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Physiological responses of white Swiss chard (*Beta vulgaris* L. subsp. *cicla*) to saline and alkaline stresses

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Abstract

Swiss chard (*Beta vulgaris* L. subsp. *cicla*) is widely cultivated as a green vegetable. We compared the effects of saline and alkaline conditions on growth, relative water content (RWC), chlorophyll (Chl) *a* and *b*, net photosynthetic rate (P_n), ionic balance, glycinebetaine (GB) and proline to understand the physiological adaptive mechanisms of Swiss chard to alkaline conditions. Although RWC was higher under alkaline conditions, the reduction of plant growth was greater (8.7% and 41.6% at 50 and 100 mM) than that under saline conditions (1.7% and 26.7% under 50 and 100 mM), indicating that Swiss chard was more sensitive to alkaline stress and the growth reduction might have been caused by high pH, CO_3^{2-} and HCO_3^- toxicity rather than water stress in Swiss chard under alkaline conditions. The significant reductions of Chl *a* (27% and 46.7%), Chl *b* (74.4% and 83%), P_n (59.8% and 91.6%), water use efficiency (33.2% and 83.9%) and K^+ content (82.0% and 83.8%), and severe ionic imbalance (Na^+/K^+: 36.9- and 50.2-fold compared with that of the control) resulted in the serious inhibition of Swiss chard growth under 50 and 100 mM alkaline conditions. GB and proline were increased under both conditions. GB content was significantly higher under 50 mM saline conditions than under 50 mM alkaline conditions, but this was not found for proline content, suggesting that the contribution of proline to alkaline tolerance was less important and GB plays an important protective role in the mechanism of alkaline tolerance for Swiss chard.

Keywords: Alkaline stress; *Beta vulgaris*; Glycinebetaine; K^+; Proline; Saline stress; Na^+
Abbreviations: Chl *a* and *b*- chlorophyll *a* and *b*; C_i- intracellular CO_2 concentration; GB- glycinebetaine; g_s- stomatal conductance; LA- leaf area; N- nitrogen; P_n- net photosynthetic rate; RGR- relative growth rate; RWC- relative water content; T_r- transpiration rate; WUE- water use efficiency.

Introduction

Plant production is inhibited in response to different environmental stresses such as salinity, drought, extreme temperature and high light intensity. Salinity is a widespread adverse environmental problem globally. Although the world's land surface constitutes about 13.2×10^9 hectares, only 1.5×10^9 hectares (about 11.4% of the total areas) are currently cultivated. Of this cultivated land, about 0.34×10^9 hectares (23%) are saline and another 0.56×10^9 hectares (37%) are sodic (Tanji, 1990). Therefore, it is very important to study plant nutritional physiology under saline conditions and to improve plant salt tolerance for the improvement of crop production in saline- and alkaline-prone areas.

It is well known that salt stress induces various biochemical and physiological responses in plants and affects almost all plant functions, including photosynthesis, growth and development (Nemoto and Sasakuma, 2002). The main consequences of plant exposure to salt stress are water deficit and ion excess (Greenway and Munns, 1980), which causes osmotic stress and ion imbalance (Hasegawa et al., 2000). Stomatal closure due to water deficit leads to the formation of reactive oxygen species (ROS), which are highly toxic and can damage many important cellular components (Fu and Huang, 2001; Bor et al., 2003**)**. The effects of hyperosmotic and hyperionic stress can limit plant growth or result in plant death. However, a high level of injury could be avoided by the accumulation of osmotic adjustment factors such as soluble carbohydrates, proline and GB, and/or lowering of the toxic concentration of ions in the cytoplasm by the restriction of Na^+ influx or its sequestration into the vacuole and/or its extrusion (Hasegawa et al., 2000; Neto et al., 2004; Farooq and Azam, 2006; Ghoulam et al., 2002). GB and proline are two major osmotic adjustment factors that accumulate in a variety of plant species in response to environmental stresses such as drought, salinity, extreme temperatures, UV radiation and heavy metals (Ashraf and Foolad, 2007). Furthermore, GB is abundant mainly in chloroplasts and protects membrane structures (Crowe et al., 1992), protects cytoplasm and chloroplasts from Na^+ damage (Rahman et al., 2002), maintains photosynthetic efficiency (Ashraf and Foolad, 2007) and can scavenge reactive oxygen species. The level of accumulated GB is correlated with the degree of salt tolerance (Saneoka et al., 1995; Sakamoto and Murata, 2002). Proline increases proportionally faster than other amino acids under water stress (Bates et al., 1973), and it was found that exogenous proline improved the growth of salt-stressed tobacco cell cultures (Hoque et al., 2007), and may improve the adaptation of *Pancratium maritimum* L. to salt stress (Khedr et al., 2003). Although proline accumulation is used as a parameter of selection for salt stress tolerance, it cannot be regarded as a marker for salt tolerance (Misra and Gupta, 2005).

