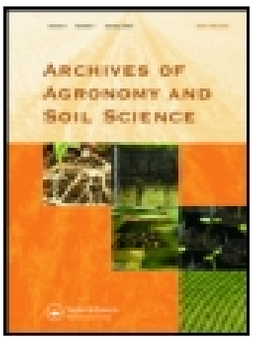

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Sulfur metabolism in *Allium cepa* is hardly affected by chloride and sulfate salinity

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ABSTRACT

Salinity as a major agricultural problem can affect crop growth and quality. Onion (*Allium cepa* L.) plant contains a wide variety of sulfur-containing compounds which may be involved in plant protection against salt stress. In the current study, a similar reduction in growth caused by chloride and sulfate salts was observed when onion was exposed to equimolar concentrations of Na^+. Also, no difference was observed for shoot/root ratio and dry matter content of roots and shoots. Plants accumulated Na^+ and the respective anions (chloride and sulfate) which in turn caused changes in the content of other nutrients. The content of potassium and calcium was decreased more than the other elements by both sodium salts. Sulfate salinity resulted in substantial increase in total sulfur and sulfate content but chloride salinity affected neither the total sulfur nor sulfate content of the roots and shoots, only in onion exposed to 200 mM chloride salt, those of roots and shoots were reduced. Furthermore, the water-soluble non-protein thiol content as well as the content of alliin remained rather unaffected. In conclusion, either salts affected the uptake and distribution of sulfate in onion, but had no or only a minor effect on the plant sulfur metabolism.

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Allium cepa; alliin; chloride salinity; sulfur metabolism; sulfate salinity

Introduction

Crop production is restricted by various biotic and abiotic environmental stress factors. Amongst abiotic stresses, salinity is considered one of the most limiting factors for productivity of agricultural crops around the world (Subramanyam et al. 2010; Chaudhary and Sharma 2014). Salinity disrupts ion homeostasis and the water potential of the plant, which may negatively affect metabolism and growth (Greenway and Munns 1980; Mansour et al. 2000; Munns 2002; Tester and Davenport 2003). Salinity contributes to reduced growth rate and changes in leaf color as well as resulting in smaller leaves, shorter length, fewer leaves, reduction in length and mass of the roots (Mathur et al. 2006; Zhao et al. 2007; Houimli et al. 2008; Gama et al. 2009; Rui et al. 2009; Memon et al. 2010). Salt stress alters photosynthesis and causes reduction in chlorophyll content due to enzymatic chlorophyll degradation (Sultana et al. 2000; Xu et al. 2000; Misra et al. 2006). Also, salinity is resulted in decrease in the rate of nutrient uptake in plants (Murillo-Amador et al. 2007; Taffouo et al. 2009). Ionic effects are manifested more generally in leaf and meristem damage (Shannon and Grieve 1999). However,

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the impact of salinity on plants depends on species, variety, growth stage, environmental factors and the type of salt (Yadav et al. 2011). Na^+ is considered to be the most toxic ion in salinity stress (Dubey 1997; Hasegawa et al. 2000). Despite the fact that salt stress is generally due to high NaCl levels, in many areas plants may have to cope with Na_2SO_4 salinity (Chang et al. 1983; Keller et al. 1986; Garcia and Hernandez 1996). Some plant species such as wheat (Datta et al. 1995), wild potato (Bilski et al. 1988), barley (Huang and Redmann 1995), creeping screwbean (Reginato et al. 2014), and cabbage (Paek et al. 1988) were shown to be more susceptible to Na_2SO_4 than to NaCl salinity. In *Brassica rapa* seedlings, sulfate salts (Na_2SO_4 and K_2SO_4) were more toxic than chloride salts (NaCl and KCl) (Reich et al. 2016), and this toxicity is strongly ameliorated by calcium supplemented to the nutrient solution (Reich et al. 2018). However there is still relatively little known about the mechanism of sulfate toxicity in plants.

Sulfur is an essential macronutrient to plants with various functions in metabolism and growth and which has a tight regulation of uptake and assimilation (Droux 2004; Saito 2004; Takahashi et al. 2011; Aghajanzadeh et al. 2016). It is taken up as sulfate from the root medium and reduced via several enzymatic steps to the amino acids cysteine and methionine that are important for structure and function of proteins (Wirtz and Hell 2006). Subsequently, these amino acids are present in a variety of other reduced sulfur compounds such as glutathione which is involved in antioxidative defense and detoxification of heavy metals, as well as the phytosulfokines, some hormones, flavonoids and sulfolipids (Mugford et al. 2009; Takahashi et al. 2011; Park et al. 2012). In addition, cysteine functions as the reduced sulfur donor for the synthesis of glucosinolates as a secondary sulfur compounds in Brassicaceae (Halkier and Gershenzon 2006; Kopriva et al. 2012) which play a role as antioxidants during stress (Fatma et al. 2014), and are putative defense compounds against herbivores (Danner et al. 2015).

