Chapter 6

Metabolism of atmospheric sulfur gases (H$_2$S, SO$_2$) in *Allium cepa*

**Abstract.** The impact of atmospheric sulfur gases was studied in onion (*Allium cepa* L.). The occurrence of toxic effects of H$_2$S in onion depended not only on the atmospheric H$_2$S level but also on the duration of the exposure. Prolonged exposure (38 days) of onion to $\geq 0.3$ µl l$^{-1}$ H$_2$S resulted in a strong reduction in shoot biomass production. H$_2$S exposure resulted in a decrease in the organic N/S ratio at all levels (0.15 to 0.6 µl l$^{-1}$ H$_2$S), which could be attributed to an increase in the pool of secondary sulfur compounds and not to changes in the sulfolipid content. The latter even decreased upon H$_2$S exposure when expressed on a lipid basis. SO$_2$ exposure resulted in an enhanced content of sulfate and total sulfur in the shoot, whereas roots were not affected. In contrast to exposure to H$_2$S, SO$_2$ exposure did not result in an increase in non-protein organic (secondary) sulfur compounds, which showed that these compounds only were a sink pool for reduced atmospheric sulfur, when both the uptake of sulfate by the roots and its reduction in the shoot were by-passed.

**Introduction**

Generally, sulfate taken up by the roots is used as the main source of sulfur for plants and the uptake, transport and subcellular distribution of sulfate are mediated by specific sulfate transporter proteins (Hawkesford, 2003; Hawkesford *et al*., 2003a; Buchner *et al*., 2004a,b). The uptake of sulfate by the roots and its transport to other plant parts are highly regulated and the affinity of the sulfate transporters towards sulfate is high; a maximum uptake and transport rate is generally already reached at $\leq 0.1$ mM sulfate (Chapter 4, Durenkamp and De Kok, 2004; Hawkesford and Wray, 2000; Buchner *et al*., 2004a). The expression and activity of the sulfate transporter proteins, as well as the activity of the enzymes of the sulfate reduction pathway, strongly depend on the sulfur nutritional status of the plant (Buchner *et al*., 2004a). Prior to its incorporation into organic compounds, sulfate needs to be reduced to sulfide, a process that primarily takes place in the chloroplasts. Subsequently, sulfide is incorporated into cysteine, the precursor for most other organic sulfur compounds (Chapter 8, Fig. 1). In most plants the predominant proportion of the organic sulfur is present in the protein fraction as cysteine and methionine residues (up to 70 % of total S), however, species like onion also may contain high amounts of secondary sulfur compounds. Part of the organic sulfur is present in the lipid fraction; in general sulfoquinovosyldiacylglycerol (SQDG) appears to be the predominant
plant sulfolipid and it accounts for 1 to 6 % of total S (Heinz, 1993; De Kok et al., 1997; Benning, 1998; Harwood and Okanenko, 2003).

In spite of their potential phytotoxic effects, foliarly deposited atmospheric sulfur gases as H$_2$S and SO$_2$ can also be used as sulfur source for growth, and they even may be beneficial if the sulfate supply to the roots is limited (Chapter 4, Durenkamp and De Kok, 2004; De Kok et al., 2000, 2002a,b). Due to the impermeability of the cuticle, H$_2$S and SO$_2$ are taken up via the stomates and their uptake is both dependent on the stomatal conductance and on the internal (mesophyll) resistance towards these gases (De Kok et al., 1998, 2002a,b). The uptake of H$_2$S is largely determined by the internal resistance, viz. the rate of metabolism of the absorbed sulfide into cysteine (Chapter 8, Fig. 1). The rate of uptake depends on the activity of O-acetylsereine(thiol)lyase and the availability of its substrate O-acetylsereine (Stuiver and De Kok, 2001) and it shows saturation kinetics with respect to the atmospheric H$_2$S level, which can be described by Michaelis-Menten kinetics (Chapter 3, Durenkamp and De Kok, 2002; De Kok et al., 1998; Stuiver and De Kok, 2001). In contrast to H$_2$S, the uptake of SO$_2$ is largely determined by the stomatal conductance, since the internal resistance to SO$_2$ is low due to its high solubility and hydration in the cell sap. In general, there is a linear relation between the uptake of SO$_2$ and the level in the atmosphere (De Kok and Tausz, 2001). Although SO$_2$, via sulfite, can directly be used in the sulfate reduction pathway, the greater part is oxidized to sulfate and transferred into the vacuole, especially at levels exceeding the sulfur requirement for growth (Chapter 8, Fig. 1). Atmospheric sulfur gases have shown to be a useful tool to study sulfate uptake and sulfur assimilation by providing an extra source of sulfur taken up by the shoot, beyond the existing controls of sulfate uptake by the roots.

