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Atmospheric H₂S and SO₂ as sulfur sources for *Brassica juncea* and *Brassica rapa*: Regulation of sulfur uptake and assimilation

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ABSTRACT

Brassica juncea and *Brassica rapa* were able to utilize foliarly absorbed H₂S and SO₂ as sulfur source for growth and resulted in a decreased sink capacity of the shoot for sulfur supplied by the root and subsequently in a partial decrease in sulfate uptake capacity of the roots. Sulfate-deprived plants were able to utilize foliarly absorbed H₂S and SO₂ as sole source for growth. However, the decrease in shoot to root partitioning and the upregulated sulfate uptake capacity upon sulfate-deprivation was hardly affected by H₂S or SO₂ exposure, indicating a poor shoot to root interaction between sulfur assimilation in the shoot and the regulation of the sulfate transporter activity in the root. Root transporter activity was regulated by the in situ sulfate rather than the water-soluble non-protein thiols (glutathione) concentration. There was also no direct relation between the thiol levels and the transcript levels of the enzymes involved in the sulfate reduction pathway in either roots or shoots of *B. juncea* and *B. rapa*. In sulfate-sufficient plants, both H₂S and SO₂ exposure resulted in strongly decreased transcript levels of APS reductase in the shoot of both species. Sulfate deprivation resulted not only in strongly enhanced transcript levels of APS reductase in both shoots and roots of the plants, but also in enhanced transcript levels of ATP sulfurylase and APS kinase in the shoots. H₂S and SO₂ exposure also resulted in a decrease in transcript levels of APS reductase in the roots and shoots of sulfate-deprived plants, but remained significantly higher than that in sulfate-sufficient plants. The transcript levels of ATP sulfurylase, APS kinase and sulfite reductase were not affected in both sulfate-sufficient and sulfate-deprived plants upon H₂S and SO₂ exposure.

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1. Introduction

Sulfate taken up by the root is generally the primary source of sulfur for plants. At a whole plant level its uptake, distribution, reduction and assimilation are well regulated and coordinated, and are adjusted to the sulfur requirement for growth (Hawkesford and De Kok, 2006). Distinct sulfate transporter proteins are involved in the uptake of sulfate by the root, transport to the shoot and subcellular distribution; the expression and activity of many of these transporters is strongly affected by plant nutritional status (Buchner et al., 2004; Hawkesford and De Kok, 2006; Koralewska et al., 2007, 2008, 2009a,b; De Kok et al., 2012). The major proportion of sulfate taken up by the root is reduced to sulfide in the chloroplasts of the shoot and subsequently incorporated into cysteine, the precursor or sulfur donor for synthesis of other organic sulfur compounds in plants (Hell, 1997; Saito, 2004;

Hawkesford and De Kok, 2006). The root plastids also contain all enzymes of the sulfate reduction pathway, although their contribution to the whole plant sulfate reduction budget is rather unclear (Hawkesford and De Kok, 2006). Sulfate needs first to be activated to adenosine 5'-phosphosulfate (APS) by ATP sulfurylase (ATPs) prior to its reduction to sulfite by APS reductase (APR; Hell, 1997; Saito, 2004). The expression and activity of APR is controlled by the plant sulfur status and is one of the key regulating enzymes of the sulfate reduction pathway (Hell, 1997; Saito, 2004; Hawkesford and De Kok, 2006). The sulfite formed is subsequently reduced to sulfide by sulfite reductase (SiR) and is subsequently incorporated into cysteine by O-acetylserine (thiol) lyase (Hell, 1997; Saito, 2004). Some APS is further phosphorylated to 3'-phosphoadenosine 5'-phosphosulfate (PAPS) by APS kinase, which is the major sulfur group donor in sulfotransferase reactions, e.g. the synthesis of sulfolipids and the sulfate moiety of glucosinolates; the latter compounds are major secondary sulfur compounds in Brassicaceae (Mugford et al., 2009, 2010; Kopriva et al., 2012).

SO₂ and H₂S are potentially phytotoxic, although in polluted areas the foliar absorption of these atmospheric sulfur gases may

