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Full Length Article

Physiological Responses of Alfalfa to High-level Salt Stress: Root Ion Flux and Stomatal Characteristics

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Abstract

Alfalfa is an important salt-tolerant leguminous forage-plant in salinity areas worldwide, but its performance in high level of salt stress cannot meet the satisfactory requirement. Especially, the short-term response of alfalfa to high-level salt stress is still not clear. In the present study, thirty-day-old alfalfa Gongnong No. 1 (*Medicago sativa* L. cv. Gongnong No.1) seedlings were exposed to NaCl treatments at concentrations of 0 (control), 50 (moderate level), 150 (high level), and 250 mM (extremely high level). Twenty-four hours after salt stress treatment, with the increase of NaCl level plant height was slightly decreased but both shoot biomass and root length were substantially declined to a dramatic extent. Also decreased was root K^+ concentration. In contrast, both Na^+ concentration and ratio of K^+/Na^+ showed increased trends. Root K^+ flux was determined using non-invasive micro-test technique (NMT) around apical root tips, wherein a clear K^+ influx was observed at the rate of about 0.5 nmol cm^{-2} s^{-1} under the condition without salt stress, while under salt stress at the rate of 2–3 nmol cm^{-2} s^{-1} did occur K^+ efflux. Accordingly, stomatal length and breadth and stomatal aperture breadth decreased with the increase of NaCl concentration, while stomatal aperture density increased with time in the first 24 h after NaCl treatment. In conclusion, as a species-specific test, alfalfa is sensitive to high-level salinity with NaCl concentrations above 150 mM in the first 24 h post salt-exposure. The key mechanism was found to be presented as the pressed stamatal conductance induced by K^+-Na^+ unbalance which was caused by root K^+ efflux.

Keywords: Salinity; Non-invasive micro-test technique; *Medicago sativa* L. cv. Gongnong No. 1; Potassium

Introduction

Salt stress is one of the most serious environmental factors limiting plant growth and crop productivity especially in arid and semi-arid regions (Bertrand *et al.*, 2015). At present, almost half of world's irrigated lands and at least 20% of global cultivated lands are suffering from salinity (Qiao *et al.*, 2014). In China, saline and alkaline soils occupy a large area of 6.7×10^6 ha, which accounts for approximately 7% of total cultivated lands (Peng *et al.*, 2008). Furthermore, salt accumulation accompanied with water resource depletion caused by unsustainable irrigation aggravates spread of salinity in agricultural systems of arid and semiarid regions in recent years (Endo *et al.*, 2014). High-level salt concentration in soils has been an issue that cannot be evasive for crop production in saline regions. For example, mean salt concentration in grassland-soils of the Songnen Plains, one of the main forage centers in China, is more than 0.7%, equaling about ~120 mM NaCl in leaching liquors from local soils (Peng *et al.*, 2008). With regard to the harmful impact of high-level salt stress on grass production, salt-stressed plant physiological performance is an important theoretical reference to cope with high-level salt stress (e.g., Anower *et al.*, 2013; Bai *et al.*, 2013; Wang *et al.*, 2014).

When plants are suffering salt stress they normally show growth reduction at two phases: the exogenous phase of water stress caused by salt accumulation outside roots, and the endogenous phase of injury caused by inherent salt accumulation (Munns, 2002). In both phases, exogenous salt impacts physiological acts in a short-term from hours to days. In the exogenous phase, the osmotic gradient induced by salt concentrations results in an efflux of water but the meanwhile salt ions flow as an influx (Chaudhuri and Choudhuri, 1998); in the endogenous phase inherently accumulated salts have rapid, transient, but reversible effects on photosynthesis-involved activities for both foliar physiologies and enzymes (Hernández and Almansa, 2002; Dinler *et al.*, 2014). In the first 24 h after salt stress, drastic physiological changes have been induced even prior to the emergence of apparent symptom. However, these changes appeared to have distinct varieties among species with contrasting salt-tolerant abilities (Golombek and Lűdders, 1998; Chaudhuri and Choudhuri, 1998). As a mediating response, active oxygen species (AOS) is activated to generate so as to initiate the inhibiting systems to defend against salt stress (Alscher *et al.*, 1997; Noctor and Foyer, 1998; Shalata and Tal, 1998). It has been established that

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this defensive response can be activated efficiently in the first 24 h after salt stress (Hernández and Almansa, 2002), the mechanism for ion flux characteristics during this short term, however, has not been well decumented, possibly due to the obstacle of determining means.

