

## Dual inoculation with *Pseudomonas fluorescens* and arbuscular mycorrhizal fungi increases phosphorus uptake of maize and faba bean from rock phosphate

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### Abstract

The process of combining microbial inoculants is a promising method to effectively improve nutrient availability in soil. In two pot experiments the single and combined application of *Pseudomonas fluorescens* (PF, strain DR54) and arbuscular mycorrhizal fungi (AMF) were evaluated in terms of their effects on phosphorus (P) nutrition of plants supplied with rock phosphate (RP). Faba bean and maize plants were cultivated for 55 days in the first and 45 days in the second experiment under semi-controlled conditions in Mitscherlich pots containing 6 kg of a loamy sand. Five treatments were evaluated for each crop including a control without any application (Con), RP, RP+PF, RP+AMF, and RP+PF+AMF. After plant harvest nutrient concentration in plant tissue (P, N, Mg, K), contents of plant available P ( $P_{di}$ ) and pH values of soil were analysed. Beans were more efficient than maize in using P from rock phosphate. Highest P uptakes of plants and values of  $P_{di}$  in soil were found in the combined RP+PF+AMF treatment. Elevated  $P_{di}$  and lower pH values compared to the control after sole AMF inoculation indicate a direct P mobilization by AM fungi.

*Keywords: AMF, Pseudomonas, rock phosphate, phosphorus mobilization*

### Zusammenfassung

#### **Eine kombinierte Ausbringung von *Pseudomonas fluorescens* und Mykorrhiza-Pilzen führt bei Mais und Bohne zur Erhöhung der P-Aufnahme aus Rohphosphaten**

Ein kombinierter Einsatz von Mikroorganismen ist eine effektive Methode um die Verfügbarkeit von Nährstoffen im Boden zu erhöhen. In 2 Gefäßversuchen wurde die Einzel- und Kombinationswirkung von *Pseudomonas fluorescens* (PF, Stamm DR54) sowie von arbuskulären Mykorrhiza-Pilzen (AMF) in Bezug auf die Phosphor (P) – Aufnahme von Pflanzen nach Zufuhr von Rohphosphat (RP) untersucht. Faba-Bohnen und Mais wurden für 55 Tage im ersten und 45 Tage im zweiten Experiment in Gefäßen mit 6 kg lehmigen Sandboden unter teil-kontrollierten Bedingungen kultiviert. Fünf Varianten wurden etabliert: RP, RP+PF, RP+AMF und RP+PF+AMF sowie eine Kontrolle (con) ohne Zufuhr von Mikroorganismen oder RP. Nach der Ernte der Pflanzen wurden deren Nährstoffkonzentration (P, N, Mg, K) sowie der pflanzenverfügbare P-Gehalt ( $P_{di}$ ) und der pH-Wert des Bodens bestimmt. Die Bohne war bezüglich der Nutzung von P aus Rohphosphat effektiver als der Mais. Der größte Anstieg der P-Aufnahmen der Pflanzen und des  $P_{di}$ -Gehaltes des Bodens wurde in der Variante RP+PF+AMF ermittelt. Auch nach einer Einzelbeimpfung mit AMF stieg der  $P_{di}$ -Gehalt des Bodens an verbunden mit einer Verringerung des pH-Wertes, was auf eine direkte P-Mobilisierung durch die AMF hindeutet.

*Schlüsselwörter: AMF, Pseudomonas, Rohphosphat, Phosphormobilisierung*

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## Introduction

The direct application of rock phosphate (RP) as a fertilizer is an easy and comparatively cheap way to add P to soils. However, the solubility of phosphorus (P) from phosphate rock is low. Vassilev et al. (2001) reported that the amount of P released from directly applied RP may be too low to provide sufficient P for crop needs.

On the other hand, plants have different strategies to mobilize P and may take up P from less soluble sources (Eichler-Löbermann et al., 2008). Legumes can be particularly well-suited for the use of RP. Mnkeni et al. (2000) reported that legumes are more efficient than cereals at utilizing P from RP. Adams and Pate (1992) found positive results for lupin and Ae et al. (1990) for pigeon pea. The enhanced solubilization of RP was mainly due to excretion of organic acids and the subsequent mechanisms of acidification, chelation, and exchange reactions (Reyes et al., 2001; Dakora and Phillips, 2002).

