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Plant growth promotion by *Bacillus amyloliquefaciens* FZB45 depends on inoculum rate and P-related soil properties

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Abstract We have studied the effect of soil P-related properties and inoculum concentration on plant growth promotion by the phytase-producing strain *Bacillus amyloliquefaciens* FZB45. The response (shoot fresh weight/P_i content) of a mycorrhizal-independent plant, Chinese cabbage, was evaluated in a soil with well-defined P-related properties. Two inoculum concentrations were evaluated under four P regimes: no P addition, inorganic P, and two levels of phytate. Significant interaction between P regime and bacterial inoculation was found. FZB45 only promoted plant growth and P uptake at the higher rate of phytate, confirming that phytase activity is its major mechanism and that is limited by soil phytate availability. The effect caused by the lower inoculum concentration was superior than that by the higher, suggesting the simultaneous involvement of a direct effect. This effect was confirmed by a soilless test, which showed a hormone-like response. FZB45 produced IAA in vitro, but its role is to be determined. These results demonstrate that soil modulates the performance of plant growth-promoting rhizobacteria in a specific manner, consistent to the mechanisms of action involved. Determination of those mechanisms and their modulating factors helps predict conditions where plant growth promotion will result, an important step in increasing the consistency of PGPR.

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Introduction

A major limitation for using plant growth-promoting rhizobacteria (PGPR) in agriculture is inconsistent results when plants are grown in soil. Knowledge of mechanisms of action and how they interact with soil properties would help optimize results and predict outcomes, thereby reducing variability (Richardson 2001; Compant et al. 2005; Richardson et al. 2009). This is particularly important for inoculants based on strains of aerobic endospore-forming bacteria, which, because of their high feasibility to be formulated, represent most of the PGPR-based products that are commercially available (Mathre et al. 1999; Kloepper et al. 2004; Ongena and Jacques 2008).

Production of phytohormones and increased plant nutrient uptake are commonly referred to as putative PGPR-mechanisms in bacilli. Several plant hormones, such as auxins (Asghar et al. 2002), gibberellins (Joo et al. 2005), and cytokinins (García de Salamone et al. 2001) are thought to be related to growth promotion. However, evidence supporting the role of those hormones in plant growth promotion by PGPR under natural conditions is still scarce. Production of IAA is the most studied case and, although not yet tested in soil, analyses of mutants suggest its biological significance in plant growth promotion by bacilli (Idris et al. 2007) and non-bacilli PGPR (Dobbelaere et al. 1999; Patten and Glick 2002). IAA accumulation in the rhizosphere has also been linked to the inoculum density of the applied PGPR strain (Dobbelaere et al. 1999).

Regarding increased plant nutrient uptake, the vast majority of the PGPR research focuses on P solubilization (Rodriguez and Fraga 1999; Richardson 2001; Gyaneshwar et al. 2002; Goldstein and Krishnaraj 2007). One of the most abundant organic molecules containing unavailable P is phytate. Phytate can account for 20% to 50% of soil organic P (Richardson et al. 2007) and can contain an amount of P equivalent to two thirds of the P applied each year in fertilizer (Mullaney and Ullah 2007). Because plants depend on microorganisms to hydrolyze phytate (Richardson et al. 2007), phytases have been suggested to be useful for solubilizing P from phytate in soil and making this element available for plant uptake (Findenegg and Nelemans 1993; Tarafdar and Marschner 1995; Richardson 2001; Richardson et al. 2001; Idriss et al. 2002; Yip et al. 2003; George et al. 2005c; Unno et al. 2005).

Most of the experiments involved in this research have been conducted in the absence of soil, and when done in its presence, plant responses are less consistent and largely dependent on soil type (Richardson 2007; Richardson et al. 2009). A considerable body of information suggests that P-related soil properties such as P and phytate content, phosphorus-fixation capacity, and pH have a major influence on plant responses (George et al. 2005a, 2005b, 2006; Richardson 2007). The details and extent of this influence are still poorly understood, especially in the case of phytase-producing PGPR, where most of these aspects remain to be explored (Richardson 2001; Richardson 2007). For instance, Idriss et al. (2002) provided evidence that phytase activity of *Bacillus amyloliquefaciens* FZB45, a trait that is also present in the closely related and commercialized strains FZB24 and FZB42, is important for plant growth stimulation under phosphate limitation. Experiments comparing culture filtrates of the wild-type strain to those of a phytase-deficient mutant clearly indicated that, in presence of phytate, phytase production by FZB45 promoted growth of corn seedlings. However, these experiments were conducted using a soilless gnotobiotic system, because of which those authors cautioned that this study should be considered as a starting point and called for verification under conditions that better mimic the natural environment.

