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Calcium ameliorates the toxicity of sulfate salinity in *Brassica rapa*

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ABSTRACT

Salinity stress in Brassica, often only associated with osmotic effects and the toxicity of Na^+, was more severe when applied as Na_2SO_4 than as NaCl, indicating that SO_4^{2-} ions had toxic effects as well. Application of 10 mM calcium in the form of $CaCl_2$ in the growth medium of plants only slightly ameliorated growth impairment by NaCl and KCl, but almost completely prevented negative effects of Na_2SO_4 and K_2SO_4 on plant biomass production. This effect was calcium specific, as $MgCl_2$ ameliorated sulfate toxicity to a much lower extent. This sulfate toxicity coincided with a strong decrease in the plant content of calcium and manganese upon sulfate salinity. Application of $CaCl_2$ largely alleviated this decrease, however, it did not prevent the higher tissue concentration of sulfate. $CaCl_2$ prevented the increase in organic sulfur compounds presumably by reducing of relative gene expression of ATP-sulfurylase (ATPS) and adenosine 5′-phosphosulfate reductase (APR) indicating a possible regulation of sulfate assimilation by calcium. The upregulation of the genes encoding for Group 4 sulfate transporters (Sultr4;1 and 4;2) upon sulfate salinity, was absent in the presence of $CaCl_2$. Therefore, additional calcium may facilitate an increased vacuolar capacity for sulfate accumulation.

1. Introduction

Although salt stress is an increasing problem for cropproduction and although much research has been carried out on the phenomena of salt stress and tolerance in plants, progress in increasing crop salt tolerance via breeding remains rather limited. As an alternative to breeding salt tolerant crops, changes in agricultural practice and fertilization could ameliorate salt stress in cropping systems. The addition of a surplus of calcium was shown to ameliorate growth inhibition of crops by salt stress in the beginning of the last century (Kearney and Cameron, 1902; Kearney and Harter, 1901), and since then a number of studies on different crop species have shown similar results and characterized the effect under more controlled conditions (LaHaye and Epstein, 1969; Cramer et al., 1990; Lopez and Satti, 1996; Kaya et al., 2003). Calcium is an essential macronutrient with many vital and beneficial functions in plants. Due to their common positive charge, a competition of sodium with calcium and potassium is widely proposed to be one of the main causes for salt stress. Amelioration of salt stress by calcium has, up to now, almost exclusively been related to sodium toxicity, due to the fact that sodium as a cation competes with calcium in cell walls and membranes and therefore disturbs their function as selective barriers (Cramer et al., 1985; Lynch et al., 1987; Rengel, 1992). This may cause a loss of potassium from roots, which may be partly prevented by addition of calcium (Shabala, 2000). The important role of calcium for potassium/sodium homeostasis under salt stress is widely accepted (Epstein, 1998; Volkmar et al., 1998).

Salt stress caused by NaCl prevails in most salt affected soils, however, plants often have to deal with other salts, such as Na_2SO_4 (Garcia and Hernandez, 1996) and many areas in the world are dominated by sulfate salts (Chang et al., 1983; Keller et al., 1986). Such an excess of sulfate salts may occur in volcanic soils, in marine soils (as sea water contains high amounts of sulfate), in agricultural soils irrigated with saline water or may be caused by anthropogenic inputs from industry or deposition of atmospheric sulfur gases (Moss, 1978; Nriagu, 1978; Freedman and Hutchinson, 1980; Chang et al., 1983). In many plant species sulfate salinity appeared to be more toxic than chloride salinity (Eaton, 1942; Paek et al., 1988; Bilski et al., 1988; Datta et al., 1995; Renault et al., 2001). Recently, Reich et al. (2017) observed both Na_2SO_4 and K_2SO_4 showed a higher toxicity in *Brassica rapa* than NaCl and KCl). The sulfate toxicity coincided with a stronger decrease of the

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tissue content of calcium, magnesium and manganese than upon compared to chloride salinity, however, this difference appeared to be too small to explain the higher toxicity of sulfate over chloride and rather; the upregulation of genes for the Group 4 sulfate transporters (Sultr4;1 and 4;2) was suggested to play a key role under sulfate toxicity (Reich et al., 2015, 2017).

Is has been observed that calcium may ameliorate salinity stress, though in these studies only NaCl and Na_2SO_4 salts were used (e.g. Cramer and Spurr, 1986; Bilski et al., 1988; Reginato et al., 2014). The experimental set-up described by Reich et al. (2017) was used in the present study to test the differences in the amelioration of NaCl, KCl, Na_2SO_4 and K_2SO_4 salinity by addition of calcium. The hypothesis was that if calcium amelioration was related only to sodium toxicity, it should be less effective under Na_2SO_4 than under NaCl toxicity, as the first is mainly caused by sulfate toxicity but the latter by sodium toxicity (Reich et al., 2017). Furthermore, if the amelioration was restricted to sodium toxicity, additional calcium should not have an effect on plants grown in K_2SO_4, which was also shown to cause severe growth inhibition in contrast to KCl. Growth and physiological parameters were used as indicators for the toxicity of the different salts. From the current study it was evident that calcium ameliorates the toxicity of sulfate salinity but not chloride salinity in *B. rapa*. The results provide new insights in the mechanisms of sulfate toxicity and in the specificity of calcium in amelioration of stress caused by different salts.

2. Material and methods

2.1. Plant material and growth conditions

Seeds of *B. rapa*, cv. Komatsuna (Van 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 $Ca(NO_3)_2.4H_2O$, 1.25 mM KNO_3, 0.25 mM KH_2PO_4, 0.5 mM $MgSO_4.7H_2O$, 89 11.6 μM H_3BO_3, 2.4 μM $MnCl_2.4H_2O$, 0.24 μM $ZnSO_4.7H_2O$, 0.08 μM $CuSO_4.5H_2O$, 0.13 μM $Na_2MoO_4.2H_2O$ and 22.5 μM Fe^{3+} -EDTA) in 30l containers (20 sets per container, three plants per set; Supplementary Fig. 1) in a climate-controlled room. Relative humidity was 60–70 % and the photoperiod was 14 h at a photon fluence rate of 300 ± 20 μ mol m^{-2} s^{-1} (400–700 nm) at plant height, supplied by Philips GreenPower LED lamps (deep white/red 120). Day/night temperatures were 21/18 °C. Ten-day old seedlings were grown without additional salt for three days, and subsequently salt concentrations were gradually increased during the following three days. For NaCl and KCl the steps were 25, 50 and 100 mM, and for Na_2SO_4 and K_2SO_4 the steps were 12.5, 25 and 50 mM. For half of the plants an additional 10 mM $CaCl_2$ was added to the nutrient together with the first addition of salt. Seedlings were grown in the final concentrations for five more days and then harvested. Roots were separated from the shoots, weighed and stored at either −20 °C or −80 °C, depending on the requirements for further analysis. For determination of the mineral nutrient content, plant tissue was dried at 80 °C for 24 h and stored in a desiccator for further use.

