# Sodium hydrosulfide modifies the nutrient ratios of soybean (*Glycine max*) under iron deficiency

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## Abstract

Iron (Fe) deficiency in calcareous soils is a major limiting factor which influences production and yield of field crops. The present study investigated the effect of NaHS, a donor of $H_2S$, which is emerging as a potential signaling molecule, on the nutrient ratios of soybean (*Glycine max* L.) under Fe deficiency. Soybean seedlings with and without NaHS were subjected to Fe deficiency and Fe sufficiency for 18 d. Subsequently, we determined the biomass of seedlings, chlorophyll concentration, Fe concentration, as well as the ratios of carbon (C), nitrogen (N), phosphorus (P), and potassium (K). The growth of soybean seedlings was inhibited by Fe deficiency. However, under Fe deficiency the application of NaHS increased the biomass as well as the Fe, N, P, and K concentrations compared to the controls. Furthermore, our results also show that the application of NaHS affected the ratios of C : N, C : P, C : K, N : P, N : K, and P : K in soybean seedlings under Fe deficiency and sufficiency. $H_2S$ played an important role in promoting the growth of soybean seedlings by enhancing the accumulation of nutrients under Fe deficiency.

**Key words:** *Glycine max* / nitrogen / nutrient limitation / phosphorus / potassium

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## 1 Introduction

Iron (Fe) is an important essential element, which plays a key role in the fundamental processes of plant physiology, such as photosynthesis and chlorophyll biosynthesis. Frequently, plants exhibit yellowing of the new leaves and interveinal chlorosis, inhibited nutrient absorption, resulting in nutrient metabolic disorders (*e.g.*, C, N, and P), and inhibition of plant growth, because Fe is unavailable in soils (*Jeong* and *Connolly*, 2009; *Santos* et al., 2015). Calcareous soils are predominant in many agricultural areas. Therefore, Fe availability has become one of the main factors that limit the production of field crops (*Rodríguez-Lucena* et al., 2010). Moreover, it is estimated that dietary iron deficiency can affect 30–60% of the world's population (*Graham* et al., 2001). Especially for Asian and African countries, iron deficiency often results in maternal death, because many people in these areas depend on plants for their daily iron supply (*Graham* et al., 2001). Therefore, strategies are being developed to fortify major food crops with Fe to improve the demand of the population (*White* and *Broadley*, 2009; *White* and *Brown*, 2010). Increasing the ability of plants to take up Fe under adverse conditions can solve those problems. Thus, understanding how plants adapt to Fe deficiency can bring new strategies to maintain normal growth and development of crops.

It is well known that carbon (C), nitrogen (N), phosphorus (P), and potassium (K) are the principal chemical elements for the growth and development of plant, and these elements play a vital role in plant functions (*Ågren*, 2008; *Güsewell*, 2004; *Cernusak* et al., 2010; *Fan* et al., 2015). Carbon is the most important constituent of the plant structure and about 50% of the dry weight of plant comes from C. N is a component of proteins, which plays a vital role in all enzyme activities. P is involved in the production and transfer of energy in plant cells (*Ågren*, 2004, 2008; *Pugnaire*, 2001). Additionally, P and N are the most important structural elements in nucleic acids (*Ågren*, 2008). These three elements of C, N and P have strong intrinsic coupling on the basis of their biochemical and physiological functions in plants. Moreover, the growth and development of plants require relatively large amounts of N and P. Therefore, N and P are macronutrients and cannot be replaced by other chemical elements in the process of plant metabolism (*Ågren*, 2008). Additionally, *Aerts* and *Chapin III* (1999) reported that the stoichiometry of C : N : P can reflect the growth rate of a plant. Moreover, the ratios of C : N, N : P, and C : P also could indicate the growth status of plants and corresponding metabolic conditions (*Ågren*, 2008; *Song* et al., 2014). For example, previous studies showed that C : N and C : P can effectively reflect the growth status and health conditions of plants, and there were negative correlations be-

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tween the growth rates of plants and ratios of C : N and/or C : P (*Elser* et al., 2000; *Güsewell*, 2004). During plant growth, the change of the N : P ratio in leaves is deemed to be closely related to the limitation of nutrients and that it might change the species competitiveness relying on the growth rate and lifestyle of plants (*Elser* et al., 2000; *Güsewell*, 2004). Moreover, when simultaneously absorbing N and P, the ratios of C : N and C : P reflect the ability of plants to take up C, and the ratio of N : P represents the dynamic equilibrium between plant nutrition demands and soil nutrients (*Elser* et al., 1996; *Güsewell*, 2004; *Elser* and *Hamilton*, 2007). In addition, K plays an important role in regulating the plant water economy by affecting stomatal function and osmotic control (*Babita* et al., 2010). Moreover, K can increase the intensity of photosynthesis, promote the formation of starch and sugar in plants, enhance the crop resistance to biotic and abiotic stresses, and improve the absorption and utilization of N (*Sangakkara* et al., 2000; *Babita* et al., 2010; *Rivas-Ubach* et al., 2012). However, the effects of Fe deficiency on the ratios of C, N, P, and K in plants are not well explained. Therefore, studies on plant growth strategies and their adaptability to Fe deficiency in the environment are required.

In plants, a large number of nutrients of senescing tissues can be removed and transported to other tissues (*Yang* et al., 2016). Moreover, different leave blades have different biological functions in plants (*Yan* et al., 2014). Old leaves can export nutrients to new leaves that can use these nutrients required for plant growth and development. However, few study focused on the nutrient ratios of plants, especially for different leave blades under environmental stress conditions.

Hydrogen sulfide ($H_2S$) is a biologically active gaseous molecule, which has become a hot topic of interest in plant research. Recently, $H_2S$ has received increasing attention, because $H_2S$ at lower concentrations plays an important role in plant response to various biotic and abiotic stresses. For instance, it was reported that boron (B), salinity, aluminum (Al), chromium (Cr), copper (Cu), Zinc (Zn), and cadmium (Cd) toxicities were alleviated by a low concentration of $H_2S$ in plants (*Wang* et al., 2010; *Zhang* et al., 2010; *Dawood* et al., 2012; *Chen* et al., 2013; *Sun* et al., 2013; *Liu* et al., 2016). Our previous study showed that $H_2S$ could improve the adaptation of *Zea mays* seedlings to Fe deficiency through regulating Fe uptake, transport, and accumulation of plant tissues (*Chen* et al., 2015b). More importantly, our previous study also revealed that $H_2S$ could enhance the photosynthesis and growth of plants through promoting chloroplast biogenesis, the expression of enzymes involved in photosynthesis, and thiol redox modification in *Spinacia oleracea* seedlings (*Chen* et al., 2011). Recently, *Jin* et al. (2017) reported that $H_2S$ mediated $K^+$, $Ca^{2+}$, and $Cl^-$ ion fluxes inducing stomatal closure in response to drought stress in *Arabidopsis thaliana*. However, the impact of $H_2S$ on the absorption of nutrients and their ratios in plants grown under Fe deficiency was not clear and the work in this area has so far been very limited.

In order to understand the effects of $H_2S$ and Fe deficiency on the nutrient ratios of plants, we selected soybean (*Glycine max* L.) plant, which is sensitive to Fe deficiency, as an experimental plant. In the present study, we hypothesized that (1) Fe deficiency results in differences of the C, N, P, and K concentrations, as well as the ratios of C : N, C : P, N : P, C : K, N : K, and P : K in soybean seedlings; (2) Fe deficiency could significantly influence the Fe concentrations of leaves, stems, and roots of soybean seedlings; (3) $H_2S$ regulates the response of plant nutrient ratios to Fe deficiency. The main purpose of our study was to determine how $H_2S$ improves the adaptation of soybean seedlings to Fe deficiency and what is the related mechanism?

## 2 Material and methods

### 2.1 Plant growth and treatments

Seeds of soybean (*Glycine max* L.) were sterilized with 75% alcohol for 30 s and then with 10% sodium hypochlorite for 4 min, and finally rinsed six times with distilled water. The seeds were germinated in 1 : 1 mixture of vermiculite and soil. Ten days days after germination, plants were transferred from soil to the culture solution in plastic containers which can accommodate six soybean seedlings. The composition of nutrient solution was as following: 1 mM $KNO_3$, 0.5 mM $MgSO_4$, 0.25 mM $KH_2PO_4$, and 0.5 mM $Ca(NO_3)_2$; 10 μM $H_3PO_4$, 0.5 μM $MnSO_4$, 0.5 μM $ZnSO_4$, 0.1 μM $(NH_4)_6Mo_7O_{24}$, 0.1 μM $CuSO_4$, and 50 μM Fe(III)-EDTA. The pH of the nutrient solution was 5.8 and the nutrient solution was changed every 2 d. Plants were grown in a growth chamber with a relative humidity of 80%, light/dark regime of 15/9 h, a photosynthetically active radiation (PAR) of 190 μmol $m^{-2}$ $s^{-1}$, and temperature of 21/27°C (night/day).

