Atmospheric NH3 deposition, S and N metabolism in curly kale
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Chapter 8.

Atmospheric NH₃ and H₂S as sole N and S sources for growth in B. oleracea

Abstract

Brassica oleracea L. was exposed simultaneously to 4 µl l⁻¹ NH₃ and 0.15 µl l⁻¹ H₂S for one week, in the presence and absence of nitrate and sulfate in the root environment. Growth parameters, total N and S contents, thiol and OAS contents, free amino acid content and protein composition were measured. Unexposed plants, which were deprived of nitrate or sulfate for a week became N deficient, but not yet S deficient. Apparently, the plants were able to remobilize and assimilate the abundant vacuolar sulfate to maintain growth during this period. In the absence of nitrate and sulfate, combined NH₃+H₂S exposure was beneficial for B. oleracea, since the atmospheric N and S sources were used for growth. However, exposure to both atmospheric nutrient sources did not change biomass allocation in favor of the root in the nitrate- and sulfate-deprived plants. Biochemical analysis revealed that nitrogen nutrition, either by nitrate supply to the root or NH₃ exposure to the shoot, mainly affected total N and free amino acid content, whereas sulfate supply to the roots and H₂S exposure mainly affected total S and thiol content. The lack of correlation between changes in thiol and OAS pools, under different atmospheric conditions, suggests that their role in the coordination between the nitrate and sulfate metabolic pathways is limited.
Introduction

Organic C, reduced N and S forms are required for protein synthesis. Coordination of the assimilatory pathways of nitrate and sulfate reduction is thus necessary, so that the appropriate proportions of amino acids are available for protein synthesis (Brunold, 1993).

A strong interaction between N and S assimilatory pathways has been established by Reuveny et al. (1980), in which the availability of sulfate affected not only sulfur assimilation but also nitrogen assimilation and vice versa. Under sulfate deprivation, an increase in ATP sulfurylase (APS) activity and mRNA levels together with an accumulation of amino compounds and a decrease in nitrate reductase (NR) activity were found (Giordano et al., 2000; Migge et al., 2000; Prosser, 2001). Under nitrogen deprivation, a reduction in APS and adenosine 5'-phosphosulfate reductase (APR) activities and mRNA levels were observed (Brunold and Suter, 1984; Koprivova et al., 2000). Recently, microarray analysis in Arabidopsis thaliana revealed nitrate as a putative inducer in sulfate uptake by its effect on the gene expression of the sulfate transporters (Wang et al., 2003).

The synthesis of cysteine not only represents the incorporation of reduced inorganic sulfur into the first stable organic molecule, which is comparable to incorporation of ammonia by glutamine synthetase (Hell et al., 2002), but it also has a central role in both N and S metabolic pathways, since it is the major direct interaction between metabolism of sulfur and nitrogen. Furthermore, cysteine is the precursor of numerous key metabolites as glutathione, phytochelatins and methionine as well as secondary N- and S-compounds. Cysteine biosynthesis consists of a two-step process. Serine and acetyl coenzyme A are condensated by serine acetyltransferase (SAT) to form O-acetylserine (OAS). This intermediate reacts with sulfide to form cysteine, a reaction that is catalyzed by O-acetylserine (thiol)lyase (OAS-TL) (Hell et al., 2002). An adequate N and C supply is necessary for serine synthesis, which makes cysteine biosynthesis an opportunity for the coordination of S assimilation with C and N metabolism (Hawkesford, 2000). In plants, SAT and OAS-TL are associated in the bi-enzyme complex cysteine synthase (Hess et al., 2004). The formation of the cysteine synthase complex is a reversible process, and therefore, this complex might play a role as a metabolic sensor and a regulatory center for the S metabolic pathway (Hell et al., 2002; Hesse et al., 2004).

The ability of using H$_2$S as a sole S source for growth in Brassica oleracea is a well-documented characteristic (De Kok et al., 1997, 1998, 2000; Stuiver and De Kok, 2001). Plant shoots form a sink for H$_2$S. H$_2$S is taken up via the stomata, showing saturation
kinetics with respect to the atmospheric H$_2$S concentrations, which can be described by the Michaelis-Menten equation. H$_2$S is metabolized with high affinity into cysteine and subsequently into other S metabolites. It has been established that the maximum uptake rates of H$_2$S vary between different plant species (De Kok et al., 2002b; Durenkamp and De Kok, 2002). An atmospheric H$_2$S level $\geq 0.075$ µl l$^{-1}$ is already sufficient to cover the organic sulfur requirement of B. oleracea (Buchner et al., 2004).

