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Positive legacy effects of grass-legume mixture leys on phosphorus uptake and yield of maize weaken over the growing season

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ABSTRACT

Context or problem: Soil legacy effects of plants are important in facilitating or inhibiting subsequent crop growth. However, in a pasture-crop rotation system, the impact of legume-grass mixtures and fertilization management via legacy effects on the follow-on crop growth is not clear.

Objective or research question: It is important to investigate how soil effects produced by different plant mixtures and fertilization management influence the growth of subsequent crops to design a sustainable cropping system. **Methods:** In the field, we conditioned 144 pasture plots with different ratios of legumes (L) and grasses (G) (L:G ratios of 3:7, 4:6, 5:5, 6:4, and 7:3), and the reference monocultures and 4 levels of phosphorus fertilizer (0, 9, 18, or 27 kg P ha⁻¹) for 5 years and sowed maize after removing the pasture to explore the legacy effects of legume-grass ley pastures on subsequent crop production.

Results: Our results showed that a proportion of 30% of legumes in the legume-grass fully mixed intercropping gave the highest positive legacy effect among the mixtures on maize growth at the jointing stage (V6) and big trumpet stage (V12), and this effect was comparable with the effect of a legume monoculture for the yield and P uptake of maize. The phosphorus fertilizer treatments did not directly affect subsequent crop growth. Path analysis showed that the legacy effects of pastures depended on the growth stage of maize and gradually weakened. Soil NH₄⁺-N and AM fungi affected phosphorus uptake of maize at the V6 stage, while at the V12 stage, only the abundance of AM fungi was associated with P uptake. No direct effect was observed at the grouting stage (R4).

Conclusions: We conclude that the seeding ratio of a previous plant community has significant effects on the soil biotic and abiotic properties that impact the performance of the subsequent crop and that legacy effects weakened during maize growth.

Significance: Our results reveal that the crop performance depends on legacies from previous cropping systems and their growth stage. Legacy effects on the subsequent crop were affected by previous different functional group seeding ratios, a finding that is extremely useful for practical management and design of pasture-crop rotation system.

1. Introduction

To meet the food needs of the world's rapidly growing population, modern intensively managed cropping systems often invest large amounts of chemical fertilizers to increase productivity (Sebilio et al., 2013) causing severe negative environmental impacts such as groundwater pollution (Ledgard et al., 2009) and greenhouse gas emissions (Shcherbak et al., 2014). Grain production has doubled in the past 50 years (Tilman et al., 2002) but the cropping systems became

increasingly simplified and intensified (Aguilar et al., 2015; Barzman et al., 2015). Cropping systems diversification is considered to be an important way for the sustainable development of cropping systems by enhancing complex interactions and synergies that optimize ecosystem functions and processes (Martin et al., 2020). Previous studies have used multi-variety mixtures, mixed cropping or intercropping, or introduction of cover crops and pasture in the crop rotation to enhance the diversification of cropping systems and increase production (Schipanski et al., 2014). Well-managed ley pasture could provide beneficial services

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including improved soil structure and increased forage and livestock production for the pasture-crop rotation system (Martin et al., 2020).

Legume-grass mixture is considered to be a good option in a ley pasture system as it includes two complementary functional groups whereby legumes can fix nitrogen (N) while grasses can utilize that N and enhance productivity (Martin et al., 2020). Legume-grass mixture leys with 30–70% legume are effective in fixing enough N to gain maximum yield (Nyfeler et al., 2011). N rhizodeposition of legumes accounts for more than 70% of total plant N, and changes the N transfer belowground by root or root exudate decomposition to benefit non-legumes (Fustec et al., 2010). However, the optimal legume-grass seeding ratio in ley pastures that could benefit the subsequent crop most is largely unknown. A recent study showed a positive legume-legacy effect of a legume-grass mixture with 50% legume on the subsequent grass crop (Fox et al., 2019). Soil legacies may vary among pastures with different plant species composition and abundance. A previous study showed that different ratios of grasses and herbs resulted in different abundances of soil fungal pathogens that negatively affected subsequent plants of the same functional plant type. In plant communities, particularly those with a higher proportion of grasses, there tends to be an accumulation of fungal pathogens associated with grasses. This accumulation contributes to a decrease in grass abundance in succeeding communities (Heinen et al., 2020). Thus, it is important to investigate how soil legacy effects produced by different plant mixtures influence the growth of the subsequent crops to design sustainable cropping systems.

Besides soil biotic properties, soil abiotic properties such as soil nutrient availability also play an important role in the creation of a legacy effect on crop performance which can influence the nutrient uptake of subsequent crops (in 't Zandt et al., 2020). Fertilizer management can directly influence nutrient availability and indirectly alter soil microbiome composition such as increasing the abundance of plant growth-promoting rhizobacteria (PGPR) to create positive legacies for subsequent crops (Chen et al., 2019b; Jing et al., 2022). The positive N-legacy of legumes in mixed grassland has been extensively validated (Fox et al., 2019). However, phosphorus (P) is often limiting in agricultural soils (Sattari et al., 2012) and plays a fundamental role in plant growth and development (Raghothama and Karthikeyan, 2005; Condon et al., 2013). Continuously intensive fertilizer input and the high sorption capacity of phosphorus lead to residual phosphorus accumulation in the soil (Condon et al., 2013). Legacy-P stocks in agricultural soils may be sufficient to sustain global crop yields for about 100 years without yield declines when P fertilizer is withheld (Zhu et al., 2018). Microorganisms generate extracellular enzymes to fulfill their elemental needs and address stoichiometric imbalances in resources (Mooshammer et al., 2014). A limitation in phosphorus was observed to enhance phosphatase activity, thereby expediting soil phosphorus cycling (Marklein and Houlton, 2012). However, how soil legacies of different legume-grass mixtures with phosphorus fertilizer application in the previous stage affect the establishment of subsequent crops and their nutrient acquisition remains largely unknown.

A previous study found that legume-grass fully mixed intercropping especially in a legume (L) to grass (G) seeding ratio of 3:7 (L:G 3:7) can create overyielding and transgressive overyielding than other mixtures and monocultures (Bi et al., 2019). Whether the most productive legume-grass fully mixed intercropping could benefit the subsequent crop (in our case forage maize, *Zea mays* L.) is not known. In this study, we investigated the legacy effects of legume-grass fully mixed intercropping with different legume-grass seeding ratios and phosphorus fertilizer application rates (P levels) on maize. We hypothesized that: 1) More N is made available by the previous legume than by the grass component of the ley. 2) The composition of the conditioning plant communities creates different microbial soil legacies, and specifically different levels of accumulation of beneficial microorganisms such as arbuscular mycorrhizal fungi (AMF). 3) Increased extracellular enzyme activities by species mixtures than monocultures have a positive legacy

effect through promoting nutrient uptake in subsequent crops.

2. Materials and methods

2.1. Experimental set-up

A field experiment was conducted at the Station of the China Forage and Grass Research System in Yinchuan, Ningxia (38° 33'N, 106° 3'E, 1348 m a.s.l., mean annual temperature: 10.6 °C, mean annual precipitation: 232 mm). The monthly rainfall and temperature during the growing season in 2021 are shown in Fig. S1. The soil was identified as sierozem soil in the Chinese soil classification, equivalent to aridisols in the USDA Soil Taxonomy (State Soil Survey Service of China 1998; Soil Survey Staff of USDA 2006) (Table S1).