2.2. Maximum quantum efficiency of photosystem II (Fv/Fm) and pigment content

Prior to harvest, *Fv/Fm* of leaves in dark adapted conditions was determined in the morning prior to the onset of the light period (PAM 2000, Walz, Effeltrich, Germany). For determination of pigment content, frozen shoots were homogenized in 100% acetone by using an Ultra Turrax (10 ml g^{-1} fresh weight) and centrifuged at 30,000*g* for 20 min. The chlorophyll $a + b$ content in the supernatant was determined according to Lichtenthaler (1987).

2.3. Sulfate and free amino acids content

Sulfate was extracted from frozen plant material in water and determined refractometrically after separation by HPLC (Reich et al., 2017). 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.

2.4. Water-soluble non-protein thiol content

For 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 N_2 one hour prior extraction to remove oxygen. After filtering through one layer of Miracloth the extract was centrifuged at 30,000 *g* for 15 min at 0 °C. Thiol content in the supernatant was determined colorimetrically at 413 nm after addition of 5,5′-dithiobis[2-nitrobenzoic acid] (De Kok et al., 1988).

2.5. Mineral nutrient composition

For determination of mineral nutrient content, 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 atomic emission spectrometry (ICP-AES) analysis. Repeat samples were carried out every 10 samples; blanks and standard reference were used for quality control. The sample introduction system consisted of a micromist glass concentric nebulizer, quartz Scott-type double-pass spray chamber at 2 °C, and nickel sample (1 mm) and skimmer (0.4 mm cones). Operating parameters were optimized daily using a tune solution containing 1 μg l^{-1} cerium, lithium, tellurium, and yttrium. Other instrument conditions were radiofrequency forward power of 1,550, sample depth of 8.0 mm, carrier gas flow rate of 0.89l min^{-1}, reaction gas flow rate of 4 ml min^{-1} (H_2) or of 4.5 ml min^{-1} (helium). An internal standard (500 μg l^{-1} germanium) was used to correct for signal drift.

2.6. RNA isolation and expression of the genes encoding the Group 4 sulfate transporters and sulfur assimilatory enzymes

Total RNA was isolated by a modified hot phenol method (Verwoerd et al., 1989). Frozen ground plant material was extracted in hot (80 °C) phenol/extraction buffer (1:1, v/v), 1 g ml^{-1}. The extraction buffer contained 0.1 M Tris − HCl, 0.1 M LiCl, 1% SDS (w/v), 10 mM EDTA, pH 8.0). After mixing, 0.5 ml of chloroform–isoamyl alcohol (24:1, v/v) was added. After centrifugation (13,400 × *g*) for 5 min at 4 °C, the aqueous phases were transferred to new tube. After adding an equal volume of chloroform and isoamyl alcohol, the total RNA was precipitated by 4 M LiCl overnight at 4 °C. Total RNA was collected and washed with 70% ethanol. Possible genomic DNA contamination was removed with a DNAase treatment step (Promega, USA). Phenol–-chloroform–isoamyl alcohol and chloroform–isoamyl alcohol were used for further purification and total RNA was precipitated by ethanol and dissolved in diethylpyrocarbonate-treated water. The quantity and quality of RNA was checked using ThermoNanoDrop 2000 and RNA in each sample was adjusted to the same concentration. The integrity of RNA was checked by electrophoresis by loading 1 μg RNA on a 1% TAE-agarose gel.

DNA-free intact RNA (1 μg) was reverse transcribed into cDNA with oligo-dT primers using a first strand cDNA synthesis kit (Promega, USA) according to the manufacturer-supplied instructions. Subsequently, the cDNA was used as a template in real-time PCR experiments with gene-specific primers. To design primers for the genes of the Sultr4;1, Sultr 4;2 and sulfur assimilatory enzymes such as ATP-sulfurylase (ATPS; EC

Table 1
List of primer sequences for qPCR analysis.

Gene	Primer sequences (5'-3')	
	Forward	Reverse
Sultr4;1	GAGGAGGTTTGGGAATAACG	AATCGCAACCCACTATACAC
Sultr4;2	CTCTCTGGCACTACGTTTG	AATAGCCGGAGAAGAAGAAG
ATPS	TTYGCKTTCCAGCTWAGG	AGGGTTTTTGWATCCCATCTC
APR	GTATGTTTCWATWGGGTGTGAG	CTYCTTGATGTTCCCTTTGTG
ACT2	AGCAGCATGAAGATCAAGGT	GCTGAGGGATGCAAGGATAG

2.7.7.4) and adenosine 5′-phosphosulfate reductase (APR; EC 1.8.99.2), the coding sequences of *Arabidopsis thaliana* genes were used to query homologous *B. rapa* sequences, which are available in the *B. rapa* genome sequence portal http://www.brassica-rapa.org. The full length sequences of these genes can be found under the following accession numbers: Sultr4;1 (XM009123507 and NM121358.2), Sultr 4;2 (Sultr4;2 XM009136985 and NM112087.2), ATPS (XM009147003, XM009150169, XM009103518 and XM009151241) and APR (XM009116311, XM009138987 and XM009125111). Relative transcript levels were normalized based on expression of the *A. thaliana* actin 2 (ACT2) gene as a reference gene (Marmagne et al., 2010; Sheng et al., 2016). To design primers, *A. thaliana* ACT2 genes (NM-112764.3) were used to query homologous *B. rapa* (JN120480.1) sequences. Gene-specific primer sets are listed in Table 1. RT-PCR was performed on an Applied Bio Systems' 7300 real-time PCR system using the SYBR Green master mix kit (Thermo Scientific) based on manufacturer's instructions. The transcript level of the target gene and actin was measured using the comparative Ct method (Schmittgen and Livak, 2008). Analysis of qPCR data was performed using three independent RNA preparations from separate plant tissues.

2.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). A one-way analysis of variance (ANOVA) was performed and the treatment means were compared using Tukey's HSD all-pairwise comparisons at the $p < 0.01$ level as a post-hoc test.