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substantially contribute to sulfur nutrition of plants (Haneklaus et al., 2003, 2007; De Kok et al., 2007). Although, it remains unclear as to what extent metabolism of the absorbed sulfur gases contributes to their detoxification, there is not always a clear-cut transition in the level/rate of their metabolism and their phytotoxicity (De Kok et al., 2009). Both SO_2 and H_2S may directly enter the sulfate reduction pathway and may be nutritionally beneficial, especially when the sulfur supply to the root is limited (De Kok et al., 2007). The pattern of uptake of SO_2 and H_2S by the foliage appears to be quite different. Both SO_2 and H_2S are taken up via the stomates and conductance is the major diffusion barrier for the uptake of these gases by the foliage (Lendzian, 1984). In general at ambient levels, there is a linear relation between foliar uptake and the atmospheric concentration of SO_2 (De Kok, 1990; De Kok and Tausz, 2001; Yang et al., 2006a; De Kok et al., 2007). The stomatal resistance appears to be the limiting factor in the foliar uptake of SO_2 , since the internal mesophyll resistance to this sulfur gas is very low. SO_2 is highly soluble in the aqueous phase of the mesophyll cells and after reaction with water yields in bisulfite, which is either oxidized to sulfate (non-enzymatically and enzymatically) or reduced and assimilated in the chloroplasts (De Kok, 1990; De Kok and Tausz, 2001; De Kok et al., 2007). The uptake of H_2S however, appears to be limited rather by the mesophyll than stomatal resistance, at least at higher ambient levels, since the foliar uptake of H_2S showed saturation kinetics with respect to the atmospheric concentrations. The internal mesophyll resistance to H_2S appears largely to be determined by the rate of metabolism into cysteine and subsequent assimilation into other organic sulfur compounds (De Kok et al., 1989, 1991, 1997, 1998, 2002, 2007; De Kok and Tausz, 2001; Stuiver and De Kok, 2001). It was observed that the foliar H_2S uptake kinetics were the result of the activity of *O*-acetylserine (thiol) lyase, the affinity of this enzyme for H_2S and the in situ availability of *O*-acetylserine (De Kok et al., 1989, 1991, 1997, 2002, 2007; Stuiver and De Kok, 2001). Moreover, differences in H_2S uptake rates between species coincided with their differences in requirement of sulfur for growth (De Kok et al., 1997, 2002).

SO_2 and H_2S impact studies have been shown to be a useful tool to get more insight into the whole plant regulation of sulfate uptake and assimilation. Brassicaceae are characterized by their high requirement of sulfur for growth and in general seedlings appear to be resistant to the toxic effects of SO_2 and H_2S (De Kok et al., 2007; Yang et al., 2006b). Atmospheric levels up to $0.25 \mu\text{l l}^{-1}$ hardly affected biomass production and total sulfur content of the plants, the latter indicated an close interaction between atmospheric and pedospheric sulfur utilization (De Kok et al., 1997; Buchner et al., 2004b; Yang et al., 2006a,b; Koralewska et al., 2008; Aghajanzadeh et al., 2014; Shahbaz et al., 2014). For instance, H_2S exposure of Brassica resulted in a partial decrease in the uptake of sulfate by the root and down-regulation the sulfate reduction pathway in the shoot by reducing the expression and activity of APS reductase (De Kok et al., 1997; Westerman et al., 2000a,b, 2001a,b; Buchner et al., 2004; Koralewska et al., 2008; Shahbaz et al., 2014). Upon sulfate deprivation, Brassica seedlings were able to transfer to foliarly absorbed SO_2 and H_2S as the sole source of sulfur for growth (Stuiver and De Kok 2001; Buchner et al., 2004; Yang et al., 2006a,b; Koralewska et al., 2008; Aghajanzadeh et al., 2014; Shahbaz et al., 2014). Continuous exposure to atmospheric levels of $\geq 0.06 \mu\text{l l}^{-1}$ (levels which regularly occur in polluted areas) substantially contribute to sulfur nutrition of Brassica (Buchner et al., 2004; Yang et al., 2005, 2006a,b; De Kok et al., 2007; Koralewska et al., 2008; Aghajanzadeh et al., 2014; Shahbaz et al., 2014).

In the current study, the interaction between pedospheric sulfate and atmospheric SO_2 and H_2S nutrition was studied, and the impact on sulfate uptake capacity of the root, sulfur metabolite

contents and the transcript levels of genes involved in the sulfate reduction pathway were studied in seedlings of two Brassica species, *Brassica juncea* and *Brassica rapa*. These two species strongly differ in levels and composition of secondary sulfur compounds, viz. glucosinolates in their shoots (Aghajanzadeh et al., 2014, 2015). The aim of the study was to obtain insights into (i) the whole plant regulation of sulfur uptake and assimilation in Brassica and (ii) the significance of shoot to root signaling therein.

