agronomy

MDPI

Article

Clover Species Specific Influence on Microbial Abundance and Associated Enzyme Activities in Rhizosphere and Non-Rhizosphere Soils

Martin Brtnicky [1,2,3], Antonin Kintl [1,4], Tereza Hammerschmiedt [1,2], Adnan Mustafa [5,*], Jakub Elbl [4,6], Jiri Kucerik [3], Tomas Vyhnanek [7], Jiri Skladanka [8], Igor Hunady [4] and Jiri Holatko [1,*]

1 Department of Agrochemistry, Soil Science, Microbiology and Plant Nutrition, Faculty of AgriSciences, Mendel University in Brno, Zemedelska 1, 613 00 Brno, Czech Republic; Martin.Brtnicky@seznam.cz (M.B.); kintl@vupt.cz (A.K.); tereza.hammerschmiedt@mendelu.cz (T.H.)
2 Department of Geology and Soil Science, Faculty of Forestry and Wood Technology, Mendel University in Brno, Zemedelska 3, 613 00 Brno, Czech Republic
3 Institute of Chemistry and Technology of Environmental Protection, Faculty of Chemistry, Brno University of Technology, Purkynova 118, 612 00 Brno, Czech Republic; kucerik@fch.vut.cz
4 Agricultural Research, Ltd., Zahradni 400/1, 664 41 Troubsko, Czech Republic; jakub.elbl@mendelu.cz (J.E.); hunady@vupt.cz (I.H.)
5 Biology Center CAS, SoWa RI, Na Sadkach 7, 370 05 Ceske Budejovice, Czech Republic
6 Department of Agrosystems and Bioclimatology, Faculty of AgriSciences, Mendel University in Brno, Zemedelska 1, 613 00 Brno, Czech Republic
7 Department of Plant Biology, Faculty of AgriSciences, Mendel University in Brno, Zemedelska 1, 613 00 Brno, Czech Republic; tomas.vyhnanek@mendelu.cz
8 Department of Animal Nutrition and Forage Production, Mendel University in Brno, Zemedelska 1, 613 00 Brno, Czech Republic; jiri.skladanka@mendelu.cz
* Correspondence: adnanmustafa780@gmail.com (A.M.); jiri.holatko@mendelu.cz (J.H.)

Abstract: Legume cultivation, especially the clover species, has shown promoting effects on soil biological properties. However, the ways in which various clover species contribute to beneficial plant-rhizosphere soil interactions have remained neglected in the past. Therefore, we performed a field experiment to assess and compare the species-specific influence of five different clover species on plant traits, microbial soil health indicators, namely soil enzymes, microbial biomass and abundance and their potential nutrient cycling abilities under rhizosphere and non-rhizosphere soils. For this, soil samples from bulk soil and rhizosphere of each clover species were collected and analyzed for soil enzymes including β-glucosidase, arylsulfatase, phosphatase, N-acetyl-β-D-glucosaminidase, and urease and microbial communities' abundance. Results revealed that the soil biological properties were more affected in the rhizosoil than in the bulk soil, although the individual legume crop variants differed in the rate and extent of the differential impact on either rhizosoil or bulk soil. The most significantly affected species-specific properties were ammonium oxidizing bacteria and phosphorus-solubilizing microbiota in the rhizosoil of white clover and alsike clover variants, whereas the least impact was exerted by sweet clover. The biological properties of rhizosoil showed a significant effect on the plant qualitative and quantitative properties. We further detected antagonism among N and P + K transfer from the rhizosoil to plants, which influenced above ground and root biomass. Overall, these results suggest that the positive effects of clover species cultivation on rhizosphere soil properties are species specific.

Keywords: rhizosoil; bulk soil; soil microbiota; soil enzymes; plant biomass; plant nutrients

check for updates

Citation: Brtnicky, M.; Kintl, A.; Hammerschmiedt, T.; Mustafa, A.; Elbl, J.; Kucerik, J.; Vyhnanek, T.; Skladanka, J.; Hunady, I.; Holatko, J. Clover Species Specific Influence on Microbial Abundance and Associated Enzyme Activities in Rhizosphere and Non-Rhizosphere Soils. *Agronomy* **2021**, *11*, 2214. https://doi.org/10.3390/agronomy11112214

Academic Editor: Jianjun Yang

Received: 10 September 2021
Accepted: 27 October 2021
Published: 31 October 2021

1. Introduction

Global food demand has been expected to increase continuously in the coming decades due to enormous growth in human population and due to increasing incomes around the globe [1]. Crop intensification associated with the agricultural revolution have paved

the way to supply food for increasing population [2,3]. However, intensive agricultural practices have resulted in declined soil fertility that ultimately limits sustainable crop production [4,5]. The increase of the crop yield per unit area has now become a great challenge for the scientific community. In this regard, the application of synthetic fertilizers has gained popularity among farmers, but the continuous use of fertilizers results in environmental pollution, and loss of biodiversity and soil quality [6]. Therefore, the adaptation of suitable strategies is required to enhance the crop production by increasing soil quality and preserving biodiversity in a sustainable way.

The cultivation of legumes in this respect is a widely accepted win-win strategy worldwide. The clover (*Trifolium*) and closely related genera are the legumes of great agricultural importance both as fodders in livestock production [7] and feedstock biomass in the green bio refinery [8] worldwide. The main feature which favors clover as an advantageous cover crop [9] and intercrop is the ability to provide N due to its N_2 fixation ability [10]. The N_2 fixation is facilitated by the symbiotic rhizobacteria-*Rhizobium leguminosarum bv. trifolii* for clovers, *Sinorhizobium meliloti* for sweet clover [11]. The interaction between the legume roots and rhizobacteria is mediated and regulated by extracellular signaling [12]. This legume-microbes signaling is facilitated through polysaccharides, secreted proteins, quorum-sensing via N-acyl homoserine lactone (AHL), and compounds involved in plant cytokinin signaling [13]. However, the efficiency and quality of transfer and response of plant to the rhizobia signaling may be affected by the diversity, abundance, and activity of the other plant-associated bacteria, which can either support the production or destroy signal AHL [14]. The plant-associated bacteria are present in variable numbers and taxa in the "rhizosoil" of legume plants.

The term "rhizosoil" refers to an area of soil in close proximity (0.39–0.64 mm) to the roots [15] that is directly affected by root secretions and associated soil microorganisms. It is affected by loosely separated plant cells the rhizodeposition [16,17], and proteins and sugars released by the roots i.e., root exudates [18]. This leads to more complex interactions that significantly affect (apart from rhizobia-legume interaction) plant growth and microbial competition in general. Most of the nutrient transfer between plants and the microflora takes place directly in the rhizosoil, which is thus distinguished from the distal soil of the bulk on the level of the microbial diversity [19], physiology, nutrient fluxes, and content of both macro- and microelements [20]. The principal differences between the rhizosphere soil (rhizosoil) and bulk soil (non-rhizosphere soil) are generally reported [21] as having differential microbial community profiles depending on the soil space (rhizosphere × bulk) [22] or different adaptation of rhizobia to variable environments of rhizosoil and bulk soil [23]. The significance and perseverance of the specific effect of rhizosoil community composition enables prolonged determination of substantial differences present within distinct soil [24], influenced by a defined agricultural treatment.

Nevertheless, the evidence of different plant species able hosting specific microbial communities when grown on the same soil [25] proved that plants can shape their rhizosphere microbiome [26,27]. The effect of different legume species grown under treatment affecting their specific rhizosphere community on the crop yield, soil community and properties, was already referred [28–30]. The authors of these studies found that mixed strain inoculation increased a higher total plant yield [28], legume-based mixture-treated soil (inoculated with commercial inoculum) showed prolonged induced effect on proportion of plants forming nodules and plant biomass when applied to the four legume species [30]. However, none of these works focused on the impact of diverse legume species-driven rhizosphere soil microbial community on nutrient content, enzyme activities, total microbial abundance, and activity. Therefore, in this work we focused on the evaluation of species-specific effect of various types of legumes on the soil biological properties, nutrient transfer, and plant qualitative and quantitative properties in the agriculture system of cover crops (in one-seasoned small scale plot experiment). We assume that various legume species determined a different soil nutrient composition due to the rhizodeposition, root exudation, and dynamic interplay in root−soil−microbiome interactions which

mediate changes in the nutrient fluxes [31]. We further presume that the differences in rhizosoil may be non-correlative or even divergent to the nutrient transfer in bulk soil. We hypothesized that,

- The biological properties of rhizosoil would exhibit generally stronger relation or correlation among each other (due to less diverse and more specified microbial community) than the biological properties of bulk soil (which likely exert much higher microbial diversity).
- The nutrient content of plant biomass would correlate significantly more positively with the biological properties of rhizosoil (due to the mutual interaction) than with biological properties of bulk soil (because of weak or no interaction).
- There would be a significant species-specific effect of tested types of legumes on both biological properties of soil (enzyme activity, microbial abundance) and nutrient content in the plant biomass.

2. Materials and Methods

2.1. Site Description and Experimental Setup

A small-scaled plot experiment under field conditions was carried out at the locality near Troubsko, Czech Republic 49°10′25, 34 N, 16°29′49, 03 E (Figure 1). In terms of agroecological division, the experimental area is characterized by mild warm/ mild dry climate with an altitude of 287 m a. s. l., average annual temperature of 8.95 °C, and an average annual rainfall of 525.6 mm (1981–2010), see (Supplementary Materials, Table S1). Geologically this area is the part of Bohemian Massif region, having the soil type silt to silty loam. The soil was characterized as Haplic luvisol according to FAO soil classification [32], in more detail in [33].

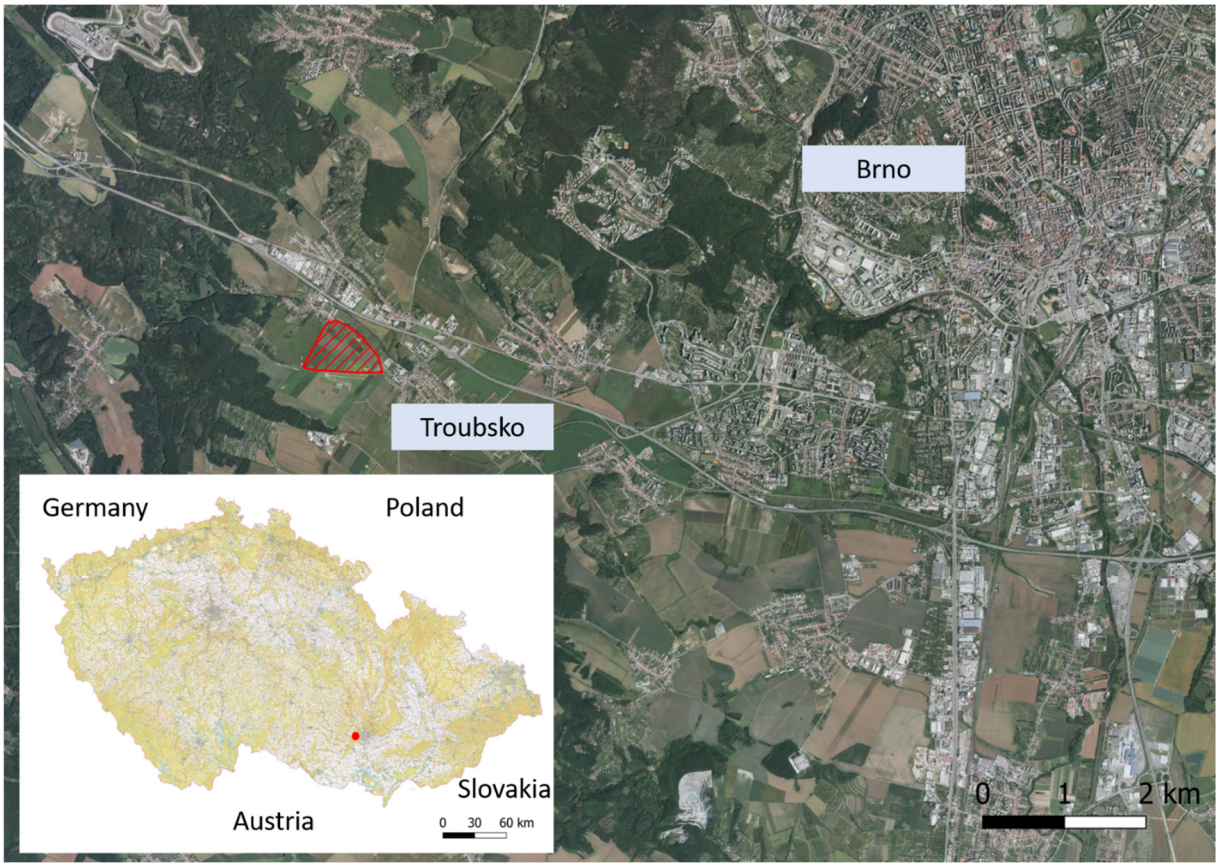

Figure 1. Location of the experimental site at the Troubsko, Czech Republic.

