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Effect of *Bacillus subtilis* QST713 and *Trichoderma asperellum* T34 on P uptake by wheat and how it is modulated by soil properties

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Abstract

Objectives The effect on P uptake by plants after inoculation with P-mobilizing microorganisms may be modulated by soil properties, including natural microbiota. However, to put this theory into practical use, research is needed to shed new light on the soil factors which affect the capability of improving P nutrition in plants. The aim of this study was to assess how two P-mobilizing microorganisms, *Trichoderma asperellum* T34 and *Bacillus subtilis* QST713, influence P uptake by wheat plants in different soils; this will allow us to identify the soil properties which affect the efficiency of P nutrition in plants.

Materials and methods. In a completely randomized experiment, wheat was grown in 12 pots in a growing chamber in soil with Olsen P values ranging from 4.8 to 8.7 mg kg^{-1}. The plants were inoculated with 3 treatments: T34, *B. subtilis* and a non-inoculated control.

Results and discussion. Overall, *B. subtilis* was more effective in increasing plant P uptake and in mobilizing soil P (measured as Olsen P values) than T34. In some soils, *B. subtilis* was the only treatment which increased Olsen P in the rhizosphere after cultivation. However, the effect of both microorganisms differed depending on the soil. For *B. subtilis*, phytase hydrolysable P, Olsen P, carbonates, the Fe_{ca}/Fe_{cbd} ratio and citrate soluble P accounted for 92 % of the variation in P uptake in inoculated plants (compared with the non-inoculated control). Most of these soil properties also accounted for 87 % of the variation in the levels of shoot DM in *B. subtilis* inoculated plants compared with shoot DM in the control plants. In addition, Olsen P, the Fe_{ca}/Fe_{cbd} ratio and phytase hydrolysable P in the NaOH extracts accounted for 82 % and 74 % of the variation in the effect of T34 on P uptake and shoot DM, respectively. Overall, the lower the initial Olsen P in the soil, the higher the P uptake caused by microorganisms.

Conclusions. The initial availability of P and organic P in soil, in addition to other properties affecting P dynamics in the soil, may explain the triggering and efficiency of the P-mobilizing mechanisms in microorganisms. These are crucial in explaining the potential benefits to crops and, as a result, their practical use as a bio-fertilizer.

Keywords Iron oxides • Olsen P • Organic P • P availability • P-mobilizing microorganisms

1 Introduction

Only a small portion of the total P present in soil is readily available to plants and microorganisms (Hisinger 2001; Delgado and Scalenghe 2008; Richardson et al. 2011). This is the result of P reactions in soils, and leads to the low efficiency of P fertilizer applied to agricultural land (Saavedra and Delgado 2005; Ryan et al., 2012). As a result, most of the accumulated P applied to the soil remains in non-bioavailable forms (Delgado and Torrent 1997; Recena et al. 2017), which constitutes a "legacy" (Withers et al. 2014) which makes no contribution whatsoever to agricultural productivity. This P legacy includes recalcitrant inorganic P (e.g. metal phosphates) and organic P (non-readily hydrolysable). Enhancing the use of these non-readily available forms by plants, and achieving greater efficiency in the use of P as a fertilizer (Cordell et al. 2009; Ryan et al. 2012) would lead to a more sustainable use of this non-renewable strategic resource (Ryan et al. 2012; Withers et al. 2014). To achieve this, more rational fertilization schemes (Recena et al. 2016) need to be designed, as well as the use of more efficient biological resources, such as crops with an increased capacity to use P (Shen et al. 2011; Kochian 2012, Heppell et al. 2015). Among the biological resources, we should also consider inoculation with microorganisms able to mobilize both the native P and the 'P legacy', as well as any insoluble sources of P applied as fertilizer (Jones and Oburger 2011; Owen et al. 2015; García-López et al. 2016).

The use of P-mobilizing microorganisms has gained in popularity over the last few decades as a means of increasing agricultural productivity and sustainability in P-poor soils, and the literature published on this topic is abundant (Kim et al. 1998; Gunes et al. 2009; Singh and Reddy 2011; Nakayan et al. 2013). However, agriculture needs integrated, cost-effective measures, and the exclusive use of microorganisms to improve P acquisition by plants is unrealistic without the additional benefits from inoculation to

control plant disease or promote plant growth in other ways. For this reason, biological control agents which are also effective in mobilizing nutrients are being increasingly used (de Santiago et al. 2013), as they allow for an integrated management of plant diseases and nutritional deficiencies, which are two major factors limiting agricultural productivity (Vassilev et al. 2006; de Santiago et al. 2011; 2013). In this regard, *Trichoderma* spp. is a well-known biocontrol agent which also has proved effective as a plant growth-promoting microorganism (Harman and Bjorkman 1998; Vassilev et al. 2006). In addition, *Trichoderma asperellum* T34 has proved effective in increasing Fe (de Santiago et al. 2009; 2011) and P uptake by plants (García-López et al. 2015). *Bacillus subtilis* is a plant growth-promoting bacteria, and some of its isolates have been found to enhance P uptake by plants (Orhan et al. 2006; Mena-Violante and Olakle-Portugal 2007; García-López et al. 2016; García-López and Delgado 2016) and to be an effective biocontrol agent of plant diseases (Lim and Kim 2010; Lahlali et al. 2011).

