



Tailored cover crop mixtures enhance soil phosphorus cycling but not wheat yield in a short-term column experiment

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Abstract

Background and aims Phosphorus (P) accumulation as “legacy P” in soils presents a challenge and an opportunity for sustainable agriculture. Cover crops (CCs) have been investigated for their P-acquisition strategies, but studies mainly focus on single species. We examined whether CC mixtures enhance P cycling compared to individual CCs by improving P uptake, soil P dynamics, and supporting subsequent wheat growth.

Methods The CC species *Avena strigosa* Schreb. (black oat), *Phacelia tanacetifolia* Benth. (phacelia), *Trifolium alexandrinum* L. (berseem clover), *Fagopyrum esculentum* Moench (buckwheat), *Linum usitatissimum* L. (linseed), *Guizotia abyssinica* [Lf] Cass. (ramtil) and *Sinapis alba* L. (white mustard)

were cultivated individually or in mixtures in columns containing low-P soil over two 80-day cycles. After incorporating CC biomass, summer wheat was grown to maturity. Measurements included CC biomass and P uptake, soil pH, potential phosphatase activity, P fractions, and wheat P uptake and yield.

Results Five out of seven CC mixtures showed higher biomass than single species, and all mixtures achieved greater P uptake. Mixtures increased alkaline phosphatase activity compared to fallow and single CCs. After the wheat cycle, soil treated with mixtures had the highest labile P and reduced moderately labile P by ~35% more than single CCs. Nevertheless, wheat P uptake and yield remained largely unaffected.

Conclusion CC mixtures improved P mobilization, uptake, and phosphatase activity compared to single CCs, but this did not translate into improved wheat performance. The findings highlight the potential of CC mixtures to mobilize P, suggesting enhanced P use efficiency and soil P availability.

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Introduction

To supply the growing global demand for food, fiber and energy, phosphorus (P) fertilization has considerably increased in the past decades, reaching over 20 Mt P y⁻¹ in 2023 (USGS 2024a). However, P fertilization is often inefficient because phosphates can bind to soil clay minerals, Fe- and Al-(oxy)hydroxides, as well as precipitate with calcium depending on soil characteristics (Gérard 2016; Xiong et al. 2022). These processes result in the accumulation of sparingly bioavailable forms in the soil, termed “legacy P” (Sharples et al. 2013), which cannot be immediately accessed by crops (Richardson et al. 2011). Phosphorus fertilizer use is projected to increase from ~20 Mt in 2023 to ~22 Mt in 2050 (USGS 2024b), which will further increase the legacy P pool in agricultural soils. For improving P efficiency and cycling in agroecosystems it is vital to successfully explore this P pool, thereby reducing the need for P fertilizer inputs and P losses to the environment.

Cover cropping has long been recognised for its benefits, such as increasing the soil organic matter content, promoting nutrient cycling, reducing N leaching, and protecting soil from erosion and weed pressure (Fageria et al. 2005; Lichtfouse 2010; Thapa et al. 2018; Abdalla et al. 2019). Additionally, some cover crop (CC) species are considered efficient P miners (Hallama et al. 2019), contributing to P cycling through mechanisms such as increased root volume and specialized root architecture, rhizosphere acidification, exudation of organic acids, carboxylates, and phosphatases, as well as colonization with arbuscular mycorrhiza fungi (AMF) and stimulation of microbial activity. After die-off, the CC biomass is incorporated into the soil as litter and undergoes microbial mineralization (McLaughlin et al. 1988), which releases orthophosphates that can be taken up by subsequent cash crops, thereby contributing to P cycling (Eichler-Löbermann et al. 2008; Rosner et al. 2018; Hallama et al. 2019; Hansen et al. 2022). However, studies relating cover cropping to P availability and cycling have yielded varying results, ranging from detrimental (Damon et al. 2014; Almeida et al.

2019) to beneficial (Little et al. 2004; Njeru et al. 2014).

Most studies focus on the effects of pure CC stands in mobilizing sparingly available soil P. However, cultivating CC mixtures instead of single species has gained popularity worldwide (Elhakeem et al. 2019), as both scientific and practical agronomic literature have provided evidence that such systems tend to promote sustainability by increasing soil microbial diversity and activity (Lange et al. 2015), improving chemical, physical and biological soil health indicators (Heuermann et al. 2022; Gentsch et al. 2024) and showing a more stable year-to-year productivity (Elhakeem et al. 2019; Florence and McGuire 2020; Restovich et al. 2022; Zhang et al. 2023) compared to single species or fallow land (Elhakeem et al. 2019; Beillouin et al. 2021; Reiss and Drinkwater 2022). Furthermore, the incorporation of diverse plant residues into the soil can enhance microbial diversity and activity, thereby accelerating the turnover of organic material (Drost et al. 2020).

While the use of CC mixtures is a widespread and well accepted practice, P cycling and mobilization is rarely mentioned or considered as an additional benefit when tailoring such mixtures, despite growing evidence that tailoring CC mixtures by combining complementary P-acquisition traits could enhance P cycling and soil P status (Little et al. 2004; Hallama et al. 2019). Due to the limited number of studies on these effects, there remains a notable gap in our understanding of the interactions between CC species grown in mixtures and their impact on P acquisition, mobilization and cycling, which may benefit subsequent crops.

To contribute to closing this gap, we tailored seven CC mixtures, each combining three species with complementary characteristics to enhance P acquisition strategies. Our aim was to determine whether these mixtures could synergistically improve P uptake and, after incorporation of their biomass into the soil, enhance the P acquisition and yield of the subsequent spring wheat crop. We hypothesized that in comparison to fallow and single CC species, CC mixtures would: 1) have greater biomass production and P uptake 2) access soil P forms of lower lability more efficiently, thus improving soil P bioavailability and 3) increase P acquisition and yield of wheat through more efficient P cycling.

Material and methods

Experimental setup

We conducted an experiment under controlled conditions (greenhouse and climate chamber) to compare the impact of two cycles of single CC species, tailored mixtures and a fallow control treatment on soil P cycling and their influence on P uptake in a subsequent summer wheat (*Triticum aestivum* L. var. KWS EXPECTUM) cycle. Acrylic columns with a diameter of 23 cm and a height of 45 cm were used. To mimic the P availability gradient of a soil profile as well as to improve water drainage in the column, the bottom ~30 cm of each column was filled with 15 kg of a 1:1 (w/w) mixture of a subsoil collected from the 25–45 cm layer of a silty cambisol (IUSS Working Group 2014) from an arable plot located in Korning, Austria, (sieved to ≤ 1 cm) and quartz sand (≤ 2 mm), homogenized using a cement mixer. A 2-cm plastic mesh was placed on the surface to delineate the boundary between the subsoil and the topsoil layers. The remaining ~15 cm of the columns were filled with 7.5 kg of a topsoil, collected from the 0–15 cm surface layer of a decalcified clayey chernozem (IUSS Working Group 2014) from an arable plot in Langenlebarn, Austria. Both soils had been air-dried and sieved to a particle size of 1 cm.

To determine the maximum water holding capacity (MWHC) of the soils in the columns, 4 columns were saturated with water and left to drain. The MWHC was determined as the difference in weight between the columns with dry soil and their wet weight 24 h after saturation and drainage. Prior to establishment of the experiment, the pH of both soils (1:2.5 soil:solution [0.01 M CaCl_2]), total P (P_t , aqua regia digestion), Olsen-P (Olsen et al. 1954), soil organic carbon (SOC) through combustion using a soliTOC (Elementar Analysensysteme GmbH, Langenselbold, Germany), and texture were determined. The soil characteristics are given in Table 1.

The P fertilization history of the topsoil field reflects a transition from regular organic inputs to a period of minimal P application. For decades, the field has been managed in a crop rotation of maize every 3rd year, and sugar beet every 5th year. Until 2000, P was supplied exclusively through livestock manure, at rates of $\sim 20 \text{ t ha}^{-1}$ of fresh manure every five years, equivalent to an average of $\sim 6 \text{ kg P ha}^{-1} \text{ yr}^{-1}$. Over the past 25 years, however, manure application ceased, and the field has only received mineral P applications before maize or sugar beet crops, intending solely to replace crop P export. This prolonged period of reduce P inputs, followed by low mineral P doses has allowed the stabilization of soil legacy P over time, while phytoavailable P levels remained low.

Treatments

The treatments included a fallow control without CC cultivation, seven single CCs and seven tailored mixtures composed of three CC species, each set up in four replicates. The species tested were *Avena strigosa* Schreb. (black oat), *Phacelia tanacetifolia* Benth. (phacelia), *Trifolium alexandrinum* L. (berseem clover, referred to as clover), *Fagopyrum esculentum* Moench (buckwheat), *Linum usitatissimum* L. (linseed), *Guizotia abyssinica* [Lf] Cass. (ramtil), *Sinapis alba* L. (white mustard, referred to as mustard). All CC mixtures were tailored taking into account potential synergies in their P acquisition strategies based on previous results from studies of a wider range of CC species (Raniero and Santner 2024) and the broader literature. A comprehensive explanation of the rationale behind the tailored CC mixtures is provided in Supplementary Information S1.

Planting density was determined on an area basis to ensure adequate soil coverage, according to common regional practices in the Austrian fields where the soils were collected. Depending on the species, soil characteristics and management, plant densities

Table 1 Chemical and physical characteristics of the topsoil and the subsoil + sand mixture prior to the setup of the experiment

Soil	Collection depth cm	pH _{CaCl2}	Total P mg kg ⁻¹	Olsen P	SOC ² g kg ⁻¹	Sand g kg ⁻¹	Silt	Clay	MWHC ³ L col ⁻¹
Topsoil	0–15	6.5	702	3.33	25.3	168	388	444	10.3
Subsoil + Sand ¹	25–45	5.7	395	3.12	-	679	238	83	

¹1:1 (w/w) mixture, ²SOC: Soil organic carbon, ³MWHC Maximum water holding capacity

ranged from 200–700 plants m^{-2} . Considering the biomass production potential of each species in the same topsoil (Raniro and Santner 2024), the plant density was then converted to the pot size, resulting in the final densities used in this study. In the mixtures, the density of each species was set at one third of its individual planting density. This approach ensured that the total plant density in the mixtures remained comparable to the single-species treatments, facilitating direct comparisons between single crops and mixtures and their generalized effects. Plants were sown with a 50% higher number of seeds than the desired final plant density and were thinned eight days after sowing (DAS). The crops present in each treatment, their abbreviations, and total plant density per column are shown in Table 2.

Fertilization

No P fertilizer was applied in any of the treatments or cycles. All other nutrients were supplied to the topsoil (7.5 kg) at 5 DAS in each cycle, when each column was fertilized with N (230 mg kg^{-1}), K (310 mg kg^{-1}), Mg (45 mg kg^{-1}), S (65 mg kg^{-1}) and Ca (107 mg kg^{-1}). At 20 DAS, B (0.3 mg kg^{-1}), Cu (1.5 mg kg^{-1}), Fe (3 mg kg^{-1}), Mn (3 mg kg^{-1}), Mo (0.3 mg kg^{-1}), Zn (3 mg kg^{-1}). A second application of N (150 mg kg^{-1}) and K (50 mg kg^{-1}) was made at

50 DAS in each cycle. The nutrient forms, applied as solutions, are shown in Supplementary Table S1.

Although the nutrient doses applied were higher than the doses typically used in agricultural settings, this approach was necessary to compensate for the limited soil volume available to the plants compared to field conditions. The goal was to ensure that nutrient availability (other than P) did not limit the growth of the crops, aiming to ensure that nutrients were not limiting for the growth of the CCs and the wheat. This strategy allowed us to isolate the P effects on crop performance, focusing on gaining a mechanistic understanding of P dynamics, rather than simulating field fertilization practices.

Experimental cycles

To assess the effects of the preceding CC species and mixtures on wheat development, P acquisition, and soil P availability, two consecutive 80-days CC cycles were conducted, followed by a CC biomass soil incorporation and incubation cycle, and finally a cycle of wheat cultivation to maturity. A simplified timeline of cycles, sampling and analyses can be seen in Fig. 1.

