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Legacy effect of green manure crops fertilized with calcium phosphite on maize production and soil properties

Mario Fontana [a], Thomas Guillaume [a], Luca Bragazza [a], Saïd Elfouki [a], Mathieu Santonja [b], Alexandre Buttler [c,d], Renato Gerdol [e], Lisa Brancaleoni [e], Sokrat Sinaj [a,*]

[a] Agroscope, Field-Crop Systems and Plant Nutrition, Research Division Plant Production Systems, Route de Duillier 50, P.O. Box 1012, CH-1260, Nyon, Switzerland
[b] Aix Marseille Univ, Avignon Université, CNRS, IRD, IMBE, Marseille, France
[c] Ecole Polytechnique Fédérale de Lausanne (EPFL), School of Architecture, Civil and Environmental Engineering, Laboratory of Ecological Systems (ECOS), Station 2, CH-1015, Lausanne, Switzerland
[d] Swiss Federal Institute for Forest, Snow and Landscape Research (WSL), Site Lausanne, Station 2, CH-1015, Lausanne, Switzerland
[e] University of Ferrara, Department of Life Science and Biotechnology, Corso Ercole I d'Este 32, I-44121, Ferrara, Italy

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ABSTRACT

Recycling phosphorus (P) is crucial to meet future P demand for crop production. We investigated the possibility to use calcium phosphite (*Ca-Phi*) waste, an industrial by-product, as P fertilizer following the oxidation of phosphite (Phi) to phosphate (Pi) during green manure (GM) cropping in order to target P nutrition of subsequent maize crop. In a greenhouse experiment, four GM crops were fertilized (38 kg P ha^{-1}) with *Ca-Phi*, triple super phosphate (*TSP*) or without P (*Control*) in sandy and clay soils. The harvested GM biomass (containing Phi after *Ca-Phi* fertilization) was incorporated into the soil before maize sowing. Incorporation of GM residues containing Phi slowed down organic carbon mineralization in clay soil and mass loss of GM residues in sandy soil. Microbial enzymatic activities were affected by *Ca-Phi* and *TSP* fertilization at the end of maize crop whereas microbial biomass was similarly influenced by *TSP* and *Ca-Phi* in both soils. Compared to *Control*, *Ca-Phi* and *TSP* increased similarly the available P (up to 5 mg P kg^{-1}) in sandy soil, whereas in clay soil available P increased only with *Ca-Phi* (up to 6 mg P kg^{-1}), indicating that Phi oxidation occurred during GM crops. Accordingly, no Phi was found in maize biomass. However, P fertilization did not enhance aboveground maize productivity and P export, likely because soil available P was not limiting. Overall, our results indicate that *Ca-Phi* might be used as P source for a subsequent crop since Phi undergoes oxidation during the preliminary GM growth.

1. Introduction

Phosphorus (P) is an essential crop nutrient (Marschner, 2012) for optimum production. At global scale P consumption increased from 4.8 Tg P yr^{-1} in 1961 up to 18 Tg P yr^{-1} in 2018 and could exceed 22 Tg P yr^{-1} in 2050 (Dhillon et al., 2017; Helin and Weikard, 2019; Mogollón et al., 2018). Although the estimation of global reserves of rock phosphates is controversial (Geissler et al., 2018; Gilbert, 2009), a more sustainable use of this finite resource must be considered (Chowdhury et al., 2017; Leinweber et al., 2018). In this respect, P recycling is critical to meet P demand and to minimize the environmental impact of P fertilizers (Moeller et al., 2018). In Switzerland, the total amount of P needs in agriculture (*i.e.* ≈ 4200 t yr^{-1} in 2015) represents half of the amount of P waste (Mayer et al., 2019), the later including 300 P t yr^{-1} in the form of calcium phosphite (*Ca-Phi*).

The potential to recycle phosphite (Phi) as fertilizer is challenging because plants cannot utilize Phi directly as P source (Danova-Alt et al., 2008). In soils with low available P content, Phi additions can alter plant metabolism and, in turn, can lead to negative effect on biomass production (Avila et al., 2011; Ratjen and Gerendás, 2009; Ticconi et al., 2001). Phi oxidation represents the only way to provide a valuable P source for crops (Gómez-Merino and Trejo-Téllez, 2015) and occurs in

Abbreviations: Ca_{exch}, Exchangeable calcium; C_{mic}, Microbial carbon; C_{Org}, Organic carbon; C_{Org} Min, Mineralization of organic carbon; *Ca-Phi*, Calcium phosphite; Fe_{exch}, Exchangeable iron; GM, Green manure; N_{mic}, Microbial nitrogen; N_{Tot}, Total nitrogen; Phi, Phosphite; Pi, Phosphate; P_{mic}, Microbial phosphorus; P_{NaHCO3}, Available Phosphorus; P_{Tot}, Total phosphorus; *TSP*, Triple super phosphate.

* Corresponding author.
E-mail address: sokrat.sinaj@agroscope.admin.ch (S. Sinaj).

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soils through microbial enzymes such as Phi-dehydrogenase, alkaline phosphatase, and C–P lyase (Costas et al., 2001; Poehlein et al., 2013; Yang and Metcalf, 2004). Several decades ago, a greenhouse experiment showed that Phi can be oxidized during a crop growth to provide phosphate (Pi) for the subsequent crop (Adams and Conrad, 1953; MacIntire et al., 1950). However, this option was overlooked probably due to the detrimental effects observed on the first crop that received Phi. To avoid negative effects, Phi fertilization could be applied during green manure (GM) cultivation prior to the planting of the cash crop (Kamh et al, 1999, 2002). GM can promote P mobilization (from fertilizer or soil) while Phi could fill the gap between available P and the P requirement of a following crop (Damon et al., 2014; Fageria, 2007; Hallama et al., 2019). This practice could be acceptable provided no residual Phi is detected in the biomass of the subsequent cash crop. Because Phi is accumulated in crop biomass and can be preferentially absorbed compared to Pi, like in the maize crop (Avila et al., 2011; Schroetter et al., 2006), a complete oxidation of Phi before the cash crop cultivation is necessary. To our knowledge, the time needed for Phi oxidation has never been quantified for different soil types. In addition, the microbial activity should be assessed after Phi fertilization given that Phi is also used as fungicide (Thao and Yamakawa, 2009).

