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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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.

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

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 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 et al. 2001; Richardson et al. 2005; Patel 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 fifteen 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 10 000 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 sodium phytate per minute) per kg 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 0.1M 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 (Figure 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 (0.1M NaOH + 1 M NaCl and 1 M HCl) after crop increased at 300 mg kg^{-1} 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 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 (Figure 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} (Figure 2b). The highest pH was observed at 100 mg Fe kg^{-1}, and the lowest at 300 mg Fe kg^{-1} (Figure 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 (Figure 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 ($P < 0.001$; Figure 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 (Figure 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 (Figure 4c). On the other hand, the inorganic P in the growing media increased linearly with increased phytase activity in the rhizosphere (Figure 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 (Adam and Pate 1992). P concentrations in plant tissues and P uptake were similar to those in other studies using inorganic phosphate in similar 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 (Figure 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).

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. 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 (Figure 4d). In addition to this increased hydrolytic activity, organic anions such as citrate

343 increases the hydrolysis of InsP6 by competition for sorbent sites; this promotes
344 desorption of InsP6 and facilitates the enzyme-substrate interaction (Mezeli et al. 2017;
345 Celi et al. 2020). Organic anions do not have any interaction with adsorbed enzymes
346 which may lead to an increased hydrolytic activity in the solution (Mezeli et al. 2017).
347 Organic anions may also complex Fe facilitating the dissolution of Fe oxides and the
348 release of adsorbed Ins6P (Celi et al. 2020). Thus, hydrolysis of adsorbed Ins6P depends
349 to some extent on the release of organic anions by plants and microorganisms; this
350 promotes the desorption of Ins6P making it available for hydrolysis.

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

358 Our results agree with evidences suggesting that phytases may be active after
359 adsorption (Mezeli et al. 2017; Yang and Chen 2017). Their adsorption on soil minerals
360 may decrease the activity of the enzymes (George et al. 2005). However, the loss of
361 phytase activity depends on the type of mineral, with clay minerals inhibiting more the
362 activity than Fe oxides (Giaveno et al. 2010). This may be ascribed to a greater
363 modification of enzymes conformation when adsorbed on clay minerals than when
364 adsorbed on Fe-oxides (Quiquampoix 1987). To some extent, this contributes to explain
365 the hydrolytic activity observed in our media with Fe oxides. However, at least part of
366 the phytase activity may be ascribed to the liquid phase. When sorbent surfaces are
367 saturated with P, the partitioning of enzyme activity between the solution and the solid
368 phase shifts towards the solution phase (Giaveno et al. 2010), since mineral surfaces are
369 occupied by the substrate or by the hydrolyzed inorganic P. As mentioned above, it is
370 assumed a high saturation of Fe oxides by released inorganic P which may decrease
371 phytase adsorption. Furthermore, the amounts of P added as *myo*-Ins6P were enough to
372 saturate the adsorption capacity ferrihydrite assuming an adsorption capacity around 2.5
373 $\mu mol\ m^{-2}$, and a typical specific surface in synthetic ferrihydrite between 200 and 400 m^2
374 g^{-1} (Gimsing and Borggaard 2007; Wang et al. 2013). Thus, it may be assumed a relevant
375 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.

References

Adams MA, Pate JS (1992) Availability of organic and inorganic forms of phosphorus to lupins (Lupinus spp.). Plant Soil 145: 107–113.

Balwani I, Chakravarty K, Gaur S (2017) Role of phytase producing microorganisms towards agricultural sustainability, Biocatalysis and Agricultural Biotechnology 12: 23-29.

Baziramakenga R, Simard RR, Leroux GD (1995) Determination of organic acids in soil extracts by ion chromatography. Soil Biol Biochem 27: 349–356.

Bol R, Julich D, Brödlin D, Siemens J, Kaiser K, Dippold MA, Spielvogel S, ZillaT, Mewes D, von Blanckenburg F, Puhlmann H, Holzmann S, Weiler M, Amelung W, Lang F, Kuzyakov Y, Feger KH, Gottselig N, Klumpp E, Missong A, Winkelmann C, Uhlig D, Sohrt J, von Wilpert K, Wu B, Hagedorn F (2016) Dissolved and colloidal phosphorus fluxes in forest ecosystems – an almost blind spotin ecosystem research. J Plant Nutr Soil Sci 179: 425–438.

