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## Article

# Soil Enzyme Activity and Stoichiometry: Linking Soil Microorganism Resource Requirement and Legume Carbon Rhizodeposition

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**Abstract:** Legumes provide multiple ecosystem services in agricultural systems. The objectives of this study were to evaluate the influence of different legumes through C rhizodeposition on the dynamics of C, N and P in soil and on microbial communities' resource requirements. Legumes pea (*Pisum sativum* L.), faba bean (*Vicia faba* L.), white clover (*Trifolium repens* L.), crimson clover (*Trifolium incarnatum* L.) and non-legume wheat (*Triticum aestivum* L.) were grown in pots. Carbon rhizodeposition was quantified by using <sup>13</sup>CO<sub>2</sub> labeling, and six soil enzyme activities were measured: β-glucosidase (BG), arylamidase (ARYLN), N-acetyl-glucosaminidase (NAG), phosphatases (PHO) and alkaline and acid phosphatases (AKP and ACP). Enzyme stoichiometry approaches were applied. The results showed that BG, NAG and ACP activities were positively influenced by faba bean and clovers. Enzyme stoichiometry analysis revealed a limitation of microorganisms in C and P resources at the plant reproductive stage. These results were explained by plant functional traits. Plant biomass production, root total length, the ability of plants to rhizodeposit C and the C and N content of plant tissues were the main explicative factors. This study also shows that N and C nutrient supplies positively contribute to nutritional requirements and the growth of microorganisms and P availability in soil.

**Keywords:** C rhizodeposition; enzyme activities; microbial communities; C, N, P cycling; legume crops; plants functional traits



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## 1. Introduction

For several decades, the intensification of agricultural activity and the use of chemical fertilizers have generated significant environmental changes, pollution and biological disturbances [1–4]. These negative effects have resulted in the emergence of new sustainable agricultural practices aimed to reduce environmental changes and improve soil quality while ensuring food and nutritional security for a growing population [5–7]. The sustainability of agricultural systems requires better use of ecological functions and a valorization of natural resources through ecological intensification of agriculture [8–12]. Among the agroecological solutions, the integration of legumes in crop rotations contributes to the sustainability of agricultural systems. The presence of legumes in agricultural production provides multiple ecosystem services [13]. Legumes contribute to the increase in soil organic matter, improve soil structure, maintain soil biodiversity and have the ability to fix atmospheric nitrogen (N<sub>2</sub>) through root symbiosis with rhizobiaceae [14–16]. The specific use of N<sub>2</sub> fixed by legume crops for their nutrition can allow the reduction in chemical fertilization and the provision of N to succeeding crops in a rotation [17–19]. The processes

by which legumes enrich soil with N include the burial of non-harvested organs and rhizodeposition [20–22]. This process is defined as the emission of a wide range of compounds from plant roots during growth including root senescence, secretion of border cells and exudation of various compounds [23]. In addition to N compounds, rhizodeposition is also the pathway by which carbon compounds enter in the soil [24–26]. On average, 17% of carbon (C) fixed by the plant is allocated to rhizodeposition, and this figure varies widely among plant species [27]. This C may shape microbial community composition, structure and activity [28–30].

Soil microbial communities play an important role in agroecosystem functioning and are essential for plant nutrition and health [31]. They contribute to global element cycling and are involved in turnover processes of organic matter, breakdown and the formation of soil aggregates [31–34]. Soil microbial communities support 80–90% of biochemical reactions in soil [35], largely driven by the presence of enzymes catalyzing the different reactions [36–41]. The enzyme activity of rhizospheric soil is usually higher than that of bulk soil. The higher enzyme activity in the rhizosphere is due not only to the stimulation of root-related microbial activity through rhizosphere deposition but also due to the release of enzymes by the root or by lysing root cells. These enzymes usually catalyze the formation of products absorbed by plant roots or rhizosphere microorganisms [42–44]. Therefore, the enzyme activities at the plant–soil interface may reflect improvement of the highly integrated microorganism–plant associations (symbiotic and plant growth promoting rhizobacteria) and control of plant pathogens and pests. The spectrum of rhizosphere enzyme activities is considered as a footprint of plant–microorganism interactions [45]. Moreover, soil enzyme activities (including rhizosphere ones) can be useful indexes of changes occurring in the microbial functioning in soil, as affected by various and different factors [46–50]. Enzyme activities are controlled by the availability of resources in soil and plants demands. Recent studies have shown that legumes can regulate soil enzymatic activities [51–53]. Legumes such as chickpea and cowpea have been shown to increase phosphatase activities compared with non-legumes [54,55]. Additionally, it has been shown that the activities of  $\beta$ -glucosidase, N-acetyl glucosaminidase and arylamidase are greater under pea, faba bean and vetch crops than under wheat and oat crops [56,57]. Siczek et al. [58] have shown that the activities of dehydrogenase, protease and urease were higher under faba bean than wheat at the reproductive stage.

Soil extracellular enzyme activities and associated enzymatic stoichiometry are considered as sensitive indicators of nutrient availability and microbial substrate limitation [59–61]. Indeed, the partitioning of C between anabolic and catabolic processes affects the rate of C accumulation in soils [62] and also changes the levels of N and P rates, which are limiting factors for the growth of plant and microorganisms in soils [63]. The use of the C:N, C:P and N:P ratios could help to better understand resource allocation in soil–plant interactions [64,65]. Enzymatic stoichiometry ratio of C, N or P-acquiring enzymes can, thus, determine the relationships between microbial nutrient demands and soil nutrient supplies [66,67] and has been suggested as an efficient method for indicating the relative resource limitation of soil microorganisms [68–76]. A meta-analysis conducted by Sinsabaugh et al. [69] concluded that microbial C:N:P acquisition ratios converge on a 1:1:1 scale based on the activities of  $\ln(\beta\text{-1,4-glucosidase})$ :  $\ln(\text{Leucine-aminopeptidase} + \beta\text{-1,4-N-acetylglucosaminidase})$ :  $\ln(\text{phosphatase})$ . Studies dealing with enzymatic stoichiometry have highlighted that using a single enzyme activity as an indicator of nutrient dynamics in soil is not representative of the complexity of metabolic activities in agrosystems. As the degradation of organic matter in soil requires the interaction of several enzymes, it could be more effective and relevant to combine a wide variety of soil enzymes [59,77]. These enzymes include those involved in the decomposition of different substrates with varying complexity in relation to C, N and P cycling.

The objective of this study was to evaluate the influence of different forage and seed legumes through C rhizodeposition on the dynamics of C, N and P elements in terms of soil and microbial communities' resource requirements. We hypothesized the following:

(1) soil enzymatic profiles would differ between legume species, and the variability could be related to their functional trait and rhizodeposition of C and (2) soil microbial communities will be less energy limited (C) and nutrient limited (N, P) due to C rhizodeposition. Therefore, we tried to understand the determinism of enzymatic activities and microorganisms' nutrient limitation under different legumes cover crops. Enzymatic activities related to C, N and P cycling and C rhizodeposition under the different legumes crops were monitored. A study of enzymatic stoichiometry was then carried out to determine the resource limitation of the microorganisms.

## 2. Materials and Methods

### 2.1. Soil and Plant Material

Soil used in this study was a loamy soil collected at 0–20 cm depth from an arable soil (Seine et Marne, France 49°33' N, 0°46' W) and sieved at 5 mm with the following characteristics: clay 207 g·kg<sup>-1</sup>, silt 706 g·kg<sup>-1</sup>, sand 87 g·kg<sup>-1</sup>, total carbon content 9.03 g·kg<sup>-1</sup>, total nitrogen content 1.06 g·kg<sup>-1</sup> and available P (Olsen) 85.51 mg·kg<sup>-1</sup>, pH 7.85 (water).

The experiment was conducted using four legume species: pea (*Pisum sativum* L.), faba bean (*Vicia faba* L.), white clover (*Trifolium repens* L.), crimson clover (*Trifolium incarnatum* L.) and wheat (*Triticum aestivum* L.) used as a non-N<sub>2</sub> fixing species.

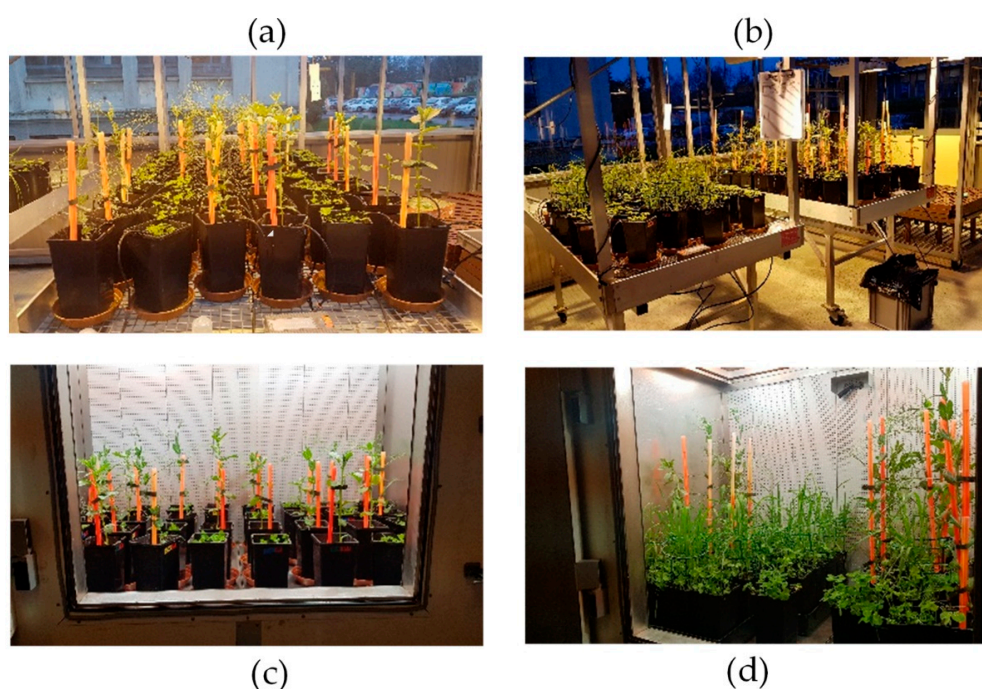
### 2.2. Growing Conditions and Experimental Design

