Complexity of nutrient use efficiency in plants
Reich, Martin

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2015

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Chapter 5

Sulfate toxicity and its amelioration by calcium in *Brassica rapa*

Martin Reich, Tahereh Aghajanzadeh, Juliane Helm\(^1\), Nieck van der Heide, Marten Staal, J. Theo M. Elzenga, Malcolm J. Hawkesford\(^2\) and Luit J. De Kok

\(^1\)Institut für Spezielle Botanik Friedrich-Schiller-Universität Jena, Philosophenweg 16, D-07743 Jena, Germany

\(^2\)Department of Plant Biology and Crop Science, Rothamsted Research, Harpenden, Herts AL5 2JQ, UK

*Manuscript in preparation*
Abstract

Sulfate is an essential nutrient in plants and as a logic consequence most research focuses on sulfate deficiency. The mechanisms behind the phytotoxicity of excess sulfate remains, however, widely unstudied up to now. To uncover the physiology and biochemistry of sulfate toxicity in plants we compared sulfate with chloride salinity, accompanied by toxic (sodium) and non-toxic (potassium) cations. Na₂SO₄ turned out to impede growth of *Brassica rapa* more than NaCl, while KCl had only a minor effect. Interestingly, also K₂SO₄ reduced plant growth more than NaCl which suggested that the higher toxicity of Na₂SO₄ was largely due to sulfate rather than sodium. Elemental analysis of plant tissue suggested that neither sodium accumulation nor the effect on beneficial macro-nutrients such as potassium or calcium could explain the increased toxicity of sulfate. As calcium is an important mediator of plasma membrane integrity and other vital functions it is of special importance under salt stress. Application of 10 mM additional calcium in the form of CaCl₂ slightly ameliorated growth impairment by NaCl and KCl but almost completely prevented all negative effects of the sulfate salts on growth and performance. This effect was calcium specific as MgCl₂ and MnCl₂ mimicked it only to a much lower extent. Sulfate accumulation was not avoided by additional calcium, which suggests a cellular protective mechanism of calcium against high tissue sulfate levels. Indeed, an increase in the levels of free amino acids and water-soluble thiols was prevented by CaCl₂ which indicated that excess cellular sulfate was not entering the chloroplasts if an excess of calcium was present. Measurements with microelectrodes at intact roots showed different responses of calcium and proton fluxes to the addition of NaCl and Na₂SO₄. Possible cellular and whole plant mechanisms of sulfate toxicity and its amelioration by calcium are discussed.
5.1 Introduction

Sulfur is an essential macronutrient for plants and a lack of it hinders optimal growth of crops. However, all minerals turn from a nutrient into a toxin, if their concentration is above a certain level (Fig. 5.1). While studies on plants under sulfate deficiency are numerous, there are less dealing with the mechanisms of toxicity caused by an excess of sulfate. Sulfate excess occurs in volcanic soils, saline or previously marine soils that are dominated by sulfate salts, in agricultural soils irrigated with saline water or is caused by anthropogenic input from industry via wet or dry deposition of atmospheric sulfur gases (Moss, 1978; Raybould et al., 1977). Additionally there were high amounts of magnesium sulfate detected on mars (Gendrin et al., 2005) which is a concern for the future development of life support systems that include plants (Visscher et al., 2010). The mechanisms of sulfate toxicity are, however, not entirely elucidated. Several intermediates of the sulfur reduction pathway are toxic (Rennenberg, 1984), but also sulfate itself was reported to have toxic effects on plant metabolism (Ryrie and Jagendorf, 1971). As salinity is a highly regarded field of research, most of the studies available on sulfate toxicity used sodium as a cation, i.e. compared Na$_2$SO$_4$ with NaCl. The susceptibility to these different salts appears to be very species dependent (Ryan et al., 1975; Renault et al., 2001), Na$_2$SO$_4$ was shown to be more toxic in e.g. wheat (Datta et al. 1995), sugar beet and tomato (Eaton 1942), wild potato (Bilski et al. 1988) barley and canola (Huang and Redmann 1995), Brassica campestris (Paek et al., 1988) and on germination of alfalfa (Redman 1974) and wheat (Hampson and Simpson 1990). Also the halophyte Prosopis strombulifera which tolerates high concentrations of NaCl is relatively sensitive to Na$_2$SO$_4$ (Reginato et al., 2014).

A possible explanation for the increased toxicity of Na$_2$SO$_4$ over NaCl is a higher phytotoxicity of sulfate compared to chloride, either caused by sulfate itself or toxic intermediates of the sulfate assimilation pathway (sulfite, sulfide). Also, detrimental effects of excess sulfate on the content of other important nutrients could play a role. For example, crystallization of the poorly-soluble Ca$_2$SO$_4$ could lead to an

![Figure 5.1: The effect of mineral nutrients on plants depending on their abundance](image-url)
internal deprivation of calcium. But also a decrease of other nutrients with a mutual positive effect under sodium toxicity, such as potassium and manganese, could be detrimental.

