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Sulfur Fertilization and Fungal Infections Affect the Exchange of H_2S and COS from Agricultural Crops

Elke Bloem,*[,†] Silvia Haneklaus,[†] Jürgen Kesselmeier,[§] and Ewald Schnug[†]

[†]Institute for Crop and Soil Science, Federal Research Centre for Cultivated Plants (JKI), Bundesallee 50, D-38116 Braunschweig, Germany

[§]Biogeochemistry Department, Max-Planck-Institute for Chemistry, P.O. Box 3060, 55020 Mainz, Germany

ABSTRACT: The emission of gaseous sulfur (S) compounds by plants is related to several factors, such as the plant S status or fungal infection. Hydrogen sulfide (H_2S) is either released or taken up by the plant depending on the ambient air concentration and the plant demand for S. On the contrary, carbonyl sulfide (COS) is normally taken up by plants. In a greenhouse experiment, the dependence of H_2S and COS exchange with ambient air on the S status of oilseed rape (*Brassica napus* L.) and on fungal infection with *Sclerotinia sclerotiorum* was investigated. Thiol contents were determined to understand their influence on the exchange of gaseous S compounds. The experiment revealed that H_2S emissions were closely related to pathogen infections as well as to S nutrition. S fertilization caused a change from H_2S consumption by S-deficient oilseed rape plants to a H_2S release of 41 pg g^{-1} (dw) min^{-1} after the addition of 250 mg of S per pot. Fungal infection caused an even stronger increase of H_2S emissions with a maximum of 1842 pg g^{-1} (dw) min^{-1} 2 days after infection. Healthy oilseed rape plants acted as a sink for COS. Fungal infection caused a shift from COS uptake to COS releases. The release of S-containing gases thus seems to be part of the response to fungal infection. The roles the S-containing gases may play in this response are discussed.

KEYWORDS: *carbonyl sulfide, fungal pathogens, hydrogen sulfide, Sclerotinia sclerotiorum, sulfur fertilization*

INTRODUCTION

The concept of an enhanced plant resistance or tolerance against fungal pathogens induced by sulfur (S) was developed in the 1990s, when lower atmospheric S inputs caused by the desulfurization of fumes led to S deficiency in many regions accompanied by increasing infection rates with fungal pathogens.[1] Since that time, many researchers have found a relationship between the S nutritional status of the crop and its susceptibility to fungal diseases as reviewed by Haneklaus and co-workers.[2] Studies of the physiological background of the higher tolerance to fungal pathogens under optimum S supply revealed several possible mechanisms, such as maintaining high levels of glutathione (GSH) and glucosinolates, the deposition of elemental S, the formation of phytoalexines and S-rich peptides and proteins, or the release of gaseous S-containing compounds such as hydrogen sulfide (H_2S).[3−13]

The release of the highly fungitoxic H_2S and its accumulation in the cells and tissues seem to be the underlying mechanism to reduce fungal diseases, particularly because the toxicity of elemental S is also believed to depend on the formation of H_2S.[14] The question remains, however, whether the H_2S concentrations are high enough to combat fungal pathogens. The release of several volatile reduced S compounds (H_2S, carbonyl sulfide, dimethyl sulfide, carbon disulfide, methyl mercaptan) from various plant species has been observed.[13] Because most measurements were conducted with plants or cut plant parts that were fed concentrated S solutions, the release of S-containing gases was most probably overestimated.[15] The second problem causing erroneous values was that measurements were conducted with S-free air and thus not reflecting the natural gas exchange of the plant.[16] Only a limited number of field data on the emission of reduced gaseous S from plants under various nutritional and plant pathological conditions in ambient air exist.[17,18] The regulatory mechanisms by which H_2S is released, the extent of H_2S emissions under natural conditions, the relationship between plant S status and H_2S emissions, and the relationship to fungal diseases have been studied only sporadically.

Carbonyl sulfide (COS) is the most abundant S gas in the atmosphere with a medium mixing ratio of 500 ppt.[19] Generally, green vegetation is a dominant sink for COS.[20,21] COS taken up into plant cells is hydrolyzed to CO_2 and H_2S by carbonic anhydrase (CA; EC 4.2.1.1), and the CO_2 is assimilated by the carboxylating enzymes ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco; EC 4.1.1.39) or phosphoenolpyruvate carboxylase (PEPCase; EC 4.1.1.31).[22−25] The CA is thus one of the H_2S-releasing enzymes and represents a link between COS and H_2S metabolism in plants. The release of H_2S from COS is not easily identified in plants as it may be consumed for the synthesis of amino acids, but can be easily detected in less complex organisms.[26,27]

The aim of our study was to investigate the impact of S fertilization and fungal attack on the exchange of H_2S and COS and relate it to changes in S metabolite concentrations in the plants. Oilseed rape was chosen as the experimental crop because former investigations revealed a release of H_2S in response to S fertilization.[17] The crops were infected by *Sclerotinia sclerotiorum* because Wang et al.[28] demonstrated that

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the susceptibility of oilseed rape against *S. sclerotiorum* decreased with increasing S application rates. Moreover, the ascomycete fungus, *S. sclerotiorum*, is a very important highly destructive, soilborne fungus that infects more than 500 plant species worldwide, causing heavy economic losses.[29] The experimental design should reveal if the gaseous exchange of H_2S and COS is involved in the direct reaction of the plant to this fungal disease. To our knowledge this is the first time that the gas exchange of H_2S and COS was investigated under conditions of fungal infections combined with severe S deficiency.

