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Assessment of Inter- and Intraspecific P Efficiency in Forage Legumes as Affected by Recycling Fertiliser

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Abstract: Legumes have a high demand for phosphorus (P) due to energetically costly biological nitrogen fixation, but they also have effective physiological and morphological strategies for P mobilization. To evaluate the inter- and intraspecific P efficiency of small-grain legumes supplied with different P recycling fertilisers, eight accessions each of alfalfa (*Medicago sativa* L.) and red clover (*Trifolium pratense* L.) were cultivated in two pot experiments under greenhouse conditions until the flowering stage. To substantiate the results, some accessions were used in both experiments. Five treatments (no P, triple-superphosphate (TSP), sewage sludge ash (SSA), biowaste compost (compost), and struvite) were considered P sources. In addition to plant P uptake, the soil P pools were analysed in detail. Red clover showed higher yields and nutrient uptakes compared to alfalfa, but intraspecific effects were marginal. The addition of P resulted only partly in an increase in yield, despite the low P content in the soil. While struvite application clearly enhanced the P uptake of the plants in both experiments, SSA application had no effect compared to the control. The same treatment effect occurs with the bio-available soil P contents, which were on average 72.6 mg kg^{-1} after struvite and 44.3 mg kg^{-1} after SSA addition. Struvite as a P source was especially effective when applied to red clover. Our study aligns with previous field results and underscores the high potential of P mobilization of small-grain legumes without pronounced inter- or intraspecific differences. While struvite is suitable as a P fertiliser, the application of SSA to legumes is not recommended.

Keywords: alfalfa; red clover; recycling fertiliser; phosphorus

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1. Introduction

Forage legumes play a crucial part in sustainable agriculture because of biological nitrogen (N) fixation (BNF) through the symbiosis with rhizobia, enhancing soil fertility and reducing the reliance on synthetic fertilisers. Alfalfa (*Medicago sativa* L.) and red clover (*Trifolium pratense* L.) as perennial small-grain legumes have been cultivated as sources of nourishing forage and protein-rich feed on a global scale [1,2]. Abundant studies have proven their benefits in crop rotations as well as beneficial traits like good persistence and wide adaptability across diverse environmental conditions [3–6].

Phosphorus (P) is crucial for the BNF due to its high energy requirement, and legume crops typically show high P demand for the synthesis of adenosine triphosphate (ATP) [7–9]. Moreover, P as a structural element of nucleic acids, coenzymes and phospholipids may limit legume growth in low-nutrient environments due to the substantial P requirement in BNF [10]. To overcome the limitation of low P availability, legumes developed strategies and mechanisms to proficiently acquire and utilize P from the soil. P deficiency can stimulate morphological alteration, for example, lateral root elongation, root hair formation, and cluster root development [11–13]. In addition, legume plants can raise the exudation of organic anions and phosphatase enzymes and mobilize sparingly available forms of soil P through desorbing and chelating [14–18].

The interspecific differences in soil P mobilization between various legume species have been verified in multiple studies, which primarily concern distinctions in root traits and the root exudations [16,19–22]. Moreover, intraspecific variabilities in P acquisition have also been examined in various legumes species [23–27]. Although several studies have described P acquisition strategies for *Trifolium* and *Medicago* species [28–30], dedicated comparisons of P uptake and efficiency between alfalfa and red clover are rare. Additionally, previous genotypic research on alfalfa and red clover accessions has focused primarily on forage yield, nutritional value and stress tolerance [31–34], while little attention has been paid to their intraspecific variations in P utilisation with different P supplies. Our previous field study directly compared P uptake and efficiency between various alfalfa and red clover accessions, as affected by long-term P management, for the first time [35], revealing low inter- and intraspecific P efficiencies. This led us to hypothesize that a greater diversity of P sources and a higher gradient of P supply is necessary to identify accessions that differ in their P efficiency.

Phosphorus in the soil is subject to numerous conversion processes, and ultimately, the availability of P in the soil is not necessarily linked to the solubility of P in the fertiliser [36]. Apart from the conventional mineral P fertilisers like triple-superphosphate (TSP), recycling fertilisers (for example, biowaste compost (compost), sewage sludge ash (SSA) and struvite) can offer potential P sources in agricultural production products and have often been described to increase crop growth. Biowaste compost has been applied in crop production within the commonly used practice of organic fertilization and P recycling in agriculture, which can improve soil's physico-chemical and biological properties and benefit plant productivity [37,38]. Sewage sludge from waste water treatment can be incinerated into SSA, eliminating organic and microbiological contaminants and resulting in more concentrated P content [39]. The median P concentration of German SSA of about 8% indicates the important potential of SSA as an alternative P source for fertilisers [40]. Although the sparingly available P forms in SSA can lead to low P fertiliser effectiveness [41,42], numerous studies have proven its beneficial effect on plant growth [43–46]. Struvite ($MgNH_4PO_4 \cdot 6H_2O$) is another promising recycling product, which is a P mineral precipitated from waste streams [47] and contains about 12% P and 6% N concentrations varying with the source and production process [48]. Although P in struvite has low solubility in water, struvite shows P fertiliser effects that are comparable with highly soluble commercial P fertilisers [49–51]. Thus, analyzing the effects of recycling fertilisers on soil P pools is also crucial to understanding the transformation processes of P applied to the soil.

In this study, two separate pot experiments were conducted in the greenhouse to investigate the inter- and intraspecific P efficiency of alfalfa and red clover as influenced by various P recycling fertilisers, aiming to (1) estimate the capacity of alfalfa and red clover to effectively utilize P from diverse P sources, (2) examine the P efficiency of different accessions of alfalfa and red clover, and (3) compare the impact of the P recycling fertilisers on crop yield, P availability, and soil P pools. We assume that (I) intraspecific differences in P uptake are more likely to be found the more the P supply in the soil differs and that (II) not only does the P amount applied (but also the type of fertiliser) influence the soil P pools and consequently the P uptake of the legumes. This study not only offers supplementary insights into the selection of alfalfa and red clover accession but also provides valuable information for the effective utilisation of recycling fertilisers as fertiliser in the cultivation of forage legumes.

2. Materials and Methods

2.1. Preliminary Experiment

In 2018 and 2019, a total of 149 alfalfa and 120 red clover accessions from the IPK Gene Bank (Satellite Collections North, Leibniz Institute of Plant Genetics and Crop Plant Research) were grown in trial fields in northeastern Germany. A systematic selection process was conducted for the accessions, taking into account various parameters including the geographical origin of the plant material, sample status, maturity group, and plant P concentration (Table 1). Ultimately, eight accessions each of alfalfa and red clover were selected as representatives. Further details regarding the accessions investigated can be found on the website of [52].

Table 1. Geographic origin, sample status (SAMPSTAT), and plant P concentration of selected alfalfa and red clover accessions.

