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Research Article

Differential Effects of Excess Potassium and Sodium on Plant Growth and Betaine Accumulation in Sugar Beet

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Abstract

The effects of long-term NaCl and KCl treatment on plant growth and betaine accumulation were investigated in sugar beet. Leaf fresh weight of the plants treated with 300 mM KCl and NaCl for 15 days were declined when compared with the control plants. Photosynthetic activity, chlorophyll content and magnesium content were decreased in the plants treated with 300 mM KCl, whereas these values were essentially maintained in the control and 300 mM NaCl treated plants. K^+ content in 300 mM KCl treated plants were significantly higher than the Na^+ content in the 300 mM NaCl treated plants. These effects were more severe in developing leaves than the mature leaves. Betaine and choline monooxygenase (CMO) accumulation levels were increased in plants exposed to 300 mM KCl and NaCl treatment, with a higher increase in NaCl treated plants. The betaine/Na^+ ratio increased during the 300 mM NaCl treatment but remained constant during the KCl treatment. Results indicate the presence of a better adaptive system to high NaCl than KCl in developing leaves of sugar beet.

Keywords: Betaine; Potassium; Salt Stress; Sodium; Sugar Beet

Introduction

Salt stress is a major factor that decreases crops yield, especially in semi-arid and arid areas (Munns and Tester 2008). World over, attempts are being made to explore and grow salt-tolerant species with potential economic and ecological value in salinized soils (Rozema and Flowers 2008, Flowers and Colmer 2015). Sugar beet is an economically important, salt-tolerant and widely distributed plant, which can grow under 300 mMNaCl treatment (Yamada et al. 2009). It accumulates glycine betaine as an osmoprotectant under NaCl stress and is an excellent model plant to study the role of betaine in regulating salt stress. In plants, betaine is synthesized via two steps of choline oxidation: choline→betainealdehyde→betaine (Rathinasabapathi et al.1997, Takabe et al. 2006). These steps are catalyzed by choline monooxygenase (CMO) and betaine aldehyde dehydrogenase (BADH), respectively, wherein the first step is rate-limiting.

Potassium (K) is ubiquitous in all higher plants and plays a vital role in a wide range of biochemical and biophysical processes. Typical K^+ concentration in growth medium is in the range of 1-2 mM (Cakmak 2005). However, most plants accumulate K^+ too much higher concentrations than required for normal metabolism (Leigh and Wyn Jones 1984), which is termed as luxury uptake (Winkler and Zotz 2010). In plants, the total K^+ is located in the cell vacuole, while a small proportion is also localized in the cytosol (Leigh and Wyn Jones 1984). In the past, extensive studies have demonstrated physiological and biochemical implications of K^+ deficiency in plants (Cakmak 2005, Ashley et al. 2006). Notwithstanding, a few studies have reported the effect of high K^+

levels in plant system (Ramos et al. 2004, Yao et al. 2010). It has been shown that 300 mM KCl for *Chenopodium album* (Yao et al. 2010) and 350-500 mM KCl for *Atriplex nummularia* (Ramos et al. 2004) were more toxic than the treatments with the same concentrations of NaCl. In agricultural land, 300 mM KCl might be too high. But, saline water, containing >400,000 ppm total salts, has been reported (Egan and Ungar 1998). In addition, over-fertilization of soils used for agricultural and horticultural purposes is a growing environmental concern. Over-use of compost, manure or other organic materials can cause adverse effects on plant growth and the environmental contamination in drinking water. Considering the remediation of soil by halophyte plants and sea water farming, high KCl treatment would be interesting. In addition, it is uncertain whether high K^+ can induce the accumulation of organic osmoprotectant.

With above background, we conducted a series of experiments to investigate the differential effects of high K^+ and Na^+ on the plant growth and betaine biosynthesis in sugar beet.