The experiment was carried out under controlled conditions in a greenhouse until the plant reproductive stage (Figure 1a,b). After seed sterilization and ten days of germination (including 2 to 3 days for seedlings emergence), seedlings were transferred to a 2 L pot (20 cm depth) containing 1.8 kg soil adjusted between 70 and 80% of the soil water holding capacity (WHC). Twenty replicates of each species were grown. The seedlings were sown according to their field sowing density adapted to the area of the pots: 380 seeds·m<sup>-2</sup> (four seedlings per pot) for wheat, 40 seeds·m<sup>-2</sup> for faba bean (one seedling per pot), 70 seeds·m<sup>-2</sup> (one seedling per pot) for pea, 500 seeds·m<sup>-2</sup> (six seedlings per pot) for white clover and 600 seeds·m<sup>-2</sup> (eight seedlings per pot) for crimson clover. The spacing varied between 3 and 6 cm between the seedlings depending on the species. The plants were grown until the reproductive stage for each species: wheat (14 weeks after transplanting), faba bean (16 weeks), pea (13 weeks) and 15 weeks for white and crimson clovers. The first harvest took place one month after the transplanting corresponding to the vegetative stage (VS), and the second harvest took place at the reproductive stage (RS) when aboveground biomass had reached more than 50% of senescence. The pots were automatically watered with a flow rate of 60 mL·pot<sup>-1</sup>·day<sup>-1</sup> and were manually adjusted to maintain the WHC between 70 and 80%. Additional light was provided by sodium lamps (400 W Philips SON T-PIA Agro, providing 400 μmol m<sup>-2</sup> s<sup>-1</sup> photosynthetic active photons) with a photoperiod of 16 h day at 20 °C and 8 h night at 18 °C.

### 2.3. <sup>13</sup>CO<sub>2</sub> Labeling of Plants

In order to estimate C rhizodeposition of the different crops, plants were introduced once a week for 24 h into a growth chamber for <sup>13</sup>CO<sub>2</sub> labeling (Figure 1c,d). This labeling started 3 weeks after transplanting and lasted for 10 weeks. The growth chamber was equipped for automatic control of light, temperature, moisture, CO<sub>2</sub> concentration and <sup>13</sup>CO<sub>2</sub> enrichment (Froids et Mesures, Beaucouze, France). The photoperiod and the day and night temperatures were similar to those used in the greenhouse. Control soil samples (five replicates) were harvested before the beginning of the labeling for <sup>13</sup>C natural abundance determination. Crop labeling lasted for 16 h per day, and the equipment was similar to that used by Cliquet et al. [78]. After labeling, the plants were taken back to the greenhouse. Before beginning labeling, the CO<sub>2</sub> concentration of the chamber was reduced to 50 ppm by compressing atmospheric air with a compressor and removing CO<sub>2</sub> with a soda cartridge that trapped CO<sub>2</sub> and reinjected air into the chamber. <sup>13</sup>CO<sub>2</sub> was then

injected into the chamber to reach a CO<sub>2</sub> concentration of 400 ppm and an <sup>13</sup>C atom excess of 4.88%. Constant CO<sub>2</sub> concentrations and <sup>13</sup>C enrichment were obtained by continuously mixing a small amount of <sup>13</sup>CO<sub>2</sub> (1 > 99%) with industrial <sup>13</sup>CO<sub>2</sub> (1.07825%<sup>13</sup>C) through the use of two mass flow controllers. These controllers were piloted by a master box (2MProcess, le Plessis-Trévis, France) connected to an infrared CO<sub>2</sub> analyzer (ADC MGA3000). The final <sup>13</sup>C levels of the atmosphere were determined by using triplicate samples of the gas inside the growth chamber collected through an exit valve at 11 a.m. and 16 p.m.



**Figure 1.** (a,b) Plant growing in greenhouse; (c,d) plants in the growing chamber.

#### 2.4. Harvest and Analyses

At both harvest points (VS and RS with 5 replicates per species at each harvest), plant shoots were cut. Roots were carefully separated from the soil by hand and washed with demineralized water. At each harvest (VS and RS), 5 more replicates were harvested to collect the entire root system. This root system was kept in an alcoholic solution and scanned the following day by using an EPSON EXPRESSION 10000XL scanner. WinRHIZO version 2007d (Regent Instruments Inc., Canada) was used to determine root traits. The harvested plant tissues were then dried at 60 °C for 72 h and weighed for dry mass determination. All tissues were then ball milled. The %C and %N were determined by using an elemental analyzer (EA3000, EuroVector, Milan, Italy) coupled with an isotope ratio mass spectrometer (IRMS, Nu Instruments, Manchester, UK). Different plant functional traits were determined: (1) total dry mass (g), (2) shoot to root ratio, the ratio of total shoot dry biomass to total root dry biomass, (3) total root length (cm) and (4) shoot and root C:N ratio.

For each harvest, soil was collected, passed through a 5 mm sieve, frozen with liquid N and stored at −80 °C prior to enzymatic activities analyses. Some soil samples were also dried, ball milled and submitted to EA-IRMS in order to determine total C, N and <sup>13</sup>C enrichment. Soil extractable phosphates were also determined by extraction in NaHCO<sub>3</sub> (P Olsen; according to ISO 11263:1994). Soil C:N, C:P and N:P ratios were then determined.

## 2.5. C Rhizodeposition Determination

Data from IRMS analyses allowed the determination of the amounts of C present in the soil compartment and were fixed during the labeling period with the following equation.

$$\text{Soil CdfR (mg)} = \left( \frac{\text{atom excess \% of soil}}{\text{atom excess \% of the atmosphere}} \right) \times \text{Soil total C (mg)} \quad (1)$$

CdfR was C derived from rhizodeposition, atom excess % of the atmosphere was 4.88% and unlabeled control compartments were used to determine the atom excess % of soil. This amount of labeled C was calculated for each harvest.

## 2.6. Enzyme Activities and Stoichiometry

The enzymes activities analyzed in this study were involved in C, N and P rich compound degradations. For the C cycle,  $\beta$ -glucosidase activity (BG; EC: 3.2.1.21) was assessed; for the N cycle, N-acetyl-glucosaminidase (NAG; EC: 3.2.1.30) and arylamidase (ARYLN; EC: 3.5.1.5) activities were evaluated. For the P cycle, total phosphatases (PHO; EC 3.1.4.1), acid and alkaline phosphatases (ACP; EC 3.1.3.2 and AKP; EC 3.1.3.1) were assessed. These enzymes activities were measured by using colorimetric substrates in 96-wells in triplicate using the procedure detailed in the ISO 20130:2018. Briefly, 4 g of fresh soil was homogenized for 10 min at 250 rpm with 25 mL of deionized water for BG, NAG and PHO activities or Trizma buffer (50 mM) at pH 7.5 for ARYLN, at pH 5.5 for ACP and at pH 11 for AKP activities. Soil solutions were incubated, respectively, with 4-nitrophenyl  $\beta$ -D-glucopyranoside 0.05 M (301.3 g·mol<sup>-1</sup>, Sigma), 4-N-acetyl- $\beta$ -D-glucosaminide 0.01 M (342.3 g·mol<sup>-1</sup>, Sigma) and 4-nitrophenylphosphate disodium salt hexahydrate 0.05 M (371.1 g·mol<sup>-1</sup>, Sigma) for PHO, ACP and AKP. The reaction was stopped with 0.5 M CaCl<sub>2</sub> and 0.1 M Tris at pH 12, each plate was centrifuged for five min at 1500 × g and absorbance was measured on a Varioskan Flash-Thermo microplate reader. The amounts of p-nitrophenol were obtained by measuring the absorbance at  $\lambda = 405$  nm, with comparison to calibration curves. For ARYLN activity, four gram soil samples were mixed for 10 min at 250 rpm with 25 mL with Trizma base (50 mM, pH 7.5). Soil solutions were incubated with L-leucine  $\beta$ -naphthylamide hydrochloride 0.008 M (292.8 g·mol<sup>-1</sup>, Sigma). The  $\beta$ -naphthylamine produced was extracted with acidified ethanol and converted to an azo compound by reacting with p-dimethylaminocinnamaldehyde (DMCA) (DMCA: 175.23 g·mol<sup>-1</sup> and ethanol 96%). The amount of  $\beta$ -naphthylamine was obtained by measuring the absorbance at  $\lambda = 540$  nm and comparing it with calibration curves. Three replicates were performed for each treatment. The activities of soil enzymes were expressed as nmoles of hydrolyzed substrate per minute per gram of dry soil. For each soil sample, the geometric mean (GMea) of enzyme activities was calculated as described by García-Ruiz et al. [79].

$$\text{GMea} = \sqrt[6]{\text{BG} \times \text{NAG} \times \text{ARYLN} \times \text{PHO} \times \text{AKP} \times \text{ACP}} \quad (2)$$

Enzymatic stoichiometry was also determined as the ratio of enzyme activities involved in C, N and P compounds degradation (C:N, C:P and N:P acquiring enzyme ratios), according to Hill et al. [71]. Two other indices described by Moorhead et al. [80] were determined. They illustrate the microbial metabolic limitations by plotting the proportional C:N vs. C:P acquiring enzymes. After connecting a line between the plot origin and point represented by these proportions, the length and angle of the vector were used to quantify relative C limitation and relative P vs. N limitations, respectively. The following equations were used:

$$\text{Vector length (L)} = \sqrt{(x^2 + y^2)} \quad (3)$$

$$\text{Vector angle (A)} = \text{DEGREES}(\text{ATAN2}(x, y)) \quad (4)$$

where  $x$  and  $y$  correspond, respectively, to acquiring enzyme C:P and C:N ratios. Longer vector length indicates greater C limitation. A vector angle  $<45^\circ$  denotes N limitation; an angle of  $>45^\circ$  denotes P limitation.