For the exogenous phase of growth decline under salt stress, external ions such as Na^+ enters into root cells, thereafter a large electrochemical gradient establishes to favour the passive entry of salt ions through a variety of cation and anion channels and/or transporters in the plasma membrane (Sun *et al.*, 2009). As a result, rapid Na^+ accumulation and a decline of K^+/Na^+ ratio were found to be generated in the first 24 h after salt stress for several vegetative and woody plants (*e.g.*, Chaudhuri and Choudhuri, 1998; Hernández and Almansa, 2002; Sun *et al.*, 2009). With regard to the approach for detecting ion flux in the first phase of 24 h after salt stress, using barley plants as materials Katsuhara *et al.* (2011) ever suggested to determine aquaporin activity to quantify the reduction of hydraulic conductivity. However, this approach received explicit queries according to the viewpoints that it failed to reflect the impact by salt stress sometimes (Maksimovic *et al.*, 2013). Instead, a non-invasive micro-test technique (NMT) successfully developed by Shabala and coadjutant colleagues (Shabala and Newman, 1997; Shabala *et al.*, 1997; Garnett *et al.*, 2001) is widely accepted due to its precise detections on the movement of plasma membrane (PM) H^+-ATPase in the Na^+/H^+ antiport according to ion kinetics in response to salt shock. Under salt stress, the pattern of short-term flux profiles of salinity ions of Na^+, K^+, and Cl^-, has been well quantified using NMT on Arabidopsis (Shabala *et al.*, 2005), Poplar (Sun *et al.*, 2009), Mangrove (Lu *et al.*, 2013), and some strains in mycorrhizal Poplar roots (Li *et al.*, 2012; Ma *et al.*, 2014). According to these studies, the short-term salt-induced efflux of intracellular K^+ was highlighted because salt impact would be intensified when K^+ loss is accompanied by accumulation of Na^+ inside the cytosol with a high level of Na^+ concentration in the range of 50–200 mM (Maathuis and Amtmann, 1999; Flowers and Hajibagheri, 2001; Britto *et al.*, 2010). However, current knowledge about NMT-detected ion flux was too independent from shoot physiologies to supply sufficient information of relations between above- and below-ground of a given plant under high-level salt stress.

In the second phase of growth reduction under salt stress, stomatal is induced to be closed by salt stress hence foliar CO_2 fixation is inhibited and CO_2/O_2 ratio declines. Stomatal conductance is heterogeneous when divided by space-scales (Xu *et al.*, 2015). At a larger scale among groups the heterogeneity of gas exchange, parameters is thought to partly depend on variations of stomatal number and size over the plant leaf (Weyers and Lawson, 1997; Mott and Buckley, 1998), while at a smaller scale of individual plant the gas exchange parameters was observed to be related to non-uniform stomata behavior of patchy stomatal closure (Mott and Buckley, 1998, 2000). These conclusions were drawn according to studies of crop and tree plants (Paz *et al.*, 2014; Amjad *et al.*, 2014; Xu *et al.*, 2015) with the correlation between stomatal closure and decline of photosynthesis (Meloni *et al.*, 2003; Xue and Liu, 2008). However, opposed viewpoints have also been put forward based on contrasting evidence (Yang *et al.*, 2007; Gazanchian *et al.*, 2007). Although leaf abaxial surface imprints have been photographed and measured to reveal morphological response of stomatal aperture to salt stress (Amjad *et al.*, 2014; Paz *et al.*, 2014), evidence from the results may lost certification to a great extent because current measures on stomatal aperture were generally conducted in days after salt stress when aperture length and breadth may have been generated as a result of multiple reversible changes (Hernández and Almansa, 2002). Thus, short-term measurements in the first 24 h after salt stress have not received deserved attention. The core concern that can reveal the mechanism of this issue may come out from the precise measures on morphology of stomatal aperture.

Alfalfa is a perennial forage characterized by high yield and production hence is known as "the queen of forage" (Wang *et al.*, 2014). Although some alfalfa cultivars were confirmed to be salt-tolerant to moderate salt stress, their tolerance, however was not so efficient when facing high-level salt stress (Chen *et al.*, 2010; Long *et al.*, 2012; Wang *et al.*, 2013). For example, alfalfa Gongnong No.1 (M*edicago sativa* L. cv. Gongnong No. 1) is a fairly moderate salt-tolerant cultivar, but it was found to have some undesirable performance in response to high-level salt stress in saline regions of Songnen Plains, Northeast China (Peng *et al.*, 2008). Additionally, many studies of salt effect on alfalfa were conducted in days or months (Table 1), quite limited evidence can be referred to for alfalfa at the species-specific level about their physiological response in a short term of the first 24 h after high-level salt stress. Therefore, in the present study, alfalfa Gongnong No. 1 was employed as research material with the objective of testing its short-term performances of ion flux and stomatal conductance in response to high-level salt stress. It was hypothesized that: (1) in the first phase of osmotic gradient established by NaCl intracellular K^+ efflux occurred, so that (2) in the second phase of inherent injure morphologies of stomatal apertures declined with time or along the salt gradient, and (3) the balance of whole-plant K^+/Na^+ ratio was broken. Data in our study have the comparing meaning for other relevant studies, and our results would supply theoretical evidence for improving alfalfa tolerance through the approach of screening alfalfa cultivars.

