Plant Soil (2021) 459:277–288
https://doi.org/10.1007/s11104-020-04764-1

REGULAR ARTICLE

The adsorbent capacity of growing media does not constrain *myo*-inositol hexakiphosphate hydrolysis but its use as a phosphorus source by plants

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Received: 15 February 2020 / Accepted: 2 November 2020 / Published online: 9 November 2020

Abstract
Aims The hydrolysis of organic P in soils is a relevant aspect contributing to the supply P to plants, which is affected by adsorbent capacity and biological properties of soils. This work aimed at studying the contribution of phytate to plant nutrition as affected by Fe oxides and phosphohydrolases releasing microorganisms in the growing medium.
Methods An experiment with cucumber and *myo*-inositol hexakiphosphate (*myo*-Ins6P) as P source was performed involving two factors: Fe oxide –ferrihydrite– rates (0, 100, 300 mg kg^{-1} of citrate–ascorbate extractable Fe), and microbial inoculation (*Trichoderma asperellum* T34, *Bacillus subtilis* QST713, and non-inoculated).
Results P uptake decreased with increased Fe oxides in the growing media. Phytase activity and organic anions concentration increased with increased Fe oxides in the media. Most of the P supplied was recovered as inorganic P at the highest Fe oxide concentration. Inoculants did not improve P uptake by plants, despite *B. subtilis* promoted an enhanced hydrolytic activity at the highest Fe oxide concentration.
Conclusions An increased adsorption capacity of the growing media restricts the use of *myo*-Ins6P as P source by plants. This was not the result of its stabilization through adsorption or a decreased hydrolytic activity, but of the adsorption of inorganic P on Fe oxides after hydrolysis.

Keywords Iron oxides · Microbial inoculants · Phytase · Phosphatase

Responsible Editor: Tim S. George.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11104-020-04764-1.

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Introduction

Phosphorus (P) is currently deemed a critical raw material for agriculture due to the limited and concentrated rock phosphate reserves (Sattari et al. 2012; Stutter et al. 2012; Faucon et al. 2015). However, P fertilization is particularly inefficient due to its reactions in soils (Hinsinger 2001; Gichangi et al. 2009; Khan and Joergensen 2009). This explains the excessive P fertilization for years, leading to an accumulation of the nutrient in soils known as legacy-P (Kleinman et al. 2015). Most of this legacy-P is poorly available to plants in particular the sizeable portion corresponding to organic P (OP) since part of applied P is incorporated into organic compounds (Stutter et al. 2012). The use by crops of this legacy-P is a crucial issue in order to reduce the dependency on mined P fertilizers (Giles et al. 2012; García-López et al. 2015; Rowe et al. 2016). On the other hand, strategies for P recycling in agriculture that lead to reducing the dependence on mined raw materials (Metson et al. 2016) will involve an increasing trend

towards the application of recycled organic materials as fertilizers that will contribute to an increased OP concentration in soils. All this reveals the need of better understanding of soils processes affecting the potential bioavailability of major organic P forms in soils.

The organic P fraction in soils is composed of different compounds that differ in solubility and bioavailability. Most organic P remains in the soil in the form of phosphate monoesters (Bol et al. 2016; Missong et al. 2016; Recena et al. 2018). Between these forms, inositol-6-phosphate (InsP6) stereoisomers are the dominant compounds. These monoesters form strong ligands with sorbent surfaces and polyvalent cations (Turner et al. 2002; Vohra and Satyanarayana 2003; Menezes-Blackburn et al. 2013; Celi et al. 2020). Consequently, insoluble Ca-, Fe-, and Al-InsP6 are continuously forming and accumulating in soil (Shang et al. 1990; Ognalaga et al. 1994; Celi et al. 1999; Giaveno et al. 2010).

The bioavailability of OP relies on the mineralization by phosphohydrolase enzymes produced by plants and microorganisms. Within this group of enzymes, phytases catalyse the hydrolysis of *myo*-inositol hexakisphosphate (*myo*-InsP6) to *myo*-inositol pentakisphosphate (*myo*-InsP5) or to lower phosphorylated *myo*-inositol phosphates (*myo*-InsP1 to *myo*-InsP4). Phytase activity may be decreased by both InsP6 and phytase adsorption on mineral surfaces; (George et al. 2004, 2007; Tang et al. 2006; Lung and Lim 2006). However, it was proved that phytase may act not only in solution, but also after adsorption to soil minerals (George et al. 2007; Giaveno et al. 2010; Yang and Chen 2017). There are however little direct evidences about how the potential adsorption of Ins6P in soil affects its use as P source by plants.

The use of Ins6P by plants depends on the exudation of organic anions, able to promote its desorption from sorbent surfaces, and phytase by roots (Richardson et al. 2000; Martin et al. 2004; Giles et al. 2012). Some rhizospheric microorganisms may contribute to improve the availability of P to plants by different mechanisms including the production of phytases (Martin 1973; Owen et al. 2015; Singh et al. 2020). Thus, the inoculation with these P-mobilizing microorganisms may be a strategy for improving the P supply to plants (Macklon et al. 1997; Richardson 2001; Richardson et al. 2005; Patel et al. 2011; Richardson et al. 2011; Balwani et al. 2017). It has been demonstrated that *Bacillus* spp. and *Trichoderma* spp. may be able to increase the use of Ins6P as P source by plants due to the production of organic anions and phytase (Fu et al. 2008; García-López et al. 2015, 2016; García-López and Delgado 2016). However, there are little evidences how these microorganisms may act in the presence of soil solid surfaces that can immobilize both InsP6 and hydrolytic enzymes.

