Sulfur Stable Isotope Discrimination in Rice: A Sulfur Isotope Mass Balance Study

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The use of sulfur (S) stable isotopes to study S metabolism in plants is still limited by the relatively small number of studies. It is generally accepted that less S stable isotope discrimination occurs during sulfate (SO$_4^{2-}$) uptake. However, S metabolism and allocation are expected to produce separations of S stable isotopes among the different plant S pools and organs. In this study, we measured the S isotope composition of the main S pools of rice plants grown under different SO$_4^{2-}$ availabilities in appropriate closed and open hydroponic-plant systems. The main results indicate that fractionation against $^{34}$S occurred during SO$_4^{2-}$ uptake. Fractionation was dependent on the amount of residual SO$_4^{2-}$ in the solution, showing a biphasic behavior related to the relative expression of two SO$_4^{2-}$ transporter genes (OsSULTR1;1 and OsSULTR1;2) in the roots. S isotope separations among S pools and organs were also observed as the result of substantial S isotope fractionations and mixing effects occurring during SO$_4^{2-}$ assimilation and plant S partitioning. Since the S stable isotope separations conserve the memory of the physiological and metabolic activities that determined them, we here underline the potential of the $^{32}$S/$^{34}$S analysis for the detailed characterization of the metabolic and molecular processes involved in plant S nutrition and homeostasis.

Keywords: fractionation, Oryza sativa L., sulfate uptake, sulfur assimilation, sulfur stable isotopes

INTRODUCTION

Since 1865, sulfur (S) has been recognized as an essential element for plant growth (Sachs, 1865; Epstein, 2000). In plants, S is found in the amino acid cysteine and methionine, short peptides, vitamins and cofactors, and secondary compounds (Takahashi et al., 2011).

Plants mainly utilize sulfate (SO$_4^{2-}$), an inorganic form of oxidized S present in the soil solution, to support their growth. SO$_4^{2-}$ is taken up by roots and allocated to various sink tissues, where it is stored in the cell vacuoles or assimilated into S organic (S$_{org}$) compounds (Saito, 2004; Takahashi et al., 2011). To accomplish the assimilation of S into biomolecules, SO$_4^{2-}$ is first activated by ATP sulfurylase to adenosine-5'-phosphosulfate (APS), which is then channeled toward reduction or sulfation (Leustek et al., 2000). Most of the APS enters the reductive pathway along which sulfite and, subsequently, sulfide are produced through two sequential reactions catalyzed by APS reductase and sulfite reductase, respectively. Sulfide is finally incorporated into O-acetylserine.
(OAS) to form cysteine in a reaction catalyzed by OAS(thiol)lyase (Takahashi et al., 2011). In the sulfation pathway, the APS is first phosphorylated by APS kinase to form 3′-phosphoadenosine-5′-phosphosulfate, the donor of SO$_4^{2−}$ for a variety of sulfation reactions catalyzed by sulfotransferases (Günl et al., 2019).

Sulfur has four stable isotopes, namely, $^{32}$S, $^{33}$S, $^{34}$S, and $^{36}$S; their relative abundances are 0.9499, 0.0075, 0.0425, and 0.0001 atom fraction, respectively (De Laeter et al., 2003). Mass differences between the S isotopes result in small but significant variations in their chemical and physical properties, which may produce considerable separation of the S isotopes during chemical reactions. The most abundant isotopes, i.e., $^{32}$S and $^{34}$S, are now commonly measured using elemental analyzers coupled with Isotope Ratio Mass Spectrometers (IRMS), and S isotope abundance is generally reported in δ notation ($\delta^{34}$S) as parts per thousand (‰) deviation relative to the Vienna-Cañon Diablo Troilite (VCDT; Coplen and Krouse, 1998) standard as follows:

$$\delta^{34}S (‰) = \frac{R_{sample} - R_{standard}}{R_{standard}} \cdot 1,000$$

where $R_{sample}$ and $R_{standard}$ are the isotope ratios ($^{34}S/^{32}S$) of the sample and standard, respectively.

Unlike what has happened with carbon and nitrogen, the natural abundance S stable isotope analysis techniques have so far scarcely been employed to study S allocation and metabolism in plants (Trust and Fry, 1992; Tcherkez and Tea, 2013), mainly due to the lack of knowledge about the $^{32}$S/$^{34}$S isotope effects occurring during S metabolism and partitioning among the different organs. Most of the irreversible reactions involving S discriminate between $^{33}$S and $^{34}$S by favoring the lighter $^{33}$S isotope, thus enriching in $^{34}$S the residual substrate molecules left behind. That is to say that irreversible reactions that do not consume all the substrate may likely produce a detectable separation of the S stable isotopes, i.e., a fractionation, at natural abundance, providing crucial insights into the understanding of S metabolic fluxes inside the plants, without the need for costly labeling experiments with radioactive ($^{35}$S) or stable ($^{34}$S) isotopes (Tcherkez and Tea, 2013).