A ley pasture system was established over 5 years (2016–2020) using a split-plot design with four species (Appendix 1): two legumes, alfalfa (*Medicago sativa*) and sainfoin (*Onobrychis vicifolia*), and two grasses, orchardgrass (*Dactylus glomerata*) and tall fescue (*Festuca arundinacea*) (Fig. 1) (Bi et al., 2019). There were nine treatments in the main plots including different legume-grass seeding ratios (L:G ratios) of 3:7, 4:6, 5:5, 6:4, and 7:3, and the four monocultures of each species (Table S2). The subplots were treated with phosphorus fertilizer (single superphosphate) including four levels (0, 9, 18, or 27 kg P ha⁻¹) (hereafter 'P levels'). Each treatment had four replicates and in total there were 144 plots with the area of each subplot being 2.0 m × 3.0 m (Bi et al., 2019). The pasture was ploughed on May 9, 2021.

After the pasture stage was completed, we removed the aboveground parts of the pasture by hand and rotovated (min-tilled) the top 5 cm with a power harrow to prepare a seedbed before sowing the maize and maize was sown on June 6, 2021. The planting density was 82,500 plants ha⁻¹ with a within-row spacing of 24 cm and 50 cm between rows. N fertilizer at 108 kg ha⁻¹ as urea and K fertilizer at 85 kg ha⁻¹ as potassium sulfate were applied 50 days after sowing (DAS) to ensure normal maize growth. In order to investigate the legacy effect of previous ley pasture and P fertilizer application on subsequent maize growth, No P fertilizer was applied during the maize growing season.

2.2. Plant and soil sampling

Before the maize was planted, four soil samples were taken from each plot using a 6-cm auger to a depth of 15 cm. The samples from each plot were thoroughly homogenized and then sieved over a 2-mm sieve. Soil samples were transported to the laboratory using an icebox. Each sample was divided into three subsamples which were allocated to the following procedures and analyses: 1) stored at -20 °C for soil water content (SWC), ammonium nitrogen (NH₄⁺), nitrate nitrogen (NO₃⁻), dissolved organic carbon (DOC), Total dissolved nitrogen (TDN), microbial biomass carbon (MBC), and microbial biomass nitrogen (MBN); 2) air-dried soil for soil pH, total carbon (TC), total nitrogen (TN), and extractable phosphate (Olsen-P); and 3) stored at -80 °C for extracellular enzyme assays and DNA extraction.

Maize was harvested three times during 2021 (June 30th, July 21st, and September 6th) at the jointing stage (V6; 50 DAS), big trumpet stage (V12; 71 DAS) and grouting stage (R4; 116 DAS) respectively. In each plot, aboveground biomass was harvested in a quadrat of 0.25 m² (6 plants) and oven-dried at 65 °C to constant weight and stored for further chemical analyses after being finely ground.

The dried plant materials were wet-digested in a mixture of HNO₃ and HClO₄ (2:1, v/v). Plant P concentrations were determined by inductively coupled plasma (ICP-OES) (AvioTM 500; PerkinElmer, Waltham, USA) (Daly and Fenelon, 2017). We then calculated P content using the following equation:

$$\text{Shoot/grain P content} = A \times B$$

where A is the aboveground biomass or grain yield, B is the P

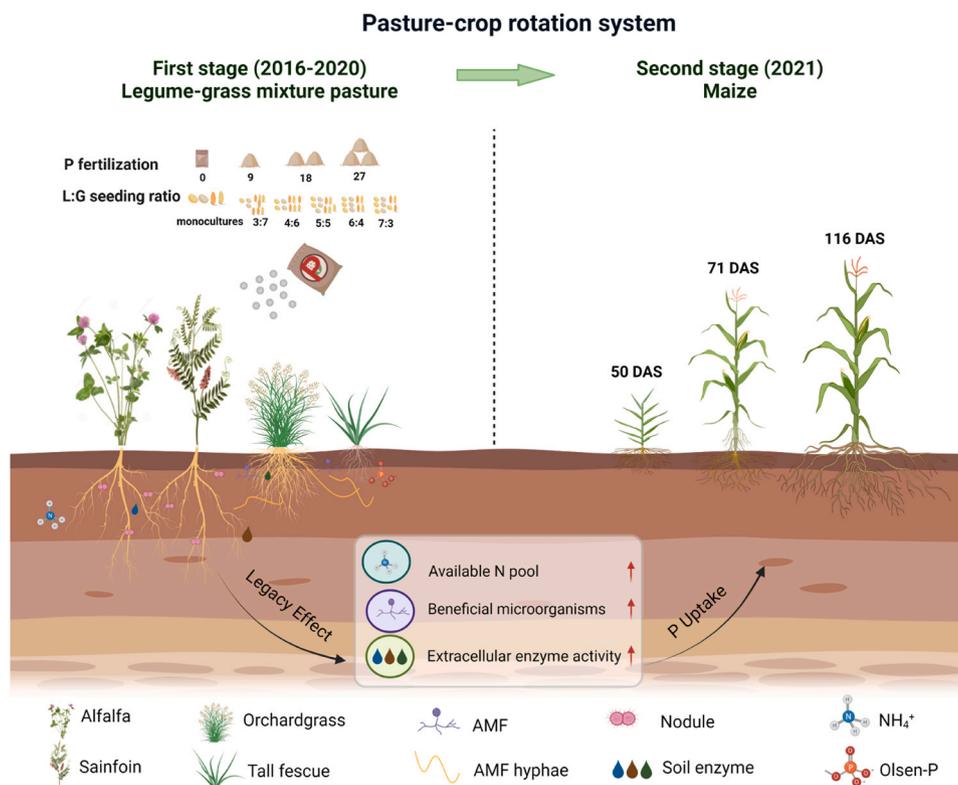


Fig. 1. Scheme of the experimental design. (1) The first stage: A 5-year-mixed pasture experiment used a split-plot design with four species: two legume species, alfalfa (*M. sativa*) and sainfoin (*O. viciifolia*), and two grass species, orchardgrass (*D. glomerata*) and tall fescue (*F. arundinacea*) (2016–2020). The main plots were treated with legume-grass seeding ratios (L:G ratios) of 3:7, 4:6, 5:5, 6:4, and 7:3, and four monocultures of each species. The subplots were treated with phosphorus fertilizer (0, 9, 18, or 27 kg P ha⁻¹). (2) The second stage: Maize was grown after removal of the pasture and sampled at 50, 71, 116 DAS to investigate the legacy effect on maize at different growth stages. We hypothesized that: 1) More N is made available by the previous legume than by the grass component of the ley. 2) The composition of the conditioning plant communities creates different microbial soil legacies, and specifically different levels of accumulation of beneficial microorganisms such as arbuscular mycorrhizal fungi (AMF). 3) Increased extracellular enzyme activities by species mixtures than monocultures have a positive legacy effect through promoting nutrient uptake in subsequent crops.

concentration of shoot or grain.