*Allium cepa* (onion) is one of the most important horticultural crops in the world. Secondary sulfur compounds (γ-glutamyl peptides and alliins) and their degradation products are responsible for the important role of *Allium* species in the food and phytopharmaceutical industry. The γ-glutamyl peptides are thought to act as precursors for the synthesis of alliins and they might have a function in the storage of sulfur and nitrogen (Randle and Lancaster, 2002; Jones et al., 2004). The likely precursors for the synthesis of γ-glutamyl peptides and alliins are the thiol compounds γ-glutamyleicysteine and glutathione, which are products of the sulfur assimilation pathway (Chapter 8, Fig. 1). In onion H$_2$S exposure resulted in an increase in sulfate, thiols and other organic sulfur compounds in the shoot. The estimated N/S ratio of the latter compounds appeared to be 2 or less (Chapter 3, 4, Durenkamp and De Kok, 2002, 2003, 2004), indicating that the increase in the organic sulfur fraction could not be explained by an increase in the protein fraction (N/S ratio of proteins is generally around 40). It needs to be evaluated whether the increase in organic sulfur compounds upon H$_2$S exposure was due to an accumulation of secondary sulfur compounds (γ-glutamyl peptides and alliins) and/or sulfolipids (Chapter 3, 4, Durenkamp and De Kok, 2002, 2003, 2004). In addition, it needs to be assessed to what extent the observed accumulation of sulfur compounds is specific for H$_2$S or the consequence of bypassing the regulatory control of the uptake of sulfate by the roots. In the present paper
the impact of H$_2$S and SO$_2$ on growth and sulfur metabolism has been compared. The significance of sulfolipids and secondary sulfur compounds as possible pool for excessive deposited atmospheric sulfur and the possible down-regulation of the sulfate reduction pathway upon H$_2$S exposure are discussed.

**Results and discussion**

**Atmospheric H$_2$S: toxin vs. nutrient**

Atmospheric sulfur gases are potentially phytotoxic, however, there is a large variation between species in the susceptibility towards these gases and the mechanisms of toxicity are still not completely understood. Like cyanide, sulfide complexes with high affinity to metallo groups in proteins (for instance heme-containing NADH oxidizing enzymes) and this reaction is probably the primary biochemical basis for the phytotoxicity of H$_2$S (Maas and De Kok, 1988; De Kok et al., 1998, 2002b). Mutagenic effects of accumulated thiol compounds (Glatt et al., 1983) or sulfide itself might also be a cause for the phytotoxicity of H$_2$S, since exposure to H$_2$S resulted in an increase in chromosomal aberrations in apical meristems and root tips (Wonisch et al., 1999a,b; Stulen et al., 2000). In general, dicotyledons are more susceptible to H$_2$S than monocotyledons, since in the latter H$_2$S hardly has direct access to the vegetation point (Stulen et al., 2000).

