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ORIGINAL ARTICLE

Sulfur starvation reduces phytosiderophores release by iron-deficient barley plants

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Abstract

The aim of the present study was to examine the effect of sulfur (S) supply on the response to iron (Fe) deficiency in graminaceous plants. Barley seedlings (*Hordeum vulgare* L. cv. Europa) were cultured hydroponically for 10 days at three S levels (0, 60 and 1200 μmol L^{-1} sulfate) with (+Fe) or without (–Fe) 100 μmol L^{-1} Fe^{III}-ethylene diamine tetracetic acid. Lowering S supply resulted in a sharp decrease in the release of phytosiderophores by Fe-deficient barley plants. Furthermore, uptake of ^{59}Fe from (^{59}Fe)-hydroxide decreased by approximately 30% when S availability was lowered; Fe deficiency caused a sharp increase in ^{59}Fe uptake that was as high as the level of S supply. The results support the view that S availability can influence either the release of phytosiderophores or the ability to take up Fe from an external solution.

Key words: barley, iron deficiency, iron uptake, strategy II, phytosiderophores.

INTRODUCTION

The secretion of phytosiderophores (PSs) from the roots to the rhizosphere is an important step in the acquisition of sparingly soluble soil iron (Fe) in graminaceous species under Fe deficiency (Strategy II) (Marschner & Römheld 1994; Römheld 1987). The most common PSs are mugineic acid (MA), deoxymugineic acid (DMA) and epi-hydroxymugineic acid (epi-HMA). Deoxymugineic acid is the first product of PSs synthesis from the precursor methionine (Mori & Nishizawa 1987) and all other PSs are derived from DMA (Ma *et al.* 1995). Methionine requirement in PSs biosynthetic pathway suggests the involvement of S metabolism in the metabolic modifications necessary to cope with Fe shortage. Furthermore, recent reports (Astolfi *et al.* 2003; Bouranis *et al.* 2003) provide data supporting mutual interference between S and Fe. In particular, our previous study showed that leaf Fe content was lower in maize (*Zea mays* L.) plants grown in S deficiency than in corresponding plants grown in the presence of the macronutrient (+S). Furthermore, imposed Fe deprivation resulted in an increase in the level of non-protein thiol compounds in both nutritive conditions (+S and –S), which is realistically explained by assuming an increased demand of reduced S for methionine and, consequently, PSs synthesis. These data provide information of several linkages between plant S nutritional status and response to Fe deficiency and, in particular, suggest that S deficiency could cause an aggravation of Fe chlorosis. The recent increase in S deficiency in many agricultural soils (McGrath & Zhao 1995) highlights the importance of this study. Despite this, very few investigations have been initiated to gain a better understanding of this aspect and the interaction mechanisms remain unclear.

In contrast, the potential to improve a plant's capability to overcome Fe deficiency by means other than satisfactory Fe supply could be an interesting rationale of this work.

We investigated the effect of S supply on the response to Fe deficiency in graminaceous plants and, in particular, we attempted to verify if inhibited release of PSs occurs in S-deficient plants. Because of the higher release rate of PSs from barley compared with other grasses, and consequently easier detection, we used barley as the model plant.

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MATERIALS AND METHODS

Growing conditions

Barley (*Hordeum vulgare* L. cv. Europe) seeds were germinated in moistened paper in the dark at 26°C for 3 days. Seedlings with roots were then transferred to plastic pots (approximately 18 seedlings in each pot) containing 2.5 L of nutrient solution (NS) (Zhang *et al.* 1991) and were cultured hydroponically for 14 days at three S application levels (0, 60 and 1200 μmol L^{-1} sulfate, i.e. S-deficient, S-intermediate and S-optimal, respectively) with (+Fe) or without (–Fe) 100 μmol L^{-1} Fe^{III}-ethylene diamine tetracetic acid (EDTA). In S-intermediate and S-deficient NS, sulfate salts (K^+, Mn^{2+}, Zn^{2+}, Cu^{2+}) were replaced by appropriate amounts of chloride salts (K^+, Mn^{2+}, Zn^{2+}, Cu^{2+}). The NS was continuously aerated and changed every 2 days. Plants were grown into a climate chamber under 200 μE m^{-2} s^{-1} light intensity and 14 h/10 h day/night regime (temperature 27°C diurnal; 20°C nocturnal; relative humidity 80%). Both leaves and roots were harvested 14 days after transfer into the NS.