In order to better understand the potential contribution of Ins6P, as major organic P form in soil, to plant nutrition, the main objectives defined in this work were the assessment of: (i) the uptake of P by plants from *myo*-InsP6; (ii) the effect of Fe oxides on phytase hydrolytic activity and P uptake by plants, and (iii) how phytase and phosphatase releasing microorganisms may affect P uptake by plants from phytate depending on the concentration of Fe oxides present in the growing medium. The uptake of Fe and Zn in plants was also studied since both nutrients may be affected by Fe oxide concentration and microbial inoculation, and both micronutrients may show an antagonistic effect with P.

Material and methods

Experimental setup

A factorial experiment with cucumber was performed. The experiment followed a completely randomized design and involved five replications and two factors:

(i) Fe–oxide concentration in the growing media (0, 100 or 300 mg kg^{-1} of citrate–ascorbate-extractable Fe), and
(ii) (ii) inoculation with microorganisms (non-inoculation, *Bacillus subtilis* QST713 and *Trichoderma asperellum* T34).

This design implies that inoculation treatments were tested at all Fe-oxide rates and allows us to study the variation ascribed to each factor, and to the interaction between both factors.

The growing medium was siliceous sand, which was sieved between 0.5- and 1-mm in order to ensure adequate aeration and hydraulic conductivity. After sieving, it was washed several times with 0.2 M Na_2CO_3 in order to disperse and remove impurities. Ferrihydrite was used as Fe oxide in the growth media (de Santiago et al. 2011). To this end siliceous sand coated with this oxide was prepared according to the procedure of Rahmatullah and Torrent (2000). The different Fe oxide

rates were achieved by including different proportions of Fe oxide coated siliceous sand in the media. The pH of the growing media was 7.5.

Cucumber plants (*Cucumis sativus* L. 'Serenade') were pot-grown in a growth chamber during 30 days after transplanting with a photoperiod of 14 h d^{-1} at a light intensity > ~ 300 μmol m^{-2} s^{-1}, a temperature of 25 °C (day) and 23 °C (night), and 65% relative humidity. Previously, seeds were germinated in peat and 15 days after germination, at the two true-leaf stage, one plant was transplanted into each pot. Polystyrene cylinders with c.a. 350 mL of volume (5.5 cm diameter and 15 cm height) were used as pots. Each pot contained 0.4 kg of growing medium. Phosphorus rate was 50 mg kg^{-1} growing media, and it was supplied in the form of phytate by irrigation with 7 mM Na *myo*-inositol hexakisphosphate (*myo*-InsP6) along the crop cycle distributed in 15 irrigations. The other essential nutrients were supplied through irrigation with a P-free Hoagland-type nutrient solution with the following composition (all concentrations in mmol L^{-1}): $MgSO_4$ (2), $Ca(NO_3)_2$ (5), KNO_3 (5), KCl (0.05), Fe- EDDHA (0.02), H_3BO_3 (0.024), $MnCl_2$ (0.0023), $CuSO_4$ (0.0005), $ZnSO_4$ (0.006), and H_2MoO4 (0.0005). pH of the nutrient solution was adjusted to 6 before irrigation. At the end of the experiment, a total of 260 mL of the nutrient solution and 16.7 mL of *myo*-InsP6 solution were applied per pot.

Inoculation with *Trichoderma asperellum* (T34) (Biocontrol Technologies, Barcelona Spain) were performed according to de Santiago et al. (2009) using conidia suspensions that were prepared according to Segarra et al. (2007). Before transplanting in pots, plant roots were immersed in a suspension of water containing 10^3 conidia per mL. In addition, after transplanting, 20 mL of a conidia suspension (2×10^5 conidia per ml) was applied to the surface of the growing medium in each pot at four points near plants (at 1 cm around the plant shoot). After both steps of inoculation, the total inoculum amounted to 10^4 conidia per g of growing media. Plant inoculation with *Bacillus subtilis* strain QST713 (Serenade Max, Bayer CropScience, Paterna, Spain) was carried out by applying 2×10^7 colony forming units (CFU) per kg of growing medium after transplanting. This was done by applying 20 mL of aqueous suspension containing 4×10^8 CFU L^{-1} on the surface of the growing medium in each pot at different points around the plants as described by García-López and Delgado (2016).

Plants analysis

After 30 days, the aerial part of the plant was harvested. Immediately after harvesting, the rhizospheric growing media was sampled according to Zhou and Wu (2012), collecting the sand adhered to the roots by shaking it off. Shoots and roots were dried at 65 °C for 48 h until constant weight, and the dry biomass (DM) determined. The dry plant material was ground, and an aliquot of 0.25 g mineralized in a furnace at 550 °C for 8 h. The ashes were dissolved in 1 M HCl by heating at 100 °C for 15 min. In the resulting solution, Fe and Zn were determined by atomic absorption spectrophotometry and P by the molybdate blue method (Murphy and Riley 1962). Certified plant material was analysed to check the complete recovery of P, Zn and Fe with this procedure. P uptake was calculated as the sum of shoot and root P after subtraction of total P contained in the seeds. P concentration in a representative sample of seeds was determined as described above for plant material.