2. Materials and methods

2.1. Plant material and H_2S and SO_2 exposure

Seeds of *B. juncea* cv. Rugosa and *B. rapa* cv. Komatsuna; Van der Wal, Hoogeveen, The Netherlands) were germinated in vermiculite in a climate-controlled room. Day and night temperatures were 22 and 18 °C (± 1 °C), respectively, relative humidity was 60–70%. The photoperiod was 14 h at a photon fluence rate of $300 \pm 20 \mu\text{mol m}^{-2} \text{s}^{-1}$ (400–700 nm) at plant height, supplied by Philips GreenPower LED (deep red/white 120) production modules. Ten day-old seedlings were transferred to an aerated 25% Hoagland nutrient solution at 0.5 mM sulfate for 3 days and subsequently transferred to fresh Hoagland nutrient solution at 0 mM sulfate (–S, sulfate-deprived; all sulfate salts were replaced by chloride salts) or 0.5 mM sulfate (+S, sulfate-sufficient) in 13 l stainless steel containers (10 sets of plants per container; three plants per set). Plants were exposed to $0.25 \mu\text{l l}^{-1}$ H_2S or SO_2 for 7 days in 150 l cylindrical stainless steel cabinets (0.6 m diameter) with a poly (methylmethacrylate) top. Sealing of the lid of the container and plant sets prevented absorption of atmospheric H_2S or SO_2 by the solution. Day and night temperatures were 24 and 20 °C (± 2 °C), respectively, and relative humidity was 40–50%. The photoperiod was 14 h at a photon fluence rate of $300 \pm 20 \mu\text{mol m}^{-2} \text{s}^{-1}$ (within the 400–700 nm range) at plant height, supplied by Philips GreenPower LED (deep red/white 120) production modules. Adjusting temperature of the cabinet wall controlled the temperature in the cabinets. The air exchange was 401 min^{-1} , whereas a ventilator stirred the air inside the cabinets continuously. Pressurized H_2S or SO_2 diluted with N_2 (1 ml l^{-1}) was injected into the incoming air stream and the concentration in the cabinet was adjusted to the desired level using electronic mass flow controllers (ASM, Bilthoven, The Netherlands). The sulfur gas level in the cabinets was monitored by an SO_2 analyzer (model 9850) equipped with a H_2S converter (model 8770; Monitor Labs, Measurement Controls Corporation, Englewood, CO, USA). Plants were harvested 3 h after the onset of the light period and the roots were rinsed in ice-cold demineralized water (for $3 \times 20 \text{ s}$). Roots were separated from the shoots, weighed, and for RNA isolation, plant material was frozen immediately in liquid N_2 and stored at -80 °C. For the measurement of the water-soluble non-protein thiol content, freshly harvested plant material was used, and for analysis of dry matter, total sulfur and sulfur content, plant tissue was dried at 80 °C for 24 h.

2.2. Total sulfur, sulfate and water-soluble non-protein thiol content

The total sulfur content was analyzed using a modification of the method as described by Jones (1995). Dried shoots and roots were pulverized in a Retsch Mixer-Mill (Retsch type MM2; Haan, Germany) and 50–150 mg of the samples was weighed into porcelain ashing trays. A 50% $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ (w/v) solution was added until saturation of the material, and the samples were dried overnight in an oven at 100 °C. Subsequently, the samples were ashed in an oven at 650 °C for 12 h. The residues were dissolved in 5 or 10 ml of 20% aqua regia (50 ml conc. HNO_3 and 150 ml conc. HCl in 1 l demineralized water) and quantitatively transferred to a

volumetric flask and made up to 50 or 100 ml with demineralized water. One SulphaVer[®] 4 Reagent Powder Pillow (HACH, Permchem[®] reagents, Loveland, USA) containing BaCl₂ was added to 10 or 25 ml of extract, and the turbidity was measured with a spectrophotometer (HACH DR/400 V, Loveland, CO, USA) at 450 nm.

For measurement of the sulfate content, pulverized dried plant material was incubated for 3–4 h in demineralized water (10 mg ml⁻¹) at 50 °C (Tausz et al., 1996; Yang et al., 2006a,b) and centrifuged at 30,000 × g for 15 min. Anions were separated by HPLC on an Agilent IonoSpher 5A anion exchange column (250 × 4.6 mm; Agilent Technologies, Amstelveen, The Netherlands) and sulfate content was determined refractometrically according to Maas et al., (1986). The HPLC system consisted of a Knauer HPLC pump model 100 and a Knauer differential refractometer model 98.00 (Knauer, Berlin, Germany). The mobile phase contained 25 mM potassium biphthalate (pH 4.3) with 0.02% NaN₃ (w/v).

Water-soluble non-protein thiols were determined colorimetrically as described by De Kok et al. (1988). Fresh plant material was homogenized in a solution containing 80 mM sulfosalicylic acid, 1 mM EDTA and 0.15% (w/v) ascorbic acid by an Ultra Turrax at 0 °C (10 ml g⁻¹ fresh biomass). Oxygen was removed from the solution by aspiration with N₂. The extract was filtered through one layer of Miracloth (Calbiochem) and then it was centrifuged at 30,000 × g for 15 min (0 °C). The total water-soluble non-protein thiol content of the supernatant was determined colorimetrically at 413 nm after reaction with 5, 5'-dithiobis [2-nitrobenzoic acid].