3. Results

3.1. Biomass production, chlorophyll content and Fv/Fm

Exposure of *B. rapa* seedlings to 100 mM NaCl and KCl and 50 mM Na_2SO_4 and K_2SO_4 resulted in a strongly reduced plant biomass production (Fig. 1). However, sulfate salinity was more toxic than chloride salinity, even at equimolar Na^+ and K^+ concentrations. The average total biomass of seedlings grown under control conditions was 1.7 g, with a reduction to 1 g by chloride salinity (40% reduction) and 0.5 g (70% reduction) by sulfate salinity, whereas shoot/root ratios were hardly affected (Fig. 1). Addition of 10 mM $CaCl_2$ to the nutrient solution alleviated the toxicity of Na_2SO_4 and K_2SO_4 salinity, with little effect on NaCl and KCl toxicity. This effect was very much calcium specific, since the ameliorating effect of the addition of 10 mM $MgCl_2$ was only minor, while $CaCl_2$ entirely prevented growth reduction by K_2SO_4 (Supplementary Fig. 2).

Chloride and sulfate salinity resulted in a decrease in the content of chlorophyll *a* + b (Fig. 1). Both the plant biomass production and the chlorophyll *a* + b content were comparable to that of the control plants upon exposure to sulfate salinity in presence of supplemental $CaCl_2$ (Fig. 1). In addition, maximum quantum yield (*Fv/Fm*) was significantly decreased by sulfate salts, and this was prevented by additional calcium (Fig. 1).

3.2. Free amino acids content

There was an increase observed in the content of free amino acids in the shoots of plants exposed to Na_2SO_4 and K_2SO_4, which was entirely prevented by additional calcium. Chloride salinity had no significant effect on free amino acid content (Fig. 2).

3.3. Total sulfur, sulfate and organic sulfur content

Total sulfur content was substantially increased by both sulfate salts. Total sulfur content in plants exposed to Na_2SO_4 was increased 2.5-fold in roots and shoot. Likewise, upon exposure of plants to K_2SO_4 salinity, total sulfur content in the roots and shoots was increased by 2 and 3.5-fold, respectively (Fig. 3). However, the strong increase of total sulfur in both roots and shoots was only partly reduced by additional calcium.

Na_2SO_4 and K_2SO_4salinity increased sulfate content in the roots 2.5 and 2-fold and in the shoots 4.5 and 2-fold, respectively (Fig. 3). The addition of supplemental $CaCl_2$ significantly affected the increased sulfate content upon sulfate salinity; it resulted in a lower increase in roots of plants exposed to Na_2SO_4 and in the shoot of plants exposed to K_2SO_4 (Fig. 3). NaCl and KCl salinity did not affect the sulfate contents in the roots and shoots, neither with nor without supplemental $CaCl_2$ (Supplementary Fig. 3).

The total sulfur and sulfate contents have been altered in such a way that the organic sulfur content, calculated by subtracting the sulfate content from the total sulfur content, of shoots of Na_2SO_4 and K_2SO_4-exposed *B. rapa* was significantly increased (Fig. 3). However, additional calcium resulted in substantial decrease of organic sulfur content in the shoots of plant exposed to both sulfate salts as well as in the roots of plants exposed to Na_2SO_4.

3.4. Water-soluble non-protein thiol content

Thiol contents in the shoots of plants exposed to Na_2SO_4 and K_2SO_4 salts were increased by 1.7 and 1.5-fold, respectively (Fig. 4). Additional calcium prevented this effect of sulfate salts, and led to thiol contents similar to plants grown under normal conditions (Fig. 4).

3.5. Mineral nutrient content

Calcium was decreased in both roots and shoots upon exposure to sulfate salts (Fig. 5). The decrease was relatively stronger in the shoots. Additional calcium prevented a decrease of tissue calcium content completely in the roots and partly in the shoots. The ameliorating effect was weaker in plants exposed to K_2SO_4. Magnesium was slightly decreased in roots of plants exposed to K_2SO_4; and additional calcium prevented this effect. In the shoots, magnesium content was strongly decreased by sulfate salts with no significant ameliorating effect of calcium. A similar result was found for potassium in plants exposed to Na_2SO_4. The strong decrease was partly prevented in the roots by additional calcium but not at all in the shoot. Potassium content in the shoot remained as low as in plants exposed only to Na_2SO_4. Plants exposed to K_2SO_4 had increased potassium contents. These were increased even more by additional calcium in roots but less so in the shoot. Additional calcium did not prevent the large increases in tissue sodium content. There was only a slight effect on the roots and no effect at all on the shoots. In contrast, additional calcium had an ameliorating effect on the slight decrease of phosphorus in the roots. The effects on micronutrients were more complex. Copper content was decreased in roots in plants exposed to sulfate salts and additional calcium resulted in even lower copper contents. In the shoot, sulfate salts had no significant effect but additional calcium slightly increased copper content. Iron content was increased in roots of plants exposed to sulfate salts and this effect was completely prevented by additional calcium. The strong decrease of manganese in both roots and shoots was only partly

