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Final report

PhD project of
P-Campus Graduate School

P efficiency of forage legumes and their capacity to utilise P from recycling products

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1 Summary and conclusions

Although legumes have a relatively high Phosphorus (P) requirement, the P supply had only small effects on alfalfa (*Medicago sativa* L.) and red clover (*Trifolium pratense* L.) yields in our study, indicating high soil P replenishment as well as high active P mobilisation by the legumes. The positive agronomic effects of organic fertilisers, especially biowaste compost, were related to their long-term effect on soil quality rather than to P supply. Struvite as P recycling product showed equivalent and even higher P efficiency than triple superphosphate (TSP). The interspecific differences between alfalfa and red clover were relatively small and the intraspecific effects were marginal. Thus, accession selection appears to be of little importance with respect to P utilisation in alfalfa and red clover cultivation, as long as the accessions used are adapted to the respective environmental conditions.

2 Introduction and objectives of PhD project

Alfalfa and red clover are perennial small grain legumes with a great importance as forage and protein-rich feed worldwide (Kulkarni et al., 2018). Due to the biological nitrogen (N) fixation (BNF) through the symbiotic association of rhizobia these crops can contribute to economic value and sustainability in plant production (Anglade et al., 2015). Numerous studies have proven the benefits of alfalfa and red clover in cereal or maize based crop rotations (Abdin et al., 2000; Coombs et al., 2017; Gaudin et al., 2014; Ketterings et al., 2015).

Plants engaged in BNF generally have a high P requirement (Broughton, 1983). The high P demand of legumes increases their sensitivity to P deficiency, which can be a major limiting factor for legume production (Lazali & Drevon, 2021). To cope with the limitation under low P availability, legumes are usually efficient in P acquisition and mobilisation from the soil through both morphological and physiological adaptation (Cheng et al., 2014; Wang et al., 2010). In addition, phosphatases can be released under P deficiency to hydrolyse and utilise organic P compounds in soil (Nannipieri et al., 2011).

In comparison to the conventional chemical P fertilisers like TSP, recycling fertilisers like biomass ash, sewage sludge ash, struvite, biowaste compost and cattle manure were proven to be an adequate P source (Eichler-Löbermann et al., 2008; Gopinath et al., 2008; Li et al., 2016; Parham et al., 2002; Requejo & Eichler-Löbermann, 2014; Uysal et al., 2014). Beside the pure P effect, many recycling fertilisers have further effects on soil quality, e.g., pH value, organic matter content, and microbial activity, which finally can also affect plant growth and development (Saha et al., 2008; Sayara et al., 2020; Silva et al., 2019).

Phosphorus acquisition strategies were described for *Trifolium* and *Medicago* species (Nazeri et al., 2014; Yang et al., 2017). However, a direct comparison of P uptake and efficiency between alfalfa and red clover is to our knowledge yet not available. Intraspecific variation of P acquisition has also been studied within different legume species like white clover, common bean, chickpea and faba bean (Gahoonia et al., 2007; Ma et al., 2009; Pang et al., 2018; Rose et al., 2010; Yan et al., 2004). Genotypic studies of alfalfa and red clover have been mainly concentrated on forage and seed yield, nutritive value and tolerance to stress (Hawkins & Yu, 2018; Li & Brummer, 2012; Vleugels et al., 2019), whereas P uptake and utilisation between alfalfa and red clover genotypes under different P supply is scarce.

To investigate the inter- and intraspecific P efficiency of alfalfa and red clover as affected by different P recycling products, we conducted a two-year consecutive field trial and two pot experiments with eight accessions each of alfalfa and red clover, aiming to 1) evaluate the P efficiency of different accessions of alfalfa and red clover, 2) explain crop induced changes of soil characteristics, and 3) assess the ability of alfalfa and red clover to utilise P from different P sources.

3 Material and methods

3.1 Accession selection

Table 1. Geographic origin, sample status (SAMPSTAT) and plant P content of selected accessions of alfalfa and red clover. Origin: Country codes according to ISO 3166; SAMPSTAT: 100 = wild; 200 = weedy; 300 = traditional cultivar/landrace; 400 = Breeding/research material; 500 = Advanced or improved cultivar; 999 = Other. Accessions with asterisks were further selected for soil sampling in different depths.