2.3. Sulfate uptake capacity

Sulfate uptake capacity was determined as described by Koralewska et al., (2007). Three sets of plants (3 plants per set) per treatment were transferred to a 25% Hoagland solution labeled with ³⁵S-sulfate (2 MBq l⁻¹) and incubated for 30 min at 25 °C at a photon fluence rate of 300 ± 20 μmol m⁻² s⁻¹ at plant height, supplied by Philips GreenPower LED (deep red/white 120) production modules. Subsequently, plants were removed and roots rinsed in ice-cold non-labeled nutrient solution (3 × 20 s). Roots and shoots were separated and digested in 1 N HCl at room temperature for 7 days. The extracts were filtered through one layer of Miracloth (Calbiochem Corporation, La Jolla, CA, USA) and 100 μl of the filtrate was mixed with 1 ml Emulsifier plus (PerkinElmer, Boston, MA, USA). Radioactivity was measured with a Liquid Scintillation counter (TRI-CARB 2000 CA Liquid Scintillation Analyzer, PerkinElmer, Waltham, MA, USA).

2.4. RNA extraction

Total RNA was isolated by a modified hot phenol method (Verwoerd et al., 1989). Frozen ground plant material was extracted in hot (80 °C) phenol/extraction buffer (1:1, v/v), 1 g ml⁻¹. The extraction buffer contained 0.1 M Tris-HCl, 0.1 M LiCl, 1% SDS (w/v), 10 mM EDTA, pH 8.0). After mixing, 0.5 ml of chloroform-isoamyl alcohol (24:1, v/v) was added. After centrifugation (13,400 × g) for 5 min at 4 °C, the aqueous phases were transferred to new tube. After adding an equal volume of chloroform and isoamyl alcohol, the total RNA was precipitated by 4 M LiCl overnight at 4 °C. Total RNA was collected and washed with 70% ethanol. Possible genomic DNA contamination was removed with a DNAase treatment step (Promega, USA). Phenol-chloroform-isoamyl alcohol and chloroform-isoamyl alcohol were used for further purification and total RNA was precipitated by ethanol and dissolved in diethylpyrocarbonate-treated water. The quantity and quality of RNA was checked using ThermoNanoDrop 2000 and RNA in each was adjusted to the same concentration. The integrity of RNA was

checked by electrophoresis by loading 1 μg RNA on a 1% TAE-agarose gel.

2.5. Real-time quantitative PCR of genes involved in sulfur reduction pathway

DNA-free intact RNA (1 μg) was reverse transcribed into cDNA with oligo-dT primers using a first strand cDNA synthesis kit (Promega, USA), according to the manufacture-supplied instructions. Subsequently, the cDNA was used as a template in real-time PCR experiments with gene-specific primers.

The primers for ATP sulfurylase (ATPS) were designed based on CDS of *B. juncea* gene (AJ223498.1) and its *B. rapa* gene orthologous (EX073466.1 and EX132953.1). Likewise, the primers for APS reductase (APR) were designed using CDS of *B. juncea* gene (AJ001207.1) and its *B. rapa* gene orthologous (EX100448.1 and EX050464.1). To design primers for APS kinase (APK), the CDS of Arabidopsis gene (AtU05238) was used to query homologous *B. juncea* (GT077720.1) and *B. rapa* (FY427499.1 and FY423472.1) sequences. Likewise, to design primers for sulfite reductase (SiR), the CDS of Arabidopsis gene (NM_120541) was used to query homologous *B. rapa* (EX130744.1, EX099351.1 and EX125480.1) and *B. napus* (EE438892.1). Expressed sequence tags (ESTs) were obtained from the publically available platform at NCBI. Gene-specific primer sets are listed in Table 1. RT-PCR was performed on Applied Bio Systems' 7300 real-time PCR system using the SYBR Green master mix kit (Thermo Scientific) based on manufacturer's instructions. The transcript level of the target gene and actin was measured using the comparative Ct method. Analysis of qPCR data was performed using three independent RNA preparations from separate plant shoots and roots.

2.6. Statistical analysis

Statistical analyses were performed using SPSS. Analysis of variance (ANOVA) was performed and the treatment means were compared using Tukey's HSD all-pairwise comparisons test at *p* = 0.05 level (treatment means of both sulfate-sufficient and sulfate-deprived plants; see figures) and at *p* = 0.05 level of significance (treatment means of sulfate-sufficient or sulfate-deprived plants; see text).

3. Results

3.1. Impact of pedospheric sulfate and atmospheric H₂S and SO₂ on growth, total sulfur, sulfate and thiol content, and sulfate uptake capacity

A 7-day exposure to 0.25 μl l⁻¹ H₂S or SO₂ did not affect plant biomass production and shoot to root biomass partitioning of *B. juncea* and *B. rapa* under sulfate-sufficient conditions (Fig. 1). Biomass production and shoot to root biomass partitioning of both species was significantly reduced after 7 days of sulfate deprivation, demonstrating that shoot growth was more affected than root

Table 1

List of primer sequences of forward and reverse strand of genes involved in sulfur assimilation pathway and actin.