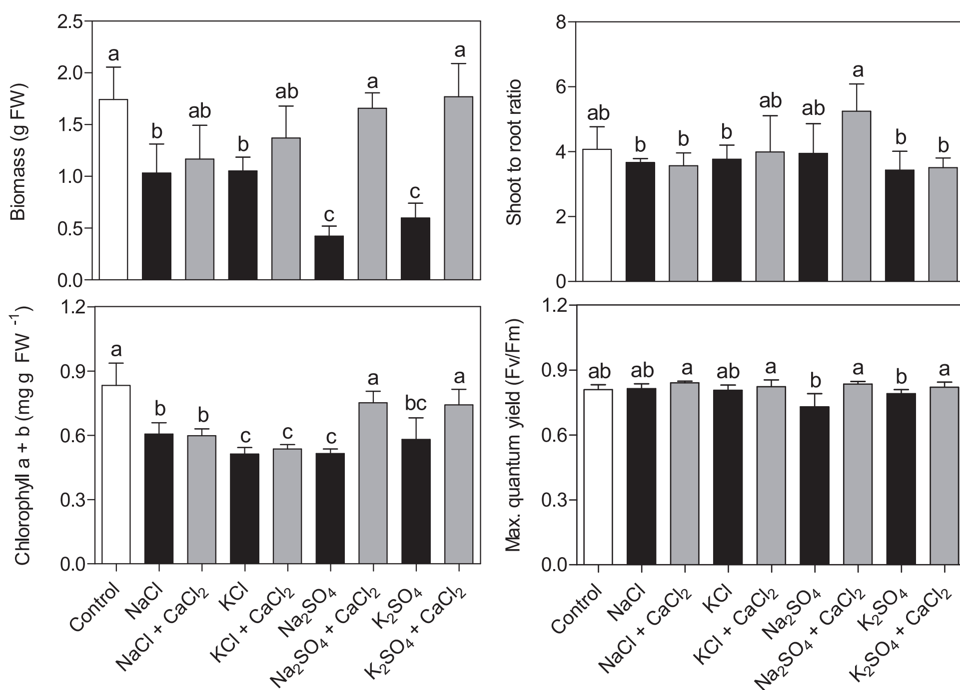

Fig. 1. Biomass, chlorophyll *a* + b and *Fv/Fm* of *Brassica rapa* seedlings exposed to 100 mM NaCl and KCl and 50 mM Na_2SO_4 and K_2SO_4 with (grey) or without (black) additional 10 mM $CaCl_2$. Data represent the mean of n measurements with three plants in each (± SD; biomass n = 15; chlorophyll *a* + b n = 3; Fv/Fm n = 10). Different letters indicate significant difference (p < 0.01; One-way ANOVA; Tukey´s HSD all-pairwise comparisons as a post-hoc test).

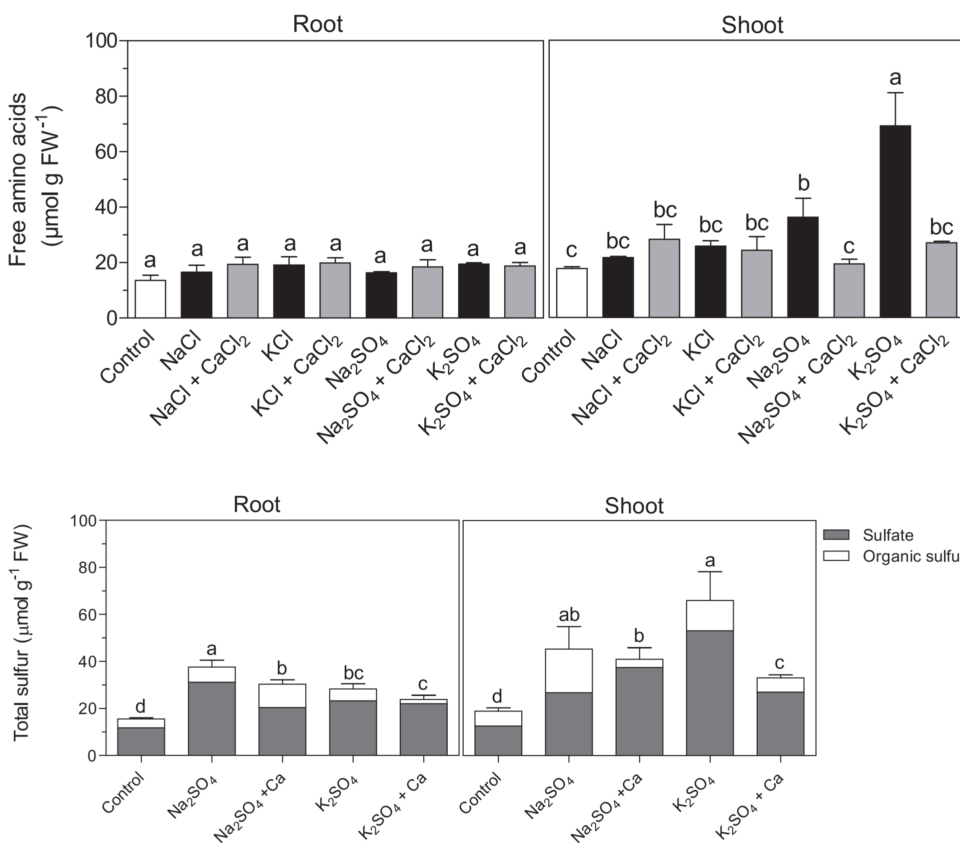

Fig. 2. Free amino acids content of shoot and roots of *Brassica rapa* seedlings exposed to 100 mM NaCl and KCl and 50 mM Na_2SO_4 and K_2SO_4 with (grey) or without (black) additional 10 mM $CaCl_2$. Data represent the mean of three measurements with three plants in (± SD). Different letters indicate significant difference (p < 0.01; One-way ANOVA; Tukey´s HSD all-pairwise comparisons as a post-hoc test).

Fig. 3. Total sulfur, sulfate and organic sulfur content of shoot and roots of *Brassica rapa* seedlings exposed to 50 mM Na_2SO_4 and K_2SO_4 with or without additional 10 mM $CaCl_2$. The sulfate and organic sulfur fraction is presented in grey and white, respectively. Data represent the mean of five measurements with three plants in each (± SD). Different letters indicate significant difference (p < 0.01; One-way ANOVA; Tukey´s HSD all-pairwise comparisons as a post-hoc test).

ameliorated by additional calcium. The decrease of molybdenum, in contrast, was slightly ameliorated only in plants exposed to K_2SO_4. The zinc remained rather unaffected; the increase by Na_2SO_4 in roots was prevented by additional calcium.

3.6. Expression of the group 4 sulfate transporters and sulfur assimilatory enzymes

The expression of the vacuolar sulfate transporters Sultr4;1 and Sultr4;2 was increased by sulfate salts (Fig. 6). This increase was more pronounced in the shoot. Additional calcium prevented this increase in all tissues and upon exposure to both sulfate salts. No significant effects were found on the expression of ATPS in roots and shoots upon

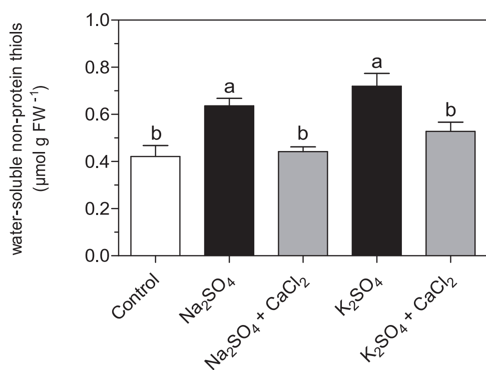

Fig. 4. Water-soluble non-protein thiols content of shoot of *Brassica rapa* seedlings exposed to 50 mM Na_2SO_4 and K_2SO_4 with (grey) or without (black) additional 10 mM $CaCl_2$. Data represent the mean of three measurements with three plants in each (± SD). Different letters indicate significant difference ($p < 0.01$; One-way ANOVA; Tukey´s HSD all-pairwise comparisons as a post-hoc test).

exposure to both Na_2SO_4 and K_2SO_4 salts. In the shoot, additional calcium appeared to reduce expression significantly for both sulfate salts (Fig. 6). APR expression was decreased by sulfate salts in both roots and shoots. Calcium had a negative effect on the expression of APR in the shoots, and resulted in a greater reduction in transcript levels of APR in plant exposed to both sulfate salts (Fig. 6).