Growing media analysis after cultivation

Enzymatic activities were determined in the rhizospheric growing media after harvest. Alkaline phosphatase activity was determined by measuring the amount of *p*-nitrophenol (PNP) released from the addition of 5 mM *p*-nitrophenylphosphate according to Tabatabai and Bremner (1969). Acid phytase activity in the growing media was determined by incubating the growing media with *myo*-inositol hexakisphosphate added as substrate for the enzyme in 2-(N-morpholino) ethanesulfonic acid (MES) buffer at a volume ratio of 1:1 at 37 °C for 60 min. The final concentrations in the assay were 15 mM MES and 2 mM *myo*-inositol hexakisphosphate, and the pH 5.5. The reaction was stopped with 10% trichloroacetic acid and the suspensions centrifuged at 10000 g for 10 min. After that, molybdate reactive P was determined in supernatants according to Murphy and Riley (1962) and phytase activity was expressed in enzyme units (amount of enzyme which releases one micromole of inorganic phosphate from *myo*-inositol hexakisphosphate per minute) per g of growing media. Since P may be adsorbed on Fe oxides, it is necessary to correct the concentration of released molybdate reactive P by an estimate of the fraction of released phosphate that is adsorbed in the medium during the hydrolysis assay. To

this end, P sorption in the plant growing media during the hydrolysis of *myo*-inositol hexakisphosphate was assessed by using controls where this organic P was replaced by KH_2PO_4 solution at 4.2 mg P L^{-1}. The fraction of this added phosphate that remains in solution allows us to estimate the fraction of hydrolysed phosphate recovered after the phytase activity assay (George et al. 2005). The average recovery for the three Fe oxides rates of added phosphate was 114% without differences between Fe oxides rates. This allows one to assume a negligible adsorption of hydrolysed phosphate due to a high saturation of Fe oxides by P. Although this phytase determination method has uncertainties such as the recovery of hydrolysed P, the pH and ionic composition different to that in the growing media, and the difference characteristics of phytases in plants and inoculants, one can assume that it will allow the assessment of the effect of Fe oxides on the hydrolytic potential of growing media.

Colony forming units (CFU) of both inoculants were determined after harvest with pyrophosphate extraction of the rhizospheric growing media. *Trichoderma spp.* CFUs were determined by dilution plating according to Chung and Hoitink (1990) using a semi-selective medium (Borrero et al. 2012). This medium has proved to be effective to measure the CFU of T34 in soil samples (de Santiago et al. 2013). *Bacillus spp*. were isolated on a nutrient–agar medium after heating the suspension at 80 °C for 10 min, according to Tuitert et al. (1998). Three plates per dilution ratio were used, and CFU were counted after 4 days. No CFUs were detected in the control treatment. In these non-inoculated pots, other *Bacillus spp.* were present, but not with the characteristic colony morphogenesis of *B. subtilis*. The density of CFU in the suspensions used for inoculation was also checked using the same procedure.

Low molecular weight organic acids in the rizospheric growing media were extracted by shaking 5 g of rhizospheric soil in 5 mL 0.1 M NaOH for 1.5 h at 4 s^{-1} (Baziramakenga et al. 1995; Radersma and Grierson 2004). The supernatant was centrifuged at 10,000 g for 10 min, filtered through a 0.45-μm cellulose filter, and the filtrate acidified to pH 2–3 with 1 M H_2SO_4. High-performance liquid chromatographic (HPLC) separation of organic acids was performed with an HPLC Varian ProStar 410 equipped with a C18 column (Varian, 250 mm × 34.6 mm, and 8 μm particle size). Elution was isocratic with 98% 5 mM H_2SO_4 at pH 2 + 2% methanol as the carrier solution at a flow rate of 0.8 mL min^{-1}, and 20 μL of injection volume. Organic anions were detected at 215 nm using a Varian 486 photo-diode array detector.

The pH of the rhizospheric growing medium was determined after extraction with water in a 1:2.5 suspension. Inorganic P in the rhizospheric growing medium after cropping was determined according Murphy and Riley (1962) after a two-step sequential extraction with 0.1 M NaOH +1 M NaCl and 1 M HCl. The first extraction step was intended to desorb P from Fe oxides, and the second step to release the remaining P —mostly precipitated P. The sequential extraction was performed on duplicate, at a 1:40 growing medium:extractant ratio, shaking at 3 s^{-1} for 17 h in an end-over-end shaker. After extraction, supernatants were obtained after centrifugation at 900 *g* during 10 min and analysed for molybdate reactive P according to Murphy and Riley (1962). P extracted with this sequential fractionation is essentially ascribed to the hydrolysis of applied *myo*-InsP6, which was the only source of P in the growing media.