Gene	Primer sequences (5'-3')	
	Forward	Reverse
ATPS	GTCTGGAGCCGATCAAG	CTTCGAGTATCGGTCATGAG
APR	CTCTTCGATGCCGTGGAG	ACCGTCCTCGTAGAAAGAGAAC
APK	GATTTGGGCTACTGGTCTTAG	TAAAGCTGAGATCACGGTTTA
SiR	GTGGTCGTGTTGGAGGTA	AGCCATTGCCGTTTGGTT
Actin	AGCAGCATGAAGATCAAGGT	GCTGAGGGATGCAAGGATAG

Fig. 1. Impact of H₂S, SO₂ and sulfate deprivation on biomass production of shoots and roots of *B. juncea* and *B. rapa*. 10 day old seedlings were grown on a 25% Hoagland solution containing 0.5 mM sulfate for 3 days and subsequently transferred to fresh 25% Hoagland solution at 0 (–S) or 0.5 mM sulfate (+S) and exposed to 0.25 μl l^{–1} H₂S or SO₂ for 7 days. The initial fresh biomass of shoots and roots in *B. juncea* were 0.044 ± 0.007 g and 0.020 ± 0.007 g, respectively. The initial fresh biomass of shoots and roots in *B. rapa* were 0.049 ± 0.012 g and 0.021 ± 0.002 g, respectively. Data on biomass production (g FW), shoot/root biomass partitioning represent the mean of 3 measurements with 9 plants in each (±SD). Means with different letters are significant different at $P < 0.05$ (Tukey's HSD all-pairwise comparisons test).

growth. The reduction in plant biomass production upon sulfate-deprivation was fully alleviated upon H₂S exposure and partially alleviated upon SO₂ exposure, showing that *B. juncea* and *B. rapa* were able to transfer to foliar absorbed sulfur instead of sulfate taken by the root as sulfur source for growth. However, the shoot to root biomass partitioning was significantly lower in H₂S- and SO₂-exposed sulfate-deprived *B. juncea* and *B. rapa* than that in sulfate-sufficient plants, both in absence and presence of these atmospheric sulfur gases. This indicated that in the absence of sulfate in the root environment, plants invested relatively more biomass in the root compared to sulfate-sufficient plants, even under circumstances in which the sulfur supply to the shoot was sufficient to maintain plant growth.

Seedlings of *B. juncea* and *B. rapa* were characterized by their high sulfate content (Fig. 2). Its content accounted for 48% and 62% of the total sulfur in roots and shoot of *B. juncea* and 54% and 45% of that in roots and shoot of *B. rapa*, respectively. H₂S and SO₂ exposure hardly affected the total sulfur and the sulfate contents of both shoots and roots of *B. juncea* and *B. rapa*. There was only a slight increase in the total sulfur content in roots of SO₂-exposed *B. juncea* plants.

H₂S and SO₂ exposure resulted in an increase in the content of the water-soluble non-protein thiol compounds in the shoots of sulfate-sufficient *B. juncea* and *B. rapa* plants, which was most noticeable upon H₂S exposure, whereas that of the roots remained unaffected (Fig. 3). Apparently, the foliar uptake resulted in an excess of reduced sulfur, resulting in enhanced levels of water-soluble non-protein thiols, presumably mainly glutathione. However, the water-soluble non-protein thiol fraction accounted only for a minor proportion of the total sulfur content in the shoot of *B. juncea* and *B. rapa* (approx. 2–4% of the total sulfur content; Figs. 2 and 3).

After 7 days of sulfate deprivation the total sulfur, sulfate and water-soluble non-protein thiol content decreased in roots and shoots of *B. juncea* and *B. rapa* (Figs. 2 and 3). The total sulfur content of H₂S and SO₂ exposure sulfate-deprived roots and shoots of both species was also much lower than that observed in sulfate-sufficient plants. However, this decrease could largely be attributed to the low content of sulfate in the roots and shoots (Fig. 2). The organic sulfur content of roots and shoots of H₂S- and SO₂-exposed *B. juncea* and *B. rapa* plants, which could be calculated by subtracting the sulfate content from the total sulfur content, was quite similar to that observed in sulfate-sufficient plants (approx. 100 μmol g^{–1} dry biomass). Evidently, at an atmospheric concentration of 0.25 μl l^{–1} the H₂S and SO₂, the foliarly absorbed sulfur was sufficient to cover the organic sulfur requirement for both shoots and roots of *B. juncea* and *B. rapa*. In both species the content of water-soluble non-protein thiol compounds of the shoots was quite similar (*B. rapa*) or somewhat higher than that of un-exposed sulfate-sufficient plants (Fig. 3). In the roots, however, the thiol contents of H₂S and SO₂ exposed sulfate-deprived *B. juncea* and *B. rapa* plants were substantially higher than that of sulfate-deprived plants, and quite similar (*B. rapa*) or two-fold lower than that observed in sulfate-sufficient plants (*B. juncea*).