In a previous study involving GM crops fertilized with *Ca-Phi*, we detected various Phi concentrations in aboveground GM biomass depending on GM species and soil type (Fontana et al., 2021). In addition, we showed that *Ca-Phi* fertilization increased available Pi at the end of GM crop due to Phi oxidation depending upon soil type. Building from our previous experimental set-up, here we specifically investigated the response of the maize crop following the incorporation of GM biomass previously fertilized with *Ca-Phi*. In particular, we measured the decomposition rate of GM residues, soil microbial biomass and enzymatic activities, soil chemistry, maize aboveground productivity and concentration of P and Phi in maize biomass. The main hypotheses of this study were: (i) the Phi concentration in GM residues affects the decomposition rate of GM residues, soil microbial biomass and enzymatic activities depending upon GM species, (ii) *Ca-Phi* fertilization affects soil available P (P_{NaHCO3}) similarly as triple super phosphate *(TSP)*, (iii) Phi concentration, contrary from GM residues, is not detected in maize aboveground biomass and (iv) *Ca-Phi* affects maize productivity in similar way to *TSP*.

2. Materials and methods

2.1. Experimental design

Four species of GM crops, followed by maize, were cultivated in a greenhouse pot experiment at Agroscope-Changins (Nyon, Switzerland). The four species used as GM crops, namely *Avena strigosa* (oat), *Brassica juncea* (mustard), *Lupinus albus* (lupine) and *Pisum sativum* (pea), were selected for their contrasted root traits and P uptake strategies (Fontana et al., 2021). Maize was selected as subsequent cash crop because it is a good indicator of the presence of Phi in the soil solution since it: (i) preferentially absorbs Phi compared to Pi, (ii) is sensitive to potential toxicity of Phi, and (iii) has a high P requirement (Avila et al., 2011; Schroetter et al., 2006).

Three fertilization treatments, namely *Ca-Phi*, *TSP* and *Control*, were applied before GM sowing. *Ca-Phi* and *TSP* fertilization rates were applied according to Swiss recommendation for maize, *i.e.* 38 kg P ha^{-1} (Sinaj et al., 2017). *TSP* granules were milled using a jaw crusher (Retsch BB50) to promote P release and dispersion within soil. As mentioned by Fontana et al. (2021), pots (9.3 L, diameter 27 cm, height 24.3 cm) have been prepared using clay and sandy soils with contrasting physico-chemical properties. The selected soils were representative of the majority of Swiss agriculture soils, which are not deficient in P. This should lower the potential toxic effect of Phi on crops as reported for P deficient soils (Avila et al., 2011; Barrett et al., 2004; Schroetter et al., 2006). At the beginning of the experiment, physico-chemical soil characteristics for sandy and clay soils were, respectively, pH: 5.8 and 7.8, clay content: 62 and 291 g kg^{-1}, sand content: 519 and 282 g kg^{-1}, organic C (C_{Org}): 16 and 19 g kg^{-1}, total N (N_{Tot}) 1.5 and 2.2 g kg^{-1}, available P (P_{NaHCO3}) 50.1 and 29.3 mg kg^{-1}, cations exchange capacity: 67.9 and 143.3 meq kg^{-1} (Fontana et al., 2021).

The aboveground biomass of GM and maize were harvested after 8 weeks *i.e.* during the flowering period. The fresh GM biomass was chopped in a bowl using pruning shears. Then, 20 g of chopped biomass was subsampled for water content determination and chemical analyses. The content of each pot (*i.e.* soil + GM root biomass) was thoroughly mixed with the rest of the chopped GM biomass before repotting. Three maize grains were then sown five days after repotting. Five days after sprouting, the two less vigorous plants were removed and left on the top of the soil. Two weeks after maize sowing, 30 kg N ha^{-1} (*i.e.* 50% NH_4/ 50% NO_3) were added to avoid nutritional stress. In addition, before maize sowing, 10 ± 0.1 g of fresh GM biomass was buried (2 cm depth) in a litterbag (mesh size = 0.45 mm). At the end of maize crop, the litterbag was recovered, oven dried (45 °C, 48 h) and weighted to determine the decomposition rate (%) of GM residues.

Daily temperature was kept between 18 °C and 25 °C to maintain optimal photosynthetic conditions for plant growth and the natural daylight was supplemented with high-pressure sodium lamps (400 W m^{-2}) from 6 a.m. to 8 p.m. when light intensity dropped below 250 W m^{-2}. Pots were watered manually to assure optimal soil moisture conditions (*i.e.* 75%–80% of the field capacity). In addition, pots were displaced every third week to prevent potential bias due to greenhouse heterogeneity.

The combination of four GM species, three fertilization treatments, two soil types, and four replicates led to a total of 96 pots. Furthermore, in order to isolate the residual effects of GM crop and GM biomass decomposition during maize growth, maize was also sown in three replicates of bare soils that were incubated during GM crop cultivation under the same greenhouse conditions and fertilization treatment, so adding 18 pots for both soils. These maize pots will be hereafter referred as "pre-incubated", whereas the pots with GM crop incorporated into the soil will be referred as "pre-cultivated".

2.2. Biomass nutrients and phi concentration measurements

The subsample of fresh GM biomass (20 g) and maize biomass were oven-dried (45 °C for 48 h) to estimate the water content before being ground using a Retsch rotor mill.

Dry ashes and total C were evaluated by calcination (480 °C for 5 h). Total N was measured after combustion using the Dumas method (Masson et al., 2010). Total P, K, Ca, Mg and Fe were determined by radial ICP-AES (Varian Vista RL Simultaneous or Varian 725 ES Simultaneous) after calcination (480 °C for 5 h) and solubilization in hydrofluoric acid (Masson et al., 2010).

Phi content in GM and maize aboveground biomass was analyzed according to the QuPPE European reference method (Anastassiades et al., 2015). Briefly, 0.5 g of dry aboveground biomass was added to 5 ml of methanol HPLC grade, acidified with formic acid (1% v/v) and subsequently vortexed for 30 s at two-time intervals within 5 min. After centrifugation at 4500 rpm for 5 min, the supernatant was injected onto a Thermo Hypercarb column (100 × 2.1 mm, granulometry = 3 μm). Then, the Phi concentration was measured using a LCMS-MS (Waters Acquity H-Class/TQ-S Micro) with a detection limit of 2 mg kg^{-1}.

2.3. Soil sampling and analysis

At the end of both GM and maize growth, four soil cores (2.5 cm diameter) were sampled along the entire depth of each pot, sieved (2 mm mesh size) and thoroughly mixed. Approximately 100 g of fresh soil was immediately stored in a cold chamber (4 °C) for enzymatic and microbial C, N and P analyses. In order to maintain similar soil storage conditions for each P fertilization treatment, each batch of analysis for

fumigation or enzymatic measurements (see description below) included one replicate of each P fertilization treatment. The remaining soil was air-dried for chemical analyses.