Cover crop cycles

The first CC cycle ran from January 14th (sowing) to April 3rd, 2023 (shoot harvest), followed by

Table 2 Cover crop species used in each treatment, treatments abbreviations, and final plant densities used in the experiment

Treatment	Black oat	Phacelia	Clover	Buckwheat	Linseed	Ramtil	Mustard	Total
Number of plants column^{-1} cycle ⁻¹								
Fallow (Control)	-	-	-	-	-	-	-	0
Black oat	15	-	-	-	-	-	-	15
Phacelia	-	12	-	-	-	-	-	12
Clover	-	-	21	-	-	-	-	21
Buckwheat	-	-	-	12	-	-	-	12
Linseed	-	-	-	-	21	-	-	21
Ramtil	-	-	-	-	-	12	-	12
Mustard	-	-	-	-	-	-	12	12
BO + LS + RT	5	-	-	-	7	4	-	16
BO + BW + MT	5	-	-	4	-	-	4	13
BO + RT + MT	5	-	-	-	-	4	4	13
LS + PH + BC	-	4	7	-	7	-	-	18
RT + PH + MT	-	4	-	-	-	4	4	12
BW + PH + BC	-	4	7	4	-	-	-	15
BW + LS + BC	-	-	7	4	7	-	-	18

Abbreviations: *BO* Black oat; *PH* Phacelia; *BC* Clover; *BW* Buckwheat; *LS* Linseed; *RT* Ramtil; *MT* Mustard

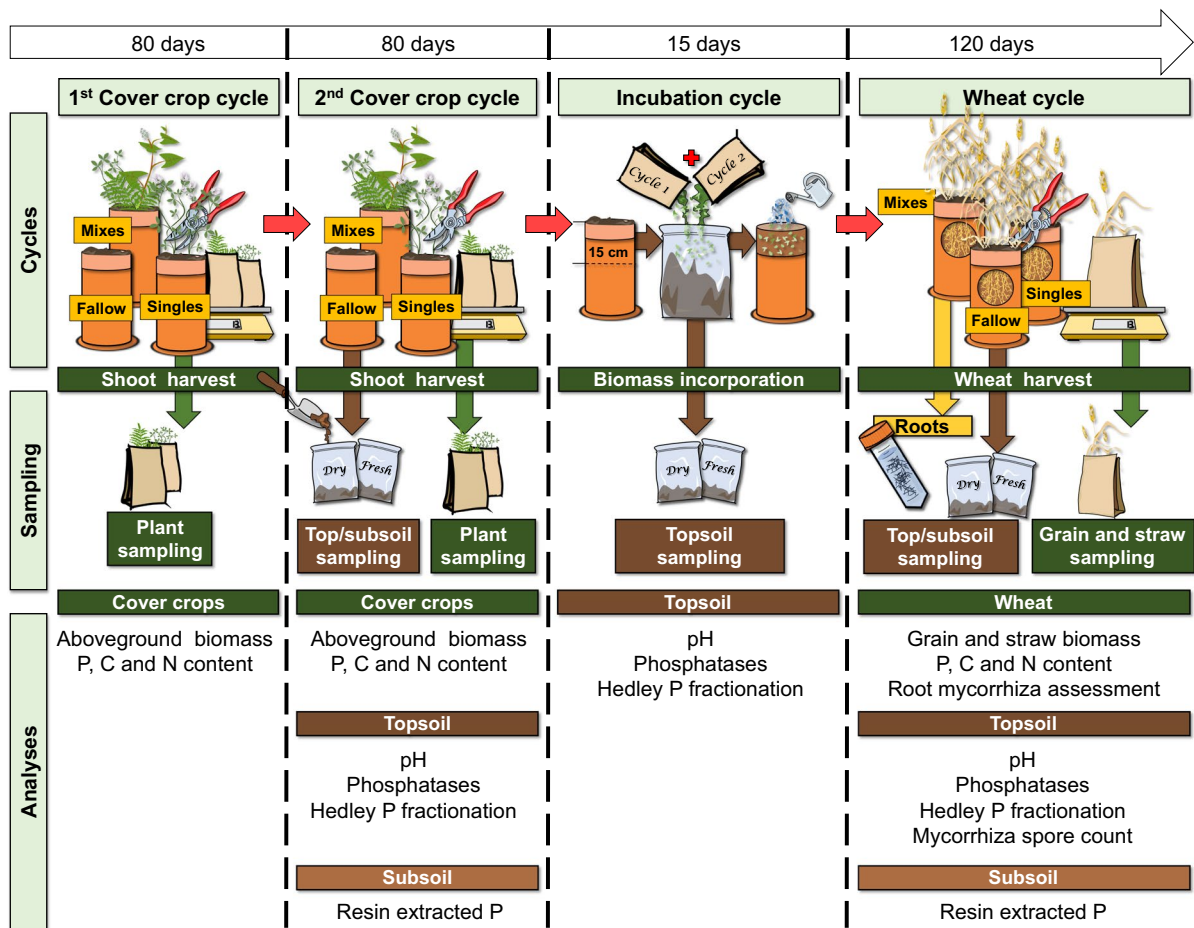


Fig. 1 A simplified timeline of the experimental cycles, samplings and analyses. All samples were taken at the end of the cycles

a second cycle sown on April 4th and harvested on June 21 st, 2023. Although it is unusual to have two CC cycles before a cash crop, this approach was necessary, because, despite the use of large soil columns, the plants had outgrown their optimal conditions after 80 days. In the region of Lower Austria, AT, CC cycles typically last between 100 and 140 days, so the second 80-day cycle allowed us to more closely align with regional practices and allowed CC roots to interact with the soil matrix for a growth period. The decision to extend the duration of the CC cycles was taken to enhance CC effects on the succeeding wheat crop, while acknowledging that this adds complexity to the system and may differ from agricultural practice. Before each cycle, large surface roots

were removed from the soil to facilitate the sowing and establishment of succeeding plants. Throughout the cycles, the greenhouse was maintained at a temperature range of 30 °C (day) to 16 °C (night), and soil humidity was kept between 60–70% MWHC by manual watering on a weight basis.

Cover crop harvest and sampling After each CC cycle, all aboveground plant parts were harvested. For the CC mixture treatments, the shoot biomass of each species was separated to determine their individual contributions to the total biomass of each mixture. The harvested biomass was oven-dried at 70 °C for 48 h, weighed, and a subsample was collected for analyses.

Cover crop biomass incubation cycle

After the second CC cycle, the total aboveground plant biomass from both cycles for the same replicates (termed “accumulated biomass” in this study) was cut to pass a 1 cm sieve and thoroughly mixed. On July 3rd, the topsoil (0 to ~15 cm) was sampled and transferred from the columns into plastic bags. The accumulated dry CC biomass from both cycles was thoroughly incorporated back into the topsoil of the same columns (replicate) in which the CC had grown by shaking the plastic bags by hand. After mixing well, the CC amended soils were returned into the columns and the soil was rewetted, keeping the soil moisture between 30 and 40% MWHC for the next 15 days to promote microbial activity and allow for biomass mineralization.

The biomass incorporated into the topsoil layer (0–15 cm) of each column would correspond to a dry biomass incorporation rate between 8 and 28 t ha⁻¹, based on a ploughing depth of 15 cm and a soil density of 1.2 g cm⁻³. Although we acknowledge that the upper limit of 28 t ha⁻¹ is exceptionally high compared to the usual 5–8 t ha⁻¹ often practiced in the region, the decision to reincorporate all the biomass produced was taken to ensure that all the P taken up by the crops was returned to the columns, thus maintaining equal P budgets across all treatments. Furthermore, applying large amounts of biomass was intended to amplify CC-related effects and provide deeper insights into shifts in soil P and organic matter dynamics.

Wheat cycle

On July 18 th, after the incubation period, 23 seeds of wheat were sown in each column at 3 cm depth, which were thinned to the desired density of 15 seeds per column at 8 DAS. This plant density was chosen to approach field conditions in the region, which often use 300–400 plants m⁻² (13–16 plants col⁻¹ in an area basis). Wheat plants were grown until maturity (120 days) in a climatic chamber with a 16 h day cycle at 21 °C with a light intensity of 150 µmol m⁻² s⁻¹ and an 8 h night cycle at 16 °C with no light, both at 50% relative humidity. Soil moisture was maintained within 60–70% MWHC through the cycles by manually watering on a weight basis.

Wheat harvest and sampling

At wheat harvest, ears and straw (leaves and stalk) were collected separately. All aboveground plant samples were oven-dried at 70 °C for 48 h and weighed for biomass determination. The wheat biomass was divided into straw and grain, the latter being separated from the ears using a mechanical thresher. The remaining ear tissue was incorporated into the respective straw samples. After harvest, thin wheat roots were sampled from the topsoil layer (0–15 cm), thoroughly washed with tap water, and stored in falcon tubes containing a 30% ethanol solution (v/v).

Plant analyses

Both CC and wheat aboveground plant samples were milled to a powder and microwave digested (HNO₃ + H₂O₂) for the determination of the P content of CC shoots, wheat shoots and wheat grain by ICP-OES (Optima 8300, PerkinElmer, Massachusetts, USA). The C and N contents of the plant tissues were determined using a CN analyzer (vario MACRO cube, Elementar Analysensysteme GmbH, Hanau, Germany). Additionally, the sampled wheat roots were used to determine AMF colonization rates. To visualize AMF structures, wheat roots were cleared in 10% KOH for seven minutes at 90 °C and rinsed with tap water (Vierheilig et al. 1998). Afterwards, roots were stained by immersion in a hot (90 °C) 5% ink-vinegar solution for 5 min. The stained roots were incubated in tap water overnight and stored in 30% ethanol. Colonization by AMF was determined under a microscope (SZ61, Olympus, Tokyo, Japan) by evaluating thirty 0.5 cm long root pieces per sample for mycorrhizal intensity and vesicle/arbuscule abundance (Trouvelot et al. 1986), using 200–400 × magnification. Roots of CCs were not collected for AMF determination due to difficulties in identifying and separating roots from different species grown in mixtures as well as the impossibility to collect representative root biomass without affecting the soil's P budget.

The phosphorus use efficiency (PUE) of wheat was calculated according to Eq. 1. The wheat harvest index and P harvest index were calculated according to Eqs. 2 and 3 respectively.

$$PUE = \frac{\text{Wheat grain yield} (g \text{ col}^{-1})}{\text{Wheat above ground P uptake} (mg \text{ col}^{-1})} \quad (1)$$

$$\text{Wheat harvest index} = \frac{\text{Wheat grain yield} (g \text{ col}^{-1})}{\text{Total above ground wheat biomass} (g \text{ col}^{-1})} \quad (2)$$

$$\text{Wheat P harvest index} = \frac{\text{P in wheat grain} (mg \text{ col}^{-1})}{\text{Total P in above ground wheat biomass} (mg \text{ col}^{-1})} \quad (3)$$

Soil sampling and analyses

Topsoil samples (0–15 cm) were collected at the end of the second CC cycle, the incubation period, and the wheat cycle. Due to the large volume of roots present in all treatments after each plant cycle, all topsoil samples were collected by gently shaking the roots into a plastic bag and were considered to be rhizosphere soil. Since no significant amounts of bulk soil remained in the columns, this method provided a more reliable sampling approach, ensuring that soil under similar root-influenced conditions was sampled at every sampling time. In addition to the topsoil samples, subsoil samples were collected after the second CC cycle and the wheat cycle. All topsoil samples were divided into two subsamples (a) and (b). Subsamples (a) were kept refrigerated (4 °C) at ambient humidity and used for the assessment of the potential phosphatase activity, as determined by the p-nitrophenol released from the soil after incubation at 27 °C (average temperature across cycles) with a p-nitrophenyl phosphate (Sigma N4645) buffered solution at pH 6.5 for acid phosphatase (ACP_{ASE}) and at pH 11.0 for alkaline phosphatase (ALP_{ASE}, Tabatabai and Bremner 1969). Additionally, both soil-only and substrate-only (soil-free) controls were included for a better control of abiotic hydrolysis, as proposed by (Daughtridge et al. 2021).

Topsoil subsamples (b) were air-dried, sieved to ≤ 2 mm and further divided into three subsamples (bi, bii, and biii). Subsample (bi) was used to determine soil pH (1:2.5 soil:solution [0.01 M CaCl₂]). Subsample (bii) was used to assess AMF spore density by wet sieving and decanting 10 g of soil in triplicates according to Gerdemann and Nicolson (1963) using cellulose nitrate filters (Ø

50 cm, 0.65 µm pore size, Sartorius Stedim Biotech GmbH, Göttingen, Germany). Only mature, whole spores were counted. Finally, subsample (biii) was subjected to the Hedley's soil P fractionation (Hedley et al. 1982), with modifications (Rheinheimer 2000). The fractionation allows the extraction of inorganic (P_i) and organic (P_o) P forms, which can be grouped into soil P pools of decreasing bioavailability. The labile pool contains anion exchange resin extracted P_i (AER-P) and P_i and P_o extracted using 0.5 M NaHCO₃ at pH 8.5 (NaHCO₃-P_i and -P_o). The moderately labile pool is composed of 0.1 M NaOH extracted P_i and P_o (0.1 M NaOH-P_i and -P_o) and 1 M HCl extracted P_i (HCl-P_i). The non-labile pool is composed of the 0.5 M NaOH extracted P_i and P_o (0.5 M NaOH-P_i and -P_o) as well as the residual P, obtained after H₂SO₄ + H₂O₂ digestion. All P determinations were performed using the phosphomolybdate blue method (Murphy and Riley 1962).

Statistical analysis

All data were tested for homogeneity of variance and normality of the residuals using the Levene and Shapiro–Wilk tests, respectively. Subsequently, an analysis of variance (ANOVA) was conducted to test for an effect of the CC treatment on the measured variables. To compare the variables topsoil pH, ACP_{ASE}, ALP_{ASE} and topsoil labile P across treatments and cycles, mixed models were applied due to the irregular intervals between sampling times and variations in biomass incorporation rates, which violated the assumption of independence of the residuals. The optimal model was chosen using the likelihood ratio test, and additional selection criteria, such as the Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC), were used when necessary. The significance of fixed effects was determined through the Wald-F test. Predicted means were calculated for all cases and treatment comparisons were conducted using Tukey's Honestly Significant Difference (HSD) test.

