

# Lupin causes maize to increase organic acid exudation and phosphorus concentration in intercropping

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## Abstract

**Purpose:** There is a need to develop agricultural practices that mobilize sparingly soluble soil phosphorus (P) due to increasing scarcity of P fertilizer. Interactions of different plant species in the rhizosphere might increase P mobilization, but the underlying mechanisms are still not fully understood.

**Methods:** We conducted a pilot study with four plant species (maize, soy, lupin, mustard) grown alone and in combination with maize (intercropping) to investigate how species interact to mobilize P from iron phosphate (FePO<sub>4</sub>). Root exudates of individual plants were collected and analyzed for low molecular weight organic acid anions (LMWOA) and pH.

**Results:** Maize increased its exudation of LMWOA and its biomass P concentration in intercropping, especially when grown together with lupin. This is the first study to show unequivocally that a high LMWOA concentration in the rhizosphere in intercropping is not only caused by high LMWOA release of the companion but also by an increased LMWOA exudation of the main crop. The high release of LMWOA was associated with a higher maize P concentration, indicating that enhanced LMWOA release in intercropping is beneficial for P acquisition of maize. Moreover, lupin and mustard mobilized more P from FePO<sub>4</sub> than maize and soy likely through high LMWOA exudation (lupin) and rhizosphere alkalization (mustard).

**Conclusion:** Taken together, we reveal that intercropping with lupin increases the release of LMWOA by maize and concurrently the maize P concentration, suggesting that intercropping is useful for the mobilization of P from FePO<sub>4</sub> because it affects the exudation of maize.

## KEYWORDS

intercropping, iron phosphate, organic acid anions, pH, phosphorus mobilization

## 1 | INTRODUCTION

Phosphorus (P) is an essential macronutrient that often limits plant growth in agriculture if not provided as fertilizer. Most P fertilizer today is produced from phosphate rock which is a finite resource that

will likely be exhausted during the next decades (Cordell & White, 2014). Thus, there is a need to reduce the reliance on phosphate rock-derived fertilizers (Ashley et al., 2011; Cordell & White, 2014). As iron phosphate (FePO<sub>4</sub>) is formed in municipal wastewater treatment plants (Wilfert et al., 2015), it could potentially

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be used as a renewable P fertilizer, thus increasing P recycling. Several studies have already investigated  $\text{FePO}_4$  as potential alternative, yet sparingly soluble P source for plants (e.g., H. Li et al., 2010; L. Li et al., 2007; Marschner et al., 2007; Pearse et al., 2007; Segal et al., 2019). Since likely only some plant species are capable of mobilizing P from  $\text{FePO}_4$ , intercropping could be beneficial for the P acquisition of associated non-P-mobilizing plant species. However, the underlying mechanisms of plant P mobilization from  $\text{FePO}_4$  are still not fully understood, especially not in intercropping.

Plants have developed several mechanisms to mobilize P from sparingly soluble sources (Hinsinger, 2001; Richardson et al., 2011; Tang et al., 2021). Among others, alkalization of the rhizosphere caused by the exudation of  $\text{OH}^-$  (or  $\text{HCO}_3^-$ ) potentially mobilizes P from  $\text{FePO}_4$  since the solubility of  $\text{FePO}_4$  increases with increasing pH (Hinsinger, 2001; Lindsay, 1979). Moreover, the exudation of low molecular weight organic acid anions (LMWOA) potentially mobilizes P from  $\text{FePO}_4$  via ligand exchange reactions by replacing P and chelating (i.e., complexing) metal cations, such as  $\text{Fe}^{3+}$ , which prevent them from precipitating with phosphate ions (Richardson et al., 2011; Y. Wang & Lambers, 2020). LMWOA possess one to three carboxyl groups ( $\text{COOH}$  or  $\text{COO}^-$ ) that are crucial for P mobilization. Generally, the P mobilization capacity of LMWOA increases with the number of carboxyl groups, making citrate very efficient in mobilizing P from  $\text{FePO}_4$  (Jones, 1998; Tsado et al., 2014; Y. Wang & Lambers, 2020). However, only some plant species exude LMWOA at high rates or change the rhizosphere pH substantially. For instance, legumes (*Fabaceae*) and especially lupins (*Lupinus* L. spp.) commonly have high LMWOA exudation, while many mustard species (*Brassicaceae*) cause rhizosphere alkalization (Marschner et al., 2007; Pearse et al., 2007; Y. Wang & Lambers, 2020).

Intercropping, that is, the simultaneous cultivation of at least two plant species, has been shown to increase grain yields as it elevates nutrient availability, and has other positive effects (L. Li et al., 2014; C. Li et al., 2020). Intercropping might also be beneficial for plant P mobilization from  $\text{FePO}_4$  since plant species with a low capacity to mobilize sparingly available nutrients might benefit from a high LMWOA exudation or changes of the rhizosphere pH by P-mobilizing companions when roots intermingle (L. Li et al., 2014; Xue et al., 2016). For instance, different lupins have been shown to solubilize sparingly available P through a high LMWOA exudation causing increased P uptake of intercropped cereals (Dissanayaka et al., 2017). Especially white lupin has been reported to chelate metal cations ( $\text{Fe}^{3+}$ ,  $\text{Al}^{3+}$ ,  $\text{Ca}^{2+}$ ) through the release of citrate, thus increasing P availability (Cu et al., 2005; Gardner et al., 1983; Gardner & Boundy 1983; H. Li et al., 2010). Moreover, different *Brassicaceae* have been shown to substantially alkalize their rhizosphere which increases P mobilization from  $\text{FePO}_4$  (Marschner et al., 2007; Pearse et al., 2007). However, P uptake of wheat was decreased or not affected by intercropping with these different *Brassicaceae* (D. Wang et al., 2007). Taken together, even though intercropping might be a promising approach to improve plant P uptake, it is still not fully understood how different plant species interact to mobilize P in intercropping.