Borrero C, Trillas I, Delgado A, Avilés, M (2012) Effect of ammonium⁄nitrate ratio in nutrient solution on control of *Fusarium* wilt of tomato *by Trichoderma* asperellum T34. Pathology 61: 132–139.

Celi L, Prati M, Magnacca G, Santoro V, Martin M (2020) Role of crystalline iron oxides on stabilization of inositol phosphates in soil. Geoderma 374 doi.org/10.1016/j.geoderma.2020.114442.

Celi L, De Luca G, Barberis E(2003) Effects of interaction of organic and inorganic P with ferrihydrite and kaolinite-iron oxide systems on iron release. Soil S*ci* 168: 479-488.

Celi L, Lamacchia S, Ajmone-Marsan F, Barberis E (1999) Interaction of inositol hexaphosphate on clays: adsorption and charging phenomena. Soil Sci 164: 574-585.

Chung YR, Hoitink HAJ (1990) Interactions between thermophilic fungi and Trichoderma hamatum in suppression of Rhizoctonia damping off in a bark compost-amended container medium. Phytopathology 80:73-77.

de Santiago A, Delgado A (2007) Effects of humic substances on iron nutrition of lupin. Biol Fertil Soils 43: 829-836.

de Santiago A, Quintero JM, Avilés M, Delgado A (2009) Effect of Trichoderma asperellum strain T34 on iron nutrition in white lupin. Soil Biol Biochem 41: 2453–2459.

de Santiago A, García-López AM, Quintero JM, Avilés M, Delgado A (2013) Effect of Trichoderma asperellum strain T34 and glucose addition on iron nutrition in cucumber grown on calcareous soils. Soil Biol Biochem 57: 598–605.

de Santiago A, Quintero JM, Avilés M, Delgado A (2011) Effect of Trichoderma asperellum strain T34 on iron copper manganese and zinc uptake by wheat grown on a calcareous medium. Plant Soil 342: 97–104

Delgado A, Scalenghe R (2008) Aspects of phosphorus transfer from soils in Europe. *J* Plant Nutr Soil *Sci* 171:552–575.

Faucon M-P, Houben D, Reynoird J-P, Mercadal-Dulaurent A-M, Armand R, Lambers H (2015 Advances and perspectives to improve the phosphorus availability in cropping systems for agroecological phosphorus management. Adv Agron 134: 1–29.

Fu S, Sun J, Qian L, Li Z (2008) Bacillus phytases: present scenario and future perspectives. Appl Biochem Biotechnol 151: 1-8.García-López AM, Avilés M, Delgado A (2015) Plant uptake of phosphorus from sparingly available P-sources as affected by Trichoderma asperellum T34. J Food Sci 24: 249–260.

García-López AM, Delgado A (2016) Effect of Bacillus subtilis on phosphorus uptake by cucumber as affected by iron oxides and the solubility of the phosphorus source. Agric Food Sci 25:216–224.

García-Lopez AM, Avilés M, Delgado A (2016) Effect of various microorganisms on phosphorus uptake from insoluble Ca-phosphates by cucumber plants. J Plant Nutr Soil Sci 179: 454–465.

George TS, Gregory PJ, Wood M, Reed D, Buresh RJ (2004) Phosphatase activity and organic acids in the rhizosphere of potential agroforestry species and maize. Soil Biol Biochem 34: 1487– 1494.

George TS, Quiquampoix H, Simpson RJ, Richardson AE (2007.) Interactions between phytases and soil constituents: implications for the hydrolysis of inositol phosphates. In Editors: Turner BL et al. (eds)Inositol phosphates: linking agriculture and the environment. CAB International, Wallingford, United Kingdom.pp. 221-241.

George TS, Richardson AE, Simpson J (2005) Behaviour of plant-derived extracellular phytase upon addition to soil. Soil Biol Biochem.37: 977–988.