Soybean seedlings were grown for 7 d, then treated with the above nutrient solution containing 0.1 or 50 μM Fe(III)-EDTA, respectively. Each treatment was run without or with 100 μM NaHS and included six replications (pots). To ensure that the Fe concentration of the nutrient solution was maintained, the solution was changed every second day. After 18 d all the samples were harvested.

### 2.2 Sample collection

The collection of all the samples from all the groups was as following: firstly, plants were taken from the solution and then rinsed three times with distilled water. Subsequently, as shown in Fig. 1, all the samples were divided for three parts including leaves, stems and roots. Leaves included leaf blade 1, 2, and 3, and stems included stem 1, 2, and 3 in our experiments (Fig. 1). Finally, these samples were placed in an oven and the temperature was set to 80°C for 3 d until a constant weight was reached. Leaves, stems, and roots were ground finely for analyzing elemental concentrations.

### 2.3 Measurements of shoot length, root length, and biomass

The shoot length and root length were measured with a ruler. The biomass was the biomass of whole seedlings and measured with an analytical balance.

 

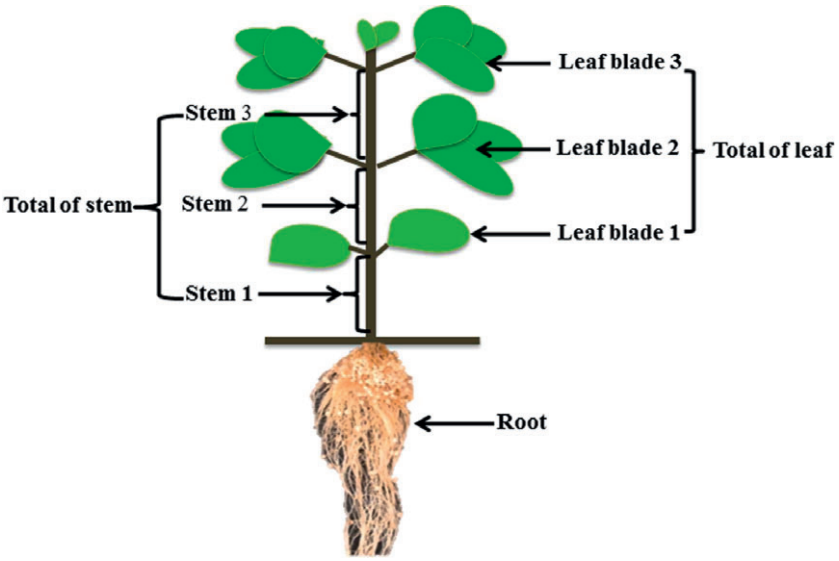


**Figure 1:** Schematic drawing of sample collection of leaves, stems, and roots of soybean seedlings grown with and without NaHS under different Fe supply. Leaves were divided into leaf blade 1, 2, and 3. Stems were divided into stem 1, 2, and 3.

### 2.4 Determination of chlorophyll concentration

The chlorophyll concentration was determined according to the method of *Lichtenthaler* (1987). Soybean leaves (0.2 g fresh weight) were powdered with liquid nitrogen and then 25 mL 80% (v/v) aqueous acetone were added into the cuvette. The chlorophyll was extracted in the dark for 48 h until completely bleached and shaken once every 30 min. The total chlorophyll concentration was calculated from the absorbance of leaf extracts at 470 nm, 646 nm, and 663 nm.

### 2.5 Determination of element concentrations

The concentration of total organic C was measured using the method of oil bath-$K_2CrO_7$ titration (*Nelson* and *Sommers*, 1982). Samples were oxidized with dichromate in the presence of $H_2SO_4$, and then heated for 5 min at 180°C, and finally titrated with $FeSO_4$.

Total N concentration was determined using Kjeldahl digestion by a Nitrogen Analyzer System (Kjeltec 8400 Auto System II, Foss Tecator AB, Höganäs, Sweden). Firstly, sample was digested using $H_2SO_4$ and then $NH_3$ was captured by $H_3BO_3$. Finally the reaction solution was titrated with HCl.

For the measurement of the total P, K, and Fe concentrations in plants, the $H_2SO_4$ : $H_2O_2$ (5 : 1) reagent mixture was used for digesting plant samples at 420°C in a digestion block. Additionally, the molybdate blue colorimetric method was used for measuring the total P concentration using a spectrophotometer (Mapada UV6300PC, Mapada, Shanghai). The concentrations of K and Fe were analyzed using an atomic absorption spectrophotometer (Hitachi Z2000, Hitachi, Japan; *Chen* et al., 2013). The C : N, C : P, C : K, N : P, N : K and P : K ratios in the leaves, stems, and roots of soybean seedlings were calculated using the values of C, N, P, and K from the samples.

### 2.6 Statistical analysis

Statistical analyses of the experimental data were calculated with the SPSS version 19.0 (SPSS, Inc., Chicago, IL). To analyze the effect of Fe deficiency on C, N, P, Fe, and K concentrations and ratios of C : N, C : P, C : K, N : P, N : K, and P : K, a two-way ANOVA was done. Significance of differences between +NaHS and –NaHS plants in all of the parameters were calculated using the independent *t*-test. The confidence level of the statistical significance was stipulated at 95%. Data are reported as means ± standard errors.

## 3 Results

### 3.1 Effect of $H_2S$ donor on plant growth and nutrient concentrations under Fe deficiency

Iron deficiency (0.1 μM Fe) significantly inhibited the growth of soybean seedlings compared with Fe sufficiency (50 μM Fe; Fig. 2A). The shoot length and root length were obviously decreased by Fe deficiency, but the application of NaHS could ameliorate Fe deficiency-induced inhibition of shoot length and root length (Fig. 2B, C). Moreover, under Fe sufficiency, NaHS also slightly increased the shoot length and root length of soybean seedlings (Fig. 2B, C). Iron deficiency significantly reduced the dry weight of soybean seedlings (Fig. 2D). However, the application of NaHS significantly alleviated the Fe deficiency-induced decrease of biomass of soybean seedlings, but had no effect on the biomass of plants under Fe sufficiency (Fig. 2D). We also found that the chlorophyll concentration under Fe deficiency was significantly decreased compared to Fe sufficiency (Fig. 2E). Similarly, the application of NaHS obviously promoted the iron deficiency-induced decrease of chlorophyll content in soybean seedlings (Fig. 2E).

Iron deficiency had significant effects on the organic C concentrations of stem and root samples, but no significant effect in leaves (Fig. 3A–C, Tab. 1). However, the C concentration was not influenced by the application of NaHS in either the leaves or stems, but showed a significant change in roots (Fig. 3A–C, Tab. 1). The C concentrations of leaves and stems were generally higher than those of roots (Fig. 3A–C). The N concentration was different from the C concentration and the N concentration went down in leaves as Fe concentrations decreased (Fig. 3D). Similarly, the N concentrations showed a significant decrease in roots under Fe deficiency compared to Fe sufficiency (Fig. 3F). The application of NaHS had an effect on the response of N concentration to Fe concentration in leaves and roots, especially for Fe deficiency (Fig. 3D–F, Tab. 1). In addition, for all treatments higher N concentrations of leaves and roots were found (Fig. 3D–F).

