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Copper-deficiency-induced phytosiderophore release in the calcicole grass *Hordelymus europaeus*

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SUMMARY

Phytosiderophore (PS) release occurs under both iron and zinc deficiencies in representative Poaceae and has been speculated to be a general adaptive response to enhance the acquisition of micronutrient metals. We tested this hypothesis within an on-going study of the role of micronutrient metal nutrition for patterns of spontaneous vegetation in relation to soil pH and carbonate content. *Hordelymus europaeus* (L.) Harz, a negative grass species commonly found on soils rich in $CaCO_3$ in Western Central Europe, was subjected to deficiencies of Fe, Zn, Mn and Cu using chelator-buffered nutrient solutions. PS release rates were determined at 3–5 d intervals during onset and development of deficiency symptoms. Plant dry matter yields and nutrient concentrations, measured at three time points were used to construct growth curves for calculation of PS release per unit root mass. In comparison with trace metal-sufficient control plants, dry matter production was markedly reduced in the Fe, Zn, Mn and Cu-deficiency treatments, with final relative yields of 6, 11, 15 and 31 %, respectively. The phytosiderophore produced under Fe- and Cu-deficiency treatments was identified, using HPLC, as desoxymugineic acid. The highest rate of PS release (18 μmol g^{-1} root d. wt in 2 h) was measured in the Fe-deficiency treatment, and there was substantial release in the Cu-deficiency treatment (7·25 μmol g^{-1} root d. wt in 2 h). No PS release above control levels (2·6 μmol g^{-1} root d. wt in 2 h) was observed in the Zn- or Mn-deficiency treatments (1·5 and 2·6 μmol g^{-1} root d. wt in 2 h, respectively). It remains to be clarified whether PS release in response to Cu deficiency is a primary reaction to the deficiency, or is caused by a chain of events similar to that observed in Zn-deficient wheat, which involves internal Fe deficiency. Our results suggest that PS release in the native plant species *H. europaeus* is a specific response to Fe and Cu deficiency and is not significantly induced in response to deficiencies of Zn and Mn. Induction of the PS mechanism in different plant species might be more diverse than previously thought.

Key words: Phytosiderophores (PS), Fe deficiency, Zn deficiency, Mn deficiency, Cu deficiency.

INTRODUCTION

Many vascular plant species are unable to colonize calcareous sites. Thus, the floristic composition of adjacent limestone and acid silicate soils differs greatly. The inability of calcifuge plants to establish in limestone sites could be related to a low capacity of such plants to solubilize and absorb Fe from these soils.

Under Fe deficiency, species of Poaceae enhance their Fe uptake by releasing non-proteinogenic amino acids, phytosiderophores (PS), from their roots which mobilize Fe from the soil by forming a chelate that is then taken up by the root (Takagi 1976; Takagi, Nomoto & Takemoto 1984; Römheld & Marschner, 1986). In previous research it was shown that calcicole grasses are better adapted to low Fe availability on calcareous sites, as a consequence of higher PS release rates and lower tissue Fe demand (Gries & Runge, 1992, 1995).

Phytosiderophore release and uptake is thought to be specific for Fe deficiency. However, a universal role of phytosiderophores in the acquisition of micronutrient metals has been proposed (Crowley, Reid & Szaniszlo, 1987), since PS form stable chelates with Zn, Mn and Cu (Nomoto, Sugiura & Takagi, 1987; Murakami *et al.*, 1989), they extract considerable amounts of Zn, Mn and Cu from calcareous soils (e.g. Treeby, Marschner & Römheld, 1989), and deficiencies of Zn, Mn and Cu are quite common on calcareous and non-calcareous soils.