Materials and Methods

Plant Material

Seeds of alfalfa Gongnong No. 1 (M. *sativa* cv. Gongnong

No. 1) were provided by Institute of Grassland Sciences, Jilin Academy of Agricultural Sciences, Gongzhuling Town, suburban Changchun City, Jilin Province, China. The experiment was conducted at April 2014 in the laboratory of Environment and Resources College, Dalian Nationalities University (39º02'N, 121º46'E). Seed were surface sterilized with 0.5% sodium hypochlorite solution for 20 min, rinsed, and sown in plastic pots (top diameter × bottom diameter × height, 18 cm × 15 cm × 13 cm) filled with humid vermiculite. Seed were germinated in the dark at 25±1ºC in a chamber room. Twenty days after germination four uniformly sized germinated seedlings were bulked and transplanted to cells (unit size: top diameter × height, 3.5 cm× 9 cm) of a quadrate foamy trait at temperature of 25/20ºC and photoperiod of 11 h/13 h for day and night, respectively. Light was supplied by plant growth lamps (Oudi Illumination Co., Huzhou City, Zhejiang Province, China) to maintain a photosynthetic photon flux density of 450 µmol m^{-2} s^{-1} measured at apical tip of seedlings. Totally 160 seedlings were transplanted. Perlite was used as substance in holes where transplanted seedlings were pretreated with nutritional solutions so as to stimulate root growth to a uniform size which facilitated following measurement on root ion flux. Nutritional composition was adapted from Wei *et al.* (2013). Briefly, mineral concentrations therein were: NH_4NO_3 4mM, K_2HPO_4 0.5 mM, KCl 0.5 mM, $CaCl_2$ 1 mM, $MgSO4.7H_2O$ 0.6 mM with other micro-elements added. Perlite-cultured seedlings were watered with 100 mL of solution per hole per day.

Salt Stress Treatment

Ten days after seedling transplanting (30 d after germination) bulks of four seedlings per tray cell were moved to another tube (top diameter × height, 2 cm × 11 cm) where conducted was salt stress treatment. Seedlings were treated as a randomly placed unit design wherein four salt treatments were employed as a series of NaCl concentrations of 0%, 0.3%, 0.9% and 1.5% (w/w) marked by the control, 50 mM, 150 mM and 250 mM NaCl treatments, respectively. As it is summarized in Table 1, the rate of 50 mM NaCl is widely adapted as the moderate stress dose in relevant studies, while the 150 mM rate is close to the highest level performed by Peng *et al.* (2008) because it simulated the mean level of salt in Sanjiang Plains where alfalfa Gongnong No. 1 is distributed. The highest rate of 250 mM is set as the target of high level of NaCl concentration reported worldwide. Totally, forty tubes were used and each ten ones from them were grouped up and marked for one treatment. Therefore, one tube was treated as a replicate (n=10) with one of the four seedlings therein as a measurement unit.

Steady-state K^+ Flux Measurement

Thus, short-term ion flux has been determined for all of H^+, Na^+, Cl^-, and K^+ in salt-stress studies (see Introduction). However, in the present study we focused on only K^+ flux because the suppression of its influx by Na^+ is evaluated to result in a great threat to K^+/Na^+ which contributes to the main significance of salt damage (Britto *et al.*, 2010) especially at high levels of Na^+ concentration over 150 mM (Jayakannan *et al.*, 2013).

K^+ flux was measured using a NMT system (University of Tasmania, Hobart, Australia). Microelectrodes were pulled and salinized with tributylchlorosilane. After backfilling, electrode tips were filled with commercially available ionophore cocktails (60031 for K^+; Fluka, Busch, Switzerland). The electrodes were mounted on a 3D-micromanipulator (MMT-5, Narishige, Tokyo, Japan), whose tips were put together and positioned 20 µm above the root surface. Root segments were mounted horizontally in a Perspex holder by using agar. The holder was immediately placed in a 4 mL measuring chamber filled with appropriate solution. The chamber was mounted on a computer-driven 3D-manipulator (PatchMan NP2, Eppendorf, Hamburg, Germany). Twenty-four hours after salt stress, root segments with apices of 1 to 2 cm were sampled by excising and used for steady-state measurements of net K^+ flux. Root segments were rinsed with distilled water and incubated in the basic measuring solution (0.5 mM KCl, 0.1 mM NaCl, 0.1 mM $CaCl_2$, and 0.1 mM $MgCl_2$) to equilibrate for 30 min. Root segments were transferred to petri-dishes containing 10 mL of fresh measuring solution. Prior to recording the flux, roots were immobilized on the bottom. The K^+ fluxes in roots were measured at 500 µm from the root apex because this is the available root apical region where exchange and flux of ions occurs stably.

During measurements, the NMT (MIFE™ tech.) software controlled PatchMan NP2 to move the electrodes between two positions, 20 and 50 µm from the root surface in a 10-s square-wave manner. The software also recorded electric potential differences from the electrodes between two positions using a DAS08 analogue to digital card (Computer Boards, USA) in the computer.

K^+ flux was measured by moving the ion-selective micro electrode between two positions close to the materials in a preset excursion (30 µm) at a programmable frequency in the range of 0.3 to 0.5 Hz. Ion-selective microelectrodes for the K^+ ions were calibrated prior to flux measurements: 0.1, 0.5, and 1.0 mM (K^+ was 0.5 mM in the measuring buffer). The K^+ electrode used for steady recordings were usually corrected two to three times by calibrations during the experiments.

Rhythmic (ultradian) flux oscillations are ubiquitous in the measured vegetative plant species. Our data show that the oscillatory periods of measured K^+ ions in alfalfa roots were usually in the range of several minutes. Therefore, K^+ fluxes were recorded for 8 to 10 min at each point, which is long enough to ensure the absence of oscillations.

