

# Utilization of organic phosphorus sources by oilseed rape, sunflower, and soybean

Hernan Belinque<sup>1</sup>, Norma Pucheu<sup>1</sup>, Norma Kerber<sup>1</sup>, and Gerardo Rubio<sup>1,2\*</sup>

<sup>1</sup> INBA, Research Institute of Agricultural and Environmental Biosciences, University of Buenos Aires, Comisión Nacional de Investigaciones Científicas y Técnicas, C1417DSE Buenos Aires, Argentina

<sup>2</sup> Soil Fertility Lab, School of Agriculture, University of Buenos Aires, Av. San Martín 4453, C1417DSE Buenos Aires, Argentina

## Abstract

We evaluated the ability of *Brassica napus* L. (oilseed rape), *Helianthus annuus* L. (sunflower), and *Glycine max* L. (soybean) plants grown inoculated with or without bacteria to utilize organic P sources. Plants were supplied with inorganic (dibasic sodium phosphate) and organic P sources (phytate and glucose phosphate) at three concentrations and grown for 40 d under sterile conditions. Three inoculation treatments were compared: control (non-inoculated plants), inoculation with *Bacillus amyloliquefaciens* BNM340, and inoculation with *Pseudomonas fluorescens* BNM296 (two bacteria with proven phytase activity). Oilseed rape, sunflower and soybean could utilize organic P sources. For example, when phytate (0.5 mM) P was used as the external P source, the increase factors over the no-P treatments were 4.5, 1.4, and 1.4 for oilseed rape, sunflower, and soybean P uptake, respectively. When glucose 1-phosphate disodium salt (G1P, 0.5 mM) was the P source, the increase factors were 8.8, 1.7, and 1.9 respectively. Positive responses to the organic P sources were found for the biomass accumulation of oilseed rape and soybean but not for sunflower. The inoculation with bacteria did not exert a promoting effect on P uptake. We demonstrate that the three species can effectively use organic P sources. The existence of crop plants that are more efficient in the utilization of different soil P sources would be particularly beneficial to improve P recycling and use of P fertilizers in agriculture.

**Key words:** *Brassica napus* / *Glycine max* / *Helianthus annuus* / phosphorus acquisition / phytate

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## 1 Introduction

Low phosphorus (P) availability is a common constraint to agricultural productivity all over the world (Lynch, 2007). For that reason, there is a clear need to explore plant strategies that have potential to increase crop yields in low P soils and to reduce the requirements of P fertilizers (Lambers et al., 2006; Richardson et al., 2011), such as the plant-driven hydrolysis of organic P. Organic P usually accounts for between 30–65% of total soil P, although some soils exhibit even higher percentages (Harrison, 1987). Based on how P is bound to carbon, soil organic P compounds are classified into phosphate esters (monoesters or diesters), phosphonates, and anhydrides (Condon et al., 2005). The monoester forms, mainly inositol phosphates, are the dominant fraction. One of the most common inositol phosphates in soils is myo-inositol hexakisphosphate (phytate; Condon et al., 2005).

Phosphate esters must first be mineralized to inorganic P to become available for plants. The process is catalyzed by extracellular enzymes released by higher plants and soil-borne organisms. Once in the soil solution, the dissolved phosphates may be either absorbed by the plant or microorganisms or subject to the normal sorption-precipitation processes with the soil matrix (George et al., 2005). Studies on the effec-

tive utilization of organic P sources have shown a high inter-specific variability in both crop and pasture species (Hayes et al., 2000; Richardson et al., 2000; Steffens et al., 2010). In wheat, Richardson et al. (2000) demonstrated a high utilization of the organic P sources glucose 1-phosphate (G1P), RNA, and ATP, but a poor utilization of phytates. Hayes et al. (2000) attributed the limited acquisition of phytate of six pasture species to the low capacity of plant roots to hydrolyze this P form and to phytate reactions with the soil matrix. Steffens et al. (2010) found an equivalent uptake of P from phytate compared to inorganic P sources for oilseed rape and a lower though important phytate uptake efficiency for maize, wheat, and rye.

Sunflower and soybean are two of the crops for which specific information about the utilization of organic P sources is lacking. These two crops and oilseed rape are among the most important oilseed crops all over the world. In Argentina, these three crops represent around two thirds of the planted area, mainly covered by highly fertile although P-deficient Mollisols (Rubio et al., 2008; Ciampitti et al., 2011).