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	200	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

In 2019, 149 alfalfa and 120 red clover accessions were cultivated for the accession selection in a preliminary experiment, which was performed based on multiple parameters including geographic origin of the plant material, sample status, plant P concentration (Table 1). Eight accessions each of alfalfa and red clover were chosen as representatives for subsequent field trial and pot experiment. More information on the accessions studied is available at the homepage of *National Inventory on Plant Genetic Resources in Germany* (https://pgrdeu.genres.de/ex-situ-bestaende/).

3.2 Field trial

A consecutive field trial was conducted from 2020 to 2021 at the experimental station of the University of Rostock, based on a long-term field experiment established in 1998. The experimental site is located in a maritime-influenced region in northeast Germany (54°3’41.47’’N; 12°5’5.59’’E). The soil at the study site is classified as Stagnic Cambisol according to the World Reference Base for Soil Resources (IUSS Working Group WRB, 2015), and the soil texture is loamy sand. The field trial was arranged in a randomised split-plot design with four blocks. Six treatments were selected for this study: a treatment without P supply (no P), with TSP, biomass ashes (ash, incinerated plant residues), cattle manure (manure), bio-waste compost (compost, sanitised compost based on green garden and landscape waste residues) and a combination of compost and TSP (compost+TSP). Seeds of each accession were sown in late April 2020. After seeding, rhizobia were applied for inoculation. Plant biomass was harvested twice a year at flowering stage, followed by determination of the biomass production, N and P concentration and uptake. After the final harvest in 2020, plants remained in the field over winter for the next consecutive cultivation year.

Soil samples at depth from 0 to 30 cm were taken for all accessions each year after the second harvest to determine double lactate extractable P (Pdl), as an indicator of plant available P in soil. Three accessions each of alfalfa and red clover were determined for enzyme activities in 0 to 20 cm soil depth in October of each year. Two accessions of each species were measured for mineral N content (Nmin) and Pdl in 0 to 30, 30 to 60 and 60 to 90 cm in October 2020 and 2021.

3.3 Pot experiment

Two rounds of pot experiment were conducted at the greenhouse of the University of Rostock in spring 2021 and 2022 with a randomised block design. The soils used were collected from a fallow field of the experimental station of the University of Rostock and classified as Stagnic Cambisol with loamy sand texture. After the removal of stone and plant residuals, 6 kg of fresh soil was filled into Mitscherlich pot. In both experiments the same four fertiliser treatments were applied, including TSP, sewage sludge ash (SSA), compost (same one as in field trial) and struvite. In addition, a control treatment without P amendment (no P) was carried out. Nutrient solution was added to the soil after fertiliser amendment to avoid nutrient deficiency during the plant growth (0.26 g N, 1 g K, 0.15 g Mg and 0.2 g S per pot)

Before the sowing, 1 cm layer of potting soil was applied on the top of the soil layer to ensure a successful germination of the seeds. In each pot experiment, seeds of five accessions each of alfalfa and red clover were sown at the end of January. After germination, 25 plants were kept in each pot for cultivation for 90 days at a day/night air temperatures of 22/18 °C. Two accession of each species were cultivated in both years to verify the results. Four replicates were prepared for all treatments. Plant biomass was harvested twice during the flowing stage, i.e., 51 and 90 days after the seeding in each pot experiment, followed by determination of the biomass production, N and P concentration and uptake. After the second harvest, soil samples were taken, air-dried, sieved (2 mm) and determined for the Pdl content. Two accessions each of alfalfa and red clover were chosen as candidates for soil P fractionation measurements.

4 Results

4.1 Field trial

Crop biomass varied primarily based on the applied fertilisers and cultivation years, with relatively small differences observed between alfalfa and red clover. The biomass for alfalfa were measured in the range of 4.7 to 5.0 Mg ha^{-1} in 2020 and 11.6 to 12.9 Mg ha^{-1} in 2021. Red clover exhibited a more pronounced intraspecific diversity, with biomass ranging from 4.1 to 5.4 Mg ha^{-1} in 2020 and 9.1 to 11.9 Mg ha^{-1} in 2021. The application of fertilisers significantly increased the average biomass of both species compared to the no P treatment. The combined treatment compost+TSP did not yield higher crop biomass compared to single compost application.