Similarly, the P concentration significantly decreased in leaves and stems under Fe deficiency (Fig. 3G, H, Tab. 1),

 

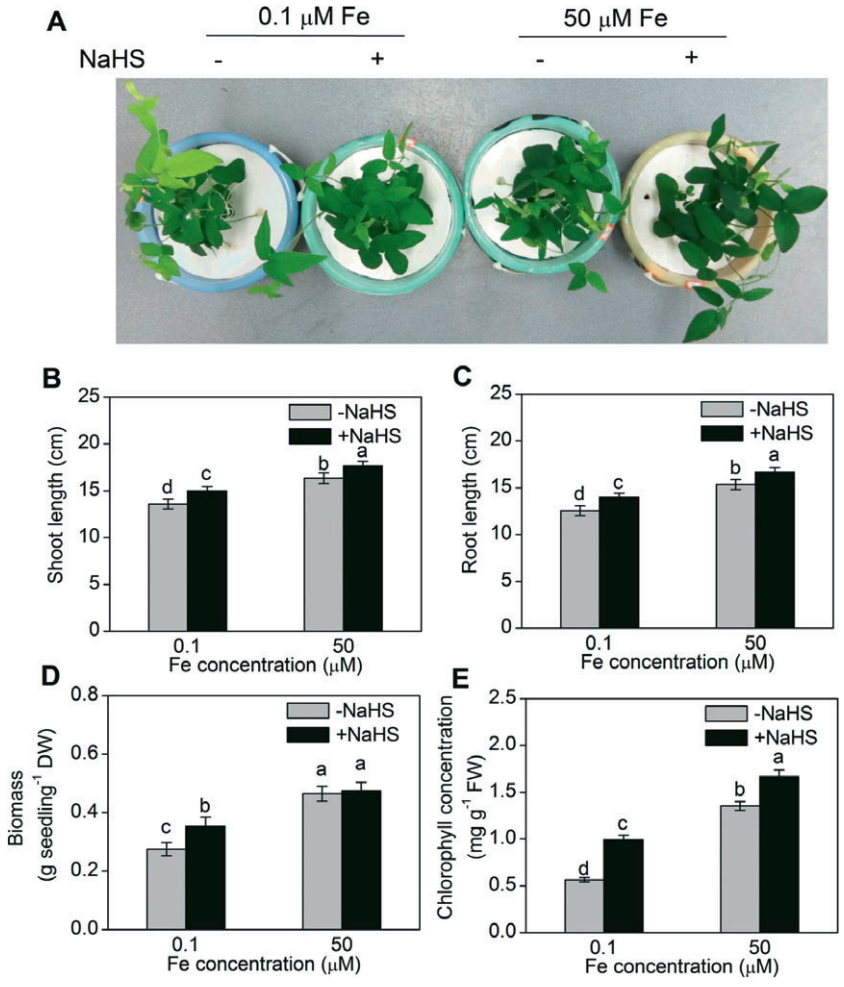


**Figure 2:** Photograph (A), shoot length (B), root length (C), biomass (D) and chlorophyll concentration (E) of soybean seedlings with and without NaHS under two Fe regimes. Each value represents the mean ± SE ($n$ = 10). Columns labeled with different letters indicate significant differences with P < 5%.

but the P concentration only showed a slight change in roots (Fig. 3I, Tab. 1). The application of NaHS significantly affected the P concentration of stems and roots (Fig. 3G–I, Tab. 1). Moreover, under Fe deficiency the NaHS-treated plants had higher P concentrations compared to the control plants (without NaHS; Fig. 3G–I). Conversely, under Fe sufficiency, the application of NaHS decreased the P concentrations of leaves and roots (Fig. 3G, I). Moreover, the P concentrations of leaves and stems were significantly lower than of roots. The K concentration showed different degrees of changes in leaves, stems, and roots under different Fe treatments (Fig. 3J–L, Tab. 1). Under Fe deficiency, the K concentration of the NaHS-treated plants significantly increased in leaves and roots (Fig. 3J, L). For all treatments, the K concentrations of leaves and stems were lower than of roots (Fig. 3J–L).

The results show that under Fe sufficiency the Fe concentrations of leaves, stems, and roots increased significantly compared to the low Fe concentration (Fig. 4A–C, Tab. 1). In addition, the application of NaHS significantly increased the Fe concentrations of leaves and roots under Fe deficiency (Fig. 4A, C), but no difference was found in stems (Fig. 4B). Moreover, under Fe sufficiency, the Fe concentrations of roots of NaHS-treated plants showed an increase compared to the control plants (without NaHS), but no significant change in leaves and stems (Fig. 4A–C). Additionally, the Fe concen-

**Table 1**: Results of two-way ANOVA for the effects of $H_2S$ (S) and Fe concentrations on C, N, P, K, and Fe concentrations of the leaves, stems, and roots of soybean seedlings.

| | Treatments | df | C concentrations | | N concentrations | | P concentrations | | K concentrations | | Fe concentrations | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | F value | *P* | F value | *P* | F value | *P* | F value | *P* | F value | *P* |
| Leaf | Fe | 1 | 0.754 | 0.405 | 216.976 | < 0.001 | 38.264 | < 0.001 | 5.97 | 0.031 | 135.448 | < 0.001 |
| | S | 1 | 0.156 | 0.7 | 56.672 | < 0.001 | 1.167 | 0.301 | 0.932 | 0.353 | 0.709 | 0.416 |
| | Fe×S | 1 | 3.839 | 0.074 | 17.498 | 0.001 | 10.416 | 0.007 | 3.399 | 0.09 | 5.019 | 0.045 |
| Stem | Fe | 1 | 7.907 | 0.016 | 1.874 | 0.196 | 19.22 | 0.001 | 0.795 | 0.39 | 48.376 | < 0.001 |
| | S | 1 | 0.214 | 0.652 | 1.593 | 0.231 | 9.254 | 0.01 | 4.392 | 0.058 | 1.775 | 0.208 |
| | Fe×S | 1 | 1.532 | 0.239 | 62.518 | < 0.001 | 0.994 | 0.339 | 2.996 | 0.109 | 0.385 | 0.546 |
| Root | Fe | 1 | 8.911 | 0.011 | 1.28 | 0.28 | 5.09 | 0.044 | 31.277 | < 0.001 | 109.092 | < 0.001 |
| | S | 1 | 6.958 | 0.022 | 13.92 | 0.003 | 9.532 | 0.009 | 4.78 | 0.049 | 21.466 | 0.001 |
| | Fe×S | 1 | 8.82 | 0.012 | 2.6 | 0.133 | 58.718 | < 0.001 | 4.819 | 0.049 | 41.78 | < 0.001 |

 

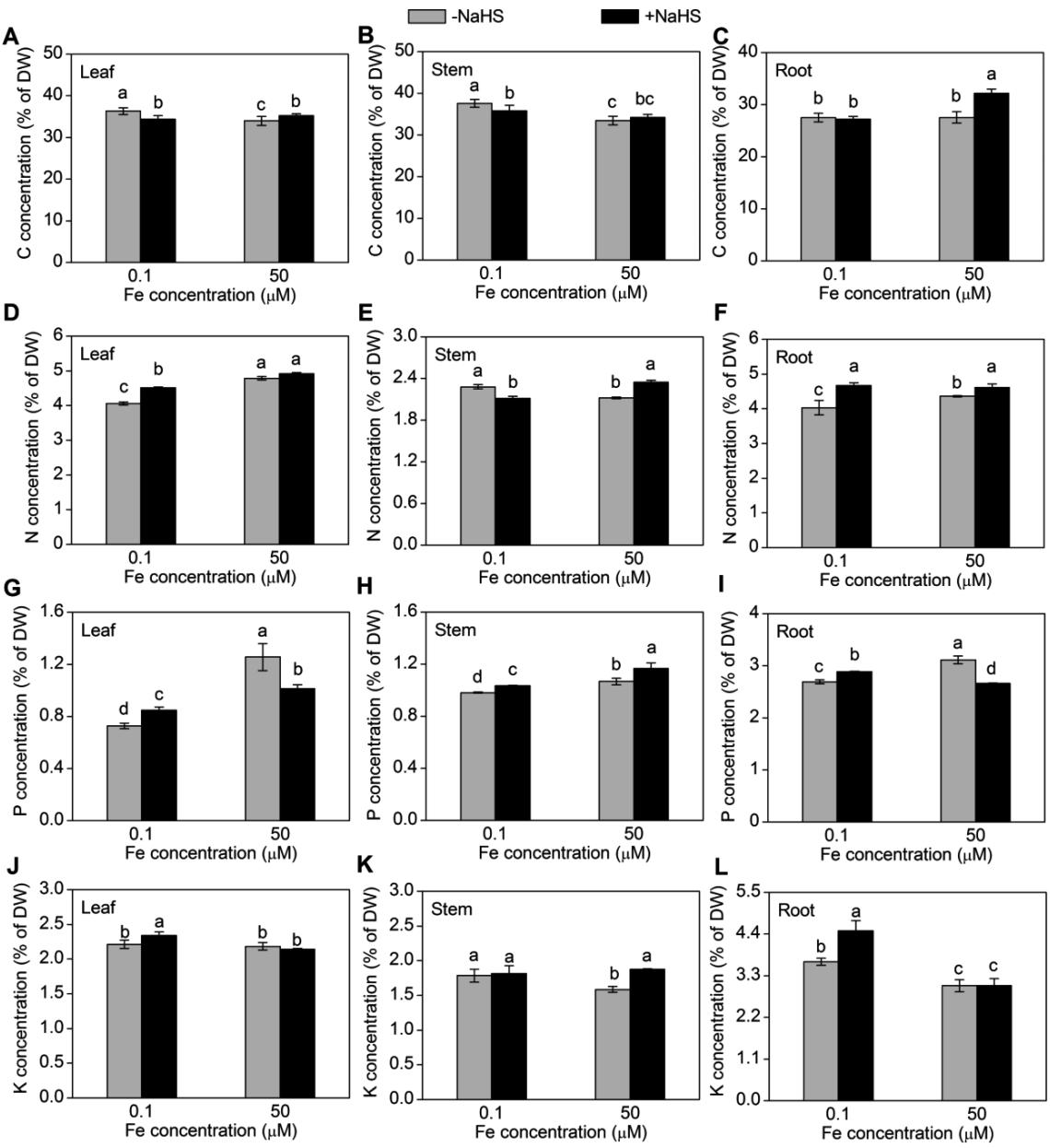


**Figure 3:** The C (A, B, C), N (D, E, F), P (G, H, I), and K (J, K, L) concentrations of leaves, stems, and roots of soybean seedlings with and without NaHS under two Fe regimes, respectively. Each value represents the mean ± SE ($n$ = 4). Columns labeled with different letters indicate significant differences with $P < 5\%$.

tration of leaves and stems was lower than in roots of soybean seedlings (Fig. 4A–C).