Onion (*Allium cepa* L.) was among the earliest cultivated crops and has important functions in food flavor and for phytopharmaceuticals (Griffiths et al. 2002; Mohamed and Aly 2008). *Allium* species such as onion, garlic, leek and chives are considered as plants with a relatively high sulfur requirement for growth, since they contain a variety of secondary sulfur compounds, namely, γ-glutamyl peptides and alliins (*S*-alk(en)yl cysteine sulfoxides (Durenkamp and De Kok 2004). The alliins and their breakdown products (e.g. allicin) are responsible for the odor and taste of *Allium* species, but they may also be involved in plant defense against insects and pathogens also serve as storage compounds for N and S (Lancaster and Boland 1990; Randle and Lancaster 2002).

In the current study, *Allium cepa*, was exposed to NaCl and Na_2SO_4 salinity in order to investigate their toxicity and their impact on sulfur metabolism.

Material and methods

Plant material and growth conditions

Seeds of onion (*Allium cepa*, cv. Rode van Florence, Van der Wal, Hoogeveen, The Netherlands) were germinated in vermiculite in a climate-controlled room. Day and night temperatures were 21 and 18°C (±1°C), respectively, relative humidity was 60–70%. The photoperiod was 14 h at a photon fluence rate of 300 ± 20 µmol m^{-2} s^{-1} (400–700 nm) at plant height, supplied by Philips GreenPower LED (deep red/white 120) production modules. Seedlings were transferred to aerated 25% Hoagland nutrient solution (pH 5.9) consisting of 1.25 mM $Ca(NO_3)_2{\cdot}4H_2O$, 1.25 mM KNO_3, 0.25 mM KH_2PO_4, 0.5 mM $MgSO_4{\cdot}7H_2O$, 11.6 µM H_3BO_3, 2.4 µM $MnCl_2{\cdot}4H_2O$, 0.24 µM $ZnSO_4{\cdot}7H_2O$, 0.08 µM $CuSO_4{\cdot}5H_2O$, 0.13 µM $Na_2MoO_4{\cdot}2H_2O$ and 22.5 µM Fe^{3+}-EDTA in 30-liter containers (20 sets of plants per container; six plants per set) for four days. Subsequently, NaCl and Na_2SO_4 salt were added to the 25% Hoagland nutrition and their concentration were gradually increased during the following three days to 100 and 200 mM NaCl, and 50 and 100 mM Na_2SO_4 and plants were exposed to these salt concentrations for 7 more days (Figure 1). Plants were harvested and root were separated from the shoot, weighed and for the measurement of the water-soluble non-

