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Phosphate solubilizing microorganisms under non-sterile conditions

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(With 2 figures)

Summary

Two species *Penicillium aurantiogriseum* and *Pseudomonas* sp. (PI 18/89) highly able to solubilize inorganic phosphates were tested for their efficiency to dissolve calcium phosphates under non-sterile conditions. In laboratory experiments plant available phosphorus was only increased when phosphate solubilizing microorganisms disposed of sufficient nutrient supply. Field studies indicate that phosphate solubilizing microorganisms were too vulnerable and unreliable to be used in agriculture. Too many biotic and abiotic factors influence outcomes of such experiments in an – up to now – unknown way, making results hardly predictable.

Key-words: phosphorus, mobilization, solubilization, microorganisms, phosphate-solubilizing microorganisms.

Phosphormobilisierende Mikroorganismen unter unsterilen Bedingungen

Zusammenfassung

Die Arten *Penicillium aurantiogriseum* und *Pseudomonas* sp. (PI 18/89) wurden als sehr effektive Lauger anorganischer Phosphate erkannt. Es wurde daher auch untersucht, inwieweit sie unter unsterilen Bedingungen in der Lage sind, Phosphor aus Kalziumphosphaten zu lösen. In Laborexperimenten wurde die Fraktion des pflanzenverfügbaren Phosphors durch die phosphatlösenden Mikroorganismen nur dann erhöht, wenn sie ausreichend mit Nährstoffen versorgt waren. Feldstudien zeigten, daß die untersuchten Mikroorganismen zu empfindlich sind, um in der Landwirtschaft eingesetzt werden zu können. Die Ergebnisse solcher Experimente werden von zu vielen biotischen und abiotischen Faktoren in einer bislang nicht geklärten Weise beeinflußt und sind daher kaum vorhersagbar.

Schlüsselworte: Phosphor, Mobilisierung, Lösung, Mikroorganismen, phosphormobilisierende Mikroorganismen.

1. Introduction

The annual world-wide yield of the mining of phosphorus ores lies in the range of $1.6 \cdot 10^{11}$ kg (FALBE and REGITZ 1991). Considering the increase in consumption workable resources will last for about 100 years (STEVENSON 1986), which means that phosphorus will be the first macronutrient to be exhausted.

P content of average soils is about 0.05 % (w/w) but as only about 0.1 % of this phosphorus is available to plants (SCHEFFER and SCHACHTSCHABEL 1992), intensive fertilization is indispensable in many agricultural soils. To avoid ecological and economical disadvantages of fertilization a great number of scientists have tried to shift the balance between the total and the plant available phosphorus in the soil to the latter fraction by means of phosphate solubilizing microorganisms (PSMs) (OSMAN et al. 1974, KUCEY 1988, ALAGAWADI and GAUER 1992). These investigations showed that phosphorus solubilization with microorganisms is a promising alternative to P fertilization (ASEA et al. 1988). Nevertheless the basic solubilization mechanisms are very complex (ILLMER and SCHINNER 1995) making the results of experiments with PSMs hardly predictable (BAREA et al. 1970). Physiological properties of PSMs and the solubilization mechanisms are dealt with in a current investigation (ILLMER and SCHINNER 1995), showing that excretion of H^+ (and not of organic acids) is most important for the solubilization of hydroxylapatite and brushite by *Penicillium aurantiogriseum* and *Pseudomonas* sp. (PI 18/89). The two investigated species were shown to be very effective in solubilizing calcium phosphates *in vitro*.

As a logical consequence the present investigation tries to clarify the question to which extent PSMs are able to maintain their properties withstanding the native microflora in a non-sterile soil. Additionally the effects of PSMs on the phosphorus fractions in non-sterile soil and on the P-uptake of plants were examined.