Alfalfa				Red Clover			
Accession	**Origin**	**SAMPSTAT**	**Plant P Concentration [mg kg^{-1}]**	**Accession**	**Origin**	**SAMPSTAT**	**Plant P Concentration [mg kg^{-1}]**
LE2812	YEM	300	4.29	LE1731	KGZ	300	3.95
LE2368	FRA	500	4.13	LE1423	FIN	400	3.67
LE2370	DNK	500	3.94	LE1391	GBR	500	3.56
LE2521	DEU	500	3.80	LE2750	HRV	100	3.43
LE713	ROU	500	3.03	LE1599	DEU	300	3.17
LE888	DEU	500	2.91	LE1775	RUS	100	2.98
LE2669	ROU	300	2.51	LE1804	SUN	999	2.83
LE2511	FRA	500	2.44	LE1937	DEU	100	2.72

Origin: Country codes (ISO 3166) [53]; SAMPSTAT: 100 = wild; 200 = weedy; 300 = traditional cultivar/landrace; 400 = breeding/research material; 500 = advanced or improved cultivar; 999: other.

2.2. Pot Experiment

Two separate pot experiments (PE) were conducted at the greenhouse of the University of Rostock with a randomized block design in spring 2021 (PE1) and 2022 (PE2) (Supplementary Figure S1). The substrate used in both experiments was soil collected from a fallow field of the experimental station of the University of Rostock (54°3′41.47″ N; 12°5′5.59″ E) with a mixture of topsoil (0 to 30 cm) and subsoil (30 to 60 cm) in a ratio of 1:2. The soil at the collection site is classified as Stagnic Cambisol according to the World Reference Base for Soil Resources [54], and the soil texture is loamy sand. The double lactate extractable P (Pdl) concentration, as an indicator of plant available P, was found to be 37.4 mg kg^{-1} in the topsoil at the collection site, indicating a suboptimal P supply for plants according to the soil P classification of the German federal state of Mecklenburg-Western Pomerania [55]. After the removal of stone and plant residuals, 6 kg of fresh soil was filled into Mitscherlich pots with a height of 31 cm and an inner diameter of 19 cm. The soil used in the pot experiment had a total P concentration of 550 mg kg^{-1}, a pH of 5.6, and an organic matter content of 2.35%. After the soil sampling in PE1, all plant residuals in pots were removed. Soils supplied with the same P amendments were mixed and refilled to respective pots and prepared for the same treatment in PE2.

In both experiments, four fertiliser treatments equivalent with 200 mg total P pot^{-1} were applied, including triple-superphosphate (TSP), sewage sludge ash (SSA), biowaste compost (compost), and struvite. In addition, a control treatment without P amendment (no P) was carried out. The TSP (Van de Reijt Meststoffen B.V., Moerdijk, The Netherlands) was produced in granular form around 3 mm. The SSA was produced and provided by Klärschlamm-Kooperation Mecklenburg-Vorpommern GmbH (KKMV, Rostock, Germany), where sewage sludge from waste water treatment was incinerated into the form of fine ash powder between 850 und 950 °C without pre- or post-treatment. The struvite (Berliner Pflanze, BWB, Berlin, Germany) with a crystal size between 1 and 2 mm was produced by Berliner Wasserbetriebe (BWB, Berlin, Germany) with the aeration of digested sludge in a reactor and precipitation by stripping CO_2 and adding mg Cl_2 [56]. Compost with a particle size between 1 to 10 mm was produced as sanitized compost based on green garden and landscape waste residues (Compost plant, Parkentin, Germany). The recycling products differed in their P concentration (Table 2). After the fertiliser's application, a nutrient solution containing N, K, mg and S (0.26 g N (NH_4NO_3), 1 g K (KCl), 0.15 g mg ($MgSO_4{\cdot}7H_2O$) and 0.2 g S ($MgSO_4{\cdot}7H_2O$)) was added to all treatments to avoid nutrient deficiency during plant growth.

Table 2. Nutritional composition (% of dry mass) of applied recycling products and TSP.

	P	N	Mg	K	Ca	Fe	Al
TSP	20.0						
SSA	11.8		1.1	0.6	10.6	23.3	2.0
Compost	0.4	2.2	0.5	0.8			
Struvite	10.0	5.0	7.2				

TSP = triple-super-phosphate, SSA = sewage sludge ash, compost = biowaste compost.

A total of eight accessions each of alfalfa and red clover were tested in PE1 and PE2, with the alfalfa accessions LE2521 and LE2669 and red clover accessions LE1731 and LE1391 being cultivated in both PE to verify the results. After the germination, 25 plants were kept in each pot for cultivation for 90 days at a day/night air temperatures of about 22/17 °C. Additional assimilation lighting was supplied to provide a total daily light period of 16 h. Plants were irrigated daily, and percolated water was collected and replenished. Weeds were manually eliminated throughout the cultivation phase. Four replicates were prepared for all treatments. Plant shoot biomass was harvested twice during the flowing stage (51 and 90 days after the seeding) in each pot experiment, where aboveground biomass was cut approximately 1 cm above the soil surface and weighed for dry weight after drying at 60 °C for seven days. After the second harvest, soil samples were taken with an auger from seven randomly selected spots in each pot and mixed carefully. Air-dried soil samples were sieved (2 mm) for standard soil P tests (Pdl).

2.3. Plant and Soil Analyses

Plant P concentration was determined after dry ashing and HCl digestion (2 g dry material digested in 20 mL of 25% boiling HCl in a 100 mL volumetric flask) via inductively coupled plasma optical emission spectroscopy (ICP-OES; ICP Serie Optima 8300DV, PerkinElmer Waltham, MA, USA). Plant N concentration was measured using a CNS Analyzer (vario EL cube, elementar, Hanau, Germany). Plant P and N uptake were determined as the product of the plant P and N concentration and the plant biomass. Plant P uptake efficiency (PUPE) was calculated as the ratio of plant P uptake and amended P in respective fertiliser. Plant P utilisation efficiency (PUTE) was calculated as the ratio of plant biomass and plant P uptake.

The soil Pdl content was measured following a modified method of [57]. In summary, 12 g air-dried soil was sieved (2 mm) and then extracted using a 150 mL solution composed of calcium lactate (0.4 M $C_6H_{10}CaO_6 \times 5H_2O$) and hydrochloric acid (0.5 M HCl) adjusted to a pH of 3.6; it was shaken overhead for 90 min with 35 rpm. Subsequently, the mixed solution was filtered (through a cellulose round filter of 3 to 5 µm) and the filtrate was measured for P concentration via ICP-OES.

The soil P fractions in combination with two accessions each of alfalfa and red clover were determined in PE1 using a modified sequential fractionation method based on the approaches from [58–60]. Alfalfa accessions LE2812 and LE2511 and red clover accessions LE1731 and LE1937 were selected as candidates based on their similar biomass production but disparate plant P concentration. Briefly, 0.5 g dry fine-ground soil was weighed into 50 mL centrifuge tubes with 30 mL deionized water. Samples were shaken overhead at 22 rpm for 18 h and then centrifugated at 3500 rpm for 20 min and decanted. Afterwards, residual soil sample was further sequentially extracted with 30 mL of 0.5 M $NaHCO_3$, 0.1 M NaOH, and 1 M H_2SO_4. No rinsing of the residual soil was performed between different extraction steps. After filtration (cellulose round filter, 3–5 µm), the total P concentration in each extract was determined by using ICP-OES.

Soil pH was measured in 0.01 M $CaCl_2$ in a soil–solution ratio of 1:2.5 using a pH electrode (pH 1100 L, VWR International, Darmstadt, Germany).