Regarding plant P concentration, red clover exhibited slightly higher average values than alfalfa in 2020, but decreased to similar levels in 2021. The combined application of compost+TSP resulted in higher P concentrations compared to the no P treatment.

Plant P uptake was strongly correlated to the crop biomass (Pearson's correlation coefficient r = 0.908 in 2020 and 0.827 in 2021, $p < 0.001$) and alfalfa showed a lower average P uptake compared to red clover in 2020, but a higher one in 2021 (Figure 1). As described for biomass, the P uptake of clover accessions showed more intraspecific differences than alfalfa in 2020 and 2021. Similar to biomass, the P uptake of both crops followed the order compost+TSP ≥ compost > manure > TSP ≥ ash > no P in 2020, but the difference was less pronounced between treatments in 2021.

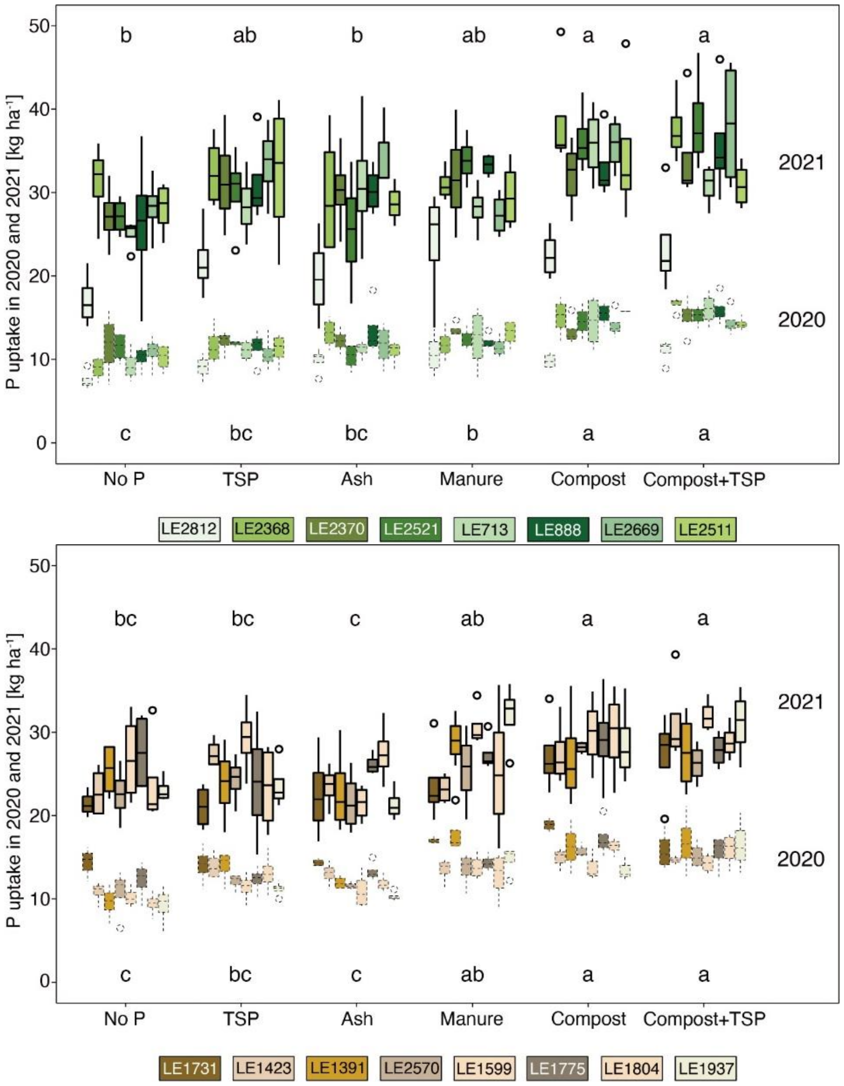

Figure 1. Plant phosphorus (P) uptake of selected accessions of alfalfa and red clover of the six studied treatments (no P, TSP, ash, manure, compost and compost+TSP) in 2020 and 2021. Alfalfa and red clover accessions are illustrated in different shades of green and brown colours, respectively. 2020 and 2021 are differentiated with dotted and solid line, respectively. Letters indicate significant difference between treatments (on average of accessions) within same plant species (Duncan's new multiple range test with $p < 0.05$). Mean ± standard deviation (n = 4)