Soil pH was determined in 1:2.5 soil-water suspensions with a pH meter (FE20-FiveEasy™, Mettler Toledo, Germany). Soil was passed through a 0.15-mm sieve for analyses of TC, TN, MBC, MBN, DOC and TDN. TC and TN were analyzed using an elemental auto-analyzer (Vario MAX CN; Elementar, Hanau, Germany). The chloroform fumigation extraction approach was applied to estimate MBC, MBN, DOC, and TDN by using a TOC/TN analyzer (Multi N/C 3100, Analytik Jena, Jena, Germany) (Vance et al., 1987). Olsen-P was determined by shaking 2.5 g of dry soil for 30 min at 20 °C in 50 mL of a 0.5 M NaHCO₃ solution (pH 8.5) as described by Bao (Bao et al., 1988) and analyzed with an ultraviolet-visible spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan).

2.3. DNA extraction and metabarcoding

DNA was extracted using the Fast DNA SPIN Kit (MP Biomedicals, CA, USA) for soil samples. The concentration and purity were measured using NanoDrop One (Thermo Fisher Scientific, MA, USA). The bacterial 16 S rRNA genes were amplified by the primer pairs 515 F (5'-GTGY-CAGCMGCCGCGGTAA-3') and 926 R (5'-CCGYCAATTYMTT-TRAGTTT-3'). Samples with bright main strips (e.g., 16 S V4: 290–310 bp/16 S V4V5: 400–450 bp) were used for further experimentation. The fungal internal transcribed spacer region of the rRNA operon was amplified by PCR using primers ITS3 (5'-GCA TCG ATG AAG AAC GCA GC-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (Tedersoo and Bahram, 2014). The size and concentration of the PCR product were detected by 1% agarose gel electrophoresis. PCR products

were mixed in equidensity ratios according to the GeneTools Analysis Software (Version 4.03.05.0, SynGene). Then, a mixture of PCR products was purified with E.Z.N.A. Gel Extraction Kit (Omega, USA). The purified amplicons were aggregated in equimolar ratios and paired-end sequenced on an Illumina Sequencer MiSeq platform (Illumina, San Diego, USA) following Guangdong Magigene Biotechnology Co., Ltd. (Guangzhou, China).

2.4. Soil enzyme activity analyses

The activities of β -D-glucoside glycohydrolase (BG), β -1,4-N-acetylglucosaminidase (NAG), L-leucine aminopeptidase (LAP) and acid phosphatase (APase) were measured using fluorogenically labeled substrates (Table S3). Soil suspensions were prepared by adding 2.75 g fresh soil (< 2 mm) into 91 mL sodium acetate buffer (pH 6.9) and shaken at 200 rpm for 30 min. Soil suspensions (i.e., 800 μ L) were added into black 96-well microplates, along with 250 μ L of 100 μ M fluorescent substrate for each target enzyme. Standard curves were determined by mixing 800 μ L of soil suspensions and 250 μ L of 4-methylumbelliferone standard substance with 0, 2.5, 5, 10, 25, 50, 75 and 100 μ M. Microplates, along with mixed solutions, were incubated at 25 °C for 4 h and subsequent reactions were terminated by adding 30 μ L of 1.0 M NaOH. Fluorescence intensities were measured using a microplate fluorimeter with 365 nm excitation and 450 nm emission filters. Activities of four hydrolytic enzymes were expressed as μ mol g⁻¹ dry soil h⁻¹.

2.5. Quantification of microbial nutrient limitation

The following methods were used to quantify microbial resource limitation. The mean ratio for C:N:P of microbes in acquiring enzyme activities is 1:1:1 (Sinsabaugh et al., 2009). The underlying principle of assessing this ratio is: in cases where microorganisms experience a deficiency in carbon (C), nitrogen (N), or phosphorus (P), they release enzymes to acquire the limited nutrients. This can result in a departure from the balanced 1:1:1 ratio of C:N:P enzyme activity (Heuck et al., 2018). The scatter plot of (LAP + NAG)/APase versus BG/(LAP + NAG) reveals four distinct regions within the plot. These regions illustrate scenarios of co-limitation by C and P, co-limitation by C and N, P limitation, and N limitation, respectively (Hill et al., 2012; Chen et al., 2019a).

2.6. Statistical analysis

The effects of the legume-grass mixture (L), phosphorus addition (P) and their interactions on aboveground biomass and grain yield, soil properties, extracellular enzyme activities and soil microbial α -diversity were analyzed using two-way analysis of variance (ANOVA). The homogeneity of variance and the normality of residuals were tested by the Levene test and Shapiro-Wilk test, respectively. One-way ANOVAs with a post-hoc Duncan's multiple range tests were performed to examine differences in soil physicochemical properties, soil extracellular enzyme activities and soil microbial α -diversity among different treatments. ANOVAs and post-hoc Duncan's multiple range tests were performed with $\alpha = 0.05$ using SPSS v. 20.0 (IBM SPSS Statistics for Windows, IBM Corp, Armonk, NY, USA).

Analysis of principal co-ordinates (PCoA) was used to explore

differences among legume-grass mixture treatments in soil microbial community composition based on weighted Bray Curtis distance. The similarity of the community composition was evaluated based on a Bray Curtis distance matrix by analysis of similarities (ANOSIM) using the vegan package of the R software. The PERMANOVA analysis and two-way ANOVA tests were employed to evaluate the effects of legume-grass mixture and P levels on community composition. Microbial correlation networks of soil bacteria and fungi were visualized using the Cytoscape (3.9.1) platforms (Shannon et al., 2003). Only the relative abundance of the operational taxonomic units (OTUs) $\geq 0.05\%$ and robust Spearman correlation scores ($|r| > 0.4$ and FDR-adjusted $p < 0.05$) were selected for network analysis by the "microeco" package in R (Liu et al., 2021). Since these topological parameters (denoting network complexity) are closely related, link density (Wagg et al., 2019) was used to denote the network complexity index. The relationship between soil physicochemical properties, soil fungal and extracellular enzyme activities, fungal network complexity and P uptake were assessed using Pearson's r correlation tests. A structural equation model (SEM) model was used to identify factors that effectively explained changes in P contents of maize and identify possible pathways controlling plant P yield under different treatments. The model was constructed using the lavaan package in R version 4.1.1 (Team 2017).

3. Results

3.1. Legacy effect of legume-grass mixtures on yield and P uptake of subsequent maize

The legacy effect of different legume-grass mixtures on aboveground biomass and grain yield of maize depended on different sowing ratios of