Collection of root exudates and preparation of phytosiderophores

On day 14 we determined PSs release by determining PSs content in root washings. Different S supplied Fe-sufficient and Fe-deficient barley plants were removed from the NS at 2 h after onset of the light period and the roots were washed two times for 1 min in deionised water. Root systems were submerged into 500 mL deionised water for 3 h with continuous aeration. Thereafter, Micropur (10 mg L^{-1}) (Roth, Karlsruhe, Germany) was added to prevent microbial degradation of PSs. Phytosiderophores in root washings were determined by direct injection (20 μL) using the anion exchange HPLC (high performance liquid chronatography) system with post-column orthophtaldialdehyde (OPA) derivatisation described by Neumann *et al.* (1999).

Measurements of ^{59}Fe uptake

To measure the capability of the root apparatus to absorb ^{59}Fe from ^{59}Fe-hydroxide, roots of intact barley plants were washed with micronutrient-free NS for 30 min and then transferred to beakers containing 200 mL of a freshly prepared micronutrient-free NS (uptake solution) buffered at pH 7.5 with 10 mmol L^{-1} Hepes-KOH. ^{59}Fe-hydroxide was prepared as previously described (Cesco *et al.* 2000) by dissolving $^{59}FeCl_3$ in water and than adding KOH 1N to increase the alkaline pH (specific activity 123 KBq μmol Fe^{-1}); the experiment was started by the addition of 1 mL of suspension containing ^{59}Fe-hydroxide (2 μmol Fe) into the uptake solution and lasted 24 h. To evaluate the capability of root apparatus to take up Fe–PSs complexes, 10 μmol L^{-1} Fe-free DMA was added to the uptake solution of a set of Fe-sufficient plants. After the uptake period, the plants were transferred to a freshly prepared ^{59}Fe-free NS for 10 min and then harvested. The root extraplasmatic ^{59}Fe pool was removed using 1.2 g L^{-1} sodium dithionite and 1.5 mmol L^{-1} 2,2′-bipyridyl in 1 mmol L^{-1} $Ca(NO_3)_2$ under N_2 bubbling according to the method described by Bienfait *et al.* (1985); the treatment was repeated 3 times. Roots and shoots were oven-dried at 80°C, weighed, ashed at 550°C and suspended in 1% (w/v) HCl for ^{59}Fe determination by liquid scintillation counting. The ^{59}Fe uptake rate, measured as μmol ^{59}Fe, is for the whole plant (root + shoot) and is presented per gram dry weight of roots per 24 h.

Other measurements

The concentration of chlorophyll per unit area was estimated in attached leaves by a SPAD portable apparatus (Minolta Co., Osaka, Japan) using, in particular, the first fully expanded leaf from the top of the plant.

Gas exchange measurements were made on attached leaves in the growth chamber with a portable gas exchange system using a PLC (parkinson leaf cuvette) broad leaf cuvette in closed circuit mode. Net photosynthetic rate was determined during the measurements. Experiments were done at ambient CO_2 concentration and the relative humidity prevailing in the growth chamber. Measurements were taken in leaves illuminated for 4–5 h.

Iron content in leaves was determined after dry ashing (500°C) of leaf material and 1:30 HCl extraction using atomic absorption spectrometry (AAS).

The total soluble sugar content of roots and leaves was determined after hot water extraction following the method described by Stephan and Rudolph (1984) with minor modifications. Plant material was homogenised to a fine powder with liquid nitrogen. Distilled water (100°C) was added to aliquots of the powdered tissue (500 μL mg^{-1} fresh weight) and homogenates were incubated for 10 min at 80°C. Insoluble material was removed by 10 min centrifugation in a microliter centrifuge at 16,500 *g* and the pellet was then re-extracted with 500 μL boiling water as described above. After a second centrifugation step, the supernatant was used for spectrophotometric determination of reducing sugars and sucrose according to Blakeney and Mutton (1980).