Both H₂S and SO₂ exposure resulted in a significant decrease in the sulfate uptake capacity of roots of both species at sulfate-sufficient conditions (approx. 30%; Fig. 4). Apparently, foliar deposition of atmospheric sulfur gases resulted in a down-regulation of the activity of the sulfate transporters. The sulfate uptake capacity of roots of sulfate-deprived *B. juncea* and *B. rapa* plants was 1.5-fold and 2-fold higher than that of sulfate-sufficient plants. Upon H₂S and SO₂ exposure the sulfate uptake capacity of roots of sulfate-deprived plants was not decreased, and was even slightly further increased. Despite the fact that plants were able to transfer to foliar absorbed sulfur instead of sulfate taken up by the

Fig. 2. Impact of H₂S, SO₂ and sulfate deprivation on total sulfur and sulfate content of shoots and roots of *B. juncea* and *B. rapa*. For experimental details, see legends of Fig. 1. Data on sulfur and sulfate content (μmol g⁻¹ DW) represent the mean of three measurements with nine plants in each (±SD). Means with different letters are significant different at $P < 0.05$ (Tukey's HSD all-pairwise comparisons test).

root as their sulfur source for growth, the sulfate uptake capacity of the root remained strongly enhanced. Evidently, upon sulfate deprivation, H₂S and SO₂ exposure did not result in a down-regulation of the activity of the sulfate transporters.

3.2. Impact of pedospheric sulfate and atmospheric H₂S and SO₂ on the transcript levels of the enzymes of the sulfate reduction pathway

Some of the enzymes of the sulfate reduction pathway were differently expressed in *B. juncea* and *B. rapa*. In *B. juncea*, the transcript level of ATP sulfurylase (catalyzes the activation of sulfate) in roots was substantially higher than that observed in

roots of *B. rapa*. Even more pronounced was the higher transcript levels of APS kinase in both roots and shoot of *B. juncea*. This enzyme is important for the synthesis of secondary sulfur compounds. The transcript levels of ATP sulfurylase, APS kinase and sulfite reductase in roots and shoots of both species were not affected by a 7-day exposure to H₂S and SO₂ (Fig. 5). However, the transcript level of APS reductase in the shoots of sulfate-sufficient *B. juncea* and *B. rapa*, which is the key-regulating enzyme in the sulfate reduction pathway, was substantially decreased (up to 75%) upon H₂S and SO₂ exposure, whereas that of the roots remained unaffected.

Fig. 3. Impact of H₂S, SO₂ and sulfate deprivation on water-soluble non-protein thiol content of shoots and roots of *B. juncea* and *B. rapa*. For experimental details, see legends of Fig. 1. Data on thiols content (μmol g⁻¹ FW) represent the mean of two experiments with three measurements with nine plants in each (±SD). Means with different letters are significant different at $P < 0.05$ (Tukey's HSD all-pairwise comparisons test).

Fig. 4. Impact of H₂S, SO₂ and sulfate deprivation on sulfate uptake capacity of *B. juncea* and *B. rapa*. For experimental details, see legends of Fig. 1. Data on sulfate uptake capacity (μmol g⁻¹ FW root h⁻¹) represent the mean of 4 measurements with 3 plants in each (±SD). Means with different letters are significant different at $P < 0.05$ (Tukey's HSD all-pairwise comparisons test).

Sulfate-deprivation substantially affected the transcript levels of APS reductase, which was strongly enhanced in both shoots and roots of *B. juncea* and *B. rapa*. The expression of APR was a 5.5- and 6.4-fold enhanced in the shoots and roots of *B. juncea* and 3.5- and 4-fold in the shoots and roots of *B. rapa*, respectively (Fig. 5). Moreover, there was a slight increase in transcript level of ATP sulfurylase and APS kinase in the shoots of both species and in the roots of *B. rapa*. H₂S and SO₂ exposure resulted in a significant decrease in the transcript levels of APS reductase in both shoots and roots of sulfate-deprived *B. juncea* and *B. rapa* (up to 50%), however, they remained substantial higher than that observed in sulfate-sufficient plants. Sulfate deprivation also resulted in a significantly increased transcript level of APS kinase in shoot of both *B. juncea* and *B. rapa*, however in the roots expression was

unaffected. H₂S and SO₂ exposure of sulfate-deprived plants did not affect the transcript level of APS kinase in either species. The transcript level of sulfite reductase was not affected in sulfate-deprived *B. juncea* and *B. rapa*, and also not upon exposure to H₂S and SO₂ (Fig. 5). Evidently, APS reductase was the key enzyme, whose expression was substantially affected by changes in pedospheric and atmospheric sulfur supply.