Increased growth and yields of cereals in legume/cereal intercropping have often been explained by the P mobilization ability of legumes, for example, through the release of LMWOA, from which cereals also benefit (L. Li et al., 2014; Tang et al., 2021; Zhang et al., 2016). However, some studies found that cereals also enhanced their exudation of LMWOA in intercropping with different legumes compared to cereal monocropping. For instance, an increased concentration of different LMWOA such as malate, citrate and oxalate has been found in the rhizosphere soil of wheat in intercropping compared to monocropping when grown together with faba bean, white lupin or pea (C. Li et al., 2016; Lo Presti et al., 2021). Similarly, the malate concentration in the rhizosphere soil of maize was significantly increased by a factor of 12 in intercropping with faba bean compared to maize monocropping (H. Li et al., 2013). When maize was intercropped with alfalfa, similar findings were obtained, both in pot experiments (L. Wang et al., 2020) and in the field (Sun et al., 2020). Moreover, the LMWOA composition of maize also differed significantly between mono- and intercropping when maize was grown together with faba bean or white lupin supplied with  $\text{FePO}_4$  (H. Li et al., 2010). However, in many of these studies it cannot be excluded that LMWOA released by the companion plants diffused to the roots of the main crop because LMWOA were collected from the rhizosphere soil (H. Li et al., 2010; C. Li et al., 2016; Lo Presti et al., 2021; L. Wang et al., 2020). Thus, it is not known if the LMWOA are derived from the cereal or from the companion plant. Therefore, an experiment that unequivocally separates root exudates released by the main and the companion crop is required to enlighten the effect that increases P mobilization in intercropping.

The aim of this pilot study was to investigate P mobilization from  $\text{FePO}_4$  in intercropping. We hypothesized that maize increases its root exudation when grown together with a companion, and that maize P uptake from  $\text{FePO}_4$  is increased in intercropping compared to monocropping. To test this hypothesis, we conducted a greenhouse experiment with four different crop species, and  $\text{FePO}_4$  as the only P source. The species were maize (*Zea mays* L.), soy (*Glycine max* (L.) Merr.), blue lupin (*Lupinus angustifolius* L.) and white mustard (*Sinapis alba* L.). Maize was grown in rhizoboxes in combination with one of three companions (two individuals of different species in one rhizobox; intercropping), and in addition all species were grown alone (two individuals of the same species; monocropping). We selected contrasting companions for this experiment, two legumes (lupin and soy) and the *Brassica* white mustard. Root exudates were collected separately from all plant individuals using the soil-hydroponic-hybrid sampling approach, in contrast to previous studies that extracted LMWOA only from rhizosphere soil (H. Li et al., 2010; C. Li et al., 2016; Lo Presti et al., 2021; L. Wang et al., 2020). Root exudates were analyzed for LMWOA, pH and dissolved organic carbon (DOC). In addition, plant biomass, and P and Fe concentrations of the plants were determined.

## 2 | MATERIALS AND METHODS

### 2.1 | Experimental setup

We conducted an intercropping experiment with four plant species grown in rhizoboxes. The only P source available for the plants was iron phosphate, except for 0.5 mg P per rhizobox supplied with an inoculum (see below). Iron phosphate (iron(III) phosphate dihydrate:  $\text{FePO}_4 \times 2 \text{H}_2\text{O}$ ; Sigma-Aldrich, Merck KGaA) was thoroughly mixed to the mineral substrate, amounting to 422 mg P per rhizobox (88.8 mg P  $\text{kg}^{-1}$  substrate). The mineral substrate consisted of 20% (vol.) perlite and 80% (vol.) quartz sand, from which 50% (wt.) had a grain size of 0.1–0.4 mm and 50% (wt.) had a grain size of 0.7–1.2 mm. A mix of micronutrients (RADIGEN<sup>®</sup> Micronutrient mixed fertilizer; Terraflor GmbH) was added to the mineral substrate (900 mg rhizobox<sup>-1</sup>), containing 5.0% MgO, 2.0% Fe, 1.5% Cu, 1.0% Mn, 0.8% Mo, 0.6% B and 0.5% Zn (further nutrients were supplied with a P-free nutrient solution as described below). The mineral substrate was filled into the rhizoboxes to a final bulk density of 1.1 g  $\text{cm}^{-3}$  which equals 4.75 kg of mineral substrate (dry weight [DW]) per rhizobox. Rhizoboxes were made of PVC and had an inner size of 49.2 × 29.3 × 3.0 cm (h × w × d).

In each rhizobox, two plants were sown at a distance of 15 cm. We used the following plant species: maize (*Z. mays* L. cv. Golden Bantam; Bingenheimer Saatgut AG), soy (*G. max* (L.) Merr. cv. Lica, Marktgesellschaft der Naturland Bauern AG), blue lupin (*L. angustifolius* L. cv. Rumba; Templiner Kräutergarten) and white mustard (*S. alba* L.; Bingenheimer Saatgut AG). All species were cultivated in monocropping, that is, two plant individuals of the same species. In addition, maize was cultivated in intercropping, that is, maize was grown together with one out of three companions (soy, lupin and mustard) in one rhizobox. All seeds except mustard were soaked in water for 24 h before seeds of a consistent size were sown in the rhizoboxes. Each of the seven combinations (maize/maize, soy/soy, lupin/lupin, mustard/mustard, maize/soy, maize/lupin, maize/mustard) was replicated four times (except for soy and mustard in monocropping of which one rhizobox each failed), resulting in a total of 26 rhizoboxes. The plants were sown in March and harvested in May 2020 after 70 days.

The rhizoboxes were placed in an open greenhouse at the University of Bayreuth under ambient conditions (i.e., around 20°C day temperature, automatic aeration above 23°C, no artificial light, automatic shading in case of strong sun exposure). The rhizoboxes were placed in a randomized block design on a wooden rack that kept them inclined by 50° throughout the experiment, and they were rearranged randomly after 5 weeks. The inclination of the rhizoboxes made the roots grow along the bottom wall of the rhizoboxes, which made it possible to remove the entire plant at harvest with very limited damage to the root system.

An inoculum was applied to the mineral substrate at the beginning of the experiment to introduce a soil microbial community. For this purpose, fresh soil (with a loamy sandy texture) was sampled from an agricultural field (for details see Schwerdtner & Spohn, 2021).

The soil was sieved (<2 mm), mixed with tap water (1:2) and shaken on an overhead shaker for 1 h before being filtered through cellulose filters (Rotilabo<sup>®</sup>, type 113P; Carl Roth GmbH & Co. KG). The filtrate was mixed with tap water to a final soil:water ratio of 1:4 and stored at 20°C over night before being applied to all rhizoboxes. The final soil inoculum had the following chemical properties ( $\text{l}^{-1}$  inoculum): 15.3 mg organic C, 4.4 mg N, 0.9 mg P and pH 7.9. Each rhizobox received 507 ml of soil inoculum and 140 ml tap water to adjust the mineral substrate to 75% water holding capacity (WHC). Priorly, the WHC of the mineral substrate was determined gravimetrically. For this purpose, an aliquot of the mineral substrate was oversaturated with water, drained for 24 h on a sand bath and weighed before and after drying at 105°C.