The homeostasis of intracellular ion concentration is fundamental to the physiology of living cells and a low cytosolic Na^+/K^+ ratio is important for maintaining cellular metabolism (Zhu, 2003). However, external excess Na^+ negatively impacts on intracellular K^+ influx and when it accumulates at high levels, results in ion imbalance and toxicity to enzymes in living cells (Hasegawa et al., 2000). K^+ plays an active role in maintenance of the photosynthetic apparatus and deficiency of K^+ reduces photosynthetic activity, Chl content and translocation of fixed carbon (Szczerba et al., 2009).

Halophytic plants can take up and accumulate Na^+ in their vacuoles and use it as an osmotic adjustment factor. Halophyte membrane lipids may also be adapted to prevent salt leakage (Glenn and Brown, 1999).

Swiss chard (*Beta vulgaris* L. subsp. *cicla*) is a glycophytic member of the Chenopodiaceae that is distributed all over the world and used as a green vegetable. There are a few reports about Swiss chard resistance to salt stress (Keeling and Ireland, 2009; Yousif et al., 2010), and Swiss chard showed marked osmotic adjustment and accumulation of proline and inorganic ions under salt stress (Ghoulam et al., 2002). However, information about alkaline stress on crop plants, particularly Swiss chard, is lacking.

The objective of this study was thus to evaluate the effects of saline and alkaline stresses on growth, photosynthesis, water relations, ion balance and osmotic adjustment in this plant, and to investigate the differences of its saline and alkaline stress tolerance.

Results

Plant growth

The growth of Swiss chard plants under saline and alkaline conditions was very interesting. The plant dry weights (DW) decreased with increasing saline and alkaline concentrations, and percentage reductions were 1.7% and 26.7% under 50 and 100 mM saline conditions, and 8.7% and 41.6% under 50 and 100 mM alkaline conditions compared with that of the control, respectively (Fig. 1). The RGR and LA decreased under both conditions, and the reduction was greater under alkaline conditions, particularly at 100 mM (Fig. 2).

Relative water content

Saline conditions caused a highly significant decrease in RWC, which decreased gradually with increasing neutral salt concentration. Alkaline conditions also induced gradual reduction of RWC, but its value was higher than those under saline conditions at the same concentration (Fig. 3).

Chlorophyll content

The Chl *a* content was not affected by saline conditions, but was gradually reduced by alkaline conditions with increasing alkaline salt concentrations. The Chl *b* content was significantly and gradually reduced under saline conditions, and sharply decreased at both concentrations under alkaline conditions. Therefore, the Chl *a*/*b* (Chl *a*/*b*) ratio increased at both concentrations under saline conditions, and was markedly increased under alkaline conditions; it was 1.31- and 1.54-fold under 50 and 100 mM saline conditions, and 3.28- and 3.20-fold under 50 mM and 100 mM alkaline conditions compared with that of the control, respectively (Fig. 4).

Photosynthesis

P_n, g_s and T_r were decreased under saline and alkaline conditions. C_i was not affected under saline conditions or under 50 mM alkaline conditions, but it increased under 100 mM alkaline conditions. WUE decreased with increasing neutral and alkaline salt concentrations and reductions were greater under alkaline conditions, particularly under 100 mM alkaline conditions (Table 1).

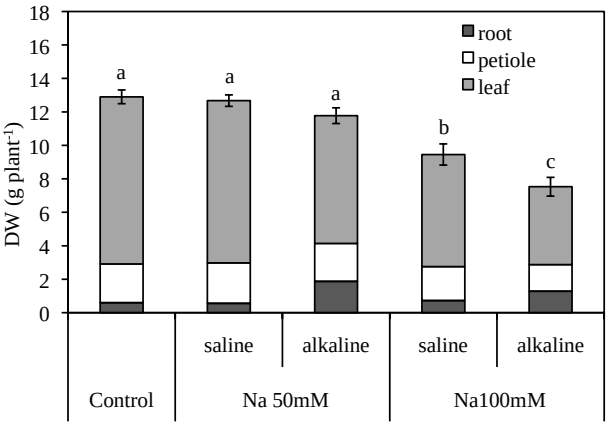

Fig 1. Effects of saline and alkaline stresses on dry weight (DW) of Swiss chard. The values are means (±SE) of three replicates.