4. Discussion

Growth of plants exposed to 50 mM Na_2SO_4 and K_2SO_4 was inhibited roughly twice as much as growth of plants exposed to 100 mM NaCl and KCl (Fig. 1). As previously discussed (Reich et al., 2017), several mechanisms may be responsible for the higher toxicity of sulfate over chloride in *B. rapa*. A differential accumulation of sodium was not a possible explanation. Plants showed similarly increased sodium contents, with slightly less sodium in plants exposed to Na_2SO_4. In addition, stronger negative effects of sulfate salts on the content of other important nutrients were excluded as a cause, as changes were either not sulfate-specific or only minor. Therefore, it was assumed that toxic effects of sulfate are the most likely cause of the severe toxicity of Na_2SO_4 and K_2SO_4. One of the most remarkable findings was that the

Fig. 5. Mineral nutrient composition of shoot and roots of *Brassica rapa* seedlings exposed to 50 mM Na_2SO_4 and K_2SO_4 with (grey) or without (black) additional 10 mM $CaCl_2$. Data represent the mean of five measurements with three plants in each (± SD). Different letters indicate significant difference ($p < 0.01$; One-way ANOVA; Tukey´s HSD all-pairwise comparisons as a post-hoc test).

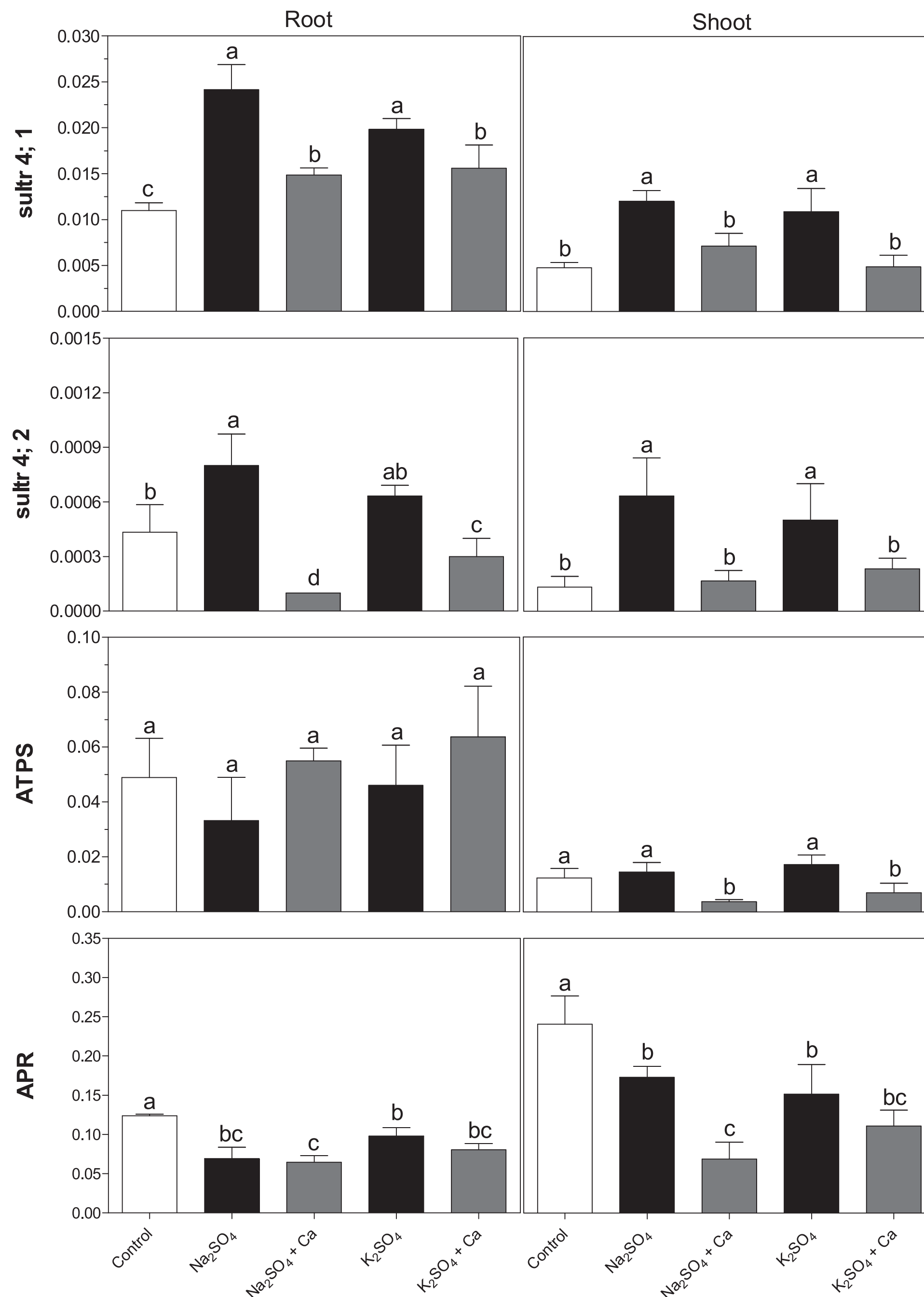

Fig. 6. Relative gene expression of the vacuolar sulfate transporters and sulfur assimilatory enzymes in shoot and roots of *Brassica rapa* seedlings exposed to 50 mM Na_2SO_4 and K_2SO4 with (grey) or without (black) additional 10 mM $CaCl_2$. Data represent the mean of three measurements with three plants in each (± SD). Different letters indicate significant difference ($p < 0.01$; One-way ANOVA; Tukey´s HSD all-pairwise comparisons as a post-hoc test). ATPS = ATP sulfurylase; APR = adenosine 5′-phosphosulfate reductase.

genes for vacuolar sulfate transporters were upregulated upon exposure to sulfate salts (Reich et al., 2017).