Rhizoboxes were watered every second day with tap water to 75% WHC as measured by weight. For this purpose, each rhizobox was weighed and water was added until the desired weight (which is the sum of the weights of the rhizobox, the mineral substrate and the water amount equivalent to 75% WHC) was reached. In addition, a P-free nutrient solution was applied regularly. For this purpose, an adapted Ruakura solution (Smith et al., 1983) was used where  $\text{KH}_2\text{PO}_4$  was substituted by  $\text{KNO}_3$  and  $\text{K}_2\text{HPO}_4$  by  $\text{K}_2\text{SO}_4$ . The final nutrient solution applied to the rhizoboxes contained ( $\text{l}^{-1}$ ): 220 mg  $\text{Mg}(\text{NO}_3)_2 \times 6 \text{H}_2\text{O}$ , 746 mg  $\text{Ca}(\text{NO}_3)_2 \times 4 \text{H}_2\text{O}$ , 377 mg  $\text{NH}_4\text{NO}_3$ , 189 mg  $\text{KNO}_3$ , 367 mg  $\text{K}_2\text{SO}_4$ , 27 mg  $\text{Na}_2\text{SO}_4$ , and 15 mg NaCl. In total, 15.8 mg N  $\text{kg}^{-1}$  substrate were applied in the form of inoculum and nutrient solution.

At harvest, 10 weeks (70 days) after emergence, root exudates were collected, and plants were analyzed for biomass production and P and Fe concentrations (see below).

### 2.2 | Root exudate collection

Root exudates were collected in sterile deionized water using the soil-hydroponic-hybrid sampling approach (Oburger & Jones, 2018). For this purpose, the bottom walls of the rhizoboxes were opened, and plants were removed as carefully as possible to prevent root damage. Roots were gently shaken and washed with deionized water to remove adhering substrate particles and potential metabolites (Oburger & Jones, 2018). The entire root system of the intact plant was then transferred to a sterile beaker that was filled with a known volume of sterile deionized water (between 75 and 175 ml) so that roots were completely submerged. We used sterile deionized water instead of a  $\text{CaCl}_2$  solution since this reduces the background matrix for the analyses while not altering exudation patterns (Egle et al., 2003; Oburger & Jones, 2018). Three blanks, that is, three beakers with known volumes of sterile, deionized water but without plants, were also included and treated in the same way as all beakers. Plants in beakers were stored at 20°C in a climate chamber with artificial lighting (650  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) for 4 h. Subsequently, plants were removed from beakers, and beakers were swayed to homogenize the exudates in the solution. The exudate solutions were filtered through 0.2  $\mu\text{m}$  syringe filters and three aliquots were frozen for

subsequent analyses of LMWOA, pH and DOC. All plants were sampled in a way ensuring that exudate collection took place during peak metabolic activity, that is, collection started  $3.5 \pm 1$  h after sunrise, as recommended in Oburger and Jones (2018). Since we used a relatively short exudate collection period and maintained very similar temperatures during plant growth and exudate collection, we assume that plant metabolism and, therefore, exudation patterns do not differ between growth and sampling conditions (Oburger & Jones, 2018).

### 2.3 | Biomass analyses

After the plants were removed from the exudate collection beakers, aboveground biomass (AGB) was separated from belowground biomass (BGB), dried at  $60^\circ\text{C}$ , weighed and milled. BGB was washed again with deionized water, dried at  $60^\circ\text{C}$ , weighed and milled.

The biomass samples (AGB and BGB of each plant) were analyzed for total P and Fe concentrations after pressure digestion in concentrated nitric acid using an inductively coupled plasma-optical emission spectroscopy (Vista-Pro radial, Varian Inc.).

### 2.4 | Exudate analyses

LMWOA were analyzed using high-performance liquid chromatography-mass spectrometry (HPLC-MS). For this purpose, the exudate samples were loaded on an HPLC RP-C18 column (Luna Omega 1.6  $\mu\text{m}$  PS C18, 100  $\text{\AA}$ ,  $100 \times 2.1$  mm, Phenomenex Inc.; operated as part of an Ultimate 3000 HPLC, Thermo Fisher Scientific GmbH) which was connected to a Q Exactive mass spectrometer (Thermo Fisher Scientific GmbH) equipped with a hybrid quadrupole orbitrap mass analyzer (maximum mass range 50–6000 Da, resolution 140,000 @  $m/z=200$ ). A 10 min isocratic elution with pure water (HPLC-grade, spiked with 0.2% formic acid) at a flow rate of  $0.3 \text{ ml min}^{-1}$  was applied. Mass spectra were acquired after electrospray ionization (ESI negative) in full scan mode ( $50 < m/z < 750$ ) recording the total ion current. For evaluation (i.e., identification and integration/quantitation) of the LMWOA, their characteristic mass traces were used (Supporting Information: Table S1).

The pH of the exudate solution was measured with a pH electrode (WTW SenTix 51; Xylem Analytics GmbH & Co. KG).

DOC was analyzed using a Total Carbon Analyzer (TOC-TN Analyzer, multi N/C 2100; Analytik Jena GmbH).

### 2.5 | Calculations

The total biomass (TBM) was calculated as the sum of the DWs of AGB and BGB for each plant. The P and Fe concentrations of the TBM (plant P concentration in  $\text{mg P g}^{-1}$  TBM and plant Fe concentration in  $\text{mg Fe g}^{-1}$  TBM) were calculated based on the

element concentrations of AGB and BGB and the DW of AGB and BGB for each plant. The element concentrations were used as means of assessing the nutritional status of the plants since the element concentrations mainly depend on the element availability in the growth medium (Mengel et al., 2001). To additionally show the total uptake of P and Fe by the plants, plant P and Fe contents (in  $\text{mg plant}^{-1}$ ) were calculated by multiplying the plant element concentrations with the TBM.

The DOC concentration in the exudate solution (in  $\text{mg l}^{-1}$ ) was multiplied by the volume of sterile deionized water in which roots were submerged to correct for the different volumes and gain results in mg per plant.