4. Discussion

At a whole plant level, the uptake of sulfate by the roots, further distribution and subsequent assimilation is highly regulated and is coordinated and balanced by the actual sulfur requirement for biomass production (Hawkesford and De Kok, 2006). In general,

Fig. 5. Impact of H₂S, SO₂ and sulfate deprivation on transcript levels of ATP sulfurylase, APS reductase, APS kinase and sulfite reductase in shoots (above x axis) and roots (below x axis) of *B. juncea* and *B. rapa*. For experimental details, see legends of Table 1. Relative gene expression of these genes was determined by qRT-PCR and the mRNA levels were compared to actin. Data on relative expression represent the mean of 3 measurements with 3 plants in each ($\pm$ SD). Means with different letters are significant different at $P < 0.05$ (Tukey's HSD all-pairwise comparisons test).

the plant nutrient requirement ($\mu\text{mol g}^{-1} \text{ plant day}^{-1}$) at the vegetative stage may be derived from the growth rate ($\text{g g}^{-1} \text{ day}^{-1}$) times the mineral nutrient content of the plant ($\mu\text{mol g}^{-1} \text{ plant}$; De Kok et al., 2000; Durenkamp and De Kok, 2004). Brassica species are considered to have a high sulfur requirement, since they are characterized by a high growth rate (exceeding $35\% \text{ day}^{-1}$ at the used experimental conditions) and relatively high sulfur content. *B. juncea* had a somewhat slower rate of biomass production than *B. rapa* (Fig. 1; Aghajanzadeh et al., 2014).

From the current and previous results it was evident that seedlings of *B. juncea* and *B. rapa* were able to utilize foliarly absorbed atmospheric H₂S and SO₂ as sulfur sources for growth, whereas it did hardly affect the shoot and root dry matter content (Aghajanzadeh et al., 2014). Under sulfate-deprived conditions, an atmospheric level of $0.25 \mu\text{l l}^{-1}$ of these gases appeared sufficient to maintain a plant growth rate similar to that of sulfate-sufficient conditions. Despite the differences in patterns and kinetics in the foliarly uptake of H₂S (determined by the rate of its metabolism

into cysteine) and SO₂ (determined by the chemical/physical properties of the gas; De Kok et al., 2007), there were hardly differences in effects on plant response, under both sulfate-sufficient and sulfate-deprived conditions. Apparently not only the absorbed H₂S, but also SO₂, directly entered the sulfur assimilatory pathway without negative side effects.

H₂S and SO₂ exposure hardly affected the total sulfur and sulfate contents of shoots and roots *B. juncea* and *B. rapa* in sulfate-sufficient plants. Brassica species contain high levels of secondary sulfur compounds, viz. glucosinolates, whose levels were substantially higher in shoots of *B. juncea* than that of *B. rapa*. However, it has been observed that glucosinolates did not function in the storage of excessive sulfur and their composition was unaffected upon exposure to H₂S and SO₂ (Aghajanzadeh et al., 2014, 2015). Atmospheric sulfur deposition may result in a decrease in sulfate uptake by the root (Herschbach et al., 1995a,b; De Kok et al., 1997, 2007; Westerman et al., 2001a,b). This decrease was facilitated by a down-regulation of the expression and activity of Sultr1;2, a high affinity sulfate transporter responsible to the primary uptake of sulfate by the root of Brassicaceae at sulfate-sufficient conditions (Buchner et al., 2004; De Kok et al., 2007; Koralewska et al., 2008; Shahbaz et al., 2013). It has been suggested that this sulfate transporter might function as a sensor of the sulfur status of the plant (Zhang et al., 2014). From the current study it seems that H₂S and SO₂ had a comparable impact on the sulfate uptake capacity of *B. juncea* and *B. rapa*. The observed partial decrease in sulfate uptake capacity of the roots indicated, that even under sulfate-sufficient conditions, both *Brassica* species transferred from sulfate taken up by the root to sulfur absorbed by the shoot as sulfur source for growth. In *B. juncea* and *B. rapa*, 60% and 46% of the total sulfur in the plant was present as sulfate. This indicated that a complete down-regulation of sulfate uptake was not necessary and that the foliar uptake and assimilation of H₂S and SO₂ at 0.25 μl l⁻¹ was sufficient to cover the organic sulfur requirement for growth of these species. The signalling mechanism between the shoot and root and the interaction between the foliar uptake of atmospheric sulfur gases and the uptake of sulfate by the root is still unknown. It has been suggested that glutathione might function in the inter-organ regulation between the assimilation of sulfate in the shoots and the expression and activity of the sulfate transporters in the root, moreover it has been suggested that also high levels of sulfate might down-regulate sulfate transporters (Rennenberg et al., 1989; Davidian et al., 2000; Vauclare et al., 2002; Hawkesford and De Kok, 2006). It was clear from the present study that H₂S and SO₂ exposure did not affect the thiol and sulfate levels of roots in both *B. juncea* and *B. rapa*. Physically removing the shoots of *B. pkinensis* the shoots resulted in a 5-fold lower sulfate uptake capacity of the roots, demonstrating that the rate of sulfate uptake is largely determined by the sink capacity of the shoot for sulfur (Koralewska et al., 2009b). Metabolism of foliarly absorbed sulfur might similarly decrease the sink capacity of the shoot for sulfate supplied by the root resulting in the observed decrease in sulfate uptake capacity in *B. juncea* and *B. rapa*.