### 2.7. Statistical Analysis

Statistical analyses were carried out by using the software R (R Development Core Team, 3.5.0 version). Data were analyzed by ANOVAs after verifying data normality (Shapiro–Wilk test, 95%) and variance homogeneity (Bartlett’s test, 95%). Multiple mean comparisons were then carried out with the Tukey test. The effects of the interaction between the plant species and the two development stages (VS and RS) on the measured parameters were tested by using two-way ANOVA. Correlations between plant functional traits, soil characteristics, enzyme activities and stoichiometry components (enzyme, soil C:N:P ratios and vectors) were assessed with Pearson’s correlation test. Principal component analyses (PCA) were performed in order to analyze the changes in all the measured data to evaluate the effects of the species and their development stage on soil enzymes activities and microbial resources requirement. The Kaiser–Meyer–Olkin measure (KMO) of sampling adequacy and Bartlett’s test of sphericity were used to test the validity of PCA.

## 3. Results

### 3.1. Soil Characteristics

The total soil C and N contents and available P were determined at the two development stages using an isotope ratio mass spectrometer (IRMS) and P Olsen extraction, respectively. The results are presented in Table 1 and showed little variations among plant species regarding total soil C and N contents at the vegetative stage (VS). However, the values of C and N contents varied slightly at the reproductive stage (RS). Indeed, higher soil C and N were observed under crimson clover and the lowest ones under pea and wheat. Regarding phosphorus, P Olsen showed a very different variation pattern and was strongly dependent on the plant phenological stage. At VS, P Olsen was higher under legume crops (on average  $149.85 \pm 4.49 \text{ mg}\cdot\text{kg}^{-1}$ ) compared to wheat ( $143.53 \pm 3.31$ ). At RS, P Olsen decreased under all crops in comparison to VS. This decrease was more pronounced under faba bean (from  $147.84 \pm 3.21$  to  $100.69 \pm 5.75 \text{ mg}\cdot\text{kg}^{-1}$ ), white clover (from  $150.92 \pm 4.16$  to  $88.63 \pm 5.14 \text{ mg}\cdot\text{kg}^{-1}$ ) and crimson clover (from  $155.48 \pm 7.86$  to  $92.96 \pm 1.91 \text{ mg}\cdot\text{kg}^{-1}$ ). The amount of C, N and P elements were used to determine soil C:N, C:P and N:P ratios. The soil C:N ratios were on average close to  $8.57 \pm 0.79$  and similar for the two development stages, with few differences observed between species (legumes vs. wheat). Soil C:P ratios were higher under wheat than legumes at VS, while at RS, faba bean and the two clovers presented the highest C:P ratios. The same variation patterns were observed for soil N:P ratios.

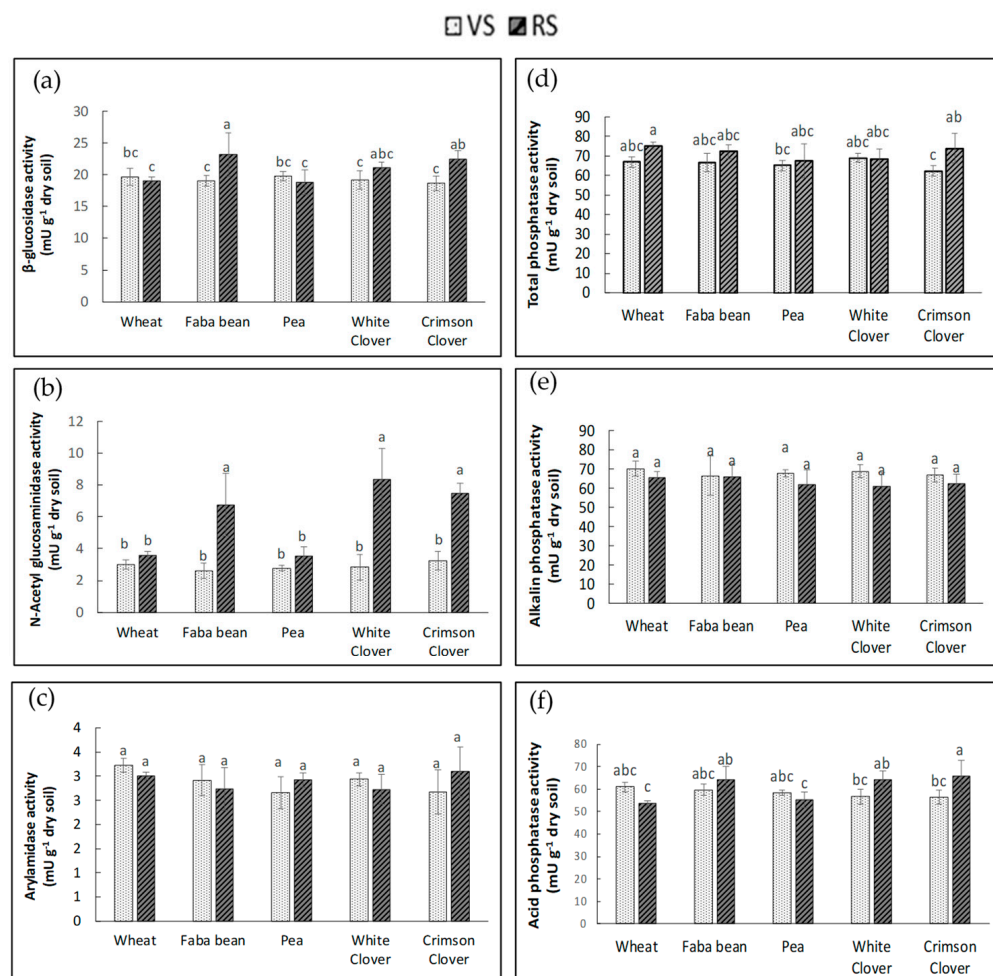
### 3.2. Enzymatic Activities

Different enzymatic activities related to C, N and P biogeochemical cycles were measured in the soils under the different plant species at two development stages. The six tested enzymes did not respond in similar ways under the plant species. The BG activity varied between  $18.66 \pm 1.15$  and  $23.15 \pm 3.41 \text{ nmol PNP/min/g dry soil}$  under the different plants and the two development stages (Figure 2a). At VS, this activity was similar in all soils, and it was equivalent to  $19.24 \pm 1.11 \text{ nmol PNP/min/g dry soil}$  on average. This activity increased significantly between VS and RS under faba bean and crimson clover.

**Table 1.** Soil chemical characteristics under all studied plants species at the two development stages.

|                                     | Total C (g·kg <sup>-1</sup> ) |                 | Total N (g·kg <sup>-1</sup> ) |                | P Olsen (mg·kg <sup>-1</sup> ) |                | C:N                |               | C:P                  |               | N:P                  |                |
|-------------------------------------|-------------------------------|-----------------|-------------------------------|----------------|--------------------------------|----------------|--------------------|---------------|----------------------|---------------|----------------------|----------------|
|                                     | VS                            | RS              | VS                            | RS             | VS                             | RS             | VS                 | RS            | VS                   | RS            | VS                   | RS             |
| Wheat                               | 11.77 ± 0.18 b                | 11.37 ± 0.28 ab | 1.77 ± 0.47 b                 | 1.29 ± 0.02 ab | 143.5 ± 3.3 a                  | 137.7 ± 2.3 b  | 6.94 ± 1.56 a      | 8.80 ± 0.28 a | 82.1 ± 1.8 b         | 82.6 ± 3.1 b  | 12.40 ± 3.36 b       | 9.39 ± 0.24 b  |
| Faba bean                           | 11.64 ± 0.22 ab               | 11.73 ± 0.10 bc | 1.36 ± 0.10 a                 | 1.33 ± 0.03 b  | 147.8 ± 3.2 ab                 | 100.6 ± 5.7 a  | 8.56 ± 0.52 b      | 8.78 ± 0.18 a | 78.8 ± 2.3 ab        | 116.8 ± 6.0 c | 9.22 ± 0.59 a        | 13.30 ± 0.56 c |
| Pea                                 | 11.25 ± 0.36 a                | 11.01 ± 0.24 a  | 1.29 ± 0.11 a                 | 1.28 ± 0.04 a  | 151.5 ± 3.5 ab                 | 130.9 ± 10.5 b | 8.73 ± 0.56 b      | 8.58 ± 0.28 a | 74.3 ± 3.0 a         | 84.5 ± 7.0 b  | 8.54 ± 0.80 a        | 9.83 ± 0.57 b  |
| White clover                        | 11.51 ± 0.18 ab               | 11.81 ± 0.26 cd | 1.25 ± 0.02 a                 | 1.34 ± 0.02 b  | 150.9 ± 4.1 ab                 | 88.6 ± 5.1 a   | 9.14 ± 0.11 b      | 8.81 ± 0.14 a | 76.3 ± 2.9 a         | 133.6 ± 6.4 d | 8.35 ± 0.37 a        | 15.16 ± 0.65 d |
| Crimson clover                      | 11.39 ± 0.26 ab               | 12.20 ± 0.29 d  | 1.33 ± 0.07 a                 | 1.39 ± 0.03 c  | 155.4 ± 7.8 b                  | 92.9 ± 1.9 a   | 8.54 ± 0.60 ab     | 8.74 ± 0.12 a | 73.4 ± 3.6 a         | 131.4 ± 3.3 d | 8.62 ± 0.49 a        | 15.02 ± 0.30 d |
| Plants species × development stages | <i>F</i> = 9.35 ***           |                 | <i>F</i> = 5.43 **            |                | <i>F</i> = 55.42 ***           |                | <i>F</i> = 5.17 ** |               | <i>F</i> = 93.85 *** |               | <i>F</i> = 29.64 *** |                |

VS: Vegetative stage; RS: Reproductive stage. Statistical data are expressed as means ± SD, (*n* = 5). Means in a column followed by the same letter are not significantly different (*p* < 0.05), different letters mean significant difference according to the Tukey test. Two-way ANOVA analysis (*F* values) showing the effect of the interaction between the plant species and the two development stages on soil chemical characteristics. \*\* *p* ≤ 0.01; \*\*\* *p* ≤ 0.001.