MATERIALS AND METHODS

Experimental Design of the Greenhouse Experiment. A bifactorial greenhouse experiment was conducted with summer oilseed rape (*Brassica napus* var. Heros). The effect of S fertilization (0 and 250 mg S per pot) and inoculation with *S. sclerotiorum* was investigated on the gaseous exchange of H_2S and COS and on the thiol content in plant material. The experiment was conducted in Mitscherlich pots containing 8 kg of pure sand. Eight plants per pot were planted, and all treatments were grown with four repetitions. S was added as K_2SO_4, and K was balanced by applying adequate rates of KCl. All other nutrients were applied in physiologically optimum rates, split in two doses, before planting and at stem elongation.

The pathogen infection was performed two times, first at stem elongation (BBCH 33−34 according to Stauß et al.[30]) and second at flowering (BBCH 65). The pathogen *S. sclerotiorum* was previously cultivated for 6 days on potato dextrose agar plates. Infection was performed by fixing an infected agar plug 5 mm in diameter in a leaf axil. Half of the eight plants per pot were infected. In each pot four plants were left without infection as "noninfected plants". The real control plants without any pathogen exposure were grown in a separate chamber to prevent infection but under the same conditions (Figure 1). The gaseous S emissions were measured 1, 2, 3, and 6 days

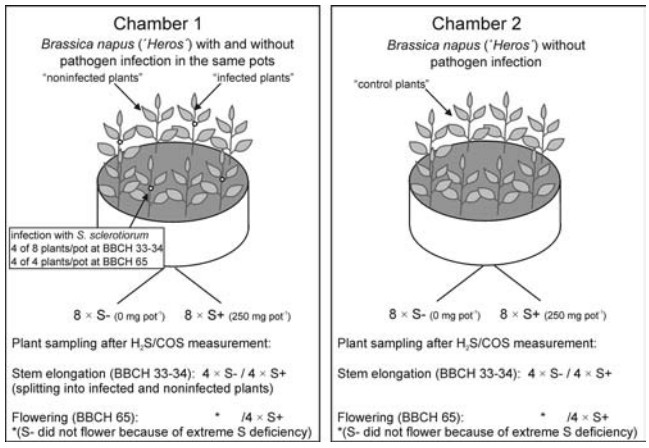

Figure 1. Experimental design of the greenhouse experiment.

after infection. The second infection was carried out only with plants that received S fertilization, because only these plants started to flower. Four plants were removed from the pots at elongation stage to leave enough room for the remaining four plants. At flowering these were infected three times each to maximize gas emission. The gaseous S emissions were recorded on five consecutive days after infection.

After the last emission measurements, the plants were dissected in leaves and stems at stem elongation stage and in leaves, stems, and flowers at flowering stage and then frozen in liquid nitrogen before freeze-drying. The dry weight of the plant parts was determined as a reference base for quantitative gas values. In the infected plants, the stem parts where the agar plug was originally set were sampled separately from the rest of the stem material, subsequently referred to as stem parts of the infection site. A visual scoring of the disease severity was performed directly at the infection site 6 days after infection on a scale from 0, indicating no infection, via 1 for mild infection, 3 for a medium strong infection, to 5, which represented a strong infection on all infection sites.

Chemical Analysis. For the extraction of total S, 0.5 g of dry plant material was digested with 4 mL of HNO_3 plus 1 mL of H_2O_2 in a microwave oven (CEM/MDS-2100, Kamp-Lintfort, Germany) at 950 ± 50 W for a total of 57 min. A pressure of 1.37 bar was reached at 20% of the power in 15 min and was kept for another minute; then the pressure was further raised in 15 min to 2.96 bar at full power and kept again for 1 min and, finally, in 15 min to 9.99 bar at full power and kept for another 10 min. Then the samples were allowed to cool for 20 min and, afterward, the digest was filled to 50 mL. The total S was analyzed by inductively coupled plasma−optical emission spectrometry (Spectro Flame M120S, Kleve, Germany).

Sulfate extraction was carried out according to the method of Novozamsky et al.,[31] and the measurement was performed by ion chromatography (Methrom 761 Compact IC equipped with a Metrosep Anion Dual 2 column).