2. Materials and methods

For detailed information about the two forest soils, the isolation of phosphate-solubilizing microorganisms, the optimization of culture conditions, applied media etc. see ILLMER and SCHINNER (1992). A description of the sites is given elsewhere (Anon. 1993), whereas some basic soil properties of soil M (for Möggers) and N (for Nenzing, both sites located in the Austrian Alps) are summarized in table 1.

Table 1

Soil properties of soil M (Möggers) and N (Nenzing); CEC in meq 100 g DM^{-1}, humus and N in %; Al, total phosphorus (Pt) and plant available phosphorus (Pp) in µg gDM^{-1}

site	soil type	humus type	humus	Pt	Pp	Al	pH	CEC	C:N	N
M	pseudogley	mull	12.4	345	0.58	474	4.6	36.7	18.4	0.36
N	pararendsina	mull	9.3	270	0.74	137	4.2	30.2	17.0	0.30

All experiments throughout the investigation were carried out with two PSMs which had been isolated from forest soil: *Penicillium aurantiogriseum* (Dierchx) and *Pseudomonas sp.* (PI 18/89) (ILLMER and SCHINNER 1992).

Culture conditions

For cultivation of PSMs medium *NM8* was used, containing glucose 2 g, sucrose 2 g, NH_4NO_3 373 mg, $MgSO_4 \cdot 7H_2O$ 410 mg, KCl 295 mg, NaCl 200 mg, $Fe^{II}Cl$ trace, soil extract 25 ml. Compounds were dissolved in 975 ml of distilled

water and pH was adjusted to 7.0. As sole phosphorus sources 150 mg hydroxyl-apatite [$Ca_5(PO_4)_3OH$] and 150 mg brushite [$CaHPO_4 \cdot 2H_2O$] were sterilized separately and then mixed with 1000 ml of the autoclaved medium from above. Solubility products of these two compounds are $1.5 \cdot 102^{-112}$ and $2.2 \cdot 10^{-7}$ respectively (EHRLICH 1990), although especially the values given for apatite vary to a great extent (WIER et al. 1971). For the inoculation of soil and hydroponics cell suspensions were used which contained $2 \cdot 10^9$ and $8 \cdot 10^6$ cells ml^{-1} of *Pseudomonas* sp. (PI 18/89) and *P. aurantiogriseum*, respectively.

PSMs in non amended soil

5 g of non-sterile air dried soil were weighed 15 times into 100 ml Erlenmeyer flasks. In each case 2 ml of a preculture of *Pseudomonas* sp. (PI 18/89) or *P. aurantiogriseum* were put into five flasks resulting in an approximate water content of the soils of 70 %. Five flasks were applied with 2 ml of sterile *NM8* only (controls). All treatments were incubated at 25 °C for one week, compensating the daily evaporation by adding distilled water. At the end of the experiment P-fractions were determined as described below.

PSMs in amended soil

20 g (FW) of soil was weighed into nine Erlenmeyer flasks. Each flask was filled with 200 ml nutrient solution containing glucose 400 mg, sucrose 400 mg, and NH_4NO_3 75 mg. Flasks (three each) were applied with 1 ml of a preculture of *Pseudomonas* sp. (PI 18/89) or *P. aurantiogriseum* or with 1 ml of sterile *NM8*. After incubating all flasks for two weeks at 25 °C and 100 RPM samples were centrifuged and supernatant solutions were analyzed for their P contents.

Hydroponics

Glass beads (ø 2 mm) were washed in HNO_3 (10 %) and in distilled water, dried and 90 g of them were introduced into 1000 ml flasks in nine replicates. Each flask was applied with 9 ml *NM8* and was autoclaved. Three of them were inoculated with 100 µl of precultures of *Pseudomonas* sp. (PI 18/89) three with *P. aurantiogriseum* and three remained sterile. All flasks were incubated at 25 °C for four days till microbial growth was macroscopically visible. Thereupon all treatments (six inoculated and three controls) were applied with 200 non-sterile seeds of cress *(Lepidium sativum)*. After another fourteen days of incubation at room temperature plants were harvested and their dry weights and P contents were determined.