Materials and methods

Seed germination, plant materials and salt treatments

Seeds of sugar beet (*Beta vulgaris* L., cv. NK-210mm-0) were germinated on paper towels moistened with distilled water under dark conditions at 25°C. After germination, seedlings were transplanted into plastic pots (100 mL) containing sterile vermiculite. Plants were grown with half strength Murashige and Skoog solutions (½ MS) in a growth chamber set at 16 h light (25°C, 100 µE m^{-2} s^{-1})/8 h dark (20°C) cycle and 60% relative humidity (Yamada et al. 2009). When seedlings were 3 weeks old, these were transferred to the growth medium containing various concentrations of NaCl (0, 50, 100, 200, 300 and 400 mM) and KCl (0, 10, 50, 100, 200, 300 and 400 mM). The plants were allowed to grow further for next 30 days. One hundred millilitres of ½ MS solution containing various concentrations of the salts were applied to the culture medium at 2 days interval. True leaves of first, second and third pairs, designated as mature (L1), developing (L2) and young (L3) leaves, were used for further biochemical estimations. Plant cultivation was carried out at least three biological replicates.

Determination of leaf succulence and water content

The degree of leaf succulence was calculated as the ratio of initial fresh weight to the dry weight (Yao et al. 2010). Four leaf discs (2 discs from tip region, 2 discs from basal region) per leaf were cut with a cork borer (ϕ=1 cm). Leaf discs were then oven-dried for at least 5 days at 55°C in a glass Petri dish. Dry weight was determined by an analytical weighing balance (Shimadzu Co, Japan).

Measurement of total chlorophyll content and photosynthetic activity

For the measurement of chlorophyll content, 50 mg leaves were homogenized in 1.8 mL of 100% acetone. After centrifugation at 20,000 × g at 4°C rotor temperature for 15 min, the absorbance of supernatant was read at 646.6, 663.6 and 750 nm using a spectrophotometer (BioSpec-1600, Shimadzu) (Yamada et al. 2015). Photosystem II activity (PSII activity) was measured by a portable Mini PAM (Pulse Amplitude Modulation) fluorometer (PAM-2000, Walz, Germany) at 25°C using 30 min dark adapted leaves and data acquisition software (DA-2000, Walz) (Hoshida et al. 2000).

Measurement of ions and osmotic concentration

Fresh leaves (200 mg) were homogenized in eppendorf tubes 25°C, and centrifuged at 20,000 × g at 4°C rotor temperature for 15 min. Osmotic concentration in aliquots (10 µL) of the supernatant was analyzed by a vapor pressure osmometer (model 5520; Wescor, Logan, Utah, USA). Cellular ions were determined using Shimadzu Personal Ion Analyzer PIA-1000 (Shimadzu, Japan) as described previously (Waditee et al. 2007). For Na^+ and K^+ extraction, 100 mg fresh leaves were homogenized in 1 mL of sterile distilled water. For Mg^{2+} extraction, fresh leaves were dried at 60°C for 1 week. The dried leaves were ground in a mortar and 20 mg of powdered material was extracted with 13 M HNO_3 for 1 h. After air-drying, it was re-extracted with sterile distilled water. Then, the contents were centrifuged at 20,000 × g at 4°C for 15 min and the supernatant was used for the measurement of Mg^{2+}.

Analysis of betaine

Betaine was extracted as described by Waditee et al. (2005). The plant tissue (100 mg FW) was extracted in an extraction buffer (methanol:chloroform: water = 12:5:1) and centrifuged at 20,000 × g at 4°C for 15 min. The supernatant was re-extracted with a mixture containing 25% (v/v) chloroform and 37.5% (v/v) water and again centrifuged at 20,000 × g for 15 min. The supernatant, thus obtained, was dried and dissolved in 100 µL water. Betaine was measured with the time of flight mass spectroscopy (KOMPACT MALDI TOF-MS, Shimadzu/Kratos) using d_{11}-betaine as the internal standard.

CMO protein expression and statistical analysis

SDS-PAGE and immune-blot analysis were carried out according to the standard protocol as described by Waditee et al. (2005). Protein contents were determined by Bradford method (Waditee et al. 2007). All values are presented as mean±standard errors of three replicates.

