Plant and Soil **154**: 189–196, 1993.

PLSO 9966

The effect of phytase on the availability of P from *myo*-inositol hexaphosphate (phytate) for maize roots

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Received 10 February 1993. Accepted in revised form 12 June 1993

Key words: bioavailability, maize, *myo*-inositol, phosphorus, phytase, phytin, soil

Abstract

The effect of adding phytase to the root medium of maize plants on the P-availability of added *myo*-inositol hexaphosphate (phytin) has been studied in pot experiments. When 40 m*M* phytin-P in nutrient solution was incubated in quartz-sand for 15 days in the absence of plants, 80% of it could be recovered from the solution as soluble organic P. Maize plants growing on this mixture assimilated P from phytin at rates comparable to those from inorganic phosphate (Pi). At a lower addition rate (2 m*M* phytin-P) only 10% was recovered in the soil solution, and plant growth was severely limited by P. At this low phytin level, the addition of phytase (10 enzyme units per kg sand) increased the plants' dry weight yield by 32%. The relative increases of the Pi concentration in the solution and of the amount of P in the plants were even higher, indicating that the observed growth stimulation was due to an increased rate of phytin hydrolysis. The enzyme-induced growth stimulation was also observed with plants growing in pots filled with soil low in P, when phytin was added. However, on three different soils the addition rates of phytin and phytase necessary for obtaining a significant phytase effect were both about 10 times higher than those required in quartzsand. It is concluded that the P-availability from organic sources can be limited by the rate of their hydrolytic cleavage.

Abbreviation: Pi – inorganic phosphate

Introduction

In agricultural soils organic phosphorus amounts usually to 30 to 80% of total P. Salts of *myo*-inositol hexaphosphate (phytic acid) and its derivatives may represent 50% of the organic P in soils (Dalal, 1978). The abundance of *myo*-inositol phosphates in the soil seems to be due to their low solubility, their firm association with the solid phase, and to their high stability (Anderson, 1980). In this paper, the term "phytin" will be used for any form of *myo*-inositol phosphate which may be present in the soil, including the Ca and/or Mg salts of phytic acid.

Despite the quantitative importance of organic P compounds (such as phytin) in the soil, our knowledge on the extent and mechanism of their use by plants is still limited (Dalal, 1978; Tarafdar and Claassen, 1988). Several types of phosphatases, such as phytases, are able to increase the rate of the dephosphorylation (hydrolysis) of organic P-compounds. These enzymes are normally present in soils where they originate from both microorganisms and plant roots (Speir and

Ross, 1978). Most (Flaig et al., 1960; Speir and Ross, 1978) but not all (Thompson and Black, 1970) authors believe that the dephosphorylation of P from organic molecules is an indispensable step during its utilization by plant roots.

The involvement of phosphatases in the utilization of organic P compounds by plant roots is likely therefore, but little is known about possible rate limitations. Increasing the phytase activity of soils did not result in an increased level of extractable inorganic phosphorus (Pi) (Jackman and Black, 1952b). Similarly, the addition of phosphatase to the soil did not decrease its organic P content (Thompson and Black, 1970). The rate of hydrolysis of soil phytin is strongly controlled by its solubility (Jackman and Black, 1951; 1952a, b) because unsoluble phytin compounds are more resistant to enzyme attack than soluble phytin. For example, the iron and aluminium salts of phytic acid (with a low solubility) were found to be highly resistant against hydrolysis by soil microorganisms (Greaves and Webley, 1969).

Upon the addition of phytin to the soil extractable Pi levels were increased (Jackman and Black, 1952b). Apparently, added phytin is initially not in equilibrium with the soil, and is more soluble and more readily hydrolysed than indigenous soil phytin. It might be expected therefore, that phytin is most easily degraded by phytase in the soil immediately after its addition.

In the present investigation the effect of added phytase has been studied on the availability of phytin-P for maize plants. Pot experiments were conducted with either quartzsand or soil with different additions of P and phytase. When quartzsand was used, the equilibrium concentrations of inorganic and total P in the soil solution could be measured in the soil solution after its separation from the sand. With soil the effect of phytase on the availability of both indigenous soil phytin and added phytin was studied.