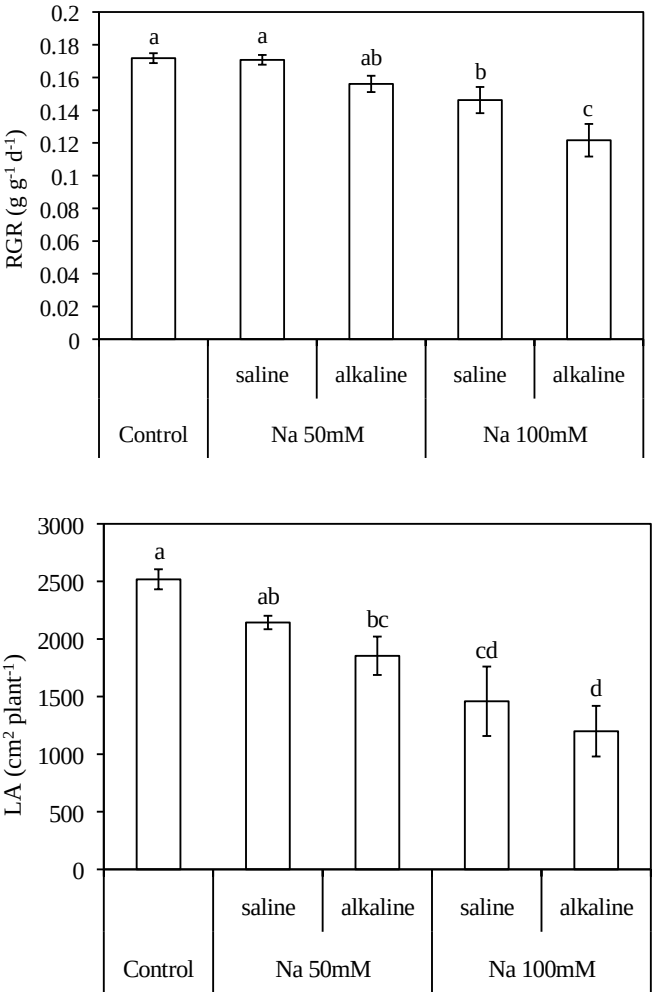

Fig 2. Effects of saline and alkaline stresses on relative growth rate (RGR) and leaf area (LA) of Swiss chard. The values are means (±SE) of three replicates.

Table 1. Effects of saline and alkaline stresses on the photosynthetic rate (P_n), stomatal conductance (g_s), intracellular CO_2 concentration (C_i) and transpiration rate (T_r) of Swiss chard. The values are means (±SE) of three replicates.

	Control	Na 50 mM		Na 100 mM	
		Saline	Alkaline	Saline	Alkaline
P_n ($\mu molCO_2\ m^{-2}\ s^{-1}$)	13.85±1.22^{a}	6.34±0.62^{b}	5.57±0.24^{b}	3.27±0.10^{c}	1.16±0.13^{d}
g_s ($molH_2O\ m^{-2}\ s^{-1}$)	0.21±0.01^{a}	0.08±0.01^{b}	0.09±0.02^{b}	0.06±0.01^{b}	0.1±0.03^{b}
C_i ($\mu molCO_2$)	232.5±10.25^{b}	212.75±25.87^{b}	223.0±13.39^{b}	249.5±20.24^{b}	319.5±8.47^{a}
T_r ($molH_2O\ m^{-2}\ s^{-1}$)	1.37±0.07^{a}	0.67±0.11^{b}	0.82±0.14^{b}	0.56±0.09^{b}	0.71±0.17^{b}
WUE (P_n/T_r)	10.16±0.81^{a}	9.46±1.53^{ab}	6.79±0.96^{bc}	5.84±1.26^{c}	1.64±0.43^{d}

Table 2. Effects of saline and alkaline stresses on the cation contents in leaves of Swiss chard. The values are means (±SE) of three replicates.

Measurements	Control	Na 50mM		Na 100mM	
		Saline	Alkaline	Saline	Alkaline
Na^+ (mg g^{-1} DW)	10.63±4.88^{e}	30.42±4.03^{d}	66.29±2.47^{b}	50.45±3.23^{c}	80.53±5.75^{a}
K^+ (mg g^{-1} DW)	56.91± 6.34^{a}	47.49±1.9^{b}	10.27±1.44^{d}	41.77±1.57^{c}	9.24±1.08^{d}
Na^+/K^+	0.17± 0.08^{c}	0.64±0.07^{b}	6.45±1.0^{a}	1.21±0.1^{b}	8.71±1.42^{a}

Table 3. Effects of saline and alkaline stresses on the contents of proline and glycinebetaine (GB) of Swiss chard. The values are means (±SE) of three replicates.

