

Effects of *Penicillium bilaii* on maize growth are mediated by available phosphorus

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Abstract

Background and aims Inoculation with *Penicillium bilaii* has been reported to increase plant growth, which is attributed to increase availability of phosphorus (P), but similar effects have been observed with no P

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limitation, suggesting that other mechanisms may be involved. The aim of this work was to evaluate the interaction between available soil P and *P. bilaii* inoculation on plant growth.

Methods Maize plants (*Zea mays*) inoculated with *P. bilaii* and non-inoculated were grown in pot experiments in three soils with different inherent P availability, and in a low P availability soil with different additions of mineral P, both with and without supplementation of all other macro and micronutrients.

Results We found a positive interaction between *P. bilaii* and available P, when other nutrients were applied, with *P. bilaii* inoculation resulting in increased root growth and thus nutrient uptake and plant growth. By contrast, when other nutrients were not supplied, little effect on plant growth or P uptake was observed, except for root length that tended to decrease in inoculated plants with P addition.

Conclusions The effects of *P. bilaii* inoculation were related to root growth and function, but were dependent on the nutrient status in the soil environment.

Keywords Phosphorus availability · *Penicillium bilaii* · Plant growth promotion · Root length · Phosphorus-solubilising microorganisms

Introduction

There is widespread interest in developing microbial-based approaches to improve the phosphorus (P) nutrition of plants in agriculture in order to reduce

dependency on use of rock mineral P fertilizers, and to improve the efficiency of fertilizer use in production systems across the globe (Richardson et al. 2011). A wide range of microorganisms with potential to increase the plant availability of P in soil have been reported, and in many cases these different bacteria and fungi have been evaluated in soil with varying success (Kucey 1983; Richardson 2001).

Penicillium bilaii is a soil fungus which has been shown to solubilise P under controlled conditions, such as calcium phosphate in agar medium (Kucey 1983) and rock phosphate when grown in liquid culture (Asea et al. 1988). In culture, *P. bilaii* has been shown to release both oxalate and citrate along with marked acidification of the growth medium (Cunningham and Kuiuack 1992). Kucey (1987) also observed an increase in NaHCO₃-extractable P in soil when inoculated with *P. bilaii*. Based on these observations, several potential mechanisms for P mobilisation in soil by microorganisms, such as *P. bilaii*, have been proposed, which include: i) the release of organic anions which act to either chelate cations that are associated with phosphate anions in soil (e.g. Ca-P, Fe-P and Al-P), or may promote directly the desorption of phosphate from the soil solid phase through ligand exchange reactions (Richardson 2001; Ryan et al. 2001; Vessey and Heisinger 2001); ii) acidification, whereby H⁺ ions released as the counter ion in response to organic anion exudation (or other anions) can increase the solubility of different forms of precipitated P (e.g. Ca-P in alkaline soils) (Illmer and Schinner 1995; De Oliveira Mendes et al. 2014); and iii) secretion of phosphatases that hydrolyse organic forms of P in soil (Tarafdar et al. 1995). Despite this, the precise mechanisms by which *P. bilaii* may promote plant growth through P mobilisation, or through other mechanisms in soil environments remain poorly understood (Leggett et al. 2015). Various studies have shown that inoculation of plants with *P. bilaii* can increase growth and yields across a wide range of different crops and in glasshouse or field conditions, including wheat (Asea et al. 1988; Gleddie et al. 1991), bean (Kucey 1987), canola (Kucey and Leggett 1989), pea (Downey and van Kessel 1990), lucerne (Beckie et al. 1998) and maize (Leggett et al. 2015). The majority of these trials have been conducted under low soil P conditions, so positive effects on plant growth are commonly attributed to a higher P availability associated with *P. bilaii* inoculation. In support of this, several studies have also observed an increase in tissue P concentration when *P. bilaii* was inoculated on

wheat and flax (Chambers and Yeomans 1991) or canola (Kucey and Leggett 1989).

By contrast, other studies have not found an increase in P content or tissue P concentration of plants, even when plant growth was increased by *P. bilaii* inoculation (Downey and van Kessel 1990; Keyes 1990; Chambers 1992; Gleddie 1992; Heisinger 1998; Vessey and Heisinger 2001). Gulden and Vessey (2000) and Vessey and Heisinger (2001) reported an increase in root length and root hair abundance in inoculated plants, which could increase plant growth by meeting the nutrient and water requirements through an increased absorptive capacity of the roots (Wang et al. 2016). This suggests that yield increase in plants inoculated with *P. bilaii* might be due to other factors that indirectly improve plant P uptake in addition to the possibility of direct P solubilisation. Sánchez-Esteva et al. (2016) similarly found root growth stimulation in wheat by *P. bilaii* as a general trend when comparing a moderately acidic and a calcareous soil. However, in a low fertility control treatment of the moderately acidic soil, a depression in root and shoot growth was observed (Sánchez-Esteva et al. 2016), which suggests a possible interaction between levels of soil nutrient availability and competition effects of *P. bilaii* on plant growth.

Lack of consistent results in growth promotion of plants inoculated with *P. bilaii* under field conditions has also been widely reported. For example, Karamanos et al. (2010) reported that *P. bilaii* inoculation increased the yield of spring wheat significantly in only five of 47 field experiments, whereas in nine cases there was a significant decrease. In this meta-analysis, response to inoculation could not be attributed to the level of soil P availability, soil organic matter content or to various climatic factors (Karamanos et al. 2010). Leggett et al. (2015) observed a higher incidence of yield increase in maize, whereby response in 71 and 79% of their small and large plots respectively, was reported (461 plots in total), with response to inoculation being more effective in fields with low or very low soil P. In contrast, in earlier studies Hnatowich et al. (1990) reported an increase in wheat grain inoculated with *P. bilaii*, but only when P was applied. In a recent rhizobox study (Gómez-Muñoz et al. 2017b), an increase in root growth vigour of *P. bilaii* inoculated maize grown in a low P soil was observed, compared to the un-inoculated control. However, there was no effect on P uptake, which lead us to question if the soil was simply too infertile to sustain a beneficial effect of *P. bilaii* inoculation. Based on this,

and the results from Sánchez-Esteva et al. (2016) we can speculate that at a more intermediate fertility level, increased root growth vigour may lead to increased P uptake, whereas at high fertility levels such effects would tend to be negated as the plants demand for P otherwise becomes non-limited. Thus, it is not clear which conditions support the beneficial effect of the fungus on plant growth and whether the level of soil P fertility is a contributing factor.

On the other hand, poor effectiveness of microbial inoculants (growth, colonisation and survival) under limited P might be associated with low competitiveness compared to the indigenous microflora due to nutrient deficiencies in the rhizosphere (Marschner 2008), or through severe P deficiency that restricts plant growth. In competition with plants at low P, the growth of microorganisms close to the root associated with root exudates and their demand for P, could result in a significant immobilisation of P. Microbes are well adapted and highly competitive at acquiring P from soil (McLaughlin and Alston 1986). Whilst immobilized P can later become available for plants through biomass turnover (Jakobsen et al. 2005), this immobilisation in the short term may restrict plant growth. Hence, to ensure the optimal growth and survival of both the inoculant and the plant, a minimum availability of soil nutrients may be needed to satisfy the requirement of both partners. While many studies have focussed on the effects of *P. bilaii* on growth promotion of different crops and conditions, no studies have systematically investigated the effects on plant growth and nutrient uptake across different levels of soil P availability with and without limitation of other plant nutrients.