Previous experiments with B. oleracea showed that NH$_3$ up to a concentration of 4 µl l$^{-1}$ could be used as nutrient for growth to a considerable extent (Chapters 5 and 6). Plant shoots form a sink for both NH$_3$ and H$_2$S, but foliar uptake of these gases occurs differently (Chapter 1). NH$_3$ may be metabolized by the GS/GOGAT cycle (Lea and Mifflin, 1974) and an increase in N-compounds is one of the best-documented effects of NH$_3$ exposure (Chapter 5 and 6). H$_2$S is metabolized with high affinity into cysteine and subsequently into other S metabolites, resulting generally in an increase of the thiol pool, mainly glutathione, as a down-stream product of cysteine biosynthesis (De Kok et al., 1998; Stuiver and De Kok, 1997). The use of atmospheric reduced N and S sources can be regarded as a valuable tool in regulatory studies and provides the ability of bypassing the regulatory control of APR and NR as rate limiting enzymes, in sulfate and nitrate assimilation, respectively.

Brassica oleracea was exposed to 4 µl l$^{-1}$ NH$_3$ and 0.15 µl l$^{-1}$ H$_2$S for one week, in the presence or absence of nitrate and sulfate in the root environment. Growth, total N and S were measured and changes in the thiol, OAS, free amino acid and protein pools were assessed. This study was aimed at 1) investigating the impact of combined NH$_3$ and H$_2$S exposure, in the absence and presence of nitrate and sulfate in the root environment, on growth of Brassica oleracea, 2) assessing to what extent combined NH$_3$ and H$_2$S exposure can replace nitrate and sulfate as nutrient source, and 3) obtaining further insight in the possible role of thiols and OAS in the coordination between N and S metabolic pathways.

**Results**

**Growth parameters as affected by NH$_3$ and H$_2$S exposure**

A 7-day nitrate deprivation resulted in a strong reduction in shoot biomass production and an increase in shoot dry matter content (DMC). Neither root biomass production nor root DMC was affected by nitrate deprivation. Shoot to root ratio was 0.5-fold lower in nitrate-deprived plants than in nitrate-sufficient plants (Table 1). A 7-day sulfate deprivation did not yet significantly affect shoot or root biomass, or the corresponding
DMC. However, shoot to root ratio was 0.7-fold lower in sulfate-deprived plants, compared to sulfate-sufficient plants (Table 1).

The effects of simultaneous nitrate and sulfate deprivation resulted in similar changes as those found for solely nitrate-deprived plants (Table 1 and 2). When plants were exposed to NH$_3$ a higher increase in shoot biomass production was observed; the shoot biomass production of -N-S plants did not differ from that found for +N+S plants. In addition, shoot DMC of -N-S plants was the same as shoot DMC of +N+S plants. Shoot to root ratio of -N-S plants increased upon NH$_3$ exposure and was similar to that of unexposed +N+S plants. NH$_3$ exposure, in combination with sufficient nitrate and sulfate, led to a higher shoot to root ratio (Table 2). H$_2$S exposure did not affect any of the growth parameters of -N-S plants, which were similar to those of the unexposed -N-S plants. The changes in growth parameters found upon simultaneous NH$_3$+H$_2$S exposure, were similar to those found upon NH$_3$ exposure (Table 2).

Total N and S contents as affected by NH$_3$ and H$_2$S exposure
Nitrate deprivation resulted in a lower total N and S content in shoot as well as root, whereas sulfate deprivation did not affect shoot or root total N content. However, shoot and root total S contents were already strongly decreased by sulfate deprivation (Table 1).