The pre-crop on the plot in 2019 was winter rye (*Secale cereale* L.). After its harvesting, the stubbles were buried to a depth of 8 cm by tillage. The experimental area (150 m^2) for each of 5 experimental crops (Table 1) was divided into 5 plots (30 m^2). Two edge plots (i.e., border plots between sown variants) were skipped, therefore, the division lead to 3 small-scale-plots per a particular crop type, each plot of 3 × 10 m.

Table 1. Experimental arrangement according to the species, variety, and seed dose per square meter.

Species (lat.)	Species	Variety	Sowing [g·m^{-2}]
Trifolium incarnatum L.	crimson clover	Kardinál	2.74
Trifolium repens L.	white clover	Rivendel (small-leafed)	0.60
Trifolium hybridum L.	alsike clover	Táborský	1.78
Melilotus albus Medik.	sweet clover	Běla (2-year)	1.71
Lotus corniculatus L.	bird's-foot trefoil	Lotar	1.60

Sowing of experimental crops to a depth of 1.5 cm and with a standard row spacing of 12.5 cm led to 24 rows per a single small-scale plot. After sowing, the land was rolled and no other agronomic practices were applied until harvesting. Harvesting and sampling for plant biomass and soil properties analyses were performed 76 days after sowing.

2.2. Soil Sampling and Processing

At the end of crop season 2020 (day 76 of the experiment), the mixed samples of soil and plant biomass were collected from 10 plants. The entire total plant biomass per a harvested area was summed up, the number of growing clover plants per square meter was not quantified. The root balls of plants for analysis were carefully dug out from the soil in each plot up to 10 cm depth and placed in the plastic bag and transported to the lab. The root ball was carefully disturbed, individual roots were placed on graph paper and the soil matter which was more than 2 mm further from the root surface, was cut off with a scalpel. This cut-off soil was collected and represented a non-rhizosphere soil (herein after referred as bulk soil) from each experimental variant: I. crimson clover, III. white clover, V. alsike clover, VII. sweet clover, IX. bird's-foot trefoil (Table 2). Subsequently, the remaining soil matter from the plant root cover was removed representing the closest layer of soil on the root surface (≤2 mm) i.e., rhizosphere soil (herein after referred as rhizosoil) variants: II. crimson clover, IV. white clover, VI. alsike clover, VIII. sweet clover, X. bird's-foot trefoil (Figure 2). All experimental sample variants, which were subjected to the analyses and determination of soil properties, are listed in the Table 2.

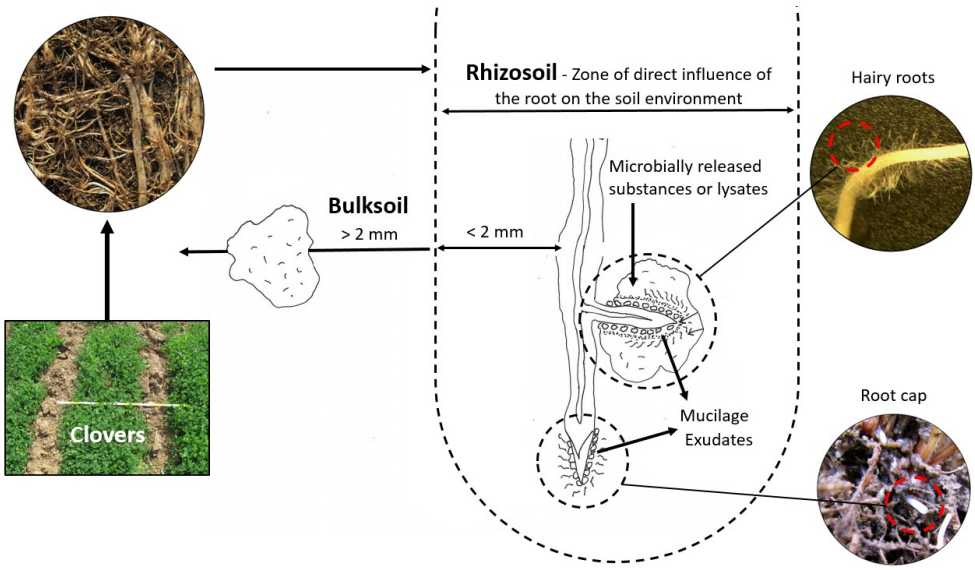

Figure 2. Pictorial representation of rhizosoil and bulk soil samplings.

Table 2. Samples according to the species and root-soil proximity.

Number	Samples	Species	Description
I.	crimson clover bulk	*Trifolium incarnatum*	soil >2 mm from the root
II.	crimson clover rhizosoil	*Trifolium incarnatum*	soil ≤2 mm close the root
III.	white clover bulk	*Trifolium repens*	soil >2 mm from the root
IV.	white clover rhizosoil	*Trifolium repens*	soil ≤2 mm close the root
V.	alsike clover bulk	*Trifolium hybridum*	soil >2 mm from the root
VI.	alsike clover rhizosoil	*Trifolium hybridum*	soil ≤2 mm close the root
VII.	sweet clover bulk	*Melilotus albus*	soil >2 mm from the root
VIII.	sweet clover rhizosoil	*Melilotus albus*	soil ≤2 mm close the root
IX.	bird's-foot trefoil bulk	*Lotus corniculatus*	soil >2 mm from the root
X.	bird's-foot trefoil rhizosoil	*Lotus corniculatus*	soil ≤2 mm close the root

Each soil sample was homogenized (bigger particles were crushed) by sieving through a 2 mm mesh and cooled down (4 °C). The samples for the enzyme activity assays and for the soil DNA isolation (and qPCR analysis) were freeze-dried [34].

2.3. Plant Biomass

The shoots were cut at the ground level, the roots were removed from the soil and washed with water [35]. Fresh aboveground biomass (AGB) and roots biomass were measured gravimetrically by weighing the shoots and cleaned roots separately on the analytical scales. The weighed shoots were dried at 60 °C to the constant weight, and dry AGB was measured gravimetrically by weighing the dried shoots on the analytical scales. Dry plant biomass (AGB or root) samples for elemental analysis were prepared using Kjeldal digestion procedure. Total nitrogen (N) content in plant biomass was determined by Kjeldahl method. Total phosphorus (P) and total potassium (K) content were analyzed by AAS-the atomic absorption spectrometer Agilent 55B AA (Agilent Technologies, Santa Clara, CA, USA).

2.4. Soil Enzyme Activities

The soil enzyme activities were measured according to [36], crudely extracted in a water soil suspension, using on-microplate design. Samples were freeze-dried before extraction. The p-nitrophenol (PNP)-derivatives of the specific soil substrates were used for UV-Vis spectrophotometric measurement at λ = 405 nm. β-glucosidase (GLU) utilized 4-nitrophenyl β-D-glucopyranoside [37], arylsulfatase (ARS) utilized potassium 4-nitrophenyl sulfate [38], phosphatase (Phos) utilized 4-nitrophenyl-phosphate disodium salt hexahydrate [39], and N-acetyl-β-D-glucosaminidase (NAG) utilized 4-Nitrophenyl-N-acetyl-β-D-glucosaminide [39]. Urease (Ure) activity was determined [40] as an amount of ammonium produced from the substrate urea, which was detected UV-Vis spectrophotometrically according to a modified Berthelot method using the reagent sodium salicylate, which form a product cyanurate (λ = 650 nm). Microplate reader Tecan Infinite 200 Pro (Tecan Group Ltd., Männedorf, Switzerland) was used for measurement. Each soil sample was measured in nine replicates. The activities were expressed in the units $nmol \cdot min^{-1} \cdot g^{-1}$.

2.5. DNA Extraction and Real-Time qPCR

A commercial E.Z.N.A.® soil DNA kit (Omega Bio-tek, Norcross, GA, USA) was used to extract DNA from approx. 0.25 g of lyophilized soil. DNA extracts were used for real-time PCR for quantification of partial bacterial (16S rDNA), fungal (18S rDNA) ribosomal RNA encoding DNA loci, and quantification of target genes vis amoA (coding for ammonium monooxygenase enzyme–indicator of ammonium oxidizing bacteria (AOB), nirS (coding for nitrite reductase enzyme–indicator of denitrification), phoD (coding for alkaline phosphatase enzyme–indicator of phosphate solubilization). Spiking with the DNA of plasmid vector pUC18 derivate was used for internal standardization for valuation of yield efficiency and contamination with PCR inhibitors of each sam-

ple. Isolated DNA was quantified on the Picodrop device (Quantica, Picodrop Limited, Cambridge, UK). SYBR-green assays were performed with CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The primers used were 1108F (5′ ATGGYTGTCGTCAGCTCGTG 3′) and 1132R (5′ GGGTTGCGCTCGTTGC 3′) for bacteria [41], FF390 (5′ CGATAACGAACGAGACCT 3′) and FR1 (5′ AICCATTCAATCGGTAIT 3′) for fungi [42], 517F (5′CCAGCAGCCGCGGTAAT 3′), AMOA1F (5′ GGGGTTTCTACTGGTGGT 3′) and AMOA1R (5′ CCCCTCKGSAAAGCCTTCTTC 3′) for ammonia-oxidizing bacterial (AOB) [43], nirSCd3aF (5′ AACGYSAAGGARACSGG 3′) and nirSR3cd (5′ GASTTCGGRTGSGTCTTSAYGAA 3′) for denitrifying microbiota (nirS) [44], ALPS-F730 (5′ CAGTGGGACGACCACGAGGT 3′) and ALPS-1101 (5′ GAGGCCGATCGGCATGTCG 3′) for phosphate solubilizers (ALPS) [45]. Primers SQP (5′ GTTTTCCCAGTCACGAC 3′) and SQPR2 (5′ CTCGTATGTTGTGTGGAA 3′) were used for qPCR quantification of pUC18-derivate DNA (internal standard). All the reagents and reactives used were of analytical quality: oligonucleotides provided by Eurofins (Luxembourg City, Luxembourg) and 5x HOT FIREPol EvaGreen qPCR Mix Plus (NO ROX) provided by Solis Biodyne (Tartu, Estonia).

2.6. Statistical Analyses

Data processing and statistical analysis were carried out with the help of the statistical program R version 3.6.1. [46], together with the additional packages "ggplot2" [47] for creating all the graphs.

One-way analysis of variance (ANOVA) was used for testing the effect of different treatments to the soil properties and to detect the statistically significant difference among the treatments it was applied the post-hoc Duncan's multiple range test from package "agricolae" [48] at the significance level of 0.05. For testing the effect of plants (as first factor) and sampling place (as second factor)—also their interaction—two-way analysis of variance was used (ANOVA). The factor level means calculating (with 95% confidence interval (CI)) was carried out by using "treatment contrast". Partial eta-squared (ηp2) from package "BaylorEdPsych" [49] was employed for measuring the effect size, and the Pearson's correlation coefficient (with 95% CI) was applied for measuring the linear dependence among the soil properties.

For modelling the relationships between the soil properties with dependence of selected treatments principal component analysis (PCA) was also used with the help of the additional packages "factoextra" [50] and "FactoMineR" [51]. The chart of the correlation matrix was created by using additional package "PerformanceAnalytics" [52].

3. Results

3.1. Plant Biomass and Nutrient Content Relation to Rhizosoil Microbial Abundance and Activity

The plant biomass was determined as aboveground (AGB) fresh and dry (shoot) values and root fresh and dry values. The significantly highest AGB fresh value was received for crimson clover, significantly the lowest two values showed sweet clover and bird's foot trefoil. Significantly increased dry AGB was received for crimson clover again, compared to the other variants, from which the significantly lowest three values belonged to sweet, white, and alsike clovers (Figure 3). The root fresh biomass was insignificantly highest in white and alsike clover variants, with significantly lowest value for sweet clover again. Root dry biomass was the lowest in the variant crimson clover and its value was significantly decreased compared to bird's foot trefoil and white clover, the highest in average (Figure 3). These findings indicated a significant disproportion in the shoot:root ratio of crimson clover, which was more than 4 for fresh and more than 7 for dry biomass. The shoot:root ratio of both fresh and dry biomass was between 2 and 3 for other clover types. The Pearson's analysis showed significant positive correlation (Figure A1) of AGB fresh and urease (r = 0.5) as well as 18S rDNA (r = 0.56) in rhizosoil. Root fresh biomass correlated (Figure A1) significantly positively with all rhizosoil microbial abundance traits

(16S and 18S rDNA, AOB, nirS, ALPS; r = 0.63–0.83) and with all rhizosolic enzymes (ARS, GLU, NAG, Phos, Ure; r = 0.62–0.92).