Soil organic carbon (C_{Org}) was determined after a sulfochromic oxidation (NF ISO 14235). The C_{Org} loss after maize cultivation relative to the initial carbon content, subsequently referred as mineralization of C_{Org} (C_{Org} Min), was calculated according to equation (1):

$$C_{Org}Min = \frac{(C_{Org\_Maize\_B} - C_{Org\_Maize\_E})}{C_{Org\_Maize\_B}} \quad (1)$$

where $C_{Org\_Maize\_B}$ and $C_{Org\_Maize\_E}$ are the concentrations of soil C_{Org} at the beginning and at the end of maize crop, respectively. Total soil N was measured using an elemental analyzer (Thermo, flash 2000) (NF ISO 13878). Available P (*i.e.* Pi) was estimated following a sodium bicarbonate (Na–HCO_3) extraction (NF ISO 11263) (P_{NaHCO3}) (Olsen, 1954). After ammonium acetate extraction, exchangeable K, Ca and Mg were measured using a Thermo Radial ICAP 6000 Series ICP-OES (Thermo Fisher Scientific, Freemont, CA, USA) (NFX 31–108).

Soil microbial C (C_{mic}), N (N_{mic}) and P (P_{mic}) measurements were performed according to chloroform fumigation method (Vance et al., 1987). Briefly, a K_2SO_4 extraction (0.5 M, 1:10) was performed to analyze total C and N of fumigated and non-fumigated soil samples using a TOC/TN auto analyzer (Shimadzu analyzer TOC-V CPH + TNM-1). Phosphorus from fumigated and non-fumigated samples was estimated using a (1:20) 0.5 M $NaHCO_3$ (pH 8.5) extraction according to Murphy & Riley (Murphy and Riley, 1962). The correction factors k_C (0.45), k_N (0.54) and k_P (0.40) were used to calculate C_{mic}, N_{mic} and P_{mic} values, respectively (Jenkinson et al., 2004).

The activity of β-glucosidase, chitinase, leucine-aminopeptidase and alkaline-phosphatase were measured as following: 1 g of fresh soil was shaken for 1 h with distilled water (ratio 1:10) and then 50 µl of methylumbelliferone or 7-amino-4-methylcoumarin was added in 200 µl of water extract (supernatant). The activities of β-glucosidase, chitinase and alkaline-phosphatase were measured on a microplate reader (FLUOstar Galaxy, BMG Labtechnologies, Offenburg, Germany) after 2 h incubation at 450 nm emission and 330 nm excitation wavelength. A similar protocol was applied to measure leucine-aminopeptidase activity except that the 50 µl of 4-methylumbelliferyl β-D-cellobioside as substrate was added to 200 µl of water extract and that the excitation wavelength was 365 nm.

2.4. Data analysis

Statistical analyses were performed using R 3.01 software (R Core Team, 2013). Normality condition was checked with shapiro. test function available in the *stats* package whenever required by the model used. If the condition was not fulfilled, then permutational tests were used. The entire dataset included three factors namely soil type, GM species (lupine, mustard, oat, pea and pre-incubated soil) used to assess GM crop effect and fertilization (*Ca-Phi*, *TSP* and *Control*). As this study focused on fertilization effect, statistical analyses were conducted with the aim to control or remove (depending on statistical test) the effects of the two other factors (*i.e.* soil type and GM species). First, adjusted R^2 and *p*-values of each factor were concomitantly quantified with each tested variable using the function rda (*vegan* package). Helmert contrasts were constructed for the effects of soil type, GM species and fertilization as explanatory variables (Legendre and Legendre, 2012). Then, statistical tests were conducted separately for each soil type since soil effect was generally significant on the tested variables. Therefore, the fertilization effect was tested on datasets including data from all GM species grown on the same soil type that consequently did not meet the condition of independence. In such case, GM species effect was previously tested (*i.e.* adjusted R^2 and *p* value) using the rda, RsquareAdj and anova functions. If species effect was not significant ($p > 0.05$), fertilization effect was tested by one-way ANOVA followed by post-hoc Tukey test with the function tukeyHSD (*stats* package) or pairwisePermutationTest (*rcompanion* package) if normality condition was not met. Otherwise, interaction between effects of species and fertilization was tested. For the not significant interaction scenario, the mixed models were performed with species effect as random factor and fertilization treatment as fixed factor using the lme function (*nlme* package). For the significant interaction scenario, the variable was analyzed separately for each species and fertilization effect was tested using a one-way ANOVA followed by post-hoc Tukey test (*e.g.* on biomass productivity, P concentration in aboveground biomass, P_{NaHCO3} and enzymatic activities).

A *t*-test was applied to compare P_{NaHCO3} values at the beginning and at the end of maize crop for each soil type, GM species and fertilization treatment. In addition, relationships between the variation of soil available P during maize crop and maize P export were tested for each soil type and GM species. These datasets combined data from the three fertilization treatments and thus did not met the independence condition. Therefore, to explain the variation of available P during maize crop, fertilization effect and maize P export were modeled concomitantly as explanatory variables using the function varpart (*vegan* package) and Helmert contrasts to account for the fertilization effect (Legendre and Legendre, 2012). In this way, the part of the variance explained by P maize export and controlled by fertilization effect (*i.e.* explained simultaneously by both explanatory variables) was quantified (adj R^2). This allowed to assess how much the causal relationship between variation of available P during maize crop and P maize export was due to the fertilization effect.

3. Results

3.1. Decomposition of green manure residues and microbial response to P fertilization

Decomposition rate of GM residues (n = 96) was better explained by species type (adj $R^2 = 0.95$, $p < 0.001$) than by soil type (adj $R^2 = 0.07$, $p < 0.01$) whereas fertilization treatment had no effect (adj $R^2 = 0.00$, $p > 0.05$). Overall, lupine residues decomposed the most whereas those of oat the least, regardless of the soil type (Table S1).

In the clay soil, decomposition of GM residues was influenced by GM species, but no effect of fertilization treatment was found (Table 1). The C_{mic} and the C_{Org}Min decreased with *Ca-Phi* compared to *Control* in pre-cultivated pots, but only C_{mic} was influenced by GM species. Like in clay soil, the decomposition of GM residues in the sandy soil was affected by GM species. If we exclude the oat treatment, whose residues decomposition was unaffected by fertilization treatments, the decomposition rates of the residues of the other GM species decreased in the *Ca-Phi* and *TSP* treatments compared to *Control*. The decomposition rate of pea residues, characterized by the highest Phi concentration, decreased largely (Table S1). In addition, P_{mic} increased with *Ca-Phi* compared to *TSP* and *Control* for pre-incubated soil, whereas it increased with *TSP* addition in pre-cultivated soil compared to *Ca-Phi* and *Control* (Table 1).