Like plants microorganisms can also improve the availability of sparingly soluble inorganic and organic phosphates by releasing substances such as organic ions, enzymes and protons. Using the microbial effects for plant production can be done by the management of existing soil microbial populations to optimize their capacity to mobilize P (Oberson et al., 2001; Khan et al., 2010) or by the application of specific microbial inoculants (Mäder et al., 2011; Krey et al., 2011). Although attempts to improve the plant nutrition using microbial inoculants has focused mainly on fungi (Wakelin et al., 2004; Relwani et al., 2008), the application of rhizobacteria has also been proposed as a component of sustainable nutrient management systems (Taurian et al., 2010). Bacteria belonging to the genera *Bacillus* and *Pseudomonas* were often found to be very efficient in liberating orthophosphate from organic and inorganic sources (Tye et al., 2002; Richardson et al., 2011).

Arbuscular mycorrhizal fungi (AMF) colonize most agricultural crops and also play an important role in P supply to plants in P deficient farming systems. The importance of AMF in P supply may be comparable to that of root hairs. Their hyphae can extend further from roots than the root hairs, which resulted in a higher soil volume that a colonized root can explore (Smith and Read, 2008). Mainly for plants with short root hairs, the AMF colonization has advantages, as it was shown for *Phaseolus* beans (Miguel, 2004).

Although the major P effect of AMF colonization is due to an increase in spatial P availability, studies have also shown a biochemical mobilization by AMF. This resulted in a better respond of plants colonized with AMF to the application of insoluble phosphate forms in comparison with non-mycorrhizal roots (Medina et al., 2006). Furthermore, AMF can protect plants against toxic elements (e.g.

Zn, Cd, and Mn) by accumulation of these in their hyphae (Clark and Zeto, 2000) and may enhance plants' tolerance against pathogen by competing with pathogenic microorganisms (Turk et al., 2006). For Faba bean root colonization by indigenous AMF increased vegetative growth and seed yield in addition to improving nodulation (Mathur and Vyas, 2000).

The development of **multifunctional microbial inoculants** is a promising method to increase the positive effects of microorganisms. This can be based on more than one effect of the single organism or on a combination of organisms (Vassileva et al., 2010). Bacterial and fungal populations can interact in the rhizosphere and stimulate plant growth and improve nutrient availability very effectively (Zaidi et al., 2003; Toljander et al., 2007). Additive effects between AMF and plant growth-promoting bacteria were observed, e.g., after the combined application of AMF and *Pseudomonas* species (Gamalero et al., 2004) or *Bacillus circulans* (Singh and Kapoor, 1999).

The main objectives of this study were to evaluate rock phosphate as a P source for maize (*Zea mays*) and bean (*Vicia faba*) in combination with single or dual application of *Pseudomonas fluorescens* (PF) and AMF. The following hypothesis was tested: Combined inoculations with P solubilizing bacteria and AMF can increase the fertilizing effects of rock phosphate.

In order to test this hypothesis under semi-controlled conditions, two outdoor pot experiments were established with naturally P poor soil. The *P. fluorescens* DR54 strain was chosen due to its effects on soil P pools in pot experiments.

## Materials and methods

### Experimental setup

Two pot experiments were conducted in a greenhouse in 2008. The soil utilized was loamy sand originating from the upper soil layer (0 to 30 cm) of a field experiment close to Rostock (North Germany, soil characteristics are given in Table 1). The dominant soil type on the field site was a Stagnic Cambisol. The trials were carried out in a complete randomized design with four replications using Mitscherlich pots containing 6 kg air-dried and sieved (10 mm) soil. Maize (*Zea mays*) and faba bean (*Vicia faba*) were cultivated in both experiments in June and August and harvested 55 and 45 days respectively after sowing. Five treatments were arranged for each crop including controls without any application (Con), rock phosphate (RP), RP+*Pseudomonas fluorescens* (RP+PF), RP+mycorrhizal inoculum (RP+AMF) and RP+PF+AMF. Eight seedlings were sown in each pot and thinned to four plants after germination.