In order to test these possibilities, the present study was conducted with concentrations of NaCl and Na$_2$SO$_4$ containing the same amount of sodium to ensure a comparable sodium toxicity (i.e. equimolar concentrations). Additionally, KCl and K$_2$SO$_4$ were used with the aim to determine the sole toxicity of the anions if not accompanied by the toxic sodium. Growth and photosynthetic performance parameters were measured, as well the accumulation of the different cations and anions to find out if a higher accumulation of sodium might explain the increased toxicity of Na$_2$SO$_4$. Furthermore, the content of other elements was determined and ion-selective microelectrodes were used to find out if Na$_2$SO$_4$ and NaCl lead to different fluxes of calcium and protons at intact roots. In another set of experiments the effect of additional calcium in the different salt stresses was examined. Calcium is known to have the potential to ameliorate salt stress (Rengel, 1992). This effect is associated with sodium toxicity, as sodium is displacing calcium from plasma membranes (Cram, 1983). If this was true, the effect of additional calcium should ameliorate stress caused by NaCl and Na$_2$SO$_4$. Effects of KCl and K$_2$SO$_4$ should remain unaltered by additional calcium, if calcium amelioration was purely related to sodium.

5.2 Material and Methods

Plant material and growth conditions

Seeds of Brassica rapa, cv. Komatsuna; Va der Wal, Hoogeveen, The Netherlands) were germinated in vermiculite. Ten day-old seedlings were transferred into a 25% Hoagland nutrient solution (pH 5.9) consisting of 1.25 mM Ca(NO$_3$)$_2$.4H$_2$O, 1.25 mM KNO$_3$, 0.25 mM KH$_2$PO$_4$, 0.5 mM MgSO$_4$.7H$_2$O, 11.6 µM H$_3$BO$_3$, 2.4 µM MnCl$_2$.4H$_2$O, 0.24 µM ZnSO$_4$.7H$_2$O, 0.08 µM CuSO$_4$.5H$_2$O, 0.13 µM Na$_2$MoO$_4$.2H$_2$O and 22.5 µM Fe$^{3+}$-EDTA in 30 liter containers (20 sets per container, three plants per set) which were placed in a climate controlled room for the duration of the experiment. Relative humidity was 60-70% and the photoperiod was 14 h at a photon fluence rate of 300 ± 20 µmol m$^{-2}$ s$^{-1}$ (within the 400-700 nm range) at plant height, supplied by Philips GreenPower LED lamps (deep white/ red 120). Day/night temperatures were 21/ 18 °C. Plants were pre-grown without additional salt for three days, then salts were gradually increased during the following three days. For NaCl and KCl the steps were 25, 50 and 100 mM and for Na$_2$SO$_4$ and K$_2$SO$_4$ the steps were 12.5, 25 and 50 mM in order to get the same concentrations of sodium. For the calcium amelioration experiments 10 mM CaCl$_2$ was added to the solution together with the first salt on day four. Plants were grown in the final concentrations for seven more days so that the total duration of the experiment was eleven days.

Growth analysis

On day eleven, plants were harvested and shoot and root fresh weight was determined. For determination of the dry matter content fresh plant tissue was dried at
80 °C for 24 h and stored in a desiccator for further use. Fresh material was stored in either -20°C or -80°C, depending on the requirements for further analysis.

**Maximum quantum yield of PS II and stomatal resistance**

On the day of harvest, maximum quantum yield of photosystem II (Fv/Fm, in dark-adapted conditions) of leaves was determined in the morning before light was switched on (PAM 2000, Walz, Effeltrich, Germany). After the light had been switched on for several hours, stomatal resistance was measured on the largest leaf (AP4 Leaf Porometer, Delta-T Devices Ltd., Cambridge, UK).

**Anions and free amino acids**

Anions (nitrate and sulfate) were extracted from frozen plant material and determined refractometrically after separation by HPLC (Aghajanzadeh et al. 2014). From the same extracts free amino acids were measured after deproteinization using a ninhydrin color reagent according to Rosen (1957) by colorimetric determination at 578 nm.

**Pigment content**

For determination of pigment content, 10 ml g⁻¹ fresh weight of 100% acetone was added to frozen shoot material. After homogenization with an Ultra Turrax the extract was centrifuged at 30,000 g for 20 minutes and chlorophyll a, b and total carotenoids were determined according to Lichenthaler (1987).

**Water soluble non-protein thiols**

For the determination of thiols, fresh plant material was used on the day of harvest and homogenized in an extraction medium (10 ml g⁻¹ fresh weight) containing 80 mM sulfosalicylic acid, 1 mM EDTA and 0.15% (w/v) ascorbic acid. Samples and extract were kept on ice and the extraction medium was bubbled with N₂ one hour prior extraction to remove oxygen. After filtering over one layer of Miracloth the extract was centrifuged at 30,000 g for 15 minutes at 0°C. Thiol content of the supernatant was determined colorimetrically at 413 nm wavelength after addition of 5,5’-dithiobis[2-nitrobenzoic acid] according to De Kok et al. (1988).

**Mineral nutrient composition**

For the determination of mineral nutrient contents, dried leaf tissues (0.2–0.5 g) were digested with 5 ml of nitric acid: perchloric acid (87:13, v/v; 70 % concentration, trace analysis grade; Fisher Scientific; Zhao et al., 1994). The minerals in the digested samples were analyzed by inductively coupled plasma
mass spectrometry (ICP-MS) and by inductively coupled plasma atomic emission spectrometry (ICP-AES) analysis as described in Chapter 4. Mineral nutrient contents are expressed on a dry weight basis (given in \( \mu\text{mol g}^{-1} \)).