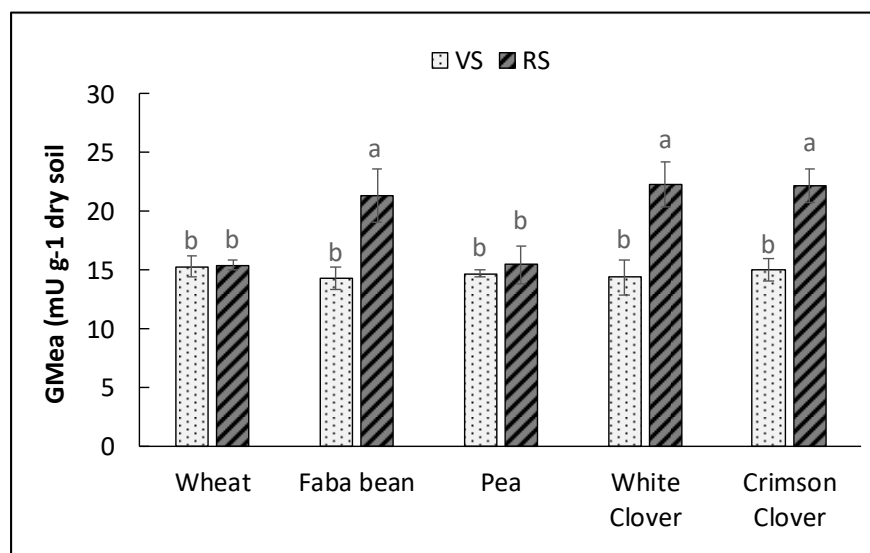
**Figure 2.** Enzyme activities under all plants species at the two development stages. (a)  $\beta$ -glucosidase, (b) N-acetylglucosaminidase, (c) Arylamidase, (d) Total phosphatases, (e) Alkaline phosphatase, (f) Acid phosphatase. VS: vegetative stage; RS: reproductive stage. Data are mean  $\pm$  SD ( $n = 5$ ). Letters indicate statistically significant difference between plants species and development stages at  $p < 0.05$  according to Tukey's test.

The NAG activity (Figure 2b) varied between  $2.62 \pm 0.47$  and  $8.33 \pm 1.96$  nmol PNP/min/g dry soil. At VS, NAG activity did not vary between plant species (it was on average equivalent to  $2.90 \pm 0.21$  nmol PNP/min/g dry soil). At RS, faba bean and the clovers showed consistently higher NAG activity values compared to VS. ( $F = 12.44$ ,  $p < 0.001$ ). The NAG activity values were three times higher at RS, such as for faba bean ( $6.76 \pm 1.99$  nmol PNP/min/g dry soil) and white and Crimson clovers ( $8.33 \pm 1.96$  and  $7.45 \pm 0.64$  nmol PNP/min/g dry soil, respectively). ARYLN activity (Figure 2c) ranged from  $2.66 \pm 0.34$  to  $3.23 \pm 0.14$  nmol PNP/min/g dry soil and was similar for both development stages (VS and RS) and plants species.

The results of the PHO, AKP and ACP activities are presented in Figure 2d–f, respectively. The three phosphatase activities showed similar ranges of variation (from  $53.83 \pm 1.18$  to  $75.12 \pm 1.78$  nmol PNP/min/g dry soil), but they presented different response patterns. The AKP activity was not affected by the two development stages (VS and RS) and plant species, while PHO and ACP activities increased significantly only under crimson clover at RS. Among phosphatase, ACP was the sole activity that varied significantly between plant species at RS. Indeed, faba bean and the clovers showed higher ACP activity compared to wheat and pea.

The geometric means (GMea) of the assessed enzymes were used as an integrating index of soil enzyme activities (Figure 3). GMea was calculated using only the three

enzyme activities that varied according to the plant's species:  $GMea = \sqrt[3]{(BG \times NAG \times ACP)}$ . At VS, no significant difference was observed between soils under different plants species with an average index of  $14.76 \pm 1.00$  nmol PNP/min/g dry soil. At RS, this index was significantly higher in soils with faba bean and white and crimson clovers ( $F = 19.54$ ,  $p < 0.001$ ). This enzyme index did not vary between the two development stages for soils under wheat and pea.



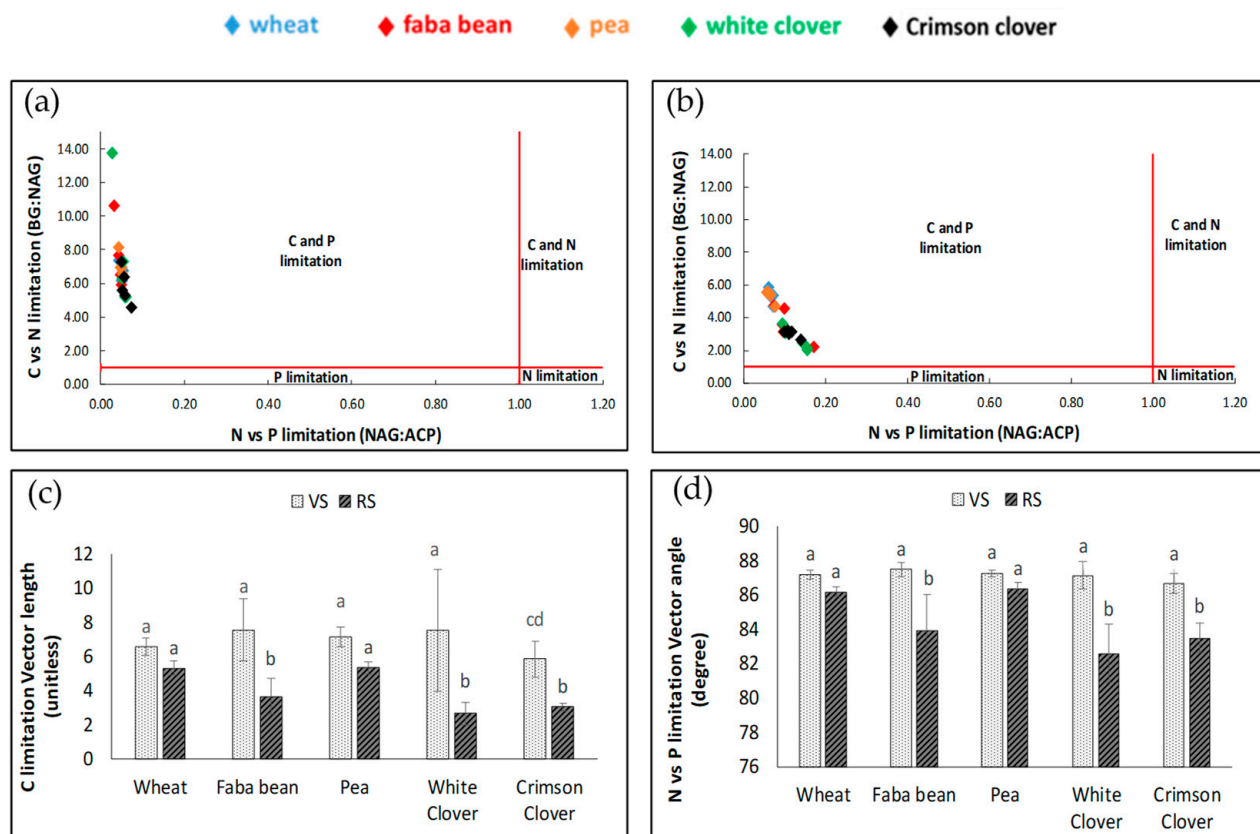
**Figure 3.** Patterns of the geometric means (GMea) of BG, NAG and ACP activities at the two development stages. BG:  $\beta$ -glucosidase; NAG: N-acetyl-glucosaminidase; ACP: acid phosphatase; VS: vegetative stage; RS: reproductive stage. GMea was calculated by using only the three enzyme activities that varied according to the plant's species.  $GMea = \sqrt[3]{(BG \times NAG \times ACP)}$ . Data are mean  $\pm$  SD ( $n = 5$ ). Letters indicate statistically significant difference between plants species and development stages at  $p < 0.05$  according to Tukey's test.