Results

Effects of KCl and NaCl on the growth of sugar beet

The inclusion of 10-50 mM NaCl or KCl in the growth medium enhanced the growth, but at higher concentrations (>300 mM), the growth was inhibited, compared with the control plants (Fig. 1). The enhanced growth of sugar beet at lower concentrations of NaCl has also been reported previously (Russell et al. 1998, Liu et al. 2008, Wu et al. 2013). The growth medium of the control

essentially did not contain Na^+, but contains about 1 mM K^+ due to a major salt, KNO_3. After 30 days, it has been found that plants treated with 400 mM NaCl survived, whereas those treated with ≥300 mM KCl were almost dead, indicating that growth inhibition by high KCl concentrations was more severe than that of NaCl.

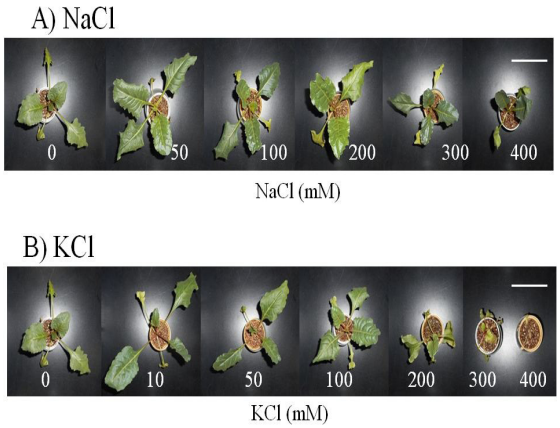

Figure 1: Photographs of sugar beet after KCl and NaCl treatments. Sugar beet was allowed to grow for 3 weeks before treating with various concentrations of NaCl and KCl. The plants were observed and photographed for next 30 days. The bar represents 10 cm.

In the following experiment, the concentration of KCl and NaCl was fixed to 300 mM and the time course of fresh weight and succulence degree of L1 (mature leaf) and L2 (developing leaf) of *B. vulgaris* were determined. Fresh weight of L1and L2 leaves was decreased when treated with 300 mM NaCl or KCl as compared to control (Fig. 2A). The degree of succulence of L1 leaves increased with increasing the time period of treatment, irrespective of the mode of treatment (Fig. 2B). In contrast, the degree of succulence of L2 leaves treated with 300 mM KCl increased significantly after 10 and 15 days as compared to control and NaCl treated plants. In control plants, leaf area of L1 remained constant whereas leaf area of L2 increased with increasing time period (data not shown). Leaf area of the plants treated with 300 mM of KCl or NaCl was lower than that of the control plants, and the decrease was more severe in KCl treated plants than in NaCl treated plants (data not shown).