**Calcium and proton fluxes at excised roots in response to NaCl and Na\(_2\)SO\(_4\)**

For the measurement of calcium and proton fluxes, roots from plants grown under control conditions were used on between day 7 and 12 after onset of the salt treatments. Excised roots with a total length of 1-2 cm from the tip were placed in a 1 ml measuring basin and immobilized using glass capillaries. Thereafter the root was covered with 1 ml of a low salt measuring solution (MS; containing 200 \( \mu\text{M} \) MgCl\(_2\), 100 \( \mu\text{M} \) KCl and 100 \( \mu\text{M} \) CaCl\(_2\)). The microelectrode was positioned at the beginning of the differentiated zone, where first root hairs started to appear (usually 3-5 mm from the root tip) and after an incubation of 1 h the response of proton and calcium fluxes to the addition of either 25 mM NaCl or 12.5 mM Na\(_2\)SO\(_4\) to the MS was recorded. The salts were added as a stock (10 \( \mu\text{l} \)) and the MS was gently mixed with a 100 \( \mu\text{l} \) pipette. Net fluxes of H\(^+\) and Ca\(^{2+}\) were measured using ion selective electrodes with the MIFE technique (Microelectrode Ion Flux Estimation; Shabala et al., 1997; Vreeburg et al., 2005; Lanfermeijer et al., 2008). Microelectrodes were pulled from borosilicate glass capillaries (GC150-10; Harvard Apparatus) and silanized with tributylchlorosilane (Fluka 90974). The H\(^+\)-selective electrodes were back filled with 15 mM of NaCl and 40 mM of KH\(_2\)PO\(_4\) and front filled with Hydrogen Ionophore II (Cocktail A; Fluka 95297). The Ca\(^{2+}\)-selective electrodes were back filled with 500 mM CaCl\(_2\) and front filled with Calcium Ionophore I (Sigma Aldrich 21192). Only H\(^+\) electrodes with a response of > 53 mV per pH unit (pH range 5.1–7.8, \( r^2 > 0.999 \)) and Ca\(^{2+}\) electrodes with a response of > 21 mV per decade (\( r^2 > 0.99 \)) were used for measurements. The reference electrode, filled with 300 mM KCl was placed in a separate compartment electrically connected with the measuring chamber via a salt bridge consisting of 300 mM (NH\(_4\))\(_2\)SO\(_4\) in 2% (w/v) agar. The electrode was positioned 10 \( \mu\text{m} \) from the root surface.

**pH of shoot and root extracts**

For pH determination in shoot and roots the same extracts were used as for anion determination (extraction in water, 10x diluted). The pH was measured using H\(^+\)-selective electrodes, as described above. The dilution was calculated converting the pH to [H\(^+\)] by \( 10^\left(-pH\right) \), dividing by 10 and
Amelioration of sulfate toxicity by calcium

Chapter 5

converting the resulting \([H^+]\) back to pH by \(-\log([H^+])\).

Ameliorating effect of other salts

To compare the effect of additional CaCl\(_2\) with the addition of other salts, the above described experiments were repeated with 10 mM CaCl\(_2\), 10 mM MgCl\(_2\) and 10 µM MnCl\(_2\) in 12 liter containers. After the same time period as above, plants were harvested and fresh weight was determined.

5.3 Results

Effect of different salts on biomass, anion accumulation and mineral nutrient content

Exposure of \textit{Brassica rapa} to the chloride and sulfate salts had different effects on growth. The lower concentrations did not show significant effects on growth while the higher concentrations led to growth impairment with the sulfate showing a stronger effect than the chloride salts (Fig. 5.2). Plants accumulated the anion present in excess but chloride to a much higher extent than sulfate (Fig. 5.3). Chloride levels under NaCl and KCl were ca. 7.5-13 fold higher in leaves and 10-15 fold higher in roots than under control conditions while sulfate levels under Na\(_2\)SO\(_4\) and K\(_2\)SO\(_4\) were only about 2.5-6 fold increased in leaves and 1.5-2 fold in roots. Sulfate levels in roots were significantly decreased by the higher concentrations of NaCl and KCl. Doubling of the external concentrations did not, or only slightly, increase tissue contents of the respective anion in roots. In shoots, contents were further increased but this increase was greater upon exposure to sulfate salts and not significant in NaCl. Nitrate levels in shoots were only significantly affected by NaCl which led to a decrease of approximately 50%. In the roots additionally KCl and 50 mM Na\(_2\)SO\(_4\) and 25 mM K\(_2\)SO\(_4\) led to a significant decrease in nitrate content.