Lysimeter

Ten polyethylen lysimeters ($l \times w \times h4 = 57 \times 37 \times 30$ cm) were used for investigating the influence of P fertilizer and PSMs on the efflux of phosphorus. Two soils (M for Möggers and N for Nenzing see Anon. (1993) and table 1), two sorts of P fertilizer (Thomasphosphate and Superphosphate) and two PSMs *(Pseudomonas* sp. (PI 18/89) and *P. aurantiogriseum)* were used for the experiment: M1: untreated control; M2: surface application of *Pseudomonas* sp. (PI 18/89) (with a rate of $6 \cdot 10^{15}$ cells m^{-2}); M3: surface application of *P. aurantiogriseum* (with a rate of $6 \cdot 10^{12}$ cells m^{-2}); M4: surface application of Thomasphosphate (25 g P m^{-2}); M5: surface application of Superphosphate (25 g P m^{-2}); N1–N5 analogous to M1–M5 with soil derived from Nenzing. P contents of fertilizers were 13 % P_2O_5 and 19 % P_2O_5 for Thomas- and Superphosphate respectively. When sufficient amounts of leachates were available sampling took place every week throughout the year. Lysimeters were located in Innsbruck (Austria) gathering rain but without direct sun exposure. Soil in the lysimeters were allowed to stabilize for 15 weeks to minimize disturbances throughout the experiment.

Field studies

Experiments were carried out in Möggers and Nenzing in Vorarlberg (Austria). At each site five squares (with an area of 25 m² each) were pegged out and treated in the manner described for lysimeters. Every six weeks soil of the A_h-horizon was sampled from both sites. The concentrations of aluminium, of the total, inorganic, organic and plant available phosphorus, the content of the organic matter and the pH were determined.

Analysis

The ignition procedure of SAUNDERS and WILLIAMS (1955) was used to determine the total (Pt), the inorganic (Pi) and calculating the difference of both the organic (Po) phosphorus. Plant available phosphorus (Pp) was extracted using LiCl (0.4 M; soil:solution = 1:20; pH 5.6). Common extracts were also tested but led to unsatisfactory results: $NaHCO_3$ (0.5 $\underline{M}$) led to muddy filtrates, which could not be overcome with carbon black. Especially in acidic soils lactate methods cause a distinct overestimation of plant available phosphorus probably because of complexation with Fe- and Al-phosphates (SCHEFFER and SCHACHTSCHABEL 1992). Phosphorus was analyzed using the Mo-blue method of OLSEN and SOMMERS (1982). Extraction of exchangeable aluminium was performed with 1 $\underline{M}$ KCl (soil:solution 1:50) according to the method of BARNHISEL and BERTSCH (1982). Aluminium was determined with AAS in the nitrous oxide-acetylene flame at 309.3 nm. Where digestion of organic material was necessary the method of STEWART et al. (1974) was applied using $HClO_4$, HNO_3 and H_2SO_4. Soil pH was determined in $CaCl_2$ (0.01 $\underline{M}$) with a soil:solution ratio of 1:3.5.

All values shown are averages of 3, 5 or 6 replicates depending on the particular experiment. Statistics (outlier exclusion, analysis of variance, Student's t-test, regression analysis) were performed on PC's using *STATGRAPHICS Statistical Graphics System*.