Giaveno C, Celi L, Richardson AE, Simpson RJ, Barberis E (2010) Interaction of phytases with minerals and availability of substrate affect the hydrolysis of inositol phosphates. Soil Biol and Biochem 42: 491-498.

Gichangi EM, Mnkeni PNS, Brookes PC. 2009. Effects of goat manure and inorganic phosphate addition on soil inorganic and microbial biomass phosphorus fractions under laboratory incubation conditions. Soil Sci Plant Nutr. 55: 764–771.

Giles CD, Richardson AE, Druschel GK, Hill JE (2012) Organic Anion–Driven Solubilization of Precipitated and Sorbed Phytate Improves Hydrolysis by Phytases and Bioavailability to Nicotiana tabacum. Soil sci 177: 591-598.

Gimsing AL, Borggaard OK (2007) Phosphate and glyphosate adsorption by hematite and ferrihydrite and comparison with other variable-charge minerals. Clays Clay Miner 55: 108–114 https://doi.org/10.1346/CCMN.2007.0550109

Hayes JE, Richardson AE, Simpson RJ (1999) Phytase and acid phosphatase activities in roots of temperate pasture grasses and legumes. Aust J Plant Physiol 26: 801–809.

Hinsinger P (2001) Bioavailability of soil inorganic P in the rhizosphere as affected by root-induced chemical changes: a review. Plant Soil 237: 173–195.

Hocking PJ (2001) Organic acids exuded from roots in phosphorus uptake and aluminium tolerance of plants in acid soils. Adv Agron 74: 63–97.

Jatuwong K, Suwannarach N, Kumla J, Penkhrue W, Kakumyan P, Lumyong S (2020) Bioprocess for Production, Characteristics, and Biotechnological Applications of Fungal Phytases. Front Microbiol 11 https://doi.org/10.3389/fmicb.2020.00188

527 Khan KS, Joergensen RG (2009) Changes in microbial biomass and P fractions in
528 biogenic household waste compost amended with inorganic P fertilizers.
529 Bioresour Technol 100: 303–309.

530 Kleinman PJA, Sharpley AN, Withers PJA, Bergström L, Johnson LT, Doody DG (2015)
531 Implementing agricultural phosphorus science and management to combat
532 eutrophication. Ambio 44: 297-310

533 Jones DL, Darah PR, Kochian LV (1996) Critical evaluation of organic acid mediated
534 iron dissolution in the rhizosphere and its potential role in root iron uptake. Plant
535 Soil 180: 57-66.

536 Liu J, Bo X, Zhao Z, Guo L (2015) Highly exposed Pt nanoparticles supported on porous
537 graphene for electrochemical detection of hydrogen peroxide in living cells.
538 Biosens Bioelectron 74: 71-77.

539 Lung SC, Lim BL (2006) Assimilation of phytate-phosphorus by the extracellular phytase
540 activity of tobacco (Nicotiana tabacum) is affected by the availability of soluble
541 phytate. Plant Soil 279: 187-199.

542 Macklon AES, Grayston SJ, Shand CA, Sim A, Sellars S, Ord BG (1997) Uptake and
543 transport of phosphorus by Agrostis capillaris seedlings from rapidly hydrolysed
544 organic sources extracted from 32 P-labelled bacterial cultures. Plant Soi. 190:
545 163-167.

546 Madrid L, Diaz-Barrientos E, Contreras MC (1991) Relationships between zinc and
547 phosphate adsorption on montmorillonite and an iron oxyhydroxide. Soil
548 Research 29: 239-247.

549 Martin M, Celi, L, Barberis E (2004) Desorption and plant availability of myo-inositol
550 hexaphosphate adsorbed on goethite. Soil sci 169:115-124

551 Menezes-Blackburn D, Jorquera MA, Greiner R, Gianfreda L, de la Luz Mora M (2013)
552 Phytases and phytase-labile organic phosphorus in manures and soils. Crit Rev
553 Environ Sci Technol 43: 916-954.