Plant N concentration and uptake were only measured in 2021. Alfalfa showed slightly higher N concentrations compared to red clover (27.6 > 26.8 g kg^{-1}), and fertilisation did not significantly affect N concentrations in either species. Plant N uptake also correlated closely with biomass ($r = 0.836$, $p < 0.001$), with alfalfa exhibiting higher N uptake than red clover (330 vs 277 kg ha^{-1}). Intraspecific differences were only found between alfalfa accessions.

Negative correlation was found between plant P uptake and Pdl with correlation factors about $r = -0.3$. Despite the higher P uptake in red clover, soil cultivated with alfalfa showed lower average soil Pdl contents (41.4 mg kg^{-1}) than that with red clover (48.1 mg kg^{-1}) in 2020 ($p < 0.05$) (Figure 2). In 2021, soil Pdl contents were generally lower than in 2020 with only small interspecific differences. The soil Pdl contents were not affected by the individual accessions within a crop species, but strongly depended on applied fertilisers and followed the same order in both cultivation years: compost+TSP > compost > manure > ash > TSP > no P.

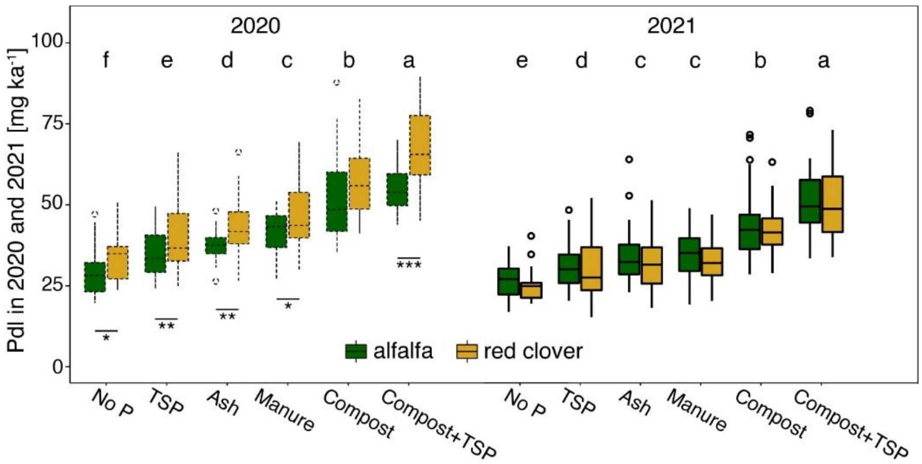

Figure 2 Double lactate extractable phosphorus (Pdl) in topsoil (0 to 30 cm) of the six studied treatments with cultivation of alfalfa (green) and red clover (brown) in 2020 (framed with dotted lines) and 2021 (framed with solid lines). Letters indicate significant difference between treatments (on average of plant species) within same year (Duncan's new multiple range test with $p < 0.05$). Whiskers and asterisks indicate significant interspecific difference within respective treatments. Mean ± standard deviation (n = 32)

Soil cultivated with alfalfa had higher average Nmin contents in soil in 2020 and 2021 (18.2 and 16.4 kg ha^{-1}, respectively) than red clover (16.2 and 13.9 kg ha^{-1}, respectively). Intraspecific differences were not detected. Fertiliser application increased Nmin content, with the largest effect observed for compost and manure.

Enzyme activities in the soil were affected by crop species and fertiliser treatments. The activities of acid phosphatase (acP) were higher in soils cultivated with red clover compared to alfalfa. Fertiliser treatments influenced the activities of dehydrogenase (DH) and alkaline phosphatase (alP), with higher activities after the application of compost and manure. However, no clear pattern was identified in the interactions between crop species and treatments.

