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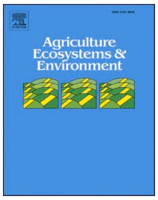

Can earthworms and root traits improve plant struvite-P uptake? A field mesocosm study

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ABSTRACT

The availability of conventional linear mineral phosphorus (P) fertilization will become lower as phosphate rock stocks are limited and strongly concentrated in a few locations. Therefore, we need to increase agronomic P use efficiency and find alternative, recycled, sources of P. Two possible solutions mentioned in the literature are (i) using struvite, a mineral circular P fertilizer; and (ii) making use of earthworm activity, which has been shown to increase P availability. Here, we study the interaction between these two approaches, with the hypothesis that earthworms could increase the P availability from the poorly soluble struvite. We set up a field-based mesocosm experiment in a sandy soil with a low agronomic P status with 13 different treatments combining three earthworms species (*Lumbricus rubellus* Hoffmeister, *Aporrectodea caliginosa* Savigny and *A. longa* Ude alone or in a threes species mixture), different P fertilizers (no P, Triple Super Phosphate (TSP) and struvite). The experiment lasted 13 months (five fertilisation-harvest cycles). We found that, in field conditions, the yield and P uptake of *Lolium perenne* did not differ between fertilization with struvite or TSP. Earthworms only played a minor role in explaining ryegrass P uptake compared to fertilisation. We did not see either positive nor negative interactions between earthworms and struvite, meaning that earthworms did not further increase the P availability from struvite. The equal performances of struvite and TSP are explained by an enhanced effort from plants to actively take up P through a modification of root traits. This includes increased arbuscular mycorrhizal fungi colonisation and the production of finer and longer roots. Our results show that struvite performs comparably to TSP under realistic field conditions, making it a viable alternative to phosphate rock-based fertilizers.

1. Introduction

Plant phosphorus (P) limitation is a major issue in many agro-ecosystems. Although a substantial amount of P can be present in soils, the large majority of it is bound to metal-(hydr)oxides and clay mineral edges or included in primary minerals and soil organic matter, making it unavailable for uptake by plants (Hesterberg, 2010). Most soils need P fertilisation to reach an optimum crop production, but current P fertilisation practices are not sustainable in the long term. Indeed, most mineral P fertiliser is extracted from phosphate rocks, which are a finite resource predicted to run out in the coming centuries (Koppelaar and Weikard, 2013). Moreover, phosphate rocks are concentrated in a limited number of countries, as more than 80 % of the global phosphate rock stock is located in only four countries (U.S. Geological Survey, 2022). While there is a strong monopoly and dependency on phosphate rock-based fertiliser, P use in agriculture needs to become both more efficient and more circular (Nesme et al., 2018; Schneider et al., 2019).

Struvite ($NH_4MgPO_4 \bullet 6\,H_2O$) is one of the key products for a more circular P fertilisation and can be recycled from wastewater. This mineral is one of the most promising circular P fertilisers to date with several industrial recovery units already running worldwide (Nageshwari and Balasubramanian, 2022). Struvite has a low solubility in water (Bhuiyan et al., 2007), which allows for a reduced environmental impact of the fertiliser, but also makes plant P uptake more challenging than with conventional P fertilisers. The literature mentions environmental benefits including less nitrogen (N) and P leaching (Ahmed et al., 2018), lower nitrous oxide emissions (Yang et al., 2023), and less P becoming directly available due to adsorption to reactive soil particles (Degryse et al., 2017). The agronomic effectiveness of struvite compared to conventional P fertilisers is still debated (Ahmed et al., 2018), partly because of the unrealistic experimental designs used in studies (*e.g.*, short experimental times, high plant density, confounding factor of soil

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P tests or high struvite application rates) (Hertzberger et al., 2020). To date, greenhouse experiments still make up the majority of struvite fertilisation trials; this may contribute to the perceived poorer agronomic performance of struvite as compared to conventional P fertilisers (Hertzberger et al., 2020; Huygens and Saveyn, 2018). The performance of struvite as compared to conventional, linear P fertilisers in field conditions still needs to be investigated.

Although in agricultural systems plants rely largely on fertiliser for P supply, biotic factors can also play an important role in P uptake in agricultural grasslands. Plants have strategies themselves to increase their P uptake, for instance through increased root exploration or symbiosis with arbuscular mycorrhizal fungi (AMF) (Richardson et al., 2009). There is a trade-off between the various strategies, meaning that plant species will usually favour one strategy over another (Honvault et al., 2021; Wen et al., 2019), although some plants like *Lolium perenne* seem to equally use multiple strategies to alleviate soil P deficient growth conditions (Ros et al., 2018; Turner and Newman, 1984). The trade-off between nutrient uptake strategies can be best illustrated by the “Root Economics Space”, a concept that describes the root system according to two gradients (Bergmann et al., 2020). The first is a conservation gradient that distinguishes fast-growing plants with a high root N concentration from slow ones with a high Root Tissue Density (RTD). The second gradient relates to collaboration with “Do-it-yourself” plants exploring the soil for nutrients by themselves, having a high Specific Root Length (SRL) as opposed to plants that rely more on AMF to acquire nutrients and thus having a larger root diameter (Bergmann et al., 2020). AMF are linked to increased plant P uptake in managed grasslands (Oelmann et al., 2021) and the collaboration between plants and AMF is highest under intermediate levels of P fertilisation (Liu et al., 2016). Arbuscular mycorrhizal fungi increased the plant P uptake under struvite fertilisation in a greenhouse study with tomato plants, likely indirectly by increasing the benefit of soil exploration in a situation where the soil solution P is low. Struvite may have produced a constant flow of available P in the soil solution through its slow dissolution of struvite, as opposed to the peaks of readily available P provided by conventional and highly soluble fertilisers (Di Tomassi et al., 2021). Although a few studies have explored the effect of struvite fertilisation on the plant root system (Di Tomassi et al., 2021; Omidire et al., 2023; Valle et al., 2022), to our knowledge none have placed their results within the frame of the P uptake strategies belowground.