The exudation of each LMWOA (in  $\mu\text{mol plant}^{-1}$ ) was calculated by multiplying the LMWOA concentrations in the exudate solution (in  $\text{mg l}^{-1}$ ) with the volume of sterile deionized water and dividing by the molar mass of the respective LMWOA. The LMWOA exudation was also divided by the DW of BGB to gain results per g root DW. The concentration of dicarboxylic LMWOA (in  $\mu\text{mol plant}^{-1}$  or  $\mu\text{mol g root DW}^{-1}$ ) was calculated as the sum of malate, malonate, succinate and fumarate. The concentration of tricarboxylic LMWOA was calculated as the sum of citrate and aconitate. The concentrations of mono- (gluconate), di- and tricarboxylic LMWOA (in  $\mu\text{mol plant}^{-1}$  or  $\mu\text{mol g root DW}^{-1}$ ) were multiplied with the number of carboxyl groups (1, 2 or 3, respectively; Supporting Information: Table S1) to calculate the total number of carboxyl groups in the exudate solution.

The pH in the exudate solution (including blanks) was converted into the  $\text{H}^+$  concentration, and subsequently the  $\text{H}^+$  concentration was multiplied by the volume of sterile deionized water and reconverted into pH to correct for the different volumes.

### 2.6 | Statistical analyses

Before all statistical analyses, normality was checked with Shapiro–Wilk normality test, and homogeneity of variances was tested with Levene's test. To test for significant differences ( $p < 0.05$ ) among maize in the different species combinations, we conducted an analysis of variance (ANOVA) followed by Tukey's post-hoc test (Tukey honestly significant difference) when normality and homogeneity assumptions were met, or a Kruskal–Wallis test followed by a post-hoc test using the criterium Fisher's least significant difference and Holm correction for  $p$  adjustment when normality and homogeneity assumptions were not met. To test for significant differences ( $p < 0.05$ ) of the single companions between mono- and intercropping, we conducted a Wilcoxon rank sum test. Further, we conducted simple regressions (i.e., linear models) to identify correlations between response variables. All statistical analyses were performed in R (version 3.5.2; R Core Team, 2018) using the packages agricolae (1.3-2; Mendiburu, 2020), car (3.0-7; Fox & Weisberg, 2019), dplyr (0.8.5; Wickham et al., 2020), ggpattern (0.4.2; FC et al., 2022) and ggplot2 (3.3.0; Wickham, 2016).

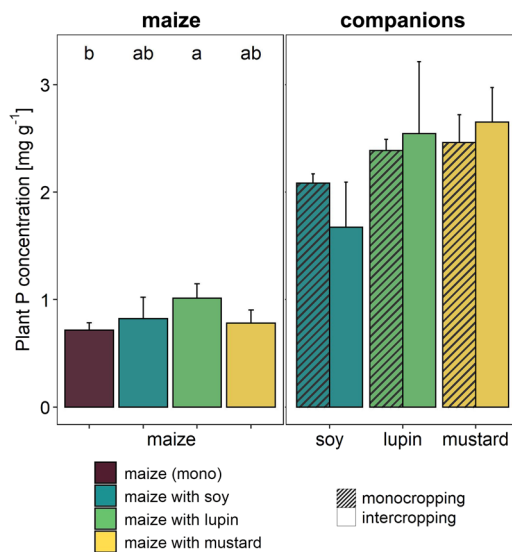
### 3 | RESULTS

#### 3.1 | Plant P and Fe

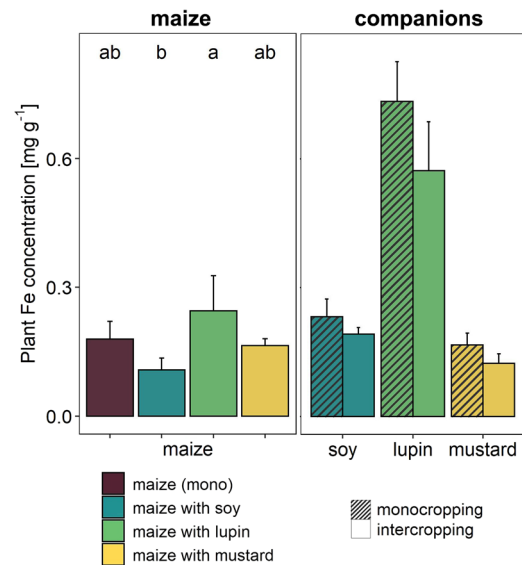
Maize P concentration was significantly increased in intercropping with lupin, by a factor of 1.4, compared to maize in monocropping ( $p = 0.045$ ; Figure 1). P concentrations of soy, lupin and mustard were higher by a factor of 2.3–3.7 compared to maize, irrespective of cropping treatment (Figure 1). Maize P content tended to be increased by a factor of 1.4 in intercropping with lupin compared

to maize monocropping, although the difference was not statistically significant ( $p = 0.187$ ; Supporting Information: Figure S1). P contents of lupin and mustard were higher by a factor of 2.4–3.5 compared to maize, irrespective of cropping treatment (Table 1; Supporting Information: Figure S1). More P was allocated in AGB than BGB by all species (Supporting Information: Tables S2–S3).

Maize Fe concentration was significantly higher in intercropping with lupin than with soy ( $p = 0.008$ ; Figure 2). It also tended to be increased by a factor of 1.4 in intercropping with lupin compared to maize monocropping, albeit not statistically



**FIGURE 1** Plant P concentration of maize (left) and companions (right). Columns show means and error bars indicate standard deviations. Different lowercase letters indicate significant differences ( $p < 0.05$ ) among maize in the different species combinations. There was no significant difference for single companions between mono- and intercropping.



**FIGURE 2** Plant Fe concentration of maize (left) and companions (right). Columns show means and error bars indicate standard deviations. Different lowercase letters indicate significant differences ( $p < 0.05$ ) among maize in the different species combinations. There was no significant difference for single companions between mono- and intercropping.

**TABLE 1** Dry weights of aboveground (AGB), belowground (BGB) and total biomass (TBM) as well as plant phosphorus content (plant P) of the four species grown in mono- and intercropping.