	Control	Na 50mM		Na 100mM	
		Saline	Alkaline	Saline	Alkaline
Proline (μmol g^{-1} DW)	4.25±0.49^{c}	6.76±1.72^{c}	7.65±0.95^{bc}	10.19±2.29^{ab}	12.16±1.61^{a}
GB (μmol g^{-1} DW)	75.07±19.19^{c}	222.36±3.02^{a}	164.49±20.96^{b}	224.03±7.85^{a}	191.79±5.30^{ab}

Ion accumulation

Na^+ content increased with increasing salinity and alkalinity, and the accumulation was higher under alkaline conditions than under saline conditions. The K^+ content decreased with increasing salinity and alkalinity, and the reductions under alkaline conditions were markedly higher than under saline conditions. Therefore, the Na^+/K^+ ratio increased with increasing salinity and alkalinity and sharply increased under alkaline conditions. It was about 37.94- and 51.24-fold under 50 mM and 100 mM alkaline conditions, and 3.76- and 7.12-fold under 50 mM and 100 mM saline conditions compared with that of the control, respectively (Table 2). Cl^- content was significantly increased under saline conditions, but was not affected under alkaline conditions (Fig. 5).

Total N, proline and GB

N content increased under saline conditions, but did not change under alkaline conditions (Fig. 6). Proline content gradually increased with increasing neutral and alkaline salt concentrations, and there were no differences between saline conditions and alkaline conditions at 50 and 100 mM. GB content significantly increased under both conditions and it was 222.4 μmol g^{-1} DW and 224.0 μmol g^{-1} DW at 50 mM and 100 mM under saline conditions, and 164.5 μmol g^{-1} DW and 191.8 μmol g^{-1} DW at 50 mM and 100 mM under alkaline conditions, respectively (Table 3).

Discussion

At the lower end of the salt-tolerance scale, *Beta vulgaris* plants are sometimes considered to be halophytes (Glenn and Brown, 1999). Halophytes demonstrate reduced growth under saline conditions because of loss of cell turgor and a decline of plant photosynthetic capacity. Swiss chard growth (LA, DW and RGR) was inhibited by salinity and alkalinity, and higher growth reduction was observed under alkaline stress than under saline stress, suggesting that this plant was affected more under alkaline conditions. This result agrees with previous studies, which reported that the extent of reduction of wheat growth under alkali stress was greater than that under salt stress (Yang et al., 2008), the injurious effect caused by alkali stress was greater than that of salt stress at the same salinity in barley (Yang et al., 2009) and the plant dry matter yield of both Foxtail millet and Proso millet decreased significantly under alkali stress conditions (Islam et al., 2011).

Decrease in RWC indicates a loss of turgor that results in limited water availability for cell extension processes (Katerji et al., 1997). In the present study, RWC was significantly reduced under both levels of saline conditions, whereas it slightly decreased under alkaline conditions. This result was partially in agreement with that of Bie et al. (2004), who reported that the water uptake of lettuce plant was not affected by $NaHCO_3$ treatments. Salinity stress often induces osmotic adjustment to ensure the maintenance of water uptake and cell turgor under stress conditions (Chaves et al., 2009), and halophytes can use Na^+ for osmotic adjustment (Glenn and Brown, 1999). Therefore, higher Na^+ content under alkaline conditions might have one function to maintain the uptake of water. On the other hand, plant responses are governed by the concentrations of ions in soil solution. Electrical conductivity (EC) of soil extracts is well correlated with their osmotic potential (Bernstein, 1975). In the present study, the EC of irrigated water was lower under alkaline conditions than under saline conditions, resulting in higher water potential of soil. Previous studies reported that pH and buffer capacity should be considered when evaluating the strengths of salt-alkali mixed stress (Li et al., 2010); high pH retards plant growth and CO_3^{2-} also has its own drastic effect on plants under Na_2CO_3 stress (Ali et al., 2004). The growth reduction was related to HCO_3^- toxicity and high pH rather than water stress or excessive Na in a $NaHCO_3$ experiment (Bie et al., 2004). In the present study, although the RWC was higher under alkaline conditions than under saline conditions, Swiss chard growth was more retarded under alkaline conditions, indicating that the injurious effect in Swiss chard under alkaline conditions might have been caused by high pH, CO_3^{2-} and HCO_3^- toxicity.