HSD values are shown as error bars of the mean values of the mixtures in the figures, to facilitate the comparison of any two mean values from any cycles after establishment. If a data point falls within the HSD error bar of another data point, they are not significantly different at the $p \leq 0.05$ significance level. In these figures,

the lines connecting each data point serve only as visual aid and do not represent the behaviour of the response variables between sampling dates. Additionally, to determine statistical differences between the single CC and mixed CC groups, an independent ANOVA was performed, considering all single and mixed species as the same treatment ($n = 28$). The significance threshold was set at $p \leq 0.05$ for all analyses. All tests were performed using RStudio version 4.2.2.

To evaluate the observations of crop mixtures against predicted values derived from their single-species components, the “components’ average” for each parameter was calculated. Therefore, the three parameter values observed for the single-CC treatments corresponding to each species in the mixtures were averaged, since each species in the mixture was sown at one third of the seed density used in its respective single-CC treatment. In addition, the standard deviations of each components’ average were calculated using the Kragten’s propagation of uncertainty method (Kragten 1994). This method circumvents the challenge of arbitrarily pairing replicates from different species, which would lead to subjective and potentially inconsistent results. Instead, the average and its uncertainty (standard deviation) were calculated independently for each species from its replicates. The propagated uncertainties account for the uncertainty in the predicted total without requiring direct replicate-to-replicate combinations. In this way, mixture averages could be statistically compared to the components’ averages using a two-sample *t*-test. The predicted values (components’ average and standard deviation) were treated as a derived estimate, while the observed values came directly from the mixture replicates. This method provides a robust framework for evaluating synergy or antagonism in crop mixtures relative to their single-species components. Specific information on Kragten’s uncertainty propagation methodology can be found in (JCGM 1995; Kreuzeder et al. 2015).

Results

Plants biomass and nutrient uptake

Cover crops

The accumulated shoot biomass of all CC treatments (Table 3) ranged from $34.8 \pm 5.2 \text{ g col}^{-1}$ for clover to $118.2 \pm 9.3 \text{ g col}^{-1}$ for buckwheat. The BW +LS

+BC treatment produced the highest accumulated biomass for the mixtures, which was statistically comparable to buckwheat and BO +BW +MT. The mixtures BO +BW +MT and BW +LS +BC had significantly higher accumulated shoot biomass production compared to the components’ averages, with an increment of $+47.7 \text{ g col}^{-1}$ and $+27.9 \text{ g col}^{-1}$, respectively. Only the mixtures BO +LS +RT and LS +PH +BC had lower biomass than their components’ averages, with losses of 2 and 4%, respectively, which were not statistically significant.

The accumulated P uptake of the CCs (Table 3) did not follow the same pattern as observed for the biomass and ranged from $100.1 \pm 19.5 \text{ mg col}^{-1}$ for clover to $251.3 \pm 18.9 \text{ mg col}^{-1}$ for BW +LS +BC. Buckwheat and mustard produced the highest biomasses for the individual CCs, but their P uptake was only significantly higher than that of the clover. The highest P uptakes observed were the BW +LS +BC and the BW +PH +BC mixtures. While the former had a significantly higher P uptake than buckwheat despite producing comparable biomass, the latter was comparable to both buckwheat and BW +LS +BC, despite its significantly lower biomass production. Significantly higher P uptake than their components’ averages was observed in all buckwheat-containing mixtures. Additional C, N and C:N ratio data from the CCs are available in the Supplementary Table S2.

Wheat biomass and nutrient concentrations

The wheat’s grain and straw biomass and P uptake following the CC cycles (single or mixtures) or fallow (control) can be seen in Fig. 2A and B, respectively. No differences in wheat grain yield were observed between any of the treatments including the control, with an overall grain yield average of $41.9 \pm 4.5 \text{ g col}^{-1}$. Wheat straw biomass differed significantly only between linseed ($59.9 \pm 2.4 \text{ g col}^{-1}$) and BO +BW +MT ($45.8 \pm 14.2 \text{ g col}^{-1}$). The control treatment (fallow) showed the lowest P uptake in wheat shoots ($107.5 \pm 6.8 \text{ mg col}^{-1}$), significantly lower than LS +PH +BC ($149.8 \pm 7.8 \text{ mg col}^{-1}$) and ramtil ($150.3 \pm 18.1 \text{ mg col}^{-1}$), which were comparable to all other treatments. No statistically significant differences were found for the total N in the wheat aboveground biomass under any treatments, with an average $0.49 \pm 0.09 \text{ g col}^{-1}$ in the straw, and $1.15 \pm 0.13 \text{ g col}^{-1}$ in the grain, for a total of $1.65 \pm 0.18 \text{ g col}^{-1}$.

Table 3 Accumulated (sum of cycles 1 and 2) cover crop shoot biomass and P uptake after two cycles of 80 days each

Single cover crops	Accumulated shoot biomass (g col ⁻¹)	Tukey ¹ HSD grouping	Cover crop mixtures	Accumulated shoot biomass				Tukey HSD grouping	Components' average (g col ⁻¹)
				Species 1	Species 2	Species 3	Total		
Clover (BC)	34.5 ± 5.25	h	LS+PH+BC	6.20 ± 1.91	31.8 ± 7.30	6.30 ± 2.56	44.2 ± 5.88	gh	46.0 ± 1.92
Linseed (LS)	47.5 ± 1.42	gh	BO+LS+RT	22.9 ± 2.61	12.2 ± 2.45	20.5 ± 3.33	55.6 ± 1.60	fg	56.8 ± 2.63
Black oat (BO)	55.2 ± 7.30	fg	BO+RT+MT	15.2 ± 1.73	12.3 ± 1.57	46.9 ± 6.95	74.4 ± 6.60	de	72.8 ± 3.84
Phacelia (PH)	56.1 ± 1.94	fg	RT+PH+MT	12.1 ± 3.56	17.1 ± 5.11	55.1 ± 4.75	84.4 ± 9.27	cd	73.1 ± 3.02
Ramtil (RT)	67.7 ± 2.56	ef	BW+PH+BC	61.8 ± 3.56	24.2 ± 5.66	11.5 ± 2.02	97.4 ± 6.75	cd	69.6 ± 3.62
Mustard (MT)	95.4 ± 8.52	c	BO+BW+MT	15.2 ± 4.65	42.5 ± 3.52	42.1 ± 8.22	99.8 ± 9.06*	bc	89.6 ± 4.86
Buckwheat (BW)	118.2 ± 9.32	a	BW+LS+BC	80.6 ± 6.57	20.5 ± 2.59	13.2 ± 0.58	114.4 ± 7.58*	ab	66.7 ± 3.60

Single cover crops	Accumulated shoot P uptake (mg col ⁻¹)	Tukey grouping	Cover crop mixtures	Accumulated shoot P uptake				Tukey grouping	Components' average (g col ⁻¹)
				Species 1	Species 2	Species 3	Total		
Clover (BC)	100.1 ± 19.5	d	LS+PH+BC	24.3 ± 6.04	112.2 ± 17.0	14.1 ± 7.13	150.5 ± 9.7	cd	144.3 ± 11.9
Linseed (LS)	162.1 ± 22.0	c	BO+LS+RT	67.3 ± 1.74	48.4 ± 13.8	46.8 ± 11.0	162.5 ± 17.7	c	158.4 ± 11.6
Black oat (BO)	149.6 ± 22.9	cd	BO+RT+MT	43.1 ± 7.00	32.8 ± 3.47	96.6 ± 8.59	172.5 ± 8.5	bc	163.9 ± 13.2
Phacelia (PH)	170.7 ± 20.3	bc	RT+PH+MT	26.6 ± 6.01	43.8 ± 10.8	127.8 ± 12.6	198.3 ± 16.0	bc	170.9 ± 12.7
Ramtil (RT)	163.4 ± 13.9	c	BW+PH+BC	128.4 ± 17.4	60.1 ± 8.79	26.5 ± 4.65	215.1 ± 19.6 *	ab	150.9 ± 11.6
Mustard (MT)	178.7 ± 29.2	bc	BO+BW+MT	37.5 ± 14.9	71.0 ± 13.5	87.3 ± 15.3	195.9 ± 31.9 *	bc	170.1 ± 14.1
Buckwheat (BW)	182.0 ± 20.5	bc	BW+LS+BC	155.6 ± 13.9	71.4 ± 5.49	24.2 ± 2.82	251.3 ± 18.9 *	ab	148.6 ± 11.9

Legend

- More than 10% higher than components' average
- Up to 10% higher than components' average
- Equal to components' average
- Up to 10% lower than components' average
- More than 10% lower than components' average

Data presented represents averages ± standard deviation. ¹ Tukey HSD grouping between each treatment ($n = 4$) is represented by the letters, where different letters represent statistically significant differences at $p \leq 0.05$. Asterisks (*) represent significant differences between the mixture averages and their components' averages in a t -test at $p \leq 0.05$. Abbreviations: BC Clover; LS Linseed; BO Black oat; PH Phacelia; RT Ramtil; MT Mustard; BW Buckwheat

The data for PUE, wheat harvest index and P harvest index are presented in Table 4. The highest wheat PUE was observed for the control, significantly higher than most treatments, except for buckwheat and BW +LS +BC, which were statistically similar. The wheat following buckwheat also showed a high harvest index, although the highest value was observed for the wheat following BO +BW +MT, statistically higher than the wheat after clover, linseed, black oat, mustard and the mixture LS +PH +BC.

Changes in topsoil pH

Topsoil pH (Fig. 3) reached its lowest value in all treatments after the CC cycles, except in the control (no CCs), where pH remained constant (6.64 ± 0.03) over the two cycles. The lowest pH values were observed under clover (5.28 ± 0.22), linseed (5.31 ± 0.13) and LS +PH +BC (5.36 ± 0.12). Only BO +RT +MT and mustard did not differ significantly from the control after both cycles, with pH values of 6.21 ± 0.15 and 6.20 ± 0.23 , respectively. After the wheat cycle, only the control treatment showed a decrease in pH to 6.34 ± 0.17 . All other treatments experienced a net alkalization after the wheat when compared to after the CC cycles, with significant

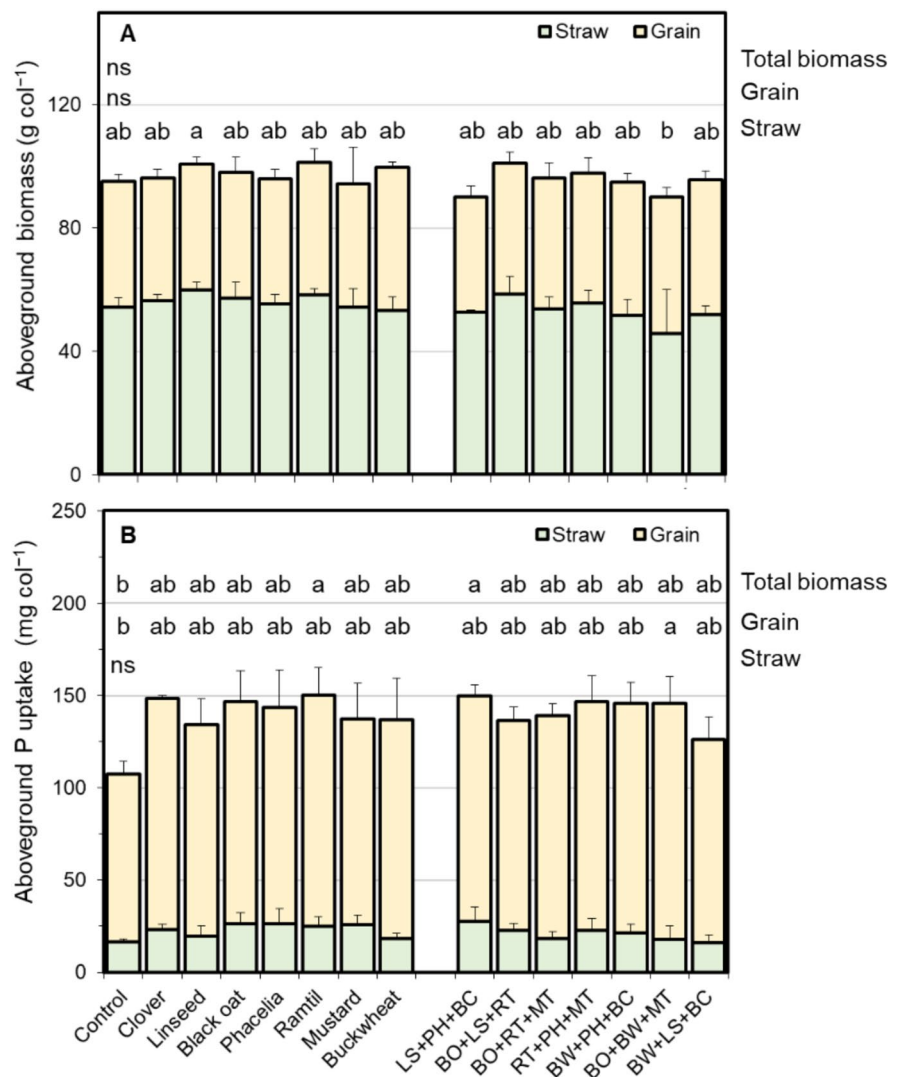
differences from the control only observed in the soils which sustained the growth and were incorporated with the biomasses of BO +LS +RT (6.06 ± 0.10), black oat (6.02 ± 0.18), clover (6.00 ± 0.08) and linseed (5.78 ± 0.15).

