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IDENTIFICATION OF SHORT-TERM Na^+ SECRETION IN SALT TOLERANT CELL LINE FROM ALFALFA CALLUS CULTURES SELECTED ON HALF-NATURAL SEAWATER MEDIUM

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Abstract

Seawater irrigation is critical for coastal agriculture development, but key mechanism of root-ion flux in the salt-tolerance of callus-cultured plant lines in response to seawater remains to be unclear. In this study, hypocotyl-calluses of *Medicago sativa* L. cv. Gongnong No.2 were cultured to generate the parent line of M_0, ten passages after which salt tolerant line of EMS-induced Ms_1 was screened by half-natural seawater (collected from Boshi Bay in Dalian City, Northeast China). Regenerated seedlings from M_0 and Ms_1 were compared for their difference of salt tolerance to seawater proportions of 50%, 30%, 10%, and 0% (Control). Factors of different callus-cultured lines and seawater treatment did not have any interactive effect on shoot height (length) and Na^+ accumulation, stomatal conductance (SC), foliar chlorophyll content. For both lines, seawater treatments at lower proportions of 10% and control tended to have more root length, shoot biomass, and stomatal density (SD), but less MDA and proline contents. Compared to seedlings from the M_0 line, relatively higher tolerance to salt of those from the Ms_1 line mainly resulted from less investment of dry-mass to root, declined SD and SC, controlled shoot Na^+ accumulation, but greater chlorophyll content and membrane-protective ability. Results obtained by Non-invasive micro-test technique (NMT) indicated that in the first 1.5 min net Na^+ flux in roots in the Ms_1 line showed highest rate and significant extrusion pattern, while some net Na^+ influx occurred in the M_0 line. In conclusion, seawater irrigation has some potential to be used in the culture of coastal alfalfa development through screening salt-tolerant callus-cultured lines. Root Na^+ efflux is a critical parameter determining their resistance to seawater salinity.

Key words: Genotype selection, Cell-cultured line screening, Salt sensitive, Ocean resource, Saline soils.

Introduction

Surface water is currently the main water source for agricultural uses in most planet regions due to its easy access and generally low cost (Cazalé *et al.*, 2009). Due to the pressure of economy and population growth on available water resource, surface water has be threatened by salinization following seawater intrusion (Fu *et al.*, 2009; Hartl *et al.*, 2011). Both of population growth and sea-water eruption seawater resulted in the pressure of fresh water erosion in coastal area, where live about 50% of the world's population (Shabala & Pottosin, 2014). As a result, seawater was suggested to be used as an alternative watering source for agricultural irrigation (Duan *et al.*, 2011).

Sea water Thus, the usage of seawater as irrigation source has been suggested to implement through desalination (Shi & Theg., 2010; Kim & An., 2013; Kumar *et al.*, 2016), but the issues of energy consumption and additive fresh water employment have yet been well resolved. Instead, to cultivate salt-tolerant plants has more practical meaning. The physiological response of salt-tolerant plants from callus culture to salt stress has been studied for several times, which were also referred to as the evidence of some given gene expressions to improve salt tolerance in recently published reports (Deng *et al.*, 2015; Hu *et al.*, 2012). Our knowledge about physiological performance of callus-cultured plants in response to seawater is mainly concentrated in foliar organs, but information about physiological changes in their root system is quite scarce (Chen *et al.*, 2006).

Under salt stress, Na^+ is the primary component imposing ionic toxicity on plants, which causes more severe damage to photosynthetic apparatus than osmotic pressure (Yarra *et al.*, 2012). During this damage process, Na^+ disrupts ion homeostasis with the generation of excess reactive oxygen species (ROS) (Zhu *et al.*, 1998). As an active response, plasma membrane (PM)-located Na^+/H^+ antiporters and vacuolar membrane (VM)-located Na^+/H^+ antiporters carry out active Na^+ extrusion from cytosol (Chen *et al.*, 2005). Recently, it was proved that the evolvement of a set of antioxidant system to scavenge ROS had some relationship to PM Na^+/H^+ antiporter (Yadav *et al.*, 2011). All these conclusions together suggest that salt-tolerance was generated through either the adjustment of root Na^+ uptake (Barragán *et al.*, 2012) or the stimulation of root Na^+ extrusion. In callus-cultured plants, traditional approach of identifying the activity of the PM H^+-ATPase has indirectly proved the potential Na^+ extrusion from roots. In addition, the non-invasive micro-test technique (NMT) enables the precise quantification of Na^+ efflux from root cells. To our knowledge, few studies have supplied direct evidence about Na^+ efflux from roots of callus-cultured plants using NMT. This is worthy to be elucidated because the of discharging Na^+ can strengthen salt-tolerance in plants to seawater through callus culture selection.