Enhanced PS release in Zn-deficient wheat has been reported by Zhang, Römheld & Marschner (1989, 1991), Cakmak *et al.* (1994) and Walter *et al.*

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(1994). However, results by Walter *et al.* (1994) show that root-to-shoot transport of Fe is impaired in Zn-deficient plants. These authors suggest that this causes Fe deficiency in the shoot, which in turn triggers production of phytosiderophores. Although this would indicate that PS release under Zn deficiency is not a specific response, it could still have ecological significance. Release of PS in response to Zn, Mn or Cu deficiency, imposed by chelator-buffered solutions, was insignificant in barley (Gries *et al.*, 1995). In conventional nutrient solutions, Zn, Mn and Cu deficiencies are difficult to impose in a time frame required for physiological experiments. This is largely caused by the ubiquitous presence of these metals as laboratory contaminants, coupled with very small plant requirements. By using chelating agents such as HEDTA, the concentrations of micronutrient metals in the nutrient solution can easily be lowered to deficient levels. The chelator can be supplied in higher concentrations than micronutrient metals, thus buffering their free activities to appropriately low levels. These so-called 'chelator–buffered' nutrient solutions (Chaney, 1988) are a useful technique to reproducibly impose different levels of micronutrient metal deficiencies within a short time (Bell, Angle & Chaney, 1991; Bell, Chaney & Angle, 1991; Parker, Aguilera & Thomason, 1992; Gries *et al.*, 1995).

The objective of this research was to evaluate the hypothesis that root exudation of chelating compounds (phytosiderophores) is a universal response mechanism of Poaceae to micronutrient metal deficiencies and is not confined to Fe deficiency. Growth and Fe-mobilizing root exudate release rates of the calcicole native species *H. europaeus* were studied under Fe, Zn, Mn or Cu deficiency. HEDTA-buffered nutrient solutions were used to impose these micronutrient metal deficiencies.

MATERIALS AND METHODS

General

Seedlings of *H. europaeus* (L.) Harz were hydroponically grown in a controlled-environment growth chamber at a PAR of approx. 300 μmol m^{-2} s^{-1} with a 16/8 h photoperiod, 75 % r.h. and day/night temperatures of 23/17 °C. Caryopses were sown in paper germination sheets and irrigated once with trace-metal-free nutrient solution (see below) and demineralized water thereafter. After 19 d, caryopses were cut off to prevent utilization of stored micronutrients and the seedlings were transferred to 3 l polyethylene containers (30 plants per container) filled with continuously aerated nutrient solution and grown for an additional 44 d under different treatments as stated below. The containers were shielded to exclude light from the solutions and root systems.

The basal nutrient solution composition was $Ca(NO_3)_2$, 2 mM; KNO_3, 1 mM; $MgSO_4$, 0·5 mM; KH_2PO_4, 0·08 mM; H_3BO_3, 10 μM; $NaMoO_4$, 0·1 μM; Mn, 0·6 μM; Cu, 2 μM; Zn, 8 μM; Ni, 0·1 μM; Fe, 75 μM; and 1 mM MES to buffer pH 6·0. The solution pH was checked every 2 d and adjusted as necessary by addition of HCl or NaOH. In control solutions, fixed free metal activities of Fe ($\log(Fe^{3+}) = -16{\cdot}5$), Zn ($\log(Zn^{2+}) = -9{\cdot}8$), Mn ($\log(Mn^{2+}) = -7{\cdot}7$), Ni ($\log(Ni^{2+}) = -14{\cdot}1$), and Cu ($\log(Cu^{2+}) = -13{\cdot}2$) were maintained by addition of HEDTA at a concentration equal to the sum of the Fe, Zn, Mn, Cu and Ni concentrations plus a 25 μM excess. Chemical speciations of the nutrient solutions were calculated using GEOCHEM–PC (Parker, Norvell & Chaney, 1995). Demineralized water and reagent-grade salts were used to prepare all solutions. Stock solutions of $Ca(NO_3)_2$, KNO_3, $MgSO_4$, KH_2PO_4 and MES were purified with ammonium pyrrolidine dithiocarbamate (Wallihan & Bradford, 1977) to remove contaminating levels of trace metals. Nutrient solutions were replaced every 4 d.