2.4. Statistical Analyses

Statistical analyses were conducted utilizing R version 4.0.4 [61] within the RStudio 2023.12.1 Build 402 development environment [62]. The data from all treatments were examined through a two-way ANOVA at both interspecific (considering fertiliser treatment and plant species as factors) and intraspecific levels (considering fertiliser treatment and accession as factors). When the impact of the factors was significant ($p < 0.05$), post hoc comparisons were executed, employing Duncan's new multiple range test ($\alpha = 0.05$) with the duncan.test function sourced from the agricolae package version 1.3-5 [63]. The assessment of normal distribution of residuals and homogeneity of variance in all statistical models was carried out using the shapiro.test and levene.test functions from the car package version 3.1-2 [64]. Should the normality assumptions not be satisfied, the data were transformed utilizing the boxcox function from the car package version 3.1-2 [64].

3. Results

3.1. Plant Yield and Nutrient Uptake

The plant biomass and nutrient uptake differed between the plant species. The application of P recycling products significantly influenced both biomass production and nutrient uptake, which are closely linked to the increased P available for plants from these products (see Section 3.2 below). In both pot experiments, red clover consistently exhibited higher average biomass production than alfalfa (16.8 vs. 14.4 g pot^{-1} in 2021, and 23.7 vs. 18.9 g pot^{-1} in 2022). In comparison to no P treatment, P supply with all amendments tested increased the alfalfa biomass in PE1 (between 14.3 and 15.4 g pot^{-1}) ($p < 0.05$), while in PE2, only TSP and struvite resulted in higher yields (Figure 1, Supplementary Tables S1 and S2). For red clover, higher yields compared to the no-P treatment were only observed when struvite was applied. Intraspecific differences were measured in alfalfa and red clover, but the difference between the accessions was more noticeable in red clover, where the red clover accession LE1391 tended to show the highest biomass (18.1 g pot^{-1} in PE1 and 25.3 g pot^{-1} in PE2).

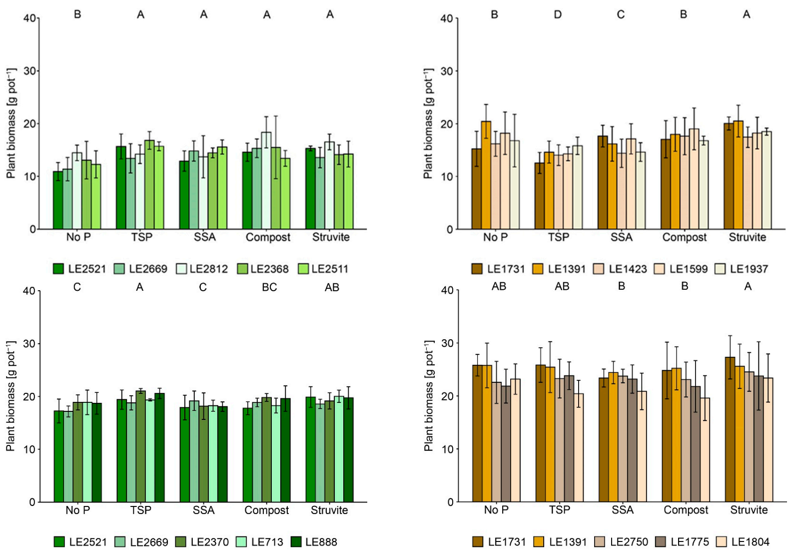

Figure 1. Plant biomass of alfalfa and red clover accessions in the five treatments (no P, TSP, SSA, compost and struvite) in PE1 (**upper part**) and PE2 (**lower part**). Different shades of green and brown colours are used to differentiate between alfalfa and red clover accessions. Letters denote significant differences among treatments (in means of accessions) (Duncan's new multiple-range test, with $p < 0.05$, $n = 4$).

The plant P concentration varied mostly in dependence on applied P fertilisers (Tables 3 and 4). In comparison to no P treatment, P application increased plant P concentration in both alfalfa and red clover in the following order: struvite $\geq$ TSP > compost > SSA. In PE1, alfalfa displayed a higher average P concentration than red clover (2.73 vs. 2.51 g kg^{-1}) ($p < 0.05$), but in PE2, similar values were measured for both species (2.44 vs. 2.41 g kg^{-1}). Among accessions, alfalfa accession LE2812 and red clover accession LE1937 exhibited the highest P concentration in 2021 (both with 2.86 g kg^{-1}).

Table 3. Phosphorus (P) concentration (Pcon) of alfalfa and red clover accessions in the five treatments (no P, TSP, SSA, compost, and struvite) in PE1.

Pcon [g kg^{-1}]		Treatment										
Species	Accession	No P *		TSP *		SSA *		Compost *		Struvite *		Average
Alfalfa	LE2812	2.59 ± 0.40	Aa	3.02 ± 0.37	Aa	2.87 ± 0.58	Aa	2.66 ± 0.12	Aa	3.16 ± 0.52	Aab	2.86 ± 0.44
	LE2521	2.47 ± 0.23	Bab	3.14 ± 0.20	Aa	2.42 ± 0.14	Bab	2.64 ± 0.43	Ba	3.24 ± 0.35	Aa	2.78 ± 0.44
	LE2511	2.12 ± 0.11	Bb	2.82 ± 0.39	Aa	2.58 ± 0.56	ABab	2.53 ± 0.24	ABa	2.97 ± 0.40	Aab	2.60 ± 0.44
	LE2368	2.46 ± 0.04	Cab	3.09 ± 0.25	Aa	2.25 ± 0.18	Cb	2.78 ± 0.17	Ba	3.09 ± 0.19	Aab	2.74 ± 0.38
	LE2669	2.26 ± 0.23	Cab	3.37 ± 0.47	Aa	2.38 ± 0.32	Cab	2.48 ± 0.21	BCa	2.80 ± 0.19	Bb	2.66 ± 0.49
	Average	2.38 ± 0.27	C	3.09 ± 0.36	A	2.50 ± 0.42	BC	2.62 ± 0.25	B	3.05 ± 0.35	A	2.73 ± 0.44
Red clover	LE1731	1.75 ± 0.13	Db	2.73 ± 0.02	ABab	2.29 ± 0.37	BCa	2.17 ± 0.13	CDb	3.05 ± 0.36	Aa	2.38 ± 0.52
	LE1423	2.03 ± 0.30	Cab	2.94 ± 0.20	Aab	2.05 ± 0.33	Cb	2.44 ± 0.18	Bb	3.24 ± 0.24	Aa	2.54 ± 0.55
	LE1391	1.80 ± 0.19	Cb	2.50 ± 0.05	Bb	1.96 ± 0.15	Cb	2.37 ± 0.22	Bb	3.02 ± 0.27	Aa	2.31 ± 0.51
	LE1599	1.86 ± 0.14	Cb	2.56 ± 0.30	Bb	2.29 ± 0.24	Ba	2.44 ± 0.20	Bb	3.24 ± 0.47	Aa	2.44 ± 0.53
	LE1937	2.26 ± 0.16	Ca	3.08 ± 0.40	ABa	2.10 ± 0.17	Cab	2.86 ± 0.26	Ba	3.53 ± 0.06	Aa	2.86 ± 0.57
	Average	1.91 ± 0.24	E	2.79 ± 0.30	B	2.13 ± 0.28	D	2.44 ± 0.28	C	3.20 ± 0.33	A	2.49 ± 0.55
Average		2.16 ± 0.35	E	2.96 ± 0.37	B	2.33 ± 0.40	D	2.54 ± 0.28	C	3.12 ± 0.34	A	2.62 ± 0.51

No P = without P amendment, TSP = triple-super-phosphate, SSA = sewage sludge ash, compost = biowaste compost. Uppercase letters denote a significant difference among treatments, and lowercase letters denote a significant difference among accessions within the same plant species (Duncan's new multiple-range test, with $p < 0.05$). Asterisks indicate significant difference between plant species within the same treatment. Mean ± standard deviation ($n = 4$).