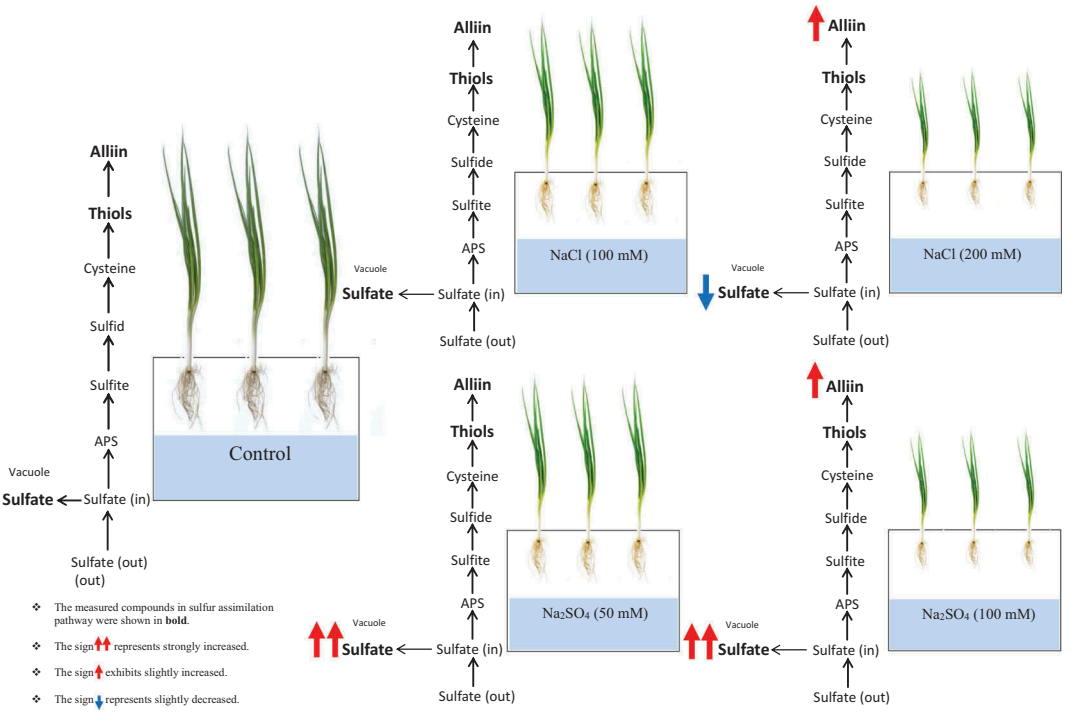

Figure 1. The scheme of *Allium cepa* growth at different concentrations of sodium salts.

protein thiol content, 54 freshly harvested plant materials (3 measurements with 18 plants in each) was used. For analysis of dry matter content 54 plant tissues (3 measurements with 18 plants in each), total sulfur and anions content 54 plant tissues were dried at 80°C for 24 h. For alliin, 54 plant materials (3 measurements with 18 plants in each) were frozen immediately in liquid N_2 and stored at −80°C.

Total sulfur content and anions content

The total sulfur content was analyzed using a modification of the method as described by Jones (1995). To measure total sulfur, 50–150 mg of the dried roots and shoots was saturated with a 50% $Mg(NO_3)_2 \cdot 6H_2O$ (w/v) solution. Then the samples were dried then ashed. The residues were dissolved in 5 or 10 ml of 20% aqua regia (50 ml conc. HNO_3 and 150 ml conc. HCl in 1 l demineralized water) and made up to 50 or 100 ml with demineralized water. One SulphaVer® 4 Reagent Powder Pillow (HACH, Permachem® reagents, Loveland, USA) containing $BaCl_2$ was added to 10 or 25 ml of extract, and the turbidity was measured with a spectrophotometer (HACH DR/400V, Loveland, USA) at 450 nm.

For measurement of the anions content, pulverized dried plant material was incubated for 3–4 h in demineralized water (10 mg ml^{-1}) at 50°C (Tausz et al. 1996; Yang et al. 2006) and centrifuged at 30,000 × *g* for 15 min. Anions were separated by HPLC on an Agilent IonoSpher 5 A anion exchange column (250 × 4.6 mm; Agilent Technologies, Amstelveen, The Netherlands) and sulfate content was determined refractometrically according to Maas et al. (1986). The organic sulfur content was calculated by subtracting the sulfate content from the total sulfur content determined in the same tissue sample.

Water-soluble non-protein thiol and alliin content

Water-soluble non-protein thiols were determined colorimetrically as described by De Kok et al. (1988). Fresh plant material was homogenized in a solution containing 80 mM sulfosalicylic acid, 1 mM EDTA and 0.15% (w/v) ascorbic acid at 0°C (10 ml g^{-1} fresh weight). Oxygen was removed from the solution by aspiration with N_2. The extract was centrifuged at 30,000 × *g* for 15 min (0°C). The total water-soluble non-protein thiol content of the supernatant was determined colorimetrically at 413 nm after reaction with 5, 5′-dithiobis [2-nitrobenzoic acid].

For the analysis of the alliin content frozen and finely ground plant material (70 mg) was extracted with 500 µl 85% (v/v) MeOH. After centrifugation at 13,000 × *g* and 4°C for 10 min. Alliin was analyzed using UHPLC/TQ-MS on an AdvanceTM-UHPLC/EVOQTMElite-TQ-MS instrument (Bruker) equipped with a C-18 reversed phase column (Kinetex 1.7 u XB-C18, 10 cm × 2.1 mm, 1.7 µm particle size, Phenomenex) by using a 0.05% formic acid in water (v/v) (solvent A), 0.05% formic acid in acetonitrile (v/v) (solvent B) gradient at a flow rate of 0.4 ml min^{-1} at 40°C. Alliin quantification was based on external standard curves for alliin (±; *S*-allyl-L-cysteine sulfoxide, Sigma-Adrich, # 74264).