Before sowing, 2 g of RP powder and 25 ml of mycorrhizal inoculum were added to the respective treatments at a depth of 10 cm. The RP contained 13.1 % of P (30 %  $P_2O_5$ ) and came from Kola Peninsula, Russia. The extractability of P in the RP powder was 6.7 % in water, and 57.3 % in ammonium citrate, whereas the pH value was 8.4. The commercial mycorrhizal product consisted of *Glomus etunicatum*, *Glomus intraradices* and *Glomus claoideum* with a spore number of  $10^5$  per l litre. The carrier material was expanded clay with a grain size of 2 to 4 mm and a pH of 7.5. Per pot 25 ml of this product was used. The PF-DR54 inoculum was prepared by growing in liquid R2A medium (Difco) at 25 °C for 36 h, suspended in 0.1 M  $MgSO_4$  buffer, washed twice and re-suspended in distilled water at about  $10^8$  cfu per ml. After appearance of the second leaf the PF suspension was applied to the respective treatments ( $10^8$  cfu per plant). The pots were irrigated with distilled water. In order to consider the possible effect of experimental time, the plants in the first experiment (June-Exp) were harvested after 55 days, the plants in the second (August-Exp) after 45 days. The plants were cut above ground, oven-dried at 60 °C for 72 h, weighed and ground for chemical analyses. At harvest soil samples were taken and dried for further analyses.

Table 1:  
Characteristics of the soil used for planting

Texture	P mg kg <sup>-1</sup>	K mg kg <sup>-1</sup>	Mg mg kg <sup>-1</sup>	OM %	pH
sandy loam	48.2	61.0	234	2.80	6.86

#### Plant and soil analyses

Shoot-biomass P concentrations were measured after dry-ashing using the vanadad-molybdate method (Page et al., 1982). Shoot biomass N concentrations were measured by using the Kjeldahl digestion method modified according to Jones et al. (1991). Nutrient uptake per pot was calculated by multiplying shoot biomass (g) with shoot nutrient concentration (mg g<sup>-1</sup>). The organic matter content in soil was determined after ashing in an oven at 550 °C. Soil contents of double lactate soluble P, K and Mg ( $P_{dl}$ ,  $K_{dl}$ ,  $Mg_{dl}$ ), as well as pH ( $CaCl_2$ ) were measured according to Blume et al. (2000). Phosphates extracted with double lactate solution represent the plant available P (Schachtschabel and Beyme, 1980).  $K_{dl}$  and  $Mg_{dl}$  concentrations were measured with a flame photometer. P shoot biomass and  $P_{dl}$  were measured with a spectral photometer at 430 nm.

#### Statistical procedure

Data were subjected to the GLM (General Linear Model) procedure of SPSS version 15. Treatment effects were tested by one-way analysis of variance followed by Duncan's multiple range test used for multiple comparisons. Mean differences were considered significantly different at  $p < 0.05$ .

#### Results and discussion

In both experiments the **yield** of maize and beans significantly increased when RP was combined with *P. fluorescens* and AMF together, whereas no yield effect was found after the RP addition only compared to the control. Single application of either AMF or Pseudomonas with RP only resulted in higher yields for beans in the June experiment, when the plants were harvested at the end of flowering stadium.

