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The Impact of Atmospheric H_2S on Growth and Sulfur Metabolism of *Allium cepa* L.

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K e y w o r d s : *Allium cepa*, onion, hydrogen sulfide, sulfur metabolism, nitrogen metabolism, H_2S deposition, alliins.

S u m m a r y

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The impact of atmospheric H_2S deposition on growth and sulfur metabolism has been studied in onion (*Allium cepa* L.). The H_2S uptake followed saturation kinetics with respect to the H_2S concentration. The maximum H_2S uptake rate (JH_2S_{max}) was approx. 1 μmol g^{-1} FW h^{-1} and the KH_2S (H_2S concentration at which $½JH_2S_{max}$ was reached) was approx. 1.5 μl l^{-1}, which demonstrated that onion had a rather high H_2S uptake rate when compared with other species. Upon exposure of onion to 0, 0.075, 0.15, 0.225 and 0.3 μl l^{-1} H_2S for two weeks, growth was only slightly reduced at 0.3 μl l^{-1} H_2S. H_2S exposure resulted in an increased content of sulfate, total thiols and total sulfur in the shoot, while that in the roots was unaltered. There was a slight increase in total nitrogen content in the shoot, whereas the content of nitrate and amino acids was hardly affected. There was a substantial increase in the organic sulfur fraction in the shoot upon H_2S fumigation, which was probably due to an increase in the alliin content.

Introduction

Atmospheric H_2S has a paradoxical impact on plants. It may negatively affect growth at atmospheric levels of 0.03 μl l^{-1} and higher and may even cause visible injury and defoliation at ≥ 0.3 μl l^{-1}, however, there is a wide variation in susceptibility between species to H_2S (DE KOK & al. 1998, 2000, 2002). On the other hand, foliarly absorbed H_2S may be utilized as sulfur source for growth and may be beneficial if the pedospheric sulfur supply is limited (DE KOK & al. 2000, 2002, WESTERMAN & al. 2000). H_2S is taken up via the stomates, metabolized with high

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affinity into cysteine and subsequently into other sulfur compounds (DE KOK & al. 1998, STUIVER & DE KOK 2001). In *Brassica oleracea* L. there was a direct interaction between foliar H_2S deposition and the uptake and metabolism of pedospheric sulfate (DE KOK & al. 2000, WESTERMAN & al. 2000, 2001a,b).

Allium cepa L. (onion) was possibly one of the first domesticated vegetables by man and it was already cultivated by the ancient Egyptians. *Allium cepa* appears to be a species with a high sink capacity for reduced sulfur. This species belongs to the genus *Allium* (about 400 species), which derives its name from an allyl group in its secondary sulfur compounds. These compounds, specific for the family Alliaceae, are known as alliins and consist of different stereoisomers of *S*-alk(en)ylcysteine sulfoxides. Cysteine is the major precursor for the synthesis of alliins (BLOCK 1992). The content of alliins in the leaves can directly be related to the sulfur status and can be affected by the sulfate supply to the roots (RANDLE & al. 1993, 1999, HANEKLAUS & al. 1997). The alliins may account for up to 80% of the total organic sulfur fraction in onion and they are metabolically inert end-products, which can not be re-metabolized as sulfur source (SCHNUG 1993).

The aim of our study is to get insight into the interaction between atmospheric and pedospheric sulfur nutrition in onion and the significance of secondary sulfur compounds as sink for foliarly absorbed sulfur gases. The present study is focused on the kinetics of H_2S uptake by onion and on the impact of H_2S fumigation on growth, the sulfur content and its distribution in both shoot and roots.

Material and Methods

Seeds of onion (*Allium cepa* L. cv. Nerato F1; Nickerson-Zwaan, Made, The Netherlands) were germinated in vermiculite in a climate-controlled room. Day and night temperatures were 20 and 17 °C, respectively, with a relative humidity of 60-70%. The photoperiod was 14 hours at a photon fluence rate of 250-300 µmol m^{-2} s^{-1} (PAR 400-700 nm).