Table 1: A summary of typical studies of salt-stress effects on alfalfa growth

Author(s) and year	Material	NaCl doses	Study type and term
Fougère *et al.* (1991)	Clonal propagation of shoot cuttings from *Medicago sativa* L.	100 and 150 mM	Lab., 14 d
Ashraf and Oleary (1994)	Two transgenic lines: M. *sativa* AZ-Germ Salt II and *M. sativa* Mesa Sirsa	0, 100 mM	Lab., 60 d
Safarnejad *et al.* (1996)	*M. sativa.*	200 mM	Lab., 14 d
Petrusa and Winicov (1997)	Tranfer alfalfa lines	171 mM	Lab., 1 d
Djilianov *et al.* (2003)	R1 to R4 and T1 of transgenic lines of M. *sativa*	0, 37.5, 75, 150 mM	Lab., ~ 30 d
Wang and Han (2007)	Two cultivars: *M. sativa* cv. Zhongmu No.1 and *M. sativa* Defor	120 mM	Lab., 15 d
Peng *et al.* (2008)	M. *sativa* cv. Gongnong No.1	32, 64, 96, 128, 160 mM	Lab., 7 d
Wang and Han (2009)	Two cultivars: *M. sativa* Zhongmu No.1 and Defor	0, 70, 140, 210 mM	Lab., 20 d
Wang *et al.* (2009)	Six cultivars: *M. sativa* cv. Xinmu No.1, Xinjiang Daye, Algonquin, Golden Empress, Victor, Northstar	200 mM	Lab., 7 d
Li *et al.* (2010)	*M. sativa*	30, 60, 90, 120, 150 mM	Lab., 14 d
Chen *et al.* (2010)	Four cultivars: *M. sativa* cv. Zhongmu No.1, Maverick, Vernal, Pioneer5446	0, 50, 100, 150, 200 mM	Lab., 15 d
Babakhani *et al.* (2011)	Two cultivars: *M. sativa* cv. Yazdi and M. *sativa* cv. Diabolourde	0, 100, 150, 200 mM	Lab., 14 d
Li *et al.* (2011)	Transgenic M. *sativa* overexpressing the *SsNHX1* gene	0, 100, 200, 300, 400 mM	Lab., 50 d
Long *et al.* (2012)	*M. sativa* cv. Zhongmu No.1	300 mM	Lab., 14 d
Anower *et al.* (2013)	Two half-sib families of *M. sativa* subsp *falcata*	40 mM	Site collected and Lab. test , 21 d
Wang *et al.* (2013)	*M. sativa* Zhongmu No.1	0, 50, 100, 150, 200, 250 mM	Greenhouse, 7 d
Bai *et al.* (2013)	Transgenic *M. sativa* overexpressing the gene of *GsCBRLK*	300 mM	Lab., 15 d
Tang *et al.* (2013)	Transgenic *M. sativa* overexpressing the gene of *GsZFP1*	250 mM	Lab., 26 d
Ashrafi *et al.* (2014)	Nine cultivars of *M. sativa*	20, 60, 120 mM	Field, 2 yr
Farissi *et al.* (2014)	Four cultivars: *M. sativa* cv. Tafilalet 1, Tafilalet 2, Demnate, and Tata	0, 100, 200 mM	Greenhouse, 45 d
Tang *et al.* (2014)	Transgenic *M. sativa* overexpressing the gene of *GsWRKY20*	250 mM	Lab., 28 d
Wang *et al.* (2014)	Transgenic *M. sativa* cv. Xinjiang Daye overexpressing the gene of *AtNDPK2*	250 mM	Lab., 21 d
Endo *et al.* (2014)	*M. sativa*	5.4, 21.6, 43.2 mM	Greenhouse, 30 d
Bertrand *et al.* (2015)	Two cultivars: *M. sativa* cv. Apica and *M. sativa* cv. Halo	0, 20, 40, 80, 160 mM	Lab., 42 d

Stomatal Aperture Morphology

As a dicotyledonous species (Bagga *et al.*, 1991), alfalfa has more stomata on the lower epidermis than on the upper epidermis. Therefore, we only measured on the abaxial surface which accounts for most of the gas exchange. After 0- (start of the measurement), 1-, 2-, 3- and 24-h of salt stresses, leaves of another seedling from the four ones in one planting tube (the first one for K^+ ion flux measurements) was sampled and cleaned for leaf surface. Then, leaves were rinsed with a phosphate buffer solution (pH=7.2) and cut into pieces. Pieces from abaxial area were sampled and fixed in 2.5% glutaraldehyde (v/v) for 4 h, then washed for 3 to 4 times using phosphate buffer remove residual glutaraldehyde. Sampled leaf pieces were dehydrated by being soaked in ethanol at series concentrations of 30%, 50%, 70%, 80%, 90%, 95% and 100% (v/v), twice for each concentration and each for 15 min. First-step dehydrated leaf-samples were then soaked in another mixture-solution for 15 min which contains 2:1 (v/v) mixture of ethanol and isoamyl acetate. Dried leaf pieces were photographed using a Leitz DMRD light microscope (Leica Mikroskopie and Systeme GmbH, Wetzlar, Germany) with an associated camera (Leica DFC 420). Stomata length was measured as the length of dumbbell-shaped cells. Stomata breadth equals the widest width vertical to length. Stomatal aperture breadth was presented as stomatal opening level. For measuring stomata aperture density, a grid of 1 mm^2 was randomly superimposed.