At each plant sampling date, the harvested plants were rinsed with demineralized water, separated into shoots and roots, dried at 60 °C for 3 d, weighed, ground, and subsamples digested in 65 % nitric acid at 180 °C. Elemental analyses were conducted by atomic absorption spectrophotometry.

Experiment 1 : growth and phytosiderophore (PS) release

The experiment consisted of a trace-metal-sufficient control and four treatments in which Fe, Zn, Mn or Cu were omitted from both the complete nutrient solutions used for seed germination, and those used for seedling growth. There were four replications per treatment. After 15, 30 and 44 d, 10 plants were harvested from each container.

Experiment 2 : diurnal cycle

Thirty d after transplanting, root exudates of Cu-deficient plants were collected for 1 h each from the onset of the light period until 1600 hours. A new set of plants was used for each collection. Chelating capacity of root exudates was determined with the Fe mobilization assay.

Determination of heavy-metal-binding activity of root exudates (phytosiderophore concentration)

Root exudates were collected every 3–5 d (experiment 1) during peak PS release, which occurred over a 4-h period starting 2 h after onset of the photoperiod. The roots were rinsed for 1 min under a gentle stream of demineralized water and the plants were then transferred to small plastic containers containing 50 ml of aerated demineralized water and

0·01 g l^{-1} of Micropur™ (Roth GmbH, Karlsruhe, Germany), a commercial bactericide used for drinking water purification. At this concentration, 10 % of that recommended by the manufacturer for water purification, the collection solution was bacteriostatic only, and had no direct effects on root growth, as observed in preliminary experiments after repeated collections at full-strength concentration. In previous experiments (Gries *et al.*, 1995) we found that in 10 % Micropur, bacterial population numbers in PS-collection solutions were approx. 10^3–10^4 per ml water, and that these collection solutions were stable for at least 2 d on the laboratory bench. PS-collection solutions were always processed immediately.

After a 2-h collection period, the solutions were filtered through Schleicher & Schuell (Dassel, Germany) type 595 filters. The concentrations of complexing agents in the root exudates were measured with an Fe-binding assay modified from Takagi (1976) (V. Römheld, pers. comm.; Gries *et al.*, 1995). In Poaceae, these are mainly phytosiderophores. On 10 of 12 d root exudates were also analysed with an Fe mobilization assay (modified from Takagi, 1976; Gries & Runge, 1995).

Iron-binding assay

In the modified Fe-binding assay, 0·5 ml of $FeCl_3$ solution (pH 2·1) was added to 10 ml of the collection solution and shaken for 15 min. After addition of 1 ml of 1 M Na-acetate buffer (ph 7), the solution was shaken for another 10 min and filtered through blue ribbon paper (type 589^3, Schleicher & Schuell) into 0·2 ml 6 N HCl. Ferric iron was reduced by addition of 0·5 ml of 8 % hydroxylamine-hydrochloride and heating to 60 °C for 20 min. The concentration of ferrous iron was then determined colorimetrically by measuring absorbance at 562 nm after adding 0·2 ml of 0·25 % ferrozine and 1 ml of 2 M Na-acetate buffer (pH 4·7). Since the phytosiderophore : substrate ratio influences the upper detection limit of the assay, all assays were carried out with $FeCl_3$ concentrations of 0·2 mM and 1·0 mM for each sample.

Iron-mobilization assay

In the modified Fe-mobilization assay, 2 ml of freshly precipitated $Fe(OH)_3$ and 0·5 ml of 0·5 M Na-acetate (pH 5·6) was added to 10 ml of the collection solution, shaken for 2 h and filtered through blue ribbon paper (589^3, Schleicher & Schuell) into 0·2 ml of 6 N HCl. Determination of ferrous iron was carried out as described for the Fe-binding assay.