Table 4. Phosphorus (P) concentration (Pcon) of accessions of alfalfa and red clover from the five studied treatments (no P, TSP, SSA, compost, and struvite) in PE2.

Pcon [g kg^{-1}]		Treatment										
Species	Accession	No P		TSP		SSA		Compost *		Struvite		Average
Alfalfa	LE713	2.05 ± 0.18	C	2.70 ± 0.08	A	2.16 ± 0.22	BC	2.37 ± 0.16	B	2.82 ± 0.25	A	2.40 ± 0.35
	LE2370	1.95 ± 0.09	D	2.60 ± 0.12	B	2.22 ± 0.14	C	2.44 ± 0.08	BC	2.83 ± 0.25	A	2.41 ± 0.34
	LE2521	2.12 ± 0.19	C	2.73 ± 0.18	AB	2.27 ± 0.11	C	2.51 ± 0.14	B	2.87 ± 0.12	A	2.50 ± 0.31
	LE888	2.02 ± 0.21	C	2.88 ± 0.30	A	2.18 ± 0.15	BC	2.31 ± 0.16	B	2.76 ± 0.17	A	2.43 ± 0.39
	LE2669	2.14 ± 0.05	B	2.77 ± 0.22	A	2.26 ± 0.07	B	2.42 ± 0.09	AB	2.73 ± 0.41	A	2.47 ± 0.32
	Average	2.06 ± 0.16	D	2.74 ± 0.20	A	2.22 ± 0.14	C	2.41 ± 0.14	B	2.80 ± 0.24	A	2.44 ± 0.34
Red clover	LE1731	2.19 ± 0.15	B	2.69 ± 0.32	A	2.26 ± 0.28	B	2.23 ± 0.21	B	2.55 ± 0.20	A	2.38 ± 0.29
	LE1391	2.12 ± 0.07	B	2.73 ± 0.24	A	2.20 ± 0.22	B	2.32 ± 0.13	B	2.79 ± 0.20	A	2.43 ± 0.33
	LE2750	2.03 ± 0.09	C	2.56 ± 0.21	AB	2.32 ± 0.04	BC	2.11 ± 0.18	C	2.81 ± 0.30	A	2.38 ± 0.34
	LE1775	2.11 ± 0.17	C	2.61 ± 0.08	AB	2.27 ± 0.23	C	2.31 ± 0.12	BC	2.74 ± 0.26	A	2.41 ± 0.29
	LE1804	2.13 ± 0.05	B	2.77 ± 0.09	A	2.30 ± 0.06	B	2.26 ± 0.28	B	2.70 ± 0.16	A	2.43 ± 0.29
	Average	2.12 ± 0.11	C	2.67 ± 0.20	A	2.27 ± 0.18	B	2.25 ± 0.18	B	2.72 ± 0.23	A	2.41 ± 0.30
Average		2.09 ± 0.14	D	2.70 ± 0.20	A	2.25 ± 0.16	C	2.33 ± 0.18	B	2.76 ± 0.23	A	2.42 ± 0.32

No P = without P amendment, TSP = triple-super-phosphate, SSA = sewage sludge ash, compost = biowaste compost. Uppercase letters denote a significant difference among treatments (Duncan's new multiple-range test, with $p < 0.05$). Asterisks indicate the significant different between plant species within same treatment. Mean ± standard deviation ($n = 4$).

Plant P uptake mainly followed plant biomass with correlation factors of r = 0.705 in PE1 and 0.815 in PE2 ($p < 0.001$). Consequently, struvite and TSP showed generally higher effectiveness in increasing P uptake compared to the no-P treatment in both experiments (Figure 2), and red clover showed a higher average P uptake than alfalfa in both experiments (42.5 vs. 39.5 mg pot^{-1} in PE1 and 57.2 vs. 46.4 mg pot^{-1} in PE2). Intraspecific differences were also measured in both experiments, with high P uptake measured for the alfalfa accession LE2812 in PE1 and red clover accessions and LE1391 in PE2. An interactive effect between treatments and plant species was detected in PE1, where red clover supplied with TSP showed lower P uptake than alfalfa (39.1 vs. 46.5 mg pot^{-1}) but showed significant higher value when fertilized with struvite (60.8 vs. 45.1 mg pot^{-1}).

The PUPE in alfalfa and red clover differed between applied fertilisers, where struvite demonstrated higher values than SSA and compost and comparable values to TSP (Supplementary Figure S2). The PUTE of alfalfa and red clover differed between treatments and fell in the following order: no P > ash > compost > TSP ≥ struvite (Supplementary Tables S3 and S4). Interspecific differences were only observed in PUTE in PE1, where red clover showed generally higher PUTE than alfalfa. No pronounced differences were measured in PUPE or PUTE.

The plant N concentration differed between the plant species (Tables 5 and 6), with alfalfa displaying higher average N concentrations than red clover in both experiments (38.8 vs. 36.5 g kg^{-1} in PE1 and 36.4 vs. 31.9 g kg^{-1} in PE2). Alfalfa accessions showed no differences in N concentration in both experiments, whereas small but significant intraspecific differences were always detected in red clover accessions.

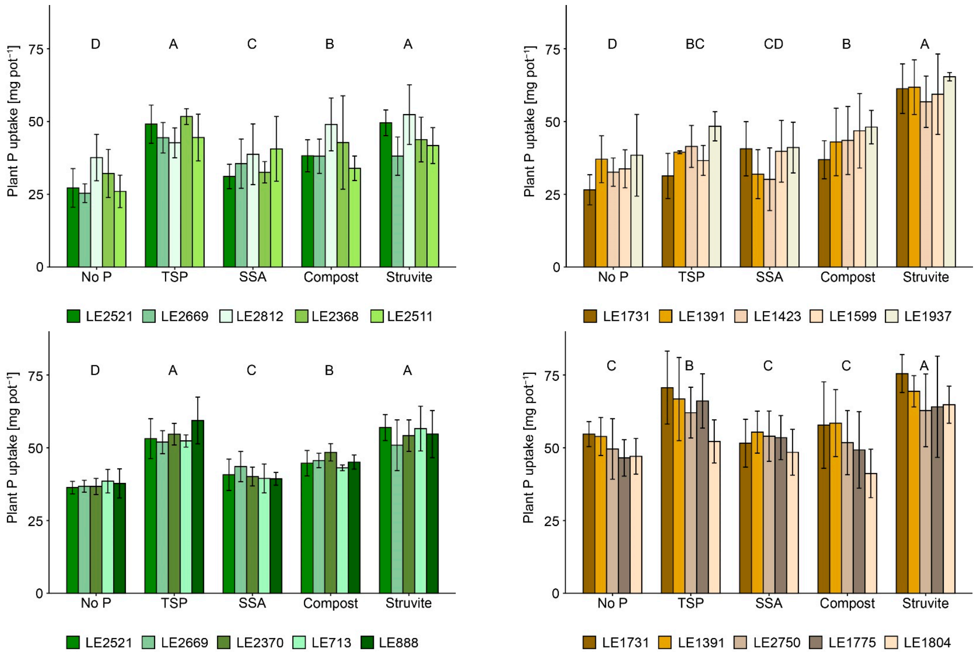

Figure 2. Plant phosphorus (P) uptake of alfalfa and red clover accessions in the five treatments (no P, TSP, SSA, compost and struvite) in PE1 (**upper part**) and PE2 (**lower part**). Different shades of green and brown colours are used to differentiate between alfalfa and red clover accessions. Letters denote significant differences among treatments (in means of accessions) within the same plant species (Duncan's new multiple-range test, with $p < 0.05$, $n = 4$).