For the clay soil, enzymatic activities were affected by fertilization treatments depending on GM species (Table 2). Notably, alkaline-phosphatase increased with *Ca-Phi* and *TSP* compared to *Control* in pre-cultivated pots with mustard and oat. However, *Ca-Phi* addition dramatically decreased alkaline-phosphatase in pre-cultivated pots with pea and in pre-incubated pots compared to *TSP* and *Control*. Leucine-aminopeptidase also decreased with *Ca-Phi* compared to *TSP* and *Control* for pots pre-cultivated with legumes, but increased for mustard treatment with *Ca-Phi* and *TSP* compared to *Control*. Similar effects were observed for β-glucosidase and chitinase activities. In contrast, enzymatic activities were poorly affected by fertilization treatments in the sandy soil (Table 2).

3.2. Fertilization effect on soil available P

For both soil types, available P generally decreased during maize

Table 1
Mean values of decomposition rate of green manure residues (Dec GM Res), soil organic C mineralization during maize crop (C_{Org} Min = C_{Org} at the beginning of maize crop - C_{Org} at the end of maize crop) / C_{Org} at the beginning of maize crop), soil microbial C (C_{mic}), N (N_{mic}) and P (P_{mic}) for pre-cultivated pots (n = 48, PC) and pre-incubated pots (n = 9, PI) in clay and sandy soils at the end of the maize crop. Species effect (Spe. Eff.) of GM is given with the adjusted R^2. Significant differences ($p < 0.05$, ANOVA) between fertilization treatments are indicated, within the same row, by different letters based on Tukey test and mixed models.

		Clay			Spe. Eff.	Sand			Spe. Eff.
		Ca-Phi	TSP	Control	Adj R^2	Ca-Phi	TSP	Control	Adj R^2
Dec GM Res (%)	PC	82	85	82	0.38***	83 B[a,b]	83 B	86 A	0.58***
C_{Org} Min (%)	PI	8	8	9	0.09	4	2	6	0.02
	PC	- 4 B	2 A	6 A		0	6	- 2	
C_{mic} (mg kg^{-1})	PI	202	283	182	0.13*	87	176	133	0.00
	PC	169 C[a]	202 B	245 A		139	145	153	
N_{mic} (mg kg^{-1})	PI	31	29	38	0.00	22	26	23	0.03
	PC	31	43	33		15	11	11	
P_{mic} (mg kg^{-1})	PI	22	26	27	0.00	17 A	6 B	6 B	0.00
	PC	28	26	29		14 A	21 B	16 A	

Linear models are significant at $p < 0.05$ (*) or $p < 0.001$ (***).
[a] Refers to mixed model performed.
[b] Oat was excluded (n = 36).

Table 2
Mean soil enzymatic activities of β-glucosidase, chitinase, alkaline phosphatase (nmol of methylumbelliferone g^{-1} soil hr^{-1}) and leucine-aminopeptidase (nmol of 7-amino-4-methylcoumarin g^{-1} soil hr^{-1}) at the end of the maize crop in pre-incubated pots (n = 9) and pre-cultivated pots in relation to GM species and soil type (n = 12). Significant differences ($p < 0.05$, ANOVA) between fertilization treatments are indicated, within the same row, by different letters based on Tukey test.

Clay												
	β-glucosidase			Chitinase			Alkaline-phosphatase			Leucine-aminopeptidase		
	Ca-Phi	TSP	Control	Ca-Phi	TSP	Control	Ca-Phi	TSP	Control	Ca-Phi	TSP	Control
Lupine	2.35 A	3.35 B	1.63 A	1.79 A	2.31 B	1.53 A	10.15	10.39	6.86	0.53 A	4.47 B	2.28 A
Mustard	3.06 A	1.95 AB	1.41 B	2.43 A	1.53 B	1.40 B	8.25 A	10.75 B	2.51 C	3.88 A	3.18 A	1.69 B
Oat	2.01	2.56	2.75	1.51[†]	1.68	2.47	8.92 A	9.93 A	4.71 B	2.76	3.99	2.63
Pea	2.42	2.95	3.35	1.98	1.88	3.24	1.06 A	9.16 B	6.01 C	0.51 A	3.93 B	4.20 B
Pre-incubated	2.56	2.23	2.51	2.08 A	1.57 B	1.92 AB	5.66 A	9.29 B	9.24 B	3.70	3.47	4.52
Sand												
	β-glucosidase			**Chitinase**			**Alkaline-phosphatase**			**Leucine-aminopeptidase**		
	Ca-Phi	*TSP*	*Control*	*Ca-Phi*	*TSP*	*Control*	*Ca-Phi*	*TSP*	*Control*	*Ca-Phi*	*TSP*	*Control*
Lupine	1.09	1.81	1.93	1.08 A	1.16 AB	1.43 B	2.21 AB	1.77 A	2.48 B	2.64 A	1.38 B	2.39 A
Mustard	1.42	1.29	1.53	1.33	1.23	1.46	2.55	2.07	2.54	2.62	1.76	2.40
Oat	1.41 A	1.34 A	1.78 B	1.38 A	1.34 A	1.72 B	2.54	2.62	2.74	2.82	2.45	2.55
Pea	1.03	1.78	1.89	0.97	1.28	1.40	1.75	2.06	2.34	1.89	2.19	1.72
Pre-incubated	1.95	1.76	1.86	1.62	1.52	1.43	2.17	2.04	2.06	1.79	1.95	1.73

crop (Fig. 1). These decreases were consistently significant for the *Control* treatment, especially for sandy soil.

For clay soil, the decrease in soil available P during maize crop for pre-incubated pots was not affected by fertilization treatments and was related to P maize export (Table 3). At the end of the maize crop, *Ca-Phi* increased soil available P compared to *TSP* and *Control* in the pre-incubated soil ($p < 0.05$, Fig. 1). For pre-cultivated pots, a greater reduction of soil available P during maize crop was observed for *Control* than for *Ca-Phi* and *TSP* and was generally not related to P maize export (Fig. 1, Table 3). Available P level was maintained during maize crop (*i.e.* decreases of available P were not significant) for pre-cultivated pots with oat and mustard fertilized with *Ca-Phi* and for pre-cultivated pots with lupine and mustard fertilized with *TSP*. For the pre-cultivated pots with oat, P_{NaHCO3} at the end of the maize crop was lower in the fertilization treatments (*Ca-Phi* and *TSP*) compared to *Control*. Pre-cultivated pots with pea exhibited a lower P_{NaHCO3} compared to the other GM species and pre-incubated pots, particularly for *TSP* treatments.