Free cysteine, γ-glutamylcysteine (γ-EC), and glutathione (GSH) were determined by high-performance liquid chromatography (Merck Hitachi, Darmstadt, Germany) according to the method of Hell and Bergmann.[32] The extraction was carried out using 20−30 mg of finely ground freeze-dried plant material and 1 mL of 0.1 M HCl containing 4% Polyvidon-25. After removal of plant debris by subsequent centrifugation, the supernatant was used for reduction in the dark with dithiothreitol. The assay contained 1 M Tris-HCl, pH 8, 10 mM dithiothreitol, 0.08 M NaOH, H_2O, and plant extract or standard, respectively. After 1 h of reduction, sulfhydryl groups were derivatized with 25 μL of 10 mM monobromobimane and subsequently stabilized by the addition of 705 μL of acetic acid (5%). The separation of cysteine and GSH was carried out by HPLC using a 250 × 4.6 mm Nova-Pak C18 column (4 μm) (Waters 044380). Fluorescence detection was used for the measurement with excitation at 380 nm and an emission wavelength of 480 nm.

H_2S and COS emissions from plants were recorded using a gas chromatographic system, which was originally developed to determine reactive S compounds in the ambient air.[33] The analytical system comprises a portable, temperature-programmable gas chromatographic system, equipped with a packed column (Carbopack BHT 100, Supelco, Bellefonte, PA, USA) and a commercially available flame photometric detector (FPD 84XO/8500, Perkin-Elmer, Norwalk, CT, USA). A dynamic open chamber system was connected to a gas collector, which allowed the collection of a defined amount of air per time unit. It was necessary to concentrate the gases because the concentrations of S gases in the atmosphere are very low. Plants were put in the dynamic open chamber system, which was illuminated by high-pressure metal halide lamps sodium-400 W bulbs at 20−24 klx, and the measurements were conducted during the day at room temperature. The gases were concentrated by collecting 5 L of air in 25 min through cryogenic collectors, which were cooled in liquid argon at −186 °C. The reduced S gases with melting points between −86 and −138 °C freeze in the collectors, whereas N_2 and O_2 pass the traps. The measuring system and the design of the cryogenic traps were described in detail by Huber and Haunold.[34] After trapping of the S gases, the cooled sampling tubes were integrated into the carrier gas stream (N_2) of the GC and measured in a two-step desorption procedure: first, the sample loop was brought from liquid argon (−186 °C) into a bath made from dry ice and ethanol at a temperature of −79 °C. At this temperature CO_2, H_2S, and COS are volatilized completely, together with all other gases present in the air. The gases were transported from the original cryogenic trap to a second cryogenic trap, the focus trap, where only these three gases were concentrated by "cold desorption" for 5 min. Next, the focus trap was transferred to warm water to start the analytical run. The cold desorption step is of great importance for the measurement of H_2S because dramatic losses of H_2S may take place if H_2S and water come into contact.

Table 1. Influence of S Fertilization on the Emission of H_2S and COS from Oilseed Rape (var. Heros) at Stem Elongation (Data Obtained from Control Plants without Fungal Infection) and on Disease Severity with *Sclerotinia sclerotiorum*

S fertilization (mg pot^{-1})	H_2S emission[a] (pg g^{-1} (dw) min^{-1})	COS emission[a] (pg g^{-1} (dw) min^{-1})	disease severity (scale 0–5)[b]
0	−90.7 b	−65.3 a	3.3 a
250	+41.4 a	−173.9 a	4.3 a
LSD $_{5\%}$	99.2	463.6	1.7

[a]Different letters mark statistically different means at the 5% level by the Tukey test. [b]0 = no infection, 1 = mild, 3 = medium, and 5 = strong infection with *S. sclerotiorum* at the infection site.

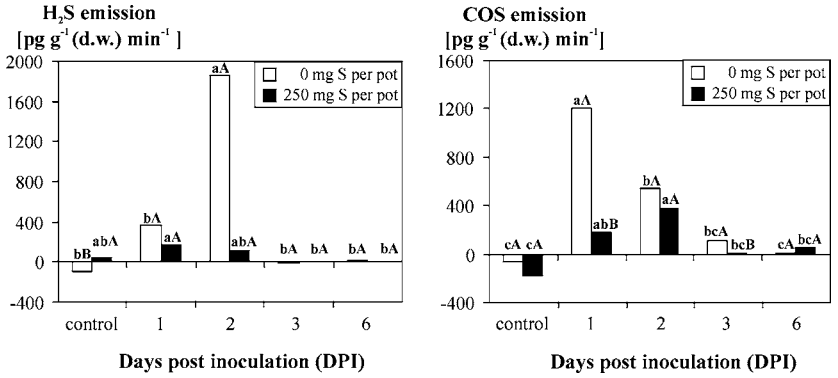

Figure 2. H_2S and COS emissions of summer oilseed rape at stem elongation in relation to infection with *Sclerotinia sclerotiorum* and S fertilization (different lower case letters indicate significant differences in the H_2S and COS emissions in relation to the days post inoculation; different upper case letters indicate significant differences in relation to S application at the $LSD_{5\%}$ level).