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 atomic emission spectrometry (ICP-AES) analysis. Blanks and standard reference material (NIST 1567, a wheat flour) 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 (1mm) and skimmer (0.4mm cones). Operating parameters were optimized daily using a tune solution containing 1 µg l^{-1} cerium, lithium, tellurium, and yttrium. An internal standard (500 µg l^{-1} germanium) was used to correct for signal drift.

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 (see figures).

Results and discussion

Impact of chloride and sulfate salinity on growth

Exposure of onion to NaCl and Na_2SO_4 salinity resulted in a decreased plant biomass production. The plant biomass was reduced by 40 and 60% at 100 and 200 mM NaCl and by 35 and 55% at 50 at 100 mM Na_2SO_4, respectively (Figure 2). The shoot/root ratio was not significantly affected by NaCl and Na_2SO_4 salinity (Figure 2). Exposure of plants to 200 mM NaCl and 100 mM Na_2SO_4 resulted in 35 and 50% increase in the root dry matter content, respectively (Figure 2). The dry matter content of the shoot was increased by 34 and 56% at 100 and 200 mM NaCl and 24 and 52% at 50 and 100 mM Na_2SO_4, respectively (Figure 2).

The toxicity of NaCl and Na_2SO_4 salinity in onion may be predominantly ascribed to Na^+, since equimolar concentrations of this cation had a similar impact on the root and shoot biomass production and the dry matter content (Figure 2). In a comparable study on the impact of chloride and sulfate salinity on *Brassica* species, in contrast, a higher toxicity of Na_2SO_4 over NaCl was observed due to the higher toxicity of sulfate over chloride at equimolar sodium concentrations (Reich et al. 2015, 2016). Sodium as the most prevalent ions in saline soils has an osmotic

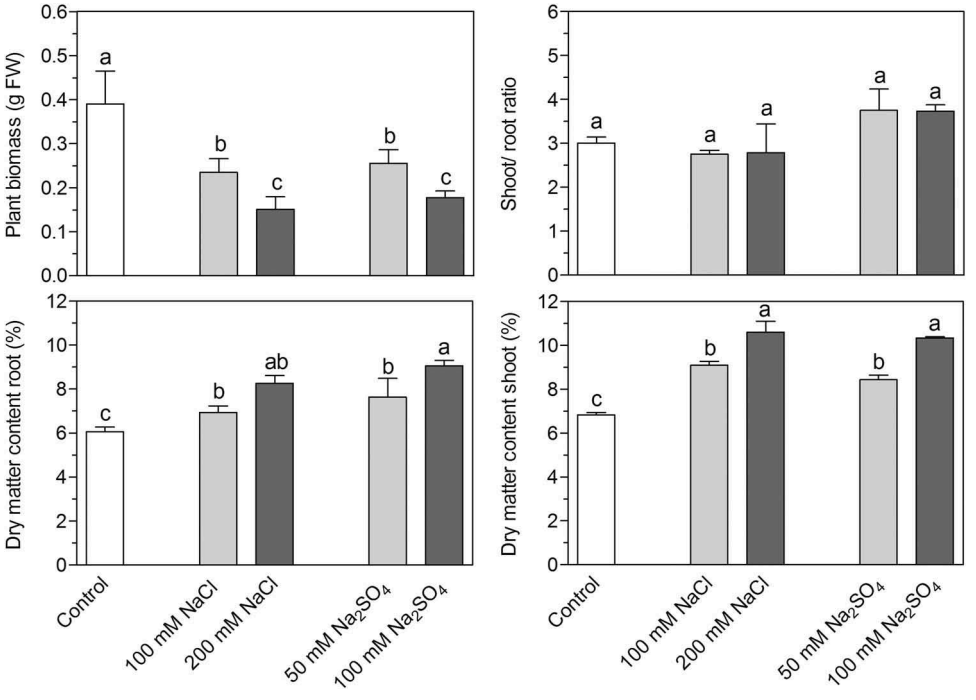

Figure 2. Impact of NaCl and Na_2SO_4 salinity on plant biomass, shoot/root ratio and dry matter content of root and shoot of *Allium cepa*. Different letters indicate significant difference ($p < 0.01$; One-way ANOVA, Tukey's HSD all-pairwise comparisons as a post-hoc test).