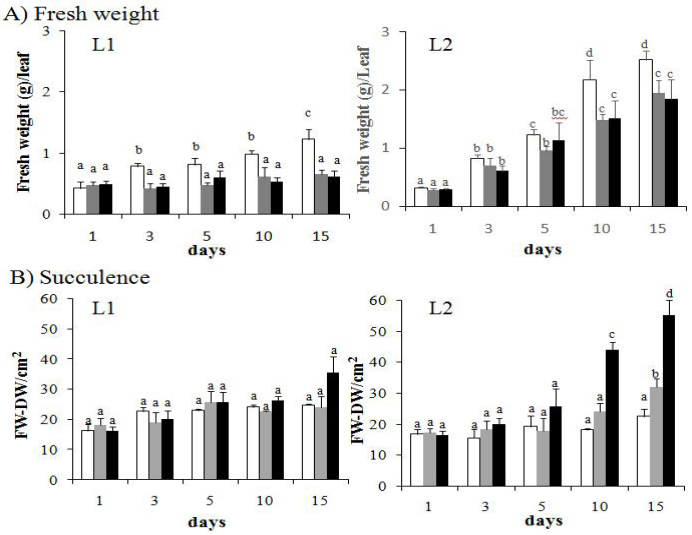

Figure 2: Time course of leaf fresh weight and succulence degree of KCl and NaCl treated *B. vulgaris*. After treating the 3 weeks old seedlings of sugar beet with 300 mM NaCl or 300 mM KCl, fresh weight and succulence degree of its mature leaf (L1) and developing leaf (L2) were measured at different time intervals. The data are presented as mean±SE (n=3). The letters above the graph represent significant difference (P≤0.05) between WT type (white bar), NaCl treated plants (gray bar) and KCl treated plants (dark bar).

High KCl decreased chlorophyll content and photosynthetic activity

The leaf color of L2 leaves treated with 300 mM NaCl was changed to yellowish green (data not shown), indicating a decrease in chlorophyll content. However, when measured, the total chlorophyll content in L1 and L2 leaves of NaCl treated plants were similar to the control leaves (Figs. 3A and 3B). In contrast, the 300 mM KCl treatment reduced the chlorophyll content up to 50% after 10 and 15 days in L2 leaves (Fig. 3C), The maximum quantum yield of PSII (F_v/F_m) was decreased after 10 days treatment with 300 mM KCl, whereas it was unaffected by the NaCl treatment (Fig. 3C).

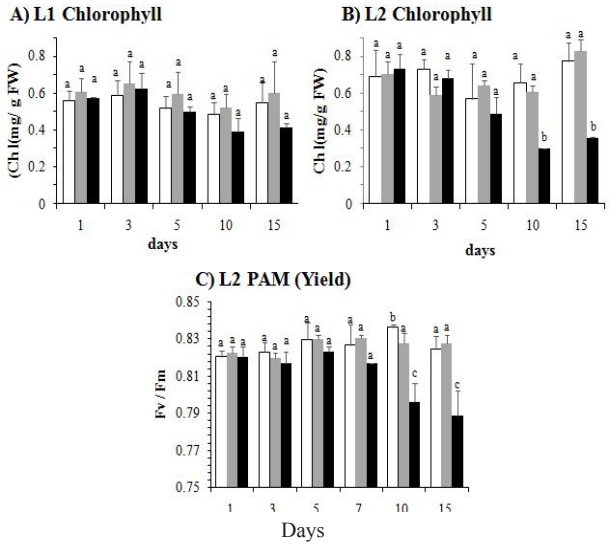

Figure 3: Time course of chlorophyll content and quantum yield of PSII photochemistry (F_v/F_m) in leaves after KCl and NaCl treatment. After treating the 3 weeks old seedlings of sugar beet with 300 mM NaCl or 300 mM KCl, chlorophyll content and quantum yield of PSII of itsmature leaf (L1) and developing leaf (L2) were measured at different time intervals. A) L1 chlorophyll content, B) L2 chlorophyll content, C) L2 PSII activity. The different letters above the bars indicate significance difference between treatments at P≤0.05 between WT type (white bar), NaCl treated plants (gray bar) and KCl treated plants (dark bar). Values are expressed as mean±SE (n=3).

Changes in cation content in response to salt stress

The content of K^+ increased with increasing the time period of exposure in both L1 and L2 leaves (Fig. 4A). After 15 days, the amount of K^+ in L1 and L2 leaves treated with 300 mM KCl was 371.2 and 674.8 µmol/gFW, respectively, thereby indicating that K^+ prefers to accumulate in developing leaves. A similar trend of changes was observed in NaCl treated plants (Fig. 4B); however, the amount of Na^+ in L1 and L2 leaves was 170.8 and 258.5 µmol/gFW, respectively, which was 50% lower than K^+.