Table 5. Nitrogen (N) concentration (Ncon) of alfalfa and red clover accessions from the five treatments (no P, TSP, SSA, compost, and struvite) in PE1.

Ncon [g kg^{-1}]		Treatment										
Species	Accession	No P *		TSP		SSA *		Compost *		Struvite		Average
Alfalfa	LE2812	40.7 ± 4.6	Aa	36.1 ± 2.2	ABa	38.6 ± 3.8	ABa	37.8 ± 4.3	ABa	34.7 ± 1.2	Bb	37.6 ± 3.7
	LE2521	42.3 ± 1.0	Aa	36.2 ± 3.9	Ba	38.9 ± 4.3	ABa	39.4 ± 3.7	ABa	38.5 ± 2.4	ABa	39.0 ± 3.6
	LE2511	40.1 ± 1.9	ABa	37.0 ± 3.1	Ba	39.0 ± 3.2	ABa	41.0 ± 2.6	Aa	37.2 ± 3.6	ABab	38.9 ± 3.1
	LE2368	40.5 ± 3.0	Aa	34.8 ± 3.8	Ba	39.7 ± 3.9	Aa	41.6 ± 3.4	Aa	39.9 ± 1.8	Aa	39.3 ± 3.8
	LE2669	41.9 ± 2.4	Aa	36.9 ± 4.4	Ba	39.7 ± 3.8	ABa	39.9 ± 1.1	ABa	38.4 ± 2.4	ABa	39.4 ± 3.2
	Average	41.1 ± 2.7	A	36.2 ± 3.3	D	39.2 ± 3.4	BC	39.9 ± 3.2	AB	37.7 ± 2.8	CD	38.8 ± 3.5
Red clover	LE1731	33.7 ± 0.8	Ba	37.5 ± 1.4	Aab	37.6 ± 2.2	Aa	35.7 ± 1.9	ABa	36.6 ± 2.0	Abc	36.2 ± 2.1
	LE1423	36.8 ± 3.0	Aa	38.4 ± 2.0	Aa	37.5 ± 4.2	Aa	39.3 ± 1.7	Aa	39.4 ± 1.1	Aa	38.3 ± 2.6
	LE1391	34.0 ± 1.9	ABa	33.7 ± 2.9	ABc	32.0 ± 1.5	Bb	36.5 ± 2.0	Aa	35.0 ± 0.7	ABc	34.2 ± 2.2
	LE1599	36.4 ± 2.6	Aa	35.2 ± 0.3	Abc	38.3 ± 1.7	Aa	35.8 ± 3.0	Aa	39.4 ± 2.3	Aa	37.0 ± 2.5
	LE1937	36.4 ± 1.1	Aa	36.1 ± 0.9	Aabc	36.1 ± 4.9	Aa	37.7 ± 0.9	Aa	37.9 ± 1.8	Aab	36.8 ± 2.6
	Average	35.4 ± 2.3	B	36.4 ± 2.3	AB	36.2 ± 3.7	AB	37.1 ± 2.3	A	37.5 ± 2.3	A	36.5 ± 2.7
Average		38.4 ± 3.8	A	36.3 ± 2.8	B	37.7 ± 3.8	A	38.6 ± 3.1	A	37.6 ± 2.5	A	37.7 ± 3.3

No P = without P amendment, TSP = triple-super-phosphate, SSA = sewage sludge ash, compost = biowaste compost. Uppercase letters denote a significant difference among treatments, and lowercase letters denote a significant difference among accessions within the same plant species (Duncan's new multiple-range test, with $p < 0.05$). Asterisks indicate significant difference between plant species within the same treatment. Mean ± standard deviation ($n = 4$).

Table 6. Nitrogen (N) concentration (Ncon) of alfalfa and red clover accessions from the five treatments (no P, TSP, SSA, compost, and struvite) in PE2.

Ncon [g kg^{-1}]		Treatment										
Species	Accession	No P *		TSP *		SSA *		Compost *		Struvite *		Average
Alfalfa	LE713	36.2 ± 2.7	Aa	37.6 ± 1.4	Aa	35.7 ± 1.4	Aab	37.7 ± 2.2	Aa	37.7 ± 1.5	Aa	36.9 ± 1.9
	LE2370	35.4 ± 2.0	Aa	37.1 ± 1.5	Aa	36.3 ± 1.3	Aab	38.0 ± 2.4	Aa	37.4 ± 1.3	Aa	36.8 ± 1.8
	LE2521	36.6 ± 3.5	Aa	36.0 ± 1.2	Aa	36.6 ± 1.5	Aa	37.1 ± 1.7	Aa	36.0 ± 1.0	Aa	36.5 ± 1.8
	LE888	34.9 ± 1.2	Ba	36.1 ± 1.5	ABa	34.3 ± 0.3	Bb	37.6 ± 2.4	Aa	36.2 ± 1.0	ABa	35.8 ± 1.8
	LE2669	37.1 ± 3.4	Aa	36.1 ± 1.0	Aa	34.8 ± 1.3	Aab	36.7 ± 1.5	Aa	36.0 ± 1.4	Aa	36.1 ± 1.9
	Average	36.0 ± 2.5	BC	36.5 ± 1.3	ABC	35.6 ± 1.4	C	37.4 ± 1.9	A	36.6 ± 1.3	AB	36.4 ± 1.8
Red clover	LE1731	31.8 ± 1.4	Aa	32.1 ± 1.8	Aa	32.4 ± 2.2	Aa	30.9 ± 1.2	Aab	31.3 ± 2.2	Aab	31.7 ± 1.7
	LE1391	32.1 ± 0.4	Aa	32.8 ± 1.6	Aa	32.1 ± 2.7	Aa	30.9 ± 1.7	Aab	32.5 ± 1.0	Aab	32.1 ± 1.6
	LE2750	30.2 ± 0.7	Bb	33.0 ± 2.5	Aa	33.5 ± 2.1	Aa	32.3 ± 0.8	ABa	31.8 ± 1.4	ABab	32.2 ± 1.9
	LE1775	31.2 ± 1.1	Aab	31.4 ± 1.8	Aa	31.2 ± 0.5	Aa	29.8 ± 0.5	Ab	30.3 ± 1.4	Ab	30.8 ± 1.2
	LE1804	31.3 ± 0.5	Bab	33.5 ± 1.1	Aa	32.6 ± 0.4	ABa	33.0 ± 2.5	ABa	33.3 ± 1.2	ABa	32.7 ± 1.5
	Average	31.3 ± 1.1	B	32.6 ± 1.8	A	32.4 ± 1.8	A	31.4 ± 1.8	B	31.8 ± 1.7	AB	31.9 ± 1.7
Average		33.7 ± 3.1	B	34.5 ± 2.5	A	34.0 ± 2.3	AB	34.5 ± 3.6	A	34.2 ± 2.9	AB	34.2 ± 2.9

No P = without P amendment, TSP = triple-super-phosphate, SSA = sewage sludge ash, compost = biowaste compost. Uppercase letters denote a significant difference among treatments and lowercase letters denote a significant difference among accessions within the same plant species (Duncan's new multiple-range test, with $p < 0.05$). Asterisks indicate significant difference between plant species within the same treatment. Mean ± standard deviation ($n = 4$).

