10.1088/1748-9326/11/3/034003

Kochewad, S. A., Meena, L. R., Devender, K.umar, Sanjeev, K.umar, Meena, L. K., Singh, S. P., and and Singh, K. (2017). Livestock based integrated farming systems for livelihood improvement of small and marginal farmers. *SAJFTE.* 3, 526–532. doi: 10.46370/sajfte.2017.v03i01.09

Kumar, S., Samal, S., Dwivedi, S. K., and Manibhushan. (2017). Enhancement in productivity and income sustainability through Integrated farming system approaches for small and marginal farmers of Eastern India: Integrated farming system approaches for small and marginal farmers of Eastern India. *J. Agri. Search.* 4, 85–91. doi: 10.21921/jas.v4i2.7777

Kumar, S., Singh, S., Meena, M., Shivani, S., and Dey, A. (2012). Resource recycling and their management under integrated farming system for lowlands of Bihar lowlands of Bihar. *Indian J. Agric. Sci.* 82, 504–510. doi: 10.56093/ijas.v82i6.18889

Kumara, O., Sannathimmappa, H., Basavarajappa, D., Danaraddi, V., Pasha, A., and Rajani, S. (2017). Integrated farming system-An approach towards livelihood security, resource conservation and sustainable production for small and marginal farmers. *Int. J. Plant Soil Sci.* 15, 1–9. doi: 10.9734/IJPSS/2017/31994

MacCarthy, D. S., Zougmore, R. B., Akponikpe, P. B. I., Koomson, E., Savadogo, P., and Adiku, S. G. K. (2018). Assessment of greenhouse gas emissions from different land-use systems: a case study of CO_2 in the Southern Zone of Ghana. *Appl. Environ. Soil Sci.* 2018, 1–12. doi: 10.1155/2018/1057242

Mahapatra, I. C., and Behera, U. K. (2011). Rice based farming systems for livelihood improvement of Indian farmer. *Indian J. Agron.* 56, 1–19. doi: 10.59797/ija.v56i1.4660

Mali, H., Kumar, A., and Katara, P. (2014). "Integrated farming system for irrigated and rainfed conditions," in *Proceedings of the National Symposium on Agricultural Diversification for Sustainable Livelihood and Environmental Security, India.*

Meena, L. R., Kochewad, S. A., Prusty, A. K., Bhanu, C., and Kumar, K. (2022). Sustainable integrated farming system model for small farm holders of Uttar Pradesh. *Indian J. Agric. Sci.* 92, 1080–1085. doi: 10.56093/ijas.v92i9.110742

Munandar, G.ustiar, F., Hayati, R., and Munawar, A. I. (2015). Crop-cattle integrated farming system: an Alternative of climatic change mitigation. *Trop. Anim. Sci. J.* 38, 95–103. doi: 10.5398/medpet.2015.38.2.95

Nurcholis, M., and Supangkat, G. (2011). *Pengembangan Integrated Farming System untuk Pengendalian Alih Fungsi Lahan Pertanian. Dalam: Prosiding Seminar Nasional Budidaya Pertanian: Urgensi dan Strategi Pengendalian Alih Fungsi Lahan Pertanian.* Bengkulu, ID.

Othman, K. (2006). "Integrated farming system and multifunctionality of agriculture in Malaysia," in *XV International Symposium on Horticultural Economics and Management 655* 291–296. Available online at: http://www.actahort.org/books/655/655-36.html. doi: 10.17660/ActaHortic.2004.655.36

Pandey, P. R., Gupta, J. K., Narvariya, R. K., Meena, S. C., and Narwariya, D. (2019). Constraints faced by farmers in adoption of integrated farming system in Vindhyan Plateau of Madhya Pradesh. *Plant Arch.* 19, 512–514.

Panwar, A. S., Ravisankar, N., Singh, R., Prusty, A. K., Shamim, M., Tripathi, D., et al. (2019). AICRP on integrated farming systems: salient achievements and future directions. *Indian J.Fertilisers* 15, 338–353.

Paramesh, V., Ravisankar, N., Behera, U., Arunachalam, V., Kumar, P., Solomon Rajkumar, R., et al. (2022). Integrated farming system approaches to achieve food and nutritional security for enhancing profitability, employment, and climate resilience in India. *Food Energy Secur.* 1:321. doi: 10.1002/fes3.321

Porpavai, S., and Marimuthu, R. (2018). Development of integrated farming system model for marginal farmers of cauvery delta zone. *Indian J. Ecol.* 45, 183–186.

Pushpa, J. (2010). Constraints in various integrated farming systems. *Agric. Update* 5, 370–374.

Rajasekaran, R., Francis, N., Mani, V., and Ganesan, J. (2023). "Proso millet (*Panicum miliaceum L.*)," in *Neglected and Underutilized Crops* (New York: Academic Press), 247–278. doi: 10.1016/B978-0-323-90537-4.00005-3

Ravisankar, N., Pramanik, S. C., Rai, R. B., Nawaz, S., Biswas, T. K., and Bibi, N. (2007).Study on integrated farming system in hilly upland areas of Bay Islands. *Indian J. Agron.* 52, 7–10. doi: 10.59797/ija.v52i1.4879

Ravisankar, N., Singh, P., Mishra, R. P., Prusty, A. K., Shamim, M., Singh, R., et al. (2019). *Annual report of AICRP on Integrated farming system.* Available online at: https://iifsr.icar.gov.in/icar-iifsr/AICRP/pdf/AICRPIFS_Annual_Report_2018-2019.pdf (accessed October 15, 2023).

Ray, M., Samanta, B., Haldar, P., Chatterjee, S., and Khan, D. K. (2012). Agriculture practices and its association with livestock in hilly areas of West Bengal. *Indian J. Hill Farm.* 25, 53–57.

Sachinkumar, T. N., Basavaraja, H., Kunnal, L. B., Kulkarni, G. N., Ahajanashetty, S., B., et al. (2012). Economics of farming systems in northern transitional zone of Karnataka. *Karnataka J. Agric. Sci.* 25, 350–358.

Sarwar, A. K. M. G., Hossain, S. Z., and Chanda, S. C. (2017). Effect of Dhaincha accessions on soil health and grain yield of rice. *J. Biosci. Agric. Res.* 13, 1140–1145. doi: 10.18801/jbar.130217.139

Shankar, K. A., Yogeesh, L. N., Prashant, S. M., Peer, P. S., and Desai, B. K. (2017). Integrated farming system: profitable farming to small farmers. *Int. J. Curr. Microbiol. Appl. Sci.* 6, 2819–2824. doi: 10.20546/ijcmas.2017.610.330

Shanmugam, R., and Ramamoorthy, K. (2014). Effect of nutrient recycling in seri-based integrated farming system on soil fertility, productivity and profitability in maize-sunflower cropping system. *Trends Biosci.* 7, 317–321.

Sharma, R. L., Abraham, S., Bhagat, R., and Prakash, O. (2017). Comparative performance of integrated farming system models in Gariyaband region under rainfed and irrigated conditions. *Indian J. Agric. Res.* 51, 64–68. doi: 10.18805/ijare.v51i1.7064

Sharma, U. C., and Sharma, V. (2018). Greenhouse gases emissions from agriculture sector in Northeastern region of India. *Acta Sci. Microbiol.* 1, 36–43.

Shyam, C. S., Shekhawat, K., Rathore, S. S., Babu, S., Singh, R. K., and Upadhyay, P. K. (2023). Development of integrated farming system model - a step towards achieving biodiverse, resilient and productive green economy in agriculture for small holdings in India. *Agron.* 13:955. doi: 10.3390/agronomy13040955

Siddeswaran, K., Sangeetha, S. P., and Shanmugam, P. M. (2012). "Integrated farming system for the small irrigated upland farmers of Tamil Nadu," in *Extended Summaries: Third International Agronomy Congress on Agriculture diversification, Climate change management and Livelihoods, New Delhi, India.*

Singh, K., Bohra, J. S., Singh, T. K., Singh, J. P., Singh, Y., and Singh, C. S. (2007). Productivity and economics of integrated farming system in irrigated agroecosystem of eastern Uttar Pradesh. *Indian J. Agron.* 52, 11–15.

Sivamurugan, A. P. (2001). *Sustainable farming system under irrigated upland situation.* Ph.D Thesis, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu.

Thavaprakaash, N., and Premavathi, R. (2019). Farmer's participatory coconut based integrated farming systems for sustainable farm productivity in high rainfall zone of Tamil Nadu. *Madras Agric. J.* 106, 576–581. doi: 10.29321/MAJ.2019.000313

Thierfelder, C., Cheesman, S., and Rusinamhodzi, L. (2013). Benefits and challenges of crop rotations in maize-based conservation agriculture (CA) cropping systems of southern Africa. *Int. J. Agric. Sustain.* 11, 108–124. doi: 10.1080/14735903.2012.703894

Uddin, M. T., and Takeya, H. (2006). Comparative study on integrated farming in Bangladesh and other countries. *Bangladesh J. Agric. Econ.* 29, 81–92.

Ugwumba, C. O. A., Okoh, R. N., Ike, P. C., Nnabuife, E. L. C., and Orji, E. C. (2010). Integrated farming system and its effect on farm cash income in Awka south agricultural zone of Anambra State, Nigeria. *Am.-Eurasian J. Agric. Environ. Sci.* 8, 1–6.

Vinodakumar, S. N., and Desai, B. K. (2017). Dairy based farming system models for livelihood security of small and marginal farmers of NE Karnataka. *Int. J. Conserv. Sci.* 5, 805–808.

Vinodakumar, S. N., Desai, B. K., Channabasavanna, A. S., Satyanarayana, R., Patil, M. G., and Patil, S. S. (2017). Relative performance of various integrated farming system models with respect to system productivity, economics and employment generation. *Int. J. Agric. Sci.* 13, 348–352. doi: 10.15740/HAS/IJAS/13.2/348-352

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Effect of plantation age on plant and soil C:N:P stoichiometry in Kentucky bluegrass pastures

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Plant and soil C:N:P stoichiometry reflects the element content and energy flow, which are important for biogeochemical cycling in ecosystems. Although plantation age has been verified to affect leaf C:N:P stoichiometry in alfalfa plants, its effect on plant and soil C:N:P stoichiometry in grass remains poorly documented. A 10-year field experiment of Kentucky bluegrass *(Poa pratensis)* was used to test how plantation age affect plant and soil C:N:P stoichiometry in a perennial rhizomatous grass pasture. This study demonstrated that leaf C:N, C:P, and N:P ratios exhibited a rapid increasing trend from 2 to 6 years of age, whereas leaf C:N showed a slight decreasing trend, and leaf C:P and N:P maintained stability from 6 to 9 years of age. Stem C:N and N:P were not different among plantation ages, while stem C:P increased from 2 to 4 years of plantation age and then maintained stability from 4 to 9 years of plantation age. Root N:P showed an increasing trend from 2 to 6 years of plantation age and relative stability from 6 to 9 years of plantation age, whereas root C:N and C:P showed decreasing trends from 2 to 9 years of plantation age. Although soil C:P did not differ among nine plantation ages, soil C:N and N:P remained relatively stable from 2 to 6 years of plantation age. However, soil C:N showed a decreasing trend, while soil N:P showed an increasing trend after 6 years of plantation age. The results from an ecological stoichiometric homeostasis analysis further showed that N in the leaf, stem, and root and P in the stem had strict homeostasis, whereas P in the leaf and root showed plastic and weakly homeostatic status, respectively. These results present a pattern concerning the plantation age in relation to plant and soil C:N:P stoichiometry in a perennial grass and provide useful information for N and P management in Kentucky bluegrass pastures.

KEYWORDS

Kentucky bluegrass, ecologic stoichiometry, plantation age, leaf, stem, root, soil

1 Introduction

Carbon (C), nitrogen (N), and phosphorus (P) are three vital elements for plants, regulating plant growth and development (Zhang et al., 2021). Soil C:N:P stoichiometry is considered to provide insight into the potential contribution of plant organics to soil fertility, as well as soil nitrogen and phosphorus availability (Pang et al., 2021). The C:N:P stoichiometries between plants and soil are suggested as a major indicator to understand their ecological functions and processes (Cao and Chen, 2017), in which plant productivity is one of the main functions of pastures (Lindsey et al., 2020).

Most previous studies have focused on the spatial variability of C:N:P stoichiometry in terrestrial ecosystems (Dijkstra et al., 2012; He and Dijkstra, 2015; Hu et al., 2019) or the responses of plant and soil C:N:P stoichiometry to biotic and abiotic disturbances (Pathak et al., 2017; Kleyer et al., 2019). Only a few studies have addressed the temporal variability of leaf C:N:P stoichiometry in shrubs (Zhang et al., 2016; Zeng et al., 2017; Yu et al., 2021) and legumes (Wang et al., 2015) to examine the relationship between leaf C:N:P stoichiometries and plantation ages, and the previous studies found that leaf N:P in *Haloxylon ammodendron* shows a rapid increase from 2 to 5 years of plantation age, but leaf C:N exhibits a decline, and they are stable after the 5-year plantation age (Zhang et al., 2016). Leaf C:N, C:P, and N:P ratios in *Caragana korshinskii* decreased with an increase in plantation ages from 10 to 30 years (Zeng et al., 2017); those ratios in *Zanthoxylum planispinum* showed no significant difference in 5-, 10- , and 20-year plantation ages, respectively (Yu et al., 2021); and those ratios in alfalfa proved to be the highest at the 8-year plantation age (Wang et al., 2015). However, how plantation age simultaneously affects the temporal variability of the plant (leaf, stem, and root), and soil C:N:P stoichiometry receives less attention. Therefore, examining the effects of plantation ages on plant and soil C:N:P stoichiometry is one of the most important approaches to understanding the effect of plantation ages on the ecological functions and processes of pastures.

In agricultural practices, perennial grasses are promising candidates for the establishment of artificial pastures with good quality and higher productivity for grazing livestock (Jones et al., 2015) or for the regeneration of vegetation populations in degraded grasslands with their high reproductivity. Many previous studies have found that the aboveground biomass of perennial grass pastures often differs during different plantation ages (Folck et al., 2023), and this difference is related to soil nitrogen availability and supplies because grass is nitrophile (Mazzucato et al., 1996). Kentucky bluegrass (*Poa pratensis*), a perennial rhizomatous grass with high tolerance to environments, has become increasingly important (Pi et al., 2015; Wu et al., 2023) because of its ability to be productive in cool temperatures and high soil moisture conditions (DeKeyser et al., 2015; Zhu et al., 2023). This grass has been widely grown in Eurasia (Cui et al., 2020), Asia (Wang C. et al., 2023), and North America (Folck et al., 2023; Toledo et al., 2023) due to its unique ecological advantage: the relatively longer time intervals between tilling, which reduce the risk of soil erosion compared to annual grass pastures (Lindsey et al., 2020).

Kentucky bluegrass is recognized as the most preferred choice for the establishment of pastures or the management of degraded grassland in the Qinghai-Tibetan plateau (Pi et al., 2015). At present, pasture areas of Kentucky bluegrass cover over 4.33 million ha in the Qinghai-Tibetan plateau. Previous studies were focused on the growth features of Kentucky bluegrass in relation to seeding time (Jing et al., 2018), the role of Kentucky bluegrass in vegetation rebuilding of "black soil type" in degraded grassland (Wen et al., 2006), the responses of reproductive phenology and vegetative growth of Kentucky bluegrass to climate change (Wei et al., 2022), and high-yield and high-quality cultivation techniques for Kentucky bluegrass (Jing et al., 2019). Whether the plantation ages affect plant and soil C:N:P stoichiometry in Kentucky bluegrass pasture remains poorly documented.

This study uses Kentucky bluegrass as a focal grass to investigate the effect of plantation ages on plant and soil C:N:P stoichiometry in a perennial rhizomatous grass pasture through an 8-year field experiment. The hypotheses are that (1) C:N, C:P, and N:P in the leaf, stem, and root of Kentucky bluegrass increase and then decrease as plantation age increases; (2) C:N, N:P, and C:P in the soil decrease with the increase in plantation ages, which can present a general pattern concerning the effect of plantation ages on plant and soil C:N:P stoichiometry in a crop and provide a reference for the nitrogen and phosphorus management of Kentucky bluegrass pastures in the Qinghai-Tibetan plateau.

2 Materials and methods

2.1 Experimental site description

The experiment site is located at the Haibei experiment station (36°59.36′N, 100°52.848′E, elevation 3,156 m) of Qinghai University in Qinghai Province, China. This station is on the eastern edge of the Qinghai-Tibetan plateau. This experiment site experiences a cold plateau continental climate without an absolute frost-free period. The average annual precipitation at this experiment station is 369.1 mm, most of which occurs from June to August of each year, corresponding with the growing season, while the mean annual evaporation is ∼1,400 mm. The average annual temperature at this experiment station is 0.5°C, with a maximum in August (19°C) and a minimum in January (−4°C; Figure 1). The soil at the experiment site is chestnut soil (Wang Y. et al., 2023), similar to Entisol in FAO classification, and this soil is the major type for crop production in this area. The soil chemical properties measured at the 0–40 cm layer are as follows: pH (8.43), soil organic carbon (6.95 g kg^{-1}), total N (0.6886 g kg^{-1}), total P (0.52 g kg^{-1}), and total K (9.84 g kg^{-1}).

2.2 Establish pastures of Kentucky bluegrass

Three field plots were established in 2014 to monitor the relationships between plant productivity and plantation ages. The plot size was 30 m × 30 m. The distance between the two plots was 200 m, which ensured that precipitation and air temperature were the same among the three plots, avoiding the biases introduced by precipitation and air temperature on plant productivity.

To quantify the relationship between aboveground biomass and stoichiometry, phosphorus is not applied to the experiment plots because a high amount could promote the rapid growth of Kentucky bluegrass roots. Therefore, 75 kg ha^{-1} of urea (N 46%) was applied to each experiment plot in 2014. Kentucky bluegrass seeds with an 88% germination rate were broadcasted in each plot on 5 April 2014. The seeding density was 3 g m^{-2}. During the experimental period of 2015–2022, the Kentucky bluegrass

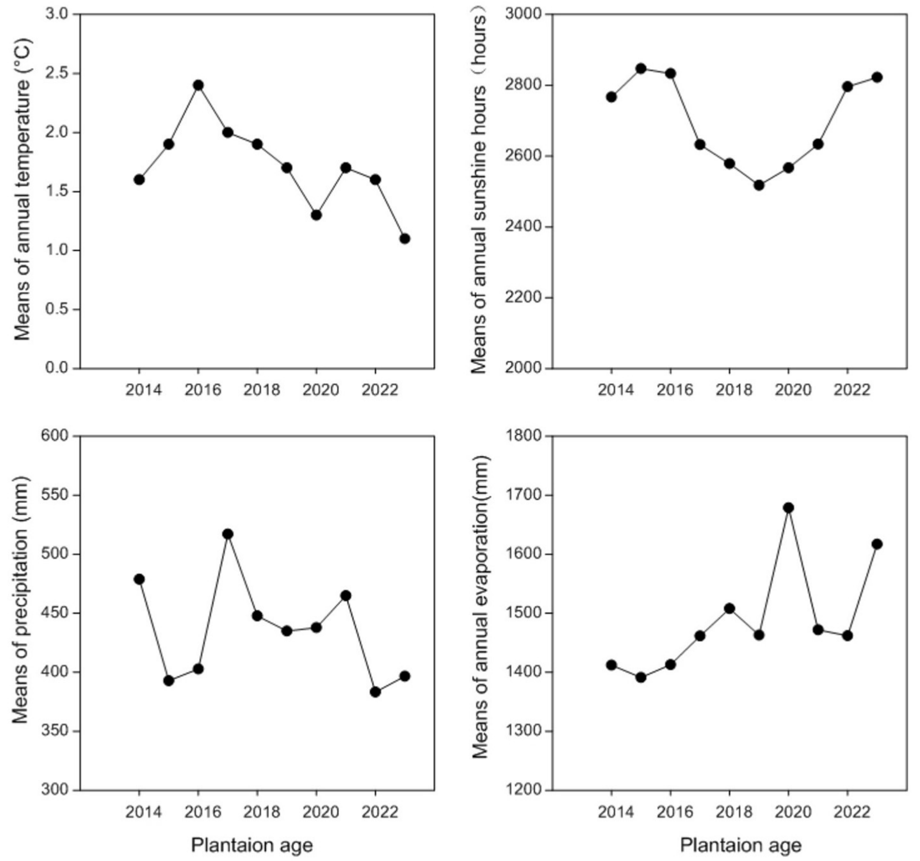

FIGURE 1
Means of annual temperature (°C), annual sunshine hours (h), annual precipitation (mm), and annual evaporation (mm) at Haibei Station during 2015–2022.

pastures were generally rainfed without external irrigation or further fertilizer application. The plots were fenced during the experiment period.

2.3 Plant and soil samplings

In the process of plant and soil sampling, three plots were considered replicates. Sampling was conducted at the flowering stages of Kentucky bluegrass in July from 2015 to 2022 (Supplementary Table S1). In each plot, five subplots with a size of 0.5 m × 0.5 m were randomly selected to sample plants (leaves, stems, and roots) and soils, and these samples were completely mixed as a composite sample to measure C, N, and P concentrations. First, at each subplot, shoots were harvested by shear and then divided into leaves and stems, in which flowers were classified as stems. Second, the root auger with a 10-cm inner diameter was used to collect a soil column with a 20-cm layer at this subplot, and this soil column was screened out of garbage and stones and then divided into root samples and soil samples using a 2-mm sieve (Duan et al., 2024). The leaves, stems, and roots were placed into envelopes, and the soil samples were collected in fabric sample bags and carried back to the laboratory. These composite samples of leaves, stems, and roots were used to measure total C, total N, and total P concentrations, and soil composite samples were used to measure organic C, total N, and total P concentrations.

2.4 Measurement of C, N, and P

All composite samples of leaves, stems, and roots were dried at 65°C until no further mass loss could be found. Soil composite samples were air-dried. The total C and total N in leaves, stems, and roots and the total N in soils were analyzed with the Dumas dry combustion method using a Flash-II EA112 Elemental Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). The total P in leaves, stems, roots, and soils was determined using the molybdenum blue colorimetric method. Soil organic C was determined using dichromate heating and oxidation method.

2.5 Estimation of ecological stoichiometric homeostasis

C:N, C:P, and N:P refer to the ratio between the total concentrations of C, N, and P in Kentucky bluegrass or in soil organics, respectively.

A regulation index (1/H) proposed by Hood and Sterner (2010) was used to quantify the degree of stoichiometric homeostasis

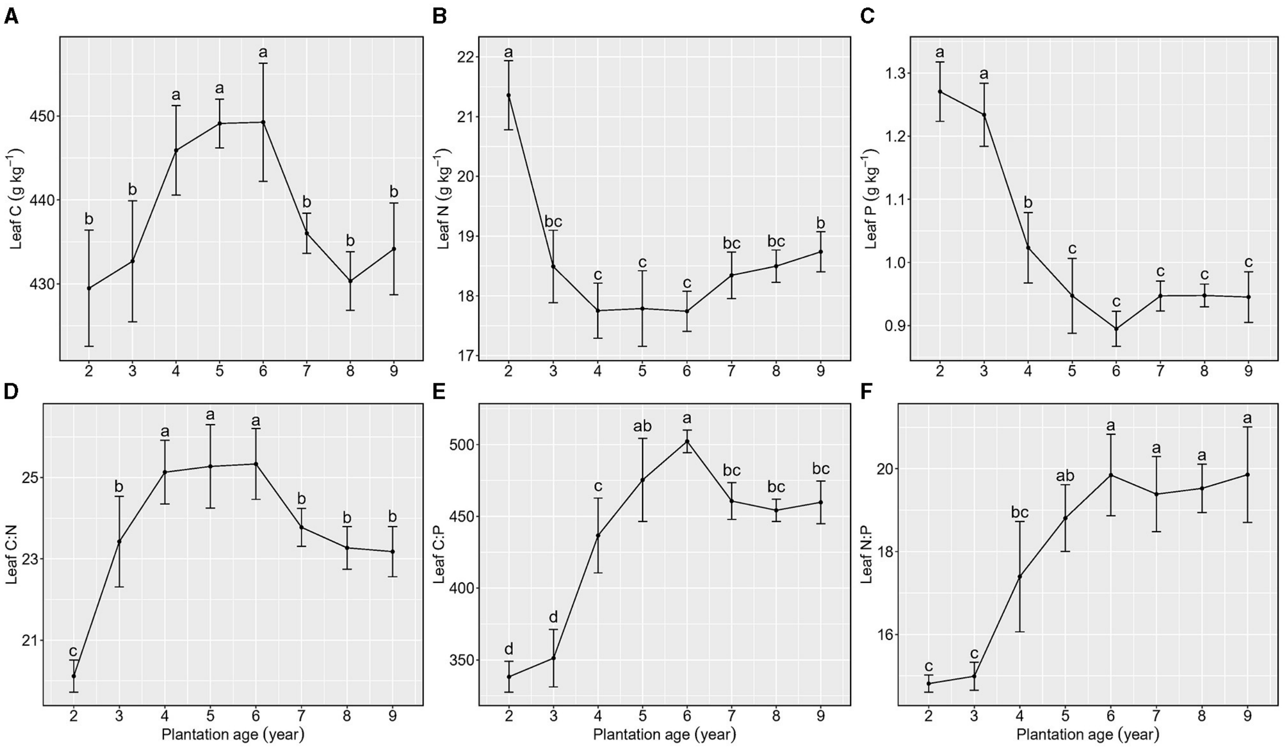

FIGURE 2
Stoichiometric characteristics of leaf C, N, and P of Kentucky bluegrass at plantation age sequences (mean ± SE; different letters denote significant differences at the $\alpha = 0.05$ level). **(A)** Leaf C, **(B)** Leaf N, **(C)** Leaf P, **(D)** Leaf C:N, **(E)** Leaf C:P, and **(F)** Leaf N:P. The error bars represent the standard deviation.

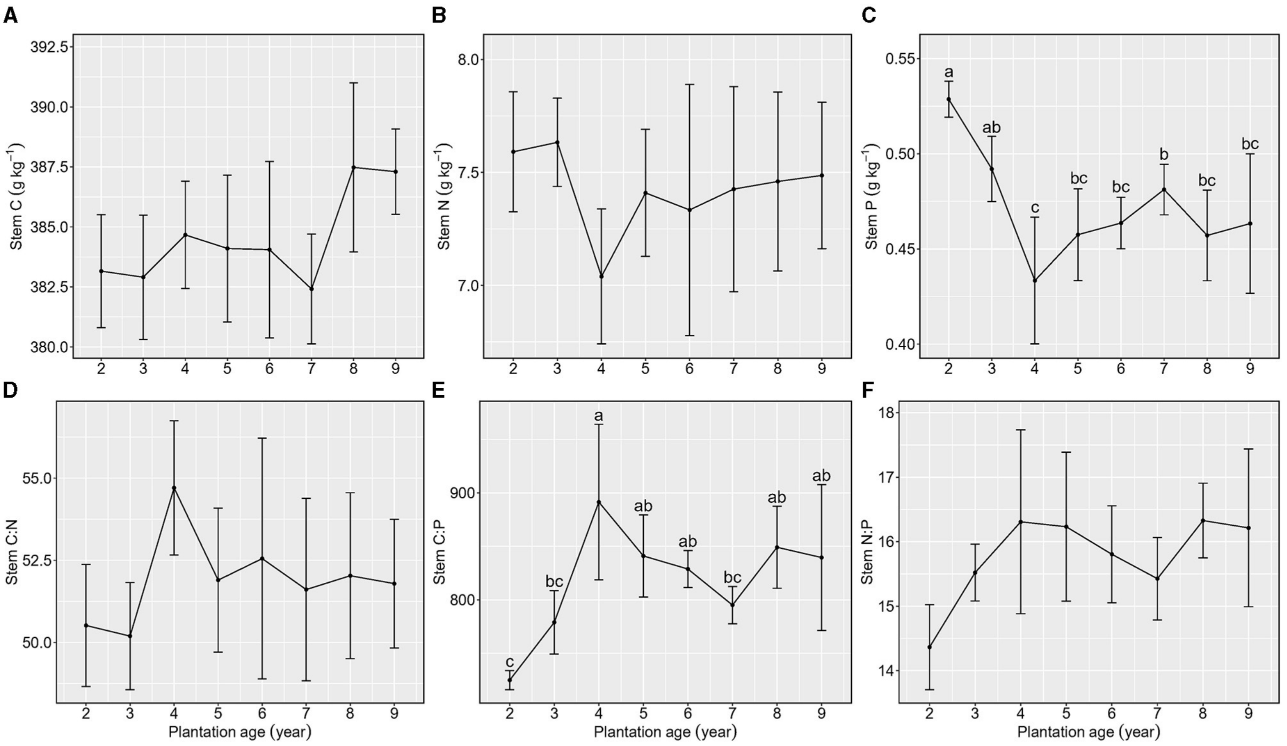

FIGURE 3
Stoichiometric characteristics of stem C, N, and P of Kentucky bluegrass at plantation age sequences (mean ± SE; different letters denote significant differences at the $\alpha = 0.05$ level). **(A)** Stem C, **(B)** Stem N, **(C)** Stem P, **(D)** Stem C:N, **(E)** Stem C:P, and **(F)** Stem N:P. The error bars represent the standard deviation.