It has been suggested that the utilization of P from inositol phosphates may be particularly effective if the activity of root phytases is complemented by phytases released by soil mi-



\* Correspondence: Dr. G. Rubio; e-mail: rubio@agro.uba.ar

croorganisms (Richardson et al., 2011). Phosphate uptake by several plant species was significantly greater in the presence of isolates of *Pseudomonas* sp. or *Bacillus* sp. that were previously selected for extracellular phytase activity (Richardson et al., 2000; Idriss et al., 2002). This suggests that the phytases released by the microorganisms may enhance the availability of organic P. However, soil microorganisms may also immobilize organic P and this has implications for competition with plants for P (Richardson et al., 2011).

In this work we tested the hypotheses that oilseed rape, sunflower, and soybean are able to utilize organic soil P sources and that this ability is further enhanced by some root-colonizing bacteria with proven phytase activity. Our experimental approach included sterile conditions to ensure that any organic P mineralization was produced by enzymes released by the plants or by the specific combination of plants and bacteria.

## 2 Material and Methods

### 2.1 Growth of plants in sterile hydroponic medium

Oilseed rape (*Brassica napus* L., cv. Filial Precoc), sunflower (*Helianthus annuus* L., cv. Paraiso 33) and soybean (*Glycine max* L., cv. FN 4.1) plants were grown under sterile conditions in tubes 3 cm wide and 50 cm (oilseed rape and soybean) or 30 cm (sunflower) tall for 40 d. Prior to sowing, tubes were autoclaved (121°C; 103 kPa) for 20 min, and seeds were surface-sterilized by three washes with 5% sodium hypochlorite, followed by rinses with sterile distilled water. Seeds were placed on a 3MM Whatman filter paper folded to form a U-shaped base that was suspended in the nutrient solution. The composition of the nutrient solution (40 mL per tube, pH 5.5) is described in Richardson et al. (2000). Two sources of organic [myo-inositol hexa-phosphoric acid dodecasodium salt (phytate), glucose 1-phosphate disodium salt (G1P)] and one of inorganic P (dibasic sodium phosphate), were used, all obtained from Sigma Aldrich Co., St. Louis, MO, USA. For each P source, three equivalent concentrations of P were evaluated: 0.1 mM, 0.5 mM, and 1.0 mM. A control treatment without P added was also included (no-P treatment). The tubes were placed on an orbital shaker at 130 rpm in a culture chamber at 25°C with light intensity (16 h d<sup>-1</sup>) of 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$  provided by fluorescent tubes (Philips TLD 865).

### 2.2 Preparation of the bacterial culture and phytase activity

*Bacillus amyloliquefaciens* BNM340 and *Pseudomonas fluorescens* BNM296 were isolated from the surface horizon of a typical Hapludoll located at Lincoln, Buenos Aires, Argentina (34°47'35.29" S; 61°33'5.34" W). Aliquots of 50  $\mu\text{L}$  from the bacterial cultures (passed through a 0.22  $\mu\text{m}$  sterile filter, Millex GP, Biopore) were incubated with 100  $\mu\text{L}$  0.4% phytate dissolved in 100 mM sodium acetate acetic acid pH 6 buffer, containing 2 mM calcium chloride (Dharmsthiti et al., 2005). Phytase activity was evaluated at 50°C with a modification of the ammonium molybdate method (Idriss et al., 2002). Meas-

urements were performed 10, 20, 30, and 60 min after the addition of the phytate. The reaction was stopped by adding ammonium molybdate vanadate. Phytase activity was calculated from the slope of the line obtained when released P was plotted against time and expressed in nmol of inorganic orthophosphate released per minute per mL of culture. Samples were centrifuged at 11,800 g for 1 min and the absorbance of the supernatant was measured at 415 nm. Absorbance data as a function of P concentration were obtained from a standard curve of potassium dihydrogen phosphate. Phytase activity was measured at different points of the growth curve of bacteria (10, 15, 25, and 35 hours) to determine at which stage of the curve the secretion of the enzyme was greatest.