Chl is an important pigment that functions in photosynthesis. In the present study, total Chl content decreased under both conditions, particularly under alkaline conditions, which led to

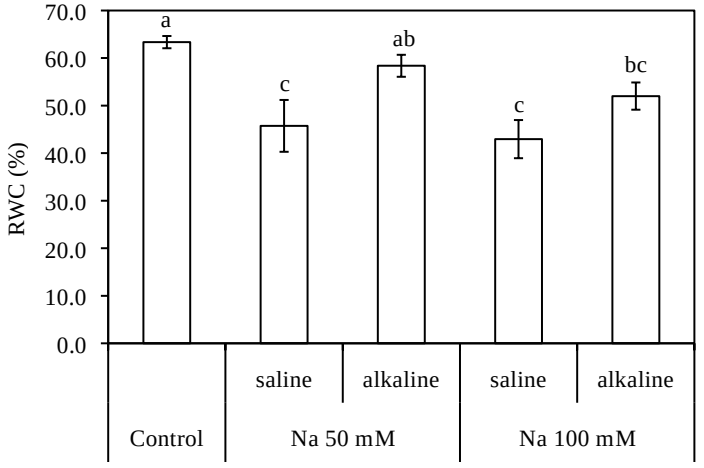

Fig 3. Effects of saline and alkaline stresses on RWC (%) of Swiss chard. The values are means (±SE) of three replicates.

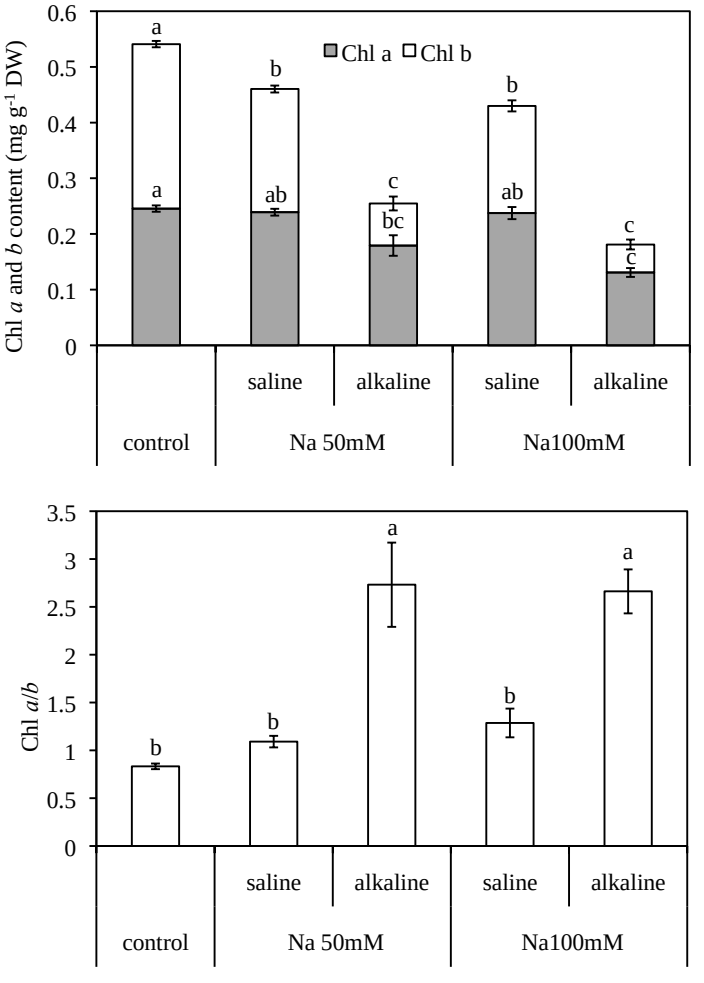

Fig 4. Effects of saline and alkaline stresses on chlorophyll (Chl) *a*, chlorophyll (Chl) *b* and chlorophyll (Chl) *a*/*b* ratio of Swiss chard. The values are means (±SE) of three replicates.

the reduction of photosynthetic activity, resulting in a greater reduction of plant growth under alkaline conditions. Szczerba et al. (2009) suggested that K^+ deficiency reduces Chl content. In a similar study, potassium deficiency decreased cotton leaf Chl content compared with sufficient potassium (Zhao et al., 2001). On the other hand, GB supply was another important factor for ALA (5-aminolevulinic acid) production (Sasaki et al., 2002), and ALA is a key precursor in the biosynthesis of Chl. In the present study, total Chl content was more reduced by alkaline stress than saline stress, which might have been caused by greater reduction of potassium and lower content of GB under these conditions. In contrast, Chl *b* was sharply decreased under alkaline conditions, which resulted in a clear increase in Chl *a*/*b* ratio. Kitajima and Hogan (2003) reported that the Chl *a*/*b* ratio increased while Chl content decreased in response to N limitation in photosynthetic leaves of tropical woody seedlings. In this study, the fact that the Chl *a*/*b* ratio was higher under alkaline conditions might have been partially caused by the lower N content compared with that under saline conditions. It is well known that a methyl group of Chl *a* is replaced by a formyl group, which is inserted by chlorophyllide *a* oxygenase (CAO), resulting in conversion into Chl *b*. Harper et al. (2004) reported that changes in Chl *b* levels in plants are regulated at the CAO mRNA level. In the present study, we suggested that the conversion from Chl *a* to Chl *b* is inhibited by alkalinity in Swiss chard, which might have resulted from the inhibition of CAO mRNA transcription.