As plant N uptake exhibited a strong positive correlation with plant biomass (r = 0.900 in PE1 and r = 0.883 in PE2, $p < 0.001$), red clover displayed a higher average N uptake than alfalfa in both experiments (611 vs. 551 mg pot^{-1} in PE1 and 755 vs. 689 mg pot^{-1} in PE2). Intraspecific differences were rarely detected (Figure 3). Compared to the no-P treatment, the application of compost and struvite resulted in higher alfalfa N uptake in both experiments, while for red clover, only struvite increased the N uptake in comparison to the no-P treatment ($p < 0.05$).

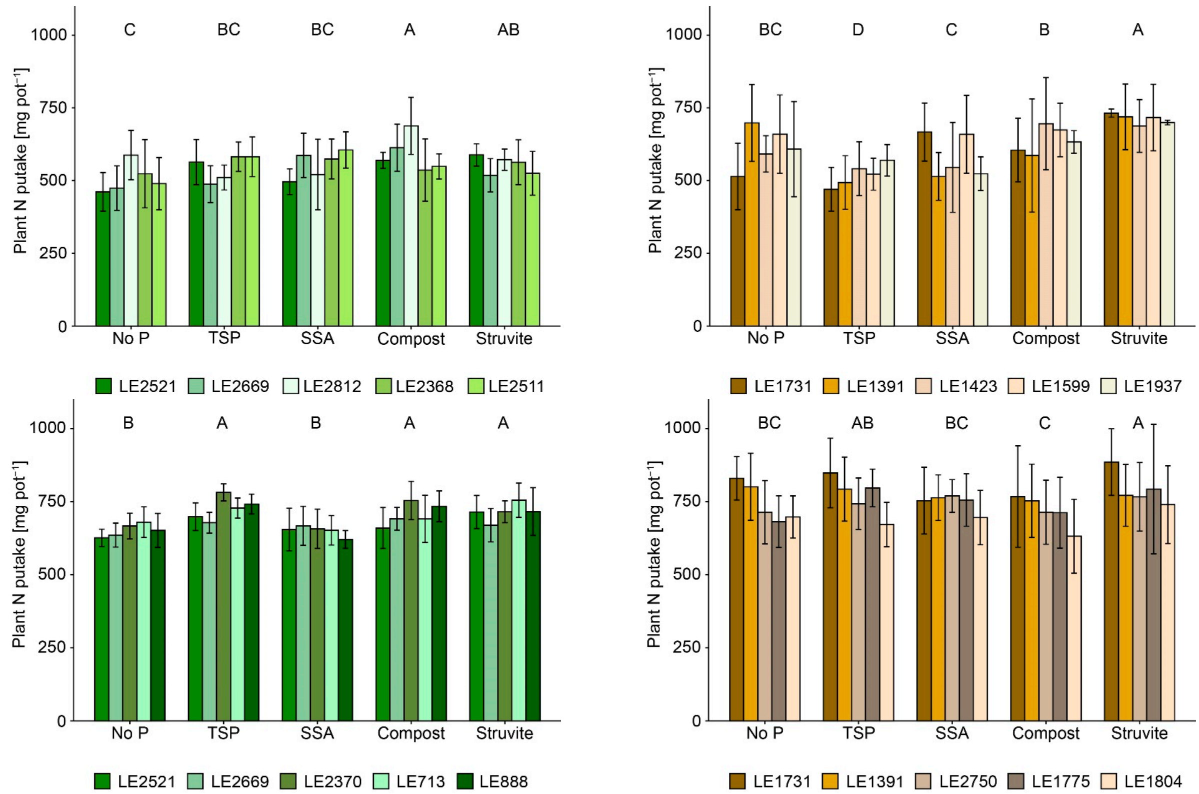

Figure 3. Plant nitrogen (N) uptake of alfalfa and red clover accessions in the five treatments (no P, TSP, SSA, compost and struvite) in PE1 (**upper part**) and PE2 (**lower part**). Different shades of green and brown colours are illustrated to differentiate between alfalfa and red clover accessions, respectively. Letters denote significant differences among treatments (using the means of accessions) (Duncan's new multiple-range test, with $p < 0.05$, $n = 4$).

Alfalfa accessions LE2521 and LE2669 and red clover accessions LE1731 and LE1391 were tested in both experiments, showing almost identical biomass and nutrient uptake in both years.

3.2. Plant Available P and P Fractions in Soil

The fertiliser treatments had a clear effect on soil P content, affecting both the Pdl and the soil P fraction, which was more pronounced than in plant characteristics.

The Pdl content remained relatively consistent between experiments, with the exception that soils treated with compost showed higher Pdl in PE2 compared to PE1 (90.8 vs. 73.8 mg kg^{-1}). The fertiliser treatment increased Pdl content compared with the no-P treatment and followed a similar pattern in both experiments, where on average, compost and struvite (82.3 and 72.6 mg kg^{-1}) increased soil Pdl content more effectively than TSP and SSA (63.3 and 44.3 mg kg^{-1}) ($p < 0.05$). Soils cultivated with alfalfa displayed a higher average Pdl content (63.4 mg kg^{-1}) than those with red clover (55.7 mg kg^{-1}) in PE1, but the values were similar in PE2 (about 62 mg kg^{-1}). No intraspecific differences between either alfalfa or red clover accessions regarding soil Pdl were detected in any experiment.

The soil P fractions were determined for soils cultivated with selected alfalfa and red clover candidates in PE1 (see Section 2.3). Significant interspecific differences between alfalfa and red clover were observed in P-H_2O (32.2 and 35.2 mg kg^{-1}) and P-NaOH (226.9 and 267.6 mg kg^{-1}). As for Pdl, no intraspecific differences were found between the accessions within either plant species. Compared to the no-P treatment, all applied fertiliser increased P-H_2O, with struvite having higher efficacy (44.4 mg kg^{-1}) than TSP (37.6 mg kg^{-1}), compost (36.0 mg kg^{-1}), and SSA (27.6 mg kg^{-1}). The application of TSP, compost, and struvite raised P-$NaHCO_3$ to a similar level (124.6, 117.8 and 117.4 mg kg^{-1}, respectively) compared to the no-P treatment (100.3 mg kg^{-1}). The P-NaOH fraction was comparably less affected by the applied fertiliser, and only struvite increased the P-NaOH of soils cultivated with red clover. Finally, the rather stable P-H_2SO_4 fraction was also affected by the fertilisers. Especially in the SSA treatment, this fraction was raised (150.7 mg kg^{-1}) compared to the no-P treatment (114.5 mg kg^{-1}).