Table 2. Influence of S Fertilization and Fungal Infection with *Sclerotinia sclerotiorum* on S-Containing Compounds in Leaves and Stems of Oilseed Rape at Stem Extension

	S fertilization[a]			infection with *Sclerotinia sclerotiorum*[a]					
parameter	0 mg pot^{-1}	250 mg pot^{-1}	$LSD_{5\%}$	control[b]	noninfected plants[c]	infected plants[d]	stem parts of infection site[e]	$LSD_{5\%}$	interaction,[f] S × inf
mineral contents (mg g^{-1} dw)									
total S (leaf)	0.74 b	5.63 a	1.04	4.28 a	3.04 ab	2.75 b		1.27	ns
total S (stem)	0.94 b	2.41 a	0.45	1.76 ab	1.77 ab	1.53 b	2.13 a	0.64	ns
SO_4-S (leaf)	0.11 b	1.34 a	0.30	0.73 a	0.74 a	0.83 a		0.36	ns
SO_4-S (stem)	0.48 b	1.03 a	0.39	1.44 a	0.40 b	0.47 b	0.82 b	0.55	ns
thiols (nmol g^{-1} dw)									
leaf cysteine	37.3 b	232.0 a	43.7	236.0 a	76.1 b	114.2 b		53.5	***
leaf γ-EC	87.6 a	39.8 b	16.0	88.4 a	60.4 b	38.9 c		19.6	ns
leaf GSH	276.3 b	2370.3 a	236.7	1383.1 b	922.2 c	1851.3 a		290.0	***
stem cysteine	41.0 b	168.3 a	31.4	153.8 a	102.4 b	95.3 b	86.0 b	44.4	*
stem γ-EC	113.3 a	14.5 b	19.6	81.1 a	56.2 ab	31.8 b	78.4 a	27.7	ns
stem GSH	384.4 b	2638.6 a	316.7	1373.5 b	1300.6 b	2118.3 a	1446.6 b	447.8	ns

[a]Different letters indicate statistically different means of metabolite concentrations in relation to S fertilization or infection at the 5% level by the Tukey test. [b]Control plants were grown separate from infected plants. [c]Control plants were grown together with infected plants. [d]Plants were artificially infected. [e]Infection site of the stem showed visible symptoms of fungal infection. [f]Significance levels obtained from standard statistics were coded in the following way: ns, not significant; *, significant, $p < 0.05$; **, highly significant, $p < 0.01$; ***, very highly significant, $p < 0.001$.

For calibration, gaseous standards (Vici Metronics, Santa Clara, CA, USA) were used. Calibration curves were recorded over ranges of 0.5–7 ng of H_2S and 1–15 ng of COS. The system used in this study had detection limits of 52 pg of H_2S and 251 pg of COS per sample. The disadvantage of the system is the time-consuming sampling and measurement, which strictly limits the number of samples to not more than 20 per day. Furthermore, it is necessary to measure the ambient air in addition to the plant samples to monitor net H_2S emission or uptake as the open chamber system is ventilated with ambient air.

Statistical Analysis. Two-way ANOVA was used to analyze the results, and means were compared by the Tukey test at the 5% probability level.

RESULTS AND DISCUSSION

The exchange rates of S gases between plants and the atmosphere are affected by many factors. Besides the role of the plant species itself, the most important environmental

	S_{total} Leaf	SO_4-S Leaf	Cysteine Leaf	γ-EC Leaf	GSH Leaf	H_2S exchange 1 DPI[a]	COS exchange 1 DPI[a]	H_2S exchange 2 DPI[a]	COS exchange 2 DPI[a]
SO_4-S Leaf	**0.805** ***								
Cysteine Leaf	**0.914** ***	**0.715** ***							
γ-EC Leaf	**-0.379** *	**-0.530** **	ns						
GSH Leaf	**0.716** ***	**0.843** ***	**0.623** ***	**-0.685** ***					
H_2S 1 DPI[a]	ns	ns	ns	**-0.417** *	ns				
COS 1 DPI[a]	**-0.514** *	**-0.418** *	**-0.536** **	ns	ns	**0.675** ***			
H_2S 2 DPI[a]	ns	ns	ns	ns	ns	ns	**0.442** *		
COS 2 DPI[a]	ns	ns	ns	**-0.463** *	ns	**0.495** *	**0.751** ***	ns	
Disease severity	ns	ns	ns	**-0.613** **	ns	ns	**0.433** *	ns	**0.502** *

Figure 3. Correlations (r) of S-containing compounds in leaves of oilseed rape with H_2S and COS gas exchange and fungal infection with *Sclerotinia sclerotiorum* at stem elongation. (Significance levels obtained from standard statistics were coded in the following way: ns, not significant; *, significant, $p < 0.05$; **, highly significant, $p < 0.01$; ***, very highly significant, $p < 0.001$).

triggers are the ambient air concentration of the S gases, the photosynthetically active radiation, temperature, and humidity.[35−37] In the present study the S status of the plants and fungal infection were investigated as further important factors.