Significant higher pH levels compared to the components' averages were observed for BO +RT +MT, RT +PH +MT and BW +LS +BC after the CC cycles. In addition, the mixtures LS +PH +BC, BW +PH +BC and BW +LS +BC showed higher soil pH after the wheat cycle when compared to their components' averages.

Mycorrhizal root colonization and spore density

Mycorrhizal colonization rates in the roots of wheat were evaluated, however, either the AMF structures were completely absent, or only remnants of old or decayed structures were detected. Consequently, the dataset lacks sufficient validity and, thus, is not reported. Nevertheless, the density of AMF spores after wheat was assessed (Supplementary Figure S1) and varied from 964 ± 367 to 3307 ± 1130 spores kg⁻¹ of soil, under BO +LS +RT and LS +PH +BC, respectively. All clover-containing mixtures showed particularly high spore counts, which

Fig. 2 Wheat above-ground biomass (**A**) and P uptake (**B**) following cover crop cycles and biomass incorporation. Error bars represent standard deviation ($n = 4$). Different letters in the same row represent significant differences, and “ns” means no significance in the HSD Tukey test at $p \leq 0.05$, where the first row (from top to bottom) represents the total biomass, the second represents the grain and the third represents the straw. Abbreviations: BC: Clover, LS: Linseed, BO: Black oat, PH: Phacelia, RT: Ramtil, MT: Mustard, BW: Buckwheat



were significantly higher than those of BO +LS +RT, buckwheat (998 ± 147 spores kg^{-1}) and black oat (1015 ± 254 spores kg^{-1}). Only LS +PH +BC increased spore count significantly when compared to the control (1480 ± 297 spores kg^{-1}), but no spore density was significantly lower than the latter. The discussion on CC effects on AMF can be found in Supplementary Information S2.

Potential activity of phosphatases

The potential activities of acid and alkaline phosphatases can be seen in Figs. 4 and 5, respectively. The control treatment had the lowest and constant activity levels for both phosphatases before the wheat,

at an average (CC cycles and incubation) of 113.7 ± 12.1 $\text{mg h}^{-1} \text{kg}^{-1}$ for ACP_{ASE} and 83.2 ± 12.4 $\text{mg h}^{-1} \text{kg}^{-1}$ for ALP_{ASE} . Significant peaks in the potential activity of both phosphatases were observed for all other treatments after the CC cycles and the biomass incubation period. After the CC cycles, four individual crops (mustard, phacelia, buckwheat and black oat) and four mixtures (BO + RT + MT, RT + PH + MT, BO + BW + MT, BW + PH + BC) showed significantly higher ACP_{ASE} than the control. Among those, the only significant difference was for the black oat (292.7 ± 112 $\text{mg h}^{-1} \text{kg}^{-1}$), which was lower than BO + RT + MT (457.3 ± 64.0 $\text{mg h}^{-1} \text{kg}^{-1}$). Furthermore, nine treatments had significantly higher ALP_{ASE} than the control after the CC

Table 4 Comparative analysis of wheat's P use efficiency, harvest indices after cover crop cycles and soil amendments with individual cover crop species and mixtures

Treatments	PUE (g mg ⁻¹)	Tukey HSD grouping	Harvest index	Tukey HSD grouping	P harvest index	Tukey HSD grouping
Control	0.380 ± 0.010	a	0.430 ± 0.015	ab	0.845 ± 0.016	NS
Clover	0.268 ± 0.013	bc	0.413 ± 0.019	b	0.845 ± 0.018	
Linseed	0.307 ± 0.037	abc	0.405 ± 0.018	b	0.853 ± 0.026	
Black oat	0.280 ± 0.023	bc	0.415 ± 0.010	b	0.822 ± 0.027	
Phacelia	0.289 ± 0.040	abc	0.423 ± 0.009	ab	0.818 ± 0.032	
Ramtil	0.288 ± 0.019	abc	0.425 ± 0.021	ab	0.833 ± 0.024	
Mustard	0.291 ± 0.080	abc	0.418 ± 0.050	b	0.808 ± 0.053	
Buckwheat	0.345 ± 0.049	ab	0.466 ± 0.014	ab	0.866 ± 0.021	
LS + PH + BC	0.250 ± 0.026	c	0.415 ± 0.025	b	0.816 ± 0.048	
BO + LS + RT	0.311 ± 0.014	abc	0.420 ± 0.020	ab	0.835 ± 0.019	
BO + RT + MT	0.307 ± 0.043	abc	0.441 ± 0.024	ab	0.871 ± 0.020	
RT + PH + MT	0.289 ± 0.036	abc	0.431 ± 0.033	ab	0.847 ± 0.037	
BW + PH + BC	0.297 ± 0.015	abc	0.455 ± 0.007	ab	0.853 ± 0.019	
BO + BW + MT	0.306 ± 0.042	abc	0.502 ± 0.093	a	0.877 ± 0.043	
BW + LS + BC	0.348 ± 0.030	ab	0.456 ± 0.028	ab	0.873 ± 0.018	

Values represent averages ± standard deviation ($n = 4$). Letters represent significant differences according to Tukey's HSD test at $p \leq 0.05$. *NS* Non-significant. Abbreviations: *BC* Clover; *LS* Linseed; *BO* Black oat; *PH* Phacelia; *RT* Ramtil; *MT* Mustard; *BW* Buckwheat

cycles. These were BO + RT + MT, buckwheat, BO + BW + MT, RT + PH + MT, mustard, BW + PH + BC, BW + LS + BC, BO + LS + RT and phacelia, within the range of $303.3 \pm 33.0 \text{ mg h}^{-1} \text{ kg}^{-1}$ (BO + RT + MT) to $203.1 \pm 61.1 \text{ mg h}^{-1} \text{ kg}^{-1}$ (phacelia).

After biomass incubation, all treatments further increased the potential activity of both phosphatases. Mustard had the highest ACP_{ASE} after this cycle ($567.8 \pm 37.6 \text{ mg h}^{-1} \text{ kg}^{-1}$), while only clover, ramtil, and linseed did not differ significantly from the control. The relative increase in ALP_{ASE} in this period was even greater, with some treatments more than doubling their potential activity. Of the seven treatments with comparably high and significantly greater ALP_{ASE} than all others, five were mixtures, with only mustard and buckwheat showing such high ALP_{ASE} activities. The mixture LS + PH + BC was the only one comparable to the control treatment, along with the single clover, linseed, black oat and ramtil.

After the wheat, only the control's potential phosphatase activities increased, although not significantly in relation to the incubation period, reaching $98.7 \pm 9.47 \text{ mg h}^{-1} \text{ kg}^{-1}$ for ALP_{ASE} and $169 \pm 19.3 \text{ mg}$

$\text{h}^{-1} \text{ kg}^{-1}$ for ACP_{ASE} . All other treatments showed a reduction in potential ACP_{ASE} and ALP_{ASE} . While the control's ACP_{ASE} was higher than that of linseed ($134.8 \pm 7.49 \text{ mg h}^{-1} \text{ kg}^{-1}$), clover ($114 \pm 16.5 \text{ mg h}^{-1} \text{ kg}^{-1}$) and ramtil ($112 \pm 6.35 \text{ mg h}^{-1} \text{ kg}^{-1}$) after wheat, they were not statistically different. The mixture LS + PH + BC was the only treatment which had previously higher ACP_{ASE} than the control but was no longer significantly different after wheat. For ALP_{ASE} only linseed and clover did not differ from the control. Buckwheat and mustard were still among the highest for ALP_{ASE} after wheat, but all other single crops were below the CC mixtures.

The treatment BO + RT + MT was the only to promote higher ACP_{ASE} than its components' averages in all sampling points, while BW + LS + BC was statistically higher only after incubation and the wheat cycle. Furthermore, only the mixtures LS + PH + BC and BO + LS + RT did not promote statistically different ALP_{ASE} levels compared to their components' averages in any sampling point, while all other mixtures showed significant differences, especially after the incubation cycle.

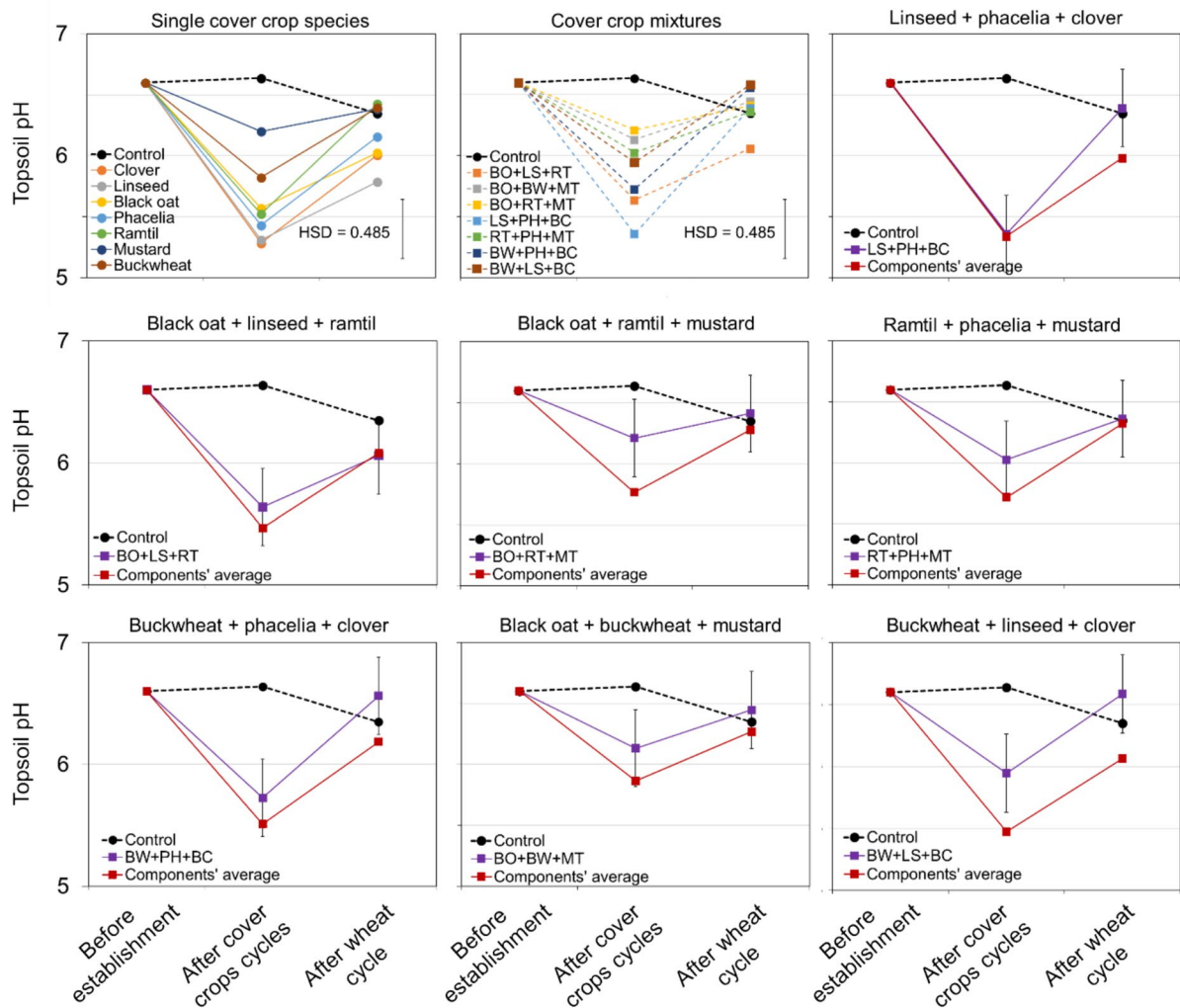


Fig. 3 Changes in topsoil pH at different sampling points under fallow, single cover crops and cover crop mixtures. The red lines show the average values of the three single crops composing each mixture for comparison. Lines connecting data points are for visual aid only. Error bar represents the

Tukey test's HSD value, which allows the comparison of any two data points except "before establishment" ($n = 4$). Abbreviations: BC: Clover, LS: Linseed, BO: Black oat, PH: Phacelia, RT: Ramtil, MT: Mustard, BW: Buckwheat

Changes in soil P pools and fractions

The topsoil used in this experiment contained $702 \text{ mg kg}^{-1} \text{ P}_i$, ~7% of which was in the labile P pool, ~24% in the moderately labile P pool and ~69% in the non-labile P pool. All treatments significantly reduced the labile and moderately labile topsoil P pools after the wheat cycle (end of the experiment). The non-labile pool remained constant throughout the experiment, and no differences were observed between treatments. The full dataset on topsoil P fractions after CC cycles,

biomass incubation, and non-labile P fractions after CC cycles and wheat, can be found in the Supplementary Tables S3, S4, and S5, respectively.