The efficiency of microorganisms in improving plant nutrition may be constrained by environmental conditions, particularly by soil properties (de Santiago et al. 2013). In fact, a wide range of environmental and management factors may affect the final efficacy of any fungal or bacterial strain utilized as an inoculant. First, the chemical properties of the soil have proved to affect their potential benefits (Enkhtuya et al. 2000; Radersma and Grierson 2004). Nutrient availability in the soil is also considered a crucial factor in how microbial inoculants enhance plant nutrition (García-López et al. 2013; Owen et al. 2015). Furthermore, the size and competitiveness of indigenous microbial populations may also be vital for their success in getting established (Adholeya et al. 2005; Vassileva et al. 2010; Owen et al. 2015). However, it is not always clear which factors most influence the successful use of microbial inoculants, and recent studies have revealed that inoculation is not always affected by nutrient availability or native microbial communities in soil

(Köhl et al. 2016). Most of the studies reporting the benefits of a given P-mobilizing microorganism have been performed on a specific type of soil. Thus, new research is required on the soil factors affecting the success of P-mobilizing microorganisms as bio-fertilizers in order to support their practical use. This success may not only be dependent on the establishment of the inoculant in the rhizosphere, but also on the microbial mechanisms for solubilizing inorganic P or hydrolyzing organic P. These mechanisms have been shown not only to depend on the microorganism, but also to be triggered by particular conditions in the plant growing medium (García-López et al. 2016).

This work was aimed at studying the potential benefits of inoculation with two P-mobilizing microorganisms, *Trichoderma asperellum* T34 and *Bacillus subtilis* QST713, on P uptake by wheat plants in different soils; it will allow us to identify the soil properties governing their efficiency in contributing to P nutrition in plants. These two free-living organisms were selected because, based on previous evidence (García-López et al. 2016), their P-mobilizing abilities are expected to be affected differently by soil properties. Both are commercial strains which are effective in promoting plant growth and biocontrol of some relevant soil-borne diseases and have proven effective in mobilizing soil P (García-López et al. 2015; 2016; García-López and Delgado 2016). Particular emphasis will be paid to soil properties which are usually determined in routine soil fertility studies which focus on applying practical results to assess the potential efficiency of these microorganisms.

2 Materials and methods

2.1 Soils

A set of 12 soil samples were selected for this study encompassing representative soils from different agricultural lands from Spain and including different orders according to

Soil Taxonomy (Soil Survey Staff, 2010): Mollisols, Entisols, Inceptisols, Alfisols, and Vertisols. This selection included soils representative of the Mediterranean climate (Recena et al. 2016) under different agricultural uses (Table 1). Samples were collected from the surface (at a depth of 20 cm) and ground into 6 mm clumps for plant cultivation; for soil analysis, a portion of the soil was ground to 2 mm.

2.2 Soil analysis

The general soil properties were assessed by the following methods: a densimeter for particle size analysis (Gee and Bauder 1986), dichromate oxidation for soil organic matter (SOM) (Walkley and Black 1934) and the calcimeter method for total Ca carbonate equivalent (CCE). In addition, electrical conductivity (EC) and pH were measured at a soil:water ratio of 1:2.5. The nutrient availability index measurements were: (i) extraction with diethylenetriaminepentaacetic acid (DTPA) for micronutrients according to Lindsay and Norvell (1978), and (ii) bicarbonate extraction for P according to the Olsen method (Olsen et al., 1954). Iron ascribed to poorly crystalline Fe oxides was determined after reduction with citrate-ascorbate (Fe_{ca}), and a reduction with citrate-bicarbonate-dithionite was carried out sequentially to assess the amount of Fe ascribed to crystalline oxides (Fe_{cbd}) (de Santiago and Delgado 2006). In both extracts, Fe was determined by atomic absorption spectrometry.

The main P fractions in the soils were studied. The total inorganic P (Pi) was determined as the molybdate reactive P (MRP) after extraction with 0.5 M H_2SO_4, and total organic P as the increase in 0.5 M H_2SO_4 extractable MRP after soil calcination (Kuo 1996). The main P fractions were studied according to the sequential fractionation scheme described by Recena et al. (2015). According to this scheme, the main fractions identified are: (i) adsorbed P plus soluble metal phosphates (the two first steps, NaOH and citrate-

bicarbonate extractions), (ii) P ascribed to hydroxyapatite (citrate extractable, Pc), (iii) P occluded in poorly crystalline and crystalline Fe oxides (reductant soluble, citrate-ascorbate and citrate-bicarbonate-dithionite extractable, respectively), and (iv) residual Ca phosphates (acetate and HCl extractable). Phytase hydrolysable P was determined in all the P fractions, as described by Recena et al. (2016); after enzyme hydrolysis, MRP was determined by the colorimetric method of Murphy and Riley (1962).

