Research Topics

Cover crops and green manures: providing services to agroecosystems

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Cover crops and green manures: providing services to agroecosystems

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Table of contents

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Editorial: Cover crops and green manures: providing services to agroecosystems

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Editorial on the Research Topic
Cover crops and green manures: providing services to agroecosystems

1 Introduction

Soil health is the capacity of a soil to function within natural or managed ecosystem boundaries, sustain plant and animal productivity, maintain or enhance water and air quality, and promote plant and animal health (1). Soil health indicators are closely linked to the capacity of the soil to provide essential ecosystem services, which contribute significantly to human well-being. Some ecosystem services are food and fiber production, biodiversity conservation, nutrient cycling, and carbon sequestration. However, the conversion of native grasslands into croplands, a process known as "agriculturization," has led to the depletion of soil organic carbon content, one of the key components of soil health (2). Consequently, agriculturization has negatively impacted on the ability of the soil to provide these vital ecosystem services.

Adopting sustainable management practices helps alleviate or even reverse the negative impact of agriculturization on the ecosystem services provision. Sustainable management practices should be considered based on their economic viability, social responsiveness, and environmental impact, complexifying their feasibility on agricultural production systems. Cover crops and green manures are usually called "service crops" to highlight the multiple ecosystem services they offer to the agroecosystems (3). Thus, integrating cover crops and green manures into crop rotations appears to be a promising sustainable management strategy.

The global adoption of cover crops and green manures in cash crop rotations has recently increased. However, possible trade-offs between their benefits and side effects may hinder their widespread use. Additionally, the limited adoption of cover crops by farmers can be attributed to a lack of information regarding the ecosystem services these crops provide across diverse climate and soil conditions, as well as variations in cover crop species, management practices, and production systems. The objective of this Research

Topic was to address these research gaps by compiling studies that provide innovative insights into the inclusion of cover crops in agroecosystems, focusing on their relationships with: i) nutrient cycling, soil fertility, and plant nutrition; ii) soil quality indicators and overall soil health; iii) cash crop productivity and quality; iv) soil biology and biodiversity; and v) greenhouse gas emissions and carbon sequestration.

2 This Research Topic

Different services provided by cover crops and green manures across various agroecosystems were highlighted in this Research Topic. In their investigation of weed suppression, Wirén et al. emphasized the effectiveness of both single and mixed cover crop species in managing weed populations. This aspect is crucial for reducing reliance on chemical herbicides and supporting more sustainable farming practices. Complementing this Research Topic, García et al. demonstrated that incorporating Canavalia as a green manure increased tobacco (*Nicotiana tabacum* L.) yields compared to a fallow, with this effect further enhanced through integration with arbuscular mycorrhizal fungi (AMF), allowing for reductions in mineral fertilizer use. This synergy illustrates the potential of integrated practices to maximize crop productivity while decreasing dependence on synthetic fertilizers.

Nutrient cycling and soil fertility were the ecosystem services mainly addressed in this Research Topic. Cover crops modify nutrient cycling in the systems by acting as catch crops during fallow periods and releasing the nutrients after their termination. In this context, Couëdel et al. showed that crucifer-legume mixtures optimized nutrient uptake during fallow periods compared to sole cover crops. Although some negative competition for specific nutrients was observed, the overall compatibility of these mixtures enhanced the provision of ecosystem service by reducing nutrient losses, mainly through leaching and/or runoff, and promoting nutrient recycling for the following cash crop. Similarly, Yang Y. et al. revealed that leguminous cover crops improved soil phosphorus availability and citrus growth in phosphorus-deficient soils, further highlighting the importance of these crops in nutrient management strategies. Moreover, Yang X. et al. showed in a comparative analysis of cover crops under different tillage methods that hairy vetch outperformed other species in maize (*Zea Mays* L.) grain yield, while providing valuable nitrogen credits upon termination. These findings underscore the critical role of cover crops in promoting nutrient cycling and their potential as a nutrient management strategy for achieving sustainable agricultural practices.

Environmental benefits of green manures were another important topic discussed in this Research Topic. Lyu et al. provided a detailed review of the impact of green manure application on soil nitrogen transformation and cash crop nitrogen uptake and utilization. The authors argued that the total or partial replacement of inorganic nitrogen fertilizers with green manures prevented nitrate leaching and ammonia volatilization, while the effect on N_2O emissions depended on cover crop management and environmental conditions. Moreover, they emphasized the importance of aligning nitrogen mineralization and immobilization with the nutrient demand of cash crops to improve nitrogen use efficiency and reduce potential losses. Additionally, Cafaro La Menza and Carciochi characterized various cover crop species and related them to their main contributions to agroecosystems. These authors stated that grasses are suitable for soil erosion control, weed suppression, and enhancing carbon balances, while legumes like vetch are essential for nutrient recycling and adding nitrogen to cropping systems. This synthesis-analysis highlighted that the selection of cover crop species should be based on the specific ecosystem services needed in the agroecosystem.

To properly evaluate the benefits of including cover crops and green manures in agroecosystems, a long-term assessment should be undertaken to avoid erratic responses from specific years. In this context, Wang et al. demonstrated that extending mulching duration in orchard systems significantly increased soil nitrogen and phosphorus levels, along with microbial activity. This finding highlighted the long-term benefits of green manures as a sustainable soil management practice.

In integrated crop-livestock systems, farmers are often tempted to use cover crops as animal feed. Although, by definition, this could not strictly be considered a cover crop (as cover crops are defined as species that grow between two cash crops and are not grazed or harvested), it is a possibility in current cropping systems. In this regard, Fu et al. highlighted the drawbacks of mowing ryegrass (*Lolium perenne* L.), which could lead to declines in soil nutrient content, enzyme activities, and microbial community composition, despite increases in cash crop yield. These results underscore the complexity of managing cover crops and the challenges in determining the appropriateness of this practice within cropping systems.

3 Concluding remarks/ future directions

The studies compiled in this Research Topic showed the multifaceted role of cover crops and green manures in enhancing agroecosystem services. However, assessing their impact is inherently complex, as it is influenced by various factors. While some benefits, such as improved nutrient cycling and soil health, are consistently observed, others may vary significantly based on local environmental conditions and agricultural practices. To effectively evaluate the contributions of cover crops and green manures, a holistic approach seems essential. This approach should encompass not only the immediate agronomic benefits but also the broader ecosystem services they provide with a focus on the long-term.

In conclusion, this Research Topic serves as a valuable resource for advancing our understanding of cover crops and green manures, facilitating more informed decisions regarding their adoption and maximizing their benefits while minimizing potential trade-offs. As we continue to explore the potential of these crops, it is imperative to evaluate them with a comprehensive perspective, ensuring that we consider economic, social, and environmental aspects.

Author contributions

WC: Writing – original draft, Writing – review & editing. JG: Writing – review & editing. NW: 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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Living grass mulching improves soil enzyme activities through enhanced available nutrients in citrus orchards in subtropical China

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Living grass mulching (LGM) is an important orchard floor management that has been applied worldwide. Although LGM can effectively enhance soil nutrient availability and fertility, its effects on microbial-mediated soil nutrient cycling and main drivers are unclear. Meanwhile, the variation of enzyme activities and soil nutrient availability with LGM duration have been rarely studied. This study aims to explore the effects of mulching age and soil layer on enzyme activities and soil nutrients in citrus orchards. In this study, three LGM (*Vicia villosa*) treatments were applied, i.e., mulching for eight years, mulching for four years, and no mulching (clean tillage). Their effects on the enzyme activities and soil nutrients were analyzed in different soil layers of citrus orchards in subtropical China, i.e., 0-10, 10-20, and 20-40 cm. Compared to clean tillage, mulching for four years had fewer effects on enzyme activities and soil nutrients. In contrast, mulching for eight years significantly increased available nitrogen (N), phosphorus (P) nutrients, β-glucosidase, and cellobiohydrolase activities in the soil layer of 0-20 cm. In the soil layer of 0-40 cm, microbial biomass carbon (C), N, P, N-acetylglucosaminidase, leucine aminopeptidase, and acid phosphatase activities also increased ($P < 0.05$). Mulching for eight years significantly promoted C, N, and P-cycling enzyme activities and total enzyme activities by 2.45-6.07, 9.29-54.42, 4.42-7.11, and 5.32-14.91 times, respectively. Redundancy analysis shows that mulching treatments for eight and four years had soil layer-dependent positive effects on soil enzyme activities. Microbial C and P showed the most significant positive correlation with enzyme activities, followed by moisture content, organic C, and available N ($P < 0.05$). Available nutrients contributed almost 70% to affect enzyme activities significantly and were the main drivers of the enzyme activity variation. In summary, LGM could improve soil enzyme activities by increasing available nutrients. The promotion effect was more significant under mulching for eight years. Therefore, extending mulching age and improving nutrient availability are effective development strategies for sustainable soil management in orchard systems. Our study can provide valuable guidelines for the design and implementation of more sustainable management practices in citrus orchards.

KEYWORDS

living grass mulching, soil enzyme activity, soil nutrient, mulching age, soil layer, orchard

Introduction

Orchards have been widely maintained worldwide and have become an essential part of agriculture owing to the tremendous economic value of the fruit (Rey, 2011; Zhao et al., 2021). The acreage of orchards has increased by approximately 22% since 2000 worldwide (FAO, 2020). Soil is the foundation of ensuring orchard productivity and promoting ecosystem stability. Agricultural management regimes largely affect soil properties and biochemical processes (Jia et al., 2022). Orchard management practices vary across regions, resulting in differential impacts on soil quality (e.g., physicochemical and biochemical properties) (Demestihas et al., 2017; Xiang et al., 2023). China has the largest orchard area in the world (Xiang et al., 2022). Although orchards have contributed significantly to improving the total vegetation coverage in China, understory management of orchards is still lagging (Wei et al., 2017). Clean tillage (total weeding control) is a popular orchard floor management practice in China (Wang et al., 2015). Implementing clean tillage management in orchards can speed up soil organic matter's mineralization and decomposition and exacerbate the decrease of enzyme activities and soil microbial community diversity (Wang et al., 2009; Vignozzi et al., 2019; Xiang et al., 2022). Optimal management practices, such as living grass mulching (LGM), have been promoted to curb soil degradation in orchards (Rumpel et al., 2020). However, farmers and decision-makers have not realized its potential impacts on the orchard ecosystems, resulting in the slow implementation of optimal practices (Wei et al., 2017). Therefore, a critical assessment of soil characteristics responding to orchard floor management is necessary to realize sustainable utilization of orchard soils.

LGM is a soil management method that replaces whole-garden or inter-row bare soil with sod cultivation (Atucha et al., 2013; Wang et al., 2015; Taguas et al., 2017). Previous research has shown that LGM may alter many aspects of soil properties in orchards, such as soil physical properties (Haruna et al., 2020), soil organic carbon (SOC) stocks (Xiang et al., 2022), soil nutrient contents (Wei et al., 2017), soil biological activities (Ramos et al., 2011), and soil microbial community composition (Wang et al., 2022). Thus, LGM is very likely to affect the ecosystem functions of orchards (Wei et al., 2018). Soil enzymes can directly mediate the catabolism of soil organic and mineral components and are crucial in biogeochemical cycles within terrestrial ecosystems (Margida et al., 2020; Liu et al., 2022). Soil enzyme activity is more sensitive to soil quality changes compared with physicochemical properties (e.g., soil nutrient content and organic matter) (Luo et al., 2018). It can be considered an early warning indicator of soil system changes (Utobo and Tewari, 2015). LGM has been reported to increase the activities of soil enzymes, such as urease and phosphatase activity (Xiang et al., 2023). However, some studies have reported opposite results or no effects (Solanki et al., 2019; Adetunji et al., 2021). Kumar et al. (2022) also pointed out that the positive effects of mulching measures on soil enzyme activity could be enzyme-specific. Furthermore, LGM affects soil enzyme activity by changing soil properties (e.g., temperature, pH, soil bulk density, water content, and nutrient content) (Burns et al., 2016). LGM can improve the exogenous input of soil organic matter and enzyme activity, thus accelerating organic matter degradation and soil nutrient mineralization and improving soil nutrient levels. This indicates that enzyme activity has a positive correlation with organic matter (Zheng et al., 2018). However, exceptions exist (Sun et al., 2021). Burns et al. (2013) found that increased soil nutrients promoted nutrient uptake by microorganisms and thus reduced related catalytic enzyme activities. This indicates that soil enzyme activity is mainly affected by soil nutrient availability. Moreover, a relatively high element content in soils can also promote other elements' use by extracellular enzymes (Sinsabaugh, 2010). Therefore, inter-element coupling increases the difficulty in determining the mechanism of enzyme activity changes under orchard floor management. These inconsistent results highlight the call for more cases to better understand the soil biological properties in orchards in response to LGM.

The effects of LGM on soil biological properties may be a long-term process. Due to strong anthropogenic disturbance, short-term LGM may not cause significant changes in orchard soil properties (Wang et al., 2020a). Nevertheless, most studies have focused on the effects of short-term LGM on orchard soil biological properties. The use of LGM for a different number of years in the orchard has been rarely studied. Moreover, various biotic and abiotic factors affecting soil enzyme activity vary with soil layers (Sun et al., 2021). Generally, enzyme activity decreases with increasing soil layer depth (Stone et al., 2014). However, orchard disturbances (e.g., fertilization, irrigation, and understory maintenance) have more direct and significant effects on the topsoil than on the subsoil (Sun et al., 2021). Some studies have reported that enzyme-associated mineralization rates of deep soil mineral nitrogen (N) or carbon (C) in subsoil were close to or higher than those in topsoil under environmental variations (Schnecker et al., 2015; Wang et al., 2019). Hence, enzyme activity variations in different soil layers of orchards due to LGM remain unclear. Evaluating the dynamics of enzyme activities and soil nutrients under different mulching ages and soil layers is necessary in order to better understand the biochemical processes under LGM.

Citrus is the fruit tree with the largest planting area in the world. It can promote regional economic development and ecological environment (Tu et al., 2021). The Three Gorges Reservoir area (TGRA) in China is one of the optimal citrus production regions worldwide due to its unique natural resources and ecological conditions (Xia et al., 2015). Currently, clean tillage is commonly adopted for citrus orchard floor management, resulting in soil degradation problems such as soil compaction and decreasing organic matter (Liang and Li, 2019). This study aims to provide a beneficial biological approach for improving soil nutrient cycling efficiency and quality in citrus orchards. In this study, *Vicia villosa* (a leguminous plant) was selected as mulching grass to investigate enzyme activities and soil nutrients in different soil layers (0-40 cm) under clean tillage and two mulching ages (four and eight years), respectively. This plant has strong adaptability, high N fixation capacity, high coverage, a shallow root system, and no need to cut. Specifically, the objectives of this paper include (i) analyzing the impacts of mulching ages and soil layers on enzyme activities and soil nutrients, (ii) revealing the correlation of enzyme activities with soil properties, and (iii) identifying the key factors influencing soil enzyme activities in regards to mulching ages and soil layers.

Materials and methods

Study site

Field experiments were performed in a citrus orchard on a sloping site in Zigui County, Hubei Province, China (110°40’ E, 31°4’ N). The local climate belongs to the subtropical monsoon climate. The annual average temperature and precipitation were 16.7 °C and 1013.1 mm, respectively. The soil is purple and mainly has a sandy loam texture. Since the 2000s, citrus has been continuously cultivated in the study area.

The citrus had a planting density of about 825 plants ha^{-1}: plant spacing, 3 ± 0.5 m; row spacing, 3.5 ± 0.5 m. The main fertilizer used was mixed fertilizers containing 22% N, 6% P_2O_5, and 11%K_2O (about 3300 kg ha^{-1}). The fertilizers were used three times a year, including one base fertilizer and two top dressings.

Experimental design

A species of *Vicia villosa* (VV) was used as mulching grass in the citrus orchard and sown initially using the full mulching method (45 kg ha^{-1}) in September 2013 and 2017, respectively. Then, the grass grew naturally and was not cleaned. The weeds in the citrus orchard with clean tillage were manually removed. Other field management practices (e.g., fertilization type and time) at these experimental sites were the same. Therefore, the experimental site with grass mulching included mulching for four years (VV_4) and eight years (VV_8) by 2021. The experimental site without mulching (i.e., clean tillage) was taken as the control (CT). At each site, every two plots had a distance of more than 50 m. Three replicates were used for analysis (Table 1).

Soil sampling

Soil samples were obtained from citrus orchards with different LGM ages in March 2021. Soils were collected from three soil layers (i.e., 0-10, 10-20, and 20-40 cm) in each plot using a five-point sampling method and combined into one mixed sample by soil layer. A total of 27 soil samples (three

TABLE 1 The basic information of experimental plots with living grass mulching.

Plots	Altitude (m)	Slope (°)	Area (m^2)	Planting density of VV (kg ha^{-1})	Aspect
VV_8	220	20	200	45	south
VV_4	220	20	200	45	south
CT	220	20	200	0	south

mulching ages × three soil layers × three replicates) were collected. Stones and large roots were removed from the fresh soil. Then, the collected soil samples were sieved using a 2-mm sieve and split into halves. One part was stored at 4°C to determine soil moisture content, microbial biomass, and enzyme activities. The other part was naturally air-dried to determine the physicochemical properties of soil samples.

Soil physicochemical properties and microbial biomass

The soil sample was dissolved into water (soil:water = 1:2.5) to determine soil pH. Total nitrogen (TN), SOC, and total phosphorus (TP) were calculated employing the Kjeldahl method, the $K_2Cr_2O_7$-H_2SO_4 oxidation method, and acid melt-molybdenum, antimony, and scandium colorimetry, respectively (Zheng et al., 2020). Based on the modified alkaline hydrolysis diffusion method and the Olsen method, alkali-hydrolyzed nitrogen (AN) and available phosphorus (AP) were analyzed (Wang et al., 2016). Based on the chloroform fumigation-extraction method, soil microbial biomass nitrogen (MBN), carbon (MBC), and phosphorus (MBP) were measured (Brookes et al., 1985; Vance et al., 1987). Soil moisture content (MC) was measured using the ring sampler method.

Soil enzyme activities

Six soil enzymes were selected, including β-glucosidase (BG), cellobiohydrolase (CB), N-acetylglucosaminidase (NAG), leucine aminopeptidase (LAP), acid phosphatase (APH), and phenol oxidase (POX). Their activities were measured using microplate fluorimetry (German et al., 2011; Zhou et al., 2020). Soil suspensions were obtained by adding fresh soil (equivalent to 1g of dry soil) into 125 mL sodium acetate buffer (50 mmol L^{-1}; pH = 5.0-6.6) and stirring for 1 min. Soil suspensions (200 μL) and corresponding enzyme substrates (i.e., 7-amino-4-methylcoumarin [AMC for LAP] and 4-methylumbelliferone [MUB for BG, CB, NAG, and APH], 50 μL, 200 μmol L^{-1}) were combined in eight sample assay wells of 96-well microplates. Then, the microplates were incubated in the dark at 25 °C for 3 hours. Fluorescence at 365 nm excitation and 450 nm emission filters was determined based on a microplate fluorometer (SpectraMax i3x, Molecular Devices, Beckman Coulter, CA, USA). The soil suspension (600 μL) and the substrate (L-3,4-dihydroxyphenylalanine, DOPA, 150 uL, 25 mmol L^{-1}), were mixed and then added to the 96-well microplates in order to determine the POX activity. The microplates were incubated for an hour with shaking. The absorbance was measured at 465 nm. The activities of the six enzymes were measured in the unit of nmol h^{-1} g^{-1} soil.

The C, N, and phosphorus (P)-cycling enzyme activity and total enzyme activity were calculated using the normalization method. The geometric mean (GM) was calculated to evaluate the enzyme activities with different functions and the total enzyme activities (Eqs. (1-4); Raiesi and Salek-Gilani, 2018):

$$\mathbf{GMC} = \sqrt[n1]{\prod\nolimits_{i=1}^{n1} \mathbf{EC_i}} \tag{1}$$

$$\mathbf{GMN} = \sqrt[n2]{\prod\nolimits_{i=1}^{n2} \mathbf{EN_i}} \tag{2}$$

$$\mathbf{GMP} = \sqrt[n3]{\prod\nolimits_{i=1}^{n3} \mathbf{EP_i}} \tag{3}$$

$$\mathbf{GM} = \sqrt[n1+n2+n3]{\prod\nolimits_{i=1}^{n1} \mathbf{EC_i} \times \prod\nolimits_{i=1}^{n2} \mathbf{EN_i} \times \prod\nolimits_{i=1}^{n3} \mathbf{EP_i}} \tag{4}$$

where GMC, GMN, and GMP indicate C, N, and P-cycling enzyme activities, respectively; EC_i, EN_i, and EP_i are the normalized value of enzyme i in C, N, and P-cycling, respectively; $n1$, $n2$, and $n3$ are the number of enzymes in C, N, and P-cycling, respectively; GM indicates the total enzyme activity. In this study, $n1 = 3$ (POX, BG, and CB), $n2 = 2$ (NAG and LAP), and $n3 = 1$ (APH).

Statistical analysis

Statistical analysis was performed using R v.3.6.1. First, Tukey's HSD tests and one-way and two-way analysis of variance (ANOVA) were performed to evaluate the variations of soil enzyme activities, microbial biomass, and physicochemical properties under different mulching ages, different soil layers, and their interactions. Then, Pearson's correlation analysis was used to determine the relationships of soil enzyme activities and other soil properties. Univariate and multivariate (stepwise) linear regression analyses were performed to determine the magnitude of the interaction between other soil properties and the individual soil enzyme activity. Finally, based on the above information, redundancy analysis (RDA) was used to simultaneously examine all soil enzyme activities and the influence of other soil properties. To remove collinearity among variables, a Monte Carlo permutation test (999 permutations) and variance inflation factor inspection (VIF < 5) were used to identify effective variables. Then, the impact of other soil properties on soil enzyme activity was investigated. The importance ranking of the main influencing factors of soil enzyme activity was further determined using hierarchical partitioning (Lai et al., 2022).

Results

Effects of mulching age and soil layer on soil properties

Table 2 shows the effects of mulching age and soil layer on orchard soil properties. The soil layer significantly influenced all microbial biomass and soil physicochemical properties ($P < 0.05$). The mulching age significantly affected all microbial biomass and all soil physicochemical properties except for TN and TP. Their two-way interaction only significantly affected AP.

Soil physicochemical properties generally varied with mulching ages in the three soil layers (especially at 0-10 cm) (Figure 1). SOC, TN, AN, pH, and MC progressively increased with increasing mulching ages in all soil layers (Figures 1A–C, F, G). TP and AP decreased under VV_4 and then significantly increased under VV_8 (Figures 1D, E). In comparison to the CT treatment, the VV_8 treatment substantially improved AN (69.08%) and AP (144.96%) at 0-10 cm, SOC at 20-40 cm (89.29%), and MC in all three soil layers (31.29%, 30.58%, and 26.10%) ($P < 0.05$) The VV_4 treatment only significantly increased MC by 12.77% at 0-10 cm ($P < 0.05$). TN, TP, and pH had no significant increase under different mulching ages in each soil layer.

MBC, MBN, and MBP also showed an upward trend with mulching ages in all soil layers, except for MBP at 0-10 cm (Figure 2). MBC at three soil layers under the VV_4 treatment was slightly higher than that under the CT treatment, while the increase was not significant (Figure 2A). MBN at 10-20 cm and MBP at 20-40 cm under the VV_4 treatment significantly increased by 57.36% and 404.50%, respectively, compared to those under the CT treatment ($P < 0.05$; Figures 2B, C). MBC, MBN, and MBP under the VV_8 treatment in all soil layers significantly increased by 245.72-733.85%, 83.82-186.57%, and 124.56-522.61%, respectively, compared to those under the CT treatment ($P < 0.05$; Figure 2).

The soil microbial biomass and physicochemical properties showed a similar trend with the soil layer (Figures 1, 2). In general, except for pH and MC, all other biochemical properties decreased with the soil layer depth (i.e., 0-10 cm > 10-20 cm > 20-40 cm). Remarkably, MBN was the highest at 10-20 cm ($P < 0.05$).

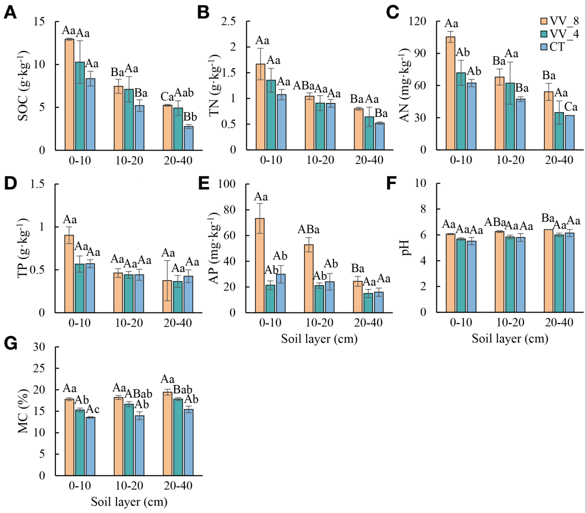

FIGURE 1

Soil physicochemical properties under different mulching ages within the different soil layers in citrus orchards. **(A–E)** The contents of soil organic carbon, total nitrogen, alkali-hydrolyzed nitrogen, total phosphorus, available phosphorus. **(F)** pH. **(G)** Soil moisture content. Values are the mean ± standard error ($n = 3$). Capital letters represent significant differences in different soil layers under the same mulching age. Lowercase letters represent significant differences under different mulching ages in the same soil layer based on Tukey's tests and one-way ANOVA ($P < 0.05$). VV_8, mulching for eight years; VV_4, mulching for four years; CT, clean tillage; SOC, soil organic carbon; TN, total nitrogen; AN, alkali-hydrolyzed nitrogen; TP, total phosphorus; AP, available phosphorus; MC, moisture content.

Effects of mulching age and soil layer on soil enzyme activities

From Table 3, the mulching age significantly affected all soil enzyme activities ($P < 0.05$). The soil layer significantly influenced all soil enzyme activities except for POX. The combined effect of mulching age and soil layer only significantly affected LAP, APH, GMN, GMP, and GM ($P < 0.05$). The mulching age and soil layer significantly affected C, N, and P-cycling and total enzyme activities ($P < 0.001$).

The soil enzyme activities were ranked in descending order in terms of mulching age and soil layer: VV_8 > VV_4 > CT; 0-10 cm > 10-20 cm > 20-40 cm (Figure 3). The BG activity (0-10 cm) and CB activity (10-20 cm) under the VV_8 treatment were 1.22 and 3.90 times greater than those under the CT treatment ($P < 0.05$),

TABLE 2 Statistical differences (*F*-values and significance level) between means of soil properties by two-way ANOVA with mulching age and soil layer.

Factor	SOC	TN	AN	TP	AP	pH	MC	MBC	MBN	MBP
Mulching age (A)	6.04**	3.46	7.18**	1.21	25.24***	5.10*	40.97***	187.87***	26.57***	14.05***
Soil layer (L)	24.11***	15.32***	13.18***	6.84**	12.05***	4.14*	9.71***	5.61*	82.93***	12.69***
A*L	0.47	0.55	0.73	1.23	3.82*	0.22	0.28	0.16	1.94	2.74

SOC, soil organic carbon; TN, total nitrogen; AN, alkali-hydrolyzed nitrogen; TP, total phosphorus; AP, available phosphorus; MC, moisture content; MBC, MBN, and MBP represent microbial biomass carbon, nitrogen, and phosphorus, respectively. *, **, and *** represent $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. The data is F value.

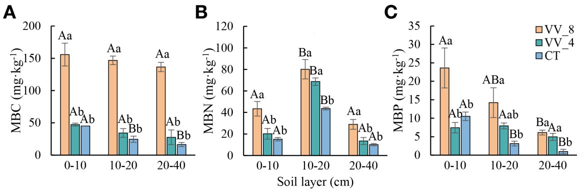

FIGURE 2
Soil microbial biomass in different soil layers under different mulching ages in citrus orchards. **(A-C)** The contents of soil microbial biomass carbon, nitrogen, and phosphorus. Values are the mean ± standard error (*n* = 3). Capital letters represent significant differences under the same mulching age in different soil layers. Lowercase letters represent significant differences under different mulching ages in the same soil layer based on Tukey's tests and one-way ANOVA ($P < 0.05$). VV_8, mulching for eight years; VV_4, mulching for four years; CT, clean tillage; MBC, MBN, and MBP represent microbial biomass carbon, nitrogen, and phosphorus, respectively.

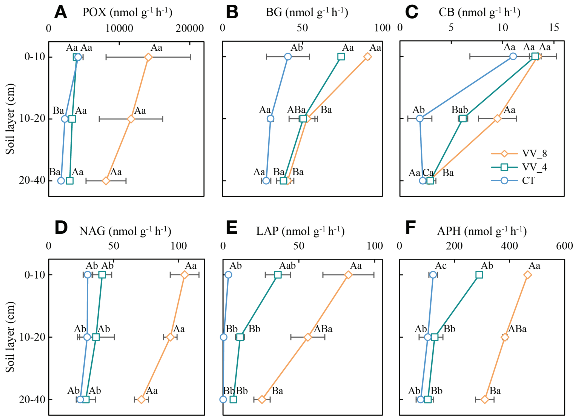

FIGURE 3
Soil enzyme activities under different soil layers and mulching ages in citrus orchards. **(A–F)** The activities of phenol oxidase, β-glucosidase, cellobiohydrolase, N-acetylglucosaminidase, leucine aminopeptidase, acid phosphatase. Values are the mean ± standard error (*n* = 3). Capital letters represent significant differences under the same mulching age in different soil layers. Lowercase letters represent significant differences under different mulching ages in the same soil layer based on Tukey's tests and one-way ANOVA ($P < 0.05$). VV_8, mulching for eight years; VV_4, mulching for four years; CT, clean tillage; POX, phenol oxidase; BG, β-glucosidase; CB, cellobiohydrolase; NAG, N-acetylglucosaminidase; LAP, leucine aminopeptidase; APH, acid phosphatase.

respectively. The NAG, LAP, and APH activities under the VV_8 treatment in all soil layers were 1.94-2.49, 22.17-223.77, and 2.71-3.00 times greater than those under the CT treatment, respectively ($P <$ 0.05). Furthermore, compared to the CT treatment, the VV_4 treatment only significantly increased the activities of BG (81.16%) and APH (136.77%) at 0-10 cm ($P < 0.05$).

The activities of GMC, GMN, GMP, and GM increased with mulching age in all soil layers. Under the VV_8 treatment, they were 2.45-6.07, 9.29-54.42, 4.42-7.11, and 5.32-14.91 times higher than those under the CT treatment, respectively ($P < 0.05$). The VV_4 treatment significantly enhanced GMC activity at 10-20 cm, GMN and GMP activities at 0-10 cm, and GM activity at 0-10 cm and 20-40 cm by 259.84%, 296.44%, 215.55%, 216.45%, and 360.98%, respectively ($P < 0.05$). GMC, GMN, GMP, and GM activities declined as the soil layer got deeper. Under the VV_8 and VV_4 treatments, these activities were much higher at 0-10 cm compared to those at 20-40 cm. The CT treatment only significantly changed GMN (Figure 4).

Main influencing factors of soil enzyme activity

The correlation analysis reveals that the enzyme activity generally exhibited significant positive correlations with MBC, MBP, AN, and AP ($P < 0.001$). SOC and TN also had a positive and significant correlation with all enzyme activities ($P < 0.001$; Figure 5A), except for POX and APH activities. Under the VV_8 treatment, the correlation of BG, NAG, APH, and GMP activities with MBP was significantly positive ($P < 0.05$). The correlation of BG, CB, and GMC activities with SOC was significantly positive ($P < 0.001$). LAP activity showed a significant positive correlation with TN ($P < 0.001$). N and P-cycling enzyme activities exhibited a significant positive correlation with AN and AP ($P < 0.05$; Figure 5B). BG activity was positively associated with AN, SOC, TN, and TP under the VV_4 treatment ($P < 0.05$; Figure 5C). The enzyme activities exhibited a significant positive correlation with soil C and N nutrients under the CT treatment ($P < 0.05$; Figure 5D). Univariate and stepwise regression analyses found similar results to explain the relationship between soil enzyme

TABLE 3 Statistical differences (*F*-values and significance level) between means of soil enzyme activities by two-way ANOVA with mulching age and soil layer.

Factor	POX	BG	CB	NAG	LAP	APH	GMC	GMN	GMP	GM
Mulching age (A)	9.39**	18.18***	3.67*	57.07***	38.67***	145.58***	29.56***	126.91***	145.58***	132.83***
Soil layer (L)	1.02	24.10***	28.98***	3.84*	11.79***	28.83***	21.43***	21.33***	28.83***	43.42***
A*L	0.25	2.50	1.25	0.88	3.32*	4.21*	2.20	4.80**	4.21*	7.18***

POX, phenol oxidase; BG, β-glucosidase; CB, cellobiohydrolase; NAG, N-acetylglucosaminidase; LAP, leucine aminopeptidase; APH, acid phosphatase ; GMC, GMN, and GMP represent C, N, and P-cycling enzyme activities, respectively; GM, total enzyme activities. *, **, and *** represent $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. The data is *F* value.

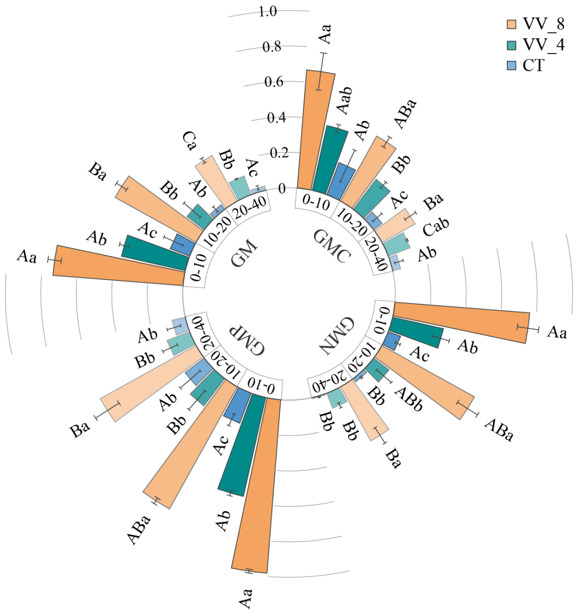

FIGURE 4
Soil C-cycling (GMC), N-cycling (GMN), P-cycling (GMP), and total (GM) enzyme activity in different soil layers under different mulching ages in citrus orchards. Values are the mean ± standard error (n = 3). Capital letters represent significant differences under the same mulching age in different soil layers. Lowercase letters represent significant differences under different mulching ages in the same soil layer based on Tukey's tests and one-way ANOVA (P < 0.05). VV_8, mulching for eight years; VV_4, mulching for four years; CT, clean tillage.

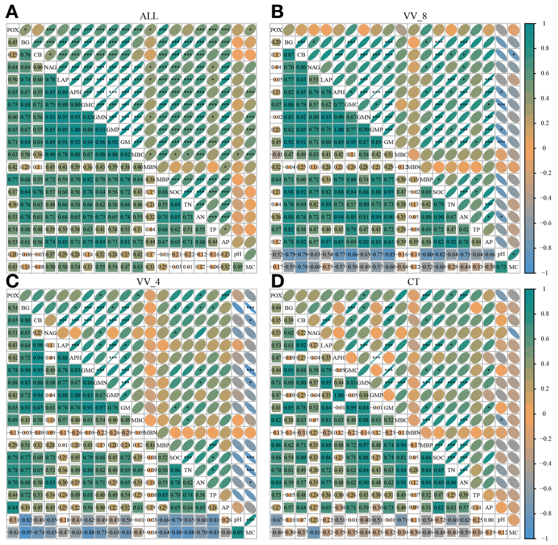

FIGURE 5
Correlation of soil enzyme activities with other soil properties. **(A)** All treatments. **(B)** The treatment of mulching for eight years. **(C)** The treatment of mulching for four years. **(D)** The treatment of clean tillage. VV_8, mulching for eight years; VV_4, mulching for four years; CT, clean tillage; POX, phenol oxidase; BG, β-glucosidase; CB, cellobiohydrolase; NAG, N-acetylglucosaminidase; LAP, leucine aminopeptidase; APH, acid phosphatase; GMC, GMN, and GMP represent C, N, and P-cycling enzyme activities, respectively; GM, total enzyme activities; MBC, MBN, and MBP represent microbial biomass carbon, nitrogen, and phosphorus, respectively; SOC, soil organic carbon; TN, total nitrogen; AN, alkali-hydrolyzed nitrogen; TP, total phosphorus; AP, available phosphorus; MC, moisture content. * and *** represent $P < 0.05$ and $P < 0.001$.

activities and other soil properties. MBC, MBP, SOC, AN, and AP had significant and strong univariate relationships with each enzyme activity (P < 0.01; Figure 6). Also, stepwise regression output showed that the standardized regression coefficients of MBC, MBP, SOC, AN, and AP were statistically significant (P < 0.05; Table 4) and had higher absolute values. These results indicate that the response of soil enzyme activity to MBC, MBP, SOC, AN, and AP was more sensitive, that is, these five biochemical properties had a greater positive effect on soil enzyme activity.

The two main axes (RDA) 1 and 2 were selected with an explanation of 63.87% and 3.95%, respectively (Figure 7). RDA shows that soil properties showed positive effects on enzyme activities (Figure 7). The soil properties were ranked in descending order in terms of importance: MBC (16.40%) > MBP (13.18%) > MC (11.15%) > SOC (9.09%) > AN (6.33%) > AP (5.57%) > TN (5.15%) > pH (3.09%) > MBN (1.54%). The response of soil enzyme activities to the top five soil properties was significant (P < 0.05; Table 5). MBC had the longest arrow line and the highest explanation amount. This indicates that MBC was the most important influencing factor. The available nutrients that had significant impacts, i.e., MBC, MBP, and AN, accounted for nearly 70% of the indices that significantly influenced soil enzyme activities (Table 5).

In general, MBC and MC mainly affected the activities of GMN (LAP), GMP (APH), and POX. MBP mainly affected GM, GMC (BG and CB), and NAG activities. BG and CB activities were also affected by SOC and AN. In addition, the effects of soil properties on soil enzyme activities varied with mulching ages and soil layers. The VV_8 treatment positively affected all enzyme activities at all soil layers, especially at 0-20 cm. The VV_4 treatment only positively affected BG and CB activities at 0-10 cm.

Discussion

Effects of different mulching ages on soil enzyme activities

LGM could improve soil nutrients and enzyme activities by increasing SOC input from root exudates and above-ground residues (Qian et al., 2015; Kader et al., 2017). We found that

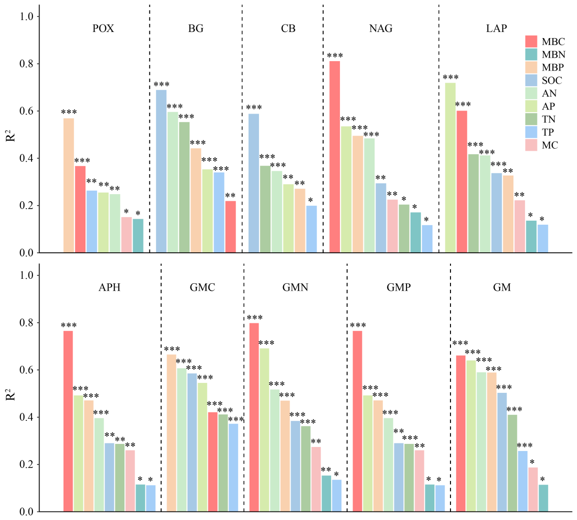

FIGURE 6
The adjusted R^2 of univariate regression analysis for soil enzyme activities and soil physicochemical properties and microbial biomass. POX, phenol oxidase; BG, β-glucosidase; CB, cellobiohydrolase; NAG, N-acetylglucosaminidase; LAP, leucine aminopeptidase; APH, acid phosphatase; GMC, GMN, and GMP represent C, N, and P-cycling enzyme activities, respectively; GM, total enzyme activities; MBC, MBN, and MBP represent microbial biomass carbon, nitrogen, and phosphorus, respectively; SOC, soil organic carbon; TN, total nitrogen; AN, alkali-hydrolyzed nitrogen; TP, total phosphorus; AP, available phosphorus; MC, moisture content. *, **, and *** represent $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

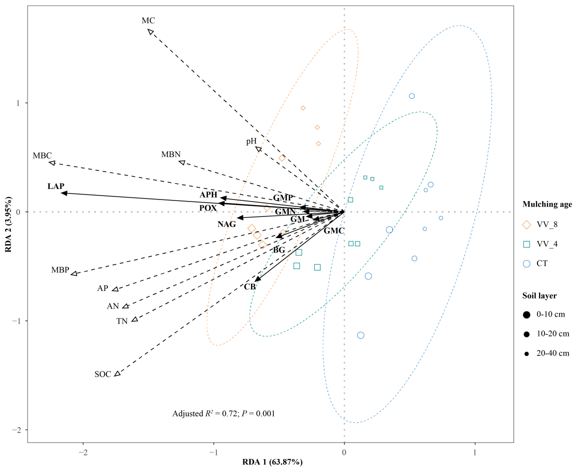

FIGURE 7
Redundancy analysis (RDA) ordination plot of enzyme activities constrained by physicochemical properties and microbial biomass that significantly explained variation. VV_8, mulching for eight years; VV_4, mulching for four years; CT, clean tillage; POX, phenol oxidase; BG, β-glucosidase; CB, cellobiohydrolase; NAG, N-acetylglucosaminidase; LAP, leucine aminopeptidase; APH, acid phosphatase; GMC, GMN, and GMP represent C, N, and P-cycling enzyme activities, respectively; GM, total enzyme activities; MBC, MBN, and MBP represent microbial biomass carbon, nitrogen, and phosphorus, respectively; SOC, soil organic carbon; TN, total nitrogen; AN, alkali-hydrolyzed nitrogen; AP, available phosphorus; MC, moisture content.

LGM's effects on enzyme activity were closely correlated with mulching ages and soil layers. The BG and APH activities at 0-10 cm were notably higher under the VV_4 treatment than the CT treatment (Figure 3). The BG activity was an important indicator reflecting the quality of organic matter and C sink level (Cenini et al., 2016). BG can hydrolyze cellobiose to generate glucose and provide metabolites for soil microorganisms (Singhania et al., 2013). After short-term mulching with *Vicia villosa*, cellulose and other β-1, 4-glucan polymers dominated the soil organic matter input and directly acted on the topsoil. Thus, the secretion of hydrolase enzymes (especially BG) was improved. These enzymes were the most closely related to the organic matter formed by the decomposition of herbaceous

TABLE 4 The standardized regression coefficient of multivariate (stepwise) linear regression analysis.

Variables	R^2	MBC	MBN	MBP	SOC	TN	AN	TP	AP	pH	MC
POX	0.57***	0.23	0.16	**0.77*** **	0.24	0.17	0.11	0.06	0.03	0.21	0.26
BG	0.69***	0.17	0.08	0.23	**0.84*** **	0.17	0.26	0.14	0.12	0.13	0.20
CB	0.59***	0.04	0.01	0.06	**0.78*** **	0.10	0.18	0.01	0.10	0.14	0.07
APH	0.80***	**0.78*** **	0.05	0.12	**0.23* **	0.04	0.09	0.003	0.004	0.01	0.10
NAG	0.87***	**0.74*** **	0.07	0.04	0.02	0.15	**0.30** **	0.01	0.01	0.15	0.01
LAP	0.77***	0.21	0.02	0.02	0.21	0.25	0.19	0.09	**0.77*** **	0.03	**0.26* **
GMC	0.81***	0.07	0.07	**0.50*** **	**0.50*** **	0.08	0.10	0.06	0.09	**0.25* **	0.14
GMN	0.90***	**0.58*** **	0.01	0.07	0.09	0.01	**0.24* **	0.02	**0.26* **	0.07	0.14
GMP	0.80***	**0.78*** **	0.05	0.12	**0.23* **	0.04	0.09	0.003	0.004	0.01	0.10
GM	0.83***	**0.63*** **	0.08	0.19	**0.46*** **	0.04	0.20	0.10	0.18	0.13	0.17

POX, phenol oxidase; BG, β-glucosidase; CB, cellobiohydrolase; NAG, N-acetylglucosaminidase; LAP, leucine aminopeptidase; APH, acid phosphatase; GMC, GMN, and GMP represent C, N, and P-cycling enzyme activities, respectively; GM, total enzyme activities; MBC, MBN, and MBP represent microbial biomass carbon, nitrogen, and phosphorus, respectively; SOC, soil organic carbon; TN, total nitrogen; AN, alkali-hydrolyzed nitrogen; TP, total phosphorus; AP, available phosphorus; MC, moisture content. *, **, and *** represent $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. Numbers in bold font represent the variables entered into the model, and the numbers in regular font represent variables removed from the model.

TABLE 5 Significance test results and importance ranking of soil properties.

Soil properties	Importance	Percentage of soil properties in total variation	*P*
MBC	1	16.40	0.012*
MBP	2	13.18	0.012*
MC	3	11.15	0.020*
SOC	4	9.09	0.016*
AN	5	6.33	0.046*
AP	6	5.57	0.068
TN	7	5.15	0.064
pH	8	3.09	0.132
MBN	9	1.54	0.212

*indicates $P < 0.05$. MBC, microbial biomass carbon; MBP, microbial biomass phosphorus; MC, moisture content; SOC, soil organic carbon; AN, alkali-hydrolyzed nitrogen; AP, available phosphorus; TN, total nitrogen; MBN, microbial biomass nitrogen.

residues (Sinsabaugh et al., 2008). This is consistent with the finding of Feng et al. (2021). They reported that a short-term leguminous grass mulching system increased the BG activity compared with the non-mulching treatment, indicating the increased C inputs from grass mulching which could stimulate microbial activity. The increase of APH activity after short-term mulching may be related to P sequestration in herbs and fruit trees. In the initial mulching stage (the VV_4 treatment), soil P decreased at 0-10 cm (Figures 1, 2) due to the competition for P between fruit trees and herbs. This may induce short-term P limitation in the soil (Deng et al., 2017). The result also agreed with the study by Chen et al. (2020). Therefore, fruit trees may release more root exudates to stimulate microbial activity and increase P secretion to alleviate P limitation.

Under the VV_8 treatment, the enzyme activities significantly increased, especially N-cycling (NAG and LAP) and P-cycling (APH) enzyme activities (Figures 3, 4). After long-term mulching with *Vicia villosa*, the enzyme activity was higher due to increased soil organic matter by herbaceous residue accumulation. Increased C input could weaken microbial C limitation and increase unstable components of SOC (Kalinina et al., 2019). Thus, the formation and release of enzymes were accelerated, and enzyme activities were effectively promoted. NAG and LAP were mainly involved in soil N transformation (Cenini et al., 2016) and chitin and peptide decomposition, respectively. The increased activities of these two enzymes may be attributed to the enhanced N fixation capacity of legumes and the weakened P limitation after long-term mulching. The increase in N availability promoted the N-cycling enzyme activities. The increase in the NAG activity may be due to the increase in the number of fungi caused by the long-term accumulation of herbaceous residues (Ramos-Zapata et al., 2012; Wang et al., 2020b) since chitin mainly exists in fungal cell walls and animal exoskeletons (Zheng et al., 2018). Wang et al. (2020b) showed that long-term mulching promoted the increase of NAG activity. However, another study stated that NAG was not directly affected by the decomposition of crop residues. Thus, long-term mulching did not affect its activity (Zheng et al., 2018). In addition, the continuous increase of APH indicates that the soil still showed P deficiency, although P limitation was alleviated after long-term mulching. Therefore, to satisfy tree growth needs, long-term accumulation of herbaceous residues still continuously promoted the conversion of organic P to inorganic P (Singh et al., 2018), thus maintaining a high APH activity.

Responses of soil enzyme activities in different soil layers to living grass mulching

The enzyme activity generally decreases with the deepening of soil layers (Stone et al., 2014). The topsoil is more conducive to promoting enzyme activity than the subsoil (Uksa et al., 2015; Avazpoor et al., 2019). The enzyme activities under LGM in this study also decreased with increasing soil depth (Figures 3, 4), which agreed with the findings of Sun et al. (2021). The result was related to grassroot distribution and nutrient input of surface residues. The grassroots were mainly distributed between 0-20 cm (Liu et al., 2018). The effect of surface residues on soil nutrients (i.e., C turnover and N and P mineralization) directly acted on the topsoil (Moradi et al., 2017). Therefore, the activities of five hydrolase enzymes (except for NAG) under LGM were significantly lower in the subsoil than in the topsoil (Figures 3, 4). POX activity had no significant changes among different soil layers.

Under LGM, the difference in soil enzyme activity at different soil layers may be due to various interactions between enzymes and microbial populations. LGM could increase the overall C metabolic activity due to increased soil organic matter input (Qian et al., 2015). This enhancement effect may be related to increased soil bacteria. The increase of microbes may be associated with certain enzyme secretion (i.e., BG and CB), thus enhancing soil C-cycling. Moreover, the N-fixation of the legume herb mulching promoted an increase in soil N metabolism and stimulated protein production of N-cycling bacterial communities. Thus, the activities of related enzymes, such as LAP, were enhanced. Some studies have shown that the alpha diversity of bacteria was lower in the subsoil than in the topsoil due to decreased oxygen and a low-nutrient environment in the deep soil (Wang et al., 2020a). Therefore, the enzyme activities were related to increased bacteria and were much higher in the topsoil than in the subsoil. As mentioned above, NAG mainly hydrolyzed chitin secreted by fungal cell walls. However, related studies have shown that fungal alpha diversity was not significantly different among soil layers (Wang et al., 2020a). Fungi may have high adaptability to LGM-induced environmental changes. This was one of the possible reasons for the insignificant differences in the NAG activity at different soil layers. In addition, the correlation between the enzyme activity and enzyme producers was weaker for oxidase than hydrolase (A' Bear et al., 2014). Thus, the POX activity had no significant differences at soil layers.

Factors mediating soil enzyme activity under living grass mulching

Soil enzyme activity is influenced by biotic and abiotic factors (Jian et al., 2016), such as soil nutrients, microbial biomass, and moisture content. Under LGM, soil organic matter increased with the continuous input of root litter and surface residues (Wei et al., 2017). The increased soil organic matter could promote microbial activity and extracellular enzyme secretion. Thus, soil enzyme activity had a positive correlation with organic matter. In this study, SOC had the most significant positive correlation with the C-cycling enzyme activity. This indicates that SOC was the key factor influencing the C-cycling enzyme activity (Figures 5, 7). Compared to TN, AN also significantly positively affected BG and CB activities. The results show that available nutrients had stronger effects than total nutrients. Sun et al. (2021) also found that soil enzyme activity was more easily affected by available nutrients. Conversely, some studies have found that the C-cycling enzyme activity was significantly positively affected by TN (A'Bear et al., 2014; Qian et al., 2015; Zheng et al., 2018).

In this study, N and P-cycling enzyme activities were mainly positively affected by MBC and MBP, followed by AN (Figure 7). The result indicates that some biotic factors (e.g., microbial biomass) were more critical to soil enzyme activities. The soil microbial biomass is not only a key and highly active pool for storing soil nutrients but also a sensitive microbial activity indicator to reflect soil quality (Muñoz et al., 2017). Under long-term mulching, surface residues can provide microorganisms with sufficient metabolic substrates, promote the absorption and utilization of C, P, and other elements by microorganisms, and then accelerate the secretion of N and P-cycling enzymes. Some studies also found that MBC was positively correlated with soil enzyme activity (Bowles et al., 2014; Sun et al., 2021).

We also found that MC positively affected soil enzyme activities (Figure 7). Moisture is an essential determinant of soil enzyme activity, which increases with soil MC (Baldrian et al., 2013). LGM could enhance soil porosity and promote water infiltration and storage (Blanco-Canqui et al., 2011; Basche et al., 2016). Soil structure gradually improved with mulching ages. The increase in enzyme activity may be correlated to the enhancement of permeability and agglomeration ability (Roldán et al., 2005). In addition, soil moisture is essential in maintaining MBC (Kader et al., 2017). This study demonstrated a significant positive correlation between MBC and MC (Figures 5, 7). Therefore, the positive effect of MC on enzyme activity may be attributed to the mediating role of MBC.

In addition, except for soil physicochemical properties, soil enzyme activities were also affected by other factors, e.g., climate, soil type, and management measure. Previous studies have found that soil enzyme activities can be affected by climate, i.e., temperature and precipitation (Zhou et al., 2013; Jian et al., 2021). The effects of temperature on soil enzyme activities were directly correlated with the variations in the kinetic characteristics of enzymes (Steinweg et al., 2013). The increase in temperature can generally enhance C and N-cycling enzyme activities (Wallenstein et al., 2009). However, some studies have shown opposite conclusions (A'Bear et al., 2014). Soil moisture closely related to precipitation was positive with enzyme activities, which was another important factor affecting enzyme activities (Steinweg et al., 2012). Soil enzyme activity was also affected by soil type, which may be closely related to unique soil properties, such as texture (Acosta-Martínez et al., 2007; Štursová and Baldrian, 2010; Zhang et al., 2017). Moreover, management measures, such as fertilization and mulching materials and methods, can also affect soil enzyme activity in the orchard ecosystem (Kader et al., 2017; Zheng et al., 2020). Previous studies found that green organic manure significantly increased soil enzyme activity compared to inorganic fertilization (Piotrowska and Wilczewski, 2012). Orchard grass (Gramineae) with high C/N can improve the C-cycling enzyme activity while grass (Leguminosae) with low C/N can promote the N-cycling enzyme activity (Wang et al., 2020b). The mixture of legumes and other grasses was more conducive to promoting soil enzyme activity compared to single grass mulching (Chavarría et al., 2016). In this study, consistent management measures were adopted at all of the experimental plots to avoid the impact of human interference and then highlight the effect of mulching age on soil enzyme activities. This study focused on the influence of soil biological and physicochemical properties on enzyme activities. In our previous research, the effects of cultivated grass (*Vicia Villosa*) and natural grass (*Galium spurium* and *Stellaria media*) on soil enzyme activities were preliminarily compared. The results show that the improvement of soil enzyme activities by leguminous grass mulching was higher than that by natural grass mulching (Wang et al., 2023). This result emphasized the importance of selecting appropriate grass types in the orchard ecosystem. Therefore, in future research, different influencing factors should be further studied to implement more sustainable practices.

Conclusion

In general, long-term mulching (the VV_8 treatment) effectively improved soil nutrient and enzyme activity levels. However, short-term mulching (the VV_4 treatment) had fewer effects on soil biochemical properties. The improvement effect of living grass mulching on soil enzyme activities was soil layer-dependent. Long-term mulching can affect deeper soil layers than short-term mulching. Compared to other soil properties, available nutrients (i.e., MBC, MBP, and AN) had significant effects on enzyme activities. Thus, soil enzyme activities could be improved through enhanced available nutrients. In addition, this study indicates that increasing nutrient availability by extending mulching age can be an effective strategy for sustainable soil management in orchard systems.

Data availability statement

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

Author contributions

NW: conceptualization, investigation, data curation, methodology, formal analysis, visualization, writing - original Draft, writing - review and editing. LL: formal analysis, visualization, writing - review and editing. MG: data collection, writing - review and editing. ZJ: investigation, writing - review and editing. JH: investigation. HC: visualization. WX: resources, supervision, project administration. CL: conceptualization, supervision, project administration, funding acquisition. All authors have read and approved the manuscript.

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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

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Effect of planting and mowing cover crops as livestock feed on soil quality and pear production

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Introduction: The increasing demand for animal-products has led to an increasing demand for livestock feed. Using cover crop as green manure in orchards is an effective measure to improve fruit yield and quality. However, the effect of mowing cover forage crops as livestock feed on soil quality and crop production is unclear.

Method: Therefore, a 4-year field experiment, which included two treatments, was conducted in pear orchards in Luniao County, China: natural grass (NG) and planting and mowing forage crop ryegrass as livestock feed (MF).

Results: Under MF treatment, most soil nutrient content, especially alkalihydrolysable N (AN), total phosphate (TP), available phosphate (AP), and microbial biomass phosphate (MBP), had decreased significantly ($P<0.05$), while β-D-glucosidase (BG, C-cycle enzyme) and soil C limitation at 10–20 cm depth and P limitation at subsoil (20–40 cm) was increased. In addition, the soil bacterial community component in topsoil (0–10 cm and 10–20 cm) and fungal community component in topsoil and subsoil were changed in the MF treatment. Network analysis showed that MF treatment had a lower edge number in topsoil but the community edge numbers increased from 12794 in NG to 13676 in MF in subsoil. The average weight degree of the three soil layers in MF treatment were reduced, but the modularity had increased than that in NG. For crop production, MF treatment was 1.39 times higher in pear yield and titratable acids (AC) reduced from 0.19% to 0.13% compared with NG. These changes were more associated with the indicators at the subsoil, especially for TP, AN, pH, and F-NMDS1 (non-metric multidimensional scaling (NMDS) axis 1 of fungi).

Discussion: These results provide data support for the feasibility of planting and mowing forage crops as livestock feed on orchards as well as a new idea for the integration of crop and livestock.

KEYWORDS

community component, different depth, mowing forage crop, pear yield, soil nutrient content, titratable acids

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1 Introduction

To meet the demand of the affluent population for animal-sourced food, animal products have rapidly increased in the recent decades (Raney et al., 2009). In developed countries, over half of the protein was supplied by animal products and a sharp increase was experienced in developing countries (FAO, 2019). To ensure the supply of these animal products, the number of feedlots is rapidly increasing, which means more forage should be supplied. At the same time, humans are facing a great challenge regarding food supply to ensure food security. It has been determined that the total grain production needs to be increased by 60%–110% to meet the demand in 2050 when compared with the current levels (Tilman et al., 2011). Therefore, it is critical to provide sufficient feed for livestock while not compromising food security. At present, grass is a vital forage for livestock production, accounting for approximately 50% of the total livestock intake (Hasha, 2002; Herrero et al., 2013). However, in China, the world's largest market for animal products, grassland productivity has dropped significantly in recent years, resulting in the great disparity between the supply and demand of forage across different seasons and regions (Li et al., 2016; Jin et al., 2021). Thus, exploring different grass planting patterns can effectively solve the insufficient feed problem.