Onion and related *Allium* species, as monocotyledons, were not very susceptible to the toxic effects of H$_2$S (Chapter 3, 4, Durenkamp and De Kok, 2002, 2003, 2004). Short-term exposure (one week) up to 0.6 µl l$^{-1}$ H$_2$S, a level which by far exceeds the sulfur requirement for growth, did not result in a reduction of growth in onion (Chapter 4, Durenkamp and De Kok, 2004). However, prolonged exposure to the same range of H$_2$S levels for 38 days resulted in a substantial decrease in biomass production and a slight increase in dry matter content in onion shoots at levels $\geq$ 0.3 µl l$^{-1}$ H$_2$S (Fig. 2). Apparently, the occurrence of toxic effects of H$_2$S in onion depended not only on the atmospheric H$_2$S level but also on the duration of the exposure. The latter might be due to a cumulative effect of sulfide or produced toxic metabolites for instance in meristematic tissue. Prolonged exposure to H$_2$S resulted in an increased content of sulfate and other sulfur-containing compounds, as illustrated by a maximal 5-fold increase in the total sulfur content of the shoot upon exposure up to 0.6 µl l$^{-1}$ H$_2$S (Fig. 2). The organic N/S ratio was decreased at all levels of H$_2$S exposure, independent of the effects of H$_2$S phytotoxicity (Fig. 2). The decrease in the organic N/S ratio could be attributed to an increase in non-protein organic (secondary) sulfur compounds, which pool might be a sink for reduced sulfur (Chapter 3, 4, Durenkamp and De Kok, 2002, 2003, 2004). Prolonged H$_2$S exposure also resulted in an enhancement of nitrogen-containing compounds in the shoot, which possibly was the consequence of a disturbed metabolism and/or an alternation in tissue and shoot development.
Fig. 2. Impact of prolonged H$_2$S exposure on growth and sulfur and nitrogen metabolism in shoots of onion (Allium cepa L. cv. Nerato). Seedlings were grown in vermiculite for two weeks and subsequently transferred to a regular potting soil and exposed to 0, 0.15, 0.3 and 0.6 µl l$^{-1}$ H$_2$S for 38 days (prolonged exposure). Data represent the mean of three measurements with five plants in each (± SD).
Atmospheric H$_2$S could be used as a sulfur source for growth in onion, especially when the sulfate supply to the roots was deprived (Chapter 4, Durenkamp and De Kok, 2004). However, upon prolonged exposure H$_2$S appeared to be phytotoxic and it reduced biomass production.

**Impact of H$_2$S exposure on sulfolipids**

The main plant sulfolipid sulfoquinovosyldiacylglycerol (SQDG) is synthesized from UDP-sulfoquinovose and diacylglycerol with sulfite as the likely sulfur precursor (Sanda et al., 2001; Harwood and Okanenko, 2003). Sulfite is synthesized from APS by APS reductase and this enzyme is the predominant site of regulatory control of the sulfate reduction pathway (De Kok et al., 2002a; Vauclare et al., 2002). The sulfolipid content of the shoot (expressed on a lipid basis) decreased upon exposure to H$_2$S (Table 1), which could be caused by a down-regulation of the sulfate reduction pathway and by a subsequent decrease in sulfite production, the sulfur precursor of SQDG (Sanda et al., 2001). This suggestion is supported by observations in *Brassica oleracea*, where a similar decrease in sulfolipid content (expressed on a lipid basis) was observed upon H$_2$S exposure (De Kok et al., 1997). The sulfate reduction pathway is known to be down-regulated via APS reductase upon H$_2$S exposure in *Brassica oleracea* (Westerman et al., 2001b). Since the sulfolipid content was not increased upon exposure to H$_2$S, sulfolipids did not act as a sink pool for atmospheric reduced sulfur.

The total lipid content of the shoot was increased upon exposure to H$_2$S, which could not be explained by an increase in either sulfolipid or pigment content (Table 1). It needs to be evaluated to what extent this increase in lipid content upon H$_2$S exposure can be attributed to changes in the overall structure and/or composition of membranes. Another option for the increase in total lipid content could be the formation of vesicles containing secondary sulfur compounds, as suggested by Turnbull et al. (1981). The possible enhancement of the secondary sulfur compounds content in the shoot might be accompanied with a subsequent increase in vesicle formation resulting in an increase in the total lipid content. The latter was not observed in *Brassica oleracea* (De Kok et al., 1997), since in this species an accumulation of secondary sulfur compounds was absent upon H$_2$S exposure (Westerman et al., 2001a).