and an ionic component of toxicity for plants (Munns and Tester 2008). The osmotic component leads to a water deficit in the plant (Levitt 1980), while sodium taken up in excess causes physiological damage inside of the plant. Both sodium salts entered the onion plant in the current study and caused reductions in growth which might be due to a disturbance of membrane integrity and function, an interference with internal balance of solutes and the uptake of other nutrients and a toxicity for several cellular processes (Greenway and Munns 1980; Marschner 1995; Reginato et al. 2014). High cytosolic concentrations of Na^+ may also disrupt K^+ homeostasis, which is often considered to be the primary cause of its toxicity under saline conditions (Kronzucker and Britto 2011). The observed increase in dry matter content upon chloride and sulfate salinity might be due to a decrease water content caused by the osmotic effect of the salts. Another reason could be the accumulation of inorganic ions in the vacuoles and organic solutes for osmotic adaptation (Reinoso et al. 2005; Xu et al. 2008). Overall, the impact of the different salts on growth parameters were very similar which is in contrast to the results of comparable experiments with *Brassica rapa* (Reich et al. 2016).

Impact of chloride and sulfate salinity on anions and mineral nutrient content

Chloride salinity resulted in a strongly enhanced chloride content of both root and shoot of onion. Exposure of plants to 100 mM NaCl resulted in a 4- and 5-fold increase in the chloride content of the root and shoot, respectively, however, the content was not further increased at 200 mM NaCl (Figure 3). NaCl salinity affected neither the total sulfur nor sulfate content of the root and shoot at 100 mM, but the total sulfur content was decreased in shoots at 200 mM (Figures 3 and 4). A voltage-dependent anion channels mediate transportation of Cl^- and SO_4^{2-} into root cells (Frachisse et al. 1999). Decrease in total sulfur may is due to interactions between sulfate and