Material and methods

In experiment 1 the equilibrium concentrations of Pi and total P were measured after incubating pots with quartz-sand mixed with nutrient solution for 15 days in the absence of plants. P additions were chosen to result in final P-concentrations in the soil solution of 0, 10, 20 and 40 m*M* for phytin, and 2 m*M* for KH_2PO_4. The five P-treatments were combined with three different ammonium levels, viz. 0, 10 and 25% of the total N concentration in the nutrient solution (which was constant at 15 m*M*). There were no replications.

P-uptake has been measured in parallel pots with maize plants grown for 15 days. The same combinations of P-additions and ammonium concentrations were used, but each treatment was in duplo.

In experiment 2 the effect of phytase has been studied on the P-availability of phytin-P in sand culture. Plants were grown for 20 days on quartzsand supplied with no phosphate, 2 m*M* phytin-P, or 2 m*M* NaH_2PO_4. Where no P or 2 m*M* NaH_2PO_4 was added no phytase was supplied. At 2 m*M* phytin, however, 0, 0.01 or 1 μL phytase solution was added per pot. Each treatment was replicated four times. In experiment 3 the effect of phytase on P availability has been studied with three different soils, low in P (Table 1). Plants were grown for 19 days on three different soils at P-applications of 0, 320, 1000 and 3200 μmol P per pot, added as either NaH_2PO_4 or phytin. Where phytin was added, phytase was applied at a rate of 1 or 10 μL per pot. Each treatment was replicated four times.

The following methods were applied in all experiments:

Quartz-sand and soil preparation. Pots of about 1.3 l were filled with 1 kg acid-washed quartzsand, mixed with nutrient solution of the following final composition (in m*M*): $Ca(NO_3)_2$ 5.0, KNO_3 5.0, $MgSO_4$ 2.0, and the trace elements (in mg L^{-1}) Fe 4.6 (as EDTA complex), B 0.5, Mn 0.5, Zn 0.05, Cu 0.02, and Mo 0.001. In

Table 1. Main characteristics of the soils used

Name	Characteristic	pH(H_2O)	PAL[a]	Water[b]
Heide	limed sandy heath soil	5.5	5	200
Loess	P-poor loess subsoil	6.0	3	240
Nhasamba	sandy tropical soil	5.6	6	216

[a] Ammonium lactate extractable P (mg/100g soil).
[b] Water needed to reach 60% of the waterholding capacity (mL kg^{-1} dry soil).

experiment 2 the pH of the nutrient solution was stabilized by the addition of 2 m*M* 2-(N-morpholino)ethanesulfonic acid (MES-NaOH) buffer, pH 5.5. The final water content of the sand was 160 mL per kg dry sand.

For the soil experiments the pots were filled with 1.0 kg (Loess and Nhasamba) or 1.1 kg soil (Heide). Each pot was fertilized with 1.1 g calcium ammonium nitrate (27% N).

Addition of Pi, phytin and phytase. Stock solutions (between 20 and 64 m*M* in the different experiments) of inorganic phosphate (NaH_2PO_4) and Ca-phytate (Sigma; containing 1.5 mol Ca per mole phytic acid) in water were prepared. The phytin stock solution was brought to pH 5.5 with NaOH. In order to reach the final P-concentrations, equivalents of these solutions were added to the pots during filling.

Phytase was obtained from Gist-brocades (Delft, The Netherlands) in a stabilized solution. One mL of this solution had an activity of 10 000 units, one unit being defined as the amount of enzyme hydrolysing 1 μmol phytate per minute from a 1 m*M* Na-phytate solution at 37°C, pH 5.5. During filling of the pots between 0.01 and 10 000 μL of this solution was added to the soil in a total volume of 10 or 15 mL water and mixed thoroughly. When 10 μL enzyme or less was added per pot, the addition was repeated weekly, by adding the same amount of enzyme to the water supplied to the plants (see below).

Plant growth conditions. After the addition of nutrients, phytin and phytase, the total water content of the sand or soil was brought to 60% of waterholding capacity. Two maize plants (*Zea mays* L., var. LG11) per pot were sown, other pots remained free of plants. All pots were transferred into a climate chamber at 25°C (dew-point 22.5°C), with 16 hours light (136 Watt/m^2) per day.

The water content of the sand and soils was maintained at its original value by daily additions of demineralized water.

Harvest and analyses. At the time of harvest, plant shoots were cut at soil level. In the sand experiments (experiments 1 and 2) the roots were collected and rinsed with water. For the determination of P-concentrations, soil solution was sucked out of the sand under vacuum and filtered if turbid. Pi was determined by the molybdenum blue assay, total soluble P by inductively coupled plasma (ICP-) atomic emission spectrometry. Soluble organic P was calculated as the difference.