Statistical analysis

An analysis of variance (ANOVA) was performed using the general linear model procedure in Statgraphics Centurion XVI (StatPoint 2013). Previously, normal distribution and homoscedasticity were assessed by the Shapiro-Wilk and Levenne tests, respectively. A two-way ANOVA was performed to study the effect of Fe rates and inoculation microorganisms, which were considered fixed factors, on studied variables. This model allows us to assess the effect of main factors and their interactions. When interactions were not significant, the effect of one factor did not depend on the level of the other factor. In this case, mean comparison for the different levels of the significant factor should be performed (Seltman 2018). When interactions were significant the effect of main factors cannot be assessed since the effect of one factor depends on the level of the other. In this case, a one-way ANOVA with the combination of both factors was performed since (de Santiago et al. 2013). Mean comparison was performed according to the Tukey test ($P < 0.05$). Regressions were also performed by using the same software.

Results

Iron oxides in the growing media significantly affected dry biomass (DM) production, either shoots, roots or

total (Fig. 1 and Table S1). Dry biomass in all the plant organs significantly decreased with increased concentration of Fe oxides in the growing media. Moreover, the total amount of P in plants, the concentration of P in aerial parts, total Fe in roots, the concentration of Fe in roots and shoots, and total Zn in shoots were affected by Fe oxide rate. The total P accumulation in shoots and roots, and P uptake also decreased with increased Fe oxides in the growing media (Tables 1 and S1). On the other hand, Fe concentration in plant organs increased with increased Fe oxide concentration, while only the lowest Fe oxide concentration promoted total Fe in roots significantly higher than in control without Fe oxides (Table 1 and S1). Nevertheless, non-significant differences in total Fe in shoots and roots for the two levels of Fe oxides in the growing media were observed. Zn accumulation decreased significantly at the highest Fe oxide concentration when compared with control without Fe oxides (Table 1).

The effect of Fe oxides on inorganic P in the growing media estimated as the sum of two consecutives extractions (0.1 M NaOH +1 M NaCl and 1 M HCl) after crop increased at the highest rate of ferrihydrite when compared with medium without Fe oxides (Table 1). With this Fe oxide concentration, sequential extraction recovered as inorganic P (molibdate reactive phosphorus –MRP) around 80% of P supplied as phytate, most recovered in the first fractionation step (65% of total recovery; data not shown). In the medium without Fe oxides and in that with

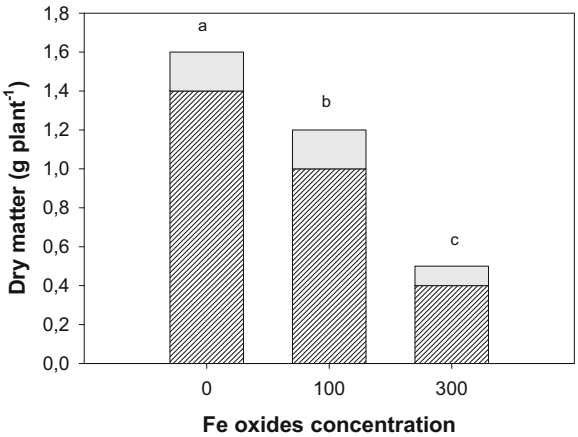

Fig. 1 Effect of the different Fe oxides concentration on dry matter (DM) in shoots and roots of cucumber plants. In each column, grey color corresponds to DM in roots and grated to DM in shoots. The effect of Fe oxide rate was significant according to ANOVA ($P < 0.0001$). Means with different letter were significantly different according to the Tukey's test at $P < 0.05$

100 mg Fe kg^{-1} this recovery amounted to 20 and 40% of the supplied P, respectively (Table 1). Phytase activity only increased significantly (by three times) in the medium with the highest Fe oxide concentration relative to the medium without Fe oxides (Fig. 2a). This activity was not affected by inoculation with microorganisms. Iron oxides in the growing media significantly increased the accumulation of organic acids in the rizhosphere, without differences between 100 and 300 mg Fe kg^{-1} (Fig. 2b). The highest pH was observed at 100 mg Fe kg^{-1}, and the lowest at 300 mg Fe kg^{-1} (Fig. 2c). Significant interactions between the two factors ($p = 0.0177$), inoculation and Fe oxides, were observed for phosphatase activity since it only increased with the simultaneous application of *B. subtilis* and Fe oxides at 300 mg Fe kg^{-1} in growing media (Fig. 3). Although colony forming units were observed in the rhizosphere after harvest (Table 1), overall, inoculation had non-significant effects on studied variables.

Inorganic P in the growing media at harvest explained 91% of variation in the P uptake by plants ($P < 0.001$; Fig. 4a); this uptake, however decreased with increasing inorganic P in the media. Phosphorus uptake by plants also decreased linearly with increased Fe concentration in plants ($Y = 4–0.8X$; $R^2 = 0.91$; $P < 0.001$). Phosphorus uptake by plants decreased with increased phosphatase activity in the rhizosphere at harvest (Fig. 4b). However, in the growing medium with the highest Fe oxide concentration, increased phosphatase activity did not correspond to a decreased P uptake. As observed for phosphatase, P uptake decreased with increased phytase activity in the rhizosphere (Fig. 4c). On the other hand, the inorganic P in the growing media increased linearly with increased phytase activity in the rhizosphere (Fig. 4d).

Zinc concentration in shoots decreased with increased inorganic P in the growing media at harvest ($Y = 13–0.1\ X$; $R^2 = 0.75$; $P < 0.005$). However, this relationship was different depending on the inoculation, it being more significant in the case of T34 ($R^2 = 0.99$; $P < 0.05$; not shown).