Soil biota play an important role in the soil P cycle (Alori et al., 2017; Le Bayon and Milleret, 2009) and may have an impact on plant P uptake from struvite. Struvite fertilisation led to a more diverse and active microbial community compared to Mono-Ammonium Phosphate (MAP) and Simple Superphosphate (SSP) in a lettuce pot experiment (Mancho, 2023). Another relevant group of soil biota that can affect P availability is earthworms. Earthworm presence can increase the soil P availability through mineralisation of organic P source and desorption of mineral P source in relation to their effect on the soil pH and reactive surface area (Le Bayon and Milleret, 2009; Vos et al., 2022a), but this is highly context and species-dependent. Earthworms are commonly categorized into three distinct groups reflecting their feeding guilds: epigeic species, eating and leaving in the litter at the soil surface; endogeic, geophagic species living buried in the soil; and anecic species, leaving in deep permanent galleries but feeding on the surface litter (Bouché, 1977; Örley, 1885). While casts from earthworms belonging to the three feeding guilds were richer in readily available P than the bulk soil (Vos et al., 2019), under field conditions only two species (*Lumbricus terrestris* Linnaeus and *Aporretodea longa* Ude) have been proven to improve grass P uptake (Vos et al., 2022b). Beyond mobilising soil P, earthworms are able to enhance the availability of P from mineral fertilisers with a low solubility such as phosphate rock (Atmaca, 2021; Mackay et al., 1983). Their composted casts (EcoTea, Canada) have been shown to increase the effectiveness of struvite to fertilise oat in controlled conditions (Hernández Jiménez et al., 2020). The chemical impact of their casts is one way that earthworm could impact dissolution. Another way could be linked to the bioturbation of the soil they inhabit which could move struvite potentially closer to plant’s root as shown with other less soluble fertilisers (Mackay et al., 1983). Although soil biota seem to improve the fertilising performances of struvite in controlled conditions, to our knowledge the link between struvite and biota has not been studied under field conditions before.

This study has the objective to understand what biotic factors influence struvite effectiveness as a fertiliser. We hypothesised that: (1) in the field conditions, struvite is as effective as Triple Superphosphate (TSP) to fertilise plants; (2) earthworms enhance struvite dissolution and thus its effectiveness; and (3) struvite fertilisation stimulates plants to develop different P acquisition strategies, thus affecting their root traits. We tested these hypotheses with ryegrass in a 13-month field-mesocosm experiment using a sandy soil with a low agronomic P status and three different earthworm species.

2. Materials and methods

2.1. Soil

The soil was collected from the topsoil of a field in Achterberg, the Netherlands (51°59′32.28” N, 5°35′1.37” E) that received no phosphorus fertilisation for a period of 25 years. Over the last few years, this field has been conventionally managed with respect to fertilisation and crop rotation but still remains P-deficient. Prior to excavation, it was used to grow potatoes. Details on the soil characteristics are given in Table 1. Briefly, it was a noncalcareous sandy soil with a pH of 5.8 The agronomic soil P status was classified as “low” in the Dutch P fertiliser recommendation system (Commissie Bemesting Grasland en Voedergewassen, 2017), which is based on P-CaCl$_2$ as an intensity soil P test method and P-AL as a quantity method (Reijneveld et al., 2022). For P-CaCl$_2$, soil was extracted with 0.01 M CaCl$_2$ to determine the size of the readily available soil P pool (Houba et al., 2000), whereas soil was extracted with ammonium lactate (P-AL) (Egnér et al., 1960) to determine the sum of readily available P plus labile soil P (van Doorn et al., 2023). The P-CaCl$_2$ of the soil used here was 0.2 mg·kg^{-1} and P-AL was 116 mg·kg^{-1}. The amount of reversibly adsorbed P (P-ox) as determined by soil extraction with 0.2 acid ammonium oxalate (Schwertmann, 1964) was rather low with 268 mg·kg^{-1}. The summed amount of Fe- and Al-(hydr)oxides ([Fe+Al]-ox), which are simultaneously extracted with P-ox by acid ammonium oxalate, was 69.3 mmol kg^{-1}. The P loading α, which was calculated as the molar ratio of P-ox versus [Fe+Al]-ox (van der Zee and van Riemsdijk, 1988), was 0.12. This P loading α can be considered as low when compared to a large set of representative Dutch agricultural topsoils (Koopmans et al., 2006). The soil texture was measured using the pipet method (Houba et al., 1997). Soil organic matter was measured through loss on ignition at a temperature of 500 °C (Hoogsteen et al., 2015). Ammonium, nitrate, dissolved organic

Table 1
Characteristics of the soil used here. DON: Dissolved organic nitrogen.