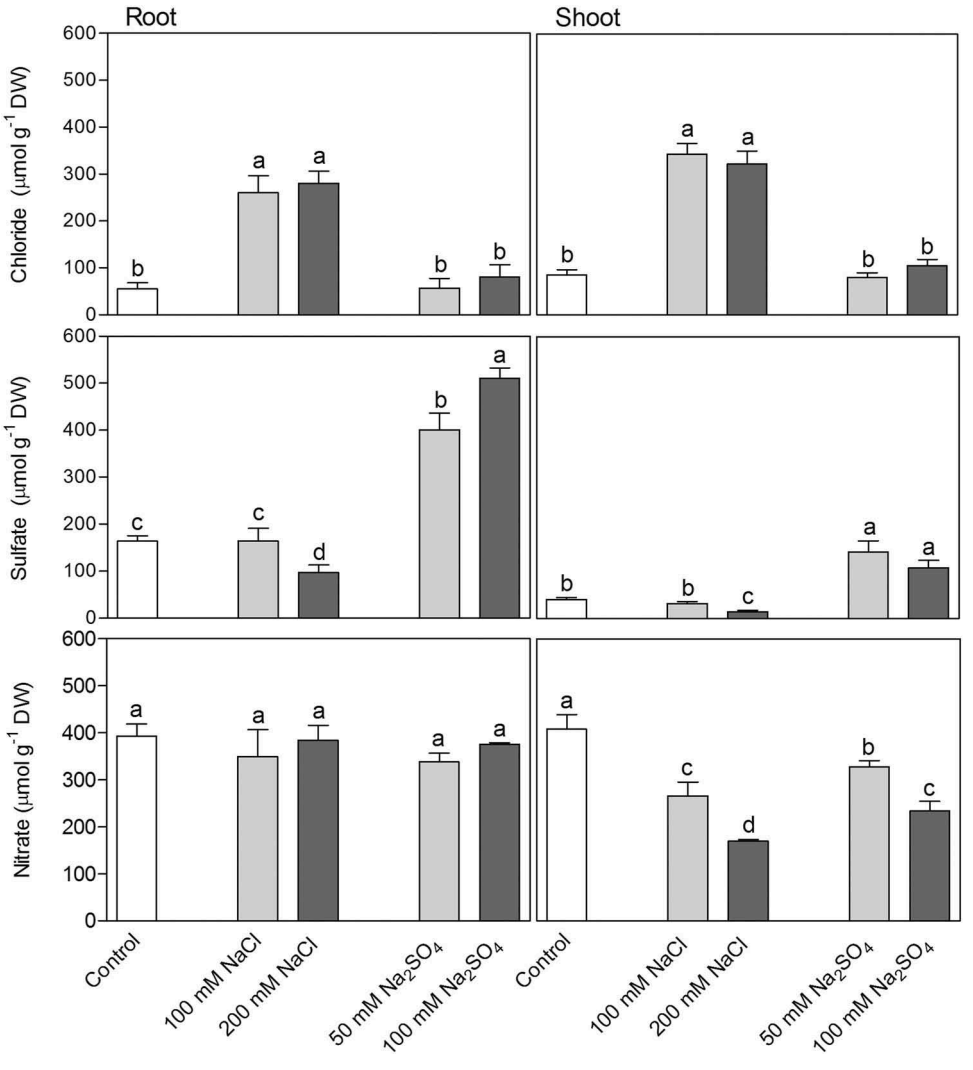

Figure 3. Impact of NaCl and Na_2SO_4 salinity on chloride, sulfate and nitrate content of root and shoot of *Allium cepa*. Different letters indicate significant difference ($p < 0.01$; One-way ANOVA, Tukey's HSD all-pairwise comparisons as a post-hoc test).

chloride which might be caused by the competition for anion adsorption sites in the root cell. In addition, it has been proposed that the rate of diffusion of the monovalent Cl^- ion is greater than the diffusion rate of the bivalent SO_4^{2-} (Beringer et al. 1992).

In onion, the major proportion of sulfur is accumulated as sulfate in both shoot and roots (Figures 3 and 4) which was similar to *Brassica* species where sulfate content accounted for up to 60 to 80% of the total sulfur (Aghajanzadeh et al. 2014; Westerman et al. 2001; Castro et al. 2003; Yang et al. 2006) . Sulfate salinity resulted in an up to 3-fold and 1.5-fold increase in the total sulfur content of the root and shoot, respectively (Figure 4). This increase can mostly be ascribed to an accumulation of sulfate (Figure 3). Exposure of plants to 50 and 100 mM Na_2SO_4 resulted in a 2.5 and 3-fold increase in sulfate content of the root and 2 and 2.5-fold increase of its content of the shoot, respectively (Figure 3). The sulfate content of the root was 5-fold higher than that of the shoot, which may indicate that the translocation of sulfate from the root to the shoot of onion was restricted upon sulfate salinity. This differed from observations in *Brassica*, where upon sulfate

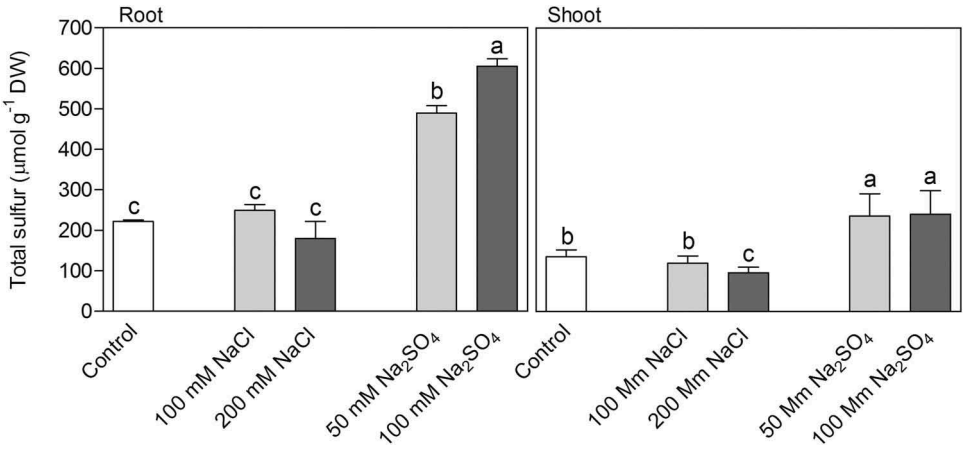

Figure 4. Impact of NaCl and Na_2SO_4 salinity on mineral nutrient composition of root and shoot of *Allium cepa*. Different letters indicate significant difference ($p < 0.01$; One-way ANOVA, Tukey's HSD all-pairwise comparisons as a post-hoc test).

salinity the highest content of sulfate was observed in the shoot (Reich et al. 2015, 2016). The chloride content of both root and shoot was not affected by sulfate salinity (Figure 3).