Growth Measurements and Chemical Analysis

Twenty four hours after salt stress the last two seedlings were sampled for measurements of growth and chemical analysis. One seedling was excised to sections of shoot and root, which were subsequently measured for height and root length, respectively. Sections of shoot and root were both oven-dried at 150°C for 15 min then at 70°C for 2 d then measured for dry weight. Oven-dried roots were powered using a mortar and pestle. Approximately 10 mg powder was digested in 1 M HCl over night. Then sample was then spun for 3 min at 11, 000 × *g*. The concentrations of Na^+ and K^+ in the digested samples were measured using a flame photometer (6400A, Shandong, China).

Statistical Calculation and Analysis

The K^+ ion flux rate was calculated using Fick's law of diffusion:

$$J = - D (dc / dx) \quad (1)$$

Where J is the ion flux in the x direction, dc represents the ion concentration difference, dx is the microelectrode movement between two positions, dc/dx is the ion concentration gradient and D represents the ion diffusion coefficient in a particular medium.

Potential conductance index (*PCI*) was calculated as:

$$PCI = (S_L)^2 \times S_D \quad (2)$$

Where, S_L is stomatal length, and S_D is stomatal density.

All data were analyzed using SPSS® software (IBM, NYSE, USA). Tests for normality and constant variance were performed and no transformations were necessary. A one-way analysis of variance (one-way ANOVA) was performed for data analysis. When the effects were

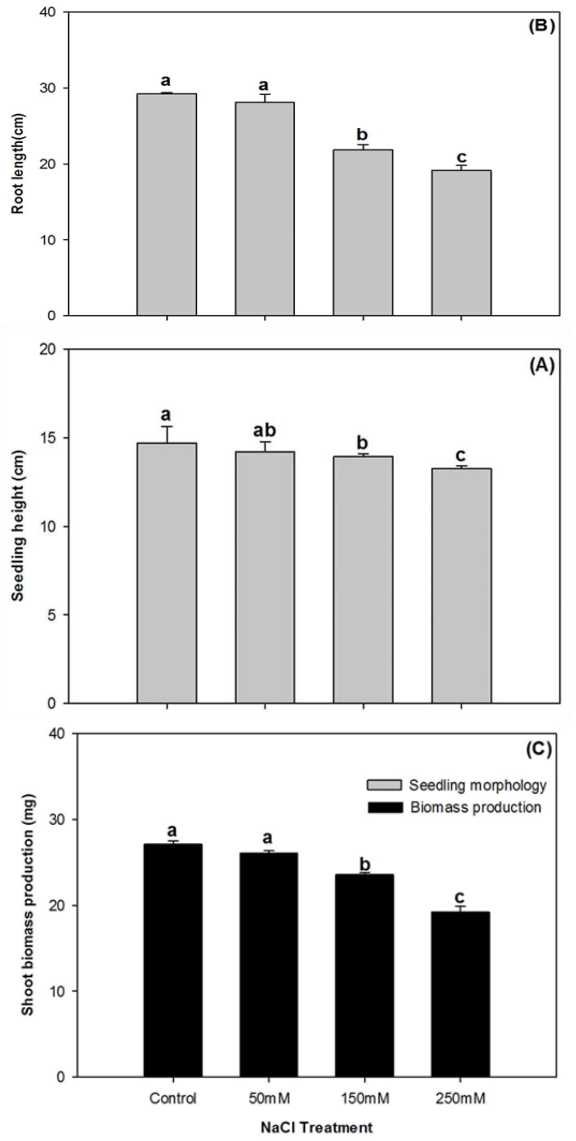

Fig. 1: Seedling height (A), root length (B), and shoot biomass production (C) of alfalfa Gongnong No.1 subjected to NaCl treatments of concentrations at 0 (Control), 50, 150, and 250 mM. Different letters indicate significant differences according to LSD test at 0.05 level

indicated to be significant by ANOVA, means (±*SD*) were ranked according to LSD test at α = 0.05. Pearson correlations were performed to test the relationships between stomatal morphological parameters and concentrations from NaCl treatments, and experiment hours.

Results

Seedling Growth and Shoot Biomass Production

Both seedling height and root length declined with the increase of rates of NaCl treatments (*Sig.* <0.0001; Fig. 1A, B). However, results were not different between the control and the 50 mM treatments. Similar as results of seedling height and root length, shoot biomass production also declined with increase of rates of NaCl treatments. Biomass in the 150 mM and 250 mM were lesser than those in the control and the 50 mM treatments ($p < 0.0001$; Fig. 1C).

Na^+ and K^+ Accumulations and K^+/Na^+ Balance

Root Na^+ accumulation increased with the rate of NaCl treatments, but did not increase at NaCl concentrations above 150 mM (Sig. <0.0001; Fig. 2A). In contrast, K^+ accumulation decreased with increase of rate of NaCl concentration ($p < 0.0001$; Fig. 2B). As a result, K^+/Na^+ ratio also decreased with increase of rate of NaCl treatments from 6 to less than 2 although results did not differ between the 150 mM and the 250 mM treatments ($p < 0.0001$; Fig. 2C).

Results of NMT indicated that values of K^+ flux was negative in the control treatment, suggesting an absolute influx therein ($p < 0.0001$; Fig. 3). In the treatments where seedlings were exposed to NaCl treatments, values of K^+ flux turned to be positive and showed an increased trend with rate of NaCl treatments. However, percentile-values fluctuated in the 150 and 250 mM treatments and difference between them was not significant.