2.3 Experimental setup

The experiment was completely randomized and consisted of three replications performed twice at different times under the same growing conditions in a growing chamber. Wheat plants were sown in 12 pots of soil and inoculated with microorganisms (*Trichoderma asperellum* T34, *B. subtilis* QST713 and a control without inoculation). Each replication consisted of one pot containing one plant of wheat.

The seeds were pre-germinated in petri dishes at 8º C and darkness for 15 d, and afterwards germinated in perlite. The seedlings were transplanted to pots 15 days after germination and grown on for 33 additional days in 106 mL cylindrical pots (diameter 3 cm; height 15 cm), each containing 110 g of soil. Previously, the roots were thoroughly washed with deionized water to eliminate any residue of perlite.

Inoculation with T34 (Biocontrol Technologies, Barcelona, Spain) was carried out in two steps: by immersing plant roots in a suspension of water containing 10^6 conidia per L before transplanting, and by applying by 10^7 conidia per kg of growing medium after transplanting (de Santiago et al., 2009). The latter step involved the application of 20 mL of conidial suspension in water ($2 \cdot 10^8$ conidia L^{-1}) prepared according to Segarra et al. (2007) on the surface of each pot at four different points around the plants. Inoculation with the *B. subtilis* strain QST713 (Serenade Max, Bayer CropScience, Paterna, Spain)

was performed by adding $2 \cdot 10^7$ colony forming units (CFU) per kg of growing medium after transplanting by applying 20 mL of aqueous suspension containing $4 \cdot 10^8$ CFU L^{-1} on the surface of each pot at different points around the plants (García-López and Delgado 2016).

During the experiment, the plants grew in the growing chamber under constant controlled environmental conditions, with a photoperiod of 14 h, 23°C/20°C day/night temperature, 65% RH, and 22 W m^{-2} light intensity. The plants were fertigated 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_2MoO_4 (0.0005). pH of the nutrient solution was adjusted to 6 before irrigation.

2.4 Soil analysis after cultivation

Immediately after the plants were harvested, the rhizospheric soil was sampled according to Zhou and Wu (2012), taking the soil adhering to the roots and collected by shaking it off from the roots in air. β-glucosidase activity and alkaline phosphatase activity were determined in the rhizospheric soil just after soil sampling. The former was measured as the amount of pNP (*p*-nitrophenol) formed from PGN (p-nitrophenyl-b-D-Glucopiranoside) according to Eizavi and Tabatabai (1988), and the latter, as the amount of pNP released from 5 mM *p*-nitrophenyl phosphate using the method suggested by Tabatabai and Bremner (1969). Since it is a soil quality indicator which is highly sensitive to changes in soil and which usually increases with the soil microbial biomass (Stott et al. 2010), β-glucosidase activity was also studied.

Colony forming units (CFU) were determined by dilution plating after sodium pyrophosphate extraction of the rhizospheric soil. *Trichoderma* spp. was isolated by using

199 Chung and Hoitink (1990)'s semi-selective medium as described by Borrero et al. (2012).
200 This medium has proved effective to measure the CFU of T34 in soil samples (de
201 Santiago et al. 2013). *Bacillus* spp. were isolated on a nutrient–agar medium after heating
202 the suspension at 80 ºC for 10 min, according to Tuitert et al. (1998). The assessment of
203 *B. subtilis* CFUs was carried out on the basis of its particular colony morphogenesis,
204 showing a complex architecture with aerial projections that serve as preferential sites for
205 sporulation (Aguilar et al. 2007). Three plates per dilution ratio were used, and CFU were
206 counted after 4 days. Despite being microorganisms that can be present in the rhizosphere,
207 no CFU were detected in the control. In these non-inoculated pots, other Bacilli were
208 present, but not the characteristic colony morphogenesis of *B. subtilis*. The density of
209 CFU in suspension used for inoculation was also checked using the same procedure.
210 Organic anions from rhizospheric soil were also measured, since their exudation
211 is a P-mobilizing mechanism in some microorganisms (García-López et al. 2016). These
212 anions were extracted by shaking 5 g of rhizospheric soil in 5 mL 0.1 *M* NaOH for 1.5 h
213 at 4 s^{-1} (Baziramakenga et al. 1995; Radersma and Grierson 2004). After extraction, the
214 suspensions were centrifuged at 10,000 *g* for 10 min, the supernatant filtered through a
215 0.45 µm cellulose filter and the filtrate acidified to pH 2–3 with 1 *M* H_2SO_4. High
216 performance liquid chromatographic (HPLC) separation of organic acids was performed
217 with an HPLC Varian ProStar 410 equipped with a C18 column (Varian, 250 mm x 34.6
218 mm, and 8 µm particle size). Elution was isocratic with 98 % 5 mM H_2SO_4 at pH 2 + 2
219 % methanol as the carrier solution at a flow rate of 0.8 mL min^{-1}, and 20 µL of injection
220 volume. Organic anions were detected at 215 nm using a Varian 486 photo-diode array
221 detector. Standard solutions of acids (acetic, oxalic, citric, malic, fumaric and succinic)
222 were prepared as individual stock solutions, using Sigma acids (Sigma, Barcelona,

Spain). Olsen P (Olsen et al. 1954) and the pH by extraction with water at a 1:2.5 ratio were determined in the rhizospheric soil.

