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Mobilization of iron by phytosiderophores as affected by other micronutrients

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Abstract

It has been shown previously (Treeby *et al.*, 1989) that phytosiderophores, released by roots of iron deficient grasses (Gramineae), mobilize from calcareous soils not only iron (Fe) but also zinc (Zn), manganese (Mn) and copper (Cu). Mobilization of Fe may therefore be impaired by other micronutrient cations. This has been studied in both, model experiments with Fe hydroxide and with a calcareous soil (15% $CaCO_3$, pH 8.6) amended with micronutrients as sulfate salts.

Mobilization of Fe from Fe hydroxide by phytosiderophores (epi-3-hydroxymugineic acid) was not affected by the addition of $CaCl_2$, $MgSO_4$ and $MnSO_4$, slightly inhibited by $ZnSO_4$ and strongly inhibited by $CuSO_4$. In a calcareous soil amended with increasing levels of $ZnSO_4$, $MnSO_4$ and $CuSO_4$, mobilization of Fe by phytosiderophores remained uneffected by Zn and Mn amendments but was progressively impaired by increasing levels of Cu amendment, correlated with corresponding enhancement of Cu mobilization.

High concentrations of $ZnSO_4$ and $MnSO_4$ and relatively high concentrations of $CuSO_4$ were required for inhibition of Fe mobilization by phytosiderophores. It is therefore concluded that in most calcareous soils phytosiderophores efficiently mobilize Fe, and that phytosiderophores play an important role in Fe acquisition by grasses grown on calcareous soils.

Introduction

Phytosiderophores released by roots of grasses (Gramineae) in response to Fe (Marschner *et al.*, 1986; Takagi 1976) and Zn deficiency (Zhang *et al.*, 1989) have a high affinity to Fe^{III} (Nomoto *et al.*, 1987). The mobilization of Fe from sparingly soluble Fe^{III} hydroxide by phytosiderophores is due to chelation without involvement of reduction of Fe^{III} (Kissel, 1987).

Based on the published data on stability constants of phytosiderophores (Nomoto *et al.*, 1987; Sugiura *et al.*, 1981) and the activities of cations in the soil solution of calcareous soils, Crowley *et al.* (1987) postulated that phytosiderophores may not mobilize Fe in soils, but rather Zn and Cu. This assumption was in part supported experimentally showing that phytosiderophores mobilize not only Fe but also Zn, Cu and Mn in calcareous soils (Marschner *et al.*, 1989; Takagi *et al.*, 1988; Treeby *et al.*, 1989).

The present investigation was therefore undertaken to study in more detail the effect of various divalent cations (Cu^{2+}, Zn^{2+}, Mn^{2+}, Ca^{2+} and Mg^{2+}) on the Fe mobilization by phytosiderophores from both, freshly precipitated Fe^{III} hydroxide (according Takagi, 1976) and a calcareous soil (according Awad *et al.*, 1988).

Materials and methods

Plant culture for phytosiderophore production

Seeds of barley (*Hordeum vulgare* L. cv Europa) were germinated in the dark for 3 days in quartz sand moistened with saturated $CaSO_4$ solution. Thereafter the seedlings were transferred to a continuously aerated, Fe free nutrient solution of the following composition: 2.00 m*M* $Ca(NO_3)_2$, 0.75 m*M* K_2SO_4; 0.50 m*M* $MgSO_4$; 0.5 m*M* KH_2PO_4; 0.10 m*M* KCl; 1 μM H_3BO_3; 0.5 μM $MnSO_4$; 0.5 μM $ZnSO_4$; 0.2 μM $CuSO_4$; 0.01 μM $(NH_4)_6Mo_7O_{24}$. The plants were grown under controlled environmental conditions (day/night 16/8 h; light intensity 220 μE m^{-2} s^{-1} (fluorescent tubes Silvania, cool white FR 96T 12); temperature 25°C/23°C; relative humidity 70–80%. The nutrient solutions were changed every second day.