Alfalfa is an important forage crop with high values and admissive suitability to be utilized in semi-arid environments where limited rain or irrigation water is available. The increasing fresh water scarcity drives the saline water to be an alternative source of agricultural irrigation for the alfalfa culture (Kumar *et al.*, 2016). General saline irrigation for alfalfa includes drainage waters (Bonza & Michelis, 2011) and brackish ground waters (Bertrand *et al.*, 2015) from saline water table. Due to the ability of alfalfa to produce forage in saline environments, its forage production was compared with some other halophytes irrigated using seawater (Cornacchione & Suarez, 2015). These comparisons arouse an interest of the possibility to irrigate

alfalfa using seawater according to the reality that some natural alfalfa communications inhabit within coastal regions and work as the main forage feeding livestock (Dong, *et al.*, 2011). Hence, theoretical work needs to focus on the available approach to enable alfalfa to establish with seawater. Screening the callus-cultured generations may be an applicative way to improve resistance of alfalfa to seawater salinity. Root ion flux characteristics need to be identified to reveal the core determination of alfalfa grown with seawater salinity.

Coastal agriculture contributes to a large proportion of the whole agricultural system in China, whereas 20 million ha of mud flats distribute along 18 thousands km of coastal areas (Aldesuquy *et al.*, 2013). Due to the limit of irrigating with seawater to salt-tolerant crops, current irrigation of coastal agriculture is still performing the fresh water transported form inland, or most coastal lands may have to be tuned barren (Ashrafi *et al.*, 2015). Dalian is a moderately developed coastal city in Northeast China, where the use of seawater in water-saving agriculture has ever been determined (Endo *et al.*, 2014). Alfalfa Gongnong (*Medicago sativa* L. cv. Gongnong) is a widely distributed in Northeast China (Jiang *et al.*, 2011), and it is sensitive to high-level of salinity in regional soils (Flowers, 2004). In the present study, the alfalfa cultivar Gongnong was employed as the research material for screening salt-tolerant cell lines from callus cultures by half natural seawater medium. The objective is to identify the physiological mechanism of advances in salt resistance of callus-generated mutant compared to parent plants. Specially, the short-term Na^+ flux characteristics were measured using NMT between the mutant and parent plants. It was hypothesized that: relative to the parent plants under salt stress, seawater-screened salt-tolerant mutant (i) obtained better growth but showed prospective transpiration performance, and (ii) had higher rate of short-term Na^+ efflux out of root cells with less foliar Na^+ accumulation at a longer term.

Materials and Methods

Seawater sampling: Seawater samples were collected in the ocean area about 2 km away from shore-sides at Boshi Bay (39º03'41" N, 121º78'22" E), along the southern coast from the west towards east of Jinzhou District. Totally 20 samples were collected in two round-trips at the speed of 15 km h^{-1} (Fig. 1). Seawater in the sampling area may have received the least contaminations caused by fast industrial development in Dalian in recent years. Each seawater sample was dredged at the depth of about 1.5 m beneath the sea-surface by a 1-L pot; thereafter at least 600 ml seawater was collected and sealed for one time in plastic bottles and stored at 0-4ºC until determination. Seawater salinity was determined by an ultraviolet (UV) spectroscopy method with the UV absorption in the 190-250 nm specifically described by DiNoto and Mecozzi. The averaged salinity level was determined to be 2.98% and each of the seawater samples was determined to be 2.99%, 2.99%, 2.97%, 2.98%, 2.98%, 2.98%, 2.98%, 2.98%, 2.99%, 2.98%, 2.98%, 2.98%, 2.98%, 2.98%, 2.99%, 2.98%, 2.98%, 2.99%, 2.98%, 2.98% in sampled seawater. The osmotic potential was comparable to NaCl at concentration of 3.0 % (~500 mM) in extracts from saline soil solutions. According to (Peng *et al.*, 2008), this seawater salinity level equals about 4.2 times of the highest salinity stress level in soils naturally distributed alfalfa in Northeast China.