3. Results

PSMs in non amended soils

Plant available phosphorus was increased through *Pseudomonas* sp. (PI 18/89) and *P. aurantiogriseum* by 6 % and 9 % respectively, but as increases were only significant at p <0.1 and not at p <0.05, results are not shown.
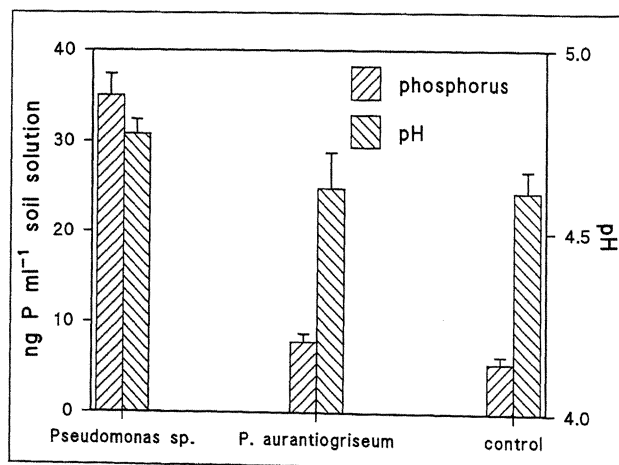

Figure 1: Phosphorus concentration and pH in solution of soil supplemental amended with nutrients and inoculated with phosphate solubilizing microorganisms. Phosphorus concentration: Pseudomonas sp. (PI 18/89 > control***: P. aurantiogriseum > control**; differences concerning pH are not significant

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Figure 2: Phosphorus efflux of phosphorus out of lysimeters during 32 weeks after a preincubation of 15 weeks
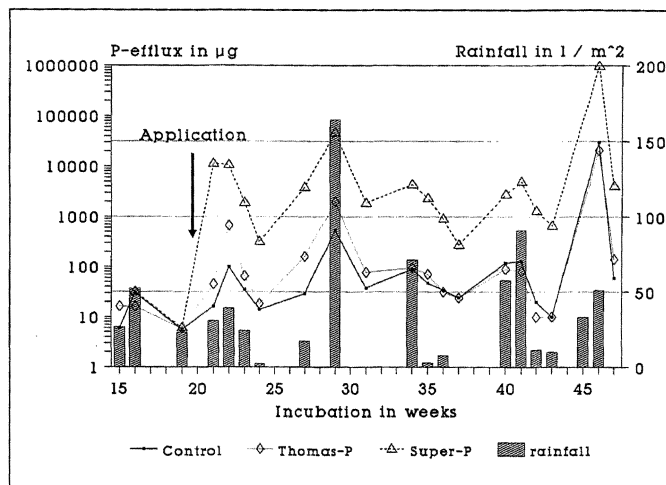
PSMs in amended soils

Contrary to uninoculated samples soluble phosphorus was highly significantly increased by means of *Pseudomonas* sp. (PI 18/89) and *P. aurantiogriseum* independent of pH (fig. 1).

Hydroponics

The experiment was repeated four times in all, because no uniform trend was detectable although experimental conditions were identical. Three of four experiments showed a highly significant (p <0.001) increase of P-contents of plants via inoculation with *P. aurantiogriseum*, but in a further repeat the opposite was detected. Cultures to which *Pseudomonas* sp. (PI 18/89) were applied had a higher, a lower and twice an equal P content in comparison to the control. Biomass itself was in most cases decreased by the addition of PSMs.

Lysimeter

No differences were detected when comparing the P-efflux from lysimeters amended with PSMs and control so that these values are omitted in figure 2. This lack of a significant difference might be caused by the suboptimal climatic conditions for growth of the microorganisms (average temperatures in Innsbruck decreased from 9.1 °C (October) to − 2.2 °C (January) and then rose again to 16.5 °C in May) on the one hand and by the high P-fixing capacity of forest soils on the other hand.

Contrary to arrangements with PSMs P-efflux was increased highly significantly (p <0.001; soil M and N) and significantly (p <0.05; only soil M) by the addition of Super- and Thomasphosphate respectively. This outcome in itself was not surprising but the scale was. When Superphosphate was added, about 90 and 50 mg P lysimeter⁻¹ were washed out from soil M and N respectively over a period of 32 weeks as compared to values between 0.6 and 1.2 mg P lysimeter⁻¹ in all other arrangements. These measurements indicate that the outflow is increased up to 150 fold values out from the top 30 cm when intensively fertilized with soluble phosphates.