Sulfate uptake and allocation in plants involve a family of SO$_4^{2−}$ transporter proteins whose activities are tightly regulated and coordinated with those of the assimilation pathways to control plant S homeostasis (Buchner et al., 2004; Gigolashvili and Kopriva, 2014; Sacchi and Nocito, 2019; Takahashi, 2019). A few pioneering studies indicated that a less S isotope discrimination occurs during SO$_4^{2−}$ uptake since the isotope composition measured for plant total S (Stot) is typically depleted in $^{34}$S by 1−2‰ with respect to that measured for the SO$_4^{2−}$ source feeding the plants (Mekhtiyeva, 1971; Krouse et al., 1991). In contrast, less is known about the S isotope composition of the SO$_4^{2−}$ ions in the plant tissues, which should reflect the metabolic activities in which SO$_4^{2−}$ is involved as a substrate. Although the isotope effects linked to SO$_4^{2−}$ metabolism largely remain to be investigated in plants, it is possible to suppose that reductive SO$_4^{2−}$ assimilation fractionates against $^{34}$S, since it involves changes in the covalent bonding of the S atoms (Rees, 1973). Significant isotope effects have been reported for bacterial SO$_4^{2−}$ reduction, which enriches both the sulfide produced in the lighter $^{32}$S isotope and the remaining SO$_4^{2−}$ in the heavier $^{34}$S isotope (Thode et al., 1949; Kemp and Thode, 1968).

This study presents a detailed study on the dynamics of S stable isotopes occurring in appropriate closed or steady-state hydroponic-plant systems to dissect the $^{32}$S/$^{34}$S isotope effects associated with SO$_4^{2−}$ uptake, allocation, and metabolism in rice plants. In this study, we also provided the first complete S isotope mass balance in rice which considers organic and inorganic S pools in roots and shoots.

**MATERIALS AND METHODS**

**Plant Material and Pre-growing Conditions**

Rice (*Oryza sativa* L. cv. Vialone Nano) carropys were surface sterilized with 70% (v:v) ethanol for 1 min, washed three times with sterile deionized water, and finally sown on filter paper saturated with deionized water to be incubated in the dark at 26°C. After 7 days, seedlings selected for uniform growth were transferred into 3-L plastic tanks (18 seedlings per tank), containing the following complete nutrient solution: 1.5 mM KNO$_3$, 1 mM Ca(NO$_3$)$_2$, 100 µM MgSO$_4$, 250 µM NH$_4$H$_2$PO$_4$, 25 µM Fe-EDTA, 46 µM H$_3$BO$_3$, 9 µM MnCl$_2$, 1 µM ZnCl$_2$, 0.3 µM CuCl$_2$, 0.1 µM (NH$_4$)$_6$Mo$_7$O$_24$, and 30 µM Na$_2$O$_7$Si (pH 6.5). Seedlings were kept for a 14-days pre-growing period in a growth chamber maintained at 26°C and 80% relative humidity during the 16-h light period and at 22°C and 70% relative humidity during the 8-h dark period. The photosynthetic photon flux density was 400 µmol m$^{-2}$ s$^{-1}$. Nutrient solutions were renewed two times a week to minimize nutrient depletion. At the end of the pre-growing period, roots were gently washed for 30 min in 3 L of deionized water (>18.2 MΩ cm). Plants were then transferred into fresh solutions and used in two different experimental setups (A and B). The parts of the plants were sampled, frozen in liquid N$_2$, and stored at −80°C for further analysis.

**Experimental Setup and Tissue Sampling**

In experimental setup A, pre-grown rice plants were transferred into fresh complete nutrient solutions and then grown further, under the same conditions described before, for 3–11 days, not renewing the growing media. Both plants and nutrient solutions were sampled at the beginning of the experiment and every day (starting from the third day).

In experimental setup B, pre-grown rice plants were transferred into fresh complete nutrient solutions (+SO$_4^{2−}$) or fresh minus sulfate nutrient solutions (−SO$_4^{2−}$), in which an equimolar amount of MgCl$_2$ replaced MgSO$_4$. Plants were grown under these conditions for 48 or 72 h by renewing the growing media every day.

In both the experimental setups, before sampling, plant roots were washed for 30 min in 3 L of deionized water to remove SO$_4^{2−}$, which was not absorbed, from the root apoplast. After
washing, plants were gently blotted with paper towels, shoots were separated from roots, and then, both were frozen in liquid N₂ and stored at −80°C for further analysis.

**Xylem Sap Sampling**
In each sampling period (experimental setup B in the presence of SO₄²⁻), the shoots of four rice plants were cut at 1 cm above the roots with a microtome blade to collect, with a micropipette, the xylem sap exuded from the lower cut surface during a 90-min period (Maghrabi et al., 2021).

**Preparation of Samples for Sulfur Isotope Analysis and Quantitative Determination of the Sulfur Pools**
Frozen samples were ground to a fine powder using mortar and pestle in liquid N₂ and stored frozen in a cryogenic tank.