The Pdl and Nmin in lower soils depths were measured for selected plots, considering only two accessions of each species The average Pdl content of both years decreased with increasing soil depths. Neither interspecific nor intraspecific differences in the subsoil were detected regarding the Pdl content. However, the application of compost and manure increased Pdl levels in the 30 to 60 cm depth range. In the 60 to 90 cm depth range, fertiliser treatments did not affect Pdl content. The crops had a clear impact on Nmin in the deeper soil layers. Clearly higher Nmin contents were found in the red clover plots than in the alfalfa plots in the soil depth of 60 to 90 cm.

4.2 Pot experiment

The plant biomass differed mainly in dependence on cultivation years and plant species. In comparison to 2021, plant biomass increased in the second pot experiment in 2022 and the difference between alfalfa and red clover was more pronounced in 2022 (14.1 vs. 16.9 g pot^{-1} in 2021 and 18.9 vs. 23.7 g pot^{-1} in 2022). Within both plant species, differences were measured between alfalfa and red clover accessions. However, intraspecific diversity was more noticeable in red clover, where the accession LE1391 had higher biomass in both pot experiment. In comparison to no P treatment, all P amendments increased the biomass of alfalfa to similar levels in 2021, but only TSP and struvite showed positive effects in 2022. For red clover, only struvite was found to increase the biomass compared to no P treatment in 2022, whereas the application of SSA and TSP decreased the biomass production of red clover.

The plant P concentration varied mostly in dependence on applied P fertilisers. In 2021 alfalfa was measured with higher average P concentration than red clover (2.73 vs. 2.51 g kg^{-1}), but decreased to similar values in 2022 (2.44 vs 2.41 g kg^{-1}). Within plant species, intraspecific differences were only detected in alfalfa and red clover accessions in 2021. In comparison to no P treatment, applied P fertilisers increased the plant P concentration of both alfalfa and red clover, where higher P concentrations were measured in TSP and struvite treatment.

Plant P uptake was highly correlated to the plant biomass ($r = 0.705$ in 2021 and 0.815 in 2022, $p < 0.05$), and alfalfa showed a lower average P uptake than red clover in both 2021 and 2022 (Figure 3). Similar to biomass, P uptake of red clover accessions showed more pronounced intraspecific differences than alfalfa, with accessions LE1391 exhibiting higher P uptake in both 2021 and 2022. The applied fertiliser increased the plant P uptake of alfalfa and showed same pattern in both years: struvite = TSP > compost > SSA > no P. Similar higher P uptakes with struvite and TSP application were also measured in red clover in 2022.

The Pdl content followed a similar pattern in both years, with the exception that soils with compost application had higher Pdl in 2022 in comparison to 2021 (Figure 4). No interspecific or intraspecific differences were detected in any pot experiment. The application of compost and struvite increased soil Pdl content more effectively than TSP, compared to no P treatment.