In plants exposed to NaCl, the chloride content did not significantly increase further when external concentrations were doubled while in KCl a further increase of ca. 30% was observed in both shoot and roots. Sulfate content in shoots of plants exposed to Na\(_2\)SO\(_4\) and K\(_2\)SO\(_4\) almost doubled, when the concentrations of the salts in the medium was doubled. In the roots, in contrast, sulfate levels remained on the same level (Fig. 5.3). Nitrate content in shoots was highly variable, but addition of sulfate salts did not result in significant changes. The nitrate in shoots exposed to NaCl was, however, significantly lower if tested separately against the control with an unpaired Student’s t-test. In the roots, all chloride salts and 50 mM Na\(_2\)SO\(_4\) decreased nitrate content significantly (Fig. 5.3). Slightly more sodium was
Figure 5.2: Biomass and dry matter content of shoots and roots of *Brassica rapa* exposed to two levels of NaCl, KCl, Na$_2$SO$_4$ and K$_2$SO$_4$ salinity. Lower concentrations were 25 mM for Na$_2$SO$_4$ and K$_2$SO$_4$ and 50 mM for NaCl and KCl (pattern) and higher concentrations were 50 mM for Na$_2$SO$_4$ and K$_2$SO$_4$ and 100 mM for NaCl and KCl. Data represent the mean of five measurements with three plants in each (± SD). Values with different letters are significantly different from each other (p < 0.01; One-way ANOVA; Tukey as a post-hoc test).

Figure 5.3: Anion content of shoots and roots of *Brassica rapa* seedlings exposed to two levels of NaCl, KCl, Na$_2$SO$_4$ and K$_2$SO$_4$ salinity. Lower concentrations were 25 mM for Na$_2$SO$_4$ and K$_2$SO$_4$ and 50 mM for NaCl and KCl (pattern) and higher concentrations were 50 mM for Na$_2$SO$_4$ and K$_2$SO$_4$ and 100 mM for NaCl and KCl. Data represent the mean of four measurements with three plants in each (± SD). Values with different letters are significantly different from each other (p < 0.01; One-way ANOVA, Tukey as a post-hoc test; for nitrate in shoot: 50 mM and 100 mM NaCl, as well as 50 mM K$_2$SO$_4$ are significantly different from Control with p < 0.05).
accumulated in plants exposed to Na$_2$SO$_4$ than in plants exposed to NaCl, but in shoots only if contents are expressed on a fresh weight basis (Fig. 5.4). Sulfur content was slightly, but non-significantly, decreased in the chloride salts and 2-3 fold increased in the sulfate salt treatments. In roots, K$_2$SO$_4$ led to a significantly higher sulfur content than Na$_2$SO$_4$. Potassium content was increased more by KCl than by K$_2$SO$_4$ in shoot and roots. In the shoot, however, the results was reversed if expressed on a fresh weight basis. Potassium content was strongly and similarly decreased by the sodium salts but without. Calcium content was strongly decreased by all salts.

Magnesium content was decreased by ca. 50% in the shoot without significant differences between the different salts. In the roots, magnesium content was stronger decreased by the sulfate salts. Phosphorus content was only decreased by the salts if expressed on a dry weight basis, while on a fresh weight basis there was even an increase in shoots of plants exposed to the sulfate salts. Due to large variance there were no significant differences found for the content of copper, except a significant decrease in roots of plants exposed to NaCl. For iron content, no significant differences were found. Manganese content was strongly decreased by all salts and more by sulfate salts in shoots if expressed on a dry weight basis. In roots, a stronger decrease by sulfate salts is not significant due to the large variance in the control (due to one exceptionally low value). If tested separately from the control, the differences were highly significant and revealed that manganese levels in plant roots treated with chloride salts was 5-fold higher than in roots treated with sulfate salts. Molybdenum content was decreased by all salts, without significant differences if expressed on a fresh weight basis. Zinc content was ca. 1.5 to 3-fold increased by all salts (although not always significant) and more by sulfate salts, if expressed on a fresh weight basis.

**Effects of additional calcium**

The addition of 10 mM CaCl$_2$ to the growing medium ameliorated growth impairment caused by the higher concentrations of the salts. While the effect was rather small for chloride salts the strong growth reduction by sulfate salts was completely prevented (Fig. 5.8). Additional CaCl$_2$ had also a slightly but significantly positive effect on plants grown without salt (not shown). The content of chlorophyll a and b was decreased by all salts (Fig. 5.9) and only in the sulfate salts, additional CaCl$_2$ led to chlorophyll levels similar to that of control plants. Maximum quantum yield ($F_v/F_m$) was only reduced by Na$_2$SO$_4$ and this decrease was prevented by additional CaCl$_2$ (Fig. 5.9). Stomatal resistance was slightly increased by Na$_2$SO$_4$ and by
Figure 5.4: The effect of the different salts on the content of macronutrients in shoot of Brassica rapa seedlings. Data represent the mean of five measurements with three plants in each (± SD). Values with different letters are significantly different from each other (p < 0.01; One-way ANOVA, Tukey as a post-hoc test).
Figure 5.5: The effect of the different salts on the content of macronutrients in roots of *Brassica rapa* seedlings. Data represent the mean of five measurements with three plants in each (± SD). Values with different letters are significantly different from each other (p < 0.01; One-way ANOVA, Tukey as a post-hoc test).
Figure 5.6: The effect of the different salts on the content of micronutrients in shoots of *Brassica rapa* seedlings. Data represent the mean of five measurements with three plants in each (± SD). Values with different letters are significantly different from each other (p < 0.01; One-way ANOVA, Tukey as a post-hoc test).
Figure 5.7: The effect of the different salts on the content of micronutrients in roots of *Brassica rapa* seedlings. Data represent the mean of five measurements with three plants in each (± SD). Values with different letters are significantly different from each other (p < 0.01; One-way ANOVA, Tukey as a post-hoc test).
NaCl and KCl with additional CaCl$_2$. Additional CaCl$_2$ did not prevent accumulation of sulfate in the plants exposed to Na$_2$SO$_4$ and K$_2$SO$_4$ although it was significantly lower in shoots under K$_2$SO$_4$ and in roots under Na$_2$SO$_4$ exposure (Fig. 5.10). Free amino acids and water-soluble thiols were increased in shoots by both sulfate salts and additional CaCl$_2$ prevented this increase entirely (Fig. 5.10 and 5.11).