2.5 Plant analysis

The plants were harvested 33 days after transplanting them into pots. They were sampled with the roots, which were separated from the soil and thoroughly washed. After that, shoots and roots were separated, and both organs dried to constant weight (48 h) in a forced-air oven at 65 ºC. After drying and determination of the dry matter (DM) in each organ, the dry plant material was ground and an aliquot of 0.25 g mineralized in a furnace at 550 ºC for 8 h. The resulting ashes were dissolved in 1 M HCl by heating at 100 ºC for 15 min. In the remaining solution, P was determined by Murphy and Riley (1962)'s colorimetric procedure. Certified plant material was also analyzed in parallel to confirm the complete recovery of nutrients with this procedure. The total P uptake by plants was calculated as the amount of P present in shoots and roots minus that in seeds.

2.6 Statistical analysis

An analysis of variance (ANOVA) test was performed using the General Linear Model procedure in Statgraphics Centurion XVI (StatPoint, 2013) to identify the effects of the factors studied on the different variables measured in the experiments. Previously, normal distribution and homoscedasticity were assessed by the Kolmogorov–Smirnov and Levenne tests, respectively. If necessary, the data were potentially transformed to meet both criteria ($transformed\ data = data^{-b}$, with b as the slope of the relation between the logarithms of standard deviation and the logarithm of the mean for each treatment). Results from both replications of the experiment were jointly analyzed, with each replication deemed as a separate block in order to exclude the variation associated with

248 the repetition of the experiment (de Santiago et al. 2009). If the data transformation did
249 not meet normality or the homoscedasticity criteria, the effects of the factors was assessed
250 by the Kruskal-Wallis non-parametric test; in these cases, the effect of the interaction
251 could not be assessed. Only one factor (soil) was taken into account in the ANOVA of
252 CFU in soil because no CFUs were detected in non-inoculated soil. Means differences
253 were assessed via Tukey's test ($P < 0.05$). When interactions between factors were
254 significant, the main factors could not be assessed in a combined analysis, and it was
255 therefore not possible to compare the means of the main factors (de Santiago et al. 2013).
256 The significant interaction means that the effect of each inoculation treatment on a given
257 variable differed depending on the soil. In that case, multiple regression analysis was
258 performed by the least square method to assess which soil properties accounted for the
259 ratio of the observed value for inoculated plants to the value of non-inoculated plants
260 (control). On this relative basis, the effect of treatments can be assessed in different
261 environments (soils) (Black 1993). This analysis was carried out independently for T34
262 and for *B. subtilis*. The model dimension, i.e. number of independent variables to be
263 included, was selected according to the Akaike information criterion (AIC; Akaike 1974)
264 and the accuracy of the model checked by Mallows' *Cp* statistic (Gilmour et al. 1996).
265 As an additional requirement, independent variables included in the model were
266 significant according to the *t* statistic at $P < 0.05$. When correlated independent variables
267 were included in the models to meet the best AIC, the sign of the coefficient was assumed
268 not to have any explicative value (Krzywinski and Altman 2015). In these cases, the
269 variance inflation factor (VIF) was calculated. If VIF were lower than 5, it could be
270 supposed there was a reasonable estimation of coefficients (Marquadt 1970). In addition,
271 ridge regression was performed to check that the sign of the coefficients did not change
272 when the VIP was adjusted to near 1. If the independent variables are not correlated, it is

possible to assess the variance partitioning to evaluate the relative importance of variables in a regression model (Darlington 1968). In these cases, this was done by performing a Stepwise Regression (Pedhazur 1997). Other multiple regressions and correlation analysis were also performed, and the regression and correlation analyses were carried out using Statgraphics Centurion XVI software.

3 Results

3.1 Soil properties

The soils studied showed a wide range of properties. In particular, the properties ranged widely as regards the P cycle in the soil, with different P forms and fractions, Ca carbonate equivalent (CCE) and Fe oxides (Table 1). Regarding the Fe oxide mineralogy, Fe bound to crystalline oxides (Fe_{cbd}) was dominant, accounting on average for 86 % of Fe in oxides. Olsen P values ranged from 4.8 to 8.7 mg kg^{-1}; these values amounted to a minor fraction of the inorganic P in soils, which varied from 0.18 to 0.52 g kg^{-1}.