Species	Companion	AGB ( $\text{g plant}^{-1}$ )	BGB ( $\text{g plant}^{-1}$ )	TBM ( $\text{g plant}^{-1}$ )	Plant P ( $\text{mg plant}^{-1}$ )
Maize	Maize	$2.55 \pm 0.51$	$1.38 \pm 0.54$	$3.93 \pm 1.00$	$2.84 \pm 0.91$
	Soy	$2.81 \pm 0.84$	$1.14 \pm 0.65$	$3.95 \pm 1.49$	$3.27 \pm 1.31$
	Lupin	$3.11 \pm 0.91$	$1.50 \pm 0.50$	$4.61 \pm 1.29$	$4.56 \pm 0.95$
	Mustard	$2.68 \pm 0.89$	$1.18 \pm 0.48$	$3.86 \pm 1.35$	$3.07 \pm 1.25$
Soy	Soy	$1.87 \pm 0.41$	$0.51 \pm 0.12$	$2.38 \pm 0.53$	$4.94 \pm 0.96$
	Maize	$1.93 \pm 0.26$	$0.59 \pm 0.08$	$2.52 \pm 0.24$	$4.23 \pm 1.17$
Lupin	Lupin	$1.86 \pm 0.14$	$2.34 \pm 0.84$	$4.20 \pm 0.94$	$10.00 \pm 2.10$
	Maize	$2.49 \pm 1.93$	$1.99 \pm 1.48$	$4.48 \pm 3.39$	$10.00 \pm 4.83$
Mustard	Mustard	$2.16 \pm 0.18$	$0.67 \pm 0.04$	$2.83 \pm 0.21$	$6.92 \pm 0.26$
	Maize	$2.56 \pm 0.25$	$0.58 \pm 0.06$	$3.14 \pm 0.30$	$8.32 \pm 1.12$

Note: Numbers show means  $\pm$  standard deviations. There was no significant difference among maize in the different species combinations or for single companions between mono- and intercropping.

significantly ( $p = 0.274$ ; Figure 2). Plant Fe content of lupin was higher by a factor of 3.8 compared to maize, irrespective of cropping treatment (Supporting Information: Figure S2). More Fe was allocated in BGB than AGB by all species (Supporting Information: Tables S2–S3).

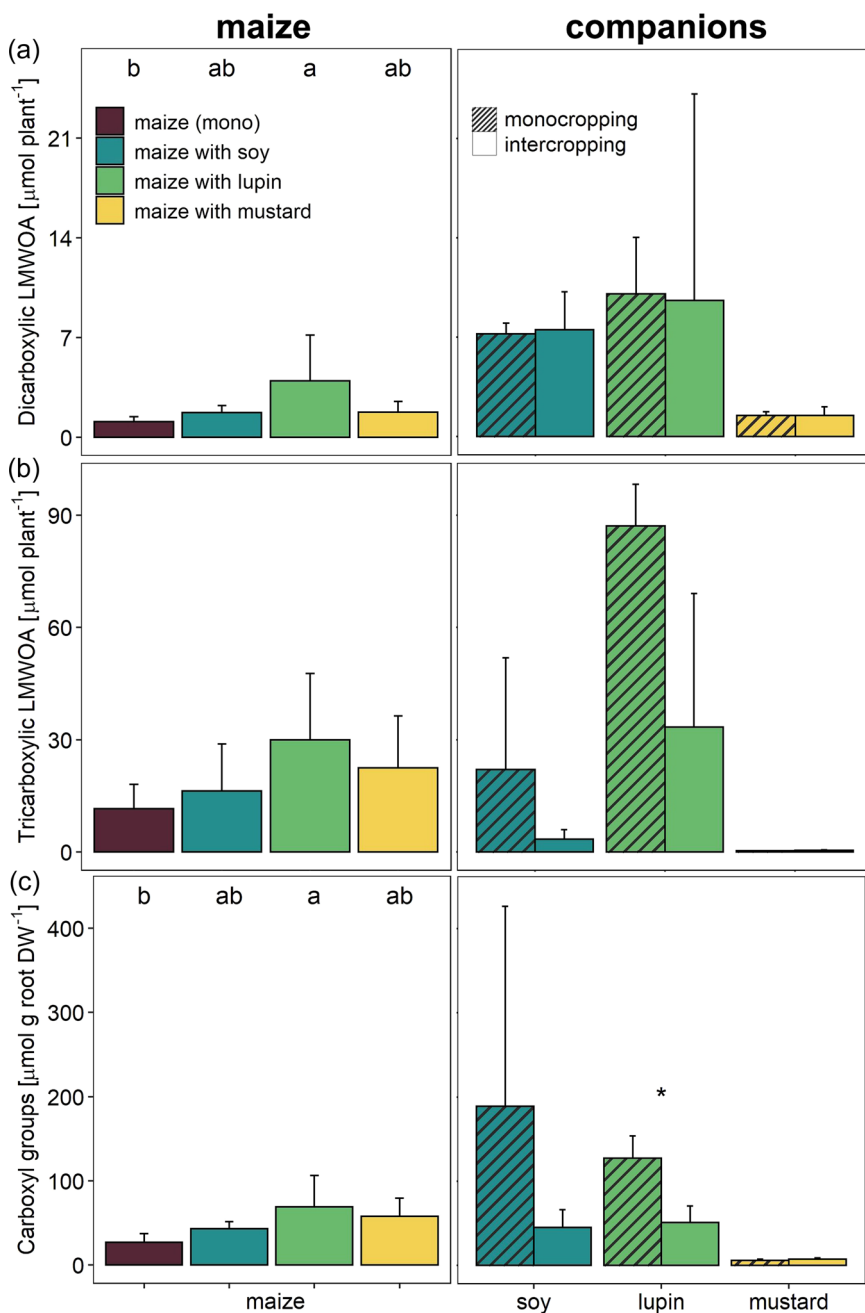
### 3.2 | Biomass

No significant difference in biomass production was found among maize in the different species combinations (Table 1). Only maize AGB and TBM in intercropping with lupin tended to be increased by a

factor of 1.2 when compared to maize monocropping, although the differences were not statistically significant ( $p = 0.765$  for AGB;  $p = 0.879$  for TBM; Table 1).

### 3.3 | Exudation of LMWOA and DOC and pH

In intercropping with lupin, dicarboxylic LMWOA exudation by maize was significantly increased by a factor of 3.6 compared to maize monocropping ( $p = 0.018$ ; Figure 3a; Table 2). In intercropping with lupin, tricarboxylic LMWOA exudation by maize tended to be increased by a factor of 2.6 compared to maize monocropping, albeit



**FIGURE 3** Dicarboxylic (a) and tricarboxylic (b) LMWOA in the exudate solution of maize (left) and companions (right), calculated per plant, as well as total number of carboxyl groups (c), calculated per gram root dry weight (see also Table 2 and Supporting Information: Table S4). Columns show means and error bars indicate standard deviations. Different lowercase letters indicate significant differences ( $p < 0.05$ ) among maize in the different species combinations, tested separately for each panel. Asterisks indicate significant differences ( $p < 0.05$ ) between mono- and intercropping of single companions. LMWOA, low molecular weight organic acid anions.

**TABLE 2** Gluconate (Glu), malate (Mal), malonate (Mao), succinate (Suc), fumarate (Fum), citrate (Cit) and aconitate (Aco) as well as the total number of carboxyl groups (COOH) and dissolved organic carbon (DOC), analyzed in the exudate solutions and calculated per plant (for data per gram root dry weight see Supporting Information: Table S4).