Plant material was digested in a mixture of H_2SO_4, Se and salicylic acid with the addition of H_2O_2, and analyzed for P by the molybdenum-blue assay.

Results

Effect of phytin dosage on P-availability in sand culture (Experiment 1).

Figure 1 shows the effect of different additions of phytin (up to 40 m*M*) and Pi (2 m*M*) on the concentration of total P and Pi in the sand solution of pots without plants. When inorganic P was added, most of it could be recovered after 15 days as soluble Pi (Fig. 1A). The missing P was probably precipitated or adsorbed to the quartzsand. Most of the phytin added was recovered as soluble organic P (Fig. 1B), i.e. only a small fraction of it was dephosphorylated spontaneously (Fig. 1A). The missing part of the added phytin-P (3-5 m*M* at the different phytin additions) was probably precipitated as an insoluble salt.

The partial replacement of nitrate by ammonium in the nutrient solution did not significantly affect the Pi concentrations in the pots without plants. Therefore for Figure 1 the values of the different ammonium levels were averaged within the P-treatments. In pots with plants, however, ammonium affected the pH and the Pi concentrations in the nutrient solution, as well as the P-uptake of the plants. Therefore for Figure 2 the data of the individual pots were used. When the uptake of P by the plants is plotted against the final concentration of Pi in the solution, then – at the same external concentration of Pi – P uptake of plants supplied with phytin exceeds that of plants supplied with Pi (Fig. 2A). However, when the uptake is plotted against the concentration of total soluble P, it appears that

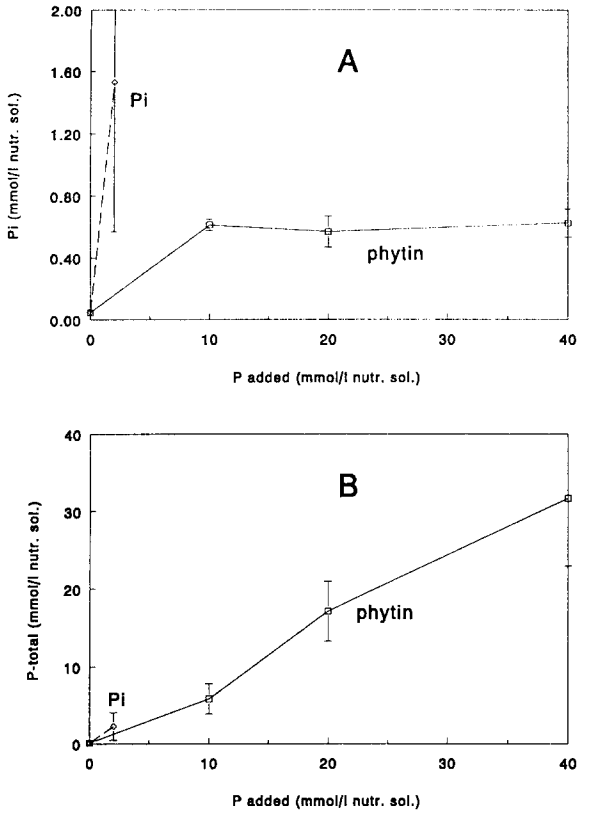

Fig. 1. Concentrations of inorganic (**A**) and total (**B**) phosphorus in the nutrient solution extracted from the sand after 15 days (experiment 1). Data from pots without plants, supplied with NaH_2PO_4 ("Pi") or phytin. Means ± standard deviations of three pots, with 0, 10, and 25% of the nitrogen in the nutrient solution supplied as ammonium.