Collection of root exudates (phytosiderophores)

Root exudates were collected at onset of visual Fe deficiency symptoms (mild chlorosis). Two hours after the beginning of the light period the roots were rinsed briefly with distilled water and placed in aerated distilled water for 4 h for collection of the root exudates. The plants were then returned to the nutrient solution. This procedure of exudate collection was repeated daily for 6–8 successive days. Immediately after collection the root exudates were concentrated to dryness under vacuum at 40°C and stored at −18°C.

Removal of heavy metals from root exudates

Heavy metal contaminations in the root exudates were removed by passing the root exudates through the selective cation exchange resins Chelite N and Chelite P (Serva Feinbiochem., Heidelberg, F.R.G.). For this the Chelite N was percolated with 1 *N* HCl and rinsed with deionized water, and the Chelite P percolated with 2 *N* HNO_3, followed by 2 *N* NaCl and then rinsed with deionized water until neutrality. The two columns were connected in series and rinsed with acetic acid/acetate buffer (0.2 *M*) of pH 4.5. The concentrated root exudates were resolved in the acetate buffer and passed through the columns overnight. The cleaned root exudates were dried under vacuum at 40°C, dissolved in a small volume of deionized water, and passed through a cation exchange resin (Amberlite IR 120, H^+ form). The fraction with phytosiderophores eluted with 1 *N* NH_4OH was collected, dried under vacuum and stored. The heavy metal content in the cleaned phytosiderophores was lower than 0.5% on molar basis. The presence of epi-3-hydroxymugineic acid as main phytosiderophore in the purified exudates of barley cv. Europa was confirmed by comparison with standards by a HPLC method (Mori *et al.*, 1987).

Determination of the phytosiderophore concentration

The phytosiderophore concentration in the cleaned exudates was determined as described by Takagi (1976) by measuring the amount of Fe (III) solubilized from Fe^{III} hydroxide. Briefly, an aliquot of the root exudate was shaken with freshly precipitated Fe hydroxide at pH 5.6 (Na acetate buffer) for 2 h at 25°C, filtered and the solubilized Fe^{III} in the filtrate determined photometrically as Fe^{II} $(Ferrozine)_2$ complex at 562 nm following the addition of a reductant (hydroxylamine hydrochloride) and Ferrozine [3-(2-pyridyl)-5,6-bis (4-phenylsulfonic acid)-1,2,4-triazine].

The effect of divalent cations on the Fe mobilization was determined by adding increasing amounts of $CuSO_4$ (0.05–0.5 μmol), $ZnSO_4$ (0.5–20 μmol), $MnSO_4$ (0.5–20 μmol), $MgSO_4$ (50–2500 μmol) and $CaCl_2$ (50–2500 μmol) to Fe^{III} hydroxide suspensions (10 μmol Fe) together with 0.5 μmol phytosiderophores. After shaking (2 h) and filtration the mobilized Fe was determined photometrically as the Fe^{II} Ferrozine complex as described above.

Characteristics and treatments of the calcareous soil

The calcareous soil used in the experiments was an Orthic Calcorthid, pH 8.6 with a $CaCO_3$ content of 14.8% (Awad *et al.*, 1988). The soil was amended by adding dissolved salts of

Table 1. DTPA extractable micronutrients of the calcareous soil amended with $ZnSO_4$ and $CuSO_4$

Amendment (mg kg^{-1} air dry soil)	DTPA extractable micronutrients (mg kg^{-1} air dry soil)			
	Fe	Zn	Cu	Mn
no (control)	6.6	1.7	1.0	12.5
13 mg Zn	4.3	5.9	1.6	6.3
5 mg Cu	5.2	7.9	4.9	13.1
20 mg Cu	5.5	7.2	22.1	13.7

$ZnSO_4$, $MnSO_4$ and $CuSO_4$. The soils were dried at room temperature and than rewetted with distilled water several days later. The control treatment was also rewetted with distilled water. The soils were kept at field capacity for a period of 6 weeks, and after airdrying sieved (0.2 mm). The DTPA extractable micronutrients were determined according to Lindsay and Norvell (1978) and are presented in Table 1.