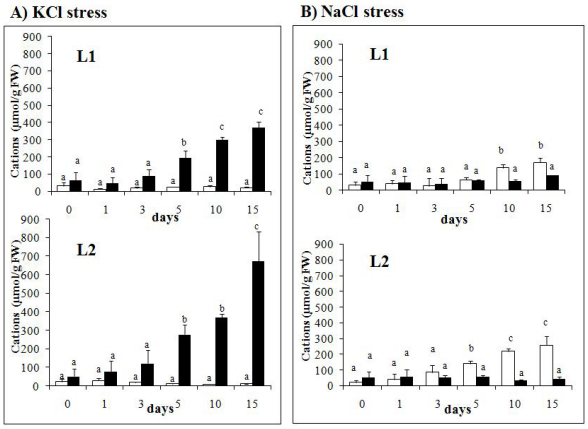

Figure 4: Time course of cation contents in leaves of KCl and NaCl treated *B. vulgaris*. After treating the 3 weeks old seedlings of sugar beet with 300 mM NaCl or 300 mM KCl, Na^+ (white bar) and K^+ (dark bar) contents were measured in its mature leaf (L1) and developing leaf (L2) at different time intervals. A) KCl stress, B) NaCl stress. The different letters above the bars indicate significant difference between means at P≤ 0.05. Values are expressed as mean±SE (n=3).

The osmolarity of L1 and L2 leaves in NaCl and KCl treated plants increased with increasing time period, whereas that in the control plants was constant. At 15 days after treatment, the osmolarity of L1 and L2 in KCl treated plants was 3.9- and 4.3- folds higher than control plants, respectively, whereas in NaCl treated plants it was 2.3- and 2.3-fold higher than in control plants (data not shown).

After 45 days of treatment with 300 mM KCl, the precipitation of salt was observed which was confirmed to be KCl based on the ionic analysis of the precipitate (data not shown). In contrast, no salt precipitation was observed in *B. vulgaris* plants treated with 300 mM NaCl, and the plants grew continuously. Since the interactive effects between excess K^+ and Mg^{2+} deficiency has been reported earlier (Pujos and Morard 1997, Farhat et al. 2013), we measured the Mg^{2+} content in L1 and L2 leaves after 5 and 15 days of salt treatments (Fig. 5). Mg^{2+} contents in L1 leaves did not show a significant change compared to the control plants. However, the

Mg^{2+} contents in L2 leaves of NaCl and KCl treated plants was reduced compared to the control plants. This reduction was more significant in the KCl treated plants after 15 days of exposure.

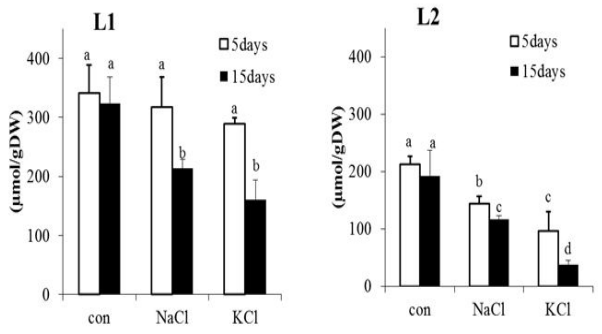

Figure 5: Mg^{2+} content in *B. vulgaris* under KCl and NaCl treatment. After treating the 3 weeks old seedlings of sugar beet with 300 mM NaCl or 300 mM KCl, Mg^{2+} content was determined at different time intervals, 5 and 15 days. Different letters above the bars indicate significantly different means at P≤0.05. Values are expressed as mean±SE (n = 3).

Excess of KCl and NaCl induce greater accumulation of betaine

Betaine content increased with increasing the incubation time of NaCl in L1, L2 and L3 leaves, and the accumulation was the highest in L3 followed by L2 and L1 (Fig. 6A). These results are in conformity with the earlier findings of Yamada et al. (2009). In this study, we found that betaine content was increased under high KCl (300 mM) conditions (Fig. 6A). Betaine content was increased with increasing the incubation time of KCl in L1, L2 and L3 leaves, and the accumulation was the highest in L3 followed by L2 and L1. These results were similar to that by the NaCl treatment, but the betaine accumulation levels were slightly lower than that of the 300 mM NaCl treated plants.