Discussion

The applied *myo*-InsP6 was used as P source by plants, in agreement with previous evidences in quartz sand without oxides (Adams and Pate 1992). P concentrations in plant tissues and P uptake were similar to those in other studies using inorganic phosphate in similar

Table 1 Effect of the main factors on concentration and total content of P, Fe and Zn in plant, MRP and CFUs in the growing media after crop

Source of variation	Plants										Growing media		
	P concentration		Total P			Fe concentation		Total Fe		Total Zn	MRP	CFU T 34	CFU *B. subtilis*
	Shoots	Roots	Shoots	Roots	P uptake[a]	Shoots	Roots	Shoots	Roots	Shoots			
	g kg^{-1}		mg plant^{-1}			mg kg^{-1}	g kg^{-1}	µg plant^{-1}	mg plant^{-1}	µg plant^{-1}	mg kg^{-1}		
Fe rate (mg kg^{-1})													
0	2.4 b	3.5	3.2 a	0.6 a	3.8 a	65 b	0.4 c	91	0.1 b	11 a	12 b	7062	813,788
100	2.3 b	3.7	2.3 b	0.5 a	2.8 b	85 b	1.4 b	82	0.2 a	10 ab	21 b	5955	863,173
300	3.6 a	4.6	1.0 c	0.2 b	1.1 c	239 a	2.7 a	63	0.1 ab	7 b	41 a	6095	603,725
Inoculation													
B. subtilis	3.0	4.2	2.1	0.5	2.3	143	1.3	71	0.1	9	31	–	–
T 34	2.8	3.4	2.5	0.5	2.8	143	1.6	89	0.1	10	22	–	–
Control	2.6	4.1	2.0	0.4	2.4	110	1.5	76	0.1	10	22	–	–

Means $n = 15$ for Fe rate, and 15 for inoculation

MRP phosphorous molibdate reactive, *CFU* colony forming units

Only effects of factors have been considered when the factor was not involved in a significant interaction

Means followed by different letters within a column are significantly different according to the Tukey test ($P < 0.05$) for each factor

[a] Estimated as the total P in aerial parts and roots minus P present in seeds

experimental setups with the same crop (e.g. García-López et al. 2015). Without Fe oxides, the P uptake by plants (3.8 mg per plant) accounted for around 20% of P applied as *myo*-InsP6. This reveals a significant hydrolysis of *myo*-InsP6 in the growing media. However, this hydrolysis was unaffected by microbial inoculation, likely reveling the capacity of plant phytases to hydrolyze these organic P compounds (Hayes et al. 1999; George et al. 2004). The lack of effect of microbial inoculants cannot be ascribed to a failure in the rhizosphere colonization since significant CFUs were detected at harvest (Table 1).

Overall, Fe oxides in the growing media strongly and negatively affected plant development and P uptake. The decreased DM yield with Fe oxides should be mainly ascribed to a decreased P availability to plants. Although there was not a decrease in P concentration in plant tissues, total P in plants and P uptake decreased with increasing Fe oxides in the media by the same magnitude as for total DM yield (Fig. 1). This reveals that P availability in the growing media decreased, but the decreased DM accumulation did not lead to decreased P concentration in plant tissues (even there was an increase at the highest Fe oxide concentration in the growing media). It is well-known that part of adsorbed P on Fe oxides remains unavailable to plants (Delgado and Scalenghe 2008). Thus, Fe oxides considerably reduced the efficiency of applied P (García-López and Delgado 2016). In our case, P source was added as *myo*-InsP6 that is adsorbed on ferrihydrite (Celi et al. 2003). It is assumed that this adsorption protects *myo*-InsP6 from enzymatic hydrolysis leading to its accumulation in soil (Stutter et al. 2015) and its decreased use as P source by plants. However, our results contradict this assumption since significant amounts of inorganic P were recovered from the growing media after harvest with the sequential chemical fractionation which is assumed to release most of the inorganic P retained in the media (Table 1). In particular, most of the P applied as *myo*-Ins6P was recovered as inorganic P at the highest Fe oxide rate in the growing media. Despite this evident hydrolysis, most of the inorganic P in the media was not available to plants due to its adsorption on oxides after hydrolysis. The reduced availability of adsorbed inorganic P was ascribed to the initial negligible saturation of sorbent surfaces by P, which implies that a significant portion of P was adsorbed on high-energy sites (Shao et al. 2006).