554 Metson GS, MacDonald GK, Haberman D, Nesme T, Bennett EM (2016) Feeding the
555 corn belt: Opportunities for phosphorus recycling in US agriculture. Sci Total
556 Environ 542: 1117–1126

557 Mezeli MM., Menezes-Blackburn D, George TS, Giles CD, Neilson R, Haygarth PM
558 (2017) Effect of citrate on Aspergillus niger phytase adsorption and catalytic
559 activity in soil. Geoderma 305: 346–353.

560 Missong A, Bol R, Willbold S, Siemens J, Klumpp E (2016) Phosphorus forms in forest
561 soil colloids as revealed by liquid-state 31P-NMR. J Plant Nutr Soil Sci 179:159–
562 167.

563 Montilla I, Parra MA, Torrent J (2003) Zinc phytotoxicity to oilseed rape grown on zinc-
564 loaded substrates consiting of Fe oxide-coated and calcite sand. Plant Soil 257:
565 227-236.

566 Mullaney EJ, Ullah AHJ (2003) The term phytase comprises several different classes of
567 enzymes. Biochem Bioph Res Co 312: 179–184

568 Murphy J, Riley JP (1962) A modified single solution method for the determination of
569 phosphate in natural waters. Anal Chim Acta 27: 31–36.

570 Ognalaga M, Frossard E, Thomas F (1994) Glucose-1-phosphate and myo-inositol
571 hexaphosphate adsorption mechanisms on goethite. Soil Sci Soc Am J 58: 332-
572 337

573 Oh B, Choi W, Park S. Kim Y, Oh TK (2004) Biochemical properties and substrate
574 specificities of alkaline and histidine acid phytases. Appl Microbiol Biotechnol
575 63: 362–372 https://doi.org/10.1007/s00253-003-1345-0

576 Owen D, Williams AP, Griffith GW, Withers PJA (2015) Use of commercial bio-
577 inoculants to increase agricultural production through improved phosphrous
578 acquisition. Appl Soil Ecol 86: 41–54.

579 Patel DK, Murawala P, Archana G, Kumar, GN (2011) Repression of mineral phosphate
580 solubilizing phenotype in the presence of weak organic acids in plant growth
581 promoting fluorescent Pseudomonads. Bioresour Technol 102: 3055–3061.

582 Quiquampoix H (1987) A stepwise approach to the understanding of extracellular enzyme
583 activity in soil I. Effect of electrostatic interactions on the conformation of a β-D-
584 glucosidase adsorbed on different mineral surfaces. Biochimie 69: 753–763.

585 Radersma S, Grierson PF (2004) Phosphorus mobilization in agroforestry: Organic
586 anions, phosphatase activity and phosphorus fractions in the rhizosphere. Plant
587 Soil 259: 209–219.

588 Rahmatullah, Torrent J (2000) Phosphorus dynamics and uptake by wheat in a model
589 calcite-ferrihydrite system. Soil Science 165: 803–812.

590 Recena R, Cade-Menun BJ, Delgado A (2018) Organic Phosphorus Forms in Agricultural
591 Soils under Mediterranean Climate. Soil Sci Soc Am J 82:783-795

592 Richardson AE, Lynch JP, Ryan PR, Delhaize E, Smith FA, Smith SE, Ryan MH,
593 Veneklaas EJ, Lambers H, Oberson A, Culvenor RA, Simpson RJ (2011) Plant
594 and microbial strategies to improve phosphorus efficiency in agriculture. Plant
595 Soil 349: 121–156.

596 Richardson AE, George TS, Hens M, Simpson RJ (2005) Utilization of soil organic
597 phosphorus by higher plants. Turner BL, Frossard E, Baldwin DS, editors, eds,
598 Organic Phosphorus in the Environment. CABI, Wallingford, UK, pp 165–184

599 Richardson AE, Hadobas PA, Hayes JE (2000) Acid phosphomonoesterase and phytase
600 activities of wheat (Triticum aestivum L.) roots and utilization of organic
601 phosphorus substrates by seedlings grown in sterile culture. Plant Cell Environ
602 23: 397-405.