![Figure 5.8: The effect of NaCl, KCl, Na$_2$SO$_4$ and K$_2$SO$_4$ on biomass and dry matter content of shoot and roots of Brassica rapa seedlings with or without additional 10 mM CaCl$_2$. Data represent the mean of n measurements with three plants in each (± SD; biomass n = 15; dry matter content n = 5). Values with different letters are significantly different from each other (p < 0.01; One-way ANOVA; Tukey as a post-hoc test).](image-url)
Response of calcium fluxes and pH at roots to NaCl and Na$_2$SO$_4$

Addition of 12.5 mM Na$_2$SO$_4$ and 25 mM NaCl caused an immediate efflux of calcium (Fig. 5.13). Although both salts contained equimolar concentrations of sodium, NaCl caused a much higher efflux than Na$_2$SO$_4$. The peak responses (the sum of all measuring points during 10 min after addition) was about three times higher in NaCl. Within a few minutes the efflux vanished and the steady response 20-30 min after salt addition (Fig. 5.13) did not differ significantly from the flux before addition nor between the two different salts. Calcium concentration at the root surface increased simultaneously with the observed efflux and mimicked the different response to the two salts. The pH at the root surface responded conversely to NaCl and Na$_2$SO$_4$ addition. While the former salt caused a decrease, the latter led to an increase (Fig. 5.14). Both responses showed a peak followed by a phase of decline but reached a stable value different from that before the addition. Fig. 5.14 shows the response of root surface pH to 25 mM NaCl and 12.5 mM Na$_2$SO$_4$. While it decreased in response to NaCl it increased if Na$_2$SO$_4$ was added.
Chapter 5  
Amelioration of sulfate toxicity by calcium

Figure 5.10: The effect of NaCl, KCl, Na$_2$SO$_4$ and K$_2$SO$_4$ on contents of sulfate and free amino acids in shoots and roots of *Brassica rapa* seedlings with or without additional 10 mm CaCl$_2$. Data represent the mean of n measurements with three plants in each (± SD; sulfate n = 4-7; amino acids n = 3). Values with different letters are significantly different from each other (p < 0.01; One-way ANOVA; Tukey as a post-hoc test).

Figure 5.11: The effect of NaCl, KCl, Na$_2$SO$_4$ and K$_2$SO$_4$ on the content of water-soluble non-protein thiols in shoots of *Brassica rapa* seedlings with or without additional 10 mm CaCl$_2$. Data represent the mean of three measurements with three plants in each (± SD). Values with different letters are significantly different from each other (p < 0.01; One-way ANOVA; Tukey as a post-hoc test).
Figure 5.12: Comparison of the amelioration potential of calcium, (10 mM CaCl$_2$) magnesium (10 mM MgCl$_2$) and manganese (10 µM MnCl$_2$) on growth of of Brassica rapa seedlings exposed to 50 mM K$_2$SO$_4$. Data represent the mean of ten measurements with three plants in each (± SD). Values with different letters are significantly different from each other (p < 0.01; One-way ANOVA; Tukey as a post-hoc test).

pH of shoot and root extracts

The pH of shoot extracts of plants exposed to the sulfate salts was ca. 0.5 units higher than the one of control plants. Plants exposed to additional CaCl$_2$ showed a pH closer to the control level (Fig. 5.15). No significant differences were found in root extracts.

Ameliorating effect of other salts

It was tested if 10 mM MgCl$_2$ and 10 µM MnCl$_2$ would have similar or different effects on growth of plants under toxic sulfate concentrations. Both compounds ameliorated growth impairment caused by 50 mM K$_2$SO$_4$ but to a much lower extent than CaCl$_2$ (Fig. 5.12).
Figure 5.13: The effect of addition of 25 mM NaCl (red) and 12.5 mM Na$_2$SO$_4$ (blue) on calcium fluxes (upper and median panel) and calcium concentration (lower panel) at the surface of excised roots of *Brassica rapa* seedlings grown under control conditions. All values are normalized relative to the average of 10 min prior to addition. The grey shade in the upper and lower panels represents the SE. The median panel shows the peak response (left) of the 10 min directly after and the steady response 20-30 min after salt addition (right). In both cases the mean of the sum of all values recorded in the respective time frame ±SE is shown and the p-values of an unpaired-student t-test are given. Negative values are per definition an efflux (upper and median panel). n = 4.
Figure 5.14: The effect of addition of 25 mM NaCl (red) and 12.5 mM Na$_2$SO$_4$ (blue) on pH at the surface of excised roots of *Brassica rapa* seedlings grown under control conditions. Values are normalized relative to the average of 10 min prior to addition. The grey shade in the upper and lower panels represents the SE. n = 5.