Upon western blotting, no CMO bands were detected in the control plants, whereas these were detected in the L1 and L2 leaves of KCl and NaCl treated plants (Fig. 6B). However, the band intensity of L1 leaves was higher than that of the L2 leaves, and the band intensity of KCl treated plants was slightly less than that of NaCl treated plants. The ratio of betaine/Na^+ was increased during the treatment, whereas the ratio of betaine/K^+ remained nearly constant (Fig. 6C).

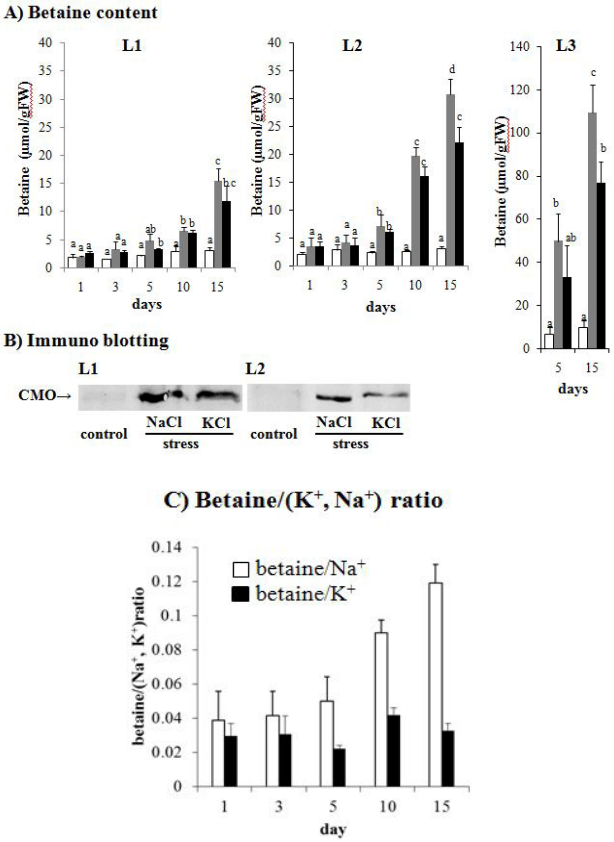

Figure 6: Changes in CMO and betaine content in leaves of *B. vulgaris* after KCl and NaCl treatments. A) Time course of betaine content in leaves after KCl or NaCl treatment. Three week old seedlings of sugar beet were grown in the presence of 300 mM KCl or 300 mM NaCl for 1, 3, 5, 10, and 15 days. Then, betaine was extracted from the leaves and measured as described in materials and methods. Within each figure panel, different letters above the bars indicate significantly different means at P≤0.05. Values are expressed as mean±SE (n=3). B) Immunoblotting of CMO. Three-week old seedlings treated with 300 mM KCl or 300 mM NaCl for 15 days were harvested. The proteins were extracted from leaves and anal-

ysed SDS-PAGE. BvCMO was detected by immuno-blot analysis using the antibodies raised against spinach CMO. C) Changes of betaine/cation ratio. The betaine/K^+ and betaine/Na^+ ratios were calculated based on the data of (A) and (B) and their time course changes were represented.

Discussion

The present data clearly shows that 300 mM KCl concentration resulted in the significant accumulation of K^+, i.e. 371 and 675 µmol/g FW in mature (L1) and developing (L2) leaves of *B. vulgaris*, respectively which corresponds to 2.2 to 2.6-fold higher accumulation than that of Na^+. Previously, Ramos et al. (2004) reported 1.3-fold higher accumulation of K^+ than Na^+ in the leaves of *Atriplex nummularia* at 350 mM KCl. This indicates that sugar beet accumulates more K^+ and lesser Na^+ than *A. nummularia*. Using 10 mM of KCl or NaCl, Reimann and Breckle (1993) demonstrated that K^+ uptake in *C. album* and *C. schraderianum* is much higher than that of Na^+. The accumulation levels of Na^+ in *C. album* and *C. schraderianum* were relatively low compared to halophilic chenopod *Atriplex prostrata* (Reimann and Breckle 1993). The present study suggests that the selectivity for Na^+ and K^+ uptake in sugar beet is similar to that of *C. album* and *C. schraderianum* than the halophilic relative *Atriplex* spp.