To calculate PS release rates based on root dry weight, root weights were interpolated between the four harvest dates. Preliminary experiments as well as results from the control treatments showed there was negligible carryover of HEDTA to the collection solutions. Moreover. simple calculations assuming a maximum carryover of 1 ml g^{-1} root of 25 μM HEDTA show that contaminating HEDTA in the collection solution would result in a maximum background iron mobilization of 0·025 μmol g^{-1} root, well below the 1–18 μmol g^{-1} root measured in the experiments. Concentrations of complexing agents in the root exudates from Zn-deficient plants were also checked with a Zn-mobilization assay (Zhang *et al.*, 1989). The results indicated that compounds that would form stable complexes with Zn, but not with Fe, were not present in root exudates of Zn-deficient or control plants. Identification of PS of Fe-deficient and Cu-deficient plants as deoxymugineic acid was confirmed by HPLC analysis (Kawai *et al.*, 1987).

Statistics

Values presented are means of four and three replicates for experiments 1 and 2, respectively. Error bars indicate ± 1 SE. ANOVA using SAS PROC GLM (SAS Institute, Cary, NC, USA) was carried out for total plant dry matter and root weight ratio for each harvest in experiment 1. For these variables, least significant differences ($P = 0{\cdot}05$) for each harvest are indicated as vertical bars in Figures 1 and 2.

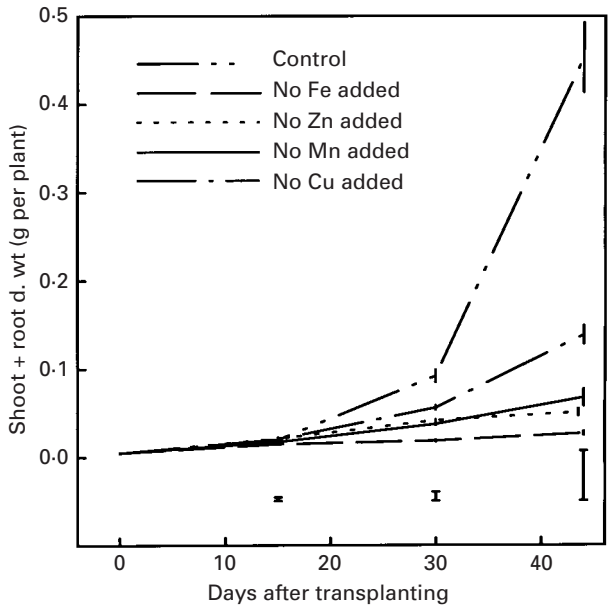

Figure 1. Dry matter yield of shoots and roots of *H. europaeus* plants subjected to deficiencies of Fe, Zn, Mn or Cu using HEDTA chelator-buffered nutrient solutions. Control plants were grown in complete nutrient solutions with Fe supplied at log Fe^{3+} activity (μM) = −16·5. Error bars indicate ± SE for plants harvested from four replicate containers. Small bars indicate LSD for each harvest date.

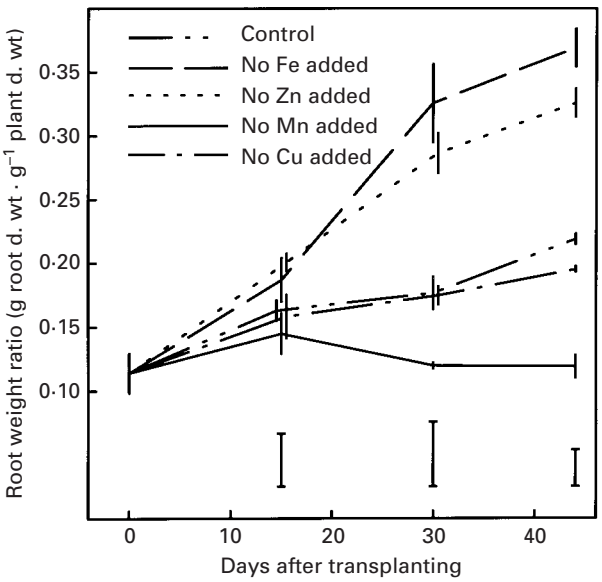

Figure 2. Root weight ratio of *H. europaeus* plants subjected to deficiencies of Fe, Mn or Cu using HEDTA chelator-buffered nutrient solutions. Control plants were grown in complete nutrient solutions with Fe supplied at log Fe^{3+} activity (μM) = −16·5. Error bars indicate ± SE for plants harvested from four replicate containers. Scale bars indicate LSD for each harvest date.