Soil labile P pool and fractions

Significant reductions in the total topsoil labile P pool ($\text{AER-P} + \text{NaHCO}_3\text{-P}_i$ and -P_o , Fig. 6) after the CC cycles were observed for all treatments except the control, in which labile P remained constant until the wheat cycle, at an average of $51.4 \pm 0.94 \text{ mg kg}^{-1}$.

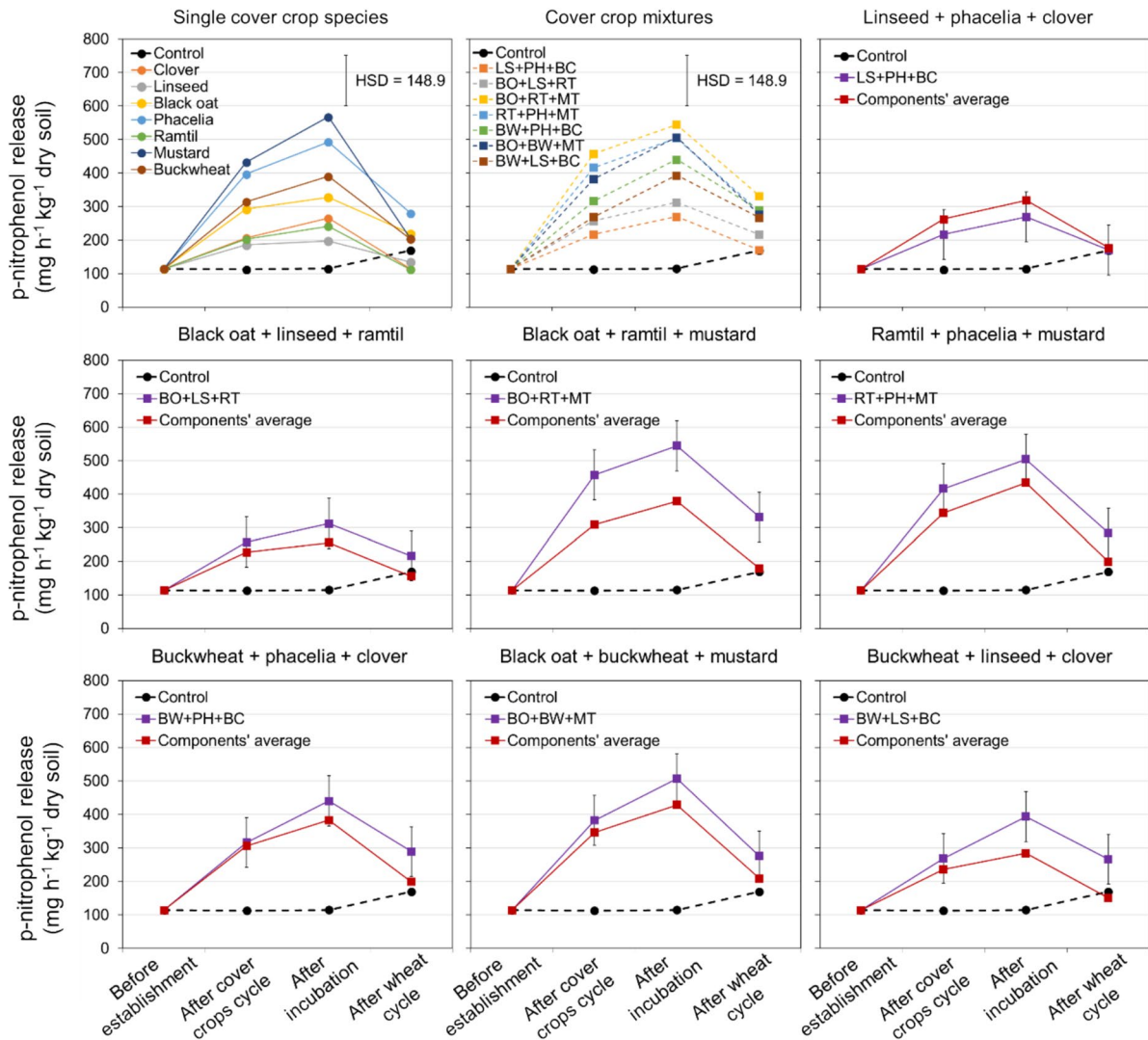


Fig. 4 Potential activity of acid phosphatases (mg of p-nitrophenol released in $\text{h}^{-1} \text{kg}^{-1}$ dry soil) as affected by fallow, single cover crops and cover crop mixtures over the different experimental cycles. The red lines show the average values of the three single crops composing each mixture for comparison.

Labile P reduction ranged between $-11.2 \pm 2.67 \text{ mg kg}^{-1}$ (RT + PH + MT) and $-22.0 \pm 2.28 \text{ mg kg}^{-1}$ (BW + LS + BC). At this stage, individual crops had reduced labile P by an average of $-15.3 \pm 1.65 \text{ mg kg}^{-1}$ and CC mixtures by an average of $-14.4 \pm 3.84 \text{ mg kg}^{-1}$. After biomass incorporation and the 15-day incubation period, all CC treatments increased their labile P, with LS + PH + BC ($+49.2 \pm 3.64 \text{ mg kg}^{-1}$), RT + PH + MT ($+48.2 \pm 0.44 \text{ mg kg}^{-1}$), BW + PH + BC ($+45.6 \pm 2.09 \text{ mg kg}^{-1}$), clover ($+45.5 \pm 1.85$

Lines connecting data points are for visual aid only. Error bar represents the Tukey test's HSD value, which allows the comparison of any two data points except "before establishment" ($n = 4$). Abbreviations: BC: Clover, LS: Linseed, BO: Black oat, PH: Phacelia, RT: Ramtil, MT: Mustard, BW: Buckwheat

mg kg^{-1}) reaching labile P levels statistically comparable to the control, which remained constant.

After the wheat cycle, the total topsoil labile P changes ranged between $-4.96 \pm 2.84 \text{ mg kg}^{-1}$ (LS + PH + BC) and $-21.5 \pm 0.96 \text{ mg kg}^{-1}$ (mustard) in comparison to the soil at the beginning of the experiment. At this stage, soils treated with single crops had, on average, a net labile P of $-16.3 \pm 4.36 \text{ mg kg}^{-1}$ compared to the starting soil, while mixtures showed a significantly lower average net decrease of

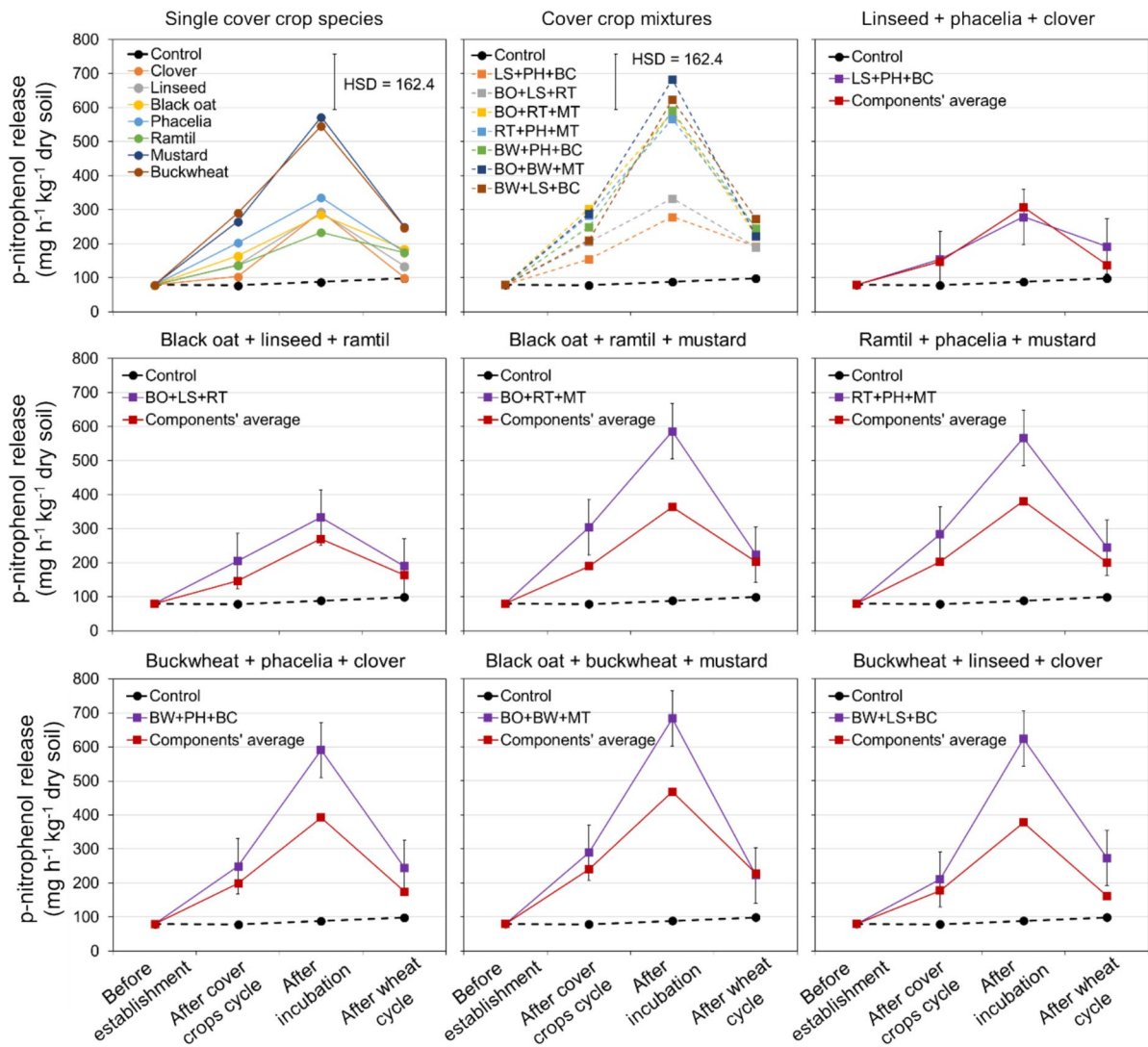


Fig. 5 Potential activity of alkaline phosphatases (mg of p-nitrophenol released in $\text{h}^{-1} \text{kg}^{-1}$ dry soil) as affected by fallow, single cover crops and cover crop mixtures over the different experimental cycles. The red lines show the average values of the three single crops composing each mixture for comparison. Lines connecting data points are for visual aid only. Error

bar represents the Tukey test's HSD value, which allows the comparison of any two data points except "before establishment" ($n = 4$). Abbreviations: BC: Clover, LS: Linseed, BO: Black oat, PH: Phacelia, RT: Ramtil, MT: Mustard, BW: Buckwheat

$-11.0 \pm 4.10 \text{ mg kg}^{-1}$. At the end of the experiment, the CC mixtures promoted 6 out of the 7 highest labile P values observed, with clover being the only individual CC comparable to them.

After the wheat, significantly higher levels of topsoil labile P were observed for most mixtures when compared to their components' averages. Exceptions were BO + LS + RT and BW + LS + BC, in which they were comparable. Interestingly,

compared to their components' averages, BW + LS + BC had significantly lower topsoil labile P, while RT + PH + MT was higher for all cycles, and LS + PH + BC was higher after incubation and the wheat cycles.

Additionally, subsoil AER-P (Supplementary Figure S2) was determined. After the CC cycles, the clover, buckwheat BW + LS + BC and the black oat did not significantly reduce AER-P in the subsoil.