The effects of simultaneous nitrate and sulfate deprivation, resulted in similar effects on total N and S content as those described for solely nitrate-deprived plants (Table 1 and 2). NH$_3$ exposure led to a 1.5 to 2-fold increase in shoots total N for +N+S and -N-S plants, respectively. Root total N was increased in -N-S plants, but unaffected in +N+S plants. Shoot and root total S were not affected in -N-S plants by NH$_3$ exposure (Table 2). H$_2$S exposure did not markedly affect shoot or root total S contents, which were similar to those found in unexposed plants. The main effect of simultaneous NH$_3$+H$_2$S exposure was the increase in shoots total N content (Table 2).

Thiol and O-acetyl serine contents as affected by NH$_3$ and H$_2$S exposure
Shoot thiol content was decreased by nitrate (1.8-fold) as well as sulfate (2.6-fold) deprivation. Similar changes were observed for root thiol content (Table 1). Shoot OAS content was strongly increased by sulfate deprivation and decreased by nitrate deprivation. Root OAS content was unaffected by nitrate deprivation but increased by sulfate deprivation (Table 1).

A simultaneous nitrate and sulfate deprivation led to a decrease in shoot thiol content. Exposure of nitrate- and sulfate-deprived plants to H$_2$S and NH$_3$+H$_2$S resulted in an
increase in shoot thiol content to a similar or even higher value than that of unexposed nitrate- and sulfate-sufficient plants (Table 2). Shoot OAS content of -N-S plants was the same in unexposed and H2S-exposed plants. NH3 exposure of -N-S plants resulted in a strong OAS accumulation in the shoot (Table 2).

Root thiol content of -N-S plants was always lower compared to +N+S plants, under all atmospheric conditions (Table 2).

**Impact of NH3 and H2S exposure on free amino acids content**
The free amino acids content was only affected by nitrate deprivation in shoot as well as root; it was decreased. Sulfate deprivation did not have yet an effect on the free amino acids content (Table 1). Simultaneous nitrate and sulfate deprivation also resulted in a decrease in free amino acids content. Free amino acids content increased upon NH3 and NH3+H2S exposures, for both +N+S and -N-S plants but was not strongly affected by H2S exposure (Table 2).

![Fig. 1. Composition of the soluble protein fraction of B. oleracea exposed to NH3, H2S and H2S+NH3, in the presence and absence of nitrate (a), sulfate (b) and nitrate + sulfate (c) supply to the root. Protein extracts were prepared as described in Chapter 2, separated in 10 % SDS-PAGE and stained with Coomassie Brilliant Blue.](image)

**Impact of NH3 and H2S exposure on the composition of soluble proteins**
The shoot soluble protein content decreased 4-fold by nitrate deprivation, but was not affected by sulfate deprivation after 7 days (Table 1). Upon NH3 and NH3+H2S exposure, a slight increase in shoot soluble protein content was found for +N+S as well as -N-S plants. H2S exposure did not affect shoot protein content (Table 2). When the soluble protein extracts were separated by SDS-PAGE, no differences in protein composition were found upon nitrate (Fig. 1a) or sulfate (Fig. 1b) deprivation, but
simultaneous nitrate and sulfate deprivation led to 2-fold decrease of Rubisco (50 kD), in unexposed- and H₂S-exposed plants (Fig. 1c).

**Table 1.** Growth and metabolite content of *B. oleracea* seedlings upon nitrate or sulfate deprivation.

Biomass (biomass production, g FW) and DMC (dry matter content, %). (S) Shoot, (R) Root, (P) Plant. Plants were pre-treated with nitrate and sulfate for one week, and subsequently deprived for 1 week. For detailed experimental conditions, see Chapter 2. Thiol and OAS contents are expressed in nmol g⁻¹ FW; protein in µg g⁻¹ FW and other data are expressed in µmol g⁻¹ FW. Data on growth represents the mean of 2 experiments, with 3 measurements, with 3 plants in each (±SD); biochemical data represents the means of 3 measurements with 3 plants in each (±SD). Different letters indicate significant differences between treatments (+NO₃⁻ and -NO₃⁻; +SO₄²⁻ and -SO₄²⁻), for each parameter (P<0.01, Students t-test).

<table>
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<tr>
<th>Growth</th>
<th>+ NO₃⁻</th>
<th>- NO₃⁻</th>
<th>+ SO₄²⁻</th>
<th>- SO₄²⁻</th>
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Table 2. Growth and metabolic analysis of *B. oleracea* seedlings upon nitrate or sulfate deprivation and exposed to H$_2$S and NH$_3$.