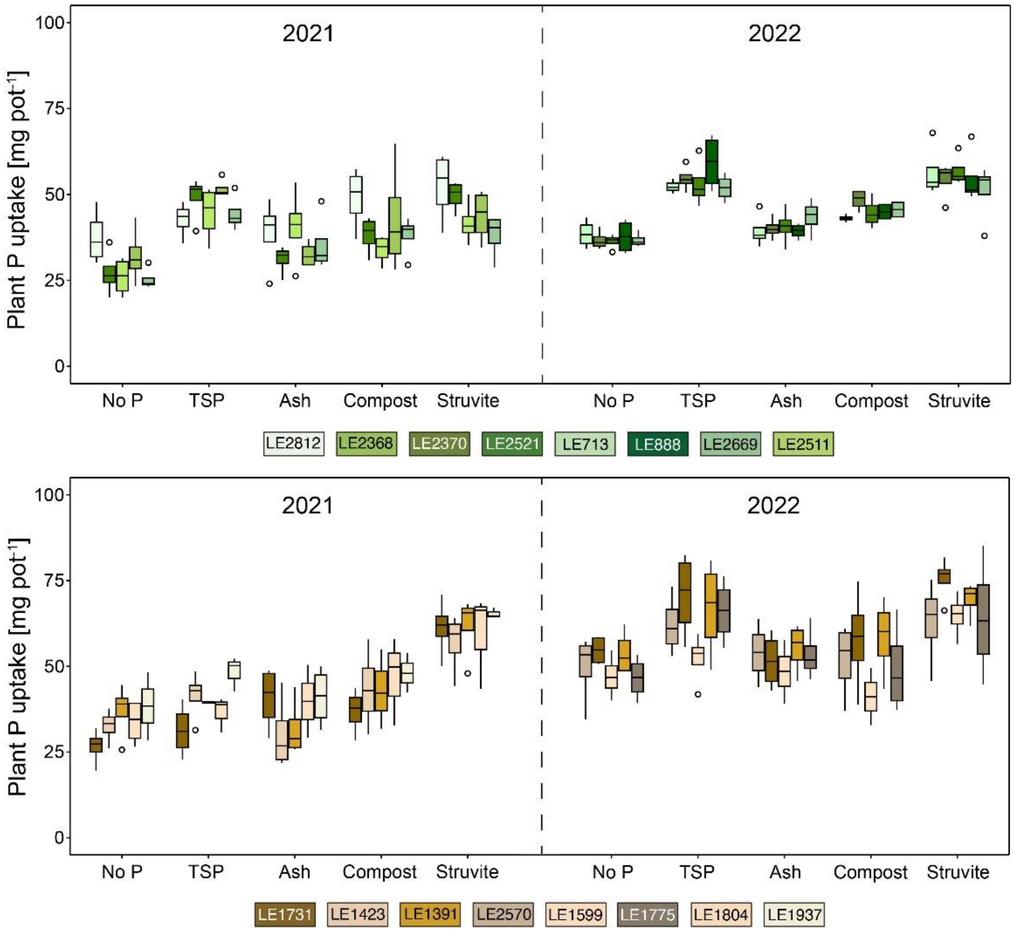

Figure 3 Plant phosphorus (P) uptake of selected accessions of alfalfa and red clover of the five studied treatments (no P, TSP, SSA, compost and struvite) in pot experiment 2021 and 2022. Alfalfa and red clover accessions are illustrated in different shades of green and brown colors, respectively. Letters indicate significant difference between treatments (on average of accessions) within same plant species (Duncan's new multiple range test with $p < 0.05$). Mean ± standard deviation (n = 4)

The soil P fractions were determined for soils cultivated with selected alfalfa and red clover candidates in the first pot experiment (see Section 3.3). Significant interspecific differences were measured in P-H_2O with no P treatment and P-NaOH with TSP or compost treatment. No intraspecific differences were measured between alfalfa and red clover accessions. In comparison to no P treatment, all applied fertilisers increased P-H_2O, with struvite showing highest efficacy. The application of TSP, compost and struvite raised P-$NaHCO_3$ to a similar level. The P-NaOH fraction was not affected by fertiliser, and P-H_2SO_4 was increased by he application of SSA and compost.