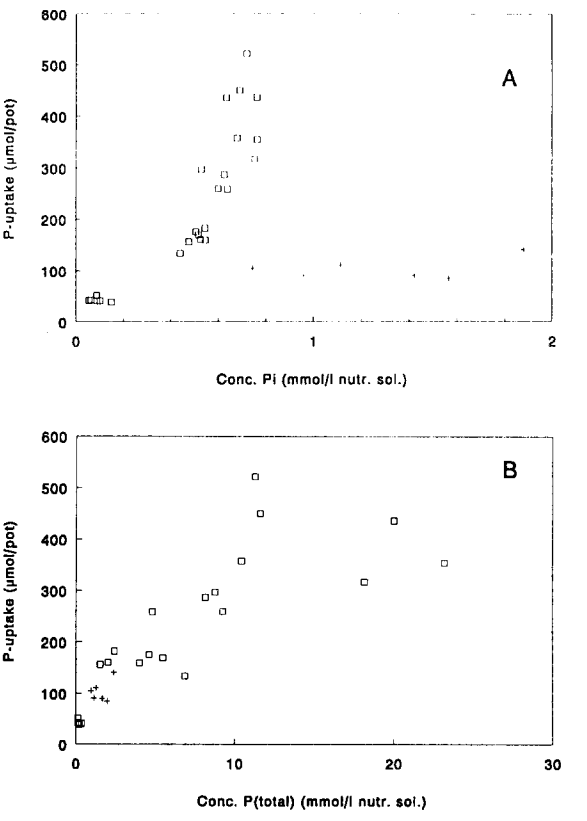

Fig. 2. P uptake of maize plants grown for 15 days on sand with nutrient solution supplied with phytin (□) or Pi (+) as a function of the concentration of Pi (**A**) or total P (**B**) in the nutrient solution at the end of the experiment. Each point represents the uptake of 2 plants grown on the same pot. Nutrient solutions contained 0, 10 or 25% of total N in the form of ammonium, the rest as nitrate. P addition rates were the same as indicated in Figure 1.

inorganic and organic P are taken up with a similar efficiency (Fig. 2B).

The concentrations of P in the plants were high in this experiment (up to 400 mmol/kg dry matter) and there was no significant relationship between the P-concentrations in the plants and their respective dry matter yields, i.e. the growth of the plants was not limited by P.

Effect of phytase on P-availability in sand culture (Experiment 2).

In order to detect a possible rate limitation in P-uptake, in the following experiment phytin was supplied at a rate of 2 m*M* P only, corresponding with 320 µmol P per pot. The resulting concentrations of soluble P in the soil solution were about 0.25 m*M* P(total) and 0.05 m*M* Pi, as measured in pots without plants and without addition of phytase. These values were obtained after 6 days and did not change significantly until the end of the experiment. P-uptake (Fig. 3B) and P-concentrations of the plants were low as well, and growth was limited by P.

When 2 m*M* Pi was applied instead of phytin, the Pi concentrations in the nutrient solution were considerably higher (Fig. 3A), plants took up more P (Fig. 3B) and showed higher internal P concentrations (not shown) and better growth (Fig. 3C).

When phytin was the P-source the addition of 1 µL phytase per pot resulted in a nearly 10-fold