For sandy soil, the available P levels were never maintained during maize crop and the decreases were generally higher for *Ca-Phi* and *TSP* than for *Control*. However, no difference in the decrease of available P during maize growth was observed between *Ca-Phi* and *Control* for pre-cultivated pots. The relationship between the decrease in available P during maize crop and P export was significant only for pre-incubated pots and was largely controlled by fertilization effect (Table 3). The decrease in available P during maize crop growth was influenced more by fertilization treatments for the pre-incubated than for pre-cultivated pots. Finally, both *Ca-Phi* and *TSP* increased soil available P at the end of the maize crop in pre-incubated and pre-cultivated pots compared to *Control* ($p < 0.05$, Fig. 1).

3.3. Productivity and nutrient content of aboveground maize biomass

Aboveground maize biomass was three times higher on sandy soil (*i.e.* 44.5 ± 16.5 g pot^{-1}) than on clay soil (15.1 ± 6.7 g pot^{-1}, $p < 0.001$, Fig. 2). For clay soil, GM species effect was significant (adj $R^2 = 0.42$, $p < 0.001$) and the highest productivity was observed for pots pre-cultivated with pea (Fig. 2). For sandy soil, species effect was also significant (adj $R^2 = 0.82$, $p < 0.001$) and the highest productivity was observed for pre-incubated pots followed by pre-cultivated pots with pea, lupine, mustard and oat. A negative effect of P fertilization was observed for aboveground biomass productivity of pots pre-cultivated with pea in clay soil.

For the entire dataset (n = 114), the P content in maize aboveground biomass was influenced more by soil type (adj $R^2 = 0.57$, $p < 0.001$) than by GM species (adj $R^2 = 0.14$, $p < 0.001$). Fertilization, on the other hand, had no effect (adj $R^2 = 0.00$, $p = 0.90$), except for pre-cultivated pots with pea in clay soil and with mustard and oat in sandy soil (Table 4). Compared to *Control* in *Ca-Phi*, P concentration in aboveground biomass of maize was lower for clay soils and higher for sandy soils ($p < 0.001$). Compared to *Control* in *Ca-Phi*, the lower maize

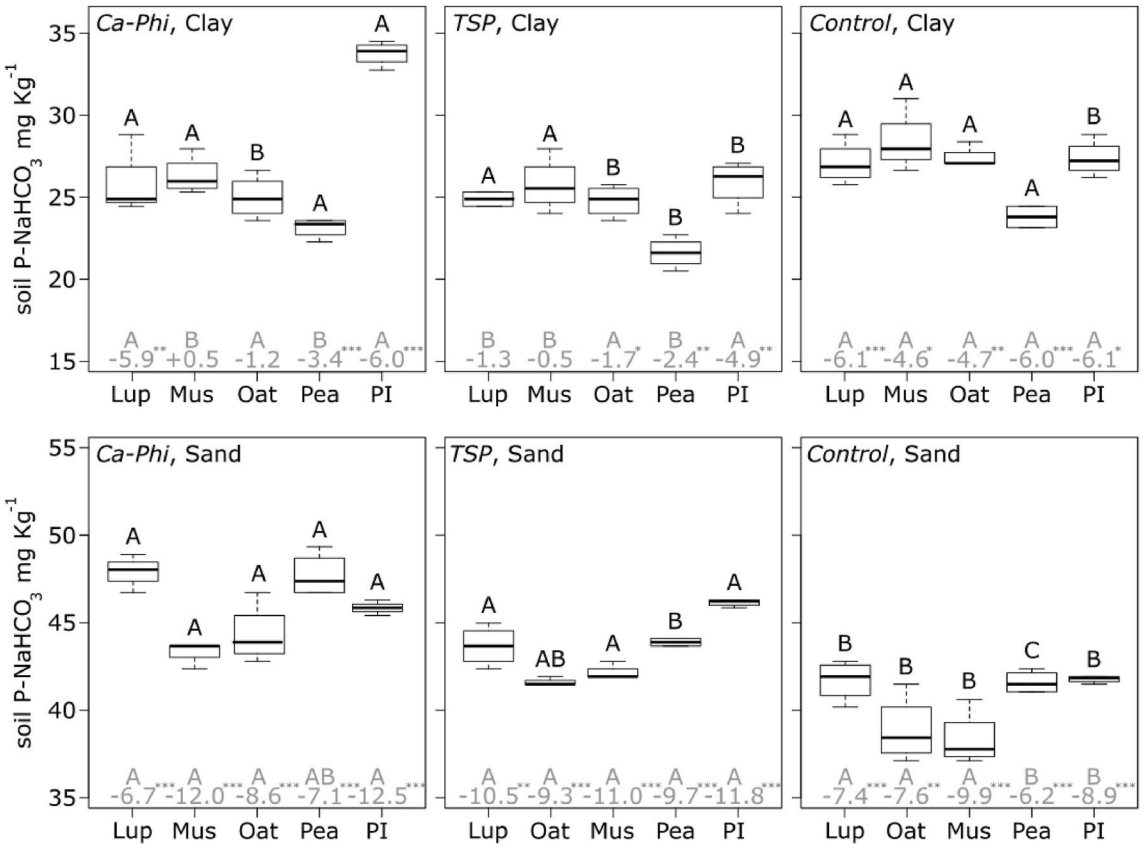

Fig. 1. Boxplot of soil available P and, below, variation in soil available P during maize crop (*i.e.* P–$NaHCO_3$ at the beginning of maize crop minus P–$NaHCO_3$ at the end of maize crop) for pre-cultivated pots with oat, lupine, mustard and pea and for pre-incubated soil (PI) in clay and sandy soils. Different uppercase letters indicate significant differences ($p < 0.05$, ANOVA and Tukey test) of soil available P between treatments (*i.e. Ca-Phi*, *TSP* and *Control*) for the same soil type and pre-cultivated GM species. For variation in available P during maize crop, significant differences (*t*-test) during maize crop growth are indicated at $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***) and uppercase letters indicate significant differences between treatments for the same soil type and pre-cultivated GM species ($p < 0.05$, ANOVA and Tukey test).

