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BRIEF COMMUNICATION

Sufficient sulfur supply promotes seedling growth, alleviates oxidation stress, and regulates iron uptake and translocation in rice

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Abstract

We investigated the effect of sulfur (S) supply on growth, oxidative stress, and iron uptake and transport in rice (*Oryza sativa* L.) seedlings using a hydroponic culture with four S concentrations (0, 1.75, 3.5, and 7.0 mM). The length and fresh mass of seedlings were enhanced with the increased S concentration. In addition, the content of thiobarbituric acid reactive substances (TBARS) in rice leaves was the highest when no S was added to the nutrition solution and gradually declined with the increasing S supply. The higher S nutrition reduced the amount of Fe plaque on rice roots and increased Fe content in roots and leaves. The content of nicotianamine was significantly higher in rice roots under the S deficiency, whereas the reverse trend was observed in rice shoots. Taken together, the sufficient S nutrition promoted growth of rice, reduced oxidative stress, and ensured normal Fe uptake and distribution.

Additional key words: nicotianamine, *Oryza sativa*, TBARS.

Iron is essential element for plant growth, but its solubility is generally low in upland soils, making it scarcely available to plant roots. In such situations, plants have developed specialized systems to optimize Fe uptake either by expression of ferric-chelate reductase (strategy I) or by secretion of phytosiderophore (strategy II) from roots (Morrissey and Guerinot 2009, Curie and Briat 2003). On the other hand, flooding of paddy fields often leads to a reductive dissolution of Fe oxides and release of ferrous ions to the soil solution, thus Fe availability may be significantly different from upland soils. In order to avoid oxidative stress caused by Fe^{2+} accumulation, rice roots release oxygen to the rhizosphere resulting in precipitation of iron oxide/ hydroxide on the root surface (Chen *et al.* 2006). Absorption and transport of Fe in plants is tightly regulated to avoid harmful effects by moving it symplastically in the form of Fe-nicotianamine (NA) through the interconnected cytoplasm of roots and shoots (Hell and Stephan 2003).

Another important plant nutrient S is contained in a variety of cellular components and plays critical roles in a number of cellular processes such as redox cycles, detoxification of heavy metals and xenobiotics, and metabolism of secondary products (Hell 1997, Leustek *et al.* 1999). Recent studies showed that both S deficiency and S overabundance are present in soils due to various S inputs from fertilizer applications, atmospheric deposition, and anti-pollution measures (Wang *et al.* 2004, Nziguheba *et al.* 2005, Zhou *et al.* 2005). In addition, during rice growth, paddy fields are alternately flooded and drained making redox conditions fluctuate, and S is cycled between sulfate (SO_4^{2-}) and sulfide (S^{2-}) (Hu and Xu 2002). These situations may affect

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Abbreviations: Cys - cysteine; NA - nicotianamine; SIM - selective ion monitor; TBA - thiobarbituric acid; TBARS - TBA reactive substances; TCA - trichloroacetic acid; UPLC - ultra performance liquid chromatography.

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availability of other elements including Fe (Morrissey and Guerinot 2009, Fan *et al.* 2010).

In recent years, remarkable progress has been made in understanding interactions between S and Fe, especially the impact of S on the mobility of Fe in plants (Masuda *et al.* 2008, Wirth *et al.* 2009, Zuchi *et al.* 2012). Sulfur in soil may affect Fe uptake in rice through the following three ways: *1*) it can regulate formation of Fe plaque on the root surface (Fan *et al.* 2010, Gao *et al.* 2010), *2*) it can influence Fe uptake (Howeler 1973), and *3*) it can influence formation of phytosiderophore (Marschner and Marschner 2012). Sulfur can increase Fe transport in the xylem and phloem (Na and Salt 2011) as well as accelerate activation of deposited Fe in the apoplast (Toulon *et al.* 1992). In addition, methionine is precursor of NA that appears to play a role in trace element homeostasis in plants. Nicotianamine does not contain S, but its biosynthesis can be affected by the content of cysteine which contains the thiol group (Na and Salt 2011). Researchers have demonstrated that NA is strongly linked with Fe transport in the rice phloem (Inoue *et al.* 2008, Lee *et al.* 2009). However, how S affects NA synthesis, Fe transport, oxidative stress and growth inhibition in rice still needs to be explored.

The objective of this study was to investigate the impact of S on rice growth, oxidative stress, and Fe distribution. In addition, we measured NA content in various parts of rice to confirm its impact on Fe transport.