Agricultural systems are complex and involve a high number of factors. Soil is recognized as a major factor affecting bacterial physiology (Vilain et al. 2006), phytase behavior (George et al. 2005b), and availability of phosphate and phytate (Fox 1981; Celi et al. 1999; Celi and Barberis 2007). Therefore, plant response to the inoculation with P-solubilizing PGPR, in general, and with phytase-producing PGPR, in particular, is expected to be modulated by soil properties, especially those related to P. In addition, soil-independent factors, e.g., bacterial production of phytohormones and concentration-dependent effects, may play a role simultaneously. Thus, understanding the effect of these factors on PGPR performance is a necessary step in the process of PGPR technology implementation. The aim of the present report was to study the effect of soil P-related properties and inoculum concentration on plant growth promotion by the phytase-producing strain *B. amyloliquefaciens* FZB45. This was studied using an experimental system consisting of a mycorrhizal-independent plant, Chinese cabbage, and a soil with well-defined P-related properties. Plant growth and P_i content were evaluated under different regimes of soil P_i and phytate, both in relation to soil P-fixing capacity, and with different inoculum concentrations.

Materials and methods

Soil characterization

The B horizon of a never-fertilized ultisol from Alabama (USA) was selected because of its naturally low P content and low level of organic matter. Soil analysis was routinely done in the Soil Testing Laboratory at Auburn University. This was classified as belonging to soil group 2, which corresponds to a loamy soil with light clays. Chemical analysis, which was performed using Mehlich-1 extract and determination by inductively coupled argon plasma spectrophotometry, showed the following parameters: pH 6.1, organic matter 1.4%, and nutrient content (mg kg^{-1}): P, 3, K, 17, Mg, 55, and Ca, 440. Dolomitic lime (1 g kg^{-1}) was added to the soil in order to increase Ca and Mg contents and reaching pH 6.5. A mixture 1:1 (*w*/*w*) soil/sand was used for all the experiments. A P-sorption curve establishing the relationship between P applied and P in soil solution was prepared for this mixture (Fox and Kamprath 1970; Nair et al. 1984). For this purpose, both 1.5 g of 4 mm-sieved air-dried soil and 1.5 g of sand (3 g soil mixture total) were put into 50 ml polypropylene tubes. Thirty milliliters of 0.01 M $CaCl_2{\cdot}2H_2O$ containing proper amounts of KH_2PO_4 were added into separate tubes in order to reach concentrations equivalent to 0, 5, 10, 15, 20, 25, 30, 40, and 50 mg P kg^{-1} soil. Two drops of chloroform were added into each of the tubes which were then placed horizontally in an orbital shaker for 24 h at room temperature and 150 rpm. After incubation, samples were centrifuged for 5 min at 2,000×*g* and filtered through filter paper Whatman No. 1. P in solution was determined using molybdate-blue method (Murphy and Riley 1962).

Bacterial inoculum preparation

The strain *B. amyloliquefaciens* FZB45 (Idriss et al. 2002) was kindly provided by Dr. Rainer Borriss (Institut für

Biologie, Humboldt Universität, Berlin). A bacterial spore suspension was prepared by growing the strain on a modified medium to stimulate sporulation. One liter of medium contained proteose peptone (vegetable, Fluka), 3.3 g; beef extract powder, 1.0 g; D-lactose monohydrate, 5.0 g; NaCl, 5.0 g; K_2HPO_4, 2.0 g; KCl, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; $MnSO_4$, 10 mg; and agar, 18 g. After incubation for 7 days at 28°C, spores were harvested from medium surface using 5 ml of sterile distilled water (SDW) per Petri dish. This suspension was centrifuged at 2,000×*g* for 10 min, the supernatant was discarded, and the pellet was resuspended in sterilized distilled water. This suspension was finally pasteurized for 15 min at 80°C and its concentration was determined by plate counting. The spore suspension was stored at 4°C until use, and the spore viability was confirmed at the moment of inoculation.