The P_n, g_s, C_i, T_r and WUE were almost the same between the 50 mM saline conditions and the 50 mM alkaline conditions. However, P_n and WUE were more reduced, and C_i was markedly increased under 100 mM alkaline conditions compared with those under 100 mM saline conditions. The degree of salt-induced reduction in photosynthetic capacity depends on the area of photosynthesizing tissue (leaf area), photosynthetic pigments (Chl *a* and *b*), and stomatal and non-stomatal factors that affect CO_2 assimilation (Dubey, 2005). In the present study, g_s values were almost the same at 100 mM Na levels under the saline and alkaline stresses. However, LA and Chl *a* and *b*, particularly Chl *b*, were more inhibited at 100 mM under alkaline conditions, which resulted in lower photosynthetic rate and photosynthetic WUE under these conditions. C_i increased at 100 mM under alkaline conditions, indicating that carbon fixation was inhibited under such conditions. One report demonstrated that K-deficient leaves had less intercellular space and fewer chloroplasts in the mesophyll cells of cotton plant (Zhao et al., 2001). On the other hand, GB is abundant mainly in chloroplasts (Crowe et al., 1992) and maintains photosynthetic efficiency (Ashraf and Foolad, 2007), and the level of accumulated GB is correlated with the degree of salt tolerance (Sakamoto and Murata, 2002). In the present study, the marked reduction of K^+ content might have led to less intercellular space and fewer chloroplasts, which caused the lower GB content under 100 mM alkaline conditions, and finally resulted in the higher C_i and inhibition of plant growth.

The similarity of the hydrated ionic radii of Na^+ (1.65~2.05 Å) and K^+ (2.35~2.66 Å) makes it difficult to discriminate between them, and ion ratios in plants are altered by the influx of Na^+ through K^+ pathways (Blumwald, 2000). The Na^+ content increased but K^+ content decreased as neutral and alkaline salts were added in the nutrient solution, which implies that there was competitive inhibition for the absorption of K^+ in Swiss chard.

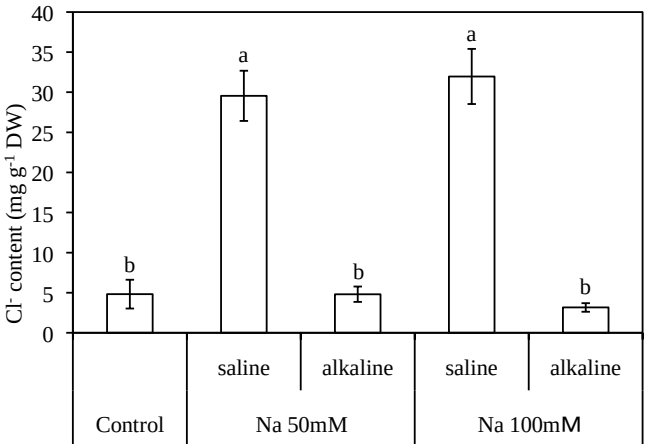

Fig 5. Effects of saline and alkaline stresses on Cl^- content in leaves of Swiss chard. The values are means (±SE) of three replicates.

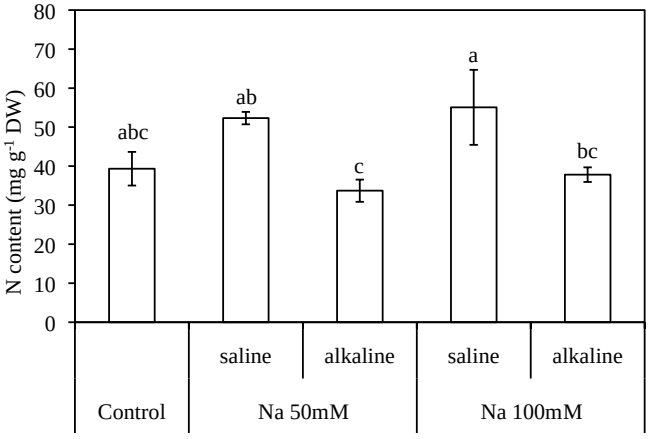

Fig 6. Effects of saline and alkaline stresses on nitrogen (N) content in leaves of Swiss chard. The values are means (±SE) of three replicates.