Exchange of H_2S and COS in Relation to S Supply and Fungal Infection at Stem Elongation. The release of H_2S by plants was positively affected by S fertilization (Table 1). Without S fertilization the plants took up H_2S from the ambient air as indicated by the negative emission values, whereas they emitted H_2S when they were fertilized with S. Without fungal infection, the plants acted as a sink for COS, and the S nutritional status did not significantly affect the COS uptake. Therefore, we conclude that the uptake of COS is most likely not regulated by the S status of the plant, whereas the uptake of H_2S is directly linked to the S nutritional status of the crop.

Fungal infection with *S. sclerotiorum* caused an increase in H_2S release and a shift from COS uptake to COS emission (Figure 2). Already 1 day after infection, the H_2S emissions of S-fertilized plants increased 4-fold from 41.4 to 165.9 pg H_2S g^{-1} (dw) min^{-1}. This increase was even stronger in the S-deficient control, in which infection increased the H_2S emission to a maximum value of 1842 pg H_2S g^{-1} (dw) min^{-1} (Figure 2). These results are in accordance with the regulation of a H_2S-releasing enzyme, L-cysteine desulfhydrase, which is repressed with increased S fertilization and up-regulated by fungal infection.[38,39] Similarly, COS uptake shifted to a maximum emission of 1207 pg COS g^{-1} (dw) min^{-1} in infected S-deficient plants and 382 pg COS g^{-1} (dw) min^{-1} in S-fertilized plants. This result is a strong indication that the release of H_2S and COS is a specific response to fungal infection and not a mechanism of disposal of excess S.

It has to be noted that the measurements showed an extremely high variation, particularly 2 days after infection. This variation is probably technical rather than biological. Due to analytical restrictions the time discrepancy between two measurements (~30 min) may miss effects such as short bursts of H_2S or COS as a reaction to the infection with fungal pathogens.

At stem elongation, total S as well as sulfate-S content increased significantly with S fertilization (Table 2), and the unfertilized plants showed strong symptoms of S deficiency. Infection with *S. sclerotiorum* caused a significant decrease in total S in leaves as well as in sulfate-S in stems. This loss of S can be caused by the release of gaseous S compounds such as H_2S after fungal infections.[17,38] Thiol concentrations were determined in relation to S fertilization and fungal infection, as cysteine plays a central role in the release of H_2S. In addition, GSH is an important metabolite in stress response to biotic and abiotic stress and is thus likely to react to fungal infection with *S. sclerotiorum*.[40,41] Accordingly, Kruse et al.[42] have shown that the concentrations of cysteine as well as GSH in *Arabidopsis thaliana* increased in response to fungal infection. In the present study, cysteine and GSH concentrations in leaf and stem of oilseed rape increased with S fertilization, whereas γ-EC, the precursor of GSH, decreased. In leaves and stems, fungal infection caused a decrease in cysteine and γ-EC concentrations but 34 and 54% increases in GSH levels, respectively. Cysteine decreased by >50% in leaves and by 38% in stems of infected plants, indicating a consumption of cysteine in response to the pathogen infection.

The shift in the thiol concentrations was lower directly at the site of infection: here, only the cysteine concentration, reduced by 44%, differed significantly from the control plants. Interestingly, also the noninfected plants next to the infected plants showed significant changes in the thiol contents compared to the control plants that were spatially separated. Cysteine and γ-EC contents were significantly reduced in leaves. GSH levels, which were increased in the infected plants, were reduced in the noninfected plants next to infected ones. Levels of all three compounds were reduced in the stem parts,

too, but the decrease was less pronounced. These changes in the noninfected plants next to infected plants were unexpected and indicate a regulation, probably triggered by gaseous transmitters such as H_2S. Indeed, several studies indicated that H_2S functions as an endogenous signal molecule in plants, for example, inducing L-cysteine desulfhydrase upon pathogen attack, increasing abiotic stress tolerance, and regulating flower senescence.[38,43−45] Zhang et al.[44] demonstrated that H_2S protects plants from oxidative stress by increasing the activity of enzymes involved in detoxification of reactive oxygen species, such as superoxide dismutase and catalase and by lowering activity of lipoxygenases.

The correlations between the release of gaseous S compounds and the S-containing plant metabolites as well as disease severity were determined to unravel possible links between these processes (Figure 3). Correlations between S-containing compounds, gas emissions, and infection were stronger in leaf material than in stems; therefore, only leaf data are presented in Figure 3. As expected, strong correlations were found between total S and S-containing metabolites, as well as between cysteine and GSH and GSH and its precursor γ-EC.

Correlations between metabolite levels and emissions of H_2S and COS are presented for 1 and 2 days after infection because at this time the burst of H_2S and COS release occurred (Figure 2). The emission of H_2S was negatively correlated with the γ-EC concentrations and positively correlated with COS release. The γ-EC concentration decreased with infection with a concomitant increase in GSH. Most likely, both reactions occur at the same time, the release of H_2S and the conversion from γ-EC into GSH. A direct relationship is not likely as no correlations were found for H_2S emissions and γ-EC 2 days after infection.