The objective of this study was to investigate whether there is an interaction between soil fertility level, especially available soil phosphorus, and the impact of *P. bilaii* on plant growth. We hypothesised that: 1) at low soil fertility and P availability, inoculation may stimulate root growth, but not affect P uptake of shoot biomass, due to nutrient limitation, 2) at intermediate fertility and P levels, P uptake and biomass production may be increased in response to inoculation, 3) the addition of P without other nutrients to a low fertility soil will not improve maize growth in inoculated plants due to nutrient limitation and competition between plants and *P. bilaii*, and 4) at high P and fertility levels, no benefits of *P. bilaii* inoculation would be expected to be observed.

Materials and methods

Pot experiments

Soil was collected from a long-term fertilization trial (CRUCIAL), located on the experimental farm of the University of Copenhagen, Denmark (55°40′51.7″N, 12°16′35.8″E). This field experiment was initiated in 2003 and has mainly been cropped with spring cereals. The soil type was a sandy loam (clay 12.6%, silt 14.3% and sand 69.8%) Luvisol (FAO classification). In the present study the soils were chosen according to their P content and availability, using soils with low fertility and P availability (L), unfertilised and cropped since initiation of the trial in 2003, a cattle slurry treatment (around 50 tons slurry ha⁻¹ y⁻¹, annually) with medium fertility and P availability (M) and a solid cattle manure treatment with relatively high annual input (around 85 tons solid manure ha⁻¹ y⁻¹) and thus high fertility and P availability (H). The main properties of these soils are presented in Table 1 and more information for the soils can be found in López-rayo et al. (2016) and Gómez-Muñoz et al. (2017a).

Three growth chamber pot experiments with maize (*Zea mays*, variety Ambition from Limagrain A/S, Horsens, Denmark) were set up using similar environmental conditions. The variety Ambition was selected due to its early vigour and being well adapted to a wide range of soil conditions. Maize plants were inoculated with *Penicillium bilaii* using a solution prepared from a commercial product, JumpStart® Wetttable Powder, 2015

Table 1 Initial soil pH, loss on ignition, total N, total C, total P, inorganic N (ammonium + nitrate) and water-extractable P in the low (L), medium (M) and high (H) P soils used in the experiments

Properties	Soil		
	L	M	H
pH	7.2 ± 0.2	7.3 ± 0.3	7.0 ± 0.1
Loss on ignition (g kg ⁻¹)	51 ± 6	53 ± 5	70 ± 5
Total N (g kg ⁻¹)	1.64 ± 0.01	1.83 ± 0.2	2.38 ± 0.2
Total C (g kg ⁻¹)	14.6 ± 0.16	16.6 ± 1.92	23.8 ± 2.08
Total P (mg kg ⁻¹)	430 ± 11	580 ± 22	666 ± 26
Inorganic N (mg kg ⁻¹)	6.8 ± 0.9	8.7 ± 1.4	17.4 ± 2.7
Water-extractable P (mg kg ⁻¹)	2.93 ± 0.29	13.8 ± 1.0	33.1 ± 1.7

Values indicate mean ± standard deviation (n = 4)

(PROD.# 7065102-705 Novozymes BioAg Limited, Saskatoon, Canada).

For experiment 1, the three different soils (low, medium and high P availability) were mixed with a P-free nutrient solution (see below) (L_{Nut} , M_{Nut} and H_{Nut} , respectively) and sown with maize seeds either non-inoculated or inoculated with *P. bilaii* ($L_{Nut}Pbi$, $M_{Nut}Pbi$ and $H_{Nut}Pbi$). In this experiment (6 treatments), eight replicates were set up per treatment (48 pots in total), four of which were harvested 28 days after sowing (DAS) and the rest at 38 DAS, although results from the second harvest only are reported. For experiment 2, only soil with low P availability (L) was used, but with different additions of mineral P fertiliser (supplied as triple super phosphate, TSP). TSP (18% P content), was applied at 4 different levels of 0, 20, 40 and 60 mg P kg^{-1} and pots were sown with non-inoculated (L, L20, L40 and L60) or *P. bilaii* inoculated maize seeds (LPbi, L20Pbi, L40Pbi and L60Pbi). In this experiment, no other macro and micronutrients were added, only Milli Q® water was added during growth. For experiment 3, the setup was the same as used for experiment 2, but in this case all the other macro and micronutrients were supplied with a P-free modified Hoagland nutrient solution applied at the beginning of the experiment. Nutrients were added at a rate of 150 mg N kg^{-1} , 112 mg K kg^{-1} , 30 mg Ca kg^{-1} , 0.15 mg Cu kg^{-1} , 15 mg Mg kg^{-1} , 0.30 mg Zn kg^{-1} , 0.014 mg Mo kg^{-1} , 0.30 mg Fe kg^{-1} , 0.22 mg B kg^{-1} and 0.45 mg Mn kg^{-1} . Each treatment was replicated 4 times (giving $4 \times 2 \times 4 = 32$ pots for each of the two stages) and plants were harvested at 28 DAS for both experiment 2 and 3.

In all of the experiments, each pot contained 600 g of air dry and sieved soil (<4 mm) that was mixed with 200 g sand (3:1; soil:sand) in a plastic bag to increase the recovery of intact roots at harvest (Wang et al. 2016). In treatments where P was applied as mineral fertiliser (TSP), the required amounts (20, 40 and 60 mg kg^{-1}) were added as a finely ground powder and mixed with the soil. In all cases, the moisture content was adjusted to 40% of field capacity (corresponding to 12.6%, 14.5 and 15.9% gravimetric content for L, M and H, respectively) and the soil mixture was pre-incubated at 20 °C for seven days.

On the day of sowing, each pot (0.8 kg) was filled with the soil mixture and two maize seeds (previously washed with distilled water) were sown in the centre of each pot at 2 cm depth, and after germination one of the seeds was removed. *P. bilaii* inoculum was also added

on the day of sowing by providing a spore suspension on top of the seed at a rate of 7.2×10^5 CFU seed⁻¹.

The experiments were carried out under controlled conditions in a growth chamber with day/night of 16/8 h and temperature levels of 19/15 °C, and humidity levels of 65/72%, respectively, and a light intensity of 400 μE . Water content was corrected every two days to reach 40% of field capacity during the first week of growing, and the water content was then increased to and maintained thereafter at 60%.

Shoot and root analysis

Shoot height was recorded manually approximately every two days, starting five DAS in all experiments. At harvest, the soil was washed off the roots and the roots were washed and scanned using the STD4800 Epson scanner and analysed using WinRHIZO software (Regent Instruments Inc. 2005). Root length was measured for coarse (>0.6 mm) and fine (<0.6 mm) roots. After scanning, roots and the shoots were dried at 70 °C to determine dry matter biomass. Shoots and roots were subsequently milled and digested with HNO₃ and H₂O₂ before analysis by ICP-OES Agilent 5100 to estimate macro and micronutrient contents.

Soil sampling and analysis

On the day of harvest, soil from each pot was homogenised and soil samples were taken and analysed for water-extractable P (WEP) by extracting with MilliQ water (1 g:60 ml), shaking for 1 h and then filtering through a 0.45 μm filter (Van der Paauw 1971). The extracts were analysed for orthophosphate content using the molybdenum blue method with flow injection analysis (FIA star 5000 flow injection analyser, Foss Analytical, Denmark).