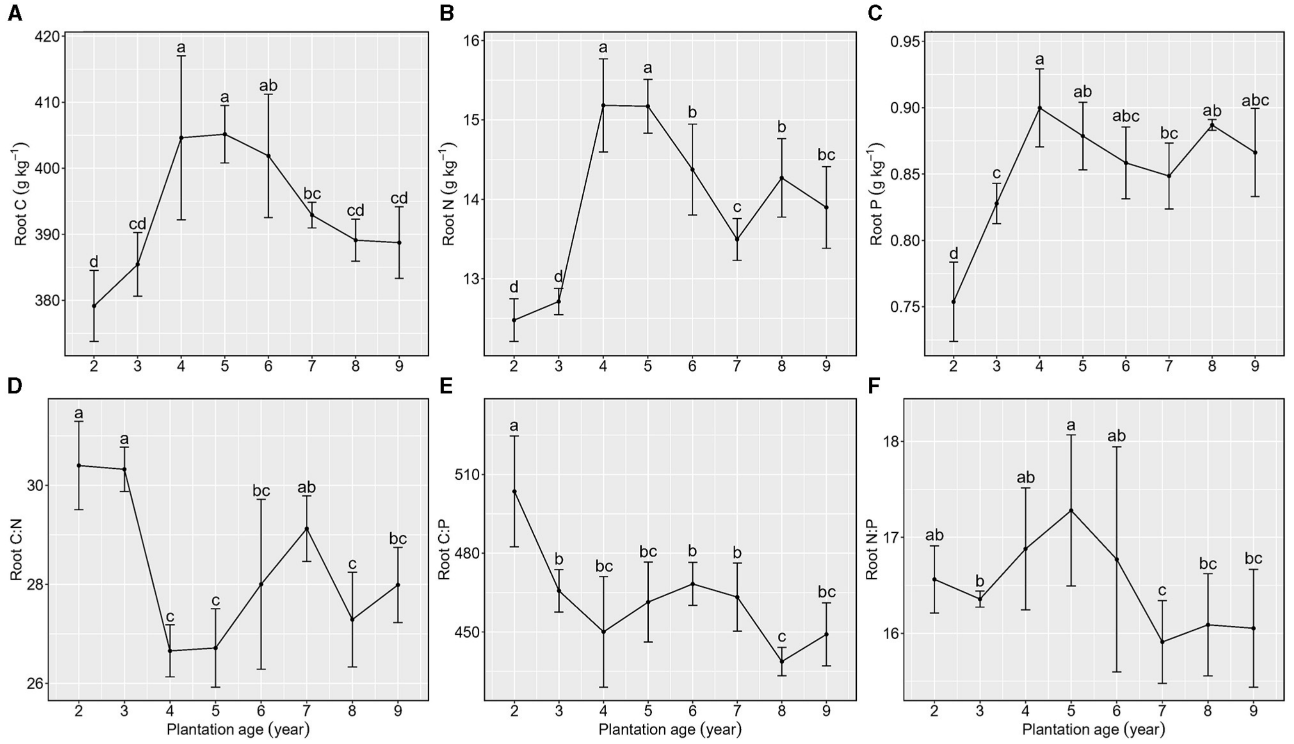

FIGURE 4
Stoichiometric characteristics of root C, N, and P of Kentucky bluegrass at plantation age sequences (mean ± SE; different letters denote significant differences at the $\alpha = 0.05$ level). **(A)** Root C, **(B)** Root N, **(C)** Root P, **(D)** Root C:N, **(E)** Root C:P, and **(F)** Root N:P. The error bars represent the standard deviation.

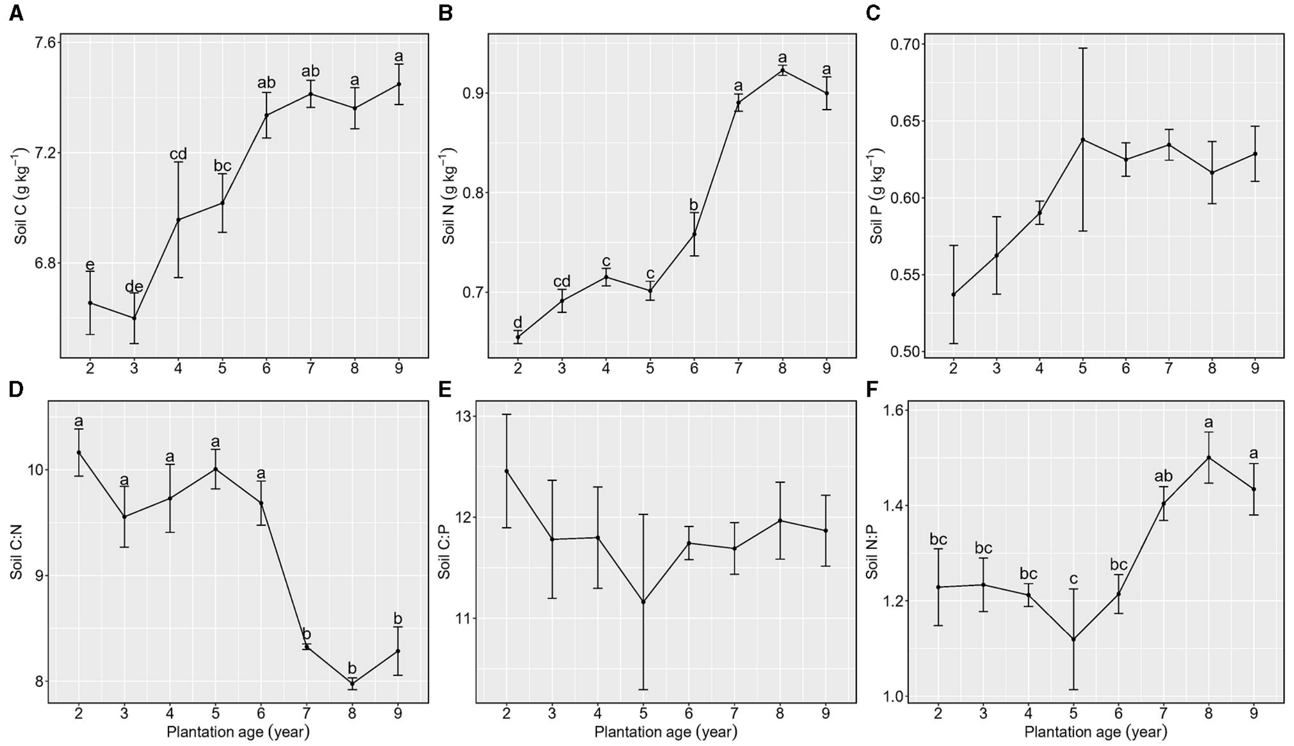

FIGURE 5
Stoichiometric characteristics of Soil C, N, and P of Kentucky bluegrass at plantation age sequences (mean ± SE; different letters denote significant differences at the $\alpha = 0.05$ level). **(A)** Soil C, **(B)** Soil N, **(C)** Soil P, **(D)** Soil C:N, **(E)** Soil C:P, and **(F)** Soil N:P. The error bars represent the standard deviation.

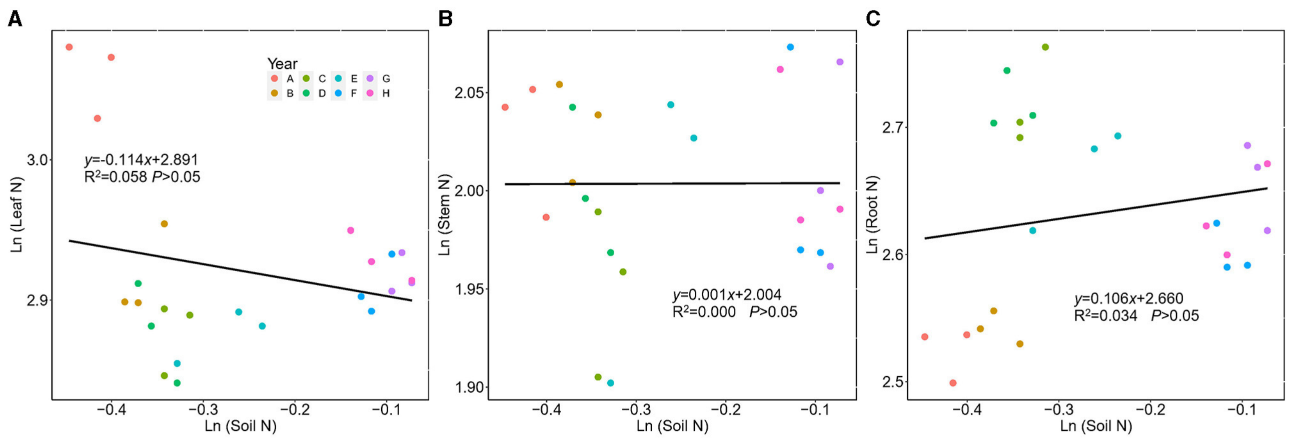

FIGURE 6
Regressions between ln-transformed N concentration in Kentucky bluegrass leaf, stem, root, and soil from 2 to 9 years of plantation age. $\ln(y) = 1/H \times \ln(x) + \ln c$; 1/H indicates the degree of stoichiometric homeostasis. **(A)** Leaf, **(B)** Stem, and **(C)** Root.

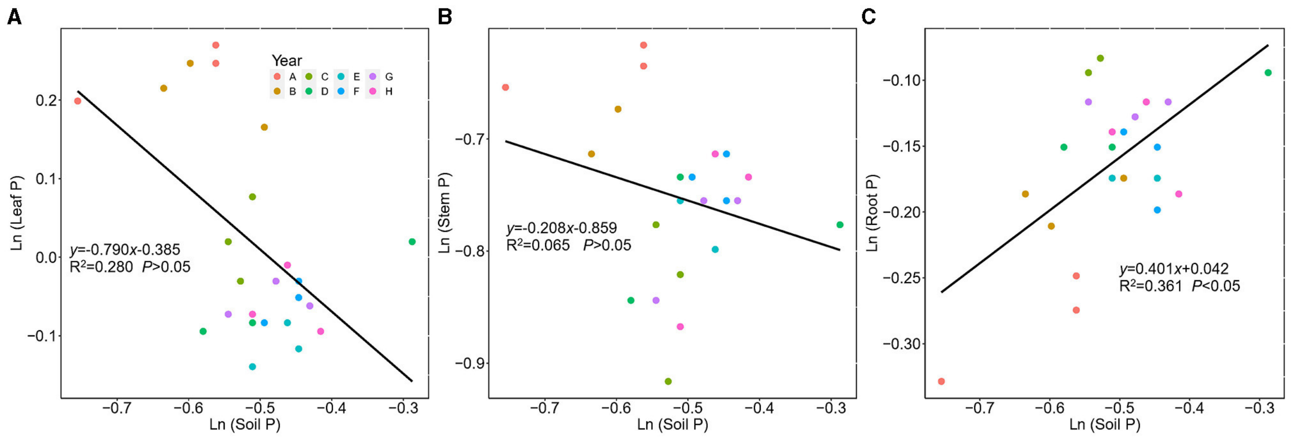

FIGURE 7
Regressions between ln-transformed P concentration in Kentucky bluegrass leaf, stem, root, and soil from 2 to 9 years of plantation age. $\ln(y) = 1/H \times \ln(x) + \ln c$; 1/H indicates the degree of stoichiometric homeostasis. **(A)** Leaf, **(B)** Stem, and **(C)** Root.

in an organism. This index was calculated by the following equation: $\ln(y) = \ln c + 1/H \times \ln(x)$, where x represents the soil nutrient stoichiometry (e.g., N or P) and y represents the nutrient stoichiometry of leaf, stem, and root. Regressions were used to estimate the relationship between soil and plant (leaf stem and root). The regression coefficient was used to produce new datasets, and these new datasets were used to calculate the regulation index (1/H). Based on a previous study (Persson et al., 2010), the regulation index was interpreted as follows: $0 < 1/H < 0.25$, "homeostatic"; $0.25 < 1/H < 0.5$, "weakly homeostatic"; $0.5 < 1/H < 0.75$, "weakly plastic"; $1/H > 0.75$, "plastic"; and $1/H \geq 1$, "not homeostatic."

2.6 Data analysis

The data were transformed when necessary to meet normality and homogeneity. Repeated measures analysis of variance (ANOVA) using least significant difference (LSD) was used to determine whether the plantation age affected the total C, total N, and total P of the plant, the total N, total P, and organic C of the soil, and the plant and soil C:N:P stoichiometry. A linear model (LM) was used to examine the regression between soil and plant (leaf, stem and root) in 95% confidence intervals. All analyses were carried out in R 4.3.2, R Foundation for Statistical Computing, Vienna, Austria.

3 Results

3.1 Effect of plantation ages on leaf C:N, C:P, and N:P

Total C concentration in leaves increased and then decreased as the plantation age increased, reaching its peak when plantation ages ranged from 4 to 6 years (Figure 2A), whereas total N and

P concentrations in leaves first decreased and then maintained a relatively stable level with the increase in plantation ages, and there were no significant differences observed when plantation ages increased from 5 to 9 years (Figures 2B, C).

Leaf C:N, C:P, and N:P exhibited various patterns as the plantation age increased (Figures 2D–F). Leaf C:N increased from 2 to 4 years of plantation age, maintained a relatively stable level from 5 to 6 years of plantation age, and then decreased from 7 to 9 years of plantation age. Leaf C:P increased from 2 to 6 years of plantation age, decreased from 6 to 7 years of plantation age, and maintained a stable level from 7 to 9 years of plantation age. Leaf N:P increased from 2 to 5 years of plantation age and then maintained stability from 6 to 9 years of plantation age.

3.2 Effect of plantation ages on stem C:N, C:P, and N:P

Total C and N concentrations in stems were not different among different plantation ages (Figures 3A, B), whereas total P concentration in stems showed a decreasing trend with the increase in plantation age (Figure 3C).

Stem C:N and N:P were not different among plantation ages, and their averages were 51.91 ± 2.33 and 15.78 ± 0.95, respectively (Figures 3D, E), whereas stem C:P increased from 2 to 4 years of plantation age and then maintained a stable level from 4 to 9 years of plantation age (Figure 3F).

3.3 Effect of plantation ages on root C:N, C:P, and N:P

With the increase in plantation age, total C, N, and P concentrations in roots exhibited a similar unimodal distribution, approximately peaking from 4 to 5 years of plantation age.

Root C:N exhibited a reduction from 2 to 3 years of plantation age, an increase from 3 to 6 years of plantation age, and relative stability from 6 to 9 years of plantation age, whereas root C:P showed a decreasing trend but with a stable phase. Root N:P showed a reduction from 2 to 3 years of plantation age, an increasing trend from 3 to 6 years of plantation age, and relative stability from 7 to 9 years of plantation age (Figure 4).

3.4 Effect of plantation ages on soil C:N, C:P, and N:P

Soil organic C, total N, and total P initially showed increasing trends from 2 to 5 years of plantation age, and then, they were relatively stable after 6 or 7 years of plantation age.

Soil C:P was not different among the nine plantation ages. In the increasing process of plantation age, soil C:N showed a slight decreasing trend and then maintained a relatively stable level, whereas soil N:P showed a slight increasing trend and then maintained a relatively stable level, in which the 7-year plantation age of Kentucky bluegrass was the inflection point for soil C:N and N:P (Figure 5).

3.5 Ecological stoichiometric homeostasis

N concentration and P concentration in plants and soil were found to differ in ecological stoichiometric homeostasis. No significant correlation was found between plant N (leaf, stem, and root) and soil N. The 1/H values of leaf, stem, and root N were 0.114, 0.001, and 0.106, respectively, indicating that N in the leaf, stem, and root had strict homeostasis (Figure 6).

The leaf, stem, and root P showed different stoichiometric homeostasis (Figure 7). The stem P exhibited homeostasis with a 1/H value of 0.208, the leaf P showed plastic status with 1/H of 0.790, whereas the root P exhibited weak homeostasis with a 1/H value of 0.401.

4 Discussion

Pastures with a single crop are verified to be usually degraded as the plantation age increases (Wang et al., 2015; Zhang et al., 2016). Previous studies have examined the relationship between leaf C:N:P stoichiometry (in alfalfa of legumes or in *H. ammodendron* of Chenopodiaceae) and plantation ages or stand ages. This study uses Kentucky bluegrass as a focal crop to examine the effects of plantation ages on C:N:P stoichiometry in leaves, stems, roots, and soils in perennial rhizomatous grass pastures, which can present a pattern of pasture degradation with the increase in plantation age from a N or P limitation perspective.

This study indicates that the C:N ratio in leaves increases from 2 to 6 years of plantation age and decreases from 6 to 9 years of plantation age; the C:N ratio in roots decreases from 2 to 5 years of plantation age, increases from 5 to 7 years of plantation age, and decreases after 7 years of plantation age; whereas the C:N ratio in stems does not differ in plantation ages. These aspects contradict the first hypothesis in this study.

The C:N ratio in leaves in the increasing process of plantation ages is dependent on the changes in leaf C and N concentration from 2 to 9 years of plantation age. The change in C concentrations in leaves may be ascribed to the photosynthetic capacity of Kentucky bluegrass among different plantation ages (Wang C. et al., 2023). The higher plant density in the early stages of plantation often limits the spatial distribution of light (Aerts, 1996; Xu et al., 2023), wherein lower-height plants may not receive sufficient light for photosynthesis and carbon sequestration (Poorter et al., 2012), resulting in the lower leaf C in 2–3 years of plantation age. When the plantation age ranges from 4 to 6 years, plant density often decreases because of self-thinning (Walter et al., 2023), which enables plants to receive more light, resulting in higher photosynthesis with a high C concentration in leaves. When plantation ages are over 6 years, biological processes may decline for perennial plants (Constable and Bange, 2015), leading to a weakened growth potential for Kentucky bluegrass (Yang et al., 2019), negatively contributing to leaf C concentration. In addition, Kentucky bluegrass uptakes soil nitrogen to supply plant growth, and the soil nitrogen can be gradually consumed with the increase in plantation age (Han et al., 2019). The decrease in soil nitrogen concentration is insufficient to meet plant requirements, leading to a decrease in leaf nitrogen concentration (Shi et al., 2015; Wang C. et al., 2023) when plantation age is below 6 years. However, when the plantation age is over 6 years, the accumulation of plant residues gradually increases soil nitrogen, and this added nitrogen can be released into the soil again (Hu et al., 2019; Zhang et al., 2021), which can provide relatively sufficient soil nitrogen for plant uptake, leading to an increase in leaf N when plantation age ranges from 6 to 9 years. With a simultaneous change in leaf C and leaf N in the increasing process of plantation age, leaf C:N increases from 2 to 6 years of plantation age and decreases from 6 to 9 years of plantation age. However, root C:N decreases from 4 to 9 years of plantation age, and this is because N is stored in roots during the period of well-developed roots (Yan et al., 2016) because perennial grass can develop well-rooted over a long plantation age (Robinson et al., 2010; Liu et al., 2018). In general, root C:N is considered an important indicator of the plant growth rate, in which a higher C:N ratio leads to a lower growth rate of perennial plants (Xiao et al., 2021; Wu et al., 2023). Therefore, these results indicated that leaves contribute to more N accumulation for Kentucky bluegrass plants during 2 to 3 years of plantation age, while roots contribute to more N accumulation for Kentucky bluegrass from 4 to 9 years of plantation age. A lower C:N ratio after 4 years of plantation age indicates that litter decomposition and nutrient return become fast (Ning et al., 2021; Wang C. et al., 2023), and this would be conducive to greater nutrient transfer to the soil from 4 to 9 years of plantation age.

Leaf and root C:P show different changeable trends with the increase in plantation age, in which leaf C:P increases and root C:P decreases as plantation age increases, and these are mainly regulated by C and P concentrations in leaf and root. The C concentration in leaves and roots was discussed in the earlier paragraph. The leaf P showed a gradually decreasing trend with the increase in plantation ages, which was mainly caused by soil phosphorus consumption and plant nutrient allocation (Zhang et al., 2021). In the early plantation stages, perennial plants may prioritize the allocation of nutrients to leaves to enhance photosynthesis (Robinson et al., 2010; Poorter et al., 2012), resulting in a higher phosphorus concentration in leaves. In contrast to early plantation stages, Kentucky bluegrass with long-term plantation ages may allocate more nutrients for roots and rhizomes (Jing et al., 2018), resulting in a further decrease in leaf P and an increase in root P.

Leaf N:P ranges from 14.873 to 15.032 between 2 and 3 years of plantation age and exceeds 16 from 4 to 9 years of plantation age, indicating that Kentucky bluegrass is co-limited by nitrogen and phosphorus (Wu et al., 2023) in 2–3 years of plantation age and becomes limited by phosphorus from 4 to 9 years of plantation age. These aspects imply that P is very important to the management of Kentucky bluegrass pasture when the plantation age is considered in practice.

This study also finds that soil C:N increases as the plantation age of Kentucky bluegrass increases. Kentucky bluegrass can sequester CO_2 from the atmosphere, and then, the fixed C can be stored in soil by plant litter (Cotrufo et al., 2013; Jackson et al., 2017) because plant tissues are the principal sources of soil C in terrestrial ecosystems (Yuan et al., 2021). In this case, soil carbon (C) content demonstrates a positive correlation with the age of Kentucky bluegrass plantations, attributed to increased plant biomass. Especially, a significant increase in soil C concentration is observed at the 4-year plantation age, explained by variations in litter quantity and quality (De Deyn et al., 2008; Cotrufo et al., 2013). Reduced litter quantity between 2 and 3 years of plantation age corresponds to diminished organic matter input, thereby decreasing soil organic carbon levels. Moreover, poorer quality litter, characterized by higher C:N ratios during the 2- and 3-year plantation age periods, tends to retard organic matter decomposition (Yuan et al., 2021), in contrast to later plantation stages (from 4 to 9 years of plantation age), fostering a low organic matter content with accelerated decomposition rates during early plantation phases. However, leaf C:N is lower at 2–3 years of plantation age than that during 4–9 years of plantation age, and this contradicts evidence for lower quality plant litter alleviating the decomposition rate of organic matter. In fact, plant roots generate a greater contribution to soil C formation than aboveground litter because roots can immediately interact with the surrounding soil minerals, microbes, and aggregates, which usually form soil C (Sokol and Bradford, 2019; Sokol et al., 2019). Soil N increases greatly from 6 to 9 years of plantation age and the increase in soil nitrogen caused by plant litter can compensate for the soil nitrogen loss due to plant uptake. Although Kentucky bluegrass litter can replenish soil N pools, it can absorb more N from soils because Kentucky bluegrass is nitrophile, and this process often delays soil N accumulation. Thus, soil N storage by plants shows a hysteresis effect compared to soil C accumulation. In this study, soil C and N concentrations exhibit an increase from 2 to 6 years of plantation age, which leads soil C:N to maintain relative stability. When the plantation age is over 7 years, the soil N concentration shows a great increase and soil C concentration shows relative stability, which causes soil C:N to show a great decrease when the plantation age is over 7 years.

Soil N:P increases with the increase in plantation ages. An increase in soil N:P can be explained by the gradual increase in soil N because soil total P is not changeable among nine plantation ages (Figure 4). However, soil N:P is <10 among nine plantation ages, indicating that the growth of Kentucky bluegrass may be limited more by N than by P (Bui and Henderson, 2013). However, soil N:P becomes higher when the plantation age is over 7 years, suggesting that soil N limitation for plant growth may be alleviated due to higher soil N and its availability with lower soil C:N (Bengtsson et al., 2003). These results demonstrate that Kentucky bluegrass pasture can improve the C sequestration potential and provide a good habitat for plant growth when the plantation age is over 7 years.

This study further finds that N in the leaf, stem, and root and P in the stem show a “homeostatic” status, and P in the root shows a “weakly homeostatic” status, whereas P in the leaf shows a “plastic” status, demonstrating that Kentucky bluegrass may maintain leaf P nutrient stability by modulating the P nutrient in the stems (Xiao et al., 2021). These results imply that soil P is more important in regulating the health growth of Kentucky bluegrass than soil N, which provides precise information for N and P management in perennial rhizomatous grass pastures.

5 Conclusion

This study used Kentucky bluegrass as a focal crop to investigate the plant (root, stem, and leaf) and soil C:N:P stoichiometry to understand the stoichiometric homeostasis as plantation age increases. This study found that plantation age had significant effects on plant (root, stem, and leaf) and soil C:N:P stoichiometry. Leaf C:N, C:P, and N:P increased from 2 to 6 years of plantation age, whereas leaf C:N decreased and leaf C:P and N:P maintained stability from 6 to 9 years of plantation age. Stem C:P increased and then maintained stability. Root N:P increased from 2 to 6 years of plantation age and maintained stability from 6 to 9 years of plantation age, whereas root C:N and C:P decreased from 2 to 9 years of plantation age. Soil C:N and N:P were found to maintain stability from 2 to 6 years of plantation age, and soil C:N was found to decrease and soil N:P was found to increase after 6 years of plantation age. The N in the leaf, stem, and root and the P in the stem had the "homeostatic" status, and P in the root had the "weakly homeostatic" status, whereas the P in the leaf had the "plastic" status. These results present a pattern concerning the effect of plantation age on plant (leaf, stem, and root) and soil C:N:P stoichiometry in a crop and suggest that Kentucky bluegrass pastures with plantation age over 6 years can improve soil quality and nutrient availability for plant growth.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

XW: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Writing—original draft. KH: Data curation, Investigation, Methodology, Writing—original draft, Visualization. QW: Data curation, Formal analysis, Visualization, Writing—original draft. WL: Methodology, Project administration, Resources, Writing—original draft. XP: Data curation, Formal analysis, Visualization, Writing—original draft. ZG: Conceptualization, Methodology, Supervision, Writing—original draft, Resources.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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References

Aerts, R. (1996). Nutrient resorption from senescing leaves of perennials are there general patterns. *J. Ecol.* 84, 597–608. doi: 10.2307/2261481

Bengtsson, G., Bengtson, P., and Månsson, K. F. (2003). Gross nitrogen mineralization-, immobilization-, and nitrification rates as a function of soil C/N ratio and microbial activity. *Soil Biol. Biochem.* 35, 143–154. doi: 10.1016/S0038-0717(02)00248-1

Bui, E. N., and Henderson, B. L. (2013). C: N: P stoichiometry in Australian soils with respect to vegetation and environmental factors. *Plant Soil* 373, 553–568. doi: 10.1007/s11104-013-1823-9

Cao, Y., and Chen, Y. M. (2017). Coupling of plant and soil C:N:P stoichiometry in black locust (*Robinia pseudoacacia*) plantations on the Loess Plateau, China. *Trees* 31, 1559–1570. doi: 10.1007/s00468-017-1569-8

Constable, G. A., and Bange, M. P. (2015). The yield potential of cotton (*Gossypium hirsutum* L.). *Field Crops Res.* 182, 98–106. doi: 10.1016/j.fcr.2015.07.017

Cotrufo, M. F., Wallenstein, M. D., Boot, C. M., Denef, K., and Paul, E. (2013). The Microbial Efficiency-Matrix Stabilization (MEMS) framework integrates plant litter decomposition with soil organic matter stabilization: do labile plant inputs form stable soil organic matter? *Glob. Change Biol.* 19, 988–995. doi: 10.1111/gcb.12113

Cui, J., Shah, S., Fahad, S., and Chen, Y. (2020). A review on kentucky bluegrass responses and tolerance to drought stress. *Abiotic Stress Plants* 1, 1–10. doi: 10.5772/intechopen.93812

De Deyn, G. B., Cornelissen, J. H., and Bardgett, R. D. (2008). Plant functional traits and soil carbon sequestration in contrasting biomes. *Ecol Lett.* 11, 516–531. doi: 10.1111/j.1461-0248.2008.01164.x

DeKeyser, E., Dennhardt, L., and Hendrickson, J. (2015). Kentucky bluegrass (*Poa pratensis*) invasion in the northern great plains: a story of rapid dominance in an endangered ecosystem. *Invasive Plant Sci. Manage.* 8, 255–261. doi: 10.1614/IPSM-D-14-00069.1

Dijkstra, F. A., Pendall, E., Morgan, J. A., Blumenthal, D. M., Carrillo, Y., Lecain, D. R., et al. (2012). Climate change alters stoichiometry of phosphorus and nitrogen in a semiarid grassland. *New Phytol.* 196, 807–815. doi: 10.1111/j.1469-8137.2012.04349.x

Duan, Y. Y., Zhang, J., Jiang, Z., Wei, X. X., Guo, Z. G., and Liu, H. X. (2024). Partial root-zone drying (PRD) leads to lower carbon retention in the soil-plant systems of alfalfa. *Irrig Sci.* 42, 45–56. doi: 10.1007/s00271-023-00902-6

Folck, A. J., Bigelow, C, A., Jiang, Y., and Patton, A. (2023). Growth analysis of Kentucky bluegrass cultivars from six classification groups. *Agrosyst. Geosci. Environ.* 6:e20444. doi: 10.1002/agg2.20444

Han, X. Y., Gao, G. Y., Li, Z. S., Chang, R., Jiao, Y., and Fu, L. (2019). Effects of plantation age and precipitation gradient on soil carbon and nitrogen changes following afforestation in the Chinese Loess Plateau. *Land Degrad. Dev.* 30, 2298–2310. doi: 10.1002/ldr.3422

He, M. Z., and Dijkstra, F. A. (2015). Phosphorus addition enhances loss of nitrogen in a phosphorus-poor soil. *Soil Biol Biochem.* 82, 99–106. doi: 10.1016/j.soilbio.2014.12.015

Hood, J. M., and Sterner, R. W. (2010). Diet mixing: do animals integrate growth or resources across temporal heterogeneity? *Am. Soc Nat.* 176, 651–663. doi: 10.1086/656489

Hu, C. C., Lei, Y. B., Tan, Y. H., Sun, X. C., Xu, H., Liu, C. Q., et al. (2019). Plant nitrogen and phosphorus utilization under invasive pressure in a montane ecosystem of tropical China. *J Ecol.* 107, 372–386. doi: 10.1111/1365-2745.13008

Jackson, R. B., Lajtha, K., Crow, S. E., Hugelius, G., Kramer, M. G., and Piñeiro, G. (2017). The ecology of soil carbon: pools, vulnerabilities, and biotic and abiotic controls. *Annu. Rev. Ecol. Evol. Syst.* 48, 419–445. doi: 10.1146/annurev-ecolsys-112414-054234

Jing, M. L., Wang, Y. L., Ma, Y. S., and Li, S. X. (2019). Optimus conditions of *Poa pratensis L.* cv. Qinghai cultivation in Qilian mountain. *Acta Agr. Bor. Occ. Sin.* 28, 31–40. doi: 10.7606/j.issn.1004-1389.2019.01.005

Jing, M. L., Wang, Y. L., Ma, Y. S., Li, S. X., and Yang, H. Z. (2018). Effects of sowing dates on growth of *Poa pratensis L.* cv. Qinghai. *Acta Agr. Bor. Occ. Sin.* 27, 1754−1762. doi: 10.7606/j.issn.1004-1389.2018.12.007

Jones, M. B., Finnan, J., and Hodkinson, T. R. (2015). Morphological and physiological traits for higher biomass production in perennial rhizomatous grasses grown on marginal land. *Glob. Change Bio.* 7, 375–385. doi: 10.1111/gcbb.12203

Kleyer, M., Trinogga, J., Cebrián-Piqueras, M. A., Trenkamp, A., Fløjgaard, C., Ejrnæs, R., et al. (2019). Trait correlation network analysis identifies biomass allocation traits and stem specific length as hub traits in herbaceous perennial plants. *J. Ecol.* 107, 829–842. doi: 10.1111/1365-2745.13066

Lindsey, A. J., Thoms, A. W., and Christians, N. (2020). Kentucky bluegrass and bermudagrass rooting response to humic fertilizers during greenhouse establishment. *Agron. J.* 112, 3396–3401. doi: 10.1002/agj2.20199

Liu, Z. Y., Taogetao, B. Y., Sun, J., Hugjiltu, M., and Li, X. L. (2018). Plant sizes mediate mowing-induced changes in nutrient stoichiometry and allocation of a perennial grass in semi-arid grassland. *Ecol. Evol.* 8, 3109–3118. doi: 10.1002/ece3.3866