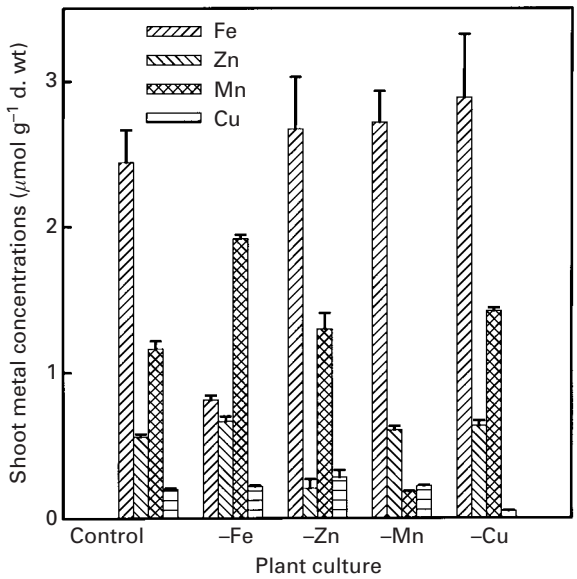

Figure 3. Fe, Zn, Mn and Cu concentrations in shoots of *H. europaeus* plants subjected to deficiencies of Fe, Zn, Mn or Cu using HEDTA chelator-buffered nutrient solutions after 15 d of culture. Control plants were grown in complete nutrient solutions with Fe supplied at log Fe^{3+} activity (μM) = −16·5. Vertical bars indicate ± SE for plants harvested from four replicate containers.

RESULTS

Growth under Fe, Zn, Mn or Cu deficiency

The −Fe, −Zn, −Mn and −Cu treatments had dramatic effects on dry matter production (Fig. 1) of this comparably slow-growing species, and the deficiencies were induced in approx. 15 d for Fe, and between 15 and 30 d for Zn, Mn and Cu. Relative yields for the −Fe, −Zn, −Mn and −Cu treatments were 20, 45, 41 and 61 % of control, respectively after 30 d, and 6, 11, 15 and 31 % of control after 44 d.

The Cu-deficiency treatment affected shoot and root growth almost identically, whilst Fe and Zn deficiency caused an almost twofold increase in root weight ratio in comparison to the control. By contrast, Mn deficiency caused the root weight ratio to decrease (Fig. 2).

Visual symptoms

Visual chlorosis symptoms developed in Fe-deficient plants after less than 1 wk. By the end of the experiment, these plants had new foliage that was almost white in colour and had stopped growing. Plants grown without added Zn had brown necroses on the youngest leaves and some grey spots on older leaves. The foliage of Mn-deficient plants had some small brown spots and was slightly lighter green than that of the control treatment. Leaves of Cu-deficient plants were clearly lighter green in colour than control leaves and emerging leaves were more twisted than usual. Roots of Cu-deficient plants had more laterals than control roots. Colour, shape and size of root tips were similar to control roots.

Tissue analysis

Shoot concentrations of Fe, Zn, Mn or Cu were drastically reduced after 15 d of culture in the Fe, Zn, Mn or Cu-deficiency treatments, respectively (Fig. 3). The treatments did not alter shoot concentrations of the other metals except that Fe-deficient plants had 50 % higher Mn concentrations compared with control plants. Root Fe concentrations (data not shown) were threefold higher than shoot concentrations in the Fe-sufficient treatments and similar to shoot concentrations in Fe-deficient plants. Root Mn concentrations were 50 % higher in Zn-deficient compared with control plants.