Statistical analysis

All the experiments were set up under a completely randomized design. Differences between the treatments for all the variables studied were tested using two-way ANOVA (including interactions) and the Fisher post hoc test. Normality was checked in all cases and significance was accepted at $P < 0.05$. All statistical analyses were performed using the software 'R' version 3.4.1 (R Core Team 2017).

Results

Shoot growth

During the first 15 DAS, shoot lengths were similar for all the treatments and after that shoot length varied across the different treatments (Fig. 1). In experiment 1, using three soils with different inherent soil fertility and P availability (Table 1), shoot length was significantly longer for the high P soil (H) compared to the low and medium P soils (L and M, respectively), and the differences increased over the growing period (Fig. 1a). At the end of the experiment (38 DAS) maize growth was affected significantly by level of soil P availability, with shoot length being longer for $H_{Nut} > M_{Nut} > L_{Nut}$ (Table 2; Table 3, supplementary material Figure S1). Similar differences were observed for shoot biomass with values of 5.6, 1.4 and 0.87 g for H_{Nut} , M_{Nut} and L_{Nut} , respectively (Fig. 1b). Inoculation with *P. bilaii* increased the shoot length and shoot biomass significantly in medium P soil ($M_{Nut}Pbi$) compared to the same soil but without inoculation (M_{Nut}), whereas there was

no significant difference due to inoculation in the low (L_{Nut}) and high (H_{Nut}) P soils (Fig. 1b; Tables 2, 3).

In experiment 2 and 3, using the low P availability soil that was fertilized with different levels of P as TSP, shoot lengths were significantly higher when the soil was amended with macro and micronutrients (L_{Nut}) compared to soil without added nutrients (L) from experiment 2 (Fig. 1c, e; Table 3, supplementary material Figure S1). In the soil without additional nutrients, there was a small effect of P addition on shoot length (Fig. 1c; Table 3), but not for shoot biomass (Fig. 1d). Plants inoculated with *P. bilaii* showed differing shoot lengths in the L20 and L40 treatments compared to the non-inoculated treatments, with longer shoot length for L20 compared to L20Pbi and the opposite effect being observed for L40 where the shoot length was longer for L40Pbi compared to L40 (Table 3). However, these differences were not evident in shoot biomass (Fig. 1d; Table 2). With addition of macro and micronutrients in experiment 3, *P. bilaii* inoculation showed no effect on shoot length, except for the treatment with no P

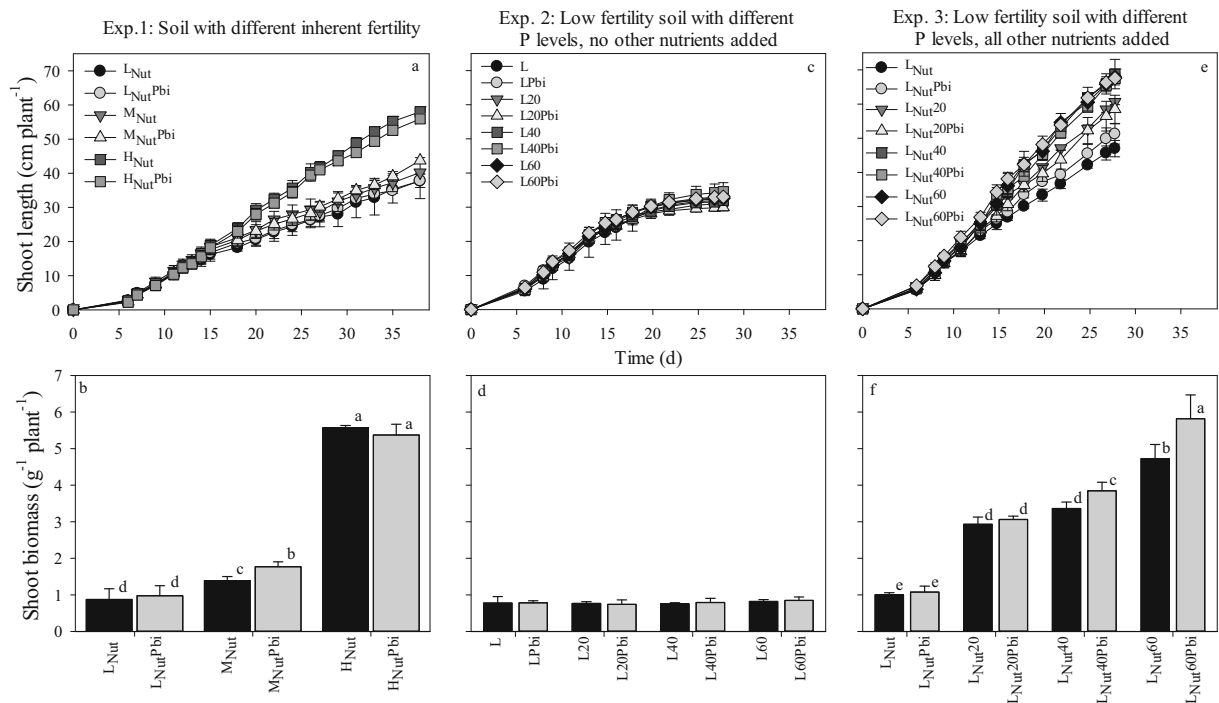


Fig. 1 Shoot length and shoot biomass for experiment 1: maize grown in low (L), medium (M) and high (H) P soil (a, b), experiment 2: maize grown in low fertility soil without adding other nutrients (c, d), and experiment 3: maize grown in low fertility soil and adding all other nutrients (e, f). For experiment

2 and 3 different rates of P were applied as TSP (20, 40 and 60 mg P kg⁻¹) and *P. bilaii* inoculation (Pbi) estimated after 38 DAS (a and b) and 28 DAS (c, d, e and f). Values indicate mean ($n = 4$) and bars denote standard deviation. Different letters in the same plot indicate significant differences between treatments ($P < 0.05$)

Table 2 Results of Factorial ANOVA *P* values for the different soil fertility levels or added P (TSP) to the low fertility soil, the effect of the *P. bilaii* inoculation (Pbi) and the interaction between both parameters

Factor	Shoot length	Shoot biomass	Root length	Root biomass	Shoot P conc.	Shoot P uptake	Shoot P uptake/root length ratio
Experiment 1: Soil with different inherent fertility							
Soil fertility	< 0.001	< 0.001	< 0.001	< 0.001	0.094	< 0.001	< 0.001
Pbi	0.613	0.489	0.056	0.052	0.060	0.038	0.710
Soil fertility x Pbi	0.083	0.138	0.606	0.030	0.274	0.786	0.462
Experiment 2: Low fertility soil with different P levels added, no other nutrients added							
TSP	0.022	0.395	0.087	0.933	< 0.001	< 0.001	< 0.001
Pbi	0.509	0.750	0.021	0.118	0.323	0.130	< 0.001
TSP x Pbi	0.003	0.909	0.028	0.753	0.088	0.313	0.149
Experiment 3: Low fertility soil with different P levels added, all other nutrients added							
TSP	< 0.001	< 0.001	< 0.001	< 0.001	0.005	< 0.001	< 0.001
Pbi	0.912	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.682
TSP x Pbi	0.104	0.013	0.001	0.006	0.265	0.003	0.217

addition where shoot length was higher in $L_{Nut}Pbi$ compared to L_{Nut} (Table 3). However, P fertilization resulted in increased shoot length in all treatments, with greatest increase occurring when higher rates of P were added (Fig. 1e, Table 2). At plant harvest, shoot lengths were similar with 40 and 60 mg P kg⁻¹ added, which were significantly higher than the shoot lengths observed for the 0 and 20 mg P kg⁻¹ treatments (Table 3). This effect of P application on shoot length was observed regardless of *P. bilaii* inoculation (Table 2). Shoot biomass was also increased with increasing rate of P addition when the soil was amended with other nutrients (Nut), but contrary to shoot length the increase in shoot biomass was enhanced by *P. bilaii* inoculation, which was significant for the $L_{Nut}40$ and $L_{Nut}60$ treatments when compared to the same treatments but without *P. bilaii* inoculation (Fig. 1f; Table 2). In the absence of added nutrients, there was no difference in shoot biomass across all P rates and no effect of inoculation (Fig. 1d).