Mazzucato, A., Falcinelli, M., and Veronesi, F. (1996). Evolution and adaptedness in a facultatively apomictic grass, *Poa pratensis* L. *Euphytica* 92, 13–19. doi: 10.1007/BF00022823

Ning, Z. Y., Zhao, X. Y., Li, Y. L., Wang, L. L., Lian, J., Yang, H. L., et al. (2021). Plant community C: N: P stoichiometry is mediated by soil nutrients and plant functional groups during grassland desertification. *Ecol. Eng.* 162:106179. doi: 10.1016/j.ecoleng.2021.106179

Pang, X. P., Yang, H., Wei, X. X., and Guo, Z. G. (2021). Effect of plateau pika (*Ochotona curzoniae*) bioturbation on soil C-N-P stoichiometry in alpine meadows. *Geoderma* 397:115098. doi: 10.1016/j.geoderma.2021.115098

Pathak, K., Nath, A. J., Sileshi, G. W., Lal, R., and Das, A. K. (2017). Annual burning enhances biomass production and nutrient cycling in degraded imperata grasslands. *Land Degrad. Dev.* 28, 1763–1771. doi: 10.1002/ldr.2707

Persson, J., Fink, P., Goto, A., Hood, J. M., Jonas, J., and Kato, S. (2010). To be or not to be what you eat: regulation of stoichiometric homeostasis among autotrophs and heterotrophs. *Oikos* 119, 741–751. doi: 10.1111/j.1600-0706.2009.18545.x

Pi, E. R., Qu, L. Q., Tang, X., Peng, T. T., Jiang, B., Guo, J. F., et al. (2015). Application of genetic algorithm to predict optimal sowing region and timing for kentucky bluegrass in China. *PLoS ONE* 10:e0131489. doi: 10.1371/journal.pone.0131489

Poorter, H., Niklas, K. J., Reich, P. B., Oleksyn, J., Poot, P., Mommeret, L., et al. (2012). Biomass allocation to leaves, stems and roots: meta-analyses of interspecific variation and environmental control. *New Phytol.* 193, 30–50. doi: 10.1111/j.1469-8137.2011.03952.x

Robinson, D., Davidson, H., Trinder, C., and Brooker, R. (2010). Root-shoot growth responses during interspecific competition quantified using allometric modelling. *Ann. Bot.* 106, 921–926. doi: 10.1093/aob/mcq186

Shi, Z. M., Tang, J. C., Cheng, R. M., Luo, D., and Liu, S. R. (2015). A review of nitrogen allocation in leaves and factors in its effects. *Acta Ecol. Sin.* 35, 5909–5919. doi: 10.5846/stxb201401240184

Sokol, N. W., and Bradford, M. A. (2019). Microbial formation of stable soil carbon is more efficient from belowground than aboveground input. *Nat. Geosci.* 12, 46–53. doi: 10.1038/s41561-018-0258-6

Sokol, N. W., Kuebbing, S. E., Karlsen-Ayala, E., and Bradford, M. A. (2019). Evidence for the primacy of living root inputs, not root or shoot litter, in forming soil organic carbon. *New Phytol.* 221, 233–246. doi: 10.1111/nph.15361

Toledo, D., Swanson, K., Meehan, M., Dahlen, C., Christensen, R., Asplin, D., et al. (2023). Agronomic and forage nutritive responses of Kentucky bluegrass dominated pastures in the northern Great Plains. *Grass Forage Sci.* 78, 268–274. doi: 10.1111/gfs.12610

Walter, G. M., Clark, J., Terranova, D., Cozzolino, S., Cristaudo, A., Hiscock, S. J., et al. (2023). Hidden genetic variation in plasticity provides the potential for rapid adaptation to novel environments. *New Phytol.* 239, 374–387. doi: 10.1111/nph.18744

Wang, C., Wang, S., Fu, B. J., Li, Z. S., and Lü, Y. H. (2023). Plantation understorey legume functional groups enhance soil organic carbon sequestration by promoting species richness. *Land Degrad Dev.* 34, 2177–2188. doi: 10.1002/ldr.4598

Wang, Y., Niu, D., Yuan, X., Guo, D., Fu, H., Elser, J. J., et al. (2023). Dominant plant species alter stoichiometric imbalances between soil microbes and their resources in an alpine grassland: Implications for soil microbial respiration. *Geoderma* 431:116336. doi: 10.1016/j.geoderma.2023.116336

Wang, Z. N., Lu, J. Y., Yang, M., and Zhang, Q. (2015). Stoichiometric characteristics of carbon, nitrogen, and phosphorus in leaves of differently aged lucerne (*Medicago sativa*) stands. *Front. Plant Sci.* 6:1062. doi: 10.3389/fpls.2015.01062

Wei, Y., Ma, Z. Y., Zhou, J. Y., and Zhang, Z. H. (2022). Experimental warming changed reproductive phenology and height of alpine plants on the Qingzang Plateau. *Chin. J. Plant Ecol.* 46, 995–1004. doi: 10.17521/cjpe.2021.0450

Wen, J. H., Ma, Y. S., Shi, J. J., Dong, Q. M., and Wang, Y. L. (2006). Study on vegetation rebuilding of "black soil type" degraded grassland with *Poa pratensis* in the riparian areas of rivers. *Grassland Turf* 2, 41–44. doi: 10.13817/j.cnki.cyycp

Wu, G., Gao, J., Li, H., Ren, F., Liang, D., and Li, X. (2023). Shifts in plant and soil C, N, and P concentrations and C: N: P stoichiometry associated with environmental factors in alpine marshy wetlands in West China. *Catena* 221:106801. doi: 10.1016/j.catena.2022.106801

Xiao, L., Bi, Y., Du, S., Wang, Y., Guo, C., and Christie, P. (2021). Response of ecological stoichiometry and stoichiometric homeostasis in the plant-litter-soil system to re-vegetation type in arid mining subsidence areas. *J. Arid Environ.* 184:104298. doi: 10.1016/j.jaridenv.2020.104298

Xu, X. P., Liu, Y. J., and Wang, J. J. (2023). Density management diagrams for *Larix gmelinii* plantations in Daxing'an mountains. *Natural Protected Areas* 3, 79–88. doi: 10.12335/2096-8981.2023070601

Yan, B. G., Ji, Z. H., Fan, B., Wang, X., He, G., Shi, L., et al. (2016). Plants adapted to nutrient limitation allocate less biomass into stems in an arid-hot grassland. *New Phytol.* 211, 1232–1240. doi: 10.1111/nph.13970

Yang, D., Song, L., and Jin, G. (2019). The soil C: N: P stoichiometry is more sensitive than the leaf C: N: P stoichiometry to nitrogen addition: a four-year nitrogen addition experiment in a *Pinus koraiensis* plantation. *Plant Soil* 442, 183–198. doi: 10.1007/s11104-019-04165-z

Yu, Y. H., Zheng, W., Zhong, X. P., and Ying, B. (2021). Stoichiometric characteristics in *Zanthoxylum planispinum* var. dintanensis plantation of different ages. *Agron. J.* 113, 685–695. doi: 10.1002/agj2.20388

Yuan, X., Chen, Y., Qin, W., Xu, T., Mao, Y., Wang, Q., et al. (2021). Plant and microbial regulations of soil carbon dynamics under warming in two alpine swamp meadow ecosystems on the Tibetan Plateau. *Sci. Total Environ.* 790:148072. doi: 10.1016/j.scitotenv.2021.148072

Zeng, Q., Lal, R., Chen, Y., and An, S. (2017). Soil, leaf and root ecological stoichiometry of *Caragana korshinskii* on the Loess Plateau of China in relation to plantation age. *PLoS ONE* 12:e0168890. doi: 10.1371/journal.pone.0168890

Zhang, J., Wang, Q., Pang, X. P., Xu, H. P., Wang, J., Zhang, W. N., et al. (2021). Effect of partial root-zone drying irrigation (PRDI) on the biomass, water productivity and carbon, nitrogen and phosphorus allocations in different organs of alfalfa. *Agr. Water Manage.* 243:106525. doi: 10.1016/j.agwat.2020.106525

Zhang, K., Su, Y. Z., Liu, T. N., and Wang, T. (2016). Leaf C:N:P stoichiometrical and morphological traits of *Haloxylon ammodendron* over plantation age sequences in an oasis-desert ecotone in North China. *Ecol. Res.* 7, 185−191. doi: 10.1007/s11284-016-1353-z

Zhu, H., Guo, P., and Li, D. (2023). Parameters of nitrogen use efficiency of Kentucky bluegrass cultivars at different N levels under deficit irrigation. *Grass Forage Sci.* 1–12. doi: 10.1111/gfs.12637

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Physio-biochemical and transcriptomic analysis of *Bacillus amyloliquefaciens* PG-4-induced salt stress tolerance in *Macrotyloma uniflorum*

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Introduction: *Macrotyloma uniflorum* is an important legume fodder crop and green fertilizer. Salinity impedes plant growth and productivity of legume crops by disrupting the ionic and osmotic balance and hormonal regulation. Plant growth-promoting rhizobacteria (PGPR) are rhizosphere bacteria that contribute to the improvement of plant growth through diverse physiological mechanisms.

Methods: In this study, the growth promoting characteristics of the isolated strain *Bacillus amyloliquefaciens* PG-4 were analyzed, and to further investigated the possible mechanism of PG-4 in mitigating the damage caused by salt stress in *M. uniflorum* plants through pot experiments.

Results: In presence of different salt levels, PG-4 showed a high potentiality to produce several plant growth promoting metabolites such as NH_3, siderophore, 1-aminocyclopropane-1-carboxylic acid deaminase (ACC-deaminase), and hydrolytic enzymes. Inoculation of the PG-4 significantly enhanced plant tolerance to salt stress, as demonstrated by promotion of plant growth (shoot and root biomass) under salt stress condition. Furthermore, PG-4 improved salt tolerance of *Macrotyloma uniflorum* seedlings by affecting the antioxidant enzymes including peroxidase (POD) and superoxide dismutase (SOD), by increasing the levels of proline, soluble sugars and chlorophyll. Treatment with PG-4 increased the K^+ content while decreased the Na^+ concentration level under salt stress. Transcriptomic analysis revealed 5525 genes were differentially expressed (PG-4-inoculated versus non-inoculated samples) at 0 mM NaCl, of which 3277 were upregulated and 2248 downregulated, while 1298 genes were differentially expressed at 100 mM NaCl, of which 819 were upregulated and 479 were downregulated. GO and KEGG enrichment analyses showed that these DEGs were significantly enriched in several terms and pathways mainly involved in the regulation of the cellular redox state, cell wall modification, metabolic adjustments, hemoglobin, biosynthesis of secondary metabolites and plant hormone signal transduction.

Discussion: These data showed that *Bacillus amyloliquefaciens* PG-4 significantly enhance salt stress tolerance in *Macrotyloma uniflorum* plants during salt stress conditions. Therefore, the results may be useful for explaining the mechanism by which PGPR inoculation regulates the salt tolerance of crops.

KEYWORDS

plant growth-promoting rhizobacteria, *Bacillus amyloliquefaciens*, *Macrotyloma uniflorum*, salt stress tolerance, transcriptome

Introduction

Soil salinity is one of the most serious problems associated with soil and is considered a major abiotic threat to agricultural production worldwide. Currently, on a global scale, 20% of the land used for agriculture is affected, and annually, 2,500–5,000 km^2 of crop production is lost annually due to salinity (Singh et al., 2022). Moreover, it has been estimated that by 2050, nearly 50% of arable land will be affected by salinity (Butcher et al., 2016). Salinity can have various effects on all plants, reducing crop growth and output by altering morphological, biochemical and physiological processes (Ali et al., 2022). An excessive accumulation of sodium (Na^+) and chloride (Cl^-) ions during salt stress causes ionic toxicity and leads to the generation of reactive oxygen species (ROS) in plant cells (Munns and Tester, 2008; Farhangi-Abriz et al., 2020). These ROS include superoxide, hydrogen peroxide, hydroxyl radicals, singlet oxygen, etc., which in excess amounts threaten plant cell integrity by initiating lipid peroxidation, damaging nucleic acids and activating programmed cell death and enzyme inhibition (Farhangi-Abriz et al., 2020). In addition, higher salt concentrations in the soil cause lower water availability, a decrease in plant nutrient uptake, and disturbed soil properties such as porosity, aeration and water conductance (Ibarra-Villarreal et al., 2021). However, in response to salt-stressed environments, plants activate various self-defense mechanisms, including osmotic adjustment, antioxidant metabolism and plant hormones (Liu et al., 2022; Wang Y. et al., 2022; Wang Q. et al., 2022). Specifically, osmotic adjustment occurs through the accumulation of organic solutes and inorganic ions in plants (Wang Y. et al., 2022; Wang Q. et al., 2022; Pistelli et al., 2023). Plant antioxidant metabolism involves nonenzymatic components such as ascorbic acid (AsA), glutathione (GSH), carotenoids and phenolic compounds, as well as enzymes such as dehydroascorbate reductase (DHAR); ascorbate peroxidase (APX); glutathione reductase; catalase (CAT); superoxide dismutase (SOD); glutathione peroxidase (GSH-Px); and glutathione S-transferase (GST), which clear excess ROS (Garcia-Caparros et al., 2021). Furthermore, plant hormones, such as ethylene, abscisic acid (ABA) and 3-indoleacetic acid (IAA), are known to endogenously regulate the homeostasis of plants and lead plants to perceive and respond to salt stress (Singh et al., 2022). However, these mechanisms are insufficient for alleviating salt stress beyond a certain limit.

To boost crop yield in salinized land, a large amount of chemical fertilizer is applied to the soil, but the increase in chemical input is not directly proportional to the increase in crop yield (Wang et al., 2018). This behavior has also led to serious soil ecological and environmental problems, including secondary salinization, soil consolidation, soil acidification and microecological imbalance (Ma et al., 2021). Furthermore, several molecular breeding and genetic engineering methods have been developed for improving crop yield under salt stress based on plant defense mechanisms. Nonetheless, these methods are expensive and time-consuming (Haque et al., 2021; Ashraf and Munns, 2022). To overcome the above issues, a new biocontrol approach has been developed to protect plants from salt stress in soil by utilizing beneficial microorganisms (Etesami and Maheshwari, 2018; Etesami and Glick, 2020). Plant growth-promoting rhizobacteria (PGPR) are an important cluster of beneficial, root-colonizing bacteria thriving in the plant rhizosphere, and positive effects on plant growth, development and metabolism (Li et al., 2020). When combined with roots and other tissues, PGPR improve the nutritional supply of crop plants through several mechanisms. PGPR have direct effects, including nitrogen fixation, phosphorus solubilization, NH_3 production, IAA, and siderophores, and indirect effects, including antioxidant defense, volatile organic compound (VOC) production, exopolysaccharide (EPS) production, and osmotic balance mechanisms for improving plant growth and enhancing tolerance against salt stress (Etesami and Maheshwari, 2018; Mohanty et al., 2021). Therefore, an increasing number of scholars are studying how soil microorganisms play a role in plant growth to achieve eco-friendly and sustainable agriculture.

Diverse members of the *Bacillus* genus, which is one of the most extensively studied genera with beneficial microorganisms in the rhizosphere, have also been shown to be involved in promoting plant growth and tolerance against environmental stresses (Egamberdieva et al., 2019; Ibarra-Villarreal et al., 2021; Patani et al., 2023). Several mechanisms underlying the interactions between plants and *Bacillus* strains under salt stress have been revealed. *B. atrophaeus* WZYH01 protected maize from salt stress by regulating the levels of plant hormones (IAA and ABA) and increasing nutrient acquisition (Hou et al., 2022). Similarly, *B. pumilus* NCT4, *B. firmus* NCT1, *B. licheniformis* LCT4, *B. cereus* LAT3, and *B. safensis* LBM4 enhanced the tolerance of tomato plants to salt stress through increased total soluble sugar, proline and chlorophyll contents (Patani et al., 2023). *B. subtilis* helps mitigate salt-induced oxidative stress in soybean plants by modulating antioxidant defense and glyoxalase systems while maintaining ion homeostasis and osmotic adjustment (Hasanuzzaman et al., 2022). Woo et al. (2020) analyzed the ameliorative effects of the *B. subtilis* strain GOT9 on *Arabidopsis thaliana* and *Brassica campestris* under salt stress. Collectively, these results show that a variety of *Bacillus* strains interact with their specific plant hosts and contribute to enhanced tolerance against salt stress. The acclimation of plants to environmental stress after PGPR induction is a complex and coordinated response involving multiple physiological, biochemical, metabolic, and molecular mechanisms. Hence, a thorough understanding of the mechanisms induced by the strain in plants is imperative for enhancing plant yield and quality, as well as sustainable agricultural development.

Legumes are susceptible to a range of abiotic threats, with salinity being one of the most critical barriers to crop yield (Yilmaz and Kulaz, 2019). Horsegram [*Macrotyloma uniflorum* (Lam.) Verdc] is a dicotyledonous plant that belongs to the Leguminosae family, extensively distributed across South Asia, Australia, West Indies and Africa. This species is cultivated mainly for providing nutritional security in the form of food, livestock supplements and green manure because of its high nitrogen availability, nutritious composition, indomitable pest resistance, and drought tolerance (Bolbhat and Dhumal, 2009), and it has been identified as a potential, future food source by the U.S. National Academy of Sciences (Kadam and Salunkhe, 1985). Like several other legume crops, *M. uniflorum* is susceptible to salinity stress. Therefore, it is necessary to improve the tolerance of *M. uniflorum* to the deleterious effects of salinity, which could be achieved by using PGPR. However, knowledge of the potential of PGPR isolates and their effects on the growth and physiological characteristics of *M. uniflorum* under salt stress remains quite limited. The present study was carried out to determine the behavior of the selected PGPR strains under salinity stress conditions as well as their role in enhancing *M. uniflorum* growth. Thus, the present study was undertaken. (a) Study of the growth-promoting traits of isolated strains, (b) identification and characterization of the isolated bacteria based on biochemical characteristics and confirmation of the bacterial genera through 16S rRNA sequence analysis, and (c) evaluation of the effects of the bacteria on the growth, physiology, and expression level of stress tolerance genes in *M. uniflorum* seedlings under salt stress. Our results not only provide valuable information for exploring the mechanism by which PGPR alleviate the osmotic and oxidative stress of salt-stressed plants but also provide an eco-friendly method for cultivating high-quality and high-yield *M. uniflorum* in salt environments, and provide theoretical reference for the biological improvement of salt soil.

Materials and methods

Materials

M. uniflorum seeds of the native cultivar Yazhou with a viability >98% were obtained from the Institute of Tropical Crop Genetic Resources, Chinese Academy of Tropical Agricultural Sciences. Healthy seeds were selected and stored in a Kraft paper bag at 4°C until use. The PGPR strain of *B. amyloliquefaciens* PG-4 (16S rRNA GenBank: PP320457) was isolated from *M. uniflorum* rhizosphere soil in Danzhou, Hainan Province, China (19°31'N, 109°34′E). The isolation procedure was the same as that described in Hmaeid et al. (2019) and Patani et al. (2023). The strain was stored in 25% glycerol solution at −80°C until use. The strain was preserved in the China Center for Type Culture Collection, with preservation numbers of CCTCC AB 2024049.

Morphological observation of strains and homology analysis of 16S rDNA sequences

After preparing a 24-h culture, the strain was smeared on slides and subsequently fixed with heat. The samples were treated with crystal violet for 1 min following rinsing with running water. The samples were again flooded with gram iodine solution for 1 min following washing with alcohol. Finally, the samples were flooded with safranin, washed with water, dried, and observed under oil immersion conditions (Masi et al., 2021).

Genomic DNA was extracted using an OMEGA Genomic DNA Extraction Kit. Next, 16S rDNA fragments were PCR-amplified with the universal primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT). The PCR mixture (30 μL) consisted of 15 μL of 2 × PCR mix, 1 μL of DNA, 1 μL of the upstream primer (10 mM), 1 μL of the downstream primer (10 mM), and 12 μL of ddH_2O. Reaction conditions: 95°C 5 min, 94°C 45 s, 55°C 45 s, 72°C 1 min 15 s, 32 cycles, 72°C 10 min. After the reaction was completed, the size and specificity of the amplified fragments were verified by 1% agarose gel electrophoresis and photographed using a gel imaging system. The PCR amplification products were purified and sequenced by Nanshan Biotech (China) Co., Ltd. The 16S rDNA sequencing results of the obtained strains were analyzed using BLAST, and the related sequences with high homology were selected for relationship analysis. A phylogenetic tree was constructed via the neighbor method in MEGA6.0 software.

Plant growth promotion assays

The plant growth-promoting properties of PG-4, including nitrogen fixation, siderophore production, NH_3 production, ACC deaminase production, cellulase production, pectinase production and amylase production, were evaluated in the presence of 0 mM, 50 mM, 100 mM and 200 mM NaCl. The determination method is described below.

Nitrogen fixation

A nitrogen (N_2) fixation test was performed in malate nitrogen-free mineral media with modifications (Baldani and Döbereiner, 1980). The inoculated media were incubated for 72 h at 30 ± 2°C. A color change from pale green to blue was considered to indicate a positive effect of N_2-fixing activity.

Siderophore production

Siderophore secretion by the isolated bacteria was assessed using blue agar plates containing chrome azurol S (CAS) (Sigma–Aldrich) (Schwyn and Neilands, 1987). A positive reaction was indicated by the formation of an orange zone around the colony, indicating siderophore excretion.

NH_3 production

NH_3 production was checked on peptone water according to Cappuccino and Sherman (1992). Fresh cultures grown in LB broth were inoculated into 10 mL of peptone water and incubated at 30 ± 2°C. After 48 h, 500 μL of Nessler's reagent was added to each tube. Ammonia production is indicated by the development of a color change from brown to yellow.

ACC deaminase production

The 1-aminocyclopropane-1-carboxylic acid deaminase activity of PG-4 was detected by using DF medium and ADF medium (Shanghai Yuanye Bio-Technology Co., Ltd., China). These media were inoculated with 1% (v/v) fresh PG-4 cultures and incubated in a shaker (180 rpm, 28°C) for 5 days. The optical density was measured at 600 nm by a spectrophotometer from three replicates; the optical density result was considered to indicate a positive result for ACC deaminase production for PG-4 that was grown in ADF media and that showed no growth on DF media.

Assay for enzyme production

Cellulase production was tested using carboxymethylcellulose (CMC) agar medium (Gupta et al., 2012). After 24 h of incubation at

30°C, the CMC plates were flooded twice: first, with an aqueous solution of Congo red (1% w/v) for 15 min, followed by 1 M NaCl for 15 min. A clear halo was considered to indicate cellulase production. Pectinase production was tested as described by Plazinski and Rolfe (1985) using ammonium mineral agar medium, with some modifications. After 3 days at 30 ± 2°C, the plates were flooded with 2% hexadecyltrimethylammonium bromide. Pectinase production was detected by clear halos around the colonies. Amylase production was determined by soluble starch agar medium. After 2 days of incubation at 28°C, the ability of PG-4 to hydrolyze amylase was determined by the appearance of a halo zone around the colonies, which was confirmed by immersion in Lugol's iodine solution for 15 min and 70% ethanol (Zhou et al., 2021). The noninoculated plates were used as controls.

Evaluation of the plant growth-promoting ability of *B. amyloliquefaciens* PG-4

The healthy seeds were surface-sterilized by immersion in 70% ethanol for 50 s, followed by rinsing three times with sterile distilled water, submerging in 10% (v/v) sodium hypochlorite for 15 min and subsequent washing 5 times with sterile distilled water. The seed germination experiment was carried out as described by Li et al. (2021) with minor modifications. The sterilized plants were completely soaked in 20 mL of PG-4 suspension ($OD_{600} = 0.6$) for 2 h to allow PG-4 to colonize the *M. uniflorum* seeds, which served as the inoculation treatment. Seeds soaked in LB media or distilled water served as uninoculated controls. Next, seeds subjected to different treatments were air-dried at room temperature and subsequently transferred to Petri dishes (10 × 10 cm) containing 0.7% water agar at 25°C under a 12 h light/12 h dark cycle for germination. Each treatment had five replicates, and each replicate contained 18 seeds. The germinated seeds were counted every day until they reached a constant number (approximately 2 days). The germination percentage, seedling plumule length, radicle length, and fresh weight were measured at 3 days after germination.

Pot experimental design

The experimental treatments included (i) no bacterial inoculum or salt stress (CN), (ii) bacterial inoculum or salt stress (BN), (iii) no bacterial inoculum with salt stress (CS), and (iv) bacterial inoculum and salt stress (BS). Surface-sterilized *M. uniflorum* seeds with normal and healthy appearances were selected. Before sowing, the seeds were inoculated by immersion for 2 h in the respective bacterial inoculum ($OD_{600} = 0.6$) or in sterile distilled water (bacterial inoculum treatment and uninoculated controls). The seeds were subsequently transferred to a separate pot (10 cm × 8 cm × 10 cm) filled with a mixture of autoclaved vermiculite and nutrient soil (1:1, v:v). After 7 and 14 days, the seedlings were thinned to one per pot and then reinoculated with 1.0 mL of the corresponding bacterial inoculum ($OD_{600} = 0.6$) (BN and BS) or sterile distilled water (CN and CS) was applied to the stem base. Salt stress was imposed beginning on day 12 by supplementing the watering solution (water or nutritive solution) with 100 mM NaCl. The plants were grown at 25°C under a 16 h light/8 h dark cycle at Hainan University (Haikou, Hainan, China). Pots were arranged in a randomized block design, and each group consisted of 8 pots. Each treatment was kept in independent trays to avoid contamination, and the trays were rotated weekly to randomize possible position effects. After 14 days of stress, the SPAD values; plant height; plant diameter; and shoot and root fresh weights (FWs) were recorded. Then, all plants were removed from the pots, immediately cleaned with distilled water, and then stored at −80°C to measure physiological characteristics. In the remaining three pots of each treatment, all the plants in the pot were removed from the pot and cleaned immediately with distilled water. Then, the samples were oven dried at 60°C for 72 h to measure their dry weight and Na^+ and K^+ concentrations.

Physiological characterization and determination of ion contents

Prior to harvest, a SPAD LD-YD chlorophyll meter (LANENDE, Shandong, China) was used to directly determine the relative chlorophyll contents of the plant leaves via soil and plant analyzer development (SPAD). The mature leaves of 10 plants were tested 3 times, and the average values were obtained (Li et al., 2020). The contents of proline (Pro), total soluble sugar (TSS) and malondialdehyde (MDA) were measured by a PRO BC0295 assay kit (Beijing Solarbio Science & Technology Co., Ltd), a BC0035 Plant Soluble Sugar Content Assay Kit (Beijing Solarbio Science & Technology Co., Ltd), and a BC0025 MDA assay kit (Beijing Solarbio Science & Technology Co., Ltd.) following the manufacturer's instructions, respectively. The activities of SOD and POD were measured by a BC0175 SOD assay kit (Beijing Solarbio Science & Technology Co., Ltd.) and a POD BC0095 assay kit (Beijing Solarbio Science & Technology Co., Ltd.) following the manufacturer's instructions, respectively. All leaves used in testing were collected from mature plants at a single location.

A fine powder of dry plant leaves was mixed with acetic acid (0.1 N) (w/v) at 90°C for 2 h, and flame emission spectroscopy was used to measure the Na^+ concentrations (Rus et al., 2001), the contents of K^+ were measured by a Potassium Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

RNA sequencing procedures

RNA was extracted from twelve samples (four treatments × three biological replicates) using a TRIzol reagent kit (Invitrogen, Carlsbad, CA, United States). Total RNA was reverse transcribed to cDNA using an NEBNext Ultra RNA Library Prep Kit for Illumina (NEB #7530, New England Biolabs, Ipswich, MA, USA). Library construction, quality detection and Illumina sequencing were carried out by Gene Denovo Biotechnology Co., Guangzhou, China. An Agilent Bioanalyzer 2,100 system was used to evaluate RNA quality, and library sequencing was performed through an Illumina NovaSeq 6,000 platform. After removing adaptors, the unknown nucleotides and those of low quality were filtered from the raw reads to obtain clean reads. All the downstream analyses were based on these clean reads. *De novo* assembly was carried out using the *de novo* assembly software Trinity.[1]

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[1] http://trinityrnaseq.sourceforge.net/

Differential expression analysis

To compare the read count values of every gene to the original expression of the gene, HTSeq (0.9.1) statistics were used to standardize the expression by using fragments for exon kilobase per million fragments mapped (FPKM) values. Genes with a false discovery rate (FDR) < 0.05 and an absolute fold change ≥ 2 were defined as differentially expressed genes (DEGs). The software package R language heatmap (1.0.8) was used for bidirectional clustering analysis of all genes for all samples. For various samples, a heatmap was generated using the Euclidean method to measure the distance to and complete relation method to the cluster according to the expression level of the same and different genes in the same sample.

Gene annotation and pathway analysis

For annotations, all unigenes that were proven to be longer than 200 bp were subjected to a BLAST search (E-value $<1e^{-5}$) against the NCBI nonredundant (NR) protein database (Pruitt et al., 2007), manually annotated and reviewed protein sequence database Swiss-Prot (Apweiler et al., 2004), Gene Ontology (GO) (Ashburner et al., 2000), Clusters of Orthologous Groups of proteins (KOG/COG) (Tatusov et al., 2000; Koonin et al., 2004), protein family (Pfam) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (Finn et al., 2008; Kanehisa et al., 2008). The Gene Ontology database[2] was used to map all the DEGs; however, Blast2GO software was used to calculate the differentially enriched genes. KEGG analysis of all the DEGs was performed by using KOBAS 2.0 software. For further analysis, significant pathways were selected.

Quantitative real-time PCR

The expression patterns of ten genes were analyzed via qRT-PCR. A pair of primers for each gene was designed using Primer 3.0 and are listed in Supplementary Table S1. qRT-PCR was subsequently performed using a Real-Time PCR System with a total reaction volume of 10 μL, which consisted of 1 μL of cDNA template, 0.5 μL of forward and reverse primers, 5 μL TB Green Premix Ex Taq II (2×) and 3 μL of sterilized ddH_2O. The PCR procedure was as follows: 95°C for 10 min; 40 cycles of 95°C for 15 s, 60°C for 59 s, and 60°C for 15 s. The expression levels of ten genes were determined based on the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008), and relative changes in gene expression were calculated from the qRT-PCR data using the TCTP gene as a reference gene.

Statistical analysis

The collected data are presented as the means of the three replicates ± SE (standard error). One-way analysis of variance (ANOVA) was used to determine statistically significant differences between the means of treatments according to Duncan's multiple range test ($p = 0.05$). All statistical analyses of data were performed using IBM SPSS Statistics 26.0. The calculation formula for Z-Score in the heat map is as follows: Z = (FPKM - mean)/standard deviation.