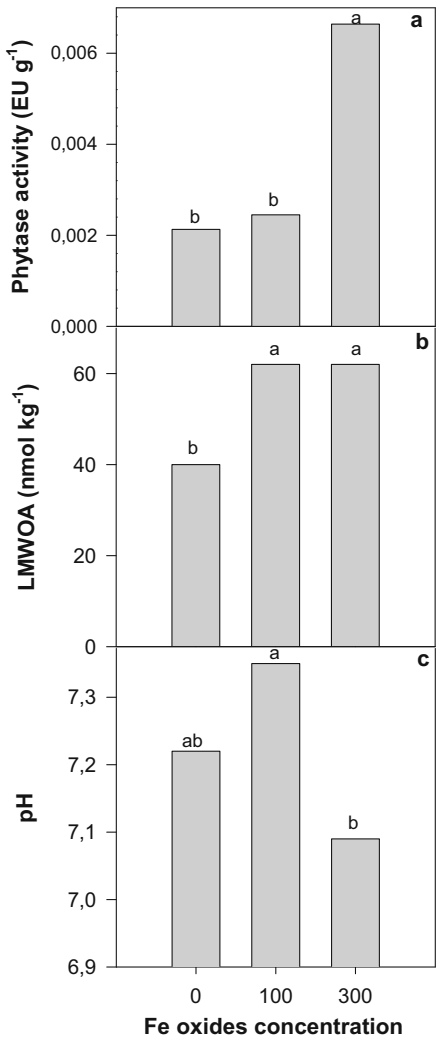

Fig. 2 **a** Effect of the different Fe oxides concentration on phytase activity, significant according to ANOVA at $P = 0.0084$, **b** on low molecular weight organic acid (LMWOA), significant according to ANOVA at $P < 0.0001$, **c** on pH in growing media after crop, significant according to ANOVA at $P = 0.011$. Means with different letter were significantly different according to the Tukey's test at $P < 0.05$

The recovery observed for inorganic P was congruent with phytase activity. Although the pH at which the phytase assay was performed and the potential adsorption of hydrolyzed P may provoke artifacts in the estimation of the real phytase activity in the media, this assay allowed us to explain the concentration of inorganic P present in the media at the end of the experiment. It should be remarked that the recovery of inorganic P added as tracer in the phytase assay was complete, as the likely consequence of a high degree of saturation by P of Fe oxides at the end of the experiment.

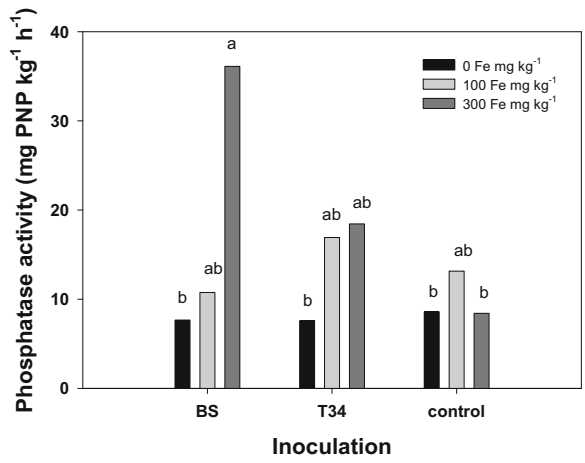

Fig. 3 Effect of the interaction between Fe oxide rate and inoculation on phosphatase activity, which was significant according to ANOVA at $P = 0.0177$. Means with different letter were significantly different according to the Tukey's test at $P < 0.05$. PNP, p-nitrophenol released

This reduces the risks of lack of accuracy in the comparisons of phytase activity between different Fe oxides rates. The highest phytase activity and inorganic P recovery were observed in the medium with the highest Fe oxide concentration (Table 1). On the other hand, the presence of Fe oxides led to an increased organic anion concentration in the media. Microorganisms and plants exudate organic anions in response to P scarcity in the growing medium (Hocking 2001; Ryan et al. 2001). Thus, conditions prone to P deficiency due to the adsorption of P on Fe oxides triggered the P mobilization mechanisms by plants and microorganisms. Under these conditions, there was a significant hydrolysis of phytate due to the increased hydrolytic activity in the media. This is evidenced by the relationship between the inorganic P recovered at the end of the experiment and phytase activity in the media (Fig. 4d). In addition to this increased hydrolytic activity, organic anions such as citrate increases the hydrolysis of InsP6 by competition for sorbent sites; this promotes desorption of InsP6 and facilitates the enzyme-substrate interaction (Mezeli et al. 2017; Celi et al. 2020). Organic anions do not have any interaction with adsorbed enzymes which may lead to an increased hydrolytic activity in the solution (Mezeli et al. 2017). Organic anions may also complex Fe facilitating the dissolution of Fe oxides and the release of adsorbed Ins6P (Celi et al. 2020). Thus, hydrolysis of adsorbed Ins6P depends to some extent on the release of organic anions by plants and microorganisms; this promotes the desorption of Ins6P making it available for hydrolysis.