5.4 Discussion

The present study showed that sulfate is not only toxic to *Brassica rapa* if accompanied by sodium but also with the non-toxic potassium as cation (Fig. 5.2). As comparative studies on salt stress almost exclusively used NaCl and Na$_2$SO$_4$, and often not with equimolar concentrations of the toxic sodium, they failed to distinguish between anion and cation toxicity. Sulfate, although an essential macronutrient, severely impaired growth at a relatively low concentration of 50 mM which led to a tissue sulfate content in leaves of ca. 30 for Na$_2$SO$_4$ and ca. 40 for K$_2$SO$_4$ and in roots of ca. 20 µmol g FW$^{-1}$. While in leaves the sulfate content approximately doubled when the external concentration was increased from 25 to 50 mM the content in roots remained constant. Xylem loading therefore was apparently not limiting the transport of the excess sulfate to the shoot and not downregulated to prevent toxic accumulation.

The key role of safe storage of excess sulfate and possible mechanisms of sulfate toxicity

The lower concentration of 25 mM Na$_2$SO$_4$ and K$_2$SO$_4$ already led to a remarkable increase in tissue levels of sulfate, but not yet significantly affected growth (Fig. 5.2 and 5.3), while the higher concentration strongly inhibited growth and led to severe injury and wilting of leaves. This phenomenon was observed to proceed relatively sudden after days of healthy growth rather than gradually. It therefore appears likely that a limited capacity of the vacuole for sulfate and a sudden efflux of stored sulfate from the vacuole into the cytosol caused the toxicity of Na$_2$SO$_4$. Sulfate efflux from the vacuole
follows a steep gradient and is therefore of passive nature (Cram, 1983) and consequently it becomes thermodynamically difficult to avoid under very high vacuolar concentrations. In a previous study, growth of Chinese cabbage was strongly impaired at 40 mM Na$_2$SO$_4$, similar to the present study (Reich et al., 2015, Chapter 4 of this thesis). Gene expression of the Sultr1;2 was further down-regulated under the level of plants grown under sufficient sulfate supply (0.5 mM) which can be interpreted as a strategy to avoid excess sulfate influx. Surprisingly this was not true for the gene expression of the tonoplast sulfate transporter Sultr4;1, which is responsible for sulfate efflux from the vacuole (Kataoka et al., 2004). Instead this transporter was increased at 40 mM Na$_2$SO$_4$. This means that a leakage of sulfate to the cytosol through the tonoplast membrane would not only be unavoidable at some point due to the great differences in concentrations but mediated by an increased transporter abundance. An explanation might be that the transcript level of the vacuolar sulfate transporters is regulated by the sulfate gradient between the cytosol and the vacuole. Under sulfate deficiency the cytosolic sulfate concentration drops and vacuolar transporters are upregulated to remobilize sulfate from the vacuoles (Kataoka et al., 2004). The same signal could be received if vacuolar sulfate concentrations are rising above cytosolic levels under an excess sulfate supply. Under normal conditions only around 1% of the total sulfur pool consists of cytosolic sulfate while the bulk is stored in the vacuole (Thoiron et al., 1981) and for this reason, total tissue concentrations are a poor predictor of cytosolic sulfate. An indication that sulfate concentrations were indeed not only increasing in the vacuole but also in the cytosol are the increased levels of thiols which are synthesized in the chloroplasts (Foyer and Halliwell, 1976).

High levels of sulfate in the chloroplasts are suggested to inhibit photophosphorylation by the coupling factor 1 (Cf1; Ryrie and Jagendorf, 1971). Later it was shown that especially sulfite, SO$_2$ and HSO$_3$ inhibit photosynthesis (Silvius et al., 1975) which fits studies that suggest that the functionality of Cf1 depends on SH-groups and their redox-state (Vallejos and Andreo, 1976; Arana and Vallejos, 1982; Nalin and McCarty, 1984). SO$_2$ fumigation led to a gradual decrease in ATP (Cerović et al., 1982). Sulfite might compete with phosphate for the functional binding sites activating ATP synthesis (Bakels et al., 1996) and on the other hand it was shown to promote the wasteful hydrolysis of ATP by activating the latent ATPase activity of the Cf1 (McCarty, 2005). As SO$_2$ is partly dissociating to sulfite which then may be reduced to sulfide or oxidized to sulfate it might have the same mechanism of toxicity as excessive sulfate in the root medium which is reduced to sulfite and sulfide. Other studies observed an inhibition of mitochondrial ATP formation by sulfite (Ballantyne, 1973). In the present study plants contained less pigments in all salts but only had a decreased $Fv/Fm$ if grown.
Amelioration of sulfate toxicity by calcium

Chapter 5

Figure 5.15: The effect of the sulfate salts with and without additional CaCl$_2$ on the pH in watery extracts of shoots and roots of Brassica rapa seedlings. Mean values ±SD are shown (n = 2-3). For each measurement, the material of three plants was pooled. Values with different letters are significantly different from each other (p < 0.01; One-way ANOVA; Tukey as a post-hoc test).

in Na$_2$SO$_4$ which can be seen as additional evidence for detrimental effects on photosynthetic functioning (Fig. 5.9). Diminished ATP availability could in turn lead to decreased translocation of sulfate into the vacuole as this process is highly dependent on sufficient ATP supply if sulfate concentrations are high (Kaiser et al., 1989).