Sulfate-deprivation generally results in multiple responses in order to optimize the potential to make sulfate uptake and use more efficient: first there is a upregulation of the expression and activity of the sulfate transporters Sultr1;1 and Sultr1;2 in the root, which may occur within a few days period, and in addition plants invest relative more in root growth resulting in a decrease in shoot/root biomass partitioning (Buchner et al., 2004; Hawkesford and De Kok, 2006; De Kok et al., 2007; Koralewska et al., 2008; Shahbaz et al., 2013). However, H₂S and SO₂ exposure hardly affected the decrease in shoot/root biomass upon sulfate-deprivation, despite the fact that an atmospheric concentration of 0.25 μl l⁻¹ was sufficient to cover the overall sulfur requirement for growth. The significance of the sulfate concentration in the root environment in

the regulation of the shoot/root biomass partitioning needs to be investigated further. There was also a poor shoot to root signaling in the regulation of the sulfate transporters in sulfate-deprived plants upon H₂S and SO₂ exposure, since the sulfate uptake capacity of the roots remained upregulated and actually was even higher than that observed in unexposed sulfate-deprived plants. The local regulation of expression and activity of the sulfate transporters in the root was not determined apparently by the level of thiols, which was somewhat lower than that observed in sulfate-sufficient plants, but by the in situ sulfate concentration in the root (Buchner et al., 2004; De Kok et al., 2007; Koralewska et al., 2008; Shahbaz et al., 2013). This indicated that the local concentration of sulfate and possibly its reduction in the plastids of the root cells are more important in the regulation of the sulfate transporters than shoot to root signaling. Similarly, it was observed that in leaf tissue of *Brassica*, the gene expression of the sulfate transporters (and that of APS reductase) was strictly controlled by the in situ sulfate concentration possibly via an interaction with the sulfate reduction in the chloroplasts (De Kok et al., 2012). It was proposed that endogenous subcellular H₂S might play a role as a gaseous transmitter in the cross-talk between the sulfate reduction pathway in the chloroplast and the transcription of the genes encoding enzymes involved in the uptake and distribution of sulfate (and possibly that of APS reductase) in the nucleus.

Exposure of plants to atmospheric H₂S generally resulted in a strong decrease in gene expression, in protein levels and in enzyme activity of APS reductase, the key-regulating enzyme in the sulfate reduction pathway in the shoots (Westerman et al., 2000a,b, 2001a,b; Durenkamp et al., 2007; Koralewska et al., 2008; Shahbaz et al., 2014). Likewise, exposure of *B. juncea* and *B. rapa* to SO₂ and H₂S also resulted in a decrease in the transcript level of APS reductase in the shoots upon exposure to SO₂ and H₂S, whereas that transcript levels of the other enzymes of the sulfate reduction pathway were hardly affected. Cysteine and glutathione are suggested to function as signals in the regulation in the expression and activity of APS reductase (Leustek and Saito, 1999; Westerman et al., 2001b; Durenkamp et al., 2007; De Kok et al., 2007), which contents are generally enhanced in shoots of SO₂ and H₂S exposed plants (Westerman et al., 2001; Yang et al., 2006a; De Kok et al., 2007; Durenkamp et al., 2007; Koralewska et al., 2008; Shahbaz et al., 2013). In the present study high levels of water-soluble non-protein thiol compounds were accompanied with low transcript levels of APS reductase in shoots of sulfate-sufficient *B. juncea* and *B. rapa*, and low thiol levels with high transcript levels in roots and shoots of sulfate-deprived plants. However, in H₂S- and SO₂-exposed sulfate-deprivation plants the transcript levels of APS reductase in roots and shoots of *B. juncea* and *B. rapa* remained substantial higher than those in sulfate-sufficient plants at comparable or only slightly reduced water-soluble thiol levels. The latter observation demonstrated that the significance of thiols viz. cysteine and glutathione in the regulation of expression needs further evaluation.

The transcript levels of ATP sulfurylase and APK kinase of shoots of H₂S- and SO₂-exposed sulfate-deprived plants also remained significantly higher than that observed in sulfate-sufficient plants. The transcript levels of APS kinase, the key enzyme in the synthesis of sulfated groups of e.g. glucosinolates, were much higher in *B. juncea* than in *B. rapa*, which coincided with the differences in glucosinolate content between these species (Aghajanzadeh et al., 2014, 2015).

5. Conclusions

It is evident that atmospheric sulfur may significantly contribute to plant sulfur nutrition, and that the metabolism of foliarly absorbed sulfur decreases the sink capacity for sulfur in the shoots resulting in a decrease in the uptake of sulfate by the roots. It was

evident that in sulfate-deprived plants there was a poor shoot to root signaling in the regulation of expression of the sulfate uptake capacity, if absorbed atmospheric sulfur gases were the sole sulfur source for growth. The significance of thiol compounds viz. cysteine and glutathione in the regulation of the expression of APS reductase needs to be evaluated further.

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