Fruit orchard is an important component of the agricultural industry in China, with approximately 1 billion hectares of land area and 32% of the total yield of the world in 2018 (FAO, 2019). However, in most commercial orchards, the inter-canopy area is usually bare soil caused by intensive management of herbicides and soil tillage (Fang et al., 2021). Intercropping cover crops with orchards can supply forage, which is a sustainable orchard management strategy. Compared with bare orchards, natural grass has a positive effect on soil physicochemical properties, microbial activities, and fruit yield and quality (Hoyt and Hargrove, 1986; Wardle et al., 2001; Milgroom et al., 2007). Monteiro and Lopes (2007) demonstrated that natural grass is more conducive to achieve a balanced supply of mineral elements and improve fruit yield and quality. In fact, many forage crops, which can be used as livestock feed, have been grown in orchards as cover crops. For example, ryegrass (*Lolium perenne L.*) was usually treated as a cover crop in fruit orchards (Wang et al., 2020; Piltz et al., 2021). After a cover crop is harvested, it is generally spread on the orchard surface as green manure. The positive effects of the long-term application of green manure on soil quality and fruit production have been reported. Green manure can directly improve soil properties and fertility and indirectly affect the activity and community structure of microorganisms to reduce the occurrence ratio of diseases, and improve yield and quality of fruit trees (Srivastavaak et al., 2007; Gomez-munoz et al., 2014; Wang et al., 2016; Deakin et al., 2018). However, the effect of planting and mowing cover crops as livestock feed on soil quality and fruit production is unclear.

Compared with northern China, the temperature and humidity conditions of pear orchards in southern China are more suitable for rapid growth of forage grass. In addition, pear (Pyrus spp.) is a vital cash crop that is widely cultivated in China. The annual ryegrass is characterized by a high grass yield and strong cold resistance, and is a common winter cover crop in the orchards of southern China (Fu et al., 2021). Thus, this study on pears was undertaken with following objectives: To 1) assess the effect of mowing ryegrass cover crops on soil quality at different depths; 2) quantify the influence of mowing cover crops on pear yield and quality; 3) analyze the correlation between soil quality indicators at different depths and pear production.

2 Material and methods

2.1 Description of study site

The study site was located at Luniao county, Zhejiang province, China (30°27′N–30°28′N, 119°43′E–119°46′E). This region is characterized by subtropical monsoon climate with a mean annual precipitation and temperature of 1350 mm and 16.0°C, respectively. The main cultivar is "Cuiguan", with an area of 533.3 ha.

The spacing in the rows and between rows of every 22-year-old tree was 4 m and 3 m, respectively. To analyze the effect of mowing cover crops as livestock feed on soil quality, and pear yield and quality, we set up two treatments since 2018: (1) natural grass (NG); and (2) planting and mowing ryegrass cover crop as livestock feed (MF). The annual ryegrass (*Lolium multiflorum Lam.*) planted in November and mowed three times in March, April, and May after the next year was the same with NG treatment. According to the local smallholders, the total N, P_2O_5, and K_2O fertilization rates in different treatments were the same, with 424.2 kg ha^{-1}, 386.4 kg ha^{-1}, and 323.4 kg ha^{-1}, respectively, and the other management was similar.

2.2 Soil sampling and determination of soil properties

Soil sampling was done in July 2021. The soil samples were collected from the same pear trees as the pear samples. The soil was divided into topsoil (0–10 cm, 10–20 cm) and subsoil (20–40 cm), and each soil sample was a mixed sample of five replications. After being mixed evenly, the soil sample was divided into three parts: the first part was stored at −80°C for the analysis of soil bacteria and fungi; the second part was used as fresh soil to measure soil microbial biomass and enzyme activity; and the last one was air-dried to determine the soil physicochemical properties.

Soil pH, soil organic carbon (SOC), total nitrogen (TN), alkalihydrolysable N (AN), total phosphate (TP), available phosphate (AP), and available potassium (AK) were measured using standard methods as described in Bao (2000). Microbial biomass carbon (MBC), microbial biomass nitrogen (MBN), and microbial biomass phosphorus (MBP) were measured using the chloroform fumigation extraction method (Brookes et al., 1985; Vance et al., 1987).

2.3 Soil enzyme activities and microbial resource limitation

C acquisition enzyme activities [β-D-glucosidase (BG)], N acquisition enzyme activities [β-Nacetylglucosaminidase (NAG)], and P acquisition enzyme activities [acid phosphatase (ACP)] were determined by the 96-well-microplate protocols German et al. (2011) and the detailed method was described in Chen et al. (2018) and Jing et al. (2017).

Microbial resource limitation was calculated using the vector analysis of ecoenzymatic stoichiometry. The length (L) and vector angle (A°) were calculated according to Moorhead et al. (2016). A relatively longer vector L means a greater C limitation. A larger Vector greater than 45° indicates P limitation, and a lower Vector less than 45° indicates N limitation. The calculation of vector L and Vector A are Equation (1) and (2):

$$\text{Vector L} = \sqrt{\left(\frac{\ln BG}{\ln ACP}\right)^2 + \left(\frac{\ln BG}{\ln NAG}\right)^2} \quad (1)$$

$$\text{Vector A}\,(\,^\circ\,) = \text{Degrees}\,(\text{ATAN2}(\left(\frac{\ln BG}{\ln ACP}, \frac{\ln BG}{\ln NAG}\right))) \quad (2)$$

2.4 Soil bacterial and fungal community composition

Soil DNA was extracted from approximately 0.5 g of soil with the FastDNA Spin Kit (MP Biomedicals, Solon, OH, USA) according to the manufacturer's instructions. The spectrophotometer (Thermo Scientific, Waltham, MA, USA) was used to analyze the concentration and quality of the DNA obtained. The 16SrRNA genes in the V4–V5 region were amplified by the primers 515F (GTGCCAGCMGCCGC GGTAA) and 907R (CCGTCAATTCMTTTRAGTTT) and the ITS1 region were amplified using the primers ITS1-F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2-2043R (5′-GCTGCGTTCT TCATCG ATGC-3′) (Xiong et al., 2017). The PCR cycle conditions were the same with that of Tang et al. (2022). Sequencing was performed by Illumina NovaSeq platform. Each representative sequence was processed using the UNITE (version 8.0, https://unite.ut.ee) and SILVA (https://www.arb-silva.de/) database. The total bacterial and fungal high-quality sequences were 2,639,562 and 2,637,285 with an average read count per sample of 87,985 (ranging from 84,087 to 91,568) and 87,909 (ranging from 83,869 to 91,799), respectively.

2.5 Analysis of pear yield and quality

Fruits were sampled simultaneously with the soil samples. Five healthy pear trees from NG and MF treatment were selected and each tree picked up eight peripheral fruits from different positions (east, south, west, and north). All fruits were taken back to the laboratory for analysis of yield and quality indicators.

Pear yield is obtained by multiplying the number of fruit per tree by the average weight per fruit used to determine fruit quality. The titratable acid (AC) and soluble solids were determined by NaOH neutralization titration method and Abbe refractometer determination method, respectively; 2–6 dichloroindophenol titration and salicylic acid method was used to determine the content of Vitamin C and soluble sugar, according to Zhang et al. (2020).

2.6 Data visualization and statistical analysis

Data processing and visualization were performed using R software (version 4.0.3), Microsoft Office Excel 2016 (Microsoft Corporation, Redmond, WA, USA) and SPSS 20.0 (SPSS Inc., Chicago, IL, USA). The differences in soil properties between soil layers, soil treatments, and their interactions were conducted using one-way analysis of variance (ANOVA) using SPSS 20.0. Significant differences were detected using a least significant difference multiple range test with $p \leq 0.05$. The difference of soil bacterial and fungal community structure was evaluated by nonmetric multidimensional scaling (NMDS), based on Bray–Curtis dissimilarities. The first 300 fungal operational taxonomic units were selected for co-occurrence network analysis to uncover the bacterial and fungal relationships. The correlations between the different components of the microbiome were derived by the "psych" package in R and correlation coefficients (R) >0.8. BH-adjusted p-values<0.05 were selected to construct the co-occurrence network and Gephi (http://gephi.github.io/) was used to generate network visualization. The relationship between soil quality indicator and pear yield and quality were performed using the "corrplot" package in R and 'randomForest' package was carried out to achieve random forest analysis.

3 Results

3.1 Soil quality

3.1.1 Soil chemical properties

Nutrient content decreased with increasing soil depth, except for soil pH and MBP (Table 1). At the same depth, there were no significant differences in TN and MBN between NG and MF treatment (Table 1). At all the three depths, the content of AN, TP, AP, and MBP under MF treatment was lower than that of NG (Table 1). Compared with NG, the SOC at 10–20 cm had significantly decreased while MBC was significantly increased in MF treatment (Table 1). The levels of soil pH at 10–20 cm and 20–40 cm depth in MF treatment were significantly higher ($P< 0.05$) than those in NG and different depths had no significant effect on soil TN and MBN (Table 1).

3.1.2 Soil enzyme activity

The activities of BG at 10–20 cm and ACP at 0–10 cm in MF were 1.6 and 1.5 times higher ($P< 0.05$) than that in NG, respectively, and no significant difference was observed on NAG (Figure 1). For stoichiometric constraint, MF treatments showed a stronger C limitation at 10–20 cm, with a longer vector L, and P limitation at 20–40 cm, as demonstrated by a vector angle >45°, compared to NG (Figure 1).

3.1.3 Soil microbial community diversity and composition

The different treatments had no significant effects on soil bacterial and fungi richness and diversity, despite an increasing tendency (Chao1 and Shannon; Table S1). However, the microbial community structure was differed between NG and MF. The results of NMDS based on Bray–Curtis dissimilarities suggested that in different treatments soil bacterial community structure of 0–10 cm and 10–20 cm and fungi community structure of 0–10 cm, 10–20 cm, and 20–40 cm were separated (Figures 2A, C).

The changes in the relative abundance of soil bacterial communities at phylum level in the 0–10 cm and 10–20 cm soil layers were similar (Figure 2A). Majority of the soil bacterial community is made up of *Proteobacteria* phyla, which accounts for 30.0% on average, with no significant differences from other groups (Figure 2B). The relative abundance (RA) of *Acidobacteria* was decreased while *Actinobacteria* was increased in 0–10 cm and 10–20 cm soil layers under MF treatment compared with NG (Figure 2B). For fungi composition, the dominant phyla included *Ascomycota*, *Basidiomycota*, *Rozellomycota, Mortierellomycota, and Chytridiomycota*, accounting for more than 70% of all fungi communities (Figure 2D). MF decreased the RA of *Basidiomycota* and *Mortierellomycota* at 0–10 cm but increased that of *Ascomycota* (Figure 2D). For the 20–40 cm soil layer, the abundance of above fungi increased in the MF treatment (Figure 2D). Compared with NG, the RA of *Chytridiomycota* was decreased at 0–10 cm and 10–20 cm depths and that at 20–40 cm depth was increased in MF (Figure 2D).

3.1.4 Soil microbial network stability

Network analysis results showed that MF had a lower edge number in 0–10 cm and 10–20 cm depth compared with NG, in which the edge numbers were 13184 and 12381, respectively (Figure 3 and Table S2). However, community edge numbers

TABLE 1 Soil chemical characteristics of different soil layers (0–10 cm, 10–20 cm, and 20–40 cm) under different treatments (NG, natural grass; MF, planting ryegrass and mowing as feed).

Indicator	NG			MF		
	0-10cm	10-20cm	20-40cm	0-10cm	10-20cm	20-40cm
pH	5.12 ± 0.33bc	5.04 ± 0.40c	5.07 ± 0.30c	5.45 ± 0.11ab	5.44 ± 0.08ab	5.55 ± 0.14a
SOC	21.49 ± 2.61a	16.14 ± 2.33b	9.80 ± 3.39c	18.50 ± 2.97ab	10.83 ± 1.20c	8.53 ± 2.58c
TN	1.34 ± 0.13a	0.95 ± 0.25bc	0.71 ± 0.16d	1.16 ± 0.20ab	0.79 ± 0.12cd	0.67 ± 0.14d
AN	185.08 ± 27.38a	127.68 ± 8.19b	79.80 ± 13.61c	136.64 ± 10.96b	99.12 ± 15.79c	50.96 ± 5.29d
TP	1.34 ± 0.20a	1.21 ± 0.18a	0.90 ± 0.06b	0.89 ± 0.10b	0.72 ± 0.14bc	0.61 ± 0.10c
AP	100.87 ± 29.75a	89.60 ± 13.81a	50.93 ± 14.40b	53.83 ± 20.34b	32.83 ± 18.68bc	10.50 ± 8.2c
AK	255.00 ± 32.79a	181.00 ± 15.65bc	151.40 ± 32.49bc	205.33 ± 67.47b	172.80 ± 53.87bc	127.2 ± 39.8c
MBC	105.9 ± 9.8a	86.9 ± 8.1b	72.3 ± 12b	104.5 ± 7a	102.3 ± 9.6a	82.5 ± 4.3b
MBN	18.5 ± 11.4a	25.4 ± 11.8a	18.8 ± 11.1a	28.6 ± 10.4a	29.5 ± 24.1a	19.8 ± 6.5a
MBP	78.3 ± 37.3a	100.7 ± 43.3a	36.5 ± 20.2b	36.7 ± 19.8b	37.4 ± 16.5b	10.8 ± 11.4c

Values in the same column following different letters suggest significant differences ($p< 0.05$). Mean ± standard deviation is presented on different treatments. SOC, soil organic carbon; TN, total nitrogen; TP, total phosphate; AN, alkalihydrolysable N; AP, available phosphate; AK, available potassium; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen; MBP, microbial biomass phosphorus.

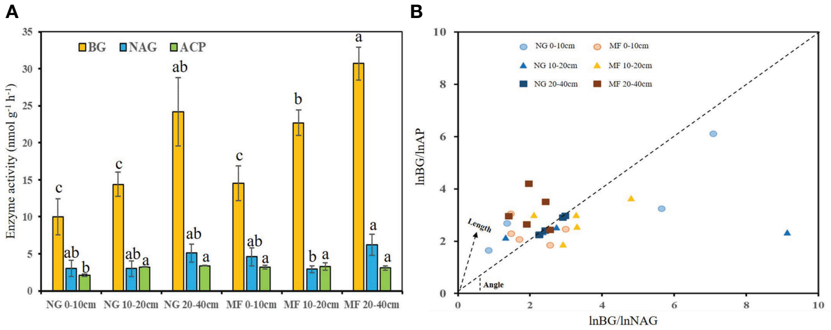

FIGURE 1
Enzyme activity **(A)** and enzyme stoichiometric constraints **(B)** of different soil layers (0–10 cm, 10–20 cm, and 20–40 cm) under different treatments (NG, natural grass; MF, planting ryegrass and mowing for feed). Changes of vector length (L) and vector angle (A∘) were calculated according to the ratios of the log transformed BG, NAG, and AP (Moorhead et al. 2016). Longer vector L indicates greater C limitation. A vector angle of < 45∘ denotes N limitation, angles > 45∘ denote P limitation. Lowercase letters a, b and c indicate a significant difference ($p < 0.05$). BG, β-D-glucosidase; NAG, β-Nacetylglucosaminidase; ACP, acid phosphatase.

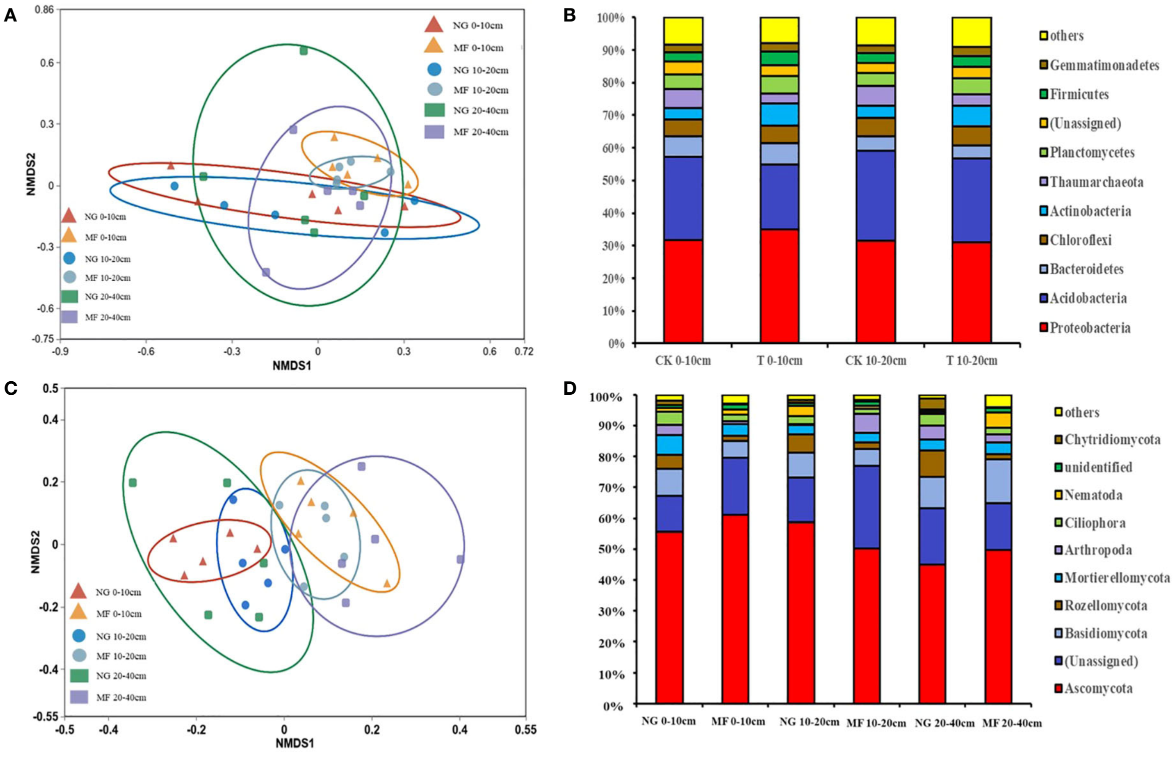

FIGURE 2
Non-metric multidimensional scaling (NMDS) based on Bray–Curtis dissimilarities **(A, C)** and relative abundance **(B, D)** of bacteria and fungi communities in different soil layers (0–10 cm, 10–20 cm, and 20–40 cm) of different treatments at phylum level. NG, natural grass; MF, planting ryegrass and mowing as feed. SOC, soil organic carbon; TN, total nitrogen; TP, total phosphate; AN, alkalihydrolysable N; AP, available phosphate; AK, available potassium; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen; MBP, microbial biomass phosphorus.

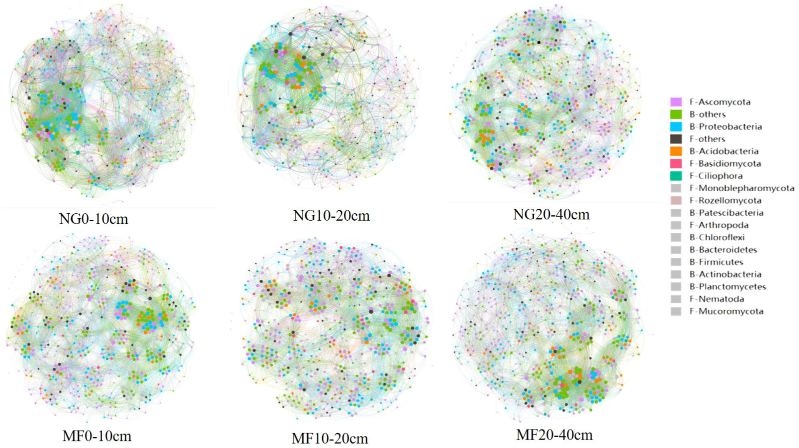

FIGURE 3
Soil bacterial and fungal co-occurrence networks between NG and MF at different depths based on Spearman's correlation coefficient (r) at the phylum level (|r| > 0.6, P-value<0.05). Node size is proportional to the connectivity (degree) of each phylum and the different colors indicate different phylum.

increased from 12794 in NG to 13676 in MF at 20–40 cm. In addition, the average weight degree of the three soil layers in the MF treatment were reduced, but the modularity was increased.

The positive and negative links between bacteria and bacteria (B-B), fungi and fungi (F-F), and bacteria and fungi (B-F) at the phylum level were analyzed (Table 2). In the three soil layers, the total proportion of positive links in MF was decreased compared with that of NG. For the different soil depths, the proportion of positive correlation on F-F increased, and that of negative correlation decreased at 0–10 cm depth; at 10–20 cm depth, the total link and negative link of F-F increased while that of B-B decreased; the negative link of B-B and B-F was increased while the positive link of F-F and B-F was decreased at 20–40 cm depth.

3.2 Pear yield and quality

Different practices had a significant effect on pear yield and quality. Pear yield under MF treatment was significantly higher than that under NG (1.39 times; Table 3). For pear quality, the content of AC was significantly decreased from 0.19% to 0.13%

TABLE 2 Numbers of links in the networks of B-B (Bacterial-Bacterial), B-F (Bacterial-Fungi), and F-F (Fungi-Fungi) obtained for soil samples under different treatments (NG, natural grass; MF, planting ryegrass and mowing as feed) at different depths (0–10 cm,10–20 cm, and 20–40 cm).

	NG0-10cm	NG10-20cm	NG20-40cm	MF0-10cm	MF10-20cm	MF20-40cm
Total links	14566	16200	12794	13814	12831	13676
Positive link	54.1	56.4	62.2	52.3	54.5	53.8
Negative link	45.9	43.6	27.8	47.7	45.5	46.2
B-B						
Positive link	2710 (18.6%)	3340 (20.6%)	2484 (19.4%)	2504 (18.1%)	2438 (19.0%)	2616 (19.1%)
Negative link	2228 (15.3%)	2502 (15.4%)	1250 (9.7%)	2033 (14.7%)	1126 (8.8%)	1930 (14.1%)
F-F						
Positive link	1117 (7.7%)	2201 (13.6%)	1976 (15.4%)	1562 (11.3%)	1821 (14.2%)	1549 (11.3%)
Negative link	1902 (13.1%)	1118 (6.9%)	1129 (8.8%)	1361 (9.9%)	1297 (10.1%)	1343 (9.8%)
B-F						
Positive link	3266 (22.4%)	3490 (21.5%)	3493 (27.3%)	3155 (22.8%)	2731 (21.3%)	3197 (23.4%)
Negative link	3343 (23.0%)	3449 (21.3%)	2462 (19.2%)	3199 (23.2%)	3418 (26.6%)	3041 (22.2%)

TABLE 3 Effect of different cultivation modes on pear yield and quality.

	Yield (t ha^{-1})	Soluble solids (%)	Titratable acids (%)	Vitamin C (mg/100g)	Soluble sugars (%)
NG	7.40 ± 1.31a	11.33 ± 0.65a	0.19 ± 0.05a	4.61 ± 2.96a	6.11 ± 1.06a
MF	10.30 ± 1.06b	11.21 ± 0.60a	0.13 ± 0.06b	4.07 ± 2.17a	6.18 ± 1.26a

Values are mean ± standard deviation from 5 replicates. Lowercase letters a and b indicate a significant difference ($p< 0.05$). NG, natural grass; T, planting ryegrass and mowing as feed.

while there were no differences in the amount of soluble solids, Vitamin C, and soluble sugars (Table 1).

3.3 Correlation of soil quality indicators, pear yield and quality

At all the three soil depths, AN, TP, and AP had a positive effect on yield, while F-NMDS1 showed a negative effect (Figure 4). In addition, F-NMDS1 positively affected AC at the three depths and AN, TP, and AP had negative effects on AC at 10–20 cm and 20-40 cm soil layers (Figure 4).

The random forest models were used to assess the percentage of the total explained variance and the importance of different variables to the change in yield and AC (Table 4). The results indicated that the relevant indicators in the 20–40 cm soil layer had the highest percentage of explained variance, with 87.4% and 58.8%. The important factors affecting the yield were TP, F-NMDS1, AN, and AP. The AC depended on AN, pH, TP, and F-NMDS1, and AN was the main factor affecting pear quality.

4 Discussion

4.1 Effect of mowing forage crop on soil quality

With the gradual increase in soil depth, the content of most soil nutrients gradually decreased, possibly as a result of fertilization management, which is basically applied to the ground surface. At the same depth, planting ryegrass can significantly increase pH in acidic soil, which similar to the findings of Zhao et al. (2022); MF treatment significantly reduced the contents of AN, TP, and AP in all soil layers, which was mainly related to the fact that ryegrass absorbs nutrients from the soil during the growth process (Liu et al., 2013). At 10–20 cm, SOC of MF treatment soil significantly decreased, but MBC increased significantly, which may be because root exudates stimulate the activity of microorganisms and promote the uptake of carbon by microorganisms.

Soil enzyme activity is an important indicator of soil nutrients cycling (Nannipieri et al., 2012; Hussain et al., 2021). MF increased soil BG, which is consistent with the results of Fernandez et al. (2016). Because BG is primarily responsible for the degradation of macromolecular compounds in plant residues (Zheng et al., 2018), the significant increase in BG in the 10–20 cm soil layer may be related to the large distribution of ryegrass roots. Chitin is mainly generated from fungal cell walls (Zheng et al., 2018), and NAG enzymes are primarily used to degrade chitin to promote nitrogen bioavailability. The insignificant differences in fungal abundance and diversity between different treatments may be an important reason for the lack of significant differences in NAG enzymes. From the perspective of enzyme stoichiometry, the MF treatment showed a greater C limitation in the 10–20 cm soil layer and a greater P limitation in the 20–40 cm soil layer which was mainly associated with the significant decrease in SOM and soil P content, respectively. Therefore, when planting ryegrass in pear orchards and mowing them as feed, measures should be taken to increase SOM in topsoil, and ensure the supply of soil phosphorus in subsoil.

Different treatments had no significant effects on soil microbial richness and diversity, but the bacterial community composition in 0–10 cm and 10–20 cm and fungi in 0–10 cm, 10–20 cm, and 20–40 cm changed greatly under different treatments. Under MF treatment, the greater abundance of *Acidobacteria* under natural grass maybe related to the diversity of plant species under this condition (Foesel et al., 2013), and the increase in abundance in *Actinobacteria* was mainly because microorganisms could only use stubborn carbon sources as carbon source as soil organic C content was decreased in MF (Mbuthia et al., 2015). For fungi community composition, *Ascomycota* and *Basidiomycota* were the most prevalent phyla, which is consistent with previous research on agricultural soil (Feng et al., 2015; Ding et al., 2017). *Ascomycota* is the most ubiquitous phylum, which can decompose the organic substrate (Ye et al., 2020) and *Basidiomycota* can produce massive fruiting bodies and cause decomposition of litter (Frac et al., 2021). *Mortierellomycota*, which plays an important role in the carbon cycle and decomposition of organic matter (Muneer et al., 2021), was found in the different treatments. These fungi play a significant role in storing mineral nutrients, metabolites, and water (Ozimek and Hanaka, 2021), and the increase of RA at 20–40 cm could be very advantageous to plants. The *Chytridiomycota* phylum acts as a bio-converter and decomposer and is a vital component in modern ecosystems (Gleason et al., 2005). The change of this fungi may have had a significant impact on ecosystem functioning.

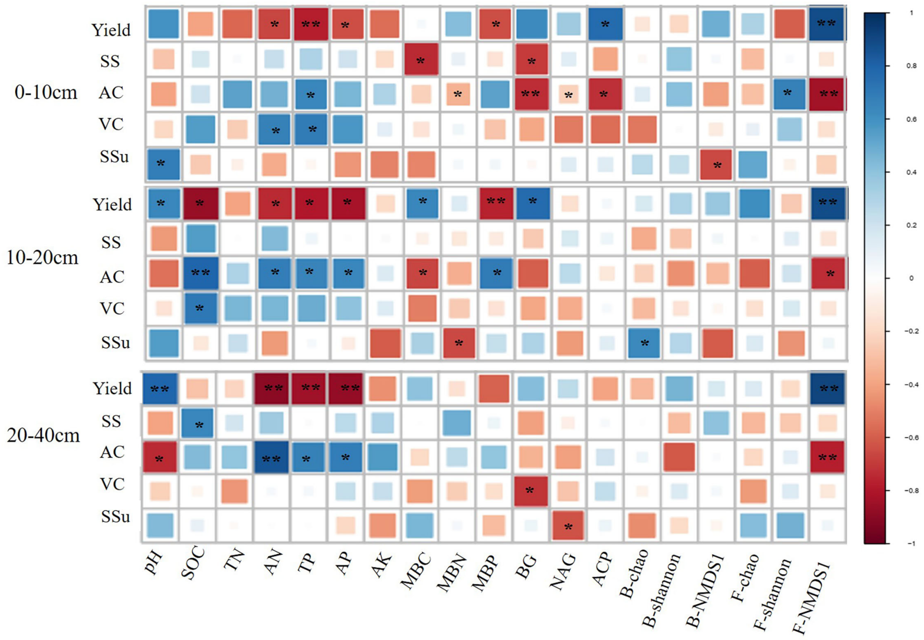

FIGURE 4
Relationship between soil quality indicators, pear yield and quality at different depths. * and ** represent the significant at 0.05 and 0.01 level, respectively. SOC, soil organic carbon; TN, total nitrogen; TP, total phosphate; AN, alkalihydrolysable N; AP, available phosphate; AK, available potassium; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen; MBP, microbial biomass phosphorus. BG, β-D-glucosidase; NAG, β-Nacetylglucosaminidase; ACP, acid phosphatase; B-chao, bacterial chao index; B-shannon, bacterial Shannon index; B-NMDS1, non-metric multidimensional scaling (NMDS) axis 1 values of the bacterial community; F-chao, fungal chao index; F-shannon, fungal shannon index; F-NMDS1, non-metric multidimensional scaling (NMDS) axis 1 values of the fungal community; SS, Soluble solids; AC, Titratable acids; VC, Vitamin C; SSu, Soluble sugars.

MF treatment had more modularity, which means it is more stable compared with NG (Deng et al., 2012). Network analysis showed that the positive links were decreased in all the three depths, which indicated that MF reduces microbial cooperation and increases competition. Compared with NG, more F-F positive links and less F-F negative links existed in the 0–10 cm depth in MF treatment, which may be because the carbon source of ryegrass treatment is single, and the fungal microorganisms cooperate to use the carbon source when decomposing. For 10–20 cm, the links of F-F were increased and that of B-B were decreased in MF, which may be related to the significant reduction of SOM in the 10–20 cm soil layer. Bacteria are generally sensitive to small molecules and readily available carbon sources, while fungi are good at utilizing carbon sources in refractory organic matter (Caesar-TonThat et al., 2010; Zhong et al., 2018). In the 20–40 cm soil layer, competition (negative links reducing and positive links increasing) among F-F, B-F, and B-B increased may be due to limited resource conditions.

4.2 Change of pear yield and quality

Cover cropping has been used as an important and effective method to improve fruit yield and quality (Fang et al., 2021). Many studies have reported that it can improve soil physical structure (Garcia et al., 2018) and enhance nutrient status (Sanchez et al., 2007; Wei et al., 2017). In the subtropics and temperate or humid zones, ground cover management had a greater benefit on fruit yield, possibly due to the formation of abundant cover crop biomass because of adequate precipitation (Fang et al., 2021). However, there is little information about the effect of mowing forage cover crop on yield and quality. Our results showed that mowing cover crop can improve pear yield and reduce AC compared with natural grass. Many studies have reported that excessive fertilizer application to pear orchards results in high soil nutrition content and negative impact on pear yield and quality, particularly in China (Fu et al., 2021; Wang et al., 2021). The results in southern China indicated that the

TABLE 4 Top four importance (percentage of increase in mean square error, %IncMSE) of variables and the percentage of the explained variance (Varex) to the change in yield and AC using the random forest models.

Depth	Rank	Yield			AC		
		Variable	%IncMSE	Var_{ex}(%)	Variable	%IncMSE	Var_{ex}(%)
0-10cm	1	F-NMDS1	7.7	24.8	BG	6.8	38.4
	2	ACP	5.2		F-NMDS1	6.6	
	3	TP	5.1		TP	4.7	
	4	AN	4.7		ACP	4.0	
10-20cm	1	AP	6.3	80.6	MBC	7.4	46.6
	2	AN	6.1		SOC	6.5	
	3	SOC	5.8		MBP	6.1	
	4	BG	5.8		AN	3.6	
20-40cm	1	TP	8.0	87.4	AN	10.2	58.8
	2	F-NMDS1	7.8		pH	7.3	
	3	AN	7.6		TP	4.7	
	4	AP	7.2		F-NMDS1	4.2	

SOC, soil organic carbon; TP, total phosphate; AN, alkalihydrolysable N; AP, available phosphate; MBC, microbial biomass carbon; MBP, microbial biomass phosphorus. BG, β-D-glucosidase; ACP, acid phosphatase; F-NMDS1, non-metric multidimensional scaling (NMDS) axis 1 values of the fungal community; SS, Soluble solids; AC, Titratable acids; VC, Vitamin C; SSu, Soluble sugars.

suitable range of soil AP for sand pears is 10–40 mg kg^{-1}, while our research found that the nutrient content under natural grass conditions is more than 50 mg kg^{-1} (Li et al., 2008; Table 1). The negative effects on pears may have resulted from too much soil nutrition content, but mowing cover crops removes some of the nutrition and reduces the soil nutrient content into a suitable range. The improvement of soil conditions may enhance fruit tree growth, resulting in higher yields. In addition, Qiu (2021) indicated that the suitable soil pH for the southern pears of China ranges from 5.6 to 7.2 and our results proved that an increase in pH can directly improve pear yield (Figure 4). At the same time, the result that ground cover management can significantly reduce fruit acidity to improve fruit quality is similar to that reported by Fang et al. (2021).

The random forest models showed that yield and AC were more associated with the relevant indicators in the subsoil (20–40 cm) rather than those in the topsoil (0–10 cm and 10–20 cm). The results of Zhang (1997) indicated that more than 70% of the roots of pear trees are mainly distributed below 20 cm, so the change of soil physicochemical properties in the subsoil layer has greater impact on pear production. Kuhn and Pedersen (2009) suggested that higher yields and quality under cover crops maybe related to suitable soil concentrations. Therefore, a decrease in AN, TP, and AP contribute to the improvement of yield and quality. In addition, F-NMDS1 has great impact on yield and quality, similar to the results of Tang et al. (2023). In general, under the condition of planting ryegrass and mowing, it is necessary to pay more attention to the changes in soil properties of the subsoil.

5 Conclusions

The results indicated that MF reduces the soil nutrient content of AN, TP, AP, and MBP at different depths, increases BG at 10–20 cm depth, and changes enzyme stoichiometric ratio (increasing the C limitation in the 10–20 cm and P limitation in the 20–40 cm layer) compared with NG. In addition, soil bacterial community component in topsoil, fungal community component in topsoil and subsoil, and the soil microbial stability were changed under MF treatment. For crop production, MF treatment showed higher pear yield and lower AC and these indicators were more related to subsoil properties (TP, AN, pH, and FNMDS1). This study revealed mowing cover cops as livestock feed can optimised soil quality and pear production especially for high soil fertility soil caused by excessive fertilization and future studies should focus on subsoil management, especially for TP and AN after planting and mowing ryegrass.

Data availability statement

The raw data presented in the study are deposited in the NCBI repository, accession numbers PRJNA910514 and PRJNA910501.

Author contributions

HF: Formal analysis, Writing - original draft. HC: Formal analysis. QM: Writing - review & editing. KH: Formal analysis. SW: Conceptualization. LW: Conceptualization. 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

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Impacts of summer-seeded legume cover crops and termination-tillage method on nitrogen availability to corn in rotation

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Summer-seeding legume cover crops can reduce erosion, mitigate nitrate leaching losses during the non-growing season, and provide bio-accumulated nitrogen (N) to the following crop. Very little information is available, however, on the most effective methods for terminating summer-seeded cover crops, or on the amount of N they can provide to subsequent crops. We therefore evaluated the impacts of selected legume cover crops and cover crop termination-tillage methods on corn (*Zea mays* L.) N credit and grain yield in a soybean (*Glycine max* Merr.) - winter wheat (*Triticum aestivum* L.)/cover crop - corn rotation over 3 consecutive years on a clay loam soil in southwestern Ontario. The cover crops were summer-seeded after wheat harvest, and included hairy vetch (*Vicia villosa* L. Roth), red clover (*Trifolium pratense* L.), white clover plus crimson clover mix (*incarnatum* and *Trifolium alba* L.), and a no cover crop control (CK). The cover crops were nested within termination-tillage method, including moldboard plow-down in fall, and herbicide spray-down in spring under strip-tillage or no-tillage then corn planting. Nitrogen fertilizer to corn was applied *via* side-dress at 200 kg N ha^{-1} to the CKs, and at 100 kg N ha^{-1} to the cover crops. Averaged over 3 years, above-ground biomass N level followed the pattern: hairy vetch > white clover plus crimson clover mix > red clover for fall plow-down termination. There were no significant differences in cover crop biomass N levels for spring termination. Corn response to cover crop was significantly affected by termination-tillage method and cover crop species, with poor corn stands after no-tillage, red clover and white plus crimson clover. Fall plow-down termination provided a range in N credit of 80-85 kg N ha^{-1}, which was significantly greater than the N credit under herbicide spray-down in spring. Under spring strip-tillage and no-tillage, hairy vetch produced significantly greater corn grain yields than red clover and white plus crimson clover mix.

KEYWORDS

cover crop, legumes, corn N credit, rotation, termination-tillage

1 Introduction

Efficient management of cover crops can increase soil organic matter, reduce soil erosion, conserve root-zone soil moisture, and enhance nitrogen (N) fertility (1–3). Legume winter-annual cover crops can also be a substantial source of N for the following crop in a crop rotation, especially in humid continental climates (4, 5). For example, Blevins et al. (6) found in Kentucky that hairy vetch (HV, *Vicia villosa* Roth) can supply about 76 kg N ha^{-1} to corn and 125 kg N ha^{-1} to grain sorghum, while Ott and Hargrove (7) found that both HV and crimson clover (CC, *Trifolium incarnatum* L.) increased average corn yields throughout Georgia. In addition, Pott et al. (8) showed that the fertilizer N replacement value of HV could reach 151 kg N ha^{-1} for corn in low fertility soils in Brazil, and Ranells and Wagger (9) showed that CC can provide up to 2/3 of the N requirement for corn in North Carolina.

However, the effectiveness of cover crops for increasing crop productivity and grain yields varies substantially with weather, length of growing season, soil type, crop species, and cover crop characteristics such as biomass production, C:N ratio and residue decomposition rate (10–12). For example, HV can release up to 80% of its accumulated biomass N within 5 weeks after termination, while CC releases about 60% in the same time period (9, 13, 14). Relative to other legume species, CC has slower N release, a narrower range of soil types where optimum performance is achieved, and a tendency to deplete soil moisture (15, 16). In a laboratory incubation study, Yang et al. (17) found that potentially mineralizable N (as % of initial N) was similar (71-75%) among shoots of CC, HV and red clover (RC, *Trifolium pratense* L.), but was substantially lower for CC roots (36.2%) than for HV and RC roots (53.0%). Some studies showed that cover crop monocultures produce greater biomass than mixtures (18–20), but others have shown that mixtures produced more biomass than their single species counterparts (21). Antosh et al. (22) found that cover crop mixtures containing hairy vetch did not accumulate as much N as hairy vetch grown alone. Hence, a legume monoculture, such as hairy vetch, may be a better strategy if the primary goal is to increase soil N.

The effectiveness of legume cover crops for producing biomass, sequestering carbon and fixing atmospheric N is impacted by the time and method of cover crop termination. Termination times range from early to late fall of the same year that the cover crop is planted, or from early spring to late spring of the following year (23, 24). Termination methods range from aggressive tillage (e.g. moldboard or chisel plowing) which incorporates most or all of the cover crop biomass into the soil, to roller-crimping or herbicide spray-down which leaves a layer of cover crop mulch on the soil surface with minimal incorporation (25–28). Generally speaking, late-terminated cover crops accumulate more biomass and biomass N than early-terminated cover crops. Clark et al. (23) found that HV terminated in early to mid-April accumulated an average of 96 kg N ha^{-1}, while late May termination accumulated up to 149 kg N ha^{-1}. Parr et al. (11) found that HV terminated in mid-April accumulated 122 to 168 kg N ha^{-1}, while late May termination produced 155 to 217 kg N ha^{-1}. Coombs et al. (24) determined in an Ontario study that RC produced significantly greater plant available N in both soil and above-ground biomass when it was spring-terminated as opposed to fall-terminated. Cover crop termination *via* tillage versus crimping or spraying affects cover crop decomposition, with N release occurring faster and more completely from cover crops incorporated into the soil than from cover crops left lying on the soil surface (29). Cover crop surface mulches, on the other hand, can be very effective at suppressing weeds and conserving root zone soil and water (30–32), but may also reduce corn germination and early growth by delaying soil dry-down and warming (33, 34). For a sandy loam soil in southwestern Ontario, Yang et al. (35) obtained an early May above-ground biomass N accumulation of 240 kg N ha^{-1} from HV, 199 kg N ha^{-1} from RC, and 119 kg N ha^{-1} from CC. In that study, when the biomass was incorporated into the soil by moldboard plowing in spring, HV and RC provided a fertilizer N replacement value (N credit) of 150 kg N ha^{-1}, and CC provided a fertilizer N replacement value of 139 kg N ha^{-1}. In a meta-analysis of 86 field trials under humid temperate climatic conditions, Bourgeois et al. (36) showed that cover crops increased average corn grain yields by 13% relative to no cover crop.

The “classic” soybean (*Glycine max* (L.) Merr) - winter wheat (*Triticum aestivum* L.) – corn (*Zea mays* L.) rotation is very popular in southwestern Ontario (37). A ttempts to include cover crops in this rotation have thus far focused largely on surface broadcast of RC seed onto frozen ground (frost-seeding). Frost seeding has met with limited success, however, as there are often insufficient freeze-thaw cycles to incorporate the seed, thereby resulting in inconsistent RC stands (38, 39). An alternative approach may be to summer-seed RC or other cover crops into post-harvest wheat stubble when the soil is warm and dry.

We are not aware of any Ontario studies evaluating the performance of summer-seeded legume cover crops after winter wheat harvest.

The objectives of this study were consequently to determine for fine-textured soils in southwestern Ontario: i) the quantity of N accumulated in the above-ground biomass of selected legume cover crops when summer-seeded into post-harvest winter wheat stubble of a soybean-wheat-corn rotation; and ii) the impacts of cover crop species and termination method on corn N credit and corn grain yield.

2 Materials and methods

2.1 Field site and experimental design

The field trial was established in 2017 at the Honourable Eugene F. Whelan Experimental Farm, Agriculture and Agri-Food Canada, Woodslee, southwestern Ontario, Canada (42° 13'N, 82°44'W). Southern Ontario has a humid continental climate with four distinct seasons, and during the study years (2017-2020), mean annual air temperature and precipitation at the site were 9.8°C and 874 mm, respectively (Table S1).

The soil was of the Brookston series (Orthic Humic Gleysol, Canadian 40) with a clay loam texture in the top 15 cm (sand = 331 g kg^{-1}, silt = 374 g kg^{-1}, clay = 295 g kg^{-1} *via* the pipette method of 41). Basic soil chemical characteristics included: pH of 6.3 (soil:water = 1:2); total carbon and total nitrogen contents of 18.8 g kg^{-1} and 1.83 g kg^{-1}, respectively, *via* dry combustion (LECO CN-2000); available phosphorus of 15.0 mg kg^{-1} *via* Olsen method (42); and available potassium of 142 mg kg^{-1} *via* ICP-ammonium acetate extraction (43).

The cropping practice at the site for at least 4 years prior to the experiment was a corn – soybean - winter wheat/cover crop rotation. During that period, each phase of the rotation was fertilized according to standard soil test recommendations (37). For this study, the site was reorganized into three adjacent fields, allowing each phase of the corn-soybean-winter wheat rotation to be present each year. During the winter wheat phase, selected legume cover crops were planted into disked winter wheat stubble shortly after winter wheat harvest in mid to late summer of 2017, 2018 and 2019 (Table 1).

The field trial was a split-plot design with 4 replications having termination-tillage as the main plot and cover crop species as the sub-plot. The termination-tillage main treatments included: i) fall termination of the cover crop by moldboard plowing after first hard (killing) frost (mid to late November), then seed-bed preparation with secondary tillage and corn planting in the following spring (CT); ii) spring herbicide spray-termination of the cover crop, then no-till corn planting (NT); and iii) spring herbicide spray-termination of the cover crop followed by spring strip tillage, and then corn planting (ST). The cover crop split-plot treatments were randomly nested within the termination-tillage treatments, and included: i) hairy vetch (HV, seeding rate = 25 kg ha^{-1}); ii) red clover (RC, seeding rate = 12 kg ha^{-1}); iii) white clover plus crimson clover (WCCC) applied as a 50:50 mix (seeding rates = 12.5 kg ha^{-1} for each); and iv) no cover crop treatment (CK). Hence, there were a total of 12 termination-tillage-cover crop combinations per block, i.e. (CT, NT, ST) × (WCCC, HV, RC, CK). The individual plots were 9.2 m wide by 30 m long for the cover crops (12 corn rows at 0.76 m spacing), and 6.1 m wide by 30 m long for the controls (8 corn rows at 0.76 m spacing). The clovers were broadcast on the surface and planter chains lightly raked the soil so the seed was just under the surface. The HV was drill-planted to 10-15 mm depth with 180 mm row spacing.

All of the cover crops are considered winter-hardy in southwestern Ontario, and considerable re-growth usually occurs in the following spring if the cover crop was not intentionally terminated in the fall. It should also be noted that herbicide termination of white clover was often only partially effective, and the WCCC treatment was consequently changed in 2019 to crimson clover only (seeding rate = 25 kg ha^{-1}), which may have impacted the final (3rd) year of the study. Seeding rates used in this study followed Ontario Ministry of Agriculture, Food, and Rural Affairs (OMAFRA) recommendations (http://www.omafra.gov.on.ca/english/crops/facts/cover_crops01/covercrops.htm).

2.2 Agronomic operations and sample collection

Detailed agronomic operations and sample collection are listed in Table 1. Accumulated above-ground biomass and biomass N in cover crops were determined from biomass samples collected by cutting the cover crop flush with the soil surface inside two demarcation frames (200 mm × 750 mm), then placing the cut material in paper bags, oven-drying (60 °C) for ~ 1 week, and then recording dry weights. Above-ground biomass and biomass N were determined in the fall from CT just before cover crop termination by moldboard plow-down to 180 mm depth (Nov. 29 2017, Nov. 19 2018, Nov. 21 2019), and in the following spring from NT and ST just before cover crop termination by herbicide spray-down (May 1 2018, May 14 2019, May 21 2020). Plow-down termination occurred in November after the first killing frost. Herbicide termination occurred in May using a mix of glyphosate (1.4 kg ha^{-1} a.i.), 2,4-D (0.56 kg ha^{-1} a.i.) and dicamba dimethylamine (0.6 kg ha^{-1} a.i.). Spring tillage occurred a few days before corn planting, and included strip-tilling the ST plots using a Pluribus-V type strip tiller (Dawn Equipment, IL, USA), plus disking, harrowing and packing the CT plots.

Corn (cultivar PO506AM) was seeded (May 31 2018, June 24 2019, June 5 2020) in 0.76 m rows at approximately 76800 seeds ha^{-1} using a Kinze no-till planter. Corn stands were determined 14 days after corn planting by counting emerged seedlings in two random 2-m row lengths per plot. Phosphorous and potassium fertilizers were applied at planting according to soil test recommendations (37). Nitrogen fertilizer (28-0-0 urea ammonium nitrate, UAN) was applied *via* side-dress (corn 6-leaf growth stage) at 200 kg N ha^{-1} to the no cover crop control plots, and at 100 kg N ha^{-1} to the cover crop plots. The N rate applied to the controls is within range of the rates typically recommended for corn production in southern

TABLE 1 Major agronomic activities.

Activity	2017-2018	2018-2019	2019-2020
Cover crop phase			
Winter wheat harvest	July 18, 2017	July 10, 2018	July 25, 2019
Cover crop seeding	July 31, 2017	July 16, 2018	Aug 7, 2019
Fall cover crop biomass collection	Nov 29, 2017	Nov 19, 2018	Nov 21, 2019
Plow-down of cover crops (CT)	Dec 1, 2017	Nov 28, 2018	Dec 6, 2019
Spring cover crop biomass collection (NT and ST)	May 1, 2018	May 14, 2019	May 21, 2020
Herbicide spray-down of cover crops (NT and ST) using Oracle @ 0.6kg/ha a.i. plus 2-4D Amine @ 0.56kg/ha a.i. plus Roundup @ 1.4 kg/ha a.i.	May 9, 2018	May 17, 2019	May 29, 2020
Corn phase			
Strip tilling before corn planting (ST)	May 29/30, 2018	June 12, 2019	June 1, 2020
Disk-harrow-pack before corn planting (CT)	May 30, 2018	June 11, 2019	June 3, 2020
Corn planting (CT, NT, ST)	May 31, 2018	June 24, 2019	June 5, 2020
Herbicide application to corn (Basagran Forte @ 0.84 kg/ha a.i.)	June 6, 2018	July 5, 2019	June 19, 2020
Corn side-dressed with 28% UAN	June 22, 2018	July 9, 2019	June 30, 2020
SPAD measurements at R1 corn stage	July 16, 2018	Aug 9, 2019	Aug 13, 2020
Corn harvest	Nov 28, 2018	Dec 13, 2019	Nov 19, 2020

Ontario (37). The cover crops received half the control N rate (i.e. 100 kg N ha^{-1}) because legume cover crops are known to accumulate about 200 kg N ha^{-1} in biomass and roots (unpublished data), and we assumed 50% of biomass N would be available to the subsequent corn crop. Corn was harvested on Nov. 28 2018, Dec. 13 2019 and Nov. 19 2020, and grain yields determined at 15.5 wt. % seed moisture content.

Composited soil core samples (0 to 300-mm depth 17.5 mm diameter, n = 10) were collected from the corn treatments (about one month after corn planting and about two weeks after side-dress N application) to determine root zone NH_4^+ and NO_3^- concentrations. The soil was occasionally too hard to sample, causing some of the collection dates to be missed or core depth to be limited to the top 150 mm. As a result, only data from top 150 mm are reported here. Corn chlorophyll status was measured at the R1 corn growth stage using a SPAD-502 leaf chlorophyll reader (Minolta Corporation, New Jersey, USA), and used as an estimate of N uptake status at the R1 corn stage.

2.3 Mineral soil nitrogen (NO_3 And NH_4^-), biomass, grain N and C analyses

Soil inorganic nitrogen (NH_4^+-N and NO_3^--N) was determined *via* the KCl extraction method of Tel and Heseltine (44) using the Lachat QuikChem 8500 Analyzer (HACH, Dusseldorf, Germany). Oven-dried biomass samples and corn grain were ground to 2 mm and total C and N contents were determined using an automatic CN analyzer (CN-2000, Leco Corp., Saint Joseph, MI, USA).

2.4 Calculation of fertilizer N replacement value by cover crops

Apparent fertilizer N replacement value, $FNRV_A$ (kg N ha^{-1}), of legume cover crop was calculated for each of the 9 cover crop-termination-tillage combinations, i.e. (CT, NT, ST) x (WCCC, HV, RC), using:

$$FNRV_A = \left(\frac{CY_{trt}}{CY_{ck}} \times 200\right) - 100 \quad (1)$$

where CYtrt (kg ha^{-1}) is corn grain yield for the selected cover crop-termination-tillage treatment combination, and CYck (kg ha^{-1}) is corn grain yield in the appropriate control, i.e. CT-CK, NT-CK or ST-CK. The numbers 200 and 100 are the amounts of fertilizer N (kg N ha^{-1}) applied to the CKs and cover crops, respectively. Hence, Eq. (1) calculates apparent corn N credit relative to the grain yield and N application rate of the controls; and as a result, FNRVA = 100 kg N ha^{-1} is obtained when CYtrt = CYck, and FNRVA = 0 kg N/ha is obtained when CYtrt = 0.5×CYck. This approach was taken because it provides a value that is both readily calculated and more relevant to grain corn producers than the usual approach, i.e. difference in total above-ground biomass N between treatment and control (45).

2.5 Statistical analyses

Analysis of variance (ANOVA) was carried out using the Statistical Analysis System (SAS, Cary, N.C.). Cover crop accumulated biomass N levels were analyzed for November and May. Biomass N, soil N, chlorophyll reading, grain yield and FNRV$_A$ were analyzed using a split-plot experimental design, with tillage-termination method treated as the main factor, cover crop type as the split factor, and year as a repeated measure. Fisher's protected least significant difference (LSD) test (α = 0.05) was used to identify significant treatment main effects.

3 Results

3.1 Air temperature and precipitation during the study period

Mean monthly air temperature and precipitation over the four-year study (2017-2020) are shown in Table S1. Year 2017 and 2020 had slightly higher average annual air temperature (10.4-10.5°C) than 2018 and 2019 (9.6-9.8°C), and 2018 and 2019 had similar air temperatures relative to the long-term average (9.6°C). The soil was periodically subjected to below-freezing temperatures during winter, with 2018 and 2019 having a colder January (-4.6 to -4.7°C) than 2020 (-1.4°C in February). These temperature differences significantly impacted cover crop re-growth and biomass accumulation in the spring. Annual precipitation for the study period was 845 mm for 2017, 922 mm for 2018, 816 mm for 2019, and 855 mm for 2020, which were all relatively close (-8% to +4%) to the long-term average (890 mm). Spring 2020 (April and May) was relatively dry (147 mm two-month total precipitation), whereas spring 2018 was somewhat wet (199 mm). Spring 2019, on the other hand, was wet enough (205 mm) to cause a 3-week delay in corn planting. In contrast to spring, summer 2020 (June to September) was substantially wetter (387 mm two-month total precipitation) relative to summer 2018 (300 mm) and summer 2019 (290 mm). These rainfall differences likely affected annual corn yields and N uptake.

3.2 Nitrogen and carbon contents in cover crop biomass

All three cover crops established well after summer-seeding into wheat stubble, and they all achieved 100% soil coverage by fall (Figure S1). Although the selected cover crops are considered winter hardy in southwestern Ontario, partial winter kill was observed in 2018 and 2019, particularly for crimson clover and hairy vetch; and this in turn affected cover crop re-growth and biomass accumulation in the following spring. Accordingly, accumulated cover crop above-ground biomass and biomass N were reported both for the first dormancy frost in late November just before plow-down (data from CT treatment only), and also for early May just before herbicide spray-down (data from ST and NT treatments pooled).

There was substantial annual variation in above-ground biomass N and C concentrations by the cover crop treatments (Figure 1). Nitrogen concentration was higher in HV (31.2 -31.7 g N kg^{-1}) than in RC and WCCC (19.9 – 27.4 g N kg^{-1}) in both fall and spring samples, and greater N concentration was found in WCCC (24.7 g N kg^{-1}) than in RC (19.9 g N kg^{-1}) in fall samples but not in spring samples (Figure 1A). All cover crops had significantly greater above-ground biomass C concentrations in spring (423 - 432 g kg^{-1}) than in fall (335 – 388 g kg^{-1}), and C concentration was higher in HV than in RC and WCCC in fall samples (Figure 1B). The November and May C:N ratios were 15.1 to 16.8 for RC and WCCC; however, the C:N ratio for HV was lower in November (12.4) and May (13.6) (Figure 1C).

Annual average above-ground biomass and biomass N varied among year, season and cover crop (Table 2). In 2017-2018 and 2019-2020, cover crop biomass was significantly lower for RC (4.0 Mg ha^{-1}) than HV (5.8 and 6.2 Mg ha^{-1}) and WCCC (6.2 and 7.2 Mg ha^{-1}) in November at the start of over-winter dormancy. November 2017 cover crop biomass N was RC (93 kg N ha^{-1})< WCCC (163 kg N ha^{-1})< HV (213 kg N ha^{-1}), and November 2018 cover crop biomass N was RC (87 kg N ha^{-1})< WCCC (136 kg N ha^{-1})< HV (184 kg N ha^{-1}). In 2019, November cover crop biomass N followed a different pattern with RC (85 kg N ha-1)< WCCC (175 kg N ha^{-1}) = HV (182 kg N ha^{-1}).