For H_2S deposition measurements two-week-old seedlings were transferred to 30 l tanks (12 plants per set, 20 sets per tank) containing a 25% Hoagland nutrient solution, pH 5.9, and grown for 10 days. H_2S uptake kinetics were measured as described by STUIVER & DE KOK 2001. Plants were taken from the nutrient solution, transferred to plastic pots filled with fine perlite, 12 plants per pot, and placed in 3 l glass cuvettes. The air flow through the cuvette was approximately 100 l h^{-1}, the temperature was 23 ± 1 °C, the relative humidity was about 40 ± 10% and the photon fluence rate was 190 ± 10 µmol m^{-2} s^{-1} (PAR 400-700 nm). The H_2S uptake rate (JH_2S) and the transpiration rate (JH_2O) were derived from the difference in concentration of H_2S and that of H_2O vapor between the inlet and outlet port of the cuvette and the total shoot fresh weight of the exposed plants, and calculated according to DE KOK & al. 1991. The apparent maximum H_2S uptake rate (JH_2S_{max}) and the atmospheric H_2S concentration at which $½JH_2S_{max}$ was reached (KH_2S) were calculated and fitted for Michaelis-Menten kinetics by Enzfitter (a Data Analysis Program by R.J. Leatherbarrow, Elsevier-BIOSOFT, Cambridge, U.K.). The ratio of H_2S deposition velocity to aqueous vapor efflux (gH_2S/gH_2O) was calculated and values were multiplied by 1.369 ($\sqrt{}$(molecular weight H_2S)/$\sqrt{}$(molecular weight H_2O), in order to correct for the differences in diffusion velocity between H_2S and H_2O (DE KOK & al. 1991, 1998).

For the assessment of the impact of H_2S fumigation on growth and metabolite content, two-week-old seedlings were transferred to stainless steel containers (19.5 x 15.0 x 45 cm; 6 plants per set, 10 sets per container) filled with a 25% Hoagland nutrient solution, pH 5.9. Containers were

placed in 150 l cylindrical stainless steel cabinets (0.6 m diameter) with a polycarbonate top, as described by STUIVER & al. 1992 and exposed to 0, 0.075, 0.15, 0.225 and 0.3 µl l^{-1} H_2S for two weeks. Day and night temperatures were 24 and 18 °C (± 1 °C) respectively, relative humidity was 40-50% and the photoperiod was 14 hours at a photon fluence rate of 300-350 µmol m^{-2} s^{-1} (PAR 400-700 nm) at plant height, with Philips HPL(R)N (400 W) as light source. Temperature was controlled by adjusting the cabinet wall temperature; the air exchange was 40 l min^{-1} and the air inside the cabinets was stirred continuously by a ventilator. Pressurized H_2S diluted with N_2 (1 ml l^{-1}) was injected into the incoming airstream and adjusted to the desired level by ASM electronic mass flow controllers (Bilthoven, The Netherlands). H_2S levels in the cabinets were controlled by an SO_2 analyzer (model 9850) equipped with a H_2S converter (model 8770, Monitor Labs, Measurement Controls Corporation, Englewood, CO 80112, USA).

Anions were determined according to MAAS & al. 1986. Shoot and roots of powdered dry material (dried at 120 °C for 18 h) were extracted for anion determination by incubating them overnight in demineralized water (50 mg in 5 ml) at 40 °C (TAUSZ & al. 1996). Homogenates were filtered through one layer of Miracloth and the filtrate was centrifuged at 30,000 *g* for 15 min (0 °C). Sulfate and nitrate in the supernatant were separated on an Ionospher 5A anion exchange column with guard column (250 x 4.6 and 30 x 3.0 mm, respectively, Varian/Chrompack Benelux, Bergen op Zoom, The Netherlands). The HPLC apparatus further consisted of a Separations high precision pump, model 300 (H.I. Ambacht, The Netherlands), provided with a Rheodyne sample injector, model 7175 (loop volume 20 µl; Cotati, CA 94928, USA), and a Knauer differential refractometer, model 98.00 (Bad Homburg, Germany). Potassium biphthalate (25 mM, pH 4.3), containing 0.02% NaN_3, was used as a mobile phase. The flow rate was 1 ml min^{-1}; detector temperature was kept at 25 °C by a waterbath. The HPLC was connected with a Shimadzu Chromatopac C-R8A data processor (Kyoto, Japan).