The emissions of COS correlated well with the concentrations of S metabolites in plants. One day after infection a negative correlation was found for COS and total S, sulfate-S, and cysteine, indicating a consumption of these S-containing compounds when COS was built. Therefore, COS is probably a product built during the decomposition of S-containing metabolites. Moreover, a positive correlation was found between the release of COS and the extent of infection with *S. sclerotiorum*. The stronger the infection, the higher was the release of COS. A decreasing extent of infection was correlated to high concentrations of γ-EC.

Changes in the Gas Exchange of H_2S and COS in Relation to Fungal Infection at Flowering. Flowering is the growth stage when the infection with *S. sclerotiorum* occurs under natural conditions, so the experiment was repeated at full flowering. Results shown in Table 3 and Figure 4 refer to plants sufficiently supplied with S as only these plants started to flower. In comparison to inoculation at stem elongation, the infection process was slower and macroscopic symptoms took longer to develop at the flowering stage. Disease severity was significantly lower at flowering with a mild to medium infection (Table 3) compared to a medium to strong infection at stem elongation (Table 1). Also, the emissions of H_2S and COS were much lower and achieved their maximum later than at stem elongation stage (Figure 4). S-containing metabolites did not change significantly due to infection with the only exception of the total S content, which surprisingly increased with infection (Table 3).

Correlations for the gaseous exchange and disease severity are presented in Table 4. As only S-fertilized plants were investigated, the relationships between the different S-containing compounds were not that pronounced. Whereas at stem elongation the release of H_2S and COS was correlated with the levels of S-containing compounds in leaves, at flowering most correlations were found between the gas release and compound concentrations in stems, especially the SO_4-S. The releases of H_2S and COS were interrelated with each other during the first 4 days after infection, and the best correlation was found 2 days after infection ($COS = 4.73 \times H_2S + 166.29$; $r^2 = 72.6\%$). The disease severity was not correlated with the gaseous release (data not shown) but was positively correlated with the total S content in leaves (Table 4). It seems, therefore, that the infection by *S. sclerotiorum* caused a higher S uptake when enough S was available. Kruse et al.[42] concluded from macroarray analysis that the S metabolism of *A. thaliana* was activated after infection with all tested pathogens; for example, transcript levels of sulfate transporters (Sultr 2;1, Sultr5;1) were 1.2−2- or 2−3-fold higher in S-fertilized and S-deficient plants, respectively. Therefore, the significantly higher total S contents in infected plants (Table 3) indicate similar activation of S uptake and metabolism in oilseed rape.

The plants showed a very different response to pathogen infection depending on the timing of infection. At stem

Table 3. Influence of Fungal Infection with *Sclerotinia sclerotiorum* on S-Containing Compounds in Oilseed Rape Leaves and Stems at Flowering

	control[a]	infected plants[a]	$LSD_{5\%}$
disease severity (scale 1−5)[b]	0.0 b	2.5 a	0.9
mineral contents (mg g^{-1} dw)			
total S (leaf)	6.6 b	12.1 a	2.9
total S (stem)	1.9 a	2.3 a	0.8
SO_4-S (leaf)	5.3 a	6.5 a	2.1
SO_4-S (stem)	1.5 a	1.2 a	0.5
thiols (nmol g^{-1} dw)			
leaf cysteine	179.7 a	147.2 a	62.6
leaf γ-EC	32.0 a	23.7 a	17.5
leaf GSH	2120.1 a	1832.4 a	466.7
stem cysteine	143.8 a	117.6 a	59.6
stem γ-EC	21.2 a	4.8 a	20.5
stem GSH	1898.7 a	1616.8 a	412.7

[a]Different letters indicate statistically different means of metabolite concentrations in relation to fungal infection at the 5% level by the Tukey test. [b]0 = no infection, 1 = mild, 3 = medium, 5 = strong infection with *Sclerotinia sclerotiorum* at the infection site.

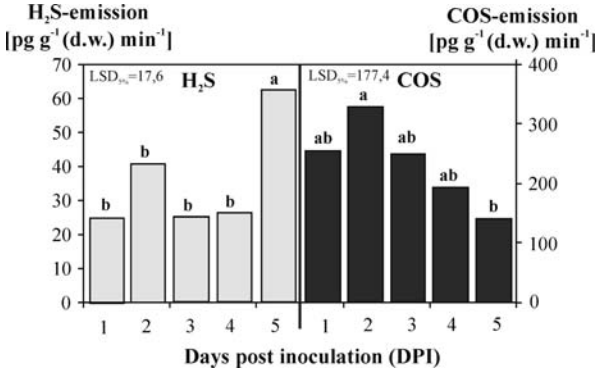

Figure 4. H_2S and COS emissions of summer oilseed rape in relation to fungal infection with *Sclerotinia sclerotiorum* at flowering (different lower case letters indicate significant differences in the H_2S and COS emissions in relation to the days post inoculation at the $LSD_{5\%}$ level).