Root chelator release

On a root d. wt basis, root chelator exudation of Fe-deficient plants reached maximum values of approx. 18 μmol g^{-1} in 2 h after 10–13 d (Fig. 4). Cu-deficient plants reached a maximum value of > 7 μmol g^{-1} in 2 h after 13 d. At that time, growth had not yet been affected by the Cu-deficiency treatment. Release rates of Cu-deficient plants remained well above control levels throughout the experiment. Chelator exudation of plants subjected to Zn or Mn deficiency (max. 1·5 and 2·6 μmol g^{-1} in 2 h, respectively) never exceeded that of the control treatment. Control plants had a maximum exudation

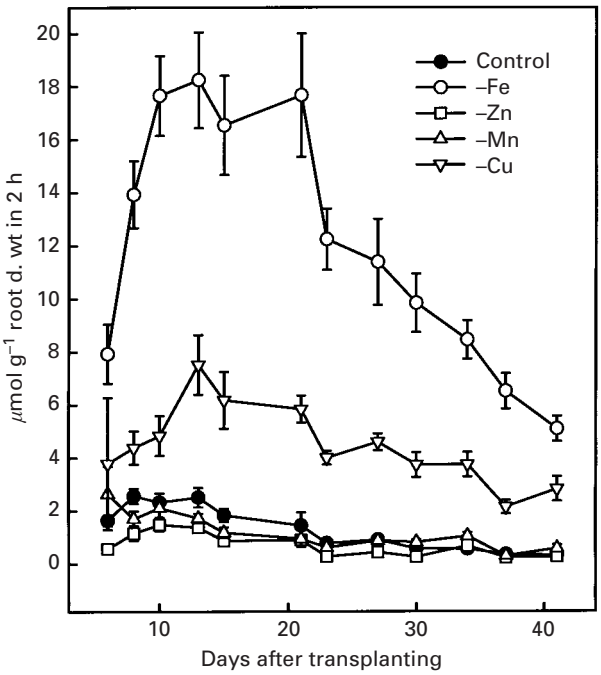

Figure 4. Fe-mobilizing exudate release rates from roots of *H. europaeus* plants subjected to deficiencies of Fe, Zn, Mn or Cu using HEDTA chelator-buffered nutrient solutions. Control plants were grown in complete nutrient solutions with Fe supplied at log Fe^{3+} activity (μM) $= -16{\cdot}5$. Phytosiderophore quantification based on measurements of Fe-mobilizing compounds using a modified assay after Takagi (1976). Vertical bars indicate $\pm$ SE for plants harvested from four replicate containers.

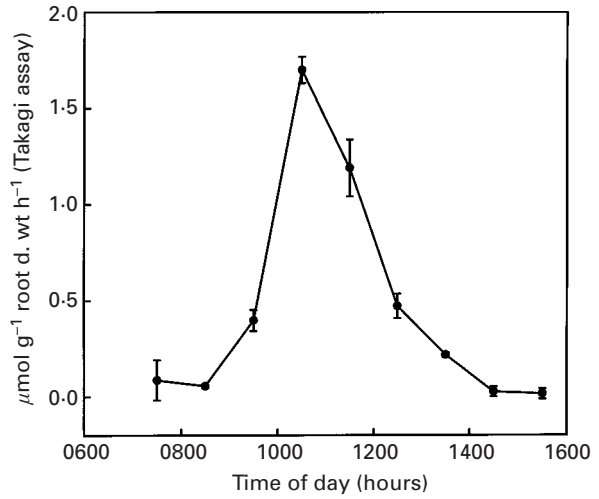

Figure 5. Time-course of Fe-mobilizing exudate release from roots of copper-deficient *H. europaeus* plants, 19 d after transplanting. Plants were subjected to Cu deficiency using HEDTA chelator-buffered nutrient solutions. The onset of the light period was 0600 hours. For each data point root exudates were collected for 60 min from new sets of plants. Phytosiderophore quantification based on measurements of Fe-mobilizing compounds using a modified assay after Takagi (1976). Vertical bars indicate $\pm$ SE for plants harvested from three replicate containers.

rate of 2·6 μmol g^{-1} in 2 h until day 13 and < 2 μmol g^{-1} in 2 h after that.