Root growth

Root length and root biomass also showed significant response to available soil P and P fertilizer addition across the three experiments. Fine (<0.6 mm) and coarse (>0.6 mm) root lengths of maize grown in high P availability soil (H_{Nut}) were approximately two-fold longer than in the medium P availability soil (M_{Nut}),

which showed significantly longer coarse root length than maize grown in low P soil (L_{Nut}) (Fig. 2a). Total root length, volume and surface area, and root biomass were also higher in soils with increasing P availability, with significant differences between the $H_{Nut} > M_{Nut} \geq L_{Nut}$ soils (Fig. 2b; Tables 2 and 3). Inoculation of plants with *P. bilaii* showed a variable response to P availability with a tendency to increase root length and biomass in the soil with low inherent P availability compared to non-inoculated treatments (Fig. 2b; Tables 2 and 3), albeit with only root biomass being significantly higher for $L_{Nut}Pbi$ treatment compared to non-inoculated L_{Nut} (Fig. 2b). On the other hand, fine and coarse root length, total root length, volume and surface area, and root biomass were similar in low P soils without macro and micronutrients addition, irrespective of P fertilizer addition (Fig. 2c, d; Table 3). However, when additional macronutrients and micronutrients were applied, fine and coarse root length and root biomass increased significantly with increasing P application (Fig. 2e, f; Tables 2 and 3). In this case there was a significant interaction with *P. bilaii* inoculation (Table 2), whereby root length and biomass were significantly increased by *P. bilaii* at the higher rates of P fertilization (40 and 60 mg P kg⁻¹) (Fig. 2f; Table 3).

The various treatments had marked effects on root morphology, as shown from the root characteristics calculated from the root image data (i.e., root volume, surface area, specific length and specific surface area,

Table 3 Shoot and root length, root volume, surface area, specific root length and specific root surface area at harvest (38 or 28 DAS for the first and second experiment, respectively) of maize grown in low (L), medium (M) and high (H) P soils with macro andmicronutrients except P (Nut) in experiment 1, or low P soil with different rates of P application as TSP (20, 40 and 60 mg P kg⁻¹) and with or without other macro and micronutrients except P added (Nut) in experiment 2 and 3, and *P. bilaii* inoculated (Pbi)

	Shoot length (cm)	Root length (cm)	Root volume (cm ³)	Root surface area (cm ²)	Specific root length (m g ⁻¹)	Specific root surface area (cm ² g ⁻¹)
Experiment 1: Soil with different inherent fertility						
L _{Nut}	37.8 ± 5.2 ^c	2738 ± 763 ^c	3.3 ± 0.7 ^c	336 ± 79 ^c	7548 ± 2089 ^a	929 ± 230 ^a
L _{Nut} Pbi	37.9 ± 2.0 ^c	3349 ± 673 ^{bc}	4.0 ± 0.9 ^c	405 ± 85 ^c	6437 ± 423 ^{ab}	778 ± 50 ^{ab}
M _{Nut}	40.8 ± 1.3 ^c	3960 ± 753 ^{bc}	5.7 ± 1.0 ^b	532 ± 93 ^b	6076 ± 950 ^{abc}	817 ± 117 ^{abc}
M _{Nut} Pbi	43.7 ± 0.9 ^b	4204 ± 154 ^{bc}	6.0 ± 0.5 ^b	561 ± 29 ^b	5672 ± 460 ^{bc}	755 ± 47 ^{bc}
H _{Nut}	58.0 ± 1.1 ^a	7540 ± 1028 ^a	10.0 ± 0.8 ^a	955 ± 102 ^a	4619 ± 486 ^c	585 ± 38 ^c
H _{Nut} Pbi	55.9 ± 0.5 ^a	8087 ± 731 ^a	9.9 ± 0.2 ^a	983 ± 57 ^a	5141 ± 315 ^{bc}	626 ± 31 ^{bc}
Experiment 2: Low fertility soil with different P levels added, no other nutrients added						
L	33.1 ± 1.2 ^{ab}	7022 ± 1459 ^{ab}	5.0 ± 1.2 ^a	660 ± 144 ^{ab}	12,301 ± 1297 ^a	1148 ± 69 ^a
LPbi	31.4 ± 1.1 ^{bc}	7580 ± 306 ^a	5.4 ± 0.6 ^a	713 ± 45 ^a	11,371 ± 419 ^{ab}	1068 ± 48 ^{ab}
L20	32.4 ± 0.2 ^b	6937 ± 582 ^{ab}	5.5 ± 0.4 ^a	687 ± 49 ^{ab}	10,697 ± 724 ^{ab}	1059 ± 44 ^{ab}
L20Pbi	29.9 ± 1.0 ^c	5513 ± 1323 ^b	4.2 ± 1.1 ^a	534 ± 131 ^b	9840 ± 927 ^b	949 ± 81 ^b
L40	32.0 ± 1.2 ^b	7484 ± 921 ^a	5.6 ± 0.2 ^a	718 ± 54 ^a	11,299 ± 600 ^{ab}	1088 ± 30 ^a
L40Pbi	34.6 ± 2.7 ^a	5473 ± 431 ^b	4.5 ± 0.4 ^a	551 ± 43 ^{ab}	10,201 ± 545 ^b	1026 ± 40 ^{ab}
L60	32.6 ± 0.7 ^b	6462 ± 718 ^{ab}	5.1 ± 0.5 ^a	637 ± 64 ^{ab}	10,337 ± 1206 ^b	1018 ± 100 ^{ab}
L60Pbi	33.1 ± 1.0 ^{ab}	6293 ± 425 ^{ab}	4.7 ± 0.3 ^a	602 ± 28 ^{ab}	10,887 ± 1041 ^{ab}	1040 ± 60 ^{ab}
Experiment 3: Low fertility soil with different P levels added, all other nutrients added						
L _{Nut}	47.0 ± 2.4 ^d	4783 ± 539 ^e	5.6 ± 0.4 ^f	572 ± 52 ^c	8572 ± 387 ^{ab}	1026 ± 21 ^a
L _{Nut} Pbi	51.3 ± 3.0 ^c	5330 ± 251 ^e	7.2 ± 0.1 ^e	692 ± 16 ^c	7798 ± 254 ^{bc}	1013 ± 7 ^a
L _{Nut} 20	60.7 ± 0.8 ^b	7526 ± 530 ^d	8.5 ± 0.4 ^d	881 ± 31 ^d	8965 ± 1140 ^a	1046 ± 83 ^a
L _{Nut} 20Pbi	58.5 ± 4.1 ^b	8205 ± 880 ^d	9.6 ± 1.1 ^{cd}	978 ± 102 ^{cd}	8832 ± 620 ^{ab}	1051 ± 42 ^a
L _{Nut} 40	68.8 ± 4.3 ^a	8535 ± 521 ^{cd}	10.8 ± 0.5 ^c	1063 ± 55 ^c	7422 ± 198 ^{cd}	925 ± 19 ^b
L _{Nut} 40Pbi	67.5 ± 1.3 ^a	11,056 ± 744 ^b	13.3 ± 0.5 ^b	1341 ± 64 ^b	7320 ± 377 ^{cd}	888 ± 31 ^{bc}
L _{Nut} 60	67.9 ± 2.3 ^a	9723 ± 376 ^{bc}	13.2 ± 0.1 ^b	1244 ± 32 ^b	6655 ± 200 ^d	852 ± 33 ^{bc}
L _{Nut} 60Pbi	67.6 ± 0.5 ^a	12,882 ± 1178 ^a	15.3 ± 0.9 ^a	1541 ± 108 ^a	6857 ± 267 ^{cd}	821 ± 6 ^c