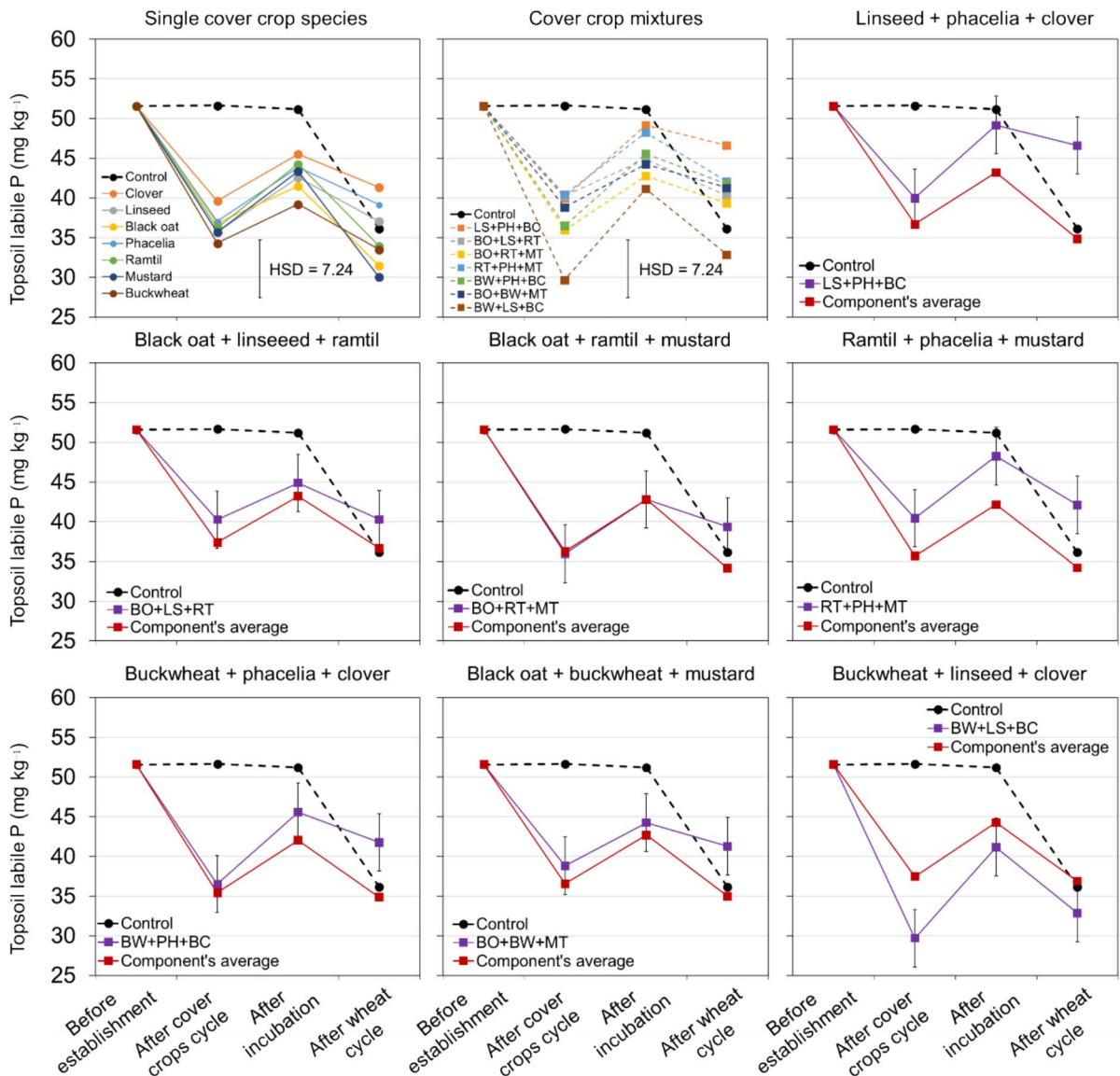


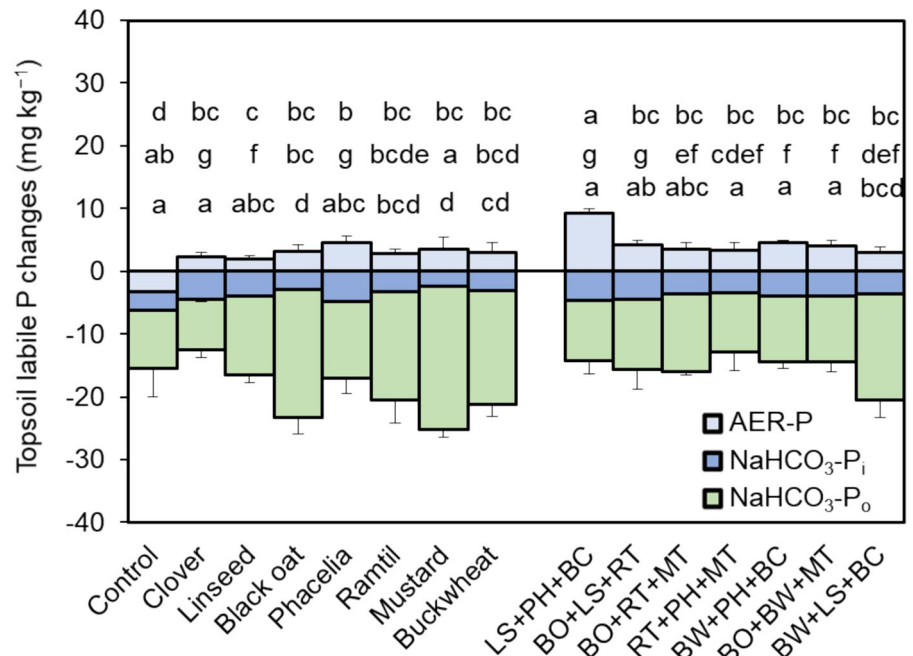
Fig. 6 Changes in topsoil labile P treated with fallow, single cover crops, cover crop mixtures at different experimental cycles. The red lines show the average values of the three single crops composing each mixture for comparison. Lines connecting data points are for visual aid only. Error bar represents

the Tukey test's HSD value, which allows the comparison of any two data points except "before establishment" ($n = 4$). Abbreviations: BC: Clover, LS: Linseed, BO: Black oat, PH: Phacelia, RT: Ramtil, MT: Mustard, BW: Buckwheat

Conversely, RT + PH + MT and its individual components promoted the highest reductions in subsoil AER-P, with the minimum value observed for the mustard, at 1.98 mg kg^{-1} . After wheat, no significant reductions in this subsoil P pool were observed compared to the results after CCs.

The specific labile topsoil P fractions were also differently affected by the treatments (Fig. 7). After the wheat cycle, significant differences in topsoil AER-P increase relative to the starting soil (5.84 mg kg^{-1}) were seen only between linseed ($+ 2.01 \pm 0.41 \text{ mg kg}^{-1}$), which was lower than phacelia ($+ 4.56$

Fig. 7 Changes in topsoil P fractions from the labile soil P pool after the wheat cycle compared to the initial soil (before establishment of the experiment). Error bars represent standard deviation ($n = 4$). Different letters in the same row represent significant differences in the Tukey test at $p \leq 0.05$ in the same order as the legend is presented. Abbreviations: BC: Clover, LS: Linseed, BO: Black oat, PH: Phacelia, RT: Ramtil, MT: Mustard, BW: Buckwheat



$\pm 1.00 \text{ mg kg}^{-1}$), which, in turn, was lower than LS + PH + BC ($+ 9.26 \pm 0.64 \text{ mg kg}^{-1}$). All other CC treatments had comparable AER-P increases and were significantly lower than LS + PH + BC. Only the control (wheat only) reduced AER-P relative to the starting soil after this cycle, by $-3.34 \pm 0.20 \text{ mg kg}^{-1}$. At this stage, soils treated with individual crops increased AER-P by $+3.06 \pm 1.29 \text{ mg kg}^{-1}$ on average, significantly lower than that promoted by the mixtures, at an average of $+4.59 \pm 2.12 \text{ mg kg}^{-1}$ in relation to the initial soil.

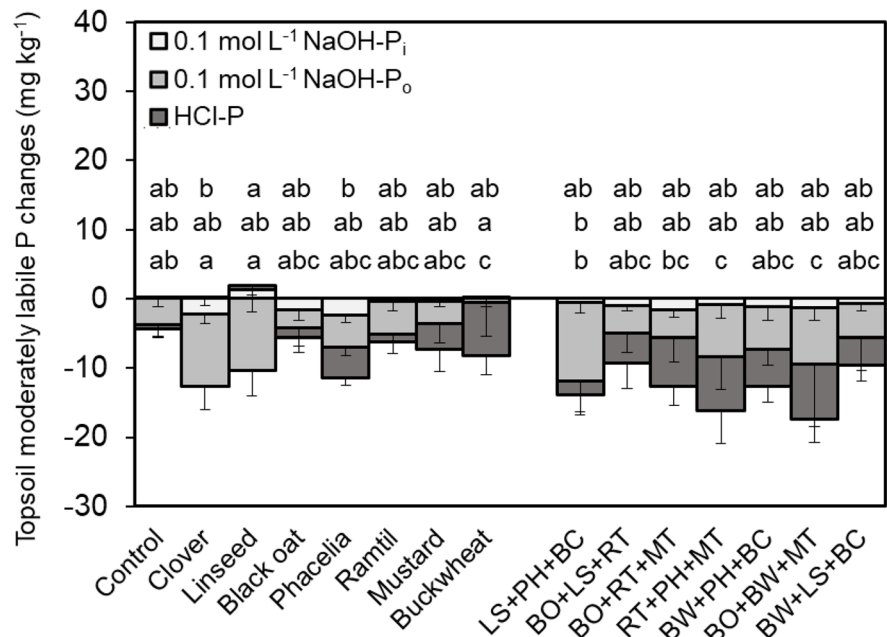
The $\text{NaHCO}_3\text{-P}_i$ and -P_o levels decreased in all treatments after wheat. Significant differences were observed in $\text{NaHCO}_3\text{-P}_i$ reductions, although these were small compared to other P pools, ranging from $-2.33 \pm 0.25 \text{ mg kg}^{-1}$ (mustard) to $-4.82 \pm 0.10 \text{ mg kg}^{-1}$ (phacelia), with an average reduction of $-3.76 \pm 0.74 \text{ mg kg}^{-1}$ across all treatments. The $\text{NaHCO}_3\text{-P}_o$ fraction decreased significantly after wheat cultivation. Notable reductions in $\text{NaHCO}_3\text{-P}_o$ were observed with mustard ($-22.83 \pm 1.22 \text{ mg kg}^{-1}$), black oat ($-20.4 \pm 2.63 \text{ mg kg}^{-1}$), buckwheat ($-18.1 \pm 1.92 \text{ mg kg}^{-1}$), and ramtil ($-17.3 \pm 3.73 \text{ mg kg}^{-1}$), all comparable to the BW + LS + BC mixture ($-16.9 \pm 2.93 \text{ mg kg}^{-1}$). On average, individual CCs reduced $\text{NaHCO}_3\text{-P}_o$ by $-15.9 \pm 5.28 \text{ mg kg}^{-1}$,

while mixtures resulted in a significantly lower reduction of $-11.4 \pm 3.12 \text{ mg kg}^{-1}$.

Moderately labile P pool

The moderately labile P pool (0.1 M NaOH-P_i and $\text{-P}_o + \text{HCl-P}$) was influenced by all treatments after wheat (Fig. 8). The control (wheat cycle only) had the least impact on this pool, resulting in a reduction of the moderately labile P level of $-4.21 \pm 2.58 \text{ mg kg}^{-1}$ compared to the initial soil. The greatest reduction in moderately labile P was observed in the BO + BW + MT treatment with an average reduction of $-17.4 \pm 10.9 \text{ mg kg}^{-1}$ compared to the soil at the start of the experiment. On average, individual crops reduced this P pool by $-8.57 \pm 4.41 \text{ mg kg}^{-1}$, about twice the reduction observed in the control, but significantly less than the average reduction by CC mixture treatments, which was $-13.1 \pm 7.21 \text{ mg kg}^{-1}$. Regarding specific fractions, changes in 0.1 M NaOH-P_i were minimal in all treatments. However, significant lower 0.1 M NaOH-P_o values were found for the buckwheat ($-0.48 \pm 4.96 \text{ mg kg}^{-1}$) compared to the LS + PH + BC ($-11.4 \pm 4.43 \text{ mg kg}^{-1}$). Similarly, HCl-P was mobilized by many treatments, with buckwheat, RT + PH + MT, and BO + BW + MT

Fig. 8 Changes in topsoil P fractions from the moderately labile soil P pool after the wheat cycle compared to the initial soil (before establishment of the experiment). Error bars represent standard deviation ($n = 4$). Different letters in the same row represent significant differences in the Tukey test at $p \leq 0.05$ in the same order as the legend is presented. Abbreviations: BC: Clover, LS: Linseed, BO: Black oat, PH: Phacelia, RT: Ramtil, MT: Mustard, BW: Buckwheat



showing significantly higher reductions compared to the control, clover, and linseed.

Single cover crops versus cover crop mixtures

Growing CC mixtures resulted in significantly greater P uptake for CCs compared to single species (Table 5). For wheat, total biomass, grain yield, and P uptake were similar between treatments, with no significant differences in PUE. Wheat following CC mixtures had a significantly higher harvest index. For N, CC mixtures accumulated significantly more N in their shoot biomass compared to single CCs. However, single CCs showed significantly higher wheat grain and straw N contents, resulting in a 12% higher total N uptake by wheat, while species mixtures allowed a better wheat N use efficiency.

The soil data comparison between the averages of single CCs and mixtures can be seen in Table 6. The intensity of the phosphatase activity peaks varied between individual species and mixtures. Between the end of the second CC cycle and the end of the biomass incubation cycle, single CCs promoted an average increase of +22 and +28% in ACP_{ASE} and ALP_{ASE} , respectively. On the other hand, CC mixtures promoted an average increase of +96% and +115%, respectively. Cover crop mixture treatments

also resulted in a higher topsoil pH after CCs and the wheat cycle.

For soil P parameters, CC mixture treatments showed significantly higher topsoil labile P after wheat and smaller reductions (Δ) in both AER-P and total labile P compared to single species, while shifts in $NaHCO_3\text{-}P_{i+o}$ fractions were comparable between the two groups. Interestingly, moderately labile P also decreased more after wheat following CC mixtures, for an average reduction of -7.9% of the total moderately labile P, with a significantly higher reduction of the HCl-P fraction, while single CCs promoted a -5.2% decrease of the total moderately labile P pool.