### 3.3. Enzyme's Stoichiometry

Enzymatic stoichiometry was also determined by using only enzymes that varied between plants species (BG, NAG and ACP), corresponding to these ratios: BG:NAG (C-acquiring enzymes vs. N-acquiring enzymes) and NAG:ACP (N-acquiring enzymes vs. P-acquiring enzymes). A correlation graph between these ratios was made to determine the resource requirements of microorganisms under the different crops according to Hill et al. [71]. A ratio equal to one was considered as a balance of nutrient demand; when above and below one we consider, for example, a transition of microorganisms from C limitation to N limitation for the ratio BG:NAG (Figure 4). This balance point at one was used as a horizontal and vertical baseline along the axis of enzyme activity ratios, and four different groups of microbial resource limitation (N limitation, P limitation, C and P limitation and N and P limitation) were categorized. At VS, microorganisms seemed to be limited in C and P (Figure 4a). The same situation was observed at RS (Figure 4b), but the C and P limitations were less pronounced (near the baselines).

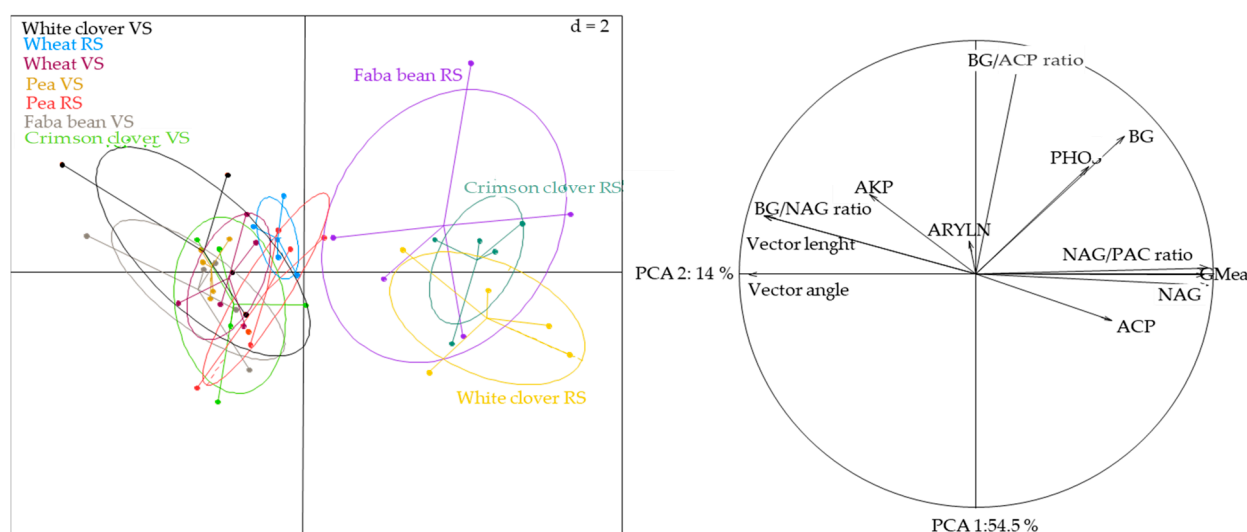
Other indexes of microbial resource requirement according to Moorhead et al. [80] are presented in Figure 4c,d. The vector length (Figure 4c) provides an indication of microbial C limitation; as this vector decreases, the C limitation becomes lower. This vector varied from  $2.67 \pm 0.67$  to  $7.56 \pm 3.56$  between species and their developmental stages ( $F = 2.82$ ,  $p < 0.05$ ). At VS, vector length varied slightly between plants (with an average of  $6.94 \pm 0.72$ ) and was lower under crimson clover. At RS compared to VS, vector length was lower under faba bean ( $3.66 \pm 1.03$ ), white clover ( $2.67 \pm 0.67$ ) and crimson clover ( $3.05 \pm 0.21$ ). These results were in accordance with the stoichiometric analysis, indicating lower C limitation under faba bean and clovers at RS compared to VS. The vector angle (Figure 4d) indicating N limitation ( $<45^\circ$ ) versus P ( $>45^\circ$ ) varied from  $82.60 \pm 1.69^\circ$  to

$87.49 \pm 0.43^\circ$  between plants and development stages ( $F = 6.83$ ,  $p < 0.001$ ), thus indicating a limitation in P. No effect of plant cover was observed at VS. Conversely, at RS, the angle was lower under faba bean and clover, indicating lower P limitation.



**Figure 4.** Enzyme stoichiometry under all studied plants species for identifying potential resource limitation at the two development stages. (a,b) Scatter graphs of enzyme stoichiometry corresponding to the ratio of BG:NAG according to the ratio of NAG: ACP at VS (a) and RS (b). BG:  $\beta$ -glucosidase; NAG: N-acetyl-glucosaminidase; ACP: acid phosphatase; VS: vegetative stage; RS: reproductive stage. To identify potential resource limitation in soil according to Hill et al. [71], 1 was used as a horizontal and vertical baseline along the axis of enzyme activity ratios, and four different groups of microbial resource limitations (N limitation, P limitation, C and P limitation and N and P limitation) were categorized. (c) Vector length and (d) vector angle calculated according to enzymes ratios. Longer vector L indicates greater C limitation. A vector angle of  $<45^\circ$  denotes N limitation; angles  $>45^\circ$  denote P limitation. Data are mean  $\pm$  SD ( $n = 5$ ). Letters indicate statistically significant difference between plants species at  $p < 0.05$  according to Tukey's test.

In order to reduce the dimensionality of the data set, a PCA was performed to highlight both the effect of plant species and their development stage on soil enzyme activities and their link to C, N and P nutrient limitation for microorganisms (Figure 5). Both the Kaiser–Meyer–Olkin measure of sampling adequacy ( $KMO = 0.578$ ) and Bartlett's test of sphericity ( $p = 0.000$ ) indicated the validity of PCA. The first two axes of PCA in Figure 5 explained 68.5% of the total variability, as illustrated by the separation of the two distinct clusters along the PCA axes. PCA clearly illustrated how the ACP, NAG, BG and PHO activities; GMea; and indexes of microbial resource requirement (based on enzymes ratios and vectors) classified the plant species according to stage development. The distribution of the clusters along the first axis showed that the faba bean and the two clovers at RS were significantly different from other modalities, which did not present any differentiation.



**Figure 5.** Principal component analysis (PCA) and correlation circle of the enzyme activities and indexes of microbial resource requirement. BG:  $\beta$ -glucosidase; NAG: N-acetyl-glucosaminidase; ACP: acid phosphatase; VS: vegetative stage; RS: reproductive stage; GMea: geometric mean.

### 3.4. Plant Traits and C Rhizodeposition

Biomass production per pot (Table 2) differed between species ( $F = 197.6$ ,  $p < 0.001$  for shoot biomass and  $F = 183.3$ ,  $p < 0.001$  for root biomass) and according to their development stage ( $F = 60.66$ ,  $p < 0.001$  for shoot and  $F = 29.69$ ,  $p < 0.001$  root). At VS, the highest shoot biomass production was observed for wheat and the lowest one for crimson clover. The biomass production of the three other plants species ranged between wheat and crimson clover. However, at RS, faba bean and the two clovers produced three and five times more aboveground biomass per pot than wheat and pea. Regarding root biomass, faba bean had the highest biomass ( $1.52 \pm 0.28$  g) at VS compared to the other plants. At RS, pea and wheat had the lowest root biomass, which was on average five times less than clovers and faba bean root biomasses. Biomass production resulted in a lower allocation of total plant root biomass under wheat and white clover at VS with higher shoot:root ratios ( $8.63 \pm 3.46$  and  $8.48 \pm 1.14$ , respectively). Conversely, at RS, pea presented the highest shoot:root ratios ( $10.98 \pm 4.79$ ).

Using WinRHIZO, the total root length was determined for both harvests (Table 2). Total root length was similar in all species at VS, but faba bean and both clover species produced much longer roots than wheat and pea did during reproductive growth.

Isotope labeling and isotope mass spectrometry (IRMS) analyses were used to determine the elemental composition of plant tissues and C rhizodeposition. Total C and N contents of the tissues were used to determine the C:N ratios of the plants (Table 3). Due to high N concentrations, regardless of development stage, lower C:N ratios were observed in legumes compared to wheat. No significant differences were observed between legume species.

Plant species rhizodeposited similar amounts of C before flowering, but faba bean and both clover species rhizodeposited more C during reproductive growth than wheat and pea did ( $F = 37.92$ ,  $p < 0.001$  between species and their developmental stage). The highest C rhizodeposition during reproductive growth was observed under crimson clover (Figure 6).

**Table 2.** Plant traits including shoot and root total dry mass per pot, shoot to root ratio and total root length for all studied plants species.