Results

Plant growth-promoting activity of the PG-4 strain

The PG-4 strain showed the potential to secrete several PGP substances in the presence of different salt concentrations (Control, 50, 100 and 200 mM), as reported in Supplementary Table S2. The PG-4 strain has the potential to fix nitrogen and produce siderophores. Spectrophotometric analysis revealed that strain PG-4 grew better on ADF media than on control DF media under different salt stress conditions, suggesting that PG-4 has the ability to produce ACC deaminase. The analysis also showed that the PG-4 strain has a widespread ability to secrete hydrolytic enzymes, including cellulase, pectinase, and amylase, under different salt stress conditions. These results suggest that the PG-4 strain may be an effective agent for supporting plant resistance to salt stress.

Growth-promoting efficacy of the PG-4 strain

The growth-promoting efficacy of PG-4 on *M. uniflorum* seeds was examined, and the results are shown in Table 1. PG-4 had a growth-promoting effect on seed germination and seedling growth, significantly increasing the germination percentage, plumule and radicle length, and fresh weight ($p < 0.05$). The average germination percentage, plumule length, radicle length, and fresh weight were 100 ± 0.00%, 2.35 ± 0.12 cm, 1.99 ± 0.09 cm, and 1.18 ± 0.02 g, respectively, which were equivalent to increases of 7.15, 20.51, 11.17, and 20.4%, respectively, relative to those of the control after PG-4 was inoculated for 3 days. Overall, these results revealed that, compared with that of the noninoculated control seedlings, PG-4 promoted the growth of inoculated *M. uniflorum* seedlings.

Identification of the PG-4 strain

The colony morphology of the PG-4 strain is shown in Figures 1A,B, and the 16S rDNA gene sequences were obtained and compared with GenBank sequences. The strain was identified as gram-positive and facultative anaerobic following the protocol described in the "Common Bacterial System Identification Manual" and "Bergey's Manual of Determinative Bacteriology" with slight modifications (Dong and Cai, 1999). The 16S rRNA amplicon sequencing results revealed a total of 1,404 bases with homology between PG-4 and *Bacillus amyloliquefaciens* NBRC 15535 (NCBI accession number: NR041455.1) of 99.3%. Their close relationship was further supported by a phylogenetic tree (Figure 1C) (Nautiyal et al., 2013; Kim et al., 2017; Liu et al., 2017; Tanaka et al., 2017; Kazerooni et al., 2021). Accordingly, the PG-4 strain was identified as *B. amyloliquefaciens*.

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[2] http://bioinfo.cau.edu.cn/agriGO/

TABLE 1 *Macrotyloma uniflorum* seed growth-promoting effects of *Bacillus amyloliquefaciens* PG-4.

Treatment	Germination (%)	Plumule length (cm)	Radicle length (cm)	Fresh weight (g)
Control	93.33 ± 2.72b	1.95 ± 0.10b	1.79 ± 0.07b	0.98 ± 0.01c
LB	96.58 ± 1.40ab	1.98 ± 0.09b	1.85 ± 0.08ab	1.12 ± 0.04b
PG-4	100 ± 0.00a	2.35 ± 0.12a	1.99 ± 0.09a	1.18 ± 0.02a

Different letters indicate significant differences at the level $p < 0.05$.

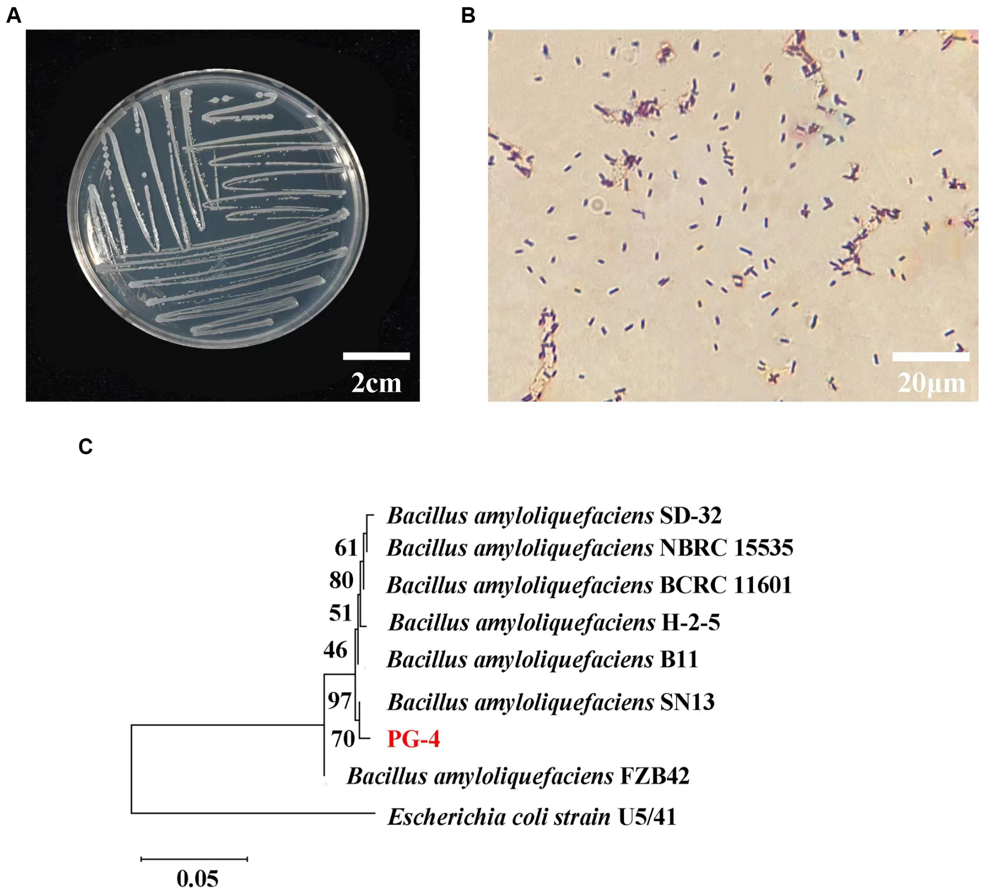

FIGURE 1
Molecular identification and biological characteristics analysis of strain PG-4. **(A)** Morphological characteristics of colony of strain PG-4. **(B)** Gram staining of strain PG-4. **(C)** A phylogenetic tree among eight *Bacillus* strains was derived from alignment of the 16S rRNA gene sequence. Scale bar, 0.05 substitutions per nucleotide position. Information on their isolation sources was obtained from the references and NCBI (BioSample).

Effects of *B. amyloliquefaciens* PG-4 on *M. uniflorum* seedlings under salt stress

After the interaction of *B. amyloliquefaciens* PG-4 with *M. uniflorum* under control conditions (without salt stress) and stress conditions (100 mM NaCl), the growth patterns of the plants were evaluated for 14 days. The growth of the plant seedlings under all treatments (CN, BN, CS, and BS) is shown in Figure 2. Greater plant growth was observed in the plants treated with PG-4 under salt stress (Figure 2A). The shoot length of the treated plants (BS) under salt stress was significantly ($p < 0.05$) different from that of the untreated control (CS) plants. The shoot length of the plants treated with CS was approximately 9.65 cm, whereas the shoot length of the BS-treated plants was approximately 10.05 cm (Figure 2B). Moreover, there was no significant ($p > 0.05$) difference in the roots of the untreated and treated plants under salt stress conditions (Figure 2C). In addition, under salt stress, compared with those in the control group without inoculation (CS), the shoot and root fresh weights of the plants treated with BS increased by 2.7 and 1.8 times, respectively (Figures 2D,E), and the shoot and root dry weights increased by 2.9 and 1.5 times, respectively (Figures 2F,G).

The chlorophyll content is an important index for predicting the health and photosynthetic capacity of plants under salt stress. Therefore, we measured the leaf chlorophyll content to examine the impact of PG-4 on the photosynthetic capacity of plants under salt stress. The results showed that salinity significantly ($p < 0.05$) reduced the chlorophyll content in the leaves of *M. uniflorum*. However, our findings also showed that, compared with that of the uninoculated control plant, PG-4 inoculation significantly increased the chlorophyll content in the plants under salt stress or those under no-salt conditions (Figure 3A).

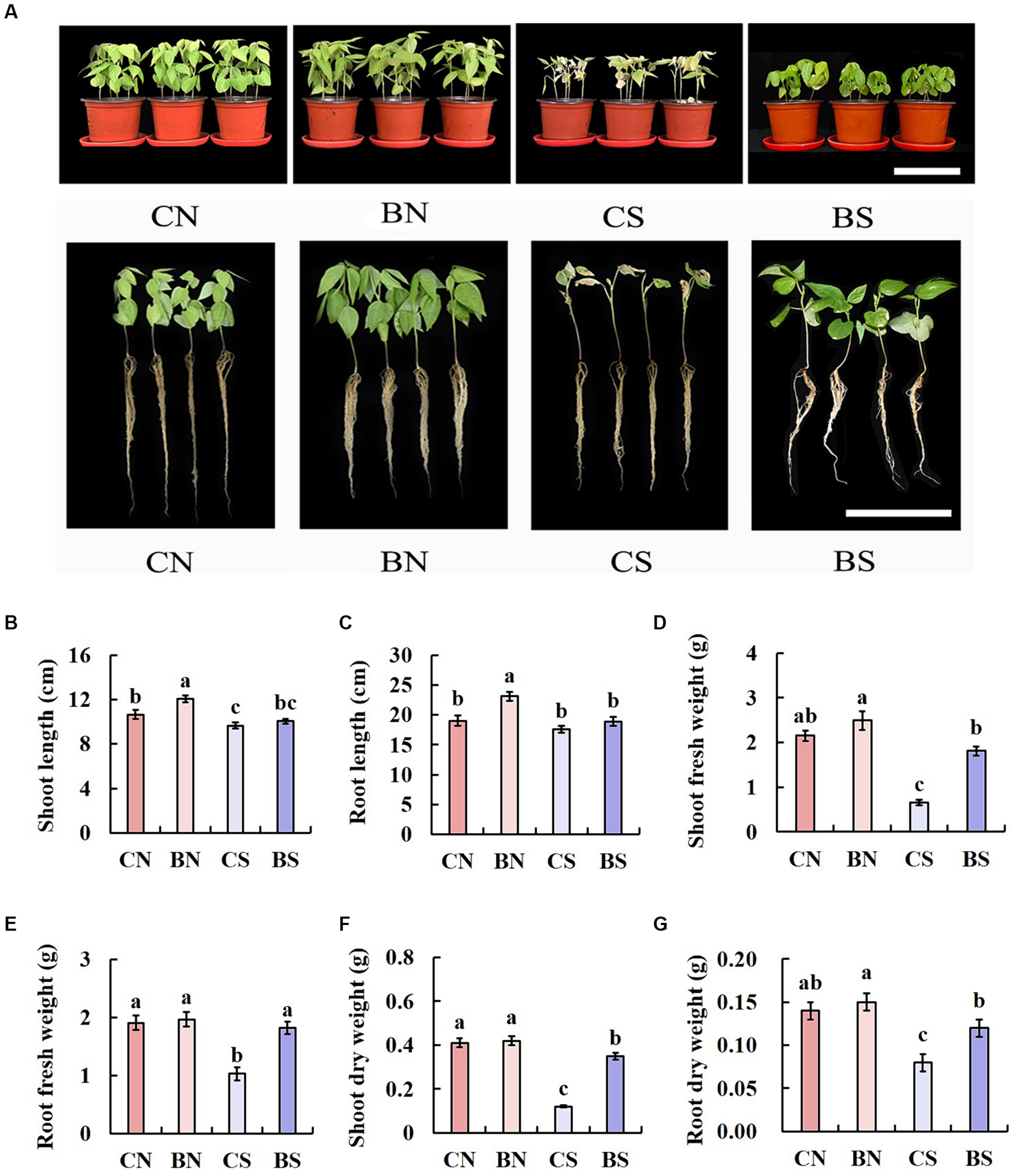

FIGURE 2
Effect of PG-4 inoculation on the growth of *Macrotyloma uniflorum* seedlings under salt stress in a pot experiment. **(A)** Growth status of *M. uniflorum* seedlings under the four treatments. Scale bar, 10 cm. **(B)** Shoot length. **(C)** Root length. **(D)** Shoot fresh weight. **(E)** Root fresh weights. **(F)** Shoot dry weight. **(G)** Root dry weight. CN, CS, BN, and BS represented the treatments of control and non-salt, control and salt, PG-4 inoculated and non-salt, and PG-4 inoculated and salt, respectively.

Salinity significantly ($p < 0.05$) increased the TSS content in the leaves of *M. uniflorum*. However, whether the plants were under salt stress or control conditions, the TSS and Pro content in the leaves of *M. uniflorum* inoculated with PG-4 was significantly higher than that in the leaves of the uninoculated control plants (Figures 3B,C). Salt stress significantly ($p < 0.05$) increased the MDA content. However, under salt stress, inoculation with PG-4 significantly reduced the MDA content in *M. uniflorum* leaves compared with that in uninoculated control plants (Figure 3D). Overall, these results indicated that inoculation with PG-4 can significantly ($p < 0.05$) reduce oxidative stress-related MDA levels and improve the salt tolerance more effectively in plants under salt stress. Furthermore, the data analysis showed that SOD and POD activity was increased by PG-4 inoculation and salinity stress, with the highest activity in BS-treated *M. uniflorum* leaves (Figures 3E,F). The analysis of Na^+ and K^+ content showed that salt stress strongly increased the absorption of Na^+ and decreased K^+ uptake. However, under salt stress, inoculation with PG-4 significantly ($p < 0.05$) increased the accumulation of potassium in plant leaves, while reduced the accumulation of sodium (Figures 3G,H). These data suggest the positive effect of *B. amyloliquefaciens* PG-4 on K^+ absorption under salt conditions.

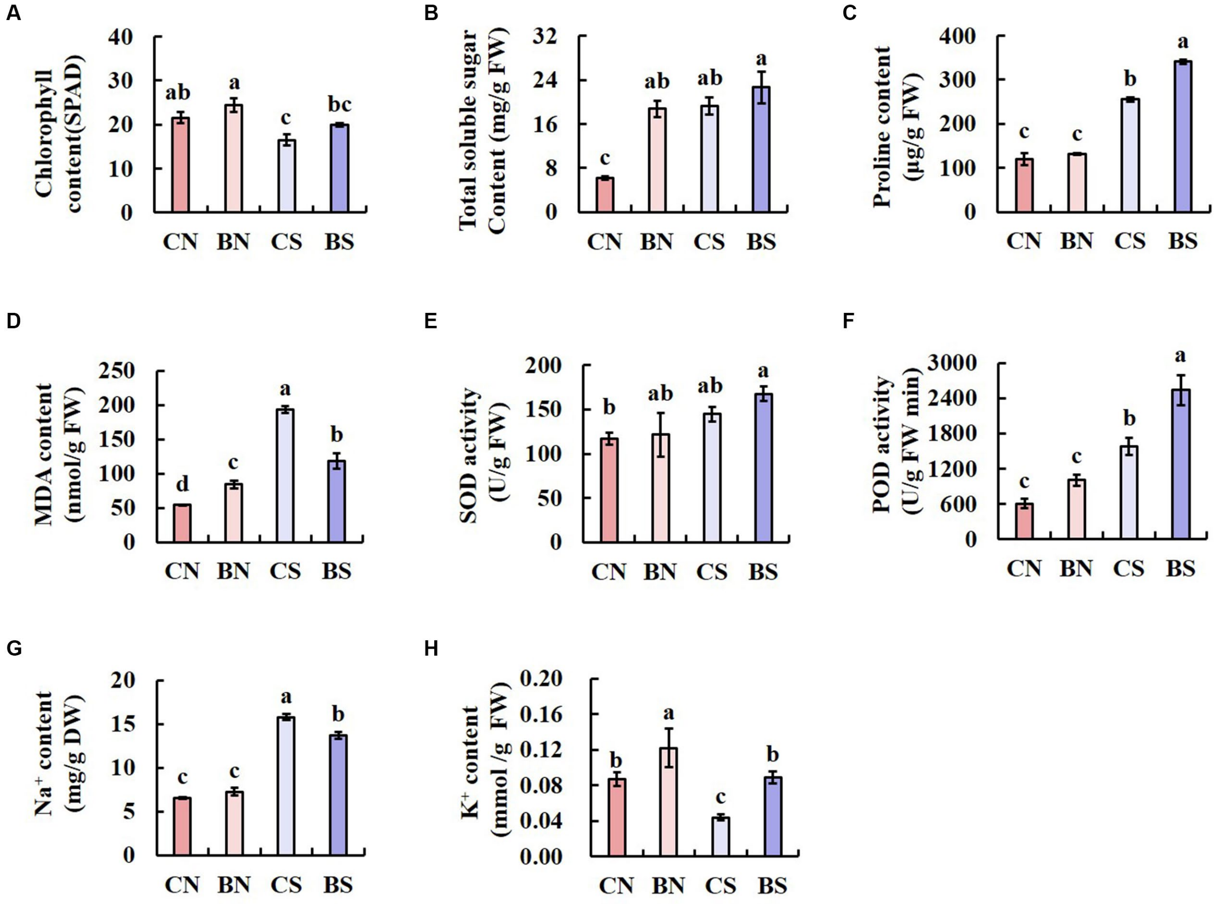

FIGURE 3
The physiological parameters of *M. uniflorum* seedlings under different treatments. Bars represent means ± SE. Different letters indicate significant differences at the level $p < 0.05$ according to Duncan's multiple range test test (n = 3 independent replicates). **(A)** Chlorophyll content. **(B)** Total soluble sugar content. **(C)** Proline content. **(D)** MDA content. **(E)** SOD activity. **(F)** POD activity. **(G)** Na^+ content. **(H)** k^+ content.

Overview of the transcriptome analysis and read assembly

To unravel the mechanism of *B. amyloliquefaciens* PG-4-mediated salt tolerance in *M. uniflorum* plants, transcriptome profiling was carried out using an RNA-Seq approach. In this study, RNA libraries were constructed and sequenced for a total of 12 samples from four groups of *M. uniflorum* samples, namely, CN, BN, CS, and BS. Among them, there were 547,313,032 original fragments. Adapter sequences and low-quality reads, at least 544,006,108 clean reads remained. Q30 (sequences with a sequencing error rate of less than 0.1%) was ≥94%, and the average GC content was 43.95% (Table 2). Trinity software was used to generate 42,179 unigenes via *de novo* transcriptome assembly. The average length of unigene is 1,314 bp (Supplementary Table S3; Figure 4A).

Functional annotation

To analyze the comprehensive functional annotation of the 42,179 unigenes, we used BLAST against four databases, namely, the NR, KEGG, KOG, and SwissProt databases. A total of 28,231 unigenes were annotated in at least one database (Figure 4B). Among them, 16,796 unigenes were assigned to 25 functional clusters according to the KOG database. "General function prediction only" (17.4%, 3,611) was the largest category (Figure 4B; Supplementary Figure S1A). In addition, we obtained functional information on the similarity between the sequences of *M. uniflorum* and related species by blasting against other plant species via the NR database. The *M. uniflorum* isoforms had the highest number of hits to *Phaseolus vulgaris* (5,810 hits), followed by *Vigna unguiculata* (4,292 hits) and *Vigna angularis* (3,364 hits) (Figure 4C).

To study the function of these genes in biological pathways, the unigenes were identified via the KEGG pathway database. A total of 6,938 unigenes were grouped into five main KEGG functional categories and 138 KEGG pathways (Figure 4D; Supplementary Table S4). A high proportion of unigenes were distributed in "Metabolism" (7,730) (Figure 4D), such as "Global and overview maps" (3,259), "Carbohydrate metabolism" (1,133), and "Amino acid metabolism" (627). To further classify the *M. uniflorum* unigenes, GO annotation was performed based on three major categories, namely, biological process, cellular component, and molecular function (Supplementary Figure S1B; Supplementary Table S5). For biological process classification, "cellular process" (14,775), "metabolic process" (12,742), and "biological

TABLE 2 Illumina-seq output statistics of 12 samples.

Sample	Raw reads	Clean reads	Clean bases/bp	Q20/%	Q30/%	GC content/%
CN 1	39,696,570	39,438,428	5,883,398,498	98.19	94.50	43.91
CN 2	45,117,204	44,824,702	6,673,992,341	98.46	95.17	44.09
CN 3	44,289,272	44,021,598	6,554,995,430	98.25	94.70	44.20
BN 1	44,653,594	44,370,548	6,596,504,239	98.04	94.18	44.11
BN 2	42,546,186	42,304,678	6,309,086,300	98.05	94.17	44.22
BN 3	50,120,930	49,829,064	7,426,054,656	98.30	94.84	44.06
CS 1	54,736,570	54,386,662	8,109,747,709	98.22	94.60	43.83
CS 2	39,172,702	38,944,782	5,803,659,457	98.12	94.34	43.37
CS 3	46,122,110	45,837,128	6,834,092,401	98.06	94.21	43.90
BS 1	39,288,066	39,072,358	5,823,173,139	98.44	95.19	44.01
BS 2	53,720,896	53,414,096	7,947,344,030	98.57	95.58	43.93
BS 3	47,848,932	47,562,064	7,094,648,863	98.31	94.84	43.82
Total	547,313,032	544,006,108	81,056,697,063	–	–	–

regulation" (4,309) were the three major categories. Unigenes involved in "binding" (12,266), "catalytic activity" (11,136) and "transporter activity" (1,807) were highly represented in the molecular function subgroups. The major categories of cellular component were "cellular anatomical entity (10,809)," "protein-containing complex (3,876)" and "virion component (101)."

Differential expression gene (DEG) and functional enrichment analysis among different treatments

The expression of these unigenes was subjected to principal component analysis (PCA) (Supplementary Figure S2A). The first two components, which explained 49.6 and 22.8% of the variation, respectively, were able to distinguish the four different treatment groups, with three biological replicates clustering together, and each group had certain differences in their gene expression profiles. A heatmap of Pearson's correlation coefficients between all sample pairs also showed that each sample was reliable with good reproducibility (Supplementary Figure S2B). Based on the read counts, DEGs were assessed with DESeq2 software. In this study, DEGs between treatments were defined on the basis of an absolute fold change ≥ 2 and a false discovery rate (FDR) < 0.05. Hierarchical clustering analysis was used to determine the expression patterns of DEGs under different experimental conditions (Figure 5A). Four different comparison groups were obtained by comparing the DEGs between different treatments: BN vs. CN, BS vs. BN, CS vs. CN, and BS vs. CS. The expression trends and numbers of DEGs identified in the different comparisons are shown in Figure 5B. There were 5,525 DEGs between the BN vs. CN groups, 3,277 upregulated genes and 2,248 downregulated genes. A total of 1,473 DEGs were found between BS vs. BN, which included 859 upregulated genes and 614 downregulated genes (Figure 5C). A total of 3,783 DEGs were found between CS vs. CN; these included 2,280 upregulated genes and 1,503 downregulated genes (Figure 5C). A total of 1,298 DEGs were found between BS vs. CS groups, which included 819 upregulated genes and 479 downregulated genes (Figure 5C). The total number of unique downregulated DEGs in the BS vs. BN comparison was 443, with 1,332 in the CS vs. CN comparison, while 171 DEGs were common between the treatments; 819 unique upregulated DEGs were found in the BS vs. BN comparison; and 1,827 were found in the CS vs. CN comparison, while 40 DEGs were common between the two treatments (Figure 5D).

Functional annotation and classification of differentially expressed genes

To further predict the DEGs induced by PG-4 inoculation and salt stress, functional annotation of the DEGs in the four groups was performed based on GO and KEGG analyses. Among the four comparative groups, BN vs. CN, BS vs. BN, CS vs. CN, and BS vs. CS, 3,904, 1,038, 2,763 and 905 DEGs, respectively, were subdivided into at least one GO term to describe their biological processes, molecular functions, and cellular components (Supplementary Figure S3). Under no-salt stress, a higher percentage of the PG-4-induced DEGs involved in plant growth regulation were found; these DEGs were associated mainly with "Cellular process," "metabolic process" and "response to stimulus" in the biological process category (Supplementary Figure S3A). According to the "response to stimulus" classification, 832 genes were changed by PG-4 inoculation under no-salt stress, whereas only 203 DEGs were regulated by salt stress in PG-4-inoculated seedlings (Supplementary Figures S3A,B). In addition, all DEGs induced by salt stress and PG-4 inoculation were mapped via KEGG to reference canonical pathways to identify the associated biological pathways (Supplementary Figure S4). A total of 347 salt stress-related DEGs in PG-4-inoculated plants were assigned to 109 pathways, which were classified into 19 KEGG pathway subcategories, of which the most common DEGs were "global and overview maps" followed by "carbohydrate metabolism," "biosynthesis of other secondary metabolites," and "translation" (Supplementary Figure S4A; Supplementary Table S6). The DEGs induced by PG-4 in salt-treated plants were assigned to 104 pathways, mainly "global and overview maps," "carbohydrate metabolism," "biosynthesis of other secondary metabolites" and "amino acid metabolism" (Supplementary Figure S4B; Supplementary Table S7).

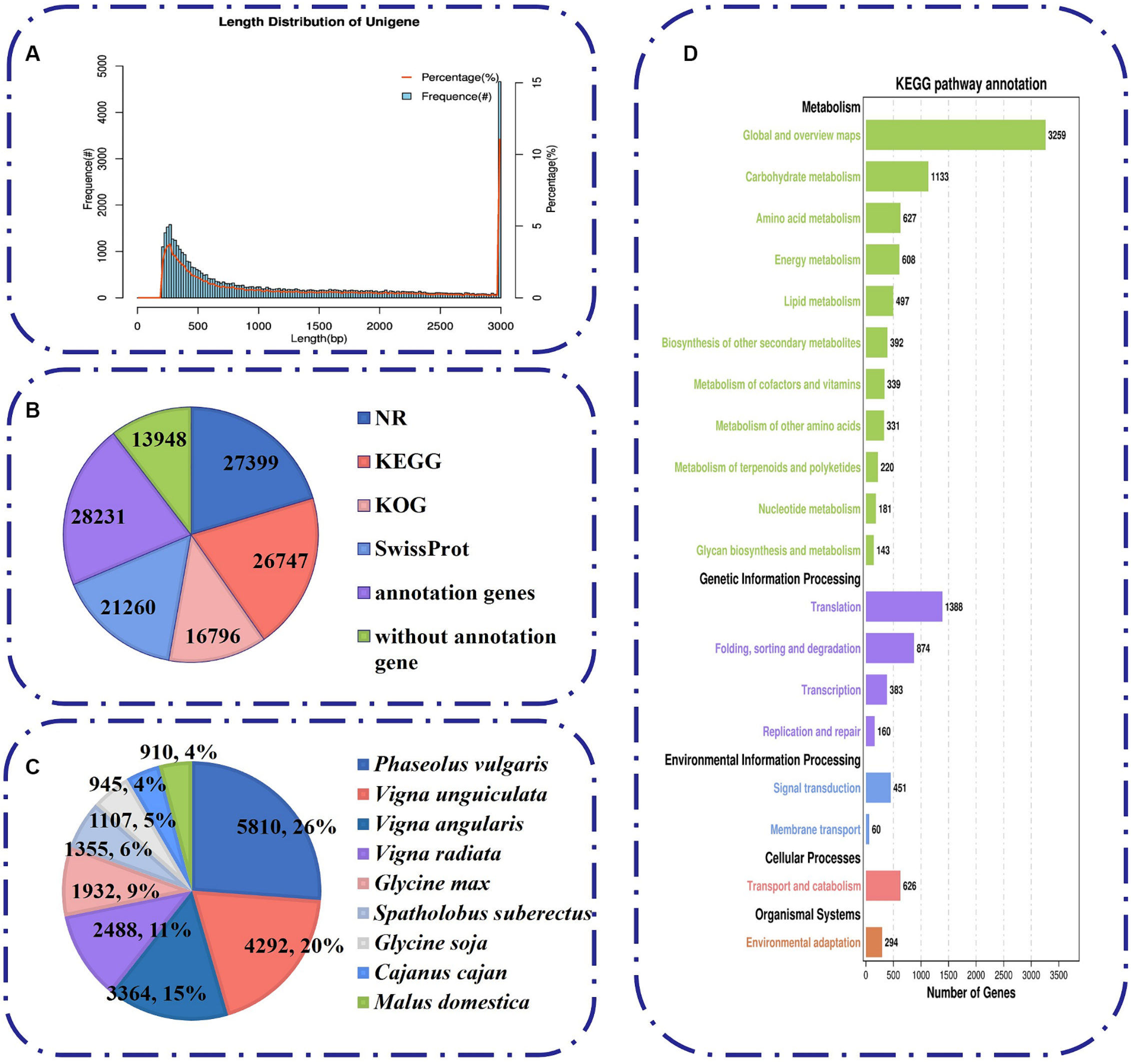

FIGURE 4
Characteristics of unigenes generated by Illumina sequencing. **(A)** The length distribution of all assembled unigenes. **(B)** The number of unigenes annotated by different databases, including NR, KEGG, KOG and Swiss-Prot. **(C)** Species distribution of the top BLAST hits for all homologous sequences. **(D)** A total of 6,938 unigenes were assigned to different KEGG terms.

Go and KEGG pathway enrichment analysis of differentially expressed genes

To elucidate the biological functions of these DEGs, the enriched GO terms were analyzed. The top 20 enriched GO terms are shown in Supplementary Figure S5. The DEGs of the different comparison groups were investigated through GO classification and KEGG enrichment. According to the GO enrichment analysis, 2,763 DEGs in the CS treatment group compared with those in the CN treatment group were significantly enriched (q-value <0.05) in 246 terms, and the most enriched GO terms that involved DEGs between CS vs. CN were related to “catalytic activity,” “oxidoreductase activity,” and “Small molecule metabolic process” (Supplementary Figure S5A). Moreover, 1,038 DEGs in the BS group compared with those in the BN group were significantly (q value <0.05) enriched in 39 terms, and the DEGs between the BS vs. BN groups were associated mainly with “heme binding,” “calcium ion binding,” “carbohydrate metabolic process” and the activity of various enzymes, such as oxidoreductase, peroxidase and glycosyltransferase (Supplementary Figure S5B). The lists of all identified significantly (q-value <0.05) enriched GO pathways are shown in Supplementary Tables S8, S9. KEGG pathway analysis was performed to explore the biological pathways represented by DEGs in the four comparison treatments. Several significant pathways were identified in the different comparisons (Figure 6). KEGG pathway enrichment revealed that the DEGs between CS vs. CN were involved primarily in “metabolic pathways,” “biosynthesis of secondary metabolites,” “phenylpropanoid biosynthesis,” “glycosylphosphatidylinositol (GPI)-anchor biosynthesis,” “circadian rhythm-plant,” “isoflavonoid biosynthesis,” “cyanoamino acid metabolism,” “alpha-linolenic acid metabolism” and “glycerolipid metabolism” (Figure 6A). The DEGs in the BS vs. BN comparison were assigned to “phenylpropanoid biosynthesis,” “plant-pathogen interaction,” “metabolic pathways,” “biosynthesis of secondary metabolites,” “pentose and glucuronate interconversions,” “starch and sucrose metabolism” and “plant hormone signal transduction” (Figure 6B). The lists of all identified KEGG pathways are shown in Supplementary Tables S10, S11.