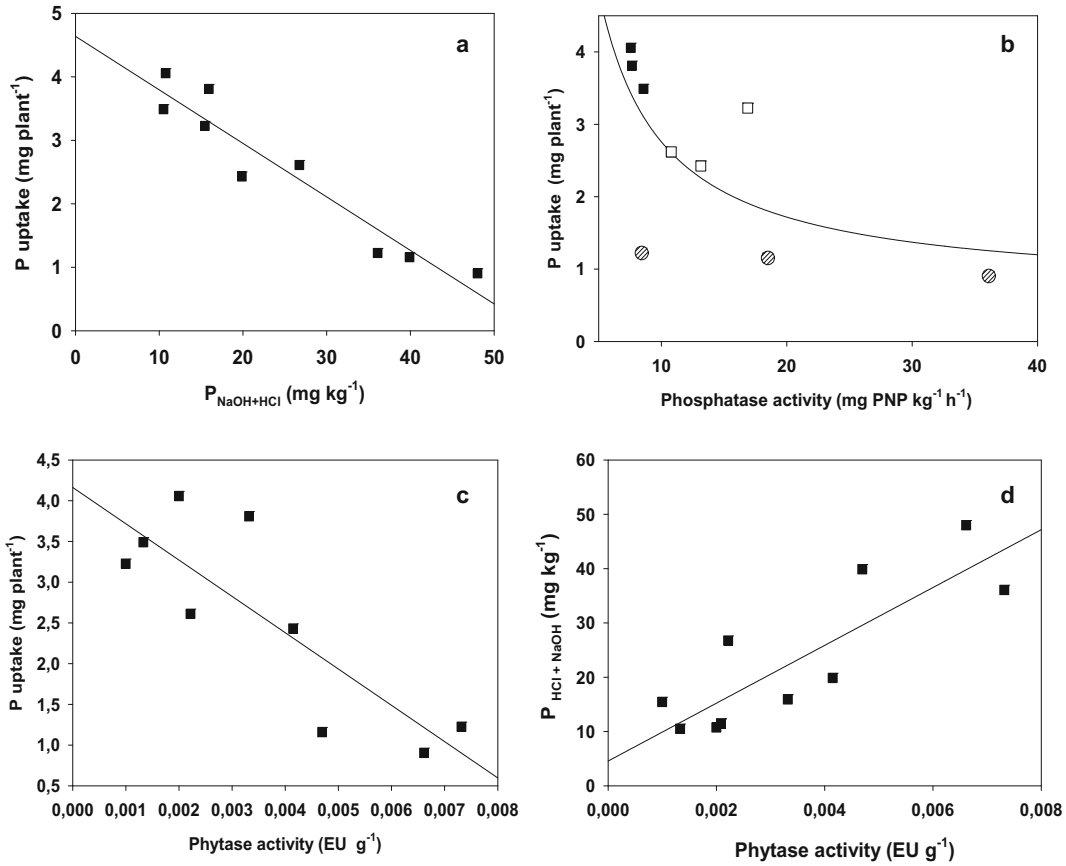

Fig. 4 **a** Relationship between P uptake by plants and molybdate reactive P in the growing media ($P_{NaOH+HCl}$) at the end of the experiment (Y = 4.6–0.1 X; $R^2 = 0.91$; $P < 0.001$); $P_{NaOH+HCl}$ is the sum of the P extracted with a sequential extraction involving 0.1 M NaOH +1 M NaCl and 1 M HCl. Each point corresponded to the mean of the five replications for each combination of the two factors (Fe oxide concentration and microbial inoculation). **b** Relationship between P uptake by plants and phosphatase activity in the growing media at the end of the experiment (Y = 1/[0.2 + 0.03 X]; $R^2 = 0.5$; $P < 0.001$). Black symbol, 0 mg Fe kg^{-1}; empty symbol, 100 mg Fe kg^{-1} and striped symbol, 300 mg Fe kg^{-1}. Each point corresponded to the mean of the five replications for each combination of the two factors (Fe oxide concentration and microbial inoculation). **c** Relationship between P uptake by plants and phytase activity in the growing media at the end of the experiment (Y = 4.1 + 4.5 × 10–2 X; $R^2 = 0.7$; $P < 0.005$). EU, enzymatic units, amount of enzyme which releases one micromole of inorganic phosphate from *myo*-Ins6P per minute. Each point corresponded to the mean of the five replications for each combination of the two factors (Fe oxide concentration and microbial inoculation). **d** Relationship between molybdate reactive P in the growing media ($P_{NaOH+HCl}$) and phytase activity in the growing media at the end of the experiment (Y = 4.6 + 5.3103 X; $R^2 = 0.71$; $P < 0.005$). $P_{NaOH+HCl}$ is the sum of the P extracted with a sequential extraction involving 0.1 M NaOH +1 M NaCl and 1 M HCl. Each point corresponded to the mean of the five replications for each combination of the two factors (Fe oxide concentration and microbial inoculation)

The increased hydrolysis of Ins6P in media with Fe oxides, however, did not lead to an increased P uptake due to the adsorption of released inorganic P on Fe oxides as mentioned above. All this may explain the apparent contradiction of a decreased P uptake by plants with increased inorganic P in the growing media (Fig. 4a). In addition, this increased phytase activity with increased P sorption capacity in the media and the adsorption of released inorganic P on oxides also explained the decreased P uptake with increased hydrolytic activity in the growing media (Fig. 4b and c).

Our results agree with evidences suggesting that phytases may be active after adsorption (Mezeli et al. 2017; Yang and Chen 2017). Their adsorption on soil minerals may decrease the activity of the enzymes (George et al. 2005). However, the loss of phytase activity depends on the type of mineral, with clay minerals inhibiting more the activity than Fe oxides (Giaveno et al. 2010). This may be ascribed to a greater

modification of enzymes conformation when adsorbed on clay minerals than when adsorbed on Fe-oxides (Quiquampoix 1987). To some extent, this contributes to explain the hydrolytic activity observed in our media with Fe oxides. However, at least part of the phytase activity may be ascribed to the liquid phase. When sorbent surfaces are saturated with P, the partitioning of enzyme activity between the solution and the solid phase shifts towards the solution phase (Giaveno et al. 2010), since mineral surfaces are occupied by the substrate or by the hydrolyzed inorganic P. As mentioned above, it is assumed a high saturation of Fe oxides by released inorganic P which may decrease phytase adsorption. Furthermore, the amounts of P added as *myo*-Ins6P were enough to saturate the adsorption capacity ferrihydrite assuming an adsorption capacity around 2.5 μmol m^{-2}, and a typical specific surface in synthetic ferrihydrite between 200 and 400 m^2 g^{-1} (Gimsing and Borggaard 2007; Wang et al. 2013). Thus, it may be assumed a relevant phytase activity in solution.