Spring re-growth had a large impact on accumulated cover crop biomass and biomass N in the following May before herbicide spray-down. In May 2018, accumulated biomass and

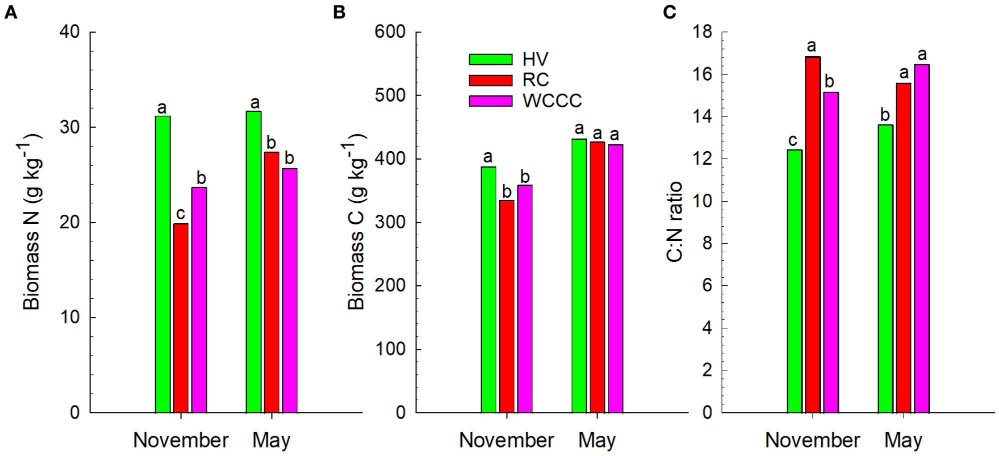

FIGURE 1
Three-year average **(A)** N content in biomass, **(B)** C content in biomass, and **(C)** C:N ratio for cover crop above-ground biomass at fall freeze-up (November) and late spring (May). HV, hairy vetch; RC, red clover; WCCC, white-crimson clover mix. Bars for the same parameter and sampling time are not significantly different (P = 0.05) if labelled with the same letter.

biomass N was HV > RC > WCCC; in May 2020, biomass N was HV > RC = WCCC, while accumulated biomass was HV > RC only; and in May 2019 HV had lower accumulated biomass and biomass N than both RC and WCCC. Over three years, HV had the greatest and RC had the lowest accumulated biomass and biomass N at freeze-up in late November; however, these differences always disappeared by early May of the following year. It was also found that WCCC and HV exhibited appreciable over-winter die-off in 2018 (HV and WCCC) and 2019 (HV), which was likely caused by cold air temperatures during December 2017 to January 2018, and during January to February 2019 (Table S1).

3.3 Impacts of cover crop species and termination-tillage method on corn stand

Corn planting date varied with spring weather, and a substantial planting delay occurred in 2019 due to wet soil conditions (Table 1). Corn emergence in CT was fast and even for all cover crops. In NT and ST, corn emerged later, and it emerged slower for RC and WCCC than for HV, especially under NT where above-ground cover crop biomass remained on the soil surface after termination by spring herbicide spray-down (Figure S2). Corn stands at 14 days after planting varied among year, season and cover crop (Figure 2). Averaged over cover crop and year, CT produced more uniform and greater corn stands (73,960 plants ha^{-1}) than ST (69,030 plants ha^{-1}) and NT (56,870 plants ha^{-1}). In NT, 3-yr average corn stands followed the pattern: CK (72,450 plants ha^{-1}) > HV (60,700 plants ha^{-1}) > WCCC (52,220 plants ha^{-1}) > RC (42,100 plants ha^{-1}); and in ST, 3-yr average corn stands followed the trend: CK (73,270 plants ha^{-1}) > WCCC (68,900 plants ha^{-1}) = RC (68,350 plants ha^{-1}) = HV (65,620 plants ha^{-1}). Corn stands were not significantly different, however, among the CT treatments (72,730 - 75,190 plants ha^{-1}). Compared to CT, overall average corn stand was 23% lower in NT and 6% lower in ST. In 2020, RC-NT had the lowest average corn stands (18860 plants ha^{-1}), which was 24% of the average corn stands in the corresponding no cover crop control (CK-NT). This indicates that cover crop species and termination-tillage method significantly impact both corn emergence and seedling establishment; i.e. no difference between cover crops and control for CT, but significant reduction in corn emergence and plant stands between cover crops and control for NT and ST.

3.4 Effects of cover crop species and termination-tillage method on available soil N and corn N status

There were no cover crop or tillage-termination method interaction effects for plant available soil N, so only main effects are reported (Table 3). The no cover crop controls (CK-CT, CK-NT, CK-ST) contained significantly more soil NH_4^+-N and total soil mineral N (NO_3^-+ NH_4^+) than the cover crop treatments in all three years; and in 2020, HV contained significantly more soil NO_3^--N than RC. Significantly more soil NO_3^--N was also found under NT and ST (60.0 and 64.7 mg N ha^{-1}, respectively) relative to CT (44.8 mg N ha^{-1}) in 2020 (data not reported), which likely reflects greater biomass N in 2020 than in the previous two years (Table 2). Although there was more mineral N under the controls due to the higher N application rate (200 kg N ha^{-1}) than under the cover crops (100 kg N ha^{-1}), N release and

TABLE 2 Above-ground crop biomass and biomass nitrogen (N) in cover crop (HV, RC, WCCC) in November 2017, 2018 and 2019 shortly before cover crop termination by moldboard plow-down (CT treatment), and in May 2018, 2019 and 2020 shortly before cover crop termination by herbicide spray-down (NT and ST treatments).

Time		2017-2018		2018-2019		2019-2020	
	Cover crop	Biomass	N content	Biomass	N content	Biomass	N content
		Mg ha^{-1}	kg N ha^{-1}	Mg ha^{-1}	kg N ha^{-1}	Mg ha^{-1}	kg N ha^{-1}
November							
	HV	5.8	213	7.4	184	6.2	182
	RC	4.0	93	6.9	87	4.0	85
	WCCC	6.2	163	7.8	136	7.2	175
	LSD(0.05)	0.7	19	ns	26	1.5	20
May							
	HV	5.0	163	3.2	77	9.3	356
	RC	3.5	104	6.2	166	7.7	206
	WCCC	2.3	61	6.2	154	8.1	210
	LSD(0.05)	0.7	23	0.7	35	1.5	52

HV, hairy vetch; RC, red clover; WCCC; white-crimson clover mix. LSD (0.05) = Protected Fisher's Least Significant Difference in a *P* value of 0.05.

availability occurs throughout the growing season in the cover crop treatments, as evidenced by the plant chlorophyll SPAD readings described below.

There were also no tillage-termination method or cover crop interaction effects for corn plant chlorophyll levels (R1 growth stage) (Table 4), while the main effects of tillage-termination method were significant in 2018 and 2020, and cover crop significantly affected chlorophyll levels in 2019 and 2020. The SPAD readings were greater for CT (55.5) than ST (53.6) in 2018, and also greater for CK (51.8) than WCCC (47.9) in 2019. In 2020, SPAD readings were greater for CT (57.4) than ST (55.3), and they were also greater for CK (58.8) than for the

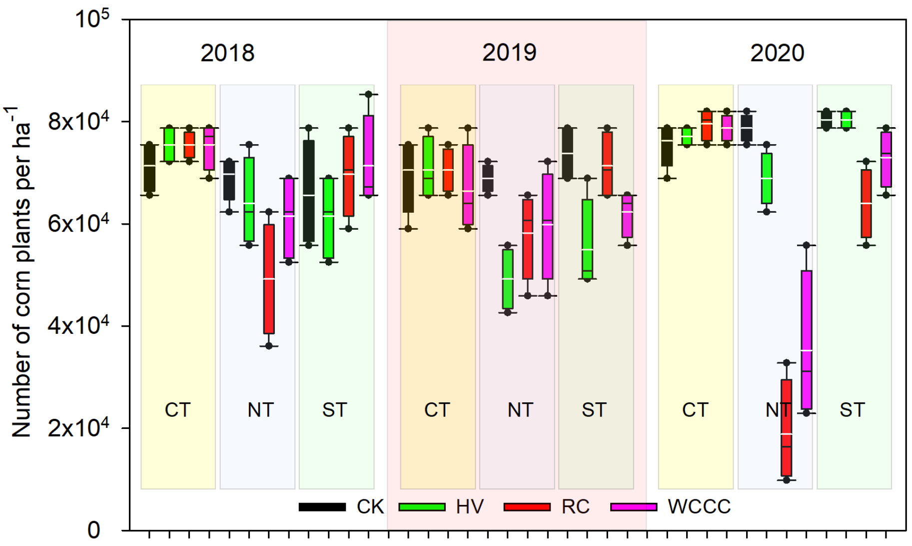

FIGURE 2
Corn stands (plant ha^{-1}, two weeks after planting) by year from 2018 to 2020 grouped by cover crop species and cover crop termination-tillage method. CT, fall moldboard plow-down termination followed by spring planting; NT, spring herbicide spray-down termination followed by no-till corn planting; ST, spring herbicide spray-down termination followed by strip tilling and corn planting; CK, no cover crop control; HV, hairy vetch; RC, red clover; WCCC, white and crimson clover mix. Boxes indicate the 25th and 75th percentile; solid dots above and below the box indicate the 90th and 10th percentile, respectively; white horizontal lines indicate mean; black horizontal lines indicate median.

TABLE 3 Effects of cover crop species and termination-tillage method on soil mineral nitrogen concentrations in the 0-150 mm depth range at one month after corn planting each year.

	2018			2019			2020		
	NO_3^-	NH_4^+	Min N	NO_3^-	NH_4^+	Min N	NO_3^-	NH_4^+	Min N
	———————————————–mg N kg^{-1}——————————————								
CK	48.9	42.1	91.0	54.9	34.7	89.5	56.2	116	172
HV	44.3	10.9	55.2	38.6	3.5	42.1	69.3	29.0	98.3
RC	48.1	16.2	64.3	37.6	4.0	41.7	45.4	31.5	76.9
WCCC	47.2	145	60.7	46.9	10.9	57.7	58.1	34.0	92.1
LSD(0.05)	11.5	17.5	26.3	18.6	13.7	29.8	13.7	24.2	32.4
P-value									
Till (T)	0.064	0.905	0.737	0.647	0.110	0.329	0.014	0.074	0.583
Crop (C)	0.857	0.003	0.042	0.218	<0.001	0.007	0.021	<0.001	<0.001
T × C	0.540	0.908	0.796	0.421	0.217	0.407	0.189	0.085	0.102

CK refers to no cover crop control + 200 kg ha^{-1} fertilizer N addition. HV=hairy vetch + 100 kg N ha^{-1} addition, RC=red clover + 100 kg N ha^{-1} addition, WCCC = white-crimson clover mix + 100 kg N ha^{-1} addition. 2020 NO_3^-N was 44.8 mg N kg^{-1} in CT versus 60.0 mg N kg^{-1} and 64.7 mg N kg^{-1} in NT and ST, respectively. LSD (0.05) = Protected Fisher's Least Significant Difference in a *P* value of 0.05.

TABLE 4 SPAD-502 chlorophyll meter readings for corn at the R1 growth stage with respect to cover crop species and termination-tillage method in 2018, 2019 and 2020.

	Chlorophyll reading (adimensional units)		
	2018	2019	2020
Cover Crop			
CK	55.0	51.8	58.8
HV	53.8	50.1	55.8
RC	54.9	49.2	55.1
WCCC	53.8	47.9	55.4
LSD(0.05)	1.9	2.9	1.9
Termination-Tillage Method			
CT	55.5	49.4	57.4
NT	53.9	50.8	56.0
ST	53.6	48.7	55.3
LSD(0.05)	1.6	2.4	1.7
P-value			
Till (T)	0.050	0.272	0.045
Crop (C)	0.419	0.023	0.002
T × C	0.765	0.217	0.885

CK = no cover crop + 200 kg N ha^{-1} addition, HV = hairy vetch + 100 kg N ha^{-1} addition, RC = red clover + 100 kg N ha^{-1} addition, WCCC = white-crimson clover mix + 100 kg N ha^{-1} addition. CT = cover crop termination by fall moldboard plow-down, then spring corn planting; NT = cover crop termination by spring herbicide spray-down and no-till corn planting; ST = cover crop termination by spring herbicide spray-down, then strip tillage and corn planting. Chlorophyll meter reading > 55 indicates an N sufficiency. LSD (0.05) = Protected Fisher's Least Significant Difference in a *P* value of 0.05.

cover crops (55.1-55.8). The SPAD readings were greater in 2018 and 2020 than in 2019.

3.5 Effects of cover crop species, termination-tillage method, and nitrogen addition on corn grain yields

Cover crop species, termination-tillage method and their interactions significantly impacted corn grain yields (Table 5). Corn grain yields from the no cover crop controls were not different among tillage-termination method in all three years, whereas grain yields in the cover crop treatments were often affected by an interaction of cover crop and tillage-termination method. Corn yields were CT > NT = ST for hairy vetch in 2018 and 2019, CT > ST > NT for red clover in 2018 and CT > NT for red clover in 2019, and CT > ST > NT for white-crimson clover mix in 2018 and 2019. In 2020, corn yields were ST > CT for hairy vetch, CT = ST > NT for red clover, and CT > NT for white-crimson clover mix. HV had similar corn yields as other cover crops under CT and ST but HV had greater yields than RC and WCCC under NT in 2018. I n 2019, corn yields were CK > RC for fall plow-down, CK > HV = RC > WCCC for spring no-tillage, and CK > RC > WCCC for spring strip tillage. And in 2020, corn grain yields were CK > cover crops for fall plow-down, CK = HV > WCCC > RC for spring no-tillage, and CK = HV > RC = WCCC for spring strip tillage.

Year-to-year variation in corn grain yields reflected changes in weather and cover crop biomass (Tables S1, 2). While 2018 and 2020 received even and timely precipitation, corn planting was delayed substantially in 2019 (June 24, 2019), relative to the other two years (May 31 2018 and June 11 2020), as 2019 had the wettest spring in several decades for southwestern Ontario. Averaged over three years and termination-tillage method, corn grain yield was greatest for CK (11.7 Mg ha^{-1}), then corn after HV (10.5 Mg ha^{-1}), then corn after RC (9.4 Mg ha^{-1}), and then corn after WCCC (9.2 Mg ha^{-1}) (Table 5). Averaged over three years and cover crop species, corn grain yields were 11.2 Mg ha^{-1} for CT > 10.3 Mg ha^{-1} for ST > 9.2 Mg ha^{-1} for NT (Table 5). Hence, cover crop termination by fall moldboard plowing generated the largest corn grain yield, and spring ST was better than NT. The 2020 corn grain yield in HV-ST (13.4 Mg ha^{-1}) was effectively equivalent to CK-ST (13.7 Mg ha^{-1}). The poorest corn yield occurred under WCCC-NT in 2019 (2.4 Mg ha^{-1}, Table 5), which were attributed to inadequate spring herbicide termination of white clover in that year.

3.6 Apparent fertilizer N replacement value, $FNRV_A$

There was a significant interaction between crop and tillage-termination method for $FNRV_A$ in each year, and $FNRV_A$ varied widely from 35 to 92 kg N ha^{-1} in 2018, from 28 to 81 kg N ha^{-1} in 2019, and from 12 to 103 kg N ha^{-1} in 2020 (Table 6). In 2018, $FNRV_A$ was similar among cover crops for CT and ST, but $FNRV_A$ was greater for HV than RC and WCCC under NT. In 2019, $FNRV_A$ was similar among cover crop species for CT and NT, however, $FNRV_A$ was greater for RC than WCCC under ST. In 2020, $FNRV_A$ was similar among cover crop species for CT, but $FNRV_A$ was greater for HV than RC and WCCC under NT and ST. Year 2019 produced the smallest mean $FNRV_A$ (53 kg N ha^{-1}) compared to 2018 (69 kg N ha^{-1}) and 2020 (73 kg N ha^{-1}). The small N credit in 2019 was likely caused by low grain yields in that year due to late planting, poor corn stands and inadequate herbicide termination of white clover (Table 5; Figure 2). High $FNRV_A$ occurred for HV in 2020 under all three termination-tillage treatments (85 – 103 kg N ha^{-1}); however, the $FNRV_A$ values were lower for RC and WCCC under NT and ST, ranging from 12 to 73 kg N ha^{-1}. The three-year average $FNRV_A$ was greatest for CT (83 kg N ha^{-1}), followed by ST (63 kg N ha^{-1}), and then NT (48 kg N ha^{-1}); and it was greatest for HV (75 kg N ha^{-1}), followed by WCCC (62 kg N ha^{-1}), and then RC (58 kg N ha^{-1}).

4 Discussion

Total global N fixation is on the order of 1.75 x 10^{11} kg N yr^{-1}, with about half (i.e. ≈ 8 x 10^{10} kg N yr^{-1} or 20-200 kg N ha^{-1} yr^{-1}) fixed biologically by leguminous plants, and the rest fixed by industrial activity including fertilizer production (46). Although farmers still predominantly use synthetically-produced N fertilizer, biologically fixed N from legume crops is being increasingly recognized as a viable N source for crop production. The ability of legume plants to fix N from the atmosphere varies with plant species, growth stage and dry matter content of plant biomass (47).

In this study, legume C:N ratios ranged from 12.3 to 16.8, which are slightly higher than those for clovers in a study conducted ~90 km northeast of our study site (9.4 to 14.2, 24), but comparable with the C:N ratios obtained for clovers (13.8 to 15.6) and HV (13.2 to 13.9) in a study conducted on a sandy loam soil about 30 km south of our study site (35). The variability in C:N ratios by crop species could be the result of differences in both development and relative plant component composition (i.e. leaves vs. stems vs. roots) at the time of sampling. Legumes with a lower C:N ratio will decompose faster and release N more quickly than legumes with higher C: N ratios (17). Therefore, terminated HV will usually release biomass N somewhat faster than terminated RC and WCCC. The amount of accumulated legume biomass can also be affected by sampling time, particularly during April and May when plant growth rate increases dramatically and often differs substantially among plant species (48). In this study, greater accumulated

TABLE 5 Corn grain yields (15.5% seed moisture content) for cover crop species and termination-tillage method (2018-2020).

	Termination-Tillage Method			LSD(0.05)
	CT	NT	ST	
	——————— Mg ha^{-1} ———————			
2018				
CK (no cover crop)	12.4	11.7	11.2	0.7
Hairy vetch	11.7	10.8	10.5	0.8
Red clover	11.9	8.4	10.5	1.2
White-Crimson clover mix	11.9	8.4	10.2	0.9
LSD(0.05)	0.7	1.1	0.9	10.8
2019				
CK (no cover crop)	10.2	10.0	9.6	0.8
Hairy vetch	9.2	7.4	7.0	1.4
Red clover	8.3	7.1	7.8	0.9
White-Crimson clover mix	9.2	2.4 [a]	6.5	1.2
LSD(0.05)	1.4	0.9	0.9	7.9
2020				
CK (no cover crop)	13.2	13.4	13.7	0.6
Hairy vetch	12.2	12.7	13.4	1.0
Red clover	12.2	7.4	11.2	1.9
White-Crimson clover mix	12.2	10.2	11.4	1.3
LSD(0.05)	1.0	1.4	0.8	11.9
3-yr average				
CK (no cover crop)	11.9	11.7	11.5	0.9
Hairy vetch	11.0	10.3	10.3	1.5
Red clover	10.8	7.6	9.8	1.1
White-Crimson clover mix	11.1	7.0	9.4	1.7
LSD(0.05)	1.1	1.4	1.3	
P-value				
	2018	2019	2020	3-year mean
Tillage (T)	<0.001	<0.001	<0.001	<0.001
Crop (C)	<0.001	<0.001	<0.001	<0.001
T × C	0.002	0.053	<0.001	0.062

CK = no cover crop + 200 kg N ha^{-1} addition, HV = hairy vetch + 100 kg N ha^{-1} addition, RC = red clover + 100 kg N ha^{-1} addition, WCCC = white-crimson clover mix + 100 kg N ha^{-1} addition. CT = cover crop termination by fall moldboard plow-down, then spring corn planting; NT = cover crop termination by spring herbicide spray-down and no-till corn planting; ST = cover crop termination by spring herbicide spray-down, then strip tillage and corn planting. [a] In 2019, apparent herbicide failure on NT-WCCC occurred, which caused significant corn yield loss. LSD (0.05) = Protected Fisher's Least Significant Difference in a *P* value of 0.05.

biomass and biomass N in 2020 is likely due to the fact that biomass sample collection in that year occurred 3 weeks later than in 2018, and one week later than in 2019 (Table 1). For example, two weeks additional growth between late April and early May increased HV biomass by 61% in North Carolina (13) and by 35% in Maryland (23).

Red clover is a long-established cover crop in southwestern Ontario, but summer-seeding into winter wheat stubble is

TABLE 6 Apparent fertilizer nitrogen replacement value, FNRV$_A$ (Eq. 1), to corn for different cover crop species and termination-tillage methods.

Year	Cover Crop Species	Termination-Tillage Method			LSD(0.05)
		CT	NT	ST	
		————————— kg N ha^{-1}———————————			
2018	Hairy vetch	89.3	74.9	69.0	14.4
	Red clover	92.1	35.4	69.5	19.5
	White-Crimson clover mix	91.6	36.2	65.2	15.4
	LSD(0.05)	12.5	17.9	10.9	
2019	Hairy vetch	80.5	44.2	37.2	28.1
	Red clover	61.8	39.8	53.5	15.3
	White-Crimson clover mix	80.3	-[a]	28.1	26.0
	LSD(0.05)	29.4	18.7	20.7	
2020	Hairy vetch	85.2	92.5	103	18.7
	Red clover	85.3	11.9	69.5	30.0
	White-Crimson clover mix	84.5	54.4	72.9	20.6
	LSD(0.05)	22.1	35.1	14.2	
3-yr average	Hairy vetch	85.0	70.5	69.7	20.2
	Red clover	79.7	29.0	64.2	12.8
	White-Crimson clover mix	85.5	45.3	55.4	16.7
	LSD(0.05)	13.8	16.4	19.3	
		P-value			
		2018	2019	2020	3-yr mean
Tillage (T)		<0.001	<0.001	<0.001	<0.001
Crop (C)		0.048	0.830	<0.001	0.002
T × C		0.016	0.104	<0.001	0.010

CK = no cover crop + 200 kg N ha^{-1} addition, HV = hairy vetch + 100 kg N ha^{-1} addition, RC = red clover + 100 kg N ha^{-1} addition, WCCC = white-crimson clover mix + 100 kg N ha^{-1} addition. CT = cover crop termination by fall moldboard plow-down, then spring corn planting; NT = cover crop termination by spring herbicide spray-down and no-till corn planting; ST = cover crop termination by spring herbicide spray-down, then strip tillage and corn planting. [a] A negative N credit was achieved due to very low corn yield so it was not include in ANOVA. LSD (0.05) = Protected Fisher's Least Significant Difference in a *P* value of 0.05.

extremely rare compared to traditional frost-seeding (e.g. 49, 50). Coombs et al. (24) found less RC biomass accumulation for late May to mid-July planting relative to mid-March to mid-April planting, which they attributed to length of available growing period. Yang et al. (35) found less biomass and biomass N by freeze-up for summer-seeded RC than HV or crimson clover. The spring and fall biomass for RC in this study (5.8 and 5.0 Mg ha^{-1}, respectively) were in the range found for an organic crop production trial on a sandy loam about 30 km south (4.1 and 6.8 Mg ha^{-1}, respectively; 35), but were greater than the amounts found for another sandy loam about 90 km northeast (0.7 and 3.1 Mg ha^{-1}, respectively; 24), and also greater than "representative" values (0.7 and 4.2 Mg ha^{-1}) reported for temperate climates (10, 51, 52). Coombs et al. (24) noted that when RC was allowed to over-winter, biomass increased relative to fall biomass for Ontario (49, 51, 53), Ohio and Michigan (45, 54). Over-winter increases in RC biomass occurred in this study, with an average fall biomass of 5.0 Mg ha^{-1} increasing to 5.9 Mg ha^{-1} by the following spring.

Hairy vetch and crimson clover are popular N sources for grain corn in the US (e.g. 11, 55). Hairy vetch is also used in Ontario because of its cold tolerance (35, 56, 57) whereas crimson clover is rarely used. The nitrogen content in HV above-ground biomass ranges from 75 kg N ha^{-1} to 288 kg N ha^{-1} at various sites in Alabama, Georgia, Kentucky, and North Carolina (11, 56, 58). Van Eerd (57) obtained relatively low biomass N (41 – 139 kg N ha^{-1}) by freeze-up for HV planted in early August in southern Ontario, as well as little re-growth in the following spring. This differs from our results, and may reflect our milder climate and/or soil differences. This study showed that HV and crimson clover performed well for accumulating above-ground biomass and biomass N in both the fall and the following spring.

Corn leaf chlorophyll level has been used for determining plant N status when a reference N level is available and all factors other than N are constant (59). SPAD-502 chlorophyll readings from the well-fertilized controls (200 kg N ha^{-1}) were used as the reference N level (60–63), and N deficiency in the cover crop treatments was deemed to occur when their SPAD readings fell below 95% of the reference. This occurred for WCCC in 2019 and for RC and WCCC in 2020. If, however, the 93% critical SPAD level of Piekielek et al. (61) is used, then none of the treatments were N deficient. The SPAD readings closely followed measured cover crop biomass N (Table 2), but showed no clear impacts on corn grain yields (Table 5). The SPAD readings at corn R1 growth stage were inconsistent with soil mineral N measured one month after corn planting (Table 3), indicating sustained release of N from legume decomposition (58, 64–66). The poor match between soil N or SPAD readings and corn yields indicates that soil N may not be a limiting factor for corn yield in this study. It appears instead that the primary factor affecting corn grain yields was corn stand number, which showed a highly significant positive correlation with corn grain yields (Figure S3).

Although results were not consistent among cover crops and years, this study never-the-less supports the general consensus that summer-seeding legume cover crops after winter wheat harvest can contribute substantial N to a succeeding corn crop in southwestern Ontario (35, 49, 57) and in the northern United States (58, 67, 68). The FNRV$_A$ in this study were comparable to the 65 to 130 kg N ha^{-1} from legume cover crops found in a Maryland study (69). The highest FNRV$_A$ (100 kg N ha^{-1}) in our study is also equivalent to that obtained in an Ontario study where oat inter-seeded with red clover provided N to a following corn crop (70). In Ohio, Ngalla and Eckert (71) compared frost-seeding red clover into winter wheat with July seeding of red clover after wheat harvest. They found that frost-seeded red clover contributed 56 to 67 kg N ha^{-1} to the following corn, but summer-seeded red clover provided effectively no N benefit because of insufficient biomass accumulation.

Partial replacement of synthetic fertilizer N with legume cover crop N often caused a substantial grain yield penalty for no-tillage, especially for RC-NT and WCCC-NT. This occurred because surface mulches, such as cover crop residues, often delay dry-down and warming of Brookston clay loam soil in southern Ontario, and this in turn reduces corn germination and early growth. Hence, RC and WCCC cover crops are not recommended for fine-textured soils under NT in southern Ontario. On the other hand, NT after HV often produced acceptable plant stands, and the higher N benefit from HV contributed to acceptable corn yields. Higher N contribution from HV was also found in Brazil, and was deemed a viable strategy for boosting corn yield under NT (72). Vaughan et al. (73) found that combining HV with NT increased the yield potential of both monoculture and rotation corn in North Carolina.

In summary, the legume cover crops, hairy vetch, red clover, crimson clover and white-crimson clover mix, could all enhance the traditional soybean – winter wheat – corn rotation in southwestern Ontario when the cover crops are summer-seeded after wheat harvest. Hairy vetch was superior to the other cover crops, however, as it generated larger amounts of biomass and accumulated greater amounts of biomass nitrogen. Further, hairy vetch is easily terminated in the fall or spring, it allows good corn emergence, and it can provide the equivalent of 85 kg N ha^{-1} of fertilizer to the corn crop. Averaged over three years and three termination-tillage systems (i.e. fall moldboard plow-down with conventional tillage, spring herbicide spray-down with strip-tillage, spring herbicide spray-down with no-tillage), corn yields following hairy vetch were 13% greater than corn yields following red clover and 15% greater than corn yields following a white-crimson clover mix. If fall termination of the cover crop is desired, summer-seeding hairy vetch or white and crimson clovers are recommended for fine-textured soils in southwestern Ontario because they accumulate more biomass relative to red clover. If strong spring re-growth is desired, hairy vetch is more desirable than red, white and crimson clovers because clovers tend to be more difficult to terminate with herbicides in the spring, and they can reduce grain yields by delaying corn emergence and reducing final corn stands.

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

XY and WR conceptualized the work; CD managed soil and crop N analysis; M-AR collected and analysed the soil and plant samples. XY planned the experiment and wrote the first draft. All authors contributed to manuscript editing and approved the final submission.

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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/fsoil.2022.1082940/full#supplementary-material

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The acquisition of macro- and micronutrients is synergistic in species mixtures: example of mixed crucifer-legume cover crops

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Cover crops are often mentioned as a way to decrease nutrient losses during the fallow period. Species mixtures of crucifer-legume have been shown to effectively take up nitrogen (N) and sulphur (S) to decrease losses due to leaching, but their ability to simultaneously take up other key macro- and micronutrients remains unknown. Our study assessed the performances of a wide variety of bispecific crucifer-legume mixtures to provide synergetic uptake of multiple nutrients to target nutrient-recycling ecosystem services. Cultivars from eight crucifer and seven legume species were tested as sole and bispecific cover crops at an experimental site near Toulouse, France. Seven macronutrients (C, N, P, K, S, Ca and Mg) and six micronutrients (B, Cu, Mn, Zn, Fe and Na) were measured in both species. Crucifer-legume mixtures showed synergetic uptake of nutrients per ha compared to sole cover crops for some nutrients through niche complementarity and facilitation processes and net competition for other nutrients. Species mixtures induced both i) higher nutrient concentrations for Mn and Fe in crucifers and Ca and B in legumes) and ii) lower concentrations for P, K, Ca and B in crucifers and Cu and Fe in legumes, indicating that the interactions differed among the species mixtures. Nevertheless, the nutrient uptake measured in shoots were always higher in species mixtures than in sole crops (LER > 1) demonstrating the compatibility and complementarity of crucifer and legume mixtures in providing multiple-nutrient catch-crop and recycling related ecosystem services. Despite overall positive interactions and synergetic complementarities, the results also highlight that some net negative competition occurred for some nutrients. Thus, further investigation is still necessary to completely understand the processes behind multiple-nutrient acquisition in species mixtures.

KEYWORDS

intercropping, cover crop, macronutrient, micronutrient, interspecific interactions, complementarity, competition, Brassicaceae-Fabaceae intercrop

Introduction

Nutrient availability is one of the main factors that influence soil fertility and plant growth in natural and agricultural systems (Halvin et al., 2005). Soil erosion and leaching are major causes of nutrient loss in agroecosystems that are often increased in intensive agricultural systems due to over-fertilisation, tillage and/or leaving soils bare during periods of high rainfall (Drinkwater and Snapp, 2007; Justes, 2017). The most important nutrients for plant growth are not equally sensitive to leaching and erosion. Macronutrient anions such as nitrate (NO_3^-) and sulphate (SO_4^{2-}) are highly mobile and leachable in soils (Thorup-Kristensen et al., 2003). Cations such as ammonium (NH_4+), calcium (Ca^{2+}), magnesium (Mg^{2+}) and potassium (K^+) can also be leached (Lehmann and Schroth, 2003), but in smaller quantities than NO_3^- (Halvin et al., 2005). While Mg^{2+} is more leachable than Ca^{2+}, loss of Mg can sometimes exceed the subsequent plant requirement (Mengel et al., 2001; van der Heijden et al., 2014). Phosphorus (P) is mostly immobile in the soil, so its leaching is negligible, but it is sensitive to erosion by runoff (Torstensson et al., 2006; Aronsson et al., 2007). Among the micronutrients, boron (B) and manganese (Mn) are susceptible to leaching, while zinc (Zn) and copper (Cu) are more tightly bound to organic matter than other micronutrients and are thus less mobile and susceptible to leaching (Halvin et al., 2005).

Under temperate conditions, heavy rainfall during the fallow period exposes bare soil to runoff and leaching between two main cash crops; this period thus has a high risk of nutrient losses (Carranca, 2012; Justes, 2017). Growing cover crops during the fallow period can provide multiple ecosystem services (Justes, 2017; Couëdel et al., 2019; Lavergne et al., 2021; Scavo et al., 2022; Van Eerd et al., 2023). For example, it is an effective solution for 1) reducing potentially leachable nutrient concentrations in the soil solution (Thorup-Kristensen et al., 2003; Constantin et al., 2010; De Notaris et al., 2018), 2) reducing the amount of water drained (Meyer et al., 2019) 3) reducing soil erosion by water and wind (Blanco-Canqui et al., 2015; Basche et al., 2016; Blanco-Canqui, 2018; Mohammed et al., 2021) and 4) improving soil physical properties (Blanco-Canqui and Ruis, 2020; Haruna et al., 2020). Besides their ability to take up nutrients from the soil, cover crops can also make nutrients available for the next crop through mineralisation of crop residues following termination (Thorup-Kristensen et al., 2003). Surprisingly, besides the well-known nitrogen (N) cycle, the potential of cover crops to take up and recycle other nutrients has rarely been quantified. The few studies that have assessed the ability of cover crops to simultaneously take up multiple nutrients revealed variability in performances among families and species (Wang et al., 2008; Wendling et al., 2016). An effective option to encourage synergetic uptake of multiple nutrients by cover crops combines a variety of families that have different characteristics and performances. Thus, species mixtures of cover crops could be a solution to increase nutrient uptake over that of sole cover crops due to niche complementarity and facilitation processes.

The review of Xue et al. (2016) highlighted that bispecific cereal-legume cash-crop mixtures grown for grains could increase the uptake of several soil nutrients through root-architecture complementary and differences in requirements between species. Studies of cash crops have shown that Zn and Fe concentrations in legumes increase when mixed with a cereal, through complementarity for resources and interspecific facilitation (Zuo and Zhang, 2008; Zuo and Zhang, 2009). However, antagonism in nutrient acquisition was also observed, *e.g* when mixed with maize, peanut had higher Fe, Zn, P and K concentrations, but lower Ca concentrations and no change in Mn or N concentrations (Inal et al., 2007). Similarly, Xia et al. (2013) observed that N, P, K, Ca and Mn concentrations in cucumber shoots and roots were significantly higher when grown in a species mixture with green garlic, but Mg concentrations were lower than those in sole crops. Such trade-offs in simultaneous nutrient acquisition require assessing cover crop mixtures to determine their potential to provide nutrient ecosystem services for leaching mitigation and recycling to the next cash crop.

Among families used in cover cropping, crucifers (Brassicaceae) are the most effective catch crops for nitrogen (N) and sulphur (S) uptake (Couëdel et al., 2018a; Couëdel et al., 2018b) while legume (Fabaceae) cover crops are especially useful for providing exogenous N to the system thanks to biological symbiotic fixation (Jensen, 1986; Tribouillois et al., 2016). Crucifer-legume mixtures grown as cover crops showed complementarity in providing ecosystem services related to N and S cycles (Couëdel et al., 2018a; Couëdel et al., 2018b). However, the only study we identified in the scientific literature that focussed on multiple-nutrient acquisition by crucifer-legume cover crop mixtures was published by Koefender et al. (2016), who showed that a mixture of radish and vetch took up more nutrients than when in sole crops and more than oat-vetch mixtures. Despite their benefits in mixtures, crucifers may be a poor companion crop for nutrient acquisition, as they cannot host arbuscular mycorrhizal fungi (AMF) and thus may negatively (or at least not positively) impact legume nutrient acquisition. Indeed, AMF facilitate acquisition of nutrients such as P, Mn, Ca, Fe and B in many mixtures of plant species (Jin et al., 2014; Teste et al., 2014). Moreover, potential negative effects of a crucifer companion crop on the acquisition of nutrients involved in symbiotic N fixation by legumes (*e.g.* P, Fe, Mo, Cu) need to be assessed (O'Hara et al., 1988; Brear et al., 2013). Legumes can be good companion crops as they facilitate the uptake of certain nutrients (*e.g.* P, K, Ca, Mg) by crucifers. Indeed, symbiotic N fixation by legumes liberates protons that acidify the rhizosphere, increasing the solubilisation of nutrients potentially available to the crucifer (Hauggaard-Nielsen and Jensen, 2005; Hinsinger et al., 2011; Tang et al., 2021).

The goal of our study was to analyse the potential of multiple-nutrient acquisition to provide multiple ecosystem services in bispecific crucifer-legume cover crop mixtures. Our specific aim is to propose a methodological analysis on a first dataset in order to illustrate an ecological theory using agronomic data, never demonstrated for crop multi-elements uptake. We believe that this novelty would be very useful in the literature as a first step to understand competition between cover crop mixtures for multi-nutrients. To this end, we tested a wide variety of species, including several cultivars. The objectives of our methodological analysis were to 1) analyse all essential macronutrients (carbon (C), P, K, Mg and Ca) and the most important micronutrients (Mn, Cu, Fe, B, Zn and

Na) and 2) assess the complementarity and facilitation performances of bispecific crucifer-legume cover crops in acquiring these nutrients. The original method used is the "4C" approach developed by Justes et al. (2021), which assesses effects of competition, complementarity, cooperation (facilitation) and compensation in species mixtures grown in agroecosystems for providing ecosystem services.

We tested two hypotheses for using cover crop sole crops and species mixtures in short (summer to mid-autumn) fallow periods:

1) The relation (complementarity or competition) between crucifers and legumes depends on the nutrient considered and the species mixed.
2) Bispecific mixtures mutualise their acquisition of multiple nutrients, unlike when the species are in sole crops, mainly due to the niche complementarity of nutrient uptake capacity and root systems when exploring the soil.

Materials and methods

Experimental design and cover crop management

A field experiment was conducted in 2014 at the Lamothe experimental farm of INP Purpan, which is located at Seysses, in south-western France (43.506° N, 1.237° E), and has a silty clay loam soil. According to the Köppen climate classification, Lamothe has an oceanic climate (temperate without a dry season but with warm summers). Cover crops were sown on 19 August and terminated on 4 November. Cumulative rainfall during the growing period was 140 mm, and the mean temperature was 19° C (see Table S1 for monthly climate averages). The soil mineral N content at sowing was 93 kg N ha^{-1} (see Table S2 for more detailed soil chemical characteristics). The experiment followed a completely randomised design with three replicates in blocks (See Figure S1 for detailed field scheme). Each elementary plot, which contained 10 rows per treatment, measured 18 m^2 (12 x 1.5 m). To avoid confounding effects of plant-plant interactions between adjacent treatments, only the six rows in the middle of each plot were harvested and used for soil measurements.

Cover crop species were selected for their ability to grow rapidly during a short fallow period in the autumn and to maximise the services related to N and S (Couëdel et al., 2018a; Couëdel et al., 2018b). Crucifer and legume species and cultivars were selected for their diversity in shoot and root architecture, sensitivity to photoperiod and precocity. All bispecific mixtures (Table S3) contained one crucifer and one legume and were designed to minimise competition according to expert knowledge and recently published information (Tribouillois et al., 2016). Therefore, only mixtures of species that had already been identified as compatible and having good growth performance when intercropped were tested, to keep the experiment within the human and financial resources available. The following five crucifer species were used: rape (*Brassica napus* L.), white mustard (*Sinapis alba* L.), Ethiopian mustard (*Brassica carinata* A. Braun.), turnip rape (*Brassica rapa* L. subsp. *oleifera*) and radish (*Raphanus sativus* L.). The following seven legume species were used: Egyptian clover (*Trifolium alexandrium* L.), crimson clover (*Trifolium incarnatum* L.), common vetch (*Vicia sativa* L.*)*, purple vetch (*Vicia benghalensis* L.), hairy vetch (*Vicia villosa* Roth.*)*, Narbonne vetch (*Vicia narbonensis* L.), soya bean (*Glycine max* (L.) Merr.) and faba bean (*Vicia faba* L.). Three additional crucifer species (Indian mustard (*Brassica juncea* L. Czern.), turnip (*Brassica rapa* L. subsp. *rapa*) and rocket (*Eruca sativa* Mill.)), along with more cultivars of the five original crucifer species, were tested only in sole crops to assess shoots and roots nutrient concentrations and uptake in a wider range of species and cultivars (Table S3). Supplementary information displaying results on this larger range of crucifer sole crops are available in Figures S1, S2 and Table S4.

Species grown as sole cover crops were sown at densities recommended for cover crops to obtain plant cover with similar densities and rapid growth and soil coverage. Sowing densities for sole cover crops were 800 seeds m^{-2} for all clover species; 150 plants m^{-2} for Ethiopian mustard; 100 plants m^{-2} for white mustard and all vetch species; 80 plants m^{-2} for rape, radish and turnip rape; 70 plants m^{-2} for soya bean and 40 plants m^{-2} for faba bean. Sowing densities for bispecific mixtures were half of the corresponding sole cover crop density of each species (50% crucifers and 50% legumes) to create a substitutive design of species mixtures. Seeds of both species were mixed in the row and sown with an experimental seeding machine, similar to a seed drill, to obtain a homogenous mixed-plant cover crop in the row to maximise plant-plant interactions.

After shallow tillage, seeds were sown 1.5-2.0 cm deep in rows 15 cm apart. The percentage of each sown species was monitored during crop emergence. Irrigation (30 mm) was applied after sowing to ensure homogeneous emergence and establishment of cover crops. Irrigation and fertilisers were not applied during the cover crop growing period to mimic "normal farming conditions". The experimental field had previously hosted crop rotations with rape, and neither the rotation in general nor the rape in particular had experienced N or S deficiency. In addition, just before the experiment, no soil-nutrient deficiencies were detected according to French soil-analysis standards (Table S2). We thus assumed that nutrients in the soil were not a limiting factor in our experiments, in particular for the cover crops, which have a shorter growing period and thus produce less biomass, which decreases nutrient requirements. An anti-grass herbicide (Axéo®) was sprayed at 1.2 litre per hectare to control a strong emergence of weeds (mainly annual grasses) in September to decrease the influence of extraneous factors.

Plant sampling

Cover crops were sampled 2.5 months after sowing, which is consistent with the usual practice of incorporating cover crops into the soil before sowing the next winter crop, as autumn is warm and rainy in this region, which promotes rapid growth of cover crops. All above-ground biomass and, for crucifers alone, root biomass, was collected from 1 m^2 per replicate (more precisely, two 0.5 m^2 quadrats were randomly put in each 12 m plot length). Roots were collected to a depth of ca. 30 cm by digging with a fork. The roots of legume species were not considered in the results due to technical difficulties in sampling fine roots in the silty clay loam soil of the study site. Most of the root biomass of legumes is composed of fine roots, while the roots of crucifers are generally tuberous, which makes them easier to collect and measure more accurately. Shoots and roots were separated using a sharp knife and secateurs, and the root systems were carefully washed with cold water to remove the remaining soil. Samples were washed, dried at 80°C for 48 h, weighed and ground to measure total C, N, S, P, K, Ca, Mg, Na, B, Cu, Fe, Mn, and Zn via elemental analyses based on the Dumas method (Elementar MicroVario Cube, Germany) for C and N, and by inductively coupled plasma - atomic emission spectroscopy (ICP-AES) for the other nutrients (at the SADEF-accredited laboratory, Aspach-le-Bas, France).

Indicators used to characterise mixture performances

The land equivalent ratio (LER), defined as the sole crop area required to reach the same biomass as a multi-species mixture or intercrop (Willey, 1979), equals the sum of the partial LER (pLER) of each mixed species. We used the LER index to assess nutrient uptake as Couëdel et al., 2018a; Couëdel et al., 2018b did for N and S acquisition, and applied here for all nutrients analysed:

$$LER = pLER\_C + pLER\_L \qquad \text{(Eq.1)}$$

$$pLER = Nutrient_{upIC}/Nutrient_{upSC} \qquad \text{(Eq.2)}$$

where pLER_C and pLER_L are the pLER of mixed crucifers and legumes, respectively, $Nutrient_{upIC}$ is the nutrient uptake of the given nutrient for a given species (crucifer or legume) in the mixture, and $Nutrient_{upSC}$ is the nutrient uptake of the same species as a sole cover crop.

LER > 1 indicates that the bispecific mixture takes up resources more efficiently than the species grown as a sole cover crop (i.e. complementarity exceeds competition). In contrast, LER< 1 indicates that competition exceeds complementarity for resource uptake, while LER = 1 indicates that the balance of plant-plant interactions is null. In a substitutive design (50% of each sole cover crop species), pLER > 0.5 indicates that, at the plant level, the species takes up more nutrients in a mixture than as a sole cover crop, while pLER< 0.5 indicates that interactions in the mixture negatively influence the species' nutrient uptake. Overall, when the two pLERs are significantly greater than 0.5, which yields a LER significantly greater than 1, complementary and facilitation processes are stronger than competition in plant-plant interactions. Inversely, when the two pLERs are less than 0.5, competition processes are stronger than complementarity for nutrient uptake (Justes et al., 2021).

Statistical analysis

Analysis of variance (ANOVA) was used to assess effects of cover crop species and cover crop type (*i.e.* sole crucifer, sole legume or mixture) on nutrient acquisition per unit area (kg ha^{-1}). Tukey's *post-hoc* test was used to distinguish differences among cover crop types for each nutrient. Normality was tested using the Shapiro-Wilk test while homoscedasticity was tested using the Bartlett's test. Percentage data were non-normal after arcsine-square root transformation, then nonparametric Wilcoxon tests were performed to test 1) differences in nutrient concentration between sole and mixed crucifers or legumes, and 2) the significance of differences in LER between cover crops and their relative difference from 1.0 (*i.e.* the net neutral effect of the 4C processes), as well as those between pLER_C and pLER_L and 0.5 (*i.e.* the net neutral effect at half density). Statistical analysis was performed using R software (R core team, 2023), and differences among treatments were considered significant at P< 0.05. Cultivar data were pooled as no significant differences in nutrient concentrations or acquisition were observed between cultivars. Results were then analysed by crop family and species to increase the robustness of the results and to highlight key points.

Results

Nutrient concentrations in crucifer and legume mixtures

Nutrient concentrations of mixed crucifers and legumes differed from those in their respective sole crops (Figure 1). For crucifers, Fe (+350% in shoots, +228% in roots in mixtures) and Mn (+40% in shoots, +200% in roots in mixtures) were the only nutrients with significantly higher concentrations in both mixed shoots and roots compared to sole cover crops (Figure 1) especially for radish and turnip rape (Table 1). Mg (+15%) and Cu (+20%) concentrations were significantly higher in crucifer mixed roots only (Figure 1; Table 1). In comparison, concentrations were significantly lower in crucifer mixture shoots than those in sole cover crops for P (-8%, especially for radish), K (-13%), Ca (-21%, except for Ethiopian mustard) and B (-25%). C (-8%) and Na (-24%) concentrations were significantly lower in crucifer mixture roots than those in sole cover crops (Figure 1; Table 1). Zn was the only nutrient with higher concentrations in crucifer mixture roots (+21%), while it was lower in mixture shoots (-10%). The concentrations of other macronutrients, such as N and S, did not differ significantly between crucifers in mixtures and sole cover crops (Figure 1; Table 1).

For legume shoots, Ca (+26%) and B (+27%) were the only nutrients with significantly higher concentrations in mixtures compared to sole cover crops (Figure 1). This pattern was observed for all legume species except for Egyptian clover and soya bean, whose Ca and B concentrations did not differ significantly between mixtures and sole cover crops (Table 1). In contrast, Cu (-12%) and Fe (-34%) were the only nutrients with significantly lower concentrations in mixed legume shoots compared to sole legume cover crops (Figure 1). For Fe, this pattern was observed in all species except faba bean, crimson clover and soya bean, for which the pattern was the opposite (Table 2). All other nutrient concentrations (C, N, S, P, K, Mg, Na, Mn and Zn) did not differ significantly between legume shoots in mixtures and in sole cover crops (Figure 1; Table 1).

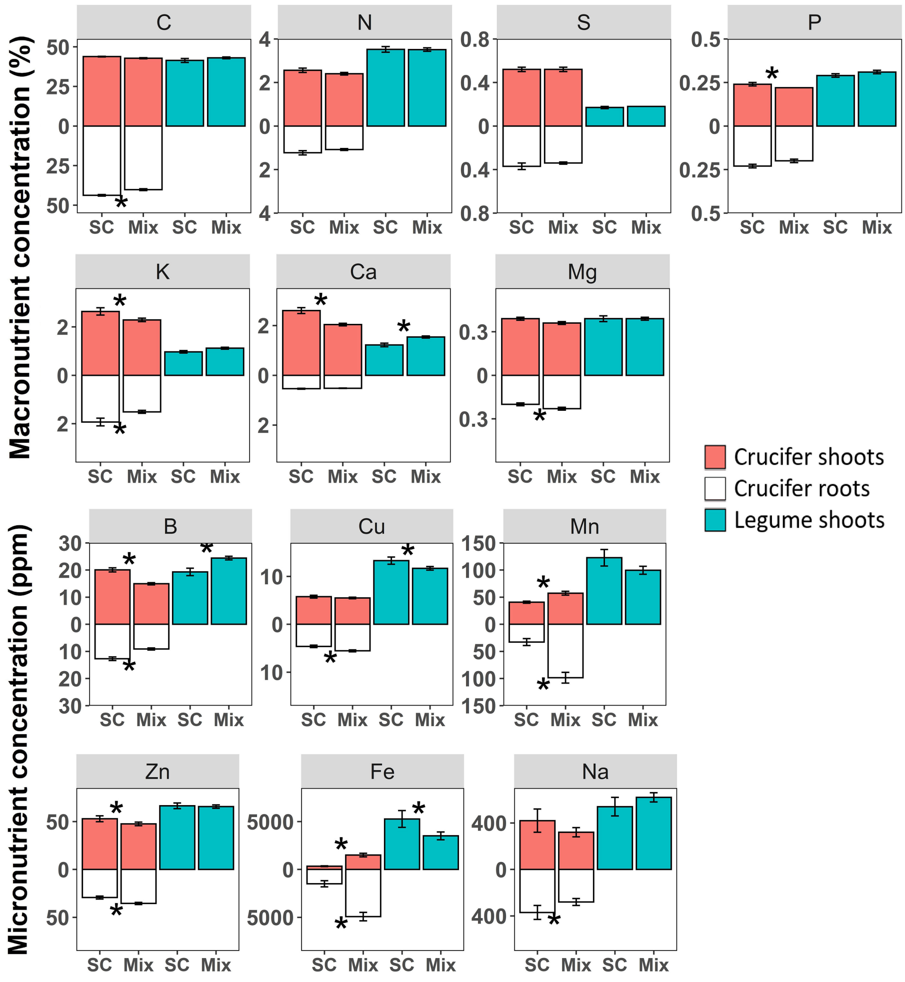

FIGURE 1
Macronutrient (%) and micronutrient (ppm) concentrations in shoots and roots of crucifers and shoots of legumes. Values correspond to the mean of sole (SC) and mixed (Mix) crucifers or legumes. Asterisks indicate a significant difference in macro- and micronutrient concentrations between a given family in a sole crop and in mixtures ($P < 0.05$). Error bars represent standard errors.

Interestingly, some differences in nutrient concentrations in mixtures compared to sole cover crops showed opposite patterns for crucifer shoots compared to legume shoots (Figure 1). Fe was the only nutrient that had significantly higher concentrations in crucifers and lower concentrations in legumes than those in the respective sole crops (Figure 1), suggesting that the crucifers may have outcompeted the legumes for Fe. In contrast, Ca and B were the only nutrients that had significantly higher concentrations in legumes and lower concentrations in crucifers than those in the respective sole crops (Figure 1), suggesting that the legumes may have outcompeted the crucifers for Ca and B.

Nutrient uptake in crucifer and legume mixtures

Nutrient uptake by cover crop mixtures was significantly higher or the same as that of the best sole cover crop family, except for Cu and Fe, for which the legume sole crop took up more nutrients than the mixtures (Figure 2). Shoot biomass, expressed as C content, was the only component that mixtures produced significantly more of (1263 kg C ha^{-1}) than crucifer sole crops (1014 kg C ha^{-1}) or legume sole crops (941 kg C ha^{-1}), especially for mixtures with turnip rape, white mustard, soya bean and Egyptian clover (Figure 2; Table 2).

Compared to crucifer sole cover crops, species mixtures (legume and crucifer combined) acquired significantly more N (+40%) and took up significantly more P (+16%), Cu (+81%), Fe (+763%), Mn (+122%) and Zn (+32%) (Figure 2). In contrast species mixtures did not acquired significantly more of these nutrients compared to legume sole cover crops (Figure 2). The N acquisition and P and Cu uptake were particularly higher in mixtures including turnip rape and white mustard compared to their corresponding sole cover crops (Table 2).

In contrast, compared to legume sole cover crops, species mixtures (legume and crucifer combined) took up significantly more S (+180%), K (+137%), Ca (+84%), Mg (+22%) and B (+26%, except for faba bean and hairy vetch), while these nutrients did not differ significantly in crucifer sole cover crops

TABLE 1 Macronutrient (%) and micronutrient (ppm) concentrations in shoots of crucifer and legume sole crops (SC) and species mixtures (Mix).

		Crucifer species					Legume species							
Nutrient	SC/ Mix	rape	white mt	Ethi. mt	turnip rape	radish	crimson C	Egyptian C	common V	purple V	hairy V	Narbonne V	faba bean	soya bean
C (%)	SC	44.5	45	44.1	43	42.6	29.4	45.4	41	47.3	38	43.1	46.2	45
	Mix	43.5	44.4	44	40.9	39.2	29.5	42.4	45.8	46.1	41.8	44.1	43	44.1
N (%)	SC	2.7	2.3	2.5	3	2.4	2.4	2.9	3.7	4.1	3.5	4.4	4.4	2.5
	Mix	2.3	2.3	2.7	2.9	2	2.1	2.6	3.9	3.9	3.4	4.3	4.1	2.9
S (%)	SC	0.52	0.43	0.62	0.53	0.51	0.12	0.14	0.17	0.23	0.17	0.2	0.23	0.15
	Mix	0.48	0.41	0.6	0.59	0.54	0.13	0.14	0.21	0.2	0.19	0.18	0.19	0.15
P (%)	SC	0.25	0.23	0.24	0.25	0.23	0.19	0.25	0.32	0.29	0.27	0.37	0.42	0.23
	Mix	0.22	0.21	0.23	0.26	0.19	0.18	0.24	0.37	0.3	0.27	0.39	0.34	0.26
K (%)	SC	2.2	2.8	1.8	3.4	3	0.71	0.59	0.93	0.99	1.2	1.1	1.1	0.87
	Mix	1.9	2.6	1.9	3.2	2.8	0.86	0.6	1.3	0.97	1.4	1.2	0.92	0.94
Ca (%)	SC	2.1	2.2	1.6	2.1	2.1	0.96	1.8	0.94	1.2	1.2	1.2	1.1	1.9
	Mix	1.5	1.8	1.7	1.9	1.4	1.2	1.8	1.4	1.4	1.8	1.3	1.3	1.9
Mg (%)	SC	0.4	0.33	0.35	0.4	0.47	0.44	0.23	0.32	0.26	0.42	0.45	0.37	0.7
	Mix	0.36	0.3	0.35	0.4	0.41	0.45	0.26	0.31	0.27	0.44	0.45	0.42	0.66
Na (ppm)	SC	230	230	870	240	550	410	810	670	490	60	690	1450	20
	Mix	150	210	670	270	510	440	820	890	470	170	900	1050	170
B (ppm)	SC	15.4	20.8	17.1	25	21.9	12.2	24.8	17.7	16.3	15.5	22.1	20.7	32.3
	Mix	11.9	18.4	15.3	19.4	16.4	13.9	24.7	26.4	19.7	24.3	27.3	19.3	33.1
Cu (ppm)	SC	4.8	8.1	5.6	6.1	4.2	12.6	13.1	11.2	12.1	12.6	18.4	21.4	9.8
	Mix	4.9	7.6	5.4	6.3	4.8	12.8	11.6	9.3	11.3	10.9	15.8	16.4	10.4
Fe (ppm)	SC	274	172.8	219.5	379	630.3	13915.7	2164.3	5569.7	2418.7	7529.7	4267	1835.3	1609.3
	Mix	1433.7	485.2	610.1	2169.3	3461.1	13595	3687.6	1118.4	2101.1	4169.3	3139.8	4553.4	2383.6
Mn (ppm)	SC	46.8	38.7	28.3	44.6	45	270.3	68.7	114.4	65.7	162.1	143.4	73.7	59.8
	Mix	61.7	42.6	34.2	69.4	79.8	250.3	91.7	51.2	64.7	109.3	135.1	136.4	78.3

(Continued)

TABLE 1 Continued

Nutrient	SC/ Mix	Crucifer species					Legume species							
		rape	white mt	Ethi. mt	turnip rape	radish	crimson C	Egyptian C	common V	purple V	hairy V	Narbonne V	faba bean	soya bean
Zn (ppm)	SC	38.5	79.6	42.9	51.3	52.7	71.2	43.1	70.9	50.8	78.2	80.5	73.7	42.9
	Mix	36.7	74.2	47	50	54.8	68	42.1	74.4	54.1	68.1	82.6	70	49.4

Each value represents the mean of species cropped as a sole crop (SC) or the mean of mixtures including the species (Mix). mt, mustard; Ethi, Ethiopian; V, vetch; C, clover.

(Figure 2; Table 2). In mixtures, soya bean and crimson clover had higher S, K, Ca and B uptake; common vetch had higher K and Ca uptake; and Egyptian clover had higher K and Ca uptake than those in their respective sole crops (Figure 2; Table 2). Mg and Na were the only nutrients with no significant difference in uptake between the mixture and crucifer and legume sole cover crops (Figure 2; Table 2).

Land equivalent ratio of nutrient uptake

The LER was significantly greater than 1 for all nutrients, indicating that the bispecific mixtures had significantly greater nutrient-acquisition capacity than sole cover crops (Figure 3). The LER of C, N, S, P, K, Ca, Mg, B, Cu and Zn was ca. 1.25, while the LER of Mn, Na and Fe was 1.53, 1.74 and 3.93, respectively. The crucifer partial LER (pLER_C) was significantly greater than 0.5 for all nutrients except Na and B, indicating that crucifers took up more nutrients in mixtures than as sole cover crops, which explained most of the high LERs (Figure 3). The legume partial LER (pLER_L) was significantly greater than 0.5 for Ca, Na and B, indicating that legumes took up more of these nutrients per plant in mixtures than as sole cover crops (Figure 3). The pLER_L did not differ significantly from 0.5 for all other nutrients indicating that mixture had no effect on legume acquisition per plant for these nutrients.

At the species level, all crucifers had a pLER_C > 0.5, except for rape for N, P, K, Ca, B, and Na (y axis, Figure 4). Crucifer species competed differently with the associated legume in mixture as shown by different pLER_L according to the crucifer included in mixture (x axis, Figure 4). For example, legumes had pLER_L< 0.5 for most nutrient when mixed with white mustard and radish while they had pLER_L > 0.5 for all nutrients when mixed with rape (x axis, Figure 4).