Field studies

Superphosphate led to distinctly increased levels of Pp in soils M and N (p <0.001). Other treatments did not show significant differences compared to

the control or to one another throughout the field studies which to our mind was again caused by the above mentioned climatic reasons.

4. Discussion

Results from experiments using non-sterile soil plus PSMs indicate that PSMs are generally able to raise the Pp-fraction in soil, but that differences are not significant except when microorganisms dispose of sufficient nutrient supply. This difference in nutrient supply might be the reason for the contradicting results presented within this investigation and also reported by various authors (MISHUSTIN and NAUMOVA 1962). YADAV and SINGH (1991) found positive effects of PSMs on Pp-fraction of soils whereas THOMAS (1985) could not detect any difference. YADAV and SINGH (1991) report that mobilization of phosphorus with PSMs is much more effective in nutrient rich than in nutrient poor soils, which again is in agreement with our results.

A great number of authors dealt with the effects of PSMs on biomass production, P-uptake and P-concentrations of plants, but again these investigations led to very contrasting results (MISHUSTIN and NAUMOVA 1962). Among others ASEA et al. (1988) and ALAGAWADI and GAUER (1992) indicate a marked increase in P uptake and biomass production of plants via inoculation with PSMs, whereas AZCON-AGUILAR et al. (1986) were not able to find such effects of PSMs. The P content of plants was mostly increased (KUCEY 1988) but again other authors report the opposite (BERTHELIN et al. 1991). Some authors indicate – like we do – that these experiments are influenced by a great number of biotic and abiotic factors (total P- and Al-content of the soil, species, age and physiological properties of the plants and microorganisms, content of organic matter, pH...) which all together lead to hardly predictable results (BAREA et al. 1970, BERTHELIN et al. 1991). Thus PSMs seem to have the ability to increase the availability of phosphorus but there must be numerous other factors that influence the effect of these organisms on plant nutrition (BAREA et al. 1970). This uncertainty makes further investigations necessary including the ones concerning plant physiological questions.

An often observed methodological mistake is worth being mentioned. Many investigations which lead to a distinct increase in P content or biomass production are carried out by comparing plants in sterile soil with plants in soil inoculated with PSMs. Firstly the extrapolation of these results to outdoor experiments is impossible because of the non-sterile conditions. Secondly nearly all rhizospheric microorganisms – not only PSMs – increase the nutrient supply of plants, which is sufficiently known and needs no further investigation.

Although lysimeter experiments showed that there is a distinct P efflux from the top 30 cm, P outflow is seldom thought to be a problem causing eutrophication of groundwater or impoverishment of soils, because soluble phosphorus is assumed to be quickly fixed in deeper soil horizons (SCHEFFER and SCHACHTSCHABEL 1992 and STEVENSON 1986). Nevertheless appreciable P loss from soil must not be thought impossible and therefore must not longer be neglected in investigations concerning the washing out of nutrients.

The lack of an influence of PSMs showed within lysimeter- and outdoor experiments agree with BANIK and DEY (1981), THOMAS (1985), GIANINAZZI-PEARSON and GIANINAZZI (1989), LEE et al. (1990). Thus in future investigations efforts should be made to clarify the contradiction which has become obvious in several studies about PSMs:

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On the one hand PSMs were shown to be very efficient in mobilizing phosphorus out of inorganic phosphates (SPERBER 1958, LOUW and WEBLEY 1959, GOLDSTEIN and LIU 1987, PATGIRI and BEZBARUAH 1990, YADAV and SINGH 1991 and ILLMER and SCHINNER 1992).

On the other hand scientists have not been able to put this ability into practice yet (BANIK and DEY 1981, THOMAS 1985, AZCON-AGUILAR et al. 1986, GIANINAZZI-PEARSON and GIANINAZZI 1989, LEE et al. 1990).

However in spite of all difficulties microbial P-mobilization in general and the clarification of the above mentioned problem should still be kept in mind as no other alternative to industrial P-fertilization via mined phophorus is in sight.

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