Discussion

Cover crops biomass and phosphorus uptake

The higher shoot biomass production of CC mixtures compared to their components' averages is a phenomenon known as overyielding (Jonsson et al. 2019). Transgressive overyielding, in turn, is when the mixture produces higher biomass than its best single component, an effect which was not observed in our trial. Both overyielding types occur when species in a mixture complement each other's growth through mechanisms such as niche differentiation (Meilhac

Table 5 Biomass production, P uptake and related parameters aggregated for single and mixed CC treatments

Parameter	Unit	Control	Single CCs	CC mixtures
Cover crops				
Accumulated ¹ biomass	g col ⁻¹	-	67.8 ± 28.1	81.5 ± 24.5
Accumulated shoot P	mg col ⁻¹	-	158.1 ± 32	192.3 ± 36*
Wheat				
Total biomass	g col ⁻¹	95.1 ± 4.32	98.1 ± 8.1	95.07 ± 8.2
Grain yield	g col ⁻¹	40.9 ± 2.2	41.6 ± 5.3	42.2 ± 4.0
Grain P	mg col ⁻¹	90.9 ± 6.73	119 ± 15.7	120 ± 11.2
Straw P	mg col ⁻¹	16.6 ± 1.6	23.5 ± 5.7	20.9 ± 6.2
Total aboveground P	mg col ⁻¹	108 ± 6.84	142 ± 18.4	141 ± 14.4
PUE	unitless	0.38 ± 0.01	0.30 ± 0.04	0.29 ± 0.04
Harvest index	unitless	0.43 ± 0.01	0.42 ± 0.03	0.44 ± 0.04*
P harvest index	unitless	0.84 ± 0.02	0.83 ± 0.03	0.85 ± 0.03

The data presented are averages ± standard deviation of all single CC species treatments combined, and all CC mixture treatments combined ($n = 28$). For the control treatment, $n = 4$. When a difference between *single cover crop species* and *mixtures* was found after an independent ANOVA at $p \leq 0.05$, the higher values observed were highlighted with an asterisk (*). ¹Accumulated refers to the average of the sum of both CC cycles

et al. 2020), or facilitative interactions (facilitation, Brooker et al. 2008). In contrast, underyielding occurs when a mixture produces less biomass than the average of its components. Parallel to the concept of overyielding, mixtures can absorb different amounts of nutrients than their components' averages or the most efficient component. To accurately describe this effect, we propose the adoption of the terms “overabsorption”, “transgressive overabsorption” and “underabsorption”.

Despite two mixtures underyielding in our experiment (LS + PH + BC and BO + LS + RT), all mixtures showed P overabsorption. The four mixtures that overyielded by more than 10% showed transgressive P overabsorption. All these mixtures contained buckwheat, mustard, or both species, suggesting synergies and facilitation of P acquisition, likely due to their high biomass production, which increases microbial activity and phosphatase exudation in the rhizosphere (Eisenhauer et al. 2017). Buckwheat is a well-known, fast-growing crop, and its high P-mobilizing potential is often attributed to its ability to acidify the rhizosphere and exude large amounts of citrate (He et al. 2002; Possinger et al. 2013; Boglaienko et al. 2014). However, in our study, soil pH under buckwheat and its mixtures showed acidification levels similar to those observed for other cover crops, suggesting that

additional mechanisms may contribute to its high P acquisition efficiency.

Interestingly, Hallama et al. (2019) reported in a meta-analysis that pure buckwheat stands had no effect on soil available P or main crop yield, while suggesting that its performance as part of mixtures warranted further investigation. Based on our shoot biomass and P uptake data, buckwheat appears to be an excellent mixture component, improving both parameters without affecting the performance of most other crops grown alongside it. The only exceptions were the P uptake of clover in the BW + PH + BC mixture, and both shoot biomass and P uptake of BO in the BO + BW + MT mixture. However, this suppression could also be due to the presence of phacelia and mustard in these mixtures, possibly exacerbated by space limitation in the columns used as experimental units.

Despite their generally good individual performance as mixture components, phacelia and mustard proved to be strong competitors in our experiment. This confirms findings of Heuermann et al. (2022), who reported that both species significantly suppressed the biomass production of companion crops when grown in mixtures. In our study, phacelia may have contributed to suppress both linseed and clover in LS + PH + BC, and clover in BW + PH + BC, the

Table 6 Phosphatase activities, P depletion and related soil parameters aggregated for single and mixed CC treatments after two 80-days cover crop cycles, a 15 day incubation and a 120-days wheat cycle

Parameter	unit	After cover crop cycles				After incubation				After wheat			
		Control	Single CCs	CC mixtures	Control	Single CCs	CC mixtures	Control	Single CCs	Control	Single CCs	CC mixtures	CC mixtures
ACP _{ASE} potential activity	mg <i>pnp</i> ¹ h ⁻¹ kg ⁻¹	112.6 ± 12.8	290.2 ± 115.0	330.8 ± 94.8	114.8 ± 13.4	354.7 ± 138.8	424.4 ± 114.4*	169.1 ± 19.3	181.1 ± 60.2	261.8 ± 58.0*			
ALP _{ASE} potential activity	mg <i>pnp</i> h ⁻¹ kg ⁻¹	78.2 ± 16.3	186.0 ± 83.1	242.1 ± 63.6*	88.3 ± 5.3	365.6 ± 146.2	522.9 ± 165.4*	98.7 ± 9.5	180.9 ± 56.6	226.8 ± 33.7*			
Topsoil pH	unitless	6.60 ± 0.02	5.59 ± 0.36	5.86 ± 0.31*	6.64 ± 0.03 n.d.	n.d.	n.d.	6.35 ± 0.17	6.16 ± 0.28	6.40 ± 0.21*			
Topsoil labile P	mg kg ⁻¹	51.7 ± 0.95	36.5 ± 2.3	37.4 ± 4.8	51.2 ± 1.0	42.9 ± 3.0	45.2 ± 3.3	36.1 ± 4.8	35.2 ± 4.4	40.8 ± 4.1*			
Topsoil labile P	mg kg ⁻¹	+ 0.08 ± 0.80	- 15.1 ± 2.3	- 14.2 ± 4.8	- 0.38 ± 1.01	- 8.67 ± 2.96*	- 6.42 ± 3.27	- 15.4 ± 4.80	- 16.3 ± 4.4	- 10.8 ± 4.1			
Topsoil ΔAER-P	mg kg ⁻¹	+ 0.12 ± 0.20	- 3.05 ± 0.51*	- 2.72 ± 0.51	- 0.03 ± 0.24	- 1.27 ± 0.43*	+ 0.14 ± 1.29	- 3.34 ± 0.20	+ 3.06 ± 1.28	+ 4.59 ± 2.12*			
Topsoil ΔNaHCO ₃ -P _i	mg kg ⁻¹	+ 0.11 ± 0.11	- 2.61 ± 0.44	- 2.88 ± 0.28	0.048 ± 0.26	- 1.63 ± 0.60	- 2.10 ± 0.32	- 2.82 ± 0.25	- 3.52 ± 0.88	- 3.94 ± 0.46*			
Topsoil ΔNaHCO ₃ -P _o	mg kg ⁻¹	- 0.20 ± 0.10	- 9.43 ± 2.68	- 8.60 ± 4.71	- 0.45 ± 0.98	- 5.77 ± 2.97	- 4.47 ± 3.06	- 9.33 ± 4.45	- 15.9 ± 5.3*	- 11.4 ± 3.1			
Topsoil moderately labile P	mg kg ⁻¹	169 ± 4.9	160 ± 5.1	157 ± 7.3 n.d.	n.d.	n.d.	n.d.	162 ± 2.6	158 ± 4.4*	154 ± 7.2			
Topsoil Δmoderately labile P	mg kg ⁻¹	+ 2.23 ± 4.94	- 6.54 ± 5.06	- 9.81 ± 7.30 n.d.	n.d.	n.d.	n.d.	- 4.28 ± 2.58	- 8.64 ± 4.41	- 13.2 ± 7.2*			
Topsoil Δ0.1 M NaOH-P _i	mg kg ⁻¹	+ 1.00 ± 0.38	+ 0.123 ± 0.803	+ 0.68 ± 0.62 n.d.	n.d.	n.d.	n.d.	0.09 ± 1.26	- 0.84 ± 1.63	- 1.06 ± 1.39			
Topsoil Δ0.1 M NaOH-P _o	mg kg ⁻¹	- 0.89 ± 3.26	- 4.98 ± 3.33	- 4.58 ± 4.65 n.d.	n.d.	n.d.	n.d.	- 3.73 ± 1.85	- 5.26 ± 4.58	- 6.61 ± 5.00			
Topsoil ΔHCl-P	mg kg ⁻¹	2.19 ± 1.70	- 1.69 ± 4.28*	- 5.92 ± 4.13 n.d.	n.d.	n.d.	n.d.	- 0.57 ± 1.19	- 2.54 ± 3.50	- 5.48 ± 3.58*			
Subsoil AER-P	mg kg ⁻¹	4.33 ± 0.12	3.15 ± 0.91	2.99 ± 0.61 n.d.	n.d.	n.d.	n.d.	4.17 ± 0.04	3.21 ± 1.00	2.98 ± 0.61			
Mycorrhiza spores	# spores kg ⁻¹	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	148 ± 297	173 ± 72.4	215 ± 110			

The data presented are averages ± standard deviation of all single CC treatments combined, and all CC mixture treatments combined ($n = 28$). For the control treatment, $n = 4$. When a difference between *single cover crop species* and *mixtures* was found after an independent ANOVA at $p \leq 0.05$, the highest values observed were marked with an asterisk (*). The Δ variables were calculated in comparison to the starting soil (before establishment) and for those, the values marked with an asterisk was that which presented the highest shift (positive or negative). n.d. = no data. ¹*pnp* is the p-nitrophenol released

latter showing a reduction in its P uptake in this mixture. Mustard, in turn, is recognized as a good P scavenger and mobilizer due to its taproots, high root hair density under low-P conditions, and by its high exudation of carboxylates and phosphatases for improved P_i and P_o acquisition (Haling et al. 2013; Hallama et al. 2019; Honvault et al. 2021; Schwerdtner et al. 2022). However, in our experiment, mustard was an even stronger competitor than phacelia, suppressing all its companion crops. This suggests that caution is warranted when including mustard in CC mixtures, as its competitive behaviour may limit the performance of other species.

Cover crop's soil phosphorus acquisition strategies

Our data show that especially mustard had a high potential activity of both phosphatases throughout the experiment, and all mustard-containing mixtures performed well for this parameter. While the mustard undoubtedly played a role in the phosphatase activity of its mixtures, the exceptional values observed under these mixtures may also be related to the presence of black oat. This crop was a weak competitor to mustard and may have promoted high phosphatase exudation in these two mixtures in an attempt to solubilize and absorb P, as most of the solution P was preferentially taken up by the competing mustard.

While CCs impacted ACP_{ASE} and ALP_{ASE} to different extents, our data revealed a pattern of higher phosphatase activity (especially ALP_{ASE}) promoted by CC mixtures in comparison to single CCs. This can be explained by the increased plant diversity, which promotes microbial activity and consequently ALP_{ASE} exudation, as has been shown by other studies (Lange et al. 2015; Shi et al. 2020; Campdelacreu Rocabrana et al. 2024). Additionally, significant peaks in both ACP_{ASE} and ALP_{ASE} were observed at 15 days after biomass incorporation in comparison to the CC cycles. This can be explained by the incorporation of large amounts of biomass, which increases microbial activity and abundance by serving as a substrate for heterotrophic microbes, and in turn leads to increased ALP_{ASE} release (Shi et al. 2020; Pokharel et al. 2020; Stegarescu et al. 2021).

The rhizosphere acidification observed after the CC cycles can be attributed to proton release by roots, which is essential for establishing an electrochemical gradient that enables nutrient uptake. This process

is particularly pronounced in low-P environments, where it also serves as a strategy to mobilize P for plant uptake (Zhang et al. 2021; Huang et al. 2023; Baccari and Krouma 2023). After the reintroduction of dry CC biomass into the soil, anions and base-forming ions return to the soil, neutralizing acids and promoting a pH increase (Yan et al. 1996; Tang and Yu 1999), which explains the higher pH observed after the wheat compared to the soil after the CCs, despite its acidifying potential as demonstrated by the control treatment.

The lowest pH values recorded were obtained after the second cycle of phacelia, linseed, clover, and their mixture (LS + PH + BC), but were only statistically different from mustard and its mixtures. Strong soil acidification by clover (Fabaceae) is a well-described phenomenon, explained mostly by this crop's ability to biologically fix atmospheric N_2 , producing H^+ as a by-product. Additionally, partial reliance on N fixation results in a lower nitrate (NO_3^-) uptake from the soil, which is absorbed through H^+/NO_3^- symporters, a mechanism consuming H^+ and thus increasing the soil pH (Rona et al. 1991; Kinoshita et al. 2023). Together, these factors result in a net release of H^+ into the soil, driving strong acidification (Haynes 1983; Monaghan et al. 1998; Tang et al. 1999b).