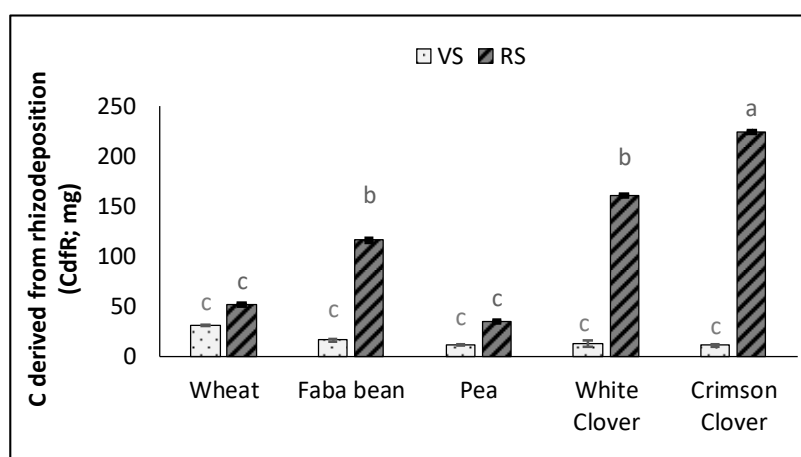
|                                     | Shoot Dry Mass (g)   |                | Root Dry Mass (g)    |               | Shoot:Root Ratio     |                | Total Root Length (cm) |                   |
|-------------------------------------|----------------------|----------------|----------------------|---------------|----------------------|----------------|------------------------|-------------------|
|                                     | VS                   | RS             | VS                   | RS            | VS                   | RS             | VS                     | RS                |
| Wheat                               | 4.14 ± 0.35 c        | 4.34 ± 0.34 a  | 0.55 ± 0.26 a        | 0.94 ± 0.09 a | 8.63 ± 3.46 b        | 4.59 ± 0.37 a  | 2435 ± 1031 a          | 8961 ± 5223 ab    |
| Faba bean                           | 2.08 ± 0.31 b        | 13.95 ± 7.77 b | 1.52 ± 0.28 b        | 6.46 ± 2.98 b | 1.37 ± 0.13 a        | 2.12 ± 0.38 a  | 4569 ± 1070 a          | 24,329 ± 13,953 b |
| Pea                                 | 2.25 ± 0.41 b        | 3.75 ± 0.97 a  | 0.55 ± 0.26 a        | 0.39 ± 0.18 a | 4.42 ± 1.03 a        | 10.98 ± 4.79 b | 4441 ± 1941 a          | 3199 ± 15,292 a   |
| White clover                        | 2.4 ± 0.24 b         | 25.88 ± 1.60 c | 0.28 ± 0.04 a        | 6.31 ± 1.66 b | 8.48 ± 1.14 b        | 4.31 ± 1.08 a  | 2379 ± 438 a           | 82,655 ± 21,032 c |
| Crimson clover                      | 1.19 ± 0.18 a        | 27.21 ± 2.03 c | 0.28 ± 0.04 a        | 5.03 ± 0.24 b | 4.19 ± 0.52 a        | 5.41 ± 0.44 a  | 2617 ± 860 a           | 60,859 ± 6339 c   |
| Plants species × development stages | <i>F</i> = 52.54 *** |                | <i>F</i> = 17.04 *** |               | <i>F</i> = 12.55 *** |                | <i>F</i> = 42.31 ***   |                   |

VS: vegetative stage; RS: reproductive stage. Statistical data are expressed as means ± SD, *n* = 5. Means in a column followed by different letters show significant differences (*p* < 0.05), different letters mean significant difference according to the Tukey test. Two-way ANOVA analysis (*F* values) showing the effect of the interaction between the plant species and the two development stages on soil chemical characteristics. \*\*\* *p* ≤ 0.001.

**Table 3.** Shoot and root C:N ratios for all studied plants species.

|                                     | Shoot C:N         |                  | Root C:N        |                |
|-------------------------------------|-------------------|------------------|-----------------|----------------|
|                                     | VS                | RS               | VS              | RS             |
| Wheat                               | 34.44 ± 2.16 c    | 115.92 ± 27.10 b | 44.75 ± 6.09 b  | 49.80 ± 9.46 b |
| Faba bean                           | 13.67 ± 1.07 a    | 33.11 ± 7.90 a   | 12.61 ± 0.29 a  | 15.61 ± 1.11 a |
| Pea                                 | 17.89 ± 3.10 b    | 48.15 ± 18.89 a  | 12.17 ± 0.31 a  | 18.19 ± 2.78 a |
| White clover                        | 12.44 ± 1.05 a    | 21.14 ± 1.90 a   | 14.37 ± 0.74 a  | 17.47 ± 1.08 a |
| Crimson clover                      | 13.05 ± 2.18 a    | 19.38 ± 1.05 a   | 14.75 ± 0.66 a  | 17.12 ± 1.01 a |
| Plants species × development stages | $F = 19.99^{***}$ |                  | $F = 0.43^{ns}$ |                |

VS: vegetative stage; RS: reproductive stage. Statistical data are expressed as means ± SD,  $n = 5$ . Means in a column followed by different letters show significant differences. ( $p < 0.05$ ), different letters mean significant difference according to the Tukey test. Two-way ANOVA analysis ( $F$  values) showing the effect of the interaction between the plant species and the two development stages on soil chemical characteristics.  $*** p \leq 0.001$ . ns: non significant.



**Figure 6.** Soil C derived from rhizodeposition (Cdfr) under all plants species at the two stages of development ( $\text{mg.pot}^{-1}$ ). VS: vegetative stage; RS: reproductive stage. Data are mean ± SD ( $n = 5$ ). Letters indicate statistical significant difference between plants species at  $p < 0.05$  according to Tukey's test.

### 3.5. Linking Plant, Soil and Microbial Components

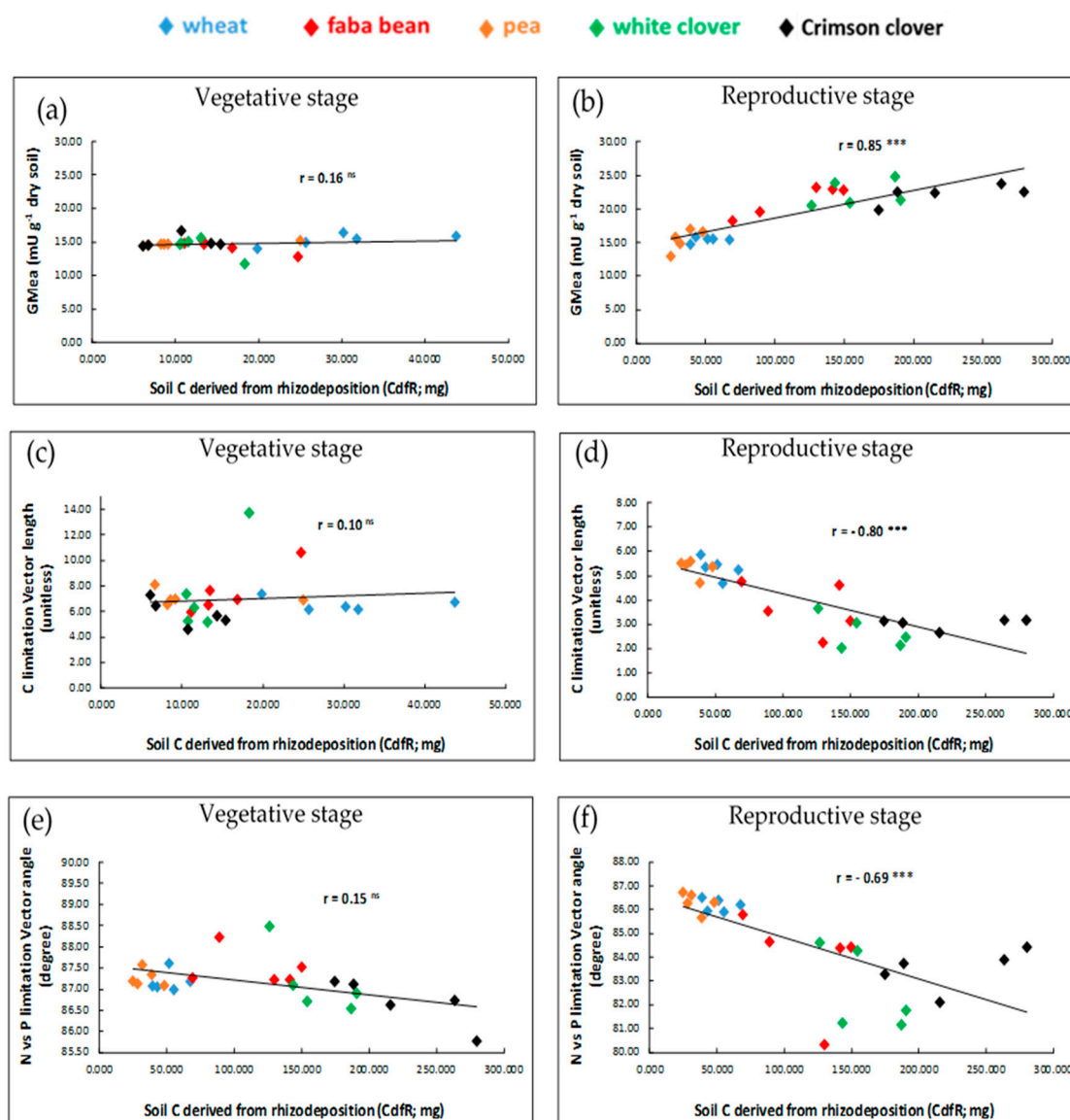
A correlation matrix was set up to analyze the link between plant traits (including rhizodeposition), soil characteristics and resource requirements of microorganisms through enzyme activities and their stoichiometry (Table 4). Plant traits linked to plant growth such as biomass production and total root length were positively correlated with soil C:P and N:P ratios as well as with enzyme activities (BG, NAG and ACP). The same observation was made with GMea. Positive correlations were also observed between soil C derived from rhizodeposition (Cdfr) and plant traits. Regarding enzyme ratios, negative correlations were observed between BG:NAG ratio and plant traits. These negative correlations were also observed with vector indexes (length and angle). Regarding C rhizodeposition, GMea was positively correlated with Cdfr, and vectors were inversely correlated with Cdfr.

Taking these correlations according to developmental stages (Figure 7), at VS, no correlation was observed between GMea and Cdfr ( $r = 0.16$ ,  $p > 0.05$ ) and similarly for vector length (C limitation;  $r = 0.10$ ,  $p > 0.05$ ) and the vector angle (N vs. P limitation;  $r = 0.15$ ,  $p > 0.05$ ). At RS, GMea was positively correlated with Cdfr ( $r = 0.85$ ,  $p < 0.05$ ). Vector length ( $r = -0.80$ ,  $p < 0.05$ ) and vector angle ( $r = -0.69$ ,  $p < 0.05$ ) were negatively correlated with Cdfr.