Species 1	Species 2	Glu ( $\mu\text{mol plant}^{-1}$ )	Mal ( $\mu\text{mol plant}^{-1}$ )	Mao ( $\mu\text{mol plant}^{-1}$ )	Suc ( $\mu\text{mol plant}^{-1}$ )	Fum ( $\mu\text{mol plant}^{-1}$ )	Cit ( $\mu\text{mol plant}^{-1}$ )	Aco ( $\mu\text{mol plant}^{-1}$ )	COOH ( $\mu\text{mol plant}^{-1}$ )	DOC ( $\text{mg plant}^{-1}$ )
Maize	Maize	0.06 ± 0.02	0.93 ± 0.29 <sup>b</sup>	0.08 ± 0.05	0.07 ± 0.03 <sup>b</sup>	0.02 ± 0.01	4.49 ± 2.94	7.09 ± 3.65	37.0 ± 20.0	2.00 ± 0.85 <sup>b</sup>
	Soy	0.09 ± 0.03	1.50 ± 0.47 <sup>ab</sup>	0.11 ± 0.02	0.10 ± 0.03 <sup>ab</sup>	0.02 ± 0.00	3.48 ± 2.40	12.89 ± 10.31	52.6 ± 38.5	3.05 ± 1.44 <sup>ab</sup>
	Lupin	0.20 ± 0.19	3.63 ± 3.14 <sup>a</sup>	0.15 ± 0.08	0.17 ± 0.05 <sup>a</sup>	0.02 ± 0.00	12.26 ± 11.84	17.68 ± 6.25	98.0 ± 59.9	5.97 ± 4.03 <sup>a</sup>
	Mustard	0.12 ± 0.04	1.54 ± 0.72 <sup>ab</sup>	0.10 ± 0.03	0.11 ± 0.03 <sup>ab</sup>	0.02 ± 0.00	7.01 ± 9.68	15.42 ± 5.68	70.9 ± 42.8	3.45 ± 1.31 <sup>ab</sup>
Soy	Soy	0.17 ± 0.05	5.68 ± 1.11	1.33 ± 0.63	0.10 ± 0.01	0.13 ± 0.02	21.98 ± 29.89	0.02 ± 0.01	80.6 ± 90.4	2.65 ± 0.67
	Maize	0.17 ± 0.02	5.53 ± 2.11	1.73 ± 0.53	0.11 ± 0.02	0.16 ± 0.06	3.04 ± 2.74	0.39 ± 0.31	25.5 ± 8.7	3.19 ± 0.59
	Lupin	0.20 ± 0.06	9.51 ± 3.87	0.28 ± 0.06	0.15 ± 0.03	0.12 ± 0.03	87.01 ± 11.14	0.05 ± 0.01	281.5 ± 39.5	5.70 ± 1.38
	Maize	0.26 ± 0.40	9.09 ± 14.17	0.28 ± 0.27	0.09 ± 0.01*	0.12 ± 0.12	32.50 ± 34.92	0.87 ± 0.88	119.5 ± 136.6	7.90 ± 12.09
Mustard	Mustard	0.04 ± 0.01	1.19 ± 0.21	0.12 ± 0.02	0.09 ± 0.02	0.08 ± 0.02	0.29 ± 0.06	0.01 ± 0.00	3.9 ± 0.7	1.13 ± 0.28
	Maize	0.04 ± 0.00	1.21 ± 0.53	0.11 ± 0.01	0.10 ± 0.03	0.08 ± 0.03	0.31 ± 0.05	0.13 ± 0.14	4.3 ± 1.1	1.05 ± 0.16

Note: Numbers show means ± standard deviations. Different lowercase letters indicate significant differences ( $p < 0.05$ ) among maize in the different species combinations. Asterisks indicate significant differences ( $p < 0.05$ ) for single companions between mono- and intercropping. The absence of letters or asterisks indicates that there was no significant difference.

not statistically significantly ( $p = 0.258$ ; Figure 3b; Table 2). The total number of carboxyl groups exuded by maize tended to be increased by a factor of 2.6 in intercropping with lupin compared to maize monocropping, albeit not statistically significantly ( $p = 0.202$ ; Table 2).

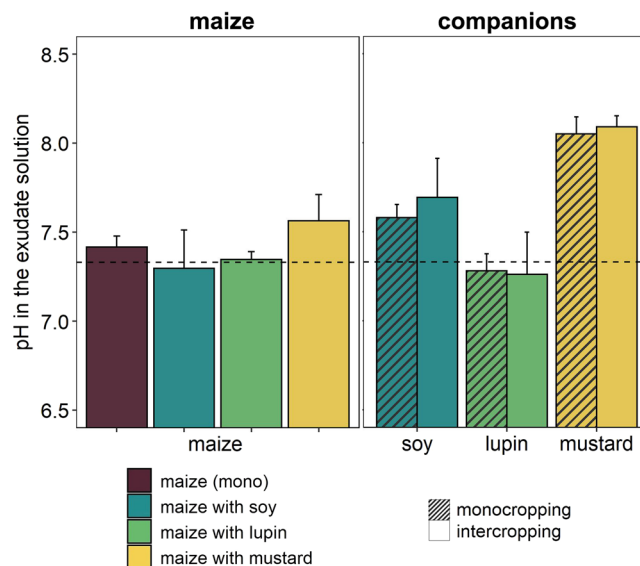
Similar trends in maize exudation were observed when LMWOA were calculated on BGB rather than plant basis (Figure 3c; Supporting Information: Table S4). The total number of carboxyl groups exuded by maize per gram root DW was significantly increased by a factor of 2.6 in intercropping with lupin compared to maize monocropping ( $p = 0.048$ ; Figure 3c). It was also slightly increased by a factor of 2.2 in intercropping with mustard compared to maize monocropping ( $p = 0.094$ ; Figure 3c). Moreover, tricarboxylic LMWOA exuded by maize per gram root DW was slightly increased by a factor of 2.6 in intercropping with lupin ( $p = 0.066$ ), and tended to be increased by a factor of 2.2 in intercropping with mustard ( $p = 0.128$ ), both compared to maize monocropping (Supporting Information: Table S4).