### 2.3 Seed inoculation

After the seeds had been disinfected, they were immersed for 15 min in a suspension of 10<sup>8</sup> colony forming units (CFU) mL<sup>-1</sup> of each of the bacterial cultures. The seeds were drained and dried in the laminar flow for 15 additional min. After the inoculation, seeds were planted in the tubes as described above. To determine the amount of bacteria finally attached to the seeds, 20–50 seeds of each species were suspended in 10 mL of sterile water and then sonicated in an ultrasonic bath for 1 min. The supernatant was serially diluted to make an agarized Petri plate count. The results were expressed as CFU seed<sup>-1</sup>. This inoculation method had been employed in previous works (e.g., Yaryura et al., 2008) showing that the method was effective to colonize the roots, as measured as both rhizoplane and rhizospheric colony counts performed 7–11 d after inoculation.

### 2.4 Determination of biomass and P concentration in plant tissue

Harvested plant material was oven-dried at 60°C for 72 h to obtain the total biomass. Subsamples (70 mg) of ground tissue were ashed at 500°C for 24 h. The ashes were dissolved in 8 mL of 0.1 M HCl and the P concentration was measured colorimetrically (Murphy and Riley, 1962). Given that the only P source available for the no-P plants was the P contained in the seeds, any positive difference in P uptake between the no-P plants and the P-added plants was assumed as an evidence of the utilization of the specific form of P added.

### 2.5 Statistical analysis

The experimental design was a completely randomized design in which each treatment was the combination of the factors P source, P dose, and bacteria. Eight replicates were performed for each culture condition of each plant. Data collected for each species were statistically analyzed by factorial ANOVA and the protected least significant difference procedure was used for mean separation when the F test was significant ( $P < 5\%$ ). Due to the large number of interactions, comparison of means within each crop was performed among all the combinations of treatments. The validity of the assumptions of variance homogeneity and normal distribution was checked for all variables.

### 3 Results

#### 3.1 Seed colonization and phytase activity of *Bacillus amyloliquefaciens* BNM 340 and *Pseudomonas fluorescens* BNM 296.

The inoculation resulted in effective colonization of the seed surface. In the case of BNM 340, the number of CFU per seed was  $5.5 \times 10^4$ ,  $4.6 \times 10^4$ , and  $5.4 \times 10^5$  for oilseed rape, sunflower and soybean, respectively. In the case of BNM 296, the number of CFU per seed was  $5.1 \times 10^3$ ,  $5.2 \times 10^5$ , and  $4.5 \times 10^6$  for each species, respectively. Both BNM 340 and BNM 296 exhibited extracellular phytase activity (Table 1). Maximal phytase activity was found after 25 h of growth, indicating the end of the logarithmic growth phase. At this time point, BNM 340 degraded four times more phytate than BNM 296 (Table 1).

**Table 1:** Phytase activity measured 10, 15, 25, and 35 h after addition of phytic acid to bacterial cultures of *Bacillus amyloliquefaciens* BNM340 and *Pseudomonas fluorescens* BNM296. Data are means of three replicates.

Growth period / h	Phytase activity / nmol P mL <sup>-1</sup> min <sup>-1</sup>	
	<i>Bacillus amyloliquefaciens</i> BNM 340	<i>Pseudomonas fluorescens</i> BNM 296
10	2.24 (0.03)	0.16 (0.01)
15	3.31 (0.11)	0.82 (0.08)
25	4.12 (0.3)	1.32 (0.15)
35	2.62 (0.3)	1.12 (0.03)

#### 3.2 Oilseed rape

The main factors P source and P concentration and most interaction terms exerted significant effects on plant P uptake (Table 2). On average, the addition of 0.1, 0.5 and 1.0 mM in-

organic P increased plant P uptake over the no-P treatment by a factor of 3.2, 9.6, and 11.9, respectively (Table 2; Fig. 1). The addition of phytate increased plant P content 1.7, 4.5, and 10.7 times and the addition of G1P 2.8, 8.8, and 9.4 times over the no-P treatment for each of the increasing P levels, respectively. Oilseed rape plants were able to utilize organic P sources, although less effectively than inorganic sources (Fig. 1). Plants took up 32–88% phytate P and 78–92% G1P relative to equivalent doses of inorganic P (range for the three P doses). These figures were calculated after subtracting the seed P. The inoculation with BNM 296 and BNM 340 had no extra benefit for plant P uptake (Table 2; Fig. 1).

Total biomass was significantly affected by P source and P concentration but not by the bacteria treatments (Table 2), although significant two-way and three-way interactions were detected (Table 2). Responses were scarce at 0.1 mM P but consistent at 0.5 and 1.0 mM P (Fig. 2; Table 2). Interestingly, increases in biomass accumulation after P addition from organic sources were equivalent or in some cases even superior to the addition of inorganic P sources.