Depletion of calcium from plasma membranes and the prevention of calcium uptake by the roots by sodium are often considered as the first and most severe toxic effects of salinity (Cramer et al., 1985; Rengel, 1992). A previous study with *B. rapa*, however, showed that a decrease in calcium content was not an exclusive feature of sodium salinity exposure, and calcium content was decreased by both chloride and sulfate salts (Reich et al., 2017). However, the ameliorating effect of calcium was only restricted to sulfate salt toxicity (Fig. 1). The severe growth inhibition caused by both Na_2SO_4 and K_2SO_4 was prevented by the addition of 10 mM $CaCl_2$ to the growth medium and resulted in maintenance of growth, chlorophyll content and maximum quantum yield at levels similar to control conditions (Fig. 1). Many investigations have shown that $CaCl_2$ plays an important role in alleviating negative consequences of many stress conditions for multiple plant species (Arshi et al., 2006; Tattini and Traversi, 2009; Shoresh et al., 2011). Increasing calcium availability may alleviate salt stress by increasing membrane integrity (Ma et al., 2005; Guimarães et al., 2011), maintaining the osmotic balance and preventing damage from cellular dehydration (Arshi et al., 2006), and postponing oxidative damage by the induction of antioxidant enzymes (Wang et al., 2009; Issam et al., 2012). Free amino acids accumulation, which was important for plant adaptation under sulfate salts stress, was reduced by supplemental addition of

$CaCl_2$. This reduction may be due to a reduction of the free amino acids biosynthesis (Jaleel et al., 2007) and/or an increase of protein biosynthesis which could be considered as an important response for maintaining the growth of sulfate stressed plants at the level of plants grown under control conditions (Fig. 1).

Plants grown in 50 mM Na_2SO_4 and K_2SO_4 showed increased levels of total sulfur, organic sulfur and water-soluble non-protein thiols content (Figs. 2–4), indicating that sulfate assimilation was affected by sulfate salts. Sulfur is essential for the synthesis of cysteine and methionine, as precursors for organic sulfur compounds, such as proteins, sulfolipids, glutathione and variety of secondary sulfur compounds (alliins, glucosinolates; Leustek et al., 2000; Durenkamp and De Kok, 2004; Kopriva, 2006). Previously, demand-driven regulation of APR has been observed in the control of the sulfate assimilation pathway (Brunner et al., 1995; Lee and Leustek, 1999; Leustek et al., 2000; Kocsy et al., 2004; Kopriva, 2006). Similarly, a surplus of sulfur compounds and/ or thiols by sulfate salts (Figs. 3 and 4) may be a reason for repression of the sulfate assimilation pathway via down regulation of APR (Westerman et al., 2001; Vauclare et al., 2002). Additional calcium prevented the increase of organic sulfur and water-soluble non-protein thiols content (Figs. 3 and 4), which may be a consequence of Ca^{2+} playing a role as a signal regulating the sulfate assimilation pathway through the reduction of relative gene expression of ATPS and APR.

In addition, the relative expression of the vacuolar sulfate transporters Sultr4;1 and 4;2 was increased by sulfate salts (Fig. 6), indicating an increased capacity for efflux of sulfate from the vacuole into the cytoplasm. In plants that received additional calcium, however, this increased expression was absent or even expression was decreased (Fig. 6). Thus sulfate was probably stored in the vacuole, leading to plants without increased organic sulfur, thiols and amino acid contents and with normal growth. If an increase of Sultr4;1 and 4;2 expression upon exposure to sulfate salts was due to a displacement of calcium from the tonoplast, this would suggest that calcium could be involved in regulation of their expression, which is an intriguing question for future studies. If cytoplasmic calcium was involved in the regulation of vacuolar sulfate transporters, an excess of sodium or potassium could disturb this regulation and lead to unfavorable up-regulation of Sultr4;1 and 4;2 and an efflux of sulfate from the vacuole. An increased capacity of the vacuole for sulfate storage due to an increased availability of calcium as a counter-ion in the vacuole appears unlikely, due to the prevailing imbalance between the two ions (Fig. 5).

However it is also possible that calcium facilitates a higher capacity of the vacuole for sulfate, for example by increasing the integrity and selectivity of the tonoplastic membrane. Calcium displacement from the membrane by sodium (Cramer et al., 1985; Läuchli, 1990) or loss of potassium might be prevented by additional calcium in the growing medium. The calcium content in plants exposed to sulfate salts was, however, still significantly decreased compared to control levels, while sodium in plants exposed to Na_2SO_4 was not lowered by calcium (Fig. 5). Furthermore, a positive effect of additional calcium on the potassium/sodium ratio as, for example, found in NaCl-stressed cotton and barley (Cramer et al., 1991), was not found for Na_2SO_4 stressed plants in the present study, as potassium was not increased by additional calcium (Fig. 5).

Another possibility is that the observed effects of calcium were actually an amelioration of calcium deficiency, caused by the exposure of plants to sulfate salts. The very low solubility of $CaSO_4$ could lead to inter- or intracellular precipitation and a consequent immobilization of calcium. This could lead to an internal calcium shortage, additional to the strong decrease of tissue calcium content caused by sodium and potassium (Fig. 5). A surplus of calcium could counteract the decrease of cellular calcium under salt stress caused by the high levels of either sodium or potassium (and potentially an additional immobilization by precipitation with sulfate), and thereby enable the fulfillment of its metabolic role, for example as cellular second messenger. Calcium was, for example, shown to be involved in the regulation of *Salt Overly Sensitive* (SOS) genes, which are crucial for an adequate stress response (Mahajan et al., 2008). If sulfate exposure would lead to an additional shortage of calcium, it would make plants more vulnerable to sodium toxicity. There was, however, no increased toxicity of sodium observed in Na_2SO_4 compared to NaCl treatments (Reich et al., 2017), and therefore the increased toxicity was attributed to sulfate. On the other hand, additional calcium could lead to more precipitation of $CaSO_4$, and therefore could decrease sulfate toxicity. Subcellular studies could clarify if and where $CaSO_4$ crystals form. Although additional calcium did not fully restore calcium levels in plants upon exposure to sulfate salts (Fig. 5), it may have increased them above a critical level necessary to maintain vital functions and optimal growth under 50 mM sulfate in the external medium. This hypothesis would, however, not explain why the effect of additional calcium is so much stronger with sulfate than compared to chloride salinity.

5. Conclusions

An explanation for the relatively high sensitivity of *B. rapa* to sulfate found in this study (and previously found by Reich et al., 2017) could be the high sulfate uptake capacity and tissue sulfate content of many *Brassica* species (Koralewska et al., 2007). Under moderate sulfur supply, these lead to the characteristically high tissue content of sulfate and reduced sulfur compounds in these species. Under an excess supply, however, it may prove to be detrimental, as it leads to toxic levels of internal sulfate. Amelioration of salt stress by calcium was not solely due to a sodium-sulfate interaction. Sulfate toxicity in *B. rapa* was, in contrast, strongly decreased by additional calcium, independent of the cation. This ameliorating effect of calcium under sulfate salt stress might be species specific and correlated with salt tolerance (Volkmar et al., 1998; Renault, 2005).