The observed increase in the non-protein organic sulfur content upon H$_2$S exposure (Chapter 3, 4, Durenkamp and De Kok, 2002, 2003, 2004) could not be attributed to changes in the content of sulfolipids. Therefore, secondary sulfur compounds appeared to be the most likely pool for excessive deposited atmospheric H$_2$S in onion.
Table 1. Impact of short-term H$_2$S exposure on pigment content in shoot and lipid content in shoot and roots of onion (*Allium cepa* L. cv. Nerato). Seedlings were grown in vermiculite for two weeks and subsequently transferred to a 25 % Hoagland nutrient solution. Four-week-old seedlings were transferred to a fresh nutrient solution and exposed to 0.3 µl l$^{-1}$ H$_2$S for one week. Data represent the mean of three measurements with 12 plants in each (± SD).

<table>
<thead>
<tr>
<th></th>
<th>0 µl l$^{-1}$ H$_2$S</th>
<th>0.3 µl l$^{-1}$ H$_2$S</th>
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</thead>
<tbody>
<tr>
<td>Shoot</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total lipid content (mg g$^{-1}$ fw)</td>
<td>3.60 ± 0.09</td>
<td>4.26 ± 0.17**</td>
</tr>
<tr>
<td>Sulfolipid content (nmol g$^{-1}$ fw)</td>
<td>89.0 ± 6.1</td>
<td>86.6 ± 2.6</td>
</tr>
<tr>
<td>Sulfolipid content (nmol mg$^{-1}$ total lipids)</td>
<td>24.7 ± 2.1</td>
<td>20.7 ± 0.2*</td>
</tr>
<tr>
<td>Sulfolipid content (nmol mg$^{-1}$ chlorophyll)</td>
<td>189 ± 5</td>
<td>187 ± 10</td>
</tr>
<tr>
<td>Total chlorophyll content (mg g$^{-1}$ fw)</td>
<td>0.47 ± 0.03</td>
<td>0.46 ± 0.01</td>
</tr>
<tr>
<td>Total carotenoid content (mg g$^{-1}$ fw)</td>
<td>0.11 ± 0.00</td>
<td>0.11 ± 0.00</td>
</tr>
<tr>
<td>Root</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total lipid content (mg g$^{-1}$ fw)</td>
<td>1.44 ± 0.12</td>
<td>1.52 ± 0.15</td>
</tr>
<tr>
<td>Sulfolipid content (nmol g$^{-1}$ fw)</td>
<td>36.5 ± 4.2</td>
<td>37.3 ± 4.2</td>
</tr>
<tr>
<td>Sulfolipid content (nmol mg$^{-1}$ total lipids)</td>
<td>25.3 ± 1.0</td>
<td>24.5 ± 0.3</td>
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</tbody>
</table>

*P < 0.05; **P < 0.01 vs 0 µl l$^{-1}$ H$_2$S; Student’s *t*-test.

**Impact of atmospheric SO$_2$ on sulfur metabolism: a comparison with H$_2$S**

In general, plant exposure to SO$_2$ results in an increase in the sulfate content and a slight increase in the thiol content (mainly glutathione) of the shoot, since part of the SO$_2$ can be assimilated into organic sulfur compounds via sulfite (De Kok and Tausz, 2001; Tausz *et al*., 2003; Yang *et al*., 2003).

Growth of onion was not affected upon a one-week exposure to 0.3 µl l$^{-1}$ SO$_2$ for one week (Table 2). An increase in the sulfate and total sulfur content of the shoot was observed upon exposure to SO$_2$ in both sulfate-sufficient and sulfate-deprived plants, whereas the content in the roots was not affected (Table 2). The increase in the total sulfur content of the shoot in sulfate-sufficient plants could solely be explained by an increase in the sulfate content (Table 2). Apparently, SO$_2$ was for the greater part oxidized to sulfate and transferred into the vacuole (Chapter 8, Fig. 1). In contrast to exposure to H$_2$S, SO$_2$ exposure did not result in a significant decrease in the organic N/S ratio of the shoot of sulfate-sufficient plants (27.7 ± 1.8 and 23.9 ± 3.5 at 0 and 0.3 µl l$^{-1}$ SO$_2$, respectively). As has been indicated above, a decrease in the organic N/S ratio upon H$_2$S exposure could likely be attributed to an increase in secondary sulfur compounds (Chapter 3, 4, Durenkamp and De Kok, 2002, 2003, 2004). These compounds only seemed to be a sink for reduced atmospheric sulfur like H$_2$S, via by-passing of the sulfate uptake in the roots and its reduction in the shoot, and not for oxidized (atmospheric) sulfur like SO$_2$. The reduction of sulfate is known to be highly regulated (De Kok *et al*., 2002a; Vauclare *et al*., 2002), in contrast to the uptake of SO$_2$, which resulted in an accumulation of sulfate upon
SO₂ exposure. Sulfate accumulation was not observed when onion was subjected to increasing levels of pedospheric sulfate, since uptake of sulfate by the roots was strictly regulated (Chapter 4, Durenkamp and De Kok, 2004; Hawkesford and Wray, 2000; Buchner et al., 2004a). A combination of H₂S exposure and different levels of pedospheric sulfate nutrition will be used to further investigate the regulation of sulfate uptake, transport, subcellular distribution and reduction through APS reductase, since these processes predominantly control the assimilation of sulfate in plants.