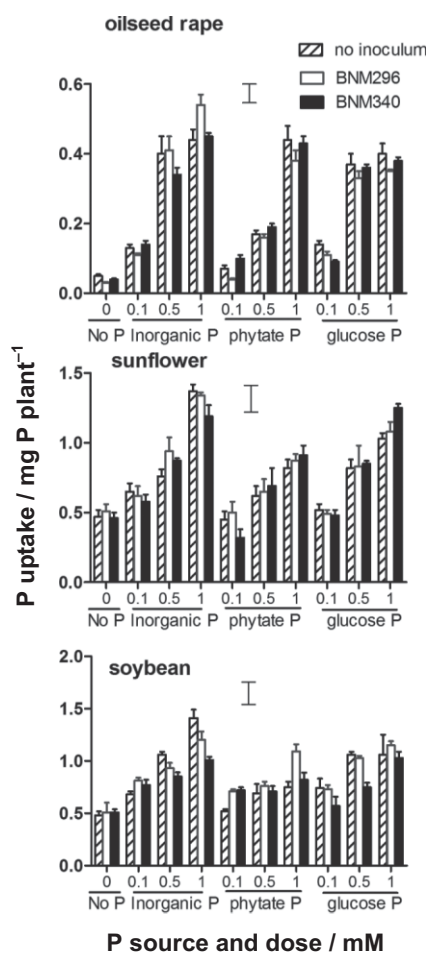
The inoculation with the bacteria BNM 296 and BNM 340 did not result in a significant benefit for plant growth (Table 2; Fig. 2). Only four and three of the treatments involving BNM 296 and BNM 340 inoculation, respectively, showed significant differences with the non-inoculated treatment (Fig. 2). However, the direction of these differences was not consistent (Fig. 2).

#### 3.3 Sunflower

The main factors P source and P concentration and the interaction between them caused significant effects on plant P uptake (Table 2). On average, the addition of 0.1, 0.5, and 1.0 mM inorganic P increased P uptake by a factor of 1.3, 1.8, and 2.7, respectively. Sunflower plants could use the organic P sources but less efficiently than inorganic phosphate; they took up 47–48% phytate P and 79–96% G1P relative to the equivalent dose of inorganic P for the 0.5 and 1 mM P doses (Table 2; Fig. 1). These calculations were made after subtract-

**Table 2:** Probability (*P*) values from analysis of variance (ANOVA) for the total biomass and phosphorus uptake. Main factors were: P source (inorganic P; phytate P; glucose P), P concentration (0.1; 0.5; 1 mM P) and bacteria (non-inoculated control; BNM 296; BNM 340).

	Total biomass			Phosphorus uptake		
	Oilseed Rape	Sunflower	Soybean	Oilseed Rape	Sunflower	Soybean
Significance level from ANOVA						
P source	0.020	0.009	< 0.001	< 0.001	< 0.001	< 0.0001
P concentration	< 0.001	0.750	< 0.001	< 0.001	< 0.001	< 0.001
Bacteria	0.082	0.006	< 0.001	0.174	0.355	< 0.001
P source × P concentration	0.234	0.143	< 0.001	< 0.001	< 0.001	< 0.001
P source × Bacteria	0.006	0.157	< 0.001	0.001	0.462	0.002
Conc × Bacteria	0.433	0.005	0.097	0.802	0.499	0.013
P source × P concentration × Bacteria	< 0.001	0.442	< 0.001	0.012	0.432	0.019