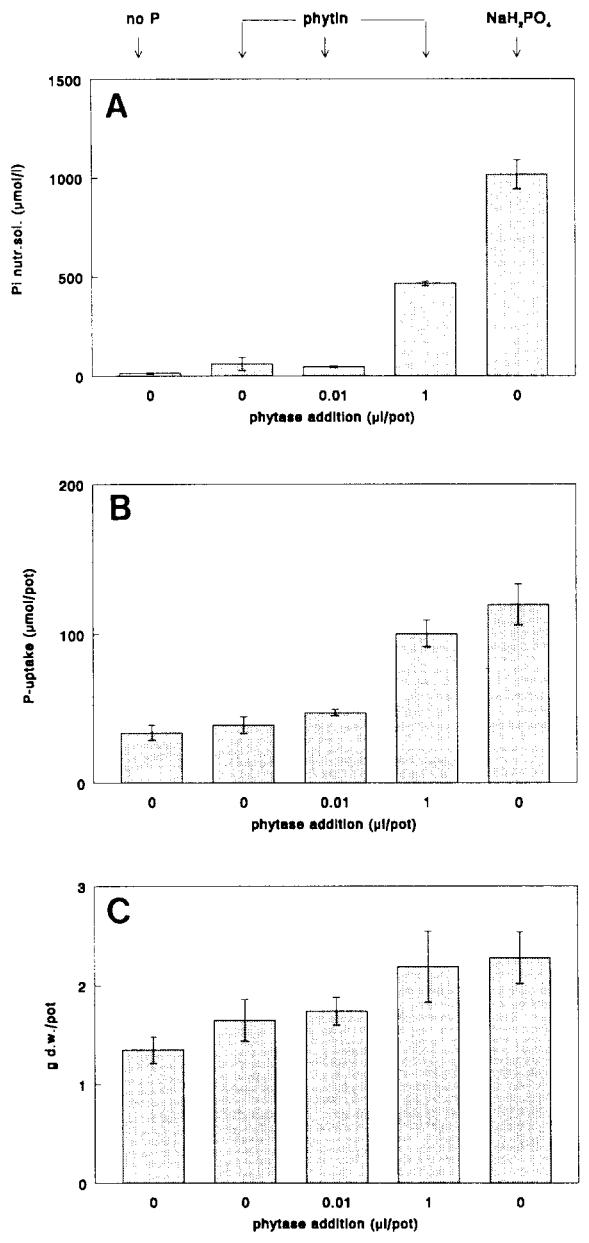

Fig. 3. Concentration of Pi in the nutrient solution at the time of harvest (**A**), and the uptake of P (**B**) and dry weight of maize plants (**C**) grown for 20 days on sand mixed with nutrient solution (experiment 2). The concentration of added P was 2 m*M* in the soil solution. Means ± standard deviations (n = 4).

increase of the concentration of Pi in the nutrient solution (Fig. 3A), a 2.5-fold increase in P-uptake (Fig. 3B), and a dry weight increase of 32% (Fig. 3C). Thus, P-uptake and growth of the maize plants was apparently limited by the rate of phytin hydrolysis. When only 0.01 µL enzyme per pot was added, the enzyme effect was visible for P-uptake only.

Effect of phytase on P-availability in three different soils (Experiment 3).

In order to investigate whether the results obtained in sand culture persist in soil, plants were grown on three different soils with different additions of phytin or NaH_2PO_4, and phytase.

Figure 4 shows the dry matter yields. From the response to the addition of NaH_2PO_4 and the low P-concentrations in plants supplied with phytin (30–50 µmol/g dry weight) it can be concluded that the growth rate of the plants was again limited by P.

In the absence of phytase the addition of phytin did not change the dry weight significantly. Neither did the addition of phytase result in an increased growth when no phytin has been added. However, when phytin was supplied at its highest rate, the addition of 1 µL phytase resulted in a significant dry weight increase on one soil (Heide), and 10 µL enzyme resulted in a positive dry weight response on all soils. In accordance, analysis of variance showed that for the total experiment there was a significant interaction between the addition of phytin and the addition of phytase in their effect on plant dry weight, with $p < 0.001$.

The amounts of phytase needed to get an enzyme effect on dry weight yield were clearly higher than in sand culture. When 320 µmol phytin was added (i.e. at the same addition rate as in experiment 2) no effect of the addition of phytase could be detected, even not at high phytase levels. The minimum addition rate of phytin for obtaining a significant phytase effect was 3200 µmol P per pot, compared to 320 µmol P in experiment 2 with quartzsand. In addition, 10 µL enzyme were needed in order to obtain an effect, compared with about 1 µL in experiment 2.

The plant availability of phytin-P in the presence of the highest phytase level (10 µL per pot) is compared with that of NaH_2PO_4 in Figure 5. The uptake of phytin-P is of the order of 10% of