Values indicate mean ± standard deviation (n = 4). Different letters in the same column indicate significant differences between treatments ($p < 0.05$)

Table 3). In particular, across the three experiments specific root length was significantly greater in all cases in low soil P availability soil (ie., L_{Nut} soil) and was highest, and least variable, across all P levels when additional nutrients were not applied (Table 3). Where nutrients were added, and in addition to the observed increases in total root length with higher P availability, this is consistent with roots becoming finer (ie, thinner roots that contribute to higher root length) in response to more limited P supply. Importantly, across the three experiments and at all P levels, inoculation of plants with *P. bilaii* had no effect on either specific root length (m g⁻¹) or specific root surface area (cm² g⁻¹) (Table 3).

Plant P uptake

At harvest maize plants grown in experiment 1 (38 DAS) showed low P concentrations (0.97 to 1.11 mg P g⁻¹ dry weight; supplementary material Table S1) regardless of the soil or P availability (L_{Nut}, M_{Nut} and H_{Nut}) and *P. bilaii* inoculation. However, and despite this suggesting P deficiency (Reuter and Robinson 1997), the different shoot biomasses observed for the treatments (Fig. 1b) resulted in very different total P contents, that were significantly higher for plants grown in the H_{Nut} > M_{Nut} > L_{Nut} soils (Fig. 3a; Table 2). Across treatments, inoculation with

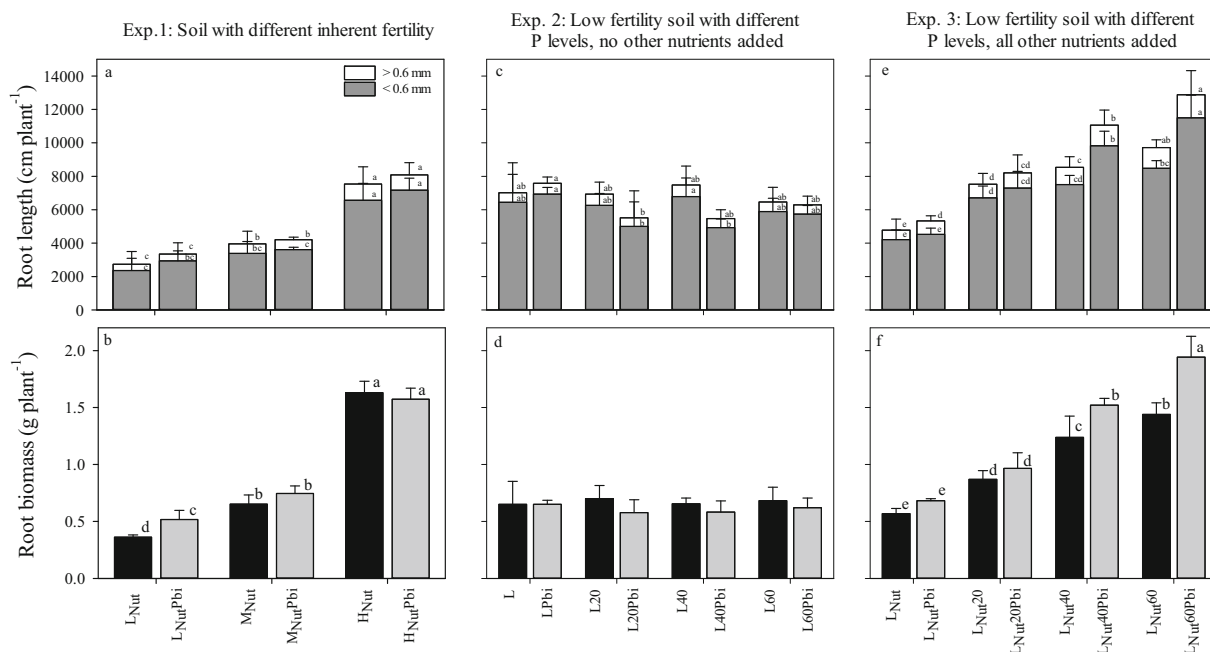


Fig. 2 Root length and root biomass for experiment 1: maize grown in low (L), medium (M) and high (H) P soil (a, b), experiment 2: maize grown in low fertility soil without adding other nutrients (c, d), and experiment 3: maize grown in low fertility soil and adding all other nutrients (e, f). For experiment

2 and 3 different rates of P were applied as TSP (20, 40 and 60 mg P kg⁻¹) and *P. bilaii* inoculation (Pbi) estimated after 38 DAS (a and b) and 28 DAS (c, d, e and f). Values indicate mean (n = 4) and bars denote standard deviation. Different letters in the same plot indicate significant differences between treatments ($P < 0.05$)

P. bilaii increased the total P uptake significantly for the three different soils (Table 2) according to the factorial ANOVA, but the pairwise comparison was only significantly different for the medium P availability soil (i.e., M_{Nut}Pbi compared to M_{Nut}; Fig. 3a).

In experiment 2, for the low fertility soil without macro and micro nutrients applied, the shoot P concentration responded to P addition (1.48 to 4.22 mg P g⁻¹ dry weight; supplementary material Table S1), being significantly higher across all rates of P addition (L < L20 < L40 < L60 treatments, respectively). A comparable increase in the total shoot P uptake in response to P fertilization was observed (Fig. 3c; Table 2) due to the same plant growth (shoot biomass, Fig. 1d) in all these treatments, whereby growth was limited by nutrients other than P, especially N which was below 8.5 mg g⁻¹ (supplementary material Table S1). Despite the reduced growth in these plants, P concentration (supplementary material Table S1) and total P uptake at the highest rate of P supply were significantly higher for plants that were inoculated with *P. bilaii* (L60Pbi compared to L60; Fig. 3c).