Property	Extractant	Unit	Value
Sand	-	%	87.7
Clay	-	%	1.6
Silt	-	%	6
Soil organic matter	-	%	3.8
pH	Calcium chloride	-	5.8
N-NH$_4$	Calcium chloride	mg·kg^{-1}	2.0
N-(NO$_3$-NO$_2$)	Calcium chloride	mg·kg^{-1}	3.9
N-DON	Calcium chloride	mg·kg^{-1}	5.1
P-PO$_4$	Calcium chloride	mg·kg^{-1}	0.2
Mg	Calcium chloride	mg·kg^{-1}	88.9
P-AL	Ammonium lactate	mg·kg^{-1}	116
P-ox	Ammonium oxalate	mg·kg^{-1}	268
Fe-ox	Ammonium oxalate	mg·kg^{-1}	1553
Al-ox	Ammonium oxalate	mg·kg^{-1}	656
P loading-α	-	-	0.12

nitrogen and magnesium in the soil solution were extracted with a 0.01 M CaCl2 solution and measured with a SFA (Houba et al., 2000). After excavation, the soil was gamma-irradiated (9 kGy, Steris, Ede, the Netherlands) to kill all earthworms and their cocoons (Nakamori et al., 2009; Zhang et al., 2016). To facilitate the recolonisation of soil by native microorganisms, the irradiated soil was inoculated with 3 % non-irradiated soil sieved over 1 mm to ensure the absence of earthworms and cocoons (Davis, 1975; Edwards and Bohlen, 1996).

2.2. Experimental design

We followed a full randomised block design (five replicates) with 13 treatments (Table 2). Eight treatments included earthworms, with either a single earthworm species (*Lumbricus rubellus* Hoffmeister, epigeic "*Lrub*"*; Aporrectodea caliginosa* Savigny, endogeic "*Acal*"; or *A. longa*, anecic, "*Alon*"*)* or a combination of these three species ("Multi"), fertilised with struvite ("Stru") or left without P fertilisation and always fertilised with N in the form of Calcium Ammonium Nitrate (CAN). In addition, we had five earthworm-free treatments: one without any N or P fertiliser ("control"), one with conventional N and P fertilisation ("TSP+CAN"), a treatment with only TSP to induce a N limitation ("Nlim"), a similar treatment with only CAN to induce P limitation ("Pli") and a treatment of struvite supplemented with CAN ("Struvite"). We used granular struvite ($NH_4MgPO_4 \bullet 6\ H_2O$) harvested from the wastewater of an experimental plant transforming soy into a low-P feed for cattle (Nuresys, Deerlijk, Belgium). The chemical composition of struvite is presented in Table S1. The struvite fertilisation was supplemented with calcium ammonium nitrate (CAN) and potassium sulphate to ensure adequate N and K availability. The alternative P fertiliser for the TSP+CANand N-lim treatments was TSP. All fertilisers were applied in a granular form, broadcasted at the soil surface. The earthworms were introduced at the rate of 15, 46 and 5 individuals per mesocosm for *L. rubellus*, *A caliginosa* and *A. longa* respectively, corresponding to the density reported per feeding guild (Frazão et al., 2017). The three species-mixture used the same earthworm density for each of the species as those as in the respective single species treatments, as these three species would inhabit different ecological niches. The earthworms were collected from a grassland in Wageningen as well as from the "earthworm hotel" (Van Groenigen, 2022) and stored in a climate-controlled room (16 °C) in buckets containing a moist sandy soil similar to the one used in the field-based mesocosm experiment, which was mixed with fresh litter. Preference was given to adult individuals but sub-adults were also used to reach the desired density. Earthworms were weighed, with voided gut (filter paper method for 48 h at 16 °C (Dalby et al., 1996)), per species and mesocosm before introduction.

The mesocosm design is shown in Fig. 1. It was composed of a 35x35x35cm PET felt bag (PLANT!T Dirt Pots, Coventry, UK) sown to a 30 cm high insect mesh. To prevent earthworms from leaving the mesocosms, a 2 cm-wide Velcro band was sown at the top, on the inside of the insect net. Each mesocosm was filled with 40 kg of the earthworm-free soil mix described above. The mesocosms were placed in the field in June 2020. The soil surrounding the mesocosms was replaced by coarse sand to reduce the possibility of earthworms invading mesocosms. A drip irrigation system was established, with the drip set at the soil surface to prevent the acceleration of the fertiliser granules dissolving due to a splash effect from the irrigation. The irrigation system was used during the dryer months of the summer to balance the draining and drying effect of the surrounding coarse sand. In July 2020, seeds of *Lolium perenne* diploid (8 $g \cdot m^{-2}$) were sown and the grass cover was left to grow for eight weeks, after which the grass was cut 5 cm aboveground and the treatments were applied. Earthworms were introduced two days prior to the fertiliser application to minimise the risk of salt stress. The fertilisation was 150 % of the national recommendation for N, P and K (Commissie Bemesting Grasland en Voedergewassen, 2017), and was split (heterogeneously so for N) per harvest cycle over the two years. Details about the fertilisation rate are given in Table S2.

The grass was harvested five times after application of the fertilisers and earthworms: in November 2020 as well as in May, July, September and October 2021. After the last harvest, the mesocosms were excavated. A soil sample of 11 cm diameter and 25 cm depth was taken in the middle of each mesocosm and washed over a 1 mm-sieve to separate the roots from the bulk of the soil. The root sample and the soil remaining in the mesocosm were hand-sorted to recover any earthworms, including mesocosms that were not inoculated with earthworms. Their guts were voided (Dalby et al., 1996) and the weight per species was subsequently measured. Each individual was also assigned to an age class (juvenile, sub-adult, or adult) depending on its morphological characteristics.