Biomass (increase in biomass production, g FW) and DMC (dry matter content, %). (S) Shoot, (R) Root, (P) Plant. Initial shoot fresh weight (g) was 0.47±0.06 and root fresh weight was 0.14±0.03. Plants were exposed to 4 μl l$^{-1}$ NH$_3$ and 0.15 μl l$^{-1}$ H$_2$S, for one week. Plants were pre-treated with nitrate and sulfate for one-week, after which the deprivation period (1 week) was imposed. For detailed experimental conditions, see Chapter 2. Thiol and OAS contents are expressed in nmol g$^{-1}$ FW; protein in μg g$^{-1}$ FW and other biochemical parameters are expressed in μmol g$^{-1}$ FW. Growth data represents the mean of 2 experiments, with 3 measurements, with 3 plants in each (±SD); biochemical data represents the means of 3 measurements with 3 plants in each (±SD). Different letters indicate significant differences between all treatments, for each parameter (P<0.01, Students $t$-test).

<table>
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<th>H$_2$S</th>
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<td>++</td>
<td>++</td>
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<td></td>
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</tr>
<tr>
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<td>1.17±0.30$^{bc}$</td>
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<tr>
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<td>17±1.4$^d$</td>
<td>12±1.3$^{bc}$</td>
<td>12±1.9$^b$</td>
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<td>0.35±0.05$^{cd}$</td>
<td>0.10±0.04$^e$</td>
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Discussion

Remobilization of N and S upon nitrate and sulfate deprivation

Plants of *B. oleracea*, grown without nitrate for one week became N deficient, whereas those grown without sulfate were not yet S deficient as shown by the inexistence of sulfur-deficiency symptoms and the lack of degradation of Rubisco large subunit (54 kD) and changes in protein composition (Fig. 1b). Differences in remobilization between nitrogen and sulfur are the most likely explanation for the difference between nitrate and sulfate deprivation, in combination with the difference in relative abundance of both nutrients in plant tissue. Previous experiments have shown that the major part of total S in *B. oleracea* is present as sulfate (up to 88 %), presumably localized in the vacuole (Chapter 3; Castro *et al.*, 2003). Apparently, the plants were able to remobilize and assimilate the sulfate to maintain growth. Cram and co-workers (Bell *et al*., 1990; Cram, 1990) proposed that the rate of transport out of the vacuole would limit the remobilization of sulfate within whole plants in conditions of sulfur deficiency. The mobilization of the vacuolar pool of sulfate has been reported to be a slow process in roots, mature leaves and particularly so in oilseed rape (Hawkesford, 2000 and references therein). However, the present results with *B. oleracea* suggest that vacuolar sulfate was released in sufficient quantities, since the abundant sulfate pool present was sufficient to support growth over a prolonged period (even up to 7 days). At the same time, the plants were not able to remobilize sufficient N for growth to maintain growth during this period. Since, after nitrate deprivation for a week, biomass production was strongly reduced, the nitrate content in the shoot was no longer detectable and the organic N had decreased by 20 % (A. Castro, unpublished results). In the present experiments a reduction in Rubisco abundance in plants simultaneously deprived of nitrate and sulfate was found (Fig. 1c), suggesting that simultaneous nitrate and sulfate deprivation acts in synergism, affecting the photosynthetic capacity of the plant, by degradation of Rubisco. Degradation of Rubisco (large subunit, 54 kD) was also found for *Lemna minor* (Ferreira and Teixeira, 1992) and in the green alga *Dunaliella salina* (Giordano *et al*., 2000) as an adaptive consequence to sulfur limitation. Changes in protein composition by both an increase and a decrease in protein abundance were found in *D. salina* (Giordano *et al*., 2000).