For S₄ total analysis, powder samples of 5 g [fresh weight (FW)] were digested at 150°C in 10 ml 2:1 (v:v) nitric:perchloric acid mixture, in order to quantitatively convert all the S forms into SO₄²⁻ (Blair and Till, 2003). Samples were then added with 1 ml of concentrated HCl and finally evaporated to dryness at 200°C to release any HNO₃ still present. The mineralized material was dissolved in 50 ml of deionized water and then brought to pH 2.0 with a tiny volume of 6 N HCl.

Sulfate was extracted from roots and shoots by homogenizing powder samples of 5 g (FW) in 50 ml of deionized water. After heating at 80°C for 40 min, the extracts were filtered and then brought to pH 2.0 with a tiny volume of 6 N HCl.

Residual nutrient solutions were boiled to evaporate water until their volumes were reduced to 50 ml. Samples were then filtered and brought to pH 2.0 with 6 N HCl.

Xylem sap samples were diluted with deionized water to a final volume of 50 ml, filtered, and then brought to pH 2.0 with a tiny volume of 6 N HCl.

Aliquots of 2 ml were collected from each diluted sample for the quantitative determination of SO₄²⁻, using the turbidimetric method described by Tabatabai and Bremner (1970). The SO₄²⁻ ions of each sample were precipitated overnight as BaSO₄ by adding 2.5 ml of a 0.5 M BaCl₂ solution. BaSO₄ was then collected by centrifugation, washed twice in 2 ml of deionized water, dried at 80°C, ground to a fine powder, and finally used for the S isotope analyses.

The amount of the S₄ organ pool of both root and shoot extracts was estimated as follows:

\[ S_{\text{org}} = S_{\text{tot}} - \text{SO}_4^{2-} \]

**Sulfur Isotope Analysis**
The δ¹³S values of samples were measured using a Flash 2000 HT elemental analyzer coupled, *via* a Conflo IV Interface, with a Delta V Advantage IRMS and interconnected to the software Isodat 3.0 (Thermo). The reaction tube, packed with tungstic oxide and copper wires separated by Quartz wool, was maintained at 1,020°C. The He carrier gas flow was 150 ml min⁻¹. The O₂ purge for flash combustion was 3 s at a flow rate of 250 ml min⁻¹ per sample. The temperature of the gas chromatography separation column was 90°C. The SO₂ reference gas pulse was introduced three times (20 s each) at the beginning of each run.

Samples (BaSO₄ precipitates and reference materials) were weighed in tin capsules. Capsules were carefully closed by folding them with cleaned tweezers and then transferred to the autosampler. The run time of the analysis was approximately 500 s for a single run. The analysis of each sample was performed five times. Calibration was performed using three secondary reference materials provided by the International Atomic Energy Agency (IAEA): IAEA-S-1 (δ¹³S = −0.30 ± 0.03‰); IAEA-S-2 (δ¹³S = 22.62 ± 0.08‰); IAEA-S-3 (δ¹³S = −32.49 ± 0.08‰). Two in-house standards were used for normalization and analytical quality assurance.

The data are reported in δ³⁴S notation, which is standardized to the VCDT international reference scale as follows:

\[ \delta^{34}S (\%) = \frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}} \cdot 1,000 \]

The mass spectrometric uncertainty (1σ) on the individual δ³⁴S measurements was better than 0.05‰.

The δ³⁴S values of the S₄ org pool were estimated by imposing the following mass balance:

\[ \delta^{34}S_{\text{S}_{\text{tot}}} \cdot S_{\text{tot}} = (\delta^{34}S_{\text{SO}_4^{2-}} \cdot \text{SO}_4^{2-}) + (\delta^{34}S_{\text{S}_{\text{org}}} \cdot S_{\text{org}}) \]

where SO₄²⁻ and S₄ tot are the amount of SO₄²⁻ and S₄ tot, respectively, measured in the same sample.

Fractionation factors (ΔL/H), i.e., in positive per mil (‰) units, were calculated by fitting an approximation of the Rayleigh equation to the data obtained by measuring δ³⁴S values of the residual SO₄²⁻ in the hydroponic solution (δ³⁴S_{SO₄²⁻_{res}}), according to Fry (2006). For these purposes, the following equation was used:

\[ \delta^{34}S_{\text{SO}_4^{2-}_{\text{res}}} = \delta^{34}S_{\text{SO}_4^{2-}_{\text{source}}} - \Delta_{L/H} \cdot \ln(f) \]

where \( f \) is the fraction of SO₄²⁻ remaining in the hydroponic solution, and \( \delta^{34}S_{\text{SO}_4^{2-}_{\text{source}}} \) is the initial S isotope composition of the S source.

Finally, the trajectories of the δ³⁴S values of the instantaneous product (S₄ tot) that forms, inside the plants, instant by instant in time were calculated using the following equation:

\[ \delta^{34}S_{\text{S}_{\text{tot}}} = \delta^{34}S_{\text{SO}_4^{2-}_{\text{source}}} - \Delta_{L/H} \cdot [1 + \ln (f)] \]

**RNA Extraction and Quantitative Real-Time PCR Analysis**
Total RNA was extracted from rice roots using TRIzol Reagent (Life Technologies Corporation, Carlsbad, CA, United States) and then purified using PureLink® RNA Mini Kit (Life Technologies Corporation, Carlsbad, CA, United States), according to the manufacturer’s instructions. Contaminant DNA was removed on-column using PureLink® DNase (Life Technologies Corporation, Carlsbad, CA, United States). The first-strand cDNA synthesis was carried out using the SuperScript™ III First-Strand Synthesis SuperMix for quantitative real-time PCR (qRT-PCR; Life Technologies...
Corporation, Carlsbad, CA, United States), according to the manufacturer’s instructions.