Shoot P concentrations were similar (1.07 to 1.12 mg P g⁻¹ dry weight) for plants grown in all treatments in

the low fertility soil that was amended with macro and micronutrients (experiment 3), with deficient concentrations (Reuter and Robinson 1997) that would be expected to regulate plant growth. Nonetheless, significantly greater growth and total shoot P uptake (Table 2) occurred with higher rates of P supply (Fig. 3e). In this case, inoculation of plants with *P. bilaii* resulted in significantly higher total P uptake (Fig. 3e) for plants grown at all rates of P addition (20, 40 and 60 mg P kg⁻¹), with both P rate and *P. bilaii* inoculation being significant, along with a significant interaction (Table 2).

In the three soils with different inherent soil P fertility (experiment 1), the ratio of P uptake per root length, as an index for root P uptake efficiency, increased with the P fertility level, whereas there was no effect by *P. bilaii* inoculation in any of the treatments (Fig. 3b). Similarly, in the low P soil fertilized with different rates of added P and all other macro and micronutrients supplied (experiment 3), the ratio of P uptake per root length was also not affected by *P. bilaii* inoculation, but was greater at higher rates of P fertilization (Fig. 3f). By contrast, in the low P availability soil fertilized with different levels of P but without additional nutrients supplied, inoculation of

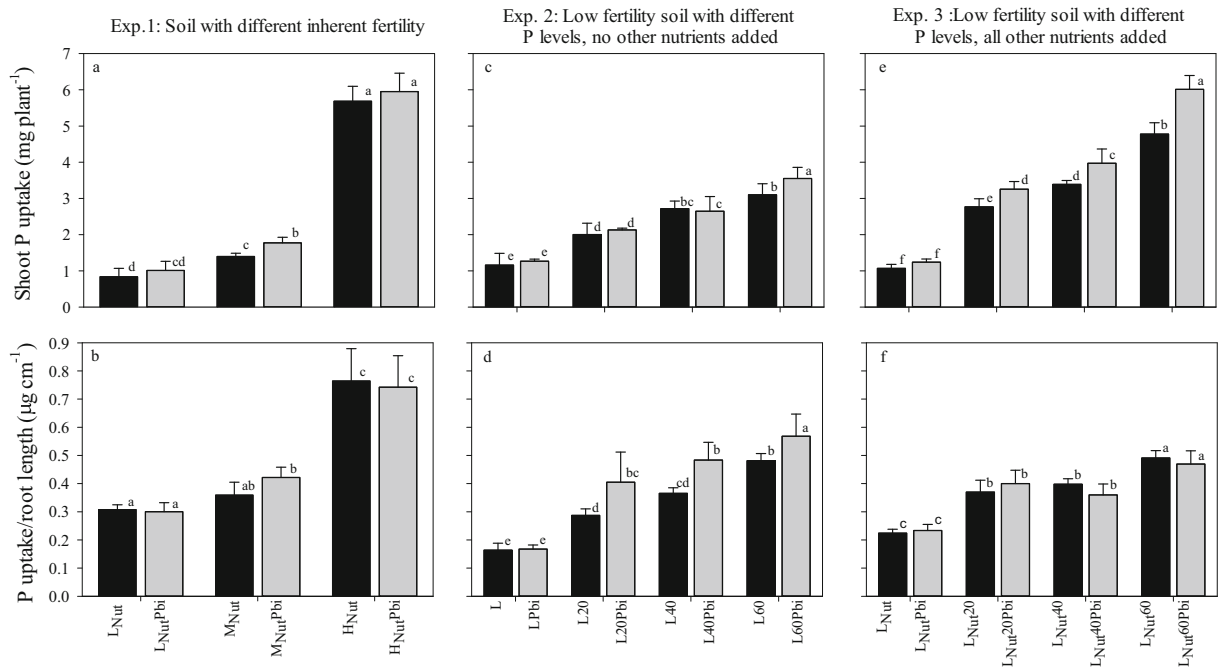


Fig. 3 Shoot P uptake and P uptake/root length ratio for experiment 1: maize grown in low (L), medium (M) and high (H) P soil (a, b), experiment 2: maize grown in low fertility soil without adding other nutrients (c, d), and experiment 3: maize grown in low fertility soil and adding all other nutrients (e, f). For experiment 2 and 3 different rates of P were applied as TSP (20, 40 and

60 mg P kg⁻¹) and *P. bilaii* inoculation (Pbi) estimated after 38 DAS (a and b) and 28 DAS (c, d, e and f). Values indicate mean (n = 4) and bars denote standard deviation. Different letters in the same plot indicate significant differences between treatments (P < 0.05)

plants with *P. bilaii* increased significantly the P uptake per root length ratio (Fig. 3d, Table 2) compared to non-inoculated plants, when P was applied at 20, 40 and 60 mg P kg⁻¹, but not when no P was applied. However, in all cases and in the three experiments the interaction between level of P availability and *P. bilaii* inoculation was not significant (Table 2).

Macro and micronutrients content

In maize grown in the three soils with different inherent fertility (experiment 1), N, Zn and Cu concentrations in shoots were reduced with the increase in P availability in the soils (L_{Nut} > M_{Nut} > H_{Nut}), but concentration of Fe in shoots was not affected (supplementary material Tables S1 and S2). At the end of the experiment, maize plants grown in experiments 1 and 3 had N, Cu and Zn concentrations that indicated potential deficiency, whereas plant growth in experiment 2 may have potentially been limited by Cu and Zn deficiency. Inoculation of plants with *P. bilaii* did not affect the macro and micronutrient concentrations in maize shoot, with the exception of Zn concentration, whereby inoculation tended to increase

Zn compared to non-inoculated plants, though this difference was significant for the low P availability soil only. By contrast, inoculation with *P. bilaii* increased the total N and K uptake in maize plants grown in the medium P soil (M_{Nut}Pbi compared to M_{Nut}), but not low or high P availability soils. In all cases there was an increase in the total macro and micronutrients uptake in maize shoot when plant were grown in the H_{Nut} > M_{Nut} > L_{Nut} soils, respectively.

In experiment 3, when all macro and micronutrients were added, concentrations of most tissue nutrients were reduced with increasing rate of P supply, whereas higher total nutrient uptake was observed. Inoculation with *P. bilaii* increased the shoot concentrations of N, K, Mg, Ca, Mn, B and Cu for plants grown in low P availability soil amended with macro and micronutrients in several instances, but not in a consistent manner across the different P treatments. Similarly, total uptake of nutrients was increased in some instances with seed inoculation of *P. bilaii*, especially at higher rates of P fertilization, due to the combined effect of biomass and nutrient concentrations (supplementary material Tables S1 and S2).

Changes in soil water-extractable P

Soil water-extractable P estimated at plant harvest was significantly higher for the high P fertility soil (H_{Nut}) compared to the low (L_{Nut}) and medium P fertility soils (M_{Nut}) (Fig. 4a). WEP at harvest time (Fig. 4a) was lower than the initial values (Table 1) for high and medium P soils, whereas there was an increase in WEP at harvest time comparing to the initial value in the low P soil. In the low P soil fertilized with different levels of P as TSP, the soil water-extractable P was also different across treatments, being greater with the higher rates of P application (Fig. 4b, c). There was however an interaction between P application level and *P. bilaii* inoculation, where soil water-extractable P was higher at lower rates of P fertilization (i.e. LPbi vs. L and L20Pbi vs. L20) without other macro and micronutrients being added (Fig. 4b). In contrast, the level of water-extractable P for plants inoculated with *P. bilaii* was lower in nutrient amended soil with the highest rate of P addition ($L_{Nut}60Pbi$ vs. $L_{Nut}60$, Fig. 4c).