Soil-plant experiment

Two-day-old seedlings of Chinese cabbage (*Brassica rapa* L. Kaboko Hybrid, Park Seed Co., Greenwood, S.C. 29647) were used in all the experiments. Seeds were disinfected by soaking in 70% ethanol for 1 min, rinsing once with SDW, then soaking again in 0.5% sodium hypochlorite for 10 min, and finally rinsing 15 times with SDW. Disinfected seeds were transferred to Petri dishes with 2% water-agar and incubated for 2 days at 28°C in the dark, until germination occurred. Then, 2-day-old seedlings were planted individually in plant growth containers which consisted of 50 ml-plastic centrifuge tubes containing 70 g of a mixture 1:1 (*w*/*w*) soil–sand. The amount of soil (air-dried and sieved at <4 mm) and sand (35 g each) to be added into each container were weighed separately and then mixed in order to ensure homogeneity. Likewise, containers were fertilized individually with 5 ml of distilled water containing NH_4NO_3, $Ca(NO_3)_2 \cdot 4H_2O$, $MgSO_4 \cdot 7H_2O$, and KCl in equivalent amounts to add 100 mg N, 100 mg Ca, 60 mg Mg, and 85 mg K kg^{-1} soil. The experiment was conducted following a 4×3 factorial arrangement. Four different P regimes were evaluated: no addition of P, 15 mg P kg^{-1} soil, 74.57 mg phytate kg^{-1} soil (equivalent to 15 mg P kg^{-1} soil, 1×), and 447.43 mg phytate kg^{-1} soil (equivalent to 90 mg P kg^{-1} soil, 6×). Phosphorus amendments were applied to each container using 5 ml of distilled water containing the respective amount of NaH_2PO_4 (source of P_i) or phytic acid dodecasodium salt hydrate from rice (Sigma P0109). Bacterial treatment was achieved at three levels: no inoculation (only SDW) and two rates of FZB45, 10^6 and 10^8 spores per seedling carried in 0.1 ml suspension. Five containers, each having a 2-day-old seedling, were used for each combination treatment, and the experiment was conducted twice. Soil moisture was kept between 40–60% of maximum water holding capacity, and plants were incubated at 25°C in a growth chamber with 16-h light and 8-h darkness for 14 days. Response variables were fresh shoot weight (FSW), shoot inorganic P content (shoot P_i content), inorganic P concentration (P_i concentration, based on fresh weight), and total P concentration (P_t concentration, based on dry weight).

Plant P extraction and determination

Whole plant P_i, which is considered to be a highly sensitive index for P nutrition, was extracted following the method by Huang et al. (2005). For this purpose, the shoot of each plant was washed in distilled water and put into 50 ml-plastic centrifuge tubes containing 15 ml 0.1 M H_2SO_4. Samples were agitated in an orbital shaker for 16 h at 160 rpm and 25°C, then heated in a water bath at 85°C for 15 min, and cooled down for P_i determination. Inorganic P (P_i) in solution was determined in the extractant by the molybdate-blue method (Murphy and Riley 1962) using a P_i standard curve also prepared in 0.1 M H_2SO_4. Inorganic P (P_i) was expressed as shoot P_i content (μg P_i per plant) and P_i concentration (mg P_i kg^{-1}, based on fresh weight). For P_t content, determination was done in leaf disks according to the method described by Aziz and Habte (1987). Briefly, a disk (0.6 cm) was taken from the upper half of the youngest fully opened leaf of each plant and transferred into a 1.5-ml microcentrifuge tube. Leaf disks were dried overnight at 70°C, weighed, and then ashed at 500°C for 3 h. These ashes were dissolved in 10 ml of distilled water and P in solution was determined by the molybdate-blue method (Murphy and Riley 1962). Total P content (P_t) content was expressed as P_t concentration (%) taking the disk dry weight as the base.

Gnotobiotic root elongation assay

The effect of two different *B. amyloliquefaciens* FZB45 inoculum sizes on root elongation of Chinese cabbage was tested under gnotobiotic conditions, following the method by Penrose and Glick (2003). Different from the mentioned method, seed disinfection and bacterial inoculation were performed as described above for the soil-plant experiment except that spores were applied on seeds instead of 2-day seedlings. Ten seed germination pouches (cyg, mega international, St. Paul, MN, USA) per treatment, with five seeds per pouch, were used. Pouches were maintained in covered transparent plastic boxes at 25°C in a growth chamber with 16-h light and 8-h darkness. Root lengths were measured 7 days after inoculation discarding those seeds that did not germinate by the second day. This experiment was conducted twice.