Stomatal Aperture Morphology

Stomatal length, breadth and aperture breadth responded to NaCl treatment from the first hour on (Table 2). Generally, parameter-values of stomatal morphologies declined with increase of concentrations of NaCl treatment, but most values in the 50 mM treatment did not show significant difference from the control treatment. Stomatal aperture density did not differed among treatments until 24 h after NaCl stress, when density declined significantly in the 150 mM and 250 mM relative to the control. *PCI* results showed the similar trend as those descried before, but 24 h after NaCl stress was higher in the 150 mM than the 250 mM treatment.

As it is presented in Table 3, Pearson correlation results indicated that most stomatal morphological parameters had a negative relationship with NaCl concentration except for the aperture density. In contrast, most parameters did not have any correlation with treating hour also except for the aperture density either. However, this parameter had a positive relationship with treating hour.

Discussion

As contributions to the scientific meaning, high level of salt stress in a short term was highlighted for alfalfa plants in the present study. For concerns of the short term issue, nearly all current studies were conducted with the aim to test the plant response to NaCl at least a week time after the commencement of salt stress. Hence, these studies did not concern the situation of short term impact of salt stress on

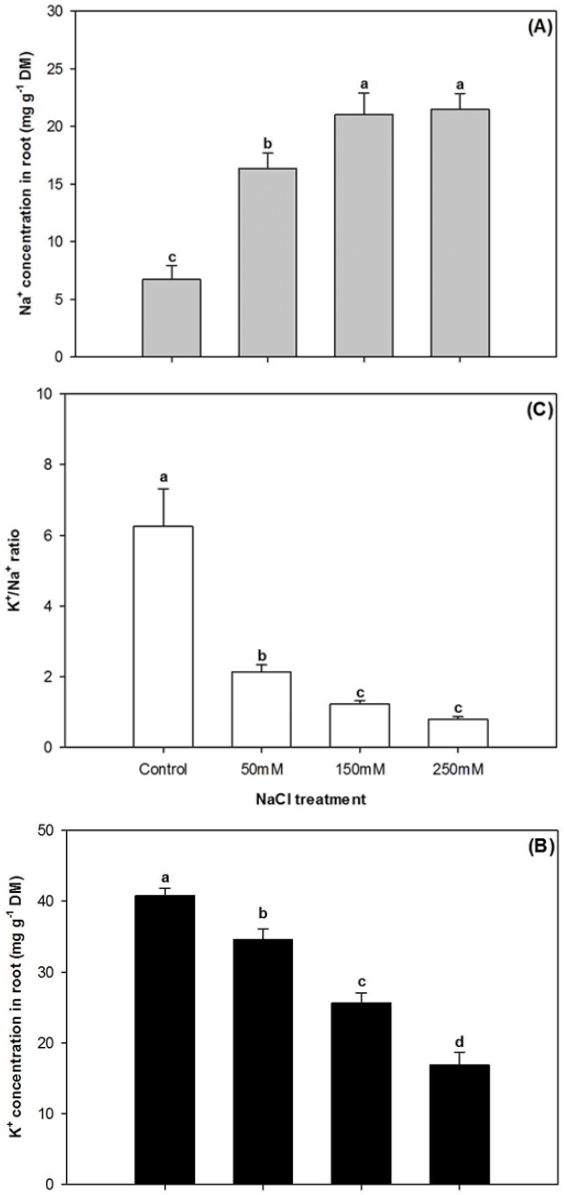

Fig. 2: Root accumulations of Na^+ (A), K^+ (B), and K^+/Na^+ ratio (C) in alfalfa Gongnong No.1 subjected to NaCl treatments of concentrations at 0 (Control), 50 mM, 150 mM, and 250 mM. Different letters indicate significant differences according to LSD test at 0.05 level

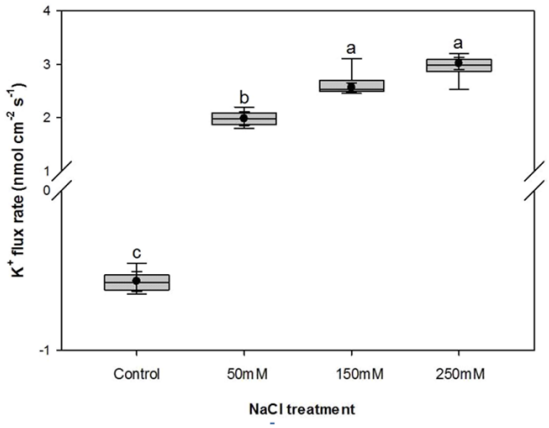

Fig. 3: Box-whisker plots of K^+ flux rate in alfalfa Gongnong No.1 subjected to NaCl treatments of concentrations at 0 (Control), 50 mM, 150 mM, and 250 mM. Boxes indicate percentiles ranged from 15% to 75%. Bars indicate scattered plots between maximum and minimum values. Dots indicate mean values. Different letters indicate significant differences according to LSD test at 0.05 level

alfalfa in hours even for those determining transgenic approaches, such Tang *et al.* (2013) and Wang *et al.* (2014). Results from a longer term observation from days to weeks would probably be misled by recoveries from Na^+ toxicity for the second time, or even the third time after salt stress (Hernández and Almansa, 2002). On the other hand, for concerns of the issue of high-level salt stress, quite few studies have taken this as their central research target. For example, as it was shown in Table 1 nearly all summarized studies were conducted in laboratory but only 25% of them performed NaCl concentrations higher than 250 mM, leaving most NaCl concentrations ranged around 150 mM which accounted the number of studies for 58% of all. Insufficient studies about high level of NaCl stress may result in an unmatched ecological implication between laboratory works and real-site conditions.