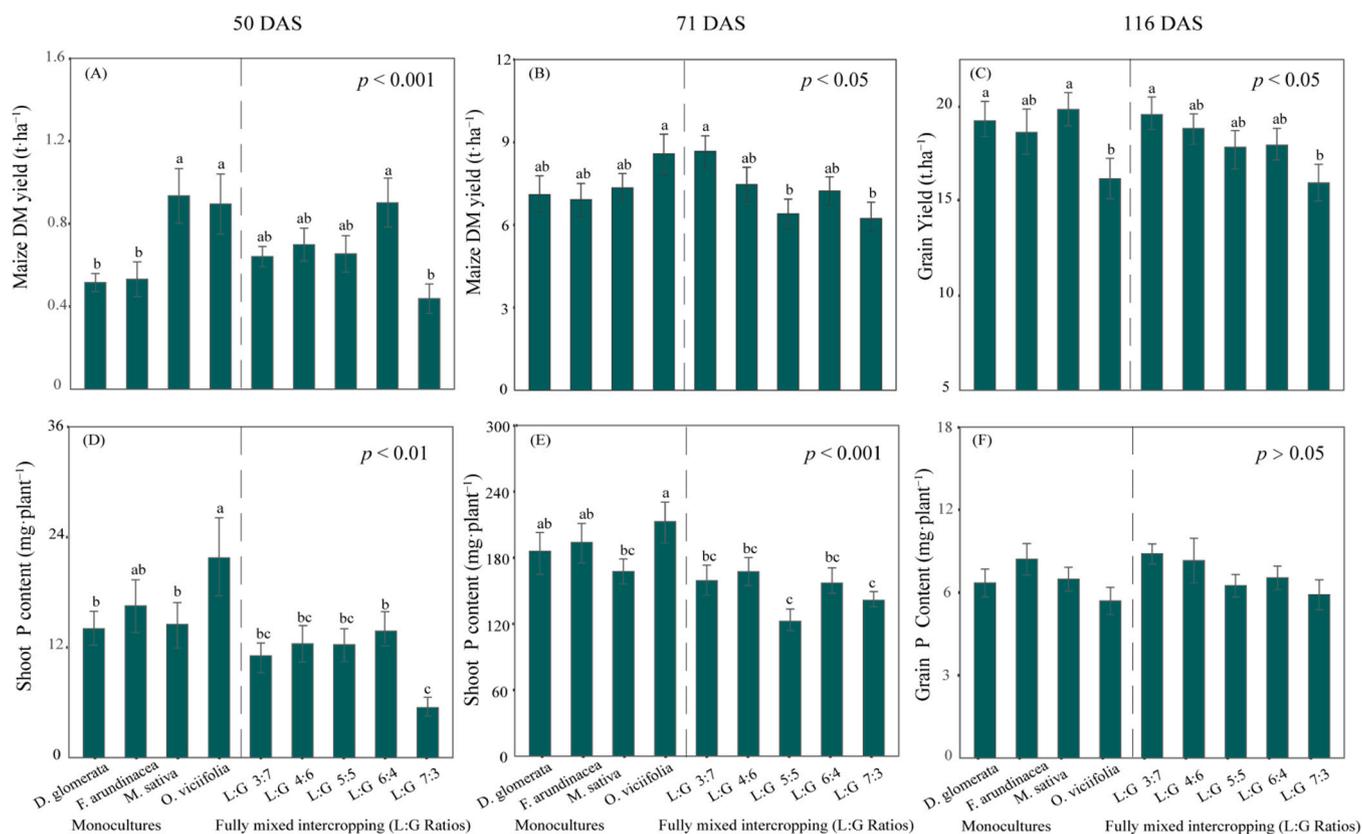


Fig. 2. The aboveground biomass in the jointing stage (V6; A), big trumpet period (V12; B) and grain yield (C), P uptake in the jointing stage (V6; D), big trumpet stage (V12; E) and grain P uptake (F) in different monocultures and fully mixed legume-grass mixtures obtained in a subsequent crop of maize across mean P rates. Values followed by the same lowercase letters are not significantly different among different cropping combinations at the 5% level by a Duncan's range test. L:G is the legume-grass seeding ratio.

legume and grass and the growth stage of maize. At 50 DAS, the aboveground biomass yield of maize under legume monocultures and mixtures with a legume proportion of 60% were significantly higher than that of grass monocultures and treatments with 70% legume (hereafter 'L:G 7:3') ($p \leq 0.001$, Fig. 2 A). There were no significant differences in yield of maize between treatments with a legume proportion of 30% (hereafter 'L:G 3:7') and L:G 7:3 at 50 DAS ($p > 0.05$, Fig. 2A). However, L:G 3:7 had the largest positive legacy effect among all legume-grass fully mixed intercropping treatments, especially significantly higher compared with the mixture of L:G 7:3 on biomass and grain yield of maize at 71 and 116 DAS ($p \leq 0.05$, Table 1, Figs. 2B and 2C). This resulted in 2.44 t ha⁻¹ or + 39% more aboveground biomass yield of maize at the big trumpet stage than for L:G 7:3 ($p \leq 0.05$, Table 1, Fig. 2 B). Aboveground biomass yield of maize after legume monocultures (average yield of two legume monocultures, hereafter 'Leg_{mono}') was 1.73 t ha⁻¹ or + 28% higher than L:G 7:3 at the big trumpet stage (71DAS, $p \leq 0.05$, Table 1). Except for *O. viciifolia* monoculture and L:G 3:7, we did not find any differences in maize yield among other monocultures and mixtures. Similarly, L:G 3:7 resulted in 5.78 t ha⁻¹ or + 33% more grain yield of maize than in L:G 7:3, while in Leg_{mono}, it was 6.22 t ha⁻¹ or + 35% more than in L:G 7:3 (116 DAS, $p \leq 0.05$, Table 1, Fig. 2 C). We did not find any differences of grain yield among other mixture treatments with L:G 3:7 or L:G 7:3. Importantly, no significant difference in aboveground biomass yield and grain yield was observed between L:G 3:7 and Leg_{mono} in any of the individual harvest.

P uptake of maize differed significantly with the legacies from different legume-grass mixtures in the jointing stage ($p \leq 0.01$, Fig. 2D) and the big trumpet stage ($p \leq 0.001$, Fig. 2 E). There was a declining trend in P uptake from 3:7–7:3 legume-grass fully mixed intercropping at big trumpet and grain stages. P content of maize after *O. viciifolia* monoculture was the highest among all monocultures at jointing stage and big trumpet stage. P content of maize in L:G 7:3 was much lower compared with other treatments and decreased by 12.5 mg P plant⁻¹, or -62.4% compared to *O. viciifolia* monoculture in the jointing stage ($p \leq 0.01$, Figs. 2D), and 42.6 mg P plant⁻¹, or -18.3% in the big trumpet stage ($p \leq 0.01$, Fig. 2E), respectively. No significant difference in grain P uptake of maize was observed. The difference between legume and grass monocultures in P uptake had also disappeared in the grain stage ($p > 0.05$, Fig. 2F).

3.2. Soil biotic and abiotic legacies with different legume-grass mixtures and phosphorus treatments

Soil biotic and abiotic legacies were mainly affected by different legume-grass mixtures rather than phosphorus treatments, and the two

Table 1

Effect of three stand types of the previous pasture indicating the legume legacy effect on the biomass yield of subsequent maize. Leg_{mono}: average of two legume monocultures, L:G 3:7: Legume-grass mixture with 30% legume seeding proportion. L:G 7:3: Legume-grass mixture with 70% legume seeding proportion. Given are predicted means (\pm SE) from regression models.