IAA determination

The capacity of *B. amyloliquefaciens* FZB45 to produce IAA was determined *in vitro*. The strain was grown for 24 h in TSB, and 20-µl aliquots were transferred into 50-ml flasks containing 10 ml of TSB supplemented to reach 0, 50, 100, 200, and 500 µg ml^{-1} of L-tryptophan (FisherBiotech, BP395). Tryptophan was added as a filtered-sterilized (0.22 µm) 2 mg ml^{-1} stock solution prepared in warm water (Patten and Glick 2002). Flasks were incubated at room temperature and 150 rpm on an orbital shaker and samples were taken 24, 48, and 72 h after inoculation. $O.D._{630}$ was recorded as an indicator of growth and an aliquot of each flask was centrifuged (5,000×*g*) to remove bacterial cells. One milliter of supernatant was mixed with 4 ml of Salkowski's reagent (150 ml of 18 M H_2SO_4, 250 ml distilled water, 7.5 ml of 0.5 M $FeCl_3 \cdot 6H_2O$) and absorbance at 535 nm was measured after 20 min (Gordon and Weber 1951; Patten and Glick 2002). IAA concentration was estimated by comparison with a standard curve prepared with Indole-3-acetic acid (Sigma I-2886).

Statistical analysis

Response data from the two plant-soil experiments were analyzed jointly as a factorial with bacterial treatment and P regime as fixed effects. The effect for experiment was extracted from the residual error term for FSW and P_t concentration and was treated as a random effect. Normality and equal variances assumptions were first evaluated using the student panel graphs generated by SAS® GLIMMIX Procedure, which was also used for all the analyses. Normal distribution was only warranted for FSW, while all the response variables involving P determination followed a log-normal distribution. As the equal variance assumption was not fulfilled for FSW and P_t concentration, the variance structures for those two response variables were modeled (*R*-side of the covariance parameters of SAS) using the group option to create homogeneous variance groups. A smaller AIC value from the 'information criteria' output and a better graphical residual distribution were considered as indicators of a good fit for the model. The residual term was the pooled residual within bacterial inoculation×P regime combination variation, as the experimental design was a CRD. For those response variables with a statistically significant interaction, all pairwise simple effect comparisons among bacterial inoculation levels within each P regime were done using the simulate adjustment of GLIMMIX procedure. Significance classes are presented in the graphs. Root length data from the two gnotobiotic assays were analyzed jointly with bacterial inoculum sizes as the only fixed effect; the effect for pouch nested within treatment (bacterial inoculum size) was treated separately as a random effect. For these data, normality and equal variance assumptions were fulfilled. Dunnett's test was used to assess the difference between each of the two bacterial rates and the untreated control.

Results

Effect of *B. amyloliquefaciens* FZB45 inoculation in soil

The P-sorption curve (Fig. 1) indicated that 15 mg P kg^{-1} was the minimum amount of P_i needed to be applied in order to increase the concentration of P_i in soil solution above 0.04 mg l^{-1}, which is considered the minimum concentration required to support suitable growth of cabbage (*Brassica oleracea*) (Hue et al. 2000). Based on this information, the response of Chinese cabbage to P fertilization in the experimental soil used was tested (data not shown). This experiment showed that application of 15, 30, and 60 mg P_i kg^{-1} soil stimulated plant growth by 57%, 77%, and 112%, respectively, compared with a non-fertilized control. Taking the previous information into account, application of 15 mg P_i kg^{-1} soil was used as the full fertilized treatment for the following experiments.

For the soil-plant experiment, factorial analysis revealed a highly significant interaction between P regime and bacterial inoculation on both FSW ($P<0.001$) and shoot P_i content ($P=0.012$; Table 1). Specifically, bacterial inoculation caused a significant increase of FSW and shoot P_i content only at the higher rate of phytate (6×, which corresponded to 447.43 mg phytate kg^{-1} soil, equivalent to 90 mg P kg^{-1} soil); whereas no effect was observed under the other three P regimes tested (Fig. 2). Although both rates of inoculums caused significant increases with respect to the non-inoculated control, the lower rate (10^6 spores per seedling) was superior than the higher (10^8 spores per seedling) for shoot fresh weight ($P=0.020$) and plant P_i content ($P=0.092$).

Neither bacterial inoculation nor its interaction with P regime had a significant effect on P_i and P_t concentrations, even though plant growth and shoot P_i content were significantly increased (Table 1). In contrast, P regime had a significant effect on all the response variables evaluated (Table 1). However, when compared in the absence of bacteria, only the addition 15 mg P_i kg^{-1} soil caused significant increases in FSW ($P<0.05$), shoot P_i content ($P<0.001$), and P_i concentration ($P<0.01$). The other two P regimes, which corresponded to the addition of two different rates of phytate, did not significantly affect any response variable ($P>0.05$).