An excess of vacuolar sulfate could also lead to the formation of toxic sulfuric acid. However, the pH in extracts of shoot and roots of plants treated with sulfate salinity was higher than in control plants (Fig. 5.15). Possibly, the high cation-anion ratio (Fig. 5.16) and translocation of sodium and potassium, respectively, to the vacuole via H$^+$/cation-antiport could have led to an increase in pH that counteracted any possible acidification. Until now, data on nutrient contents of the plants exposed to salinity and additional CaCl$_2$ is missing, but the lower pH in shoot extracts of these plants could indicate less cation uptake. Less sodium uptake could partly explain the ameliorating effect of CaCl$_2$ but it remains unclear how this effect could play a role under K$_2$SO$_4$ salinity.

Regulation of sulfate reduction under excess sulfate supply

The first step of sulfate reduction is performed by the enzyme ATP sulfurylase which activates sulfate to adenosine 5'-phosphosulfate (APS) by using ATP (reviewed by Hawkesford and De Kok (2006) Kopriva (2006)). The affinity of ATP sulfurylase for sulfate is rather low (Shaw and Anderson, 1971) which is why the concentration of sulfate in the chloroplast is assumed to be one of the rate determining steps of sulfate reduction. APS reductase (APR) is reducing
APS to sulfite and it was shown that the abundance and activity of this enzyme controls most of the sulfate flux through the pathway Vauclare et al. (2002). However, an excess of sulfate supplied by \( \text{Na}_2\text{SO}_4 \) led only to a down-regulation of the APR transcript level in the roots but to an up-regulation in the shoot (Reich et al., 2015, Chapter 4 of this thesis). This might lead to increased levels of the toxic sulfite. It appears that the regulatory function of APR is overruled at high external sulfate concentrations. De Kok and Kuiper (1986) have concluded that an excess of reduced sulfur under high external \( \text{Na}_2\text{SO}_4 \) concentrations is due to high sulfate levels at the reaction site of ATP sulfurylase.

**Cation-anion imbalance**

It had been speculated that the apparent lower membrane permeability for sulfate over chloride could lead to an imbalanced uptake of sodium and sulfate and therefore to a slower osmotic adjustment under sulfate salinity (Meiri et al., 1971). This seems to be supported by the fact that stomatal resistance is increased in \( \text{Na}_2\text{SO}_4 \) but not in \( \text{NaCl} \) in the present study (Fig. 5.9). However, it is unlikely that the strong negative effects also observed under \( \text{K}_2\text{SO}_4 \) exposure are mainly due to increased osmotic stress, also because salt concentrations were gradually increased in the present study. Additionally, the sodium-to-sulfate ratio under \( \text{Na}_2\text{SO}_4 \) salinity was not significantly different to the sodium-to-chloride ratio under \( \text{NaCl} \) salinity in shoots, if the divalency of sulfate was taken into account (Fig. 5.16).
Effects on other nutrients

Another explanation for the toxicity of excessive sulfate could be the decrease of other essential or beneficial elements. Manganese in roots was stronger decreased by sulfate than by chloride salts (Fig. 5.7) which could also be related to lower levels of the manganese superoxide dismutase which plays a crucial role in the detoxification of reactive oxygen species. For example, in the halophytic crop *Prosopis strombulifera* Na$_2$SO$_4$ led to an increase in oxidative stress while iso-osmotic concentrations of NaCl did not (Reginato et al., 2014). Complementing Chinese cabbage with the maize genes expressing copper zinc superoxide dismutase resulted in higher tolerance to both salinity and SO$_2$ (Tseng et al., 2007). However, in the present study copper was not affected and zinc was even increased by some salts (Fig. 5.6 and 5.7). K/Na and Ca/Na ratios in both shoot and roots were highly decreased by both Na$_2$SO$_4$ and NaCl but not significantly different (Fig. 5.17). Especially the K/Na ratio and potassium nutrition under salt stress in general is considered as a key parameter for salt stress and tolerance (Zhu et al., 1998; Asch et al., 2000; Cuin et al., 2008), but does apparently not explain the higher toxicity of Na$_2$SO$_4$ in *Brassica rapa*.