### 3.2 Effect of $H_2S$ donor on nutrient concentrations of different leaf blades and stem parts under Fe deficiency

To further study the effect of NaHS on nutrient balance under Fe deficiency, we analyzed the C, N, P, K, and Fe concentrations of different leaf blades and stem parts in soybean seedlings. Under Fe deficiency, the C concentrations of all leaf positions and stem parts showed no significant differences (Fig. 5A, B). In contrast, the C concentration showed a slight increase under Fe sufficiency (Fig. 5A, B). Moreover, except for the 50 μM Fe treatment, leaf blade 2 and stem 2 had a higher C concentration than other leaf positions and stem parts (Fig. 5A, B). The N concentrations under Fe deficiency showed significant increases in each leaf position of the NaHS-treated plants, and leaf blade 1 had a higher N concentration than leaf blade 2 and 3 (Fig. 5C). However, under Fe sufficiency, NaHS did not affect the N concentration of different leaf positions (Fig. 5C).

In contrast, the N concentrations of various stem parts showed different degrees of decrease in the NaHS-treated plants under low Fe concentration, but under high Fe concentration the N concentration showed increases, especially for

 

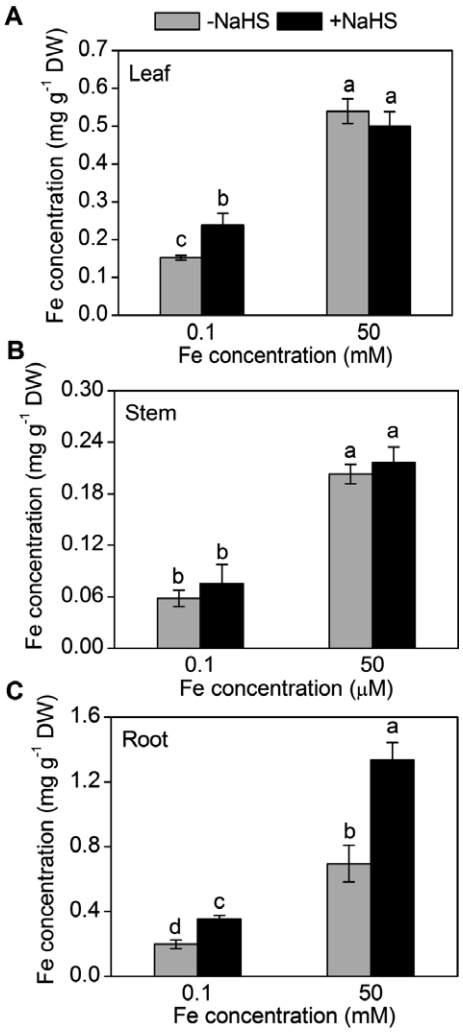


**Figure 4:** The Fe concentrations of leaves (A), stems (B), and roots (C) of soybean seedlings with and without NaHS under two Fe regimes, respectively. Each value represents the mean ± SE ($n$ = 4). Columns labeled with different letters indicate significant differences with P < 5%.

stem 1 (Fig. 5D). Interestingly, for all treatments the N concentration of stem 1 was higher than of stems 2 and 3 (Fig. 5D). The P concentrations of all leaf positions of the NaHS-treated plants grown with Fe deficiency solution showed slight increases compared to the control plants (without NaHS; Fig. 5E). Conversely, under Fe sufficiency, the P concentration of the NaHS-treated plant showed a slight decrease in the same leaf position (Fig. 5E). The application of NaHS increased the P concentration of stem 1 under Fe deficiency and sufficiency, and the P concentration of stems was 1 > 2 > 3 under all treatments except for the 50 μM Fe (Fig. 5F). The K concentration of leaves and stem was leaf 1 > 2 > 3 and stem 1 > 2 > 3 under Fe deficiency and sufficiency (Fig. 5G, H). NaHS significantly increased the K concentration of leaf 3 under Fe deficiency and also increased the K concentration of stem 3 under Fe sufficiency (Fig. 5G, H). Besides, for other treatments the change of K concentration was not significant (Fig. 5G, H). The Fe concentrations of leaves and stems were leaf 1 > 2 > 3 and stem 1 > 2 > 3 under Fe deficiency and sufficiency (Fig. 5I, J). Under Fe deficiency, the application of NaHS increased the Fe concentrations of leaf blade 1 and 2, but not of leaf blade 3 (Fig. 5I).

### 3.3 Effect of $H_2S$ donor on plant nutrient ratios under Fe deficiency

Iron deficiency significantly changed the C : N ratio in leaves and stems, but not in roots (Fig. 6A–C, Tab. 2). Under Fe deficiency, the application of NaHS significantly decreased the C : N ratio in leaves and roots, but not in stems (Fig. 6A–C, Tab. 2). However, under Fe sufficiency, the NaHS treatment had no obvious effect on the ratio of the C : N ratio in leaves, stems, and roots (Fig. 6A–C). The C : P ratio significantly declined with the increase of Fe concentration in leaves and stems, but not in roots (Fig. 6D, E, Tab. 2). In addition, under Fe deficiency, NaHS decreased the C : P ratio in leaves, stems, and roots (Fig. 6D–F). In contrast, under Fe sufficiency, the C : P ratio was increased by NaHS in leaves and roots (Fig. 6D–F). The variation in the C : K ratio in leaves,

**Table 2**: Results of two-way ANOVA for the effects of $H_2S$ (S) and Fe concentration on the ratios of C : N, C : P, C : K, N : P, N : K, and P : K in leaves, stems, and roots of soybean seedlings.

| | Treatments | df | C : N | | C : P | | C : K | | N : P | | N : K | | P : K | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | F value | *P* | F value | *P* | F value | *P* | F value | *P* | F value | *P* | F value | *P* |
| Leaf | Fe | 1 | 34.738 | < 0.001 | 42.898 | < 0.001 | 1.214 | 0.292 | 27.012 | < 0.001 | 60.638 | < 0.001 | 31.817 | < 0.001 |
| | S | 1 | 10.311 | 0.007 | 0.312 | 0.587 | 1.134 | 0.308 | 2.718 | 0.125 | 3.775 | 0.077 | 1.29 | 0.278 |
| | Fe×S | 1 | 13.52 | 0.003 | 14.96 | 0.002 | 10.749 | 0.007 | 8.453 | 0.013 | 0.003 | 0.958 | 4.72 | 0.051 |
| Stem | Fe | 1 | 13.306 | 0.003 | 29.023 | < 0.001 | 0.736 | 0.408 | 17.101 | 0.001 | 1.414 | 0.257 | 7.098 | 0.021 |
| | S | 1 | 0.841 | 0.377 | 6.239 | 0.028 | 4.286 | 0.061 | 8.03 | 0.015 | 3.672 | 0.079 | 0.227 | 0.642 |
| | Fe×S | 1 | 3.88 | 0.072 | 0.487 | 0.499 | 0.609 | 0.45 | 11.439 | 0.005 | 0.024 | 0.879 | 1.496 | 0.245 |
| Root | Fe | 1 | 0.242 | 0.632 | 2.315 | 0.154 | 63.887 | < 0.001 | 0.056 | 0.817 | 47.487 | < 0.001 | 24.867 | < 0.001 |
| | S | 1 | 3.09 | 0.104 | 10.788 | 0.007 | 0.063 | 0.807 | 21.903 | 0.001 | 0.946 | 0.35 | 4.809 | 0.049 |
| | Fe×S | 1 | 22.766 | < 0.001 | 47.159 | < 0.001 | 14.717 | 0.002 | 4.803 | 0.049 | 2.616 | 0.132 | 0.39 | 0.544 |