Here, both NaCl and Na_2SO_4 salinity resulted in a strongly reduced nitrate content of the shoot, whereas that of the root remained unaffected. Upon exposure to NaCl salinity the nitrate content of the shoot was reduced by 35 and 60% at 100 and 200 mM, respectively, whereas in Na_2SO_4 salinity its content was reduced by 9 and 43% at 50 and 100 mM, respectively (Figure 3). Several investigations showed a reduced nitrate uptake of plants upon salinity stress (Rao and Gnaham 1990; Gouia et al. 1994). Sodium salinity resulted in a strongly enhanced sodium content of both root and shoot of onion (Figure 4). In the root, exposure to 200 mM NaCl and 100 mM Na_2SO_4 led to a slightly higher increase of sodium content (Figure 4). Exposure to both sodium salts strongly decreased potassium and calcium content in shoot and roots (Figure 4). Magnesium was similarly decreased by NaCl and Na_2SO_4 in shoot. Exposure to both sodium salts significantly decreased manganese content in roots (Figure 4). Molybdenum content was decreased significantly in roots and shoot by sulfate salt. Phosphorus, copper, iron and zinc contents remained unaffected by both salts in shoot and roots (Figure 4).

The contents of potassium and calcium were further decreased than the other elements in shoot and roots by both sodium salts (Figure 4). Sodium at high concentrations can interfere with potassium and calcium uptake and competitively inhibit their influx (Botella et al. 1997; Loupassaki et al. 2002). Potassium as an abundant cation in plants is involved in generating turgor-pressure which lead to cell expansion (Yang et al. 2014). Likewise, calcium is considered as an essential nutrient for growth, development and acts as an important component of cell wall integrity and as a major secondary-messenger molecule in plants (Abdul Kader and Lindberg 2010). The reduction of tissue concentration of these ions by salt stress is therefore likely to be a main reason for reduction in growth. Overall, the effects of the two different salts on nutrient contents where quite similar. One explanation for the relatively high tolerance of onion to increased tissue sulfate concentrations (compared to other species, such as *Brassica rapa*; Reich et al. 2016) could be a higher capacity of the vacuoles for sulfate storage. Safely stored in the vacuole, sulfate cannot unfold its toxic effects in the cytosol. The relatively large vacuoles of onion plants could therefore be a reason for their comparably high tolerance to sulfate and salts in general.

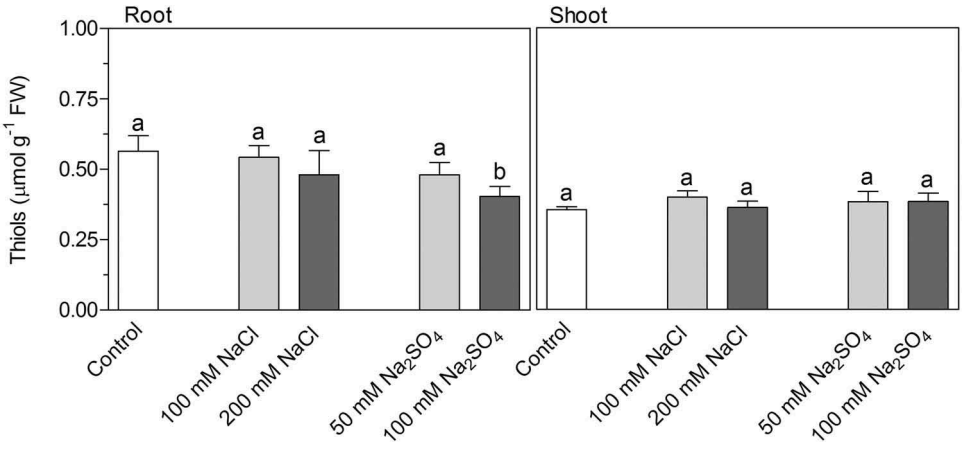

Figure 5. Impact of NaCl and Na_2SO_4 salinity on water-soluble non-protein thiols content of root and shoot of *Allium cepa*. Different letters indicate significant difference ($p < 0.01$; One-way ANOVA, Tukey's HSD all-pairwise comparisons as a post-hoc test).

Impact of chloride and sulfate salinity on water-soluble non-protein thiol content

The water-soluble non-protein thiol content (mainly glutathione) of root and shoot of onion was not affected, neither by NaCl nor by Na_2SO_4 salinity (Figure 5). Even the enhanced sulfate levels in the root and shoot upon sulfate salinity had no impact on the water-soluble non-protein thiol content (Figure 5).