Table 4. Correlations of the Release of H_2S and COS and Disease Severity with the S-Containing Compounds in Leaves and Stems of Oilseed Rape after Fungal Infection with *Sclerotinia sclerotiorum* at Flowering

		total S[a]		SO_4-S[a]		cysteine[a]		γ-EC[a]		GSH[a]	
	DPI[b]	leaf	stem	leaf	stem	leaf	stem	leaf	stem	leaf	stem
H_2S	1	ns	ns	ns	0.71**	ns	0.61*	ns	0.57*	−0.55*	ns
	2	ns	ns	ns	0.62*	ns	ns	ns	ns	ns	ns
	3	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	4	ns	ns	ns	0.56*	ns	ns	ns	ns	ns	ns
	5	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
COS	1	ns	ns	ns	0.86**	ns	0.61*	ns	0.67*	ns	0.64*
	2	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	3	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	4	ns	ns	−0.62*	0.58*	ns	ns	ns	ns	ns	0.59*
	5	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
DS[c]		0.67**	ns	ns	ns	ns	ns	ns	ns	ns	ns

[a]Significance levels obtained from standard statistics were coded in the following way: ns, not significant; *, significant, $p < 0.05$; **, highly significant, $p < 0.01$; ***, very highly significant, $p < 0.001$. [b]DPI, days after infection. [c]DS, disease severity.

elongation when the vegetative growth was important, the emission of H_2S and COS was related to S metabolism of leaves, and a higher release of gaseous S compounds caused a decrease in total S in the plant material. At flowering, when metabolites were transported into generative plant parts, an activation of the S metabolism by fungal infection resulted in accumulation of total S. However, the release of gaseous S compounds was much lower and more closely related to S compounds in the stem parts of the plants. Therefore, most likely, the timing of fungal infection is important with respect of the efficiency of S to reduce fungal infection. A comparison of plants grown under sufficient and optimum sulfate supply revealed that plants better supplied with S were able to react to an infection 1−2 days earlier with an increase in cysteine and GSH, and the authors explained the lower susceptibility of plants sufficiently supplied with S by the shorter reaction time.[42] Furthermore, studies with grapes infected by powdery mildew revealed a release of H_2S directly after fungal infection.[17] Obviously, the potential to release H_2S is important for the course of the infection.

What Can Be the Metabolic Background of the Shift from COS Sink to COS Source? The uptake of COS by plants exhibits a linear function of the ambient COS mixing ratio.[22] Different compensation points were determined as to when uptake changed into emission: 37 ppt for lichens, 53 ppt for soils, and 90−150 ppt for higher vegetation.[16,22,46] In view of these low compensation points, the emission of COS from green plants is not very likely. Nevertheless, emissions of COS from trees, with higher compensation points, were reported.[36] Kesselmeier and Merk[16] measured a significant emission of COS from rapeseed leaves at COS mixing ratios lower than 90 ppt but had no explanation for the physiological background of COS emissions. In contrast, Sandoval-Soto et al.[21] did not find any emission of COS from several tree species even below a virtual compensation point, indicating the absence of COS-producing processes.

COS is taken up by plants very effectively.[21,47,48] The CA activity is 1000-fold higher for COS than CO_2, and the S group is rapidly cleaved, reduced, and emitted in the form of H_2S; about 60% of the S taken up as COS is rapidly re-emitted as H_2S.[23−25,27] On the other hand, Bartell et al.[49] determined that only 1−2% of the S provided as COS to the soil/plant system was emitted as H_2S. This controversy can, however, be understood by taking the soil uptake into account.[46,50]

We have shown that fungal infection significantly increased the emission of S-containing gases (Figures 2, and 4) for the first 2 days after infection, and then the emission declined again rapidly. The change from a COS sink to a COS source caused by fungal infection indicates a contribution of COS metabolism to the cascade of stress-related processes. The function of COS synthesis and emission is, however, unclear. Only very limited information is available about COS synthesis in plants. It was proposed that COS can be synthesized in chloroplasts during CO_2 fixation, when during decarboxylation of phosphoenolpyruvate to acetyl-CoA thiopyruvic acid is used.[51] Moreover, isothiocyanates have been discussed as precursors for COS synthesis in plants as well as carbon disulfide and thiocarbamic acids.[52] Not only in plants but also in humans and other mammals, which produce COS endogenously, the precursors for COS synthesis are uncertain, but there is some evidence that COS is formed by the reaction of activated oxygen with methionine and cysteine.[53] In aquatic systems GSH was tested to be the most effective precursor of COS formation. The efficiency of cysteine was lower, and the addition of methionine did not result in the formation of COS.[54] Flöck et al.[54] concluded from the fact that the pH in the chloroplastic stroma is similar to ocean water (pH 8.2) that also in plants a COS formation from GSH is a possible pathway for COS release. The negative correlation between the release of COS and cysteine or γ-EC, respectively (Figure 3), indicates a contribution of these metabolites to COS release. Indeed, cysteine and GSH are key metabolites in pathogen response as their concentrations increased with all tested pathogen infections.[42]

In a study with transgenic potato lines the gaseous exchange of H_2S and COS was measured in relation to enzyme modifications related to the S metabolism of the plants.[55] The potato plants generally acted as a sink for COS, but transgenic lines overexpressing homoserine kinase (HSK) were emitting COS, indicating a strong change in the metabolism of these plants. The product of the HSK, *O*-phosphohomoserine, is reacting with cysteine to build methionine, underscoring a possible involvement of methionine in COS emission.