Table 2:

DM yields of maize and beans (g per pot) in pot experiments (sowing in June and August 2008)

Treatment	Maize		Beans	
	June	August	June	August
Con	55.5 a	38.9 a	29.6 a	18.8 a
RP	57.6 a	40.6 ab	31.6 ab	19.6 a
RP+PF	59.4 ab	40.4 ab	33 b	20.1 a
RP+AMF	57.9 a	40.9 ab	33.8 b	20 a
PR+PF+AMF	63.5 b	42.5 b	34.4 b	21.6 b

Different letters within a column indicate significant differences between the treatments;  $p < 0.05$  (Duncan), Con = control, RP = rock phosphate, PF = *Pseudomonas fluorescens*, AMF = arbuscular mycorrhizal fungi

With regard to the **P uptake**, for maize significantly higher values compared to the control were only found in the combined RP+PF+AMF treatment. For beans higher treatment effects were observed. In the June experiment P uptake by beans was raised when RP was combined with dual microorganism inoculation (RP+PF+AMF) as well as with one inoculation alone (RP+PF and RP+AMF). The RP without AMF or PF resulted in 10 % higher P uptake compared to the control, but this difference was not significant. In the August experiment however, all RP treatments resulted in higher P uptakes of beans compared to the control. This strongly indicates that beans were more efficient in using P from rock phosphate than maize. These results are in accordance with other studies emphasising

the high potential of P solubilization of legumes as stated in the introduction chapter. Missing effects of the applied *Pseudomonas* strain on maize yield are in accordance with a previous pot experiment by Krey et al. (2011). In both experiments we found higher contents of available P in soil after application of *P. fluorescens* (see below), but the yield and P uptake by maize did not increase. Although the initial soil P content was suboptimal according to the German soil classification, it can be expected that stronger yield effects of the applied *P. fluorescens* strain might have occurred in even lower soil P contents. This supposition is also supported by a strong plant-growth promotion after inoculation of maize with *Pseudomonas alcaligenes*, *Bacillus polymyxa*, and *Mycobacterium phlei* to a nutrient deficient Calcisol in a greenhouse pot experiment by Egamberdiyeva (2007). Also for AMF better results were found when the plant available P in soil was very low which went together with high percentages of root colonization (Graham and Abbott, 2000).

Positive influences of both microorganism treatments together indicate the increased effectiveness of combined

inoculation regarding the nutrient availability and plant growth, which was also shown in a study of Boer et al. (2005). Babana and Antoun (2006) found that strains of *Pseudomonas* sp. act both as plant growth promoting bacteria and mycorrhizal-helper bacteria resulting in comparable high yields of wheat plants after RP application compared to a treatment with high soluble P fertilizer.

Similar results as those for P were found for the **other nutrients**. The N uptake of maize and beans increased in relation to the control when both microorganisms together were applied (160 % and 122 %, respectively). The K and Mg uptake of both plants were also promoted when microorganisms (single or dual inoculation) were added to the soil. In particular the K uptake of maize was enhanced by microorganism application. The increase in nutrient uptake was partially higher than the increase in yields, which can be explained by higher availability of nutrients in the soil and therefore higher nutrient concentrations in plant tissue (data not shown). Also in other studies higher responses on nutrient supply were found regarding plant nutrient uptake than regarding plant yield (Eichler et al., 2004).

Table 3:

Nutrient uptake of maize and bean (mg per pot) in pot experiments (sowing in June and sowing in August 2008)

Experiment	Treatment	P uptake	N uptake	K uptake	Mg uptake
Maize					
June	Con	72.4 a	294 a	656.0 a	154 a
	RP	77.7 a	299 ab	700.8 ab	155 ab
	RP+PF	81.3 a	339 bc	759.4 b	167 ab
	RP+AMF	78.5 a	339 bc	724.4 ab	164 ab
	PR+PF+AMF	81.3 a	362 c	752.6 b	177 b
August	Con	85.7 a	371 a	956.8 a	84.1 a
	RP	93.9 a	377 a	1004 ab	85.7 a
	RP+PF	94.9 a	448 a	1150 b	93.6 ab
	RP+AMF	95.5 a	460 a	1141 b	93.4 ab
	PR+PF+AMF	87.7 a	606 b	1460 c	108 b
Faba bean					
June	Con	38.8 a	668 a	230 a	126 a
	RP	42.5 ab	674 a	246 ab	133 a
	RP+PF	48.3 bc	793 b	273 b	138 a
	RP+AMF	49.7 bc	761 b	279 bc	141 a
	PR+PF+AMF	50.5 c	815 b	310 c	141 a
August	Con	36.6 a	603 a	281 a	81.8 a
	RP	42.2 b	672 ab	286 a	84.7 a
	RP+PF	45.1 b	690 b	295 a	87.6 ab
	RP+AMF	42.4 b	685 b	295 a	87.8 ab
	PR+PF+AMF	44.5 b	738 b	321 a	99.0 b