The qRT-PCR analysis of OsSULTR1;1 (LOC_Os03g09970) and OsSULTR1;2 was performed on the first-strand cDNA in a 20 µl reaction mixture containing GoTaq® qPCR Master Mix (Promega) and the specific primers, using an ABI 7300 Real-Time PCR system (Applied Biosystems). The relative transcript level of each gene was calculated by the 2−ΔΔCt method using the expression of the OsS16 (LOC_Os11g03400) gene as reference. Primers for qRT-PCR are listed in Supplementary Table 1.

Statistical Analysis
Quantitative values are presented as mean ± SEM of three independent experiments run in duplicate (n = 3). Two distinct 3-L tanks were used for each condition analyzed in each independent experiment. ANOVA was carried out using SigmaPlot for Windows version 11.0 (SYSTAT Software, Inc., San Jose, CA, United States). The significant values were adjusted for multiple comparisons using the Bonferroni correction. The Student’s t-test was used to assess the significance of the observed differences between the values measured in root and shoot.

RESULTS
Sulfur Isotope Mass Balance in a Closed Hydroponic-Rice System (Experimental Setup A)
Potential 32S/34S isotope effects occurring during SO4 2− uptake were investigated by setting up a closed hydroponic-rice system (Figure 1A) in which a finite amount of substrate (i.e., the SO4 2− in the nutrient solution) was continuously removed from the solution, by the activity of the SO4 2− transporters of the roots, and converted into a final product (i.e., Stot). Using this system, we performed serial sacrifice experiments in which plant growth was terminated every 24 h (starting from the third day) for the S isotope analyses of both substrates and products.

During the experimental period (264 h), (i) plants continuously grown (Figure 1B) and removed 98% of the SO4 2− initially present in the nutrient solution (Figure 1C), (ii) SO4 2− absorbed was quantitatively recovered in the plants as Stot (Figures 1D,E), and (iii) no significant losses of S occurred during the growth (Figure 1E). The Stot concentration of the plants ranged from 121.2 (at the beginning of the experiment) to 98.6 µmol g−1 dry weight (DW; at the end of the experiment), while the SO4 2− concentration in the nutrient solution ranged from 100 to 65 µM, indicating that the regulation of plant S homeostasis occurred during SO4 2− absorption (Figure 1F).

Figure 2A reports δ34S data as a function of the fraction of SO4 2− remaining in the hydroponic solution (f). The δ34S of residual SO4 2− in both the hydroponic solution and plant Stot changed over time, tending toward higher values as f decreased. The δ34S values of the residual SO4 2− (δ34S_SO4 2−) increased from a minimum of −1.92‰ (at the beginning of the experiment) to a maximum of −0.21‰ (at the final sampling). In contrast, the δ34S_Stot of the plants was always lower than the δ34S_SO4 2− of the S source (−1.92 ± 0.02‰) and increased from −3.32‰ (the starting isotope composition of total plant biomass) to −2.30‰ at the final sampling, indicating that SO4 2− uptake significantly enriches plant Stot in the lighter 32S isotope. It is worth noting that, due to mass balance in the closed system, the δ34S_Stot of the rice plants tended to the δ34S_SO4 2− of the initial S source as SO4 2− concentration in the external medium approached zero, indicating that (i) SO4 2− ions in the nutrient solution were the only S source used by plants and (ii) no significant losses/fractionations of S isotopes occurred during the experiments due to H2S gaseous emission (Wilson et al., 1978; Winner et al., 1981).

The isotope effects that occurred in the closed system were analyzed using an approximation of the Rayleigh equation describing isotope partitioning between two reservoirs as one of them decreases in size (Fry, 2006). The S isotope profile of the residual SO4 2− in the nutrient solution (Figure 2A) showed a marked deviation from a typical Rayleigh enrichment (R2 = 0.79; black dashed line) due to an unexpected data point distribution at the final steps of the experiment (f ≤ 0.21). Considering the Rayleigh fractionation model, it was possible to calculate a single fractionation factor, ΔL/H = 0.48 ± 0.09‰, which describes an average of the net fractionation along the overall trajectory (profile). However, data distribution could be more appropriately described by assuming that a dual-phase Rayleigh fractionation occurred during SO4 2− uptake. In the first phase (1 ≤ f ≤ 0.31), a significant isotope fractionation against 34S-SO4 2− took place (ΔL₁/H₁ = 1.09‰), while in the second phase (f ≤ 0.21), a less pronounced isotope effect (ΔL₂/H₂ = 0.16‰) was associated with SO4 2− uptake. Figure 2A also reports the calculated trajectories of the δ34S values of Stot that forms, inside the plants, instant by instant in time from the external SO4 2− due to SO4 2− uptake; such a product is always offset in the isotope composition of the substrate (δ34S_SO4 2−) by the fractionation factor ΔL/H (Fry, 2006). In each phase (I and II), S isotope fractionation (δ34S_SO4 2−−δ34S_Stot) was practically independent of f, as can be easily observed by comparing the isotope signatures of the substrate and cumulative product for each data point.