Table 3
Variations in soil available P during maize crop growth (*i.e.* available P at the beginning minus available P at the end of the growth period) explained (adjusted R^2 and level of significance) by (i) fertilization effect and (ii) P exported by maize aboveground biomass ($P_{MaizeExp}$) for each GM species in pre-cultivated (n = 12) and pre-incubated pots (n = 9) for clay and sandy soils. The part of the variance of soil available P during maize crop growth explained by $P_{MaizeExp}$ and controlled by fertilization effect is reported in parentheses (see Materials and Methods section).

	Clay		Sand	
	Fertilization	$P_{MaizeExp}$	Fertilization	$P_{MaizeExp}$
Lupine	0.50*	0.00	0.34	0.10 (FC)
Mustard	0.52*	0.00	0.05	0.00
Oat	0.37	0.11 ([a]FC)	0.00	0.00
Pea	0.59**	0.27* (FC)	0.45*	0.15 (0.01)
Pre-incubated	0.00	0.53** (0.00)	0.70**	0.51** (0.47)

Linear models are significant at $p < 0.05$ (*) and $p < 0.01$ (**).
[a] FC indicates that the variance of the variation in available P during maize crop explained by the explanatory variable is fully controlled by the fertilization effect.

production of P fertilized clay soil pre-cultivated with pea resulted in significantly ($p < 0.05$, Table 4) lower P export in maize aboveground biomass. For pre-incubated and pre-cultivated sandy soil with mustard, oat and pea, no fertilization effect on P export was observed. Finally, no Phi was observed in aboveground biomass of maize with *Ca-Phi* for both soil types.

4. Discussion

4.1. Legacy effect of green manure fertilized with Ca-Phi on soil microbial properties

Different Phi concentrations were observed in GM residues following *Ca-Phi* fertilization depending on soil type and GM species (Table S1 and Fontana et al. (2021)). Usually, P fertilization or Phi addition result in a shift of microbial communities that can modify soil functions (Beauregard et al., 2010; Stone and White, 2012; Wongwathanarat and Sivasithamparam, 1991). Overall, our study showed that the effects of GM residues containing Phi depended on soil type and GM species. Although lower decomposition rates of GM residues were expected in the clay soil (Midmore et al., 2000), no differences between soil types were observed. In fact, decomposition rate of GM residues was mainly influenced by the GM species (Table S1) likely as a result of physico-chemical properties of GM litter (Halvorson and Smith, 1995).

In clay soil, the presence of GM residues containing Phi decreased C_{mic} amount and C_{Org}Min compared to both *TSP* and *Control* treatments highlighting the effect of Phi content in GM residues. The decrease of C_{Org} mineralization was particularly exacerbated with mustard residues (Table S1) that were characterized by the highest Phi concentration (Fontana et al., 2021). Mustard residue decomposition also resulted in higher β–glucosidase and chitinase activities for *Ca-Phi* compared to *TSP* and *Control* (Table 2). This suggests that high Phi concentration in mustard residues increased microbial C and N demands (Cañizares et al., 2011; Garcia et al., 1998; Rodriguez-Kabana et al., 1983). In addition, the strong decrease of leucine-aminopeptidase with legume residues (*i.e.*

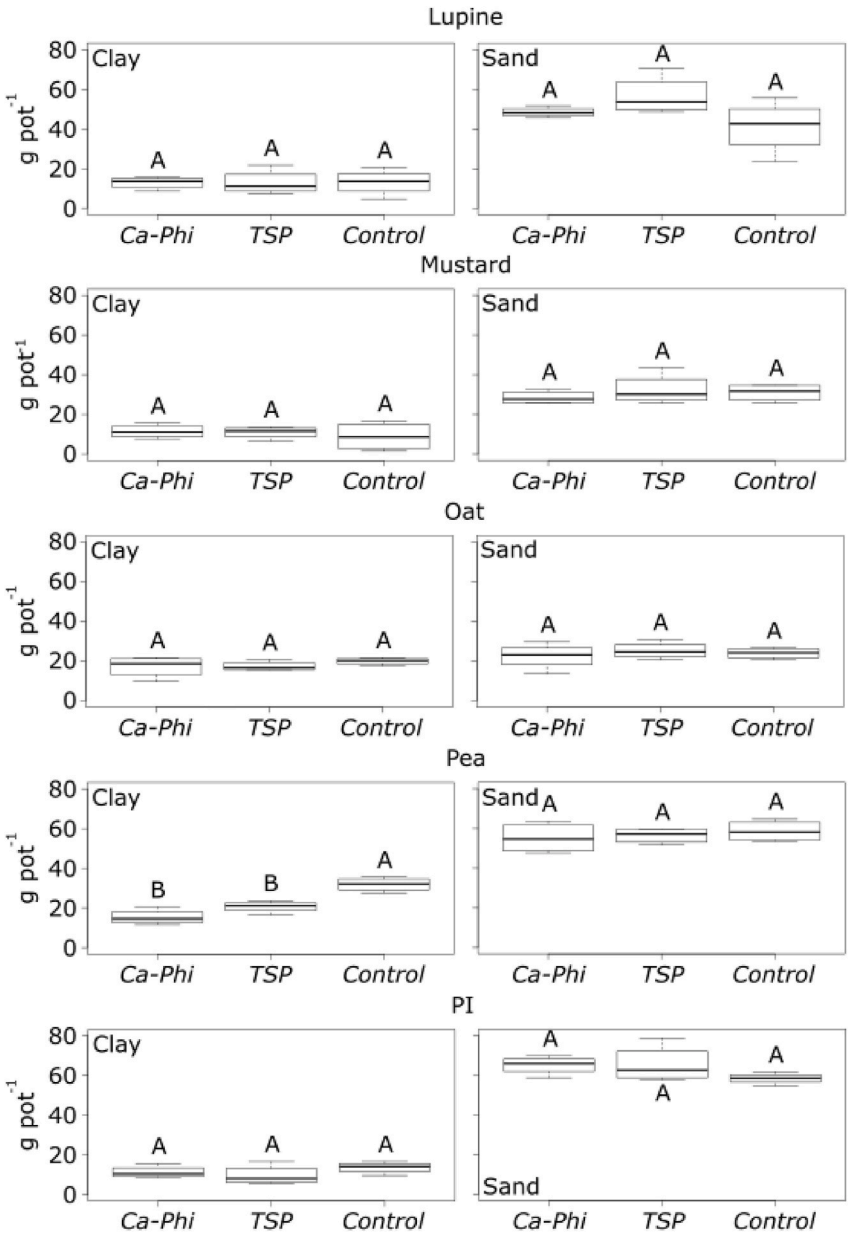

Fig. 2. Boxplot of maize aboveground biomass production (dry weight) for GM pre-cultivated pots with lupine, mustard, oat and pea in clay and sandy soils and for pre-incubated (PI) clay and sandy soils. Significant differences ($p < 0.05$, ANOVA) between fertilization treatments (*i.e. Ca-Phi*, *TSP* and *Control*) are indicated by different letters based on post-hoc Tukey tests with B < A.

lupine and pea) containing Phi (Table 2) suggests that N microbial demand was dramatically curtailed. *Ca-Phi* affected alkaline-phosphatase activity compared to *Control*, but in the opposite direction depending on the presence and the type of GM residues containing Phi. Although an increase of alkaline-phosphatase activity can be linked to Phi oxidation (Schowanek and Verstraete, 1990; Yang and Metcalf, 2004), we suspect that *Ca-Phi* effect on alkaline-phosphatase activity was likely related to the increase of organic P observed at the end of GM crop (Fontana et al., 2021; Margalef et al., 2017).