It was found that betaine synthesis was enhanced under high K^+ conditions (Figs. 6A and 6B). The betaine/K^+ ratio was almost constant (0.04) during the treatment (Fig. 6C) regardless of the significant increase of K^+ in the 300 mM KCl treated plants (Fig. 4A). In contrast, the betaine/Na^+ ratio increased with increasing the time period of 300 mM NaCl treatment, and was found to be >0.1 after 15 days of treatment (Fig. 6C). If we assume that vacuole occupy 90% of cell volume and the preferred location of Na^+ accumulation is vacuole, whereas betaine is localized in cytosol, then betaine/Na^+ ratio of 0.1 would satisfy the osmotic balance between vacuole and cytosol. By contrast, K^+ is localized in both vacuole and cytosol. Then, the accumulation of betaine in cytosol under the 300 mM KCl treatment would be lower than that by the 300 mM NaCl treatment. Lower betaine contents in the 300 mM KCl treated plants were coincided with the lower accumulation levels of CMO protein in the 300 mM KCl treated plants (Fig. 6B). Another possibility is the different sensitivity of CMO promoter to Na^+ and K^+ ions. The reason behind low betaine levels in 300 mM KCl treated plants is yet to be clarified.

Sugar beet survived even when it is subjected to high NaCl concentrations (up to 400 mM), but the 300 mM KCl treatment significantly inhibited plant growth (Fig. 1). Morphological changes, such as chlorosis (yellow-green leaf), were evidently found in plants grown under 300 mM KCl treatment, but no major changes were observed under 300 mM NaCl treatment (Fig. 3), leading to increase succulence (Fig. 2B). The leaf chlorophyll contents remained unaffected after exposing to 300 mM NaCl concentration, but decreased significantly when exposed to 300 mM KCl concentration. These observations are paralleled by similar results reported for *C. album* by Yao et al. (2010). Induction of high levels of reactive oxygen species (ROS) by high KCl treatment was demonstrated in *C. album* (Yao et al. 2010).

Mg is an important divalent cation within living cells and has a high affinity for water and forms a stable hydrate (Guo et al. 2016). In the cell, Mg exists either in the form of ions or is bound to the substances like ATP and RNA. Mg^{2+} content in the plants treated with 300 mM KCl concentrations was drastically reduced (Fig. 5), thereby indicating that high K^+ inhibits Mg^{2+} uptake in plant. It can be assumed that K^+ and Mg^{2+} may possibly compete for the channel system or transporter (Winkler and Zotz 2010).

Previously, it has been reported that Na is excluded by the root, and K is selectively taken up at high rates by the leaves of *C. album* (Reimann and Breckle 1993). The results of the present study are in conformity with those observed in *C. album*. Due to higher accumulation of K in developing leaves than in mature leaves (Fig. 4), the damage by high K was more severe in developing leaves. In the field, high salinity is mostly induced by high NaCl concentrations. However, other types of salts such as KCl and K_2SO_4 also occur naturally in the soils from different regions of the world (Egan and Ungar 1998). Heavy application of K fertilizers may also result in increased salinity of the soils (Cakmak 2013). High K uptake by the plants may result in Mg deficiency in grazing animals, which may induce disorders in the ruminant (Cakmak 2013). These facts indicate the importance to study the effects of high K concentrations on plants. In this study, we showed that the biosynthesis and accumulation of betaine in sugar beet were induced by slightly higher concentrations of KCl, but it was not sufficient for sugar beet to survive under severe KCl conditions.

Conflict of interest

The authors have no conflict of interest to declare.

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