 

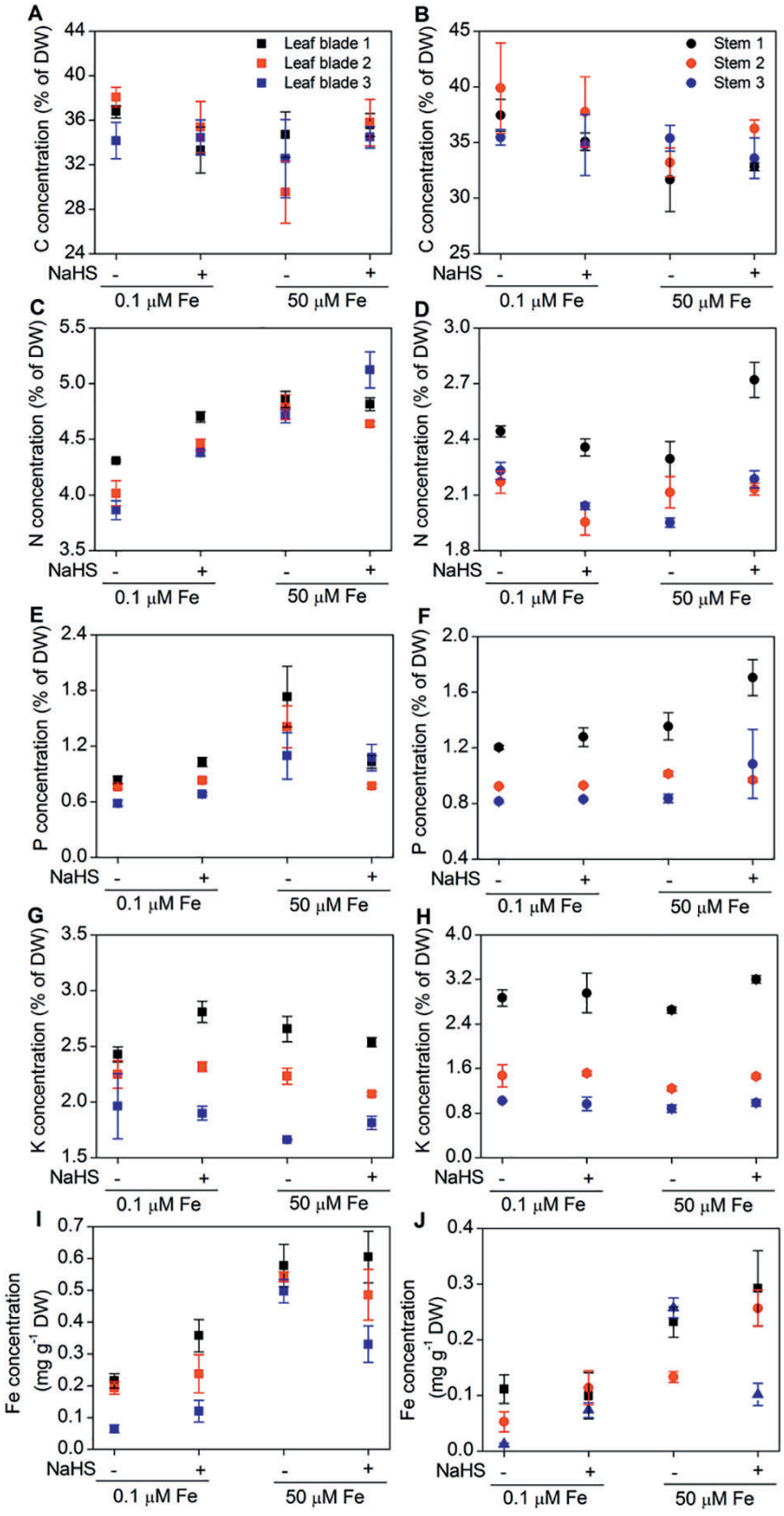


**Figure 5:** The C (A, B), N (C, D), P (E, F), K (G, H), and Fe (I, J) concentrations of leaf blades (1, 2, 3) and stem parts (1, 2, 3) of soybean seedlings with and without NaHS under two Fe regimes, respectively. Each value represents the mean ± SE ($n$ = 4).

stems and roots showed a trend of change of the C : P ratio under all treatments conditions (Fig. 6G–I). The application of NaHS decreased the C : K ratio in leaves, stems, and roots under Fe deficiency, while a high Fe concentration did not change the C : K ratio (Fig. 6G–I, Tab. 2). Iron concentrations significantly affected the N : P ratio in leaves and stems, but not in roots (Fig. 7A–C, Tab. 2). Moreover, under Fe deficiency, the application of NaHS decreased the N : P ratio in leaves and stems. In contrast, under Fe sufficiency, the presence of NaHS increased the N : P ratio in leaves and roots (Fig. 7A–C). The N : K and P : K ratios were significantly affected by Fe concentrations in leaves and roots (Fig. 7D–I, Tab. 2). In addition, the 50 μM Fe resulted in higher N : K and P : K ratios in leaves and roots of soybean seedlings relative to the 0.1 μM Fe treatment (Fig. 7D–I, Tab. 2).

## 4 Discussion

A low concentration of $H_2S$, similar to nitric oxide (NO) and carbon monoxide (CO), can act as a signaling molecule participating in various biological processes in plants (*Ali* et al., 2014; *Chen* et al., 2014; *Chen* et al., 2015a). For instance, previous studies showed that $H_2S$ enhanced the resistance of plants to abiotic stresses, such as metal toxicity, salt, and drought (*Ali* et al., 2013; *Lai* et al., 2014; *Chen* et al., 2015a; *Liu* et al., 2016). Likewise, recent studies have shown that NO, as a signaling molecule, modulated Fe uptake, translocation, and transformation in plants (*Chen* et al., 2010b). Moreover, a previous study also showed that CO played a vital role in improving the adaptation of plants to Fe deficiency (*Kong* et al., 2010). More importantly, our previous study also showed that $H_2S$ could improve the adaptation of *Zea mays* seedlings (monocotyledonous plant) to Fe deficiency through modulating Fe uptake, transport, and accumulation (*Chen* et al., 2015b). In the present study, similar to *Zea mays* as a monocotyledonous plant, under Fe deficiency the application of NaHS significantly increased the biomass, shoot length, and root length of soybean seedlings (dicotyledonous plant; Fig. 2). Also, we found that $H_2S$ alleviated the Fe deficiency-induced decrease of chlorophyll concentration in soybean seedlings (Fig. 2E). Additionally, it was found that NaHS promoted the uptake, transport, and availability of Fe in roots, stems, and leaves

 