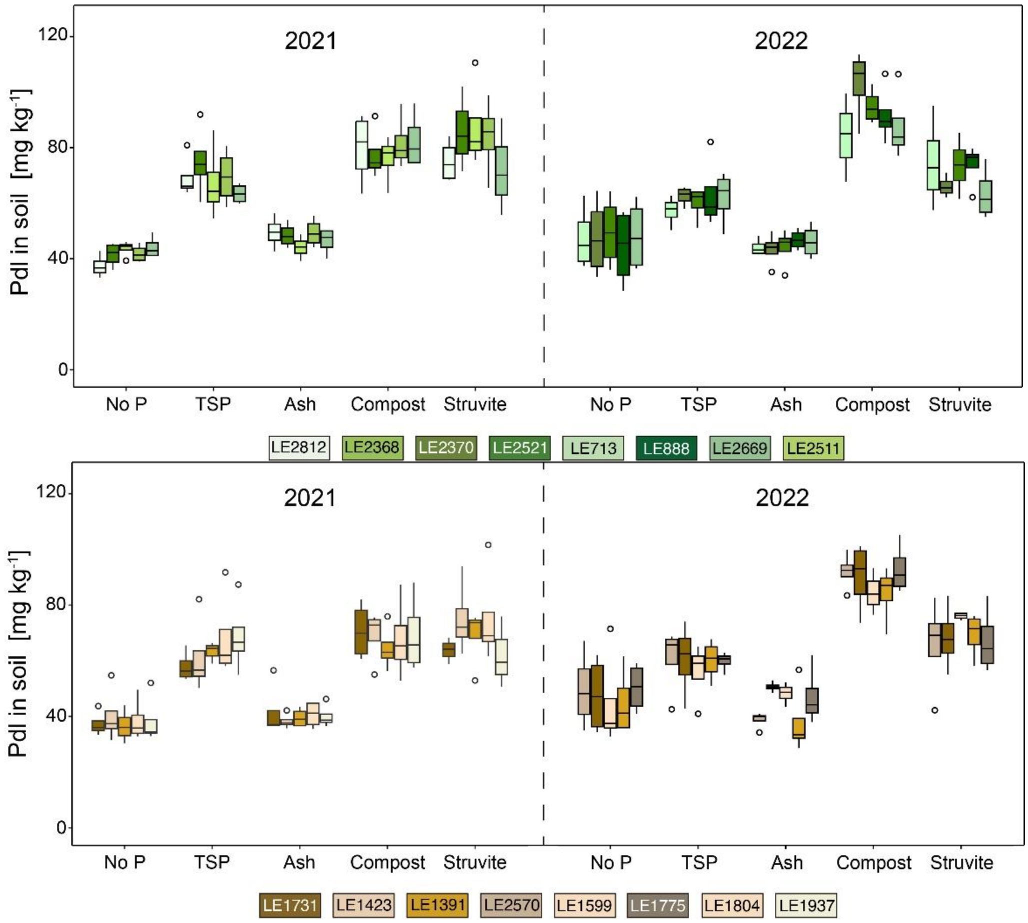

Figure 4 Double lactate extractable phosphorus (Pdl) in soils with selected accessions of alfalfa and red clover of the five studied treatments (no P, TSP, SSA, compost and struvite) in pot experiment 2021 and 2022. Alfalfa and red clover accessions are illustrated in different shades of green and brown colors, respectively. Letters indicate significant difference between treatments (on average of accessions) within same plant species (Duncan's new multiple range test with $p < 0.05$). Mean ± standard deviation (n = 4)

5 Discussion

Biomass production and nutrient uptake varied between alfalfa and red clover in the field, but the differences were relatively small and influenced by cultivation year and environmental conditions. Both species showed an increase in biomass in the second cultivation year in the field, which can be attributed to a more developed root system and sufficient precipitation in that year. Under more controlled condition, an increase of plant biomass and nutrient uptake was also observed in the second pot experiment, which could have been caused by the larger temperature differences between day and night. Compared to the field trial, interspecific biomass differences were more pronounced.

The P concentration in the plant tissues varied only slightly between the field trial and the pot experiment, the nutrient uptake was mainly dependent on biomass production. The accessions showed no pronounced differences in nutrient concentration in the field trail, although they varied widely regarding origin, sample status, and maturity group. This is in contrast to differing P concentrations measured in the preparatory experiment on two other field sites for the selection of accessions (see Section 2.1) and underlines the importance of site effects and management regarding the P accumulation in plants. Comparably, alfalfa and red clover accessions in the first pot experiment significantly differed in P concentration. More limited substrate volume in the pot might have led to a more severe P deficiency, making intraspecific P efficiency more detectable. Intraspecific differences regarding P uptake were measured between alfalfa and red clover accessions, but with varying patterns between field trial and pot experiment. Alfalfa accession LE2812 showed significantly lower P uptake than the other accessions in field trial, but a higher one in the pot experiment. Accession LE2812 is a traditional cultivar from Yemen, which was selected due to its high P concentration in biomass. Nevertheless, the environmental conditions in northeast Germany probably limited its plant growth, whereas the conditions in the greenhouse might favour its growth and biomass production and consequently increased the P uptake. Intraspecific differences among red clover accessions partly occurred, but without a consistent pattern between cultivation years in the field, which indicates the suitability for annual or perennial cultivation.