RESULTS

After 14 days of culture using six different NSs, barley plants showed changes in some macro-indices (growth and chlorophyll concentration). Figure 1 illustrates

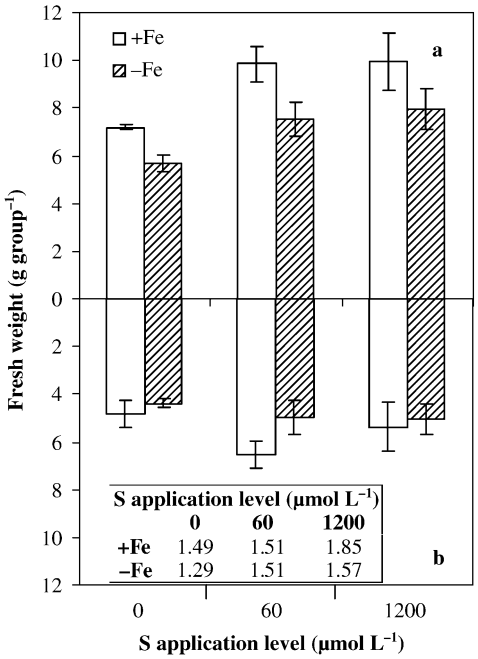

Figure 1 Fresh weight of (a) shoot and (b) root of four barley plants. Barley plants were grown in nutrient solution for 14 days with a different sulfur (S) supply (0, 60 and 1200 μmol L^{-1}) in the presence (+Fe) or absence (–Fe) of 100 μmol L^{-1} Fe^{III}-ethylene diamine tetracetic acid. Data are means ± standard error (SE) of four independent experiments with three replicates. Bars indicate SE. *In box*: shoot to root ratio.

that plants grown under S starvation (0 μmol L^{-1} sulfate) showed a significant decrease in the fresh weight of both leaves and roots, but leaf biomass was more markedly affected by S deprivation than roots. In fact, the reduction in the fresh weight was approximately 30% in leaves and only 10% in roots. Leaves from Fe-deficient plants were significantly lower in fresh weight, but there were no great differences in reduction values among barley plants with severe, slight or no S deficiency.

Chlorophyll content was determined by chlorophyll meter readings (SPAD), which provide a sensitive and accurate index of plant response to the Fe treatment, and the relative data are shown in Fig. 2a. Young, developing leaves from 14-day-old barley plants grown without sulfate in NS exhibited visible chlorosis (40% lower SPAD units). With regard to Fe deficiency, plants cultured without Fe displayed severe leaf chlorosis at harvest, with a 15–50% decrease in SPAD readings depending on S availability. In particular, chlorophyll concentrations began to decrease approximately 1 week after the onset of Fe starvation (data not shown), suggesting that Fe became a limiting factor for plant metabolism from the seventh day of hydroponic culture.

We investigated plant photosynthetic capacity and revealed that a complete lack of sulfate in the nutrient medium significantly reduced the net photosynthetic rate of Fe-sufficient plants (20% lower than the S-optimal control; Fig. 2b). Moreover, comparison of +Fe and –Fe plants showed that Fe deficiency reduced net photosynthetic rate only when plants were exposed to S-optimal and S-intermediate (60 μmol L^{-1} sulfate) conditions, by approximately 40% in both cases.

Figure 3 reveals that Fe deprivation decreased leaf Fe content and there were no significant differences in the Fe contents of leaves from plants grown on the three different Fe-deficient media (0, 60 and 1200 μmol L^{-1} sulfate). Interestingly, S availability increased leaf tissue content of Fe, which was 36 and 23% higher than in S-deficient plants at 60 and 1200 μmol L^{-1} sulfate, respectively.

We also investigated the amount of PSs released by plants in different growth conditions (Fig. 4). Phytosiderophores were only detectable in –Fe plants and the major compound was epi-HMA. Lowering S supply resulted in a sharp decrease in the amount of PSs in root exudates from Fe-deficient barley plants (75% and 25% at 0 and 60 μmol L^{-1} sulfate, respectively).