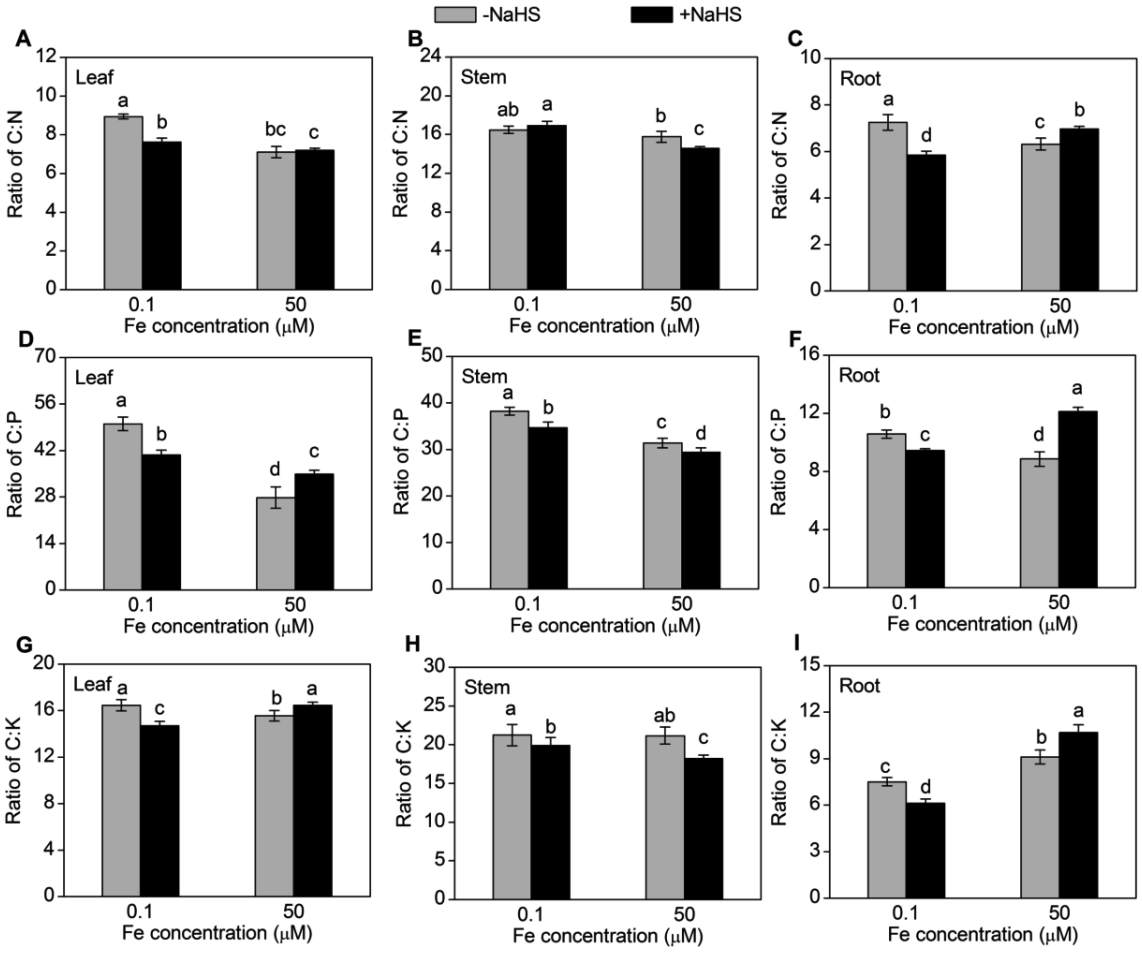


**Figure 6:** The ratios of C : N (A, B, C), C : P (D, E, F), and C : K (G, H, I) in leaves, stems, and roots of soybean seedlings with and without NaHS under two Fe regimes, respectively. Each value represents the mean ± SE ($n$ = 4). Columns labeled with different letters indicate significant differences with P < 5%.

of soybean seedlings under Fe deficiency (Fig. 4, Tab. 1). Similar results were found in our previous study that NaHS improved the adaptation of maize seedlings to Fe deficiency (*Chen* et al., 2015b). In the present study, we also analyzed the Fe concentration of different leaf blades in soybean seedlings and suggested that the application of NaHS could increase the Fe accumulation of old leaves (leaf blade 1), but not in young leaves (leaf blades 2 and 3) under Fe deficiency, suggesting that old leaves stored more Fe to meet the requirement of the growth of young leaves (Fig. 5I). Therefore, we concluded that the effects of $H_2S$ could be important for enhancing the uptake of Fe and alleviated the damage of plant Fe deficiency in calcareous soils.

Iron deficiency impairs directly the chlorophyll biosynthesis and chloroplast development, and further inhibits the photosynthetic rate of plants and the accumulation of dry matter (*Curie* and *Briat*, 2003; *Walker* and *Connolly*, 2008; *Santos* et al., 2015). Similar to the study of *Santos* et al. (2015), our results suggest that Fe deficiency significantly inhibited plant growth and resulted in the decrease of biomass and chlorophyll concentration (Fig. 2). In addition, Fe deficiency also led to a typical phenomenon of nutrient metabolic disorder (*Ågren*, 2008). The N and P concentrations are among the most important indicators of the adaption of plants to the environmental stresses (*Elser* and *Hamilton*, 2007). Additionally, a higher rate of plant growth would be mainly due to the higher N and P concentration based on the growth rate hypothesis (GRH) in plants (*Elser* et al., 1996, 2000). In the present study, Fe deficiency significantly decreased the C concentration in the roots of soybean seedlings (Fig. 3C, Tab. 1), which might have resulted in the reduction of plant biomass and inhibition of carbohydrate biosynthesis. However, NaHS did not affect the C concentration under Fe deficiency. In contrast, under Fe sufficiency the C concentration was significantly increased by NaHS, suggesting that NaHS could promote the accumulation of dry matter due to Fe sufficiency. Moreover, our results also show that the N uptake had different degrees of decline in leaves and roots under Fe deficiency compared to that of Fe sufficiency (Figs. 3D, F). Interestingly, the N concentration of both leaves and roots of NaHS-treated plants was significantly higher than in those of the control plants under Fe deficiency, suggesting that NaHS improved the adaptation of soybean seedlings to Fe deficiency through regulating the uptake of N. Previous studies showed that the N assimilation had a close relationship with photosynthesis and the activities of photosynthetic enzymes in plants (*Ågren*,

 

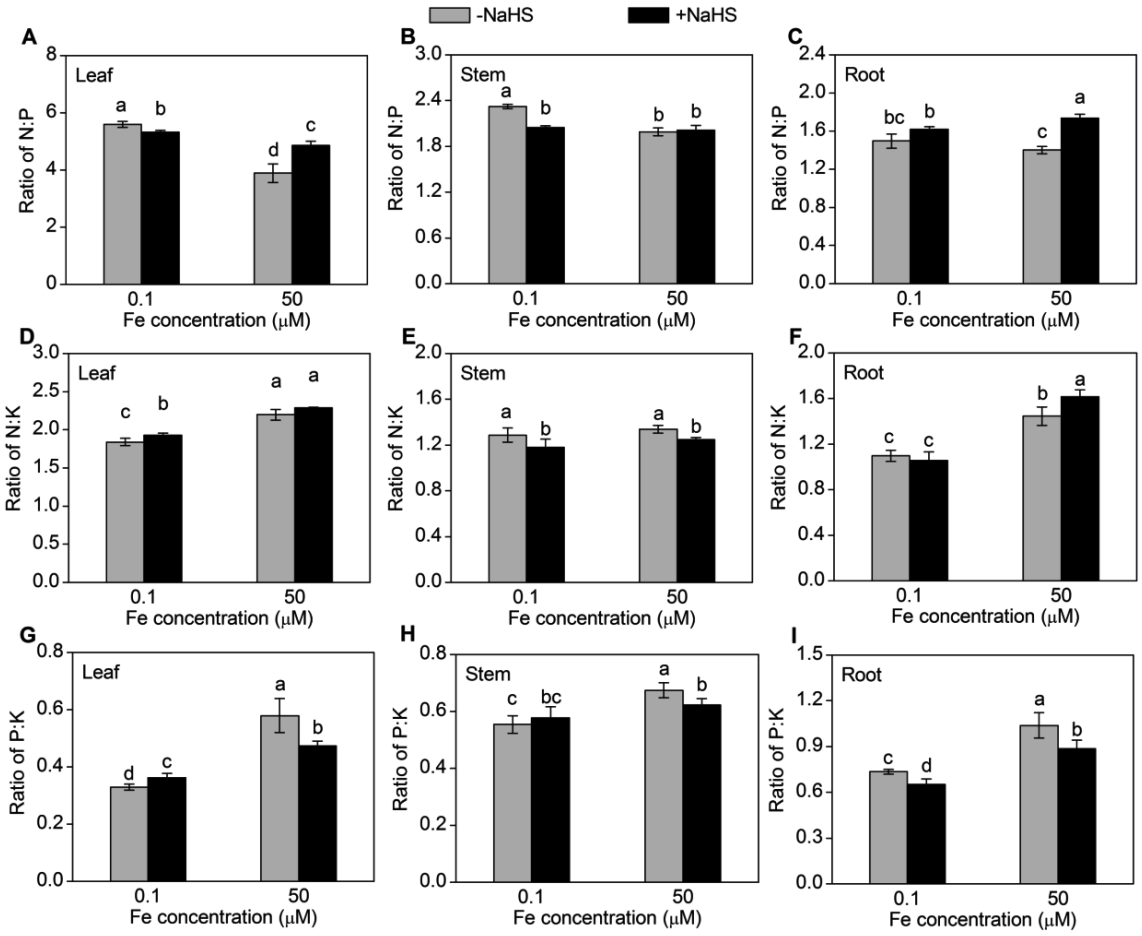


**Figure 7:** The ratios of N : P (A, B, C), N : K (D, E, F), and P : K (G, H, I) in leaves, stems, and roots of soybean seedlings with and without NaHS under two Fe regimes, respectively. Each value represents the mean ± SE ($n$ = 4). Columns labeled with different letters indicate significant differences with P < 5%.