2.3. Plant analysis

The harvested plant material from the five harvests was dried at 70 °C for 48 h. It was subsequently ground and digested using a mixture of H_2SO_4, salicylic acid, and H_2O_2 with Se (Novozamsky et al., 1983). In the digest, total N and total P were measured using a fully automated segmented flow analyzer (SFA; Skalar, SAN^{++}). Root samples of the first three blocks were carefully washed removing all soil and organic matter particles. A first subsample was weighted fresh and stained with neutral red and scanned (600 dpi, Epson V700 Photo, Suwa, Japan). The resulting images were batch analysed with Winrhizo Pro 2013e (Regent Instrument, Québec, Canada, objects smaller than 0.5 mm^2 or with a length:width ratio of less than 4 removed). The analysis was conducted on 10 diameter classes of 0.1 mm to separate finer roots from larger ones. The subsample was then dried at 70 °C for 48 h, followed by the determination of the dry weight. A second subsample was weighed fresh and stored in 60 % ethanol for subsequent AMF colonisation rate determination. The roots were stained with the ink and vinegar method (Vierheilig et al., 1998) and the level of colonisation of 100 observations across the root sample was scored from 0 to 5. The resulting colonisation

Table 2
List of treatments of the field-based mesocosm experiment.

Treatment	*L. rubellus* (indiv·mesocosm^{-1})	*A. caliginosa* (indiv·mesocosm^{-1})	*A. longa* (indiv·mesocosm^{-1})	P fertiliser	N fertiliser	K fertiliser
Control	0	0	0	-	-	-
TSP+CAN	0	0	0	TSP	CAN	K_2SO_4
Nlim	0	0	0	TSP	-	K_2SO_4
Plim	0	0	0	-	CAN	K_2SO_4
Lrub	15	0	0	-	CAN	K_2SO_4
Acal	0	46	0	-	CAN	K_2SO_4
Alon	0	0	5	-	CAN	K_2SO_4
Multi	15	46	5	-	CAN	K_2SO_4
Stru*Lrub*	15	0	0	struvite	CAN	K_2SO_4
Stru*Acal*	0	46	0	struvite	CAN	K_2SO_4
Stru*Alon*	0	0	5	struvite	CAN	K_2SO_4
StruMulti	15	46	5	struvite	CAN	K_2SO_4
Struvite	0	0	0	struvite	CAN	K_2SO_4

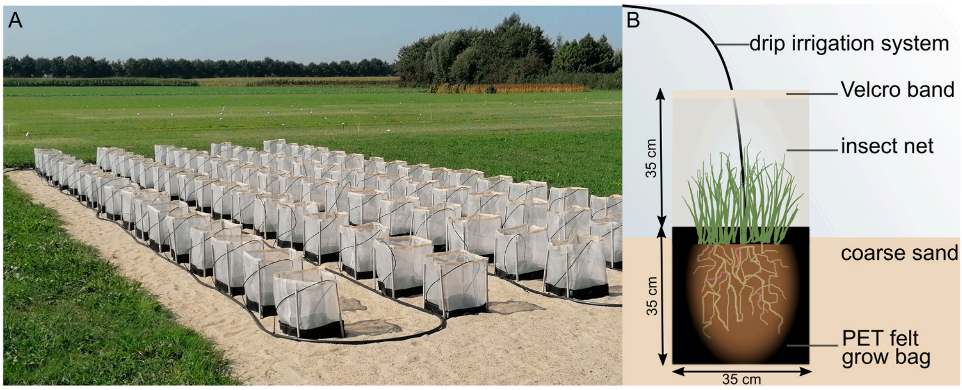


Fig. 1. Visualisation of the field-based mesocosm experiment. A: Photograph of the experiment in May 2021. B: Schematic cross-section of a mesocosm buried in the sand bed.

intensity measurement is the weighted average of the scores according to the following formula: where *ni* represent the number of observation having received the *i* score (Trouvelot et al., 1986). The dry weight of this sample was estimated using the moisture content of the first subsample. The remaining bulk of the root sample was dried at 70 °C for 48 h and weighed. The calculations of SRL and RTD were performed considering the cylinder bias (Rose, 2017).

Although a classical approach to study P fertilisation would call for soil P test, these are difficult to perform with soil that potentially still contains struvite. Indeed, the struvite remaining in the soil then partially dissolves during the extraction of many soil P test (Gu et al., 2021; Yang et al., 2023). Hence, we decided not to perform any soil P tests in this study, as the best proxy for struvite dissolution is ultimately the plant P uptake.