Among legumes, some species had a pLER_L< 0.5 such as hairy vetch (for nearly all nutrients), purple vetch (for S and Cu), faba bean (for S, P, K and Cu) and common vetch (for Mn and Fe), while other legume × nutrient combinations had a pLER_L > 0.5 (Figure 4).

None of species mixtures had both pLER_C and pLER_L< 0.5, indicating that overall competition in mixtures was never stronger than complementarity and compensation. In comparison, approximately half of the species mixtures had a pLER_L and pLER_C > 0.5, indicating that complementarity and facilitation were stronger than competition (Figure 4).

Discussion

Competition and complementarity for nutrient acquisition in species mixtures

Our study indicated that cover crop mixtures influenced nutrient acquisition of all species tested as cover crops, but the influence depended on the nutrient considered. We used the “4C concept” developed by Justes et al. (2021) to understand the main

TABLE 2 Macronutrients (kg ha^{-1}) and micronutrients (g ha^{-1}) uptake in shoots of crucifer and legume sole crop (SC) and species mixtures (Mix).

		Crucifer species					Legume species							
Nutrient	SC/ Mix	rape	white mt	Ethi. mt	turnip rape	radish	crimson C	Egyptian C	common V	purple V	hairy V	Narbonne V	faba bean	soya bean
C (kg ha^{-1})	SC	1240.2	1052.4	937.6	588.5	1251.9	630.7	1096	875.6	1586.1	1252.6	209.3	681.2	1024.9
	Mix	673.2	1042.9	666.1	567.5	880.4	437.7	709.3	538.2	804.2	522.9	131.1	336.5	726.2
N (kg ha^{-1})	SC	76.1	53.7	50.9	40.8	77	51	70.4	79.4	136.8	112.7	21.2	64.4	57.8
	Mix	37	54.7	41.4	41.7	45.4	30.8	43.1	46.4	67.4	42.8	12.9	31.8	49
S (kg ha^{-1})	SC	13.9	10.2	13.1	8	16.1	2.6	3.4	3.8	7.6	5.4	0.99	3.4	3.4
	Mix	7.6	10.1	8.9	8.6	12.4	1.9	2.3	2.4	3.4	2.2	0.53	1.5	2.5
P (kg ha^{-1})	SC	6.9	5.2	4.9	3.5	7.1	4.2	6.1	6.7	9.5	8.8	1.9	6.2	5.3
	Mix	3.5	4.7	3.5	3.7	4.3	2.7	4.1	4.4	5.1	3.2	1.2	2.7	4.5
K (kg ha^{-1})	SC	65	64.8	33.6	47.9	86.4	15.6	14.4	20.1	31.9	40.2	5.2	17.2	19.2
	Mix	30.9	62.6	29.3	44.4	60.6	12.3	10.1	14.1	15.8	15.5	3.1	6.8	15.4
Ca (kg ha^{-1})	SC	66.2	58.1	39.4	43.1	83.4	21.2	41.6	19.9	41.9	41.4	5.6	15.8	43.3
	Mix	29.1	49.8	28.6	37.9	39.6	18.1	29.3	15.1	24.1	19.3	3.6	9.1	29.6
Mg (kg ha^{-1})	SC	10.9	7.5	7.3	5.3	12.9	10.3	5.3	6.9	8.2	13.8	2.1	5.5	16.3
	Mix	5.6	7.1	5.2	5.5	8.3	6.5	4.4	3.5	4.4	5.1	1.3	3	10.6
Na (g ha^{-1})	SC	7630	6030	19590	3010	18530	9310	20720	13730	15770	2020	3790	21220	310
	Mix	2640	6010	10150	3680	11810	7050	13450	11060	8090	1480	2700	9150	3960
B (g ha^{-1})	SC	44.5	48.5	36.7	35	67	24.4	58.6	37	55.5	52.1	10.8	30.6	74.3
	Mix	18.7	43.5	22.9	25.9	34.3	21.4	41.9	29.2	33.7	24.5	7.4	14.5	53.7
Cu (g ha^{-1})	SC	13.4	18.7	11.4	8.5	12.5	30.4	32.6	23.4	40.1	40.4	10	31.3	23.2
	Mix	7.7	18.8	8.2	8.9	9.7	18.4	19.7	11	19	13.1	4.7	13.4	17.9
Fe (g ha^{-1})	SC	719.3	378.4	520.9	504.4	1790	34105	4961.1	11454	6978.9	23779.3	2023.1	2603.5	3332.5
	Mix	2010.6	1153.5	939	3274	5790.6	19208.1	6318.1	1297.4	3401.9	6019.3	1444.7	2973.8	3172.6
Mn (g ha^{-1})	SC	130.6	88.8	60.4	55.4	136.2	661	153.3	236.9	208.3	516.9	64.6	106.2	128.8
	Mix	92.4	101.3	52.5	100	142.2	356.8	156.1	55.1	106.2	152.4	43.9	90.1	119.7

(Continued)

TABLE 2 Continued

Nutrient	SC/ Mix	Crucifer species					Legume species							
		rape	white mt	Ethi. mt	turnip rape	radish	crimson C	Egyptian C	common V	purple V	hairy V	Narbonne V	faba bean	soya bean
Zn (g ha^{-1})	SC	103.7	187.7	90.1	68.7	151.4	170.4	107.3	148.1	164.1	254.5	40.4	107.6	99.6
	Mix	57	184.4	70.1	70.1	109	97.9	70.1	82.8	90.5	82.8	23.2	55	82.8

Each value represents the mean of species cropped as a sole crop (SC) or the mean of mixtures including the species (Mix). mt, mustard; Ethi, Ethiopian; V, vetch; C, clover.

species interactions in mixtures. LER exceeded 1 for all cover crop family × nutrient combinations, indicating that the overall performances of species mixtures were driven by plant-plant complementarity and cooperation (facilitation) more than competition. This validates our hypothesis that interspecific interactions between crucifers and legumes are synergistic due to niche complementary and/or facilitation processes, even if the degree of synergy depends on the nutrient. For most nutrients, an LER > 1 was due mainly to higher biomass in mixtures, with relatively constant nutrient concentrations in shoots. For a few nutrients, however, the difference in concentration was larger than the difference in plant biomass, and because biomass was higher in most mixtures, some nutrient concentrations (Mn, Fe and Na) may have driven the higher biomass in mixtures. Indeed, Fe and Mn concentrations in crucifers were much higher when grown in mixtures than as sole cover crops, which suggests 1) stronger intraspecific competition among crucifer plants in sole crops than interspecific competition with legume plants in mixtures, and/or 2) facilitation by legumes of Fe and Mn acquisition by crucifers. This agrees with results of Inal et al. (2007), who observed higher Fe concentrations in maize intercropped with a legume (peanut). To our knowledge, our study is the first to observe such a large increase in Fe and Mn in crucifers intercropped with legume cover crops. Surprisingly, crucifers in mixtures had significantly lower P, K, and Ca concentrations but the same Mg concentrations, while other authors have suggested that legumes facilitate acquisition of these nutrients, such as in cereal-legumes mixtures (Hauggaard-Nielsen and Jensen, 2005; Hinsinger et al., 2011; Tang et al., 2021). Instead, legumes seemed to compete strongly with the crucifer for Ca, as their Ca concentrations increased in mixtures. Although crucifers cannot host AMF that could facilitate acquisition of nutrients such as P, Mn, Ca, Fe and B in many types of mixtures (Jin et al., 2014; Teste et al., 2014), legumes in mixtures did not have lower P, Mn or B concentrations than those of sole legumes. Nevertheless, crucifers competed slightly or strongly with the legume for Cu and Fe, respectively, which could decrease the legume's ability to fix N (O'Hara et al., 1988; Brear et al., 2013). Interestingly, Fe concentrations in mixtures were lower in vetches and crimson clover but higher in Egyptian clover, soybean and faba bean. Unlike cereals, which exude phytosiderophores that can increase Fe and Zn bioavailability for the companion legume or crucifer (Zuo and Zhang, 2009; Xue et al., 2016; Jiao et al., 2021; Sadeghzadeh et al., 2021), crucifers do not have the same capacity to increase Fe availability in the soil. Nevertheless, when intercropped with a cereal, crucifers do not decrease Fe, Mn, Cu or Zn concentrations in the grain (e.g. Xia et al., 2013 for maize/turnip intercropping). Thus, the increase or decrease in Fe concentrations in mixed crucifers and legumes, respectively, could be due to strong competition of the crucifer with the legume more than a change in the degree of facilitation. Moreover, in basic soils (not present in our study) and unlike crucifers, most legumes release carboxylic acids that dissolve phosphate ions from bound forms, such as calcium and iron phosphates that are otherwise unavailable to plants and immobile in the soil. Consequently, legumes can then make Fe more available for the companion crucifer (Mikić et al., 2015).

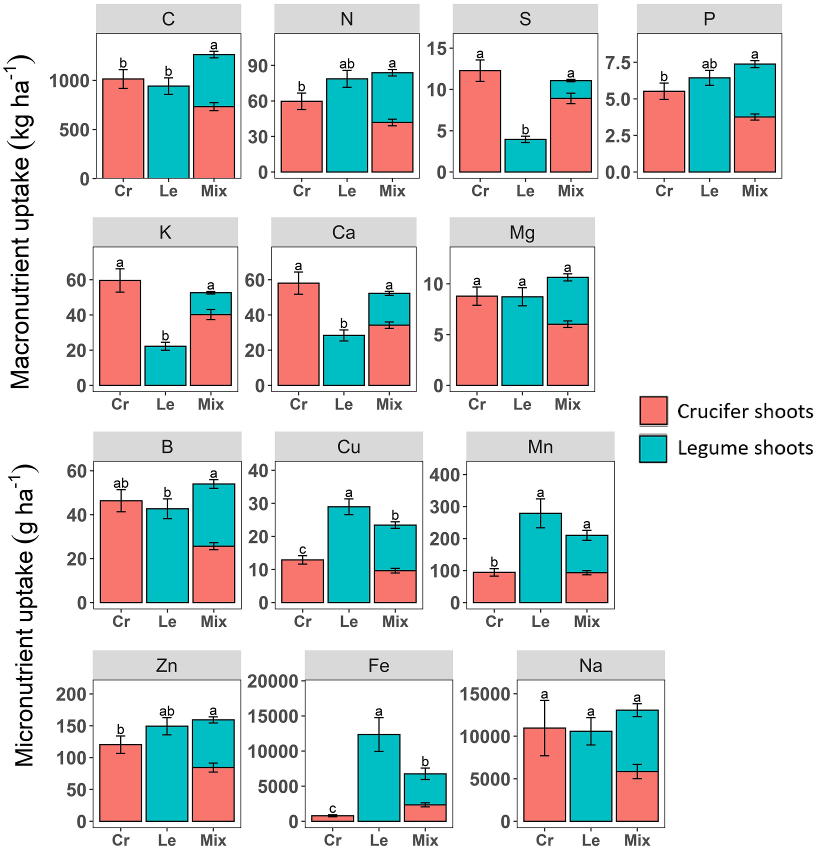

FIGURE 2

Macronutrients (kg ha^{-1}) and micronutrients (g ha^{-1}) acquired in shoots of crucifers and legumes in sole crops and species mixtures. Values correspond to the mean of crucifer (Cr) and legume (Le) sole crops or the mean of both species included in a mixture (Mix). Different letters indicate treatments with significant differences at P< 0.05. Only shoots of crucifers and legumes were included in the analysis.

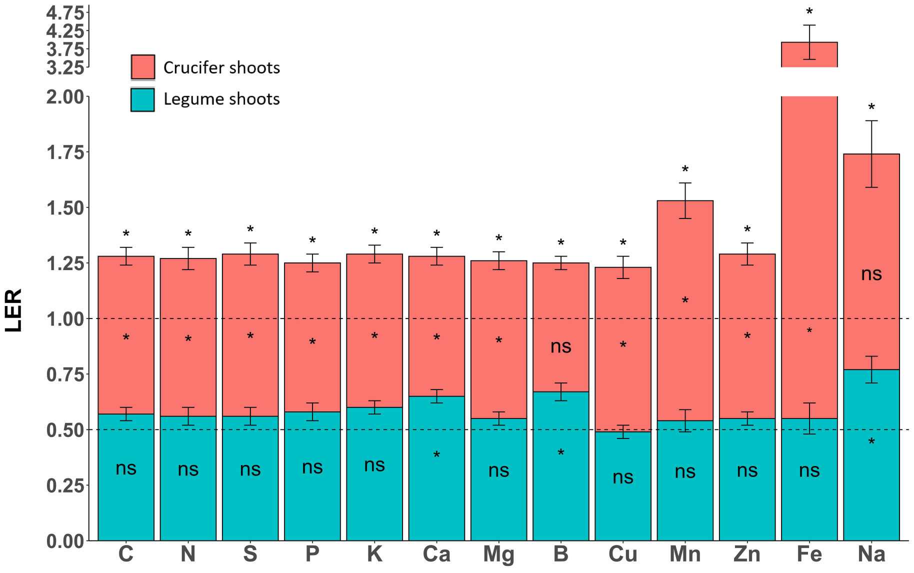

FIGURE 3

Partial land equivalent ratio (pLER) of crucifers (red) and legumes (blue). The land equivalent ratio (LER) equals the sum of the two partial LERs. Each value represents the mean of species mixtures. Asterisks indicate that pLER and LER were significantly greater than 0.5 and 1.0, respectively. Error bars represent standard errors.

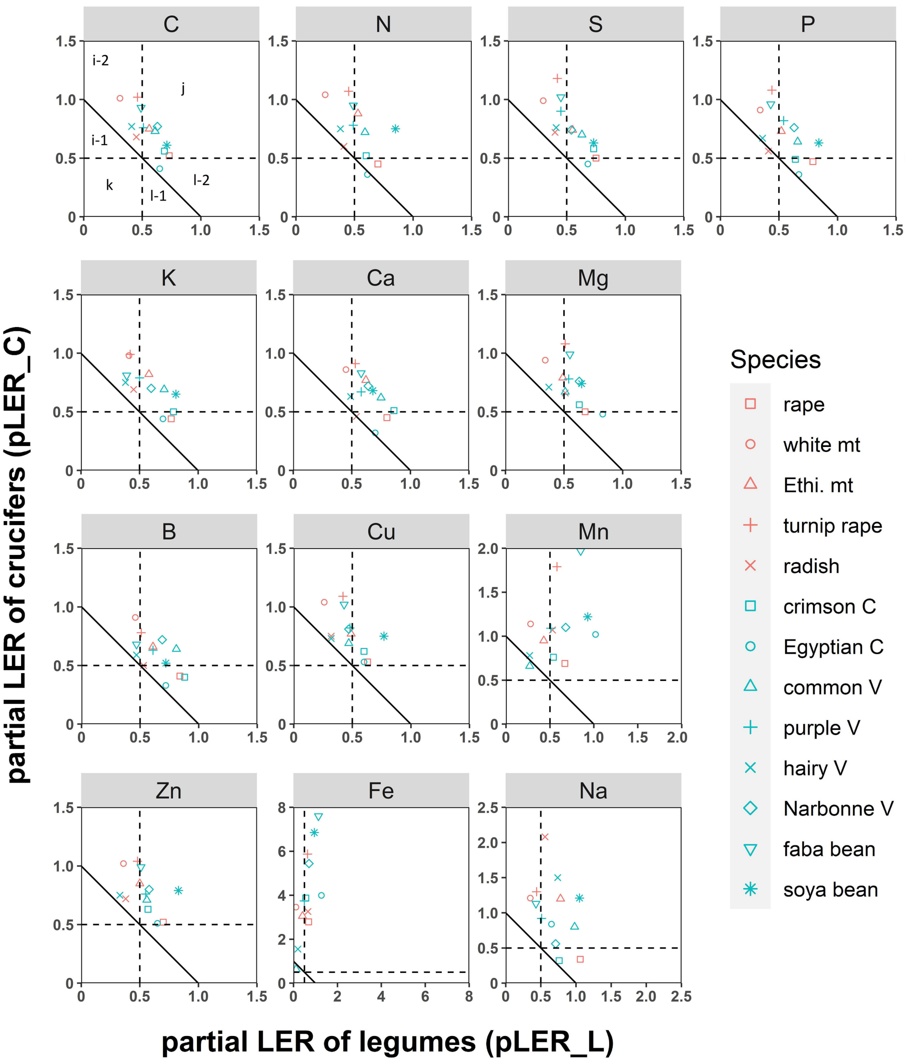

FIGURE 4
Partial land equivalent ratios (LER) for nutrient acquisition of crucifers (LERp_C) as a function of that for legumes (LERp_L). Each point represents the mean of species included in species mixtures. The solid line represents y = x (corresponding to LER = 1). Dashed lines represent pLER_C = 0.5 and pLER_L = 0.5. In area (j), both species grow better in mixtures than as sole crops, indicating that effects of species complementary and cooperation are stronger than those of competition (and vice versa in area (k)). Areas (i-1) and (i-2) indicate that the legume dominates the crucifer and vice versa in (l-1) and (l-2). Plant-plant interactions lead to an advantage of mixture in (i-2) and (l-2), but a poorly balanced mixture due to too much competition between the two species in (i-1) and (l-1). See Justes et al. (2021) for more details. mt, mustard; Ethi, Ethiopian; V, vetch; C, clover.

Cross-analysis of nutrient concentrations in plants could help detect antagonisms or facilitation between species for a given nutrient that would decrease acquisition of other nutrients and thus the performance of the entire mixture (*i.e.* Liebig's Law). Rietra et al. (2017) found synergy between P and K acquisition and antagonism between K and Mg acquisition. Our study confirms these results in crucifers, as lower K concentrations in mixtures than those in sole crops were associated with lower P concentrations but higher Mg concentrations. Nevertheless, cross-analysis of nutrient acquisition requires further studies at more sites and for additional years to definitively validate the net effect of nutrient-acquisition processes by cover crops as synergy or antagonism.

From multiple-nutrient uptake to multi-service analysis

Multiple-nutrient acquisition per unit area by cover crops is a good proxy of the performance of their catch-crop function and ecosystem service for decreasing nutrient losses through leaching and/or runoff and increasing nutrient recycling for the next cash crop. Previous research has provided limited insights into the acquisition of multiple nutrients by sole cover crops (Wang et al., 2008; Wendling et al., 2016) and even fewer studies have focused on cover crop mixtures (Koefender et al., 2016). We found that mixed crucifer and legume cover crops exhibit a synergistic effect on nutrients acquisition. Cover crop mixtures composed with legumes and non-legumes acquired as much or even more of each nutrient compared to the best-performing sole cover crops (Figure S4), as observed in previous studies of N and S elements (Couëdel et al., 2018a; Couëdel et al., 2018b). In other words, adding a legume to crucifer cover crops can increase the uptake of C, N, Fe, Cu and Mn, while adding a crucifer to legume cover crops can increase the uptake of C, S, K, Ca, Mg and B without decreasing those for other nutrients. Besides nutrients uptake synergy, biomass and C accumulated were the only components for which species mixtures performed better, indicating more photosynthesis per unit area than both sole crucifers and legumes, confirming results that highlighted higher biomass and C accumulated in mixtures than in sole crops (Wortman et al., 2012; Mortensen et al., 2021). P, Mg, B and Zn uptake were also slightly higher in mixtures, albeit not statistically significant, than in both crucifer and legume sole cover crops, suggesting potential complementarity of mixtures in providing catch-crop effects for these nutrients as well.

In addition to the multiple-nutrient catch-crop effects, multiple-nutrient recycling effects (for availability for the next crop) of cover crops are another set of ecosystem services that needs to be evaluated. Nutrient recycling have been extensively studied only for P (Takeda et al., 2009; Maltais-Landry et al., 2016; Teles et al., 2017) and a few other nutrients, such as N, S, K, Ca and Mg (Eriksen et al., 2004; Brunetto et al., 2011; Tiecher et al., 2017). Interestingly, recycling of N and S showed different patterns (Tribouillois et al., 2016; Couëdel et al., 2018a; Couëdel et al., 2018b), such as the C:N ratio was not the only driver of nutrient recycling, as supported by the key influence of the C:S ratio on S recycling. Therefore, we advocate the need for further studies to address multiple macro- and micronutrient recycling processes from cover crop decomposition and species mixtures in general. Such studies would help assessing the multi-nutrient ecosystem service effects of cover crops. Indeed, among the growing literature that assesses multiple services provided by cover crops (Schipanski et al., 2014; Finney et al., 2017; Chapagain et al., 2020), such as biocontrol, biomass production and soil C storage, nutrient ecosystem services are assessed mainly under the prism of the N cycle, while other macro- and micronutrients also generate key ecosystem services (Daryanto et al., 2018). By assessing the multi-nutrient uptake capabilities of species mixtures composed of crucifers and legumes, our study contributes valuable insights for assessing their performance across a broader spectrum of ecosystem services.

Conclusion

Overall, crucifer-legume cover crop mixtures provide synergetic multiple-nutrient catch-crop services by acquiring as much or more of each nutrient than the best sole cover crop. In species mixtures, crucifers acquired certain nutrients in much larger quantities, such as Fe (ca. four-fold) and Mn (ca. two-fold), than in sole crops. Crop nutrition status revealed strong niche complementarity and/or facilitation in crucifer-legume mixtures, in which crucifers had higher Mn and Fe concentrations, and legumes had higher Ca and B concentrations. Net competition and/or dilution effects of nutrient concentration were also identified in the cover crop mixtures tested, as legumes tended to have a negative impact on crucifer concentrations of Ca, P, K and B, while crucifers had a negative impact on legume concentrations of Cu and Fe. Our work validated the potential of species complementarity for multi-nutrient uptake using a wide range of crucifer-legume species and cultivars. However further studies should include additional sites with a variety of soil and climate conditions to further validate these first results and help design more efficient cover crop mixtures to achieve high levels of multiple nutrient-related ecosystem services under different growing conditions.

Data availability statement

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

Author contributions

LA and EJ, designed and funded the study. AC prepared the data and performed all preliminary and final analyses. All authors were involved in the interpretation of the results and contributed to writing the original version of the manuscript and improving the subsequent ones. All authors contributed to the article and approved the submitted version.

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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/fagro.2023.1223639/full#supplementary-material

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Catch crops in the Argentinean Pampas: a synthesis-analysis on nutrient characteristics and their implications for a sustainable agriculture

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Catch crops (CCs) are crops that grow between two cash crops, accumulating nutrients in their biomass and later releasing them through the decomposition of their residues. To our knowledge, no study has compared the nutrient-related traits of different CCs species. This comparison is essential for understanding the possible roles of CCs in agroecosystems and for optimizing adjustments of fertilizer rates. This study aims to: i) synthesize characteristics of the CCs, such as aboveground dry matter accumulation, nutrient [carbon (C), nitrogen (N), sulfur (S), and phosphorus (P)] concentration and accumulation, and C:nutrient ratios, ii) explore factors defining the mentioned characteristics, and iii) discuss their implications on the ecosystem services provided. By analyzing data from 52 studies, (98 site-years) in the Argentinean Pampas, we observed that grasses (excluding ryegrass) accumulated the greatest aboveground dry matter (6.08 ± 0.22 t ha^{-1}, mean ± SE) and C (2.60 ± 0.13 t ha^{-1}), making them suitable for protecting the soil surface from erosion, suppressing weeds, and improving C balances. Conversely, vetch accumulated the highest nutrient content (118 ± 5.3, 15 ± 1.5, and 14 ± 1.2 kg ha^{-1} for N, S, and P, respectively) with the lowest C: nutrient ratio (means of 15:1, 152:1, and 147:1 for N, S, and P, respectively), making it a suitable choice for recycling nutrients and providing extra N. Mixtures presented intermediate characteristics between the monocultures, allowing a balanced provision of the mentioned ecosystem services. Furthermore, the results showed that dry matter accumulation affected the nutrient-related traits analyzed, and it was related to different factors, such as CCs sowing and termination dates or precipitation. The analysis highlights the importance of selecting CCs species based on the desired ecosystem service and provides valuable information for producers as well as for modeling C balances and nutrient cycling.

KEYWORDS
cover crops, species, nitrogen, phosphorus, sulfur, carbon

1 Introduction

Many agricultural regions worldwide cultivate only one crop per year, resulting in long fallow periods with low soil cover and without vegetation (Pinto et al., 2017). This condition causes low resource use efficiency, potential water and wind erosion, and negative carbon (C) balances, contributing to reduced soil organic matter contents (Caviglia and Andrade, 2010; Caviglia et al., 2013; Andrade et al., 2017). The decrease in organic matter levels affects nutrient supply to crops through mineralization (Carciochi et al., 2018; Reussi Calvo et al., 2018; Sainz Rozas et al., 2019). Moreover, the absence of permanent crops limits nutrient recycling in most agroecosystems. For instance, water excesses during winter fallows lead to nutrient losses from the soil through leaching (Gabriel et al., 2013; Frasier et al., 2017), particularly for soil mobile nutrients such as nitrogen (N) (as NO_3^-) and sulfur (S) (as SO_4^{-2}). Additionally, erosion and runoff, promoted by the lack of soil coverage, can increase losses of low-mobile nutrients such as phosphorus (P) (De Baets et al., 2011). To mitigate nutrient losses, farmers have adopted the practice of planting crops that grow between two cash crops, reducing or eliminating fallow periods (Thorup-Kristensen et al., 2003; Rinnofner et al., 2008). These crops are known as “cover crops”, “green bridge crops”, or “catch crops” (CCs), and are not grazed (unlike forage crops), incorporated into the soil (unlike green manures), or harvested (unlike cash crops) (Thorup-Kristensen et al., 2003; Rinnofner et al., 2008; Sievers and Cook, 2018; Crespo et al., 2022; Seitz et al., 2022).

The term “CCs” is commonly used because these crops accumulate nutrients in their biomass and subsequently release them through the decomposition of their residues (Valkama et al., 2015; Kang et al., 2018). In this way, CCs play a pivotal role in nutrient recycling, diminishing nutrient losses during fallow periods and supplying nutrients to the succeeding cash crop in the sequence. This role holds various environmental and economic implications. For instance, CCs have the potential to reduce the need for N fertilizers, thereby lowering greenhouse gas emissions and mitigating eutrophication from N leaching and P runoff (Abdalla et al., 2019; Alvarez et al., 2022). Additionally, CCs could lessen dependence on external inputs such as fertilizers, thereby reducing production costs. Consequently, CCs contribute to enhancing nutrient use efficiency and establishing sustainable agroecosystems over time, helping to close nutrient cycles (Restovich et al., 2012; Daryanto et al., 2018).

The quantity and timing of nutrients supplied from CCs residues to the succeeding cash crop depend on factors such as the species, the accumulated biomass, and the C:nutrient ratio of the decomposing residue (Restovich et al., 2012; Sievers and Cook, 2018). Nevertheless, CCs may also reduce nutrient availability through immobilization, adversely affecting the nutrition of the following crop (Jahanzad et al., 2016; Sievers and Cook, 2018; Otte et al., 2019; Carciochi et al., 2021a). Besides that, CCs incorporate C into the soil, leading to an increase in soil organic C content (Duval et al., 2016; Jian et al., 2020). If the CC is a legume, it could incorporate N by biological fixation and allow for a reduction in the N fertilizer rate in the main crop. Furthermore, CCs help control weeds, improve soil structure, and diminish soil erosion and evaporation (Schipanski et al., 2014; Rimski-Korsakov et al., 2015). Conversely, some reported negative effects of CCs include reductions in cash crop productivity, often linked to the consumptive water use of CCs, particularly in semiarid environments (Blanco-Canqui et al., 2015), or the occasional increase in greenhouse gas emissions following their termination (Basche et al., 2014).

The Argentinean Pampas region covers ca. 60 Mha and accounts for 90% of the country’s grain production (Lavado and Taboada, 2009; SAGyP, 2023). Commercial arable agriculture based on nutrient extraction has been used for decades generating negative nutrient balances and soil degradation, which were magnified by the widespread cropping of soybean (*Glycine max*. (L.) Merr.) (Lavado and Taboada, 2009; Wyngaard et al., 2022). In the last 20 years, winter CCs have been slowly adopted by farmers as a possible tool for improving soil quality (Rimski-Korsakov et al., 2015; Alvarez et al., 2017). Currently, a survey conducted by the SAGyP (2023) indicates a 318% increase in the area with CCs (from 110,000 ha to 350,000 ha) in the Pampas region from 2019 to 2022. This makes the Argentinean Pampas an appropriate area to analyze the potential impact of CCs on nutrient cycling and C balance in agroecosystems.

All the benefits provided by CCs to the agroecosystems are known as “ecosystem services”. So far, the existing information about CCs in the Argentinean Pampas has been summarized by Alvarez et al. (2017) and Rimski-Korsakov et al. (2015), who focused on their effect on some ecosystem services, such as improvements in chemical and physical soil properties, crop yields, and water availability after their termination. However, no study has yet summarized CCs characteristics (i.e., dry matter and nutrient accumulation, and quality of the residues) and the possible effect on nutrient (N, S, and P) availability for the succeeding crop and C contribution to the soil. This is crucial for fine-tuning fertilizer rates, which enhances economic profits and minimizes environmental externalities, and for understanding the CCs ability to supply C while maintaining or improving the soil C balance. Therefore, for the CCs species mainly used in the Argentinean Pampas, we aim to i) synthesize characteristics of the CCs such as aboveground dry matter accumulation, nutrient (N, S, and P) concentration and accumulation, and C:nutrient ratio, ii) explore factors defining the mentioned characteristics, and iii) discuss their implications on the ecosystem services provided to the agroecosystems.

2 Materials and methods

2.1 Study area

The climate of most of the region is humid-temperate without a dry season. Annual average temperatures range between 14°C in the south to 18°C in the north. Annual precipitation has a large inter-annual variability, ranging from 500 mm $year^{-1}$ in the southwest to 1100 mm $year^{-1}$ in the northeast. Therefore, Köppen classification categorizes the region as “Cfa,” indicating a warm and temperate rainy climate (C). The precipitation is uniformly distributed throughout the year “f”, and the temperature during the warmest month is equal to or greater than 22°C, defining the thermal condition as “a” (Beck et al., 2018). The most representative great groups of soils are Argiudolls and Hapludolls (Rubio et al., 2019). Soil organic C shows a strong association with rainfall and the pH ranges from 6 to 7 in well-drained agricultural soils (Berhongaray et al., 2013).

At present, around 76% of the Argentinean Pampas is under cropping with soybean, maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), and sunflower (*Helianthus annuus* L.) being the main crops (SAGyP, 2023). Continuous cultivation of grain crops is common, and pastures or annual forages tend to disappear from rotations (Alvarez et al., 2014). The average yields of maize, soybean (1st crop), soybean (2nd crop), sunflower, wheat, and barley in this region over the last 10 years are 6.7 t ha^{-1}, 2.8 t ha^{-1}, 2.1 t ha^{-1}, 2.0 t ha^{-1}, 2.8 t ha^{-1}, and 2.4 t ha^{-1}, respectively (SAGyP, 2023). Notably, these values can be more than double in the most productive regions for each crop. Additionally, almost 90% of the agricultural production is under no-till system (ReTAA, 2023).

Crop fertilization rates in Argentina depend on the crop. According to ReTAA (2022); ReTAA (2023), soybean receives an average fertilization rate of 6 to 10 kg P ha^{-1}, and N fertilization is uncommon for this crop. In maize the average rates applied are 73 kg N ha^{-1} and 13 kg P ha^{-1}, whereas in sunflower are 20 kg N ha^{-1} and 10 kg P ha^{-1}. For winter crops such as wheat and barley, fertilizer rates range from 60 to 100 kg N ha^{-1} and 13 to 20 kg P ha^{-1}. Over the past two decades, the reduction in the proportion of grasses in crop rotations has led to decreased organic matter content in the soils (Wyngaard et al., 2022). Additionally, cation extraction with crop harvest has led to acidified soils (Sainz Rozas et al., 2011; Sainz Rozas et al., 2019; Larrea et al., 2023). Currently, most soils in the Argentinean Pampas do not exhibit potassium deficiencies, although isolated cases have been reported in the northeastern part of the region (Sainz Rozas et al., 2019; Larrea et al., 2023). Regarding micronutrients, the levels in the region are generally above critical thresholds, although there have been reports of zinc deficiency in some areas (Sainz Rozas et al., 2019; Larrea et al., 2023).

2.2 Data collection

A database with 52 studies was generated from papers published in journals and national and international conferences between the years 2004 and 2022. We conducted an online search in Scopus and Google Scholar using the keywords: “Cover crops”, “Green bridge”, “Catch crops”, “Green manures”, “Argentinean Pampas”, “Carbon”, “Nitrogen”, “Phosphorus”, “Sulfur” and “Nutrients”, both in Spanish and English. A total of 1160 studies were found with the following search equation that was applied to the title, abstract, and keywords of the publications: “Argentinean pampas” OR “Argentine pampas” and “cover crops” OR “green bridges” OR “catch crops” OR “green manures” AND “nitrogen” OR “sulfur” OR “phosphorus” OR “nutrients” OR “carbon”. Works from the proceedings of conferences/congresses of the Argentinean Soil Science Association (from 2004 to 2022) were also included. Technical bulletins from the Argentinean National Institute of Agricultural Technology and undergraduate and postgraduate theses in repositories from different universities were included in the search. In this way, it was avoided international journal publication bias, which usually published experiments with statistically significant differences among treatments. We carefully made sure to exclude any doubtful or unreliable information that could lead to misleading data. Therefore, the first step after the online search was to filter publications by title and keywords, removing 884 studies (Supplementary Figure 1). The second step was to filter by abstract, removing 936 studies in total. Finally, we filter by full text and selected 52 studies that met the following conditions: 1) were carried out under field conditions and specifying the species of CC used, 2) described characteristics of the CCs (accumulated aboveground dry matter, concentration of C, N, S and/or P in aboveground dry matter, C:nutrient ratios) and 3) specified sowing and termination dates. Therefore, 98 site-years were finally included in the dataset (Supplementary Table 1) covering a large part of the Argentinean Pampas region, where CCs are typically used Figure 1 (Pinto et al., 2017).

The information was obtained from texts, tables, and figures, using in the last case the software Get Data Graph Digitizer version 2.26.0.20 (Federov, 2013). From the collected variables, new parameters were calculated. Nutrient accumulation was computed by multiplying the nutrient concentration by the amount of aboveground dry matter. When C:nutrient ratios were not reported but the concentrations of C and nutrients were available, we calculated the ratios by dividing the concentrations of C by the concentrations of the respective nutrients.

Publications usually reported data from one single experiment, but in some cases, results from two or more experiments were included. The soils were Mollisols: Argiudolls and Hapludolls (Supplementary Table 1). In general, the experimental design used was randomized complete blocks with factorial, split-plot, or split-split-plot arrangement and 3 or 4 replicates. In all cases, data were collected from non-fertilized CCs, and their termination was accomplished through herbicide application, and in some cases, by rolling.

Five CC groups were considered for the analysis of the variables: grasses (excluding ryegrass (*Lolium* sp.)), ryegrass, vetch (*Vicia* sp.), cruciferous, and mixtures. Ryegrass was analyzed as a separate group from grasses based on a first analysis that we performed among all the grasses species, which showed significant differences between ryegrass and the other grasses on aboveground dry matter and C accumulation (data not shown). Grasses include oat (*Avena sativa* L.), barley, wheat, and common rye (*Secale cereale* L.). Mixtures consisted of vetch with oat or common rye. Cruciferous included rapeseed (*Brassica napus* L.) and radish (*Raphanus sativus* L.). Vetch was the only legume selected because the data available for other legume species were insufficient.

2.3 Data analysis

Aboveground dry matter accumulation, C, N, S, and P concentration and accumulation, and C:nutrient ratios were

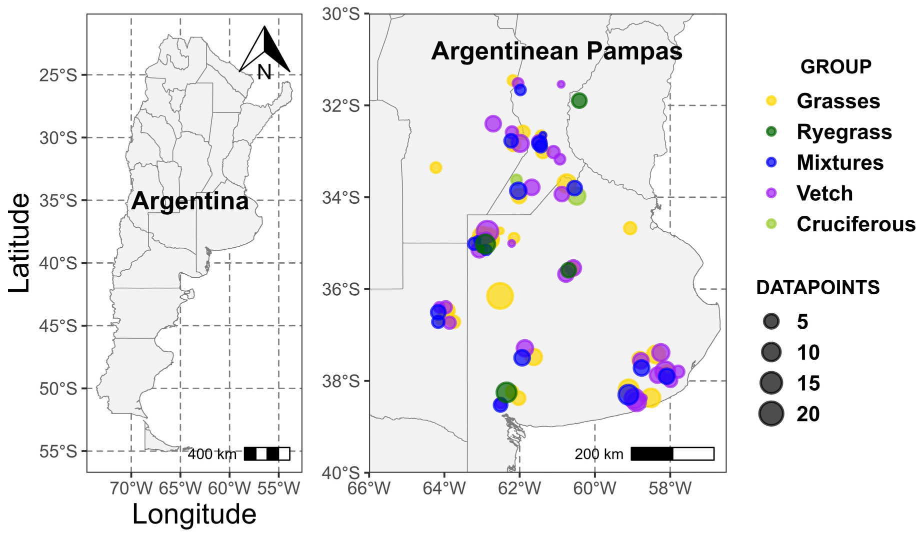

FIGURE 1
Location of the surveyed studies (n = 52; points differ a little from the actual location to avoid overlapping).

analyzed. Due to the low data availability on S and P for ryegrass (n=2) and lack of data for cruciferous, these nutrients were not analyzed in the mentioned CCs groups. The Levene's test was performed using the *car* package in R 4.2.2 software (Fox and Weisberg, 2011) for testing homogeneity of variance across CC groups. As variances were not homogenous, a model comparison was performed by the *gls* function from the *nlme* package in the R 4.2.2 software (R Core Team, 2022), adding the weights = varIdent function (Pinheiro et al., 2017). Then, an ANOVA was performed for that model using the *anova* function from the *stats* package (R Core Team, 2022). When differences among CCs groups were detected, pairwise comparisons were performed using a Tukey test (p-$value$ < 0.05).

To explain the variability observed in aboveground dry matter, a correlation analysis was performed between this variable and location, management (sowing and termination date), crop cycle duration, and the weather information available (accumulated precipitations during the CCs growing season). For this purpose, the *cor.test* procedure included in the R software was used. Finally, we analyzed the relationship between i) nutrient (N, S, and P) concentrations and aboveground dry matter, by fitting negative power functions that typically explain this relationship (Carciochi et al., 2018; Ciampitti et al., 2022), and ii) C:nutrient ratios and aboveground dry matter by fitting simple linear models. All these analyses were not conducted for ryegrass and cruciferous due to the low data availability.

3 Results

3.1 Database characterization

Catch crops were sown from early-March to late-June (without extreme dates) (Supplementary Figure 2A) and termination dates ranged from late-July to late-November (without extreme dates) (Supplementary Figure 2B). Consequently, the median CC cycle duration was 163, 162, 172, 175, and 155 days for grasses, ryegrass, mixtures, vetch, and cruciferous, respectively (Supplementary Figure 2C). Seeding rates were 25-120 kg ha^{-1} for grasses, 7-20 kg ha^{-1} for ryegrass, 7-50 kg ha^{-1} for oat or common rye plus 12-40 kg ha^{-1} vetch on mixtures, 7-45 kg ha^{-1} for vetch, and 5-20 kg ha^{-1} for cruciferous.

3.2 Aboveground dry matter and carbon accumulation

Aboveground dry matter presented a large variability both among groups and within each group, ranging from 0.38 to 17.60 t ha^{-1} (Figure 2A). The aboveground dry matter interquartile range (IQR) was 3.96-7.95 t ha^{-1} for grasses, 1.63-4.33 t ha^{-1} for ryegrass, 4.18-8.14 t ha^{-1} for mixtures, 2.80-5.41 t ha^{-1} for vetch, and 1.23-2.44 t ha^{-1} for cruciferous. Overall, aboveground dry matter was greater for mixtures and grasses (mean ± SE 6.21 ± 0.36 and 6.08 ± 0.22 t ha^{-1}, respectively) compared to vetch, ryegrass, and cruciferous (mean ± SE 4.09 ± 0.18, 3.25 ± 0.39, and 2.45 ± 0.59 t ha^{-1}, respectively).

The variability of the accumulated aboveground dry matter within each CC group (except cruciferous with little information available, n=10) was related to some of the variables analyzed (location, management, and precipitations). Aboveground dry matter accumulation was weakly correlated to the location (only positive correlations with longitude for ryegrass and vetch), positively correlated to the termination date (in all the groups), crop cycle duration (in grasses, mixtures, and vetch), and precipitation during the growing season (in grasses and ryegrass), while it was negatively correlated to the sowing date in vetch (Table 1). Moreover, based on linear relationships between aboveground dry matter accumulation and each variable, we observed that: i) one-day delay in the termination date caused

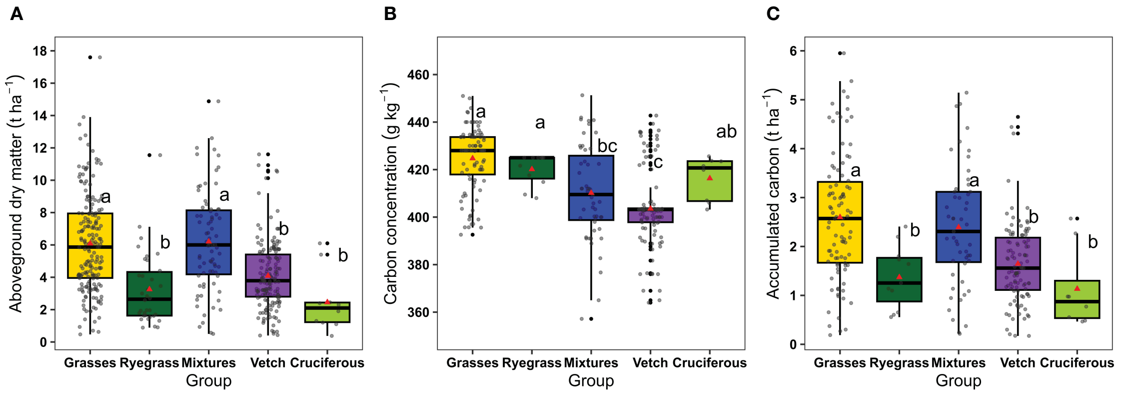

FIGURE 2
(A) Aboveground dry matter accumulated in grasses (*n*=188), ryegrass (*n*=34), mixtures (*n*=75), vetch (*n*=158), and cruciferous (*n*=10), **(B)** carbon concentration and **(C)** carbon accumulation in aboveground dry matter of grasses (*n*=97), ryegrass (*n*=11), mixtures (*n*=51), vetch (*n*=107), and cruciferous (*n*=8). Boxplots represent the 5th and 95th (whiskers), 25th (lower edge of the box) and 75th (top edge of the box) percentiles. The line inside the boxes represents the median and the red triangle inside the boxes represents the mean. Same letters indicate not significant differences among means of catch crop groups for each variable described using Tukey multiple comparison test (*p-value* < 0.05).

31.5 kg ha^{-1} (grasses; *p-value*<0.001), 22.0 kg ha^{-1} (ryegrass; *p-value*=0.049), 54.4 kg ha^{-1} (mixtures; *p-value*<0.001), and 30.9 kg ha^{-1} (vetch; *p-value*<0.001) of extra aboveground dry matter accumulation; ii) a longer cycle duration led to 23 to 28 kg ha^{-1} day^{-1} of additional aboveground dry matter accumulation in grasses (*p-value*<0.001), mixtures (*p-value*=0.006), and vetch (*p-value*<0.001), and iii) each millimeter of precipitation generated 6.5 kg ha^{-1} and 18.5 kg ha^{-1} more aboveground dry matter accumulation in grasses (*p-value*<0.001) and ryegrass (*p-value*=0.005), respectively.

Carbon concentration across groups ranged from 357 to 451 g kg^{-1} (Figure 2B). The lowest values were observed in vetch and mixtures (mean ± SE 404 ± 1.6 and 410 ± 2.8 g kg^{-1}, respectively), whereas grasses, ryegrass, and cruciferous presented the highest values (mean ± SE 425 ± 1.4, 420 ± 2.0, and 416 ± 3.3 g kg^{-1}, respectively). On the other hand, accumulated C in aboveground dry matter ranged from 0.17 to 5.95 t ha^{-1}, and similarly to aboveground dry matter, grasses and mixtures achieved the highest values (mean ± SE 2.60 ± 0.13 and 2.39 ± 0.17 t ha^{-1}, respectively), whereas vetch, ryegrass, and cruciferous the lowest (mean ± SE 1.65 ± 0.08, 1.37 ± 0.19 and 1.13 ± 0.29 t ha^{-1}, respectively) (Figure 2C).

3.3 Nitrogen, sulfur, and phosphorus accumulation and C:nutrient ratios

Nitrogen concentration, accumulation, and C:N ratio were different among CC groups (Figures 3A–C). Vetch presented the highest N concentration (mean ± SE 29.6 ± 0.8 g kg^{-1}), accumulation (mean ± SE 118.4 ± 5.3 kg ha^{-1}), and the lowest C:N ratio (mean of 15.3:1). On the contrary, grasses presented the lowest N concentration (mean ± SE 12.9 ± 0.6 g kg^{-1}), accumulation (mean ± SE 69.6 ± 4.5 kg ha^{-1}) [not differing from ryegrass (mean ± SE 58.9 ± 10.6 kg ha^{-1}) or cruciferous (mean ± SE 57.0 ± 21.1 kg ha^{-1})], and the greater C:N ratio (44.4:1) [not differing from ryegrass (29.3:1)]. Nitrogen concentration in cruciferous (mean ± SE 18.3 ± 2.3 g kg^{-1}) and mixtures (mean ± SE 17.8 ± 1.1 g kg^{-1}) was intermediate between vetch and grasses, as well as the C:N ratio (25.4:1 and 27.1:1, respectively).

Sulfur concentration varied from 0.7 to 6.2 g kg^{-1} and followed the order: vetch (mean ± SE 3.2 ± 0.2 g kg^{-1}) > mixtures (mean ± SE 2.5 ± 0.2 g kg^{-1}) > grasses (mean ± SE 1.8 ± 0.1 g kg^{-1}) (Figure 3D). There were no differences among CC groups on accumulated S (mean ± SE 13.8 ± 1.2, 18.6 ± 1.2, 14.9 ± 1.5 for grasses, mixtures and vetch, respectively) (Figure 3E). The C:S ratio presented a

TABLE 1 Pearson´s correlation coefficients between aboveground dry matter (t ha^{-1}) and the location (latitude and longitude), sowing and termination date, crop cycle duration, and precipitation during the growing season for grasses, ryegrass, mixtures, and vetch.

	Grasses (*n* = 188)	Ryegrass (*n* = 34)	Mixtures (*n* = 75)	Vetch (*n* = 158)
Latitude (°)	-0.03	0.29	0.22	-0.15
Longitude (°)	0.09	0.51**	0.02	0.22**
Sowing date (Julian days)	0.04	0.21	-0.07	-0.19 *
Termination date (Julian days)	0.36 ***	0.34 *	0.41 ***	0.35 ***
Cycle duration (days)	0.32 ***	0.25	0.33 **	0.39 ***
Precipitation (mm)	0.29***	0.52 **	0.20	0.15

*, p<0.05; **, p<0.01; ***, p<0.001.

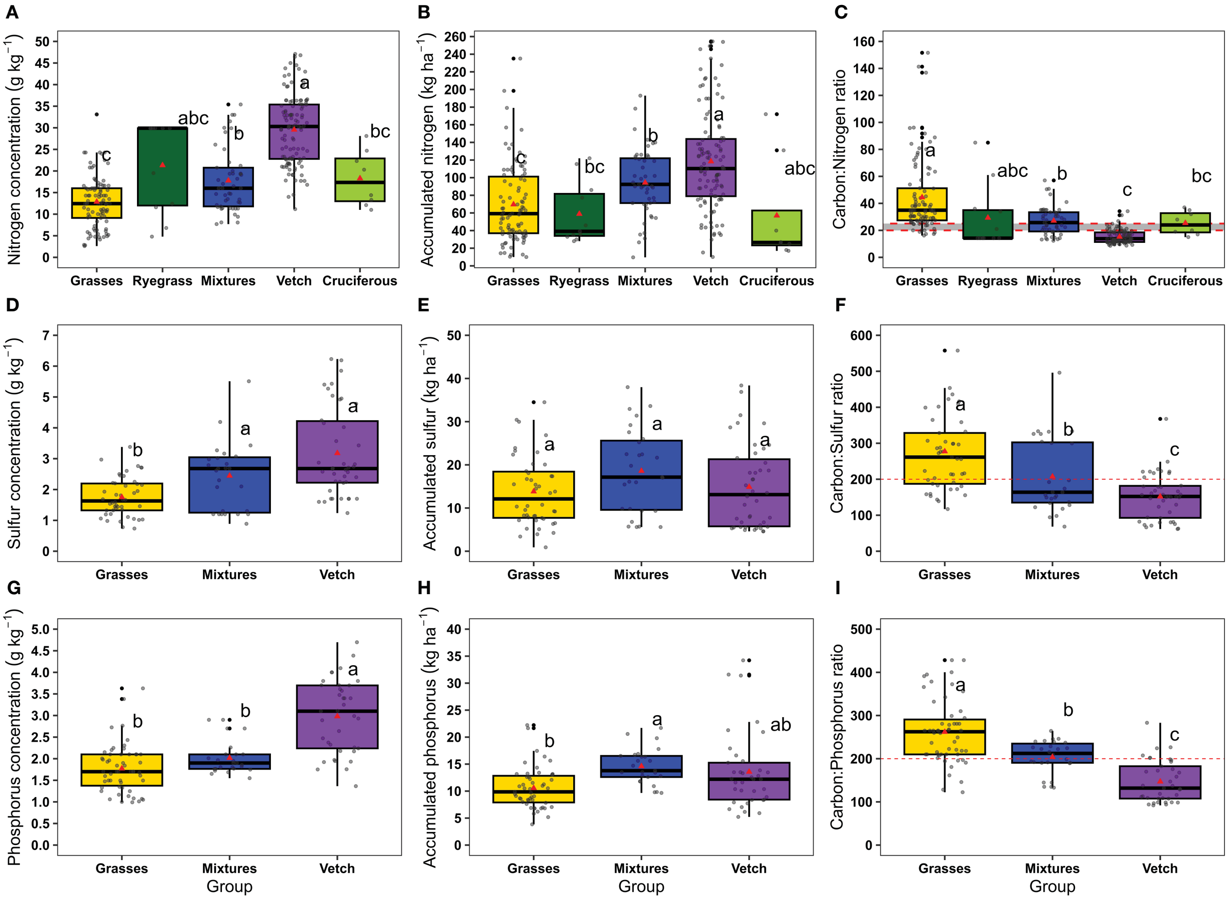

FIGURE 3
(A) Nitrogen concentration, **(B)** accumulated nitrogen and **(C)** C:N ratio in grasses (*n*=94), ryegrass (*n*=11), mixtures (*n*=49), vetch (*n*=114), and cruciferous (*n*=8). **(D)** Sulfur concentration, **(E)** accumulated sulfur and **(F)** C:S ratio in grasses (*n*=46), mixtures (*n*=27) and vetch (*n*=42). **(G)** Phosphorus concentration **(H)** accumulated phosphorus and **(I)** C:P ratio in grasses (*n*=54), mixtures (*n*=27) and vetch (*n*=37). Boxplots represent the 5th and 95th (whiskers), 25th (lower edge of the box) and 75th (top edge of the box) percentiles. The line inside the boxes represents the median and the red triangle inside the boxes represents the mean. Dashed red lines in **(C, F, I)** represent the thresholds below which mineralization is likely to occur for C:N, C:S and C:P ratios, respectively (Eriksen et al., 2004; Halvin et al., 2005; Sievers and Cook, 2018). Same letters indicate not significant differences among means of catch crops groups for each variable described using Tukey multiple comparison test (*p-value* < 0.05).3.4Nutrient concentration and C:nutrient ratios relationships with aboveground dry matter.

minimum of 61.7:1 and a maximum of 604.1:1 and followed inverse order to S concentration: grasses (277.3:1) > mixtures (207.3:1) > vetch (151.9:1) (Figure 3F).

Phosphorus concentration varied between 1.0 and 4.7 g kg^{-1} and, as observed for S, followed the order: vetch (mean ± SE 3.0 ± 0.14 g kg^{-1}) > mixtures (mean ± SE 2.0 ± 0.08 g kg^{-1}) > grasses (mean ± SE 1.8 ± 0.08 g kg^{-1}) (Figure 3G). Mixtures accumulate, on average, more P than grasses (mean ± SE 14.6 ± 0.7 vs. 10.7 ± 0.5 kg ha^{-1}) and vetch showed an intermediate P accumulation (not differing from mixtures, mean ± SE 13.6 ± 1.2 kg ha^{-1}) (Figure 3H). The C:P ratio followed the order: grasses (261.9:1) > mixtures (203.8:1) > vetch (147.0:1) (Figure 3I).

We explored the relationships between nutrient concentrations and C:nutrient ratio versus aboveground dry matter to better comprehend changes in the formers and to obtain predictive models. Nitrogen concentration in all the CCs groups and P concentration in grasses and mixtures were negatively related to aboveground dry matter, whereas there was no relationship between S concentration and aboveground dry matter (Figures 4A–C). From the negative power models fitted, it could be deduced a greater reduction in N concentration as aboveground dry matter increases for grasses and mixtures (decay rate = 0.25 and 0.24) than for vetch (decay rate = 0.09) (*p*=0.046). It should be noted that even though it was possible to fit models between N concentration and aboveground dry matter, their explanatory power was low (r^2<0.27). For P, no significant differences between grasses and mixtures were observed in the decay rate of concentration as a function of aboveground dry matter (*p*=0.150). Concerning C:nutrient ratios (Figures 4D–F), the C:N ratio was positively and linearly related to aboveground dry matter for the three CCs groups evaluated. As C concentration did not change with the amount of aboveground dry matter accumulated (*p*>0.05), the C:N behavior was opposite to that observed for N concentration [*i.e.*, C:N increment per kg ha^{-1} of aboveground dry matter was greater in grasses and mixtures (2.97 and 2.36, respectively) than in vetch (0.75)]. A positive linear relationship was also observed for C:P ratio and aboveground dry matter in grasses and mixtures, whereas C:S ratio was not related to aboveground dry matter.

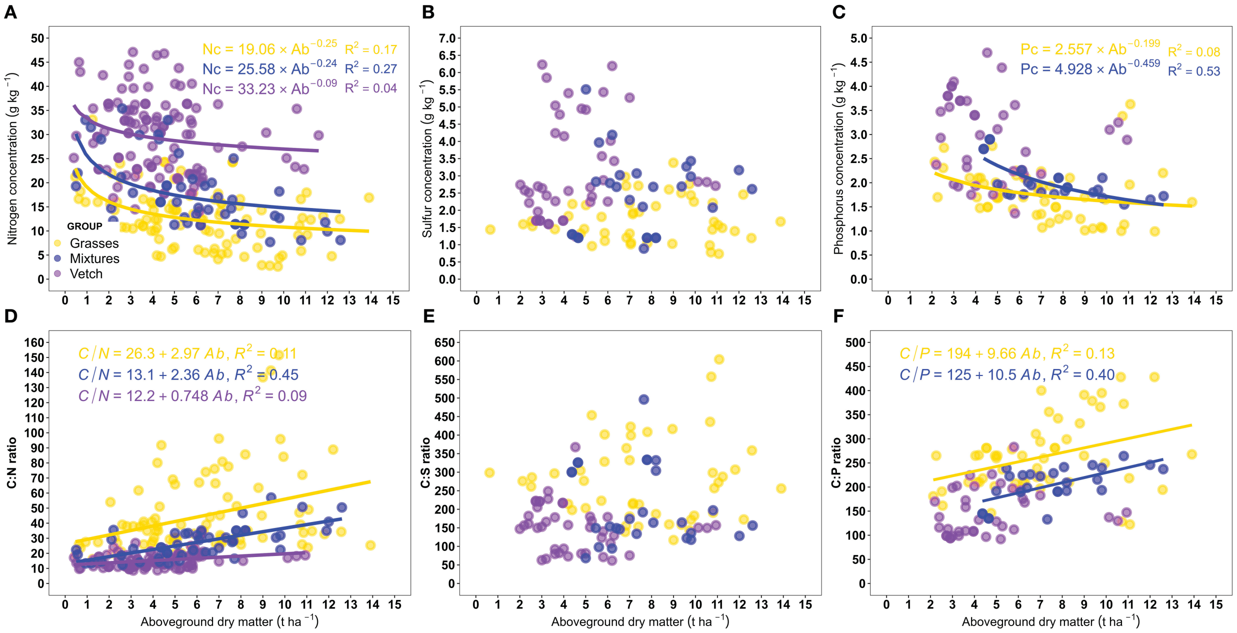

FIGURE 4
Relationship between aboveground dry matter and **(A)** nitrogen (N), **(B)** sulfur (S), and **(C)** phosphorus (P) concentrations, and **(D)** carbon (C):N, **(E)** C:S, and **(F)** C:P ratios. The lines represent significant models for each group ($p < 0.05$). For N (panels **A, D**) *n*= 114, 49, and 94, for S (panels **B, E**) *n*= 42, 27, and 46, and for P (panels **C, F**) *n*= 37, 25, and 54, for vetch, mixtures, and grasses, respectively.