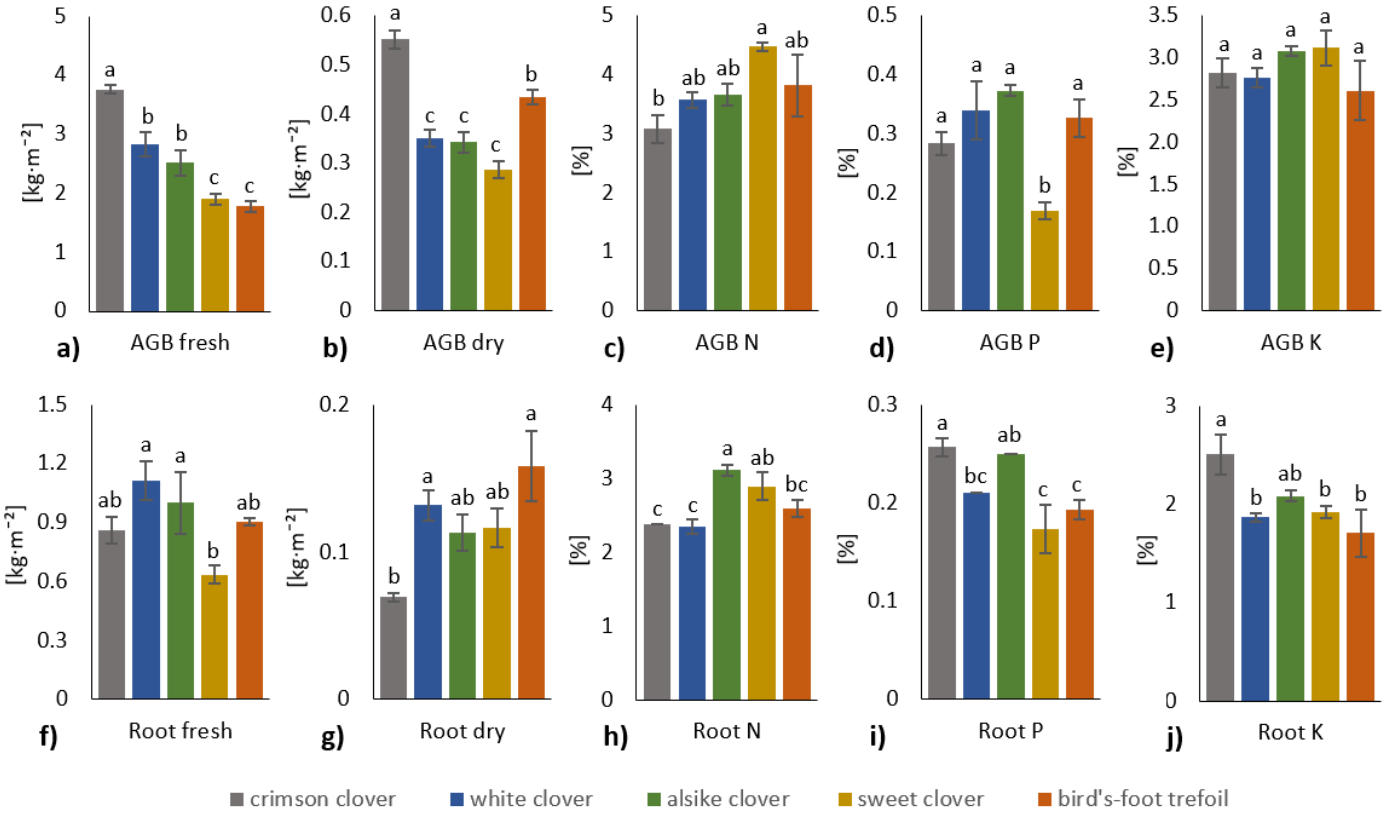

Figure 3. Plant (**a**) fresh and (**b**) dry aboveground biomass (AGB), (**f**) fresh and (**g**) dry root biomass, and (**c**–**e**, **h**–**j**) biomass nutrient (N,P,K) content in dry plant biomass of experimental variants = various types of clover/legume crops harvested after plant growth period 76 days. Values are displayed in kg·m^{-2} (AGB and root biomass) and weight per cents (% *w*/*w*), calculated as average from independent replicates (n = 3), error bars represent standard deviation. The different letters express the statistical differences at significance level $p \leq 0.05$.

The nutrient content was determined in dry plant biomass of both shoots and roots as well. AGB N was insignificantly different between all variants except of crimson clover (the lowest in average), which only was significantly decreased compared to sweet clover (Figure 3). The crimson clover's root N was together with white clover significantly lower as compared to sweet clover and alsike clover. AGB P of the sweet clover variant was on the contrary significantly decreased in comparison to the other variants, and the root P content was significantly lowered in sweet clover and bird's-foot trefoil in comparison to alsike clover and crimson clover. Only AGB K showed comparable values for all variants without statistical significance, while root K was significantly decreased in white clover, sweet clover, and bird's-foot trefoil variants compared to crimson clover (Figure 3).

The final biomass yield could be ascribed from the content of nutrients (Figures A1 and A2). Fresh AGB correlated positively with root P (r = 0.7) and root K (r = 0.74) and negatively with AGB N (r = −0.67); moreover, it correlated negatively with dry root biomass (r = −0.71). Dry AGB correlated negatively with AGB N (r = −0.66) and root N (r = −0.57) as well. Fresh root correlated positively with AGB P (r = 0.84), dry root correlated with root P (r = −0.58) and root K (r = −0.68).

The mutual correlation was found between content of nutrients too (Figures A1 and A2). AGB N correlated positively with AGB K (r = 0.56) and negatively with root P (r = −0.67) and root K (r = −0.61). AGB P correlated positively with root P (r = 0.49). Root P correlated positively with root K (0.67).

The significant correlations were obtained between nutrient content and microbial properties in the rhizosoil (Figures A1 and A2). AGB P highly positive correlated with Phos (r = 0.87), GLU (r = 0.88), NAG (r = 0.76) and with 16S rDNA (r = 0.7), 18S rDNA (r = 0.81). AGB N showed opposite correlations: moderate negative correlation with Phos (r = −0.53) and 18S rDNA (r = −0.64). Root P correlated positively to the Phos (r = 0.54), 18S rDNA (r = 0.73), AOB (r = 0.51).

The Pearson's correlation analysis equaled the putatively less significant relation between content of nutrients in plant biomass and bulk soil microbial properties (Figures 4 and 5). AGB N exerted positive correlation with NAG (r = 0.54) and negative correlation with urease (r = −0.5) and ALPS (r = −0.7). AGB P correlated negatively with AOB (r = −0.51), AGB K correlated negatively with Phos (r = −0.6). Root P correlated positively with ALPS (r = 0.54), urease (r = 0.65), and negatively with NAG (r = −0.71). Root N showed mostly negative correlation with 16S rDNA (r = −0.64), AOB (r = −0.61), nirS (r = −0.68), ALPS (r = −0.74), and ARS (r = −0.71). Root K correlated positively with AOB (r = 0.51), ALPS (r = 0.61), urease (r = 0.61), and negatively with NAG (r = −0.62).

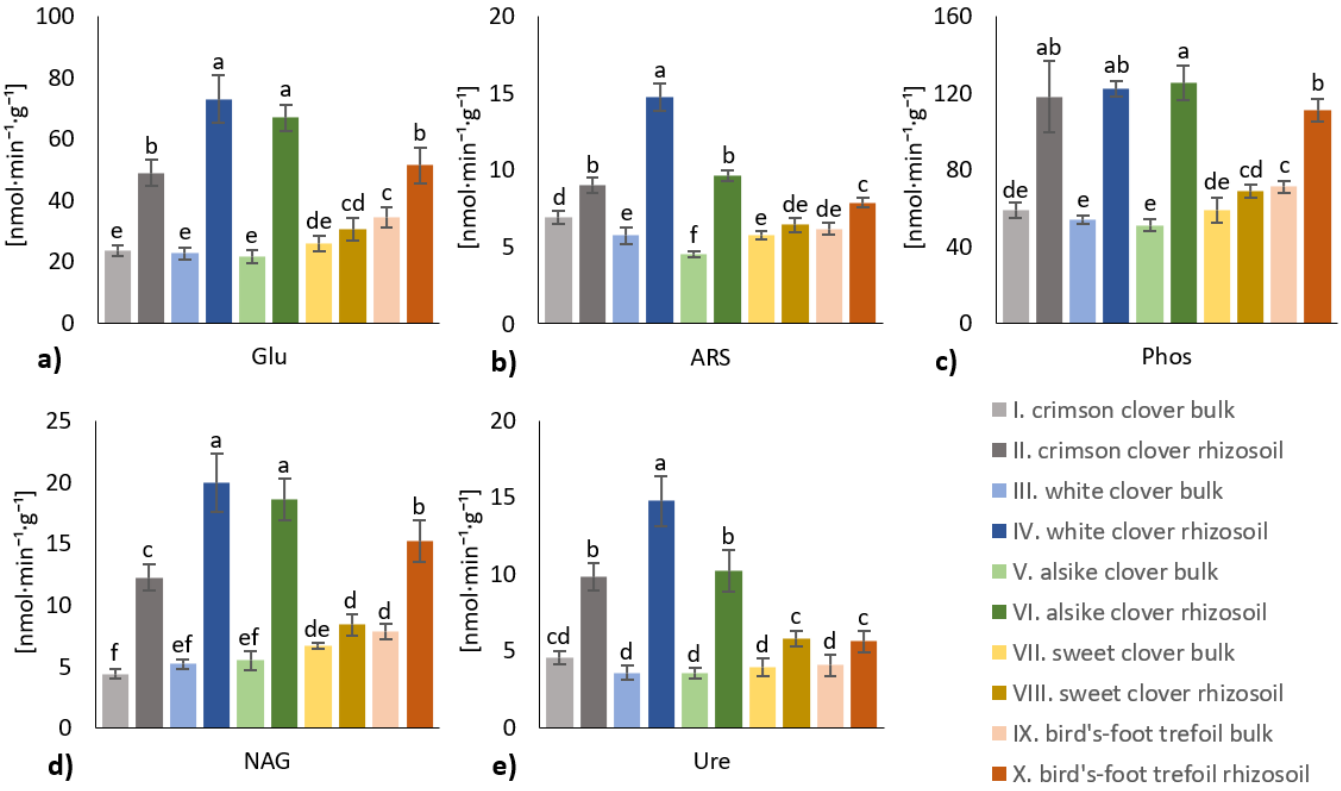

Figure 4. Soil enzyme activities in rhizosoil and bulk soil of experimental variants, collected from field cultures of various types of clover/legume crops after plant growth period 76 days: (**a**) β-glucosidase, (**b**) arylsulfatase, (**c**) phosphatase, (**d**) N-acetyl-β-D-glucosaminidase, (**e**) urease. Values (displayed in nmol·min^{-1}·g^{-1}) were calculated as average from independent replicates (n = 27), error bars represent standard deviation. The different letters express the statistical differences at significance level $p \leq 0.05$.