It could also be hypothesized that the fungus itself is responsible for the release of H_2S and COS. However, experiments with agar plates containing different concentrations of cysteine, GSH, or elemental S in the medium inoculated by *S. sclerotiorum* or measured without fungal

infection revealed no differences in the emission of H_2S and COS from plates with and without fungal infection (Bloem, unpublished data). Therefore, it is likely that the emission of H_2S and the shift from COS uptake to COS emission was caused by changes in the metabolism of the plants.

Is the Emission of H_2S or COS High Enough To Combat Fungal Pathogens? The question remains whether the change from COS uptake to COS emission is a mechanism of release of excessive S metabolite concentrations or part of the stress response to fungal pathogens. The correlations determined in this study (Figure 3; Table 4) unfortunately do not allow clear conclusions to be drawn. COS has the potential to combat fungi. It is used as a fumigant in high concentrations of approximately 80 g m^{-3} in the postharvest control of diseases as an alternative to methyl bromide.[56,57] We determined a maximum COS emission of 1207 pg COS g^{-1} (dw) min^{-1} for S-deficient plants with a medium dry weight of 1.8 g per plant. Within 24 h such a plant would emit 3.12 μg of COS. Assuming a mean plant density of oilseed rape of 60 plants m^{-2}, the COS emission would account for, at maximum, 190 μg m^{-2} in 24 h. For S-fertilized plants a maximum emission of 114 μg m^{-2} in 24 h was estimated. This emission is most likely too low to have an antifungal effect, but it is possible that higher peak emissions not captured by the measuring procedure occur, because the emission of each plant was recorded for only 25 min. Moreover, it is possible that much higher concentrations occur locally at the infection site.

Liu et al.[58] investigated the molecular response of fungal pathogens to COS. Among the up-regulated 500 transcripts, enzymes belonging to the "defense" group such as superoxide dismutase and sulfide:quinine oxidoreductase were found as well as GSH reductase. The studies of Lui et al.[58] proved the fungicidal activity of COS and allowed speculation that production of reactive oxygen species by the plant can be an important mechanism to combat fungal pathogens.

For H_2S a maximum emission of 287 μg m^{-2} in 24 h was calculated using the same estimates as for COS. This emission is most likely also too low to combat fungal disease. Fumigation experiments with *Rhizoctonia solani* showed that even very high concentrations of >30 mg L^{-1} of H_2S reduced colony growth of the fungi by only 17%, and with longer duration of fumigation an inverse effect was observed.[59] Although the significance of H_2S against fungal infections may be questioned, fungal infections obviously induce a high release of H_2S.[15,17,38] The higher total S content in leaves of infected plants at flowering most likely indicates the activation of the S metabolism by the infection, which was demonstrated in other studies, too.[60−62] Fungal infection of *Arabidopsis* caused gene expression and formation of sulfur-containing defense compounds such as camalexin, defensin, and indole glucosinolates, of which indole glucosinolates showed an early defensive role as inhibitors of host penetration and camalexin at later stages of the infection process.[60,61] An activation of cysteine and GSH metabolism was determined in tobacco plants during a compatible plant−virus interaction.[62,63] Therefore, there are other pathways connected to the S metabolism besides the release of H_2S and COS that are involved in pathogen defense.

It is also possible that the H_2S and COS released in response to fungal infection act as signaling or regulatory compounds. However, more experiments are needed to answer this intriguing question as well as to evaluate the relevance of COS and H_2S emissions after fungal attack for atmospheric chemistry.

This is particularly important because environmental changes that will cause a higher infection pressure for certain fungi may have a strong impact on atmospheric chemistry, when plants normally acting as a sink for COS change into a source.

AUTHOR INFORMATION

Corresponding Author

*E-mail: elke.bloem@jki.bund.de. Phone: ++49 531 596 2200. Fax: ++49 531 596 2199.

Notes

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ABBREVIATIONS USED

COS, carbonyl sulfide; CO_2, carbon dioxide; DPI, days post inoculation; dw, dry weight; GSH, glutathione; H_2S, hydrogen sulfide; HPLC, high-performance liquid chromatography; HSK, homoserine kinase; $LSD_{5\%}$, least significant difference at a 5% significance level; S, sulfur; γ-EC, γ-glutamylcysteine.

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