Discussion

Shoot and root growth

For the first two weeks of the three experiments, all maize plants showed similar growth as indicated by shoot length, regardless of inherent level of soil P availability, P addition or *P. bilaii* inoculation. This was probably due to the high P content of the maize seed,

which was able to satisfy plant demand until the three-leaf stage (around 13 days in these experiments), according to Barry and Miller (1989). After that, maize growth responded to the different levels of soil P availability, with the highest shoot and root lengths and biomass observed in maize grown in the inherent high P fertility soil (experiment 1), or low P soil with the highest level of P and all macro- and micronutrient supplied (experiment 3). For example, shoot and root biomass were around 5 and 2.5 times higher, respectively, when maize was grown in H_{Nut} soil compared to L_{Nut} soil treatment. However, this was not the case when no macro and micronutrients were applied to the low P soil, where no effect of P addition or *P. bilaii* inoculation was observed, most likely due to limited availability of other nutrients, mainly N, which restricted the growth and P response in these plants.

Under low P availability or with low availability of the other macro and micronutrients, inoculation of plants with *P. bilaii* did not generally increase shoot or root growth or P uptake. Other studies (eg., Wakelin et al. 2004), have reported a wheat growth promotion effect in infertile soil when inoculated with *Penicillium radicum*. In our study (experiment 1 and 3) inoculation of maize plants in the low P availability soil ($L_{Nut}Pbi$) produced longer shoot length, longer coarse root length and higher root biomass than was observed in non-inoculated plants (L_{Nut}). The ANOVA (Table 2) also indicated a significant interaction between soil fertility and *P. bilaii* for root biomass, and the sole effect of inoculation was near to significance in experiment 1, with different soil fertility levels (p -values of 0.056 and

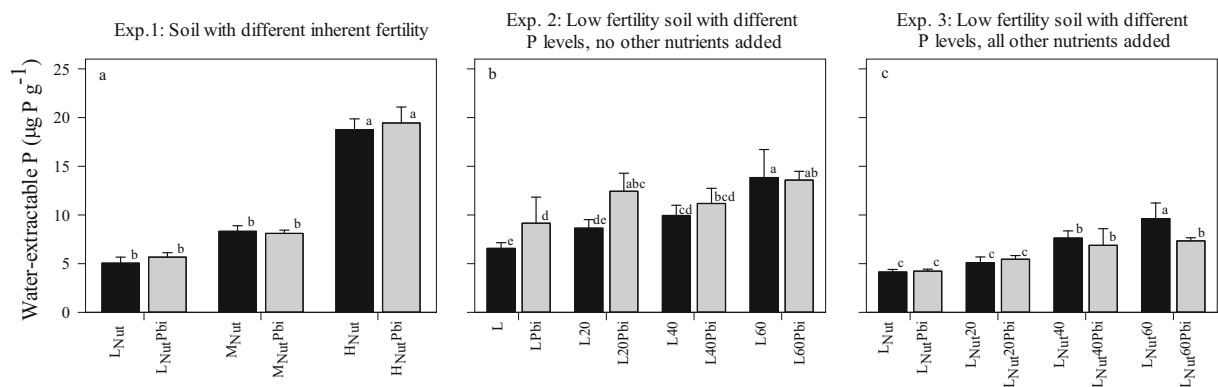


Fig. 4 Soil water-extractable P for experiment 1: maize grown in low (L), medium (M) and high (H) P soil (a), experiment 2: maize grown in low fertility soil without adding other nutrients (b), and experiment 3: maize grown in low fertility soil and adding all other nutrients (c). For experiment 2 and 3 different rates of P were

applied as TSP (20, 40 and 60 mg P kg⁻¹) and *P. bilaii* inoculation (Pbi) estimated after 38 DAS (a and b) and 28 DAS (c, d, e and f). Values indicate mean (n = 4) and bars denote standard deviation. Different letters in the same plot indicate significant differences between treatments ($P < 0.05$)

0.052, for root length and root biomass, respectively). These findings suggest that *P. bilaii* may be capable of promoting root growth even at low soil fertility levels, and thus increasing the plants absorptive capacity of the root system for immobile nutrients, such as P and Zn. Similar increases in the root length after *P. bilaii* inoculation were observed in maize by Gómez-Muñoz et al. (2017b) or in pea by Gulden and Vessey (2000) and Vessey and Heisinger (2001). Recently, Thonar et al. (2017) and Schütz et al. (2018) have also reported a plant growth promotion effect for different biofertilizers, which was mainly related to the improved root growth. It is well known that plants show adaptation in response to low P availability, including changes in root morphology or enhancing root growth to favour the capacity to explore soil (Tinker and Nye 2000; Richardson et al. 2009). It is therefore likely, that inoculation of seeds with *P. bilaii* promoted root traits that would favour P acquisition under low P availability conditions, and this observation provides some evidence in support of our first hypothesis that under low P conditions, inoculation with *P. bilaii* enhances root growth to improve P acquisition in soil.

Several other studies have reported a beneficial or variable effect of *P. bilaii* inoculation on plant growth under limited P availability in soil (Asea et al. 1988; Downey and van Kessel 1990; Wakelin et al. 2004; Leggett et al. 2015). A lack of consistent results under this condition suggests that factors other than P availability alone may influence *P. bilaii* activity in soil and its interaction with roots. Low P availability in soil could influence competition between plant roots and fungal growth, or under severe P limitation immobilised P may be less available to plants (Marschner 2008). In the present study, we observed a reduction in the shoot and root length for plants inoculated with *P. bilaii* grown in the low P soil supplied with 20 and 40 mg P kg⁻¹ when no other nutrients were applied (Fig. 2c, Table 3). Sánchez-Esteva et al. (2016) speculated that a certain critical minimum P availability in soil may be required for *P. bilaii* to have an effect on wheat plants. In the current study, we observed that a minimum P availability in soil, together with sufficient levels of macro and micronutrients, was needed to ensure the persistence and activity of *P. bilaii* in soil and to promote a significant increase in shoot and root biomass. In fact, we observed an increase in the shoot biomass and P content of maize inoculated with *P. bilaii* and grown in the medium P availability soil (M_{Nut}Pbi) when all other

nutrients were applied (Fig. 1b), though without any significant effect on the root growth in this treatment. This suggests that the maize plants achieved a higher efficiency to absorb P in this treatment when inoculated with *P. bilaii*. Similarly, in the low P soil with macro and micronutrients added, there was a significant interaction between P availability level and inoculation with *P. bilaii* (Table 2), where the latter increased shoot biomass and root length and biomass alone, and did so more with increasing levels of P (significant for L_{Nut}40Pbi and L_{Nut}60Pbi). This provides some evidence to confirm our second hypothesis that under medium P availability, inoculation with *P. bilaii* can increase plant growth. The addition of TSP supplies readily plant available P in the short term (i.e. 50% of the originally added P in TSP was measured as water-extractable), but inoculation with *P. bilaii* appeared able to either further solubilise part of the non-available P in soil or improve the uptake of already available P. Several other studies have also observed a positive effect of *P. bilaii* on plant growth when soil was fertilised with P (Kucey 1987; Kucey and Leggett 1989; Hnatowich et al. 1990; Whitelaw et al. 1997).