4 Discussion

Previous studies summarized the effect of CCs on soil water availability, soil erosion control, soil physical and chemical properties, and weed suppression, among others (Schipanski et al., 2014; Rimski-Korsakov et al., 2015; Alvarez et al., 2017; Daryanto et al., 2018; Blanco-Canqui and Ruis, 2020). To the best of our knowledge, this is one of the first investigations that synthesizes and analyzes a large dataset containing 98 site-years, allowing us to i) explore the variability on C, N, S, and P accumulation and the C:nutrient ratio in the aboveground dry matter of different CCs species, ii) identify factors explaining these variables, and iii) associate them with ecosystem services. Our study showed that grasses presented the highest biomass and C accumulation, as well as higher C:nutrient ratios. In contrast, vetch accumulated high amounts of nutrients with low C:nutrient ratios, while mixtures presented intermediate conditions between their monocultures (*i.e.*, grasses and vetch). This is highly relevant to select the appropriate CCs species based on the desired ecosystem service and to encourage the inclusion of CCs in cropping systems with negative nutrient balances and soil degradation, such as those in the Argentinean Pampas region (Lavado and Taboada, 2009).

The C:nutrient ratio of CCs residues plays a significant role in the mineralization-immobilization turnover. In this context, C:N ratios below 20 to 25:1 (Sievers and Cook, 2018), and C:P and C:S ratios below 200:1 (Eriksen et al., 2004; Halvin et al., 2005) suggest net nutrient mineralization. Hence, it is important to consider these thresholds to predict the impact of CCs on nutrient availability and the duration of soil coverage. When C:nutrient ratios suggest mineralization, net nutrient contributions to the following crop are expected, as well as a rapid decline in soil coverage over time. Conversely, in cases of immobilization, microorganisms take up nutrients from the soil to decompose the residues, thereby diminishing nutrient availability for the subsequent crop, and soil coverage is extended for a long period (Eriksen, 2008; Murungu et al., 2011; Sievers and Cook, 2018).

The overall high aboveground dry matter and C accumulation and high C:nutrient ratio in grasses indicate that they would be an appropriate option when the goal of CCs is to enhance C sequestration and maintain or even improve soil organic C stocks (Schipanski et al., 2014; Daryanto et al., 2018). In this context, after five years of using CC, Duval et al. (2016) reported greater increments in soil organic C concentration with grasses (wheat and oat) than with vetch, being the C changes in the topsoil associated with the quantity of C input. Although grasses were not the CC group with the highest nutrient accumulation, the significant amount of nutrient uptake suggests that they could prevent N and S losses, mainly through leaching, and in the case of P, through runoff (Eriksen et al., 2004; De Baets et al., 2011; Restovich et al., 2012; Gabriel et al., 2013). Moreover, the high C:nutrient ratio suggests very slow residue decomposition, leading to prolonged soil surface coverage (Eriksen, 2008; Murungu et al., 2011). Therefore, grasses would also be a suitable option for i) protecting the soil surface, reducing soil losses through water and wind erosion (De Baets et al., 2011; Vincent-Caboud et al., 2019); ii) promoting weed suppression during the CC and cash crop growing seasons (Finney et al., 2017; Vincent-Caboud et al., 2019), and iii) reducing water losses through evaporation after their termination (Restovich et al., 2012; Alfonso et al., 2020). However, it should be highlighted that the high C:nutrient ratios would cause nutrients immobilization, increasing the fertilizer rates required to satisfy the succeeding cash crop demand (Eriksen et al., 2004; Lupwayi et al., 2007; Murungu et al., 2011; Carciochi et al., 2021b).

Vetch presented the highest nutrient accumulation, indicating its key role in reducing nutrient losses during fallow periods and the associated environmental externalities (Gabriel et al., 2013; Frasier et al., 2017). Nitrogen losses by N_2O emissions and NO_3^- leaching have been questioned when analyzing the whole sequence vetch-cash crop. Meta-analyses conducted for the northern hemisphere indicate that legume CCs increase N_2O emissions compared to other species (Basche et al., 2014; Muhammad et al., 2019). However, studies conducted in the Argentinean Pampas showed no significant differences in N_2O emissions with or without CCs, including vetch (Vangeli et al., 2022; Petrasek et al., 2023). Regarding NO_3^- leaching, lower NO_3^--N content in soil was reported after vetch and other CCs, compared with a fallow condition, suggesting lower potential N losses through this mechanism (Restovich et al., 2012; Frasier et al., 2017). In addition, as 60% of the N in vetch comes from biological N fixation (Enrico et al., 2020), it is noteworthy that substantial N inputs to the systems are expected (IQR = 47.4 to 86.3 kg N ha^{-1}). The highest nutrient accumulation, along with the lowest C:nutrient ratio, suggests that vetch could rapidly release significant amounts of N, P, and S that would be available to the succeeding crop in the sequence (Eriksen, 2008; Murungu et al., 2011; Sievers and Cook, 2018; Vincent-Caboud et al., 2019). However, the observed variability in nutrients accumulation indicated that they should be quantified in each growing condition and, in combination with the nutrient requirement of the crops, adjust the nutrient rates to apply via organic or inorganic fertilizer, in order to achieve the sustainability of the agroecosystem.

As expected, mixtures containing grasses and vetch presented intermediate CCs characteristics between the respective monocultures, which agreed with previous studies (Restovich et al., 2012; Ketterings et al., 2015; Thapa et al., 2018). Therefore, mixtures could provide the different ecosystem services mentioned in a balanced way (Finney et al., 2016; Frasier et al., 2017; Vincent-Caboud et al., 2019). Especially in this CC group, the definition of their nutrient characteristics is crucial to predict the role of mixtures in the agroecosystems. For example, mixtures with low biomass accumulation, and thus low C:nutrient ratios, act as a nutrient source for the succeeding crop in the sequence, while high biomass and its associated high C:nutrient ratios, will cause nutrient immobilization and a prolonged protection of the soil surface because of their lower rate of decomposition (Eriksen et al., 2004; Lupwayi et al., 2007; Finney et al., 2016; Vincent-Caboud et al., 2019). Notably, nutrient-related traits are not only determined by biomass accumulation but also by the proportion of each species in the mixture (Hayden et al., 2014; Poffenbarger et al., 2015). Unfortunately, the available information was insufficient to include this variable in our analysis. However, we are aware that the proportion of each species should be considered to predict the behavior of mixtures (i.e., whether they are more similar to vetch or grasses) associated with the discussed ecosystem services.

As determined by Thapa et al. (2018), our observations indicate that biomass accumulation defines the magnitude of the ecosystem service provided by each CC group. In agreement with previous studies (Ketterings et al., 2015; Mirsky et al., 2017; Thapa et al., 2018), we detected factors such as the termination date, CC cycle duration, and precipitation during the CC growing season that determined the CC biomass accumulation. The location of the analyzed sites weakly explained CCs biomass accumulation, but detailed studies on CCs spatial variability are encouraged to be conducted. Contemplating the mentioned factors affecting biomass accumulation is a key point as they will influence nutrient concentrations, accumulations, and C:nutrient ratios, impacting the ecosystem services discussed. It should be highlighted that this study did not assess the C and nutrient accumulation in the roots of CCs which are known to significantly impact on the C and nutrients input to the system (Couëdel et al., 2018; Villarino et al., 2021), which deserves to be analyzed in future studies. It is worth noting that a high aboveground dry matter accumulation would not always be desirable. For example, we observed that C:nutrient ratios in grasses and mixtures increased as the dry matter accumulation also did, which would cause nutrient immobilization (Lupwayi et al., 2007; Vincent-Caboud et al., 2019). Additionally, long CCs cycle durations and late termination dates might reduce water availability for the succeeding cash crop, severely diminishing grain yield in semi-arid areas or in years with low precipitation (Meyer et al., 2019).

5 Conclusion

This synthesis-analysis offers a comprehensive understanding of nutrient and C characteristics of different CCs species grown in the Argentinean Pampas region, surpassing previous studies conducted in specific site-years. Grasses, except for ryegrass, accumulated high biomass and C, whereas vetch accumulated the highest nutrient content with the lowest C:nutrient ratios, and mixtures presented balanced characteristics. Our findings highlight the importance of selecting CCs species based on the intended ecosystem service. The information provided in this study is useful for predicting nutrient contributions from CCs to the succeeding crop and estimating the potential input of C to agroecosystems. Additional information on species such as ryegrass, cruciferous, and legumes other than vetch is limited, emphasizing the need to generate more data on these CCs.

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 authors.

Author contributions

FC and WC organized the database, performed the statistical analyses, and wrote the 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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fagro.2023.1244057/full#supplementary-material

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Drive soil nitrogen transformation and improve crop nitrogen absorption and utilization - a review of green manure applications

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Green manure application presents a valuable strategy for enhancing soil fertility and promoting ecological sustainability. By leveraging green manures for effective nitrogen management in agricultural fields can significantly reduce the dependency of primary crops on chemical nitrogen fertilizers, thereby fostering resource efficiency. This review examines the current advancements in the green manure industry, focusing on the modulation of nitrogen transformation in soil and how crops absorb and utilize nitrogen after green manure application. Initially, the influence of green manure on soil nitrogen transformation is delineated, covering processes such as soil nitrogen immobilization, and mineralization, and losses including NH_3, N_2O, and NO_3^--N leaching. The review then delves into the effects of green manure on the composition and function of soil microbial communities, highlighting their role in nitrogen transformation. It emphasizes the available nitrogen content in the soil, this article discussing nitrogen uptake and utilization by plants, including aspects such as nitrogen translocation, distribution, the root system, and the rhizosphere environment of primary crops. This provides insights into the mechanisms that enhance nitrogen uptake and utilization when green manures are reintroduced into fields. Finally, the review anticipates future research directions in modulating soil nitrogen dynamics and crop nitrogen uptake through green manure application, aiming to advance research and the development of the green manure sector.

KEYWORDS

green manure application, nitrogen management, immobilization, mineralization, soil -plant system

1 Introduction

For a substantial period preceding the widespread use of synthetic nitrogen fertilizers, green manure played a pivotal role in global food security and agricultural development (Meena et al., 2018). Even in contemporary agriculture, green manure continues to exert significant effects on soil enhancement, fertilizer substitution, and ecological protection (Spiertz, 2009). China pioneered the green manure production system before the 3rd century AD, utilizing leguminous green manure, crop rotation, and intercropping to create diverse cropping structures for fertilizing farmland (Liu et al., 2013). By the early 19th century, the practice spread from China to Europe and the Americas, evolving into cover crops. Advancements in modern science and technology have gradually unveiled the mechanisms underlying improving farmland productivity and ecological services through long-term green manure cultivation and incorporation (Fageria, 2007). Current research indicates that, as an external source of organic matter, long-term green manure incorporation primarily manifests ecological effects through improvements in soil physicochemical properties, such as aggregates and organic matter, and enhancements in biological functions, represented by microorganisms (Abbott et al., 2018; Bungau et al., 2021). Additionally, due to its intrinsic characteristics, green manure actively participates in water and nutrient regulation processes in crop-soil systems post-incorporation, contributing to energy conservation, increased yield and quality, and enhanced water and nutrient use efficiency (Zhang et al., 2010). Moreover, long-term green manure cultivation plays a positive role in preventing soil erosion and inhibiting weeds and pests in farmland (Chimouriya et al., 2018; Maitra et al., 2018).

The extended use of chemical fertilizers and continuous cropping patterns contribute to soil quality degradation, structural disequilibrium, and reduced efficiency in nitrogen absorption and utilization by crops (Bai et al., 2015; He et al., 2018; Li P. et al., 2021). Furthermore, non-point source pollution from agricultural activities, exacerbated by fertilizer applications, is evolving into an agricultural ecological predicament (Ju and Zhang, 2017). Concurrent with advancements in agricultural techniques and the promotion of sustainable development ideologies, transformative shifts are evident in global agricultural practices. Green initiatives are positioned to shape the trajectory of future agricultural endeavors (Horlings and Marsden, 2011). As a vital element of this green paradigm, green manures are gaining prominence in the roadmap for the upcoming agricultural era. This emphasis centers around adjusting crop structures, mitigating non-point source pollution, rejuvenating farmland ecosystems, integrating land cultivation with fertilization strategies, and enhancing the quality and efficacy of agricultural yields (Rees and Chow, 2005). Historically, green manure crops have been utilized as catch crops to expedite nitrogen turnover within farmland ecosystems, reinforcing the consistency and augmentation of grain outputs (Zhang et al., 2015; Zhang et al., 2016). Notably, leguminous green manure, recognized as a comprehensive bio-organic fertilizer, facilitates biological nitrogen fixation, asserting its indispensable influence in soil enrichment and moisture conservation (Kim et al., 2012). In the realm of nitrogen dynamics, green manure contributes to humus formation, mineralization, ammonification, nitrification, denitrification, and the assimilation and sequestration of accessible nitrogen by relevant functional microorganisms (Mancinelli et al., 2013). Simultaneously, the incorporation of green manures can modulate the nitrogen assimilation and deployment within principal crops by engaging in nitrogen fixation and mineralization processes (Martens and Entz, 2011; Momesso et al., 2022).

Numerous studies have explored the influence of green manure application on nitrogen absorption and utilization in cereal crops, such as wheat (*Triticum aestivum*), corn (*Zea mays*), potatoes (*Solanum tuberosum*), and rice (*Oryza sativa*) (see Figure 1 and Table 1). While these empirical findings contribute significantly to bridging theoretical gaps in the field of green manure application, there is a lack of summarized and evaluated conclusions to provide references for subsequent researchers. Drawing upon prior research, this review encapsulates the modulatory effects of incorporating green manure on key soil nitrogen transformation processes, including nitrogen fixation, mineralization, loss, crop uptake, and the role of soil microorganisms. Additionally, the study delves into nitrogen transportation and distribution within the crop root system under green manure application, elucidating the mechanisms through which green manure enhances nitrogen utilization in primary crops. This review aims to establish a foundation for advancing research on the ecological service functions associated with green manure application.

2 Response and key mechanisms of soil nitrogen transformation to green manure application

The nitrogen biogeochemical cycle involves processes such as nitrogen fixation, nitrification, denitrification, and ammonification, primarily mediated by soil microorganisms (Holz et al., 2023). Nitrogen exists in various forms in soil, classified as organic and inorganic nitrogen, with the organic content significantly surpassing the inorganic. Inorganic nitrogen is further divided into nitrate nitrogen and ammonium nitrogen based on its state (Cao et al., 2021). Nitrate nitrogen, a crucial nitrogen source for plants, is susceptible to losses through leaching and runoff because of its high mobility. Under anaerobic conditions, nitrate may be released through denitrification (Ju and Zhang, 2017). In specific scenarios, atmospheric nitrogen infiltrates the soil through fixation, transforming into inorganic nitrogen, which becomes accessible for plant uptake. This inorganic nitrogen produces compounds such as N_2O, NO, and N_2 by participating in NH_3 volatilization, nitrification, and denitrification, these nitrogenous compounds then re-enter the atmosphere, finally completing the soil nitrogen cycle (Figure 2) (Jeffrey and Carla, 2004). The processes and transformations involved in the soil nitrogen cycle are orchestrated by soil enzymes and microorganisms (Jesper et al.,

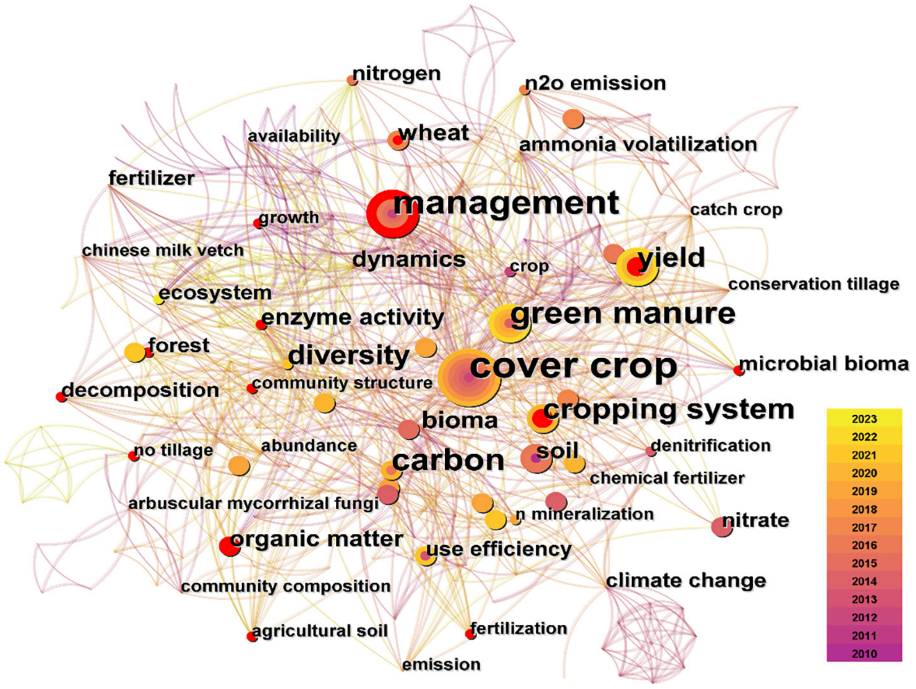

FIGURE 1
The research hotspots of green manure application in 2010 – 2023.

TABLE 1 In some countries where green manure crops are widely planted and the ecological benefits produced by green manure applications.

Green manure crops	Planting pattern	Country/ Region	Research field	Main benefits	References
Clover (Oxalis)	Rotation	Denmark	Ecological effect	Mitigating weed problems in organic annual crops	(Melander et al., 2020)
Hairy vetch (*Vicia villosa*); Yellow sweet clover (*Melilotus officinalis*)	Rotation	Norway; Ontario, Canada	Suitability of green manure crops; Economic effectiveness	Hairy vetch was rich in N and showed a high N mineralization rate; The suitability of green manure crops depends on the cultivar, sowing time, and climate region; Increase grain yield and soil nitrogen storage	(Brandsaeter et al., 2008; Yang L. et al., 2019)
Sudangrass (*Sorghum sudanese*); Cowpea (*Vigna unguiculata*); Rye (*Secale cereale*); Pea (*Pisum sativum*)	Rotation	Michigan, USA; Louisiana, USA	Maximum benefits of green manure crops Rotation; No-till and cover crops management practices	Legume green manure crops are the most reliable means to enhance cash crop yields; If soil pests are a major yield-limiting factor in cash crop production, then the use of brassica green manure should be considered	(Snapp et al., 2005; Naveen et al., 2020)
Barley (*Hordeum vulgare*); Oat (*Avena sativa*); Oilseed rape (*Brassica rapa* var.*oleifera*); Faba bean (*Vicia faba*); Lupin (*Lupinus micranthus*); Alfalfa (*Medicago sativ*)	Rotation	Sweden; Germany	Evaluating effects of introducing green manure into crop rotations; Nitrogen leaching; Net greenhouse gas balance and crop productivity	Cropping systems with legumes reduced N_2O emissions with comparable or slightly lower nitrate-N leaching and had positive phytosanitary effects	(Reckling et al., 2016)
Chickpea (*Cicer arietinum*)	Mono-culture	Algeria	The ability of fixing nitrogen	The nitrogen-fixing nodule function, germinability, and possibly photosynthesis, are revealed	(Souad Insaf et al., 2021)

(Continued)

TABLE 1 Continued

Green manure crops	Planting pattern	Country/ Region	Research field	Main benefits	References
February orchid (*Orychophragmus violaceus*); Common vetch (*Vicia sativa*); Red bean grass (*Onobrychis viciifolia Scop*)	Rotation	China	Nitrogen replacement technology, N fixation and emission reduction; Soil carbon and nitrogen sequestration	Green manure and chemical fertilizers is an efficient management approach for improving maize yields and NUE simultaneously	(Yang L. et al., 2019)

2006). Processes such as immobilization and mineralization are fundamental to the soil nitrogen cycle and have garnered considerable research attention.

2.1 Regulation of green manure application on soil nitrogen immobilization

Soil nitrogen immobilization plays a crucial role in supplying nitrogen to plants (Li Z. L. et al., 2021). Both leguminous and non-leguminous green manures, recognized for their nitrogen contributions, possess inherent abilities for nitrogen fixation. Leguminous green manures, characterized by substantial aboveground biomass, foster symbiotic interactions between their root systems and rhizobia, facilitating nitrogen fixation (Ben-Laouane et al., 2020). Upon incorporation into the field, these manures enhance soil nitrogen levels, improving nitrogen uptake and efficiency in primary crops (Coombs et al., 2017; Vincent-Caboud et al., 2019; Yang L. et al., 2019). A hectare of leguminous green manure can assimilate atmospheric nitrogen at rates ranging

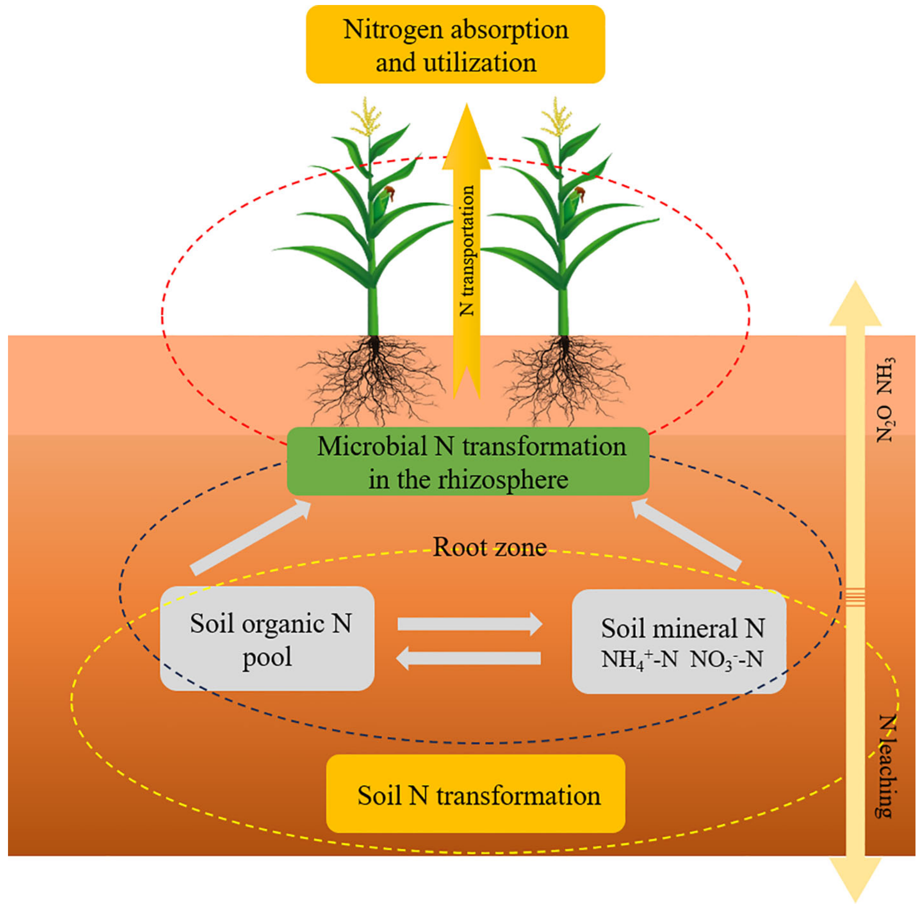

FIGURE 2
Relationship between soil nitrogen conversion and crop nitrogen uptake and utilization.

from 110 to 227 kg, potentially replacing or reducing the reliance on chemical nitrogen fertilizers (Mueller and Thorup-Kristensen, 2001; Xie et al., 2016b). According to the Food and Agriculture Organization estimates, global annual biological nitrogen fixation is approximately 130 million tons (Kakraliya et al., 2018). Leguminous plant-rhizobia symbiotic nitrogen fixation constitutes 65% – 70% of this figure, wherein the nitrogen fixed by leguminous plants can cater to 50% – 80% of the nitrogen requisites for plant development (Justes, 2018). Furthermore, both leguminous and non-leguminous green manures absorb inorganic nitrogen from soil, convert it into organic forms within the plants, and reintroduce it to the soil, contributing to soil nitrogen replenishment (Yang et al., 2022).

Soluble organic nitrogen and inorganic nitrogen are the most dynamic components in the soil matrix, available for direct uptake by plants and microorganisms or after specific transformations (Ji et al., 2018). Cultivating of green manure crops facilitates the absorption of liberated nitrogen, leading to soil nitrogen immobilization (Snapp et al., 2005). Introducing plant residues with a high C:N ratio into the soil enhances its nitrogen fixation potential (Vinther et al., 2004). Evidence suggests that soil organic carbon mass plays a pivotal role in driving soil nitrogen fixation (Cao et al., 2021). The cultivation and integration of green manures can elevate metrics such as microbial biomass carbon on the soil surface, labile organic carbon, dissolved organic carbon, and the content of resilient and tightly bound humus, thereby improving the quality of organic carbon (Figure 3) (Ye et al., 2015).

2.2 Regulation of green manure application on soil nitrogen mineralization

Nitrogen mineralization is a key player in regulating soil-active nitrogen concentrations (Ouyang et al., 2008) (Figure 4). Upon the introduction of green manure, various organic nitrogen forms undergo transformation into inorganic nitrogen through mineralization. Some of these forms are absorbed by crops, while others are adsorbed by the soil. The remaining free inorganic nitrogen volatilizes as ammonia under alkaline conditions in drylands. (Fageria and Baligar, 2005) Conversely, in paddy fields, this nitrogen produces N_2O, NO, and N_2 through the denitrification process (Mccauley et al., 2012). Empirical evidence supports the idea that soil net nitrogen mineralization increases with the incorporation of green manures (Delgado and Follett, 2011). This enhancement primarily arises from green manure meeting the nitrogen requirements of soil microorganisms, catalyzing the activation and breakdown of bound-state mineral nutrients (Kuzyakov and Xu, 2013). Specifically, in terms of soil organic nitrogen, the decomposition of green manures provides energy and carbon reservoirs for microorganisms, invigorating the metabolic functions of ammonifiers and nitrifiers. Ammonifiers convert organic nitrogen into ammonia nitrogen, while nitrifiers oxidize this ammonia nitrogen into nitrate nitrogen, further augmenting inorganic nitrogen formation.

During the initial phase of green manure decomposition (approximately 20 days), easily decomposable components are

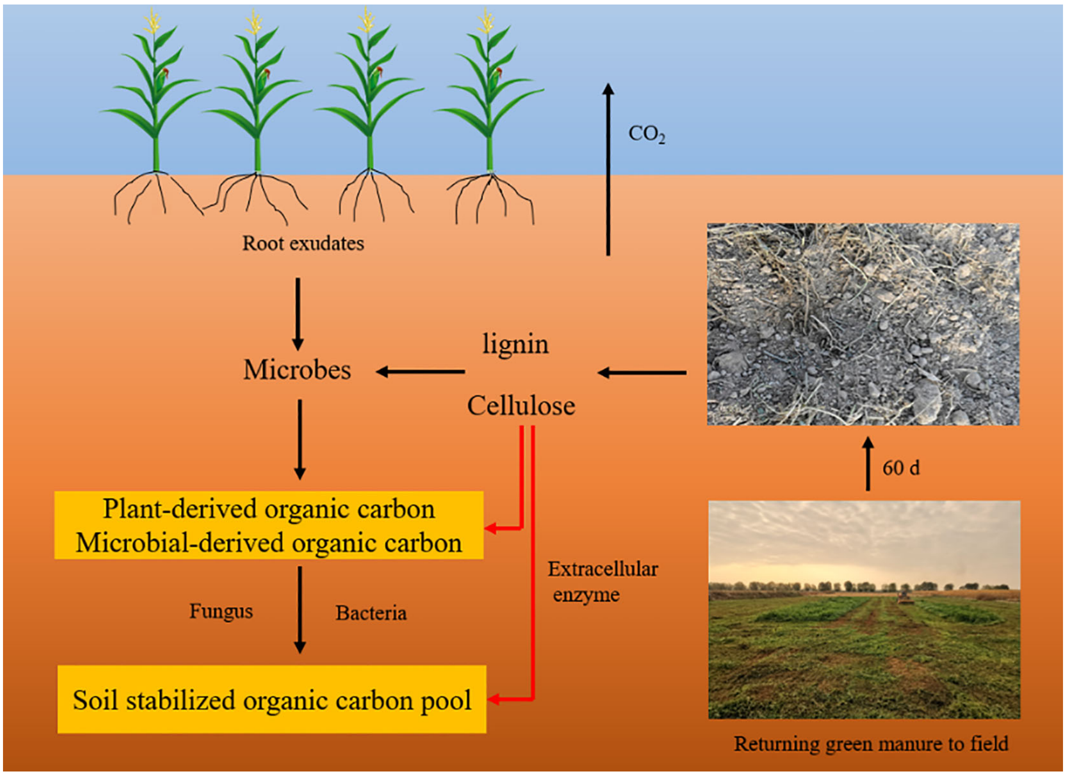

FIGURE 3
The production process of soil stable organic carbon pool after returning green manure to the field.

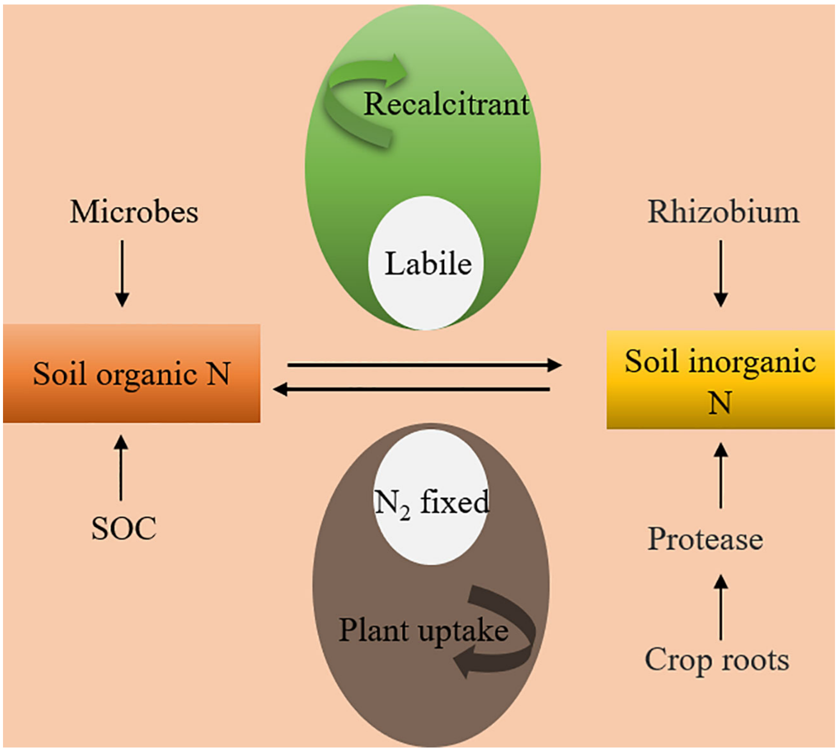

FIGURE 4
Nitrogen immobilization and mineralization process in farmland soil under green manure application.

rapidly mineralized by microorganisms, causing a significant increase in the soil's inorganic nitrogen content (Carter et al., 2014). Simultaneously, an abundance of carbon sources reinforces the nitrogen immobilization effect orchestrated by microorganisms. In the intermediate and advanced stages of green manure decomposition (approximately 20 – 60 days), the depletion of carbon sources leads to a decrease in both mineralization and immobilization effects; however, a net positive growth trajectory persists (Liu et al., 2019). Various studies suggest that soil nitrogen mineralization is driven by microorganisms, with the stoichiometry of microbial biomass influencing the intensity of nitrogen mineralization (Li et al., 2019; Li et al., 2020). For example, during the decomposition of green manures, the rate depends on the soil C:N ratio (Jahanzad et al., 2016). A lower C:N ratio accelerates decomposition and nutrient liberation, while a higher ratio slows down the breakdown of green manures (Portugal et al., 2020). Therefore, choosing leguminous plants with a balanced carbon-nitrogen ratio or a mix of leguminous and non-leguminous species can balance soil nutrient sequestration and provision. An important aspect of green manure utilization is the synchronization of dynamic nutrient release with crop nutrient requirements (Brandsaeter et al., 2008; Kandel et al., 2018). Hairy vetch and rye are the predominant green manure crops in the Midwestern United States. Sievers and Cook (2017) reported that, compared with rye, the artificial cessation of Hairy vetch growth, results in an increased nitrogen release in terms of both quantity and rate. Approximately 30 days after halting Hairy vetch growth, nearly all contained nitrogen is liberated, coinciding with the peak nitrogen assimilation phase of 8-week-old maize. By contrast, rye's nitrogen release spans a considerably longer duration, making it suitable for crops with minimal nitrogen demands. Therefore, under green manure application, aligning soil nitrogen mineralization and immobilization with crop nutrient demands can enhance efficient nitrogen absorption and utilization in subsequent crops.

2.3 Regulation of green manure application on soil nitrogen loss

2.3.1 NH_3 Volatilization

The presence of NH_3 in soil originates from the ammonification of NH_4^+-N, catalyzed by urease (Farooq et al., 2022). Decomposition of green manures, whether leguminous or non-leguminous, results in increased concentrations of soluble nitrogen, promoting the ammonification of unstable nitrogen in the short term, and causing rapid initial fluctuations in NH_3 volatilization (Janzen and Mcginn, 1991). While green manure decomposition enhances NH_3 emissions, studies have shown that ammonia volatilization from shoot decomposition post - green manure application constitutes only 0.31% the total farmland ammonia volatilization (Ferrara et al., 2021). Chemical nitrogen fertilizers remain the primary source of ammonia volatilization, and incorporating leguminous green manure with these fertilizers amplifies soil ammonia losses (Zhang et al., 2022). Introducing leguminous green manure not only boosts the mineralization potential of organic nitrogen but also enhances ammonification and nitrification of ammonium nitrogen fertilizer, resulting in elevated NH_3 emissions (Sun et al., 2021). Conversely, replacing a portion of nitrogen fertilizers with green manures can significantly reduce NH_3 volatilization (Bai et al., 2015). Rana and Mastrorilli (1998) support this, demonstrating that approximately 70% of soil ammonia in green manure-treated farmlands is discharged within 2 days post chemical nitrogen fertilizer application. The NH_3 flux regulation mechanism mirrors water vapor transport, and the decomposition of green manures releases abundant organic nutrients, such as organic carbon and nitrogen accelerating soil mineralization, reducing the interaction between ammonium nitrogen and soil colloids, increasing soil NH_4^+-N content, and catalyzing NH_3 volatilization (Kulesza et al., 2022).

2.3.2 N_2O emission

The application of leguminous green manure is widely acknowledged to increase N_2O emissions in agricultural fields. Organic cultivation soils, compared to conventionally tilled soils, exhibit higher potential denitrification rates, increased organic matter content, and heightened microbial activity (Xie et al., 2016a). However, some research suggests that leguminous green manure might attenuate N_2O emission intensity. Duan et al. (2019) propose an alternate perspective: the incorporation of leguminous green manure promotes nitrogen transformation within microorganisms, facilitating the conversion of N_2O to N_2, thereby reducing N_2O production through soil nitrification and denitrification processes. This effect is supported by increased abundance of the ammonia-oxidizing bacterium AOB-amo A and the N_2O-reducing gene nos Z. Adopting no-tillage practices and utilizing green manure plants with elevated C:N ratios can also reduce N_2O emissions, attributed to improved soil aggregate structure stabilizing volatile nitrogen (Huang et al., 2004). Integrating non-leguminous green manure with chemical nitrogen fertilizers can enhances nitrogen retention in the soil, reduces N_2O emissions from primary crop farmlands, and alleviates environmental concerns related to nitrate leaching or runoff. This is linked to a decrease in the quantity of nitrifying bacteria and a decrease in enzyme activity leading to decreased soil NO_3^--N content (Momesso et al., 2022). A global meta-analysis suggests that fields cultivated with non-leguminous green manure exhibit the highest N_2O emissions, primarily correlated with water-filled pore space (Kramer et al., 2006). In conclusion, discrepancies in N_2O emissions depend on the specific applications of green manures and varying soil environmental conditions.

2.3.3 NO_3^--N leaching

NO_3^--N, owing to its limited capacity for easy adsorption by anionic soil colloids, is the most mobile nutrient in the soil (Johnson and Cole, 1980). During rainy seasons, cultivating green manure crops can significantly mitigate nitrogen leaching, given their capacity to assimilate free mineral nitrogen from the soil (Guo et al., 2008; Basche et al., 2014). Some investigations indicate that non-leguminous cover crops have a more pronounced effect on reducing NO_3^--N leaching compared with their leguminous counterparts. On average, non-leguminous cover crops reduce leaching by 70%, whereas leguminous cover crops achieve a reduction of only 23% (Zhao et al., 2020). Campbell et al. (2008) observed negligible nitrogen leaching in soils cultivated with leguminous crops, potentially attributed to regional soil texture variations.

A global-scale meta-analysis suggests that the cultivation and application of green manure crops in primary croplands markedly decrease nitrogen leaching (Abdalla et al., 2019). Bai et al. (2015) reported that Orychophragmus violaceus, when incorporated into maize fields, minimizes nitrogen migration in maize fields to deeper soil strata,virtually eliminating nitrogen leaching. Specifically, before the application of Orychophragmus violaceus, nitrate nitrogen in primary crop soils was distributed at depths of 0 – 180 *cm*, after the application of Orychophragmus violaceus, nitrate nitrogen remained predominantly within the tillage layer (Bai et al., 2015). Other studies support the idea that the growing leguminous and graminaceous species together helps balance nitrogen supply and reduce leaching (Frasier et al., 2017). Overall, green manure crops play a crucial role in reducing NO_3^--N leaching and environmental contamination.

2.4 Response of soil microbial community characteristics to green manure application

Soil microbial communities are essential for nutrient cycling and maintaining soil ecological functions. The diversity and richness of these microbial communities serve as vital metrics for assessing the robustness of soil ecological functions (Yang X. M. et al., 2019). These microorganisms drive the material cycles of agricultural ecosystems through their involvement in soil organic matter mineralization, humus formation, decomposition, and nutrient transformation in plants (Putten et al., 2014). The degree of soil microbial development intrinsically dictates the transmutation of soil nutrients (Xu et al., 2020). Microbial decomposition is the predominant process that provides energy integral to the transformation of organic matter (Ma et al., 2023).

The introduction of green manure crops has a direct influence on soil bacterial communities and an indirect influence on these communities by altering soil characteristics (Zhang et al., 2017). Green manure application fosters enhancements in soil microbial biomass and enzyme activity, and the magnitude of these changes potentially correlates with the microbial species and quantity of the green manure used (Bowles et al., 2014; Chavarría et al., 2016). Leguminous green manures improve microbial community growth and boost soil nitrogen fixation, benefiting subsequent crops in various agricultural settings (Melander et al., 2020). After applying green manure, a notable surge in the relative abundance of Proteobacteria, Acidobacteria, Pseudomonas, and Nitrospira in the soil was observed (Gu et al., 2021). According to Ru et al. (2012), green manure, unlike inorganic fertilizers, markedly amplifies the relative abundance of Actinomycetes and Skermanella in the soil. Actinomyces, a predominant bacterial microflora ubiquitously distributed in alkaline soil, have branched mycelia that secrete hydrolases. These hydrolases break down insoluble organic substances in the soil, thereby enhancing organic mineralization, crop root development and enzyme activity, and crop nitrogen assimilation and utilization (Sanford, 2006). The introduction of green manures to fields also increases the population and vitality of arbuscular mycorrhizal fungi in the rhizosphere (Hontoria et al., 2019; Cruz et al., 2020). These fungi form symbiotic relationships with plant rhizospheres, facilitating nitrogen uptake and translocation in plants (Veresoglou et al., 2012).

Green manure application affects soil microbial communities modify the composition and diversity of soil microbial communities both directly and indirectly by enhancing the soil's physical and chemical attributes (Yang et al., 2016). Specifically, introducing green manures to fields influences the soil microbial community structure by adjusting the sources of soil carbon and nitrogen (Xie et al., 2017). While microbial biomass carbon constitutes a minor fraction of the overall soil carbon, it mirrors the microbial utilization of carbon sources for growth and reproduction, offering energy crucial for crop nutrient transformation (Pelz et al., 2005). Organic fertilizers with high C:N ratios contain low-molecular-weight organic compounds, which foster the formation of loose, porous soil aggregates favorable for microbial growth and soil nitrogen fixation (Van Zwieten et al., 2014). The degree of change in bacterial functions depends on the bacterial species and quantity of green manures applied (Mbuthia et al., 2015). Soil microbial community structure after green manure application is predominantly influenced by various factors including soil soluble organic carbon, NO_3^--N, and microbial biomass (Khan et al., 2019). This underscores that in the context of green manure deployment, the characteristics of soil microbial communities are molded by an array of determinants, including the nature and attributes of green manures, organic matter input, and soil physicochemical properties. These factors synergistically determine the composition, diversity, and functions of soil microbial communities.

3 The high-efficient nitrogen absorption and utilization mechanism of main crops under green manure application

The absorption and utilization of nitrogen in crops is a multifaceted biological and ecological process, involving nitrogen uptake within the crop rhizosphere; internal transport and transformation of nitrogen; the distribution, synthesis, and metabolism of nitrogen within the plants (Souad Insaf et al., 2021). This intricate mechanism involves various aspects such as plant physiology, genetic modulation, rhizosphere microbial interactions, and abiotic environmental determinants of the soil. The efficiency of nitrogen utilization in plants depends not only on soil nitrogen availability and uptake efficiency but also on nitrogen availability from temporary reservoirs such as proteins in source leaves, stems, or roots and the efficiency of amino acid transport (Zhu et al., 2014). Contemporary studies on the influence of exogenous organic matter on nitrogen absorption and utilization in primary crops predominantly focus on nitrogen uptake efficiency, root morphology, and nitrogen absorption and distribution.

3.1 Promotion of soil nitrogen transformation and soil nitrogen availability

The balance between mineralization and immobilization governs the dynamics and availability of soil mineral nitrogen, significantly affecting nitrogen availability in agricultural soils and its subsequent absorption and utilization by crops (Vitousek et al., 2010). After adding green manures to fields affects soil organic nitrogen components: soil microbial biomass nitrogen and mineral nitrogen show opposite trends at different stages of primary crop growth. This indicates that green manures adjust the soil nitrogen fixation-mineralization balance to meet the nitrogen needs of the primary crops (Goyal et al., 1993). When crops need nitrogen, soils abundantly provide available nitrogen. By contrast, when the crop nitrogen demand is low, inorganic nitrogen is converted to organic nitrogen in the soil. This mechanism not only reduces gaseous and nitrate losses but also improves soil quality (Robertson and Vitousek, 2009). Nitrogen reduction trials have confirmed that the addition of green manure for improved nitrogen management in agriculture increases nitrogen absorption and utilization efficiency, without reducing crop yield, when compared with conventional management practices (Ding et al., 2018). Additionally, this approach offers environmental benefits (Bai et al., 2015): first, the reduced environmental losses (such as N_2O emissions, NH_3 volatilization, and leaching) compensate for the lower soil nitrogen input; second, reintroducing green manure to the soil improves crop nitrogen uptake by enhancing the soil environment.

3.2 Promote crop yield and nitrogen uptake

In both arid regions and paddy fields, the strategic application and deployment of various green manures can significantly improve nitrogen uptake and utilization efficiency, ultimately enhancing the yield of subsequent crops (Silva et al., 2020). Long-term cultivation and incorporation of green manure have demonstrated the potential to elevate nitrogen uptake by primary crops while reducing nitrogen losses (Liang et al., 2022). Spectral image analysis conducted by Wittwer and Heijden (2020) showed that the application of leguminous green manure increased maize nitrogen absorption by 79 $kg{\cdot}ha^{-1}$. Notably, leguminous green manure crops present a promising alternative to certain chemical nitrogen fertilizers, supplementing nitrogen availability, promoting enhanced nitrogen uptake, and stimulating growth in subsequent crop stages (Abera and Gerkabo, 2021). In situations where nitrogen is reduced by 30%, compared to the sole use of chemical nitrogen fertilizers, the incorporation of non-leguminous green manure, specifically orychophragmus violaceus, resulted in a significant 9.9% and 10.2% increase in rotation maize yield and biomass, respectively, upon incorporation. Simultaneously, there was a remarkable 26.7% improvement in nitrogen utilization efficiency, highlighting the symbiotic relationship between green manure application and soil nitrogen concerning crop nitrogen uptake and utilization (Bai et al., 2015). However, disparities exist in the impact of green manure types and incorporation methods on crop yield and nitrogen dynamics. In arid irrigated regions, particularly within the vicia sativa-corn rotational framework post-wheat harvest, the consistent incorporation of green manure and residues has been shown to elevate both wheat and corn yields alongside nitrogen utilization efficiency (Lyu et al., 2020b). Notably, full green manure application yields superior outcomes (Lyu et al., 2020a). Conversely, in rainfed agricultural zones during water-deficient periods, cultivating leguminous green manure during summer fallow intervals may lead to a reduction in subsequent winter wheat yield due to green manure-induced soil moisture consumption (Han et al., 2017). However, no such yield reduction was observed upon nitrogen decrement, ensuring an enhancement in nitrogen utilization efficiency (Zhang et al., 2015). Naveen et al. (2020), however, documented a yield deficit in Pennsylvania when green manure crops were introduced during the V2 phase of corn development. This finding suggests that the optimal utility of green manure crops lies in temperate regions with copious rainfall. Danga's investigation in humid locales revealed that leguminous crops, when rotated with cereals, did not compromise the yield of subsequent crops despite their consumption of soil moisture (Danga et al., 2009). In summary, customizing the application patterns of green manure to the specificities of various agricultural contexts can optimize crop nitrogen uptake and utilization efficiency, yield, and plant nitrogen absorption.

3.3 Promote crop nitrogen transport and distribution

Nitrogen is utilized by plants through absorption, transportation, and assimilation (Mokhele et al., 2012). During the vegetative growth phase, the primary nitrogen reservoirs are roots and leaves, whereas during the reproductive phase, the primary nitrogen reservoirs are flowers, fruits, and seeds (Robe and Griffiths, 2010). In roots, nitrate absorption from the soil occurs through nitrate transport proteins (NRTs), and ammonium absorption from the soil occurs through ammonium transport proteins (AMTs) (Luo et al., 2013). After absorption, nitrogen is transported from the root to the shoots through the xylem, and within the phloem, it migrates from source leaves to sinks. After the application of ammonium-based fertilizers, most of the absorbed ammonium salts are integrated into organic compounds within the plant roots, simultaneously releasing an equivalent quantity of H^+; these H^+ reduce the pH of the surrounding soil environment, causing localized soil acidity (Raven and Smith, 1976). Some of the nitrate ions are either transported within the xylem or stored in the vacuoles of root, stem, and other storage cells, which modulate plant ion balance and osmotic pressure (Müller et al., 2004). A smaller fraction undergoes reduction to ammonia via nitrate reductase (NR) and nitrite reductase (NiR), feeding into amino acid synthesis pathways and leading to glutamine production. With the influence of transpiration, the majority of NO_3^--N is channeled via the xylem to the aboveground components of the plant. Glutamine synthase (GS), a pivotal enzyme in nitrogen assimilation, acts in conjunction with glutamate synthase (GOGAT) to convert ammonium to glutamine, and further releasing glutamate and aspartate. In essence, nitrate nitrogen is integrated into essential amino acids for assimilation by crops, a process catalyzed by nitrogen-transforming enzymes(see Figure 5).

Recent studies have made significant progress in understanding nitrogen transport, metabolism, and regulatory mechanisms. However, there is limited research on the influence of green manure affects nitrogen transport and distribution. For the pea plant (Pisum sativum), increasing amino acid accumulation and transport in the phloem can improve nitrogen absorption by roots, affecting the assimilation of available nitrogen in various plant parts such as source and sink regions (Kulesza et al., 2022). Lyu et al. (2020a) documented that full addition of leguminous green manure, as opposed to just stubble, enhances nitrogen movement from leaves to the grains of primary crops, a phenomenon also noted with non-leguminous green manures (Li T. Y. et al., 2021). Soil and plant enzymes, especially GS and nitrate reductase, play a vital role in improving crop nutrient transport, marking grain protein synthesis (Li T. Y. et al., 2021). Green manure boosts soil enzyme activities related to nitrogen absorption and use, as well as these enzymes in leaves, aiding grain protein formation and nutrient content (Fu et al., 2022). Nitrogen from decomposed green manures in soil forms a significant part of the nitrogen uptake in primary crops. In leguminous-gramineae crop rotations, 34% of gramineae crops' nitrogen uptake comes from nitrogen introduced into the soil by preceding leguminous crops (Laberge et al., 2011). Li et al. (2015) conducted field experiments in Brazil using the ^{15}N labeling technique and reported that the recovery rate of ^{15}N in corn kernels after the incorporation of vicia villosa varied between 9.8% and 10.1%, depending on the extent of vicia villosa cover.

3.4 Promote crop root development and improve rhizosphere environment

The ability of roots to absorb and transport soil nitrogen is crucial for plant nitrogen efficiency. Root growth and development determine the soil area and nutrient range accessible to plants (Fageria and Moreira, 2011). Root exudates alter the rhizosphere, affecting soil nitrogen availability (El-Shatnawi and Makhadmeh, 2001). Research on green manure and crop roots mainly focuses on the morphological attributes of the root system. There is a consensus that reintroducing green manures to fields boosts the growth of primary crop roots, enhances nutrient uptake, and increases root biomass and the root-to-shoot ratio. These benefits are linked to improved soil physicochemical properties due to green manure application (Mandal et al., 2003; Kandel et al., 2018). The interaction between mature leguminous green manure and soil microorganisms accelerates mycelial growth in the soil and decomposition of extracellular polysaccharides secreted by various microorganisms. This interaction leads to an increased production of organic acids and other root exudates, which, combined with soil aggregates, mycelia, and minerals, create a nutrient-rich, well-structured rhizosphere environment for the crop (Van Zwieten et al., 2014).

The rhizosphere environment influences nitrogen form and availability, affecting crop nitrogen absorption and utilization (Moreau et al., 2019). Introducing exogenous organic matter such as green manure significantly affects the soil-plant nitrogen

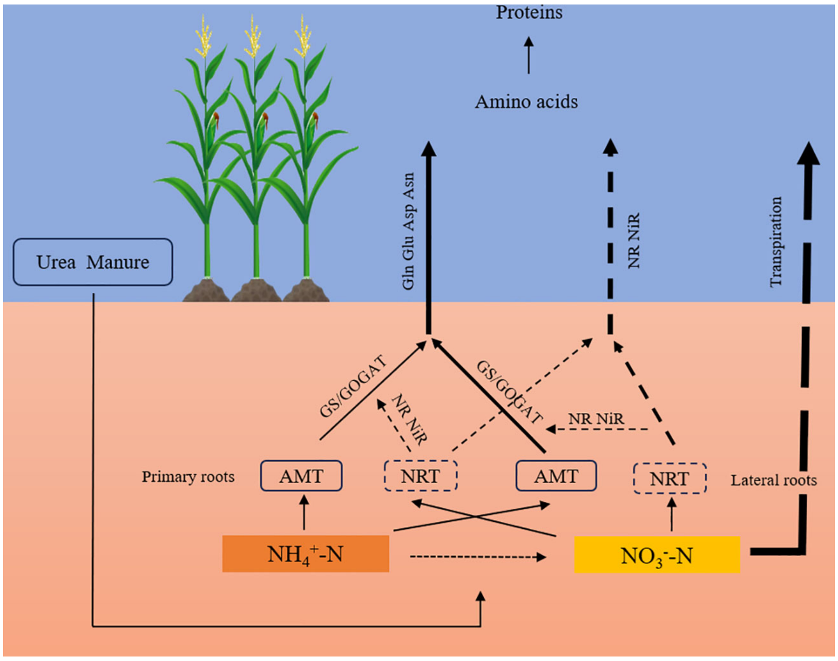

FIGURE 5
Plant nitrogen absorption and transport process.

transformation. Green manure application enhances nutrient cycling by improving soil qualities such as water-holding capacity, porosity, aggregate density, and microbial population dynamics and vigor (Letter et al., 2003; Haruna and Nkongolo, 2015). Soil moisture, temperature, and humidity are key factors influencing nitrogen absorption by the plant and transport within the plant parts (Budhar and Palaniappan, 1996). The composition and stability of soil aggregates affect soil material exchange and energy equilibrium. Notably, water-stable aggregates of size 1–10 mm are an optimal substrate for crop nutrient uptake (Tisdall and Oades, 1982; Jastrow, 1996). Lyu et al. (2022) performed a scanning electron microscopic analysis in their study and reported that, compared with just stubble application, full green manure incorporation improves soil aggregate microstructure and increases the presence of larger aggregates (Figure 6).

4 Frontier technologies and methods for exploring crop nitrogen transformation under green manure application

4.1 ^{15}N isotope tracer technology

Global agricultural scientists employ various methods to study the effects of leguminous crops. One such method is the ^{15}N labeling technique, combined with numerical models, to assess nitrogen transformation efficiency (Mary et al., 1998; Van Zwieten et al., 2014). This non-invasive method enables the contribution and distribution of different nitrogen sources in nitrogen transformation and the exchange mechanism of nitrogen between different soil components. The ^{15}N tracer technology has been vindicated to be an effective tool for investigating nitrogen mineralization and fixation, nitrogen loss means, and the relationship between nitrogen forms and plant availability (Jin-bo et al., 2013). In the late 1930s, Rittenberg et al. (1939) first applied the ^{15}N tracer technology to study biological nitrogen fixation. Mccauley et al. (2012) utilized the ^{15}N natural abundance method to determine the $\delta\ ^{15}$N of leguminous crops at various planting times, aiding farmers in selecting suitable leguminous crops for different seasons and ecological areas to optimize farmland planting systems. In China, the application of ^{15}N tracing technology focuses on nitrogen absorption, utilization, transport, and distribution in crops. (Junjun et al., 2019) examined the ^{15}N isotope composition in N_2O molecules to determine the relative contributions of denitrification and nitrification. Zhu et al. (2014) used ^{15}N labeling to study nitrogen absorption and utilization in rice following the application of Chinese milk vetch to the field. Currently, stable isotope labeling technology is primarily used to explore the nutrient flow status in soil-plant systems in agriculture, but research on the metabolic links in plants and the flow status of the whole ecosystem. For example, what is the metabolism status of green manure after it is used as forage grass in animal husbandry system?

4.2 Genomics: high-throughput sequencing technology

Since the 21st century, advancements in genomics, transcriptomics, and high-throughput sequencing technology and the development of bioinformatics have significantly propelled soil microbiology research.

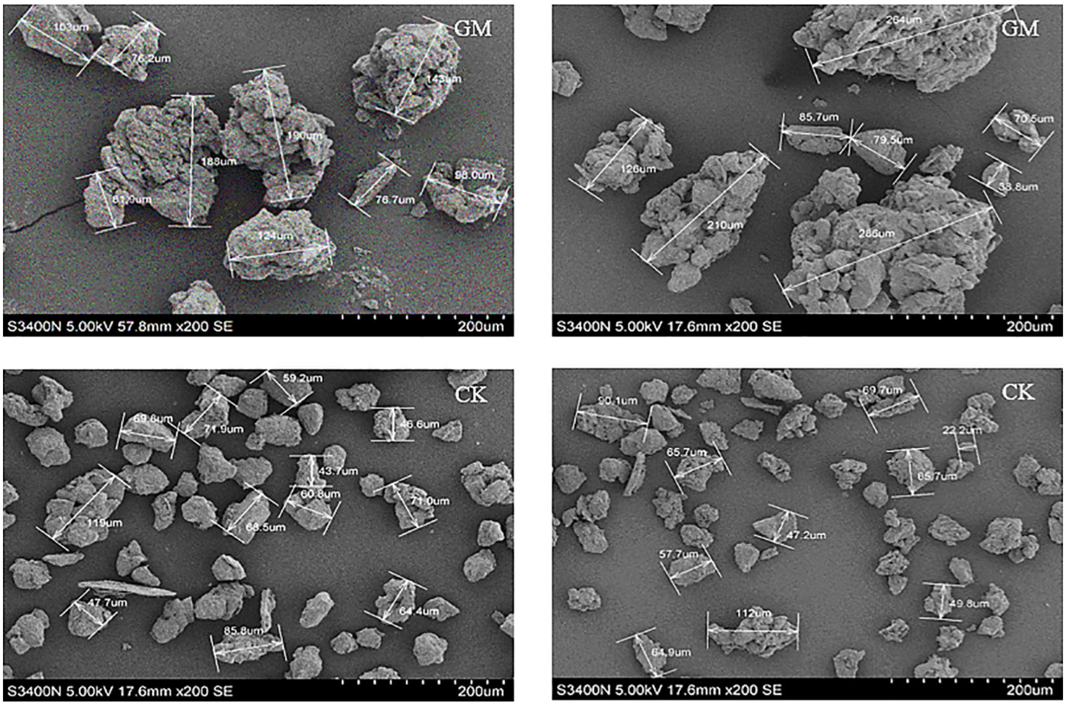

FIGURE 6
Characteristics of soil aggregate under green manure application. GM is green manure application, CK is no green manure application. The published images above are the results of research by the first author of this article and have been authorized by its publication journal and all authors.

Past studies have applied genomics to explore the genomic resources of green manure crops and understand the gene expression mechanisms, metabolic pathways, and secondary metabolites (Sathyanarayana et al., 2017). High-throughput sequencing technology, for example, has been used to examine soil bacterial communities' characteristics and functional diversity. (Liu et al., 2019) used high-throughput sequencing technology to investigate the spatial distribution patterns of functional microorganisms (AOA and AOB) involved in nitrogen transformation in the soil. They discovered that AOA abundance correlated positively with soil carbon content, while AOB abundance correlated significantly positively with soil pH. These methods offer deep insights into soil microbial community structures, functional activation of soil microorganisms under green manure application, and the role of microorganisms in soil nitrogen transformation and the regulatory mechanism underlying the gene expression of key enzymes.