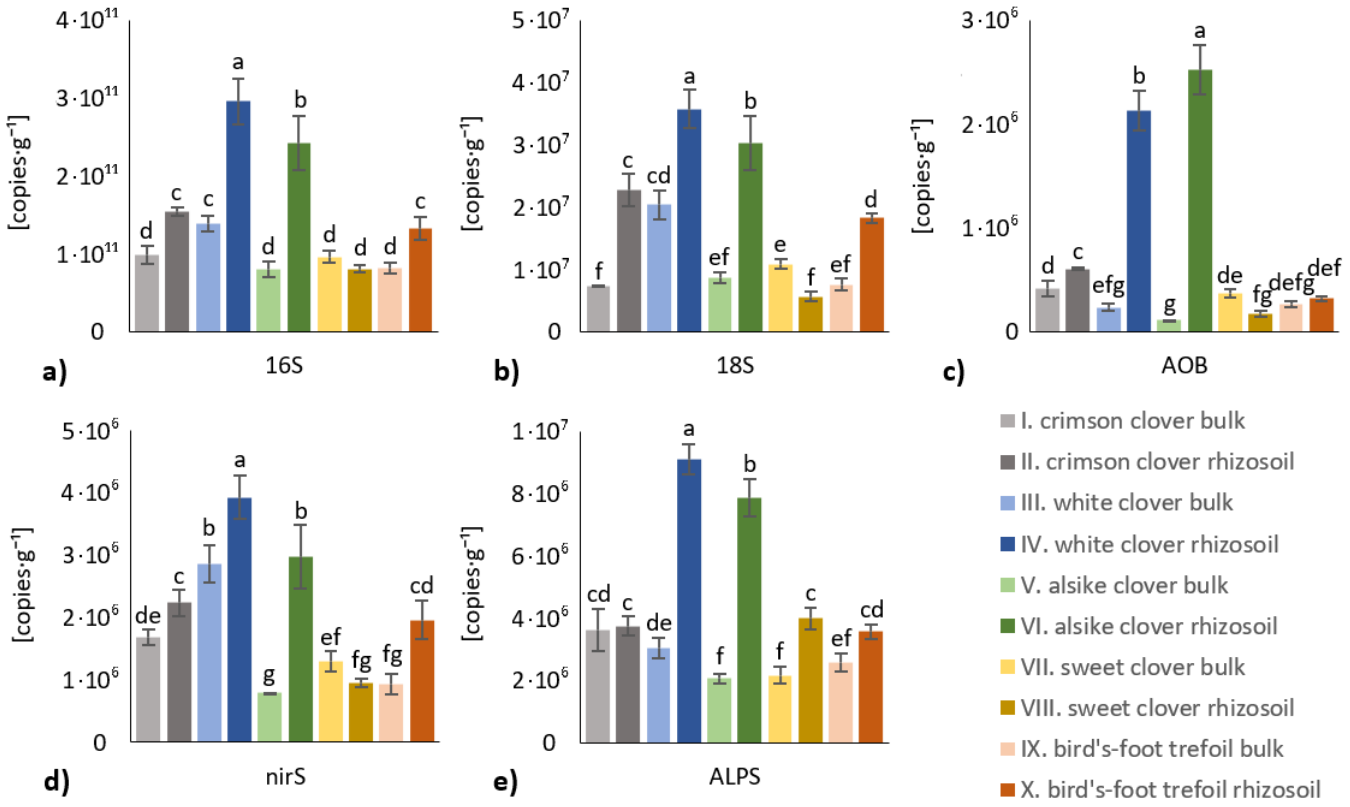

Figure 5. Microbial biomass (determined as copy number of target genes specific to functional groups) in rhizosoil and bulk soil of experimental variants, collected from field cultures of various types of clover/legume crops after plant growth period 76 days: (**a**) bacterial biomass (16S), (**b**) fungal biomass (18S), (**c**) biomass of ammonium oxidizing bacteria (AOB), (**d**) biomass of denitrifying bacteria (nirS) (**e**) biomass of phosphorus solubilizing bacteria (ALPS). Values (displayed in copies·g^{-1}) were calculated as average from independent replicates (n = 24), error bars represent standard deviation. The different letters express the statistical differences at significance level $p \leq 0.05$.

3.2. Enzymes Activities in Rhizosoil and Bulk Soil

All enzymatic activities (Figure 4) of variants crimson clover, white clover, alsike clover and bird's-foot trefoil showed significantly increased values in rhizosoil in comparison to the bulk soil. Received β-glucosidase (GLU) value were the significantly highest in white clover and alsike clover rhizosoil, compared to the other variants, from which rhizosoil GLU of crimson clover and bird's-foot trefoil were significantly increased compared to all bulk soil variants. Moreover, rhizosoil GLU values correlated (Figures A1 and A2) positively with all other enzymes (r was 0.76–0.81) and with all microbial biomass traits (r was 0.76–0.89). Whereas bulk soil GLU values correlated less strongly: positively only with Phos (r = 0.76) and NAG (r = 0.7).

N-acetyl−β-D-glucosaminidase (NAG) showed almost the same pattern: the significantly highest in white clover and alsike clover rhizosoil, rhizosoil values of crimson clover and bird's-foot trefoil variants were significantly increased compared to all bulk soil variants (Figure 4). The correlation of rhizosoil NAG (Figures A1 and A2) was positive with all enzymes (r was 0.65–0.74) and with all microbial abundance traits (r was 0.78–0.87). Bulk soil NAG correlated positively with Phos (r = 0.57).

Arylsulfatase (ARS) was detected the significantly highest in white clover rhizosoil, compared to rhizosoil crimson clover and alsike clover, which were significantly higher than ARS of bird's-foot trefoil rhizosoil. This value was significantly increased (as all other rhizosoil ARS values) compared to all bulk soil variant ARS values (Figure 4). ARS in rhizosoil correlated again broadly and positively with all enzymes (r was 0.58–0.87) and

microbial abundancies (r was 0.7–0.87). ARS in bulk correlated positively with urease (r = 0.5), AOB (r = 0.7), and ALPS (r = 0.57).

Phosphatase activity (Phos) was significantly increased in the rhizosoil variants crimson clover, white clover, and alsike clover, compared to rhizosoil value of bird's-foot trefoil, which was significantly higher compared to all Phos values from bulk soil. Rhizosoil Phos correlated positively with urease (r = 0.54) as well as all other enzymes, and highly correlated with all microbial biomass properties (r was 0.61–0.76) except of ALPS. Urease (Ure) was the significantly highest in white clover rhizosoil, in comparison to rhizosoil Ure of crimson clover and alsike clover, which values were significantly increased to urease of sweet clover and bird's-fool trefoil (Figure 4). Moreover, urease activity in rhizosoil correlated positively with enzymes and all microbial biomass traits (r was 0.73–0.86), urease in bulk soil correlated positively with AOB (r = 0.54)—Figures A1 and A2.

3.3. Microbial Biomass and Abundance in Rhizosoil and Bulk Soil

The microbial biomass of different groups of soil microorganisms was determined as copies (per gram of dry soil) of target DNA (gene, locus). The 16S rDNA, indicator of bacteria, was the significantly highest in the white clover rhizosoil as compared to all other variants, from which alsike clover rhizosoil 16S was significantly higher than rhizosoil values of crimson clover and bird's-foot trefoil and bulk soil value of white clover. The other variants were all the significantly lowest in 16S (Figure 5). Rhizosoil 16S correlated (Figures A1 and A2) positively with all other microbial abundancies (r was 0.89–0.97), bulk soil 16S correlated positively only with 18S (r = 0.8) and nirS (r = 0.91).

The 18S rDNA, indicator of fungi, showed almost the similar picture: significantly highest in the white clover rhizosoil as compared to alsike clover rhizosoil (and all others), which showed significantly increased value compared to rhizosoil crimson clover and bird's-foot trefoil and bulk soil white clover (higher than the other variants)—Figure 5. Rhizosoil 18S correlated (Figures A1 and A2) positively with all other microbial biomass traits (r was 0.8–0.93), bulk soil 18S correlated positively only with nirS (r = 0.8).

AOB monitored the biomass of ammonia oxidizing bacteria and was the significantly highest in alsike clover rhizosoil, compared to other variants with vastly lower AOB values. Except for AOB in white clover, alsike clover, and crimson clover rhizosoil, the other clover types showed comparable or significantly decreased values in rhizosoil compared to the paired bulk soil variants (Figure 5). A positive correlation (Figures A1 and A2) was found in rhizosoil for AOB with nirS (r = 0.82) and ALPS (r = 0.92) and in bulk soil between AOB and ALPS (r = 0.6).

Nitrate reductase (gene *nirS*), a determinant of denitrifying microorganisms, referred to the marking for this group nirS. The significantly highest nirS value was in rhizosoil white clover, significantly increased as compared to rhizosoil alsike clover and bulk soil white clover. These variants exerted significantly increased nirS compared to crimson clover and bird's-foot trefoil rhizosoil (Figure 4), which were on average higher than the other variants. *NirS* positively correlated with ALPS (r was 0.84 in rhizosoil, 0.52 in bulk soil)—Figures A1 and A2. Phosphorus solubilizing microorganisms ALPS (indicated by gene *phoD* coding for alkalic phosphatase) were most abundant in white clover rhizosoil, significantly increased as compared to alsike clover rhizosoil, which was significantly higher in comparison to sweet clover rhizosoil, bird's-foot trefoil rhizosoil, and both rhizosoil and bulk soil of crimson clover. The other values from bulk soil were lower in average.

4. Discussion

4.1. Plant Biomass and Nutrient Content Relation to Rhizosoil Microbial Abundance and Activity

Legume cultivation has been recognized as a win-win strategy to improve soil fertility for better production of succeeding crops [53,54]. This is because legumes have the high capacity not only to fix N for plant uptake but also due to their promoting effects on the indigenous microbial communities which ultimately results in higher crop biomass

accumulation [10,55]. These effects might be species-specific and thus determine a variable effect on plant biomass and its content of not only AGB and root N, but also other, jointly uptaken nutrients: AGB and root P, AGB and root K. Concurrently, these differences in nutrient content might be attributed to the variable traits of clover species involving differential mechanisms of nutrient transformation and uptake [55,56].

In the present study, carried out in the field conditions on small-scale-plots for 76 days, we compared the plant biomass yield, separately the aboveground biomass (shoot) and root biomass fresh and dry and correlated to the nutrient content in plant tissues. We experienced the highest fresh and dry AGB value for crimson clover, which concurrently showed significantly lowest dry root biomass. These observations were complemented with the highest average root P and K but lowest AGB and root N. This was in agreement with observed fresh AGB positive correlation with root P, root K and negative correlation with AGB N. Putatively, excessive P promoted K uptake and it was reported that such convergence improved biomass accumulation in return [57]. Root nutrient values exerted antagonism of both P and K versus N content, it might evidence a competition between acquisition of P + K nutrients and N uptake (Figure A1). We presumed that these differences in the final nutrient content values of the legume shoots and roots indicated variable uptake rates. These results agree with the previous studies [58,59].

Phosphatase activity in rhizosoil of crimson clover was, together with variants white clover and alsike clover, significantly increased compared to the other variants. Phos correlated positively with root P and negatively with AGB N, which relations supported our results. These findings, together with disproportional shoot:root ratio (>7 for dry biomass, >4 for fresh) indicated that this clover type suffered from excessive amount of soil Phos (albeit being well supplied with K) but was limited in N. We ascribed this assumption to the less extensive rooting, indicated by second lowest fresh root biomass (in average), significantly lowest dry root biomass, and apparently low AOB biomass in rhizosoil. These features anticipated putatively (proportionally to the plant biomass) decreased activity and abundance of N_2 fixing and nitrifying microorganisms. Nevertheless, the demand for N and P by crimson clover was presumably quite low and thus, resulted in enhanced biomass formation as compared to other clovers. Despite the weaker root growth of crimson clover ascribed from low biomass formation, a significant effect of plant on microbial activities and biomass in the rhizosphere was found. Except for ALPS, all values in rhizosoil were higher than in bulk soil. Therefore, the putative species-specific clover effect associated microbiota would be more pronounced in rhizosoil than in bulk soil.

Contrarily to crimson clover, sweet clover (and bird's-foot trefoil) showed significantly lowest fresh AGB and (not bird's-foot trefoil, which showed low plant water) dry AGB as well. Sweet clover root biomass was also lowest (in average) and bird's-foot trefoil exerted high dry content in both shoot and root tissues. In contrast to crimson clover, sweet clover contained the highest average AGB N, quite high root N, but the significantly lowest AGB P and lowest root P (in average). Compared to all other rhizosoil variants, the sweet clover rhizosoil showed the lowest of all enzyme activities (except of urease) and lowest microbial abundancies (except of ALPS). We assume from this that the retarded growth of sweet clover was either caused physiologically (short growth period under respective climatic conditions) or due to the limitation in P, which in return lead to excessive N. Nevertheless, reduced growth and rooting of sweet clover meant that there was not revealed any significant increase of enzyme activity (except of urease) or microbial biomass (except of ALPS) in the rhizosoil compared to the bulk soil. Moreover, 18S rDNA and AOB were significantly decreased in the rhizosoil in comparison to the bulk soil. This finding is in agreement that 18S was found to positively correlate with values of fresh AGB and root biomass, and both AGB and root P.