	Leg _{mono}		L:G 3:7	L:G 7:3
	(100%)		(30%)	(70%) ^a
Biomass / grain yield (t DM ha ⁻¹)			(t DM ha ⁻¹)	(t DM ha ⁻¹)
Jointing stage (V6)	0.92 \pm 0.28	NS ^b	0.64 \pm 0.10	0.44 \pm 0.14
Big trumpet stage (V12)	7.96 \pm 1.20	NS	8.67 \pm 1.13	6.23 \pm 1.01
Grain stage (R4)	23.77 \pm 2.83	NS	23.33 \pm 2.69	17.55 \pm 3.05
Legume legacy effect ^c (t DM ha ⁻¹)			(t DM ha ⁻¹)	(t DM ha ⁻¹)
Jointing stage (V6)			0.28	0.48
Big trumpet stage (V12)			0.71	1.73*
Grain stage (R4)			0.44	6.22*

^a Percentage of the previous pasture's legume seeding proportion

^b t-test of difference in DM yield between maize grown after L:G 3:7 and Leg_{mono}

^c Decrease in DM yield of maize grown after L:G 3:7 and L:G 7:3 as compared to maize grown after Leg_{mono}, * $p \leq 0.05$, NS $p > 0.05$

treatments did not interact (Table 2). Different legume-grass mixture treatments significantly affected the soil nutrient contents (NO₃⁻, NH₄⁺, TDN), microbial biomass (C, N), and the enzymatic activities (BG, LAP, NAG, APase) (Table 2). Only soil Olsen-P was both affected by previous P levels and legume-grass mixtures; P application rate with 27 kg P ha⁻¹ left more soil Olsen P than other P treatments. ($p \leq 0.001$, Fig. 3). In comparison to L:G 7:3, L:G 3:7 increased the soil legacy TDN (49.1%, $p \leq 0.01$), BG (146.1%, $p \leq 0.05$), LAP (72.7%, $p \leq 0.05$), APase (148.6%, $p \leq 0.05$); The *O. viciifolia* monoculture enhanced the contents of NO₃⁻ (30.4%, $p \leq 0.001$), NH₄⁺ (137%, $p \leq 0.001$), TDN (63.4%, $p \leq 0.01$), MBC (51.2%, $p \leq 0.001$), MBN (38.9%, $p \leq 0.001$), BG (168.1%, $p \leq 0.05$), LAP (53.4%, $p \leq 0.05$), NAG (231.5%, $p \leq 0.01$), APase (131.5%, $p \leq 0.05$) (Fig. S3).

3.3. Effects of legume-grass mixtures on microbial resource limitation

The three-dimensional scatter plot of enzymatic stoichiometry demonstrates that the average value of the natural logarithm of soil C:N:P acquisition enzyme ratio (ln (BG):ln (NAG + LAP):ln (APase)) deviated from the general average ratio of 1:1:1; The values were 1.21:1.60:1.49, 1.11:1.79:1.49, and 1.02:1.53:1.47 in the *O. viciifolia* monoculture, L:G 3:7, and L:G 7:3 treatments, respectively (Fig. 4A). Enzymatic stoichiometry could elucidate microbial resource limitation according to the approach reported (Cui et al., 2022a). The quadrant scatter plot of enzymatic stoichiometry demonstrated that all legume-grass mixtures were located in the P limited area (Fig. 4B), and different P levels did not show any legacy effect on the soil enzymatic activities (Table 2).

3.4. Effects of legume-grass mixture on microbial diversity and co-occurrence patterns

The α -diversity index and the community composition of bacteria were affected by different legume-grass mixtures ($p \leq 0.01$, Fig. S2), but no significant difference was observed among P levels. Two-way PERMANOVA revealed that soil bacterial β -diversity (community composition) was significantly affected by different legume-grass mixtures ($p \leq 0.001$, 11.2%, Fig. S2E). FUNGuild was adopted to annotate and screen the key ecological functions of fungal communities to further explore fungal activities for nutrient uptake in the following crop (Nguyen et al., 2016). The relative abundance of Arbuscular Mycorrhiza was higher in L:G 3:7 than in L:G 7:3 (Table S4), but statistically not significantly different.

Microbial network complexity in different legume-grass mixtures was analyzed using a robust correlation network analysis. The complexity-denoting network properties of fungal network complexity, i.e., numbers of nodes and links, the average path distance, connectedness, as well as linkage density among taxa, appeared to be higher in L:G 3:7 than in L:G 7:3 (Table S5). Here, fungal microbial network complexity was higher in L:G 3:7 than in L:G 7:3 (Fig. 5B).

3.5. Linking soil legacies with P uptake by maize

Path analysis explored the soil legacies from previously legume-grass fully mixed intercropping on the P uptake of maize (Fig. 6). There was a negative correlation between soil NH₄⁺-N, AMF, fungal network complexity and legume-grass fully mixed intercropping from ratio L:G 3:7 to L:G 7:3 among the three growth stages of maize. P content in the jointing stage of maize significantly correlated with soil NH₄⁺-N and AMF (Fig. 6A). For the big trumpet stage, only AMF has a significantly relationship with the P content (Fig. 6B). Grain P content was not impacted by the soil legacies (Fig. 6C).

4. Discussion

We found that ley pastures with different ratios of legume and grass created unique soil abiotic and biotic legacy effects via affecting AM

Table 2
The effect of different legume-grass mixtures on soil physicochemical properties, microbial biomass, and enzyme activities.

		SWC	TC	TN	NO ₃	NH ₄ ⁺	pH	DOC	TDN	MBC	MBN	BG	LAP	NAG	APase	
		%	g/kg	g/kg	mg/kg	mg/kg	(1:2.5 H ₂ O)	mg/kg	mg/kg	mg/kg	mg/kg	nmol·g ⁻¹ ·h ⁻¹				
Monocultures	D. glomerata	15.98 ±1.36b	35.71 ±0.98a	1.15 ±0.04a	8.78 ±0.85 cd	4.54 ±0.61a	8.69 ±0.03a	29.91 ±1.41a	6.01 ±0.59c	285.47 ±18.40a	19.73 ±1.36ab	84.95 ±12.45ab	269.14 ±37.28bc	31.47 ±7.03abc	971.49 ±177.51abc	
	F. arundinacea	16.68 ±1.05ab	37.03 ±1.02a	1.18 ±0.04a	6.92 ±0.54d	5.50 ±0.86a	8.72 ±0.02a	28.13 ±1.39a	6.36 ±0.86bc	304.09 ±18.47a	21.85 ±1.18a	64.55 ±8.53b	301.41 ±22.88bc	41.64 ±9.46ab	1421.72 ±303.92a	
	M. sativa	14.19 ±1.57c	35.03 ±0.95a	1.10 ±0.03a	12.19 ±0.45ab	2.87 ±0.42bc	8.66 ±0.01a	26.47 ±1.74a	7.75 ±0.83abc	210.82 ±13.69b	15.04 ±1.36 cd	139.74 ±15.06a	377.63 ±30.04ab	29.19 ±4.53abc	988.43 ±168.33abc	
	O. vicifolia	17.26 ±2.50a	37.04 ±0.65a	1.19 ±0.04a	13.07 ±0.89b	5.34 ±0.75a	8.70 ±0.02a	29.27 ±1.56a	9.33 ±0.79a	282.53 ±17.62a	17.27 ±1.03bc	145.48 ±22.70a	384.47 ±31.44ab	50.14 ±13.84a	1350.67 ±257.07ab	
Fully mixed intercropping (L: G Ratios)	L: G 3:7	13.96 ±1.70c	35.09 ±0.81a	1.11 ±0.02a	11.04 ±0.81ab	2.81 ±0.44bc	8.72 ±0.02a	32.96 ±2.51a	8.51 ±0.90ab	211.10 ±15.21b	10.26 ±0.89e	133.53 ±36.55a	433.09 ±64.10a	17.46 ±2.68c	1450.47 ±180.52a	
	L: G 4:6	14.13 ±1.40c	35.58 ±0.87a	1.16 ±0.03a	11.87 ±0.78ab	2.72 ±0.33bc	8.68 ±0.01a	27.40 ±1.66a	6.59 ±0.45bc	210.98 ±11.70b	15.84 ±1.44bcd	129.53 ±19.55a	308.41 ±28.66abc	28.88 ±7.31abc	866.84 ±151.32abc	
	L: G 5:5	14.15 ±1.07c	36.62 ±0.76a	1.14 ±0.03a	12.14 ±0.73ab	4.29 ±0.56ab	8.66 ±0.03a	30.84 ±2.59a	7.72 ±0.82abc	211.79 ±16.92b	14.41 ±1.43 cd	92.54 ±14.31b	322.73 ±27.09abc	26.22 ±5.38bc	977.87 ±80.84abc	
	L: G 6:4	13.61 ±1.88c	34.50 ±0.58a	1.11 ±0.03a	11.10 ±0.40ab	2.70 ±0.34bc	8.72 ±0.02a	26.98 ±2.42a	5.51 ±0.63c	186.70 ±12.72b	14.41 ±1.30 cd	109.23 ±17.87ab	380.90 ±44.54ab	19.21 ±3.16bc	727.45 ±183.06bc	
	L: G 7:3	13.18 ±1.08c	34.22 ±0.53a	1.12 ±0.02a	10.02 ±0.53bc	2.25 ±0.15c	8.71 ±0.01a	27.08 ±1.18a	5.71 ±0.32c	186.82 ±13.71b	12.43 ±1.72de	54.26 ±10.17c	250.67 ±41.78c	15.12 ±2.12c	583.34 ±138.57c	
	ANOVA	Legume-grass mixture (L)	0	0.180	0.441	0.000	0.000	0.500	0.371	0.003	0.000	0.000	0.010	0.025	0.003	0.045
		P levels (P)	0.883	0.897	0.905	0.222	0.538	0.973	0.866	0.452	0.709	0.626	0.666	0.724	0.928	0.986
	L × P	0.055	0.997	0.854	0.917	0.323	0.993	1	0.964	0.833	0.619	0.997	0.896	0.999	1	