4.3 Infrared spectroscopy and ^{13}C nuclear magnetic resonance technology

Infrared spectroscopy and ^{13}C nuclear magnetic resonance are can be used to study the decomposition and nutrient release of green manures. Near-infrared spectroscopy and infrared spectroscopy enable the non-destructive determination of nitrogen in soil and plant samples. These efficient and rapid detection technologies facilitate large-scale research on crop nitrogen transformationWittwer and Heijden (2020) adopted spectral image analysis showed that leguminous green manure crops can compensate for nitrogen availability, have improved nitrogen absorption, and show accelerated crop growth in later stages. The ^{13}C nuclear magnetic resonance method simultaneously monitors the distribution and transformation of carbon and nitrogen in the soil, revealing the effect of carbon-nitrogen interaction on soil nitrogen transformation. It is conducive to deeply understand the coupled cycle of soil carbon-nitrogen and promote the development of ecological agriculture. Additionally, the integration of system dynamics models, ecological models, and nitrogen cycle models helps perform numerical simulation and prediction of crop nitrogen transformation after green manure application. These models are effective because they comprehensively consider the influence of multiple factors to predict the effects of different management measures on nitrogen transformation.

5 Prospects for the regulation of soil nitrogen transformation and nitrogen absorption and utilization in crops by green manure application

Amidst global agricultural development, intensified by population growth, challenges include declining soil fertility and wastage of nitrogen resources have emerged as pressing agricultural concerns. Green manure application is an eco-friendly agronomic option, but ongoing research has revealed both existing challenges regarding green manure application and promising future opportunities. First, the high cost of green manure seeds, together with the economic inputs for sowing, incorporation, and other procedures, increases production costs. Second, despite numerous green manure varieties, only few are multi-resistant and suitable for cultivation in diverse regions. Third, how to incorporate green manure into cropping systems according to the characteristics of different ecoregions and resolve the contradictions between green manure and cash crops is a major issue for green manure cultivation and utilization. Unreasonable cultivation leads to competition for water and nutrients between green manure and cash crops, hindering the normal growth of cash crops and severely limiting the contribution of green manure to modern agriculture. Fourth, while increasing soil organic carbon content and improving soil quality and fertilizer efficiency through green manure incorporation, large amounts of greenhouse gas emissions are also generated. How to optimize cultivation methods or incorporation modes to reduce greenhouse gas emissions remains a challenge. Fifth, the fertilizer effect of green manure is slow. Not replenishing chemical fertilizers in the short term will lead to reduced yields of cash crops. Future research should focus on optimizing soil nitrogen dynamics and crop nitrogen uptake through green manure incorporation.

5.1 Deepening insights into the ecological impact of green manure application

The regulatory dynamics between green manure and soil nitrogen conversion and the crucial role of soil microorganisms in nitrogen cycling warrant in-depth exploration. Understanding the mechanisms by which nitrogen is lost through microbial processes can help reduce emissions. Knowledge of these microbial interactions under the influence of green manure can improve application methods and increase efficiency in using natural resources.

5.2 Green manure selection and distribution optimization

Different green manure crops affect soil nitrogen dynamics and crop nitrogen assimilation in various ways; therefore, choosing and arranging them wisely is important. Future research should be conducted based on the judicious selection and arrangement of green manures. Future investigations should elucidate the soil-enhancing effects of different green manures in various regions, probe the repercussions of varied green manures on soil properties under distinct planting paradigms—such as monoculture, intercropping, and mixed sowing—and create a detailed database of their soil improvement qualities of various green manures. This will provide farmers with clear guidelines to improve nitrogen efficiency and reduce nitrogen wastage.

5.3 Advancement in soil nutrient cycling and farmland ecosystem service functions

Green manure incorporation not only affects soil nitrogen dynamics and crop nitrogen availability but also interacts with other soil nutrients and ecosystem services. Future studies should consider nitrogen cycles along with other nutrient cycles, explore the effects of green manure application on soil health and ecosystem functions, and enhance the role of green manure in fostering sustainable agriculture.

6 Conclusions

Green manure crops, as environmentally friendly nitrogen sources, are cultivated in various regions globally. Both leguminous and non-leguminous varieties play a key role as intrinsic catalysts in soil nitrogen cycling. A salient characteristic of green manure application lies in its ability to maintain a balance between nitrogen fixation and mineralization, preserving soil health and providing essential nitrogen to grain crops with requisite nitrogen. Integrating green manures with chemical nitrogen fertilizers can reduce gaseous emissions of nitrogen and prevent nitrate leaching. Green manure deployment also improves the composition and functionality of soil microbial communities. Incorporating green manures into agricultural systems augments the nitrogen assimilation efficacy in primary crops in terms of crop nitrogen uptake and efficiency, evidently increasing crop yields, improving plant nitrogen uptake, facilitating nitrogen transport and distribution, enhancing root development, and regulating rhizosphere attributes such as soil aggregates. Future research could use advanced techniques such as molecular ecology to uncover the ecological mechanisms underpinning green manure application, thus enhancing farmland ecosystem services.

Author contributions

HL: Writing – original draft, Conceptualization. YL: Formal Analysis, Writing – review & editing. YW: Visualization, Writing – original draft. PW: Visualization, Writing – original draft. YS: Supervision, Writing – original draft. XY: Conceptualization, Writing – review & editing. FW: Methodology, Writing – review & editing. AY: Methodology, 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

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Green manure incorporation enhanced soil labile phosphorus and fruit tree growth

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Introduction: The incorporation of green manures substantially enhances the conversion of external phosphorus (P) fertilizers and soil-reserved P into forms readily available to plants. The study aims to evaluate the influence of green manure additions on soil phosphorus dynamics and citrus growth, considering different green manure species and initial soil phosphorus levels. Additionally, the research seeks to elucidate the microbiological mechanisms underlying the observed effects.

Methods: A citrus pot experiment was conducted under both P-surplus (1.50 $g \cdot P \cdot kg^{-1}$) and P-deficient (0.17 $g \cdot P \cdot kg^{-1}$) soils with incorporating legume (Leg), non-legume (Non-Leg) or no green manure residues (CK), and ^{18}O-P labeled KH_2PO_4 (0.5 g, containing 80‰ $\delta^{18}O_p$) was additionally introduced to trace the turnover characteristics of chemical P fertilizer mediated by soil microorganisms.

Results and discussion: In P-surplus soil, compared with the CK treatment, the Leg treatment significantly increased soil H_2O-P_i (13.6%), $NaHCO_3$-P_o (8.9%), NaOH-P_i (9.5%) and NaOH-P_o (30.0%) content. It also promoted rapid turnover of P sources into H_2O-P_i and $NaHCO_3$-P_i pools by enhancing the *phoC* (576.6%) gene abundance. In contrast, the Non-Leg treatment significantly augmented soil H_2O-P_i (9.2%) and $NaHCO_3$-P_o (8.5%) content, facilitating the turnover of P sources into $NaHCO_3$-P_i pools. Under P-deficient soil conditions, compared with the CK treatment, the Leg treatment notably raised soil H_2O-P_i (150.0%), $NaHCO_3$-P_i (66.3%), $NaHCO_3$-P_o (34.8%) and NaOH-P_i (59.0%) content, contributing to the transfer of P sources into $NaHCO_3$-P_i and NaOH-P_i pools. This effect was achieved through elevated ALP (33.8%) and ACP (12.9%) activities and increased *pqqC* (48.1%), *phoC* (42.9%), *phoD* (21.7%), and *bpp* (27.4%) gene abundances. The Non-Leg treatment, on the other hand, led to significant increases in soil $NaHCO_3$-P_i (299.0%) and $NaHCO_3$-P_o (132.6%) content, thereby facilitating the turnover of P sources into $NaHCO_3$-P_i and NaOH-P_i pools, except for the *phoC* gene abundance. Both Leg and Non-Leg treatments significantly improved citrus growth (7.3-20.0%) and P uptake (15.4-

42.1%) in P-deficient soil but yielded no substantial effects in P-surplus soil. In summary, introducing green manure crops, particularly legume green manure, emerges as a valuable approach to enhance soil P availability and foster fruit tree growth in orchard production.

KEYWORDS

orchard, phosphorus turnover, stable oxygen isotopes, microbial mobilization, cover crop

1 Introduction

The escalation of incomes and the expansion of the population over the past two decades are recognized as pivotal factors fostering the remarkable surge in global fruit consumption (Stratton et al., 2021). By the year 2021, the global expanse of orchards surpassed 64 million hectares, yielding a remarkable harvest of over 800 million tons (FAO, 2023). Phosphorus (P) holds the second position in terms of its significance among essential nutrient elements, following nitrogen (N), and assumes a critical function in the growth of fruit trees, as well as the yield and quality of fruits (Ahmed et al., 2019; Bibi et al., 2022). In order to uphold elevated plant yields and ensure global food security, approximately 19 million metric tons of phosphate rock-derived P are utilized annually for the production and application of fertilizers in agricultural systems (Chen and Graedel, 2016; Zhang et al., 2022). Nevertheless, the introduced P is readily bound by active metal ions present in soils, such as calcium (Ca^{2+}), magnesium (Mg^{2+}), iron (Fe^{3+}), and aluminum (Al^{3+}) cations, or adsorbed onto mineral surfaces. This leads to reduced P availability and diminished efficiency of P fertilizers (Fink et al., 2016b; Tian et al., 2020). The fixation and accumulation of P in soils pose a potential threat to ecological environments, including the occurrence of water eutrophication (Wang et al., 2019; Qin et al., 2020). In orchard production, these challenges are more pronounced due to the relatively low root length and density of fruit trees (typically around 2 $cm{\cdot}cm^{-3}$ for the root length to volume ratio) and their limited capacity to efficiently uptake soil nutrients (Kalcsits et al., 2020). Hence, enhancing the efficiency of P fertilizers and facilitating the conversion of accumulated P in the soil into bioavailable forms are crucial steps in promoting sustainable production and mitigating ecological risks in orchards.

Green manures, also referred to as cover crops, are generally grown at times when soil would otherwise be bare, typically in the period after a crop is harvested or the orchard alleyways (Lal, 2015). The utilization of green manure represents a significant field management strategy that can enhance the effectiveness of soil P and reduce the reliance on mineral P fertilizers (Li et al., 2015; Jamal et al., 2023; Ozbolat et al., 2023; Zhang et al., 2023). According to the study conducted by de Oliveira et al., 2017 in no-tillage onion production of Santa Catarina, Brazil, the incorporation of green manure residues has demonstrated the ability to promptly release soluble inorganic P (P_i) and elevate the availability of P in the soil. The rate of P release was contingent upon the total P content and the C/N (Carbon to Nitrogen) ratio of the residues, as well as the activities of soil microorganisms involved in P solubilization. In a 6-year comprehensive trial of commercial soybean, maize, and wheat cultivation systems in southern Brazil, Soltangheisi et al. (2018) found that continuous tillage of a variety of green manures, including common vetch (*Vicia sativa*), white lupin (*Lupinus albus*), fodder radish (*Raphanus sativus*), ryegrass (*Lolium multiflorum*), and black oat (*Avena strigosa*) were effective in utilizing moderately labile P and increasing the proportion of labile P fractions in the soils. And, white lupin (*Lupinus albus*) exhibited the highest level of improvement. Similarly, Dube et al. (2014) found comparable outcomes in their research on the addition of grazing vetch (*Vicia dasycarpa L.*) and oats (*Avena sativa L.*) to maize-based conservation agriculture systems in the Eastern Cape Province of South Africa. Corroborating these findings, Gao et al. (2016) reported similar conclusions in their studies involving the utilization of alfalfa (*Medicago sativa L*) and broad bean (*Vicia faba L.*) in rice agroecosystems in eastern China. However, there is limited information available on the specific contribution of different green manure varieties, such as legume or non-legume species, to improving P availability in orchard ecosystems and the subsequent uptake by fruit trees.

Prior investigations have validated that the impact of incorporating green manure into the soil on the active P pool can be primarily attributed to two distinct factors. Firstly, there is the release of P from the green manure itself during the process of decomposition (Karasawa and Takahashi, 2015; Dong et al., 2021). Secondly, the addition of green manure as an external carbon (C) source can stimulate the growth and activity of P-cycling microorganisms (Sarker et al., 2019). The P-cycling microorganisms were considered to make a greater contribution to the soil biological P pool in the soil (Hallama et al., 2019), and they can facilitate the transformation and circulation of soil-insoluble P through processes such as solubilization of P_i, mineralization of organic P (P_o) and accumulation and turnover of biomass P (Kafle et al., 2019). But, the impact of soil microorganisms on soil P pools is regulated by the quality of the green manure (Fink et al., 2016a; Hansen et al., 2022). Previous studies have shown that green manure residues with high P concentrations (generally refer to legume green manure) decompose faster than residues with low

P concentrations (generally refer to non-legume green manure) (Alamgir et al., 2012; Khan et al., 2022; Fontana et al., 2023). Asghar and Kataoka (2022) also indicated that the incorporation of soil with legume (*Vicia villosa*) green manure with a low C/P (Carbon to Phosphorus) ratio induced better soil P nutrition status and plant growth through increasing soil phosphatase and β-glucosidase activities and altering soil microbial community composition, compared to non-legume (*Brassica juncea L.*) green manure.

The impact of green manure on both the active P pools in the soil and the P nutrition of fruit trees is influenced not only by the C and P content of the green manure itself but also by the initial soil P status (Ullah et al., 2023). In the study by Maltais-Landry and Frossard (2015), the contribution of green manure residues to soil P fractions and a subsequent crop was similar and comparable to the effects of a water-soluble mineral P fertilizer, and with a greater contribution when soil initial available P was lower. Richardson and Simpson (2011) also indicated that under initial soil conditions of P deficiency, microorganisms have the ability to activate or deactivate various P-cycling genes and express microbial phosphatase enzymes. This activation leads to the mobilization of P pools that are not readily accessible, as opposed to initial soil conditions of P surplus. Nevertheless, there remains a lack of consensus regarding the influence of different green manure species on soil's active P pools and the phosphorus nutrition of fruit trees, particularly in the context of differing initial soil P levels. Moreover, the mechanisms underlying these effects are not well understood.

In this study, a citrus pot experiment was conducted in soils characterized by both P surplus and deficiency. Green manure residues from both legume and non-legume sources were incorporated, and ^{18}O-labeled KH_2PO_4 was introduced to trace the turnover characteristics of chemical P fertilizer facilitated by soil microorganisms. The objectives of this study were twofold: (1) to determine the magnitude of the effects of green manure additions on soil active P pools and citrus plant P uptake based on various green manure species and soil initial P status; (2) to unravel the microbiological mechanisms that underlie the aforementioned effects. Our hypothesis posited that the incorporation of green manure residues would markedly augment soil labile P pools and enhance P nutrition for citrus trees in both P-surplus and deficient soils. This effect was anticipated to occur through the stimulation of microorganisms and enzymes engaged in P cycling, leading to the conversion of accumulated soil P and chemical fertilizer P into forms readily available for crops. Additionally, we expected that the activation of legume green manures would surpass that of non-legume green manures in this context. This study can establish a theoretical foundation for the utilization of green manure in resenting a sustainable approach for the development of fruit tree production, contributing to long-term ecological balance and productivity.

2 Materials and methods

2.1 Experimental soil and crops

For the experiment, soils with both P-surplus and P-deficient were selected. These soils were obtained from Danling County (30°04′ N, 103°53′ E), in Sichuan Province of China. The P-surplus soil was collected from a well-established citrus orchard, while the P-deficient soil was obtained from a recently established citrus orchard. Based on the classification by the United States Department of Agriculture Soil Taxonomy, both the experimental soils were classified as Alfisols. Soils used in the containers were taken from the top 20 cm of soil layer in October 2020. They were sieved to < 2 mm after air-drying and removing visible plant residues and stones. The fundamental characteristics of the soil samples were presented in Table 1.

The citrus employed in the present study was of the first-year seedling stage, and the species is the Ehime mandarin 38th. The legume and non-legume green manure residues were hairy vetch (*Vicia villosa*) and rattail fescue (*Vulpia myuros (L.) C.C. Gmel.*), respectively. The green manures were freshly harvested from the field in March, 2021 and then finely chopped into 2 mm in size. The nutrient content of the green manure residues was presented in Table 2.

2.2 Experimental design and sampling

A pot-based experiment was conducted from March to September 2021 for the present study, which was carried out within a greenhouse facility located at Southwest University (29°81′ N, 106°42′ E), Chongqing, China. The experiment was designed using a completely randomized approach, considering two primary factors: (1) two treatments related to soil P levels, specifically P-surplus and P-deficient soils; (2) three treatments related to addition materials, including legume green manure (Leg), non-legume green manure (Non-Leg) and a control treatment with no addition (CK). A fixed quantity of 2 $g{\cdot}C{\cdot}kg^{-1}$ (dry soil) of green manure was added in all treatments. Due to the significant individual variations and potential systematic errors in fruit tree cultivation, each treatment was replicated five times. Each pot was filled with 10 kg of mixed soils, green manure residues and chemical fertilizers (40 $mg{\cdot}kg^{-1}$ N, 40 $mg{\cdot}kg^{-1}$ P_2O_5 and 80 mg kg^{-1} K_2O were used as the foundation fertilizers, 40 $mg{\cdot}kg^{-1}$ N were used as top-dress to ensure sufficient nutrition to citrus growth). The N, P and K

TABLE 1 The properties of P-surplus and P-deficient soils used in this experiment.

Soil properties	P-surplus soil	P-deficient soil
TP ($g{\cdot}kg^{-1}$)	1.50 ± 0.09 a	0.17 ± 0.01 b
AP ($mg{\cdot}kg^{-1}$)	274.40 ± 16.02 a	4.20 ± 0.15 b
TN ($g{\cdot}kg^{-1}$)	2.39 ± 0.09 a	0.81 ± 0.05 b
TK ($g{\cdot}kg^{-1}$)	15.28 ± 0.24 a	15.93 ± 0.41 a
SOM ($g{\cdot}kg^{-1}$)	37.40 ± 2.07 a	18.98 ± 1.66 b
pH	5.40 ± 0.19 a	4.10 ± 0.06 b

TP, soil total phosphorus; AP, soil available phosphorus; TN, soil total nitrogen; TK, soil total potassium; SOM, soil organic matter; pH, measured in 1:2.5 soil/water suspensions. The soils of this experiment were categorized according the soil nutrient classification standard for citrus orchards, with AP < 5.0 mg/kg as extremely deficient, AP 5.0 – 15.0 mg/kg as deficient, AP 15.0 – 80.0 mg/kg as moderate, and AP > 80.0 mg/kg as excessive. Values in the same row within the same parameters followed by different letters were significantly different at $p < 0.05$ according to Tukey's test.

TABLE 2 The nutrient content of the green manure residues (%, calculated by dry mass).

Green manure types	Carbon	Nitrogen	Phosphorus	Potassium	C/N ratio	C/P ratio
Leg	43.26 ± 2.83 a	3.95 ± 2.03 a	0.59 ± 0.09 a	7.09 ± 0.11 a	10.95 ± 1.24 b	73.32 ± 2.13 b
Non-Leg	43.61 ± 0.49 a	1.86 ± 0.25 b	0.31 ± 0.13 b	3.83 ± 0.06 b	23.49 ± 0.53 a	140.67 ± 4.33 a

Leg, legume green manure [hairy vetch (*Vicia villosa*)]; Non-Leg, non-legume green manure [rattail fescue (*Vulpia myuros* (L.) C.C. Gmel.)]. C/N ratio, Carbon to Nitrogen ratio in green manure residues. C/P ratio, Carbon to Phosphorus ratio in green manure residues. Values in the same column within the same parameters followed by different letters were significantly different at $p < 0.05$ according to Tukey's test.

fertilizers used in this study was urea, KH_2PO_4 and K_2SO_4, respectively. In addition, 0.5 g $KH_2P^{18}O_4$ (containing 80‰ $\delta^{18}O_p$, diluted from $KH_2P^{18}O_4$ ($^{18}O_4$, 95%), purchased from Cambridge Isotope Laboratories) were applied to each pot to track the fate of exogenous P fertilizer. Ehime mandarin 38th citrus seedlings were subsequently transplanted into pots and watered daily to 60% of the field water capacity. The position of pots switched once a week to minimize possible environmental effects.

Destructive sampling was conducted in September 2021, after citrus summer tips turned green. Plant samples were collected by separating the roots, stems, new leaves, and old leaves, following measurements of citrus height, stem thickness, and biomass. The collected plant samples were subjected to oven-drying at 105°C for 30 minutes, followed by further oven-drying at 70°C until a constant weight was achieved. Subsequently, the dried samples were ground and sieved through a 0.5 mm mesh size to determine the plant P concentration. The soil samples were divided into two portions. One portion was air-dried for the determination of total P and available P content, while the other portion was preserved at -80°C. The preserved samples were later used for analyzing phosphatase activity, the abundance of P-cycling genes, and the $^{18}O_P$ values of different forms of P_i.

2.3 Determination of P in soil and plant samples

Soil total P and available P content were determined by molybdenum antimony anti-colorimetric method after digested with NaOH and extracted by NH_4F-HCl, respectively (Murphy and Riley, 1986). Different soil P fractions were sequentially extracted following a modified Hedley method (Hedley et al., 1982). Briefly, 0.5 g dried soil was sequentially extracted with 30.0 mL Milli-Q water (most labile P), 0.5 $mol\cdot L^{-1}$ $NaHCO_3$ (pH 8.5) (labile and weakly adsorbed P), 0.1 $mol\cdot L^{-1}$ NaOH (Fe/Al oxide-bound P) and 1.0 $mol\cdot L^{-1}$ HCl (Ca-P minerals) after shaking overnight (16 h) at 25°C at 165 rpm. The supernatants were collected by centrifugation (10 min at 5000 rpm) and then filtered through 0.45 mm cellulose-acetate filters membrane to determine total P (P_t) and P_i content, the difference between P_t and P_i was taken as P_o content. Residual-P was determined by the molybdenum-antimony colorimetry after digested using the mixture of H_2O_2 and H_2SO_4.

Plant P concentration was measured by the molybdovanado phosphate method after digested in concentrated H_2SO_4 and H_2O_2 (Thomas et al., 1967).

2.4 Measurement of soil enzyme activity

Soil acid phosphatase (ACP) and alkaline phosphatase (ALP) activities were determined by the disodium phenyl phosphate colorimetric method (Jin et al., 2016). The ACP was extracted using acetate buffer at pH 5.0, while the ALP was extracted using borate buffer at pH 10.0.

2.5 Extraction of soil DNA and quantification of P-cycling genes

The fresh soil samples (0.5 g) was subjected to whole genomic DNA extraction using FastDNA ® Spin Kit (MP Biomedical, Santa Ana, USA). DNA purity and quality were detected by NanoDrop ND-2000 spectrophotometer and nucleic acid integrity was determined by agarose gel electrophoresis. The tested P-cycling genes, including *pqqC* (pyrroloquinoline-quinone synthase), *phoD* (alkaline phosphatase D), *phoC* (acid phosphatase C) and *bpp* (β-helical phytase), as well as 16S rRNA gene as a reference for bacterial abundance were quantified by quantitative PCR (qPCR) reations via QuantStudioTM 6 Flex Real-Time System (Applied Biosystems, USA). All primers used in this study were listed in Table 3. The amplification program initialized with 95°C for 10 min, following with 40 cycles of 95°C for 5 s, 58°C for 30 s and 72°C 1 min. Each PCR assay was performed in triplicate, with amplification efficiencies between 91-100%. The copy number of each target gene in soil DNA was calculated based on the standard curve and the mean Ct value. The relative abundance of P-cycling genes was calculated as the ratio of cope numbers of respective P-cycling gene and 16S rRNA gene.

2.6 Measurement of oxygen isotope ratios in phosphate

The measurement of oxygen isotope ratios in phosphate was followed the methods of Bi et al. (2018) and Jiang et al. (2017). In brief, 25.0 g freeze-dried fresh soil was sequentially extracted with H_2O, $NaHCO_3$, NaOH and HCl. Frist, the magnesium-induced co-precipitation (MAGIC) method was used to enrich PO_4^{3-} and added DAX-8 macroporous resin to eliminate the organics in samples. Then, the coprecipitation of ammonium phosphomolybdate (APM) and magnesium ammonium phosphate (MAP) method was used to further separated and purified PO_4^{3-}. After anion cation resin purified, PO_4^{3-} was converted into Ag_3PO_4 precipitation by ammonia volatilization method. Finally, the mineral structure of

TABLE 3 The primers used for quantitative qPCR and corresponding amplification cycling conditions in this experiment.

Primer set	Target gene	Amplicon length (bp)	Amplification efficiencies	Amplification cycling conditions	References
pqqC-F (CATGGCATCGAGCATGCTCC)	*pqqC*	546	94.06%	40 cycle (95°C 5 s, 58°C 30 s, 72°C 1 min)	(Meyer et al., 2011)
pqqC-R (CAGGGCTGGGTCGCCAACC)					
ALPS-F730 (CAGTGGGACGACCACGAGGT)	*phoD*	371	99.87%		(Luo et al., 2020; Sakurai et al., 2008)
ALPS-R1101 (GAGGCCGATCGGCATGTCG)					
phoC-A-F1 (CGGCTCCTATCCGTCCGG)	*phoC*	155	97.55%		(Fraser et al., 2017; Gaiero et al., 2018)
phoC-A-R1 (CAACATCGCTTTGCCAGTG)					
BPP-F (GACGCAGCCGAYGAYCCNGCNITNTGG)	*bpp*	175	91.71%		(Huang et al., 2009)
BPP-R (5′-CAGGSCGCANRTCIACRTTRTT-3′)					
338F (GGGTTGCGCTCGTTGC)	16S rRNA	191	96.95%		(Zhang et al., 2019)
518R (ATGGYTGTCGTCAGCTCGTG)					

Ag_3PO_4 was detected by X-ray diffractometer (XRD) and compared with the standard pattern to check the purity of the sample and the soil $^{18}O_P$ values were measured after the sample passing the test.

2.7 Statistical analysis

The values presented in the figures and tables are given as means ± standard errors. One-way analysis of variance (ANOVA) tests was used to examine the significant changes in soil P fractions, enzyme activities and the microbial gene abundance, citrus growth parameters and citrus P uptake under CK, Leg and Non-Leg treatments in P-surplus and P-deficient soils, respectively. Spearman correlation analysis was conducted to reveal the relationship between soil P fractions, the $\delta^{18}O_P$ values of P_i pools and phosphatase activities, P-cycling gene relative abundance in P-surplus and P-deficient soils, respectively. All the statistics analysis were performed with IBM SPSS Statistics 22.0.

To evaluate the direct and indirect factors (including green manure addition, soil phosphatase activities and P-cycling genes abundances) affecting soil P sources transformation and citrus P uptake under different treatments in P-surplus and P-deficient soils, partial least squares path models (PLS-PM) were constructed using the R 4.2.3 packages "vegan" and "plspm". And all figures in the present study were plotted using GraphPad Prism 8.0 and Adobe Illustrator 2023.

3 Results

3.1 Citrus growth and P uptake

In comparison to the control treatment (CK), the addition of green manure residues did not yield a statistically significant impact on the growth of citrus plants in P-surplus soil, except for a 5.5% increase ($p < 0.05$) in stem thickness observed under the Leg treatment (Figure 1E). However, both citrus biomass and stem thickness exhibited significant increases ($p < 0.05$) under both Leg (19.0% and 11.5%, respectively) and Non-Leg (20.0% and 16.5%, respectively) treatments in P-deficient soil (Figures 1B, F). Additionally, plant height also experienced a significant increase ($p < 0.05$) under the Non-Leg treatment, with an 11.1% increase (Figure 1D).

In P-surplus soil, the addition of legume green manure residues significantly increased P uptake of citrus roots and new leaves by 29.6% and 80.1% ($p < 0.05$), respectively (Figures 2A, G). And, a 21.3% increase ($p < 0.05$) of P uptake in citrus roots by Non-Leg treatment was also observed (Figure 2A). In P-deficient soil, there were significant increases ($p < 0.05$) in P uptake observed in citrus roots and old leaves under both the Leg and Non-Leg treatments, compared to the control treatment (CK). Specifically, there was a 21.7% increase in citrus roots and a 46.7% increase in old leaves under the Leg treatment, while the Non-Leg treatment showed a 42.1% increase in citrus roots and a 37.9% increase in old leaves (Figures 2B, F). Additionally, P uptake in new leaves significant increased ($p < 0.05$) 36.3% by Leg treatment (Figure 2H).

3.2 Soil different P fractions

In P-surplus soil, the H_2O-P_i and $NaHCO_3$-P_o content demonstrated a significant increase ($p < 0.05$) under both Leg (13.6% and 8.9%, respectively) and Non-Leg (9.2% and 8.5%, respectively) treatments. Furthermore, the NaOH-P_i and NaOH-P_o content also significantly increased by 9.5% and 30.0% ($p < 0.05$) under the Leg treatment, respectively (Table 4).

In P-deficient soil, the $NaHCO_3$-P_i and $NaHCO_3$-P_o content showed a significant increase ($p < 0.05$) under both Leg (66.3% and 34.8%, respectively) and Non-Leg (299.0% and 132.6%, respectively) treatments. Additionally, the H_2O-P_i and NaOH-P_i content also significantly increased by 150.0% and 59.0% ($p < 0.05$) under the Leg treatment, respectively (Table 4).

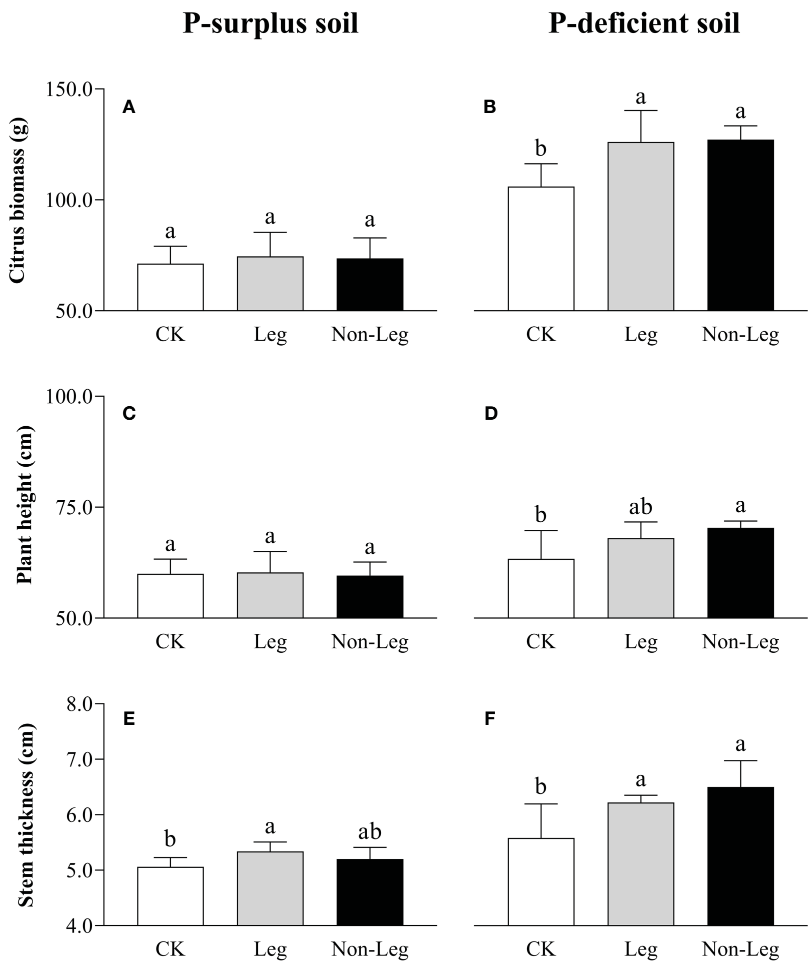

FIGURE 1
The citrus biomass **(A, B)**, plant height **(C, D)** and stem thickness **(E, F)** under different treatments in P-surplus and P-deficient soils. CK, no green manure residues addition; Leg, legume green manure residues addition; Non-Leg, non-legume green manure residues addition. Vertical bars indicated the standard errors. Different letters indicated significant differences among treatments at $p < 0.05$.

The content of HCl-P_i and Residual-P did not significantly change among the different treatments in both P-surplus and P-deficient soils.

3.3 The oxygen isotope ratios in different soil P_i pools

Green manure residues addition changed the phosphate oxygen isotope ratios of different P_i pools in both P-surplus and P-deficient soils (Figure 3). In P-surplus soil, compared with CK, the $\delta^{18}O_P$ values of $NaHCO_3$-P_i and NaOH-P_i under the Leg treatment, as well as $NaHCO_3$-P_i under the Non-Leg treatment, tended to approach or shift toward the isotopic equilibrium zone (Figure 3A). This indicated that the biological cycling of these P_i pools may be rapid and related to the oxygen isotope equilibration. In addition, the $\delta^{18}O_P$ values of HCl-P_i under the Leg treatment would be lighter than the other treatments.

In P-deficient soil, the phosphate oxygen isotope ratios of various P_i pools exhibited similar variations under both Leg and Non-Leg treatments. Compared to CK, the $\delta^{18}O_P$ values of $NaHCO_3$-P_i showed a considerable increase (shifted toward the isotopic equilibrium zone) following the addition of green manure residues (Figure 3B). This observation suggests that microorganisms play a crucial role in the turnover of $NaHCO_3$ pools.

3.4 Soil microbial activities

In P-surplus soil, the activities of ACP and ALP exhibited a significant decrease ($p < 0.05$) under the Leg than CK treatment, while no significant change was observed by treated with Non-Leg (Figures 4A, C). Conversely, in P-deficient soil, the activities of soil ACP and ALP significantly increased ($p < 0.05$) under both Leg and Non-Leg than CK treatments, with a higher increase observed under the Leg treatment (Figures 4B, D).

In P-surplus soil, the addition of green manure residues did not have a significant effect on the relative abundances of soil P-cycling genes, except for the *phoC* gene. Specifically, under the Leg treatment, the relative abundance of the *phoC* gene exhibited a significant increase ($p < 0.05$) of 576.6% compared to CK (Figures 5A, C, E, G). In P-deficient soil, compared to CK, the relative abundance of *pqqC*, *phoC*, *phoD* and *bpp* genes under the Leg treatment significantly increased (*p*

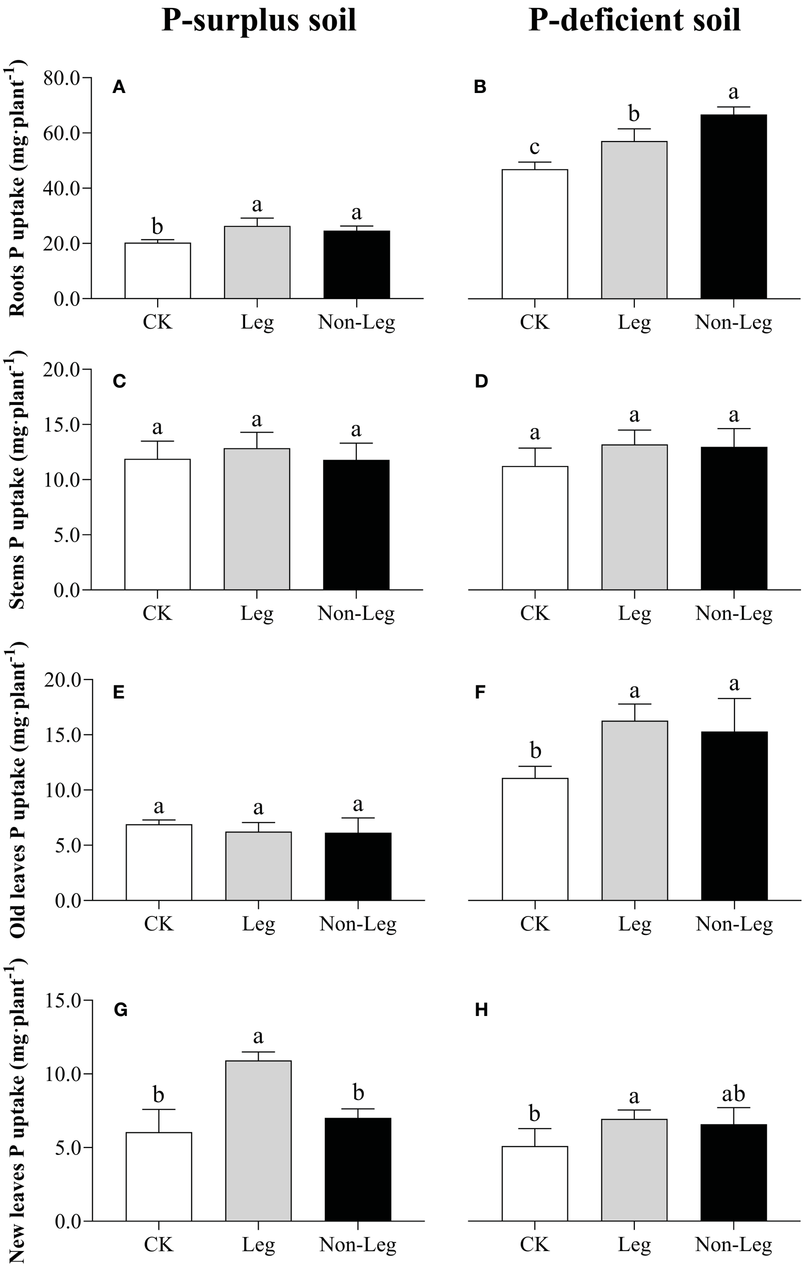

FIGURE 2
Phosphorus uptake in citrus roots **(A, B)**, stems **(C, D)**, old leaves **(E, F)** and new leaves **(G, H)** under different treatments in P-surplus and P-deficient soils. CK, no green manure residues addition; Leg, legume green manure residues addition; Non-Leg, non-legume green manure residues addition. Vertical bars indicated the standard errors. Different letters indicated significant differences among treatments at $p < 0.05$.

< 0.05) by 48.1%, 42.9%, 21.7% and 27.4%, respectively. And the relative abundance of *pqqC*, *phoD* and *bpp* genes under the Non-Leg treatment significantly increased ($p < 0.05$) by 45.1%, 33.3% and 18.6%, respectively (Figures 5B, D, F, H).

3.5 Relative contributions of various factors to soil P fractions and citrus P uptake

In P-surplus and P-deficient soils, the effects of green manure addition on soil P fractions and citrus P uptake were correlated with the majority microbial parameters in P-cycling, including enzymes activities and P-cycling genes abundances (Figure 6).

In P-surplus soil, the addition of green manure residues had a positive impact on the turnover of soil P fractions through direct effects (path coefficient = 0.59). Additionally, the influence of green manure residues on citrus P uptake was mediated indirectly through alterations in soil phosphatase activities, including both ACP and ALP (Figure 6A).

In P-deficient soil, the addition of green manure residues had a positive influence on the turnover of soil P fractions through both direct effects (path coefficient = 0.55) and indirect effects, mediated

TABLE 4 The content of soil P fractions under different treatments in P-surplus and P-deficient soils.

	P fractions (mg kg^{-1})	CK	Leg	Non-Leg
P-surplus soil	H_2O-P_i	117.78 ± 7.22 b	133.83 ± 2.84 a	128.23 ± 6.05 a
	$NaHCO_3$-P_i	370.35 ± 16.50 a	381.08 ± 3.72a	381.45 ± 6.88 a
	NaOH-P_i	565.46 ± 58.97 b	619.35 ± 14.91 a	591.40 ± 3.66 ab
	HCl-P_i	1109.28 ± 18.73 a	1086.88 ± 29.64 a	1088.63 ± 45.51 a
	$NaHCO_3$-P_o	306.70 ± 21.82 b	335.00 ± 32.99 a	332.86 ± 20.82 a
	NaOH-P_o	97.79 ± 11.08 b	126.88 ± 16.60 a	116.50 ± 6.77 ab
	Residual-P	482.36 ± 34.02 a	491.57 ± 25.96 a	490.60 ± 34.40 a
P-deficient soil	H_2O-P_i	0.06 ± 0.02 b	0.15 ± 0.04 a	0.14 ± 0.09 ab
	$NaHCO_3$-P_i	4.42 ± 0.36 c	7.35 ± 0.78 a	5.96 ± 1.03 b
	NaOH-P_i	23.09 ± 2.76 b	36.71 ± 4.47 a	24.79 ± 5.48 b
	HCl-P_i	0.87 ± 0.21 a	1.04 ± 1.04 a	0.97 ± 0.25 a
	$NaHCO_3$-P_o	0.95 ± 0.95 c	3.79 ± 0.92 a	2.21 ± 0.56 b
	NaOH-P_o	36.83 ± 2.35 a	37.98 ± 2.40 a	37.37 ± 3.63 a
	Residual-P	111.94 ± 7.32 a	113.74 ± 5.25 a	115.83 ± 4.97 a

P_i, inorganic phosphorus; P_o, organic phosphorus. CK, no green manure residues addition; Leg, legume green manure residues addition; Non-Leg, non-legume green manure residues addition. Values in the same row within the same parameters followed by different letters were significantly different at $p < 0.05$ according to Tukey's test.

by alterations in soil phosphatase activities (path coefficient = 0.49). These changes in soil phosphatase activities or soil P pools, in turn, affected citrus P uptake (path coefficient = 0.40). Additionally, citrus P uptake was also directly influenced by the addition of green manure residues (path coefficient = 1.46) (Figure 6B).

4 Discussion

4.1 The changing of soil P pools

This study confirmed green manure residues incorporation had a positive effect on the enhancement of soil P pools (Figure 6; Table 4). This effect was mediated by both green manure species and initial soil P levels, characterized that legume green manure had a superior capacity to regulate soil P pools compared to non-legume green manure, and was more prominent in P-deficient soil.

Green manure residues contain considerable amounts of nutrients and their decomposition results in the release of these nutrients into the soil, which can assist in fulfilling the nutritional requirements of crops (Calegari et al., 2013; Jamal et al., 2023). Hinsinger et al. (2011) and Pavinato et al. (2008) indicated that organic forms of P released during residues decomposition were less prone to strong adsorption on the functional groups of Fe and Al oxides and hydroxides compared to inorganic forms. And organic

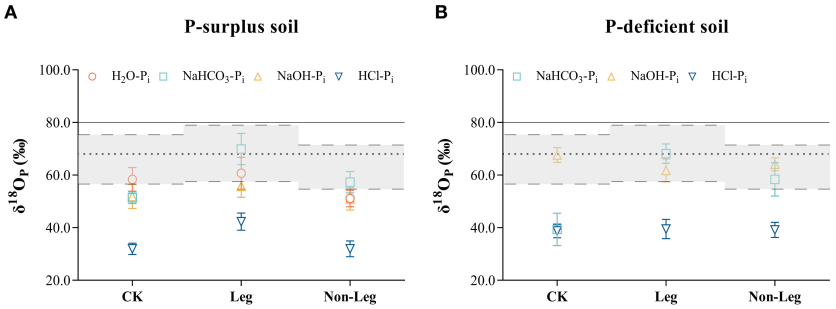

FIGURE 3
The oxygen isotope ratios ($\delta^{18}O_P$) in different soil inorganic P (P_i) pools among different treatments of both P-surplus **(A)** and P-deficient **(B)** soils. CK, no green manure residues addition; Leg, legume green manure residues addition; Non-Leg, non-legume green manure residues addition. The grey zones with long-dotted line represented equilibrium range calculated based on the monthly average highest and lowest soil temperatures and soil water isotopes values ($\delta^{18}O_W$) under different treatments during the pot experiment. The short-dotted line was the average equilibrium value calculated using the average soil temperature and average $\delta^{18}O_W$, which was included in the equilibrium zones. The $\delta^{18}O_P$ value of superphosphate was indicated with solid line. Vertical bars indicated the standard errors. The $\delta^{18}O_P$ values of H_2O were not detected in P-deficient soil in the present study.

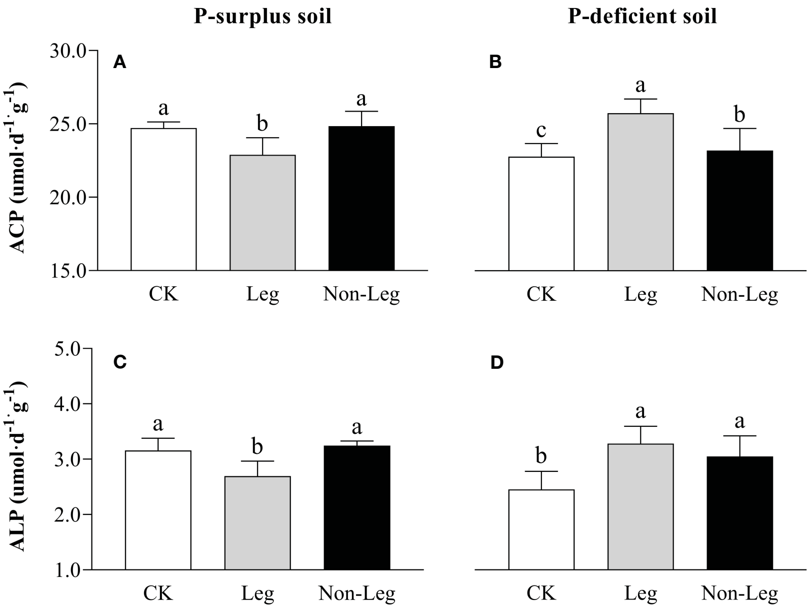

FIGURE 4
The ACP **(A, B)** and ALP **(C, D)** activities under different treatments in P-surplus and P-deficient soils. CK, no green manure residues addition; Leg, legume green manure residues addition; Non-Leg, non-legume green manure residues addition. Vertical bars indicated the standard errors. Different letters indicated significant differences among treatments at $p < 0.05$.

decomposition products can also compete for adsorption sites, thereby increasing bioavailable of soil P pool (Varela et al., 2017; Soltangheisi et al., 2020). Maltais-Landry and Frossard (2015) also reported that comparable transfer of P from green manure residues and water-soluble mineral fertilizer to the soil was observed. Consistently, Hallama et al. (2019) confirms the observation of this study, that legume green manure was found to be more nutrient efficient than non-legume green manure. This can be primarily attributed to the following reasons. Firstly, legume green manure exhibits a higher capacity for P uptake and accumulation due to its abundant biomass (Arrobas et al., 2015). In regard to the green manure materials supplied by this study, when subjected to equal carbon input (2 g·C·kg^{-1} soil), the P input of legume green manure was 270 mg·P·pot^{-1}, while the P input of non-legume green manure was 140 mg·P·pot^{-1} (Table 2). Consequently, there is a greater release of P during decomposition, thereby replenishing the labile soil P pools and promoting the growth of the main plant. Secondly, legume green manure with lower C/N and C/P ratios can more rapidly facilitate microbial decomposition and utilization, as well as stimulate the activity of P-cycling microorganisms (Truong and Marschner, 2020). This leads to the formation of greater microbial biomass P (Benitez et al., 2016), which is considered a potential active P pool in the soil (Hallama et al., 2019). These two factors collectively increased soil active P fraction (e.g. H_2O-P_i, $NaHCO_3$-P_o, NaOH-P_i and NaOH-P_o in P-surplus soil and H_2O-P_i, $NaHCO_3$-P_i, $NaHCO_3$-P_o and NaOH-P_i in P-deficient soil (Table 4) and enhanced the growth and P nutrition of citrus plants (Figures 1, 2).

The impact of green manure on soil P fractions were greatly influenced by the soil condition, particularly the size of the easily and sparingly available P pool (Damon et al., 2014). Similar to the finding of this study, the meta-analysis conducted by Hallama et al. (2019) reported that the benefits of green manure in terms of soil biological P were more pronounced in soils that had limited availability of P compared to sites with higher P availability. This study provided evidence that the contribution of green manure residues to labile soil P pools and citrus growth was comparatively limited in the context of the already abundant P availability in the soil (Figures 1, 2, 6; Table 4). This perspective was also confirmed by the research of Rick et al. (2011). Their study demonstrated that incorporation of green manure and input of rock P fertilizer had no significant impact on labile soil P fractions, wheat biomass, or P concentration due to high soil initial P availability, which already satisfied the requirements for crop growth.

4.2 The changing of soil P-cycling microorganism

There is a consensus that the incorporation of green manure residues into soil can serve as a source of C and energy for soil microorganisms (Huang et al., 2021), which in turn promotes increased metabolic activities of P-cycling microbial populations and facilitates the conversion of bio-unavailable P into bio-available forms (Pavinato et al., 2017; Zhou et al., 2018; Arruda et al., 2021). In the current study, the effects of microorganisms on soil P cycling were investigated using oxygen isotope labeling on exogenous chemical P fertilizers. This labeling technique allowed for tracking and monitoring the behavior and transformations of P_i within soil, and the closer isotope value of a P pool was to the theoretical equilibrium, the faster that the P pool was biologically cycled, indicating higher bioavailability. Conversely, if the isotope value

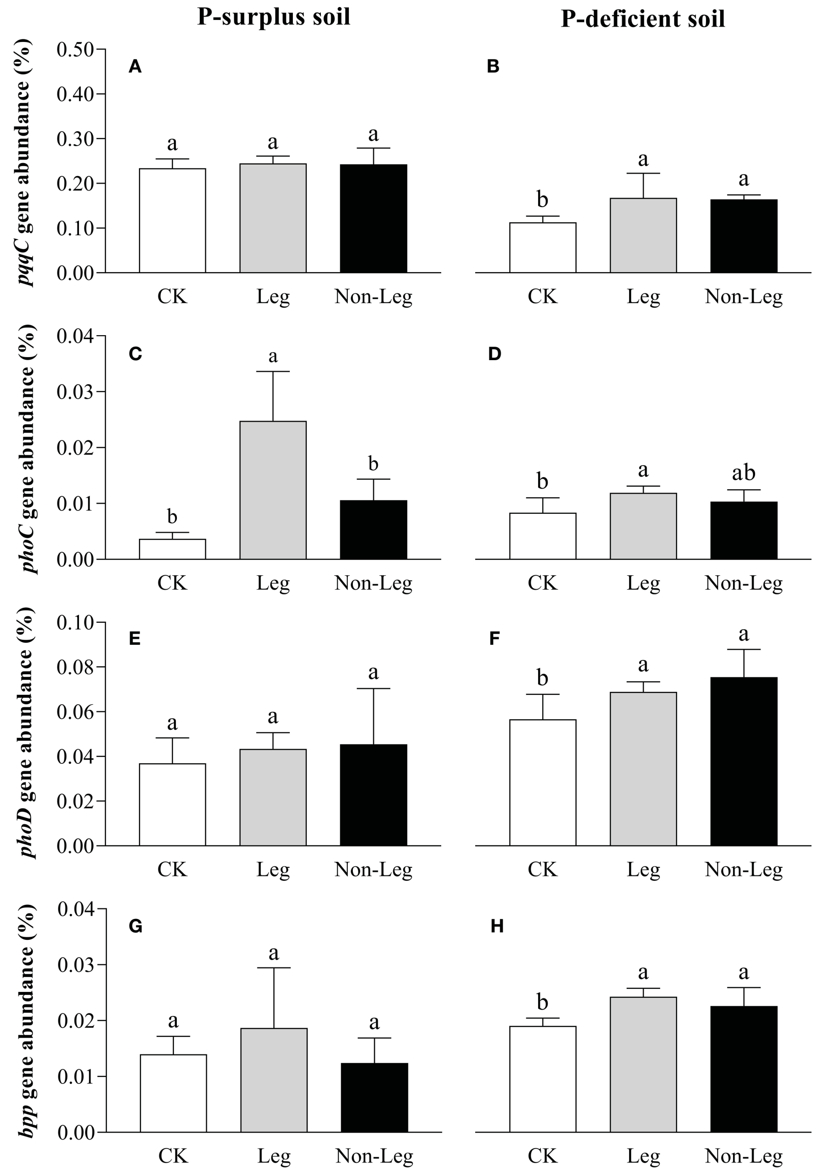

FIGURE 5
The relative abundance of *pqqc* **(A, B)**, *phoC* **(C, D)**, *phoD* **(E, F)** and *bpp* **(G, H)** under different treatments in P-surplus and P-deficient soils. The relative abundance of P-cycling genes was calculated as the ratio of cope numbers of respective P-cycling gene and 16S rRNA gene. CK, no green manure residues addition; Leg, legume green manure residues addition; Non-Leg, non-legume green manure residues addition. Vertical bars indicated the standard errors. Different letters indicated significant differences among treatments at $p < 0.05$.

deviated from the equilibrium, it suggested slower biological cycling and lower bioavailability of P (Bi et al., 2018; Tian et al., 2020). This study proved that incorporating green manure increased the conversion of chemical P fertilizer by microorganisms. In P-surplus soil, microorganisms quickly transformed P sources into H_2O-P_i and $NaHCO_3$-P_i pools under the Leg treatment and into $NaHCO_3$-P_i pools under the Non-Leg treatment (Figure 3A). In P-deficient soil, both Leg and Non-Leg treatments promoted the conversion of P sources into $NaHCO_3$-P_i and NaOH-P_i pools (Figure 3B). These findings provide direct validation of the pivotal role played by green manure in the mobilization of soil P through the activation of soil microorganisms. This activation is achieved by increasing the abundance of P-cycling bacteria and enhancing the activities of P-cycle enzymes (Piotrowska-Dlugosz and Wilczewski, 2020; Hallama et al., 2021). By promoting these microbial processes, green manure contributes to the efficient cycling and availability of phosphorus in the soil ecosystem.

In the present study, green manure residues addition had a positive effect on *pqqC*, *phoC*, *phoD* and *bpp* genes abundances and ACP and ALP activities, which was more prominent in P-deficient soil (Figures 4, 5). In response to a P_i-limited soil environment, microorganisms tend to release a higher quantity of phosphatases (Bergkemper et al., 2016). These enzymes are capable of effectively hydrolyzing ester-phosphate bonds in various phosphate-esters, thereby promoting the mineralization of soil insoluble P. This process enhances the overall effectiveness of P in the soil and

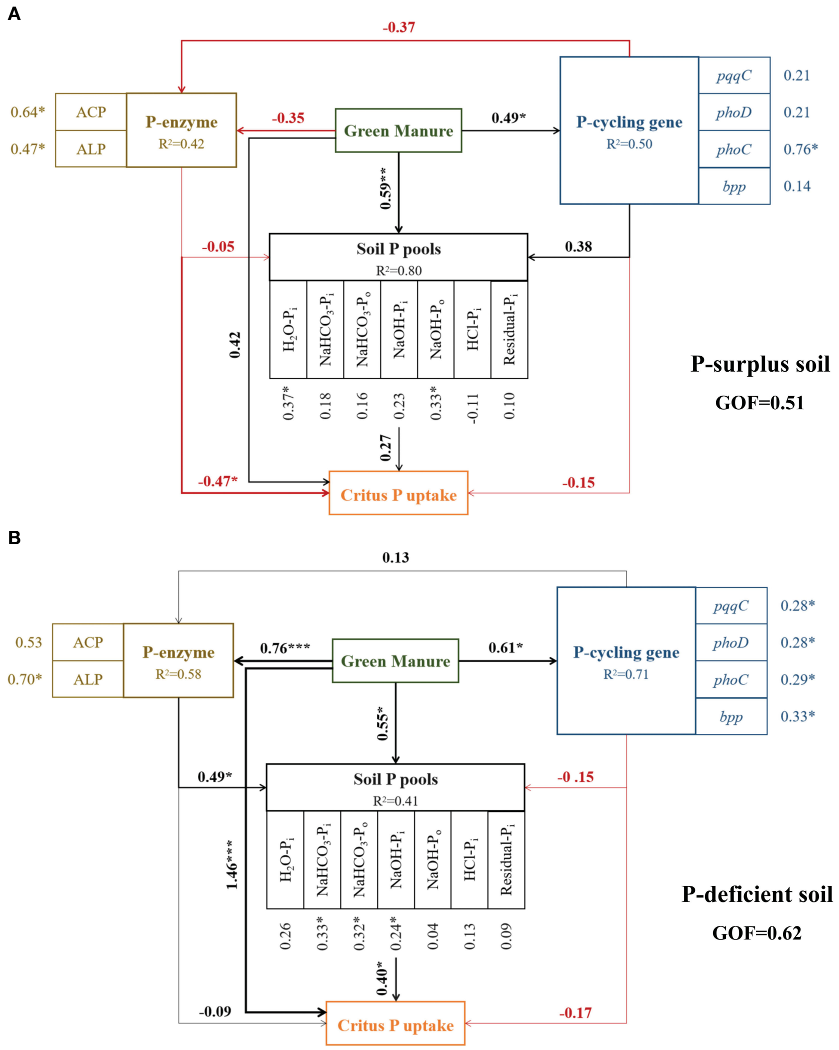

FIGURE 6
Evaluation of factors affecting soil P fractions and citrus P uptake under different treatments in P-surplus **(A)** and P-deficient **(B)** soils by partial least squares path modeling (PLS-PM). R^2 denotes the proportion of variance explained. The black and red lines indicate positive and negative relationships, respectively. The width of the arrows is proportional to the strength of the path coefficients. * indicated significant at $p < 0.05$, ** indicated significant at $p < 0.01$, *** indicated significant at $p < 0.001$.

facilitates its availability for biological uptake and cycling (Lu et al., 2022; Touhami et al., 2023).

Moro et al. (2021) also observed that in the soil with low P_i availability, microorganisms would be stimulated, especially bacteria, to synthesize large amounts of phosphatases, including acid phosphomonoesterase and phosphodiesterase, which promoted the hydrolysis of soil P_o, replenished soil active P pools and supplied plant growth. In contrast to the expected response, the incorporation of legume green manure in P-surplus soil, as observed in this study, resulted in a decrease in ACP and ALP activities (Figures 4A, C). Indeed, the study by Spohn and Kuzyakov (2013) indicated that in soils with ample P availability, soil microbial biomass P was primarily influenced by the release of plant-available P as a by-product of C mineralization. The incorporation of legume green manure in P-surplus conditions lead to an increased availability of plant-available P, reducing the need for microbial phosphatase activity (Table 4). This finding could potentially explain the observed decrease in phosphatase activities under the Leg treatments in P-surplus soil of present study (Figures 4A, C).