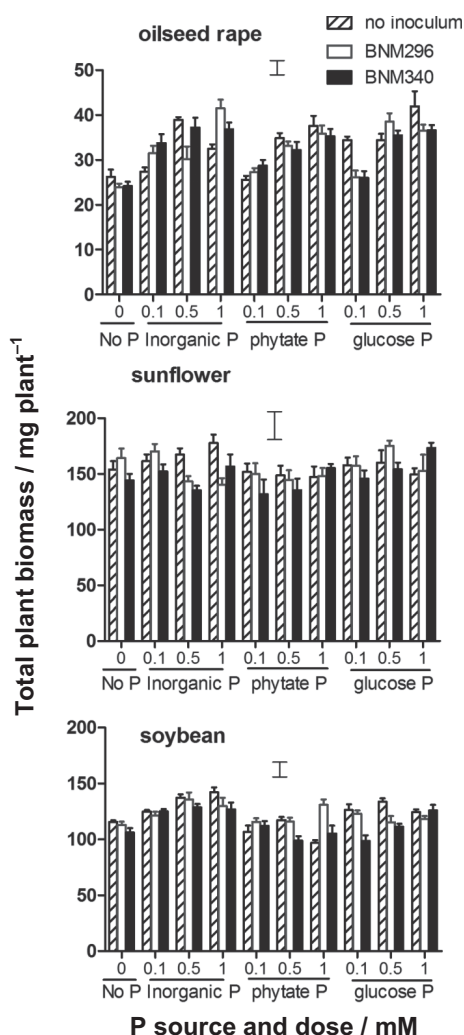
**Figure 1:** P uptake of oilseed rape, sunflower, and soybean plants as affected by P source (inorganic P; phytate P; glucose P), P concentration (0.1; 0.5; 1 mM P), and bacteria (no inoculation; BNM 296; BNM 340). Error bars denote  $\pm$  standard errors of the means. Bar marker at the center of each panel indicates LSD ( $P < 5\%$ ) for the all-pairwise comparisons test.

ing the seed P. The 0.1 mM dose was excluded because P uptake values were similar or even slightly lower than the controls with no P added. Comparing non-inoculated and inoculated plants, the only significant effect was the increase in P accumulation in plants inoculated with BNM 340 at the higher G1P level.

Overall, imposed treatments exerted no consistent effects on sunflower biomass accumulation (Table 2; Fig. 2). Only three treatments showed significant differences compared with the no-P treatment. They were the 1.0 mM dose of the inorganic P source in both the non-inoculated plants and BNM 296-inoculated plants and the 1.0 mM G1P dose applied to plants inoculated with BNM 340.

### 3.4 Soybean

All main factors and interaction terms showed significant effects on plant P uptake (Table 1). Positive responses to P began at the 0.1 mM P level but were consistent only at 0.5 and 1.0 mM P (Table 2; Fig. 1). On average, the addition of the in-



**Figure 2:** Biomass accumulation of oilseed rape, sunflower and soybean plants as affected by P source (inorganic P; phytate P; glucose P), P concentration (0.1; 0.5; 1 mM P), and bacteria (no inoculation; BNM 296; BNM 340). Error bars denote  $\pm$  standard errors of the means. Bar marker at the center of each panel indicates LSD ( $P < 5\%$ ) for the all-pairwise comparisons test.

organic P source increased P uptake over the no-P treatment by a factor of 1.5, 1.9, and 2.4 for the 0.1, 0.5, and 1.0 mM P levels, respectively. The increase factors over the no-P after the addition of phytate P averaged 1.3, 1.4, and 1.8, respectively. In the case of G1P, P uptake increased 1.4, 1.9, and 2.2 times for the range of P levels evaluated. Soybean plants could utilize the organic P sources, although less efficiently than the inorganic ones; they absorbed, on average, 51–58% phytate P and 75–98% G1P relative to the inorganic P source (on an equivalent dose basis for the three P doses). When the P came from organic sources the effect of bacteria was not consistent, although increases in P uptake were found at the 0.1 and 1.0 mM phytate-P levels.

ANOVA indicated significant effects on soybean biomass accumulation of the three main factors and most interaction terms (Table 2). Consistent responses to the addition of 0.5 and 1.0 mM inorganic P were observed but growth



responses to the organic sources were erratic and did not show a clear pattern (Fig. 2). Responses to the inoculation with bacteria were also inconsistent. From the comparison between the biomass accumulation of the non-inoculated and inoculated treatments, the detrimental effects of the inoculation (five cases) predominated over the beneficial effects (one case).