Table 2. Impact of sulfate nutrition and short-term SO₂ exposure on growth and sulfur metabolism in shoot and roots of onion (Allium cepa L. cv. Nerato). Seedlings were grown in vermiculite for two weeks and transferred to a 25 % Hoagland nutrient solution. Four-week-old seedlings were transferred to a fresh nutrient solution with 0 (-S) or 0.5 (+S) mM sulfate and exposed to 0 (-SO₂) or 0.3 (+SO₂) µl l⁻¹ SO₂ for one week. Data on fresh weight (g), sulfate and total sulfur content (µmol g⁻¹ fw) and sulfate/total sulfur ratio in shoot and roots represent the mean of four measurements with 12 or 24 (initial) plants in each (± SD). Different letters indicate significant differences between treatments (P < 0.05, Student's t-test).  

<table>
<thead>
<tr>
<th></th>
<th>Initial</th>
<th>-S</th>
<th>-S +SO₂</th>
<th>+S</th>
<th>+S +SO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Shoot</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Fresh weight</td>
<td>0.48 ± 0.05</td>
<td>1.10 ± 0.04a</td>
<td>1.12 ± 0.06ab</td>
<td>0.98 ± 0.23ab</td>
<td>1.27 ± 0.13b</td>
</tr>
<tr>
<td>Total sulfur content</td>
<td>9.0 ± 0.3</td>
<td>4.0 ± 0.3a</td>
<td>9.3 ± 0.3b</td>
<td>8.5 ± 1.2b</td>
<td>14.8 ± 1.2c</td>
</tr>
<tr>
<td>Sulfate content</td>
<td>2.6 ± 0.2</td>
<td>0.6 ± 0.0a</td>
<td>4.7 ± 0.2c</td>
<td>3.6 ± 0.5b</td>
<td>9.0 ± 0.5d</td>
</tr>
<tr>
<td>Sulfate/total sulfur</td>
<td>0.29 ± 0.03</td>
<td>0.14 ± 0.03a</td>
<td>0.50 ± 0.02c</td>
<td>0.43 ± 0.02b</td>
<td>0.61 ± 0.03d</td>
</tr>
<tr>
<td><strong>Root</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh weight</td>
<td>0.23 ± 0.02</td>
<td>0.43 ± 0.03a</td>
<td>0.42 ± 0.06a</td>
<td>0.40 ± 0.08a</td>
<td>0.46 ± 0.03a</td>
</tr>
<tr>
<td>Total sulfur content</td>
<td>9.2 ± 0.7</td>
<td>4.1 ± 0.2a</td>
<td>4.3 ± 0.6a</td>
<td>8.9 ± 0.3b</td>
<td>9.5 ± 0.4b</td>
</tr>
<tr>
<td>Sulfate content</td>
<td>5.6 ± 0.5</td>
<td>0.9 ± 0.3a</td>
<td>0.8 ± 0.3a</td>
<td>5.1 ± 0.2b</td>
<td>5.5 ± 0.2c</td>
</tr>
<tr>
<td>Sulfate/total sulfur</td>
<td>0.61 ± 0.05</td>
<td>0.21 ± 0.08a</td>
<td>0.18 ± 0.06a</td>
<td>0.58 ± 0.02b</td>
<td>0.58 ± 0.01b</td>
</tr>
</tbody>
</table>