Seeds of rice (*Oryza sativa* L. cv. N07-63) were sterilized with 3 % (m/v) NaClO for 10 min, rinsed, and germinated in an incubator in the dark. When the second leaves emerged, the seedlings were transferred into a greenhouse and cultured in a basic solution [mM]: $(NH_4)_2 \cdot SO_4$ 1.429, KH_2PO_4 0.3228, K_2SO_4 0.3506, $CaCl_2$ 0.9982, $MgSO_4 . 7\,H_2O$ 1.649, $MnCl_2 . 4\,H_2O$ 0.0101, $(NH_4)_6 \cdot Mo_7O_{24} . 4\,H_2O$ 8.091×10^{-5}, H_3BO_3 0.0180, $ZnSO_4 . 7\,H_2O$ 3.484×10^{-4}, $CuSO_4 . 5\,H_2O$ 0.0004, $FeSO_4 . 7\,H_2O$ 0.0349, $Na_2EDTA . 2\,H_2O$ 0.0396, $Na_2SiO_3 . 9\,H_2O$ 0.0001. An S concentration in this culture was 3.5 mM and it was considered as normal S supply. The seedlings were maintained at a 12-h photoperiod, an irradiance of 65.2 μmol m^{-2} s^{-1}, day/night temperatures of 32 ± 1/27 ± 1 °C, and an air humidity of 60 %.

After the third leaf fully expanded, the rice seedlings were transplanted to PVC containers with 4.5 dm^3 of the culture solution (pH 5.5 - 5.6) to grow for 7 d. The solution was renewed every two days during the whole experiment. The iron plaque was induced on rice roots by adding $FeCl_2 . 4\,H_2O$ to reach an Fe^{2+} concentration of 30.0 mg dm^{-3} which was maintained for 6 d (Chen *et al.* 2006). Then various amounts of S (as Na_2SO_4) and other chemicals were added to corresponding containers to form four treatments: S-deficiency (0 mM), S-lower (1.75 mM) than the normal concentration, S-normal concentration (3.5 mM, control), and S-higher (7.0 mM) than the normal concentration. For treatment without S, K_2SO_4, $FeSO_4$, $MgSO_4$, $ZnSO_4$, and $CuSO_4$ were replaced by appropriate amounts of KCl, $FeCl_2$, $MgCl_2$, $ZnCl_2$, and $CuCl_2$ (Zuchi *et al.* 2012) and $(NH_4)_2SO_4$ was replaced by an appropriate quantity of $Ca(NO_3)_2 . 4\,H_2O$, and no $CaCl_2$ was added in the nutrient solution. A preliminary study showed that replacing NH_4^+ with NO_3^- does not affect rice growth (data not shown). For the treatments of the S-lower and S-higher concentrations, S was added as Na_2SO_4. The rice seedlings were harvested one week later, thoroughly washed with tap water, soaked in 0.2 % (m/v) Na_2EDTA for 2 h to remove adsorbed metals on the root surfaces (He *et al.* 2007), completely rinsed with deionized water, and blotted dry with paper. The rice samples were separated into leaves, stems, and roots. One part of the samples was frozen in liquid nitrogen and stored at -20 °C for further biochemical analysis. The other part of the samples was oven-dried at 90 °C for 15 min and kept at 70 °C for 24 h to obtain a constant dry mass for subsequent Fe analysis.

Lengths and fresh masses of rice shoots and roots were measured following harvest. A 0.1 g dry sample was weighed for microwave digestion in 7 cm^3 of HNO_3. Iron content was determined by atomic absorption spectroscopy (AAS; *Hitachi z-2000*, Tokyo, Japan). The acidity of the nutrient solution was analyzed using a pH meter (*PB-21*, *Sartorius*, Goettingen, Germany) once per day before the next renewal.

Content of TBARS was measured by the method of Heath and Packer (1968). A 1.0 g fresh rice sample was ground on ice with 4.8 cm^3 of 5 % (m/v) trichloroacetic acid (TCA). The mixture was centrifuged at 12 000 *g* for 15 min and the supernatant was brought to a volume of 10 cm^3. A 2 cm^3 of supernatant was mixed with the same volume of a 0.5 % (m/v) thiobarbituric acid (TBA) solution in 10 % TCA. The mixture was heated at 95 °C for 30 min and then quickly cooled in ice. Absorbances of the reaction mixture were monitored at 532, 600, and 450 nm (at 25 °C).