3.2 Effects of soil type and microorganisms

Root DM and P concentration were significantly different between soils (Table 2; mean for each soil not shown). Significant differences between soil types were also observed in alkaline phosphatase and β-glucosidase activities, and the CFU of both microorganisms in the rhizosphere (Table 2). Inoculation with microorganisms had a significant effect on P concentration in shoots and alkaline phosphatase in the rhizospheric soil (Table 2). Both of these variables were higher in the control and with *B. subtilis* than in T34 inoculated plants (Tables 3 and 4).

Root DM was positively correlated with SOM and Olsen P, while its correlation with CCE, pH, EC, and clay content was negative (Table 5). β-glucosidase correlated

298 positively with SOM, initial Olsen P, clay, Fe in oxides (Fe_{ca} + Fe_{cbd}), citrate soluble P
299 (Pc) and organic P (OP). Alkaline phosphatase positively correlated with SOM, clay, EC,
300 Fe in oxides, Pc and OP, while it was negatively correlated with initial Olsen P and pH
301 (Table 5). Only in the case of *B. subtilis* was CFU correlated with soil properties, and it
302 was negatively correlated with initial Olsen P and positively with soil clay content (Table
303 5). In addition, 88 % of its variance was accounted for by β-glucosidase, EC, SOM and
304 clay content (Fig. 1).

305 Both enzymatic activities in the rhizosphere increased with clay content and
306 decreased with two parameters, the CCE, and the ratio of Fe in poorly crystalline oxides
307 to that in crystalline oxides (Fe_{ca}/Fe_{cbd}), according to the multiple regression analysis; in
308 addition, β-glucosidase increased with increased organic P concentrations in soil (Table
309 6). Clay was the most relevant independent variable explaining alkaline phosphatase
310 variation, and Fe_{ca}/Fe_{cbd} the most relevant variable explaining β-glucosidase variation.

311

312 **3.3 Effect of the microorganisms depending on soil**

313 The interaction between the soil and inoculation with microorganisms was significant for
314 shoot DM, P uptake by plants, Olsen P, pH and the concentration of organic anions in the
315 rhizospheric soil after cultivation (Table 2). This means that the effects of each
316 microorganism differed depending on the soil. The effect of T34 improving P uptake by
317 plants relative to the control was only significant in the soil with the highest CCE and
318 lowest content of Fe in oxides (MCC, not shown). On the other hand, *B. subtilis* increased
319 P uptake in seven soils when compared with the control. In three soils (MCC, TRB, and
320 ZMB, not shown), this microorganism increased Olsen P in rhizospheric soil after
321 cultivation; these soils were not however among those where this microorganism was
322 effective in increasing P uptake relative to control. Overall, the Olsen P decrease in the

rhizosphere after cultivation was lower with *B. subtilis* than with T34 (Table 2 and 4). For *B. subtilis*, the ratio of final Olsen P in rhizosphere to initial Olsen P increased linearly with increased CFUs ($R^2 = 0.34$; $P < 0.05$), while for T34, this ratio increased linearly with the Fe_{ca}/Fe_{cbd} ratio ($R^2 = 0.45$; $P < 0.05$).

As mentioned above, the interaction was explained on a relative basis, using the ratio of the effect of the microorganism to the effect of non-inoculated control.

In the case of *B. subtilis* for P uptake, 92 % of the variation in the ratio was explained by the phytase hydrolysable P in NaOH extracts, Olsen P, CCE, the ratio Fe_{ca}/Fe_{cbd} and citrate soluble P (Fig. 2). Most of these soil properties (including OP instead of citrate-soluble P in the model) contributed to explaining 87 % of the variation in the ratio of shoot DM in *B. subtilis* inoculated plants to shoot DM in the control (Fig. 3). Olsen P was correlated with Fe_{ca}/Fe_{cbd} ($P < 0.05$). After the VIP and the ridge equation regression were performed, the result of both multiple regressions can be considered solid enough to assess the effect of Olsen P and Fe_{ca}/Fe_{cbd}. Organic P forms contributed positively to both ratios. For P uptake and shoot DM, the ratio was related quadratically with Olsen P ($R^2 = 0.58$, $P < 0.05$; $R^2 = 0.77$, $P < 0.01$, respectively). However, when the two soils with lower Olsen P were excluded (less than 5 mg kg^{-1}), the relative effect of *B. subtilis* on P uptake and DM decreased linearly with increased Olsen P values ($R^2 = 0.49$, $P < 0.05$ and $R^2 = 0.67$, $P < 0.01$, respectively; $n = 10$). Although the differences in pH were minimal between *B. subtilis* and control, 86 % of the variance in the ratio between both treatments was accounted for by Olsen P, which contributed negatively, SOM, and organic P (Fig. 4); both of these later variables were correlated ($P < 0.05$).