Note: Abbreviations: SWC: soil water content; TC: total carbon; TN: total nitrogen; NO₃: nitrate nitrogen; NH₄⁺: ammonium nitrogen; DOC: dissolved organic carbon; TDN: Total dissolved nitrogen; MBC: microbial biomass carbon; MBN: microbial biomass nitrogen; BG: β-glucosidase; LAP: L-leucine aminopeptidase; NAG: N-acetyl-glucosaminidase; APase: acid phosphatase. The data represent the mean ± S.E; n = 16. Different lowercase letters within a column indicate significant differences between different legume-grass mixtures at the 5% level by a Duncan's range test (vertical comparison). Values under ANOVA are the probabilities (p-values) of the source of variation. L:G is the legume-grass seeding ratio.

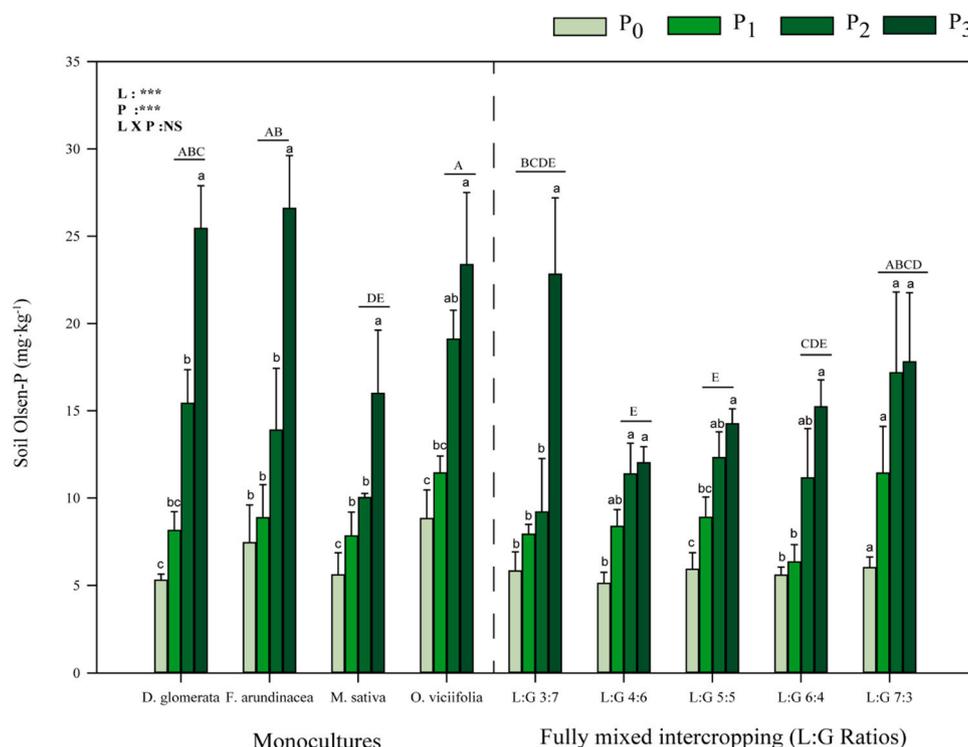


Fig. 3. The effect of different legume-grass mixtures on soil Olsen-P. L: legume-grass mixture, P: P level, NS: not significant. Different uppercase letters above bars and lowercase letters above bars indicate significant differences between different legume-grass mixture and P levels at the 5% level by a Duncan’s range test, respectively. L:G is the legume-grass seeding ratio, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, NS $p > 0.05$.

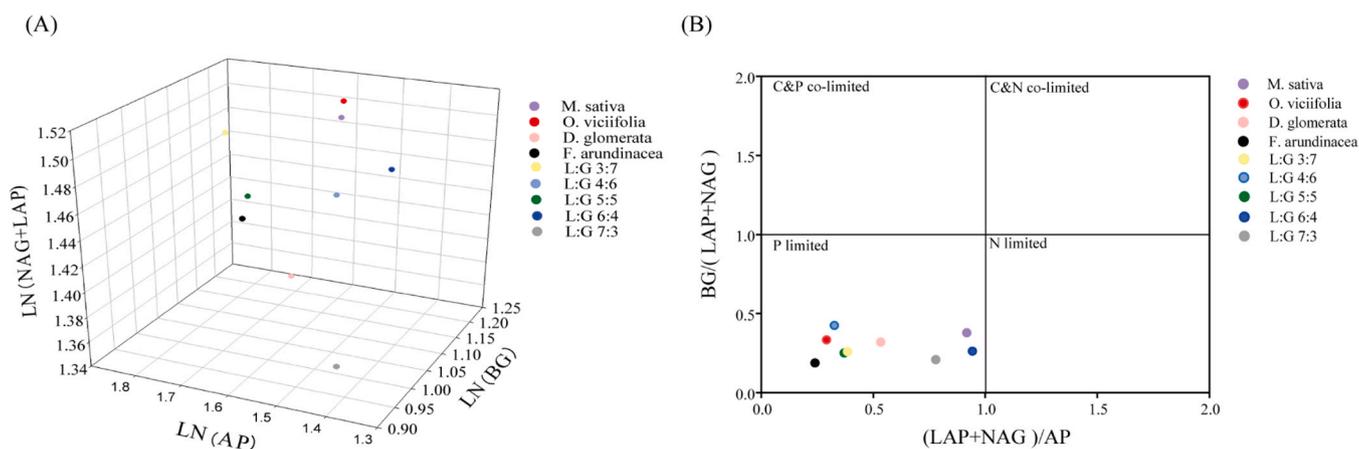


Fig. 4. Three-dimensional scatter plot of the ratio of C:N:P acquisition enzymes (A), quadrant scatter plot of enzymatic stoichiometry (B) under different legume-grass mixtures. L:G is the legume-grass seeding ratio.