The bird's-foot trefoil specific feature was the lowest shoot:root fresh biomass ratio which was about 1.9, compared to other variants with values >2.4. It was the evidence of far more (proportionally to AGB accumulation) rooting. As coupled with the highest dry AGB and root biomass, we ascribe an assumption of possible water deficiency in

the bird's-foot trefoil plants. The bird's-foot trefoil content of AGB and root K was on average the lowest compared to all other variants (Figure 3). Limited K acquisition is one of the consequences of drought [60]. The bird's-foot trefoil also exerted significantly low root P content, which positively correlated with root K and several enzyme and microbial biomass traits. Low levels of root P and K corroborated the presumption of drought stress, which was further accompanied with negative impact on microbiota in rhizosoil. Significantly decreased were enzyme activities (ARS, urease) and microbial abundancies (18S, AOB) in comparison to all variants except for sweet clover. However, similar to the crimson clover, we received a significantly positive effect of plant on values of microbial activities and biomass (except of AOB) in the rhizosphere compared to bulk soil. AOB content was very low in both rhizosoil and bulk soil, it agreed with the observed positive correlation between root K and AOB in bulk soil.

Alsike clover and white clover were very similar in their results obtained in this experiment. They both were characterized by the highest average fresh root biomass and comparably to crimson clover high dry root biomass. Fresh AGB was significantly decreased compared to crimson clover but increased compared to sweet clover and bird's-foot trefoil. Moreover, dry AGB was significantly decreased compared to the highest value of crimson clover and the bird's-foot trefoil variant–Figure 3.

These results indicated significantly enhanced growth (more significant than in crimson clover, sweet clover) of roots compared to the growth of shoots, which was evidenced by the quite low fresh biomass shoot:root ratio (2.4–2.5 both). However, the higher putative rooting of both variants was only in alsike clover plant tissues coupled with comparable (to the variants with highest values) content of AGB N, P, and K, as well as root (all three) nutrients. Compared to crimson clover, the white clover was decreased in root N, root P, root K, which finding anticipated lowered biomass formation. Nevertheless, it is difficult to explain the contrasting results of alsike clover and white clover nutrient content together with the comparable plant biomass values.

Compared to other variants, rhizosoil of both variants white clover and alsike clover showed the significantly most increased values of enzyme activities (except of Phos) and all microbial abundancies (Figures 2 and 3). Absolutely highest were the values of white clover rhizosoil (except of AOB in alsike clover) and we ascribed this feature to pronounced rooting. Plant root-promoted enhancement of soil microbiota content and activity was again evidenced by the significantly increased microbial properties in rhizosoil in comparison to the bulk soil.

The above listed differences in the plant traits of tested clover types and their correlation with diversities in rhizosoil microbial traits supported our hypothesis for a significant species-specific effect on nutrient uptake and mutual plant-soil interaction. The rhizosoil is considered more specifically affected by the used plant species than the bulk soil, due to the proximity of roots and the rate of influence in form of rhizodeposition, exudation, etc. Species-specific effects of respective taxa might be derivable from the referred differing properties (e.g., tolerance to salinity, acidic or alkaline pH, soil moisture). For instance, alsike clover and bird's-foot trefoil are especially tolerant to acidity and waterlogging [61,62], whereas sweet clover tolerates salinity and alkalinity very well, however, its tolerance to acidity is lower [63]. Compared to the sweet clover, white clover's properties are completely opposite [64]. Clovers also differ in their ability of atmospheric N fixing, the range of their field fixation capacity is 0–30 g $N \cdot m^{-2}$ [65].

4.2. Microbial Soil Propertied in Rhizosoil and Bulk Soil, and Their Relation to Plant Traits

We observed that all indicators of plant nutrient content showed correlation with the microbial abundance properties (DNA). As we hypothesized, more dynamic changes in the nutrient fluxes in rhizosoil due to the rhizodeposition [66] and root exudation [67] are the drivers of closer relation between these properties. Contrasting negative correlation of AGB N with urease, 16S, 18S, nirS in rhizosoil and with urease in bulk soil, as well as negative correlation of root N with urease, 16S, AOB, nirS in bulk soil indicated a presumed

mitigation of N acquisition by plants under enhanced N mineralization and microbial abundance in rhizosoil. While N mineralization (evidenced by NAG) in bulk soil moderately promoted N mineralization due to increased fungal biomass turnover. Higher uptake of N to the plant was putatively negatively related to the abundance and activity of soil N-transforming microbes. Presumably, assimilation of N by N_2 fixing microorganisms in counteracting to nitrification in the rhizosoil. A possible explanation could be the production of nitrification inhibitors in their root exudates [68], at the simultaneous stimulation of N_2 fixation. This finding could be also related to the referred negatively correlated clover biomass with soil indicators of N availability (Figures A1 and A2), as far as this feature was observed only in the mixed culture [69]. This fact highlights the importance of root exudates which likely modified soil properties in rhizosoil more than bulk soil, as referred [70]. For example, sweet clover contains coumarin [71] and aromatic phenolic compounds, which (when excreted by plant roots) may affect both N-fixers [72] and the other rhizobacteria. Some of these root microorganisms may counter-act as allelopathic elements [73,74] and chemically modulate final plant response to pH, osmotic potential, or complete soil microbial community [75,76].

The higher AGB N and N uptake could also interfere with P solubilization and uptake. Root P correlated with both fungal (18S rDNA) and AOB abundance, and asimilar observation was referred to the role of rhizobacteria [77] and arbuscular mycorrhiza fungi [78] in P uptake by legume crops. Arbuscular mycorrhizal fungi symbiosis has been characterized to enhance P accumulation, N nutrition, and biomass production, and was linked to rhizobacterial N fixation [78]. However, this is assuming from the results that variants with high AGB N and root N (sweet clover, alsike clover) showed either low AGB + root P (sweet clover) or comparably (to others) high values at significantly increased activity and abundance of P solubilizers in rhizosoil (alsike clover). Significantly increased (compared to others) soil Phos activity (white clover, alsike clover-Figure 4) and ALPS = P solubilizing microbes (white clover-Figure 5) in rhizosoil let us anticipate enhanced P transfer to plants, similar to results reported by reference [79]. Nevertheless, we did not corroborate this expectation. We speculated that enhanced N_2-fixation activity, the process which is highly ATP-dependent [80], induced an increased demand to plant on energy sources in form of root exudates. Subpopulations of N-transforming and P-solubilizing microorganisms are linked (to some extent) by N_2-fixators, which are also P-mineralizing, as it was referred by reference [80]. We could presume an increased demand for P for ATP generated due to enhanced C catabolism. Subsequent higher microbial acquisition of organic P made it putatively less available to Phos-mediated mineralization for plant demand and gave the explanation of observed negative correlation of AGB N and Phos.

This hypothesis of putative linkage of P uptake to ATP-dependency of N fixation could answer the observed contradiction, why the significantly increased abundance and activity of rhizosoil-associated microorganisms (indicated by Phos and ALPS) in white clover and alsike clover coupled with P uptake limited to AGB P values comparable to other clover variants with much lower biomass and activity (crimson clover, bird's-foot trefoil). Nevertheless, the higher fresh root biomass, which might indicate larger root system of the respective clover types, supported the microbes in growth and colonization significantly more efficiently not only in the rhizosoil, but also in bulk soil. White clover bulk soil values of 16S, 18S, nirS were comparable or significantly higher compared to the values of these traits in rhizosoil of variants sweet clover, crimson clover, bird's-foot trefoil.

Contrarily to the rhizosoil, the enzyme activities in the bulk soil were not all found in direct relation to the microbial soil properties (Figures A1 and A2). While GLU and NAG positively correlated with P, an antagonism with 16S, 18S rDNA, and nirS was found (Figures A1 and A2). Based on this finding, we assumed that increased denitrifying fraction of bulk soil was raised by accidental anaerobic conditions [81], and the oxygen limitation might inhibit the C mineralizing enzymes GLU and NAG due to suppression of phenolic compound breakdown–so-called enzymic latch hypothesis [82]. The GLU, Phos, and NAG

were significantly the highest in bulk soil of bird's-foot trefoil variant, whereas the ARS was the highest in the crimson clover variant.

The differences in enzyme activities among studied variants might occur either due to the large differences in microbial communities' composition [83], which are more variable in bulk soil, and partially due to the differences in C:N ratio of different clover species [84]. Moreover, it has been well established that the C:N ratio of crop residues returning in soil have strong influence of C and N cycling enzymes [85]. More specifically C cycling enzymes are positively related with C:N ratio whereas, N cycling enzymes are negatively linked to C:N ratio of the plant litter (herein after clover spp.) [86,87].

A previously published study [88] referred to higher soil nutrient contents and microbial diversity in rhizosphere soil compared to non-rhizosphere (bulk) soil, monoculture cultivation leads to differential composition of soil microbial community and to varied physicochemical properties in both rhizosoil and bulk soils. Although similarity between rhizosoil and bulk soil in some features occurred, weaker positive correlation among microbial properties was observed (Figure A1). F values of two-way analysis of variance ANOVA (Table S2) were significantly higher for the factor Sample Place compared to the factor Plant and clearly supported the observation that rhizosoil or bulk soil microbiome impacted soil properties much stronger than plant type. All microbial abundances of the bulk soil in their maximum values were in most crop variants significantly lower as compared to rhizosoil. These results are substantiated by reference [89]. Highly correlating bacterial and fungal biomass with denitrifying microorganisms were evidenced for the bulk soil of white clover (the highest nirS value), whereas the alsike clover and bird's-foot trefoil showed the smallest values of nirS in the bulk soil (Figure 5). Presumably, the microbial community of bulk soil was more abundant on the denitrifying subpopulation, thus bacterial and fungal soil biomass was reported to be positively related to the denitrifying activities in soil [90,91]. Further we observed positive relation between the ALPS and the AOB in the bulk soils of crimson clover variant and bird's-foot trefoil, which were detected as the significantly highest as compared to the bulk soil of alsike clover, which value was the lowest. Such coupled nitrification and P solubilization were reported for rhizobacteria too [92].

All observed effects were more significant in the rhizosoil than in the bulk soil. Although the previous studies [29,30] focused on various interactions and responses of various types of clovers to defined soil microbial conditions, to our best knowledge there is only one study [93], which is focused on the investigation of the rate and difference in the most specific plant-rhizobiome interaction. This interaction is much more dynamic than the plant-rhizobiome-bulk microbiome interspace relation in the bulk soil, and therefore more sensitive and responsive to the combination of soil factors (chemical, physical properties) but concurrently vastly more affected by succession of the unique microbial community of the symbiotic and mutualistic microorganisms [94]. Root-secreted substances are the substantial factor that enhance microbial activity [95,96]. Thus root-mediated microbial triggering might have increased the observed enzymes in present study (Figure 4) through enhanced microbial metabolism in search for food [97,98]. These facts further warrant the necessity of determining the effects of combinations of different clover species as well as variations of root-derived substances on an array of soil health parameters for better management of soil fertility. From this reason we consider the experimental characterization of the rhizosoil as a suitable approach for the detailed and sensitive investigation of the plant species-specific effect on the soil properties.

5. Conclusions

There are the following main conclusions resulting from the above presented outcomes of the experiment:

- The soil biological properties were generally more affected in the rhizosoil than in the bulk soil, although the individual legume crop variants differed in the rate and significance of the differential impact on either rhizosoil or bulk soil. The most

significantly affected properties were ammonium oxidizing bacteria (AOB) and P-solubilizing microbiota in the rhizosoil of white clover and alsike clover variants.
- The biological properties of rhizosoil showed generally high synergism among each other and a significant effect on the plant qualitative and quantitative properties.
- However, the antagonism among N and P + K transfer in the rhizosoil to plants in the term of nutrient uptake and acquisition by either shoots or roots of tested legume crops, and this antagonism also influenced AGB and root biomass.
- Species-specific effect of tested legume crops was evaluated: the most significant species-specific effect showed white clover and alsike clover, whereas the least significant species-specific impact exerted sweet clover.
- The experimental design may be applied as a suitable approach for the investigation of the plant species-specific effect on the soil properties.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11112214/s1, Table S1: Monthly recorded rainfalls and average temperature on Troubsko locality during the year 2020; Table S2: Results of two-way analysis of variance ANOVA.

Author Contributions: Conceptualization, M.B., A.K., and J.H.; methodology, M.B., A.K., T.H. and J.E.; software, A.M., I.H.; validation, M.B., J.E. and J.K. and T.V.; formal analysis, A.M., I.H.; investigation, A.K., J.K. and J.S.; resources, T.V., J.H.; data curation, A.M., J.E. and J.S.; writing—original draft preparation, M.B., T.H., and J.H.; writing—review and editing, A.M., J.K., T.V., I.H. and J.H.; visualization, T.H.; supervision, M.B., J.K. and J.H.; project administration, A.K.; funding acquisition, M.B., A.K. All authors have read and agreed to the published version of the manuscript.