Sulfur is essential for synthesis of the cysteine, methionine and various organic sulfur compounds, viz. glutathione, sulfolipids and a variety of secondary sulfur compounds (alliins, glucosinolates) (Leustek et al. 2000; Durenkamp and De Kok 2004; Kopriva 2006). Glutathione is considered as an essential component of the cellular anti-oxidative defense system, which limits reactive oxygen species upon exposure of plants to biotic and abiotic stresses (Noctor and Foyer 1998). It is often presumed that enhancement of the glutathione level might have adaptive value in stress tolerance of plants. However, it is evident that glutathione content of plants is often not affected or it may even decrease upon stress exposure (Tausz et al. 2004). The fact that the thiol content was not effected in onion, although the sulfate content was strongly increased suggests a tight control of sulfate reduction. This strict regulation could be another explanation for the higher tolerance of onion to an excess of sulfate compared to *Brassica rapa* (Reich et al. 2016), because the sulfur reduction pathway can also lead to an accumulation of toxic compounds such as H_2S (Rennenberg 1984).

Impact of chloride and sulfate salinity on alliin content

The alliin content in the shoot of onion was almost 4-fold higher than in root (Figure 6). It has been observed that alliins are apparently predominantly synthesized in the shoot (Lancaster et al. 1986). The alliin content of the root was only enhanced at 200 mM NaCl and 100 mM Na_2SO_4, and that of shoot was only significantly increased at 100 mM Na_2SO_4 (Figure 6). An enhanced sulfate level in the shoot upon exposure of onion plants to SO_2 did also not result in an accumulation of secondary sulfur compounds in onion (Durenkamp et al. 2005). Only if the regulation of the sulfate reduction pathway (which is localized in the chloroplast) was bypassed in onion plants when excessive reduced sulfur was supplied foliarly upon H_2S fumigation, there was a substantial accumulation of secondary sulfur compounds in the shoot (Durenkamp and De Kok 2002, 2003, 2004). H_2S fumigation also resulted in a substantial increase in the content of water-soluble non-protein thiols, viz. cysteine and glutathione, in the shoot. These thiol compounds are precursors for the synthesis of secondary sulfur compounds e.g. alliins in onion (Lawson 1996; Durenkamp and De

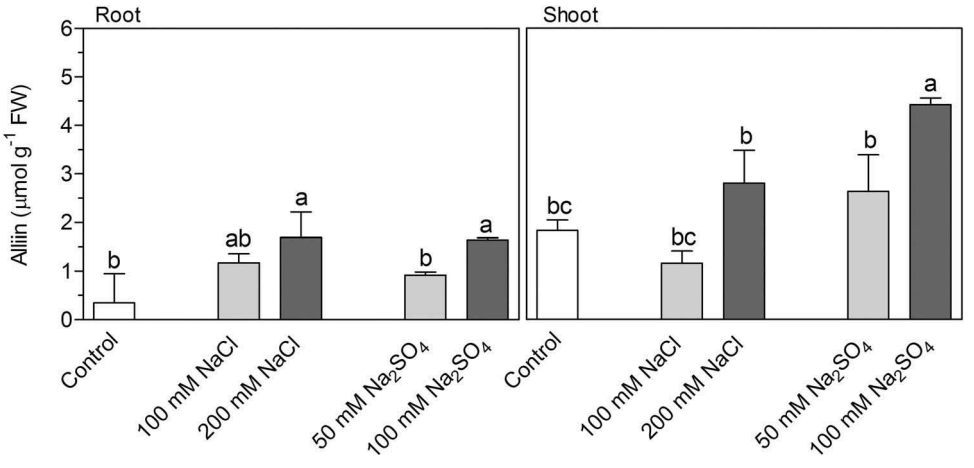

Figure 6. Impact of NaCl and Na_2SO_4 salinity on alliin content of root and shoot of *Allium cepa*. Different letters indicate significant difference ($p < 0.01$; One-way ANOVA, Tukey's HSD all-pairwise comparisons as a post-hoc test).

Kok 2002, 2003, 2004; Randle and Lancaster 2002; Durenkamp et al. 2005). Little impact of sodium salts on alliin content may reinforce strict regulation of sulfur metabolism in onion plant.

Conclusion

In current study, a similar toxicity of chloride and sulfate salts on onion has been shown when exposed to equimolar concentrations of Na^+. Furthermore, it was concluded that both salts affected the uptake and distribution of sulfate in the plant, though hardy affected sulfur reduction.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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