The results of this study, except for the *phoC* gene in P-surplus soil, showed no significant differences in the abundance of other P-cycling genes between the incorporation of legume and non-legume green manure treatments (Figure 5). This finding contradicts our understanding that organic matter with a low C/P ratio, typically found in legume green manure, has a greater capacity to stimulate P-microbial abundance compared to organic matter with a high C/P ratio, typically found in non-legume green manure (Benitez et al., 2016; Erinle and Marschner, 2020; Erinle et al., 2020). It is worth noting that the C/P ratio of the legume green manure and non-legume green manure used in this study was 73 and 141, respectively, both below 200 (Table 2). These ratios fall within the same range, which could explain the limited differences observed in P-cycling genes abundances. Alamgir et al. (2012) pointed out that crop residues with a C/P ratio above 200 tend to induce net P immobilization and depletion of P pools, while residues with a C/P ratio below 200 increase P availability and P pools. Additionally, Blanco-Canqui et al. (2015) suggested that the increase in effective phosphorus (P_o and P_i) pools through the regulation of microbial biomass and enzyme activities is primarily associated with an overall increase in soil organic matter. In other words, the exogenous inputs of C play a significant role in activating P-cycling microbial abundance. Since the amount of C inputs from different green manure sources in this study were consistent, the effects on functional gene abundances may tend to converge as well (Figure 5; Table 2).

4.3 The growth and P uptake of citrus plants

In P-deficient soil, after the incorporation of green manure residuals, the increase in soil active P pools, along with the enhanced abundance and activity of microorganisms, indirectly promoted the growth, development, and P nutrition status of citrus plants (Figures 1B, D, F, 2B, D, F, H, 6B; Table 4). However, the current research does not provide a consistent conclusion regarding the growth and P nutritional status of citrus plants when different green manure varieties are incorporated. Previous studies (Shackelford et al., 2019; Khan et al., 2022; Ullah et al., 2023) have demonstrated that non-legume green manure incorporation did not favor plant growth, while legume green manure incorporation did. The main reason for the inconsistency observed is that the chemical N supply in this study was sufficient, which may have hindered the beneficial N supply from legume green manure. Secondly, citrus plants are perennial crops and have a slower growth and development compared to annual grain crops. Their slower growth rate and development can limit the observable differences in response to different green manure treatments.

In the P-surplus soils of the present study, the addition of green manure residues did not show a significant improvement in the growth and P nutrition status of citrus plants, except for the P uptake by citrus roots (Figures 1A, C, E, 2A, C, E, G). This unexpected result can be attributed to the adequate availability of pristine soil nutrients and chemical fertilizers and the antagonistic effect of trace element uptake by citrus plants in P-surplus soils. Previous research has consistently demonstrated that high availability of P in the soil can hinder the uptake of essential trace elements such as zinc, copper, and iron by plants (Aboyeji et al., 2020; Singh et al., 2021; Stanton et al., 2022). This interference in trace element uptake can result in reduced yields and overall crop performance in various crops.

4.4 Limitations and implications

This study has provided a scientific foundation for quantifying the contributions of green manure to soil P pools and P nutrient of citrus plants, thereby enhancing production efficiency and fostering sustainable development in orchards. However, certain limitations were encountered during the experimental process. Firstly, it is essential to acknowledge that the annual citrus seedlings were employed in this experiment, which differs from the mature citrus trees typically found in actual orchard production. As a result, factors such as the kinetic uptake of soil P and the capacity to regulate the quantity, species, and activity of soil microorganisms vary between seedlings and mature trees. Consequently, the obtained results can only offer limited insights into seedling management within citrus orchards. Secondly, the environmental conditions of the pot experiment were deliberately standardized and controlled, unlike the intricate and dynamic situation present in actual orchard production. The use of potting apparatus may have restricted the growth of plants, particularly impeding root development. Research has demonstrated that P deficiencies induce changes in plant root architecture, leading to an increase in the root-to-shoot ratio, root hairs, root topsoil foraging, and root morphology (Zhang et al., 2013; Su et al., 2014; Iqbal et al., 2020). Hence, in future research endeavors, it is imperative to direct greater attention toward investigating the P uptake status of mature orchards under natural field conditions.

5 Conclusion

The present study investigated the influence of diverse green manure species on soil P dynamics and citrus growth across varying initial soil P conditions. The results demonstrated that the introduction of green manure residues positively impacted active P pools in the soil. This effect was attributed to heightened microbial and enzymatic activities involved in P cycling, leading to the conversion of both accumulated soil P and chemical fertilizer P into forms accessible to crops. Notably, legume green manures exhibited superior efficacy in regulating soil P pools, particularly in P-deficient soil. While the addition of green manure residues had limited effects on citrus growth and P uptake in P-surplus soil, its impact was significant in P-deficient soil. Interestingly, there were no substantial differences in the citrus growth and P uptake between legume and non-legume green manure addition treatments. These findings offer valuable insights for fruit farmers seeking to reduce P fertilizer inputs in orchard production, activate insoluble P in soil, mitigate soil P accumulation, and achieve ecological intensification. The study underscores the efficacy of incorporating green manure in orchards as a clean, efficient, and sustainable production model. Importantly, recommending the incorporation of legume green manure, especially in orchards with low P effectiveness, is emphasized. This recommendation aims to enhance P fertilizer utilization, facilitate the conversion of accumulated soil P into active P pools, and promote the overall growth of fruit trees.

Data availability statement

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

Author contributions

YY: Data curation, Formal Analysis, Investigation, Methodology, Software, Validation, Visualization, Writing –

original draft, Writing – review & editing. WZ: Data curation, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft. XC: Investigation, Writing – original draft. LC: Investigation, Writing – original draft. YL: Investigation, Writing – original draft. QX: Investigation, Writing – original draft. MW: Investigation, Writing – original draft. HY: Investigation, Writing – original draft. RH: Investigation, Writing – original draft. JZ: Investigation, Writing – original draft. YH: Investigation, Writing – original draft. QH: Investigation, Software, Writing – original draft. XS: Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. YZ: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Visualization, 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

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Benefits of *Canavalia ensiformis*, arbuscular mycorrhizal fungi, and mineral fertilizer management in tobacco production

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Tobacco (*Nicotiana tabacum* L.) has long been vital to Cuban agriculture, with its products renowned for their quality. Cuban tobacco is grown in soils with a long history of continuous farming using traditional fertilization methods characterized by recommended doses of mineral fertilizers. This study aims to improve the nutrition resource strategy in tobacco cultivation to ensure high yields of superior-grade tobacco leaves with adequate quality and increase fertilization efficiency. With this goal, a field experiment evaluated the traditional method of fallow with alternatives of nutrient supply systems for the production of black tobacco in Ultic Paleustalf soils. The experiment utilized *Canavalia ensiformis* (Can) treated with a mycorrhizal inoculum (AMF) based on the *Glomus cubense* strain (INCAM-4) as a preceding green manure, combined with successive mineral fertilizations for tobacco during four growing seasons in a randomized block design with factorial arrangement. Canavalia presented a positive response to mycorrhizal inoculation, significantly increasing dry biomass production (87.34%, 129.96%), mycorrhizal colonization (26.90%, 103.66%), and spore production (26.79%, 52.52%) for Can and Can+AMF treatments respectively. A biplot analysis established a strong relationship between the biomass and mycorrhizal performance of Canavalia and the growth, yield, and mycorrhizal colonization of tobacco. The results indicate that inoculated Canavalia enhances mycorrhizal performance in successional tobacco, with Can+AMF significantly increasing mycorrhization of tobacco roots by (110.06%). Moreover, the combination of Can inoculate with AMF and 75% of the recommended mineral fertilization dose consistently produced the highest tobacco yields (42.06%), growth, and mycorrhizal activity across the four years while maintaining satisfactory combustibility. In this nutrition supply system, variations of the recommended fertilizer dose significantly decreased the percentage of mycorrhizal colonization. After four growing seasons using Can + AMF and Canavalia without inoculations, soil organic matter, and availability of exchangeable calcium, magnesium, and pH increased slightly without decreasing available phosphorus and potassium contents. Consequently, we conclude that *Canavalia ensiformis*, with an inoculum based on the *Glomus cubense* strain and 75% of the recommended dose of mineral fertilizers, provides an enhanced nutrition alternative system for black tobacco production.

KEYWORDS

green manure, AMF inoculation, mineral fertilization, tobacco, nutrition system

1 Introduction

Tobacco is a highly sensitive crop to soils' nutrient deficiencies, and crop production experiences significant yield and quality fluctuations due to nutrient deficiencies or excesses (Tso, 1990; Lisuma et al., 2020). Mineral fertilizers are the most well-known and widely used method to provide crops with immediate availability and high nutrient concentrations. However, its cost is steadily increasing and is becoming unaffordable for small producers. Moreover, inadequate management of mineral fertilizers poses ecological risks, including nutrient imbalances, contamination of water sources, reduced biological activity in the soil, and excessive chemical residues in agricultural products (Kassam et al., 2013; Mishra and Arora, 2016; Hamel and Plenchette, 2017).

Nutrient supply systems are required to meet the nutritional requirements of crops as well as enhance biological mechanisms in the rhizosphere. The combination of mineral and organic fertilizers obtains favorable crop yields of superior quality, concurrently mitigating production expenses (Dai et al., 2021; Sifola et al., 2022). Simultaneously, the synergistic mechanisms maintain or improve the soil's physical, chemical, and biological characteristics (Rivera et al., 2007; Hamel and Plenchette, 2017). The use of leguminous plants contributes nitrogen to the system through biological nitrogen fixation (BNF) (Krishna et al., 2007; Sousa et al., 2016; Palmero et al., 2022; Barbieri et al., 2023). Green manures, crop rotation, and compost offer alternatives for soil management and crop nutrition (Mosquera et al., 2012; Jiang et al., 2022) (Jiang et al., 2022; Li et al., 2024). Their application in agricultural areas leads to improved soil fertility, higher organic matter content, the formation of stable aggregates, enhanced biological activity, and reduction of soil erosion reduced (Cherr et al., 2006; García Rubido et al., 2015) (Asghar and Kataoka, 2021; Voltr et al., 2021). Additionally, Recent studies indicate other beneficial results from a green manure species *Canavalia ensiformis* (Araujo et al., 2024).

Mycorrhizae provide benefits for crop growth by increasing water and nutrient absorption capacity, enhancing resilience to adverse soil conditions, and forming stable aggregates in the soil, which improve tolerance to climatic conditions and pathogens (van der Heijden et al., 2015; Kashyap et al., 2018; Li et al., 2022). Arbuscular mycorrhizal fungi (AMF) establish a symbiosis with the most economically important plant species (Willis et al., 2013), including tobacco (Subhashini, 2013) and leguminous green manures as Canavalia (Rivera et al., 2010). In this context, the use of AMF has been shown to offer several benefits for tobacco, such as inducing tolerance to Tobacco Black Shank (caused by *Phytophthora nicotianae*) (Li et al., 2023), enhancing resistance to water stress (Begum et al., 2022), and improving essential oil production, metabolism, growth and yield (Begum et al., 2021), among others.

Recent fertilization techniques involve the simultaneous use of mycorrhizal inoculants and green manure species like *C. ensiformis*, have demonstrated notable improvements in crop production and soil fertility benefits (Rivera et al., 2020). These methods, particularly when integrated into crop rotation and intercropping systems, have consistently resulted in elevated yields and satisfactory nutritional content across different crops while substantially reducing reliance on mineral fertilizer (Ben-Laouane et al., 2021; Javanmard et al., 2022). Additionally, AMF-inoculated green manure plants have proven effective in stimulating the growth of mycorrhizae associated with economically significant crops, thereby enhancing soil coverage and health (Rayne and Aula, 2020; Dong et al., 2021; Gujre et al., 2021). To the present date, there is a lack of published research regarding these co-management strategies in tobacco cultivation

Considering the low organic matter content and sandy texture of the soils dedicated to tobacco production in San Juan Martinez, Cuba, and the previously mentioned benefits from AMF, green manure, and mineral fertilization combination. This study assesses the potential benefits and feasibility of combining *C. ensiformis* and mycorrhizal inoculants with appropriate mineral fertilization doses for tobacco production. This study aims to establish a more cost-effective and sustainable integrated nutrient supply for tobacco cultivation.

2 Materials and methods

2.1 Site description

The study was conducted from 2018 to 2022 at the Tobacco Experimental Station in Cuba, 22°16'55.2"N and 83°49'19.44"W, 31 meters above sea level. This station is located within the tobacco-rich region of Vuelta Abajo, in San Juan y Martínez, Pinar del Río province. The soil in this area was classified as Ultic Paleustalfs, characterized by a slightly acidic pH and typical exchangeable Ca and Mg levels. Notably, the soil type has low organic matter content and a resident mycorrhizal population, possibly due to continuous cultivation with high doses of mineral fertilizers. Excessive fertilization also accounts for the high levels of available phosphorus and potassium in the soil (Table 1).

The average annual rainfall and monthly temperature during the experiment were typical of the climate in western Cuba, with an annual average rainfall of 1581 mm and an average monthly temperature of 25.1°C. The rainy season typically extends from May to October, accounting for approximately 81% of the annual rainfall, with an average temperature of 26.8°C.

2.2 Experimental design and tobacco management

Field experiments were conducted under conditions that resemble tobacco production environments. The fertilization treatments consisted of two factors. The first factor was the previous crop, where Canavalia with Arbuscular mycorrhizal fungi (AMF), Canavalia, and fallow plots preceded tobacco plantation The first factor was preceding crop, which included Canavalia inoculated with AMF, non-inoculated Canavalia, and fallow plots, which preceded the tobacco planting. The second factor was the macro-nutrient mineral fertilization doses, resulting in nine treatment combinations (Table 2). The experimental design followed a randomized block arrangement with a factorial A×B design, incorporating the years as the third factor (C). Each treatment had four replicates, with the same plot utilized for succession in different study years. Data from the first season were excluded to standardize the effects of the treatments on both Canavalia and tobacco, and only results from the last three seasons were presented.

Each plot had an area of 22.8 square meters (6 × 3.8 meters). Canavalia was planted in the second half of September with a spacing of 30 cm between plants, 50 cm between rows, and an approximate seeding rate of 70 kg per hectare. Fallow plots were designated as a commercial control, as tobacco growers typically allow fallow vegetation, primarily composed of *Cyperus rotundus* and *Eleusine indica*, with smaller amounts of *Amaranthus* spp., to establish before planting tobacco.

Certified *Canavalia ensiformis* seeds, as per the NRAG 193 (2011) standard, were used in this study. The methodology closely aligns with production conditions and has been tested across a wide range of experiments (Rivera et al., 2023). The certification process considers genetic and mechanical purity, germination capacity, moisture content, and phytosanitary control.

TABLE 2 Treatments studied during the experimental period.

Treatments	Previous crop	Fertilizer Percentage[1] (% RD)
Can+AMF+50% RD	*C. ensiformis* + AMF	50
Can+AMF+75% RD	*C. ensiformis* + AMF	75
Can+AMF+100% RD	*C. ensiformis* + AMF	100
Can+50% RD	*C. ensiformis*	50
Can+75% RD	*C. ensiformis*	75
Can+100% RD	*C. ensiformis*	100
Fallow+50% RD	Fallow	50
Fallow+75% RD	Fallow	75
Fallow+100% RD	Fallow	100

[1] 100% RD (Recommended Dose): 120N – 60P_2O_5 – 160K_2O – 30MgO, according to the Technical Instructions of the crop (Espino et al., 2012).

A solid arbuscular mycorrhiza inoculant based on *Glomus cubense* (INCAM-4, DAOM 241198) (Rodríguez et al., 2012), certified by the Mycorrhiza Laboratory of the National Institute of Agricultural Sciences of Cuba. The AMF had a concentration of 25 to 30 spores g^{-1} extracted by the wet sieving and decanting method (Gerdemann and Nicolson, 1963) from 50 g of inoculum.

Mass production of micorrizhal inoculum was prepared by applying *G. cubense*/INCAM-4 with a high degree of purity to seeds of *Urochloa decumbens*, which were subsequently grown in a sterile substrate following the method described by (Fernández et al., 2000). After four months, the substrate enriched with mycorrhizal propagules was extracted. This substrate mixture with mycorrhizal propagules, including mycorrhizal roots with a colonization frequency between 61% and 68%, dried at room temperature in the shade. The spore content was homogenized and adjusted to the commercial concentration of 30 spores g^{-1} (Fernández et al., 2000).

The mycorrhizal seed inoculation used the coating method with 10% of the seed weight (Fernández et al., 2000), equivalent to 10 kg ha^{-1}, preparing a homogeneous mixture of 1 kg of inoculum for every 10 kg of seeds previously moistened with 300 ml of water. Following the coating process, the seeds undergo a one-hour drying period in shaded conditions before sowing. Canavalia sowing and cultivation

TABLE 1 Some initial characteristics of the Ultic Paleustalf soil at 0–20 cm depth (average values and confidence intervals).

Item	pH	P	OM	Ca^{+2}	Mg^{+2}	Na^{+1}	K^{+1}	S	AMF spores in 50g soil
		mg kg^{-1}	g kg^{-1}	cmol kg^{-1}					
CI	5.39 ± 0.16	16.99 ± 0.85	13.6 ± 0.8	3.39 ± 0.45	1.78 ± 0.43	0.10 ± 0.01	0.46 ± 0.05	5.73 ± 0.61	55 ± 5

Chemical determinations (19): pH-KCl potentiometer: soil/solution ratio of 1:2.5; MO (organic matter) Walkey and Black method; P_2O_5 and K_2O extraction with H_2SO_4 0.05 M acid, Exchangeable cations: extraction with NH_4Ac 1 M; S: Base exchange capacity; No. Resident AMF spores (Gerdemann and Nicolson, 1963). CI confidence interval at p<0.05.

were carried out manually. The Canavalia plants were mechanically cut and incorporated into the soil at a depth of 15–20 cm at the onset of flowering, approximately 60–70 days after sowing.

The tobacco variety ‘Criollo 98’ was transplanted in the third week of December using seedlings from seedbeds. The transplanting was performed manually, with a planting spacing of 30 cm between plants and 76 cm between rows, resulting in a planting density of 38,000 plants per hectare. Each plot consisted of five rows of tobacco. Cultivation and fertilization followed the Technical Instructions for Tobacco Cultivation guidelines in Cuba, as provided by the Tobacco Research Institute (Espino et al., 2012). The recommended dose (RD) of 100% NPKMg fertilizer corresponded to 120 kg per hectare of nitrogen (N), 60 kg per hectare of phosphorus pentoxide (P_2O_5), 160 kg per hectare of potassium oxide (K_2O), and 30 kg per hectare of magnesium oxide (MgO). This fertilizer was applied in two fractions, with 40% 8–10 days after transplanting and the remaining 60% between days 18–20.

Tobacco harvesting started 55 days after transplanting and continued up to 80 days after transplanting. The harvested leaves were dried in a traditional curing house. The residual plant material from the harvest was incorporated into the soil within each plot, and the plot was left fallow until the onset of the rainy season. Then, soil preparation for the subsequent season was initiated. These practices were consistent across all treatments (Table 2).

2.3 Determinations performed on *Canavalia ensiformis*

The sampling procedure for the determinations involving *Canavalia ensiformis*used three consecutive plants selected within each plot approximately 60 days after germination from the central furrow. The sampling included plant roots and rhizospheric soil within the upper 20 cm depth in a sample composed of five sub-samples of 100 g of soil each.

The aboveground biomass (in Mg ha^{-1}) was assessed by separating the leaves and stems from the three selected plants. The fresh weight of each plant organ was measured using a precision balance (0.01 g). Subsequently, a 100 g sample from each organ was taken and dried in an oven at 70°C until a constant weight was achieved. This allowed us to determine each organ’s fresh and dry mass and the total dry mass.

Extraction of N, P_2O_5, and K_2O measured the concentration of N, P, and K in the leaves and stems of Canavalia. Nutrients (g kg^{-1}) were determined using samples collected from each plot. Subsequently, the extraction was calculated based on the data for the dry mass of each organ (in Mg ha^{-1}) and the concentration of each element (in g kg^{-1} of N, P, and K), using the following formula:

$$Extraction\ (kg\ ha^{-1}) = \sum [OB_i \cdot MC_i] \cdot fg$$

In this formula: OB represents Organ Biomass; MC represents macronutrient content in each organ; the subscript ‘i’ corresponds to leaves and stems, respectively; the factor ‘fg’ was set at 1 for N, 2.29 for P_2O_5, and 1.2 for K_2O.

The assessment of spore counts and the determination of the percentage of mycorrhizal colonization were conducted per plot using rhizospheric soil samples. Spores were quantified using the wet sieving and decanting method (Gerdemann and Nicolson, 1963) in 50 g of soil, while mycorrhizal colonization was assessed through the intercept method and staining with ‘blue ink’ (Rodríguez Yon et al., 2015) using 200 mg of rootlets extracted from each sample. The quantities of mycelia and infective roots were not determined.

2.4 Tobacco yield and characteristics

Tobacco measurements and observations were conducted on tobacco plants randomly selected from the central furrows of each plot. These assessments were carried out during the harvest of the central leaves, which occurred 70–75 days after transplanting, following the method described by Torrecilla et al. (2012). The measurements included recording the length and width of the central leaf, determining the fresh and dry mass of the central leaf using a gravimetric method with a precision analytical balance (± 0.1 mg), and estimating chlorophyll content using the SPAD-502 instrument (MINOLTA, Spectrum Technologies Inc.) (Espino et al., 2012).

The yield assessment included all the plants within the plot. It was determined as the total yield (kg ha^{-1}), comprising both superior and inferior tobacco leaves grades, following the guidelines outlined in the Technical Instructions for the Collection and Processing of Sun-Cultivated Tobacco (Fernández et al., 2004). Combustion rate determinations followed the procedures and evaluation criteria specified in the document ‘Instructions for the Assessment of Combustion Rate in Cuban Tobacco’ by Guardiola et al. (2004).

Following the harvest, soil samples were collected from the tobacco plants in each plot. These samples were evaluated for the percentage of mycorrhizal colonization in the roots (Rodríguez Yon et al., 2015), the presence of mycorrhizal spores (Gerdemann and Nicolson, 1963), and the same soil nutrient analyses that were initially conducted (Table 1).

2.5 Statistical procedure

Univariate analysis (ANOVA) with a trifactorial arrangement was performed for the variables evaluated in Canavalia and tobacco. In cases where the highest-order interaction (A × B × C) was significant, further analysis broke down the interaction by assessing the effects of different Canavalia × fertilization combinations for each year. Mean values were calculated using Duncan’s test at a $p< 0.05$ significance level. Furthermore, a multivariate Biplot analysis was conducted, integrating the variables from both tobacco and Canavalia. Regression analyses examined the relationship between yearly mycorrhizal colonization percentages and tobacco yield. Additionally, confidence intervals at $p< 0.05$ were calculated for each variable based on the initial and final soil analysis results.

3 Results

The dry biomass of Canavalia (Can) exhibited a positive response ($p < 0.05$) to the inoculation with arbuscular mycorrhizal fungi (AMF) and tobacco mineral fertilization (NPKMg) (Table 3), with an increase of 87.34% for Can and 129.96% for Can+AMF compared to Fallow. The highest dry biomass and macronutrient content ($p < 0.05$) were observed in the Canavalia treatments receiving mycorrhizal inoculations, particularly those treated with 75% and 100% of the recommended dose (RD) compared to the natural vegetation associated with fallow plots.

As expected, Canavalia inoculated with AMF treatments exhibited superior mycorrhizal functioning compared to Canavalia non-inoculated treatments, and all Canavalia treatments were notably higher than those observed in fallow natural. Similarly to the dry biomass and NPK absorption, indicators of mycorrhizal activity compared to fallow vegetation treatments increased in the number of spores by 26.79% for Can and 52.52% for Can+AMF, while for colonization, the increase was 26.90% for Can and 103.66% for Can+AMF.

Canavalia + AMF treatments presented a corresponding increase in spore numbers to AMF inoculation, but differences were not significant ($p < 0.05$) with Can+75% RD. Notably, the highest AMF colonization and number of spores ($p < 0.05$) were associated with the Can+AMF+75% RD treatment, while significant decreases were observed for lower and higher fertilizer doses (Figure 1).

The Biplot analysis indicated that the first two components accounted for a significant percentage of the experimental variance (Figure 2). Specifically, the first component explained 76% of the variance and displayed strong correlation coefficients with all Canavalia and tobacco variables, except for the yield of tobacco leaves of inferior grade, which exhibited high correlation coefficients with the second component.

TABLE 3 Aboveground dry biomass production and nutrient (NPK) uptake by Canavalia and fallow vegetation in the different treatments at the time of Canavalia cutting and fallow.

Treatments	Dry mass (Mg ha^{-1})	Total nutrient absorption (kg ha^{-1})		
	Total	N	P_2O_5	K_2O
Can+AMF +50% RD	4.25 c	131.6 c	18.76 b	98.42 c
Can+AMF +75% RD	6.09 a	209.93 a	27.02 a	140.56 a
Can+AMF +100% RD	6.00 a	194.95 a	25.55 a	132.58 a
Can +50% RD	4.08 c	126.98 c	13.93 c	90.16 c
Can +75% RD	4.06 c	116.83 c	12.88 c	89.53 c
Can +100% RD	5.18 b	164.92 b	17.29 bc	116.76 b
Fallow +50% RD	2.21 d	36.56 d	4.05 d	34.17 d
Fallow +75% RD	2.45 d	38.54 d	4.42 d	40.37 d
Fallow +100% RD	2.44 d	37.91 d	5.42 d	42.93 d
SE X	0.11	7.03	1.05	4.10

Significant interaction Canavalia × fertilization. Means with different letters in the same column differ from each other, according to Duncan's test ($p<0.05$).
AMF: *G. cubense*-INCAM-4.

Furthermore, the Can+AMF+75% RD treatment exhibited the highest values for component 1, followed by the Can+AMF+100% RD treatment, which were considerably distant from the other treatments. Following closely were the Can+AMF+50% RD

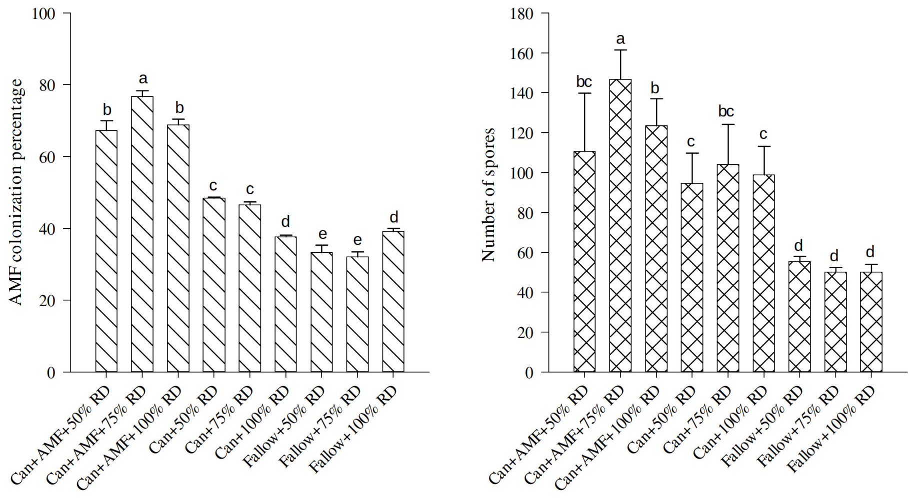

FIGURE 1
Effect of Canavalia-fertilization combinations on indicators of mycorrhizal performance in *C. ensiformis*. Significant Canavalia × fertilization interaction. SE x colonization = 0.69***, and ES x spores = 5.7*. Means with different letters within the same data series differ significantly from each other, as determined by Duncan's test ($p < 0.05$).

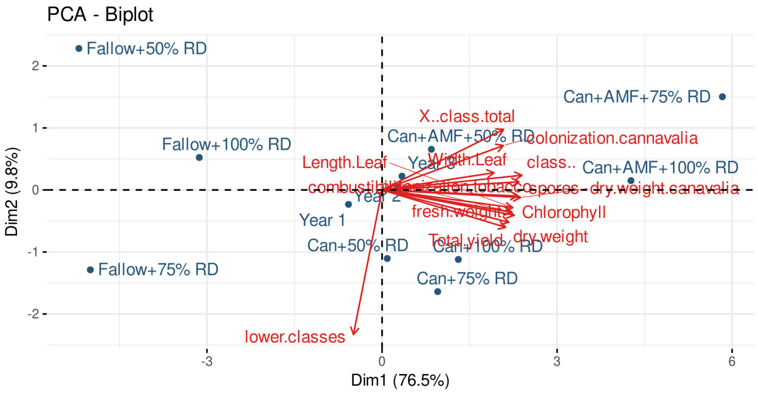

FIGURE 2
Biplot diagram integrating the evaluated variables in tobacco and canavalia.

treatment and the treatments involving Canavalia without inoculation. Among these the latter, the treatment that received 100% RD displayed the most favorable results, indicating a notable response to fertilization.

Canavalia treatments without inoculation were positioned in the first quadrant, which suggests the significant influence of component 2 on them. This association was related to higher values of leaves in inferior grades.

Lastly, treatments that exclusively received mineral fertilization exhibited the lowest values for component 1, with the treatment receiving 100% fertilization achieving the most favorable outcome. The different years showed proximity to each other, indicating minimal variations among them.

The univariate analyses indicated a significant maximum-order interaction (Canavalia × fertilization × years) for dry biomass, yield of superior leaves grade, and mycorrhizal colonization percentage. In the case of fresh biomass, chlorophyll content, total yield, yield in inferior leaves grade, and percentages in superior leaves grade, only the Canavalia × fertilization interaction factors were significant ($p< 0.05$). The leaf length responded solely to the Canavalia and fertilization factors, while the leaf width did not exhibit a response to any of the factors. Nonetheless, the highest values were consistently associated with the Canavalia plus AMF treatments.

In the case of dry biomass, yield of superior leaves grade (Table 4), and fresh total yield (Table 5), the Can+AMF+75% RD treatment presented higher means. Notably, Can+AMF+75% RD yields were higher than the values associated with the Can+AMF +50% RD treatment, and they did not exhibit significant differences when compared to the Can+AMF+100% RD treatment. The superior grade yield increased 42,6% compared to fallow 100% RD from the three seasons. In general, neither Can nor AMF inoculation significantly affected the total yield of tobacco leaves (superior grade + inferior grade). However, the specific yield of superior leaf grade was significantly higher, with an increase of

TABLE 4 Effect of Canavalia and fertilization combinations in each year on dry biomass and yield of Superior tobacco grade (g/plant).

Treatments	Dry Biomass (g ha^{-1})			Yield superior grade (kg ha^{-1})		
	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3
Can+AMF+50% RD	35.5 ab	33,0 b	32,9 c	1244.1 bc	1260.5 bc	1213.8 bcd
Can+AMF+75% RD	36,5 a	36,5 a	37,5 a	1507,5 a	1452,5 a	1493.8 a
Can+AMF+100% RD	36.3 ab	35,5 a	36.4 ab	1393 ab	1374.6 abc	1317.8 ab
Can+50% RD	35.6 ab	34.3 ab	34.3 bc	1155,8 c	1214,78 c	1125.4 cd
Can+75% RD	36,7 a	34.9 ab	35.7 ab	1218.7 bc	1259.7 bc	1308.6abc
Can+100% RD	34,1 b	35,6 a	36.6 ab	1191.6 bc	1413.9 ab	1351.9 ab
Fallow+50% RD	23,5 d	26,3 c	24,7 d	1087,8 c	943,0 d	1098.7 d
Fallow+75% RD	23,8 d	26,5 c	24,4 d	838,6d	1068,5 d	1055.4 d
Fallow+100% RD	32,0 c	32,9 b	32,8 b	1058,0 c	966.7d	1110.6 d
SE (A×B×C)		0.605*			49.59***	

Interaction Canavalia × fertilization × years significant. * significance of $p< 0.05$; *** significance at $p< 0.001$. Means with different letters in the same column differ from each other, according to Duncan's test ($p<0.05$).
Partition of Canavalia × fertilization × years (A×B×C) interaction.

TABLE 5 Effect of combinations of Canavalia and fertilization factors on fresh biomass, total and inferior grade leaves yield of dark tobacco.

Treatments	Total yield (kg ha^{-1})	Percentage of superior grade leaves	Inferior grade leaves (kg ha^{-1})	Combustibility (Seconds)
Can+AMF+50% RD	1996,8 b	62,2 bc	757,3 bc	28,50
Can+AMF+75% RD	2173,4 a	68,4 a	688,8 cd	29,67
Can+AMF+100% RD	2128,9 a	64,0 b	766,8 bc	30,00
Can+50% RD	1999,4 b	58,4 c	834,2 ab	28,75
Can+75% RD	2096,6 ab	60,4 bc	901,0 a	27,13
Can+100% RD	2098,6 ab	60,9 bc	820,6 b	29,25
Fallow+50% RD	1821,9 c	57,8 c	673,4 d	25,17
Fallow+75% RD	1893,5 c	52,5 d	906,1 a	26,50
Fallow+100% RD	1716,5 c	61,1 bc	777,0 b	26,67
SE (A×B)	31.62**	1.12**	22.24***	

Significant Canavalia × fertilization interaction** significance at $p < 0.01$; ***significance at $p < 0.001$. Unequal letters in each column lead to significant differences between treatments by Duncan's test ($p<0.05$).

20.49% for Can and 32.82% for Can+AMF compared to fallow treatments (Table 5).

From 2018 to 2022, Tobacco yields from all treatments with Canavalia demonstrated significantly higher values than treatments without Canavalia. Intermediate fertilization doses produced the highest yield values in pre-treated plots with Canavalia. Tobacco yields from Non-inoculated Canavalia treatments presented significant differences ($p < 0.05$) in contrasting fertilization rates.

On the contrary, yields of the inferior grade leaves from treatments with non-inoculated Canavalia showed the highest values, while the Fallow+50% RD treatment had the lowest values. All treatments exceeded 20 seconds regarding tobacco leaf combustibility, which meets the quality standard (Torrecilla et al., 2012) and is considered excellent.

Regarding leaf length, the highest yield values were observed when Can+AMF+75% RD fertilization (data not shown).

Furthermore, the highest percentages ($p < 0.05$) of mycorrhizal colonization in tobacco were consistently recorded in the Can+AMF+75% RD treatment across all years (Table 6). Moreover, the mycorrhizal colonization average increased by 110.06% compared to Fallow+100% RD. Treatments with Can+AMF as a precedent presented lower values, although mycorrhizal colonization was significantly higher than those treatments using non-inoculated Canavalia as a precedent, which were higher than fallow preceded tobacco cultivation. Additionally, there was a direct and linear relationship between the percentage of mycorrhizal colonization in tobacco and yield ($R^2 = 64\%$).

At the fourth season, soil analyses (Table 7) indicated that using Canavalia in preparation for tobacco crops, whether inoculated or not with AMF, led to significant increases in the organic matter content, calcium (Ca), magnesium (Mg), and exchangeable potassium (K). Moreover, Canavalia-treated plots exceeded the initial soil conditions (Table 1). There were no significant differences in pH levels, and the available phosphorus (P) contents did not exhibit significant variations relative to the initially high levels.

4 Discussion

This study indicated that Canavalia and tobacco associated with mycorrhizae positively respond to the inoculation of efficient AMF (arbuscular mycorrhizal fungi) strains. Several authors have reported positive effects in both crops, resulting in satisfactory mycorrhizal activity and enhanced potential of Canavalia as a green manure (Martín Alonso et al., 2012; Simó González et al., 2016). Additionally, AMF inoculations had enhanced tobacco yield (Subhashini, 2013).

The results highlight the positive impact of using *Canavalia ensiformis* as a precursor for tobacco cropping (Guerra et al., 2006; Espino et al., 2012), with these benefits further enhanced by inoculating

TABLE 6 Effect of combinations of Canavalia and fertilization factors in each year on mycorrhizal colonization (%).

Treatments	Year 1	Year 2	Year 3
Can+AMF +50% RD	49,65 b	52,22 b	48.54 bc
Can+AMF +75% RD	53,25 a	58,12 a	55,8 a
Can+AMF +100% RD	50.64 ab	51, 25 b	50,31 b
Can+50% RD	40,33 c	41,76 d	44.08 ef
Can+75% RD	49,42 b	48,56 c	41,57 e
Can+100% RD	42,4 c	39,11 d	46.06 cd
Fallow+50% RD	24,61 e	26,44 e	23,57 f
Fallow+75% RD	25,81 e	22,34 f	23,56 f
Fallow+100% RD	29,2 d	28,51 e	19,58 g
SE (A×B×C)		1.17***	

Significant interaction of Canavalia, Fertilization, and Years (*** indicates significance at $p < 0.001$). Unequal letters in each column indicate significant differences between treatments, as determined by Duncan's test ($p<0.05$).
Partition of Canavalia × fertilization × years (A×B×C) interaction.

TABLE 7 Effect of Canavalia applications on some soil chemical properties (0–20 cm depth) at the end of the four campaigns.

Treatments	KCl	P_2O_5	OM	Ca^{+2}	Mg^{+2}	K^{+1}
	mg $100g^{-1}$		g kg^{-1}		cmol kg^{-1}	
Canavalia+AMF	5.52 ± 0.23	40.0 ± 1.5	18.7 ± 0.5	4.22 ± 0.37	1.93 ± 0.32	0.49 ± 0.01
Canavalia	5.63 ± 0.25	40.0 ± 1.65	18.2 ± 0.3	4.17 ± 0.27	1.98 ± 0.24	0.49 ± 0.01
Fallow	5.38 ± 0.18	38.0 ± 1.05	14.5 ± 0.3	3.47 ± 0.04	1.54 ± 0.03	0.45 ± 0.01

CI confidence interval at $p<0.05$.

Canavalia. This study recorded that AMF inoculations resulted in higher biomass production, increased nutrient content, and enhanced yields of superior-grade leaves, even with lower fertilizer doses. Furthermore, the Canavalia treatments showed enhanced mycorrhizal activity, reaching colonization values approaching 60%, demonstrating strong mycorrhizal performance, as commonly observed in most crops (Rivera et al., 2023). However, additional benefits associated with mycorrhizal inoculation of Canavalia require further research on soil properties (Rivera et al., 2007).

It's important to note in this study that although non-inoculated Canavalia increased the 'resident' mycorrhiza and thus the mycorrhization of subsequent tobacco crops, it was not completely effective and showed lower performance compared to when Canavalia was inoculated. These results align with the findings of other authors (Sánchez et al., 2009; Rivera et al., 2010), who evaluated several species of non-inoculated green manures in different soil conditions and reported that although mycorrhization of the successional crop increased, it was not fully effective in enhancing crop yield.

Consistent with our findings, several publications have reported the positive impact of *C. ensiformis*, when inoculated with efficient AMF strains. Enhanced yield, nutritional status, mycorrhizal functioning, and fertilizers uptake efficiency for different succeeding crops had been reported (Martín Alonso et al., 2012; Simó González et al., 2016; João et al., 2017). This is in agreement with the effectiveness of *C. ensiformis* as a precursor for the successful mycorrhization of subsequent crops. More previous studies have indicated a lasting effect of the applied inoculant on the first crop of the succession, with intervals of no more than 30 days between the harvest of the inoculated crop and the planting of the succeeding crop (Martín Alonso et al., 2012; Simó González et al., 2016; Espinosa et al., 2018), a similar condition in our study.

While the use of *C. ensiformis* as a precursor offers multiple benefits to the agrosystem, including soil cover and increased organic matter content, soil aggregation, and biological activity (Cherr et al., 2006; Guerra et al., 2006). Canavalia's contribution to the growth and yield of tobacco has been associated with Canavalia's role in providing nitrogen (N_2) and recycling nutrients present in its biomass (Ambrosano et al., 2013; Viola et al., 2013). Green manure nutrients undergo a gradual mineralization process, and in conjunction with mineral fertilization applied to the crop, they provide the tobacco's nutrient requirements. This is especially critical in sandy loam soils, which are prone to nutrient leaching (García Rubido et al., 2015).

The benefits associated with Canavalia are further enhanced with the inoculation of efficient AMF strains. AMF increases the advantages linked to Canavalia growth and nutrient recycling and promotes more efficient biological nitrogen fixation (Larimer et al., 2014; Bulgarelli et al., 2017). Most importantly, AMF inoculations lead to effective mycorrhization of tobacco.

The higher yields with lower fertilizer doses can be attributed to the positive interaction between mycorrhizae and plant nutrition in the presence of organic residues. This interaction has been reported to increase the mineralization of residues by the microbiota in the mycorrhizosphere (Hodge and Storer, 2015; Bukovská et al., 2018). While some authors emphasized the enhancements in nitrogen absorption, once Canavalia biomass decomposes, its various nutrients become available for the crop root system. This is further facilitated by more efficient and rapid nutrient utilization from inoculated Canavalia. More, enhanced mycorrhizae activity, as reported in this study, has also been related to a greater length and number of mycorrhizal hyphae (Hodge and Fitter, 2010; Thirkell et al., 2016; Bukovská et al., 2018), and an increased quantity of mycorrhizal roots (Cheng et al., 2016).

Higher mycorrhizal activity typically results in a reduction of fertilizers. However, when high doses of fertilizers are applied, mycorrhizae growth may decline or be inhibited, and low fertilizer doses may not lead to optimal mycorrhizal functioning (Rivera et al., 2007, Rivera et al, 2020). Specifically, the utilization of the Can+AMF +75% RD treatment yielded the highest mycorrhizal colonization percentages and demonstrated the best overall performance in all variables, except for the yield of inferior-grade leaves (Bilalis et al., 2015). Lower and higher fertilizer doses in the presence of Canavalia inoculated with AMF led to a decrease in mycorrhizal activity, indicating that this dose was optimal for mycorrhizal development and the associated benefits. Higher percentages of superior-grade leaf yields were achieved with this treatment, suggesting improved nutrition and greater physiological efficiency in tobacco plants.

The fallow plot results elucidate the impact of tobacco fertilization on Canavalia's growth and development while endorsing this experimental approach's effectiveness in establishing the treatments' medium-term effects. The increases in soil organic matter and exchangeable cations, along with the sustained availability of phosphorus (P) and potassium (K), are additional benefits derived from this soil management strategy (Chu et al., 2020; Etesami et al., 2021; Shi et al., 2021). These benefits undoubtedly contribute to soil fertility improvement, reassuring that the proposed reduction in fertilizers will not lead to medium-term disadvantages, thereby enhancing agrosystem sustainability.

The high yields achieved, surpassing previous reports for the same tobacco varieties (Espino et al, 2012), in the presence of moderate fertilizer doses, promote the economic and environmental viability of employing Canavalia inoculated with efficient AMF strains as a precursor for tobacco. Consequently, it should be considered an integral component of crop production technology.

5 Conclusions

The use of *C. ensiformis* treated with an inoculum based on an efficient AMF strain (*Glomus cubense*; INCAM-4) as a precursor for tobacco cultivation not only leads to increased biomass production and nutrient availability in Canavalia but also provides an effective means of mycorrhization for the subsequent economic crop. The mycorrhizal activity in tobacco roots, coupled with the enhanced nutrient recycling from the inoculated Canavalia, resulted in greater fertilization efficiency and higher yields of superior grade and total tobacco leaves. In this case, only 75% of the recommended mineral fertilizer dose is required. This is in contrast to the fallow and mineral fertilization employed or when non-inoculated *C. ensiformis* is used. After four years of evaluations, soil properties showed improvements in soil organic matter and exchangeable cations while maintaining adequate available phosphorus levels, demonstrating that the 25% reduction in fertilization does not negatively impact soil fertility. Therefore, incorporating this nutrition strategy into tobacco crop production technology is highly recommended, and further studies should confirm the effects on different edaphoclimatic conditions.

Data availability statement

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

Author contributions

DPdL: Formal analysis, Visualization, Writing – review & editing, Conceptualization, Methodology, Writing – original draft. MGR: Conceptualization, Visualization, Writing – review & editing, Funding acquisition, Investigation, Resources, Writing – original draft. RR: Conceptualization, Funding acquisition, Investigation, Methodology, Resources, Writing – original draft. DM-C: Visualization, Writing – review & editing. YG: Visualization, Writing – review & editing, Data curation, Formal analysis.

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Conflict of interest

Publisher's note

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Improving dual cover crop mixtures to increase shoot biomass production and weed suppression potential

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Introduction: Cover crop services depend on biomass production and species composition of the cover crop stand. In this study, we investigated the interactions in shoot biomass of dual cover crop mixtures and their competitiveness to suppress weeds before winter under different growing conditions.

Methods: A field experiment was performed on sandy loam soil in Triesdorf, Germany, for two consecutive years. The cover crop species white mustard (*Sinapis alba* L.), oil radish (*Raphanus sativus* var. *oleiformis* L.), phacelia (*Phacelia tanacetifolia* J.), Egyptian clover (*Trifolium alexandrinum* L.), common vetch (*Vicia sativa* L.), field pea (*Pisum sativum* L.), and field bean (*Vicia faba* L.) were grown in pure stands or dual mixtures. Cover crops were sown in August, and shoot biomass was harvested at the end of the vegetation period. Cover crop shoot biomass was dried, and the share of each species and the sown test weed (*Brassica napus* L.) were determined.

Results: The share of shoot biomass of a cover crop species in dual-species mixtures was closely related to its shoot biomass in its pure stand. In both years, mustard and phacelia showed similar interactions in shoot biomass production when growing with a second species. Regarding competitiveness against weeds, cruciferous species outperformed other cover crop species and could suppress weeds even if they were mixed with a less competitive partner. Weed suppression of mixtures with phacelia depended on the second component in the mixture and was more effective in a dry season. Legume species, especially Egyptian clover and common vetch could not suppress weeds in a pure stand.

Discussion: Our results show that species with high weed suppression potential as single stands retain this ability in dual cover crop mixtures, regardless of the suppression potential of the second species that completes the mixture.

KEYWORDS

interspecific interaction, integrated weed management, cruciferous species, legumes, phacelia, sustainable crop production, binary mixtures, catch crops

1 Introduction

Increasing food demand, environmental constraints, and societal expectations necessitate sustainable cropping systems. One solution to these needs is to integrate cover crops into crop rotations, established mainly in periods when no main crop is growing (e.g., summer to winter). They are cultivated primarily for their soil and environmental benefits rather than for income generation. Nevertheless, cover crop adoption in Europe has steadily increased due to agricultural policies (Kathage et al., 2022; Klages et al., 2022; Peltonen-Sainio et al., 2023). However, as of 2023, the Common Agricultural Policy (CAP) reduced the general support of widespread cover cropping. While the Ecological Focus Areas program was previously the main driver of the increase in cover crop area, Good Agricultural and Environmental Conditions (especially GAEC 6, 7 & 8) now only account for a small proportion of cover crop area under average farm conditions (European Commission, 2021). Thus, the decision to grow cover crops depends primarily on national legislation and farmers´ knowledge of their agronomic and environmental benefits (Storr et al., 2019).

The provided ecosystem services range from capturing and storing nutrients to preventing nitrate leaching while increasing nutrient efficiency in crop production systems (Ilgen, 1990; Heuermann et al., 2019; Sieling, 2019; Hallama et al., 2021; Gentsch et al., 2022; Heuermann et al., 2022). They improve soil health by protecting bare soil from erosion, increasing macro-aggregate stability (Gentsch et al., 2023), improving water infiltration, and promoting soil microorganisms (Roarty et al., 2017; Restovich et al., 2019; Gentsch et al., 2020; Ruis et al., 2020; Blanco-Canqui et al., 2022; Koudahe et al., 2022). Furthermore, cover crops are a powerful tool for climate change mitigation, as they are a sufficient tool to reduce the carbon footprint in cropping systems (Schön et al., 2024) when adapted to local growing conditions (Poeplau and Don, 2015; Kaye and Quemada, 2017; Abdalla et al., 2019; Seitz et al., 2022). In addition, the establishment of cover crops, especially in simplified crop rotations, promotes the biodiversity of soil, plant, insect, and even avian communities (Axelsen and Kristensen, 2000; Wilcoxen et al., 2018; Schütz et al., 2020; Fiorini et al., 2022). Choosing the right cover crop is an important management tool to regulate pests and soil-borne plant diseases (Wnuk and Wojciechowicz-zytko, 2010; Patkowska and Konopiński, 2014; Hauer et al., 2016; Baysal-Gurel and Liyanapathiranage, 2019; Wright et al., 2019) and they are used for integrated weed management (Haramoto and Gallandt, 2004; Lemessa and Wakjira, 2014; Schappert et al., 2018; Riemens et al., 2022).

Cover crop species vary in their ability to provide ecosystem services under a given set of climatic conditions (Bodner et al., 2010; Ramirez-Garcia et al., 2015; Tribouillois et al., 2015a, 2015b; Wendling et al., 2016; Wagg et al., 2021). Therefore, increasing the number of species and, thus, the diversity of plant traits through cover crop mixtures is more likely to achieve multiple goals (Finney et al., 2017; Ranaldo et al., 2020; Reiss and Drinkwater, 2020). Identifying suitable plant measures for the targeted ecosystem services is essential for validating cover crop mixtures. The production of shoot biomass is an agronomically relevant measure of the establishment and growth of cover crops (Cottney et al., 2022). In addition to the simple on-farm evaluation of this measure, biomass serves as a source of soil organic matter and provides the potential to suppress weeds and volunteer plants (Lavergne et al., 2021; Kümmerer et al., 2023). Nevertheless, several controversial strategies for optimizing shoot biomass production have been discussed. Strategies range from the best-selected pure stand that produces the highest shoot biomass under the given conditions (Florence and McGuire, 2020) to cover crop mixtures that produce high biomass best adapted to specific climatic conditions (Wendling et al., 2019). However, the general assumption that mixtures produce higher shoot biomass than pure stands is highly dependent on the species used (Elhakeem et al., 2021), so the choice of cover crop species is crucial and requires consideration of environmental conditions and agronomic objectives (Hendrickson et al., 2021).

In field experiments, pure stands that were best adapted to the climatic conditions at the time of selection had the best shoot biomass production in the first few weeks of development but not throughout the growing season. By contrast, cover crop mixtures have a higher potential to limit the risk of crop growth failure under changing weather conditions (Heuermann et al., 2022). For this reason, developing resilient mixtures that produce high biomass yields under different climatic conditions is a significant goal. Although the goals of cover crops are well defined in practice, there currently need to be general rules about species in mixtures, how they affect each other, and the services they provide. Selection for a mixture can be based either on the performance of a species in pure stand to predict its share of shoot biomass in a cover crop mixture (Wortman et al., 2012; Wilson et al., 2019) or on the use of species that regularly dominate biomass in multi-species cover crop mixtures (Baraibar et al., 2020). However, due to their single-species performance, some dual cover crop mixtures reached higher shoot biomass than expected (Wendling et al., 2017). At best, mixtures should be designed to adapt to variable weather conditions and reach over-yielding or transgressive over-yielding (Wendling et al., 2017). This is particularly important for weed suppression (MacLaren et al., 2019; Baraibar et al., 2021), fast ground cover (Brust et al., 2014), high leaf area index, and maximum canopy height (Florence et al., 2019; Smith et al., 2020).

The present study systematically quantified the interaction between commonly used cover crop species, sown in August, in dual mixtures to improve cover crop performance in shoot biomass and weed suppression.

2 Materials and methods

2.1 Location

The field experiment was conducted in Triesdorf in southern Germany and repeated for two years, namely 2016 (49°12'33.04"N 10°39'1.25"E) and 2017 (49°12'9.99"N 10°39'33.60"E). The sites, with a mean annual air temperature of 8.7°C and annual precipitation of 674 mm (2005 - 2015), are characterized by a warm, fully humid climate with warm summer (Kottek et al., 2006). The soils are classified as a Stagnic Cambisol (IUSS Working Group WRB, 2015). The soil texture of the fields is sandy loam.

2.2 Experimental design

Seven cover crop species, namely white mustard (*Sinapis alba* L.), oil radish (*Raphanus sativus* var. *oleiformis* L.), phacelia (*Phacelia tanacetifolia* J.), Egyptian clover (*Trifolium alexandrinum* L.), common vetch (*Vicia sativa* L.), field bean (*Vicia faba* L.) and field pea (*Pisum sativum* L.) were established either as pure stands with common seed rates (grains m^{-2}) or in dual mixtures, with adjusted seed rates of 50% of its pure stand (Table 1).

Field trials were designed in a randomized complete block design with four replicates using 10.5 m × 4.5 m plots. In addition to the cover crop treatments, canola (*Brassica napus* L.) was used as a test weed (5 seeds m^{-2}) to quantify the competitiveness of the cover crops. The seeds were sown together with the cover crops.

2.3 Growing conditions and crop management

Winter barley (*Hordeum vulgare* L.*)* was the preceding crop in the field trials in both years, harvested on July 18, 2016, or July 17, 2017. After fertilizing with biogas manure (80 kg N ha^{-1} in 2016 and 60 kg N ha^{-1} in 2017 adjusted to N_{min} levels which are the calcium chloride extractable mineral N compounds (NO_3^- & NH_4^+) (Amelung et al., 2018), a three-bar tine cultivator was used for a 20 cm deep tillage, followed by seedbed preparation using a PTO harrow, and cover crops were sown on August 19, 2016, and August 17, 2017. All plots were sown with a field trial seed drill (Plotseed S, Wintersteiger, Ried, Austria) with a row spacing of 13.6 cm in a seeding depth of 2.0 cm. For quantification of shoot biomass, cover crops were harvested 89 days after sowing on November 15, 2016, and 85 days after sowing on November 9, 2017, at the end of the vegetation period using a field trial green forage harvester (Hege 212, Hege Saatzuchtmaschinen, Hohebuch, Germany).

Growing conditions for cover crop establishment were very different for the two seasons. The 2016 season was characterized by dry to moderate weather conditions, with total precipitation of 33 mm in July and 38 mm in August. Despite this low total precipitation before the experiment was started, rainfall events were reported in 2016 after sowing in a two-week pattern in September and October (Figure 1A). The monthly average temperature was July 18.7°C, August 17.8°C, September 16.1°C, and October 7.5°C. The 2017 cover crop growing season was characterized by wet weather conditions with total precipitation of 128 mm in July, 66 mm in August, 55 mm in September, and 63 mm in October. The average monthly temperature was 18.1°C in July, 17.9°C in August, 11.7°C in September, and 10.0°C in October (Figure 1B).

2.4 Plant sampling

According to the BBCH code, cover crop development was monitored until the end of the vegetation period (Meier, 2018). After field emergence, the stand density of all species was determined by counting and distinguishing the plant species in the four replicates on September 9, 2016, and September 6, 2017. Stand density was counted in each of the four replicates in two seeding rows, each for a 1-meter distance (covering 2,2% of the core plot) and converted into stand density per m^2 using the row distance of the seeder.

All above-ground biomass (fresh weight) was quantified by harvesting cover crop shoots from 11.25 m^{-2} in all four repetitions at a cutting height of 5.0 cm from each plot and weighed using a field trial green forage harvester with an integrated weighing system (Hege 212, Hege Saatzuchtmaschinen, Hohebuch, Germany). The harvester continuously collected a sample of 300 g of chopped fresh material from each plot to determine the total dry matter content after drying at 105°C until constant weight to calculate the dry matter yield based on the fresh matter yield. In addition, a second sub-sample of 4000 - 5000 g of above-ground biomass was collected from each plot to fractionate into the different plant species to determine their portion in the fresh matter shoot biomass. Subsequently, up to 300 g per species were dried at 60°C until constant weight to determine species-specific dry matter content, and the material was kept for further analysis. Finally, the dry matter yield of all species was calculated.

TABLE 1 Cover crop species, cultivars, seed rates in pure stands, and thousand-grain weight used in the field trials in Triesdorf, Germany.

Cover crop species	Latin Name	Cultivar	Seed rate	Thousand-grain weight
			[grains m^{-2}]	[g]
White mustard	*Sinapis alba* L.	Litember	250	6.6
Phacelia	*Phacelia tanacetifolia* J.	Beehappy	450	1.8
Egyptian clover	*Trifolium alexandrinum* L.	Alex	1000	2.5
Oil radish	*Raphanus sativus* var. *oleiformis* L.	Brutus	180	14.4
Common vetch	*Vicia sativa* L.	Hanka	150	51.3
Field bean	*Vicia faba* L.	Fuego	70	554.8
Field pea	*Pisum sativum* L.	Salamanca	80	182.6

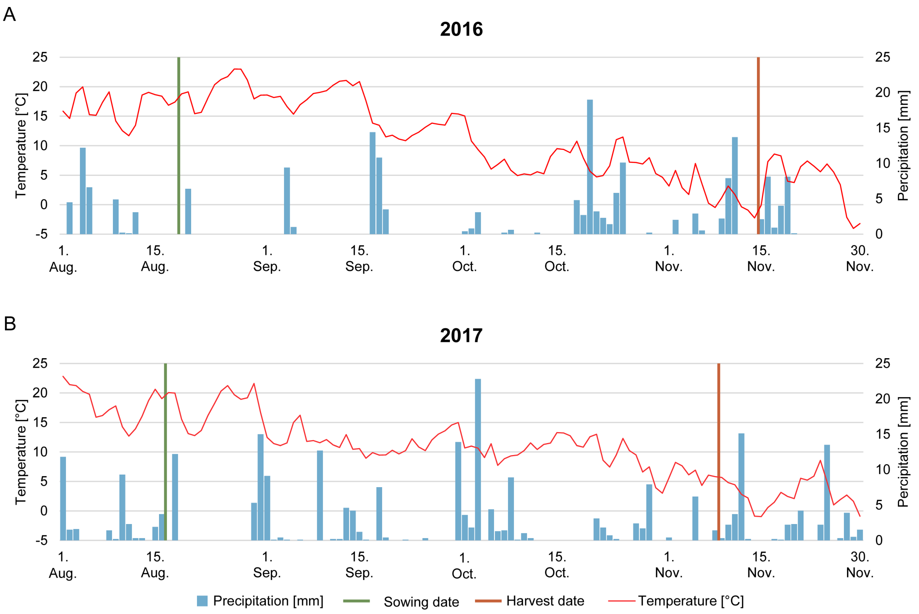

FIGURE 1
Growing conditions during the cover crop vegetation period [mean daily air temperature [°C] (red line), daily precipitation [mm] (blue bars)]. A green line indicates the time point of sowing, and a red line indicates the harvest in 2016 **(A)** and 2017 **(B)**.