In sandy soil, a decrease of GM decomposition rate was observed for *Ca-Phi* (Table 1), especially for pea containing the highest Phi concentration (Table S1), pointing out that Phi in GM residues curtailed microbial decomposition activity. This is consistent with the decrease of feeding activity also observed in a sandy soil after Phi addition (Stoven et al., 2007). However, P_{mic} with *Ca-Phi* and *Control* were not different (Table 1), suggesting that Phi in GM residues did not affect microbial P demand. In contrast, *Ca-Phi* increased P_{mic} compared to *Control* and *TSP* treatments in pre-incubated sandy soil suggesting that the P microbial demand was rather influenced by a shift of microbial activity rather than by the P availability. However, in the sandy soil *Ca-Phi* poorly affected C_{Org} mineralization, C_{mic}, N_{mic} and enzymatic activities compared to *Control* even with pea residues (Tables 1 and 2). Altogether, these results show that GM residues containing Phi influenced the microbial properties in relation to the GM species, supporting our first hypothesis. However, microbial properties (*i.e.* microbial C, N and P, enzymatic activity, or decomposition rate of GM residues) were affected differently depending on the interaction between GM species and soil type. For both soil types, the magnitude of *Ca-Phi* and *TSP* effects on microbial properties was comparable. Therefore, our study is in line with the conclusions of Stoven et al. (2007) stating that "phosphite did not harm the biocenosis of soil microorganisms".

Table 4
Mean P concentration (±sd) and P export by aboveground biomass of maize grown on pre-cultivated pots with four different species of green manure crops, namely mustard, oat, peat, and lupine (n = 12) and pre-incubated pots with no green manure crops (n = 9), according to the fertilization treatments, *i.e. Ca-Phi*, *TSP* and *Control*. Significant differences ($p < 0.05$, ANOVA) between fertilization treatments are indicated, within the same row, by different letters based on post-hoc Tukey tests and mixed models.

Maize P concentration (mg kg^{-1}) pre-cultivated with:	Clay Ca-Phi	TSP	Control	Sand Ca-Phi	TSP	Control
Lupine	5.1	4.8	5.2	2.2	2.3	2.5
Mustard	4.4	4.2	5.0	3.9 A	3.8 A	3.2 B
Oat	3.4	3.1	3.2	4.1 A	3.6 B	3.6 B
Pea	4.4 A	4.0 A	3.2 B	2.5	2.6	2.5
Pre-incubated	6.1 A	5.0 B	5.7 AB	2.1	2.0	1.9
Maize P export (mg pot^{-1}) pre-cultivated with:	Clay *Ca-Phi*	*TSP*	*Control*	Sand *Ca-Phi*	*TSP*	*Control*
Lupine	66.7	59.3	64.6	108.1 AB	129.2 A	95.7 B
Mustard	48.9	45.0	41.9	111.1	121.2	98
Oat	58.0	53.0	63.8	91.4	90.7	86.8
Pea	66.6 B	82.9 AB	100.7 A	138.5	146.4	143.5
Pre-incubated	68.3	47.5	78.7	134.8	133.1	107.9

4.2. Legacy effect of green manure fertilized with Ca-Phi on soil available P

At the end of the maize growth, soil available P was the result of cumulative effects of (i) P fertilization, an effect already present at the beginning of the maize crop (Fontana et al., 2021), and (ii) variations in available P during maize crop due to maize P export, P derived from GM residue mineralization and the exchange between available and sparingly soluble P pools.

In pre-incubated soils without decomposing GM residues, changes in available P during maize crop were mainly driven by maize P-export (Table 3). Since no effect of P fertilization was found on P maize export, fertilization effect observed at the end of GM crop (Fontana et al., 2021) persisted until the end of maize crop, leading to an increase of available P in both soil types for *Ca-Phi* fertilization, and only in the sandy soil for *TSP* fertilization (Fig. 1). This result demonstrates that *Ca-Phi* can increase the amount of available P likely due to Phi oxidation, similarly to *TSP* in sandy soil with low P fixing capacity and, to a larger extent, in clay soils with high P fixing capacity. In fact, it is well known that soils with high fixing capacity negatively affect available P readily released from *TSP* compared to P from *Ca-Phi* that is not water-soluble (Fontana et al., 2021; Morel et al., 1989).

In pre-cultivated soils with GM crops, the variation in available P during maize growth was not related to P export (Table 3) because GM residues influenced available P differently with regard to fertilization treatments, GM species and soil types (Fig. 1). In addition, the influence of GM species on P maize export (Table 4) and on soil available P (Fig. 1) were not explained by the P provided from GM species, despite their important differences on P concentration (*i.e.* 1.7–6.0 mg P kg^{-1} (Fontana et al., 2021)). In fact, P mobilization from GM residues is mainly influenced by (i) P sorption/desorption reactions, (ii) microbial activity, and (iii) the quality of GM residues (Zhu et al., 2018), all of which were differently influenced by *Ca-Phi* and *TSP* depending on soil type.