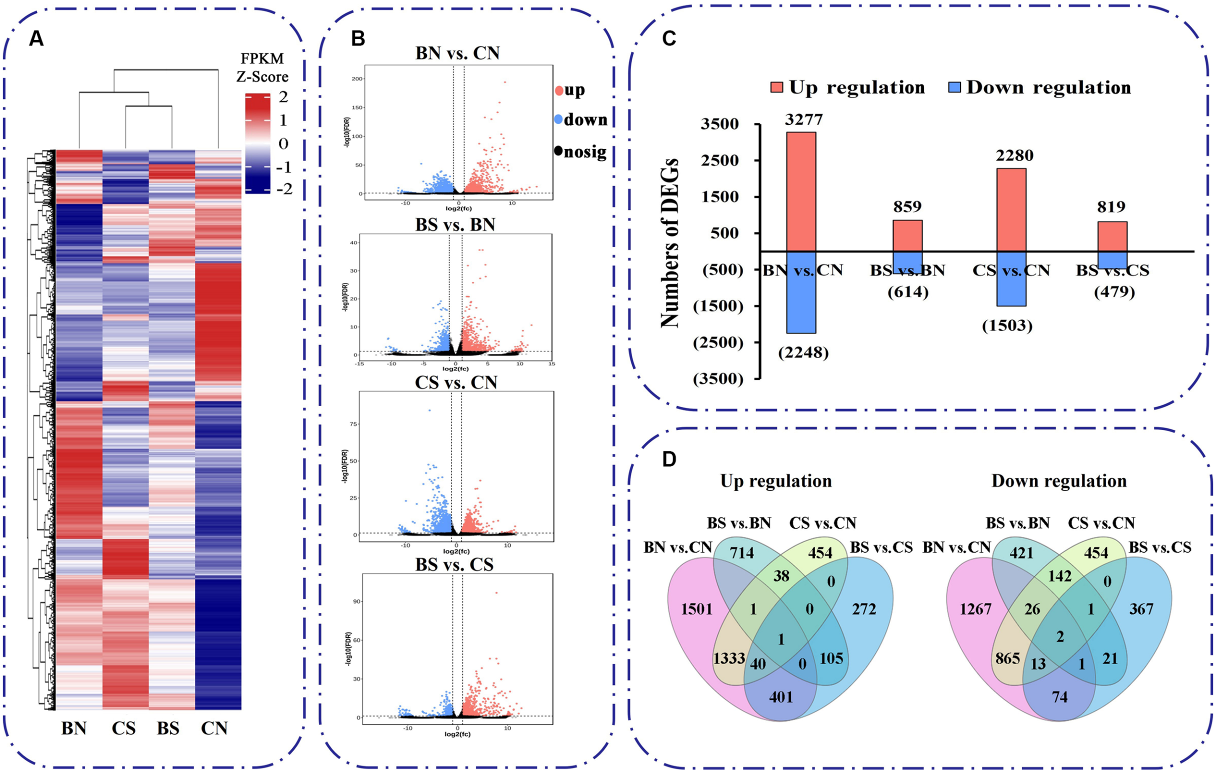

FIGURE 5
Transcriptional variations in *M. uniflorum* roots under different treatments. **(A)** Hierarchical clustrering analysis of the DEGs under different treatments. The color scale represents FPKM values in each treatment, normalized using z-score. **(B)** Significance analysis of the DEGs in different treatments by Volcano plots. **(C)** The number of up- and down-regulated genes in different treatments. **(D)** Venn diagrams showed the proportions of the up-regulated and down-regulated genes in four treatments.

Response of key unigenes to hormone signal transduction

Phytohormones, such as ethylene, IAA, cytokinin and ABA, play critical roles in eliciting a salinity stress adaptation response in plants (Singh et al., 2022). The three protein components related to auxin signal transduction are the auxin receptor-associated SCF complex (SKP1, Cullin and F-box complex), the auxin protein (Aux/IAA), which has regulatory functions, and the auxin response factor (ARF). Early auxin response genes can be divided into three categories: Aux/IAAs, GH3s, and small auxin-up RNAs (SAURs) (Hagen, 2015). The AUX/IAA, ARF, SAUR, GH3, and SAUR genes in *M. uniflorum* under salt stress were significantly downregulated to varying degrees, while these genes were upregulated by PG-4 in *M. uniflorum* under salt stress (Figure 7A; Supplementary Table S12). Three AUX/IAA genes (Unigene0023452, Unigene0023453 and Unigene0035539), one ARF gene (Unigene0030592), one SAUR genes (Unigene0028233) and one GH3 genes (Unigene0015319) were downregulated under salt stress, while they were significantly upregulated by PG-4 under salt stress. The genes Unigene0023453, Unigene0023452, and Unigene0015319 were down regulated by 2.9-, 2.5- and 2.8-fold, respectively, by salt stress, while they were significantly downregulated 2.4-, 2.4- and 1.8-fold, respectively, by PG-4 under salt stress conditions. In the ABA signaling pathway, only one PYL gene (Unigene0002166), three protein phosphatase 2C (PP2C) genes (Unigene0030091, Unigene0031036 and Unigene0031406) and one ABF gene (Unigene0003719) were differentially expressed in the BS vs. CS comparison (Figure 7B; Supplementary Table S12). The protein phosphatase 2C 51 isoform X2 (Unigene0031406) were significantly downregulated by salt stress but upregulated by PG-4 inoculation. In the BR signaling pathway, three TCH4 genes (Unigene0036355, Unigene0022458, and Unigene0027059) were significantly downregulated by 1.3-, 1.2- and 1.2-fold under salt stress, respectively, while they were significantly upregulated by 1.6-, 2.6- and 2.3-fold after PG-4 inoculation under salt stress (Figure 7C; Supplementary Table S12). A differentially expressed JAZ genes (in JA signaling pathway) were present in BS vs. CS (Figure 7D; Supplementary Table S12), and two differentially expressed TGA genes (in SA signaling pathway) were present in BN vs. CN (Figure 7E; Supplementary Table S12). Overall, these results indicated that PG-4 can affect the signal transduction process of plants in response to salt stress, which can help plants prepare for future salt stress.

Response of key unigenes to transporter proteins and key cellular metabolic processes

Among the thirteen DEGs related to ABC transporters expressed in seedlings, seven ABC transporter DEGs (Unigene0003435,

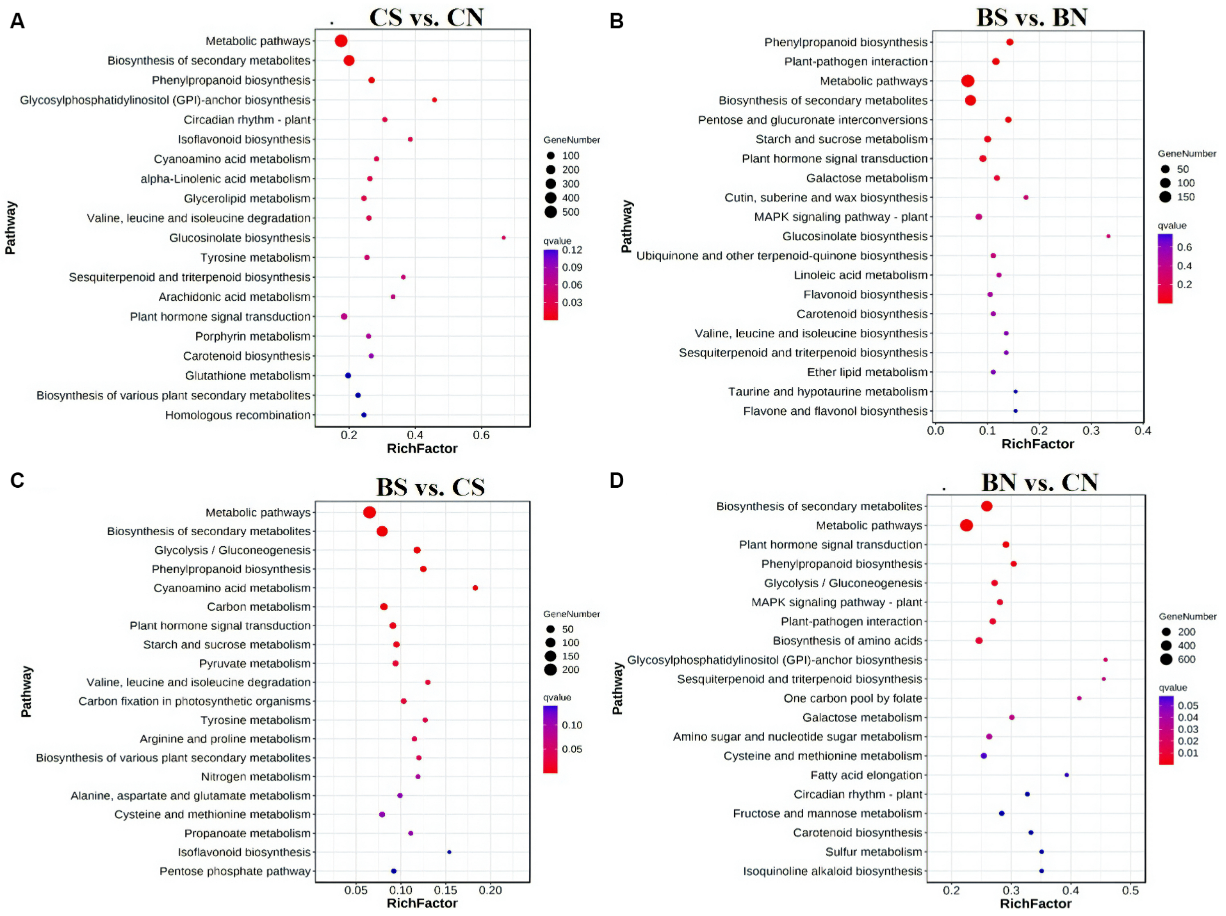

FIGURE 6
Enriched KEGG pathways analysis on of genes with significant differences between groups. **(A)** Enriched KEGG pathways of differential genes between CS and CN. **(B)** Enriched KEGG pathways of differential genes between BS and BN. **(C)** Enriched KEGG pathways of differential genes between BS and CS. **(D)** Enriched KEGG pathways of differential genes between BN and CN. False discovery rate (FDR) is the corrected *p*-value.

Unigene0006341, Unigene0019507, Unigene0022111, Unigene0022624, Unigene0027432, and Unigene0029184) were significantly downregulated, and three (Unigene0000140, Unigene0033713, and Unigene0037140) were significantly upregulated in the CS vs. CN comparison (Supplementary Figure S6A; Supplementary Table S13). In the BS vs. CS comparison, three ABC transporter DEGs (Unigene0003435, Unigene0006339, and Unigene0022513) were significantly upregulated, while only one (Unigene0020052) was significantly downregulated. In addition, the expression of the ABC transporter B family member 11 (Unigene0003435) was significantly downregulated by 1.94-fold in response to salt but significantly upregulated by 6.50-fold in response to PG-4 inoculation under salt stress (Supplementary Figure S6A; Supplementary Table S13). In soluble sugar metabolism, Unigene0038428 and Unigene0038427, which encode invertase, were significantly downregulated, and Unigene0014202, Unigene0010635, and Unigene0019276, which encode sucrose synthase, were significantly upregulated in the CS vs. CN comparison. In the BS vs. CS comparison, the granule-Unigene0003715, glucan endo-1,3-beta-glucosidase 1 (Unigene0042124) and 12 (Unigene0016553) genes were significantly upregulated (Supplementary Figure S6B; Supplementary Table S14).

Pectinesterase (EC 3.1.1.11) is an enzyme that catalyzes the hydrolysis of pectin to produce pectinic acid and methanol and can regulate cell wall strength, cell growth and stress resistance. The expression of DEGs related to pectin metabolism was significantly upregulated by PG-4 inoculation under salt conditions. The pectinesterase/pectinesterase inhibitor 47 (Unigene0031614) and pectinesterase (Unigene0035002) were significantly downregulated (1.50-fold and 1.48-fold, respectively) by salt stress, while they were significantly upregulated (1.79-fold and 1.74-fold, respectively) by PG-4 under salt stress (Supplementary Figure S6C; Supplementary Table S15). In addition to the above transcripts encoding carbohydrate-active enzymes, the xyloglucan endotransglucosylase/hydrolase protein (XTH) transcript required for cell wall synthesis and modification was also shown to be induced by PG-4 inoculation under salinity stress (Supplementary Figure S6D; Supplementary Table S16). In the BS vs. CS comparison, seven XTH DEGs (Unigene0007670, Unigene0036223, Unigene0022458, Unigene00036355, Unigene0022457, Unigene0022459 and Unigene0027059) were significantly upregulated, and only two XTH DEGs (Unigene0033881 and Unigene44199) were significantly downregulated. Overall, these results suggested that the changes in the cell wall structure of *M. uniflorum* in response to salt stress could be adjusted by the inoculation of PG-4 (Supplementary Figure S6D; Supplementary Table S16).

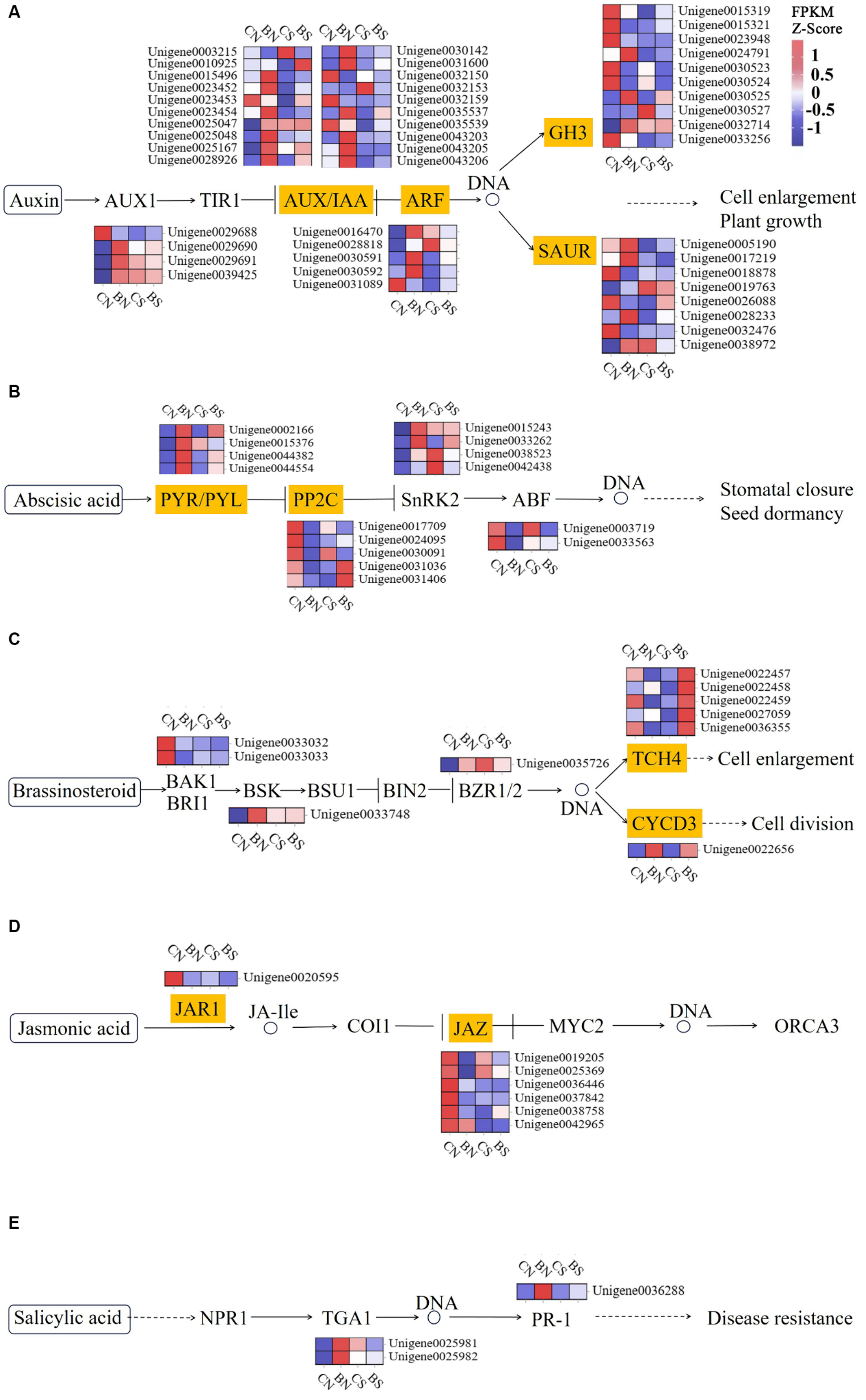

FIGURE 7

PG-4 activated plant hormone signal transduction pathway under salt stress. **(A)** Auxin signaling pathway. **(B)** ABA signaling pathway. **(C)** BR signaling pathway. **(D)** JA signaling pathway. **(E)** SA signaling pathway. The color scale represents FPKM values in each treatment, normalized using z-score.

Classification of transcription factors (TFs) under PG-4 inoculation and salt stress

In this study, 74 and 166 differentially expressed TFs were identified in the CS vs. BS comparison and the CN vs. CS comparison, respectively, and mainly belonged to the TF families APETALA2/ethylene responsive factor (AP2/ERF), WRKY, myeloblastosis protein (MYB) superfamily, NAC domain protein (NAC), basic helix–loop–helix (bHLH), and basic leucine zipper (bZIP), which are known to be associated with plant abiotic and biotic stress response and regulation (Figure 8; Supplementary Table S17). As shown in Figure 8A, these TFs were extensively upregulated by PG-4 under salinity stress, suggesting the special role of PG-4 in the transcriptional regulation of stress responses. In contrast, 60.64% of the TFs were downregulated by salinity stress, which may reflect the compromised defense response in *M. uniflorum* plants. AP2/ERF is one of the largest TF families involved in the response to salt stress (Xie et al., 2019). Among these TFs, ERF106 (Unigene0018746), PTI5 (Unigene0023663) and ERF095 (Unigene0025360) were significantly downregulated by salt treatment but significantly upregulated upon PG-4 inoculation (Figure 8B; Supplementary Table S17).

RNA-seq results validated by qRT–PCR

To verify the reliability of the RNA-seq data, we randomly selected 10 unigenes (Unigene0023113, Unigene0029497, Unigene0027804, Unigene0022458, Unigene0022457, Unigene0036457, Unigene0004787, Unigene0030152, Unigene0037512, and Unigene0027059) for further investigation via qRT-PCR (Supplementary Table S1; Supplementary Figure S7A). The results showed that the expression patterns determined via qRT-PCR were highly consistent with the RNA–seq data, with a relative R2 of 0.94 (Supplementary Figure S7B).

Discussion

To address the increasing the demand for food due to the growing world population under various abiotic stresses, a more sustainable and environmentally friendly approach is required. The use of PGPR has recently become a new option for improving salt stress tolerance in plants due to its beneficial interactions with plants (Bhat et al., 2020; Chen et al., 2021). *Bacillus* is the most common genus found in PGPR and represent the most widely used and well-studied bacteria in agricultural production (Shultana et al., 2022; Singh et al., 2022; Ditta and Ullah, 2023; Pistelli et al., 2023). Many *Bacillus* spp. have been reported to enhance plant tolerance to salinity and mitigate the salt stress-induced inhibition of plant growth. For example, Akbar et al. (2022) reported that *B. subtilis* and *B. pumilus* significantly enhanced salt stress tolerance in cotton plants under salt stress conditions. Ansari et al. (2019) suggested that the *B. pumilus* strain FAB10 contributes to salt stress alleviation in wheat plants through multiple modes of action. Similarly, *B. amyloliquefaciens* has been demonstrated to increase salt tolerance in a variety of host plants, such as *Arabidopsis* (Liu et al., 2020), maize (Chen et al., 2016), rice (Nautiyal et al., 2013) and *Codonopsis pilosula* (Han et al., 2017). In the present study, the PGP activity of *B. amyloliquefaciens* PG-4 was evaluated under detached and greenhouse conditions (Supplementary Table S2). The results indicated that the plant-beneficial effects were supported by the successful colonization of *M. uniflorum* by PG-4 under both mock and salt conditions. The ability of PGPR to alleviate salt stress damage and improve plant growth could result from the production of bioactive secondary metabolites or compounds. PG-4 produced ACC deaminase, siderophores, nitrogen-fixing enzymes and major enzymes, such as protease, amylase, and cellulase. ACC is a precursor for ethylene, one of the most important regulatory hormones for plant growth (Glick, 2014). The production of siderophores is one of the most vital mechanisms for preventing plant pathogens (Etesami and Maheshwari, 2018), suggesting the potential biocontrol function of PG-4.

Salt stress usually triggers ROS accumulation by reducing CO_2 assimilation, interfering with plant metabolism and causing ion toxicity (Chen et al., 2022; Wang Y. et al., 2022; Wang Q. et al., 2022). The rapid and excessive accumulation of ROS causes lipid peroxidation, protein denaturation, membrane damage and nucleic acid dysfunction in plants (Gupta and Pandey, 2020; Wang Y. et al., 2022; Wang Q. et al., 2022). The level of malondialdehyde (MDA), produced during oxidative damage caused by lipids (lipid peroxidation), is regarded as the main indicator of salinity-induced oxidative stress injury in plant tissues (Gupta and Pandey, 2020; Wang et al., 2021). Compared with that of control plants, the PG-4-inoculated plants presented a lower content of MDA under salt stress (Figure 3D), which agrees with the findings of previous studies (Hmaeid et al., 2019; Gupta and Pandey, 2020; Masmoudi et al., 2021). Wang Y. et al. (2022) and Wang Q. et al. (2022) also showed that *B. cereus* G2 alleviated the toxic effect of oxidative bursts in *Glycyrrhiza uralensis* Fisch. resulting from salt stress by inhibiting the production rate of $O2^-$ and correspondingly decreasing the concentration of MDA. Therefore, we speculate that the inoculation of plants with PG-4 reduces the MDA content and reduces ROS production and accumulation at the initial stage of salt stress, which might be an important mechanism for enhancing the salt tolerance of plants. Antioxidant enzymes play an important role in maintaining the normal functioning of plants by repairing and detoxifying the oxidative damage caused by stress-generated ROS (Akhter et al., 2022; Malea et al., 2022). In the antioxidant enzyme system, the conversion of superoxide anions ($O2^-$) into H_2O and H_2O_2 by SOD is the "first line of defense" for plants to cope with oxidative stress. Peroxidase (POD) is an H_2O_2 scavenging enzyme (Akhter et al., 2022; Wang Y. et al., 2022; Wang Q. et al., 2022). In the present study, compared to that in non-salt -stressed plants, the POD activity in plant leaves significantly increased after 2 weeks of salt stress (Figure 3F), indicating that salinity increases the activities of antioxidants involved in scavenging ROS (Abd_Allah et al., 2018; Wang et al., 2020). Most notably, inoculation with PG-4 significantly increased SOD and POD activity in salt-stressed *M. uniflorum* plants, enabling them to clear excess ROS and alleviate oxidative stress in plants. Similar studies by Hegazi et al. (2017) and Masmoudi et al. (2021) have shown that inoculation with beneficial microorganisms can trigger antioxidant defense mechanisms in plants to induce the synthesis of antioxidant enzymes such as SOD, POD, and CAT. Moreover, osmotic adjustment occurs by reducing osmotic potential to maintain cell volume and turgor, thereby reducing the adverse effects of salt stress by balancing

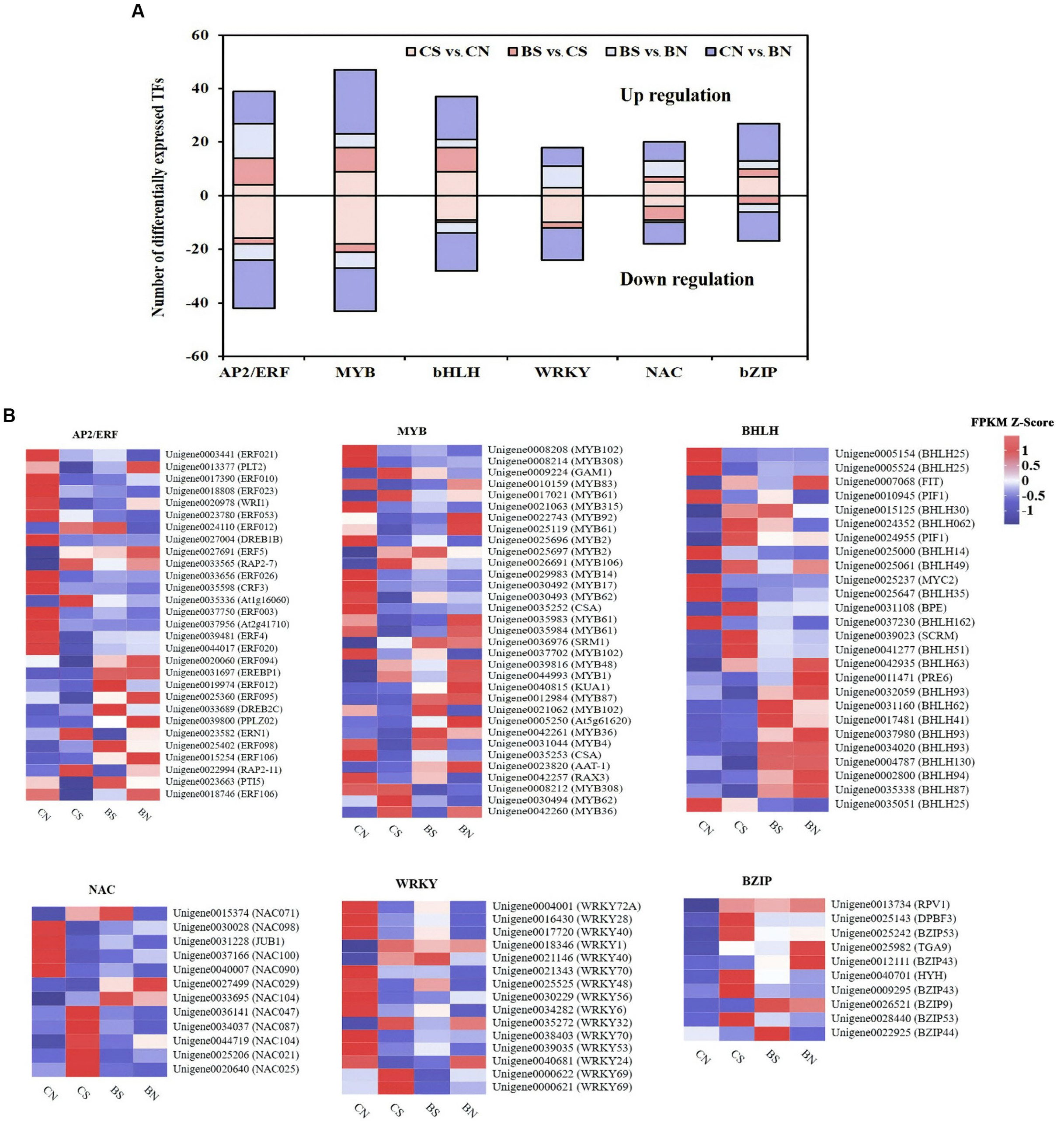

FIGURE 8
The differential expression pattern of the six TF families. **(A)** The number and expression pattern of differentially expressed TFs. The positive numbers indicate the up-regulated TFs and the negative numbers indicate the down-regulated TFs. **(B)** The differential expression pattern of the six TF families were depicted by a heatmap. The color scale represents FPKM values in each treatment, normalized using z-score.

solute potential, which subsequently promotes cell growth (Zhang et al., 2019). In the present study, the soluble sugar and proline contents in *M. uniflorum* leaves also significantly increased under salt stress (Figures 3B,C), which was consistent with previous findings in soybean (Soliman et al., 2020), maize (Wang Y. et al., 2022; Wang Q. et al., 2022) and *Pisum sativum* (Gupta et al., 2022). In particular, PG-4 further increased the proline and soluble sugar contents in salt-stressed *M. uniflorum* plants, suggesting that PG-4 could lower the osmotic potential and maintain cell turgor by promoting proline and soluble sugar accumulation, eventually helping *M. uniflorum* respond to the osmotic stress caused by salt stress. Soluble sugars and proline are involved not only in osmotic adjustment but also in scavenging ROS and protecting cells from oxidative damage (Zouari et al., 2019).

Salt stress results in the accumulation of Na^+ ions within plants (Joshi et al., 2022). Excessive Na^+ in plants competes with other ions to bind proteins, which leads to enzyme inactivation and affects the uptake of K^+ and other ions through the impact of ion balance between the two sides of the cytoplasmic membrane, causing severe ion toxicity (Zhu, 2016; Liu et al., 2022). Therefore, reducing the accumulation of Na^+ in the cytoplasm to maintain ion balance in plants is necessary for plants to reduce ion toxicity and enhance salt tolerance. We observed that salt stress led to a decrease in the K^+ concentration in the *M. uniflorum* seedlings, while inoculation with PG-4 significantly increased the K^+ concentration in the seedlings (Figures 3G,H). Our findings indicated that PG-4 reconstructs the ion balance of host plants by increasing K^+ and/or reducing Na^+ in the cycloplasm, thereby reducing the harmful effects of Na^+ on plants (Chen et al., 2016, 2017; Yilmaz et al., 2020; Shultana et al., 2022). Tank and Saraf (2010) suggested that PGPR inoculation increases the production of exopolysaccharides, which retain Na^+ in salt soil and prevent absorption by plant roots. Moreover, after inoculation with PG-4, the chlorophyll content of the *M. uniflorum* plants was significantly higher than control plants under salt stress (Figure 3A). These results may be due to the increased absorption and concentration of K^+ and reduced absorption of Na^+ by inoculation of *B. amyloliquefaciens* under salt stress, which increase the content of antioxidant compounds and improve photosynthesis (Ruíz-Sánchez et al., 2011; ALKahtani et al., 2021). PGPR can generate exopolysaccharides, which enhance soil structure, increase the availability of soil water, improving plant physiological characteristics, such as chlorophyll content, particularly under stress conditions (Tank and Saraf, 2010; ALKahtani et al., 2020).