4. Discussion

4.1. Inter- and Intraspecific Biomass Production and Nutrient Uptake

Both alfalfa and red clover developed well under the experimental conditions, and the biomass was comparable with that in other pot experiments with alfalfa [22] and red clover [65]. Red clover showed higher biomass production and nutrient uptake than alfalfa in both experiments (Section 3.1). Similar interspecific differences were also reported in previous studies under similar conditions by [66,67]. The lower biomass of alfalfa might partly be due to the pH of about 5.6 to 5.8, which is in a normal range for a loamy sand soil, but relatively low for alfalfa.

On average, alfalfa and red clover produced higher biomass in PE2 than in PE1. This was also true for alfalfa accessions LE2521 and LE2669 and red clover accessions LE1731 and LE1391, which were cultivated in both PE1 and PE2, so it is highly unlikely to be an effect of accession. Large day/night temperature differences can benefit alfalfa growth [68,69]. However, the temperature set-ups in our experiments were not that different (with a day/night temperature of 22.7/17.2 °C in PE1 and 22.5/15.7 °C in PE2). Given the relatively small variation in P concentration (Section 3.1), P uptake was primarily in independent of biomass production in our study.

The intraspecific differences were relatively small, but some accessions performed better than others. The relatively high P concentration and P uptake of alfalfa accession LE2812, a traditional cultivar from Yemen, are consistent with measurements from preparatory experiments for the accession selection (Section 2.1). This means that this accession has a high P uptake efficiency when growing under favourable conditions in terms of temperature and lighting. However, results from a field trial in north-eastern Germany show that relatively cool weather conditions can limit the growth of this accession [35]. Apart from accession LE2812, other alfalfa accessions were originally from European countries and showed similar yield and nutrient uptakes. Red clover accession LE1391, which was tested in both PE1 and PE2, showed high biomass and high nutrient uptake, which is in agreement with the result in our field study [35]. The constant performance of accession LE1391 in controlled and field conditions underlines its agronomic value, and it could be recommended as a suitable candidate for cultivation as a forage legume in Europe. That site effect and management can play important roles in the biomass production and P utilisation of alfalfa and red clover, as was previously described in other studies [33,70,71], should be considered when recommending accessions for cultivation. Overall, looking at the results from this experiment and previous studies, the P concentration in the biomass seems to play a subordinate role in the performance of the accessions under various growth conditions.

4.2. Effects of P-Recycling Fertilisers on the Performance of the Plants and on Soil Characteristics

The P-recycling fertilisers applied strongly affected plant P content and P availability in the soil. However, the yield effect of the applied fertiliser treatments was less pronounced. Even though the Pdl content in the no-P treatment (with average levels of 39.9 and 47.4 mg kg^{-1} in PE1 and PE2, respectively) ranked as suboptimal [55], the plant P concentration (2.16 mg kg^{-1} in PE1 and 2.06 mg kg^{-1} in PE2) in the no-P treatment still surpassed the critical threshold of 2.0 mg kg^{-1}, regarded as an indicator of the P deficiency of alfalfa at the early flowering stage [72]. This indicates that both alfalfa and clover were able to supply themselves sufficiently with P even in the control and underlines their ability to increase P mobilization capacity as described in the introduction.

Struvite showed the highest P fertiliser effect among the P-recycling products. The efficiency of struvite for plant P nutrition has been reported in previous studies under controlled conditions [73,74], with soybean and red clover showing stronger responses to struvite supply than cereal species. Despite being regarded as a slow-release P fertiliser [44,75], comparable or even higher PUPE was measured for alfalfa and red clover supplied with struvite than with TSP (Supplementary Figure S2). This supports the suggestion that dissolution of struvite in soil is a more accurate indicator of its effectiveness than the P solubility test in solutions [76,77]. Although the plants' P uptake was high when struvite was applied, this treatment also resulted in a higher Pdl content and P-H_2O fraction in the soil than the application of other P sources (Figures 4 and 5). Thus, it seems that struvite application together with legume cultivation is a very efficient strategy for P utilisation. Struvite solubility generally increases with decreasing pH [47]. The pH of 5.6 in soil (see Section 2.2) together with the acidification in the rhizosphere of the legumes [78,79] will have resulted in a continuous decomposition of struvite crystals and hence increased the effectiveness of struvite.

In contrast to the other recycling products, compost contains relevant amounts of N. However, the higher N supply of compost did not lead to higher N concentrations in the legumes. This underlines the slow N release from compost [80] and makes compost a suitable fertiliser for legumes. Compost application resulted in increased P contents in the labile pool in the soil, but less so than TSP and struvite (Figure 5). In addition, the supplied compost increased the readily available P fraction in soil, as the readily available P fraction usually constitutes the largest fraction in the compost [81]. The fertiliser effect of the same compost on alfalfa and red clover (as well as the effect of TSP) was also investigated in a two-year field study based on a long-term field experiment [35]. In the field, compost showed a stronger impact on yield than TSP. However, this was most likely due to the additional positive influence of compost on soil properties such as water storage capacity. These effects were less relevant in the pot experiment under controlled conditions.