Gnotobiotic root elongation assay and IAA production

In order to elucidate any direct effect of FZB45 on root development that could explain the higher increases

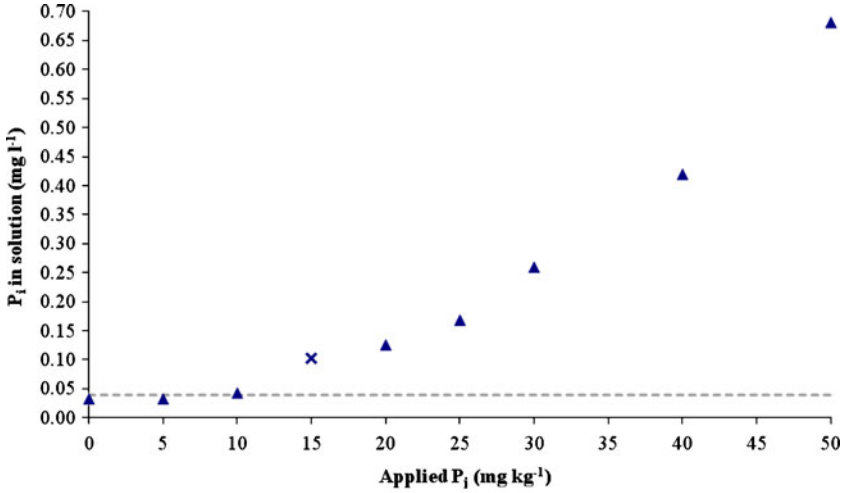

Fig. 1 Concentration of P in soil solution after application of different amounts of inorganic P to the mixture 1:1 (*w*/*w*) soil/sand used in all the experiments. Each point represents one single determination. *Dashed line* indicates the level of P in soil solution associated with 80–95% of maximum yield of cabbage (*Brassica oleracea*) (Hue et al. 2000). In a separate experiment (data not shown), application of 15 mg P_i kg^{-1} soil (diagonal cross point marker) caused a 57% increase in fresh shoot weight of Chinese cabbage (*Brassica rapa* Kaboko Hybrid) 2 weeks after planting. This level of P fertilization was used as the full fertilized treatment

observed with the lower inoculum concentration, a gnotobiotic assay was conducted in sterile seed germination pouches. In effect, only the lower bacterial rate (10^6 spores per seed) significantly promoted root elongation of Chinese cabbage one week after inoculation (P=0.007), whereas the higher rate (10^8 spores per seed) showed no difference compared with the non-inoculated control (P=0.486; Table 2). Such soil-independent root growth-promoting effect at low bacterial concentrations suggests the involvement of a hormone-like compound, probably auxin type. To further clarify this, we determined if *B. amyloliquefaciens* FZB45 was able to produce IAA, a widely known bacterially produced auxin. This strain was effectively able to produce this compound in vitro, which was additionally stimulated by the presence of tryptophan in the growth medium (Table 3). Even in absence of any tryptophan amendment, IAA was detected in the medium after 48 h; however, production of this compound was increased with addition of more than 200 μg tryptophan ml^{-1}, being detected as soon as 24 h after inoculation of the medium. In general, at all the three reading times, the addition of tryptophan to the medium stimulated a higher production of IAA, reaching five- to sixfold increases for the highest level tested, 500 μg tryptophan ml^{-1}.

Discussion

In our system, *B. amyloliquefaciens* FZB45 promoted plant growth and P nutrition in soil. These effects, however, only occurred under conditions conducive for phytase activity, supporting the conclusion that phytase activity is the major mechanism for plant growth promotion by this strain. Additionally, FZB45 exerts a direct mechanism on plant growth, probably by IAA production, which creates a concentration-dependent response to inoculation and interacts with phytase-mediated effects.