603 Richardson AE (2001) Prospects for using soil microorganisms to improve the acquisition
604 of phosphorus by plants. Austr J Plant Physiol 28: 897–906.

605 Rowe H, Withers PJA, Baas P, Chan NI, Doody D, Holiman J, Jacobs B, Li HG (2016)
606 Integrating legacy soil phosphorus into sustainable nutrient management
607 strategies for future food, bioenergy and water security. Nutr Cycl Agroecosys
608 104: 393–412.

609 Ryan PR, Delhaize E, Jones DL (2001) Function and mechanism of organic anion
610 exudation from plant roots. Annual Review of J Plant Physiol Pathol Plant J Mol
611 Biol 52: 527–560.

612 Sánchez-Rodríguez AR, del Campillo MC, Torrent J (2013) Phosphate aggravates iron
613 chlorosis in sensitive plants grown on model calcium carbonate–iron oxide
614 systems. Plant Soil 373: 31–42.

615 Sattari SZ, Bouwman AF, Giller KE, van Ittersum MK (2012) Residual soil phosphorus
616 as the missing piece in the global phosphorus crisis puzzle. Proc Natl Acad Sci
617 US 109: 6348–6353.

618 Seltman HJ (2018) Experimental design and analysis. Available on line at
619 http://www.stat.cmu.edu/~hseltman/309/Book/Book.pdf

620 Shao XH, Xing CH, Du ST, Yu CY, Lin XY, Zhang YS (2006) Phosphorus Adsorption
621 Saturation of Ferrihydrate as an Index of Phosphorus Availability to Paddy Rice,
622 J Plant Nutri 29: 1187-1197 DOI: 10.1080/01904160600767245

623 Singh B, Boukhris I, Pragya, Kumar V, Yadav AN, Farhat-Khemakhem A, Kumar A,
624 Singh D, Blibech M, Chouayekh H, Alghamdi OA (2020) Contribution of
625 microbial phytases to the improvement of plant growth and nutrition: A review.
626 Pedosphere 30: 295–313.StatPoint Technologies (2013) Statgraphics Centurion
627 XVI. Warrenton.

628 Stutter MI., Shand CA, George TS, Blackwell MSA, Bol R, MacKay RL, Richardson
629 AE, Condron LM, Turner BL, Haygarth PM (2012) Recovering phosphorus from
630 soil: a root solution? Envi Sci Techn 46: 1977−1978.

631 Stutter M, Stand C, George T, Blackwell M, Dixon L, Bol R, MacKay R, Richardson A,
632 Condron L, Haygarth P (2015) Land use and soil factors affecting accumulation
633 of phosphorus species in temperate soils. Geoderma 257–258, 29–39

634 Segarra G, Casanova E, Bellido D, Odena MA, Oliveira E, Trillas I (2007) Proteome
635 salicylic acid and jasmonic acid changes in cucumber plants inoculated with
636 Trichoderma asperellum strain T34. Proteomics 7: 3943–3952

637 Shang C, Huang PM, Stewart JWB (1990) Kinetics of adsorption of organic and inorganic
638 phosphates by short-range ordered precipitate of aluminium. Can J Soil Sci 70:
639 461-470

640 Stutter M, Stand C, George T, Blackwell M, Dixon L, Bol R, MacKay R, Richardson A,
641 Condron L, Haygarth P (2015) Land use and soil factors affecting accumulation
642 of phosphorus species in temperate soils. Geoderma 257–258: 29–39

643 Tabatabai MA, Bremner JM (1969) Use of p-nitrophenyl phosphate for assay of soil
644 phosphatase activity. Soil Biol Biochem 1: 301–307.

645 Tang J, Leung A, Leung C, Lim BL (2006) Hydrolysis of precipitated phytate by three
646 distinct distinct families of phytases. Soil Biol Biochem 38: 1316-1324

647 Tran TT, Hashim SO, Gaber Y, Mamo G, Mattiasson B, Hatti-Kaul R (2011)
648 Thermostability alkaline phytase from Bacillus sp. MD2: Effect of divalent metals
649 on activity and stability. J Inorg Biochem 105: 1000–1007

650 Tuitert G, Szczech M, Bollen GJ (1998) Suppression of Rhizoctonia solani in potting
651 mixtures amended with compost made from organic household waste.
652 Phytopathology 88: 764–773.