On the relative basis defined above, Olsen P, Fe_{ca}/Fe_{cbd} and phytase hydrolysable P in NaOH extracts accounted for 82 % and 74 % of the variation in the effect of T34 on P uptake and shoot DM, respectively (Figures 2 and 3). In spite of the correlation of Olsen

P and Fe_{ca}/Fe_{cbd}, their sign in both multiple regressions can be considered accurate according to the results of the ridge regressions. Above 5 mg kg^{-1} of Olsen P, the relative effect of T34 on shoot DM decreased linearly as the Olsen P values increased ($R^2 = 0.41$, $P < 0.05$, $n = 10$). The relative effect of T34 on pH was explained by Olsen P and the sum of phytase hydrolysable P in all the fractions in the sequential scheme used (Fig. 4).

4 Discussion

4.1 Effect of microorganisms on plant development and P uptake

Trichoderma asperellum T34 led to lower P concentration in shoots than *B. subtilis* and the non-inoculated control. The effect was not ascribed to a dilution effect due to increased DM accumulation in the shoots (which was not significantly different from other treatments), or to an overall decreased P uptake. In addition, P concentration and DM in roots were not affected by inoculation with microorganisms. On the contrary, the decreased P concentration in shoots with T34 may reveal some change in the translocation of P from roots to shoots caused by this microorganism. Alternatively, this lower P concentration in T34 treated plants when compared with other treatments may be also the partial consequence of a lower phosphatase activity in the rhizosphere of T34-inoculated plants. Although this microorganism produces P-hydrolytic enzymes, the hydrolytic activity may be constrained by certain properties of the plant growing media (García-López et al. 2015).

The effect of microorganisms on P uptake and plant development measured as shoot DM yield varied, depending on the soil. In some soils, inoculation did not increase either variable, but this was not due to a failure in the colonization of rhizosphere by the microorganisms, since CFU of both *B. subtilis* and T34 were detected in significant concentrations in all the inoculated pots. The soil properties affected the CFU of *B.*

subtilis, but not that of T34. The density of this latter microorganism was not correlated with biochemical or physicochemical properties of soil within the range studied. In the case of *B. subtilis*, CFU was negatively correlated with β-glucosidase activity at the end of the experiment. This proved that the density of this microorganism decreased with increased general microbial activity in soil. In this regard, the competition between the studied strain of *B. subtilis* and other microorganisms has proved to be a pivotal issue in explaining its benefits in the inoculated growing media (Gossen et al. 2016). However, for both microorganisms, there was no relationship between CFU and shoot DM yield or P uptake by plants. Thus, the density of microorganisms was not a significant factor explaining its potential benefits on plants.

Overall, *B. subtilis* was more effective in increasing P uptake than T34, as revealed by the value of the ratio of its effect to the effect of the non-inoculated control. For the former inoculant, the ratio was above 1 in most of the soils, meanwhile for T34 it was below 1 in most of the cases (Fig. 2). The benefits of *B. subtilis* on P uptake when compared with T34 could be ascribed to an increased mobilization of soil P, as revealed by the lower decrease in Olsen P in rhizospheric soils with *B. subtilis* relative to T34. Furthermore, in three of the soils, *B. subtilis* increased Olsen P in rhizospheric soil, thus revealing its potential for increasing soil P availability to plants in soil. The mobilization of P by *B. subtilis* depended on the density of the microorganism, as revealed by the relationship between the ratio of final Olsen P in the rhizosphere to the initial Olsen P and CFUs. However, P uptake was not related to Olsen P or CFUs in the rhizosphere after cultivation. This may point to the idea that that not only P mobilization but also other factors affecting P absorption by plants can affect the final P uptake (García-López et al. 2016). In the case of T34, P mobilization increased as the ratios of Fe in poorly crystalline oxides increased as compared to that in crystalline oxides (Fe_{ca}/Fe_{cbd}). This likely reveals

398 that P can be more easily mobilized by this microorganism when bound to poorly
399 crystalline oxides.
400 Both microorganisms were effective in increasing DM yield in the majority of
401 soils (Fig. 3). The growth-promoting effect of *B. subtilis* may be related at least partially
402 to improved P nutrition. In the case of T34, other factors probably contribute to its
403 growth-promoting effect. It is worth noting that the Olsen P of the soils studied were in
404 general below the threshold value for fertilizer response (Recena et al. 2016), and
405 consequently, the DM yield must be limited by low P availability in the soil. Thus, an
406 improved P uptake by plants should be reflected in a greater DM yield. This is supported
407 by the properties explaining the relative effect of both microorganisms when compared
408 with the non-inoculated control: for P uptake and shoot DM, the explicative variables in
409 the multiple regression models were almost the same and were related to the P
410 biogeochemistry in the soil (Figures 2 and 3).
411
412 **4.2 Soil properties affecting the action of microorganisms**
413 In previous studies, the action of *B. subtilis* was shown to be unrelated to the level of P
414 availability in the artificial plant-growing medium (García-López and Delgado 2016).
415 However, in the soils studied here, the effect of both microorganisms on P uptake and
416 shoot DM yield was affected by the initial Olsen P. Both microorganisms promoted
417 increased P uptake the more the values of Olsen P in the soil decreased. This agrees with
418 the negative sign of Olsen P in the multiple regressions (Fig. 2 and 3), and with the linear
419 relationship of their relative effect on P uptake and shoot DM with Olsen P (above 5 mg
420 kg^{-1}). In addition, the population density of *B. subtilis* was negatively correlated with
421 initial Olsen P. This likely reveals that the microorganism was more competitive in soils
422 with lower initial P availability level. This is in agreement with the known fact that