Free amino acid content was determined according to ROSEN 1957. Shoot and roots of frozen fresh material were homogenized in demineralized water at 0 °C with an Ultra Turrax for 15 s (1 g in 10 ml; STUIVER & al. 1992). Homogenates were filtered through one layer of Miracloth, incubated in a boiling waterbath for 10 min and the filtrate was centrifuged at 30,000 *g* for 15 min (0 °C). Free amino acid content of the supernatant was measured by ninhydrin colorimetric determination at 579 nm.

Analysis of the total sulfur content of shoot and roots was performed using a modification of the method as described by JONES 1995. Samples were dried at 120 °C for 18 h, pulverized by a Retsch microdismembrator (type MM2; Haan, Germany) and 50-100 mg of the samples was weighed into porcelain ashing trays. 50% $Mg(NO_3)_2 \cdot 6\ H_2O$ was added to saturate the material, after which it was dried in an oven at 100 °C overnight. The samples were ashed in an oven at 650 °C for 12 h. The residue was dissolved in 10 ml 20% aqua regia (50 ml HNO_3 and 150 ml HCl in 1 l distilled water) and the volume was adjusted to 100 ml with distilled water. One SulfaVer®4 Reagent Powder Pillow (HACH, Permachem® reagents, Loveland, USA) containing $BaCl_2$ was added to 25 ml of the mixture and the turbidity was measured on a spectrophotometer (HACH DR/400V, Loveland, USA) at 450 nm.

Total nitrogen content of shoot and roots was determined with the Kjeldahl method according to BARNEIX & al. 1988. All nitrogen was reduced to NH_4^+ by boiling the tissue in concentrated H_2SO_4 in the presence of a catalyzer. NH_4^+ was determined colorimetrically at 410 nm after reaction with Nessler's reagent.

The organic N and S content was calculated by subtracting the inorganic N and S content from the total N and S content.

For measurements of.the total thiol content, shoot and roots were extracted after STUIVER & al. 1992 and the content of total water-soluble non-protein sulfhydryl compounds was measured according to DE KOK & al. 1988. The relative growth rate (RGR) was calculated from the ln-transformed plant fresh weights (HUNT 1982).

Data were statistically analyzed using an unpaired Student's t-test and a F-test for linearity.

Results and Discussion

The uptake of gases by plant shoots is predominantly determined by both the diffusive conductance of the stomates and the mesophyll (internal) resistance (DE KOK & TAUSZ 2001). The uptake of H_2S by plant shoots appears to be largely dependent on the mesophyll resistance, *viz.* the rate of H_2S metabolism into cysteine and subsequently into other sulfur compounds (DE KOK & al. 1998, 2000, 2002, DE KOK & TAUSZ 2001). The maximum uptake rates of H_2S differ considerably among species (DE KOK & al. 2002).