2008; *Cernusak* et al., 2010). Our previous study found that NaHS promoted photosynthesis and gene expression of photosynthetic enzymes in plants, which possibly had a strong relation with the N assimilation (*Chen* et al., 2011, 2015b). Moreover, Fe deficiency resulted in a decrease of photosynthetic rate and caused the inhibition of N absorption by plants (*Santos* et al., 2015), which is consistent with our results. In addition, the application of NaHS increased the N accumulation of old leaves (leaf blade 1) and young leaves (leaf blades 2 and 3) under Fe deficiency (Fig. 5C).

The nutrient allocation of cellular components was affected by the consequential changes of N and P concentrations in plants (*Chen* et al., 2010a; *Song* et al., 2014). Moreover, the activity of phosphatase in roots was stimulated by N availability, which could potentially improve the uptake of P (*Pugnaire*, 2001; *Rivas-Ubach* et al., 2012). In the present study, the P concentration of leaves and stems significantly decreased under Fe deficiency (Fig. 3G, H). However, the P concentration of the NaHS-treated plants was higher than that in the control plants in leaves, stems, and roots under Fe deficiency (Fig. 3G–I). These results show that NaHS might drive the uptake of nutrients to enhance the adaptation of plants to Fe deficiency. Moreover, K, that is involved in the regulation of stomatal movement and plant osmotic control (*Reich* and *Oleksyn*, 2004), plays an important role for the growth and development of plants. In the present study, Fe deficiency significantly increases the K concentration of roots, but not of leaves and stems, suggesting that Fe deficiency is one of the most important limiting factors for the growth and development of plants, and shows a higher internal requirement for K under this environmental stress condition (*Cakmak*, 2005). Interestingly, our results also found that NaHS significantly increased the K concentration of roots compared to the control plants under Fe deficiency (Fig. 3L). This agrees with our previous study in which NaHS increased the K concentration of barley seedling roots treated with high salinity (*Chen* et al., 2015a). Additionally, the variation of C concentration in different leaf blades is different from other nutrition elements. For instance, the order of C concentration in different leaf blades under Fe deficiency was 2 > 1 > 3, but under Fe sufficiency the order was 1 > 3 > 2 (Fig. 5A), suggesting that Fe deficiency induced the transfer of the C from old leaves to young leaves. Unlike the leaves, in the different stems, the order of C concentration under Fe deficiency was 2 > 1 > 3, but under Fe sufficiency it was 3 > 2 > 1 (Fig. 5B). However, the order of the other four elements N, P, K, and Fe in different leaf blades and stems was 1 > 2 > 3, for either Fe deficiency or suffi-

 

ciency, suggesting that old tissues accumulate more nutrients (Fig. 5C–J). Moreover, NaHS treatment did not significantly change the order of these nutrient element concentrations in different leaf blades and stems of soybean seedlings.

A higher growth rate of plant would be mainly accompanied by lower ratios of C : N and C : P according to the growth rate hypothesis (GRH) (*Elser* et al., 1996; *Song* et al., 2014). Here, our results showed that the NaHS-treated plants had lower C : N and C : P ratios in the leaves and roots than the control plants under Fe deficiency, and this could partly explain the greater biomass of the NaHS-treated plants growing under Fe deficiency (Fig. 6, Tab. 2). The ratio of leaf N : P is an important index to determine the nutrient limitation. When the plants were N-limited, the N concentration was lower, but the P concentration was significantly higher. Under N limitation, a lower N : P ratio was observed, which was mainly caused by a higher P concentration. In the present research, the N : P ratios in the roots of Fe deficiency soybean showed no significant differences between the NaHS-treated and control plants (Fig. 7C, Tab. 2), while in the leaves and stems the N : P ratio of the NaHS-treated plants was significantly lower than that of the control plants under Fe deficiency (Fig. 7A, B). This is likely to indicate that under these two treatments the P deficiency significantly inhibited the shoot growth of the control plants, because P plays a vital function in protein and nucleic acid synthesis.

## 5 Conclusion

The results show that Fe deficiency inhibited the growth and development of soybean plants. The application of NaHS alleviated Fe deficiency by enhancing the absorption of C, N, P, and K, thereby improving the adaptation of plants to Fe deficiency in calcareous soils.

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## References

*Aerts, R.*, *Chapin III, F. S.* (1999): The mineral nutrition of wild plants revisited: a re-evaluation of processes and patterns. *Adv. Ecol. Res.* 30, 1–67.

*Ågren, G. I.* (2004): The C:N:P stoichiometry of autotrophs—theory and observations. *Ecol. Lett.* 7, 185–191.

*Ågren, G. I.* (2008): Stoichiometry and nutrition of plant growth in natural communities. *Annu. Rev. Ecol. Evol. Sys.* 39, 153–170.

*Ali, B.*, *Song, W. J.*, *Hu, W. Z.*, *Luo, X. N.*, *Gill, R. A.*, *Wang, J.*, *Zhou, W. J.* (2014): Hydrogen sulfide alleviates lead-induced photosynthetic and ultrastructural changes in oilseed rape. *Ecotox. Environ. Safe.* 102, 25–33.

*Ali, S.*, *Farooq, M. A.*, *Hussain, S.*, *Yasmeen, T.*, *Abbasi, G. H.*, *Zhang, G.* (2013): Alleviation of chromium toxicity by hydrogen sulfide in barley. *Environ. Toxicol. Chem.* 32, 2234–2239.

*Babita, M.*, *Maheswari, M.*, *Rao, L. M.*, *Shanker, A. K.*, *Rao, D. G.* (2010): Osmotic adjustment, drought tolerance and yield in castor (*Ricinus communis* L.) hybrids. *Environ. Exp. Bot.* 69, 243–249.

*Cakmak, I.* (2005): The role of potassium in alleviating detrimental effects of abiotic stresses in plants. *J. Plant Nutr. Soil Sci.* 168, 521–530.

*Cernusak, L. A.*, *Winter, K.*, *Turner, B. L.* (2010): Leaf nitrogen to phosphorus ratios of tropical trees: experimental assessment of physiological and environmental controls. *New Phytol.* 185, 770–779.

*Chen, A.*, *Zeng, G.*, *Chen, G.*, *Zhang, C.*, *Yan, M.*, *Shang, C.*, *Hu, X.*, *Lu, L.*, *Chen, M.*, *Guo, Z.*, *Zuo, Y.* (2014): Hydrogen sulfide alleviates 2, 4-dichlorophenol toxicity and promotes its degradation in *Phanerochaete chrysosporium*. *Chemosphere* 109, 208–212.

*Chen, J.*, *Wang, W.-H.*, *Wu, F.-H.*, *You, C.-Y.*, *Liu, T. W.*, *Dong, X. J.*, *He, J.-X.*, *Zheng, H.-L.* (2013): Hydrogen sulfide alleviates aluminum toxicity in barley seedlings. *Plant Soil* 362, 301–318.

*Chen, J.*, *Wang, W.-H.*, *Wu, F.-H.*, *He, E.-M.*, *Liu, X.*, *Shangguan, Z.-P.*, *Zheng, H.-L.* (2015a): Hydrogen sulfide enhances salt tolerance through nitric oxide-mediated maintenance of ion homeostasis in barley seedling roots. *Sci. Rep.* 5. DOI: 10.1038/srep12516.

*Chen, J.*, *Wu, F.-H.*, *Wang, W.-H.*, *Zheng, C.-J.*, *Lin, G.-H.*, *Dong, X.-J.*, *He, J.-X.*, *Pei, Z.-M.*, *Zheng, H.-L.* (2011): Hydrogen sulphide enhances photosynthesis through promoting chloroplast biogenesis, photosynthetic enzyme expression, and thiol redox modification in *Spinacia oleracea* seedlings. *J. Exp. Bot.* 62, 4481–4493.

*Chen, J.*, *Wu, F.-H.*, *Shang, Y.-T.*, *Wang, W.-H.*, *Hu, W.-J.*, *Simon, M.*, *Liu, X.*, *Shangguan, Z.-P.*, *Zheng, H.-L.* (2015b): Hydrogen sulphide improves adaptation of *Zea mays* seedlings to iron deficiency. *J. Exp. Bot.* 66, 6605–6622.

*Chen, M. M.*, *Yin, H. B.*, *O'Connor, P.*, *Wang, Y. S.*, *Zhu, Y. G.* (2010a): C: N: P stoichiometry and specific growth rate of clover colonized by arbuscular mycorrhizal fungi. *Plant Soil* 326, 21–29.

*Chen, W. W.*, *Yang, J. L.*, *Qin, C.*, *Jin, C. W.*, *Mo, J. H.*, *Ye, T.*, *Zheng, S. J.* (2010b): Nitric oxide acts downstream of auxin to trigger root ferric-chelate reductase activity in response to iron deficiency in *Arabidopsis*. *Plant Physiol.* 154, 810–819.