423 increased P availability in the soil decreases the populations of oligotrophic bacteria such
424 as *B. subtilis* through its effect on root exudates (Koyama et al. 2014).

425 The positive effects of microorganisms on P uptake and shoot DM yield increased
426 with increased concentrations of phytase-hydrolysable P in NaOH extracts. This leads us
427 to assume that the benefits of both microorganisms were at least partially due to the
428 hydrolysis of organic P, which is consistent with their well-known hydrolytic enzyme
429 production (García-López et al. 2015; 2016). However, it is likely that for T34, other
430 enzymes different to the phosphatase assayed, probably phytases (García-López et al.
431 2015), contributed to this effect. In addition, the total organic P in the soil helps to explain
432 the positive effects of *B. subtilis* on shoot DM. According to Recena et al. (2016; 2017),
433 organic P can be a source of P for plants. All this evidence points to the fact that the
434 positive contribution of these microorganisms to P uptake by plants can be ascribed at
435 least partially to the hydrolysis of non-readily available organic P forms.

436 The mineralogy of Fe oxides was significant in explaining the effects of both
437 microorganisms on P uptake and shoot DM yield. Here, the ratio of Fe ascribed to poorly
438 crystalline oxides to that ascribed to crystalline oxides (Fe_{ca}/Fe_{cbd}) contributed to
439 explaining the effects of both microorganisms. This ratio has proved crucial in accounting
440 for the amount of P available to plants, since P concentration in the soil solution is
441 expected to increase with increased ratios, as the affinity of P for poorly crystalline oxides
442 is lower than that for crystalline oxides (Recena et al. 2017). Thus, P uptake by plants is
443 expected to be enhanced when Fe_{ca}/Fe_{cbd} is increased. This makes the contribution of the
444 microorganisms studied to P uptake at increased ratios of poorly crystalline oxides to
445 crystalline oxides less evident (Fig. 2).

446 The relative effect of *B. subtilis* on P uptake and shoot DM was negatively affected
447 by CCE. Thus, the soil buffering capacity constrained the benefits of this microorganism.

448 This leads us one to conclude that P mobilization by *B. subtilis* could be ascribed to the
449 acidification of the rhizosphere, in agreement with previous evidence (García-López and
450 Delgado 2016). This acidification may contribute to dissolving any precipitated metal
451 phosphates. However, the contribution of poorly soluble Ca phosphates (citrate soluble,
452 Pc) to P uptake seemed to be negative. This probably indicates that, when these forms
453 were abundant, other forms such as organic P were preferentially mobilized. In addition,
454 the phosphatase activity for all treatments correlated negatively with CCE, which also
455 goes towards explaining the negative effect of this compound on the effect of *B. subtilis*.
456 Furthermore, in the case of T34, its relative effect when compared with control was not
457 affected by CCE, which is perhaps caused by a decreased contribution of acidification
458 mechanisms on P release by this microorganism, as observed by García-López et al.
459 (2016).
460 The acidification capacity of both microorganisms was also affected by the soil
461 properties; it increased as initial Olsen P decreased and fell when OP or the sum of
462 phytase hydrolysable P in all the fractions rose (Fig. 4). This likely reveals that initial P
463 availability level and concentration of hydrolysable organic P are factors explaining the
464 triggering of P mobilization based on acidification. In particular, acidification fell when
465 there was an increased concentration of potentially hydrolysable organic P.
466 Enzymatic activities in the rhizosphere were also affected by soil properties. Here,
467 both actions studied increased with SOM. This was probably due to the positive effect of
468 SOM on microbial biomass (Moreno et al. 2016) and root development. As expected,
469 phosphatase correlated negatively with initial Olsen P, revealing that this mechanism was
470 triggered less as P availability in the soil increased. This activity increased with OP, which
471 probably indicates that hydrolytic activity may be triggered by the presence of substrate,
472 in agreement with the previous evidence observed for T34 (García-López et al. 2015).

When non-phytoavailable, poorly soluble Ca phosphates are the dominant P forms in the plant growing media, an increased phosphatase activity has also been observed (García-López et al. 2015) which may contribute towards explaining the positive correlation observed between this activity and citrate-soluble P.