Maize exuded mainly aconitate and citrate, irrespective of cropping treatment. Soy in monocropping and lupin in both cropping treatments exuded mainly citrate, while soy in intercropping exuded mainly malate and citrate. Mustard exuded mainly malate, irrespective of cropping treatment (Table 2). Intercropping maize with the different companions also affected the composition of LMWOA exuded by maize. When maize was grown with lupin, the contribution of citrate and malate to the total number of carboxyl groups increased, while the contribution of aconitate decreased compared to maize monocropping. In contrast, when maize was grown with soy or mustard, the contribution of aconitate increased and the contribution of citrate decreased compared to maize monocropping (Table 2; contributions not shown).

DOC exudation of maize was significantly increased by a factor of 3.0 in intercropping with lupin compared to maize monocropping ( $p = 0.030$ ; Table 2). The pH in the exudate solution of mustard was higher by 0.6 pH units compared to all other species, irrespective of cropping treatment (Figure 4). Mustard and, to a lesser extent, soy increased the pH in the exudate solution compared to the blanks (Figure 4).

### 3.4 | Correlations

The total number of carboxyl groups exuded by maize was positively related with maize biomass, and the correlation was stronger for maize AGB ( $R^2 = 0.582$ ;  $p < 0.001$ ) and TBM ( $R^2 = 0.487$ ;  $p = 0.002$ ) than maize BGB ( $R^2 = 0.248$ ;  $p = 0.029$ ; Figure S3). We also found a positive correlation between the total number of carboxyl groups exuded by maize and the total plant P content per rhizobox which is the sum of maize P content and companion P content ( $R^2 = 0.578$ ;  $p = 0.003$ ; Supporting Information: Figure S4a). Further, maize P content increased with increasing LMWOA exudation by maize ( $R^2 = 0.407$ ;  $p = 0.005$ ; Supporting Information: Figure S4b).



**FIGURE 4** Mean pH in the exudate solution of maize (left) and companions (right). Columns show means and error bars indicate standard deviations. The dashed line shows the solution pH of blanks. There was no significant difference among maize in the different species combinations or for single companions between mono- and intercropping.

## 4 | DISCUSSION

We found that lupin caused maize to increase its root exudation and its biomass P concentration in intercropping. This is the first study to show unequivocally that a high LMWOA concentration in the rhizosphere in intercropping is not only caused by high LMWOA release of the companion but also by an increased LMWOA exudation of maize when grown together with lupin. In addition, our study suggests that P from  $\text{FePO}_4$  was likely mobilized via LMWOA exudation (particularly by lupin) and rhizosphere alkalinization (by mustard).

### 4.1 | Intercropping caused maize to increase root exudation and P concentration

We found that maize exuded generally more DOC (Table 2) and LMWOA (Figure 3) in intercropping with lupin than in maize monocropping, irrespective of the calculation basis (per plant or per g root DW) (Figure 3; Table 2 and Supporting Information: Table S4). These findings indicate that lupin caused maize to increase specific root exudates. This might, however, not be specific to lupin since DOC and LMWOA exudation by maize also tended to be enhanced when maize was grown together with mustard (compared to maize monocropping). Moreover, DOC and LMWOA exudation by maize were not significantly different among the intercropped maize plants in intercropping with lupin, soy and mustard (Table 2 and Supporting Information: Table S4; Figure 3). An increased LMWOA exudation by



maize in intercropping with lupin, faba bean or alfalfa compared to maize monocropping has been reported earlier (H. Li et al., 2010, 2013; Sun et al., 2020; L. Wang et al., 2020). However, these previous studies explained the elevated LMWOA concentrations mainly by diffusion of LMWOA from legumes to maize roots, and their analysis of rhizosphere soil did not allow a distinction between the exudates of the two different plants (H. Li et al., 2013; C. Li et al., 2016). In contrast, our results show that exudation patterns of maize changed through intercropping. Since we collected the root exudates of both species separately from different plants and not from rhizosphere soil, we can exclude that the exudates diffused from companion roots to maize roots.

The main reason for the high LMWOA exudation of maize in intercropping with lupin is likely that maize tended to produce the largest biomass in this species combination, which was likely associated with more carbon being available for LMWOA synthesis (Curl & Truelove 1986; Dechassa & Schenk 2004). This is supported by a positive correlation between maize biomass and maize LMWOA exudation (Supporting Information: Figure S3). A positive relationship between maize biomass and maize exudation has been reported earlier (Groleau-Renaud et al., 1998). However, the higher maize biomass alone does not fully explain the enhanced LMWOA exudation by maize in intercropping since the relationship between maize biomass and LMWOA exudation was not very strong ( $R^2 = 0.487$ ; Supporting Information: Figure S3). Moreover, maize TBM tended to be slightly increased only in intercropping with lupin (Table 1), whereas the LMWOA exudation by maize also tended to be slightly enhanced in intercropping with mustard (Table 2; Figure 3) where the maize biomass was not increased.

A second reason for the high LMWOA exudation of maize in intercropping was likely P (and Fe) deficiency. Maize has been found earlier to respond to P (and Fe) deficiency by exuding higher amounts of the same LMWOA for which we found the largest increases in intercropping (Carvalho et al., 2011; Gaume et al., 2001). The exudation of LMWOA is commonly increased in response to P (and Fe) deficiency by many plant species (Canarini et al., 2019; Spohn et al., 2020; Y. Wang & Lambers, 2020). Hence, the increased LMWOA exudation by maize in intercropping compared to monocropping might additionally be explained by high interspecific competition for P (and Fe) since the companion plants took up more P than maize. Lupin took up 3.5 times more P than maize (Table 1; Supporting Information: Figure S1), which likely decreased soil P availability and thus triggered increased LMWOA exudation by maize (Figure 3). Further, mustard took up 2.7 times more P than maize (Table 1; Supporting Information: Figure S1), which likely decreased P availability in the rhizoboxes and promoted the slight increase in LMWOA exudation by maize in intercropping with mustard compared to maize monocropping (Figure 3). This is also indicated by our finding that the total number of carboxyl groups exuded by maize was positively correlated with the total P content, that is, the sum of maize P content and companion P content (Supporting Information: Figure S4a). Moreover, lupin took up significantly more Fe than all other species (Supporting Information: Figure S2), which likely caused

Fe scarcity in the rhizosphere and further triggered increased LMWOA exudation by maize (Figure 3), as discussed above for P. LMWOA exudation of maize was likely more affected by lupin than mustard and soy (Figure 3) since mustard and soy took up substantially less Fe than lupin (Supporting Information: Figure S2). Thus, taken together, it might be the combined effect of improved maize growth and high interspecific competition for P (and Fe) in intercropping that caused maize to increase its root exudation in intercropping compared to maize monocropping. However, maize plants in monocropping likely also competed for P (and Fe) without affecting maize LMWOA exudation. Thus, it could also be the specific exudation profile of lupin that triggered maize to increase its exudation. Further research is, therefore, needed to examine in depth the underlying mechanisms of increased maize exudation in intercropping, and to test whether this is species-specific.