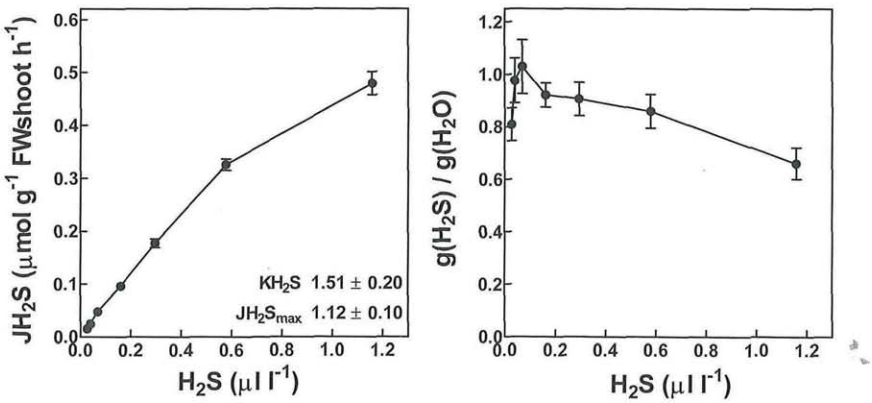

Fig. 1. Kinetics of H_2S uptake in shoots of onion. Two-week-old seedlings were grown on a 25% Hoagland nutrient solution at 24 °C for 10 days after which the H_2S uptake (JH_2S) was measured at various atmospheric H_2S levels. The mean transpiration rate (JH_2O) was 18.6 ± 0.8 mmol g^{-1} fresh weight h^{-1}. KH_2S and JH_2S_{max} are expressed as µl l^{-1} and µmol g^{-1} fresh weight h^{-1}, respectively. Data represent the mean of 4 measurements with 12 plants in each (± SD). Repeating the experiment gave approx. the same results (1.38 and 1.04 for KH_2S and JH_2S_{max}, respectively).

Similar to observations with other species, the H_2S uptake by shoots of onion followed saturation kinetics with respect to the H_2S concentration and the saturation kinetics fitted well with the Michaelis-Menten equation. The maximum H_2S uptake rate (JH_2S_{max}) was 1.1 µmol g^{-1} FW h^{-1} and the H_2S concentration at which $\frac{1}{2}JH_2S_{max}$ was reached (KH_2S) was 1.5 µl l^{-1} H_2S (Fig. 1), which showed that onion had a rather high capacity for H_2S uptake and a higher KH_2S value when compared with other species (DE KOK & al. 2002). The ratio of H_2S deposition velocity to aqueous vapor efflux (gH_2S/gH_2O) was calculated to obtain insight into the factors determining H_2S uptake in the shoots of onion. The gH_2S/gH_2O ratios remained constant and close to 1 at levels up to 0.3 µl l^{-1} H_2S, which indicated that uptake of H_2S was limited by its diffusion through the stomates only and that the mesophyll (internal) resistance was close to zero (Fig. 1).

Onion is a rather slow growing species with a RGR of approx. 9.5% day^{-1} (on a plant fresh weight basis at a day/night temperature of 24/18 °C) and appeared to be resistant to relatively high levels of H_2S (Table 1). The plant biomass production was hardly affected upon a two-week exposure to atmospheric H_2S levels up to 0.3 µl l^{-1}. Shoot/root ratio and dry matter content in shoot and roots were not affected upon H_2S fumigation (Table 1).

Table 1. The impact of H_2S exposure on growth of onion. Two-week-old seedlings were grown on a 25% Hoagland nutrient solution at 24 °C for two weeks and exposed to 0, 0.075, 0.15, 0.225 and 0.3 µl l^{-1} H_2S. Data on fresh weight (g) and dry matter content (DMC) represent the mean of 6 and 3 measurements, respectively, with 6 plants in each (± SD). RGR (% day^{-1}) was calculated using the ln-transformed plant fresh weight over a two-week interval as described by HUNT 1982. There were no significant differences between treatments ($P<0.05$, Student's *t*-test).

	0 µl l^{-1} H_2S	0.075 µl l^{-1} H_2S	0.15 µl l^{-1} H_2S	0.225 µl l^{-1} H_2S	0.3 µl l^{-1} H_2S
Fresh weight					
Shoot	0.31± 0.07	0.32 ± 0.07	0.34 ± 0.05	0.31 ± 0.07	0.26 ± 0.05
Roots	0.12 ± 0.02	0.13 ± 0.03	0.13 ± 0.02	0.12 ± 0.02	0.11 ± 0.02
S/R ratio	2.55 ± 0.30	2.55 ± 0.32	2.55 ± 0.31	2.48 ± 0.21	2.36 ± 0.24
RGR					
Plant	9.5 ± 1.0	9.9 ± 1.4	10.5 ± 1.5	9.7 ± 1.2	8.7 ± 1.0
DMC					
Shoot	6.59 ± 0.65	6.39 ± 0.54	6.65 ± 0.80	6.51 ± 0.48	6.65 ± 0.58
Roots	4.46 ± 0.49	4.21 ± 0.35	4.19 ± 0.55	4.22 ± 0.50	3.99 ± 0.36