Calcium ameliorates sulfate toxicity

Depletion of calcium from plasma membranes and the prevention of its uptake by the roots by sodium are often considered as one of the first and most severe toxic effects of salinity (Cramer et al., 1985; Rengel, 1992). In the present study however, the decrease in calcium content was not an exclusive
feature of sodium salinity. Calcium content was decreased by all salts but in roots slightly more by sulfate salts (Fig. 5.4). These difference appear to small to explain the extreme differences in growth. Surprisingly, addition of 10 mM CaCl$_2$ to the growing medium prevented negative effects of Na$_2$SO$_4$ on growth and performance while not significantly increasing growth under NaCl (Fig. 5.8). Even more surprising was that the ameliorating effect of calcium was strongest in the sulfate salts and thus also ameliorated growth impairment caused by K$_2$SO$_4$. The above described mechanisms of sulfate toxicity are therefore presumed to have a strong connection to internal calcium homeostasis. As calcium is known to stabilize membrane structure and facilitate membrane function and selectivity, it might also lead to a higher capacity of the vacuole for sulfate. The fact that total sulfate levels in shoots remain high in plants with additional calcium but levels of thiols and free amino acids are normal supports this hypothesis (Fig. 5.11 and 5.12). Another possibility is an internal deprivation of calcium caused by the formation of Ca$_2$SO$_4$ crystals. Ca$_2$SO$_4$ has a very low solubility and crystallization would make essential calcium unavailable for vital functions. Additionally, depending on the extent of crystallization this could also physically disrupt membranes. Ca crystals in general have been shown to form in many plant leaves but their physiological function is still a point of discussion. Ca$_2$SO$_4$ crystals are relatively rare and only described in some studies. Intriguingly, Huttunen et al. (1990) showed that such crystals form if conifers are subjected to acid rain (H$_2$SO$_4$ and HNO$_3$) which could mean that crystal formation could also be a protective response to excessive sulfate. Additional calcium, as added in the present study, could promote the formation of Ca$_2$SO$_4$ crystals and bind toxic sulfate. In Tamarix aphylla spherical aggregates of Ca$_2$SO$_4$ crystals were found in mesophyll vacuoles (Storey and Thomson, 1994). If the formation of Ca$_2$SO$_4$ crystals play a detrimental or protective role during sulfate toxicity has to be clarified with microscopic studies. Also MgCl$_2$ slightly ameliorated the effect of K$_2$SO$_4$ on growth which shows that also the potentially positive effects of additional magnesium are not an exclusive feature under sodium or chloride toxicity (Slabu et al., 2009). But neither additional MgCl$_2$ nor MnCl$_2$ showed the same ameliorating potential as CaCl$_2$ under sulfate salinity (Fig. 5.12).

Increased loss of calcium from intact roots seems not to be an explanation of the increased toxicity of Na$_2$SO$_4$ over NaCl. Actually, addition of NaCl to roots caused a higher efflux of calcium than addition of Na$_2$SO$_4$ (Fig. 5.13). This may have two different reasons. One is that sodium is taken up faster if NaCl is supplied due to the higher permeability of the membrane for chloride compared to sulfate. The fast decay of the efflux peak, however, could be an indication that a major proportion of the measured calcium derives from the cell wall and quickly dissolves from it due to the sudden increase in
external sodium. If this was the case, the anion specific difference could again have different reasons. Very low solubility of CaSO$_4$ could lead to an immediate precipitation of calcium with sulfate if Na$_2$SO$_4$ is added. However, the increase of calcium at the root surface after addition of NaCl is around 1 mM which is still far below the maximum solubility of CaSO$_4$ (ca. 2 g l$^{-1}$ at 20°C). A more likely explanation could be the different response of the root surface pH to the addition of the different salts (Fig. 5.14). NaCl caused and efflux of protons while Na$_2$SO$_4$ caused an influx. The latter is in accordance to the response to MgSO$_4$ shown in Chapter 2 of this thesis, which is caused by the uptake of sulfate via a sulfate/proton-symport. Binding of positive (and especially divalent) ions to the apoplast depends on the cation exchange capacity which in turn is governed by the pH. At a low pH, i.e. a high concentration of protons, cation exchange capacity increases and e.g. calcium is quickly replaced by protons. The low pH caused by sudden sodium influx under NaCl addition could favor calcium release from the apoplast while the increased pH caused by sulfate uptake under Na$_2$SO$_4$ addition stabilizes or even increases calcium binding to the apoplast. Therefore, less calcium would be dissolved form the apoplast if sodium is accompanied by sulfate. If this is true, the peak response right after salt addition could resemble apoplastic calcium while the steady response after relaxation of the peak could resemble the calcium continiously lost from the symplast (Fig. 5.13). But in this phase the calcium flux does not significantly differ from the flux prior to salt addition. No drastic differences in tissue content of sodium and calcium were observed (Fig. 5.4) which suggests that the difference in calcium efflux found after an immediate salt addition are not maintained over a longer period of exposure.

5.5 Conclusions

Despite the relatively high levels of sulfate accumulated by Brassica species, sulfate salts turned out to be more toxic to Brassica rapa than chloride salts. This toxicity was mainly caused by sulfate itself, rather than an increased toxicity of sodium. Additional calcium protected plants from sulfate toxicity without avoiding high tissue sulfate concentrations. One possible explanation is that the vacuolar capacity for sulfate is increased by additional calcium. Another possibility is the formation of Ca$_2$SO$_4$ crystals that could be responsible for the toxicity of excessive sulfate or play a protective role by binding sulfate into a non-toxic form. Both possibilities are supported by the fact that amino acid and thiol contents in shoots remain at the same level as in control plants which is an indication that the additional sulfate is not reaching the chloroplasts. The strong ameliorating effect of sulfate toxicity by high
calcium bears possible implications for agricultural areas subjected to high sulfate levels.

References