The results delivered further indications for the mechanisms behind sulfate toxicity, suggesting that sulfate toxicity was related to the upregulation of vacuolar sulfate transporters. The upregulation was prevented by high calcium levels, which presumably facilitated an increased sulfate storage in the vacuoles. Additional calcium prevented the increase in reduced sulfur compounds by decreasing relative gene expression of APR and ATPS, indicating a regulation of sulfate assimilation by calcium.

Conflict of interest

We declare that we have no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jplph.2018.08.014.

References

Arshi, A., Abdin, M.Z., Iqbal, M., 2006. Effect of $CaCl_2$ on growth performance, photosynthetic efficiency and nitrogen assimilation of *Cichorium intybus* L. grown under NaCl stress. Acta Physiol. Plants 28, 137–147.
Bilski, J.J., Nelson, D., Conlon, R.L., 1988. The response of four potato cultivars to chloride salinity, sulfate salinity and calcium in pot experiments. Am. Potato J. 65, 85–90.
Brunner, M., Kocsy, G., Ruegsegger, A., Schmutz, D., Brunold, C., 1995. Effect of chilling on assimilatory sulphate reduction and glutathione synthesis in maize. J. Plant Physiol. 146, 743–747.
Chang, C., Sommerfeldt, T.G., Carefoot, J.M., Schaalje, G.B., 1983. Relationships of