Funding: The work was supported by the project of Technology Agency of the Czech Republic TH03030236 and by Ministry of Education, Youth and Sports of the Czech Republic, grant number FCH-S-21-7398.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: The authors thank Tivadar Baltazar for his expert advice in statistical analysis.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

AGB	Aboveground biomass
AGB N	nitrogen content in dry AGB
AGB P	phosphorus content in dry AGB
AGB K	potassium content in dry AGB
AHL	N-acyl homoserine lactone
AOB	ammonia oxidizing bacteria
ALPS	phosphorus solubilizing microbes
ARS	arylsulfatase
FAO	Food and Agriculture Organization of the United Nations
GLU	β-glucosidase
NAG	N-acetyl-β-D-glucosaminidase
nirS	nitrate reductase/denitrifiers
PCA	principal component analysis
Phos	phosphatase
r	Pearson's correlation coefficient
root N	nitrogen content in dry root biomass
root P	phosphorus content in dry root biomass
root K	potassium content in dry root biomass
Ure	urease

Appendix A

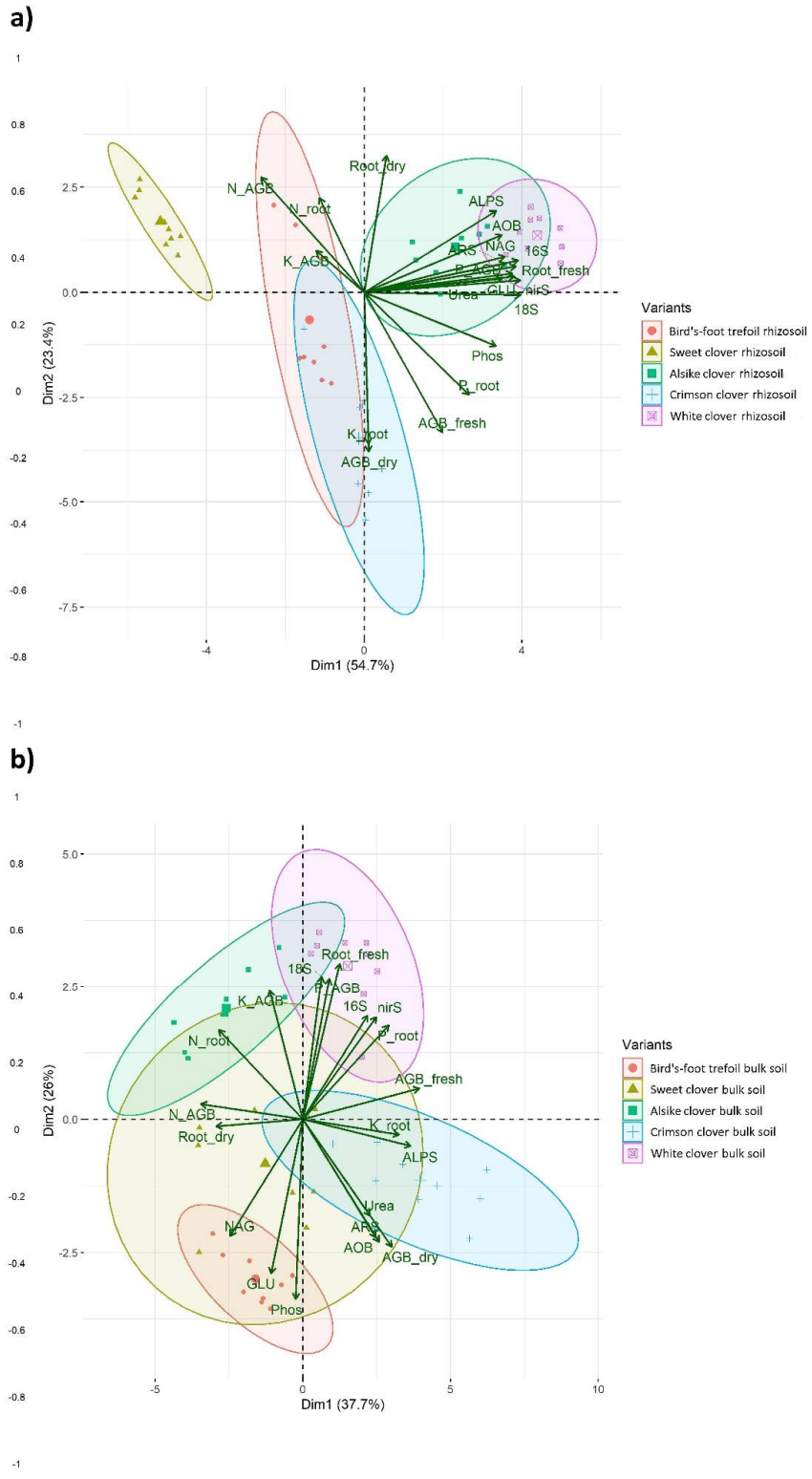

Figure A1. PCA biplot analyses of the properties of rhizosoil (**a**) and bulk soil (**b**) and plant biomass. Abbreviations are: (AGB-fresh, dry); fresh and dry aboveground biomass, (root-fresh, dry); fresh and dry root biomass, (N-, P-, K- AGB or root); biomass nutrient (N,P,K) in AGB or roots, (16S, 18S, AOB, *NirS*, ALPS); bacterial, fungal, ammonium oxidizing bacteria, denitrifying bacteria and phosphorus solubilizing bacteria and (Glu, ARS, Phos, NAG, Ure); β-glucosidase, arylsulfatase, phosphatase, N-acetyl-β-D-glucosaminidase, and urease enzymes, respectively.

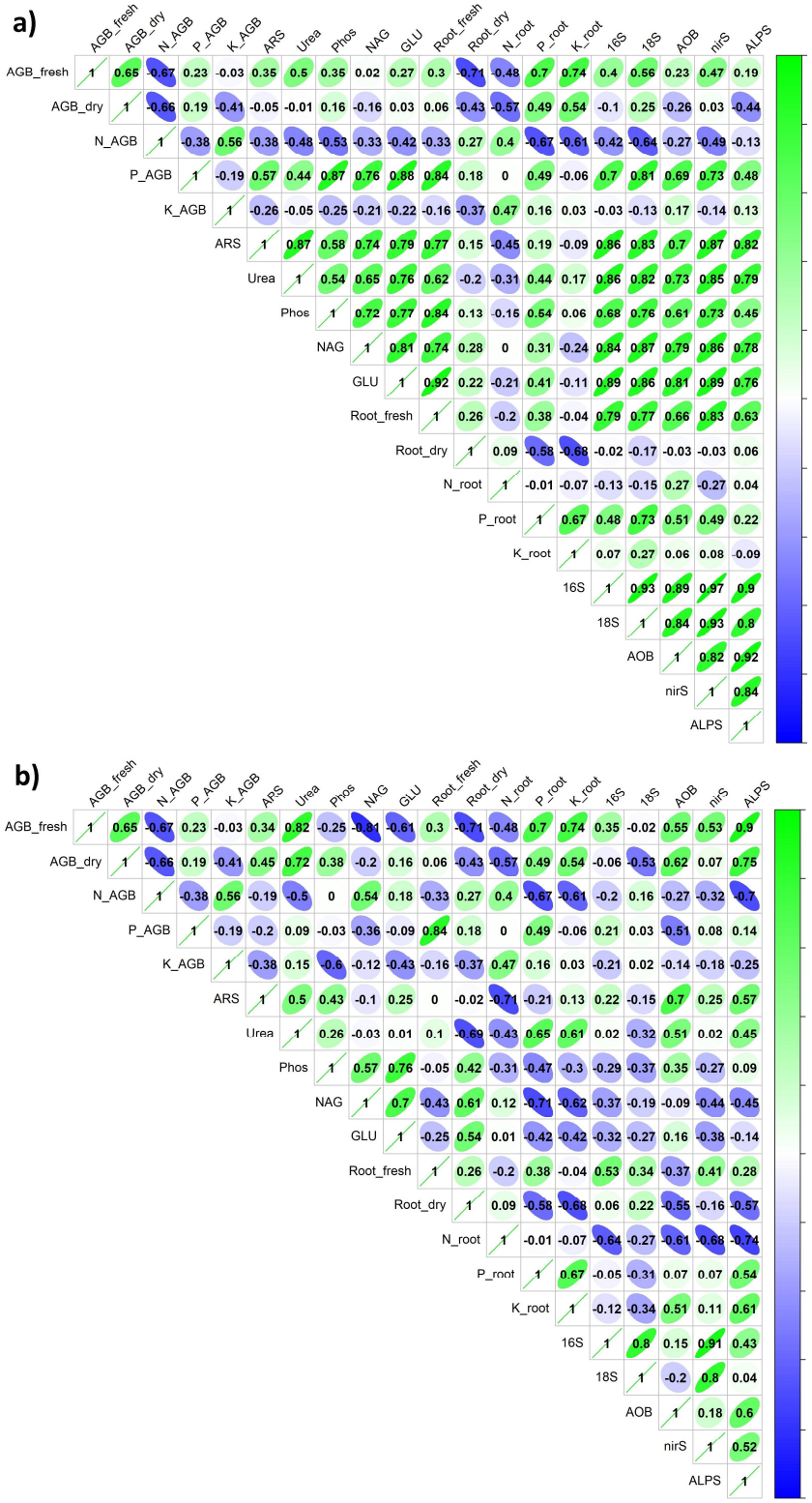

Figure A2. Pearson's correlation analyses of the properties of rhizosoil (**a**) and bulk soil (**b**) and plant biomass. Abbreviations are: (AGB-fresh, dry); fresh and dry aboveground biomass, (root-fresh, dry); fresh and dry root biomass, (N-, P-, K- AGB or root); biomass nutrient (N,P,K) in AGB or roots, (16S, 18S, AOB, *NirS*, ALPS); bacterial, fungal, ammonium oxidizing bacteria, denitrifying bacteria and phosphorus solubilizing bacteria and (Glu, ARS, Phos, NAG, Ure); β-glucosidase, arylsulfatase, phosphatase, N-acetyl-β-D-glucosaminidase, and urease enzymes, respectively.