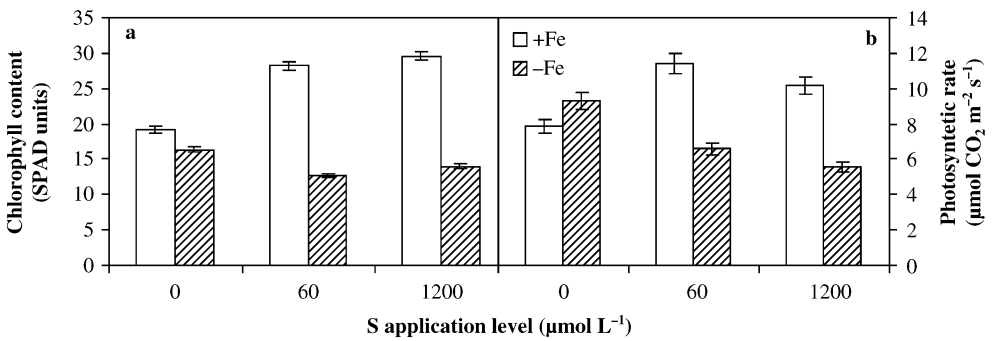

Figure 2 (a) Chlorophyll content and (b) photosynthetic capacity of barley leaves. Plants were grown hydroponically for 14 days at three sulfur (S) application levels (0, 60 and 1200 μmol L^{-1} sulfate) supplied (+Fe) or not (–Fe) with 100 μmol L^{-1} Fe^{III}-ethylene diamine tetracetic acid. SPAD readings were made using the first fully expanded leaf from the top of the plant. Data are means ± standard error (SE) of four independent experiments with three replicates. Bars indicate SE.

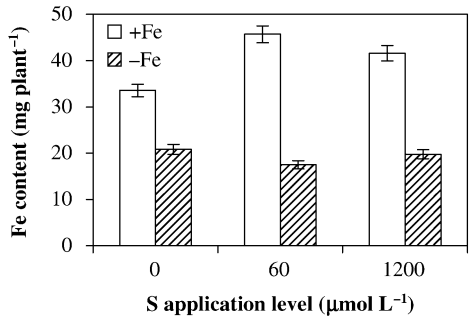

Figure 3 Iron content in leaves of barley plants grown hydroponically for 14 days at three sulfur (S) application levels (0, 60 and 1200 μmol L^{-1} sulfate) with (+Fe) and without (–Fe) 100 μmol L^{-1} Fe^{III}-ethylene diamine tetracetic acid. Data are means ± standard error (SE) of four independent experiments with three replicates. Bars indicate SE.

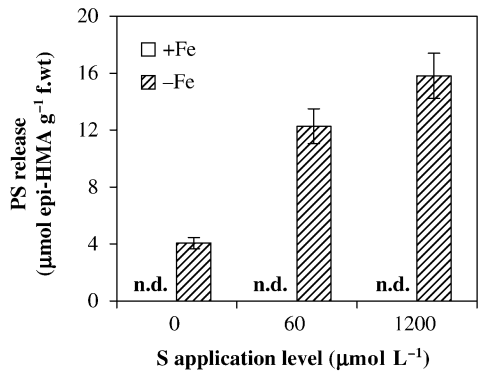

Figure 4 Phytosiderophores (PSs) (epi-hydroxymugineic acid [epi-HMA]) release by barley plants grown hydroponically for 14 days at three sulfur (S) application levels (0, 60 and 1200 μmol L^{-1} sulfate) supplied (+Fe) or not (–Fe) with 100 μmol L^{-1} Fe^{III}-ethylene diamine tetracetic acid. PSs release was measured by determining the PSs content in root washings. Data are means ± standard error (SE) of four independent experiments with three replicates. Bars indicate SE. f.wt, fresh weight; n.d., no data.

Figure 5 shows data from the analysis of concentrations of total sugars in leaf and root tissues. Sugar concentrations in leaves were almost unchanged by 60 μmol L^{-1} sulfate in NS, but sugars in the root tissue were higher in plants from S-deprived NS (0 μmol L^{-1} sulfate) than in those at 60 and 1200 μmol L^{-1} sulfate. Furthermore, Fe deficiency reduced sugar concentrations in the leaves of plants grown at 60 and 1200 μmol L^{-1} sulfate, but not when sulfate was completely omitted from the NS.