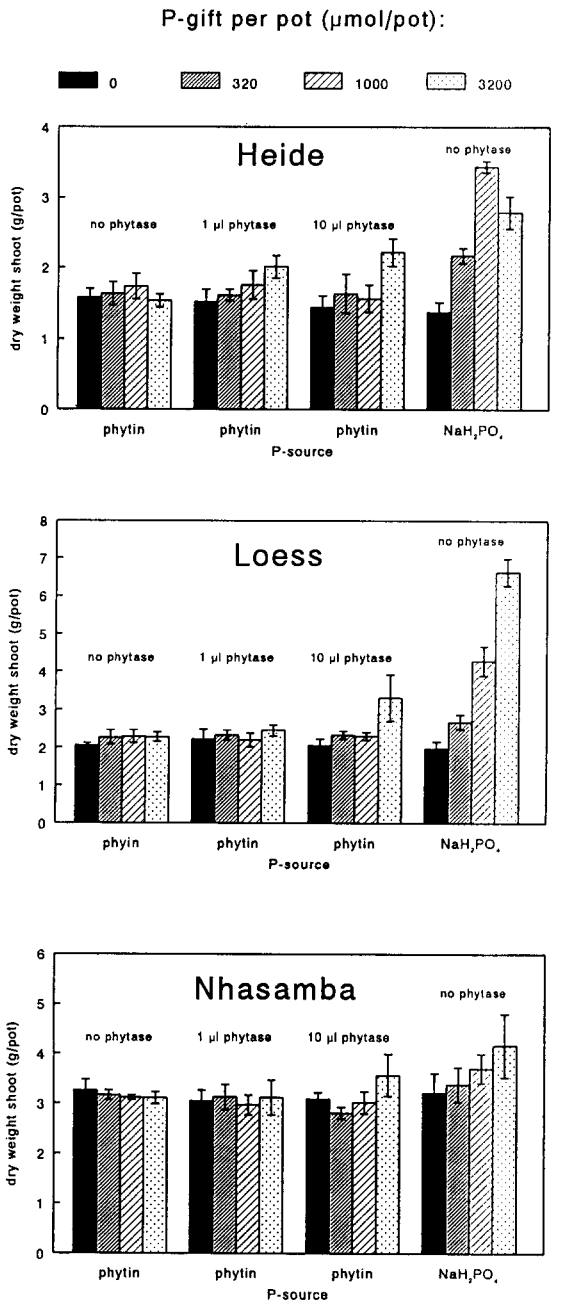

Fig. 4. Dry weight of the shoots of maize grown on three different soils for 19 days, as affected by P-source, phytin addition rate and by the addition of phytase (experiment 3). Means ± standard deviations (n = 4).

that of added Pi. The slope of the lines in Figure 5 indicates that about 15% of applied NaH_2PO_4, but only 1-2% of the applied phytin P, was recovered in the plant shoots.

Discussion

In experiment 1, plants were grown in the absence of added phytase and the solubility of phytin largely determined its bioavailability. This is in line with the view generally held in the literature (e.g. Adams and Pate, 1992). When high rates of phytin are supplied, high concentrations of soluble phytin appear in the soil solution and plants can use it easily as a P-source.

In this experiment no constant equilibrium concentration was established at the different phytin doses. This can be explained by the fact that – on a molar basis – the total amount of P supplied as phytin at all levels of application greatly exceeded that of Ca + Mg. Precipitation of phytin will have reduced the concentration of Ca and Mg in the nutrient solution down to low values, the excess of phytin remaining soluble in the nutrient solution.

Under the conditions of experiment 1 (characterized by an excess of phytin over Ca and Mg and no Al or excess Fe present) the amount of soluble phytin in the soil solution exceeded that of inorganic P considerably. Plant uptake and internal plant concentrations were high and the rate of plant growth was not limited by P. The high availability of phytin-P for plants in nutrient solutions (Rogers et al., 1940), even under aseptic conditions (Flaig et al., 1960) contrasts with its low availability in soil (Martin and Cartwright, 1971; Wild and Oke, 1966) which might be due to the different availabilities of precipitating cations in nutrient solutions and soil solutions.

In experiment 2, a considerably lower addition of phytin-P was employed and the rate of hydrolysis limited the rate of P-uptake by the plants, in line with the conclusion of Flaig et al. (1960).

Tarafdar and Claassen (1988) found that the amount of Pi hydrolysed from phytin by the acidic plant phosphatase associated with the roots of clover, grown under sterile conditions, exceeded the amount taken up by the plants by a factor of 20. However, the conditions of their experiment resembled those of the present experiment 1, where there was no limitation imposed by the availability of phytin-P, due to its