Crop effects on soil characteristics were found for Pdl in the topsoil (0 to 30 cm) and Nmin in subsoils (30 to 60 and 60 to 90 cm) in the field. Red clover resulted in higher soil Pdl contents in 2020 than alfalfa, while both crops had similar P uptakes, suggesting active P mobilisation processes by red clover. However, this mobilisation could not by explained by phosphatases activities. Alfalfa cultivation resulted in lower Nmin contents in deeper soil layers compared to red clover, possibly due to differences in rooting patterns and N utilisation from deeper soil layers. Crop effects on Pdl were measured in the first pot experiment, with red clover showing lower soil Pdl than alfalfa, which can partially by explained by the higher P uptake by red clover.

The applied organic fertiliser usually increased plant biomass and P uptake more effectively than inorganic ones compared to no P treatment in the field trial, suggesting positive impacts on soil quality like soil organic matter, storage of water and biological activity. However, the same effect was not observed in the pot experiment, since this positive impact is likely attributed to long-term processes. Nmin content in the field soil was not significantly affected by the organic fertilisers, indicating that the supplied N was mainly incorporated into the soil organic matter. Organic fertiliser application stimulated microbial activity in the field, as reflected by increased enzyme activities in the soil. P recycling products SSA, compost and struvite showed the ability to increase plant biomass, P concentration and P uptake compared to the no P treatment. However, when comparing with TSP, the most commonly used chemical fertiliser, only struvite exhibited similar or higher efficacy. All P amendments increased the soil Pdl content, with compost and struvite resulting in higher Pdl content than SSA. This is consistent with the results in P fractionations where compost and struvite demonstrated a superior capability to raise the labile P than SSA. The high amount of Fe-P and Al-P in SSA can explain the relatively low plant available P and a higher stable P fraction in the soil compared with the other treatments.

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Attachment

Posters and presentations:

- Poster at International P-Campus Symposium 2019, Rostock, Germany
- Presentation at International P-Campus Symposium 2020, Rostock, Germany
- Poster at International P-Campus Symposium 2021, Rostock, Germany
- Poster at 4th Phosphorus in Europe Research Meeting 2021, virtual meeting
- Presentation at 34th Meeting of the EUCARPIA Fodder Crops and Amenity Grasses Section 2021, Freising, Germany
- Poster at 63. Tagung der Gesellschaft für Pflanzenbauwissenschaften e. V. 2021, Rostock, Germany
- Poster at International P-Campus Symposium 2022, Rostock, Germany
- Poster at European Society for Agronomy XVII. Congress 2022, Potsdam, Germany
- Presentation at 18th European Workshop on Phosphorus Chemistry 2022, Rostock, Germany
- Poster at BonaRes Conference 2023, Berlin, Germany

Public relations:

- IPK Institutstag 2022, Gatersleben, Germany
- "Lange Nacht der Wissenschaft" 2023, Rostock, Germany

Publications:

- Hu, Y., Jarosch, K. A., Kavka, M., & Eichler-Löbermann, B. (2022). Fate of P from organic and inorganic fertilizers assessed by complementary approaches. Nutrient Cycling in Agroecosystems, 124(2), 189–209. https://doi.org/10.1007/s10705-022-10237-x
- Hu, Y., Dehmer, K. J., Willner, E., Bachmann-Pfabe, S., & Eichler-Löbermann, B. (2021). Phosphorus utilisation capacity of forage legumes from recycling products. Exploiting Genetic Diversity of Forages to Fulfil Their Economic and Environmental Roles: Proceedings of the 2021 Meeting of the Fodder Crops and Amenity Grasses Section of EUCARPIA, 37–40. https://doi.org/10.5507/vup.21.24459677.09
- Hu, Y., Dehmer, K. J., Willner, E., & Eichler-Löbermann, B. (2023). Specific and Intraspecific P Efficiency of Small-Grain Legumes as Affected by Long-Term P Management. Agronomy, 13(3), 900. https://doi.org/10.3390/agronomy13030900