Aiming to decompose the two phases into their physiological and molecular components, we performed a transcriptional analysis of OsSULTR1;1 and OsSULTR1;2, i.e., the main rice genes reasonably involved in SO4 2− uptake (Godwin et al., 2003; Kumar et al., 2011; Figure 2B). Results revealed that the transition from the two phases was associated with significant changes in the ratio between the transcript levels of the two genes: the OsSULTR1;2 transcript was always independent of f and was more abundant than the OsSULTR1;1 transcript during the first phase (1 ≤ f ≤ 0.31), while the OsSULTR1;1 transcript level rapidly increased in the second phase (f ≤ 0.21), when the SO4 2− concentration in the nutrient solution became limiting for plant growth [(SO4 2−) ≤ 37 µM; Figure 2B].

Sulfur Isotope Mass Balance in a Whole Plant: Steady-State vs. Sulfur Starvation (Experimental Setup B)
The possible 32S/34S isotope effects associated with both S partitioning among plant organs and cell metabolism were investigated by comparing plants pre-grown in complete nutrient
solutions and then continuously maintained on media containing SO$_4^{2-}$ or deprived of SO$_4^{2-}$ for 72 h (experimental setup B). Nutrient solutions were changed every day to minimize the changes in the isotope signature of the S source ($-1.92 \pm 0.02\%$) due to fractionation associated with SO$_4^{2-}$ uptake.

Results showed that the S isotope composition of the whole plants did not significantly change over time since similar $\delta^{34}\text{S}_{\text{S}_\text{tot}}$ values were measured at each time period (0, 48, and 72 h) in both of the growing conditions (Figure 3). At the beginning of the experiment (0 h), the S$_\text{tot}$ of the whole plants was significantly depleted in $^{34}\text{S}$ by $-1.40 \pm 0.08\%$ relative to the S source (Figure 3).

Plants maintained in hydroponic solutions containing SO$_4^{2-}$ grew linearly in the observation period (Figure 4A). As expected, the concentrations of SO$_4^{2-}$, S$_\text{tot}$, and S$_\text{org}$ did not significantly change in both root and shoot over time (Figures 4B,C). The invariance of each S pool was associated with the invariance of their isotope signatures, indicating that plants reached metabolic and isotope steady-states (Figure 5). The S$_\text{tot}$ of root and shoot was significantly depleted in $^{34}\text{S}$ by $-1.94 \pm 0.08\%$ and $-1.09 \pm 0.09\%$, respectively, relative to the S source (Figure 5A); moreover, $\delta^{34}\text{S}_{\text{S}_\text{tot}}$ values were significantly lower in the root than in the shoot in all the conditions analyzed (Figure 5A). In the root, the SO$_4^{2-}$ pools were significantly ($P < 0.001$) $^{34}\text{S}$-depleted relative to the S source, while in the shoot, they were significantly ($P < 0.001$) $^{34}\text{S}$-enriched relative to the same S source (Figure 5B). The S$_\text{org}$ pool of both root and shoot were significantly $^{34}\text{S}$-depleted with respect to the S source; interestingly, both the S$_\text{org}$ pools were also significantly $^{34}\text{S}$-depleted with respect to their relative SO$_4^{2-}$ pools of the cells (Figure 5C). Finally, no differences were found in comparing the $\delta^{34}\text{S}_{\text{SO}_4^{2-}}$ values of the SO$_4^{2-}$ pools in the xylem sap and in the whole root system (Figure 5D).

In contrast, SO$_4^{2-}$-deprived plants dynamically allocated S previously absorbed during the preliminary growth phase, preserving both the overall S isotope signature (Figure 3) and the total amount of S$_\text{tot}$ over time (Figure 4). However, due to both continuous growth (Figure 4D) and S allocation processes, the S$_\text{tot}$ concentration in rice organs changed over time, decreasing linearly in both root ($R^2 = 0.993$; Figure 4E) and shoot ($R^2 = 0.999$; Figure 4F). The SO$_4^{2-}$ concentration sharply decreased over time in both root and shoot due to SO$_4^{2-}$