Mobilization of Fe from soils

The experimental systems used to assay heavy metal mobilization from soils has been described by Awad *et al.* (1988). Briefly, 0.2 g of air dry soil was added to 5 mL of 10^{-4} *M* $CaSO_4$ in a 16 mm diameter dialysis tube which was placed in 95 mL of 10^{-4} *M* $CaSO_4$ containing 0.5 g air dried Chelite N (H^+) resin beads (0.3–0.8 mm particle size) for adsorption of the mobilized metals. Metal free phytosiderophores (epi-3-hydroxymugineic acid) were added to the external solution to a final concentration of 10^{-5} *M*, and both the external solution and the solution in the dialysis tubes were continuously mixed by aeration. After 24 h of mobilization the dialysis tubes were removed, the external solution decanted and the resin beads rinsed several times with deionized water which was added to the decanted solution. The external solution was then dried under vacuum, dissolved in 10 mL of 3% HNO_3. The resin beads were dried at 90°C, ashed at 550°C for 12 h, dissolved with 1 mL of 33% HNO_3, dried at 30°C and then dissolved in 10 mL of 3% HNO_3. The heavy metal contents were determined on a Beckman Directly Coupled Plasma Spectrophotometer. In principal the amounts of micronutrient in solution and on the resin beads were similar as shown in a comprehensive study by Treeby *et al.* (1989). Therefore in this paper only the data of the solutions are presented. For all treatments there were 5 replicates, and mean values were separated by Duncans Multiple Range Test.

Results

Mobilization of Fe from Fe^{III} hydroxide

Phytosiderophores are highly efficient in mobilizing Fe from sparingly soluble inorganic Fe^{III} hydroxide in absence of other divalent cations (Fig. 1). Increasing amounts of $CuSO_4$ had a strong inhibitory effect on the Fe mobilization by phytosiderophores. Already 0.05 μmol $CuSO_4$ decreased the Fe mobilization to 88% and with 0.5 μM $CuSO_4$ (Cu: phytosiderophore ratio 1:1) the Fe mobilization dropped to 25% of the control (no $CuSO_4$ addition).

Compared to $CuSO_4$ increase in $ZnSO_4$ concentrations inhibited much less the Fe mobilization by phytosiderophores (Fig. 2). The addition of 0.5 μmol $ZnSO_4$ (Zn: phytosiderophore ratio 1:1) decreased the Fe mobilization by phytosiderophores to 86%. About forty times more $ZnSO_4$ (20 μmol) was required to obtain a similar inhibitory effect on the Fe mobilization as by the addition of 0.5 μmol $CuSO_4$.

In contrast to $CuSO_4$ and $ZnSO_4$ the Fe

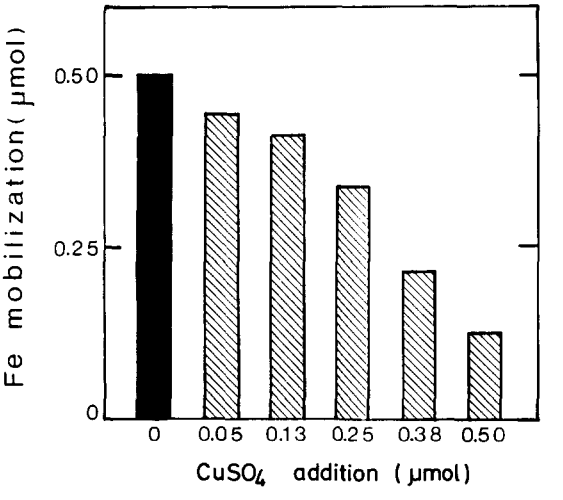

Fig. 1. Effect of increasing concentrations of $CuSO_4$ on the Fe mobilization by phytosiderophores (0.5 μmol) from freshly precipitated Fe^{III} hydroxide.