Exposure of onion seedlings to atmospheric H_2S resulted in an increased content of sulfate, thiols and total sulfur in the shoots. There was a slight increase in total nitrogen content of the shoot, whereas that of nitrate and amino acids was hardly affected. Metabolite content of the roots was not affected upon H_2S exposure. At 0.3 µl l^{-1} H_2S sulfate and total sulfur contents in the shoot were increased with 6.9 and 17.7 µmol g^{-1} FW, respectively, whereas the total nitrogen content in the shoot increased with 29 µmol g^{-1} FW and the nitrate content was not significantly affected (Fig. 2). This demonstrated that in particular for sulfur the greater proportion of the increase in its content was due to an increase in the organic sulfur fraction. In unexposed plants the organic N/S ratio was around 41, predominantly representing the molar ratio of N/S in the proteins. However, upon H_2S exposure the organic N/S ratio decreased with the H_2S concentration, which likely has to be attributed to an increase in non-protein organic sulfur compounds (Fig. 3). It has been observed that onion has a sink capacity for sulfur which is metabolized into secondary sulfur compounds *viz.* alliins (RANDLE & al. 1993, 1999, HANEKLAUS & al. 1997). The molar ratio of N/S in alliins and its precursors is ≤ 2. The present data provide circumstantial evidence that a great part of the increase in total sulfur content upon H_2S exposure may be attributed to an accumulation of alliins or its precursors. The impact of H_2S exposure on alliin

synthesis will further be investigated. The experimental techniques will have to be optimized, since alliin compounds are rather unstable during extraction and subject to degradation.