## 4 Discussion

Oilseed rape, sunflower, and soybean effectively utilized organic sources of P. This was consistently demonstrated by the higher P accumulation of the plants supplied with phytate and G1P compared to the no-P plants. In some cases, these positive responses were accompanied by the accumulation of biomass (Table 2; Fig. 1)

Plants utilized phytate with a relatively high efficiency, presumably by enzymatic hydrolysis of the substrate. For example, for the intermediate P dose (0.5 mM P) oilseed rape, sunflower, and soybean took up on average 39, 46, and 51% phytate P, respectively, compared to equivalent doses of inorganic P. Our results support the evidence that some cultivated species can acquire P directly from phytate in contrast to other species that cannot. Barley, oat (Tarafdar and Claassen, 1988), lupin (Adams and Pate, 1992), sugar beet, and buckwheat (Steffens et al., 2010) are among the plants with proven phytate P utilization. Steffens et al. (2010) also observed that oilseed rape can acquire phytate P. In contrast, phytate P did not improve plant P nutrition in subterranean clover, white clover, burr medic, ryegrass, wallaby grass, phalaris (Hayes et al., 2000) and wheat (Richardson et al., 2000; George et al., 2008; Steffens et al., 2010). G1P was utilized with a higher efficiency than phytate. Compared to equivalent doses of inorganic P, oilseed rape, sunflower, and soybean absorbed 92, 96, and 98% G1P, respectively, for the intermediate P dose (0.5 mM P). This agrees with most studies reporting a high availability of this organic P source (e.g., Hayes et al., 2000; Richardson et al., 2000; George et al., 2008). However, since G1P is a minor component of soil P, the contribution of this fraction to plant P nutrition is expected to be poor.

Oilseed rape accumulated more P and biomass in response to the addition of P than sunflower and soybean (Table 2; Figs. 1 and 2). This can be attributed to the fact that oilseed rape has the smallest seed size among the three species and, thus, a low amount of seed P reserves. George et al. (2008) also observed that the variation in organic P accumulation was correlated with seed P content.

The inoculation resulted in effective colonization of the seed surface. The values were either equivalent or higher than those obtained by Richardson et al. (2000) with *Pseudomonas* strain CCAR59. We found that the non-significant effects of the inoculation with either BNM296 or BNM 340 on P uptake largely predominated over the beneficial or detrimental effects (Fig. 1). Out of the 30 comparisons between non-inoculated and inoculated plants [(3 P doses  $\times$  3 P sources  $\times$  3 species) + (1 no-P  $\times$  3 species)], only five cases of statistically significant effects of the inoculation treatment were found

(four increases and one decrease in plant P accumulation). In terms of biomass accumulation, the detrimental effects predominated over the beneficial ones (i.e., increases in plant growth). These results indicate that BNM296 or BNM 340, despite having phytase activity, did not contribute significantly to plant P acquisition. Probably, the extracellular phytase released by plants roots to the external medium was sufficient to satisfy their demand of P. An alternative explanation is the need of a microbial loop to enhance P availability from phytate, as proposed by Irshad et al. (2012). These authors observed that the bacterial ability to degrade phytate should be combined with the action of bacterial grazers, like nematodes, to release the nutrients retained by the bacteria.

Whether the observed ability to utilize both G1P and phytate P remains functional under field conditions will depend on factors such as the availability of soil inorganic (Yun and Kaeppler, 2001; Tarafdar and Claassen, 2005) or organic P (Findenegg and Nelemans, 1993), and the enzyme and substrate interactions with the soil matrix (George et al., 2005; Richardson et al., 2007). The effective utilization of soil organic P would be impeded in inorganic P-rich soils, because phosphates are directly available to plants. It would be also precluded under very low organic P concentrations in the soil solution, as was clearly observed in our and other experiments (e.g., Findenegg and Nelemans, 1993). The question about what the real concentration of soil organic P is that roots face in the soil is crucial. However, this information is scarce and supposedly highly variable (Martin, 1970; Condron et al., 2005). On the other hand, access to organic P sources could be restricted in high sorptive soils due to the high affinity between both inositol phosphates and phytases and the soil solid phase (George et al., 2005).

The experiments presented here demonstrated that oilseed rape, sunflower, and soybean can effectively use organic P sources. An intriguing question is why some plants have the ability to utilize organic P and others do not. One straightforward possibility is that they evolved in environments with different availability of soil organic P, thus, experiencing a different evolutionary pressure. Anyway, the existence of crop plants that are more efficient in the utilization of different soil P sources would be particularly beneficial to improve the recycling of P and the use of P fertilizers (Rubio et al., 2012). This is relevant given the nonrenewable nature of the external sources of P and the potential adverse effects of excess P in agricultural systems. Since organic P is neglected by some routine soil P testing methods (Steffens et al., 2010), the present results support the need of research focused on improving soil P test methods in order to include the plant-available organic P.

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