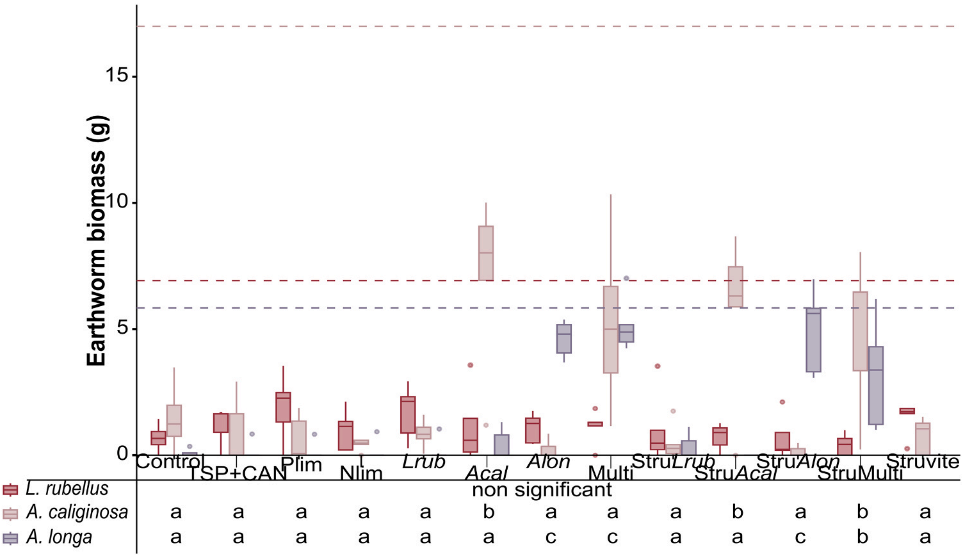
2.4. *Statistical analysis*

The statistical analyses were conducted in R (R Core Team, 2022). Earthworm species biomass per treatment was tested with an ANOVA and Tukey's Least Significant Difference post-hoc test when normality assumption were respected (*L. rubellus*), or with the Kruskal-Wallis test followed by Dunn's post-hoc test with the Benjamini-Hochberg procedure when the assumption of normality was violated (*A. caliginosa*, *A. longa*). Since earthworm communities differed at the end of the experiment as compared to the communities we set at the beginning, we used a modelling approach to analyse the data. The total P uptake was modelled according to the earthworm biomass and density per species, as well as the N and P fertilisers with the *MuMIn* package (Bartoń, 2023). We ranked all the possible multiple linear regression models according to their Akaike Information Criteria (AIC). Any model with an AIC value within two units of the best AIC value is considered as good as the best model. We thus selected all models with an AIC value within two units from the best AIC value and averaged them into one model, as model averaging is considered the best practice (Lukacs et al., 2010). All models selected for the averaging were given the same weight. There was no block effect and so this was not further used in the analyses.

3. Results

3.1. *Earthworm biomass and density*

The earthworm biomass per species at the end of the field-based mesocosm experiment is shown in Fig. 2. In the treatments they were introduced in, the biomass of earthworm species were 20, 30 and 80% of the original biomass at the end of the experiment for *L. rubellus*, *A. caliginosa* and *A. longa,* respectively. The density of earthworms



	Control	TSP+CAN	Plim	Nlim	*Lrub*	*Acal*	*Alon*	Multi	Stru*Lrub*	Stru*Acal*	Stru*Alon*	StruMulti	Struvite
L. rubellus	non significant												
A. caliginosa	a	a	a	a	a	b	a	a	a	b	a	b	a
A. longa	a	a	a	a	a	a	c	c	a	a	c	b	a

Fig. 2. Earthworm biomass per treatment for the three species at the end of the field-based mesocosm experiment. The table shows the results of the ANOVA and Tukey test. Dashed lines correspond to the original average biomass of earthworms in treatments where they were applied. lim: limitation, *Lrub*: *L. rubellus*, *Acal*: *A. caliginosa*, *Alon*: *A. longa*, Multi: combination of the three earthworm species, Stru: struvite.

followed the same pattern with 30, 50 and 90% of the original density remaining at the end of the experiment respectively for *A. caliginosa L. rubellus* and *A. longa* (Figure S1). *A. longa* and *A. caliginosa* were mostly found back in the treatment were they were introduced. However, at the end of the experiment, *L. rubellus* biomass was distributed equally in all mesocosms, regardless whether it was initially introduced or not (Fig. 2).

3.2. Plant phosphorus uptake

The plant P uptake over the course of the five harvests is shown in Fig. 3. Struvite supplemented with CAN resulted in the same plant P uptake as the positive control combining TSP and CAN, reaching 5.6 $g{\cdot}m^{-2}$ on average, which was significantly higher than the P-limited treatment (Fig. 3A). The N-limitation control had the same P uptake as the negative control, which was 30 % of the uptake of the struvite and TSP+CAN treatments. The N:P ratio according to the fertilisation regime confirm that these two treatments were the most N-limited (Figure S5). To assess the potential impact of earthworms on grass P uptake, we used an average model approach (see Section 2.4). The average model presented in Fig. 3B is the result of the weighted average according to the AIC value of a list of the best models. Our results highlight that fertilisers, but not earthworms, significantly contribute to the final model. Nonetheless, earthworm parameters were selected in individual models included in the average model and thus appear in that model. Thus, although none of the earthworm parameters significantly contributed to P uptake as modelled through the average model, their presence in it indicates that they do have an effect, albeit small and unsignificant in comparison to fertilisers. There was no significant interaction between earthworm species and fertilisation treatments. Similar results were found for the aboveground grass biomass (Figure S3).

3.3. AMF colonization and root morphology

The results of the AMF colonisation analysis are presented in Fig. 4. Plants with adequate N and P fertilisation, *i.e.* the struvite and TSP+CAN treatments, were less colonised by AMF compared to plants that were limited by either of both elements, although this was not statistically significant. Still, the struvite treatment colonisation was significantly much higher (7.7 fold) than that of the TSP+CAN treatment, while having a similar P uptake. There was a significant negative correlation between AMF colonisation and the P uptake (Spearman rank correlation, rho= −0.367).

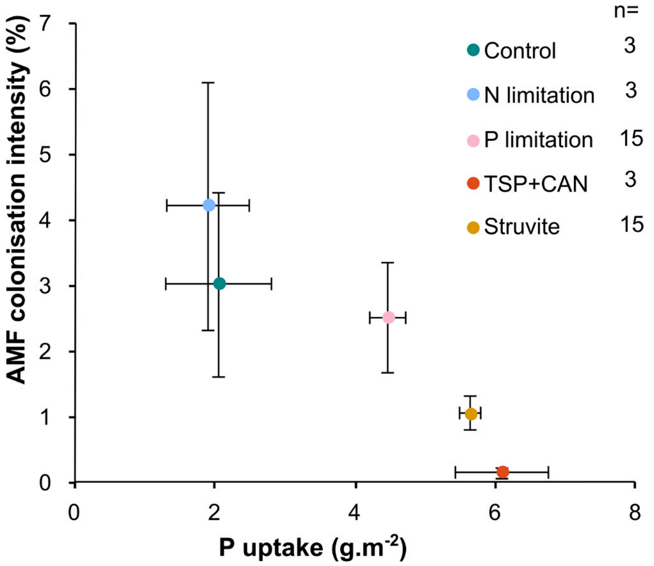


Fig. 4. AMF colonisation intensity according to the total P uptake. Colours indicate the different fertilisation regimes. P limitation contains the Plim, Lrub, Acal, Alon, Multi treatments. Struvite contains the StruLrub, StruAcal, StruAlon, AtruMulti and Struvite treatments. TSP: Triple superphosphate, CAN: Calcium Ammonium Nitrate.