In recent years, researchers have gradually begun to study the effect of PGPR on plant molecular responses through the use of the transcriptome method, with the final objective of improving plant salt tolerance (Wang Y. et al., 2022; Wang Q. et al., 2022; Xiao et al., 2022). The investigation of the transcriptome-wide response of *M. uniflorum* leaves revealed that PG-4-initiated salt tolerance is likely attributed to the regulation of several key biological processes, such as stress signaling components and regulators, plant hormone signal transduction and putative regulators of cell wall organization. Previous studies have shown that PGPR can enhance antioxidant enzyme activity and enhance host plant antioxidant capacity by inducing the upregulation of antioxidant enzyme-encoding genes (Khanna et al., 2021; Nivetha et al., 2021; Maleki et al., 2023). In the present study, GO terms related to the cellular redox state, such as "oxidoreductase activity," "response to oxidative stress," "antioxidant activity" and "catalytic activity," were significantly enriched with DEGs in the BS vs. CS comparison, which was in line with the effects of PG-4 on the enhancement of antioxidant enzyme activity in *Macrotyloma uniflorum* under salt stress. Moreover, GO terms related to hemoglobin, such as "iron ion binding," "heme binding" and "tetrapyrrole binding," were significantly enriched (Supplementary Figure S5). Hemoglobin and its catalyzed products can act as strong antioxidant enzymes against various abiotic stresses, as well as inducers that function in different developmental processes, including adventitious and lateral root development (Zhao et al., 2008; Xu et al., 2011). These results suggested that PG-4 may ameliorate the negative effects of salinity stress on *Macrotyloma uniflorum* by enhancing its ROS scavenging capacity.

The plant cell wall is the first site at which plants perceive and respond to salt stress (Kaashyap et al., 2018). The stress signal triggers cell wall remodeling to maintain flexibility and protect the cell against ionic imbalance (Kaashyap et al., 2018; Colin et al., 2023). Cell wall modification under abiotic stress conditions is accomplished by modulating the cross-linking of cellulose, pectins and lignin. Xyloglucans are involved in modulating these cross-links and regulating cell wall metabolism processes (Kaashyap et al., 2018; Ganie and Ahammed, 2021). Xyloglucans are linked to at least two cellulose molecules and are reported to remodel stomatal cell walls to prevent excess water loss during osmotic stress (Gall et al., 2015; Byrt et al., 2018). Recently, studies have indicated that XTH participates in plant cell wall construction and modification and that the overexpression of XTH confers increased plant tolerance under high salinity conditions (Zhang et al., 2021). Xyloglucan-modifying enzymes reportedly impart salt and drought tolerance when they are overexpressed in pepper and wheat plants in response to osmotic stress and salt stress (Abuqamar et al., 2013). Kaashyap et al. (2018) reported that the upregulation of xyloglucan genes in the tolerant genotype clearly suggested that their activity in 'cell wall loosening' may impart salt stress tolerance to chickpea plants. In the present study, GO terms related to the cell wall, such as "pectinesterase activity," "cell wall organization," "cell wall modification" and "cell wall organization or biogenesis," were also significantly enriched with DEGs in the CS vs. BS comparison, "xyloglucan: xyloglucosyl transferase activity" and "xyloglucan metabolic process" were significantly enriched with DEGs in the CS vs. BS comparison (Supplementary Figure S5). Under salinity stress, the expression levels of DEGs encoding pectinesterase and xyloglucan endotransglucosylase/hydrolase proteins were mainly downregulated. However, the addition of PG-4 facilitated the upregulation of these transcripts under salinity stress (Supplementary Figures S6C,D). This result indicated that there was a connection between the cell wall synthesis and modification induced by PG-4 inoculation and plant tolerance of salt stress.

PGPR play a key role in regulating plant signal transduction under salt stress. Liu et al. (2017) reported that FZB42 might use the ethylene and jasmonic acid transduction pathways to induce systemic salt tolerance in *Arabidopsis* plants. Lastochkina et al. (2017) reported that *B. subtilis* enhances salt stress tolerance in wheat by enhancing JA signaling. *B. pumilus* enhances the salt tolerance of cotton plants and is related to SA signaling (Akbar et al., 2022). In this study, the auxin, cytokinin, gibberellin, abscisic acid, ethylene, brassinosteroid, JA and salicylic acid signaling pathways were triggered, as reflected in the DEGs (Figure 7). These genes might be involved in the plant mechanism underlying the salt stress adaptation conferred by PG-4.

TFs play important roles in regulating gene expression, biotic and abiotic stress responses, and signal transduction by acting as gene activators, repressors, or both (Zhang et al., 2021; Wang Y. et al., 2022; Wang Q. et al., 2022). The roles of TFs in salt stress tolerance have been clearly demonstrated in important plants, such as pepper (*Capsicum frutescens* L.) (Wang Y. et al., 2022; Wang Q. et al., 2022), asparagus (*Asparagus officinalis*) (Zhang et al., 2021), rice (Tang et al., 2019), and tomato (*Solanum lycopersicum*) (Devkar et al., 2020). For example, Gruber et al. (2009) identified 46 TFs as salt-inducible genes in *Medicago truncatula* roots, including members of the AP2/ERF, WRKY, zinc-finger bZIP, zinc finger, GRAS, ZIM, Bhlh, MYB and bZIP families. In this study, several of the TFs belonging to the AP2/ERF, MYB, bHLH, WRKY, NAC, and bZIP families were downregulated by salt stress and upregulated by PG-4 under salt stress (Figure 8; Supplementary Figure S17), indicating the critical involvement of

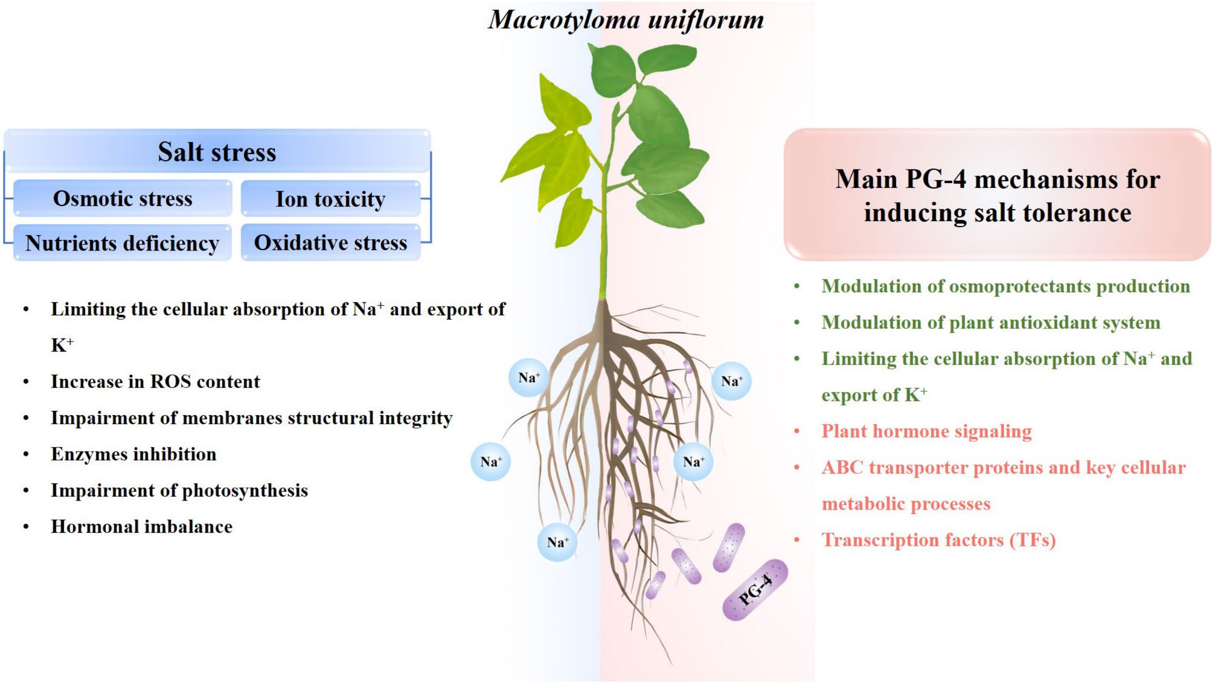

FIGURE 9
General overview of salt stress effects on plants (left) and PG-4 mechanisms involved in salt-stress tolerance (right) in *Macrotyloma uniflorum* seedlings. Green texts represent the physiological mechanism, while red texts represent the molecular mechanism by which PG-4 enhances the salt tolerance of *M. uniflorum* seedlings.

these TFs in the salt stress response and the important roles of PG-4 in the development of salt tolerance in plants.

Conclusion

The application of PGPR plays a crucial role in advancing agricultural resilience and productivity. PGPRs markedly enhance soil health, nutrient assimilation, and plant development, culminating in increased crop yields and a diminished dependency on chemical fertilizers, and its application is of great significance for sustainable agricultural. Furthermore, PGPRs are promising tools for increasing crop salt stress resistance. In the present study, the beneficial effects of *B. amyloliquefaciens* PG-4 on *M. uniflorum* plants under salt stress were evaluated. As shown in Figure 9, PG-4 significantly mitigated the deleterious effects of salt stress on *M. uniflorum* plants by improving biomass growth, increasing chlorophyll content, limiting the cellular absorption of Na^+ and export of K^+," and maintaining membrane integrity by reducing lipid peroxidation and increasing antioxidant production. Additionally, our transcriptome analysis revealed several key mediators of PG-4-induced transcriptional regulation in *M. uniflorum*, including plant hormone signal transduction, cell wall synthesis and modification and transporters. Moreover, transcription factors may also play important roles in mitigating the detrimental effects of salt stress on *M. uniflorum*, such as MYB, AP2/ERF, and bHLH. Our findings are helpful for providing important insights into the salt tolerance mechanism induced by PGPR and for providing a solid basis for future studies on the enhancement of legume forage tolerance to salinity stress, and providing theoretical references for bio-mediated soil improvement.

Data availability statement

The data presented in the study are deposited in the SRA: https://www.ncbi.nlm.nih.gov/sra/PRJNA1075426, accession number PRJNA1075426.

Author contributions

YW: Writing – original draft, Writing – review & editing. CG: Writing – review & editing. YX: Writing – review & editing. XL: Resources, Supervision, Writing – review & editing. DY: Resources, Supervision, Writing – review & editing. QW: Writing – review & editing. HL: Writing – review & editing. YZ: Conceptualization, Supervision, Writing – review & editing. RZ: Conceptualization, Supervision, Writing – review & editing. KL: Conceptualization, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2024.1386079/full#supplementary-material

References

Abd_Allah, E. F., Alqarawi, A. A., Hashem, A., Radhakrishnan, R., al-Huqail, A. A., al-Otibi, F. O. N., et al. (2018). Endophytic bacterium *Bacillus subtilis* (BERA 71) improves salt tolerance in chickpea plants by regulating the plant defense mechanisms. *J. Plant Interact.* 13, 37–44. doi: 10.1080/17429145.2017.1414321

Abuqamar, S., Ajeb, S., Sham, A., Enan, M. R., and Iratni, R. (2013). A mutation in the *expansin-like A2* gene enhances resistance to necrotrophic fungi and hypersensitivity to abiotic stress in *Arabidopsis thaliana*. *Mol. Plant Pathol.* 14, 813–827. doi: 10.1111/mpp.12049

Akbar, A., Han, B., Khan, A. H., Feng, C., Ullah, A., Khan, A. S., et al. (2022). A transcriptomic study reveals salt stress alleviation in cotton plants upon salt tolerant PGPR inoculation. *Environ. Exp. Bot.* 200:104928. doi: 10.1016/j.envexpbot.2022.104928

Akhter, M. S., Noreen, S., Ummara, U., Aqeel, M., Saleem, N., Ahmed, M. M., et al. (2022). Silicon-induced mitigation of NaCl stress in barley (*Hordeum vulgare* L.), associated with enhanced enzymatic and non-enzymatic antioxidant activities. *Plan. Theory* 11:2379. doi: 10.3390/plants11182379

Ali, B., Wang, X., Saleem, M. H., Sumaira, H., Hafeez, A., Afridi, M. S., et al. (2022). PGPR-mediated salt tolerance in maize by modulating plant physiology, antioxidant defense, compatible solutes accumulation and bio-surfactant producing genes. *Plan. Theory* 11:345. doi: 10.3390/plants11030345

ALKahtani, M. D. F., Fouda, A., Attia, K. A., al-Otaibi, F., Eid, A. M., Ewais, E. E.-D., et al. (2020). Isolation and characterization of plant growth promoting endophytic bacteria from desert plants and their application as bioinoculants for sustainable agriculture. *Agronomy* 10:1325. doi: 10.3390/agronomy10091325

ALKahtani, M., Hafez, Y., Attia, K., al-Ateeq, T., Ali, M. A. M., Hasanuzzaman, M., et al. (2021). *Bacillus thuringiensis* and silicon modulate antioxidant metabolism and improve the physiological traits to confer salt tolerance in lettuce. *Plan. Theory* 10:1025. doi: 10.3390/plants10051025

Ansari, F. A., Ahmad, I., and Pichtel, J. (2019). Growth stimulation and alleviation of salinity stress to wheat by the biofilm forming *Bacillus pumilus* strain FAB10. *Appl. Soil Ecol.* 143, 45–54. doi: 10.1016/j.apsoil.2019.05.023

Apweiler, R., Bairoch, A., Wu, C. H., Barker, W. C., Boeckmann, B., and Ferro, S. (2004). Uni Prot: the universal protein knowledgebase. *Nucleic Acids Res.* 32, 115D–1119D. doi: 10.1093/nar/gkh131

Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., et al. (2000). Gene ontology: tool for the unification of biology. *Nat. Genet.* 25, 25–29. doi: 10.1038/75556

Ashraf, M., and Munns, R. (2022). Evolution of approaches to increase the salt tolerance of crops. *Crit. Rev. Plant Sci.* 41, 128–160. doi: 10.1080/07352689.2022.2065136

Baldani, V. L. D., and Döbereiner, J. (1980). Host-plant specificity in the infection of cereals with *Azospirillum* spp. *Soil. Biolo. Biochem.* 12, 433–439. doi: 10.1016/0038-0717(80)90021-8

Bhat, M. A., Kumar, V., Bhat, M. A., Wani, I. A., Dar, F. L., Farooq, I., et al. (2020). Mechanistic insights of the interaction of plant growth-promoting rhizobacteria (PGPR) with plant roots toward enhancing plant productivity by alleviating salinity stress. *Front. Microbiol.* 11:1952. doi: 10.3389/fmicb.2020.01952

Bolbhat, S. N., and Dhumal, K. N. (2009). Induced macromutations in horsegram [*Macrotyloma uniflorum* (lam.) Verdc]. *Legum. Res.* 32, 278–281.

Butcher, K., Wick, A. F., DeSutter, T., Chatterjee, A., and Harmon, J. (2016). Soil Salinity: A Threat to Global Food Security. *Agron J*, 108, 2189–2200. doi: 10.2134/agronj2016.06.0368

Byrt, C. S., Munns, R., Burton, R. A., Gilliham, M., and Wege, S. (2018). Root cell wall solutions for crop plants in saline soils. *Plant Sci.* 269, 47–55. doi: 10.1016/j.plantsci.2017.12.012

Cappuccino, J. C., and Sherman, N. (1992). Ammonia Production. *Microbiol. A Lab. Manual*, 3rd edn. Redw, City, CA, USA: Benjamin/Cummings Pub Co., pp. 125–179.

Chen, J. T., Aroca, R., and Romano, D. (2021). Molecular aspects of plant salinity stress and tolerance. *Int. J. Mol. Sci.* 22:4918. doi: 10.3390/ijms22094918

Chen, G., Han, H., Yang, X., Du, R., and Wang, X. (2022). Salt tolerance of rice is enhanced by the *SS3* gene, which regulates ascorbic acid synthesis and *ROS* scavenging. *Int. J. Mol. Sci.* 23:10338. doi: 10.3390/ijms231810338

Chen, L., Liu, Y., Wu, G., Veronican Njeri, K., Shen, Q., Zhang, N., et al. (2016). Induced maize salt tolerance by rhizosphere inoculation of *Bacillus amyloliquefaciens* SQR9. *Physiol. Plantarum* 158, 34–44. doi: 10.1111/ppl.12441

Chen, L., Liu, Y., Wu, G., Zhang, N., Shen, Q., and Zhang, R. (2017). Beneficial rhizobacterium *Bacillus amyloliquefaciens* SQR9 induces plant salt tolerance through spermidine production. *Mol. Plant Microbe In.* 30, 423–432. doi: 10.1094/MPMI-02-17-0027-R

Colin, L., Ruhnow, F., Zhu, J. K., Zhao, C., Zhao, Y., and Persson, S. (2023). The cell biology of primary cell walls during salt stress. *Plant Cell* 35, 201–217. doi: 10.1093/plcell/koac292

Devkar, V., Thirumalaikumar, V. P., Xue, G. P., Vallarino, J. G., Turečková, V., Strnad, M., et al. (2020). Multifaceted regulatory function of tomato SlTAF1 in the response to salinity stress. *New Phytol.* 225, 1681–1698. doi: 10.1111/nph.16247

Ditta, A., and Ullah, N. (2023). "Perspectives of using plant growth-promoting rhizobacteria under salinity stress for sustainable crop production" in *Plant Stress Mitigators*. eds. M. Ghorbanpour and M. A. Shahid (Cambridge, MA: Academic Press), 231–247.

Dong, X., and Cai, M. (1999). *Handbook for the identification of common bacteria*. Beijing: Science Press 353–365

Egamberdieva, D., Wirth, S., Bellingrath-Kimura, S. D., Mishra, J., and Arora, N. K. (2019). Salt-tolerant plant growth promoting rhizobacteria for enhancing crop productivity of saline soils. *Front. Microbiol.* 10:2791. doi: 10.3389/fmicb.2019.02791

Etesami, H., and Glick, B. R. (2020). Halotolerant plant growth–promoting bacteria: prospects for alleviating salinity stress in plants. *Environ. Exp. Bot.* 178:104124. doi: 10.1016/j.envexpbot.2020.104124

Etesami, H., and Maheshwari, D. K. (2018). Use of plant growth promoting rhizobacteria (PGPRs) with multiple plant growth promoting traits in stress agriculture: action mechanisms and future prospects. *Ecotox. Environ. Safe.* 156, 225–246. doi: 10.1016/j.ecoenv.2018.03.013

Farhangi-Abriz, S., Tavasolee, A., Ghassemi-Golezani, K., Torabian, S., Monirifar, H., and Rahmani, H. A. (2020). Growth-promoting bacteria and natural regulators mitigate salt toxicity and improve rapeseed plant performance. *Protoplasma* 257, 1035–1047. doi: 10.1007/s00709-020-01493-1

Finn, R. D., Tate, J., Mistry, J., Coggill, P. C., Sammut, S. J., Hotz, H. R., et al. (2008). The Pfam protein families database. *Nucleic Acids Res.* 32, 138D–1141D. doi: 10.1093/nar/gkh121

Gall, H. L., Philippe, F., Domon, J. M., Gillet, F., Pelloux, J., and Rayon, C. (2015). Cell wall metabolism in response to abiotic stress. *Plan. Theory* 4, 112–166. doi: 10.3390/plants4010112

Ganie, S. A., and Ahammed, G. J. (2021). Dynamics of cell wall structure and related genomic resources for drought tolerance in rice. *Plant Cell Rep.* 40, 437–459. doi: 10.1007/s00299-020-02649-2

Garcia-Caparros, P., De Filippis, L., Gul, A., Hasanuzzaman, M., Ozturk, M., Altay, V., et al. (2021). Oxidative stress and antioxidant metabolism under adverse environmental conditions: a review. *The Bot. Rev.* 87, 421–466. doi: 10.1007/s12229-020-09231-1

Glick, B. R. (2014). Bacteria with ACC deaminase can promote plant growth and help to feed the world. *FEMS Microbiol. Rev.* 169, 30–39. doi: 10.1016/j.micres.2013.09.009

Gruber, V., Blanchet, S., Diet, A., Zahaf, O., Boualem, A., Kakar, K., et al. (2009). Identification of transcription factors involved in root apex responses to salt stress in *Medicago truncatula*. *Mol. Gen. Genomics.* 281, 55–66. doi: 10.1007/s00438-008-0392-8

Gupta, S., and Pandey, S. (2020). Enhanced salinity tolerance in the common bean (*Phaseolus vulgaris*) plants using twin ACC deaminase producing rhizobacterial inoculation. *Rhizosphere* 16:100241. doi: 10.1016/j.rhisph.2020.100241

Gupta, A., Rai, S., Bano, A., Sharma, S., Kumar, M., Binsuwaidan, R., et al. (2022). ACC deaminase produced by pgpr mitigates the adverse effect of osmotic and salinity stresses in *Pisum sativum* through modulating the antioxidants activities. *Plan. Theory* 11:3419. doi: 10.3390/plants11243419

Gupta, P., Samant, K., and Sahu, A. (2012). Isolation of cellulose-degrading bacteria and determination of their cellulolytic potential. *Int. J. microbiol.* 2012:578925. doi: 10.1155/2012/578925

Hagen, G. (2015). Auxin signal transduction. *Essays Biochem.* 58, 1–12. doi: 10.1042/bse0580001

Han, Q. Q., Wu, Y. N., Gao, H. J., Xu, R., Paré, P. W., Shi, H., et al. (2017). Improved salt tolerance of medicinal plant *Codonopsis pilosula* by *Bacillus amyloliquefaciens* GB03. *Acta Physiol. Plant.* 39, 1–7. doi: 10.1007/s11738-016-2325-1

Haque, M. A., Rafii, M. Y., Yusoff, M. M., Ali, N. S., Yusuff, O., Datta, D. R., et al. (2021). Advanced breeding strategies and future perspectives of salinity tolerance in rice. *Agronomy* 11:1631. doi: 10.3390/agronomy11081631

Hasanuzzaman, M., Raihan, M. R. H., Nowroz, F., and Fujita, M. (2022). Insight into the mechanism of salt-induced oxidative stress tolerance in soybean by the application of *Bacillus subtilis*: coordinated actions of osmoregulation, ion homeostasis, antioxidant defense, and methylglyoxal detoxification. *Antioxidants* 11:1856. doi: 10.3390/antiox11101856

Hegazi, A. M., El-Shraiy, A. M., and Ghoname, A. A. (2017). Mitigation of salt stress negative effects on sweet pepper using arbuscular mycorrhizal fungi (AMF), *bacillus megaterium* and brassinosteroids (BRs). *Gesunde Pflanz.* 69, 91–102. doi: 10.1007/s10343-017-0393-9

Hmaeid, N., Wali, M., Mahmoud, O. M. B., Pueyo, J. J., Ghnaya, T., and Abdelly, C. (2019). Efficient rhizobacteria promote growth and alleviate NaCl-induced stress in the plant species *Sulla carnosa*. *Appl. Soil Ecol.* 133, 104–113. doi: 10.1016/j.apsoil.2018.09.011

Hou, Y., Zeng, W., Ao, C., Luo, Y., Wang, Z., Hou, M., et al. (2022). *Bacillus atrophaeus* WZYH01 and *planococcus soli* WZYH02 improve salt tolerance of maize (*Zea mays* l.) in saline soil. *Front. Plant Sci.* 13:891372. doi: 10.3389/fpls.2022.891372

Ibarra-Villarreal, A. L., Gándara-Ledezma, A., Godoy-Flores, A. D., Herrera-Sepúlveda, A., Díaz-Rodríguez, A. M., Parra-Cota, F. I., et al. (2021). Salt-tolerant *bacillus* species as a promising strategy to mitigate the salinity stress in wheat (*Triticum turgidum* subsp. *durum*). *J. Arid Environ.* 186:104399. doi: 10.1016/j.jaridenv.2020.104399

Joshi, S., Nath, J., Singh, A. K., Pareek, A., and Joshi, R. (2022). Ion transporters and their regulatory signal transduction mechanisms for salinity tolerance in plants. *Physiol. Plantarum* 174:e13702. doi: 10.1111/ppl.13702

Kaashyap, M., Ford, R., Kudapa, H., Jain, M., Edwards, D., Varshney, R., et al. (2018). Differential regulation of genes involved in root morphogenesis and cell wall modification is associated with salinity tolerance in chickpea. *Sci. Rep.* 8:4855. doi: 10.1038/s41598-018-23116-9

Kadam, S., and Salunkhe, D. K. (1985). Nutritional composition, processing, and utilization of horse gram and moth bean. *Crit. Rev. Food Sci. Nutr.* 22, 1–26. doi: 10.1080/10408398509527416

Kanehisa, M., Araki, M., Goto, S., Hattori, M., Hirakawa, M., Itoh, M., et al. (2008). KEGG for linking genomes to life and the environment. *Nucleic Acids Res.* 36, D480–D484. doi: 10.1093/nar/gkm882

Kazerooni, E. A., Maharachchikumbura, S. S., Adhikari, A., Al-Sadi, A. M., Kang, S. M., Kim, L. R., et al. (2021). Rhizospheric *Bacillus amyloliquefaciens* protects *Capsicum annuum* cv. Geumsugangsan from multiple abiotic stresses *via* multifarious plant growth-promoting attributes. *Front. Plant Sci.* 12:669693. doi: 10.3389/fpls.2021.669693

Khanna, K., Kohli, S. K., Sharma, P., Kour, J., Singh, A. D., Sharma, N., et al. (2021). "Antioxidant potential of plant growth-promoting rhizobacteria (PGPR) in agricultural crops infected with root-knot nematodes" in *Antioxidants in plant-microbe interaction*. eds. A. K. Singh, S. Kumar and T. Sinha (Berlin: Springer)

Kim, M. J., Radhakrishnan, R., Kang, S. M., You, Y. H., Jeong, E. J., Kim, J. G., et al. (2017). Plant growth promoting effect of *Bacillus amyloliquefaciens* H-2-5 on crop plants and influence on physiological changes in soybean under soil salinity. *Physiol. Mol. Biol. Pla.* 23, 571–580. doi: 10.1007/s12298-017-0449-4

Koonin, E. V., Fedorova, N. D., Jackson, J. D., Jacobs, A. R., Krylov, D. M., Makarova, K. S., et al. (2004). A comprehensive evolutionary classification of proteins encoded in complete eukaryotic genomes. *Genome Biol.* 5, R7–R28. doi: 10.1186/gb-2004-5-2-r7

Lastochkina, O., Pusenkova, L., Yuldashev, R., Babaev, M., Garipova, S., Blagova, D. Y., et al. (2017). Effects of *Bacillus subtilis* on some physiological and biochemical parameters of *Triticum aestivum* L. (wheat) under salinity. *Plant Physiol. Bioch.* 121, 80–88. doi: 10.1016/j.plaphy.2017.10.020

Li, G. E., Kong, W. L., Wu, X. Q., and Ma, S. B. (2021). Phytase-producing *Rahnella aquatilis* JZ-GX1 promotes seed germination and growth in corn (*Zea mays* L.). *Microorganisms* 9:1647. doi: 10.3390/microorganisms9081647

Li, Y., Li, W., Hu, D., Shen, P., Zhang, G., and Zhu, Y. (2020). Comparative analysis of the metabolome and transcriptome between green and albino zones of variegated leaves from *Hydrangea macrophylla* 'Maculata' infected by hydrangea ringspot virus. *Plant Physiol. Bioch.* 157, 195–210. doi: 10.1016/J.Plaphy.2020.10.012

Liu, S., Hao, H., Lu, X., Zhao, X., Wang, Y., Zhang, Y., et al. (2017). Transcriptome profiling of genes involved in induced systemic salt tolerance conferred by *Bacillus amyloliquefaciens* FZB42 in *Arabidopsis thaliana*. *Sci. Rep.* 7:10795. doi: 10.1038/s41598-017-11308-8

Liu, S., Tian, Y., Jia, M., Lu, X., Yue, L., Zhao, X., et al. (2020). Induction of salt tolerance in *Arabidopsis thaliana* by volatiles from *Bacillus amyloliquefaciens* FZB42 via the jasmonic acid signaling pathway. *Front. Microbiol.* 11:562934. doi: 10.3389/fmicb.2020.562934

Liu, Y., Xun, W., Chen, L., Xu, Z., Zhang, N., Feng, H., et al. (2022). Rhizosphere microbes enhance plant salt tolerance: toward crop production in saline soil. *Comput. Struct. Biotec.* 20, 6543–6551. doi: 10.1016/j.csbj.2022.11.046(30)

Ma, T., Zeng, W., Lei, G., Wu, J., and Huang, J. (2021). Predicting the rooting depth, dynamic root distribution and the yield of sunflower under different soil salinity and nitrogen applications. *Ind. Crop. Prod.* 170:113749. doi: 10.1016/j.indcrop.2021.113749

Malea, P., Kokkinidi, D., Kevrekidou, A., and Adamakis, I. S. (2022). The enzymatic and non-enzymatic antioxidant system response of the seagrass *Cymodocea nodosa* to bisphenol-a toxicity. *Int. J. Mol. Sci.* 23:1348. doi: 10.3390/ijms23031348

Maleki, M., Fatehi, F., and Ghorbanpour, M. (2023). "PGPR reduces the adverse effects of abiotic stresses by modulating morphological and biochemical properties in plants" in *Plant Stress Mitigators*. eds. A. Vaishnav, S. S. Arya and D. K. Choudhary (Singapore: Springer)

Masi, C., Gemechu, G., and Tafesse, M. (2021). Isolation, screening, characterization, and identification of alkaline protease-producing bacteria from leather industry effluent. *Ann. Microbiol.* 71, 1–11. doi: 10.1186/S13213-021-01631-X

Masmoudi, F., Tounsi, S., Dunlap, C. A., and Trigui, M. (2021). Endophytic halotolerant *Bacillus velezensis* FMH2 alleviates salt stress on tomato plants by improving plant growth and altering physiological and antioxidant responses. *Plant Physiol. Bioch.* 165, 217–227. doi: 10.1016/j.plaphy.2021.05.025

Mohanty, P., Singh, P. K., Chakraborty, D., Mishra, S., and Pattnaik, R. (2021). Insight into the role of PGPR in sustainable agriculture and environment. *Front. Sustain. Food Syst.* 5:667150. doi: 10.3389/fsufs.2021.667150

Munns, R., and Tester, M. (2008). Mechanisms of salinity tolerance. *Annu. Rev. Plant Biol.* 59, 651–681. doi: 10.1146/annurev.arplant.59.032607.092911

Nautiyal, C. S., Srivastava, S., Chauhan, P. S., Seem, K., Mishra, A., and Sopory, S. K. (2013). Plant growth-promoting bacteria *Bacillus amyloliquefaciens* NBRISN13 modulates gene expression profile of leaf and rhizosphere community in rice during salt stress. *Plant Physiol. Bioch.* 66, 1–9. doi: 10.1016/j.plaphy.2013.01.020