A low cytosolic Na^+/K^+ ratio is important for maintaining cellular metabolism (Zhu, 2003). A high cytosolic Na^+/K^+ ratio results in injury to cells and tissues of plants (Blumwald, 2000; Matoh et al., 1987), leading to nutritional imbalance (Peng et al., 2004) and toxicity to enzymes (Hasegawa et al., 2000). In the present study, the Na^+ content surrounding the rhizosphere was the same between saline conditions and alkaline conditions. However, Na^+ content was higher and K^+ content was much lower under alkaline conditions, leading to greater induction of Na^+/K^+ under alkaline conditions than under saline conditions, which resulted in toxicity for the growth of Swiss chard. Glenn and Brown (1999) reported that Na^+ content in the cytoplasm could be maintained at non-toxic levels through the operation of ion translocation at the plasma membrane, to import Cl^- into the cell and to export Na^+. In the present study, the fact that the Na^+ content under saline conditions was lower than that under alkaline stress might have been caused by higher Cl^- content in leaf tissues. Under alkaline conditions, the Cl^- content was not affected, although greater induction of Na^+ accumulation occurred, resulting in a sharp reduction of K^+ content.

Proline and GB are two major organic osmolytes that accumulate in a variety of plant species in response to environmental stresses (Ashraf and Foolad, 2007). Previous study reported that the salinity-induced increase in proline content was always higher in the sensitive genotype than in the tolerant one in sorghum genotypes (Lacerda et al.,2001). Islam et al.(2011) also indicated that the proline content of saline and alkaline stress-sensitive plants of Foxtail millet was significantly higher than that of tolerant plants of Proso millet under saline and alkaline conditions. The GB content was significantly increased in both leaves and roots, but proline content was not significantly affected by salinity in algarrobo (Meloni et al., 2004). In the present study, both proline and GB contents were induced under saline and alkaline conditions. However, proline content was almost the same between saline and alkaline conditions, whereas GB content was significantly higher under 50 mM saline conditions than under 50 mM alkaline conditions. This result suggested that the contribution of proline to the alkaline tolerance seemed to be less important in Swiss chard, and GB played an important protective role in the mechanism of salt tolerance for Swiss chard. GB was discovered in sugar beets in the 19th century, and spinach can accumulate relatively high levels of GB in its chloroplasts (Sakamoto and Murata, 2002). In the present study, Swiss chard, which comes from the same family as spinach and sugar beets, accumulated a relatively high level of GB under control conditions, and levels were increased under both test conditions. However, enhanced values were not like those of proline, which showed almost the same levels between saline and alkaline conditions. GB was instead induced more under saline conditions. GB is an N-trimethylated amino acid, and synthesized via two distinct pathways from two distinct substrates: choline and glycine (Sakamoto and Murata, 2002); it was shown to increase in leaf tissues with increasing N application under water-stress conditions (Saneoka et al., 2004). The fact that GB content was lower under alkaline conditions than under saline conditions might be because the total N was lower under alkaline conditions. On the other hand, a stable tissue pH is necessary for plants to maintain normal metabolism (Yang et al., 2007). Previous studies have suggested that the tissue pH values in shoots of *Kochia sieversiana* (Yang et al., 2007), barley (Yang et al., 2009) and *Lathyrus quinquenervius* (Zhang and Mu, 2009) were the same as in the control. Under alkaline conditions, a lack of external protons might inhibit the pathway for the synthesis of GB from choline, which has a hydroxyl group. At the same time, the higher RWC value under alkaline conditions is helpful to reduce the GB content under alkaline conditions. GB plays an important role as a compatible solute in plants under various types of environmental stress (Sakamoto and Murata, 2002), and is commonly referred to as an osmoprotectant (Ashraf and Foolad, 2007). On the other hand, exogenous GB significantly reduced Na^+ accumulation and maintained higher K^+ content in salt-stressed plants (Lutts, 2000; Ashraf and Foolad, 2007). In the present study, the fact that Na^+ content increased more and K^+ content decreased more under alkaline conditions than under saline conditions might have been due to the lower GB content under alkaline conditions.

Materials and methods

Plant materials

White Swiss chard seeds (*Beta vulgaris* L. subsp. *cicla* (L) cv. Natsuna), obtained from Utane Seed Co., Ltd. (Utsunomiya, Japan), were sown in seedbeds filled with a soil mixture of field soil and perlite (2:1 v/v). The seedbeds were kept in greenhouse conditions with natural light and temperature, and were sufficiently watered with basal nutrient solution every two days. The nutrient solution contained 4.2 mM NO_3-N, 0.3 mM NH_4-N, 0.2 mM P_2O_5, 1.1 mM K_2O, 0.4 mM MgO, 1.0 mM CaO, 5.3 µM MnO, 5.4 µM B_2O_3, 12.1 µM Fe, 0.1 µM Cu, 0.3 µM Zn and 0.08 µM Mo.