The “Root Economics Space” is a theoretical space drawn by the first two dimensions of a PCA build with, vertically, the RTD opposing the root N content and horizontally the SRL opposing the average root diameter of multiple plant species (Bergmann et al., 2020). Our dataset did not include the root N content so we used a variation of the RES built only with the SRL, RTD and average diameter to differentiate between ryegrass rooting strategies (Fig. 5). Using these traits, we could identify the conservation and collaboration gradients, which together explain 98 % of the variability of the three root traits included. Fig. 5A shows the position of the various fertilisation treatments in the root economics space. While the control and N limitation treatments were in the

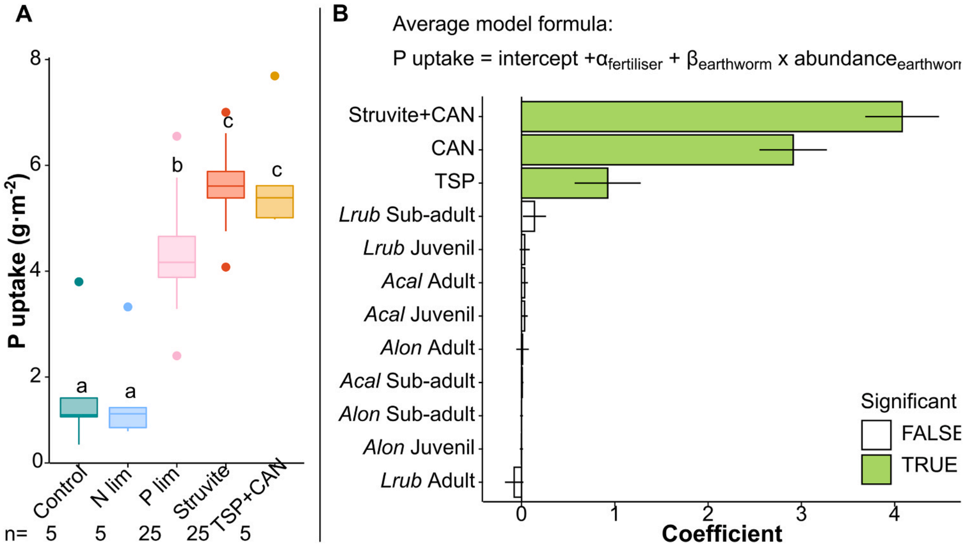


Fig. 3. Phosphorus uptake of grass as impacted by fertilisation and earthworms density per age class. (a) Phosphorus uptake according to fertilisation regimes. (b) Coefficients of parameters included in the average model of P uptake. Significance (TRUE or FALSE) refers to the t-test testing if coefficient are significantly different from 0. For further details, see section2.4. TSP: Triple superphosphate, CAN: Calcium Ammonium Nitrate, lim: limitation, *Lrub*: *L. rubellus*, *Acal*: *A. caliginosa*, *Alon*: *A. longa.* P lim contains the Plim, Lrub, Acal, Alon, Multi treatments. Struvite contains the StruLrub, StruAcal, StruAlon, AtruMulti and Struvite treatments.