Effects of NH$_3$ and H$_2$S exposure on biomass production and allocation

Exposure to NH$_3$ of nitrate- and sulfate-deprived *B. oleracea* plants resulted in a considerable increase in shoot biomass production. When plants were exposed to NH$_3$
and to NH$_3$+H$_2$S, shoot biomass production was 86% and 78%, respectively, of that of unexposed nitrate- and sulfate-deficient plants. Atmospheric NH$_3$ at a concentration of 4 µl l$^{-1}$, therefore, was able to replace nitrate as nutrient to a considerable extent. This is in agreement with previous findings (Chapter 5 and 6), and theoretical calculations on the possible contribution of NH$_3$ to the N budget of plants (Stulen et al., 1998). Root biomass production of the nitrate- and sulfate-deficient plants, which was higher than that of the nitrate- and sulfate-deficient plants, however, was not affected by exposure to NH$_3$ or NH$_3$+H$_2$S. Also, sulfate deprivation for a week did not yet affect the shoot biomass production of *B. oleracea* but the shoot to root biomass partitioning was lower, due to a higher biomass allocation to the root (Table 1). The latter was also previously observed by Buchner et al. (2004) and Stuiver and De Kok (1997). Even though *B. oleracea* apparently was able to use both NH$_3$ and H$_2$S as nutrient source for growth, exposure did not change biomass allocation in favor of the root in the nitrate- and sulfate-deprived plants. Other experiments with *B. oleracea*, exposed to H$_2$S and SO$_2$, showed similar results; the decrease in shoot to root ratio upon sulfate deprivation was hardly affected (Buchner et al., 2004; Yang et al., 2003, 2005). The patterns of changes in root development upon nutrient deprivation are mediated by nutrient-specific signal transduction pathways, which sense the external and/or internal nutrient concentrations (López-Bucio et al., 2003). The present experiments showed for N- as well as S nutrition that there is no direct signaling from the shoot to the root involved in regulating biomass allocation to the root. Whether the external or internal nitrate or sulfate concentration in the root (or a specific part of the root) itself is the sensing factor, is still an open question.

**Role of thiols and OAS in the coordination between N and S metabolic pathways**

Previous studies with two cultivars of *B. oleracea* showed that the thiol pool responded rapidly to H$_2$S exposure (cv. Arsis, A. Castro, unpublished results; cv. Bornick, Westerman et al., 2000). Over a concentration range of 0.15-1.2 µl l$^{-1}$ H$_2$S, the response was induced after 4 h, and the thiol pool reached its maximum after 12 h of exposure, after which it was maintained for up to one week (A. Castro, unpublished results). In the present study, the thiol pool increased 1.4-fold upon H$_2$S fumigation for one week (Table 2), which is in agreement with results from Buchner et al. (2004) for *Brassica oleracea*, at lower H$_2$S concentrations (0.075 µl l$^{-1}$) and Riemenschneider et al. (2005) for *Arabidopsis thaliana*. The effect of H$_2$S on the thiol pool can be explained by the direct incorporation of H$_2$S into cysteine and subsequently glutathione (De Kok et al., 1998, 2000). Simultaneous sulfate and nitrate-deprivation led to a decrease in the thiol pool, which was increased upon NH$_3$ and H$_2$S exposure (Table 2). H$_2$S exposure did not affect
shoot O-acetylserine (OAS) levels (A. Castro, unpublished results; Table 2), but sulfate deprivation led to a strong increase in OAS levels, probably by inhibition of cysteine biosynthesis. Combined nitrate and sulfate deprivation resulted in a decrease in OAS levels, while NH₃ exposure in combination with nitrate and sulfate deprivation resulted in an accumulation of OAS (Table 2). The latter might have been caused by an accumulation of amino compounds, in particular serine, the N precursor for OAS biosynthesis. In nitrate- and sulfate-deprived plants exposed to H₂S+NH₃, OAS levels were restored (Table 2), since the biosynthesis of cysteine is possible by H₂S incorporation into sulfide.

By its central position between both metabolic pathways and due to its sensitivity to manipulations in N and S nutrition, a role of OAS as regulator of a large number of genes, and in the metabolic signaling pathway involved in the coordination between N and S metabolic pathways has been assumed (Hawkesford, 2000; Hell et al., 2002; Droux, 2003; Hesse et al., 2004; Hirai et al., 2003). However, the lack of correlation between changes in the thiol and OAS pools found in the present experiments suggest a limited role of OAS in the coordination between both metabolic pathways. This is in agreement with the suggestion of Hawkesford and De Kok (2006), that OAS accumulation reflects an imbalance of nitrogen and sulfur nutrition, rather than an early metabolic signal in fine-tuning these pathways.