fungal abundance and soil nutrient availability. Overall, the legacy effect of the ley pasture with an L:G ratio of 3:7 performed the best among all mixtures on maize growth. Path analysis was conducted to investigate the relationship between soil legacies and P uptake in different legume-grass mixtures and showed that the legacy effect was mainly through soil $\text{NH}_4^+\text{-N}$ and AM fungal relative abundance affecting the P uptake in the jointing stage. In the big trumpet stage, only AM fungal relative abundance correlated with the P uptake, but such effect was not found at the grain stage.

Soil abiotic legacy may affect nutrient uptake of subsequent plant by altering root density in different soil nutrient availability (in ‘t Zandt et al., 2020). Soil Olsen-P were significantly affected by previous P levels and different legume-grass mixture treatments, but the distinct difference did not affect the subsequent P uptake and growth of maize. Critical

soil Olsen-P value for maize growth is different in different regions. A previous study used three models (linear-linear, linear-plateau and Mitscherlich models) and obtained an average value of $15.3 \text{ mg P kg}^{-1}$ (Tang et al., 2009), and the value in Europe countries was on average 18 mg P kg^{-1} (Nawara et al., 2017). In the present study, we found that the soil Olsen-P under all legume-grass fully mixed intercropping was lower than that value (Fig. 3) indicating that the soil legacy available phosphorus was insufficient to meet the growth of maize. Compared to monocultures, the yields of mixtures over past five years were significantly higher, resulting in lower remaining P stock (Bi et al., 2019). Moreover, the enzymatic stoichiometry analysis showed that soil microorganisms were all in the P limited area under legume-grass mixtures that resulted in competition between microorganisms and plants for nutrients (Fig. 4B). Competition for phosphorus between soil

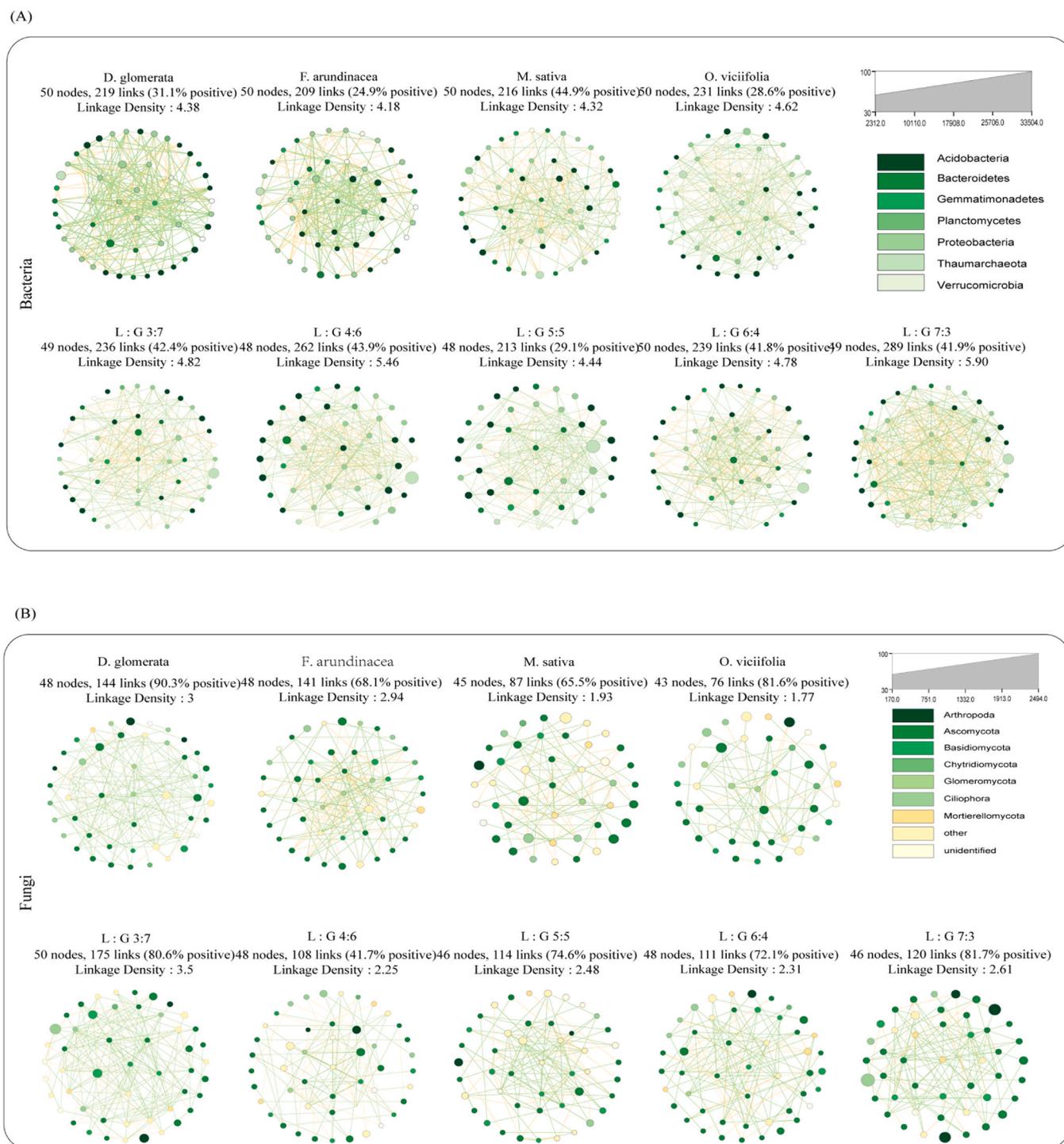


Fig. 5. Microbial correlation networks of soil bacteria (A) and fungi (B) under different legume-grass mixtures; L:G is the legume-grass seeding ratio. Nodes indicate individual operational taxonomic units (OTUs), while links represent a significant correlation between OTUs. The size of a node is proportional to the relative abundance of OTUs, and the width of each link is proportional to the Spearman's correlation coefficient. Green and orange links represent positive and negative relationships, respectively.