Nivetha, N., Lavanya, A. K., Vikram, K. V., Asha, A. D., Sruthi, K. S., Bandeppa, S., et al. (2021). "PGPR-mediated regulation of antioxidants: prospects for abiotic stress management in plants" in *Antioxidants in plant-microbe interaction*. eds. A. K. Singh, S. Kumar and T. Sinha (Berlin: Springer)

Patani, A., Prajapati, D., Ali, D., Kalasariya, H., Yadav, V. K., Tank, J., et al. (2023). Evaluation of the growth-inducing efficacy of various *bacillus species* on the salt-stressed tomato (*Lycopersicon esculentum* mill.). *Front. Plant Sci.* 14:1168155. doi: 10.3389/fpls.2023.1168155

Pistelli, L., Libik-Konieczny, M., and Hasanuzzaman, M. (2023). Plant-microbe interactions and their role in salinity tolerance. *Front. Plant Sci.* 14:1142563. doi: 10.3389/fpls.2023.1142563

Plazinski, J., and Rolfe, B. G. (1985). Analysis of the pectolytic activity of Rhizobium and Azospirillum strains isolated from Trifolium repens. *J. Plant Physiol.* 120, 181–187. doi: 10.1016/S0176-1617(85)80021-3

Pruitt, K. D., Tatusova, T., and Maglott, D. R. (2007). NCBI reference sequence (ref Seq): a curated non-redundant sequence database of genomes, transcripts and proteins. *Nucleic Acids Res.* 35, D61–D65. doi: 10.1093/nar/gkl842

Ruíz-Sánchez, M., Armada, E., Muñoz, Y., García de Salamone, I. E., Aroca, R., Ruíz-Lozano, J. M., et al. (2011). *Azospirillum* and arbuscular mycorrhizal colonization enhance rice growth and physiological traits under well-watered and drought conditions. *J. Plant Physiol.* 168, 1031–1037. doi: 10.1016/j.jplph.2010.12.019

Rus, A., Yokoi, S., Sharkhuu, A., Reddy, M., Lee, B. H., Matsumoto, T. K., et al. (2001). *AtHKT1* is a salt tolerance determinant that controls Na^+ entry into plant roots. *P. Natl. Acad. Sci. USA.* 98, 14150–14155. doi: 10.1073/pnas.241501798

Schmittgen, T. D., and Livak, K. J. (2008). Analyzing real-time PCR data by the comparative *CT* method. *Nat. Protoc.* 3, 1101–1108. doi: 10.1038/nprot.2008.73

Schwyn, B., and Neilands, J. B. (1987). Universal chemical assay for the detection and determination of siderophores. *Anal. Biochem.* 160, 47–56. doi: 10.1016/0003-2697(87)90612-9

Shultana, R., Zuan, A. T. K., Naher, U. A., Islam, A. M., Rana, M. M., Rashid, M. H., et al. (2022). The PGPR mechanisms of salt stress adaptation and plant growth promotion. *Agronomy* 12:2266. doi: 10.3390/Agronomy12102266

Singh, P., Choudhary, K. K., Chaudhary, N., Gupta, S., Sahu, M., Tejaswini, B., et al. (2022). Salt stress resilience in plants mediated through osmolyte accumulation and its crosstalk mechanism with phytohormones. *Front. Plant Sci.* 13:1006617. doi: 10.3389/fpls.2022.1006617

Soliman, M., Elkelish, A., Souad, T., Alhaithloul, H., and Farooq, M. (2020). Brassinosteroid seed priming with nitrogen supplementation improves salt tolerance in soybean. *Physiol. Mol. Biol. Plants* 26, 501–511. doi: 10.1007/s12298-020-00765-7

Tanaka, K., Fukuda, M., Amaki, Y., Sakaguchi, T., Inai, K., Ishihara, A., et al. (2017). Importance of prumycin produced by *Bacillus amyloliquefaciens* SD-32 in biocontrol against cucumber powdery mildew disease. *Pest Manag. Sci.* 73, 2419–2428. doi: 10.1002/ps.4630

Tang, Y., Bao, X., Zhi, Y., Wu, Q., Guo, Y., Yin, X., et al. (2019). Overexpression of a MYB family gene, *OsMYB6*, increases drought and salinity stress tolerance in transgenic rice. *Front. Plant Sci.* 10:168. doi: 10.3389/fpls.2019.00168

Tank, N., and Saraf, M. (2010). Salinity-resistant plant growth promoting rhizobacteria ameliorates sodium chloride stress on tomato plants. *J. Plant Interact.* 5, 51–58. doi: 10.1080/17429140903125848

Tatusov, R. L., Galperin, M. Y., Natale, D. A., and Koonin, E. V. (2000). The COG database: a tool for genome-scale analysis of protein functions and evolution. *Nucleic Acids Res.* 28, 33–36. doi: 10.1093/nar/28.1.33

Wang, Y., Huang, L., Du, F., Wang, J., Zhao, X., Li, Z., et al. (2021). Comparative transcriptome and metabolome profiling reveal molecular mechanisms underlying *OsDRAP1*-mediated salt tolerance in rice. *Sci. Rep.* 11:5166. doi: 10.1038/s41598-021-84638-3

Wang, D., Lu, X., Chen, X., Wang, S., Wang, J., Guo, L., et al. (2020). Temporal salt stress-induced transcriptome alterations and regulatory mechanisms revealed by Pac bio long-reads RNA sequencing in *Gossypium hirsutum*. *BMC Genomics* 21, 1–15. doi: 10.1186/s12864-020-07260-z

Wang, Q., Peng, X., Lang, D., Ma, X., and Zhang, X. (2022). Physio-biochemical and transcriptomic analysis reveals that the mechanism of *Bacillus cereus* G2 alleviated oxidative stress of salt-stressed *Glycyrrhiza uralensis* Fisch. Seedlings. *Ecotox. Environ. Safe* 247:114264. doi: 10.1016/j.ecoenv.2022.114264

Wang, Y., Sun, Q., Liu, J., Wang, L., Wu, X., Zhao, Z., et al. (2022). *Suaeda salsa* root-associated microorganisms could effectively improve maize growth and resistance under salt stress. *Microbiol. Spectr.* 10, e01349–e01322. doi: 10.1128/spectrum.01349-22

Wang, W., Wu, Z., He, Y., Huang, Y., Li, X., and Ye, B. C. (2018). Plant growth promotion and alleviation of salinity stress in *Capsicum annuum* L. by *bacillus* isolated from saline soil in Xinjiang. *Ecotox. Environ. Safe* 164, 520–529. doi: 10.1016/j.ecoenv.2018.08.070

Woo, O. G., Kim, H., Kim, J. S., Keum, H. L., Lee, K. C., Sul, W. J., et al. (2020). *Bacillus subtilis* strain GOT9 confers enhanced tolerance to drought and salt stresses in *Arabidopsis thaliana* and *Brassica campestris*. *Plant Physiol. Biochem.* 148, 359–367. doi: 10.1016/j.plaphy.2020.01.032

Xiao, X., Wang, Q., Ma, X., Lang, D., Guo, Z., and Zhang, X. (2022). Physiological biochemistry-combined transcriptomic analysis reveals mechanism of *Bacillus cereus* G2 improved salt-stress tolerance of *Glycyrrhiza uralensis* Fisch. Seedlings by balancing carbohydrate metabolism. *Front. Plant Sci.* 12:712363. doi: 10.3389/fpls.2021.712363

Xie, Z., Nolan, T. M., Jiang, H., and Yin, Y. (2019). AP2/ERF transcription factor regulatory networks in hormone and abiotic stress responses in Arabidopsis. *Front. Plant Sci.* 10:228. doi: 10.3389/fpls.2019.00228

Xu, S., Hu, B., He, Z., Ma, F., Feng, J., Shen, W., et al. (2011). Enhancement of salinity tolerance during rice seed germination by presoaking with hemoglobin. *Int. J. Mol. Sci.* 12, 2488–2501. doi: 10.3390/ijms12042488

Yilmaz, Y., Erdinc, C., Akköprü, A., and Kıpçak, S. (2020). Use of plant growth promoting rhizobacteria against salt stress for tomato (*Solanum lycopersicum* L.) seedling growth. *Acta Sci. Pol. Hortorum. Cultus.* 19, 15–29. doi: 10.24326/asphc.2020.6.2

Yilmaz, H., and Kulaz, H. (2019). The effects of plant growth promoting rhizobacteria on antioxidant activity in chickpea (*Cicer arietinum* L.) under salt stress. *Legum. Res.* 42, 72–76. doi: 10.18805/LR-435

Zhang, X., Gao, H., Liang, Y., and Cao, Y. (2021). Full-length transcriptome analysis of asparagus roots reveals the molecular mechanism of salt tolerance induced by arbuscular mycorrhizal fungi. *Environ. Exp. Bot.* 185:104402. doi: 10.1016/j.envexpbot.2021.104402

Zhang, F., Wang, Y., Liu, C., Chen, F., Ge, H., Tian, F., et al. (2019). *Trichoderma harzianum* mitigates salt stress in cucumber *via* multiple responses. *Ecotox. Environ. Safe.* 170, 436–445. doi: 10.1016/j.ecoenv.2018.11.084

Zhao, L., Gu, R., Gao, P., and Wang, G. (2008). A nonsymbiotic hemoglobin gene from maize, *ZmHb*, is involved in response to submergence, high-salt and osmotic stresses. *Plant Cell Tiss. Org.* 95, 227–237. doi: 10.1007/s11240-008-9436-3

Zhou, H., Ren, Z. H., Zu, X., Yu, X. Y., Zhu, H. J., Li, X. J., et al. (2021). Efficacy of plant growth-promoting bacteria *Bacillus cereus* YN917 for biocontrol of rice blast. *Front. Microbiol.* 12:684888. doi: 10.3389/fmicb.2021.684888

Zhu, J. K. (2016). Abiotic stress signaling and responses in plants. *Cell* 167, 313–324. doi: 10.1016/j.cell.2016.08.029

Zouari, M., Hassena, A. B., Trabelsi, L., Rouina, B. B., Decou, R., and Labrousse, P. (2019). "Exogenous proline-mediated abiotic stress tolerance in plants: possible mechanisms" in *Osmoprotectant-mediated abiotic stress tolerance in plants: Recent advances and future perspectives*. eds. M. A. Hossain, V. Kumar, D. J. Burritt, M. Fujita and P. S. A. Mäkelä (Cham: Springer)

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Forage yield, competition, and economic indices of oat and common vetch intercrops in a semi-arid region

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Intercropping of annual favorable legumes with grains to produce forage is utilized extensively worldwide to improve resource use efficiency. To identify the best intercropping system for a semi-arid region of China, intercrops of oat (*Avena sativa* L.) and common vetch (*Vicia sativa* L.) at five planting proportions and oat and common vetch monocrops were produced over the 2011 and 2012 growing seasons in Xifeng, northwest China. Several indices were used to evaluate yields, competitive interrelationships between the two crops, and economic returns. The oat mono-crop had the highest dry matter yield (6.51 t ha^{-1}), while the oat–common vetch intercrop, with an 80: 20 planting ratio, produced the highest crude protein production (696 kg ha^{-1}). The land equivalent ratio (1.167), relative crowding coefficient (2.445), and actual yield loss (0.750) were more favorable for the oat–common vetch intercrop at a seeding ratio of 20: 80. The oat–common vetch intercrop at a seeding ratio of 20: 80 showed the highest values for monetary advantage index (35.51). Overall, the autumn-sown 20: 80 oat–common vetch intercrop was more productive from resource utilization and economic perspectives.

KEYWORDS

actual yield loss, competitive, intercropping, forage, land equivalent ratio, monetary advantage

1 Introduction

Intercropping of grain and Leguminosae has many advantages over monocultures, such as better use of light, water (Luo et al., 2016; Tamburini et al., 2020; Liu H. et al., 2023), and nutrients, higher yields than those from legume monocultures, increased feed value (Pinto et al., 2022), reduced nitrous oxide emissions from soil (Shen et al., 2018), and better control of the occurrence of pests and weeds (Gronle et al., 2015; Maitra et al., 2021; Koskey et al., 2022). It also increases soil organic matter (Cong et al., 2014). Intercropping is more below-ground competitive and intercropping than monocropping, thus changing the resource playing field (Liu X. et al., 2023). Therefore, intercropping is receiving increasing attention because of its potential to increase agricultural production's sustainability (Andersen et al., 2007; Duchene et al., 2017).

The ratio of each species in a mixed sowing system, and the growth conditions affect the efficiency of intercropping (Caballero and Goicoechea, 1986). For example, oat–pea intercrops fell short of achieving any grain yield advantage in soil with low organic matter content

(Neugschwandtner and Kaul, 2014). Interspecific competition is one of the factors that affect the component species to determine their yields in a mixture, compared with those from monocultures (Caballero et al., 1995). Traditionally, the aggressivity index (A) indicated the relative yield of one crop over a companion crop in an intercropping system (McGilchrist, 1965). More recently, the land equivalent ratio (LER) is now the predominant metric used to evaluate competitiveness (Agegnehu et al., 2006; Esmaeili et al., 2011). Compared with the LER, the actual yield loss (AYL) index provides more accurate information regarding the competition among and within the component crops, as well as the behavior of each species in the intercropping system. This is because AYL emphasizes the productivity of each plant, whereas LER merely considers the yield per unit land area (Banik et al., 2000). In addition to dry matter (DM) yield, the efficiency of environmental resource use and economic profitability should also be considered (Ghosh, 2004; Midya et al., 2005; Gitari et al., 2020). None of the competitiveness indices provide insights into the economic advantages of intercropping systems.

The Loess Plateau is a large geographical region (62,000 km^2) in northwestern China (Figure 1). Despite the challenging climate, this region is home to some 80 million people who depend on traditional rain-fed farming techniques and play a vital role in China's food production. Winter wheat (*Triticum aestivum* L.) is the main crop in traditional cropping systems. The harvest takes place in late June or early July, and there is a period of summer fallow from July to September. Hence, the overall effectiveness of utilizing precipitation is frequently diminished due to the significant evaporation of moisture to the exposed soil throughout the idle season (Zixi et al., 1994; Chen et al., 2023). Total effective precipitation (TEP) is typically as low as 82%, whereas planting forage crops followed by winter wheat can increase TEP to 97% and is encouraged (Fengrui et al., 2000).

In addition, crop-livestock systems are essential to dryland agriculture, providing food security and livelihood options for people. Livestock are also a vital source of income for the local farmers and government aims to double small ruminant numbers and expand the area of forage through the "Six Million" project in the Longdong Loess Plateau (Malézieux et al., 2009). With livestock production expanding in this region, a challenge currently faced by farmers is to choose profitable annual forage crops stored by hay or silage to solve the feed deficit during winter and early spring. While the right choice of annual forage crops may depend on unpredictable nature of precipitation in this region and subsequent winter wheat. Intercrop of cereals and legumes is not only increasing water and land use efficiency (Zhu et al., 2022; Raza et al., 2023), enhancing soil carbon and nitrogen (Jensen et al., 2020), enrichment of microbial communities (Lai et al., 2022), but also stimulating subsequent wheat yield under rainfed conditions (Scalise et al., 2015). Therefore, in this study, oat (*Avena sativa* L.), which has a higher dry matter when sown in summer fallow than in spring on the Loess plateau (Zhang Y, et al., 2015), was intercropped with common vetch (*Vicia sativa* L.) at five different seeding ratios. Competition indices were calculated for each of the intercrops, as well as economic and yield indices. These indices have not been used previously to evaluate competition among different seeding ratios or to evaluate economic advantages of oat–common vetch intercrops. We hypothesized that an intercrop of oat and common vetch with high dry matter, crude protein (CP) yield and economic benefit could be used as hay for livestock production during the summer fallow period on the Loess Plateau.

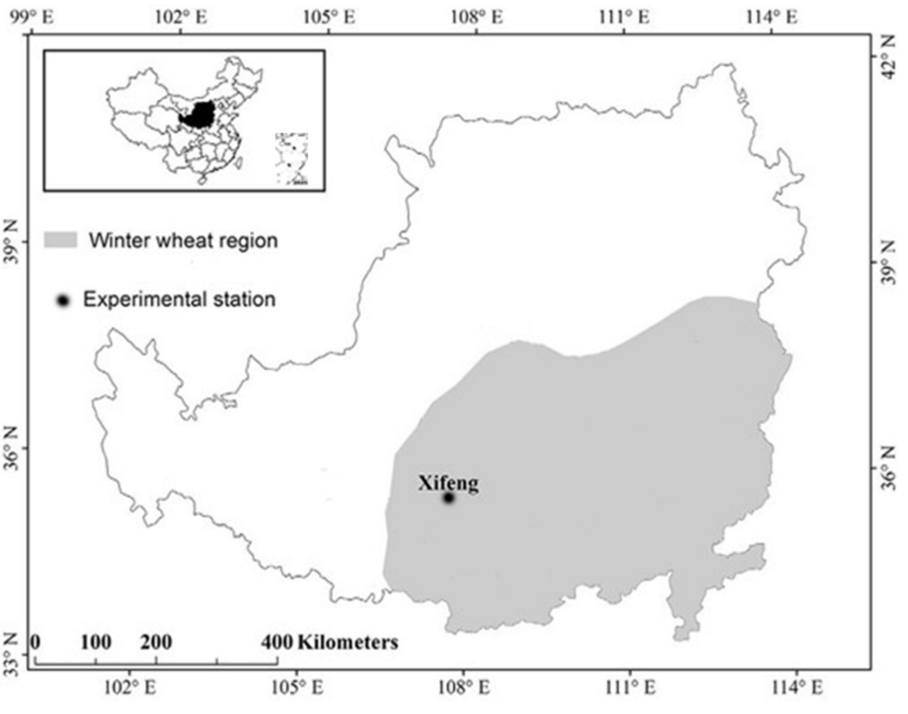

FIGURE 1
Location of experimental station on the Loess Plateau, northwest China.

The objectives of this 2-year study were as follows: (1) to evaluate the forage and protein yields of oat and vetch grown in monocultures and intercrops in a semi-arid region; (2) to examine the effect of competition between oat and common vetch in this intercropping system; and (3) to assess overall productivity, competition, and economic parameters for each of the intercrops and monocrops.

2 Materials and methods

2.1 Site description, experimental design, and measurements

This study was conducted over the 2011 and 2012 growing seasons at the Loess Plateau Research Station of Lanzhou University (35°40′ N, 107°51′ E, elev. 1,298 m) located at Xifeng, Gansu Province, northwest China. The region is characterized as a semi-arid zone, with mean annual precipitation of 548 mm concentrated in July–September. Average annual pan evaporation is 1,504 mm, about three times higher than the precipitation. Average annual mean temperature is 8.3°C, and mean temperatures in the hottest (July) and coldest (January) months are 21.3°C and −5.3°C, respectively. Average annual solar radiation is 5,489 MJ m^{-2}. The mean length of the annual growing season is 255 d. The dominant soil type is sandy loam with an average field water-holding capacity of 0.22 kg·kg^{-1} and a wilting point of 0.07 kg·kg^{-1}. In this area, 0–10 cm soil organic carbon 9.6 g·kg^{-1}, available nitrogen 23.6 mg·kg^{-1}, available phosphorus 6.2 mg·kg^{-1}, total nitrogen 0.7 mg·kg^{-1}. The organic carbon of 10–20 cm soil was 10.2 mg·kg^{-1}, available nitrogen 31.5 mg·kg^{-1} and available phosphorus 7.2 mg·kg^{-1}.

The seeding rate for monocultures of oat (cultivar No. 2 Qingyin) and common vetch (cultivar No. 3 Lanjian) was 165 (about 495 seeds m^{-2}) and 105 kg ha^{-1} (about 200 seeds m^{-2}), respectively. The intercrops were sown in a replacement series using the following oat–common vetch seeding ratios (%): 80: 20, 67: 33, 50: 50, 33: 67 and 20: 80, corresponding to 396-40, 332-66, 248-100, 163-134 and 99-160 seeds m^{-2}, with row spacing of 30 cm. The seeds of both kinds were planted at the same time. The research methodology employed a randomized complete block approach consisting of four separate replications. The dimensions of the land were 4 meters by 6 meters. A 15-hp cultivator was used to prepare the seedbed down to a measurement of 20 cm in depth. Planting was carried out in a single fade using a 4-coulter plot drill and seeds were deposited at a depth of 4 cm. On 7 July 2011 and 12 July 2012, crops were seeded manually. Based on local fertilizer recommendations, nitrogen and phosphorus were applied prior to sowing at rates corresponding to 46 kg N and 100 kg P_2O_5 per hectare, respectively. Weeds were effectively controlled in all plots through human hoeing. The climatic conditions during the 2-year study period are shown in Figure 1.

Plants in a 0.75 m^2 sampling area within every plot were removed manually to ground level on October 25, 2011, and on October 21, 2012, when common vetch was at the pod-filling stages and oat was at the kernel milk stage. Samples were separated into component species and weighed. To assess the dry matter (DM) yield, subsamples (0.5 kg fresh weight) of each species from each plot were dried in a hot air draft oven at a constant temperature of 65°C until they reached a stable weight.

2.2 Crop nitrogen concentration and protein yield

The sub-samples utilized for DM measurements were pulverized using a Wiley mill to achieve a particle size small enough to pass through a 1-mm screen. The Kjeldahl method was employed to ascertain the aggregate nitrogen content in intact plant samples (Black, 1965). The crude protein (CP) is determined by multiplying the amount of nitrogen by an amount of 6.25 (Jones, 1931). Crop protein yield (kg ha^{-1}) for this intercropping system was calculated as follows:

$$\text{Crop protein yield} = \text{N\%oat} \times 6.25 \times \text{DM}_{\text{oat}} + \text{N\%vetch} \times 6.25 \times \text{DM}_{\text{vetch}}.$$

2.3 Competition indices

Multiple concurrence assessments were used to compute the possible advantages of intercropping and the influence of inter-species rivalry in the mixture. The LER was used as the criterion to assess the advantage of a mixed stand containing both oat and common vetch as desired species (Osiru and Willey, 1972). The LER was calculated as follows:

$$LER = LER_{oat} + LER_{vetch}$$

$$LER_{oat} = \frac{Y_{OV}}{Y_O} \quad LER_{vetch} = \frac{Y_{VO}}{Y_V}.$$

where Y_O and Y_V are the yields of oat and common vetch in a pure stand, and Y_{OV} and Y_{VO} are the DM yields of oat and common vetch in any one mixture.

The relative crowding coefficient (K) quantifies the degree of dominance of one species over another in a mixture (Lithourgidis et al., 2011), and was calculated as follows:

$$K = K_{oat} \times K_{vetch}$$

$$K_{oat} = \frac{Y_{OV} Z_{VO}}{(Y_O - Y_{OV}) Z_{OV}} \quad K_{vetch} = \frac{Y_{VO} Z_{OV}}{(Y_V - Y_{VO}) Z_{VO}}$$

Z_{OV} and Z_{VO} represent the proportional proportions of oat and common vetch that are seeded in a mixture. When the multiplication of the two coefficients (K_{oat} and K_{vetch}) exceeds one, it results in a yield advantage. When the value of K is one, there is no advantage in terms of yield. There is a yield disadvantage when K is lower than one.

Aggressivity (A) was calculated as follows:

$$A_{oat} = \left(\frac{Y_{OV}}{Y_O Z_{OV}}\right) - \left(\frac{Y_{VO}}{Y_V Z_{VO}}\right)$$

If the value of A_{oat} is 0, it indicates that both crops have equal competitiveness. If the value of A_{oat} is positive, it indicates that oat is the dominating species. Conversely, if A_{oat} is negative, it suggests that oat is the species being dominated. Aggressivity of common vetch was determined in the same way.

$$A_{vetch} = \left(\frac{Y_{VO}}{Y_V Z_{VO}}\right) - \left(\frac{Y_{OV}}{Y_O Z_{OV}}\right)$$

Partial real yield loss (AYL_{oat} or AYL_{vetch}) represents the relative change in yield, either loss or gain, of each species when planted as intercrops compared to their yield in a monoculture. The AYL was calculated as follows (Banik et al., 2000):

$$AYL_{oat} = \left\{\left[\frac{Y_{OV} \,/\, Z_{OV}}{Y_O \,/\, Z_O}\right] - 1\right\}$$

$$AYL_{vetch} = \left\{\left[\frac{Y_{VO} \,/\, Z_{VO}}{Y_V \,/\, Z_V}\right] - 1\right\}$$

$$AYL = AYL_{oat} + AYL_{vetch}$$

2.4 Economic indices

The Monetary Advantage Index (MAI) offers insights into the economic benefits of the combining system. The MAI was calculated as follows:

$$MAI = \left(Y_{OV} P_{oat} + V_{VO} P_{vetch}\right) \times \frac{LER - 1}{LER}$$

A higher MAI score corresponds to a more profitable cropping scheme (Ghosh, 2004). The intercropping advantage (IA) was computed utilizing the subsequent equation (Banik et al., 2000).

$$IA_{oat} = AYL_{oat} \times P_{oat}$$

$$IA_{vetch} = AYL_{vetch} \times P_{vetch}$$

$$IA = IA_{oat} + IA_{vetch}$$

where P is the hay price, average hay procurement price per ton: common vetch = €55, oat = €43 (Lithourgidis et al., 2006).

2.5 Statistical analyses

An integrated analysis of variance (ANOVA) was conducted to independently examine the dry matter (DM), concentration, and yields of CP in both sole crops and intercrops, considering data from many years. Data were analyzed employing the mixed model feature in SPSS version 16.0 (SPSS Inc., Chicago, IL, United States) utilizing an autoregressive covariance structure. Year was a random effect, seeding ratio was a fixed effect. Bartlett's test was used to examine the homogeneity of variances. The competition and economic indices were differentiated using the least significant difference (LSD) test with a significance level of 0.05.

3 Results

3.1 Variations in yearly precipitation

The precipitation patterns observed throughout crop growth (July–October) in both 2011 and 2012 closely resembled the historical average but with some end-of-season variations (Figure 2). Since there were no significant effects between years, minor rainfall and temperature differences had no effect on DM or index measurements.

3.2 Aboveground dry matter

The analysis of variance for above ground dry matter (ABDM) data of oat and common vetch indicated notable variations exist among seed ratios. However, there were no significant effects on yield for either year or year x seed ratio (Table 1).

As expected, the ABDM yield was significantly higher for the oat monoculture than for the common vetch monoculture and the oat–common vetch intercrops ($p < 0.05$). As the ratio of common vetch increased in intercrops, the ABDM yield of oats decreased. For example, the oat ABDM was 6.51 t ha^{-1} in the monoculture but decreased with higher proportions of common vetch in the mixture (2.15 and 2.24 t ha^{-1} in the 33:67 and 20:80 oat–common vetch intercrops, respectively). Common vetch showed an ABDM advantage when sown in a mixture. Common vetch constituted 49 and 55% of the AGDM in the 33:67 and 20:80 oat–common vetch intercrops, respectively (Table 1).

3.3 Crude protein percentage and yield

We determined the CP concentration (%) and protein yield (kg ha^{-1}) for each of the monocrops and intercrops (Table 1). The lowest CP concentration was in the oat monoculture (8.12%), and the highest CP concentration was in the common vetch monoculture (14.88%). However, the common vetch monoculture had the lowest protein yield (496 kg ha^{-1}) because of its low DM yield (3.34 t ha^{-1}). In the intercrops, the CP percentage increased as the ratio of common vetch in the intercrops increased. The maximum protein output was in the 80:20 oat–common vetch intercrop (696 kg ha^{-1}). This was because of the high dry matter yield of the 80:20 oat–common vetch intercrop (6.39 t ha^{-1}).

3.4 Competition indices

The values for the competition indices K (Table 2) and A (Table 3) indicated that oat was the stronger competitor in the oat–common vetch intercrops. The values of A and K for oat differed significantly among seeding ratios. As evident from the formula for A, the values

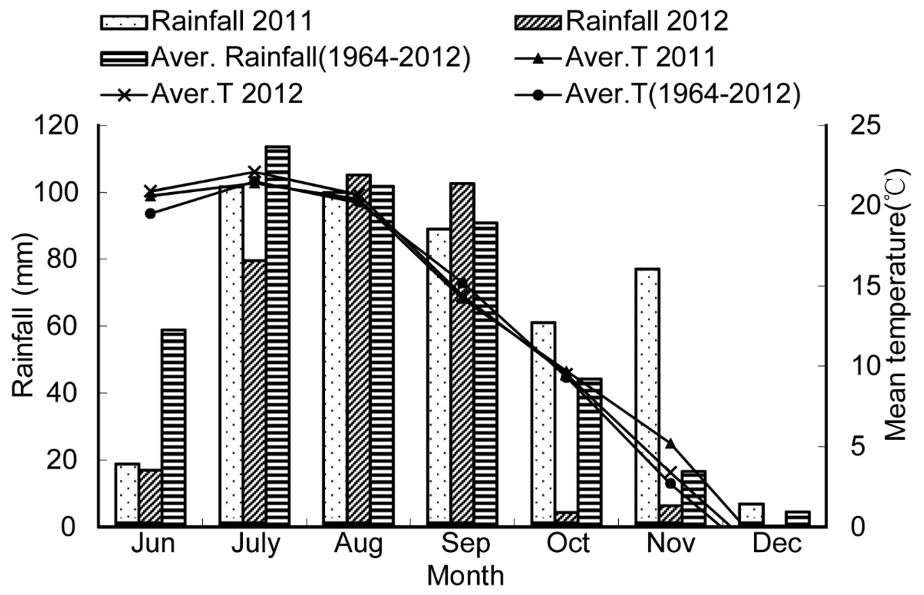

FIGURE 2
Monthly rainfall and mean air temperature (T) during 2011 and 2012 growing seasons at Qingyang Research Station, Gansu, China.

TABLE 1 Forage and protein yield of oat (O) and common vetch (V) monocultures and intercrops at different seeding ratios.

Treatment (seeding ratio)	Above-ground dry matter yield (t ha^{-1})			Crude protein (%)	Protein yield (kg ha^{-1})
	Oat	Vetch	Total		
O:V(100:0)	6.51 (0.82)	0.00	6.51 (0.82)	8.12 (1.28)	528 (5)
O:V(80:20)	5.76 (0.37)	0.62 (0.10)	6.39 (0.40)	10.91 (1.75)	696 (24)
O:V(67:33)	4.81 (0.56)	0.95 (0.17)	5.76 (0.60)	10.12 (2.34)	642 (20)
O:V(50:50)	3.18 (0.31)	1.52 (0.11)	4.70 (0.23)	12.03 (1.65)	565 (21)
O:V(33:67)	2.15 (0.23)	2.14 (0.37)	4.30 (0.51)	13.12 (1.89)	563 (21)
O:V(20:80)	2.24 (0.32)	2.75 (0.33)	4.99 (0.49)	13.81 (2.68)	654 (18)
O:V(0:100)	0.00	3.34 (0.56)	3.34 (0.56)	14.88 (2.87)	496 (18)
$LSD_{0.05}$	0.75	0.94	1.04	1.65	141

Values shown are averaged over two growing seasons (2011 and 2012). Standard errors are shown in parentheses.

for A of oat and common vetch are numerically identical but differ in their sign. In this experiment, A_{oat} was positive (indicating dominance) in the 80:20, 67:33, and 50:50 oat–common vetch intercrops. The highest A_{oat} value was in the 20:80 oat–common vetch intercrop (Table 3). The values of K, which represent crowding, were notably higher for oat than for common vetch and did not show a smooth trend with respect to the range of seed ratios. However, the K_{oat} values were highest when oat was a minor or major mixture component, and lower when oat and common vetch were mixed in similar proportions (Table 2).