Table 1 shows that in Fe-sufficient barley plants the uptake of ^{59}Fe from (^{59}Fe)-hydroxide decreased by approximately 30% by lowering S availability. Iron deficiency caused an increase in ^{59}Fe uptake, which was greater the higher the level of S supplied. The addition of Fe-free DMA to the uptake solution of a set of Fe-sufficient plants enhanced the rates of ^{59}Fe uptake from

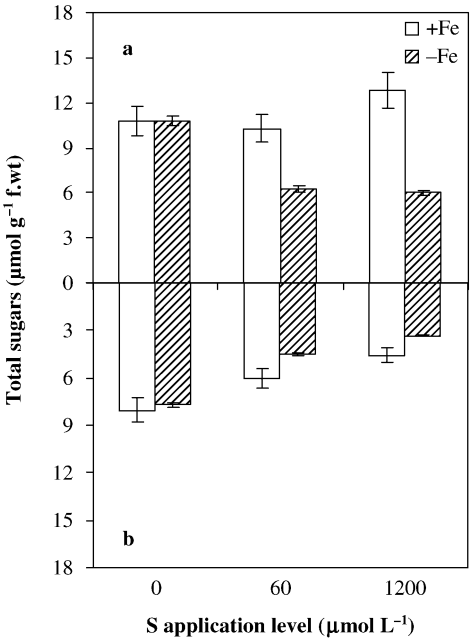

Figure 5 Total soluble sugar content of (a) leaves and (b) roots of barley plants grown hydroponically for 14 days at three sulfur (S) application levels (0, 60 and 1200 μmol L^{-1} sulfate) supplied (+Fe) or not (–Fe) with 100 μmol L^{-1} Fe^{III}-ethylene diamine tetracetic acid. Data are means ± standard error (SE) of four independent experiments with three replicates. Bars indicate SE.

(^{59}Fe)-hydroxide, particularly in plants adequately supplied with sulfate (1200 μmol L^{-1}).

DISCUSSION

The present study provides direct evidence that different S availability in the growth medium can affect the response to Fe deficiency in the graminaceous species *Hordeum vulgare* L.

Plant yield responses to S supply showed a classic behavior extensively reported in the literature (Robinson 1994), which was reflected by a decrease in the shoot to root fresh weight ratio, particularly evident in plants with severe S deficiency (Fig. 1). Furthermore, in S- and Fe-deprived plants the shoot to root ratio decreased by nearly the same extent as for S-deprived plants.

The most typical visual symptom of S deficiency is leaf chlorosis (Marschner 1995). From our data it appears that only severely S-deficient plants show a statistically significant reduction in chlorophyll content and associated leaf chlorosis, whereas under moderate S deficiency these symptoms are less detectable. A significant decrease in chlorophyll level in Fe-deficient leaves has also been widely reported (Marschner 1995). This is consistent with our observations that from approximately

Table 1 Uptake of ^{59}Fe from ^{59}Fe-hydroxide by 14-day-old barley plants grown in nutrient solution at three sulfur application levels (0, 60 and 1200 μmol L^{-1} sulfate) with (+Fe) or without (–Fe) 100 μmol L^{-1} Fe^{III}-ethylene diamine tetracetic acid

Pre-culture (μmol L^{-1})	Uptake (μmol ^{59}Fe g^{-1} root dry weight h^{-1})	
	Without exogenous DMA	With 10 μmol L^{-1} exogenous DMA
1200 SO_4^{2-} with 100 Fe	1.44 ± 0.01	6.73 ± 0.04
1200 SO_4^{2-} without Fe	7.07 ± 0.63	
60 SO_4^{2-} with 100 Fe	1.08 ± 0.01	4.02 ± 0.52
60 SO_4^{2-} without Fe	4.64 ± 0.25	
0 SO_4^{2-} with 100 Fe	1.12 ± 0.06	4.32 ± 0.18
0 SO_4^{2-} without Fe	3.25 ± 0.24	

Iron was supplied as ^{59}Fe-hydroxide; where described, Fe-free deoxymugineic acid (DMA) was added at a final concentration of 10 μmol L^{-1}. Data are mean ± standard error of four independent experiments with three replicates.