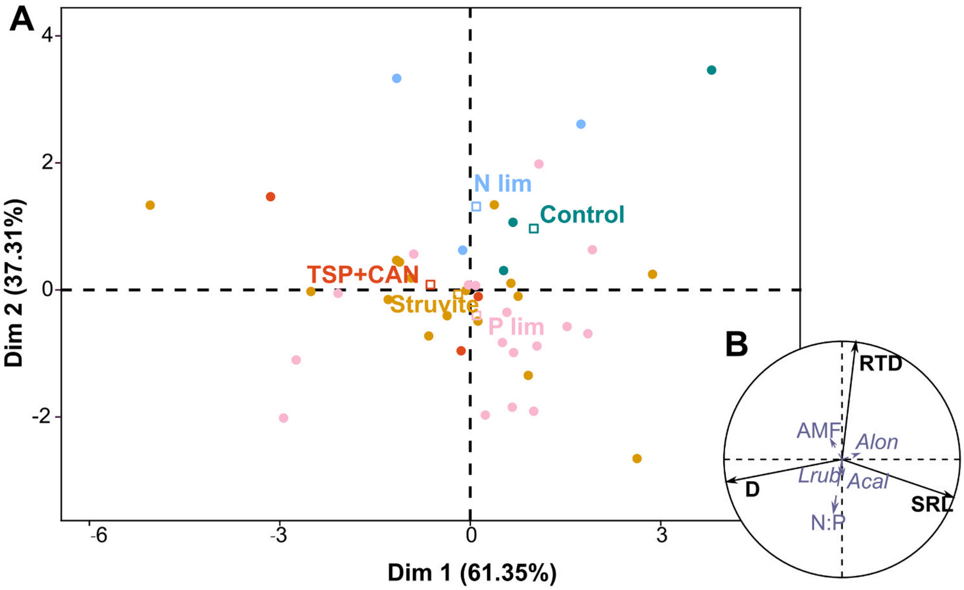


Fig. 5. Fertilisation treatments on the root economics space. (A) Individual graph of the PCA according to their fertilisation regime. (B) Variable graph of the PCA. Active variables are in black and supplementary variables are in grey. Traditionally, the root economics space is defined by four poles: along the collaboration gradient (here horizontal), the "do-it-yourself" pole (here SRL) opposes the "outsourcing" pole (here D) while along the conservation gradient (here vertical), the "slow" pole (here RTD) opposes the "fast"pole (here N:P). TSP: Triple superphosphate, CAN: Calcium Ammonium Nitrate, D: root diameter, RTD: Root Tissue Density, SRL: Specific Root Length, lim: limitation, *Lrub*: *L. rubellus*, *Acal*: *A. caliginosa*, *Alon*: *A. longa*. P lim contains the Plim, Lrub, Acal, Alon, Multi treatments. Struvite contains the StruLrub, StruAcal, StruAlon, AtruMulti and Struvite treatments.

"slower" part of the space, the other treatments that did receive N fertilisation were close to the middle of the conservation gradient, with the TSP+CAN, struvite and P limitation treatments ranging from slower to faster, respectively. The treatments range from TSP+CAN, struvite, N and P limitations and control along the collaboration gradient from "outsourcing" to "do-it-yourself", respectively. Fig. 5B shows the correlation of active and passive variables with the two PCA axes. The N:P ratio in shoots was correlated with the conservation gradient towards the "fast" pole. Earthworm species biomasses' correlations to either PCA axis were negligible.

4. Discussion

4.1. Struvite is just as effective a fertiliser as TSP

We hypothesised that struvite would be just as effective a fertiliser as TSP in field conditions. This was indeed the case as there was no significant difference in P uptake between the two treatments (Fig. 3). Field experiments with struvite, compared to greenhouse pot experiments, tend to yield better results than with a conventional P counterpart regarding aboveground biomass but poorer regarding P uptake (Hertzberger et al., 2020; Huygens and Saveyn, 2018). This could be related to the slower plant growth in the field versus in the greenhouse. Although struvite dissolution is impacted by temperature, the parameter limiting its dissolution might be the surface of contact between struvite and the soil (Ariyanto et al., 2017; Bhuiyan et al., 2007; Degryse et al., 2017). Plants on the contrary, are heavily impacted by temperature and thus the environmental conditions from the greenhouse or the field will have a large impact on how fast they grow and how fast fertilizer need to be able to provide nutrients to avoid limitation (Sato and Ito, 1969).

Moreover, irrespectively of their growing conditions, grass trials tend to show a higher agronomic efficiency of struvite for biomass production, and a significantly higher P uptake than when using a conventional phosphate rock-based fertiliser (Huygens and Saveyn, 2018). Although *L. perenne* is very sensitive to P fertilisation compared to other grasses (Ros et al., 2018), it is less dependent on the timing of the P supply in the form of P fertilisation as opposed to other crops such as maize for which grains are the main production target and that require a high P uptake early on to reach optimum grain yield (Bindraban et al., 2020; van Duijnen et al., 2021). This explains why grasses usually reach a similar yield and a higher phosphorus use efficiency when fertilised with "precipitated phosphate salts" *sensu* Huygens and Saveyn, (2018) as compared to conventional fertilisers (Huygens and Saveyn, 2018). Our results thus demonstrate that slow release of P from struvite appears to be an effective fertilisation strategy for grass biomass production as it led to the same P uptake as TSP that has a high solubility. To understand the similar P uptake from these two fertilisers with different dissolution rate, we must investigate belowground.

4.2. Earthworms only played a minor role in explaining plant performances

A critical point to assess any earthworm effect is the retrieval of earthworms from the mesocosms where they were inoculated (Vidal et al., 2023). In our mesocosm field experiment, the earthworm treatments we set up were not maintained as expected during the experiment (Fig. 2). Earthworms were introduced in October, which is rather late in the season and may be quite close to their period of reduced activity for the winter (Edwards, 2004). Where *A. caliginosa* and *A. longa* seemed to have been fit enough to survive the winter, this might not have been the case for *L. rubellus*. As an epigeic species, *L. rubellus* has an ecological selection strategy of type r (Pianka, 1970), meaning the survival of the species relies on the production of a large number of individuals with short life spans. This could explain the decrease of the earthworm biomass in mesocosms where *L. rubellus* was introduced (i.e. high mortality). Because of the significant change in the distribution of *L. rubellus*, it is not appropriate to look at results of our experiment through the lens of earthworm treatments as they are. Instead, we used the parameters describing the earthworm community at the end of the experiment to investigate possible earthworm effect on the system.

We hypothesised that earthworms would enhance the dissolution and thus the effectiveness of struvite., In our field-based mesocosm experiment earthworms did not appear to increase struvite dissolution, although earthworms have been shown to indirectly increase the dissolution of phosphate rock pellets (Mackay et al., 1983) and the compost of their casts increases the effectiveness of struvite in greenhouse conditions (Hernández Jiménez et al., 2020) Toxicity tests have shown that *Eisenia fetida* does not avoid nor seek struvite (Rastetter et al., 2017) and unpublished observations in our group seem to confirm this for the three earthworm species used in this experiment. Mackay et al. (1983) described the interaction of earthworms with phosphate rocks via two pathways: the incorporation of pellets into the soil through bioturbation and the increased dissolution through ingestion. Although these two pathways significantly improved the availability of phosphate rocks in their pot study, the earthworm density per kg of dry soil of their incubation trial was 20 times higher. It is possible that at such an unrealistic density, earthworms might have an effect on struvite dissolution (Vidal et al., 2023), but they did not at field density, which we used in our experiment.