As we hypothesized, growth of alfalfa Gongnong No. 1 declined 24 h after salt stress, symptomized as wilted shoot erection and reduced root proliferation. This concurs with findings of juvenile seedlings of fig (Golombek and Lűdders, 1998) and jute (Chaudhuri and Choudhuri, 1998). Declined growth of alfalfa was caused by depressed photosynthesis through decrease of shoot biomass production (Fig. 1C). A little surprisingly, both measures of root length and shoot biomass production revealed insignificant differences between the control and the 50 mM treatment, which suggested that in regions where alfalfa Gongnong No. 1 is distributed, such as Sanjiang Plains, depressed performance of alfalfa Gongnong No. 1 under low-level salt stress of 50 mM NaCl may not be generated in a short term of hours (Peng *et al.*, 2008). In contrast, synchronous declines of seedling height, root length, and shoot biomass production in the 150 and 250 mM treatments presented a compensatory explanation of declined growth of alfalfa Gongong No. 1 to month-long term study in Peng *et al.* (2008), which was a short-term result caused by high-level salt stress of higher than 150 mM NaCl.

On basis of confirmed decline of growing status in our study, the explanation of two phases of mechanism analysis put forward by Munns (2002) was also capable for alfalfa. After salt stress treatment was commenced, an osmotic gradient of Na^+ was established in 24 h, hence Na^+ accumulation in root immediately showed straightaway

Table 2: Responses of stomatal characteristics (μm) to treatments of Control (0 mM), 50, 150, and 250 mM NaCl, determined at the start of the experiment and 1, 2, 3, and 24 h later of the start

NaCl Treatment	Time of treatment (h)				
	0	1	2	3	24
Stomatal length/ breadth ratio					
Control	13.1a/10.2x	13.0a/10.2x	12.9a/10.3x	13.1a/10.1x	13.1a/10.2x
50 mM	13.1a/10.2x	12.7ab/7.7y	12.4ab/6.4y	11.9b/5.4y	12.6ab/8.3y
150 mM	13.1a/10.2x	12.0bc/6.4z	11.0bc/5.2yz	9.7c/4.4y	11.7b/6.8yz
250 mM	13.1a/10.2x	11.7c/5.8z	10.3c/4.8z	9.7c/3.0z	9.8c/5.3z
Stomatal aperture breadth (μm)/ density (No. mm^{-2})					
Control	2.8a/170x	2.6a176/x	2.2a/170x	1.7a/173x	2.9a/172z
50 mM	2.8a/170x	2.5a/176x	1.8b/176x	1.3b/179x	2.3b/198yz
150 mM	2.8a/170x	1.4b/176x	0.9c/177x	0.7c/178x	1.5c/205xy
250 mM	2.8a/170x	1.1b/173x	0.7c/173x	0.5c/175x	0.9d/217x
Potential conductance index (μm^2 cm^{-2})					
Control	291.74a	297.44a	282.90a	296.89a	295.17a
50 mM	291.74a	283.87a	270.62a	253.48b	314.34a
150 mM	291.74a	253.44ab	214.17b	167.48c	280.62a
250 mM	291.74a	238.63b	184.73b	165.79c	206.99b

Note: Different letters of a, b, c and x, y, z indicated significant differences for a column of results in response to the four NaCl treatments

responses to NaCl treatments (Fig. 2A). As a comparing meaning, short-term hyper-accumulation of Na^+ subjected to 24 h of salt stress was also observed on vegetative and woody plants (Chaudhuri and Choudhuri, 1998; Hernández and Almansa, 2002; Sun *et al.*, 2009). Our results compensated the alfalfa response of Na^+ accumulation to former studies at the species-specific level, meanwhile confirmed the potential Na^+ toxicity by hyper-accumulation in dicotyledon. According to Hernández and Almansa (2002) over-loaded Na^+ in a stressed plant can be diluted in the second 24 h after salt stress, which has been confirmed in pea leaves. Thereafter, short-term Na^+ toxicity harms caused by hyper-accumulation may also be reversible in alfalfa, in spite the mediating approach and manipulation need more work to confirm.

As a micro-scale response to Na^+ stress at about ~200 mM of NaCl level (Maathuis and Amtmann, 1999; Flowers and Hajibagheri, 2001; Britto *et al.*, 2010), a definite K^+ efflux was confirmed by NMT for alfalfa (Fig. 3). Surprisingly, K^+ flux rate did not change between the high levels of salt stress at NaCl rates of 150 mM and 250 mM (Fig. 3), but root K^+ leachate was intensified in the high level of Na^+ stress of 250 mM compared to that of 150 mM (Fig. 2B). This contradiction was caused by declined root length in high levels of salt stress (Fig. 1B) which stimulated K^+ efflux cm^{-2} when K^+ efflux rate was unchanged. Negative values in the controlled alfalfa illustrating K^+ influx rate ranged from 0.3 to 0.6 nmol cm^{-2} s^{-1} with a mean of about 0.5 nmol cm^{-2} s^{-1}, which was comparable with transient results of non-inoculated roots in poplar (Ma *et al.*, 2014) and untreated roots in Arabidopsis mutants (Shabala *et al.*, 2005) but was much lower than those in salicylic acid treated Arabidopsis roots (Jayakannan *et al.*, 2013). Hence, when detecting K^+ flux rate under the condition without salt stress, homogenous results seemed to be obtained under the similar condition, but had quite little influence on plant species.