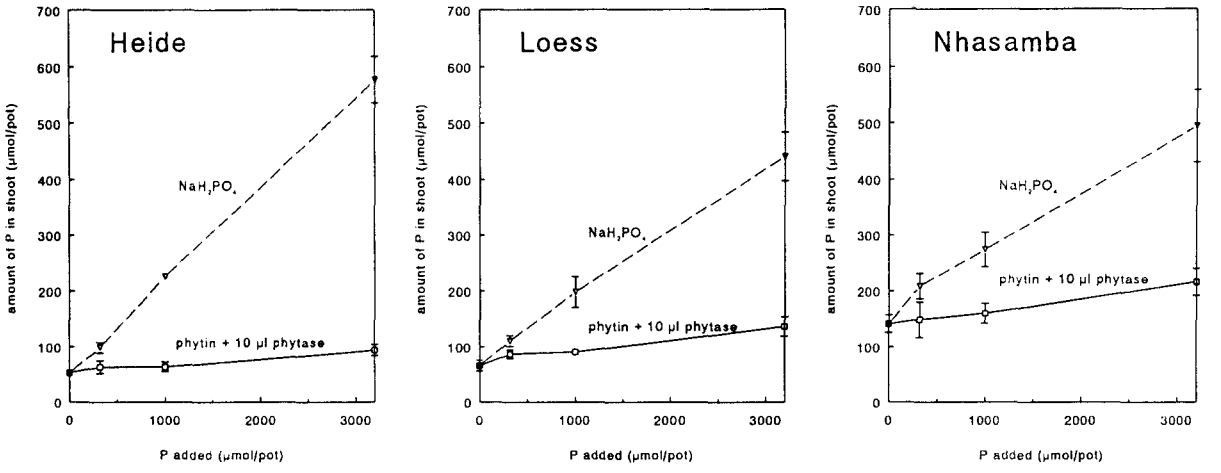

Fig. 5. Amount of P in maize shoots grown on three different soils for 19 days, as affected by the P-source and the addition of phytase (experiment 3). Means ± standard deviations (n = 4).

abundance in the solution. Evidently, the rate of hydrolysis is not only dependent on the activity of the enzyme but also on the availability of its substrate.

The present observation that the plants take up more P when the level of Pi is increased by the enzyme (Fig. 3) does not rule out the possibility of an uptake of unhydrolyzed organic P. On basis of the results from experiment 3 (see below) it might be inferred, however, that such a mechanism, if it exists, would be immaterial in the case of phytin assimilation from soil.

In a root substrate containing a solid phase, the root phosphatases may not easily reach the organic P located outside the rhizosphere (Helal and Sauerbeck, 1984). In accordance, an exhaustion of organic P has been measured in the rhizosphere (Tarafdar and Jungk, 1987). Because the phytase activity in the rhizosphere is high (Tarafdar and Claassen, 1988) and the addition of phytase to the soil stimulates the uptake of phytin-P nevertheless (Figs. 3 and 4), it may be supposed that in the present experiments 2 and 3 phytin located outside the rhizosphere has contributed significantly to the P-supply of plants. In accordance, the mean concentration of total soluble P in pots without plants on day 6 has been determined to be 1.6 m*M* for the NaH_2PO_4 treatment, 0.25 m*M* for the phytin treatment without phytase, and 1.5 m*M* for the phytin treatment with 1 µL phytase. Apparently, the hydrolysis of phytate caused by the addition of phytase has resulted in a drastic increase in the solubility and mobility of P in the quartzsand.

In soil, the plants' assimilation of phytin-P was also stimulated by phytase, although this was less pronounced than in quart-sand. This was presumably due to the different equilibrium concentrations of Pi and phytin established in soils as compared with quartzsand, caused by the absence of Al and excess Fe in the nutrient solution. In addition, enzymes in the soil may be inactivated by means of sorption or by the presence of inhibitory agents. Finally, the indigenous phytase-activity of the soils may have contributed to the difference in response between soil and quartz-sand. It should be noticed that the highest rates of phytase addition used in experiments 2 and 3 were probably both suboptimal, because no saturation of the response curves is indicated.

Added phytase did not stimulate P-uptake and growth when no phytin was added to the soil. This is in line with the results of Jackman and Black (1952b). The highest enzyme activity added to the soils in our experiments (100 unit/kg soil) was of the same order of magnitude as the activity normally found in soils (Speir and Ross, 1978).

In order to see whether indigenous soil-P would be hydrolysed by a much higher level of phytase, 10 ml enzyme was applied per kg soil in another experiment. This addition corresponded

to an increase of the endogenous enzyme activity of the soil by three orders of magnitude. In three of the soils tested, there was no response in P-uptake, but in one soil (Nhasamba) the enzyme addition increased P-uptake of the maize plants very significantly (from 90.8 ± 17.3 to 196.5 ± 15.8 μmol per pot at $n = 4$). Unfortunately, this result did not conclusively demonstrate an increase in the rate of soil-P hydrolysis, because together with the enzyme preparation, 153 μmol P was added to the pots. Nevertheless, a recovery of 69% ((196.5-90.8)/153) for added P is incredibly high, considering the P-recoveries of 10 and 1.5% for Pi and phytin, respectively, in the same soil (Figure 5). Therefore, the dramatic increase in the enzyme level in this last experiment may have resulted in an increase in the hydrolysis rate of soil-phytin in this particular soil. However, because of the extremely high enzyme activity needed in order to obtain this effect, it is probably of little practical importance.

Acknowledgements

The gift of phytase by Gist-brocades is gratefully acknowledged.

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Section editor: A C Borstlap