In our field-based experiment the earthworm effect is small and not significantly contributing to the final average model as opposed to fertilisers (Fig. 3). Yet, they were selected in the average model, meaning that they are important to explain plant P uptake. The lack of significance might be due to several factors. First, the co-linearity among earthworm variables: this was due to the nature of the variables included in the model (age classes) and to the presence of multiple earthworm species in several of the mesocosms. This co-occurrence was expected from the treatments where the three species were initially introduced, but was exacerbated by the invasion of *L. rubellus* and to some extent of *A. caliginosa* in many mesocosms. Reducing the number of earthworm variables would have reduced the co-linearity but also reduced the fit of the model, so we chose to keep the earthworm data detailed per species and life-stages in the model. Another factor influencing the lack of significance might have been the duration of the experiment. In a field mesocosm experiment, Vos et al. (2022) did find a significant effect of *Lumbricus terrestris* and *A. longa* on the P uptake of *L. perenne* over a longer experimental period (three years and fifteen harvests). Increasing the length of the experiment might have revealed a significant earthworm effect, but it would also have increased the risk of further homogenisation of the earthworm communities by invasion and reproduction in mesocosms that were initially earthworm-free. For future field experiment investigating the effect of earthworms on the agroecosystem, scientists may want to base their experimental design on Bayesian statistics instead of frequentists statistics. Doing so would mean increasing from the start the variability of earthworm communities instead of fruitlessly trying to keep fixed earthworm communities in field conditions.

To sum up, we reject our hypothesis: earthworms did not enhance struvite effectiveness in our field-based experiment. However, they did have a small (albeit not significant *stricto sensu*) effect on the plant P uptake.

4.3. Struvite's effectiveness is related to a shift to a P-limited plant growth strategy

We hypothesised that struvite fertilisation stimulates plants to develop different P acquisition strategies, thus affecting their root traits. Plants of the P-limitation treatment engaged more in collaboration with AMF (Fig. 4) and had thinner, longer and lower-density roots (Fig. 5) compared to roots of the TSP+CAN treatment. Although the P applied in the form of struvite and TSP+CAN were the same and resulted in equally similar P uptake (Fig. 3), the plants treated with struvite had a significantly higher AMF colonisation, which was about half of that of P-limited plants but 7.7 times higher than that of the TSP+CAN treatment (Fig. 4). Morphological root traits were also intermediate between those of the TSP+CAN treatment and the P-limitation treatment (Fig. 5, S4). The explanation for this likely resides in the properties of struvite. As opposed to TSP which has a high solubility thereby facilitating a fast released of P to the soil solution, struvite is poorly soluble leading to a lower rate of P release, which means that plants may also have to rely on other P sources such as the release of residual P residing within the soil solid phase to fully meet their P needs (Bogdan et al., 2021; Degryse et al., 2017). The soil used in our experiment had little readily available P for plants to take up (Table 1) as well as a relatively high P buffering capacity, which probably resulted in a limiting amount of P available at time locally in the soil solution so strategies to increase both the soil exploration and the unlocking of phosphorus from the solid phase had to be used (Wen et al., 2022). Here we were able to demonstrate that struvite-treated plants relied more heavily on an enhanced soil exploration through mycorrhizal association as well as increased root length and so we accept our hypothesis. Plants may also have used some nutrient mining strategies *sensu* Wen et al. (2022) although we did not measure it. Overall, struvite P fertilisation, by being released at a sub-optimal rate for the plants, promoted an increased reliance on plant-led P uptake whereas TSP+CAN did not promote the same self-reliance. Struvite, in addition to being a more sustainable fertiliser (Remy and Ruhland, 2006), leads to a more resilient grass crop against P shortages.

We used the “Root Economics Space” concept to visualise the root traits of *L. perenne* under different fertilisation regimes. This tool served the visualisation purpose well and although we could not include the root N content, the “fast” pole of the conservation gradient was still defined as intended since the aboveground plant N:P ratio was negatively correlated with the conservation gradient (Fig. 5B). Omitting the root N content, however, has reduced the variability of the data included in the PCA and has likely resulted in the very high variability explained through the root economics space: 98 % instead of 73.4 % for the root economics space of global species (Bergmann et al., 2020). The variability of the data will have been further reduced by the inclusion of only one plant species (albeit under different fertilisation regimes) when the root economics space designed by Bergmann et al. (2020) contained 748 plant species. Yet, the positions of the various fertilisation treatments on this gradient were still compelling here as they clearly ranged from fully readily available nutrients on the left to a strong nutrient limitation on the right. With the need to increase nutrient use efficiency in agrosystems, having a tool helping to visualise the root response to a given fertiliser is valuable, as plants relying more on their own nutrient acquisition strategies while still agronomically performing to the best standards is a step towards more sustainable agroecosystems.

5. Conclusions

Our field-based mesocosm experiment showed that struvite was just as good a P fertiliser as a conventional P fertiliser. Furthermore, struvite, by being intrinsically a slow-release fertiliser, triggered plant P acquisition strategies and thus resulted in more AMF colonisation and morphological root changes that are reflective of moderate P limitation without having an impact on either the yield or the P uptake of *L. perenne*. Although earthworms have been shown to impact plant P uptake in controlled conditions, their role was only minor in explaining grass P uptake in our field conditions in comparison to the major role of P fertilisers.

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CRediT authorship contribution statement

Laura Ferron: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jan Willem Van Groenigen:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Gerwin Koopmans:** Writing – review & editing, Supervision, Methodology. **Alix Vidal:** Writing – review & editing, Supervision, Project administration, Methodology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.agee.2024.109255.

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