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Atmospheric H_2S and SO_2 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 H_2S and SO_2 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 H_2S and SO_2 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 H_2S or SO_2 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 H_2S and SO_2 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. H_2S and SO_2 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, APK kinase and sulfite reductase were not affected in both sulfate-sufficient and sulfate-deprived plants upon H_2S and SO_2 exposure.

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 take up by the root is reduced to sulfide in 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).

SO_2 and H_2S 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 $\mu l\,l^{-1}$ 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., 2004,b; 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 $\geq 0.06\ \mu l\,l^{-1}$ (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 ± 20 $\mu mol\,m^{-2}\,s^{-1}$ (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 $\mu l\,l^{-1}$ 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 ± 20 $\mu mol\,m^{-2}\,s^{-1}$ (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 40 l 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 × 20 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% $Mg(NO_3)_2{\cdot}6H_2O$ (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