Unexpectedly, the experiments conducted by Idriss et al. (2002) using corn seedlings grown in nutrient solution and inoculated with FZB45 spores could not be reproduced in

Table 1 *P* values from the analysis of variance for the effect of *Bacillus amyloliquefaciens* FZB45 inoculation, different P regimes, and their interaction on fresh shoot weight, inorganic (P_i), and total (P_t) phosphorus content of Chinese cabbage grown in soil for 2 weeks under growth chamber conditions

Factor	Fresh shoot weight	Shoot P_i content	P_i concentration	P_t concentration
P regime	0.011*	<0.001*	<0.001*	<0.001*
Bacterial inoculation	<0.001*	0.109	0.716	0.752
P regime×bacterial inoculation	<0.001*	0.012*	0.782	0.222

Bacterial inoculation was done at two different rates (10^6 and 10^8 spores per seedling). P regimes included application of P_i and two rates of phytic acid. Data are from two separate experiments, each having five replicates per treatment combination. *P* values followed by an asterisk are considered to be statistically significant

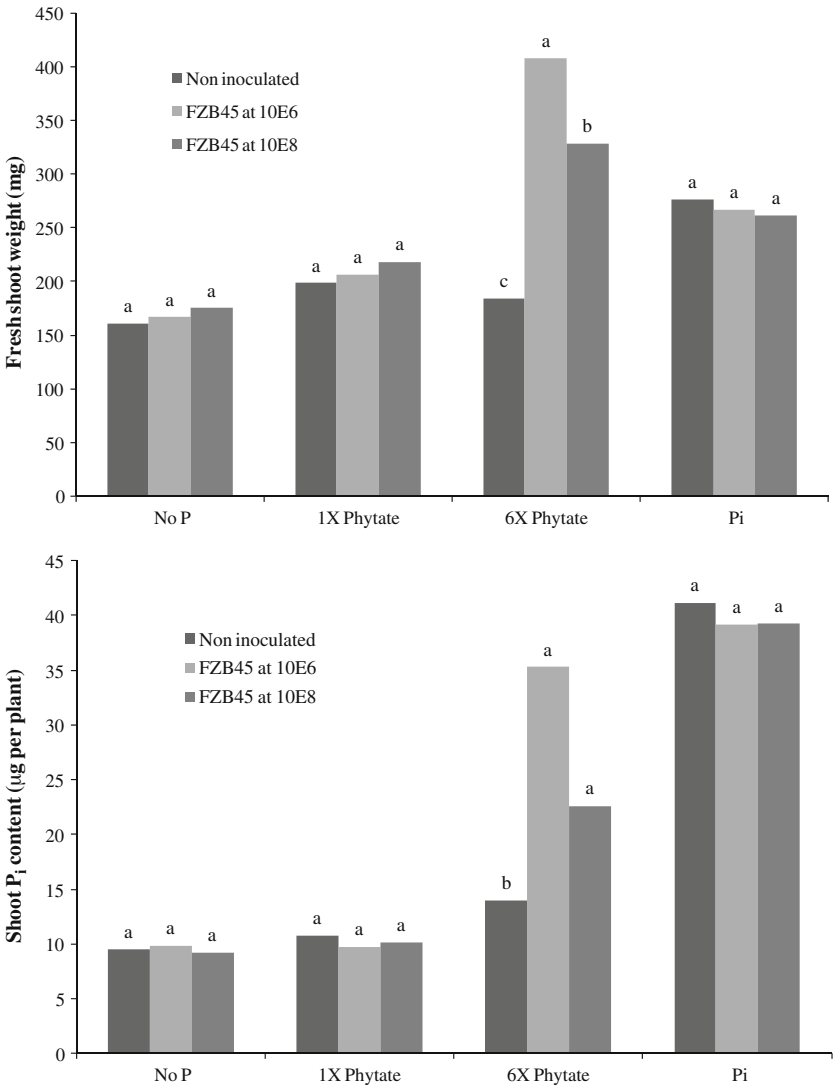

Fig. 2 Effect of the inoculation with two different rates of *Bacillus amyloliquefaciens* FZB45 under four different P regimes on fresh shoot weight and plant inorganic P content of Chinese cabbage grown in soil for 2 weeks under growth chamber conditions. Averages are expressed as the least squares means. Those with the same letter within the same P regime are not significantly different (P value>0.05) according to simulate adjustment of GLIMMIX procedure. Interactions were significant for both response variables and P values for the F tests are shown in Table 1. Inoculations were performed as spore suspensions and are expressed as spores per seedling. Data are from two separate experiments, each having five replicates per treatment combination. Plant P_i content was analyzed as $\log_e$-transformed data and had 101 degrees of freedom, while fresh shoot weight had 97

our laboratory (data not shown). The addition of phytate to the nutrient solution resulted in chlorosis and severe calcium deficiency symptoms, which was consistent with the formation of a white precipitate of phytate and Ca

Table 2 Least squares means (LS mean), standard errors (SE), and probability of difference to the untreated control (Dunnett's test) for two concentrations of *Bacillus amyloliquefaciens* FZB45 on root length of Chinese cabbage under gnotobiotic conditions 1 week after seed inoculation