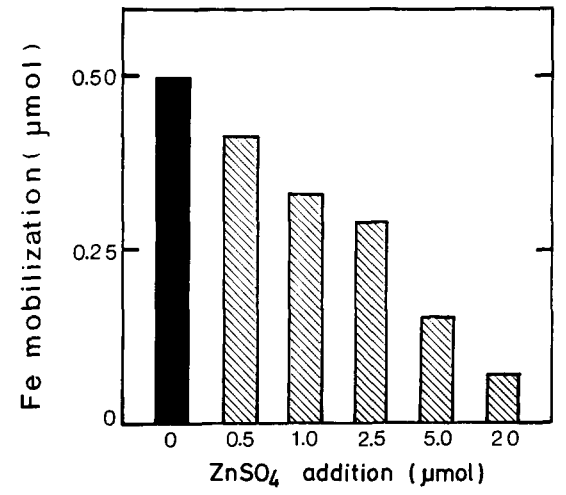

Fig. 2. Effect of increasing concentrations of $ZnSO_4$ on the Fe mobilization by phytosiderophores (0.5 μmol) from freshly precipitated Fe^{III} hydroxide.

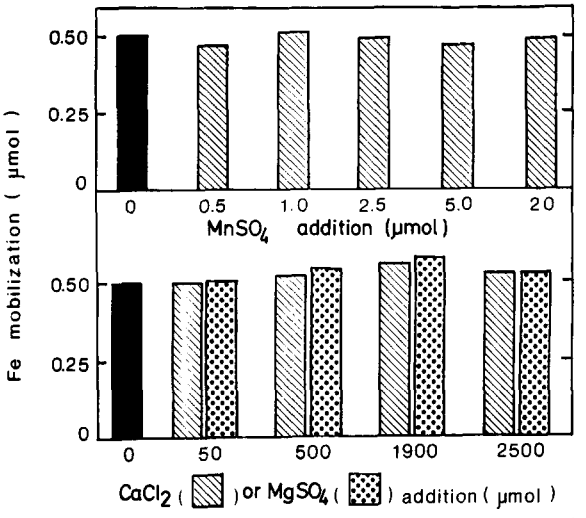

Fig. 3. Effect of increasing concentrations of $MnSO_4$ (upper) and of $CaCl_2$ and $MgSO_4$ (lower) on the Fe mobilization by phytosiderophores (0.5 μmol) from freshly precipitated Fe^{III} hydroxide.

mobilization by phytosiderophores from freshly precipitated Fe^{III} hydroxide was not inhibited by $MnSO_4$, $MgSO_4$ or $CaCl_2$ (Fig. 3), inspite of 40 fold excess of Mn or 5000 fold excess of Ca and Mg.

Mobilization of Fe from a calcareous soil

Phytosiderophores enhanced solubility of Fe by a factor between 2 and 3 in the non-amended as

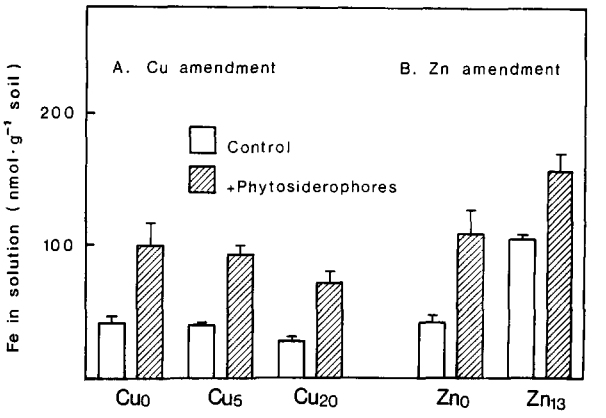

Fig. 4. Effect of amendments of a calcareous soil with $CuSO_4$ ($Cu_0 = 0$; $Cu_5 = 5$ mg, $Cu_{20} = 20$ mg Cu kg^{-1} soil) and Zn ($Zn_0 = 0$; $Zn_{13} = 13$ mg Zn kg^{-1} soil) on the mobilization of Fe by 10^{-4} *M* $CaSO_4$ (control) and by 10^{-5} *M* phytosiderophores. Vertical bars represent the SE (n = 5).