On a total plant d. wt basis, maximum phytosiderophore (PS) release rates were 0·40, 4·27, 0·28, 0·39 and 1·13 μmol g^{-1} in 2 h for the control, $-$Fe, $-$Zn and $-$Cu treatments, respectively, and these maximum rates occurred on days 8, 13, 10, 6 and 13, respectively.

Chelator concentrations of root exudates from Fe-deficient plants measured by the Fe-binding assay were on average 65 % higher than concentrations measured by the Fe-mobilization assay. For root exudates from Cu-deficient plants, the ratio between the two assays was $1{\cdot}73 \pm 0{\cdot}14$ and was thus similar to that for Fe-deficient plants. This indicates that the chelator composition of root exudates from Cu-deficient plants was similar to that of Fe-deficient plants.

Time course of root chelator release from Cu-deficient plants

Exudation of chelators from roots of Cu-deficient plants (Fig. 5) began 2 h after onset of the light period, which was at 0600 hours. Exudation peaked after *c.* 5 h of light, and ceased after 8 h of light. This time course is identical to the diurnal pattern of PS release in response to Fe deficiency in *H. europaeus* (Gries & Runge, 1992) and other grass species (e.g. Takagi *et al.*, 1984; Zhang *et al.*, 1989).

DISCUSSION

Phytosiderophore (PS) release in *H. europaeus* was rapidly induced in response to both Fe and Cu deficiencies. This is the first reported case of Cu-deficiency-induced PS release in grasses. Fe- and Cu-deficient plants were able to maintain release rates well above background levels even when growth was reduced to 6 or 31 % of the control, respectively.

In contrast to the response that was induced under Fe and Cu deficiencies, there was no effect of Zn or Mn deficiency on root exudation of PS in this native species. In this regard, the HEDTA chelator-buffered nutrient solutions employed in this study proved to be very useful for imposing micronutrient-metal deficiencies, especially for slow-growing native plant species such as *H. europaeus*. The inherent RGR of *H. europaeus* is lower than that of wheat or barley. Therefore, it was expected that it would take a much longer time for micronutrient deficiencies to develop in this species. However, the plants in the metal-deficiency treatments progressed from normal growth to severely deficient in < 30 d. For the induction of Cu deficiency this is in agreement with Gries *et al.* (1995) but contrary to the findings of Bell *et al.* (1991), who suggested that Cu deficiency could only be obtained using a combined BPDS-HEDTA chelator-buffered system. Identification of PS in root exudates from both Fe-deficient and Cu-deficient *H. europaeus* plants as deoxymugineic acid was confirmed by HPLC analyses. Theoretically, other non-specific chelators such as citric acid, which

has some affinity for Cu and is present in root exudates of Fe- and Cu-deficient *H. europaeus* (data not shown), could contribute to the Fe-mobilizing capacity of root exudates under Cu deficiency. Whilst the Fe-binding assay does detect citric acid, preliminary experiments showed that in the Fe-mobilization (Takagi) assay, solubilization of Fe from $Fe(OH)_3$ precipitate by citric acid is marginal. We thus assume that Fe mobilization by root exudates from Cu-deficient plants measured with the Takagi assay was by PS. The Fe-mobilization assay typically yields lower values for chelator concentrations than the Fe-binding assay. This is mainly due to absorption of PS by the large surplus of amorphous $Fe(OH)_3$ and is probably mediated by polysaccharides in root exudates (W. Hördt, pers. comm.). The fact that almost the same constant ratio between the two assay methods was found for root exudates from both Fe-deficient and Cu-deficient plants suggests that the same chelators are produced under Cu deficiency as under Fe deficiency.