Phosphatase activity correlated positively with the soil P adsorption capacity, measured as the amount of clay or Fe in oxides. Several studies have demonstrated that the presence of clay or iron oxides enhanced particular enzyme activities (Bayan and Eivazi 1999; Allison 2006; Shahriari et al. 2010). In addition, a decreased P concentration in soil solution with increased adsorption capacity in the soil may trigger the hydrolytic activity observed previously by García-López et al. (2015) in pure cultures of T34. Since the P concentration in the soil increased as Fe_{ca}/Fe_{cbd} increased, this explains the negative contribution of this ratio to the phosphatase activity in the rhizosphere (Table 6).

5 Conclusions

The effect of inoculation with both microorganisms on plant P uptake and DM yield, compared with the non-inoculated control, was not explained by their density assessed by CFU or by the biochemical properties ascribed to microbial activity in the soil. The lower the initial Olsen P and ratio of poorly crystalline Fe oxides to crystalline oxides in the soil, the higher the relative effect of both microorganisms. In addition, higher concentrations of phytase-hydrolysable P led to both microorganisms improving P uptake and DM yield, compared with the control. Although the potential effects on the microbial community structure were not within the scope of this study, the soil properties which accounted for the triggering and efficiency of P-mobilizing mechanisms of these microorganisms were, in fact, the most important factors in explaining their potential benefits on the P nutrition of crops.

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FIGURE CAPTIONS

Figure 1. Estimation of colony forming units (CFU) of *Bacillus subtilis* QST713 as a function of electrical conductivity (EC), β-glucosidase activity (Glu), organic matter (SOM) and clay content in soil. All the variables used in the model were significant at $P < 0.05$. Significant correlations were observed between clay and EC ($P < 0.05$). The variance inflation factor was always lower than 5. The sign of the coefficients did not change when a ridge regression was performed with VIP adjusted to 1.

Figure 2. a) Estimation of the ratio of P uptake *in Bacillus subtilis* QST713 inoculated plants to that in the non-inoculated control, as a function of phytase-hydrolysable P in NaOH extracts of the sequential fractionation scheme (NaOH-P_{phyt}), Olsen P, Ca carbonate equivalent (CCE), the ratio of Fe in poorly crystalline oxides to that in crystalline oxides (Fe_{ca}/Fe_{cbd}) and citate soluble P in the sequential fractionation scheme (Pc); b) Estimation of the ratio of P uptake in *Thricoderma asperellum* T34 inoculated plants to that in the non-inoculated control, as a function of phytase-hydrolysable P in NaOH extracts of the sequential fractionation scheme (NaOH-P_{phyt}), Olsen P, and the ratio of Fe in poorly crystalline oxides to that in crystalline oxides (Fe_{ca}/Fe_{cbd}). All the variables used in the model were significant at $P < 0.05$.
Significant correlations were observed between Olsen P and Feca/Fecbd, and NaOH-Pphyt and Pc ($P < 0.05$). The variance inflation factor was always lower than 5. The sign of the coefficients did not change when a ridge regression was performed with VIP adjusted to 1.

Figure 3. a) Estimation of the ratio of shoot dry matter (DM) *in Bacillus subtilis* QST713 inoculated plants to that in the non-inoculated control, as a function of Olsen P, total organic P (OP), Ca carbonate equivalent (CCE), the ratio of Fe in poorly crystalline oxides to that in crystalline oxides (Fe_{ca}/Fe_{cbd}), and phytase-hydrolysable P in NaOH extracts of the sequential fractionation scheme (NaOH-P_{phyt}); b) Estimation of the ratio of P uptake in *Thricoderma asperellum* T34 inoculated plants to that in the non-inoculated control as a function of Olsen P, the ratio of Fe in poorly crystalline oxides to that in crystalline oxides (Fe_{ca}/Fe_{cbd}), and phytase-hydrolysable P in NaOH extracts of the sequential fractionation scheme (NaOH-P_{phyt}). All the variables used in the model were significant at $P < 0.05$. Significant correlations were observed between Olsen P and Feca/Fecbd ($P < 0.05$). The variance inflation factor was always lower than 5. The sign of the coefficients did not change when a ridge regression was performed with VIP adjusted to 1.

Figure 4. a) Estimation of the ratio of pH in rhizosphere *in Bacillus subtilis* QST713 inoculated pots to that in non-inoculated pots, as a function of Olsen P, soil organic matter (SOM), and total organic P (OP); b) Estimation of the ratio of pH in rhizosphere in *Thricoderma asperellum* T34 inoculated pots to that in the non-inoculated pots, as a function of Olsen P, and the sum of phytase-hydrolysable P in all the extracts of the sequential fractionation scheme (P_{phyt}); Olsen P accounted for 54 % of the variance explained by the model, and P_{phyt} 46 %. All the variables used in the model were significant at $P < 0.05$. Significant correlations were observed between OP and SOM ($P < 0.05$). The variance inflation factor was always lower than 5. The sign of the coefficients did not change when a ridge regression was performed with VIP adjusted to 1.