At harvest, the iron plaque deposited on the root surface was extracted using the dithionite-citrate-carbonate method of Claff *et al* (2010). Fresh roots were incubated for 2 h at 20 °C in 80 cm^3 of a solution (pH 6.5) containing 0.03 M sodium citrate and 0.125 M sodium bicarbonate, with the addition of 1.6 g of sodium dithionite. After filtration, the extraction solution was analyzed for Fe as described above.

The samples (0.20 g) previously stored in the dark at -20 °C were ground in liquid N_2, and NA was extracted using 0.3cm^3 of ultrapure water at 80 °C for 30 min and then centrifuged at 13 400 *g* for 10 min. Calibration curves of NA standards were prepared daily at 1, 10, 50, 100, 500 nM concentrations using NA stock solutions (kindly given by Prof. Luqing Zheng). All the supernatants were filtered through 0.22 μm nylon membranes (*King-ber*, Tianjin, China). A liquid chromatography/mass spectrometry (LC/MS) system consisted of an ultra performance liquid chromatography

(UPLC; *Dionex*, *Thermo*, IL, USA) coupled with an *LTQ Orbitrap XL* mass spectrometer (*ThermoFisher Scientific*, Massachusetts, USA). Instrument control was through *Tune 2.6.0* and *Cheomeleon* programs. A UPLC column utilized was *Hypersil GOLD C18* (100 × 2.1 mm, 3 µm particle sizes; *ThermoFisher Scientific*). A UPLC mobile phase A was 0.1 % (v/v) formic acid in water, and B was 100 % acetonitrile. Separation was carried out in 6.0 min at a flow rate of 0.2 $cm^3 \cdot min^{-1}$ under the following conditions: 0 - 1.5 min, 2.0 % B; 1.5 - 2.5 min, 95.0 % B; 3.0 - 3.1 min, 2 % B; 3.1 - 6.0 min 2 % B. A column oven temperature was set at 35 °C, and an auto sampler temperature was set at 10 °C. An injection volume was 5×10^{-6} dm^3. The ion source was equipped with an electrospray ionization probe. A capillary temperature was 300 °C, and a source voltage was set at 4 kV. The sheath gas was set at 35 arb, the aux gas was set at 10 arb. Accurate mass spectra were recorded using a selective ion monitor (SIM) mode at an m/z value of 304.14. External mass calibration of the *Orbitrap* was performed once a week to ensure an accuracy < 5 µg dm^{-3}. Data were processed using the *Xcalibur* software.

The *PASW Statistics v.17.0* (*SPSS*, Chicago, USA) and *SigmaPlot* were used for statistical analyses and figure drawing, respectively. One-way *ANOVA* and Duncan's test were used to detect significant ($P < 0.05$) effects of the S treatments on the Fe, NA, and TBARS content in the shoot and root tissues.

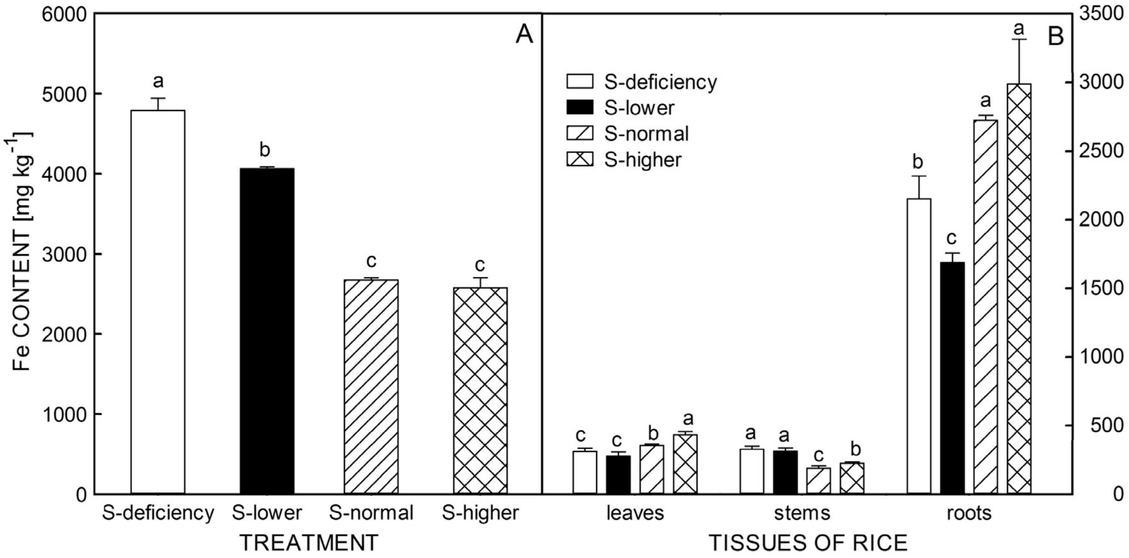

Fig. 1. The content of Fe in iron plaque (*A*) and in tissues of rice plants (*B*) grown in nutrient solutions at four different S concentrations (S-deficiency, 0 mM; S-lower, 1.75 mM; S-normal, 3.5 mM; and S-higher, 7.0 mM, respectively). Means ± SE, $n = 3$. Different letters indicate significant differences among treatments ($P < 0.05$).