Several studies using wheat (*Triticum aestivum* and *Triticum durum*) have demonstrated that PS release is enhanced under Zn deficiency (Zhang *et al.*, 1989, 1991) and can reach levels comparable to those of PS release by Fe-deficient barley (Cakmak *et al.*, 1994). Zinc-deficiency-induced PS release was also found in two native grasses (Cakmak *et al.*, 1996). However, in our experiment, exudation of chelating compounds under Zn or Mn deficiency occurred at very low rates that were similar to, or lower than, background release rates by putatively Fe-sufficient plants. This is also true when differences in root weight ratios between treatments are taken into account by calculating chelator-release rates on a total d. wt basis rather than on the more common root d. wt basis.

The PS response in Zn-deficient wheat is probably due to impaired Fe metabolism, which only indirectly induces PS release (Walter *et al.*, 1994). Theoretically, the same could be true for the response to Cu deficiency that we observed. However, there are no indications that a disturbance of Fe metabolism could have caused PS release in our experiments. Tissue analysis of Cu-deficient plants did not show any unusual patterns such as shoot or root Fe deficiency or Fe hyperaccumulation. Moreover, PS release was induced at an early growth stage, before symptoms of deficiency developed, and before growth was affected. By contrast, in a previous experiment (data not shown) PS release by moderately Fe-deficient *H. europaeus* plants did not exceed 2·6 μmol g^{-1} root d. wt in 2 h, which is only one-third of the release rate of Cu-deficient plants in this present experiment, even though yield of these moderately Fe-deficient plants was reduced by 10 % after 11 d and by 16 % after 18 d. It thus appears that induction of PS release under Cu deficiency occurs at an even earlier stage of the deficiency than under Fe deficiency. The hypothesis that PS release is a physiological response to Cu deficiency is further supported by the fact that Cu-deficient plants were able to maintain high rates of PS exudation throughout the experiment even when growth was reduced to less than one-third of the control. Also, the diurnal pattern of PS release under Cu deficiency (Fig. 5) was identical to that known from Fe-deficient *H. europaeus* plants (Gries & Runge, 1992). In combination, these findings suggest that PS release in response to Cu deficiency is a well regulated mechanism.

In barley, uptake rates of PS-complexed Cu are 10-fold lower than those of the PS-Fe complex (Ma *et al.*, 1993), suggesting that the PS system functions primarily for Fe transport. This preferential recognition of Fe-PS complexes remains to be examined for *H. europaeus*. Nonetheless, even 10-fold lower uptake rates of Cu-PS could still be sufficient to meet plant Cu demand. Based on calculations of plant yield and tissue Cu concentration, the quantities of chelators released under Cu deficiency greatly exceed the Cu uptake rate required for normal growth. A Cu uptake rate of 0·13 μmol g^{-1} root d. wt d^{-1} would suffice to maintain a tissue Cu concentration of 0·2 μmol g^{-1} d. wt at a RGR of 0·1 g g^{-1} d^{-1}, assuming a root weight ratio of 0·15 g g^{-1}. If it is assumed that similar quantities of PS are produced during the second 2 h of the peak release period, the maximum daily capability for PS release would exceed 14 μmol g^{-1} root.

Studying metal extraction by PS from a wide range of calcareous and non-calcareous soils we found that PS preferentially mobilize Fe but also significant quantities of Zn and Cu from soils (data not shown). Considering that plant Cu demand is much lower than Fe demand, the amounts of Cu mobilized appeared sufficient to meet plant requirements for this metal. This suggests that PS release would be an advantage for plants growing on soils low in available Cu.

In conclusion, this research demonstrates that PS release in response to deficiencies of micronutrient metals is not restricted to Fe and Zn, but might be more widespread than previously thought. The mechanism seems to be specific for Fe and Cu deficiencies, but not for Zn or Mn deficiency in *H. europaeus*. Release of PS under Cu deficiency could have an ecological significance, regardless of whether it is indirectly caused by impaired metabolism, or as a specific response mechanism. This question needs further study. Also, it remains to be examined whether Cu-deficiency-induced PS release is a general phenomenon in native grass species.

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