microorganisms and plant roots occurs mostly under low phosphorus conditions (Clausing and Polle, 2020; Bargaz et al., 2021). Thus, soil Olsen-P and APase showed significant differences but no relationship was found with maize P uptake (Fig. 6). To promote optimal plant growth in crop rotation systems, it is essential to improve the monitoring and supplementation of phosphorus nutrients. The path analysis showed that soil $\text{NH}_4^+\text{-N}$ impacted maize growth at jointing stage. Previous studies have demonstrated that $\text{NH}_4^+\text{-N}$ uptake by maize is important to

promote its growth, especially in the jointing stage (Garnett et al., 2015; George et al., 2016). When legume forage or legume-grass mixtures are grown, the effective N pool in the soil from plant residues available for uptake by the subsequent crop may increase (Walker et al., 2017; Fox et al., 2019). One possible explanation is that the yield of L:G 3:7 treatment was significantly higher than that in L:G 7:3 and other treatments over the last five years while L:G 3:7 also may have more below-ground biomass. This larger amount of plant residues may result

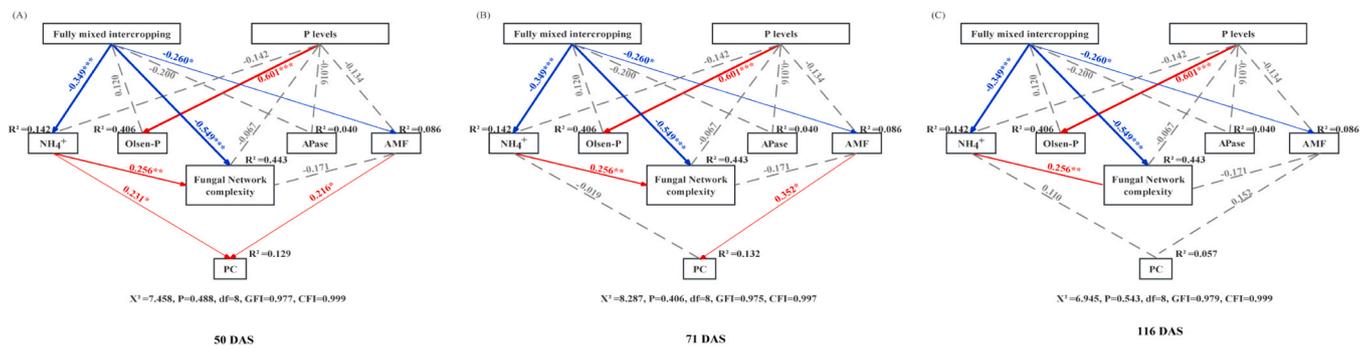


Fig. 6. Plant P contents in the jointing stage (V6; A), big trumpet stage (V12; B) and grain P content (R4; C) of maize are differentially affected by soil abiotic and biotic legacies. Path analyses show the relationships between soil NH_4^+ , Olsen-P, APase, AMF at the end of pasture and P contents in follow on crop of maize. PC: plant content. Path coefficients of SEM model are reflected by the numbers next to the single-headed arrows (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$), blue and red arrows indicate positive and negative effects, respectively.

in higher amounts of NH_4^+ -N left in the soil (Lüscher et al., 2014; Bi et al., 2019). Furthermore, the average ratio of soil C:N:P acquisition enzymes of legume-grass fully mixed intercropping was 1.00:1.45:1.32, which suggests that the microbes were limited by N and P (Fig. 4A). Similarly, a recent study found that the C:N:P acquisition enzyme ratio with 1.04:1.11:1.00 indicated microbial N limitations (Cui et al., 2022b). Meanwhile, the activity of soil β -glucosidase, L-leucine aminopeptidase and phosphatase, which are related to organic matter decomposition and the mineralization of N and P (Zhang et al., 2019), were higher in L:G 3:7 than in L:G 7:3 after the pasture stage was finished (Table 2), indicating the available N and P might have been higher in L:G 3:7. Thus, more soil available nutrients from L:G 3:7 can promote subsequent maize growth. Moreover, we also found that soil NH_4^+ -N was positively correlated with plant P uptake at the jointing stage. NH_4^+ -N uptake by maize may lead to a decrease in pH of the maize rhizosphere, which can enhance P mineralization and indirectly promote plant growth (Jing et al., 2012). However, we did not observe this positive relationship in the big trumpet period of maize, which may be due to the uptake of legacy N by the crop and the addition of nitrogen fertilizer at the jointing stage offsetting the residual NH_4^+ -N legacy effect. Despite soil NH_4^+ -N, our results also indicate that the legacy effect may be gradually weakened or even disappear as the subsequent plant grows.

In addition to the abiotic properties, soil biotic properties also contributed to the legacy effects on plant performance. We found that the soil bacterial community diversity and composition were significantly affected by legume-grass mixtures but were less effective than the fungal community in influencing growth of the subsequent crop (Fig. S2 and Table S4). This is consistent with previous findings that fungal communities play an important role in legacy effects by increasing the accumulation of grass-associated fungal pathogens or mutualists (Heinen et al., 2020). We found a negative relationship between the fungal network complexity and the mixed sowing ratio from L:G 3:7 to L:G 7:3 which showed that the fungal network in L:G 3:7 was more complexity than L:G 7:3 (Fig. 6 and Table S5). Moreover, the positive effect of plant diversity on AMF community diversity enables AMF derived from plant mixtures to promote plant growth (Van der Heijden et al., 1998; Hahl et al., 2020; Wang et al., 2021). AMF fungal acquires carbon and improves plant access to soil phosphorus and nitrogen (Johnson, 2010), thereby promoting plant nutrient uptake. We found that L:G 3:7 had higher AM fungal abundance than L:G 7:3 (Table S4) indicating previous pasture with 30% legume may be more effective in accumulating beneficial mutualists. P uptake was significantly positively correlated with the AM fungal abundance at the jointing and big trumpet stages of maize (Fig. 6, A and B), which indicates that the soil legacy AM fungal play a comparable role to improve the nitrogen and phosphorus acquisition of the plants as previous work using inoculation AMF species *Funneliformis mosseae* (Li et al., 2022). We therefore conclude that a properly designed pasture can increase the accumulation of mutualists

such as AMF and promote nutrient uptake in the subsequent crop.

5. Conclusion

Our study showed that the ratio between different legumes and grasses in the ley pasture mediates biotic and abiotic soil legacy, and these legacies influence subsequent crop performance in grass-crop rotations. The results demonstrate that 30% legume seeding proportions in the previous legume-grass fully mixed intercropping had positive legacy effect on the yield of the subsequent crop by enriching beneficial soil legacies (more NH_4^+ -N and microbial enzymes in P mineralization and high relative abundance of AMF) compared with 70% legume seeding proportion in the legume-grass mixture, and the positive legacy was comparable with legume monocultures. Furthermore, these effects may gradually decrease or even disappear as the subsequent crop grows. Our results reveal that the crop performance depends on legacies from previous legume-grass seeding ratios. Legacy effects on the subsequent crop were affected by previous different functional group seeding ratios, a finding that is extremely useful for practical management and design of pasture-crop rotation system.

CRedit authorship contribution statement

Jingying Jing: Writing – review & editing, Supervision, Formal analysis, Conceptualization. **Paul C Struik:** Writing – review & editing, Formal analysis, Conceptualization. **Yingjun Zhang:** Funding acquisition, Supervision, Conceptualization. **Hongqian Yu:** Investigation, Data curation. **Yixian Bi:** Methodology, Conceptualization. **Yujuan He:** Writing – original draft, Methodology, Visualization, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.fcr.2024.109434](https://doi.org/10.1016/j.fcr.2024.109434).

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