References

1. West, P.C.; Gerber, J.S.; Engstrom, P.M.; Mueller, N.D.; Brauman, K.A.; Carlson, K.M.; Cassidy, E.S.; Johnston, M.; MacDonald, G.K.; Ray, D.K.; et al. Leverage points for improving global food security and the environment. *Science* **2014**, *345*, 325–328. [CrossRef] [PubMed]
2. Ahmad, M.; Naseer, I.; Hussain, A.; Zahid Mumtaz, M.; Mustafa, A.; Hilger, T.H.; Zahir, Z.A.; Minggang, X. Appraising Endophyte—Plant Symbiosis for Improved Growth, Nodulation, Nitrogen Fixation and Abiotic Stress Tolerance: An Experimental Investigation with Chickpea (*Cicer arietinum* L.). *Agronomy* **2019**, *9*, 621. [CrossRef]
3. Rafique, M.; Naveed, M.; Mustafa, A.; Akhtar, S.; Munawar, M.; Kaukab, S.; Ali, H.M.; Siddiqui, M.H.; Salem, M.Z.M. The Combined Effects of Gibberellic Acid and Rhizobium on Growth, Yield and Nutritional Status in Chickpea (*Cicer arietinum* L.). *Agronomy* **2021**, *11*, 105. [CrossRef]
4. Bationo, A.; Lompo, F.; Koala, S. Research on nutrient flows and balances in west Africa: State-of-the-art1Paper contributes to EC INCO-DC project IC18-CT96-0092 (Spatial and temporal variation of soil nutrient stocks and management in sub-Saharan African farming systems).1. *Agric. Ecosyst. Environ.* **1998**, *71*, 19–35. [CrossRef]
5. Wik, M.; Pingali, P.; Broca, S. *Global Agricultural Performance: Past Trends and Future Prospects*; World Development Report; World Bank: Washington, DC, USA, 2008.
6. Verma, P.; Yadav, A.N.; Kazy, S.K.; Saxena, A.K.; Suman, A. Elucidating the diversity and plant growth promoting attributes of wheat (Triticum aestivum) associated acid tolerant bacteria from southern hills zone of India. *Natl. J. Life Sci.* **2013**, *10*, 219–226.
7. Refsgaard, K.; Halberg, N.; Kristensen, E.S. Energy utilization in crop and dairy production in organic and conventional livestock production systems. *Agric. Syst.* **1998**, *57*, 599–630. [CrossRef]
8. Andersen, M.; Kiel, P. Integrated utilisation of green biomass in the green biorefinery. *Ind. Crop. Prod.* **2000**, *11*, 129–137. [CrossRef]
9. Snapp, S.S.; Swinton, S.M.; Labarta, R.; Mutch, D.; Black, J.R.; Leep, R.; Nyiraneza, J.; O'Neil, K. Evaluating cover crops for benefits, costs and performance within cropping system niches. *Agron. J.* **2005**, *97*, 322–332. [CrossRef]
10. Fustec, J.; Lesuffleur, F.; Mahieu, S.; Cliquet, J.-B. Nitrogen rhizodeposition of legumes. A review. *Agron. Sustain. Dev.* **2010**, *30*, 57–66. [CrossRef]
11. Poole, P.; Ramachandran, V.; Terpolilli, J. Rhizobia: From saprophytes to endosymbionts. *Nat. Rev. Microbiol.* **2018**, *16*, 291–303. [CrossRef] [PubMed]
12. Ali, M.A.; Naveed, M.; Mustafa, A.; Abbas, A. The Good, the Bad, and the Ugly of Rhizosphere Microbiome. In *Probiotics and Plant Health*; Springer: Singapore, 2017; pp. 253–290.
13. Downie, J.A. The roles of extracellular proteins, polysaccharides and signals in the interactions of rhizobia with legume roots. *FEMS Microbiol. Rev.* **2010**, *34*, 150–170. [CrossRef]
14. Veliz-Vallejos, D.F.; Kawasaki, A.; Mathesius, U. The Presence of Plant-Associated Bacteria Alters Responses to N-acyl Homoserine Lactone Quorum Sensing Signals that Modulate Nodulation in Medicago Truncatula. *Plants* **2020**, *9*, 777. [CrossRef] [PubMed]
15. Ortas, I. Determination of the extent of rhizosphere soil. *Commun. Soil Sci. Plant Anal.* **1997**, *28*, 1767–1776. [CrossRef]
16. Hütsch, B.W.; Augustin, J.; Merbach, W. Plant rhizodeposition—An important source for carbon turnover in soils. *J. Plant Nutr. Soil Sci.* **2002**, *165*, 397–407. [CrossRef]
17. Elbl, J.; Zahora, J. *The Comparison of Microbial Activity in Rhizosphere and Non-Rhizosphere Soil Stressed by Drought*; Mendel Univ Brno, Fac Agronomy: Brno, Czech Republic, 2014.
18. Walker, T.S.; Bais, H.P.; Grotewold, E.; Vivanco, J.M. Root exudation and rhizosphere biology. *Plant. Physiol.* **2003**, *132*, 44–51. [CrossRef]
19. Turner, T.R.; Ramakrishnan, K.; Walshaw, J.; Heavens, D.; Alston, M.; Swarbreck, D.; Osbourn, A.; Grant, A.; Poole, P.S. Comparative metatranscriptomics reveals kingdom level changes in the rhizosphere microbiome of plants. *ISME J.* **2013**, *7*, 2248–2258. [CrossRef] [PubMed]
20. Kemp, J.; Lotter, D.; Meyer, A.; Kleinert, A.; Pérez-Fernández, M.; Valentine, A. Variation in rhizosphere nutrient cycling affects the source of nitrogen acquisition in wild and cultivated Aspalathus linearis (N.L.Burm.) R.Dahlgren plants. *Appl. Soil Ecol.* **2018**, *130*, 26–33. [CrossRef]
21. Dennis, P.G.; Miller, A.J.; Hirsch, P.R. Are root exudates more important than other sources of rhizodeposits in structuring rhizosphere bacterial communities? *FEMS Microbiol. Ecol.* **2010**, *72*, 313–327. [CrossRef] [PubMed]
22. de los Reyes, A.M.M.; Ocampo, E.T.M.; Manuel, M.C.C.; Mendoza, B.C. Analysis of the Bacterial and Fungal Community Profiles in Bulk Soil and Rhizospheres of Three Mungbean [Vigna radiata (L.) R. Wilczek] Genotypes through PCR-DGGE. *Int. Lett. Nat. Sci.* **2020**, *77*, 1–26. [CrossRef]
23. Di Cenzo, G.C.; Checcucci, A.; Bazzicalupo, M.; Mengoni, A.; Viti, C.; Dziewit, L.; Finan, T.M.; Galardini, M.; Fondi, M. Metabolic modelling reveals the specialization of secondary replicons for niche adaptation in Sinorhizobium meliloti. *Nat. Commun.* **2016**, *7*, 12219. [CrossRef]
24. Kravchenko, A.N.; Negassa, W.C.; Guber, A.K.; Hildebrandt, B.; Marsh, T.L.; Rivers, M.L. Intra-aggregate Pore Structure Influences Phylogenetic Composition of Bacterial Community in Macroaggregates. *Soil Sci. Soc. Am. J.* **2014**, *78*, 1924–1939. [CrossRef]
25. Hartmann, A.; Schmid, M.; van Tuinen, D.; Berg, G. Plant-driven selection of microbes. *Plant Soil* **2009**, *321*, 235–257. [CrossRef]
26. Grayston, S.J.; Wang, S.Q.; Campbell, C.D.; Edwards, A.C. Selective influence of plant species on microbial diversity in the rhizosphere. *Soil Biol. Biochem.* **1998**, *30*, 369–378. [CrossRef]

27. Berendsen, R.L.; Pieterse, C.M.J.; Bakker, P. The rhizosphere microbiome and plant health. *Trends Plant Sci.* **2012**, *17*, 478–486. [CrossRef]
28. Svenning, M. Competition for Nodule Occupancy Between Introduced Strains of Rhizobium leguminosarum Biovar trifolii and its Influence on Plant Production. *Ann. Bot.* **2001**, *88*, 781–787. [CrossRef]
29. Saeed, Q.; Xiukang, W.; Haider, F.U.; Kučerik, J.; Mumtaz, M.Z.; Holatko, J.; Naseem, M.; Kintl, A.; Ejaz, M.; Naveed, M.; et al. Rhizosphere Bacteria in Plant Growth Promotion, Biocontrol, and Bioremediation of Contaminated Sites: A Comprehensive Review of Effects and Mechanisms. *Int. J. Mol. Sci.* **2021**, *22*, 10529. [CrossRef] [PubMed]
30. Roberts, R.; Jackson, R.W.; Mauchline, T.H.; Hirsch, P.R.; Shaw, L.J.; Doring, T.F.; Jones, H.E. Is there sufficient Ensifer and Rhizobium species diversity in UK farmland soils to support red clover (Trifolium pratense), white clover (T. repens), lucerne (Medicago sativa) and black medic (M. lupulina)? *Appl. Soil Ecol.* **2017**, *120*, 35–43. [CrossRef]
31. de la Fuente Canto, C.; Simonin, M.; King, E.; Moulin, L.; Bennett, M.J.; Castrillo, G.; Laplaze, L. An extended root phenotype: The rhizosphere, its formation and impacts on plant fitness. *Plant J.* **2020**, *103*, 951–964. [CrossRef]
32. FAO. World Reference Base for Soil Resources. In *International Soil Classification System for Naming Soils and Creating Legends for Soil Maps*; Food and Agriculture Organization of the United Nations: Rome, Italy, 2014.
33. Brtnicky, M.; Hammerschmiedt, T.J.; Elbl Kintl, A.; Skulcova, L.; Radziemska, M.; Latal, O.; Baltazar, T.; Kobzova, E.; Holatko, J. The Potential of Biochar Made from Agricultural Residues to Increase Soil Fertility and Microbial Activity: Impacts on Soils with Varying Sand Content. *Agronomy* **2021**, *11*, 1174. [CrossRef]
34. Capolongo, A.; Barresi, A.A.; Rovero, G. Freeze-drying of lignin peroxidase: Influence of lyoprotectants on enzyme activity and stability. *J. Chem. Technol. Biotechnol.* **2003**, *78*, 56–63. [CrossRef]
35. Iocoli, G.A.; Zabaloy, M.C.; Pasdevicelli, G.; Gomez, M.A. Use of biogas digestates obtained by anaerobic digestion and co-digestion as fertilizers: Characterization, soil biological activity and growth dynamic of *Lactuca sativa* L. *Sci. Total. Environ.* **2019**, *647*, 11–19. [CrossRef]
36. ISO_20130. *Soil Quality—Measurement of Enzyme Activity Patterns in Soil Samples Using Colorimetric Substrates in Micro-Well Plates*; International Organization for Standardization: Geneva, Switzerland, 2018.
37. Verchot, L.; Borelli, T. Application of -nitrophenol (NP) enzyme assays in degraded tropical soils. *Soil Biol. Biochem.* **2005**, *37*, 625–633. [CrossRef]
38. Whalen, J.K.; Warman, P.R. Arylsulfatase activity in soil and soil extracts using natural and artificial substrates. *Biol. Fertil. Soils* **1996**, *22*, 373–378. [CrossRef]
39. Jackson, C.R.; Tyler, H.L.; Millar, J.J. Determination of microbial extracellular enzyme activity in waters, soils, and sediments using high throughput microplate assays. *J. Vis. Exp.* **2013**, *2013*, e50399. [CrossRef]
40. Cordero, I.; Snell, H.; Bardgett, R.D. High throughput method for measuring urease activity in soil. *Soil Biol. Biochem.* **2019**, *134*, 72–77. [CrossRef] [PubMed]
41. Amann, R.I.; Ludwig, W.; Schleifer, K.H. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Rev.* **1995**, *59*, 143–169. [CrossRef]
42. Vainio, E.J.; Hantula, J. Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified ribosomal DNA. *Mycol. Res.* **2000**, *104*, 927–936. [CrossRef]
43. Rotthauwe, J.H.; Witzel, K.P.; Liesack, W. The ammonia monooxygenase structural gene amoA as a functional marker: Molecular fine-scale analysis of natural ammonia-oxidizing populations. *Appl. Environ. Microbiol.* **1997**, *63*, 4704–4712. [CrossRef] [PubMed]
44. Kandeler, E.; Deiglmayr, K.; Tscherko, D.; Bru, D.; Philippot, L. Abundance of narG, nirS, nirK, and nosZ genes of denitrifying bacteria during primary successions of a glacier foreland. *Appl. Environ. Microbiol.* **2006**, *72*, 5957–5962. [CrossRef]
45. Hu, Y.; Xia, Y.; Sun, Q.; Liu, K.; Chen, X.; Ge, T.; Zhu, B.; Zhu, Z.; Zhang, Z.; Su, Y. Effects of long-term fertilization on phoD-harboring bacterial community in Karst soils. *Sci. Total. Environ.* **2018**, *628–629*, 53–63. [CrossRef]
46. R_CORE_TEAM. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2020.
47. Wickham, H. *ggplot2: Elegant Graphics for Data Analysis*; Springer-Verlag: New York, NY, USA, 2016.
48. Mendiburu, D.F. *Agricolae: Statistical Procedures for Agricultural Research*; R Package Version 1.2-3; 2015. Available online: http://CRAN.R-project.org/package=agricolae (accessed on 29 October 2021).
49. Beaujean, A.A. *R Package for Baylor University Educational Psychology Quantitative Courses*; BaylorEdPsych; 2012. Available online: https://rdrr.io/cran/BaylorEdPsych/ (accessed on 29 October 2021).
50. Kassambara, A.; Mundt, F. *Factoextra: Extract and Visualize the Results of Multivariate Data Analyses*; 2017. Available online: https://rpkgs.datanovia.com/factoextra/index.html (accessed on 29 October 2021).
51. Lê, S.; Josse, J.; Husson, F. FactoMineR: An R Package for Multivariate Analysis. *J. Stat. Softw.* **2008**, *25*, 1–18. [CrossRef]
52. Peterson, B.G.; Carl, P. *Performance Analytics: Econometric Tools for Performance and Risk Analysis*; 2020. Available online: https://rdrr.io/cran/PerformanceAnalytics/ (accessed on 29 October 2021).
53. Thilakarathna, M.S.; Papadopoulos, Y.A. Nitrogen fixation and transfer of red clover genotypes under legume-grass forage based production systems. *Nutr. Cycl. Agroecosyst.* **2016**, *106*, 233–247. [CrossRef]
54. Sekeran, U.; Loya, R.J. Intercropping of kura clover (*Trifolium ambiguum* M. Bieb) with prairie cordgrass (*Spartina pectinata* Link.) enhanced soils biochemical activities and microbial community structure. *Appl. Soil Ecol.* **2019**, *147*, 1–10. [CrossRef]