Plant P uptake

At harvest, maize plants grown in low, medium and high P soil (experiment 1) and low P soil with macro and micronutrients added (experiment 3) showed low P concentration around 1 mg P g⁻¹ (supplementary material Table S1); even at increasing P levels. This suggests that across all these treatments P remained as a limiting factor for the maize growth over the growing period of around one month. This means that with the experimental setup used, we were not able to fully test our fourth hypothesis that at high P availability and soil fertility levels (i.e., not limiting for plant uptake), no benefits of *P. bilaii* inoculation will be observed. Interestingly, under our experimental conditions, significant increases in shoot and root biomass and total shoot P uptake were observed in the inoculated treatments that received 40 and 60 mg P kg⁻¹ with all other nutrients added (L_{Nut}40Pbi and L_{Nut}60Pbi), along with a significant interaction for these parameters. Without knowing whether the highest level of P application that we used was sufficient to indeed fully satisfy plant demand, this observation is somewhat contrary to our hypothesis above, and as such further investigation is required.

According to the factorial ANOVA, *P. bilaii* inoculation increased P concentration in the treatments receiving all the other nutrients and varying levels of P (experiment 3). However, the increases were marginal (supplementary material Table S1), and only significant in the pairwise tests for plants grown in the $L_{Nut}20Pbi$ and $L_{Nut}60Pbi$ treatments. An increase was also observed in the $L60Pbi$ treatment. For two of the three treatments ($L60Pbi$ and $L_{Nut}20Pbi$) there was no difference in shoot or root growth, suggesting that soil P availability in these treatments was increased by inoculation with *P. bilaii*.

In the low P soil with all the nutrients added and P supplied at 40 and 60 mg P kg⁻¹ the inoculation with *P. bilaii* increased significantly P uptake, which is related to the higher shoot and root growth reported for these treatments. Similar increases in P uptake after *P. bilaii* inoculation have been previously reported in wheat and flax (Asea et al. 1988; Chambers and Yeomans 1991) and in canola (Kucey and Leggett 1989). By contrast, others studies have not found an increase in P uptake of plants stimulated in growth following inoculation with *P. bilaii* (Downey and van Kessel 1990; Keyes 1990; Chambers 1992; Gledie 1992; Heisinger 1998; Vessey and Heisinger 2001) or with different biofertilizers, revealing the limited capacity of biofertiliser to influence plant P uptake (Lekfeldt et al. 2016). Furthermore, it is evident that the mechanisms behind the improvement of the growth in inoculated plants are unclear, but according to our results, soil nutrient availability in general and in particular P, seems to have an important role in regulating the efficacy of the *P. bilaii* inoculation in plants.

In maize grown in low P soil without the addition of macro and micronutrients (experiment 2), the P concentration increased with increasing P fertilisation, since growth was not P limited, at least at higher levels of P supply. Similar results were observed for P uptake. These results reveal that the plants are able to accumulate P in shoots proportionally to the P availability in soil (Haling et al. 2016). Although root growth in inoculated plants was reduced, presumably due to nutrient competition in low P soil without the addition of macro and micronutrients, the inoculated plants were able to increase the amount of P uptake per unit of root. In these treatments, P uptake/root length ratio increased in *P. bilaii* inoculated plants compared to non-inoculated plants, which resulted in a higher P content in the plant together with limited root growth.

Micro and macronutrients uptake

The concentrations of macro and micronutrients decreased with the increase of P in soil, due to the dilution effect as result of the higher plant growth (experiment 1 and 3). In maize plants grown in experiment 1 and 3, potential Cu and Zn deficiency was evident at the end of the experiment (Campbell 2009), which may have limited the plant growth in the later stages of plant growth. However, the amount of macro and micronutrients applied at the beginning of the experiment would be sufficient to satisfy plant demand for sufficient time to allow differences between treatments with and without P source or *P. bilaii* inoculation to be observed. However, the total macro and micronutrients uptake was significantly higher in the high P soils (experiment 1) and low P soil when higher rates of P were added (experiment 3). Similarly to P uptake, N and K uptake were increased in the medium P availability soil ($M_{Nut}Pbi$, experiment 1) and $L_{Nut}20Pbi$ and $L_{Nut}40Pbi$ (experiment 3) when plants were inoculated with *P. bilaii* compared to non-inoculated plants, probably just reflecting that a higher biomass results in a higher assimilation of the other nutrients if available.

Marschner (2008) stated the importance of having sufficient amount of nutrients in soil to favour microbial growth and thus P solubilization. Consistent with this, plants grown in the low P soil with no addition of macro and micronutrients (experiment 2), had lower content of N, Fe, Zn and Cu regardless of P fertilization or *P. bilaii* inoculation, whereby at the end of the experiment concentrations of N, Zn and Cu in the plant tissues were below the sufficient ranges proposed by Campbell (2009). This nutrient limitation resulted in reduced growth of maize plants, which would most likely impede any possible benefit from *P. bilaii* inoculation. Thus confirming our third hypothesis that under nutrients limitations *P. bilaii* did not benefit plant growth, most likely due to nutrients competition between plant and microorganism.

Changes in soil water-extractable P

Soil water-extractable P estimated at plant harvest responded to the background P availability in soil or the soluble P inputs supplied. In the medium and high P soil, there was a decline in WEP comparing the initial values of WEP (Table 1) with the WEP estimated at harvest time (Fig. 4), which can be associated with the

plant P uptake. Whereas in the low P soil, plant growth and uptake was so poor, that this did not lower WEP, rather the soil disturbance and higher temperature in the growth chamber caused net soil organic P mineralisation and therefore a slight increase in WEP (Bünemann et al. 2016). However, and despite the high P availability in these soils, maize plants showed P deficiency in these treatments, confirming that availability of other nutrients, and not only P availability, regulated plant growth and P uptake in this study. In the low P soil without other nutrients supplied, the inoculation with *P. bilaii* increased soil water-extractable P especially in those treatment where 0 and 20 mg P kg⁻¹ were supplied. However, this higher P availability did not result in a higher P uptake due to the severe N limitation reported in these plants, which restricted plant growth. These results support that *P. bilaii* inoculation may increase P availability in soil, as previously reported (e.g. Kucey 1987). However, it remains unclear why the high P availability supports increased plant P uptake in some cases only.

Conclusions

Growth of maize plants responded to P availability when all other nutrients required for plant growth were also adequately available. In this case, a positive interaction between inoculation of maize seeds with *P. bilaii* and P level was generally observed. Increased shoot biomass of inoculated plants grown in soil with high levels of available P through fertilisation, was related to an increase in root length and biomass. This indicates that higher P uptake was due to increased root growth and exploration, as we did not observe any evidence that *P. bilaii* mobilised any soil P under these conditions. When no other nutrients were added, plant growth was limited and was generally not enhanced by either P fertilisation or *P. bilaii* inoculation, which reduced root growth that was most likely associated with nutrient competition between plant and microorganisms. However, the P-uptake per root length increased as a result of *P. bilaii* inoculation at increasing P levels, when other nutrients were not added. This was attributed to the root length, which tended to decrease in inoculated plants with P addition, which resulted in an overall increase in P uptake per unit of root length in soils that were limited in N but not for P. Collectively, this indicates that the effects of *P. bilaii* inoculation were related to root

growth and function, although the response was highly dependent on the nutrient status in the soil environment.

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