2.5 Analysis of interactions and competitiveness between cover crop species

Shoot biomass in mixtures was compared to the shoot biomass yields of pure stands of each species, which were used to analyze interactions in dual mixtures (De Wit, 1960). A graph was created to show the interspecific interactions in shoot biomass to visualize the effect of dual mixtures compared to single species. The measurements of the pure stands are shown as columns on the outer left and right sides of the graph (Figures 2A, B). The measurements of the shoot biomass in the dual mixtures of the two sown species at a seeding rate of 50% of the species´ pure stand are shown in between as two individual columns. While the bar on the middle left side shows the estimated shoot biomass based on half of the shoot biomass yields of the two pure stands, the bar on the middle right side displays the measured shoot biomasses of the dual mixture of the field trial. Two lines have been added to better visualize the differences between estimated and measured above-ground biomass yields. The black-dotted line represents the estimated yield, while the red line displays the measured shoot biomass yield (Wendling et al., 2017; Bernardo, 2020) (Figures 2A, B). When mixtures outperform even the highest-yielding pure stand, the interaction of the two species is described as transgressive over-yielding (De Wit, 1960; Hooper and Dukes, 2004;

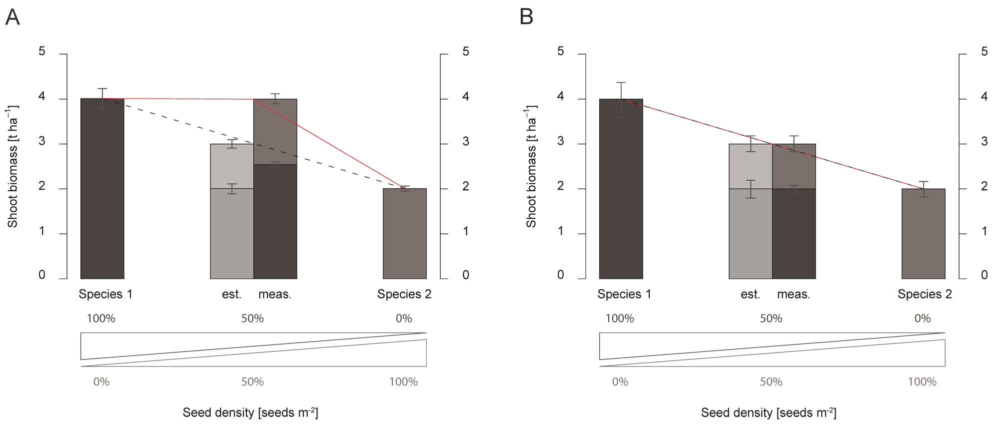

FIGURE 2
Examples demonstrating over-yielding **(A)** and additive effects **(B)** in dual mixtures. The plots show schematically shoot biomass production [t ha^{-1}] of pure stands of the two species (1 & 2) used in the dual mixture. The estimated shoot biomass (est.) based on half of the pure stands´ single species above-ground biomass of all four repetitions is displayed in the middle-left bar. In comparison, the measured shoot biomass (meas.) of the dual mixture at the end of the vegetation period is shown in the middle-right bar. Error bars represent standard deviation.

Hector, 2006; Wendling et al., 2017). If the measured total shoot biomass yield of the mixture is less than the highest shoot biomass of the pure stand but exceeds the expected shoot biomass of the mixture (Figure 2A), the interaction of the two species is called over-yielding (Hooper and Dukes, 2004; Hector, 2006; Wendling et al., 2017; Couëdel et al., 2018b; Elhakeem et al., 2021). Alternatively, the shoot biomass yield of the mixture is equal to the estimated shoot biomass derived from the pure stand yield. In this case, the interaction of the two species is described as an additive effect (Figure 2B). If one plant species impedes another's growth and the measured shoot biomass is lower than the calculated one, these are antagonistic effects.

Oilseed rape was selected as a test weed in the experiment because of its strong competitiveness and its similar use in other studies (Krato and Petersen, 2012; Gregoire et al., 2021). Besides the test weed and cover crop shoot biomass, other weed species were not considered due to their negligible proportion of the shoot biomass. The percentage of test weed shoot biomass refers to its proportion in the total shoot biomass. The competitiveness of cover crop treatments to suppress test weeds is described by the total dry matter shoot biomass (i.e., cover crops and weeds) harvested before winter, minus the percentage of test weed shoot biomass (Di Bella et al., 2021), customized to: Equation 1.

$$\begin{aligned} &\mathit{Competitiveness\ \ of\ \ cover\ \ crops\ \ treatment}\ \ (\%) \\ &\quad = 100 - \mathit{Test\ \ weed\ \ shoot\ \ biomass}\ \ (\%) \end{aligned}$$

2.6 Statistical analysis

Statistical analysis was conducted using R version 4.3.1 (R Core Team, 2021). A mixed model accounting for block and year effects was fitted using the *lmer* function from the "lme4" package (Bates et al., 2015) to model the dry matter yield of the cover crops. Since analysis of variance (ANOVA) of this model showed a significant interaction of cover crop species and year, we decided to analysis each year with a separate model accounting for the block effects (treated as random). Residuals of the models were checked for normal distribution and heteroscedasticity with diagnostic plots before performing an ANOVA (Kozak and Piepho, 2018). If needed, observed values were transformed to meet this assumption.

Models were fitted at three different levels for each year: at the total plot level, at the species level, and with total plot values of observed and expected mixtures. Block effects were included as random effects, and at the species level, the interaction of species and cover crop treatment and the effect of the plot (both treated as random) were included. If the ANOVA was significant ($p < 0.05$), analyses were performed for each species without adjustment of p-values (Fisher´s LSD test) at the plot yield level. Analysis at the species level was also performed separately for each combination of species with Tukey's adjustment of p-values (Tukey's HSD test) due to 4 groups (species 1 & 2 in pure stand and their share in the mixture) being compared, using the 'emmeans package' (Lenth, 2017). A two-sample t-test was performed for each mixture to compare the actual measured total yield of a mixture with the estimated total yield. Linear regression analysis was performed to test the effect of cover crop shoot biomass on test weed shoot biomass. Prior to analysis, the data were checked for zero inflation using the R package "Performance" (Lüdecke et al., 2021).

3 Results

Multi-species cover crop mixtures are supposed to improve cropping systems and agronomic ecosystem services such as weed suppression. For this reason, the shoot biomass of cover crops in pure stands or dual mixtures was determined under two different conditions. While the 2016 growing season was characterized by moderate precipitation, growing conditions in 2017 were influenced by high precipitation and unlimited water supply.

3.1 Interactions in dual cover crop mixtures on shoot biomass formation

All species were grown as pure stands or in mixtures with adjusted seeding rates (50% of pure stands Table 1) to evaluate the interactions between different species in dual mixtures compared to pure stands. In the first step, mustard-containing cover crops were tested based on mustards' historical relevance in cover cropping.

3.1.1 Dual mixtures based on mustard

In 2016, mustard produced 4.37 t ha^{-1} shoot biomass in a pure stand, while the second species tested in a dual mixture, oil radish, had 3.71 t ha^{-1} when grown in a pure stand (Figure 3A). Consequently, the estimated yield of the mustard and oil radish mixture (half of the shoot biomass yields of both species) reached 4.04 t ha^{-1} in 2016. The dual mixture achieved above-ground biomass of 4.44 t ha^{-1}, which was not significantly higher than the estimate, thus indicating an additive effect on shoot biomass (Figure 3A). Interestingly, oil radish had the highest proportion of shoot biomass in the mixture, exceeding its estimated yield (Figure 3A), indicating a higher interspecific competitiveness than mustard. This result was reproduced under the non-water limiting conditions in 2017. However, the mixture's yield increase exceeded mustard's shoot biomass, although the differences were not statistically significant (Figure 4A).

When mustard was grown with phacelia, the measured above-ground biomass yield was equal to that of the pure stands (Figure 3B). Mustard reached a higher proportion of shoot biomass than estimated based on the performance of the pure stands of both species. However, the measured above-ground biomass of the dual mixture showed no advantage over the pure stands or the estimated mixture. Thus, it resulted in an additive effect, which was also observed in the wet conditions of 2017, even though the proportion of phacelia's shoot biomass was lower in the dual mixture (Supplementary Figure S1A).

Overyielding was observed in 2016 when mustard was grown in a dual mixture with the legume Egyptian clover and common vetch. Clover alone had a shoot dry matter yield of only 0.48 t ha^{-1},

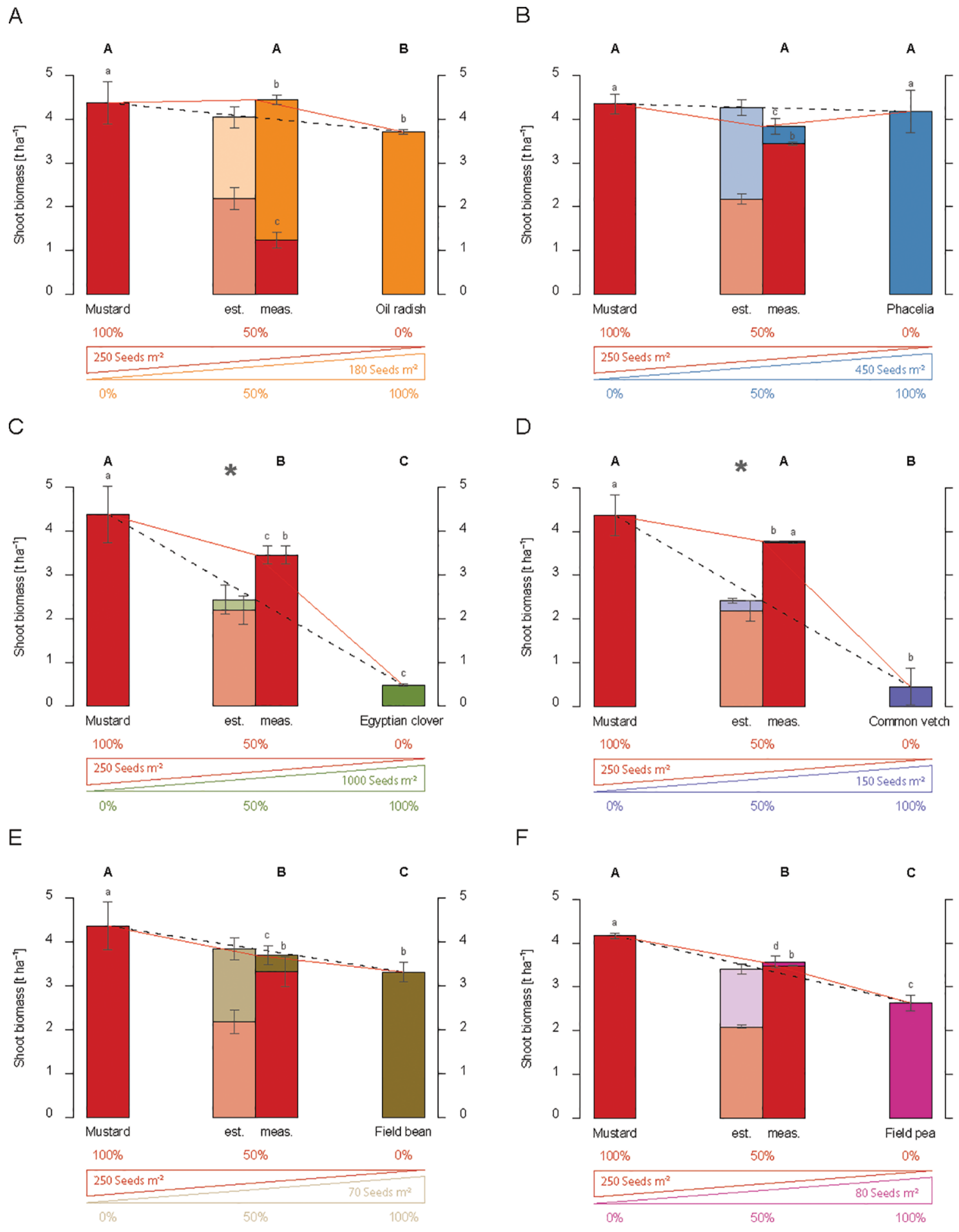

FIGURE 3
Shoot biomass production (dry matter) [t ha^{-1}] of dual mixtures of cover crops consisting of mustard and a second species, namely oil radish **(A)**, phacelia **(B)**, Egyptian clover **(C)**, common vetch **(D)**, field bean **(E)**, or field pea **(F)** compared to pure stands in 2016. The plots show results for pure stands of the two species used in the dual mixture, the estimated shoot biomass (est.) based on 50 % of each species in pure stand in relation to the measured shoot biomass (meas.) of the dual mixture at the end of the vegetation period, and the proportion of the individual species in shoot biomass of the mixture. The bars show means with standard deviations, n=4. Different capital letters indicate the significant differences in total biomass yield of treatments at $p < 0.05$ by Fisher LSD-Test, while different lower-case letters indicate significant differences in the total biomass of different species at $p < 0.05$ by Tukey's HSD-Test. Significant differences between estimated and measured shoot biomass of dual mixtures are indicated by an asterisk (*, t-test, $p < 0.05$).

and the estimated yield of the mixture was 2.43 t ha^{-1}. In the field trial, the measured shoot biomass of the Egyptian clover and mustard mixture exceeded the estimated shoot biomass and significantly overreached it by 1.02 t ha^{-1} in 2016. However, Egyptian clover contributed only 0.001 t ha^{-1} to the shoot biomass in this mixture (Figure 3C), and mustard reached 78.8% of the yield level of its pure stand even when half of the seed density was applied. A similar over-yielding effect was observed in the mustard-common vetch mixture. Here, the measured shoot biomass exceeded the estimated shoot biomass significantly by as much as 1.55 t ha^{-1} (Figure 3D).

These results suggest that combining mustard and legumes leads to an overyielding effect. However, this is in contrast to the fact that a combination of mustard with the legume field bean or field pea only led to additive effects in 2016 (Figures 3E, F), even though the above-ground biomass of field bean (3.31 t ha^{-1},

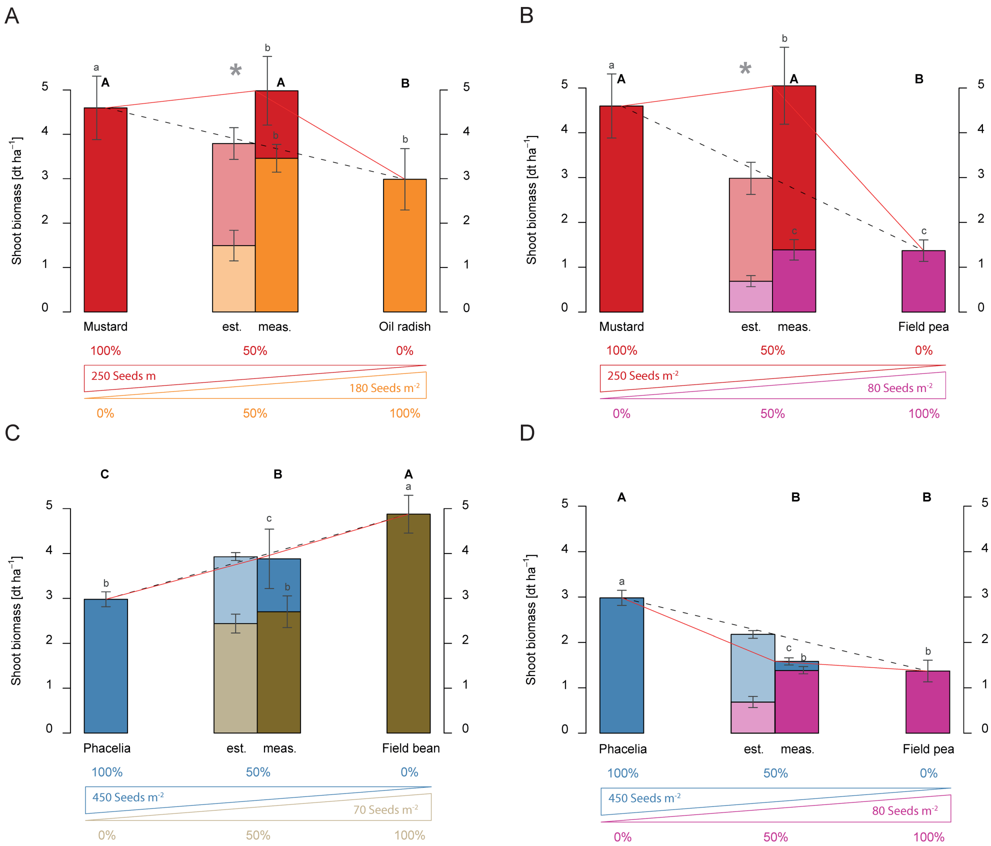

FIGURE 4

Shoot biomass production (dry matter) [t ha^{-1}] of dual mixtures of cover crops consisting of mustard and a second species, namely oil radish **(A)**, and field pea **(B)**, or phacelia in combination with field bean **(C)** or field pea **(D)** on shoot biomass production [t ha^{-1}] compared to pure stands in 2017. The plots show results for pure stands of the two species used in the dual mixture, the estimated shoot biomass (est.) based on 50 % of each species in pure stand in relation to the measured shoot biomass (meas.) of the dual mixture at the end of the vegetation period, and the proportion of the individual species in the shoot biomass of the mixtures. The bars show means with standard deviations, n=4. Different capital letters indicate the significant differences in total biomass yield of treatments at $p < 0.05$ by Fisher LSD-Test, while different lower-case letters indicate significant differences in the total biomass of different species at $p < 0.05$ by Tukey's HSD-Test. Significant differences between estimated and measured shoot biomass of dual mixtures are indicated by an asterisk (*, t-test, $p < 0.05$).

Figure 3E) and field pea (2.64 t ha^{-1}, Figure 3F) was apparently higher than that of Egyptian clover (0.48 t ha^{-1}, Figure 3C) or common vetch (0.45 t ha^{-1}, Figure 3D). Despite the comparatively high shoot biomass of field beans and field peas, the proportion of these crops in the above-ground biomass of the mixtures was surprisingly low (Figures 3E, F). These observations were stable for field beans over the years or for weather conditions.

Comparable observations were made for the grain legume field pea. The mixture with mustard showed 0.19 t ha^{-1} above the estimate in 2016, and mustard produced 97.7% of the biomass (Figure 3F). In 2017, the non-limiting water conditions changed the biomass proportions. The above-ground biomass of the mustard–field pea mixtures was 2.10 t ha^{-1} higher than the estimate. This resulted in total shoot biomass of 5.02 t ha^{-1} (Figure 4B) and an over-yielding effect. Interestingly, the proportion of field pea shoot biomass in the mixture (1.43 t ha^{-1}) was on the same level as the total shoot biomass of the field peas in the pure stand (1.36 t ha^{-1}).

These results indicate that the interactions between mustard and a second species and its impact on shoot biomass are rather species-specific than plant family-specific. However, the effects on the above-ground biomass yield of mustard-based mixtures tested were highly comparable under different growing conditions.

3.1.2 Dual mixtures based on phacelia

To investigate if the interactions between species tested are species-specific, mustard was replaced by the less competitive cover crop species phacelia in dual mixtures (Figure 5). Compared to the results of mustard-containing mixtures (Figure 3), the legumes showed increased competitiveness against phacelia. The small-seeded legumes Egyptian clover and common vetch contributed a slightly higher proportion of shoot biomass in the mixtures (Figures 5A, B) than in the dual mixtures with mustard, and the share of field beans and field peas in dual mixtures with phacelia was markedly higher (Figures 5C, D).

Over-yielding was observed when phacelia was combined with Egyptian clover. The measured shoot biomass exceeded the estimated shoot biomass significantly by 1.45 t ha^{-1} in 2016 (Figure 5A). A similar effect occurred in the phacelia-common

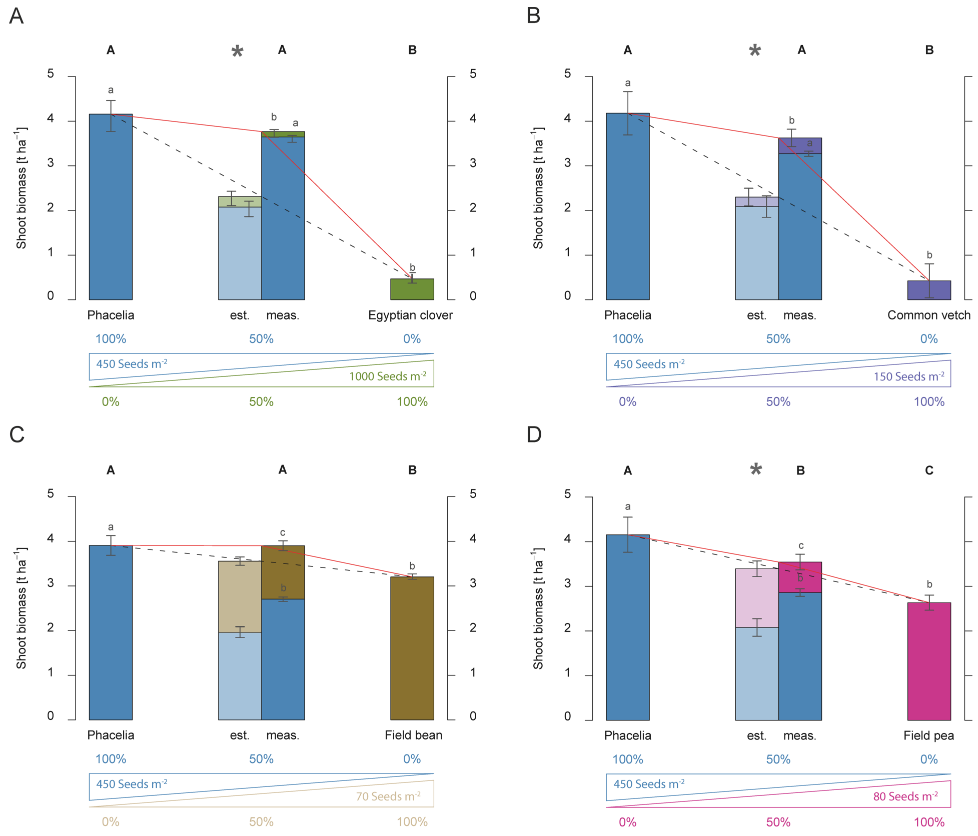

FIGURE 5

Shoot biomass production (dry matter) [t ha^{-1}] of dual mixtures of cover crops consisting of phacelia and a second species, namely Egyptian clover **(A)**, common vetch **(B)**, field bean **(C)**, and field pea **(D)** compared to pure stands in 2016. The plots show results for pure stands of the two species used in the dual mixture, the estimated shoot biomass (est.) based on 50 % of each species in pure stand in relation to the measured shoot biomass (meas.) of the dual mixture at the end of the vegetation period, and the proportion of the individual species in shoot biomass of the mixture. The bars show means with standard deviations, n=4. Different capital letters indicate the significant differences in total biomass yield of treatments at $p < 0.05$ by Fisher LSD-Test, while different lower-case letters indicate significant differences in the total biomass of different species at $p < 0.05$ by Tukey's HSD-Test. Significant differences between estimated and measured shoot biomass of dual mixtures are indicated by an asterisk (*, t-test, $p < 0.05$).

vetch mixture; the measured shoot biomass surpassed the estimate significantly by 1.32 t ha^{-1} (Figure 5B). In phacelia-field bean mixtures, only an additive effect was observed regardless of the water condition (Figures 5C, 4C). Field beans contributed a larger share of shoot biomass of 1.11 t ha^{-1} in the mixture with phacelia than 0.30 t ha^{-1} in a mixture with mustard (Figure 3E). In contrast to 2016 (Figure 5C), field beans contributed 2.85 t ha^{-1} under non-water-limited conditions (Figure 4C). So, field beans provided the majority of shoot biomass of the 4.00 t ha^{-1} in 2017.

Combining phacelia and field peas instead of field beans led to slight overyielding effects in shoot biomass formation, with a total yield of 3.54 t ha^{-1} in 2016 (Figure 5D). Surprisingly, a suppressive effect was observed in the phacelia-field pea mixture under water-sufficient conditions in 2017 (Figure 4D). In contrast to the mustard-field pea mixture (Figure 4B), phacelia contributed only a small portion of the shoot biomass in the phacelia-field pea mixture (Figure 4D), while under the water-limited conditions in 2016, phacelia produced more than 80% of the shoot biomass of the mixture (Figure 5D). Nevertheless, the most striking observation was that dual mixtures of legumes with phacelia showed similar shoot biomass yield response with mustard.

3.1.3 Single plant development in dual mixtures

So far, this work has focused on the total shoot biomass yield (dry matter) of the mixtures and the species involved in pure stands. We then shifted the focus to individual plants to examine the interactions between species in mixtures in more detail. One measure of the intraspecific competition of a species is the average weight of individual plants. Mustard showed significantly higher single-shoot weights (dry matter) when grown with Egyptian clover, common vetch, field bean, field pea or phacelia than with oil radish (Figure 6A). In contrast, phacelia (Figure 6B) significantly decreased single-shoot weights in mixtures with oil radish and mustard compared to those with Egyptian clover, field bean, or field pea. Similar observations were made for single-plant shoot weight of mustard or phacelia under water-sufficient conditions in 2017, except for field pea treatments (Supplementary Figure S2). One explanation may lie in the generally poor phacelia and field pea

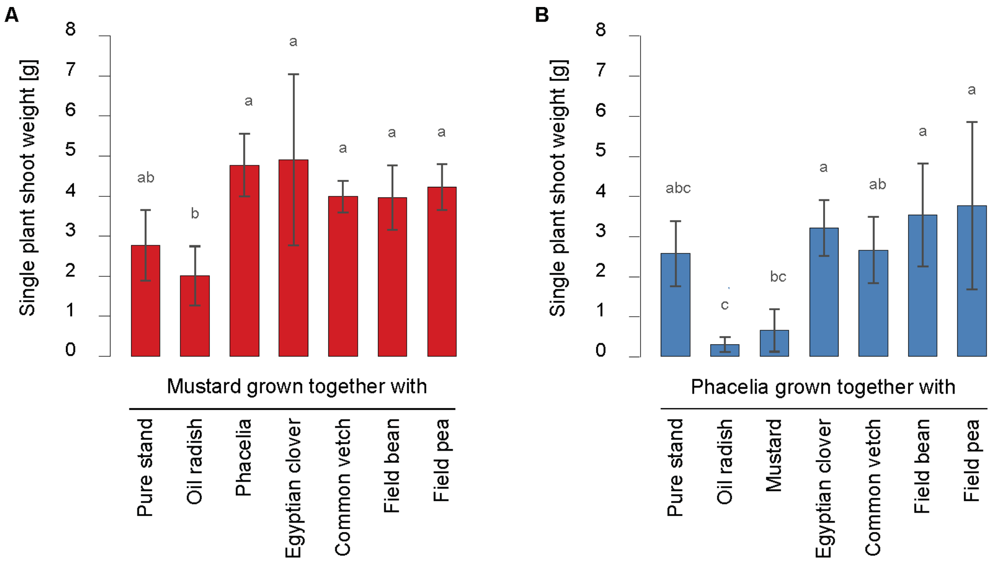

FIGURE 6
Single plant shoot weight (dry matter) [g] of mustard **(A)** and phacelia **(B)** in pure stand and dual mixtures with oil radish, Egyptian clover, common vetch, field bean, and field pea in 2016. Bars show means with standard deviations, n=4; different letters indicate significant differences among treatments at $p < 0.05$ by Tukey's HSD-Test.

growth under water-sufficient conditions in 2017. In addition, field emergence was determined to rule out the possibility that plant density differences affected individual plants´ growth conditions (Supplementary Table S1), and no statistically significant differences between treatments were found.

3.1.4 Interactions in mix containing legumes only

So far, only mixtures between legumes and non-legumes have been investigated. Following the observation that small-seeded legumes, such as Egyptian clover and vetch, caused over-yielding effects in dual mixtures with non-legume species (Figures 3C, D, 5A, B), it was tested whether this observation can also be made in combination containing small-seeded legumes and grain legumes. Significant differences were found between estimated and measured shoot biomass with an overyielding effect of 0.83 t ha^{-1} when the small-seeded legume common vetch was combined with field bean (Figure 7A). The increased yield of field beans caused the overyielding effect compared to the estimate. However, with 3.16 t ha^{-1}, the above-ground biomass was highest in the pure stand of field beans. A similar result was found for the common vetch-field pea mixture. Here, an overyielding effect was observed with a difference of 0.53 t ha^{-1} between measured and estimated shoot biomass (Figure 7B). An additive effect was also observed when the field beans and field peas were grown together (Figure 7C). Here, the above-ground biomass yield of the two species and their expected share in the mixture corresponded to the estimate.

Taken together, the interactions among cover crop species mainly resulted in an additive or overyielding effect on above-ground biomass. Interestingly, observations in mixtures showed comparable interaction patterns regardless of whether phacelia or mustard was grown with a second species. Additive effects were observed, especially when grain legumes were added, while overyielding occurred when small-seeded legumes complemented the mixture.

3.2 Competitiveness of cover crops against weeds

Weed suppression is one management service that cover crops provide in crop rotations. We evaluated the competitiveness of cover crop treatments against weeds by determining the shoot biomass of oilseed rape as test weed at cover crop harvest at the end of the vegetation period.

A negative correlation was observed between the above-ground dry matter biomass of cover crops and the weed under water-limited conditions in 2016 (Figure 8A) but not under sufficient water supply in 2017 (Figure 8B). The cover crop shoot biomass in 2016 explained 72% ($p < 0.001$) of the variance in test weed shoot biomass. A species-specific pattern was evident for pure stands (marked with crosses). The small-seeded legumes Egyptian clover and common vetch did not suppress weeds sufficiently. In contrast, the grain legumes field peas and even more field beans were able to reduce weed shoot biomass. However, the test weed shoot biomass was higher than 0.5 t ha^{-1}. Best results in weed suppression were obtained with phacelia, mustard, or oil radish, where the test weed no longer developed.

As shown earlier, over-yielding effects were observed in some dual mixtures. We therefore tested whether the increased above-ground biomass would also lead to increased weed suppression. Dual mixtures consisting only of legumes (marked with grey dots) showed better weed suppression (Figure 8A), although weed suppression was significantly lower than in other dual mixtures (Figure 8C). Mixtures of phacelia with other cover crops (marked with squares) showed that all legumes tested decreased the competitiveness compared to the pure phacelia stand (Figure 8A). This even held for the grain legumes field bean and field pea, which produced much more biomass than small-grained legumes. Combining phacelia with mustard or oil radish outcompeted the weeds. On the other hand, mustard (marked with triangles) suppressed the weeds, regardless of which other species were grown in the dual mixture.

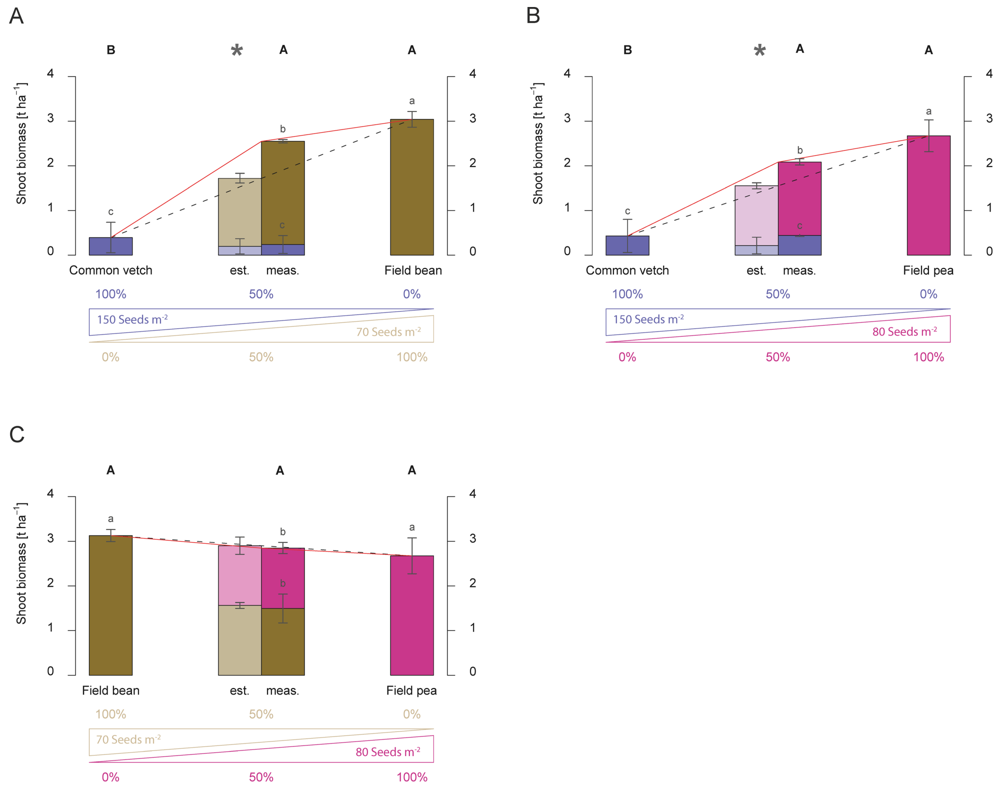

FIGURE 7

Shoot biomass production (dry matter) [t ha^{-1}] of dual mixtures of cover crops consisting of legumes compared to pure stands in 2016. Mixtures combine common vetch and field bean **(A)**, common vetch and field pea **(B)**, and field bean and field pea **(C)**. The plots show results for pure stands of the two species used in the dual mixture, the estimated shoot biomass (est.) based on 50 % of each species in pure stand in relation to the measured shoot biomass (meas.) of the dual mixture at the end of the vegetation period, and the proportion of the individual species in shoot biomass of the mixture. The bars show means with standard deviations, n=4. Different capital letters indicate the significant differences in total biomass yield of treatments at $p < 0.05$ by Fisher LSD-Test, while different lower-case letters indicate significant differences in the total biomass of different species at $p < 0.05$ by Tukey's HSD-Test. Significant differences between estimated and measured shoot biomass of dual mixtures are indicated by an asterisk (*, t-test, $p < 0.05$).

The competitiveness of cover crop treatments against weeds can also be described by the percentage of cover crop shoot biomass to the whole shoot biomass of the treatment. The cruciferous species oil radish and white mustard and the non-cruciferous species phacelia had a high competitiveness of 99.2 to 100.0% in a pure stand (Figure 8C). Pure stands of field beans and field peas had a lower competitiveness of 92.3% compared to cruciferous species. Generally, pure stands of Egyptian clover (38.5%) and common vetch showed significantly lower competitiveness (28.2%, Figure 8C) than the other cover crops in pure stands.

Combinations with cruciferous species attained a high competitiveness of 99.0% to 100.0% (Figure 8C). In contrast, the competitiveness decreased in dual mixtures of phacelia with Egyptian clover (96.1%), common vetch (97.4%), field bean (96.1%), and field pea (96.0%). Mixtures containing only legumes showed the lowest competitiveness in dual mixtures. Compared to other dual mixtures, combinations of common vetch and field bean (84.8%), common vetch and field pea (85.4%), and field bean and field pea (90.6%) showed significantly lower competitiveness against the test weed, compared to mixtures containing cruciferous species and mixtures of phacelia with field peas or common vetch (Figure 8C).

Under sufficient water conditions in 2017, no correlation was observed between cover crop shoot biomass and test weed shoot biomass. However, competitive species such as oil radish, mustard, and phacelia competed well with the test weed, regardless of whether it was grown in pure stands or mixtures (Figure 8B). A closer look at the competitiveness showed that phacelia had significantly lower competitiveness in the pure stand than mustard or oil radish under the conditions of 2017 (Supplementary Figure S3). Nevertheless, similar patterns were observed in 2016 and 2017.

In summary, the ability of competitive species to compete against the test weed was not hampered when dual mixtures with less competitive species were formed. However, unlike shoot biomass, no synergistic effects in suppression performance were observed between the species used.

4 Discussion

The integration of cover crops into cropping systems offers many benefits in terms of ecosystem services and crop management. These benefits are further enhanced when

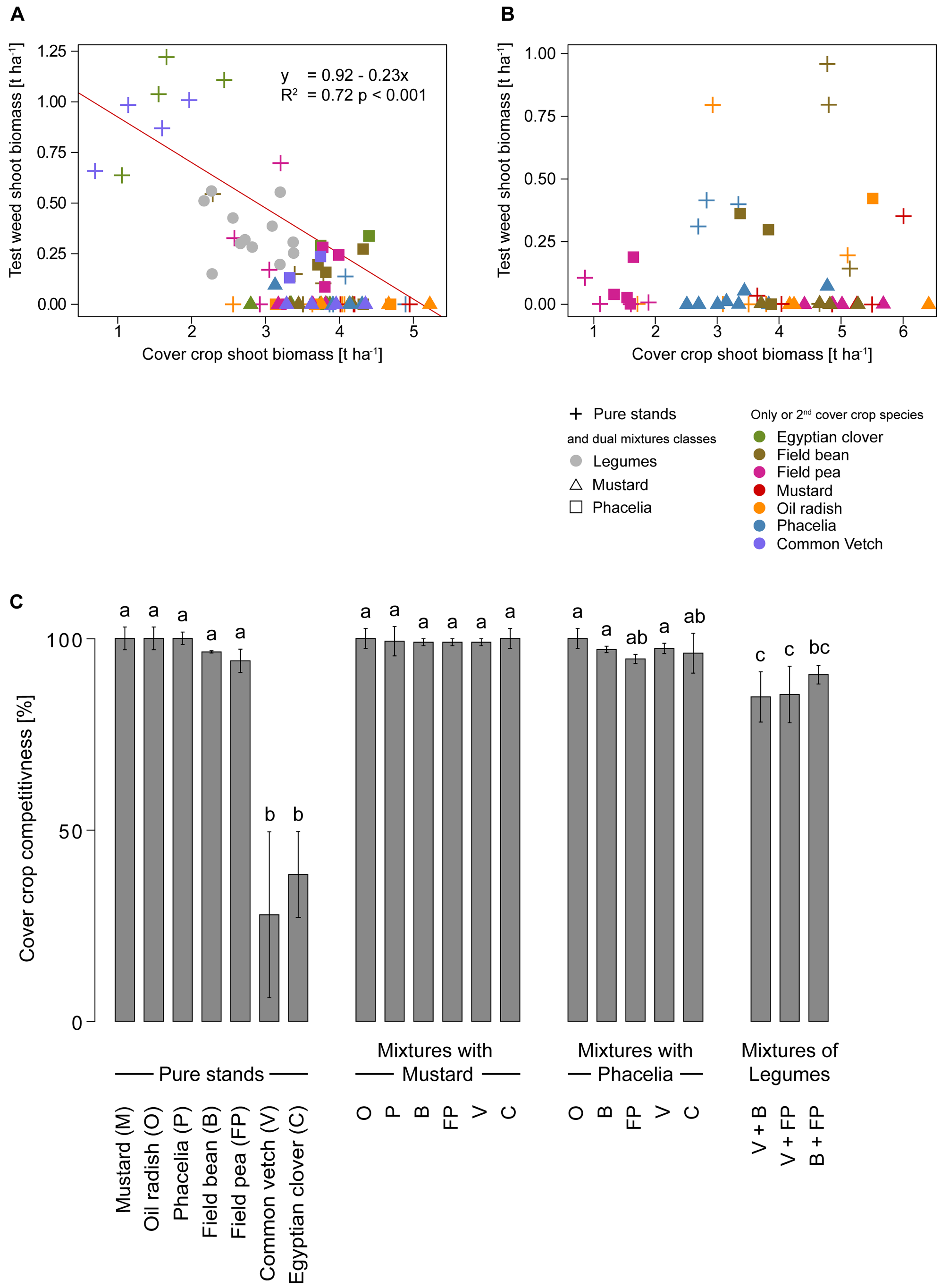

FIGURE 8

Correlation between cover crop shoot biomass (dry matter) [t ha^{-1}] in pure stand (crosses) or dual mixtures containing legumes only (dots), mustard (triangle), phacelia (square), with a second partner (color code) and test weed shoot biomass (dry matter) [t ha^{-1}] in 2016 **(A)** and 2017 **(B)**. Color code represents the species of the pure stand or the second partner in the dual mixture and the influence of cover crops pure stands and dual mixtures on competitiveness **(C)** in 2016. Bars show means with standard deviations, n=4; different letters indicate significant differences among treatments at $p < 0.05$ by Tukey's HSD-Test.

combined with other techniques, such as reduced tillage (Groß, 1996; Wittwer et al., 2017; Abdalla et al., 2019; Alletto et al., 2022). On the other side, it becomes more challenging to control volunteer plants and weeds. In conventional farming systems, volunteer seed germination is often controlled with non-selective herbicides before planting to maintain cover crop benefits and reduced tillage intensity. However, this strategy has been criticized from several perspectives. A promising strategy is to control volunteer plants and weeds by growing cover crops during the off-season. This approach requires high competitiveness of cover crop mixtures by using species that produce more above-ground biomass or better compete with weeds (Büchi et al., 2018; Grosse and Heß, 2018; Schappert et al., 2018; Schmidt et al., 2019; Bunchek et al., 2020). Therefore, the present study aimed to identify mechanisms in shoot biomass formation in dual cover crop mixtures of different plant species and their ability to suppress weeds.

4.1 Impact of interactions between species in cover crop mixtures on shoot biomass is species-specific

Plants growing in a mixed stand compete for space, light, water, and nutrients. This becomes even more important when combining species with different characteristics, as in cover crop mixtures. Nevertheless, interactions among species can also promote plant growth (Baeumer and De Wit, 1968; Heuermann et al., 2019). One hypothesis as to why mixtures can produce more shoot biomass than pure stands assumes that competitive species benefit from less competitive partners in mixtures. However, in contrast to previous studies (Finney et al., 2017; Wendling et al., 2017), we did not observe significantly higher shoot biomass yields than in pure stands, described as transgressive over-yielding under the water-limited conditions in 2016 (Figures 3, 5 & 7), but in mustard - oil radish and mustard – field pea mixtures under the water-sufficient conditions in 2017 (Figures 4A, B). Nevertheless, an overyielding effect was evident in 2016 when the competitive species mustard was combined in dual mixtures with less competitive species such as Egyptian clover and common vetch (Figures 3C, D). Similar observations were made for phacelia. While phacelia is not as competitive as mustard (Figure 3B), the number of less competitive partners leading to over-yielding effects was even higher and included field beans in addition to Egyptian clover and common vetch (Figures 5A–C). Even when dual mixtures were designed among much less competitive species, such as field bean or field pea, the combinations with even less competitive species, such as common vetch, resulted in an over-yielding effect (Figures 7A, B).

Compared to the estimated shoot biomass based on the pure stand, the shoot biomass of the competitive partner (mustard, phacelia, and field bean) increased significantly in dual mixtures with weaker species, not only in dual mixtures showing over-yielding. For mustard, this observation is consistent with other studies showing its high competitiveness in dual mixtures with legumes (Wortman et al., 2012; Couëdel et al., 2018b). However, our results suggest that this is wider than legumes. Mustard also increased shoot biomass relative to the estimate in mixtures with phacelia (Figure 3B). There is a clear indication that this effect generally works, as shown for phacelia against less competitive species such as Egyptian clover, common vetch, and field bean (Figures 5A–C), or field bean against common vetch (Figure 7A). Moreover, this effect is even reversible when these previously competitive species are combined with an even more competitive species, such as oil radish in the case of mustard (Figure 3A), mustard in the case of phacelia (Figure 3B), or mustard and phacelia in the case of field bean (Figures 3E, 5C). When placed as the weaker partner in another mixture, the former dominant species showed less shoot biomass than the estimate based on pure stand yields.

The mode of action of the increasing above-ground biomass of the competitive species in a mixture is unlikely due to the promotive effect of nitrogen fixation of the less-competitive partner because it was also observed with non-legume partners. Non-legume species increased the growth of more competitive partners, even if they belonged to the same family and shared similar root properties for nutrient acquisition or pest control, such as oil radish and mustard (Figure 3A). The promotive effect was also observed even if only legumes, such as field beans and common vetch, were combined (Figure 7A). This observation is even more significant because similar results were observed under the water sufficient conditions in 2017, and transgressive over-yielding even occurred for the mustard-field pea mixture when mustard was the dominant partner (Figure 4B) and the mustard-oil radish mixture when mustard became the weaker partner of the mixture (Figure 4A).

Another mechanism is explained by a niche differentiation leading to a complementary effect or the dominance of a higher-yielding species, known as a selection effect (Loreau and Hector, 2001). Studies by Elhakeem et al. (2021) and Couëdel et al. (2018b) observed both effects in mixtures containing oil radish. These findings might further indicate that the competitive species mustard, oil radish, and phacelia generated higher shoot biomass through a selection effect. In another experiment, higher crop growth rates and higher crop nitrogen uptake rates of mustard than phacelia and mustard were measured across different environments (Tribouillois et al., 2015a).

Another approach to further investigate the mechanism of increased above-ground biomass of the competitive species by companion crops is to shift the focus from total shoot biomass in the stand to the single plant weight. Both mustard and phacelia decreased shoot weight per plant when combined with more competitive species (Figures 6A, B). Mustard reduced shoot formation by over one-fourth when combined with oil radish (Figure 6A) and phacelia by factor 4 when grown together with mustard (Figure 6B). However, the seeding rate was reduced by half for all species in the dual mixture. The situation was quite different when tested species acted as the competitive partner in a mixture. They showed an apparent increase in the shoot weight per plant regardless of whether the less competitive partner was a legume or not (Figures 6A, B). Based on these observations, the main advantage of the weak partner, besides increasing diversity, appears to be its retarded development or the small plant size, resulting in higher availability of space and resources for the rapidly developing competitive partner. Supposing the advantage is only based on more space and resources per plant, the effect must also be present when reducing seed density. However, a reduction in the seeding rate did not increase the above-ground biomass in the species tested (data not shown). This is also consistent with Wendling et al. (2017), who showed that responses to sowing rate adjustments are species-specific and dependent on the fertilization level.

The stimulatory effect on the single plant weight of a competitive partner in a mixture was observed regardless of whether the combination resulted in over-yielding (Figures 3C, D, 5A–C) or not (Figures 3B, E, F, 5D). There is a clear indication that over-yielding is only possible if the weak partner reduces its proportion of shoot biomass compared to the estimate. However, this conclusion does not align with the transgressive over-yielding effects observed under water-sufficient conditions in 2017 (Figure 4). While the radish-mustard mixture followed the pattern of 2016 and mustard significantly reduced its shoot growth under these conditions (Figure 4A), the mustard-pea mixture showed a significant increase in shoot biomass of both species compared to the estimate (Figure 4B).

One explanation can be found in the favorable starting conditions for field peas in 2017 compared to 2016. Moderate temperatures and sufficient soil moisture provided perfect conditions for germination and seedling development, even for field peas, which have a high water demand during germination (Raveneau et al., 2011; Tribouillois et al., 2016). For this reason, an equal development of mustard and field peas during early development could lead to similar competitiveness. These observations align with other studies that report transgressive overyielding of mustard-field pea mixture under non-water-limited conditions (Wendling et al., 2017). While the non-limited water conditions promoted field peas, they hampered the development of phacelia (Figures 4C, D compared to 5C, D). Thus, the mixture of phacelia and field beans showed only an additive effect in 2017 (Figure 4C) instead of an over-yielding effect in the water-limited conditions in 2016. The retarded performance of phacelia under water-sufficient conditions in 2017 was also obvious in the phacelia-field pea mixture, resulting even in a suppressive effect (Figure 4D). However, besides phacelia-containing mixtures, the weather conditions had no significant effect on the observed patterns of interactions in the mixtures.

Taken together for autumn-grown cover crops in the two tested environments, over-yielding in cover crop mixtures is likely to occur when two characteristics of the combined species are met: (i) Species must differ in competitiveness. The present study allows competitiveness to be ranked from the strongest to the weakest species from oilseed radish > mustard > phacelia > field bean > field pea > common vetch > Egyptian clover. (ii) The shoot biomass production in pure stands of the less competitive partner must be lower by at least a factor of two than that of the competitive species. However, the system has only been tested for dual mixtures. It should also be noted that phacelia significantly reduces biomass and competitiveness, especially in wet years.

4.2 Improved cover crop mixtures can suppress weeds and volunteer plants

A large above-ground biomass is often proposed as the primary property of a cover crop in weed control (Hoffman et al., 1993; Gerhards and Schappert, 2020). In this study, a negative correlation between the shoot biomass of the cover crop and the shoot biomass of the test weed oilseed rape was only observed in one of the two experimental years (Figures 8A, B). While cover crop shoot biomass was important for weed suppression under water-limited conditions in 2016 (Figure 1A), biomass production was not the mode of action under sufficient water supply in 2017 (Figure 1B). This is consistent with previous observations, which showed no correlation between cover crop shoot biomass and weed suppression (Schappert et al., 2019b; Smith et al., 2020). However, in both years, a suppressive effect was observed for some of the cover crop species or mixtures tested.

Pure stands of mustard and phacelia have been well described to reduce weed shoot biomass more effectively than small-seeded legumes under European field conditions (Brant et al., 2009; Brust et al., 2011). In the present study, the cover crops mustard, oil radish, and phacelia showed the best competitiveness against weeds regardless of the weather conditions. However, the suppressive effect was weaker for phacelia and showed a larger variation in the case of mustard and oil radish under wet conditions in 2017 (Figure 8B). In general, these observations were confirmed by other studies, which also found that pure stands of mustard, oil radish, and phacelia were able to suppress weeds effectively (Brust et al., 2014; Schappert et al., 2019a; Cottney et al., 2022; Tadiello et al., 2022), even under similar growing conditions as in southern Germany in 2016 and 2017 (Schappert et al., 2019a).

Other tested cover crop species, such as field bean and field pea, showed lower competitiveness than cruciferous species. At the same time, common vetch and Egyptian clover failed to outcompete weeds (Figure 8A). Previous studies reported the low competitiveness of pure legume stands against weeds compared to phacelia and oil radish (Cottney et al., 2022). An explanation for this is that the conditions of the locations may not fit this species. However, in northern Italy, Egyptian clover and common vetch as pure stands showed similar low biomass production (Tadiello et al., 2022) as in this study (Figures 3C, D, 5A, B), and hence lower competitiveness against weeds than mustard (Figure 8A). Our results are consistent with studies from Canada, which have shown that legumes have a lower competitiveness against weeds compared to crucifer or forb species (Wagg et al., 2021). Thus, neither the climatic conditions nor the daylength effect cause a low level of competitiveness of the small-seeded legume species.

The use of mixtures is often discussed as a strategy to increase the competitiveness of cover crops, as the species then complement each other in their suppressive effect on weeds. In our study, this hypothesis can only be confirmed in some cases: While mustard alone perfectly suppressed the test weed oilseed rape (Figures 8A, B) and showed a competitiveness factor of almost 100% (Figure 8C; Supplementaryray Figure S3), the addition of other species to form dual mixtures neither increased nor decreased the ability of mustard (Figure 8), regardless of whether such dual mixtures lead to an over-yielding effect (Figure 3). Other studies have shown similar results. For instance, the over-yielding of cover crop mixtures compared to the best-performing pure stands did not lead to benefits in weed suppression or other ecosystem services (Smith et al., 2014, 2020). The question then arises whether increasing the mixtures' biodiversity affects weed suppression. Schappert et al. (2019b) showed that mixtures containing three or more cover crop species have a reduced ability to suppress weeds compared to pure cruciferous crops. Lower soil coverage and shoot biomass best explained the lack of competitiveness.

Regarding weed suppression, the beneficial effects of competitive cover crops like mustard are rapid field emergence and soil coverage (Brust et al., 2014). These benefits are still present in dual mixtures containing mustard. Other mechanisms of action, such as allelopathic effects via released glucosinolates, were found to be 20% lower in dual mixtures than in pure crucifer stands (Couëdel et al., 2018a). For phacelia, the choice of a partner is more important, even though phacelia showed high competitiveness (99.2%, Figure 8C) under dry growing conditions (Brust et al., 2014; Schappert et al., 2018). In dual mixtures with legumes, phacelia could not fully compensate for the low competitiveness of legumes (Figure 8C), which slightly increased the observed test weed shoot biomass (Figure 8A). Whether this was due to the lower competitiveness of phacelia compared to mustard (Figure 3B), the tendentially lower biomass of the phacelia-legume mixtures (Figure 5), the lack of allelopathic effects (Couëdel et al., 2018a), or further characteristics of the mixture partners (Heuermann et al., 2023) could not be determined here. It remains to be noted that the mixtures showing over-yielding effects (Figures 5A–C) did not perform better in weed suppression (Figure 8).

A remaining open question is whether legumes, in general, are less competitive against weeds than non-legumes. Legume-only combinations were designed to investigate this further. All mixtures tested showed weaker competitiveness against test weeds than cruciferous species or phacelia (Figures 8A, C). Small-seeded legumes such as Egyptian clover and common vetch showed weak weed suppression and weak competitiveness even when combined with grain legumes with higher weed suppression, such as field bean or field pea (Figures 8A–C). Explanations for weak weed suppression by mixtures with legumes, especially small-seeded legumes, may be found in lower growth rates of the legumes compared to other species (Wendling et al., 2016) or poor performance under conditions of lower precipitation and temperature (Lavergne et al., 2021). Although the shoot biomass increased when two legumes were grown in mixtures, their competitiveness did not reach the level of the dual mixtures or the pure stands of the crucifer species (Figure 8).

In conclusion, our results indicate that weed suppression by cover crop mixtures is best achieved by the inclusion of a competitive species such as oil radish or mustard that effectively suppresses weeds even in combination with less competitive species such as field bean, field pea, common vetch, or Egyptian clover under different weather conditions. The use of phacelia is rather recommended under dry conditions. Under water-sufficient conditions, a mixture containing grain legumes as competitive partners, such as field beans or field peas, also performs satisfactorily.

5 Conclusion

In agricultural practice, cover crop mixtures are designed mainly through trial and error, consuming field trial resources to find the best-performing combinations. This study systematically evaluated two-component mixtures under two contrasting weather conditions. Our results show that competitive species in cover crop mixtures are essential for biomass production and weed suppression. Biomass formation showed over-yielding effects when competitive species, such as oil radish, mustard, and phacelia, and to a lower extent, field beans were combined in dual mixtures with species that form less above-ground biomass, such as common vetch or Egyptian clover. By contrast, additional species did not significantly enhance competitiveness against the test weed. Generally, the most competitive cover crops were the dominant ones, such as oilseed radish or mustard, while phacelia performed better, especially in dry conditions. For adaptions strategies, this means that cruciferous species such as mustard and oil radish form the basis of cover crop mixtures in crop rotations without oilseed rape. In oilseed rape rotations, the basic component is phacelia. The other species completing the mixtures must either have a high level of weed suppression or produce significantly less biomass than the main species to induce over-yielding effects in the cover crop mixture. Future research must determine which non-legumes are suitable as weak components to create optimized cover crop mixtures in legume-free rotations with high weed suppression potential. Generally, breeding programs to optimize species for cover crop mixtures and the identification of suitable seeding ratios for mixtures will be promising future strategies.

Data availability statement

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

Author contributions

JG: Conceptualization, Data curation, Formal analysis, Methodology, Visualization, Writing – original draft, Writing – review & editing. RK: Data curation, Visualization, Writing – review & editing. DH: Validation, Writing – review & editing. NG: Data curation, Formal analysis, Writing – review & editing. DS: Writing – review & editing. UF: Writing – review & editing. GG: Writing – review & editing. Nv: Writing – review & editing. BB: Conceptualization, Formal analysis, Methodology, Supervision, 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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fagro.2024.1416379/full#supplementary-material

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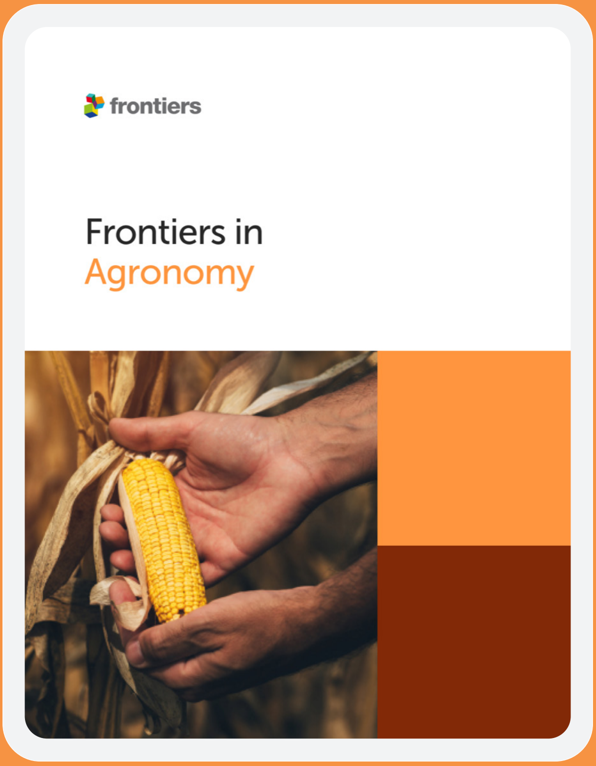

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