**Table 4.** Pearson correlation matrix showing plant traits, enzyme activities and stoichiometry and soil component relationships among the entire experiment.

|                | Shoot<br>Dry Mass | Root Dry<br>Mass | Shoot:Root | Root<br>Length   | CdFR             | Soil C:N       | Soil C:P         | Soil N:P         | ACP             | NAG              | BG              | GMea             | BG:NAG           | BG:ACP | NAG:ACP          | Vector<br>Length | Vector<br>Angle |
|----------------|-------------------|------------------|------------|------------------|------------------|----------------|------------------|------------------|-----------------|------------------|-----------------|------------------|------------------|--------|------------------|------------------|-----------------|
| Shoot dry mass | 1.00              |                  |            |                  |                  |                |                  |                  |                 |                  |                 |                  |                  |        |                  |                  |                 |
| Root dry mass  | <b>0.87 ***</b>   | 1.00             |            |                  |                  |                |                  |                  |                 |                  |                 |                  |                  |        |                  |                  |                 |
| Shoot:Root     | −0.16             | <b>−0.39 **</b>  | 1.00       |                  |                  |                |                  |                  |                 |                  |                 |                  |                  |        |                  |                  |                 |
| Root length    | <b>0.94 ***</b>   | <b>0.84 ***</b>  | −0.23      | 1.00             |                  |                |                  |                  |                 |                  |                 |                  |                  |        |                  |                  |                 |
| CdFR           | <b>0.94 ***</b>   | <b>0.81 ***</b>  | −0.16      | <b>0.86 ***</b>  | 1.00             |                |                  |                  |                 |                  |                 |                  |                  |        |                  |                  |                 |
| Soil C:N       | 0.14              | 0.17             | −0.12      | 0.17             | 0.14             | 1.00           |                  |                  |                 |                  |                 |                  |                  |        |                  |                  |                 |
| Soil C:P       | <b>0.95 ***</b>   | <b>0.88 ***</b>  | −0.21      | <b>0.90 ***</b>  | <b>0.93 ***</b>  | 0.15           | 1.00             |                  |                 |                  |                 |                  |                  |        |                  |                  |                 |
| Soil N:P       | <b>0.84 ***</b>   | <b>0.75 ***</b>  | −0.12      | <b>0.77 ***</b>  | <b>0.81 ***</b>  | <b>−0.32 *</b> | <b>0.88 ***</b>  | 1.00             |                 |                  |                 |                  |                  |        |                  |                  |                 |
| ACP            | <b>0.61 ***</b>   | <b>0.63 ***</b>  | −0.19      | <b>0.55 ***</b>  | <b>0.65 ***</b>  | −0.11          | <b>0.62 ***</b>  | <b>0.64 ***</b>  | 1.00            |                  |                 |                  |                  |        |                  |                  |                 |
| NAG            | <b>0.87 ***</b>   | <b>0.81 ***</b>  | −0.17      | <b>0.83 ***</b>  | <b>0.87 ***</b>  | 0.15           | <b>0.88 ***</b>  | <b>0.76 ***</b>  | <b>0.56 ***</b> | 1.00             |                 |                  |                  |        |                  |                  |                 |
| BG             | <b>0.67 ***</b>   | <b>0.76 ***</b>  | −0.16      | <b>0.54 ***</b>  | <b>0.65 ***</b>  | 0.09           | <b>0.64 ***</b>  | <b>0.56 ***</b>  | <b>0.59 ***</b> | <b>0.57 ***</b>  | 1.00            |                  |                  |        |                  |                  |                 |
| GMea           | <b>0.90 ***</b>   | <b>0.87 ***</b>  | −0.18      | <b>0.83 ***</b>  | <b>0.91 ***</b>  | 0.11           | <b>0.91 ***</b>  | <b>0.81 ***</b>  | <b>0.70 ***</b> | <b>0.97 ***</b>  | <b>0.72 ***</b> | 1.00             |                  |        |                  |                  |                 |
| BG:NAG         | <b>−0.69 ***</b>  | <b>−0.62 ***</b> | 0.09       | <b>−0.66 ***</b> | <b>−0.71 ***</b> | −0.04          | <b>−0.73 ***</b> | <b>−0.65 ***</b> | <b>−0.42 **</b> | <b>−0.84 ***</b> | <b>−0.33 *</b>  | <b>−0.82 ***</b> | 1.00             |        |                  |                  |                 |
| BG:ACP         | 0.19              | 0.26             | −0.01      | 0.10             | 0.13             | 0.22           | 0.14             | 0.03             | <b>−0.29 *</b>  | 0.13             | <b>0.59 ***</b> | 0.16             | 0.03             | 1.00   |                  |                  |                 |
| NAG:ACP        | <b>0.83 ***</b>   | <b>0.75 ***</b>  | −0.15      | <b>0.79 ***</b>  | <b>0.82 ***</b>  | 0.17           | <b>0.84 ***</b>  | <b>0.72 ***</b>  | <b>0.40 **</b>  | <b>0.98 ***</b>  | <b>0.50 **</b>  | <b>0.92 ***</b>  | <b>−0.84 ***</b> | 0.20   | 1.00             |                  |                 |
| Vector lenght  | <b>−0.69 ***</b>  | <b>−0.62 ***</b> | 0.09       | <b>−0.65 ***</b> | <b>−0.71 ***</b> | −0.04          | <b>−0.73 ***</b> | <b>−0.65 ***</b> | <b>−0.42 **</b> | −0.84            | <b>−0.33 *</b>  | <b>−0.82 ***</b> | <b>0.99 ***</b>  | 0.03   | <b>−0.84 ***</b> | 1.00             |                 |
| Vector angle   | <b>−0.83 ***</b>  | <b>−0.75 ***</b> | 0.15       | <b>−0.79 ***</b> | <b>−0.82 ***</b> | −0.17          | <b>−0.84 ***</b> | <b>−0.71 ***</b> | <b>−0.40 **</b> | <b>−0.98 ***</b> | <b>−0.51 **</b> | <b>−0.92 ***</b> | <b>0.84 ***</b>  | −0.20  | <b>−0.99 ***</b> | <b>0.84 ***</b>  | 1.00            |

BG:  $\beta$ -glucosidase; ACP: acid phosphatase; NAG: N-acetyl-glucosaminidase; GMea: geometric mean; CdFR: carbon derived from rhizodeposition. Significant correlations are shown in bold (\*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ ; \*\*\*  $p \leq 0.001$ ;  $n = 50$ ).



**Figure 7.** Pearson correlation of soil C derived from rhizodeposition with (a,b) GMea of enzymes activities. BG:  $\beta$ -glucosidase; NAG: N-acetyl-glucosaminidase; ACP: acid phosphatase; (c,d) vector length; (e,f) vector angle at vegetative and reproductive stage. Significant correlations ( $p < 0.05$ ;  $n = 25$ ) are shown by \*\*\*  $p \leq 0.001$ .

#### 4. Discussion

In this study, the objective was to evaluate the influence of different forage and seed legumes through C rhizodeposition on the dynamics of C, N and P elements in the soil and microbial communities resource requirements. The variation patterns of enzyme activity and microorganism limitations on C, N and P nutrients were linked to plant functional traits and C rhizodeposition. This reflects strong dependence on microbial communities and their activities on C, N and P sources in soils.

##### 4.1. Variability of Enzymatic Activities According to Plant Cover Crops

As postulated in our first hypothesis, this study shows that soil enzymatic activities may differ strongly between legume crops. Soil enzymes have been reported as useful soil-state bioindicators because they provide information on soil's ability to perform biogeochemical reactions [81,82]. We have chosen to measure  $\beta$ -glucosidase (BG),

N-acetyl- $\beta$ -glucosaminidase (NAG), arylamidase (ARYLN) and phosphatases activities (PHO, AKP and ACP), which decompose various substrates with varying complexity [81]. The BG enzyme is related to the C cycle, acting in the cleavage of cellobiose into glucose molecules [82]. The ARYLN enzyme catalyzes the hydrolysis of N-terminal amino acids from arylamides [83]. The NAG enzymes catalyze the hydrolysis of chitin, which is important in C and N cycling in soils. Both ARYLN and NAG activities are associated with microbial N acquisitions, and they play major roles in N mineralization in soils [84,85]. On the other hand, phosphatases are closely related to P mineralization.

This study revealed that both plant species and plant age can modify soil enzymatic activities. At the end of crop growth, compared to wheat and pea, faba bean and the two clovers increased the activities of BG, NAG and ACP involved in the degradation of C, N and P resources, respectively. Similarly, an integrative enzyme index calculated using BG, NAG and ACP activities through the geometric mean [79] showed higher levels of enzyme activities in the soil of these three legumes compared with wheat and pea. These results are in agreement with other works showing high enzyme activities in legume rhizosphere [86–88]. Maltais-Landry [56] also showed a stimulating effect of legumes on BG and NAG activities with species such as pea, faba bean and vetch compared with wheat and oats. It has also been shown that, in addition to BG, the presence of legumes increases the activity of ACP [89]. Similarly, Nuruzzaman et al. [90] demonstrated increasing ACP activity in the rhizosphere of lupin followed by faba bean, pea and wheat. These results highlighted the stimulation of legume crops on enzyme activities and partially confirmed the first hypothesis. However, it should be noted that pea, which is a legume crop, did not induce a significant difference on the enzymatic activities compared to wheat. This could be explained by the pea variety used and the functional traits developed by this species. During this study, pea exported most of the C and N resources to the aboveground part (data not shown) and produced less root biomass. A lower rhizodeposition of C was observed for pea and wheat compared with the other legume species; therefore, less resources were available in the belowground part compared to the other legumes.