3.5 Economic indices

The values for AYL calculated for mixture components varied with seeding rate and were positive for oat and negative for common vetch, indicating a general yield loss for common vetch in intercrops. The LER_{oat} values exhibited a positive correlation with the proportion of oat in intercrops, while the LER_{vetch} values showed a positive correlation with the proportion of common vetch in intercrops. As year effects were not statistically significant, the average LER for the 2011 and 2012 growing seasons was used to calculate MAI. For the mixtures, both AYL and LER showed similar trends to that of K with respect to seeding ratios, with the lowest values for the 50:50 mixtures (Table 3).

The economic indices IA and MAI varied significantly with the seeding rate. Positive IA values were obtained for oat at seeding ratio 80:20 and 67:33 and the highest IA value was for the 80:20 oat–common vetch intercrop. The IA values for common vetch were all negative except that for the 20:80 mixture. This value was smaller than those obtained for oat so that the total IA followed the same pattern as that of IA_{oat}. The highest overall IA (0.254) was for the 80:20 oat–common vetch intercrop. Similar to the trends in yield (Table 1), the MAI showed positive values for the oat–common vetch intercrops with seeding ratios of 80:20, 67:33, and 20:80 (18.54, 5.90, and 35.51, respectively).

TABLE 2 Land equivalent ratio (LER) and relative crowding coefficient (K) for monocrops and oat (O)–common vetch (V) intercrops with different seeding ratios.

Treatment (seeding ratio)	Land equivalent ratio			Relative crowding coefficient		
	LER_{oat}	LER_{vetch}	LER	K_{oat}	K_{vetch}	K
O:V(80:20)	0.885 (0.060)	0.186 (0.020)	1.070 (0.056)	1.920 (0.178)	0.912 (0.060)	1.751 (0.152)
O:V(67:33)	0.739 (0.040)	0.284 (0.017)	1.023 (0.042)	1.394 (0.615)	0.807 (0.082)	1.125 (0.537)
O:V(50:50)	0.488 (0.057)	0.455 (0.022)	0.944 (0.055)	0.955 (0.238)	0.835 (0.073)	0.798 (0.215)
O:V(33:67)	0.330 (0.046)	0.641 (0.091)	0.971 (0.130)	1.001 (0.161)	0.878 (0.232)	0.879 (0.386)
O:V(20:80)	0.344 (0.066)	0.823 (0.052)	1.167 (0.093)	2.098 (0.939)	1.165 (0.183)	2.445 (0.812)
$LSD_{0.05}$	0.13	0.15	0.20	0.45	0.22	0.86

Values shown are averages from two growing seasons (2011 and 2012). Standard errors are shown in parentheses.

TABLE 3 Aggressivity (A) and actual yield loss (AYL) for oat (O)–common vetch (V) intercrops at different seeding ratios.

Treatment (seeding ratio)	Aggressivity		Actual yield loss		
	A_{oat}	A_{vetch}	AYL_{oat}	AYL_{vetch}	AYL
O:V(80:20)	0.671 (0.050)	−0.671 (0.050)	0.106 (0.015)	−0.072 (0.019)	0.034 (0.010)
O:V(67:33)	0.401 (0.087)	−0.401 (0.087)	0.103 (0.060)	−0.138 (0.051)	−0.035 (0.004)
O:V(50:50)	0.017 (0.003)	−0.017 (0.003)	−0.023 (0.014)	−0.090 (0.043)	−0.113 (0.096)
O:V(33:67)	−0.320 (0.049)	0.320 (0.049)	0.001 (0.001)	−0.044 (0.014)	−0.043 (0.026)
O:V(20:80)	−0.590 (0.041)	0.590 (0.041)	0.720 (0.329)	0.029 (0.015)	0.750 (0.034)
$LSD_{0.05}$	0.32	0.32	0.41	0.03	0.49

Values shown are averages from two growing seasons (2011 and 2012). Standard errors are shown in parentheses.

4 Discussion

4.1 Aboveground dry matter

Intercropping is becoming more popular in sustainable agricultural methods, because of its potential to increase the efficiency of resource use. In previous studies on intercropping systems, it has been reported that oat dominated pea (*Pisum sativum* L.) (Neugschwandtner and Kaul, 2016), at 66:33 common vetch–barley (*Hordeum vulgare* L.) intercropping (Osman and Nersoyan, 1986), 55:45 common vetch–oat intercrop (Dhima et al., 2007) and 75:25 oat–faba bean (*Vicia faba* L.) intercropping (Dhima et al., 2014) had higher yield than their respective monocrops. These findings suggested that the maximum DM yields depend both on the species and on the seeding ratio. However, Another study found that the seeding ratios had no impact on the dry matter yields in combinations of pea and cereals, such as wheat or oat (Carr et al., 1998).

In this study, the contribution of common vetch to DM yield decreased with higher proportions of common vetch in the intercrops. This may have been because of competition between oat and common vetch in the intercrops, given that all the competition indices pointed out that oat was the prevailing species in the mixture. One possible explanation is that the tillering capacity of oat made it more competitive than common vetch (Lithourgidis et al., 2011). Another possible explanation is that oats grow faster initially and have a higher plant height than the intercropping common vetch. Putting the intercropped common vetch a disadvantage in terms of light (Feng et al., 2015).

The mechanisms that explain over-yielding intercropping are generally attributed to specific mutual complementarity (Dong et al., 2018) and beneficial interactions among species (facilitation) in resource use (Stomph et al., 2020). Cong et al. (2014) showed that facilitation of P, Fe, Mn and Zn acquisition is a potentially important cause of overyielding in annual intercropping systems. Cereal/legume intercropping improves phosphorus acquisition (Hinsinger et al., 2011).

4.2 Crude protein

The concentration of CP is a crucial nutritional key qualities of forage crops and is commonly utilised to assess forage systems, particularly intercropping systems (Yolcu et al., 2009). The CP concentration was shown to increase in intercrops due to the legume contribution (Bedoussac et al., 2014). In the Mediterranean environment, pure common vetch or higher proportions of common vetch intercropped with oat showed higher CP yields (Lithourgidis et al., 2006), because of the low proportion of protein in the ABDM of oat (Lithourgidis et al., 2011). However, Li et al. (2006) found no notable disparities in nitrogen intake between intercropping systems that combine legumes and cereals, and the traditional practice of growing these crops separately. The study found that the common vetch monocrop had the highest CP concentration at 14.88%. Another study found that CP yields were greatest with a 3:1 ratio of oats to common vetch, increasing 21.3 and 6.1% over oats alone and wild peas alone, respectively (Qu et al., 2022). However, in this semi-arid region under summer-sowing conditions, the highest CP yield (696 kg ha^{-1}) was in the oat–common vetch intercrop with an 80:20 seeding ratio. This was because of the high DM yield of this intercrop.

4.3 Competition indices

The LER is an indicator of the effectiveness of environmental resource use in intercropping compared to monocropping (Willey and Rao, 1980). Intercropping benefits species' development and productivity when the LER is greater than one; Intercropping has a negative effect on the growth and yield of crops grown in mixtures when the LER is less than one (Reddy and Chetty, 1984; Ofori and Stern, 1987). Dhima et al. (2007) reported LER values ranging from 1.05 to 1.09 in a mix of common vetch with wheat, triticale (×Triticosecale Witt mark), barley, and oat at common vetch–cereal ratios of 55:45 and 65:35. LER for maize and cowpea (*Vigna unguiculata* L., Walp) intercropping exceeded 1 (1.91 and 1.53) and found that the intercrops were more stable than monocrops (Dimande et al., 2024). In another study, the land productivity of intercrops was 12–32% higher than those of monocrops (Chapagain and Riseman, 2014; Xu et al., 2021). In the present study, the oat–common vetch intercrop at a seeding ratio of 20:80 showed the highest LER value (1.17); This suggests that to attain the same yield as an intercrop, a solo cropping system would need to occupy an area that is 17% greater (Midya et al., 2005). Nassab et al. (2011) reported that a 67:33 mixture of maize and sunflower (*Helianthus annuus* L.) showed a higher LER value than those of other mixtures and monocrops. Wang et al. (2021) found that in oat and common vetch intercropping, LER was highest when oats were sown at a rate of 50%, which was 11–57% higher than other sowing rates. In the present study, the LER value for the 50:50 oat–common vetch intercrop was lower than one, suggesting strong competition between the two crops in this mixture.

In the intercrop mixtures, the K_{oat} values were higher than the K_{vetch} values, which indicates that oat was more competitively effective than vetch under these conditions. However, Dhima et al. (2007) report that *K* was higher for common vetch than for cereals in the mixture of vetch with wheat or triticale (65:35 common vetch–cereal). The *K* values exhibited a comparable pattern to the LER values in this investigation. In all mixtures, the *K* value was greater than one, indicating that there was a yield advantage of intercropping (Willey and Rao, 1980; Banik et al., 2000; Ghosh, 2004). The *K* value was above two in the 67:33 and 20:80 oat–common vetch mixtures, indicating a substantial yield increase from intercropping.

The A values indicated which species was dominant in the intercrops. Common vetch was the dominant species (as indicated by positive A_{vetch} values) only when it was the main component in the mixture (67% or 80%) (Table 3). Similar results have been reported previously for common vetch–wheat and common vetch–triticale mixtures (65:35) (Dhima et al., 2007). The study showed that the difference in aggression to competition ratios between oats and common wild pea was not significant under the mixed cropping system (Zhu et al., 2022). In the present study, oat was dominant in the oat–common vetch intercrops with seeding ratios of 80:20, 67:33, and 50:50. Cereals like maize, sorghum (*Sorghum bicolor* L) Moench, and pearl millet (*Pennisetum americanum* L) were also reported to be dominant in groundnut–cereal intercropping systems (Ghosh, 2004).

AYL offers more accurate data on both inter-and intraspecific rivalry among part crops and the actions of the various species in intercropping systems, in comparison to other competition indices (Banik et al., 2000). Positive AYL values signify a benefit, whereas negative AYL values signify a drawback in intercrops when the primary goal is to compare production on a per-plant basis (Zhang Q, et al., 2015). Dhima et al. (2007) reported negative AYL values for common vetch–triticale (65:35), common vetch–barley (65:35) and common vetch–oat (55:45) intercrops. Neugschwandtner and Kaul (2014) reported that 75:25, 50:50, and 25:75 oat–pea intercrops did not show yield advantages when grown in fertile soil. Takim (2012) studied maize–cowpea intercrops (67:33, 50:50, and 33:67) and reported negative AYL values for intercropped cowpea ranging from −0.257 to −0.813, indicating a yield loss of 25.7–81.3%, compared with that of a cowpea monocrop. In the present study, the AYL values for common vetch were negative and ranged from −0.044 to −0.138, indicating a yield loss of 4.4–13.8% in intercrops. Although the partial AYL of oat was positive except in the 50:50 oat–common vetch intercrop, the AYL was negative except in the 20:80 and 80:20 oat-common vetch intercrop, and this was insufficient to offset the decrease in crop productivity caused by the presence of common vetch in the mixture.

4.4 Economic indices

Dhima et al. (2007) reported a maximum MAI value of 13.47 for a 20:80 oat–common vetch mixture, while the maximum yield advantage was obtained for a 45:55 oat–common vetch mixture. In the present study, the most advantageous mixtures were the 20:80 oat–common vetch intercrop, followed by the 67:33 oat–common vetch intercrop (IA values of 0.254 and 0.99, respectively; Table 4). Lithourgidis et al. (2011) reported that a 20:80 cereal–pea intercrop

TABLE 4 Intercropping advantage (IA) and monetary advantage index (MAI) for oat–common vetch intercrops at different seeding ratios.

Treatment (seeding ratio)	Intercropping advantage			*MAI*
	IA_{oat}	IA_{vetch}	*IA*	
O:V(80:20)	0.294 (0.033)	−0.040 (0.005)	0.254 (0.010)	18.54 (2.66)
O:V(67:33)	0.176 (0.085)	−0.077 (0.027)	0.099 (0.001)	5.90 (1.02)
O:V(50:50)	0.007 (0.005)	−0.050 (0.024)	−0.043 (0.014)	−13.18 (2.24)
O:V(33:67)	−0.141 (0.062)	−0.025 (0.016)	−0.165 (0.005)	−6,28 (0.15)
O:V(20:80)	−0.259 (0.145)	0.016 (0.003)	−0.242 (0.042)	35.51 (2.44)
$LSD_{0.05}$	0.18	0.17	0.15	7.63

Values shown are averages from two growing seasons (2011 and 2012). Standard errors are shown in parentheses.
Average hay procurement price per ton: common vetch = €55, oat = €43.

provided the maximum economic profit, consistent with our results. In line with our findings, Ghosh (2004) observed that greater LER and K values were linked to notable economic advantages, as indicated by larger MAI values.

The outcomes of our study indicate that the practice of planting oat and common vetch together at various seeding ratios has an impact on the dry matter yield of each species, the degree of rivalry between the two species, and ultimately the economic viability of the intercropping system. By planting oats and common vetpea in different proportions, the method of increasing the yield of annual forage crops is realized to solve the problem of local feed shortage in winter and early spring. In future studies, we can explore the mixed sowing mechanism of annual forage crops and understand the principle of mixed sowing to increase yield, so as to select more mixed crops and clarify the breeding direction of future mixed crops.

On the Loess Plateau, oat–common vetch at a seeding ratio of 20:80 is the optimal intercrop in terms of balancing the nutritive value, competition between species, and economic returns. The results of this study illustrate how yield, competition, resource use, and economic indices can be used to compare different intercrops and identify which systems have the best overall value.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

YJ: Writing – original draft, Writing – review & editing. QZ: Conceptualization, Data curation, Writing – original draft. FM: Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Agegnehu, G., Ghizaw, A., and Sinebo, W. (2006). Yield performance and land-use efficiency of barley and faba bean mixed cropping in Ethiopian highlands. *Eur. J. Agron.* 25, 202–207. doi: 10.1016/j.eja.2006.05.002

Andersen, M. K., Hauggaard-Nielsen, H., Weiner, J., and Jensen, E. S. (2007). Competitive dynamics in two- and three-component intercrops. *J. Appl. Ecol.* 44, 545–551. doi: 10.1111/j.1365-2664.2007.01289.x

Banik, P., Sasmal, T., Ghosal, P. K., and Bagchi, D. K. (2000). Evaluation of mustard (Brassica compestris Var. Toria) and legume intercropping under 1: 1 and 2: 1 row-replacement series systems. *J. Agron. Crop Sci.* 185, 9–14. doi: 10.1046/j.1439-037X.2000.00388.x

Bedoussac, L., Journet, É.-P., Hauggaard-Nielsen, H., Naudin, C., Corre-Hellou, G., Prieur, L., et al. (2014). "Eco-functional intensification by cereal-grain legume intercropping in organic farming systems for increased yields, reduced weeds and improved grain protein concentration" in *Organic farming, prototype for sustainable agricultures.* eds. S. Bellon and S. Penvern (Dordrecht: Springer), 47–63.

Black, C. A. (1965). Method of soil analysis part 2. *Chem. Microbiol. Proper.* 9, 1387–1388,

Caballero, R., and Goicoechea, E. L.. (1986). Utilization of winter cereals as companion crops for common vetch and hairy vetch. Proceedings of the 11th General Meeting of the European Grass Fed.

Caballero, R., Goicoechea, E. L., and Hernaiz, P. J. (1995). Forage yields and quality of common vetch and oat sown at varying seeding ratios and seeding rates of vetch. *Field Crop Res.* 41, 135–140. doi: 10.1016/0378-4290(94)00114-R

Carr, P. M., Martin, G. B., Caton, J. S., and Poland, W. W. (1998). Forage and nitrogen yield of barley—pea and oat—pea intercrops. *Agron. J.* 90, 79–84. doi: 10.2134/agronj1998.00021962009000010015x

Chapagain, T., and Riseman, A. (2014). Barley–pea intercropping: effects on land productivity, carbon and nitrogen transformations. *Field Crop Res.* 166, 18–25. doi: 10.1016/j.fcr.2014.06.014

Chen, J., Chen, Y., Wang, K., Wang, G., Junhua, W., and Zhang, Y. (2023). Differences in soil water storage, consumption, and use efficiency of typical vegetation types and their responses to precipitation in the loess plateau, China. *Sci. Total Environ.* 869:161710. doi: 10.1016/j.scitotenv.2023.161710

Cong, W.-F., Hoffland, E., Li, L., Six, J., Sun, J.-H., Bao, X.-G., et al. (2014). Intercropping enhances soil carbon and nitrogen. *Glob. Chang. Biol.* 21, 1715–1726. doi: 10.1111/gcb.12738

Dhima, K. V., Lithourgidis, A. S., Vasilakoglou, I. B., and Dordas, C. A. (2007). Competition indices of common vetch and cereal intercrops in two seeding ratio. *Field Crop Res.* 100, 249–256. doi: 10.1016/j.fcr.2006.07.008

Dhima, K. V., Vasilakoglou, I. B., Xh Keco, R., Dima, A. K., Paschalidis, K. A., and Gatsis, T. D. (2014). Forage yield and competition indices of faba bean intercropped with oat. *Grass Forage Sci.* 69, 376–383. doi: 10.1111/gfs.12084

Dimande, P., Arrobas, M., and Rodrigues, M. Â. (2024). Intercropped maize and cowpea increased the land equivalent ratio and enhanced crop access to more nitrogen and phosphorus compared to cultivation as sole crops. *Sustain. For.* 16:1440. doi: 10.3390/su16041440

Dong, N., Tang, M.-M., Zhang, W.-P., Xing-Guo Bao, Y., Wang, P. C., and Li, L. (2018). Temporal differentiation of crop growth as one of the drivers of intercropping yield advantage. *Sci. Rep.* 8:3110. doi: 10.1038/s41598-018-21414-w

Duchene, O., Vian, J.-F., and Celette, F. (2017). Intercropping with legume for agroecological cropping systems: complementarity and facilitation processes and the importance of soil microorganisms. A review. *Agric. Ecosyst. Environ.* 240, 148–161. doi: 10.1016/j.agee.2017.02.019

Esmaeili, A., Sadeghpour, A., Hosseini, S. M. B., Jahanzad, E., Chaichi, M. R., and Hashemi, P. M. (2011). Evaluation of seed yield and competition indices for intercropped barley (*Hordeum vulgare*) and annual medic (*Medicago scutellata*). *Int. J. Plant Prod.* 5, 395–404,

Feng, X. M., Yang, Y., Ren, C. Z., Yue Gao, H., and Zeng, Z. H. (2015). Effects of legumes intercropping with oat on photosynthesis characteristics of and grain yield. *Acta Agron. Sin.* 41, 1426–1434. doi: 10.3724/SP.J.1006.2015.01426

Fengrui, L., Songling, Z., and Geballe, G. T. (2000). Water use patterns and agronomic performance for some cropping systems with and without fallow crops in a semi-arid environment of Northwest China. *Agric. Ecosyst. Environ.* 79, 129–142. doi: 10.1016/S0167-8809(99)00149-8

Ghosh, P. K. (2004). Growth, yield, competition and economics of groundnut/cereal fodder intercropping systems in the semi-arid tropics of India. *Field Crop Res.* 88, 227–237. doi: 10.1016/j.fcr.2004.01.015

Gitari, H. I., Nyawade, S. O., Kamau, S., Karanja, N. N., Gachene, C. K. K., Raza, M. A., et al. (2020). Revisiting intercropping indices with respect to potato-legume intercropping systems. *Field Crop Res.* 258:107957. doi: 10.1016/j.fcr.2020.107957

Gronle, A., Lux, G., Böhm, H., Schmidtke, K., Wild, M., Demmel, M., et al. (2015). Effect of ploughing depth and mechanical soil loading on soil physical properties, weed infestation, yield performance and grain quality in sole and intercrops of pea and oat in organic farming. *Soil Tillage Res.* 148, 59–73. doi: 10.1016/j.still.2014.12.004

Hinsinger, P., Betencourt, E., Bernard, L., Brauman, A., Plassard, C., Shen, J., et al. (2011). P for two, sharing a scarce resource: soil phosphorus acquisition in the rhizosphere of intercropped species. *Plant Physiol.* 156, 1078–1086. doi: 10.1104/pp.111.175331

Jensen, E. S., Carlsson, G., and Hauggaard-Nielsen, H. (2020). Intercropping of grain legumes and cereals improves the use of soil N resources and reduces the requirement for synthetic fertilizer N: a global-scale analysis. *Agron. Sustain. Dev.* 40:5. doi: 10.1007/s13593-020-0607-x

Jones, D. B. (1931). *Factors for converting percentages of nitrogen in foods and feeds into percentages of proteins, vol. 183.* Washington, DC: US Department of Agriculture.

Koskey, G., Leoni, F., Carlesi, S., Avio, L., and Bàrberi, P. (2022). Exploiting plant functional diversity in durum wheat–lentil relay intercropping to stabilize crop yields under contrasting climatic conditions. *Agronomy* 12, 210–224. doi: 10.3390/agronomy12010210

Lai, H., Gao, F., Hao, S., Zheng, P., Li, Y., and Yao, H. (2022). Nitrogen distribution and soil microbial community characteristics in a legume–cereal intercropping system: a review. *Agronomy* 12:1900. doi: 10.3390/agronomy12081900

Li, L., Sun, J., Zhang, F., Guo, T., Bao, X., Smith, F. A., et al. (2006). Root distribution and interactions between intercropped species. *Oecologia* 147, 280–290.

Lithourgidis, A. S., Vasilakoglou, I. B., Dhima, K. V., Dordas, C. A., and Yiakoulaki, M. D. (2006). Forage yield and quality of common vetch mixtures with oat and triticale in two seeding ratios. *Field Crop Res.* 99, 106–113. doi: 10.1016/j.fcr.2006.03.008

Lithourgidis, A. S., Vlachostergios, D. N., Dordas, C. A., and Damalas, C. A. (2011). Dry matter yield, nitrogen content, and competition in pea–cereal intercropping systems. *Eur. J. Agron.* 34, 287–294. doi: 10.1016/j.eja.2011.02.007

Liu, X., Jiao, Y., Zhao, X., Yu, X., Zhang, Q., Li, S., et al. (2023). Root architecture of forage species varies with intercropping combinations. *Agronomy* 13, 2223–2234. doi: 10.3390/agronomy13092223

Liu, H., Struik, P. C., Zhang, Y., Jing, J., and Stomph, T.-J. (2023). Forage quality in cereal/legume intercropping: a meta-analysis. *Field Crop Res.* 304:109174. doi: 10.1016/j.fcr.2023.109174

Luo, S., Lingling, Y., Liu, Y., Zhang, Y., Yang, W., Li, Z., et al. (2016). Effects of reduced nitrogen input on productivity and N 2 O emissions in a sugarcane/soybean intercropping system. *Eur. J. Agron.* 81, 78–85. doi: 10.1016/j.eja.2016.09.002

Maitra, S., Hossain, A., Brestic, M., Skalicky, M., Ondrisik, P., Gitari, H., et al. (2021). Intercropping—a low input agricultural strategy for food and environmental security. *Agronomy* 11, 343–370. doi: 10.3390/agronomy11020343

Malézieux, E., Crozat, Y., Dupraz, C., Laurans, M., Makowski, D., Ozier-Lafontaine, H., et al. (2009). Mixing plant species in cropping systems: concepts, tools and models: a review. *Agron. Sustain. Dev.*, 29, 43–62. doi: 10.1007/978-90-481-2666-8_22

McGilchrist, C. A. (1965). Analysis of competition experiments. *Biometrics* 21, 975–985. doi: 10.2307/2528258

Midya, A., Bhattacharjee, K., Ghose, S. S., and Banik, P. (2005). Deferred seeding of blackgram (*Phaseolus mungo* L.) in rice (*Oryza sativa* L.) field on yield advantages and smothering of weeds. *J. Agron. Crop Sci.* 191, 195–201. doi: 10.1111/j.1439-037X.2005.00157.x

Nassab, A. D., Mohammadi, T. A., and Kaul, H.-P. (2011). Competition and yield in intercrops of maize and sunflower for biogas. *Ind. Crop. Prod.* 34, 1203–1211. doi: 10.1016/j.indcrop.2011.04.015

Neugschwandtner, R. W., and Kaul, H.-P. (2014). Sowing ratio and N fertilization affect yield and yield components of oat and pea in intercrops. *Field Crop Res.* 155, 159–163. doi: 10.1016/j.fcr.2013.09.010

Neugschwandtner, R. W., and Kaul, H.-P. (2016). Concentrations and uptake of macronutrients by oat and pea in intercrops in response to N fertilization and sowing ratio. *Arch. Agron. Soil Sci.* 62, 1236–1249. doi: 10.1080/03650340.2016.1147648

Ofori, F., and Stern, W. R. (1987). Cereal–legume intercropping systems. *Adv. Agron.* 41, 41–90. doi: 10.1016/S0065-2113(08)60802-0

Osiru, D. S. O., and Willey, R. W. (1972). Studies on mixtures of dwarf sorghum and beans (*Phaseolus vulgaris*) with particular reference to plant population. *J. Agric. Sci.* 79, 531–540. doi: 10.1017/S0021859600025910

Osman, A. E., and Nersoyan, N. J. E. A. (1986). Effect of the proportion of species on the yield and quality of forage mixtures, and on the yield of barley in the following year. *Exp. Agric.* 22, 345–351. doi: 10.1017/S0014479700014599

Pinto, P., Cartoni-Casamitjana, S., Cureton, C., Stevens, A. W., Stoltenberg, D. E., Zimbric, J., et al. (2022). Intercropping legumes and intermediate wheatgrass increases forage yield, nutritive value, and profitability without reducing grain yields. *Front. Sustainable Food Syst.* 6, 13–29. doi: 10.3389/fsufs.2022.977841

Qu, J., Li, L., Bai, J., Chen, G., Zhang, Y., and Chang, Q. (2022). Influence of different proportion intercropping on oat and common vetch yields and nutritional composition at different growth stages. *Agronomy* 12:1908. doi: 10.3390/agronomy12081908

Raza, M. A., Zhiqi, W., Yasin, H. S., Gul, H., Qin, R., Rehman, S. U., et al. (2023). Effect of crop combination on yield performance, nutrient uptake, and land use advantage of cereal/legume intercropping systems. *Field Crop Res.* 304:109144. doi: 10.1016/j.fcr.2023.109144

Reddy, M. N., and Chetty, C. R. (1984). Staple land equivalent ratio for assessing yield advantage from intercropping. *Exp. Agric.* 20, 171–177. doi: 10.1017/S0014479700003288

Scalise, A., Tortorella, D., Pristeri, A., Petrovičová, B., Gelsomino, A., Lindström, K., et al. (2015). Legume–barley intercropping stimulates soil N supply and crop yield in the succeeding durum wheat in a rotation under rainfed conditions. *Soil Biol. Biochem.* 89, 150–161. doi: 10.1016/j.soilbio.2015.07.003

Shen, Y., Sui, P., Huang, J., Wang, D., Whalen, J. K., and Chen, Y. (2018). Greenhouse gas emissions from soil under maize–soybean intercrop in the North China plain. *Nutr. Cycl. Agroecosyst.* 110, 451–465. doi: 10.1007/s10705-018-9908-8

Stomph, T. J., Dordas, C., Baranger, A., de Rijk, J., Dong, B., Evers, J., et al. (2020). Designing intercrops for high yield, yield stability and efficient use of resources: are there principles? *Adv. Agron.* 160, 1–50. doi: 10.1016/bs.agron.2019.10.002

Takim, F. O. (2012). Advantages of maize-cowpea intercropping over sole cropping through competition indices. *J. Agric. Biodivers. Res.* 1, 53–59.

Tamburini, G., Bommarco, R., Wanger, T. C., Kremen, C., Van der Heijden, M. G. A., Liebman, M., et al. (2020). Agricultural diversification promotes multiple ecosystem services without compromising yield. *Sci Adv.* 6:eaba1715. doi: 10.1126/sciadv.aba1715

Wang, S., Chen, G., Yang, Y., Zeng, Z., Yuegao, H., and Zang, H. (2021). Sowing ratio determines forage yields and economic benefits of oat and common vetch intercropping. *Agron. J.* 113, 2607–2617. doi: 10.1002/agj2.20647

Willey, R. W., and Rao, M. R. (1980). A competitive ratio for quantifying competition between intercrops. *Exp. Agric.* 16, 117–125. doi: 10.1017/S0014479700010802

Xu, R., Zhao, H., Liu, G., You, Y., Ma, L., Liu, N., et al. (2021). Effects of nitrogen and maize plant density on forage yield and nitrogen uptake in an alfalfa–silage maize relay intercropping system in the North China plain. *Field Crop Res.* 263:108068. doi: 10.1016/j.fcr.2021.108068

Yolcu, H., Polat, M., and Aksakal, V. (2009). Morphologic, yield and quality parameters of some annual forages as sole crops and intercropping mixtures in dry conditions for livestock. *J. Food Agric. Environ.* 7, 594–599,

Zhang, Y., Liu, J., Zhang, J., Liu, H., Liu, S., Zhai, L., et al. (2015). Row ratios of intercropping maize and soybean can affect agronomic efficiency of the system and subsequent wheat. *PLoS One* 10:e0129245. doi: 10.1371/journal.pone.0129245

Zhang, Q., Tian, L., Liu, Y., Jiang, H., and Shen, Y. (2015). Yield and water use efficiency of annual forage crops on the loess plateau. *Arid Zone Studies* 32:7,

Zhu, S.-G., Cheng, Z.-G., Yin, H.-H., Zhou, R., Yang, Y.-M., Wang, J., et al. (2022). Transition in plant–plant facilitation in response to soil water and phosphorus availability in a legume-cereal intercropping system. *BMC Plant Biol.* 22:311. doi: 10.1186/s12870-022-03706-6

Zixi, Z., Stewart, B. A., and Xiangjun, F. (1994). Double cropping wheat and corn in a sub-humid region of China. *Field Crop Res.* 36, 175–183. doi: 10.1016/0378-4290(94)90109-0