Rice growth was negatively affected when S supply was not adequate, and 0 and 1.75 mM S caused a significant decrease in shoot length and fresh mass, whereas doubled S (7.0 mM) stimulated rice growth (Fig. 1 Suppl.).

In order to understand how S supply affected acidity of the nutrient solution and so Fe absorption, we measured the culture pH and found that it decreased across all the treatments, but the rates were higher in the normal and higher S treatments, dropping from 5.52 ± 0.01 to 3.84 ± 0.03 in the normal S in 3 d. The acidification of the growth solution was much lesser in the case of the S deficiency and lower S. The solution pH decreased from 5.52 ± 0.03 to 4.97 ± 0.01 at 0 mM S and from 5.53 ± 0.04 to 4.57 ± 0.02 at 1.75 mM S (Fig. 2 Suppl.).

The oxidative stress (the content of TBARS) was higher in leaves than in stems and roots. The most severe oxidative stress was observed under the S deficiency, which resulted in 79.4, 33.2, and 25.7 % increases in TBARS content in leaves, stems, and roots, respectively, compared to the control (Fig. 3 Suppl). The TBARS content in rice leaves and roots decreased as S supply increased.

Iron was distributed on the root surface as well as in the tissues of the root, stem and leaf. The quantity of iron plaque at 0 mM S was the highest, about 1.8-times higher than at 3.5 mM S (Fig. 1*A*). Increasing S in the solution inhibited Fe adsorption on the surface of rice roots but promoted its uptake by roots except for the 1.75 mM S treatment (Fig. 1*B*). However, although the Fe content of roots was higher when more S was added, translocation of Fe from roots to shoots did not increase. On the contrary, the translocation factor (Fe content in shoots/Fe content in roots) decreased from 29.8 to 22.1 % with the increase of S supply.

The NA content in rice roots and shoots showed contrasting trends with the different S supplies. Significantly more NA was synthesized in the deficient and lower S treatments (0 and 1.75 mM) in rice roots (Fig. 2*C*). In rice leaves and stems, however, the NA content was higher when S in the growth medium was 3.5 mM or higher (Fig. 2*A*,*B*).

In graminaceous plants, an adequate S nutrition is not required only for biomass production but also for Fe uptake and assimilation (Hell and Stephan 2003, Zuchi *et al.* 2012). However, previous studies were focused on Fe deficiency responses (*e.g.*, Zuchi *et al.* 2012). The effects of S nutrition on Fe accumulation remain unclear in rice when Fe is in excess, which often occurs in flooded paddy soils due to dissolution of Fe oxides. Thus in the present study, we investigated the effect of different S supplies on Fe absorption and distribution in rice.