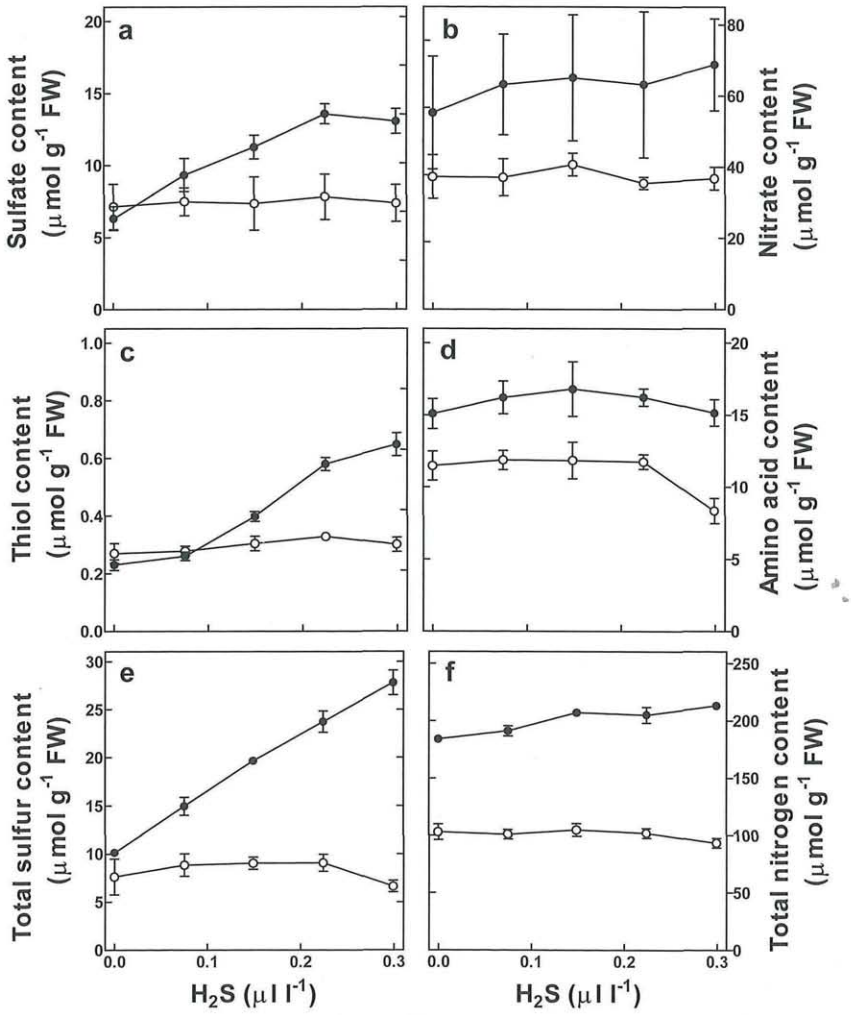

Fig. 2. The impact of H_2S exposure on the sulfate, nitrate, thiol, amino acid, total sulfur and total nitrogen content of onion. Two-week-old seedlings were grown on a 25% Hoagland nutrient solution at 24 °C for two weeks and exposed to 0, 0.075, 0.15, 0.225 and 0.3 µl l^{-1} H_2S. The sulfate content (a), the nitrate content (b), the total thiol content (c), the free amino acid content (d), the total sulfur content (e) and the total nitrogen content (f) of shoot (closed circles) and roots (open circles) are expressed in µmol g^{-1} FW. Data on sulfate, nitrate, free amino acid, total sulfur and total nitrogen represent the mean of 3 measurements with 36 plants in each (± SD). Total nitrogen and total sulfur contents increased linearly with the H_2S concentration ($P<0.05$, F-test). Data on the total thiol content represent the mean of 4 measurements with 6 plants in each (± SD).

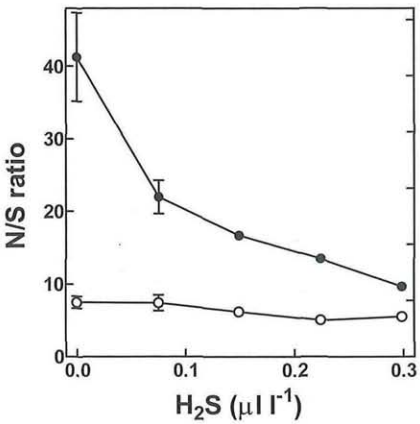

Fig. 3. The impact of H_2S exposure on the organic and inorganic N/S ratio of onion. The organic (closed circles) and inorganic (open circles) N/S ratio's (± SD) were derived from data presented in Fig. 2.

H_2S had a strong impact on thiol levels of the shoot. At 0.3 µl l^{-1} H_2S the thiol content in the shoot was 2.8 times higher than that in the shoot of the control plants but at 0.075 µl l^{-1} H_2S it was only 1.1 times higher, indicating that at low levels of H_2S the incorporation of cysteine (and glutathione) into other metabolic compounds, possibly alliins, was high and prevented a substantial accumulation of thiols (Fig. 2c). Future studies will include measurements on the composition of the thiol pool, since H_2S exposure may not only affect the content but also the composition of the thiol pool (BUWALDA & al. 1993, POORTINGA & DE KOK 1997, TAUSZ & al. 1998, WESTERMAN & al. 2000).

In *Brassica oleracea* there was a strong interaction between the uptake and metabolism of atmospheric H_2S and pedospheric sulfate. Upon H_2S exposure the sulfate uptake was downregulated, whereas the total sulfur content was hardly affected (DE KOK & al. 2000, WESTERMAN & al. 2001a). It appeared from our experiments that the sulfur metabolism in onion was differently regulated since the total sulfur content increased linearly with the H_2S concentration.

The interaction between atmospheric and pedospheric sulfur nutrition and its consequences for alliin synthesis will further be evaluated.

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