The lower pH shifts observed for the mustard and its mixtures were expected based on previous results (Raniero and Santner 2024), which showed no pH changes by mustard in comparison to the same control soil. Other studies even reported that mustard has an alkalinizing effect (Schwerdtner et al. 2022), which may be due to increased ammonification or retarded nitrification in this species (Heuermann et al. 2022). Brassicaceae are known to exude compounds such as glucosinolates (Halkier and Gershenzon 2006), the degradation products of which can lead to a suppression of nitrifying bacteria in the soil (Bending and Lincoln 2000). As the dominant CC in the BO + RT + MT and RT + PH + MT mixtures, the mustard suppressed the growth and likely the root activity of its companions, resulting in a higher pH level in the mixtures than their components' averages. However, a significant pH reduction relative to the control was observed in both mixtures, likely related to the effects of the companion crops. For example, phacelia's reportedly high competitiveness (Heuermann et al. 2022) may have had an impact on soil pH even when suppressed by mustard.

Cover crop effects on soil phosphorus status

Cover crop soil P mobilization and uptake resulted in a reduction of the soil labile P pool to varying degrees. This was expected since the labile pool is characterized by P forms of high solubility and bioavailability which are easily accessible to crops (Gatiboni and Condron 2021). The higher average reliance of single CCs on taking up P labile P suggests this pool played a greater role in their nutrition in comparison to mixtures. In contrast, the higher access to moderately labile P by mixtures could be explained by the complementary P acquisition strategies present in mixed cropping systems. Different species may access P from a wider range of pools by utilising diverse root traits, exudates, and associated microbial communities (Haling et al. 2013; Honvault et al. 2021; Raniro and Santner 2024), allowing them to mobilise P from less readily available sources, such as moderately labile P pools, thereby reducing their reliance on the labile pool (Teles et al. 2017; Doydora et al. 2020; Gatiboni et al. 2021).

This diversification of P acquisition strategies offers significant advantages, especially when CC mixtures are cultivated over multiple years. By accessing more strongly bound P pools, mixtures may help not only to keep P availability high, but also to slow down the progressive transformation of P into less available forms. Over time, this could enhance the efficiency of fertilizer P utilization, reducing the need for repeated high fertilizer inputs and contributing to a more sustainable management of soil P resources. These potential benefits underscore the importance of further research to understand how long-term cultivation of CC mixtures influences P cycling and availability, as well as its wider implications for improving P-use efficiency in agricultural systems.

Cover crop effects on wheat performance

Cover crop mixtures have been shown to have a higher nutrient carry-over potential when compared to pure stands (Heuermann et al. 2022), releasing nutrients more efficiently for the succeeding crops. This is in line with our results, which showed that after the 15-day biomass incubation period, three mixtures (LS + PH + BC, RT + PH + MT and BW

+ PH + BC) and only the single crop clover released the most P back into the soil. In these treatments, labile P levels were no longer significantly different from the control, likely due to rapid mineralization of their biomass and efficient P release during decomposition. Interestingly, all these mixtures contained phacelia, and two of them contained clover, suggesting an elevated P cycling potential, especially as mixture components.

Despite significant differences observed in several CC and soil parameters after the CC cycles, wheat biomass production, grain yield, P uptake and PUE were only minimally affected by the CC treatments. We propose two explanations for this. First, two fertilized CC cycles resulted in the production and incorporation of large amounts of CC biomass, which may not be normally achievable under field conditions. This high organic C input likely stimulated microbial growth and activity, increasing microbial competition with wheat for available P. This may have led to microbial P immobilization, limiting the net P availability and yield response of wheat. Secondly, the P release from the CC residues was too slow during the critical early stages of wheat growth, when P demand is highest, particularly during tillering and stem elongation (Römer and Schilling 1986; Rodríguez et al. 1999; Meier et al. 2024). After the 15-day incubation period, the labile P derived from the biomass may begin to decrease due to sorption reactions, as observed by Raniro et al. (2025). Subsequently, the more lignified biomass starts to decompose, and its delayed P dissolution may not be rapidly available for plant uptake.

These dynamics may have resulted in low available P levels in the soil at the stage of highest wheat demand, driving the reduction of its yield potential and hence P demand. This was evidenced by the visual signs of P deficiency in the older leaves of wheat in all treatments, such as stunted growth and purple-coloured leaves, which suggest that P was a limiting factor for crop growth and productivity. Later in the cycle (grain filling), P demand is minimal and uptake is reduced due to reduced root activity and beginning root senescence, thus any additional P released from the CC residues would have contributed minimally to further P uptake, as recently shown for maize (Ampong et al. 2024). Nevertheless, the control treatment was the only one that showed significantly lower P uptake of wheat compared to ramtil and LS

+PH + BC, as well as promoting the lowest average PUE observed. Therefore, cover crops (whether pure or mixed) may be an effective strategy to mobilize P and make it more available to subsequent crops, since it allowed higher P uptake by wheat than the fallow control.

These different P availabilities are also related to soil pH effects, affected by rhizosphere mechanisms and biomass incorporation. While wheat promoted soil acidification, evidenced by a pH reduction of 0.29 ± 0.15 in the control treatment, its effect was not sufficient to overcome the pH increasing potential of the added biomass, resulting in a higher final pH than those after the CC cycles in all treatments except the control. This can be explained by the characteristics of the incorporated biomass, such as alkalinity (Noble et al. 1996; Tang et al. 1999a; Tang and Yu 1999), base generating cation concentrations and their ion release patterns (Pocknee and Sumner 1997), all of which can neutralize soil acidity.

The drastic reduction in ACP_{ASE} and ALP_{ASE} after wheat for all CC treatments can be explained by two mechanisms. Firstly, wheat is known to show only minor phosphatase activity and abundance beyond the immediate root surface, as ACP_{ASE} are tightly associated with the cell wall (Richardson et al. 2000; Staudinger et al. 2022). Secondly, soil was sampled after wheat maturity, a stage in which plants are senescing, and root and microbial activity is greatly reduced (Siqueira et al. 2021). However, it is interesting to note that despite the great reduction observed after the wheat cycle, six treatments (five of which are CC mixtures) still had a significantly higher ACP_{ASE} activity than the control, while ALP_{ASE} was higher for all treatments except clover and linseed, with only mustard and buckwheat as single crops among the highest values recorded. This can be explained by the high root biomass density produced by single CCs and mixtures, which promotes higher biological activity despite the absence of living plants.

Soil phosphorus status after the wheat cycle

In our experiment, the significant increases in AER-P observed after the wheat cycle in soils where CCs had been previously cultivated and incubated can be explained by the decomposition of plant residues, which release P back into the solution. Combined with an asynchrony between P release from CC

residues and wheat demand, this allowed the accumulation of more labile P in some treatments, explaining the higher soil P levels observed at the end of the experiment. Here, however, the total CC biomass produced by each treatment was incorporated, representing an incorporation of different P quantities. This setup ultimately resulted in significantly different AER-P increases from different crops, with the highest increase observed for the LS + PH + BC mixture and the lowest for linseed.

Additionally, the lower $NaHCO_3-P_o$ observed for individual CC-treated soils after wheat in comparison to the mixture-treated soils can be explained by two mechanisms. Firstly, the higher biomass-derived P incorporated into the soil via mixtures may have contributed to buffer the reduction in labile P_o by directly increasing this pool. Secondly, these mixtures tended to decompose faster in the soil (Raniero et al. 2025), which may have released more P_i into the soil that can be directly taken up by plants, reducing the need for mineralizing P_o to supply the initial demand. This shows that CC mixtures can have a higher potential in maintaining soil labile P_i and P_o when compared to single crops, which, despite limited evidence for benefits in wheat in the short-term (one cycle), warrants further investigation in multiple years and field trials. The potential for continued CC biomass decomposition and P release beyond the timeframe of this experiment is further discussed in Supplementary Information S3.

The increases in labile P observed for both single and mixed CCs can also be explained through their uptake of moderately labile P ($0.1\text{ M NaOH-}P_i$ and $-P_o$, and $HCl-P_i$). The significant reduction of moderately labile P by the single CCs tested contrasts with our previous results, which showed no changes in that P pool, attributed to the smaller pot size (2 kg) and shorter trial length (one cycle of 108 days) used, which were not sufficient to induce such P mobilization in the soil (Raniero and Santner 2024). However, reports on moderately labile P uptake by CC species are available in literature (Dube et al. 2014; Teles et al. 2017; Soltangheisi et al. 2020). In this experiment, three crop cycles for a total of 295 days, conducted in large columns, resulted in greater plant growth than in our previous experiment, inducing significant labile and moderately labile P reductions not only by single and mixed CCs, but also by the wheat, although to a lesser extent as shown by the control treatment.

The significantly higher moderately labile P decreases under mixtures compared to single CCs and the wheat alone can be explained by several mechanisms. Firstly, the higher average biomass produced by the mixtures allowed a higher labile P uptake, thus promoting P mobilization from less labile P fractions. Secondly, diversity of living plants as well as in the composition of decomposing litter has been shown to increase microbial biomass and activity (Lange et al. 2015; Chen et al. 2019; Drost et al. 2020). These effects can work synergistically, as stimulation of diverse microbial communities can lead to higher biomass-derived P_i dissolution and P_o mineralization to meet microbial demand, potentially increasing P availability and plant productivity (Chen et al. 2022).

Overall, CC mixtures mobilized more P from the moderately labile pool, facilitated wheat access to similar amounts of P when compared to the control and single CCs, and resulted in higher available P levels at the end of the experiment, providing indirect but strong evidence for enhanced P cycling through the soil–plant system. Despite this, the non-labile P pool was not successfully accessed by any treatments due to the highly recalcitrant nature of the phosphates it contains. However, this pool has been shown to be accessed by certain CCs depending on species, soil characteristics and fertilization history, and time-scales (Merlin et al. 2016; Doydora et al. 2020; Gati-boni et al. 2021).

The role of cover crops in soil legacy phosphorus mobilization

The immediately plant-available P (Olsen P) in the topsoil used was only 3.33 mg kg^{-1} , well below the P fertility thresholds for wheat in European soils, which range from 12 to 24 mg kg^{-1} Olsen P (Jackson et al. 1991; Speirs et al. 2013; Nawara et al. 2017; Steinfurth et al. 2022). Therefore, the low Olsen P level in our experiment likely compromised wheat productivity substantially, which could be mitigated through fertilization. However, in a closed-system experiment (sealed pot) with the same soil, the addition of 60 mg kg^{-1} P as triple superphosphate (TSP) increased the total labile P pool by $\sim 15 \text{ mg kg}^{-1}$ after 45 days of incubation, implying that $\sim 75\%$ of the P applied was shifted into less available fractions (Raniero et al. 2025). Therefore, meeting wheat's P requirements with TSP would likely lead to an increase of

the soil legacy P, mostly as moderately and non-labile fractions.

After the CC cycles, single species had reduced moderately labile P by $\sim 3.9\%$, while CC mixtures promoted a significantly greater reduction of $\sim 5.8\%$. In the wheat-only (fallow control) treatment, moderately labile P was reduced by $\sim 2.6\%$. In that sense, the use of CCs, and more specifically CC mixtures, could be an important tool to improve the fertilization efficiency in the medium to long term, reducing the rate of P accumulation in fertilized fields. Furthermore, combining efficient P-mining CC mixtures with more efficient P fertilization strategies (e.g. banded application) could result in a net legacy P decrease overtime, representing a successful exploration of soil legacy P.

Conclusions

Our findings confirm hypotheses (1) and (2), as CC mixtures were generally more efficient in utilizing moderately labile soil P, reducing this pool by an average of $-13.1 \pm 7.21 \text{ mg kg}^{-1}$, compared to $-8.57 \pm 4.41 \text{ mg kg}^{-1}$ for single species. However, we reject hypothesis (3), as CC mixtures did not significantly increase wheat P acquisition (average $141.9 \pm 16.5 \text{ mg col}^{-1}$) or yield compared to single CCs and fallow (average $41.9 \pm 4.7 \text{ g col}^{-1}$). While CC mixtures promoted increases of soil phosphatase activity by up to 115% (alkaline phosphatases) compared to the control, this did not translate into immediate yield gains, suggesting that the benefits of cover cropping may be more evident in long-term soil fertility and health.

Our findings confirm that CCs, and especially CC mixtures, were efficient at enhancing soil legacy P cycling and highlight the importance of strategic species selection and management to balance compatibility, competitive dynamics, and overall effectiveness in CC mixtures. Integrating CCs with fertilization strategies such as localized or banded applications could enhance their P-mobilizing effects, leveraging the legacy P pool while reducing P accumulation. Long-term field research is essential to evaluate the sustained impact of CC mixtures on soil P availability, nutrient cycling, and soil health. Additionally, integrating CC mixtures into best-practice farming systems can help maximize P efficiency and promote sustainable soil management.

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Author contributions Henrique Rasera Raniero was responsible for the study's conceptualization, methodology, formal analysis, investigation, data curation, visualization and writing of the original draft. Jakob Santner contributed to the study's conceptualization, methodology, and participated in reviewing and editing the manuscript. Karin Hage-Ahmed provided resources and contributed to reviewing and editing the manuscript. Christiana Staudinger and Dorette S. Müller-Stöver provided supervision and contributed to reviewing and editing the manuscript.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests The authors declare no competing interests.

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