Studies have fully confirmed that the main mechanism

Table 3: Two-tailed *Sig.* values from Pearson correlations between stomatal morphologies of length, breadth, aperture breadth, density, and potential conductance index (PCI), and concentration of NaCl, or and treating hours

Stomatal parameters	NaCl concentration	Treating hour
Stomatal length	-0.647**	-0.14
Stomatal breadth	-0.611**	-0.043
Aperture breadth	-0.582**	-0.02
Aperture density	0.288	0.754**
PCI	-0.620**	0.112

Note: Data with different letters in the same column are significantly different at 0.05 level

to cope with mediating salt stress can be referred to as controlling the uptake of Na^+ and maintaining the K^+/Na^+ balance (Shi *et al.*, 2002; Shabala *et al.*, 2013; Amjad *et al.*, 2014). As an interactive result of K^+ efflux and hyper-accumulation of Na^+, ratio of K^+/Na^+ in alfalfa Gongnong No. 1 declined remarkably in high level of NaCl treatments of 150 mM, which was not statistically different from that of 250 mM (Fig. 2C). This was mainly resulted in by Na^+ accumulation, but partly impacted by K^+ concentration declines in high levels of NaCl treatments, although alfalfa Gongnong No. 1 is evaluated to be a moderate salt-tolerant cultivar. The unbalanced Na^+-K^+ relationship presented a severe maladjustment of alfalfa Zhongmu No. 1 to high level of salt stress at concentrations higher than 150 mM. Declined K^+/Na^+ ratio was also a result of a relative failure of competition for binding sites of K^+ on the PM which were occupied by Na^+. Efflux of K^+ and decreased K^+ accumulation in xylem were responsible for weakened stomatal conductance and subsequently pressed photosynthesis. Therefore, according to our results about K^+/Na^+ ratio, the sensitivity of this cultivar to salt stress may be need to be re-considered, at least at a short term scale.

Negative relationships between stomatal morphologies and NaCl concentration (Table 3) suggested a clear response of stomatal closure to salt in a short term for alfalfa

Gongnong No. 1. This response has also been confirmed by many former studies on other plants (Paz *et al.*, 2014; Amjad *et al.*, 2014; Xu *et al.*, 2015). Stomatal closure partly explained our conjecture of declined photosynthesis presented as declined growth due to pressed CO_2 exchange. Surprisingly, aperture density had no relationship with NaCl concentration. These findings, together with the positive relationship between aperture density and treating hour, explained heterogeneity of stomatal conductance at the space scale (Xu *et al.*, 2015). At larger scales, the heterogeneity of gas exchange resulting from variations of stomatal number and size over the plant leaf (Weyers and Lawson, 1997; Mott and Buckley, 1998) was actually originated from short term out-sync; in contrast, at smal scales the gas exchange parameters led to by patchy stomatal closure (Mott and Buckley, 1998, 2000) was a result of time dynamic in hours. Specially, *PCI* has been well established as a sensitive parameter to quantify leaf conductance (De Costa *et al.*, 2000; Batos *et al.*, 2010). Its negative relationship with NaCl concentration suggested a potential risk of short term salt stress on conductance, therefore a short term threat of water stress can be one of causes contributing to salt stress. Null correlation between *PCI* and time was partly attributed to the participation of aperture density, which failed to be correlated with NaCl concentration but positively related to time. All results we described above can be concluded as a representation of integrated stomatal responses to short term NaCl, which revealed that: salt induced stomatal aperture to be closed, or to show chosing-trends, but more apertures were also induced to be involved in, hence potential stomatal characteristics did not show any clear responses in the first hours after salt stress.

Conclusion

Our study highlights the importance of high level of salt stress on alfalfa. Also, we compensate current evidence using alfalfa at the species-specific level in a short term of 24 h after salt stress. With the increase of NaCl level, height slightly decreased while root length and shoot biomass production showed dramatic declines in 24 h after salt stress. Na^+ accumulation increased with salt stress level, but results between the 150 mM and 250 mM levels were not statistically different, neither were results about K^+/Na^+. The use of NMT enables precise measure on K^+ flux, which was formed as influx at a rate of ~0.5 nmol cm^{-2} s^{-1} under the condition without salt stress and formed as efflux at rates from 2 to 3 nmol cm^{-2} s^{-1} under high levels of salt stress of 150 and 250 mM. The relationship between stomatal morphologies and NaCl concentration or treating time supplies a deeper thinking about stomatal behavior. For the species of alfalfa Gongnong No. 1, its early performance 24 h after high level of salt stress at concentrations higher than 150 mM did not perform well. Therefore, use and development of this cultivar in severe salinity conditions needs to screen for more generations or to employ new approaches, such as salt-tolerant functional gene over-expression.

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