*Curie, C.*, *Briat, J. F.* (2003): Iron transport and signaling in plants. *Annu. Rev. Plant Biol.* 54, 183–206.

*Dawood, M.*, *Cao, F.*, *Jahangi, M. M.*, *Zhang, G.*, *Wu, F.* (2012): Alleviation of aluminum toxicity by hydrogen sulfide is related to elevated ATPase, and suppressed aluminum uptake and oxidative stress in barley. *J. Hazard. Mater.* 209, 121–128.

*Elser, J. J.*, *Dobberfuhl, D. R.*, *MacKay, N. A.*, *Schampel, J. H.* (1996): Organism size, life history, and N:P stoichiometry. *BioScience* 46, 674–684.

*Elser, J. J.*, *Hamilton, A.* (2007): Stoichiometry and the new biology: the future is now. *PLoS Biol.* 5. DOI: 10.1371/journal.pbio.0050181.

*Elser, J. J.*, *Sterner, R. W.*, *Gorokhova, E.*, *Fagan, W. F.*, *Markow, T. A.*, *Cotner, J. B.*, *Harrison, J. F.*, *Hobbie, S. E.*, *Odell, G. M.*, *Weider, L. J.* (2000): Biological stoichiometry from genes to ecosystems. *Ecol. Lett.* 3, 540–550.

*Fan, H.*, *Wu, J.*, *Liu, W.*, *Yuan, Y.*, *Hu, L.*, *Cai, Q.* (2015): Linkages of plant and soil C:N:P stoichiometry and their relationships to forest growth in subtropical plantations. *Plant Soil* 392, 127–138.

*Graham, R. D.*, *Welch, R. M.*, *Bouis, H. E.* (2001): Addressing micronutrient malnutrition through enhancing the nutritional quality of

 

staple foods: Principles, perspectives and knowledge gaps. *Adv. Agron.* 70, 77–142.

*Güsewell, S.* (2004): N:P ratios in terrestrial plants: variation and functional significance. *New Phytol.* 164, 243–266.

*Jeong, J.*, *Connolly, E. L.* (2009): Iron uptake mechanisms in plants: Functions of the FRO family of ferric reductases. *Plant Sci.* 176, 709–714.

*Jin, Z.*, *Wang, Z.*, *Ma, Q.*, *Sun, L.*, *Zhang, L.*, *Liu, Z.*, *Liu, D.*, *Hao, X.*, *Pei, Y.* (2017): Hydrogen sulfide mediates ion fluxes inducing stomatal closure in response to drought stress in *Arabidopsis thaliana*. *Plant Soil* 419, 141–152.

*Kong, W.-W.*, *Zhang, L.-P.*, *Guo, K.*, *Liu, Z.-P.*, *Yang, Z.-M.* (2010): Carbon monoxide improves adaptation of Arabidopsis to iron deficiency. *Plant Biotechnol. J.* 8, 88–99.

*Lai, D.*, *Mao, Y.*, *Zhou, H.*, *Li, F.*, *Wu, M.*, *Zhang, J.*, *He, Z.*, *Cui, W.*, *Xie, Y.* (2014): Endogenous hydrogen sulfide enhances salt tolerance by coupling the reestablishment of redox homeostasis and preventing salt-induced $K^+$ loss in seedlings of *Medicago sativa*. *Plant Sci.* 225, 117–129.

*Lichtenthaler, H. K.* (1987): Chlorophylls and carotenoids: pigments of photosynthetic membranes. *Methods Enzymol.* 148, 350–383.

*Liu, X.*, *Chen, J.*, *Wang, G.-H.*, *Wang, W.-H.*, *Shen, Z.-J.*, *Luo, M.-R.*, *Gao, G.-F.*, *Simon, M.*, *Ghoto, K.*, *Zheng, H.-L.* (2016): Hydrogen sulfide alleviates zinc toxicity by reducing zinc uptake and regulating genes expression of antioxidative enzymes and metallothioneins in roots of the cadmium/zinc hyperaccumulator *Solanum nigrum* L. *Plant Soil* 400, 177–192.

*Nelson, D. W.*, *Sommers, L. E.* (1982): Total Carbon, Organic Carbon, and Organic Matter, in Page, A., Miller, R., Keeney, D. (eds.): Methods of Soil Analysis: Chemical and Microbiological Properties. SSSA, Madison, WI, USA, pp. 539–579.

*Pugnaire, F. I.* (2001): Variability of inorganic nutrient concentrations in leaves. *New Phytol.* 150, 506–507.

*Reich, P. B.*, *Oleksyn, J.* (2004): Global patterns of plant leaf N and P in relation to temperature and latitude. *P. Natl. Acad. Sci. USA* 101, 11001–11006.

*Rivas-Ubach, A.*, *Sardans, J.*, *Pérez-Trujillo, M.*, *Estiarte, M.*, *Peñuelas, J.* (2012): Strong relationship between elemental stoichiometry and metabolome in plants. *P. Natl. Acad. Sci. USA* 109, 4181–4186.

*Rodríguez-Lucena, P.*, *Ropero, E.*, *Hernández-Apaolaza, L.*, *Lucena, J. J.* (2010): Iron supply to soybean plants through the foliar application of IDHA/$Fe^{3+}$: effect of plant nutritional status and adjuvants. *J. Sci. Food Agri.* 90, 2633–2640.

*Sangakkara, U. R.*, *Frehner, M.*, *Nösberger, J.* (2000): Effect of soil moisture and potassium fertilizer on shoot water potential, photosynthesis and partitioning of carbon in mungbean and cowpea. *J. Agron. Crop Sci.* 185, 201–207.

*Santos, C. S.*, *Roriz, M.*, *Carvalho, S. M. P.*, *Vasconcelos, M. W.* (2015): Iron partitioning at an early growth stage impacts iron deficiency responses in soybean plants (*Glycine max* L.). *Front. Plant Sci.* 6. DOI: 10.3389/fpls.2015.00325.

*Song, M.*, *Chai, Q.*, *Li, X.*, *Yao, X.*, *Li, C.*, *Christensen, M. J.*, *Nan, Z.* (2014): An asexual Epichloë endophyte modifies the nutrient stoichiometry of wild barley (*Hordeum brevisubulatum*) under salt stress. *Plant Soil* 387, 153–165.

*Sun, J.*, *Wang, R.*, *Zhang, X.*, *Yu, Y.*, *Zhao, R.*, *Li, Z.*, *Chen, S.* (2013): Hydrogen sulfide alleviates cadmium toxicity through regulations of cadmium transport across the plasma and vacuolar membranes in *Populus euphratica* cells. *Plant Physiol. Biochem.* 65, 67–74.

*Walker, E. L.*, *Connolly, E. L.* (2008): Time to pump iron: iron-deficiency-signaling mechanisms of higher plants. *Curr. Opin. Plant Biol.* 11, 530–535.

*Wang, B.-L.*, *Shi, L.*, *Li, Y.-X.*, *Zhang, W.-H.* (2010): Boron toxicity is alleviated by hydrogen sulfide in cucumber (*Cucumis sativus* L.) seedlings. *Planta* 231, 1301–1309.

*White, P. J.*, *Broadley, M. R.* (2009): Biofortification of crops with seven mineral elements often lacking in human diets—iron, zinc, copper, calcium, magnesium, selenium and iodine. *New Phytol.* 182, 49–84.

*White, P. J.*, *Brown, P. H.* (2010): Plant nutrition for sustainable development and global health. *Ann. Bot.* 105, 1073–1080.

*Yan, Z.*, *Kim, N.*, *Han, W.*, *Guo, Y.*, *Han, T.*, *Du, E.*, *Fang, J.* (2014): Effects of nitrogen and phosphorus supply on growth rate, leaf stoichiometry, and nutrient resorption of *Arabidopsis thaliana*. *Plant Soil* 388, 147–155.

*Yang, J.*, *Worley, E.*, *Ma, Q.*, *Li, J.*, *Torres-Jerez, I.*, *Li, G.*, *Zhao, P.-X.*, *Xu, Y.*, *Tang, Y.*, *Udvardi, M.* (2016): Nitrogen remobilization and conservation, and underlying senescence-associated gene expression in the perennial switchgrass *Panicum virgatum*. *New Phytol.* 211, 75–89.

*Zhang, H.*, *Hu, L.-Y.*, *Li, P.*, *Hu, K.-D.*, *Jiang, C.-X.*, *Luo, J.-P.* (2010): Hydrogen sulfide alleviated chromium toxicity in wheat. *Biol. Plant.* 54, 743–747.

 