In spite of the supply of iron (Fe-EDDHA) with the nutrient solution to avoid Fe deficiency, Fe concentration in shoots and roots increased with ferrihydrite in the growing media (Table 1). This oxide is known to be a source of Fe for plants (de Santiago and Delgado 2007). The exudation of organic anions may contribute to Fe uptake by plants (García-López et al. 2015) through the formation of plant-available organic-Fe^{3+} complexes in the rhizosphere (Jones et al. 1996). The release of organic anions may also contribute to the release and uptake of P by plants (García-López and Delgado 2016). However, P uptake decreased with increased Fe in plants. This may be explained by two mechanisms: (i) the known antagonistic effect between both nutrients, and (ii) the decrease in the efficiency of Fe mobilization mechanisms from oxides by plants when there is a high saturation of sorbent sites by P (Sánchez-Rodríguez et al. 2013). This latter mechanism may explain the negative correlation between P uptake and Fe uptake despite the enhancement of mechanisms such as the organic anion exudation able to mobilize both nutrients.

In the case of Zn, its uptake by plants decreased with increased inorganic P in the growing media. This may be ascribed to two potential reasons: (i) inorganic P increased at increased Fe oxide concentration in the media, and Fe oxide is a Zn sorbent surface which constraints its absorption by plants (Montilla et al. 2003) and (ii), an increased P adsorption may lead to an enhanced Zn adsorption on oxides (Madrid et al. 1991; Liu et al. 2015). All this reveals that dynamics of Ins6P in growing media with high P sorption capacity and the mechanisms involved in its use by plants and microorganisms may have consequences on the Fe and Zn availability to plants.

Limitations in the method for assessing phytase activity in the growing media may mask differences between *B. subtilis* QST713 and *T. asperellum* T34 inoculated media. Histidine acid phosphatases (HAPs) from fungi are acidic, thus the activity was determined at a suitable pH (5.5), while its activity may be minimal at the pH (7.5) of the growing media (Tang et al. 2006; Mezeli et al. 2017; Singh et al. 2020). On the other hand, phytases from *Bacillus subtilis* (β-propeller phytases type –BPPs) are alkaline (Singh et al. 2020). Thus, the activity was not determined at a suitable pH, while the pH of the growing media was optimal (Tang et al. 2006). Thus, phytase activity determination method may overestimate the phytase activity promoted by T34, and underestimate that promoted by *B. subtilis* QST713. In addition, HAPs have broader specificity for substrates than BPPs, which essentially hydrolyses Ca-phytates (Mullaney and Ullah 2003; Oh et al. 2004; Jatuwong et al. 2020) likely formed in the media. Final product is *myo*-Ins1P in the case of HAPs, and *myo*-Ins3P for BPPs, which may show different adsorption dynamics, different interaction with organic anions, and different sensitivity to other phosphatases present in the media. This complex set of factors involved makes difficult the comparison of phytase activities between *T. asperellum* T34 and *B. subtilis* QST713. The inoculation with both microorganisms did not lead to differences in measured phytase activity and benefits on growth or P uptake by plants relative to non-inoculated media. Perhaps, with a high restriction of P availability to plants due to the Fe oxides in the media, their potential effects are not evident. However, differences between inoculants were not significant in media without Fe oxides. Another possible explanation is that the characteristics of the growing media in terms of factors affecting both phytases (pH, sorbent surfaces, ionic composition, ionic strength, and dynamics of added *myo*-Ins6P) (George et al. 2005; Tran et al. 2011; Mezeli et al. 2017; Celi et al. 2020) and the different catalytic products did not lead to promote benefits to plants when compared with non-inoculated media.

Bacillus subtilis QST713 increased phosphatase activity in the rhizosphere with the highest Fe oxide concentration in the growing media. Thus, this

microorganism contributes to an increased hydrolyzing capacity in growing media with high P sorption capacity.

Conclusions

Although phytate was used as P source by plants, P uptake decreased with increased Fe oxides in the growing media. This reduction was not ascribed to a decreased hydrolytic activity since P mobilization strategies, i.e. organic anion exudation and phytase activity, increased with increased Fe oxide concentration in the media. Most of the P added as phytate was recovered as inorganic P in the growing media after harvest at the highest Fe oxide concentration. Thus, the negative effect of Fe oxide on P uptake was the consequence of inorganic P adsorption after hydrolysis. Although inoculants did not improve P uptake, *Bacillus subtilis* enhanced hydrolytic activity at the highest Fe oxide concentration.

Acknowledgements This work was funded by the Spanish Ministry of Economy, Industry, and Competitiveness and the European Regional Development Fund of the European Union through the National Research, Development and Innovation Program (Plan Estatal I + D + i, Project AGL2017-87074-C2-1-R). The authors thank the Agricultural Research Service of the University of Seville (SIA) for technical assistance and access to experimental facilities.

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