Treatment	LS mean	SE	Dunnett's P value
Control	6.85	0.26	
FZB45 at 10E6	7.97	0.26	0.007
FZB45 at 10E8	7.23	0.25	0.486

Inoculations were performed as spore suspensions and are expressed as spores per seed. Data are from two separate experiments, each having five plants per pouch and ten pouches per treatment. F test had a P=0.011 and degrees of freedom for error=56

$(NO_3)_2{\cdot}4H_2O$ (a component of the nutrient solution). Inositol phosphates are strong chelators, which would explain the precipitate (Grynspan and Cheryan 1983). Possibly the corn hybrid used in our tests was more sensitive to calcium deficiencies or to the components in the liquid medium than that used by Idriss et al. (2002). These observations, along with a low response to P fertilization in the soil used in our experiments, caused us to reject the use of corn as the model plant for our experimental system. Chinese cabbage proved to be an excellent choice because of its fast and homogeneous growth as well as its high suitability for P nutrition assays due to the small size of seeds and sensitive response to changes in soil P availability (Huang et al. 2005). Also, unlike tomato (which was also highly responsive to P addition) and corn, Chinese cabbage is independent of mycorrhizal associations (Habte 2000), thereby avoiding potential interference by plant uptake of soil P.

Our results support the conclusion that phytase activity is the major mechanism by which *B. amyloliquefaciens*

Table 3 Production in vitro of IAA by *Bacillus amyloliquefaciens* FZB45 in the presence of various concentrations of tryptophan

Tryptophan concentration (μg ml^{-1})	IAA production (μg ml^{-1} per OD_{630} unit)		
	24 h	48 h	72 h
0	0.0±0.0	0.6±0.0	0.8±0.0
50	0.0±0.0	0.9±0.3	1.1±0.2
100	0.0±0.0	1.2±0.1	1.7±0.1
200	0.4±0.2	1.6±0.1	2.3±0.1
500	0.5±0.1	3.4±0.4	4.2±0.3

Average ± standard error from three replications

FZB45 promotes plant growth. No effect of inoculation was observed without phytate addition, neither under P-limited conditions nor full P fertilization (Fig. 2). Thus, under our experimental conditions, FZB45 only increased the growth of Chinese cabbage when phytate was available in soil. This, along with the fact that P was the only limiting nutrient in soil, strongly suggests that the beneficial effect of inoculation was due to P solubilization through the strain's phytase activity. This also indicates that any other beneficial trait of FZB45 was secondary and not biologically significant in the absence of phytate, whether P_i content in soil was high or low. It is important to mention that phytase activity could also increase the availability of phytate-chelated nutrients such as Ca^{+2}, Mg^{+2}, or Zn^{+2}, an effect that cannot be ruled out. However, this is an unlikely explanation for the observed growth response as soil fertilization was previously standardized to have sufficient elements (except P), and no deficiency symptoms were observed for any of those elements, even when phytate was added.

Phytase has been shown to solubilize sufficient P to increase plant growth (Richardson 2001). Our results suggest that FZB45's phytase activity solubilized enough P to significantly increase plant growth and shoot P_i content. This effect was significant on shoot fresh weight, even though fresh measurements commonly require more replications than dry weight to detect significant differences as observed in root determinations (Bashan and de Bashan 2005). Here, it is important to take into account that it would be expected more variation in fresh root samples than in shoots and that residual analysis of our experiments discarded any bias by showing a random distribution of the uncontrolled variation. The absence of treatment effects on P concentrations indicates that both plant growth and shoot P_i content increased at similar rates causing a dilution effect, which could be expected for early plant growth under very limited soil P content.

Studies relating phytase activity and plant growth promotion have included different approaches such as addition of purified microbial phytases (Findenegg and Nelemans 1993), inoculation with phytase-producing microorganisms (Tarafdar and Marschner 1995; Richardson et al. 2001; Idriss et al. 2002; Unno et al. 2005), and the use of transgenic plants expressing microbial phytases (Yip et al. 2003; George et al. 2005c). Among these reports, plant growth increases by inoculation with phytase-producing bacteria are the fewest so far. To our knowledge, besides the previously mentioned study with FZB45 on corn in nutrient solution (Idriss et al. 2002), only three other reports have linked plant growth promotion with bacterial production of phytase. Two of these reports tested bacterial strains with natural phytase production in soilless systems (Richardson and Hadobas 1997; Richardson et al. 2001; Unno et al. 2005), while the third report was conducted in soil and used a genetically modified strain expressing a phytase of fungal origin (Li et al. 2007).