The variation pattern in enzymatic activities according to the plant species seems to be explained by plant traits. Indeed, plant traits defined as morphological and physiological characteristics are studied in relation to many processes and ecosystem services [91]. Considered as important regulators of ecosystem processes, the functional traits of plants influence soil characteristics, soil resources dynamic and the abundance and diversity of microorganisms [92,93]. The differences observed between legumes and cereals are related to particular physiological functioning that allows them to fix atmospheric  $N_2$ , to have N-rich tissues and to enrich the soil in N such as those reported in the literature [20,94,95]. For example, ACP activity in the soil is produced and released by both roots and microorganisms. Similarly to proteins, phosphatases have relatively high N concentrations and may represent a significant investment of N. Therefore, increased N availability may raise the extracellular phosphatase activity of plants [96] and especially of legumes due to their N-rich tissues, which can explain the difference with wheat. In our study, positive correlation was observed between root N content (data not shown) and ACP activity ( $r = 0.65$ ,  $p < 0.001$ ), which is in line with the previous statement and confirms the observation made by Maltais-Landry. [56]. A positive correlation was found between root N content and BG ( $r = 0.76$ ,  $p < 0.001$ ) as well as NAG ( $r = 0.79$ ,  $p < 0.001$ ), highlighting the contribution of the physiological trait of legumes relative to the increase in soil enzyme activities of different enzymes in soil.

In this study, plant primary production (above and belowground biomass) was positively correlated with GMea ( $r = 0.90$ ,  $p < 0.001$  and  $r = 0.87$ ,  $p < 0.001$ , respectively). The level of soil enzyme activities also appeared to increase with root length ( $r = 0.83$ ,  $p < 0.001$ ). Forage legumes, which produce long and thin roots, increase the volume of soil exploration. According to previous studies [97,98], this property contributes by indirectly regulating the decomposition of soil organic matter (SOM) by destabilizing soil matrix, which protects organic carbon in aggregates. In contrast, faba bean produces large root diameter,

and it can be hypothesized that faba bean could impact soil structures such as the forage legumes. These traits contribute to the increased availability of resources [97] for eventual degradation by enzymes. Faba bean, white clover and crimson clover, which presented higher relative growth rates (RGR) between VS and RS (data not shown) and the longest roots, stimulated markedly soil enzyme activities. These species with greater N-rich tissues had the lowest C:N ratios. Taken together, their characteristics are consistent with traits of exploitative species that grow rapidly, with high tissue and exudate quality in contrast to conservative species that grow slowly and have low tissue and exudate quality [99–101]. These exploitative species are also associated with microbial communities dominated by bacteria and higher tissues decomposition rates due to their chemical composition and lower C:N ratio [102,103]. In this study, legumes species (except pea) presented the lowest C:N ratio in their tissues compared to wheat, and shoot C:N ( $r = -0.66$ ,  $p < 0.001$ ) and root C:N ( $r = -0.56$ ,  $p < 0.01$ ) were negatively correlated with GMea at RS.

Another important physiological trait is the ability of plants to input C in soil through the process of rhizodeposition. Soil C derived from rhizodeposition (CdfR) was positively correlated with GMea ( $r = 0.91$ ,  $p < 0.001$ ) and with each enzyme's individual activity (BG; NAG; ACP). Clovers (particularly crimson clover) and faba bean rhizodeposited the most C into the soil and resulted in higher levels of enzyme activities. This C source represents an important energy supply for the activity of microorganisms, which is essential in nutrient cycling [24,100,104,105]. Rhizodeposition also has a positive priming effect on SOM decomposition, and some studies have shown the positive effect of root C supply on enzyme activities in the soil [106–108]. Since the supply of C from the roots to the soil is rich in sugar compounds, it has been shown that BG activities is positively influenced by the presence of glucose-rich exudate [109]. Root C contributes to the substrate supply for the activity of some enzymes and could be used as a good indicator to evaluate the impact of different plant species on soil enzyme activities [100,110].

#### 4.2. Microbial Resource Requirement under the Different Crops

In addition to the determination of the variation of enzymatic activities in soil, the second objective of this study was to better understand the resource requirements of microorganisms under different plant species. Enzymatic activity being a response to metabolic needs, different indexes including measured activities and inspired by ecological stoichiometry were determined [64,65,111]. The approach detailed in Hill et al. [71], including enzyme ratios BG:NAG (C limitation vs. N limitation) and NAG:ACP (N limitation vs. P limitation), was used to evaluate C, N and P limitations of microorganisms under different plant cover crops according to their stage of development. In the present study, P and C limitations were less pronounced at RS, especially under faba bean and clovers. This was confirmed using other indexes. In fact, these three plants species presented the lowest vector length (indicating C limitation) and vector angle (corresponding to P limitation when angle  $> 45^\circ$ ). The variation in C and P limitation of microorganisms among plants is explained by their functional traits. The lower C and P limitations under faba bean and both clover species are related to their strong growth, root length and their ability to rhizodeposit C in the soil. During VS, no significant correlations were observed between plant traits and measured limitation indexes, showing that these plants had little impact on soil activities during vegetative growth. However, at RS, vector length and angle were negatively correlated with plant traits. More precisely, when the amounts of C rhizodeposited by plants increased, the vectors length and angle decreased, showing that the microorganisms were less limited in C and P. The lower resource limitation also seemed to be influenced by the N and C content of the roots. Indeed, vector length was negatively correlated with the N and C content of the roots ( $r = -0.73$ ,  $p < 0.001$  and  $r = -0.78$ ,  $p < 0.001$ , respectively). The same observations were made for vector angle with  $r = -0.59$ ,  $p < 0.001$  for roots N and  $r = -0.64$ ,  $p < 0.001$  for roots C. Taken together, these results confirm that plant cover crops modulate enzymatic activities and microorganism growth, particularly in the context of legume cultivation. Indeed, changes in plant communities can influence

microorganism and enzyme activities [49,112–114]. This finding is consistent with previous works showing that plant resources and their stoichiometry may strongly influence microbial traits [63,64,115,116]. Using soil enzyme vector analysis, Xiao et al. [61] indicated that the microbial community was co-limited by C and P during secondary plant succession in a natural grassland. These limitations were mainly associated with soil nutrient status.

The resource limitation of microorganisms is essentially related to the availability and stoichiometry of these resources in soil [61,67], and these resources are influenced by the plant species. In this study, vector length was negatively correlated with soil C:P ( $r = -0.72$ ,  $p < 0.001$ ) and soil N:P ( $r = -0.65$ ,  $p < 0.001$ ). The same observations were made with vector angle. Faba bean and clover species had the highest soil C:P and N:P. Accordingly, Cui et al. [67] found similar correlations between C and P limitation of microorganisms (vectors length and angle) and soil resources in alpine ecosystem. The ability of legumes such as faba bean and clovers to enrich soil with N and C explains the lower microorganisms' C and P limitation under these plants compared to others. Since the production of enzymes is made from amino acids, their synthesis requires a cost in N, C and also small amounts of P [64,117]. These elements must be available for the synthesis of enzymes. According to Allison and Cheaters [118], a microbe absorbing 100 units of P and allocating one unit to the production of enzymes would need 200 units of C and 57 units of N due to the C:N:P stoichiometry of enzymes. Thus, when C and N are in abundance, enzyme production could favor the acquisition of P resources. Based on a model developed by Allison and Cheaters [118], the addition of C, N and P substrates results in different ways changes can occur during microorganism growth, rate of enzyme synthesis and nutrient availability. Indeed, various substrate availability resulted in a decrease in the amounts of C-acquiring, N-acquiring and P-acquiring enzymes. The increase in C substrate would increase the growth of microorganisms and the synthesis of P and N enzymes. Consequently, microorganisms may switch to N limitation when C is in excess. Conversely, when N is present in excess, there is a strong synthesis of C and P acquiring enzymes that become limiting when related to N. These observations are in line with the results of this study, as C and N supply by plants under the faba bean and clovers mainly favored the decrease in P limitation at the end of plant growth. Our results show that the decrease in C limitation may be related to the increase in C availability through plant rhizodeposition. However, C limitation seems to be more complex, since at a certain threshold of C in the soil, there was a shift from C limitation to N limitation [119]. The increasing input of C and N under faba bean and clovers also contributes to greater microbial growth.

## 5. Conclusions

This study confirms the impact of plant species and their development stage on soil enzyme activities in relation to the dynamics of C, N and P. BG, NAG and ACP enzyme activities were positively influenced by the presence of faba bean and clovers compared to pea and wheat. A stoichiometric analysis of enzymes activities revealed a limitation of microorganisms in C and P resources at the end of plant growth, but strong differences were observed between legume species. These limitations in C and P were lower in the presence of faba bean and forage legumes (white clover and crimson clover). The variations of enzyme activities and C, N and P microorganism limitations were explained by plant functional traits. Indeed, plant biomass production, total root length, the ability of plants to rhizodeposit C and C and N contents of plant tissues were the main explicative factors of the observed variations. Faba bean and clovers were more effective in C rhizodeposition than compared to pea and wheat. It is known that N and C nutrient supplies positively contribute to nutritional requirements and growth of microorganisms and P availability in soil. The stoichiometric approach used in this study and by several authors is a useful tool for linking different levels of biological organization in order to better understand soil/plant/microorganisms interactions. This study also highlighted the strong connection between C, N and P nutrient cycling in soil and the factors modulating nutrients demand and the production patterns of soil enzymes.

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