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**FIGURE 1** Time course of S partitioning in a closed hydroponic-rice system. (A) Experimental setup: A finite amount of sulfate (SO$_4^{2-}$) is continuously removed from the hydroponic solution and converted into the total S of the plant (S$_\text{tot}$). (B) Plant total biomass accumulation. (C) Residual SO$_4^{2-}$ in the hydroponic solution over time. (D) S$_\text{tot}$ accumulation over time. (E) Residual SO$_4^{2-}$ in the hydroponic solution vs. plant S$_\text{tot}$ accumulation. (F) Plant S$_\text{tot}$ concentration vs. SO$_4^{2-}$ concentration in the hydroponic solution. Data are means ± SE of three independent experiments run in duplicate ($n = 3$).
assimilation (Figures 4E,F). In fact, the concentration of the \( S_{\text{org}} \) in the root slightly decreased over time from 7.26 ± 0.41 to 4.36 ± 0.14 \( \mu \text{mol g}^{-1} \text{ FW} \), while in the shoot, it remained relatively constant. The \( S_{\text{tot}} \) isotope composition of both root and shoot did not change over time (Figure 6A) and was significantly \( ^{34}S \)-depleted relative to the \( S \) source. As previously observed, the \( \delta ^{34}S_{S_{\text{tot}}} \) values were significantly lower in the root than in the shoot (Figure 6A). Differentiably, the \( SO_4^{2−} \) pools of both root and shoot became progressively enriched in \( ^{34}S \) over time. It is worth noting that the most pronounced changes in the \( SO_4^{2−} \) isotope composition were observed in the shoot: the maximum variations observed at 72 h were 2.70 ± 0.05\% and 6.71 ± 0.19\% for root and shoot, respectively (Figure 6B). The \( S_{\text{org}} \) pools of both roots and shoot were significantly \( ^{34}S \)-depleted compared to the \( S \) source; their \( \delta ^{34}S_{S_{\text{org}}} \) values changed differently over time since, in the root, they increased moving from 0 to 48 h and then remained constant at 72 h, while in the shoot, a significant increase was observed when moving from 48 to 72 h (Figure 6C).

**DISCUSSION**

It is generally assumed that terrestrial plants assimilate \( S \) from the soil (\( SO_4^{2−} \)) and from the atmosphere (\( SO_2 \)) with less \( S \) isotope fractionation since the foliar \( \delta ^{34}S \) values are generally intermediate between those of the soil and the atmosphere or near to one extreme (Kennedy and Krouse, 1990; Krouse et al., 1991). However, the correct evaluation of the isotope effects due to \( S \) acquisition needs a direct comparison between the isotope compositions of the \( S_{\text{tot}} \) of a whole plant and the \( S \) source used by the same plant, since the \( \delta ^{34}S_{S_{\text{tot}}} \) value of a single plant organ may result from fractionations and mixing effects occurring during \( SO_4^{2−} \) uptake, assimilation, and partitioning.

To address the \( S \) isotope effects during \( S \) acquisition, we performed an \( S \) isotope mass balance in a closed system, in which the accumulation of \( S_{\text{tot}} \) in the plants is considered as the result of the continuous consumption of a unique and finite \( S \) source (\( SO_4^{2−} \)) initially present in a hydroponic solution (Figure 1A). In such a model system, if fractionation occurs, the enrichment in a given isotope in one part of the system results in its depletion in the other, so that isotopic mass balance is always maintained (Fry, 2006).

Our data indicate that isotope discrimination against \( ^{34}S \) occurred during \( SO_4^{2−} \) uptake, which resulted in transient lighter \( S \) isotope compositions of the plants and concomitant \( ^{34}S \) enrichments of the residual \( SO_4^{2−} \) in the hydroponic solution (Figure 2A). Interestingly, fractionation exhibited two phases characterized by distinct fractionation factors \( [\Delta_1(L/H)] \) and \( [\Delta_2(U/L)] \) that can be considered as “isotope phenotypes” reflecting plant physiological adaptation to the \( SO_4^{2−} \) concentrations in the nutrient solution, which changed during the experiment (Figures 1F, 2B). The maximum fractionation observed was associated with \( f \) values ranging from 1 to 0.31 (corresponding to external \( SO_4^{2−} \) concentrations ranging from 100 to 50 \( \mu \text{M} \)), while the minimum isotope effect was associated with the smallest \( f \) values, when the concentration of \( SO_4^{2−} \) in the nutrient solution became critical (≤37 \( \mu \text{M} \)) and was potentially able to induce an array of \( S \)-deficiency physiological responses (Maruyama-Nakashita et al., 2003), including changes in the expression of the root high-affinity \( SO_4^{2−} \) transports, \( OsSULTR1;1 \) and \( OsSULTR1;2 \), involved in \( SO_4^{2−} \) uptake (Figure 2B). Although a certain degree of
FIGURE 3 | Experimental setup and \(^{32}\)S/\(^{34}\)S isotope effects associated with S partitioning and metabolism in rice plants. Plants were pre-grown in complete nutrient solutions and then continuously maintained on media containing \(\text{SO}_4^{2-}\) or deprived of \(\text{SO}_4^{2-}\) for 72 h (experimental setup B). \(S_{\text{tot}}\), total S amount in a whole plant; \(\delta^{34}\text{S}_{\text{S}\text{tot}}\), S isotope composition of the whole plant; \(\Delta\), \(^{34}\text{S}\) depletion relative to the S source (\(\delta^{34}\text{S}_{\text{SO}_4^{2-}\text{source}} = -1.92 \pm 0.02 \%oo\)). Data are means ± SE of three independent experiments run in duplicate (\(n = 3\)).