7 days onward Fe-deprived conditions resulted in a rapid development of leaf chlorosis.

We investigated if leaf chlorosis was associated with reduced plant photosynthetic capacity. There was a statistically significant reduction of photosynthetic activity in S-deprived plants, but surprisingly no significant difference was found between plants grown at 60 and 1200 μmol L^{-1} sulfate. In addition, we found that Fe starvation reduced photosynthetic rate in S-limited and S-sufficient plants by almost the same extent.

Data show that the imposition of an intermediate sulfate supply (60 μmol L^{-1}) did not result in the appearance of the most common S deficiency symptoms (reduction in chlorophyll content and in photosynthetic capacity), which suggests that these plants were able to grow in a nearly optimal way at a lower S nutritional level.

Data reported in Figure 3 reveal a relationship between S availability and leaf Fe content. Increased availability of S in the growth medium caused a significant increase in leaf Fe content in Fe-sufficient plants. This result is consistent with previous reports. Bouranis *et al.* (2003) showed that S-deficient maize plants presented lower values of leaf Fe concentration compared with S-sufficient plants. Similar results were reported in our previous work (Astolfi *et al.* 2003), which showed that leaf Fe content was lower in maize plants grown in S deficiency than in corresponding plants grown in the presence of the macronutrient (+S). The effect of S deprivation on Fe content might be explained, at least in part, by considering that grasses utilize Strategy II for Fe acquisition and that methionine is required in PSs biosynthetic pathway (Mori & Nishizawa 1987). Thus, any reduction in S availability could result in a reduced methionine pool and PSs production. The above considerations are further supported by the analysis of the amount of PSs released from plants under different S availability. Data from the present study show that only severe S deprivation will limit PSs release and probably affect Fe mobilization by PSs.

Interestingly, it has been previously shown that the availability of sulfate in Fe-deficient growth medium can also affect accumulation (Kuwajima & Kawai 1997) of PSs and incorporation of ^{14}C of glucose into PSs in Fe-deficient barley roots. Complementary analyses of PSs concentrations in the root tissue are underway to clarify the question of whether under severe S deficiency, PSs release is limited by reduced production of PSs and/or by impairment of the PSs export mechanism.

It is unlikely that lower PSs release in S-deficient plants is limited by carbohydrate supply. In fact, data from the total sugars analysis show that sugar concentrations are mainly affected in the shoot tissues. In contrast, sugar levels were almost unaffected by both S and Fe deficiency in roots where PSs are effectively produced (Nakanishi *et al.* 1999). This might be explained considering that Fe deficiency reduced the shoot to root ratio. As a result, a decrease in sugar concentrations in leaves could be in support of increased root growth under Fe-deficient conditions. This is consistent with previous studies (Koch *et al.* 1996; Thoiron & Briat 1999) that have shown a correlation between Fe deficiency and an increase in root sugar levels, probably driven by reorientation of photoassimilate partitioning in favor of roots. Therefore, it is possible that a decrease in leaf biomass could lead to an adjustment in photosynthetic activity rate, producing a decrease in leaf sugar concentrations. This result differs from that reported by Thoiron and Briat (1999), who showed that leaf sucrose content remained unchanged under Fe deficiency. However, other authors (Arulanantham *et al.* 1990) recorded a decrease in sucrose accumulation in leaves of sugar beet plants under moderate or severe Fe deficiency.

Finally, we found that an intermediate supply of S (60 μmol L^{-1} sulfate), which did not affect either plant biomass production or photosynthesis, reduced the uptake rate of Fe (–40% and –25% with and without PS, respectively) with respect to the S-optimal condition. This

finding cannot be attributed to limitation of photosynthesis or the availability of carbohydrates because the photosynthetic capacity of Fe-deficient plants was independent of S application and intracellular sugar concentrations even increased with declining S supply.

The results from the present study support the view that the ability to cope with Fe deficiency in Strategy II plants is modified by S nutrition. In particular, S availability can affect the Fe nutritional status at multiple control levels, influencing either the release of PSs or the capability to take up Fe from the external solution. Further investigations are attempting to elucidate this strong relationship.

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