653 Turner BL, Baxter R, Whitton BA (2002) Seasonal phosphatase activity in three
654 characteristic soils of the English uplands polluted by long-term atmospheric
655 nitrogen deposition. Environ Pollut 120: 313-317.

656 Vohra A, Satyanarayana T (2003) Phytases: microbial sources, production, purification,
657 and potential biotechnological applications. Crit Rev Biotechnol 23: 29-60.

658 Wang X, Li W, Harrington R, Liu F, Parise JB, Feng X, Sparks DL (2013) Effect of
659 ferrihydrite crystallite size on phosphate adsorption reactivity. Environ Sci
660 Technol 47: 10322-10331

661 Yang XZ, Chen LJ (2017) Distribution of exogenous phytase activity in soil solid–liquid
662 phases and their effect on soil organic P hydrolysis. J Plant Nutr Soil Sci 180: 39–
663 48, https://doi.org/10.1002/jpln.201600421, 2017.

664 Zhou X, Wu F (2012) Dynamics of the diversity of fungal and Fusarium communities
665 during continuous cropping of cucumber in the greenhouse. FEMS Microbiol Ecol
666 80: 469–478

667 **FIGURE CAPTIONS**

668 **Fig 1.** Effect of the different Fe oxides concentration on dry matter (DM) in shoots and
669 roots of cucumber plants. The effect of Fe oxide rate was significant according to
670 ANOVA ($P < 0.0001$). Means with different letter were significantly different according
671 to the Tukey´s test at $P < 0.05$.

672

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

678

679 **Fig 3.** Effect of the interaction between Fe oxide rate and inoculation on phosphatase
680 activity, which was significant according to ANOVA at $P = 0.0177$. Means with different
681 letter were significantly different according to the Tukey´s test at $P < 0.05$.

682

683 **Fig 4. (a)** Relationship between P uptake by plants and molybdate reactive P in the
684 growing media ($P_{NaOH+HCl}$) at the end of the experiment ($Y = 4.6 - 0.1\ X$; $R^2 = 0.91$;
685 $P<0.001$); $P_{NaOH+HCl}$ is the sum of the P extracted with a sequential extraction involving
686 0.1 M NaOH + 1 M NaCl and 1 M HCl. Each point corresponded to the mean of the five
687 replications for each combination of the two factors (Fe oxide concentration and
688 microbial inoculation). **(b)** Relationship between P uptake by plants and phosphatase
689 activity in the growing media at the end of the experiment ($Y = 1/[\ 0.2 + 0.03\ X]$; $R^2 =$
690 0.5; $P<0.001$). Black symbol, 0 mg Fe kg^{-1}; empty symbol, 100 mg Fe kg^{-1} and striped
691 symbol, 300 mg Fe kg^{-1}. Each point corresponded to the mean of the five replications for
692 each combination of the two factors (Fe oxide concentration and microbial inoculation).
693 **(c)** Relationship between P uptake by plants and phytase activity in the growing media at
694 the end of the experiment ($Y = 4.1 + 4.5x10–2\ X$; $R^2 = 0.7$; $P<0.005$). EU, enzymatic
695 units, amount of enzyme which releases one micromole of inorganic phosphate from
696 sodium phytate per minute. Each point corresponded to the mean of the five replications
697 for each combination of the two factors (Fe oxide concentration and microbial

698 inoculation). **(d)** Relationship between molybdate reactive P in the growing media
699 ($P_{NaOH+HCl}$) and phytase activity in the growing media at the end of the experiment (Y =
700 4.6 + 5.3 103 X; R^2 = 0.71; P<0.005). $P_{NaOH+HCl}$ is the sum of the P extracted with a
701 sequential extraction involving 0.1 M NaOH + 1 M NaCl and 1 M HCl. Each point
702 corresponded to the mean of the five replications for each combination of the two factors
703 (Fe oxide concentration and microbial inoculation).