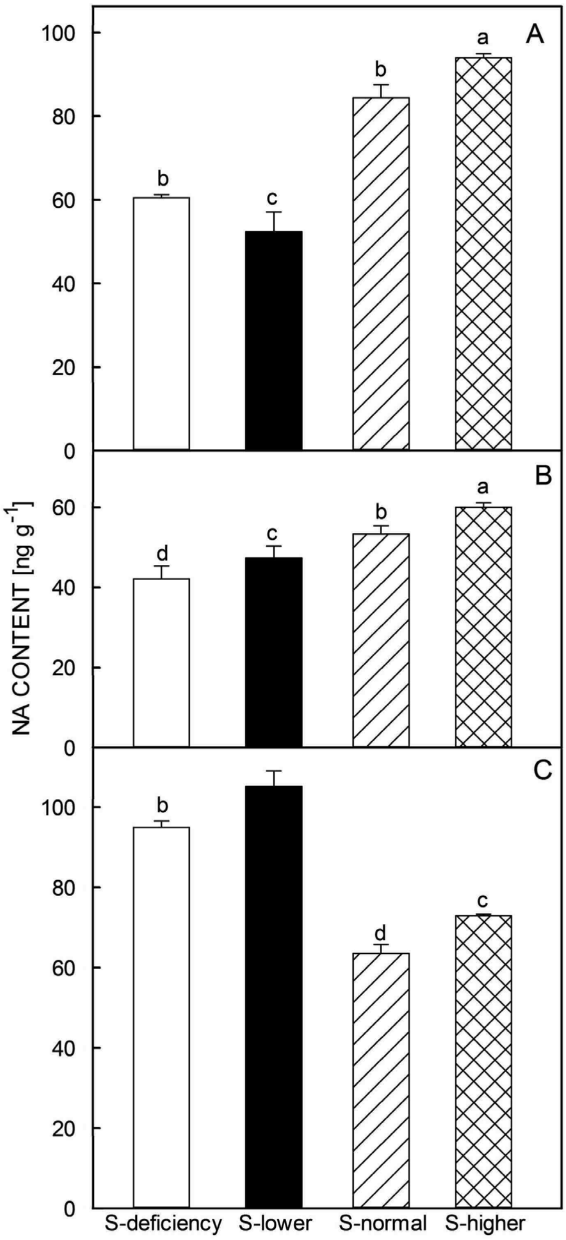

Fig. 2. The nicotianamine content in leaves (*A*), stems (*B*), and roots (*C*) of rice grown in nutrient solutions at four different S concentrations (S-deficiency, 0 mM; S-lower, 1.75 mM; S-normal, 3.5 mM; and S-higher, 7.0 mM, respectively). Means ± SE, *n* = 3. Different letters indicate significant differences among the treatments ($P < 0.05$).

The growth of rice was clearly impaired by the S deficiency (0 and 1.75 mM) with a significantly lower fresh mass and length of seedlings than under the 3.5 mM S (Fig. 1 Suppl.). When S supply was low, the oxidative stress was also more severe in all the parts of rice, especially in leaves (Fig. 3 Suppl.). These results are in accordance with plant responses to S deficiency reported in the literature (Robinson 1994). The S application at 7.0 mM further enhanced the rice growth (Fig. 1 Suppl.) and reduced the TBARS content (Fig. 3 Suppl.) suggesting that the higher S might be beneficial for rice. These findings are similar with results reported by Wu *et al.* (2014) who showed that sulfur supply increases dry masses of roots, stems, leaves, and grains of rice.

In this paper, we also found that the S nutrition was pivotal for an optimal Fe uptake and distribution in rice. Firstly, the S deficiency resulted in buildup of Fe plaque on the rice roots leading to a lower Fe content in the roots and leaves (Fig. 1*B*). It has been observed that the symplastic movement of Fe is assisted by NA in plant roots, thus when Fe requirement in rice shoots becomes strong in the case of S deficiency, more NA is synthesized in roots to make the Fe more mobile (Hell and Stephan 2003). The increased S supply also affected the pH of the nutrient solution. Replacement of NH_4^+ with NO_3^- at the zero S supply slowed down the acidification of the nutrient solution (Fig. 2 Suppl.). The low pH might lead to a partial dissolution of the Fe plaque on the root surface (Fig. 1*A*) and a subsequent higher Fe uptake (Fig. 1*B*). It was also found that Fe uptake is significantly enhanced in wheat receiving S (Zuchi *et al.* 2012). Nevertheless, their results were obtained under the conditions of Fe deficiency. Therefore, an adequate S nutrition is needed for the normal Fe absorption in rice and this is independent of Fe supply.

The variations of NA in rice roots and shoots followed contrasting trends (Fig. 2). In the shoots, NA synthesis was promoted by the S application. In addition, the quantities of Fe and NA were significantly correlated in leaves ($r = 0.905$, $P < 0.01$, $n = 12$). This is necessary because rice leaves are much more sensitive to oxidative stress than roots (Fig. 3 Suppl.), so in order to avoid an increase of membrane peroxidation (represented by TBARS), Fe is complexed with chelators such as NA in rice (Rellán-Álvarez *et al.* 2008). In the roots, however, the amount of NA was significantly higher under the S deficiency (Fig. 2). Interestingly, the content of NA was also significantly correlated with the translocation factor of Fe ($r = 0.925$, $P < 0.01$, $n = 12$) suggesting that NA synthesis in rice roots was induced by the

S deficiency to facilitate Fe transport towards shoots to meet the Fe requirement in rice.

In conclusion, this study demonstrates that when Fe was sufficient in the nutrient solution, an adequate S supply promoted rice growth, reduced oxidative stress, and regulated Fe uptake and translocation in rice through NA synthesis. More studies are needed to determine the details of NA generation, the quantity of Fe-NA complexes under various S concentrations, and their relations with Fe mobility in rice.

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