Stress treatments

Six-week-old uniform seedlings were transplanted into 3 L plastic pots filled with a soil mixture of granite regosol soil and perlite (2:1 v/v). Each pot had one plant and was considered as one replication with 3 replications per set. One set was used for growth index determination at the beginning of treatments, and the remaining sets were treated under saline and alkaline conditions. For treatments, seedlings were irrigated with adjusted basal nutrient solution containing 50 and 100 mM neutral salt mixture of NaCl and Na_2SO_4 (9:1 molar ratio) (Zhang and Mu, 2009) under saline conditions, and 50 and 100 mM alkaline salt mixture of $NaHCO_3$ and Na_2CO_3 (9:1 molar ratio) (Zhang and Mu, 2009) under alkaline conditions. Control plants were irrigated by adjustment of basal nutrient solution. The pH values of irrigated solution were 6.53 and 6.57 at 50 and 100 mM saline treatments, and 9.07 and 9.19 at 50 and 100 mM alkaline treatments, respectively. In addition, the EC (mS m^{-1}) values of irrigated solution were 702 and 1230 at 50 and 100 mM saline treatments, and 533 and 903 at 50 and 100 mM alkaline treatments, respectively.

Plant harvesting and growth measurements

Plants were harvested upon emergence of the cotyledon after two weeks of treatments and washed using tap water at first, and then using distilled water. After removing moisture with tissue paper, plant tissue was carefully separated into leaf and petiole and frozen directly in liquid nitrogen, then freeze-dried and measured for its DW. The LA was measured using a leaf area meter (AMM-5 type leaf area meter, Hayashi-Denko, Tokyo, Japan). RGR was calculated using the method of Hunt (1990). The RWC was measured as described by Turner (1981).

Physiological index analysis

P_n, g_s, C_i and T_r of leaves were measured using a portable photosynthesis system equipped with a leaf chamber and a portable open flow infrared gas analyzer (LI-6400, LI-COR Biosciences, Lincoln, NE, USA) at 14:00-15:00 on the 14th day after the last watering before plant harvesting. The photosynthetically active radiation (PAR) was 1200 µmol m^{-2} s^{-1}, the relative humidity was 80% and the ambient CO_2 concentration was set at 350 µmol mol^{-1}. The temperature of leaf surfaces was 25°C and the flow of air was 500 mL s^{-1}. Chl *a* and *b* were determined following the measures described by Arnon (1949). For cations, around 20 mg of finely ground powder was digested by nitric acid-hydrogen peroxide, and then the Na^+ and K^+ concentrations were determined using a flame photometer (ANA-135, Eiko Instruments Inc., Tokyo, Japan). For anions, about 0.02 g of dry sample of plant tissue was homogenized in distilled water (used for HPLC) at 100°C for 1 h, and then centrifuged. The upper extracted solution was used to determine the concentrations of Cl^-, by ion chromatography. The N concentration was determined by the Kjeldahl method after digestion with sulfuric acid. GB was purified by Dowex-1-Cl^- ion-exchange chromatography and Dowex-50-H^+ ion-exchange chromatography from the leaf methanol extraction after separation by chloroform and water, and eluted from the column by Dowex-50-H^+ ion-exchange chromatography with 6 M NH_4OH. The dried NH_4OH eluant was extracted with 10 mL of acetonitrile:methanol (20:1, v/v) to remove protein (Yang et al., 1995), and then GB was analyzed by HPLC (Gulliver system, Jasco Co., Tokyo, Japan) using a Shodex NH_2P-40 4E column (Saneoka et al., 2004). The proline was also extracted using methanol and then measured following the ninhydrin method described by Bates et al. (1973) using L-proline as a standard.

Statistical analysis

The experiment was setups as a completely randomized design with both species and five salinity and alkali levels. All data were examined by one-way ANOVA using Statistical Package for the Social Sciences (SPSS) for Windows. Values are given as mean ± standard deviation (SD) and multiple comparisons of means of data between different salinity treatments within the plants were performed using Duncan′s test at the 5% significance level.

Conclusion

Although the RWC under alkaline conditions was higher due to the lower electrical conductivity in soil than that under saline conditions, the Swiss chard growth was more inhibited, suggesting that the injurious effect in Swiss chard under alkaline conditions might be related to high pH, CO_3^{2-} and HCO_3^- toxicity. Proline content was greater in leaves under alkaline conditions that caused the greatest reduction in growth, whereas GB content was higher in leaves under saline conditions than that under alkaline conditions, suggesting that proline does not play an important role in the mechanism of alkaline tolerance for Swiss chard and GB plays an important protective role in Swiss chard in a salt environment.

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