55. Kiers, E.T.; West, S.K.; Denison, R.F. Maintaining cooperation in the legume-rhizobia symbiosis: Identifying selection pressures and mechanisms. In *Nitrogen-Fixing Leguminous Symbioses*; Springer: Dordrecht, The Netherlands, 2008; pp. 59–76.
56. Silveira, M.L.; Kohmann, M.M. Maintaining soil fertility and health for sustainable pastures. In *Management Strategies for Sustainable Cattle Production in Southern Pastures*; Academic Press: London, UK, 2020; pp. 35–58.
57. Chtouki, M.; Naciri, R.; Soulaimani, A.; Zeroual, Y.; El Gharous, M.; Oukarroum, A. Effect of Cadmium and Phosphorus Interaction on Tomato: Chlorophyll a Fluorescence, Plant Growth, and Cadmium Translocation. *Water Air Soil Pollut.* **2021**, *232*, 11. [CrossRef]
58. Zhang, F.; Niu, J.; Zhang, W.; Chen, X.; Li, C.; Yuan, L.; Xie, J. Potassium nutrition of crops under varied regimes of nitrogen supply. *Plant Soil* **2010**, *335*, 21–34. [CrossRef]
59. Lee, Y.; Hwanga, T.Y.; Lee, S.; Shinogi, Y.; Oh, T.K.; Sung, J. Nutrient-Specific Variation of C-N Metabolism in the Leaves and Roots of Bell Pepper (*Capsicum annunm*. L) in Response to Macronutrient Deficiency. *J. Fac. Agric. Kyushu Univ.* **2020**, *65*, 25–34. [CrossRef]
60. Subbarao, G.V.; Yoshihashi, T.; Worthington, M.; Nakahara, K.; Ando, Y.; Sahrawat, K.L.; Rao, I.M.; Lata, J.-C.; Kishii, M.; Braun, H.-J. Suppression of soil nitrification by plants. *Plant Sci.* **2015**, *233*, 155–164. [CrossRef]
61. Dear, B.; Moore, G.; Hughes, S. Adaptation and potential contribution of temperate perennial legumes to the southern Australian wheatbelt: A review. *Aust. J. Exp. Agric.* **2003**, *43*, 1–18. [CrossRef]
62. ABMI_Website, 3.8.2021. Alberta Biodiversity Monitoring Institute: Alsike Clover (Trifolium hybridum). Available online: https://abmi.ca/home/data-analytics/biobrowser-home/species-profile?tsn=99003745 (accessed on 29 October 2021).
63. Rogers, M.E.; Colmer, T.D.; Frost, K.; Henry, D.; Cornwall, D.; Hulm, E.; Deretic, J.; Hughes, S.R.; Craig, A.D. Diversity in the genus Melilotus for tolerance to salinity and waterlogging. *Plant Soil* **2008**, *304*, 89–101. [CrossRef]
64. ABMI_Website, 12.8.2021. Alberta Biodiversity Monitoring Institute: White Clover (Trifolium repens). Available online: https://abmi.ca/home/data-analytics/biobrowser-home/species-profile?year=2016&tsn=99003747 (accessed on 29 October 2021).
65. IAEA_TECDOC. *Improving Yield and Nitrogen Fixation of Grain Legumes in the Tropics and Sub-tropics of Asia*; No. 1027; International Atomic Energy Agency: Vienna, Austria, 1998.
66. Lynch, J.M.; Whipps, J.M. Substrate flow in the rhizosphere. *Plant Soil* **1990**, *129*, 1–10. [CrossRef]
67. Roque-Malo, S.; Woo, D.K.; Kumar, P. Modeling the Role of Root Exudation in Critical Zone Nutrient Dynamics. *Water Resour. Res.* **2020**, *56*, 23. [CrossRef]
68. Wang, M.; Zheng, Q.; Shen, Q.; Guo, S. The critical role of potassium in plant stress response. *Int. J. Mol. Sci.* **2013**, *14*, 7370–7390. [CrossRef]
69. Blesh, J. Functional traits in cover crop mixtures: Biological nitrogen fixation and multifunctionality. *J. Appl. Ecol.* **2018**, *55*, 38–48. [CrossRef]
70. Qi, X.; Wang, E.; Xing, M.; Zhao, W.; Chen, X. Rhizosphere and non-rhizosphere bacterial community composition of the wild medicinal plant Rumex patientia. *World J. Microbiol. Biotechnol.* **2012**, *28*, 2257–2265. [CrossRef]
71. Schlosse, G. On influence of external conditions on content of coumarin and related compounds in leaves of *Melilotus albus*. *Z. Pflanzenzucht.* **1968**, *59*, 327.
72. Sugiyama, A.; Shitan, N.; Yazaki, K. Involvement of a Soybean ATP-Binding Cassette-Type Transporter in the Secretion of Genistein, a Signal Flavonoid in Legume-Rhizobium Symbiosis. *Plant Physiol.* **2007**, *144*, 2000–2008. [CrossRef]
73. Barazani, O.; Friedman, J. Allelopathic bacteria and their impact on higher plants. *Crit. Rev. Microbiol.* **2001**, *27*, 41–55. [CrossRef]
74. Cheng, F.; Cheng, Z. Research Progress on the use of Plant Allelopathy in Agriculture and the Physiological and Ecological Mechanisms of Allelopathy. *Front Plant Sci* **2015**, *6*, 1020. [CrossRef]
75. Li, Y.-P.; Feng, Y.-L.; Kang, Z.-L.; Zheng, Y.-L.; Zhang, J.-L.; Chen, Y.-J. Changes in soil microbial communities due to biological invasions can reduce allelopathic effects. *J. Appl. Ecol.* **2017**, *54*, 1281–1290. [CrossRef]
76. Liu, J.; Ge, X.; Fan, X.; Liu, H.; Gao, Y.; Ren, A. The Inhibitory Effect of Endophyte-Infected Tall Fescue on White Clover Can Be Alleviated by Glomus mosseae Instead of Rhizobia. *Microorganisms* **2021**, *9*, 109. [CrossRef]
77. Korir, H.; Mungai, N.W.; Thuita, M.; Hamba, Y.; Masso, C. Co-inoculation Effect of Rhizobia and Plant Growth Promoting Rhizobacteria on Common Bean Growth in a Low Phosphorus Soil. *Front. Plant Sci* **2017**, *8*, 141. [CrossRef]
78. Hack, C.M.; Porta, M.; Schäufele, R.; Grimoldi, A.A. Arbuscular mycorrhiza mediated effects on growth, mineral nutrition and biological nitrogen fixation of Melilotus alba Med. in a subtropical grassland soil. *Appl. Soil Ecol.* **2019**, *134*, 38–44. [CrossRef]
79. Orhan, E.; Esitken, A.; Ercisli, S.; Turan, M.; Sahin, F. Effects of plant growth promoting rhizobacteria (PGPR) on yield, growth and nutrient contents in organically growing raspberry. *Sci. Hortic.* **2006**, *111*, 38–43. [CrossRef]
80. Ahemad, M.; Kibret, M. Mechanisms and applications of plant growth promoting rhizobacteria: Current perspective. *J. King Saud Univ.-Sci.* **2014**, *26*, 1–20. [CrossRef]
81. Keiluweit, M.; Wanzek, T.; Kleber, M.; Nico, P.; Fendorf, S. Anaerobic microsites have an unaccounted role in soil carbon stabilization. *Nat. Commun.* **2017**, *8*, 1771. [CrossRef]
82. Freeman, C.; Ostle, N.J.; Fenner, N.; Kang, H. A regulatory role for phenol oxidase during decomposition in peatlands. *Soil Biol. Biochem.* **2004**, *36*, 1663–1667. [CrossRef]
83. Brennan, E.B.; Acosta-Martinez, V. Cover cropping frequency is the main driver of soil microbial changes during six years of organic vegetable production. *Soil Biol. Biochem.* **2017**, *109*, 188–204. [CrossRef]

84. Ranells, N.N.; Wagger, M.G. Nitrogen-15 recovery and release by rye and crimson clover cover crops. *Soil Sci. Soc. Am. J.* **1997**, *61*, 943–948. [CrossRef]
85. Tian, L.; Shi, W. Short-term effects of plant litter on the dynamics, amount, and stoichiometry of soil enzyme activity in agroecosystems. *Eur. J. Soil Biol.* **2014**, *65*, 23–29. [CrossRef]
86. Dai, X.; Zhou, W.; Liu, G.; He, P.; Liu, Z. Soil C/N and pH together as a comprehensive indicator for evaluating the effects of organic substitution management in subtropical paddy fields after application of high-quality amendments. *Geoderma* **2019**, *337*, 1116–1125. [CrossRef]
87. Yang, Y.; Ji, R.; Zhang, H.; Christie, P.; Feng, G.; Li, X.; Gai, J. Stoichiometric analysis of an arable crop-soil-microbe system after repeated fertilizer and compost application for 10 years. *J. Soils Sediments* **2021**, *21*, 1466–1475. [CrossRef]
88. Li, Z.; Zu, C.; Wang, C.; Yang, J.; Yu, H.; Wu, H. Different responses of rhizosphere and non-rhizosphere soil microbial communities to consecutive Piper nigrum L. monoculture. *Sci. Rep.* **2016**, *6*, 35825. [CrossRef]
89. Herman, D.J.; Johnson, K.K.; Jaeger, C.H.; Schwartz, E.; Firestone, M.K. Root Influence on Nitrogen Mineralization and Nitrification in Avena barbata Rhizosphere Soil. *Soil Sci. Soc. Am. J.* **2006**, *70*, 1504–1511. [CrossRef]
90. Laughlin, R.J.; Stevens, R.J. Evidence for Fungal Dominance of Denitrification and Codenitrification in a Grassland Soil. *Soil Sci. Soc. Am. J.* **2002**, *66*, 1540–1548. [CrossRef]
91. Bedmar, E.; Bueno., E.; Correa., D.; Torres, M.; Delgado, M.; Mesa, S. Ecology of Denitrification in Soils and Plant-Associated Bacteria. In *Benef. Plant-Microb. Interact.*; Gonzalez, M.B.R., Gonzalez-Lopez, J., Eds.; CRC Press: Boca Raton, FL, USA, 2013; pp. 165–182.
92. Li, X.; Petersen, S.O.; Sørensen, P.; Olesen, J.E. Effects of contrasting catch crops on nitrogen availability and nitrous oxide emissions in an organic cropping system. *Agric. Ecosyst. Environ.* **2015**, *199*, 382–393. [CrossRef]
93. Shi, S.; Nuccio, E.E.; Shi, Z.J.; He, Z.; Zhou, J.; Firestone, M.K. The interconnected rhizosphere: High network complexity dominates rhizosphere assemblages. *Ecol. Lett.* **2016**, *19*, 926–936. [CrossRef] [PubMed]
94. Tkacz, A.; Cheema, J.; Chandra, G.; Grant, A.; Poole, P.S. Stability and succession of the rhizosphere microbiota depends upon plant type and soil composition. *ISME J.* **2015**, *9*, 2349–2359. [CrossRef] [PubMed]
95. Cheng, W.X.; Kuzyakov, Y. Root effects on soil organic matter decomposition. In *Roots and Soil Management: Interactions between Roots and the Soil*; Zobel, R.W., Wright, S.F., Eds.; Agronomy Monograph No. 48; ASA-CSSA-SSSA: Madison, WI, USA, 2005; pp. 119–143.
96. Nannipieri, P.; Giagnoni, L.; Renella, G.; Puglisi, E.; Ceccanti, B.; Masciandaro, G.; Fornasier, F.; Moscatelli, M.C.; Marinari, S. Soil enzymology: Classical and molecular approaches. *Biol. Fertil. Soils* **2012**, *48*, 743–762. [CrossRef]
97. Blagodatskaya, E.; Kuzyakov, Y. Mechanisms of real and apparent priming effects and their dependence on soil microbial biomass and community structure: Critical review. *Biol. Fertil. Soils* **2008**, *45*, 115–131. [CrossRef]
98. Mason-Jones, K.; Kuzyakov, Y. Soil Biology & Biochemistry "Non-metabolizable" glucose analogue shines new light on priming mechanisms: Triggering of microbial metabolism. *Soil Biol. Biochem.* **2017**, *107*, 68–76.