In the pre-cultivated clay soil, the decrease of available P at the end of GM crop fertilized with P was not observed at the end of the maize crop likely due to a loss of organic carbon that competed with P for sorption sites (Fontana et al., 2021; Von Wandruszka, 2006). The absence of differences in available P between fertilization treatments can be explained by a higher decrease of available P during maize crop growth for the *Control* compared to P fertilization treatments (Fig. 1). This suggests that P mobilization from GM residues was affected differently depending on fertilization treatment. P fertilization increased and decreased Fe_{exch} and Ca_{exch}, respectively, compared to *Control* regardless of GM species (Table S2). This likely modified P sorption through possible interferences between P, Ca and Fe as suggested by the negative correlation between Fe_{exch} and Ca_{exch} (*Pearson correlation coefficient* $r = -0.94$, n = 60) and the positive one between Fe_{exch} and available P (*Pearson correlation coefficient* $r = 0.70$, n = 60). The same observation was reported by Maftoun and Moshiri (2008) for a soil type similar to our clay soil. In addition, microbial activity was likely modified by P fertilization that influenced C_{Org} mineralization and enzymatic activities compared to *Control* (Tables 1 and 2) and, in turn, available P. At the end of maize crop, *Ca-Phi* and *TSP* generally increased alkaline-phosphatase activity compared to *Control*, suggesting a stronger mobilization of organic P that was accumulated during GM crop after P fertilization (Table 2; Fontana et al. (2021)). The decreases in available P during maize corresponded to only 39% and 37% of maize P export for *Ca-Phi* and *TSP*, respectively, while it reached 83% for *Control* ($p < 0.05$, data not shown). This indicates that more P from sparingly available pool(s) was absorbed by maize following P fertilization compared to *Control*.

In pre-cultivated pots with sandy soil, the low P fixing capacity and the very high available P may have oversaturated P fixing sites (Defra, 2010; Morel et al., 1989), leading to a strong response of P fertilization in relation to the amount of P input (Cao et al., 2012; Fontana et al., 2021; Shepherd and Withers, 1999). Although the lower decomposition rate of GM residues with *Ca-Phi* compared to *Control* suggests that the quality of GM residues was affected (Table 1), variation in available P during maize was not affected by *Ca-Phi* fertilization (Fig. 1). Therefore, the fertilization effect was still present during maize, and consequently *Ca-Phi* and *TSP* increased available P at the end of both GM and maize (Fig. 1 and Fontana et al., 2021). As a result, our second hypothesis was validated only for the sandy soil.

4.3. Legacy effect of green manure fertilized with Ca-Phi on maize aboveground biomass

We did not detect any Phi in maize aboveground biomass for both soil types, indicating that the Phi previously added in the soil and present in the GM crops (Table S1) was not transferred to the maize. Probably Phi underwent oxidation or was unavailable to the maize. These results validate the third hypothesis of this study. Our results are in line with previous studies reporting a time span of few weeks to oxidize added Phi (in a water-soluble form) in reconstructed sandy soils (Ouimette and Coffey, 1989; Stoven et al., 2007).

Generally, *Ca-Phi* and *TSP* did not affect either the aboveground productivity of maize, or the P export in aboveground biomass for each soil type. This is likely because P was not limiting due to the high available P content in both soils (Defra, 2010; Fontana et al., 2021). Instead, maize aboveground productivity was higher in sandy than in clay soil, due to different soil physical properties, as already observed during the previous growth of GM crops (Fontana et al., 2021). Additionally, we observed that the aboveground productivity of maize in clay soil was higher for pots pre-cultivated with pea and oat (Fig. 2), *i.e.* two GM species characterized by higher specific root lengths enhancing physical fertility (Faucon et al., 2017; Miller et al., 2003; Puget and Drinkwater, 2001; Wendling et al., 2017). Therefore, *Ca-Phi* fertilization did not hamper the positive rotational effect of GM on maize. In the sandy soil, N availability was controlled by GM species type, as supported by the relationship between maize productivity and N content in GM aboveground biomass (*i.e.* N export) (Table S3). Although the symbiotic process of N fixation was probably hindered during GM crop due to *Ca-Phi* fertilization (Fontana et al., 2021), the cultivation of GM legumes (*i.e.* Lupine and Pea), characterized by higher biomass N content (Büchi et al., 2015; Jemo et al., 2006), improved the subsequent maize productivity with or without *Ca-Phi* fertilization (Fig. 2). These

findings validate the fourth hypothesis of this study. Our results suggest that the cultivation cycle of the GM crop is sufficient to oxidize the added Phi and that the positive rotational effect expected after GM crops is not negatively affected by *Ca-Phi* fertilization.

5. Conclusion

Phi was detected in the aboveground biomass of GM crops previously fertilized with *Ca-Phi*, but not in the subsequent maize. This suggests that Phi was not present in the soil solution during maize growth. Higher P_{NaHCO3} values at the end of the GM and maize in response to the addition of *Ca-Phi* indicate that Phi was oxidized and partly available for crops. Eight weeks seems adequate to potentially oxidize Phi in both soil types cultivated with different GM species as well as in bare soil. *Ca-Phi* fertilization increased P_{NaHCO3} similar to *TSP* in a soil with low P fixing capacity (sandy soil). *Ca-Phi* fertilization increased P_{NaHCO3} more compared to *TSP* in a soil with high P fixing capacity (clay soil). This is likely due to the lower water solubility of *Ca-Phi* compared to *TSP*, a characteristic that could favor a longer availability of P. Overall, our results suggest that *Ca-Phi* could be used as a valuable P source for crops.

The incorporation of GM residues containing Phi had different effects on soil microbial properties depending on soil type and GM species. In clay soil, GM residues containing Phi decreased the C_{Org} mineralization and C_{mic} content similarly for all GM species. In sandy soil, the presence of Phi in GM residues decreased their decomposition rate except for oat. In addition, enzymatic activities were affected by *Ca-Phi* and *TSP* fertilizations, particularly in the clay soil, but differently depending on GM species. Overall, the amount of *Ca-Phi* applied in our study does not seem harmful for the biological fertility of soil during maize growth. The effect of *Ca-Phi* on microbial biomass and enzymatic activities was similar to that of *TSP*, but lower when compared to the effect associated with soil type and GM species. In addition, *Ca-Phi* did not impair the positive rotational effect of the GM crop on the productivity of the following maize except for pea on clay soil. Future investigations are necessary to test if Phi can be leached and to what extent *Ca-Phi* fertilization can affect soil microbial functional groups, in addition to quantifying the Phi oxidation kinetic related to soil properties.

Credit author statement

Mario Fontana: Conceptualization, Methodology, Data curation, Investigation, Visualization, Formal analysis, Writing - original draft. Thomas Guillaume: Writing - review & editing. Saïd Elfouki: Investigation, Formal analyses. Luca Bragazza: Writing - review & editing. Mathieu Santonja: Writing - review & editing. Alexandre Buttler: Writing - review & editing. Renato Gerdol: Writing - review & editing. Lisa Brancaleoni: Writing - review & editing. Sokrat Sinaj: Project administration, Funding acquisition, Resources, Conceptualization, Methodology, Data curation, Writing - review & editing.

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.

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Appendix A. Supplementary data

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