well as in the Cu and Zn amended calcareous soil (Fig. 4). Copper amendment slightly decreased (5–30%) the Fe mobilization in both the absence and presence of phytosiderophores. In contrast and against expectation $ZnSO_4$ amendment (13 mg Zn kg^{-1} soil, corresponding to about 40 kg Zn/ha) enhanced the Fe mobilization both without and with phytosiderophores by a factor of about 2 (Fig. 4). Amendments with Mn were without effect on Fe mobilization by phytosiderophores (data not shown).

Discussion

The extent to which the various heavy metal (micronutrient) cations affect the Fe mobilization by plant-borne chelators reflect their affinity to phytosiderophores. In our experiments the phytosiderophore epi-3-hydroxymugineic acid was used. A structurally related phytosiderophore, mugineic acid, has been shown to have affinities for heavy metal cations decreasing in the order $Cu^{2+} > Fe^{3+} \gg Zn^{2+} \gg Mn^{2+}$ (Nomoto *et al.*, 1987). In principle, these different affinities are also reflected in the extent to which Fe mobilization by the phytosiderophore epi-3-hydroxymugineic acid from freshly precipitated Fe^{III} hydroxide was affected by $CuSO_4$, $ZnSO_4$ and $MnSO_4$ (Figs. 1 and 2). However, the inhibi-

tory effect of $CuSO_4$ (Figs. 1, 4) on Fe mobilization by phytosiderophores was less than expected from the stability constants (Crowley *et al.*, 1987; Nomoto *et al.*, 1987).

Mobilization of Fe by phytosiderophores from Fe^{III} hydroxide was only slightly depressed by $ZnSO_4$. In the calcareous soil phytosiderophores increased Fe solubility by a similar amount (50–60 nmol Fe g^{-1} soil) in the soil without as with Zn amendment (Fig. 4). The unexpected increase in Fe solubility after Zn amendment may be the result of replacement effects of exchangeable Fe from organic matter or cation exchange sites (Shuman, 1988).

In agreement with the stability constants calculated by Nomoto *et al.* (1987) no inhibitory effect of the addition of Mn on Fe mobilization by phytosiderophores neither from freshly precipitated Fe^{III} hydroxide (Fig. 3) nor from soil was found. The increased mobilization of Mn by phytosiderophores in a calcareous soil, as has been found by Treeby *et al.* (1989) and Marschner *et al.* (1989), was probably not due to the chelation of Mn by phytosiderophores, but the result of reduction of Mn^{IV} to Mn^{II} with corresponding oxidation of the phytosiderophores. Mobilization of Mn, as result of reduction of Mn^{IV} to Mn^{II} by root exudates, for example, by amino acids, organic acids or sugars is a well established phenomenon (Godo and Reisenauer, 1980; Marschner, 1988; Uren and Reisenauer, 1988). In view of the fact that Ca^{2+}, Mg^{2+} and Mn^{2+} had no inhibitory effect, and Cu^{2+} and Zn^{2+} only relatively small ones on the Fe mobilization by phytosiderophores it is concluded that phytosiderophores play an important role in the acquisition of Fe from soils by graminaceous plants. This conclusion is supported by data of Takagi *et al.* (1988) and Shi *et al.* (1989). Only in soils contaminated with high levels of Cu, the Fe mobilization by phytosiderophores may be severely impaired and lead to Fe deficiency in grasses (Bergmann, 1988).

The data presented also suggest that the release of phytosiderophores by roots of grasses under Zn deficiency (Zhang *et al.*, 1989) can be considered as ecological advantage for grasses grown on calcareous soils not only for the Fe acquisition but also for acquisition of Zn.

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