electrical conductivity with total dissolved salts and cation concentration of sulfate-dominant soil extracts. Can. J. Soil Sci. 63, 79–86.
Cramer, G.R., Spurr, A.R., 1986. Responses of lettuce to salinity. I. Effects of NaCl and Na_2SO_4 on growth. J. Plant Nutr. 9, 115–130.
Cramer, G.R., Läuchli, A., Polito, V.S., 1985. Displacement of Ca^{2+} by Na^+ from the plasmalemma of root cells a primary response to salt stress? Plant Physiol. 79, 207–211.
Cramer, G.R., Epstein, E., Läuchli, A., 1990. Effects of sodium, potassium and calcium on salt-stressed barley. I. Growth analysis. Physiol. Plant. 80, 83–88.
Cramer, G.R., Epstein, E., Läuchli, A., 1991. Effects of sodium, potassium and calcium on salt-stressed barley. Physiol. Plant. 81, 197–202.
Datta, K.S., Kumar, A., Varma, S.K., Angrish, R., 1995. Differentiation of chloride and sulphate salinity on the basis of ionic distribution in genetically diverse cultivars of wheat. J. Plant Nutr. 18, 2199–2212.
De Kok, L.J., Buwalda, F., Bosma, W., 1988. Determination of cysteine and its accumulation in spinach leaf tissue upon exposure to excess sulfur. J. Plant Physiol. 133, 502–505.
Durenkamp, M., De Kok, L.J., 2004. Impact of pedospheric and atmospheric sulphur nutrition on sulphur metabolism of *Allium cepa* L., a species with a potential sink capacity for secondary sulphur compounds. J. Exp. Bot. 55, 1821–1830.
Eaton, F.M., 1942. Toxicity and accumulation of chloride and sulfate salts in plants. J. Agric. Res. 64, 357–399.
Epstein, E., 1998. How calcium enhances plant salt tolerance. Science 280, 1906–1907.
Freedman, B., Hutchinson, T.C., 1980. Pollutant inputs from the atmosphere and accumulations in soils and vegetation near a nickel-copper smelter at Sudbury, Ontario, Canada. Can. J. Bot. 58, 108–132.
Garcia, C., Hernandez, T., 1996. Influence of salinity on the biological and biochemical activity of a calciorthird soil. Plant Soil 178, 255–263.
Guimarães, F.V.A., de Lacerda, C.F., Marques, E.C., de Miranda, M.R.A., de Abreu, C.E.B., Prisco, J.T., Gomes-Filho, E., 2011. Calcium can moderate changes on membrane structure and lipid composition in cowpea plants under salt stress. Plant Growth Regul. 65, 55–63.
Issam, N., Kawther, M., Haythem, M., Moez, J., 2012. Effects of $CaCl_2$ pretreatment on antioxidant enzyme and leaf lipid content of faba bean (*Vicia faba* L.) seedlings under cadmium stress. Plant Growth Regul. 68, 37–47.
Jaleel, C.A., Manivannan, P., Sankar, B., Kishorekumar, A., Gopi, R., Somasundaram, R., Panneerselvam, R., 2007. Water deficit stress mitigation by calcium chloride in *Catharanthus roseus*: effects on oxidative stress, proline metabolism and indole alkaloid accumulation. Colloids Surf. B Biointerfaces 60, 110–116.
Kaya, C., Ak, B.E., Higgs, D., 2003. Response of salt-stressed strawberry plants to supplementary calcium nitrate and/or potassium nitrate. J. Plant Nutr. 26, 543–560.
Kearney, T.H., Cameron, F.K., 1902. The effect upon seedling plants of certain components of alkali soils. USDA Office of the Secretary Report 71. pp. 1–60.
Kearney, T.H., Harter, L.L., 1901. The Comparative Tolerance of Various Plants for the Salts Common in Alkali Soils. No. 113. US Government Printing Office.
Keller, L.P., McCarthy, G.J., Richardson, J.L., 1986. Mineralogy and stability of soil evaporites in North Dakota. Soil Sci. Soc. Am. J. 50, 1069–1071.
Kocsy, G., Szalai, G., Galiba, G., 2004. Effect of osmotic stress on glutathione and hydroxyl methyl glutathione accumulation in wheat. J. Plant Physiol. 161, 785–794.
Kopriva, S., 2006. Regulation of sulfate assimilation in Arabidopsis and beyond. Ann. Bot. 97, 479–495.
Koralewska, A., Posthumus, F.S., Stuiver, C.E.E., Buchner, P., Hawkesford, M.J., De Kok, L.J., 2007. The characteristic high sulfate content in *Brassica oleracea* is controlled by the expression and activity of sulfate transporters. Plant Biol. 9, 654–661.
LaHaye, P., Epstein, E., 1969. Salt toleration by plants: enhancement with calcium. Science. 166, 395–396.
Läuchli, A., 1990. Calcium, salinity and the plasma membrane. In: Leonard, R.T., Hepler, P.K. (Eds.), Calcium in Plant Growth. The American Society of Plant Physiologists, Rockville, MD, pp. 26–35.
Lee, S.M., Leustek, T., 1999. The effect of cadmium on sulfate assimilation enzymes in *Brassica juncea*. Plant Sci. 141, 201–207.
Leustek, T., Martin, M.N., Bick, J.A., Davies, J.P., 2000. Pathways and regulation of sulfur metabolism revealed through molecular and genetic studies. Annu. Rev. Plant Physiol. Plant Mol. Biol. 51, 141–165.
Lichtenthaler, H.K., 1987. Chlorophylls and carotenoids: pigments of the photosynthetic biomembranes. Methods Enzymol. 148, 350–382.
Lopez, M.V., Satti, S.M.E., 1996. Calcium and potassium-enhanced growth and yield of tomato under sodium chloride stress. Plant Sci. 114, 19–27.
Lynch, J., Cramer, G.R., Läuchli, A., 1987. Salinity reduces membrane-associated calcium in corn root protoplasts. Plant Physiol. 83, 390–394.
Ma, R., Zhang, M., Li, B., Du, G., Wang, J., Chen, J., 2005. The effects of exogenous Ca^{2+} on endogenous polyamine levels and drought resistant traits of spring wheat grown under arid conditions. J. Arid Environ. 63, 177–190.
Mahajan, S., Pandey, G.K., Tuteja, N., 2008. Calcium-and salt-stress signaling in plants: shedding light on SOS pathway. Arch. Biochem. Biophys. 471, 146–158.
Marmagne, A., Brabant, P., Thiellement, H., Alix, K., 2010. Analysis of gene expression in resynthesized Brassica napus allotetraploids: transcriptional changes do not explain differential protein regulation. New Phytol. 186, 216–227.
Moss, M., 1978. Sources of sulfur in the environment: the global sulfur cycle. In: Nriagu, J.O. (Ed.), Sulfur in the Environment, Part 1: The Atmospheric Cycle. Wiley, New York, pp. 23–50.
Nriagu, J.O., 1978. Production and uses of sulfur. In: Nriagu, J.O. (Ed.), Sulfur in the Environment, Part 1: The Atmospheric Cycle. Wiley, New York, pp. 1–21.
Paek, K.Y., Chandler, S.F., Thorpe, T.A., 1988. Physiological effects of Na_2SO_4 and NaCl on callus cultures of *Brassica campestris* (Chinese cabbage). Physiol. Plant. 72, 160–166.
Reginato, M., Sosa, L., Llanes, A., Hampp, E., Vettorazzi, N., Reinoso, H., Luna, V., 2014. Growth responses and ion accumulation in the halophytic legume *Prosopis strombulifera* are determined by Na_2SO_4 and NaCl. Plant Biol. 16, 97–106.
Reich, M., Aghajanzadeh, T., Stuiver, C.E.E., Koralewska, A., De Kok, L.J., 2015. Impact of sulfate salinity on the uptake and metabolism of sulfur in Chinese cabbage. In: De Kok, L.J., Hawkesford, M.J., Rennenberg, H., Saito, K., Schnug, E. (Eds.), Molecular Physiology and Ecophysiology of Sulfur. Springer International Publishing, pp. 227–238.
Reich, M., Aghajanzadeh, T., Helm, J., Parmar, S., Hawkesford, M.J., De Kok, L.J., 2017. Chloride and sulfate salinity differently affect biomass, mineral nutrient composition and expression of sulfate transport and assimilation genes in *Brassica rapa*. Plant Soil 411, 319–332.
Renault, S., 2005. Response of red-osier dogwood (*Cornus stolonifera*) seedlings to sodium sulphate salinity: effects of supplemental calcium. Physiol. Plant. 123, 75–81.
Renault, S., Croser, C., Franklin, J.A., Zwiazek, J.J., 2001. Effects of NaCl and Na2SO4 on red-osier dogwood (Cornus stolonifera Michx) seedlings. Plant Soil 233, 261–268.
Rengel, Z., 1992. The role of calcium in salt toxicity. Plant Cell Environ. 15, 625–632.
Rosen, H., 1957. A modified ninhydrin colorimetric analysis for amino acids. Arch. Biochem. Biophys. 67, 10–15.
Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative CT method. Nat. Protoc. 3, 1101–1108.
Shabala, S.N., 2000. Ionic and osmotic components of salt stress specifically modulate net ion fluxes from bean leaf mesophyll. Plant Cell Environ. 23, 825–837.
Sheng, X.G., Zhao, Z.Q., Yu, H.F., Wang, J.S., Zheng, C.F., Gu, H.H., 2016. In-depth analysis of internal control genes for quantitative real-time PCR in *Brassica oleracea* var. botrytis. Genet. Mol. Res. 15 (3), 15038348.
Shoresh, M., Spivak, M., Bernstein, N., 2011. Involvement of calcium mediated effects on ROS metabolism in the regulation of growth improvement under salinity. Free Radic. Biol. Med. 51, 1221–1234.
Tattini, M., Traversi, M.L., 2009. On the mechanism of salt tolerance in olive (*Olea europaea* L.) under low- or high-Ca^{2+} supply. Environ. Exp. Bot. 65, 72–81.
Vauclare, P., Kopriva, S., Fell, D., Suter, M., Sticher, L., von Ballmoos, O., Kra¨henbuhl, U., Op den Camp, R., Brunold, C., 2002. Flux control of sulphate assimilation in *Arabidopsis thaliana*: adenosine 5'-phosphosulphate reductase is more susceptible than ATP sulphurylase to negative control by thiols. Plant J. 31, 729–740.
Verwoerd, T.C., Dekker, B.M., Hoekema, A., 1989. A small-scale procedure for the rapid isolation of plant RNAs. Nucleic Acids Res. 17, 2362.
Volkmar, K.M., Hu, Y., Steppuhn, H., 1998. Physiological responses of plants to salinity: a review. Can. J. Plant Sci. 78, 19–27.
Wang, Y., Yang, Z.M., Zhang, Q.F., Li, J.L., 2009. Enhanced chilling tolerance in *Zoysia matrella* by pre-treatment with salicylic acid, calcium chloride, hydrogen peroxide or 6-benzylaminopurine. Biol. Plant. 53, 179–182.
Westerman, S., Stulen, I., Suter, M., Brunold, C., De Kok, L.J., 2001. Atmospheric H_2S as sulphur source for *Brassica oleracea*: consequences for the activity of the enzymes of the assimilatory sulphate reduction pathway. Plant Physiol. Biochem. 39, 425–432.
Zhao, F., McGrath, S.P., Crosland, A.R., 1994. Comparison of three wet digestion methods for the determination of plant sulphur by inductively coupled plasma atomic emission spectroscopy (ICP-AES). Commun. Soil Sci. Plant Anal. 25, 407–418.