To analyze the isotope effects occurring during S partitioning and metabolism, we carried out experiments aimed at the following: (i) preventing possible perturbations due to the continuous changes of the \(\delta^{34}\text{S}_{\text{SO}_4^{2-}}\) values of the external solution and (ii) obtaining rice plants with the same overall S isotope composition (Figure 3). In these experiments, plants can be considered systems continuously supplied by an S source that does not change in concentration and isotope composition.

As previously described, plants continuously grown in the presence of \(\text{SO}_4^{2-}\) reached metabolic and S isotope steady-states characterized by the invariance of the concentration and the isotope signature of each S pool. It should be noted that the S isotope distribution between root and shoot observed in this study strongly differs from the pattern described by Tcherkez and Tea (2013), concerning the S natural isotope composition in different organs (roots, leaf, stem, glumes, and grains) of mature wheat. Such a discrepancy might depend on the growth conditions (closed hydroponic-plant system vs. field) or, more likely, on the different S nutritional status and/or growth stage.
of the plants considered in the two studies since S stable isotope separations conserve the memory of the physiological and metabolic activities that determined them. Finally, differences may also originate from the different distribution of the S assimilation enzymes in the leaf, since rice assimilates SO\textsubscript{4}\textsuperscript{2−} mainly in the bundle sheaths, while other species, also in the mesophyll (Hua et al., 2021).

The isotope composition of the SO\textsubscript{4}\textsuperscript{2−} pools of the root was lighter relative to the S source but heavier with respect to the expected composition calculated according to the isotope discrimination occurring during SO\textsubscript{4}\textsuperscript{2−} uptake at high external concentrations [i.e., δ\textsuperscript{34}S\_SO\textsubscript{4}\textsuperscript{2−} > δ\textsuperscript{34}S\_SO\textsubscript{4}\textsubscript{source}−Δ\textsubscript{1}(L/H)]. Interestingly, SO\textsubscript{4}\textsuperscript{2−} translocation from root to shoot did not discriminate the S isotopes since no differences were found when comparing the isotope signatures of the SO\textsubscript{4}\textsuperscript{2−} ions in root and xylem sap (Figure 5). However, the SO\textsubscript{4}\textsuperscript{2−} pools of the shoot were significantly δ\textsuperscript{34}S-enriched with respect to the SO\textsubscript{4}\textsuperscript{2−} pools of both root and xylem sap. This was likely due to SO\textsubscript{4}\textsuperscript{2−} assimilation that, favoring the lighter 32S isotope, causes a 34S enrichment of the residual SO\textsubscript{4}\textsuperscript{2−} ions left behind. The occurrence of an S isotope separation during SO\textsubscript{4}\textsuperscript{2−} assimilation is consistent with the observation that the S\_org pools of the shoot were significantly depleted in 34S relative to both the SO\textsubscript{4}\textsuperscript{2−} pools of the shoot and the S source. Since the aerial portion of the plant is fed by the SO\textsubscript{4}\textsuperscript{2−} ions continuously translocated from root to shoot and the S\_tot of the shoot was lighter relative to the SO\textsubscript{4}\textsuperscript{2−} coming from the root, we can reasonably suppose that a non-negligible portion of the δ\textsuperscript{34}S-enriched SO\textsubscript{4}\textsuperscript{2−} of the shoot is translocated to the root. Thus, the isotope signature of the SO\textsubscript{4}\textsuperscript{2−} pool of the root could be the result of mixing effects due to the overall S isotope circulation, SO\textsubscript{4}\textsuperscript{2−} uptake, and local S assimilation. Assuming that during the S isotope steady-state, (i) the δ\textsuperscript{34}S\_SO\textsubscript{4}\textsuperscript{2−} values measured in the root are mainly influenced by root SO\textsubscript{4}\textsuperscript{2−} uptake and SO\textsubscript{4}\textsuperscript{2−} translocation from shoot to root, and (ii) the S isotope composition of the instantaneous SO\textsubscript{4}\textsuperscript{2−} that continuously enters the root cells should theoretically differ from the S source by the fractionation factor Δ\textsubscript{1}(L/H), so that

\[
δ\textsuperscript{34}S\_SO\textsubscript{4}\textsuperscript{2−}\_\text{root} = δ\textsuperscript{34}S\_SO\textsubscript{4}\textsuperscript{2−}\_\text{source} − Δ\textsubscript{1}(L/H)
\]

We can estimate the maximum amount of SO\textsubscript{4}\textsuperscript{2−} that, coming from the shoot, is translocated and accumulated into the root (defined as SO\textsubscript{4}\textsuperscript{2−}\_StoR) by imposing the following mass balance:

\[
δ\textsuperscript{34}S\_SO\textsubscript{4}\textsuperscript{2−}\_\text{root} = δ\textsuperscript{34}S\_SO\textsubscript{4}\textsuperscript{2−}\_\text{shoot} + δ\textsuperscript{34}S\_SO\textsubscript{4}\textsuperscript{2−}\_\text{StoR}
\]

where δ\textsuperscript{34}S\_SO\textsubscript{4}\textsuperscript{2−}\_\text{root} is the steady-state isotope composition of the SO\textsubscript{4}\textsuperscript{2−} pool of the root, SO\textsubscript{4}\textsuperscript{2−}\_\text{root} is the total amount of the

![Figure 4](https://example.com/figure4.png)
SO$_4^{2-}$ measured in the root, and $\delta^{34}$S$_{SO_4^{2-}}^{\text{shoot}}$ is the isotope composition of the SO$_4^{2-}$ ions coming from the shoot. Solving the equation for the unknown SO$_4^{2-}$$_{\text{StoR}}$ reveals that, in our conditions, 21.7% of the steady-state SO$_4^{2-}$ pool of the rice root is inherited from the shoot.