Different letters within a column indicate significant differences between the treatments;  $p < 0.05$  (Duncan), Con = control, RP = rock phosphate, PF = *Pseudomonas fluorescens*, AMF = arbuscular mycorrhizal fungi

As expected, due to the longer vegetation time and higher sunlight intensity the plant yields of the June experiment were higher than the yields of the second experiment in August.

Positive effects of microorganisms were observed regarding the **soil values**. In the combined treatment RP+PF+AMF the highest  $P_{di}$  values were found, regardless of the high P removal by plants in this variant. Application of RP together with only one inoculant resulted in higher P values mainly in the first experiment with the longer experimental time. Probably the second experiment was too short to make the microorganism effect visible. RP as a fertilizer is known to dissolve slowly and needs a long time to release P available for plants. Truong et al. (1978) studied changes in the estimated relative agronomic effectiveness (RAE) of RP with time. The RAE coefficients changed considerably between 1 and 4 months for most RP.

Table 4:

Plant available P contents in soil (P<sub>di</sub> mg per kg) and pH values in pot experiments (sowing in June and sowing in August 2008)

Experiment	Treatment	Maize		Faba bean	
		P <sub>di</sub> in soil (mg kg <sup>-1</sup> )	pH	P <sub>di</sub> in soil (mg kg <sup>-1</sup> )	pH
June	Con	42.8 a	6.93 c	46.1 a	6.93 c
	RP	45.9 ab	6.82 bc	51.8 b	6.93 c
	RP+PF	49.9 bc	6.08 a	53.7 b	6.87 c
	RP+AMF	52.8 c	6.56 b	56.4 b	6.57 b
	PR+PF+AMF	51.6 c	5.98 a	55.3 b	6.06 a
August	Con	47.0 a	6.94 b	48.6 a	6.93 b
	RP	50.2 a	6.93 b	50.8 ab	6.90 b
	RP+PF	53.4 a	6.56 b	53.8 ab	6.68 b
	RP+AMF	51.5 a	6.49 ab	54.5 ab	6.54 ab
	PR+PF+AMF	53.5 a	6.08 a	55.6 b	6.19 a

Different letters within a column indicate significant differences between the treatments; p < 0.05 (Duncan), Con = control, RP = rock phosphate, PF = *Pseudomonas fluorescens*, AMF = arbuscular mycorrhizal fungi

On average, for beans slightly higher soil  $P_{di}$  values were measured compared to maize which is in relation to the lower P removal by bean plants.

Generally the pH was lower in the treatments with the microorganism inoculation and may have resulted in a higher release of orthophosphate ions from RP. The lowering effect on pH by microorganisms and following an increasing availability of P is mainly due to excretion of organic acids and protons (Bünemann et al., 2004; Stevenson, 2005). Maliha et al. (2004) found that pH of bacterial broth dropped significantly (5.96 to 2.75) compared to the control.

Between the two cultivated plants only slight differences in soil reaction were measured. Therefore, the solubilizing effect of beans on RP cannot be explained by the pH values. However, the pH might have been lower in the direct rhizosphere zone, where organic acids and protons are released.

## Conclusion

Combined inoculation of bacterial and fungal populations can improve nutrient availability and plant growth very effectively. According to our results, a combination of *P. fluorescens* and AMF fungi of the genus *Glomus* can improve the P nutrition from RP more effectively than single applications of one of the microorganism preparations. Generally, both microorganism treatments showed the same efficacy. Higher  $P_{di}$  and lower pH values in AMF treatments compared to the control suggest a direct P mobilization resulting in the best effects for combined inoculation.

The partly diverse effects of the microorganism application on plant and soil parameters underline the complexity of the system. Field experiments are necessary to test the efficacy of the inoculants under more practical conditions.

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