Studies of P solubilization in general, including phytase activity, should take into account fundamental principles of soil fertility. For example if plant growth is to be promoted through increases in P uptake, native soil P content should be low enough for the plant to respond to its increase. Additionally, the content of this element must be the only limiting factor for plant growth. Some of the failures in promotion of plant P uptake by P-solubilizing PGPR are found to be associated with soil P contents sufficiently high to support satisfactory plant growth (de Freitas et al. 1997) or with the presence of an additional limiting factor which hinders the response to soil P increases (Fernández et al. 2007). Our study met these criteria, thereby confirming the role of in-plant growth promotion by FZB45.

Another essential consideration in these kinds of studies is that soil will affect all the components of the system, producing a very different behavior from that seen in vitro. First of all, soil will influence the global physiology of the bacterium (Vilain et al. 2006), which, in FZB45, conditions the phytase gene expression (Makarewicz et al. 2008). In addition, individual factors that will vary from soil to soil such as C (Jorquera et al. 2008) and P (Makarewicz et al. 2006) availability also determine the production of bacterial phytases. Likewise, soil properties will interact with phytate (substrate) (Celi and Barberis 2007), phytase (enzyme) (George et al. 2007a), and phosphate (product) (Fox 1981). In our experiment, response was obtained only with addition of 447 mg phytate kg^{-1} soil, which is in the range reported for naturally occurring phytate concentrations in soils (0.3–987 mg kg^{-1} soil) (Turner et al. 2002). However,

the absence of response under the lower phytate content suggests that adsorption and precipitation limited phytate availability in soil. Phytate, like phosphate, is adsorbed to soil colloids (Celi et al. 1999), and this was previously shown to limit the response of plants to inoculation with phytase-producing rhizobacteria (Richardson et al. 2001). On the other hand, it is known that the efficiency of phytases of fungal origin is affected by adsorption to soil colloids (George et al. 2005b). This depends on the phytase's physicochemical properties (George et al. 2007b), and thus, specific information for *Bacillus* phytases is required. These phytases possess a low sequence homology with other phytases (Fu et al. 2008) and a unique requirement of Ca^{2+} for stabilization and activity (Oh et al. 2001), which could confer on them a particular behavior.

In addition to producing phytase, FZB45 also displayed a direct, soil-independent mechanism for growth promotion of Chinese cabbage. This was suggested by the concentration-dependent effect of inoculation in the plant-soil experiment (Fig. 2) and confirmed by the root pouch assay (Table 2). Production of IAA is a highly likely candidate to explain these observations because it affects root elongation and is involved in the concentration-dependent plant response to inoculation with rhizobacteria (Dobbelaere et al. 1999). Also, in this study FZB45 produced IAA in vitro (Table 3), and previously, its culture filtrates caused an IAA-like effect on corn coleoptiles (Krebs et al. 1998). However, to test the biological significance of this IAA production, creation of defective mutants will be required. Results such as no response to inoculation when phytate was not added and the better response to the lower inoculum rate when phytate was present suggest an interaction between the direct and the phytase-mediated effect. A hypothesis explaining this interaction is that FZB45's direct effect promotes root elongation, which allows a greater exploration of soil and increased nutrient uptake when soil P is limiting and its concentration is increased via phytase activity. Promotion of root growth by PGPR is extensively reported (Glick et al. 1999, 2007). However, root response to soil P content and PGPR inoculation simultaneously has not yet been explored.

An ongoing challenge for the widespread use of PGPR-based biofertilizers in agriculture is to increase their consistency of performance. Achieving this goal requires not only to understand the plant bacterial-interaction, but also to determine the environmental factors affecting that interaction. The results of this study demonstrate that soil modulates plant response to inoculation with PGPR in a specific manner, consistent to the mechanisms of action involved. In addition, this study shows that soil-mediated and soil-independent bacterial effects can interact and affect plant growth simultaneously. Field evaluations during longer periods of plant growth are still required to determine the results under agronomic conditions. Since field trials make control of some variables difficult, experiments can be designed according to the results obtained here, taking into account the actual levels of P and phytate in soil and evaluating the inoculum survival. Clear determination of the mechanisms of action and their modulating factors helps predict conditions where plant growth promotion will result. Hence, such knowledge will be an important step to increasing the consistency of growth promotion by PGPR.

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