Although less information is currently available on the long-distance transport of SO$_4^{2-}$ from shoot to root, we can reasonably suppose that such an activity may involve the phloem and specific isoforms of SO$_4^{2-}$ transporters mediating the loading of SO$_4^{2-}$ into the sieve tubes (Takahashi, 2019). Feeding experiments with $^{35}$SO$_4^{2-}$ performed on Arabidopsis (Yoshimoto et al., 2003) support our finding, indicating the retranslocation of SO$_4^{2-}$ as an important activity in controlling root SO$_4^{2-}$ homeostasis and S isotope composition.

In contrast, during the growing period in the absence of SO$_4^{2-}$, rice plants can be considered closed systems assimilating the SO$_4^{2-}$ ions previously absorbed during the preliminary growth phase and allocating the S$_{\text{org}}$ pools to optimize the distribution of the limited S resources between root and shoot. It is worth noting that in these conditions, the invariance of the S$_{\text{tot}}$ isotope composition of both root and shoot was associated with dramatic changes in the isotope composition of the relative SO$_4^{2-}$ and S$_{\text{org}}$ pools (Figure 6), mainly caused by the $^{32}$S/$^{34}$S isotope effects occurring during SO$_4^{2-}$ assimilation. During the observation period, plants rapidly consumed the available SO$_4^{2-}$ pools: at the end of the experiment, the overall S$_{\text{org}}$ pool was about 94% of the S$_{\text{tot}}$. The S isotope mass balance that was carried out considering the overall SO$_4^{2-}$ and S$_{\text{org}}$ pools (Table 1) revealed that continuous S assimilation progressively enriched both the overall S$_{\text{org}}$ pool in the lighter $^{32}$S isotope and the residual SO$_4^{2-}$ in the heavier $^{34}$S isotope, producing an apparent isotope separation that was closely dependent on the severity of the imposed S starvation, as indicated by calculated $\Delta$ values ($\Delta = \delta^{34}$S$_{\text{Sorg}} - \delta^{34}$S$_{\text{SO}_4^{2-}}$).
that ranged from $-3.29 \pm 0.40$ (at the beginning of the experiment) to $-7.80 \pm 0.18^{\%} \text{h}$ (at 72 h). As expected, the most pronounced isotope separations were observed in the shoot, confirming the prominent role of the rice aerial portion in $\text{SO}_4^{2-}$ assimilation and S allocation (Takahashi et al., 2011).

CONCLUSION AND PERSPECTIVES

Our results provide an overview of the $^{32}\text{S}/^{34}\text{S}$ isotope effects occurring during $\text{SO}_4^{2-}$ uptake, partitioning, and metabolism in rice. The main results clearly show that $\text{SO}_4^{2-}$ uptake discriminates against $^{34}\text{S}$, enriching plant total biomass in the lighter $^{32}\text{S}$ isotope relative to the S source. The S isotope discrimination observed during $\text{SO}_4^{2-}$ acquisition closely depends on the amount of $\text{SO}_4^{2-}$ in the growing medium, as well as on the plants’ molecular and physiological responses aimed at optimizing S nutrition under different environmental conditions. Although further experiments will be necessary to directly measure the isotope effect associated with the activity of a single $\text{SO}_4^{2-}$ transporter, we can reasonably conclude that $\text{OsSULTR1;1}$ and $\text{OsSULTR1;2}$ differently discriminate against $^{34}\text{S}$, producing S isotope phenotypes closely dependent on their relative expression.

Results also indicate that the steady-state S isotope composition of the different S pools of both root and shoot mainly results from the substantial S isotope fractionations occurring during $\text{SO}_4^{2-}$ assimilation and mixing effects due to the overall isotope circulation inside the whole plant. Finally, the extreme variability of the S isotope phenotypes observed under various S conditions underlines the potential of the $\delta^{34}\text{S}$ analysis to provide information for further detailed characterization of the metabolic and molecular processes involved in plant S homeostasis, as well as of the plant S systemic fluxes occurring in different nutritional and environmental conditions, since the S stable isotope separations conserve the memory of the activities that determined them.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

VC, MC, GS, and FN designed the experiments. VC, MM, and MC performed the experiments. FN analyzed the data and wrote the manuscript. All authors revised the manuscript draft and approved the final version.
ACKNOWLEDGMENTS

The music of Gustav Mahler's IX Symphony inspired this study. We thank Clarissa Lancilli for her precious support during the revision of the manuscript.

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