Our finding that maize P concentrations were significantly increased in intercropping with lupin compared to maize monocropping (Figure 1) indicates that maize P acquisition benefited from the presence of lupin. Similar beneficial effects of intercropping with lupin on maize biomass production and P uptake have been reported before and were related to a high LMWOA exudation by lupin that mobilized P from sparingly available P sources from which intercropped maize also benefited (Dissanayaka et al., 2017). Our results show that maize did not only benefit from the high LMWOA release by lupin, but also increased its own exudation. Direct evidence that maize P was increased due to the enhanced exudation of maize rather than of lupin cannot be provided by our pilot study. However, we found a positive correlation between LMWOA exudation by maize and maize P content (irrespective of companion species). This indicates that maize P content likely increased with an increasing number of carboxyl groups exuded by maize (Supporting Information: Figure S4b). However, the LMWOA concentration alone does not fully explain maize P contents since the relationship between maize exudation and maize P contents was not very strong ( $R^2 = 0.407$ ; Supporting Information: Figure S4b), probably because LMWOA are only efficient in P mobilization once a specific threshold concentration in the rhizosphere is reached (McKay Fletcher et al., 2021). Root intermingling might, therefore, also play an important role since intercropped species could exude LMWOA into the same soil regions. This would create even higher LMWOA concentrations in these regions causing significant P mobilization which likely results in P facilitation in intercropping (McKay Fletcher et al., 2021). Thus, the enhanced maize P acquisition from  $\text{FePO}_4$  in intercropping with lupin can potentially reduce the reliance of agricultural production on phosphate rock-derived fertilizers.

Further, our results show no intercropping effect of mustard on maize P uptake, indicating that mustard was a strong competitor for P that did not facilitate P uptake of maize. This is supported by a previous study reporting that several *Brassica* genotypes benefited from intercropping with wheat in terms of P uptake and biomass production, whereas growth and P uptake of intercropped wheat were rather negatively affected by the *Brassicaceae*, suggesting that wheat was a poorer competitor for P than the *Brassicaceae* (D. Wang et al., 2007).

Similarly, our results show no significant effect of intercropping on maize P uptake in intercropping with soy (Figure 1; Table 1). Soy took up substantially less P than lupin and mustard (except for mustard in monocropping; Table 1). We further found that soy exuded substantially less tricarboxylic LMWOA than lupin (Figure 3b), and that the rhizosphere alkalization by soy was lower than by mustard (Figure 4). This indicates that LMWOA exudation and pH changes by soy were not high enough to have beneficial effects on maize P or Fe acquisition. This is in accordance with a meta-analysis reporting a low P mobilization ability to soy (Tang et al., 2021).

## 4.2 | P mobilization by the companions

P concentrations of all companions (soy, lupin and mustard) were substantially higher than of maize (Figure 1) indicating their competitive advantage in P acquisition. Our findings suggest that lupin mobilized P from  $\text{FePO}_4$  through a high exudation of LMWOA (Figure 3). The efficiency of different LMWOA to mobilize P depends, among other factors, on the type of LMWOA (Jones, 1998; Tsado et al., 2014; Y. Wang & Lambers, 2020). Lupin released mainly citrate and, to a lesser extent, malate (Table 2), which is in accordance with earlier findings (Egle et al., 2003; Pearse et al., 2007). Both citrate and malate have been found to efficiently mobilize P from  $\text{FePO}_4$  (Dissanayaka et al., 2017; Jones, 1998), which might, at least partially, explain the high P concentration of lupin (Figure 1). This is in accordance with former experiments relating the biomass and P content of lupin to a high LMWOA exudation (Dissanayaka et al., 2017; Lelei & Onwonga, 2014). Especially the citrate exudation by white lupin has been reported to chelate metal cations ( $\text{Fe}^{3+}$ ,  $\text{Al}^{3+}$ ,  $\text{Ca}^{2+}$ ), thus increasing P availability (Cu et al., 2005; Gardner et al., 1983; Gardner & Boundy, 1983; H. Li et al., 2010).

Our findings further suggest that mustard mobilized P from  $\text{FePO}_4$  through rhizosphere alkalization (Figure 4). Since the solubility of  $\text{FePO}_4$  increases with increasing pH (Hinsinger, 2001; Lindsay, 1979), the rhizosphere alkalization by mustard likely solubilized  $\text{FePO}_4$  thereby rendering P available, which explains the relatively high biomass P concentration of mustard (Figure 1). Moreover, the rhizosphere alkalization by mustard is in accordance with former experiments showing that *Brassica* genotypes increase the rhizosphere pH resulting in enhanced P mobilization from  $\text{FePO}_4$  (Marschner et al., 2007; Pearse et al., 2007). Oilseed rape, for instance, mobilized more P from  $\text{FePO}_4$  than wheat and different legumes (Pearse et al., 2007). However,  $\text{FePO}_4$  precipitates in soil might include isomorphous substitutions which likely change the solubility compared to pure crystalline minerals which were used in the present study (Lindsay et al., 1989).

## 5 | CONCLUSIONS

Our pilot study challenges the common view that legume/cereal intercropping is advantageous over monocropping due to the high nutrient mobilization capacity of legumes (e.g., through high LMWOA

exudation) from which the cereals simply benefit. Instead, it suggests that cereals themselves increase their LMWOA exudation in intercropping. Further, we showed that maize increases its P concentration in intercropping with lupin indicating that maize P acquisition benefited from the presence of lupin, which might be associated with the increased LMWOA release of both species. Thus, our results provide new insights into the mechanisms underlying P facilitation in intercropping which should be reaffirmed with larger sample sizes and additional plant species.

## AUTHOR CONTRIBUTIONS

Ulrike Schwerdtner and Marie Spohn designed the study. Ulrike Schwerdtner performed the greenhouse experiment. Ulrike Schwerdtner and Ulrike Lacher performed plant and exudate analyses. Ulrike Schwerdtner wrote a first draft of the manuscript, Ulrike Schwerdtner and Marie Spohn worked on the manuscript. All authors read, contributed to, and approved the final version of the manuscript.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

The data generated and analyzed during the current study are available from the corresponding author on reasonable request.

## ETHICS STATEMENT

The authors confirm that they have adhered to the ethical policies of the journal.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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