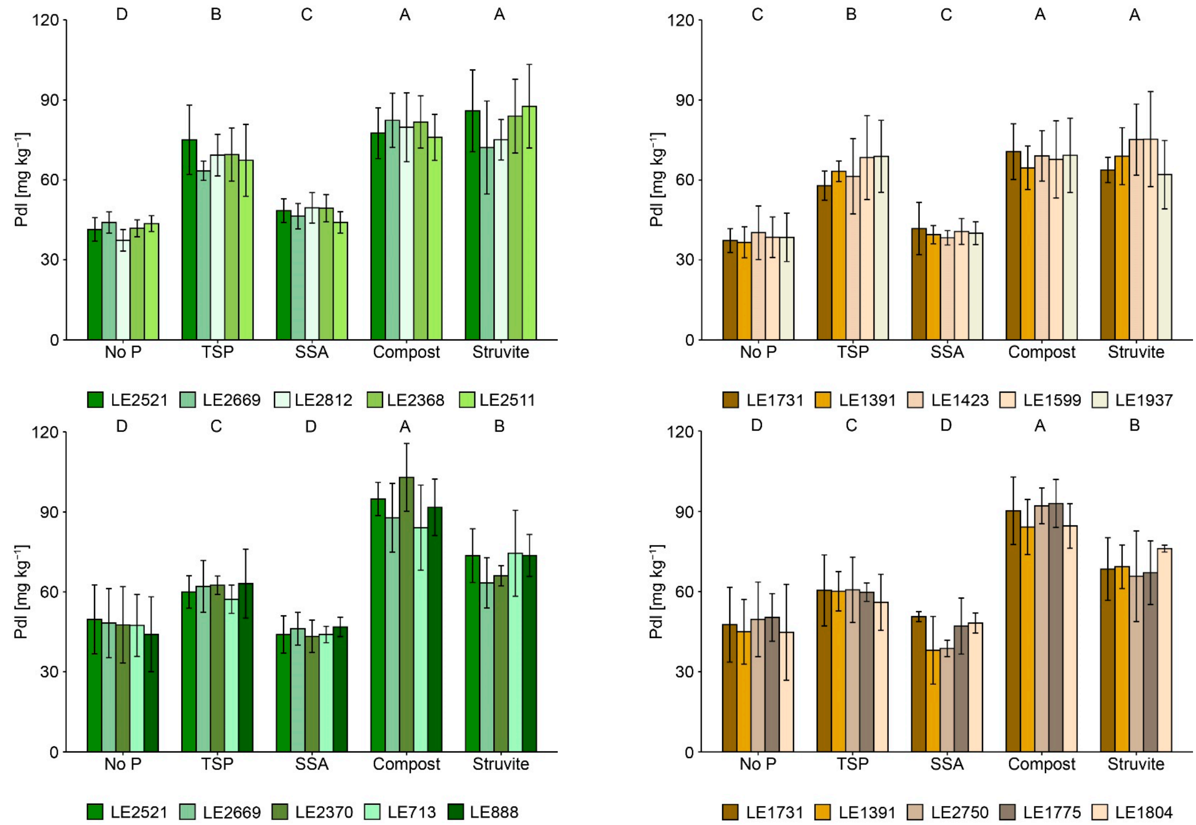

Figure 4. Double lactate extractable phosphorus (Pdl) in soil of alfalfa and red clover accessions in the five treatments (no P, TSP, SSA, compost and struvite) in PE1 (**upper part**) and PE2 (**lower part**). Different shades of green and brown colours are used to differentiate between alfalfa and red clover accessions. Letters denote significant differences among treatments (in means of accessions) (Duncan's new multiple-range test, with $p < 0.05$, $n = 4$).

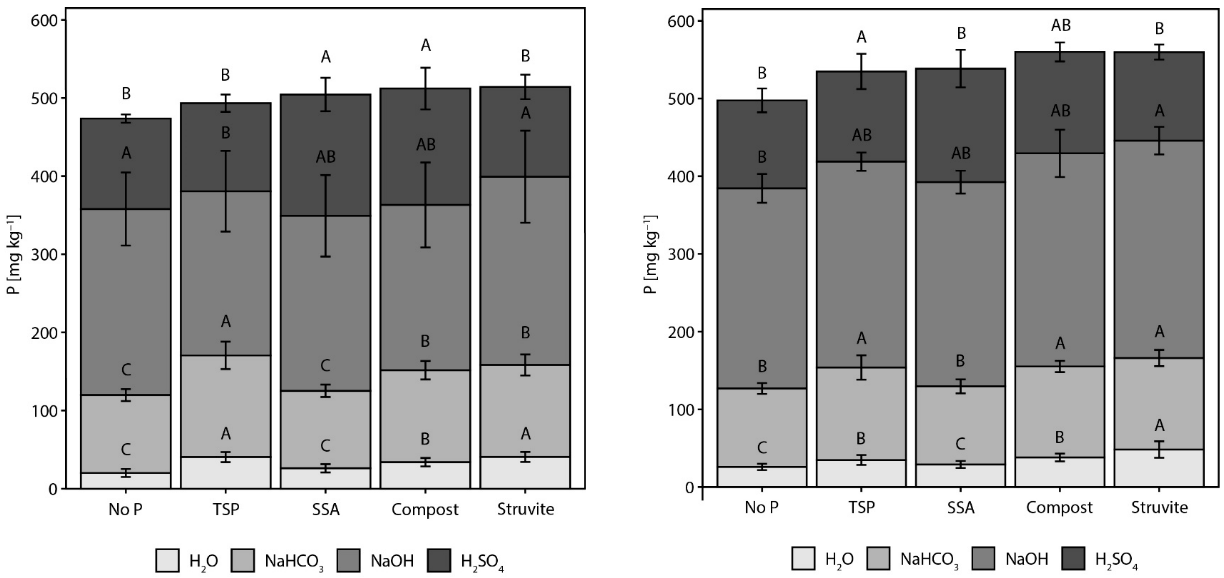

Figure 5. Phosphorus (P) concentrations of P-H_2O, P-$NaHCO_3$, P-NaOH and P-H_2SO_4 in soil of five treatments (no P, TSP, SSA, compost, and struvite) for alfalfa (**left**) and red clover (**right**) in PE1. Letters denote significant difference between treatments (in means of accessions) (Duncan's new multiple-range test, with $p < 0.05$, $n = 8$).

The P available to plants in soil supplied with compost significantly increased in PE2 in comparison to PE1 (see Section 3.2). Here, the compost used in PE2 was the same as in PE1, having only been stored for one additional year. The aging of this compost may have led to changes in the properties and chemical composition of the compost [82], where the low-molecule organic acids produced by degradation of easily degradable organic matter and enzymes exuded by microorganisms can mineralize organic P and increase the P availability in compost [83,84].

The SSA applied in our study usually resulted in the lowest P fertiliser effect of all P-recycling products. Plant biomass, P concentration, and P uptake with SSA application barely exceeded levels shown with no P treatment. Despite SSA having a P concentration comparable to struvite (as shown in Table 2), the notably high Fe content in SSA (Table 2) may result in the amendment of hardly soluble P forms in the soil, consequently reducing plant P availability in soil [45]. Additionally, the presence of Fe and Ca in SSA may contribute to increased P sorption and the formation of less available P forms in soil compared to other treatments [85]. As SSAs have a liming effect [86], the increased pH value in soil can counteract the acidification of the rhizosphere as a means of P mobilization by legumes. As a result of these factors, the marginal increase in the Pdl content and P-$NaHCO_3$ fraction after SSA application, along with a substantial increase in the P-H_2SO_4 fraction, collectively reflect the limited effectiveness of SSA as P fertiliser.

5. Conclusions

The recycling fertilisers had a clear effect on soil P contents, but the legumes' biomass was only partly affected, underlining the high active P mobilization in soil by the legumes. A clear P fertiliser effect was found for struvite, while SSA showed limited effectiveness as a P source for legumes. Red clover partly outperformed alfalfa in biomass production and nutrient uptake under our experimental conditions, but intraspecific differences were rather small. Thus, the selection of effective P sources appears to be of greater importance for the P nutrition and biomass production of alfalfa and red clover than the selection of the variety to be cultivated.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14050901/s1, Figure S1: Alfalfa and red clover accessions in greenhouse; Table S1: Plant biomass of alfalfa and red clover accessions in the five treatments (no P, TSP, SSA, compost and struvite) in PE1; Table S2: Plant biomass of alfalfa and red clover accessions in the five treatments (no P, TSP, SSA, compost, and struvite) in PE2; Figure S2: Phosphorus uptake efficiency (PUPE) of alfalfa and red clover accessions in the five treatments (TSP, SSA, compost and struvite) in PE1 (upper part) and PE2 (lower part); Table S3: Phosphorus utilisation efficiency (PUTE) of alfalfa and red clover accessions in the five treatments (no P, TSP, SSA, compost, and struvite) in PE1; Table S4: Phosphorus utilisation efficiency (PUTE) of alfalfa and red clover accessions in the five treatments (no P, TSP, SSA, compost, and struvite) in PE2.

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