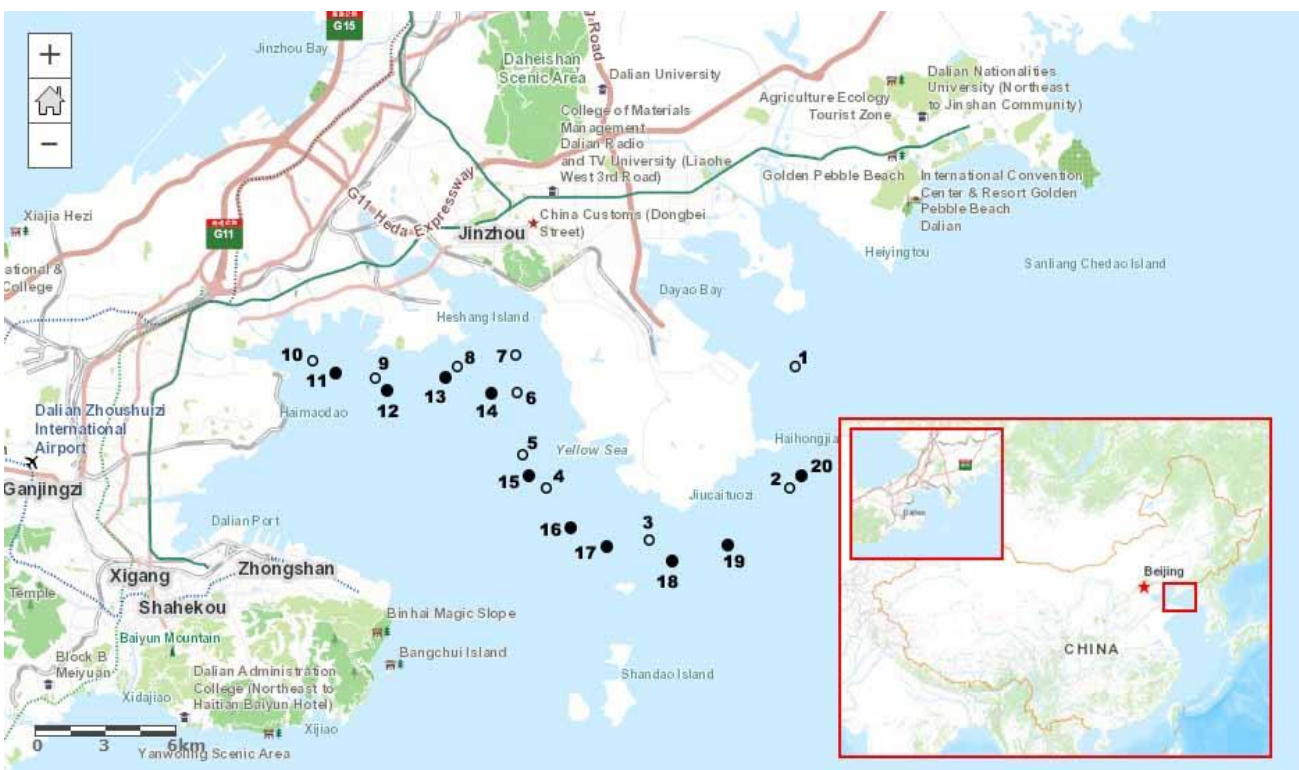

Fig. 1. Seawater sampling points in Boshi Bay, Jilin Zhou District, Dalian City, China. Dots with different styles indicate sampling trip-routes. Maps are made by screen shots from the open website of ArcGis® on-line (https://www.arcgis.com/home/).

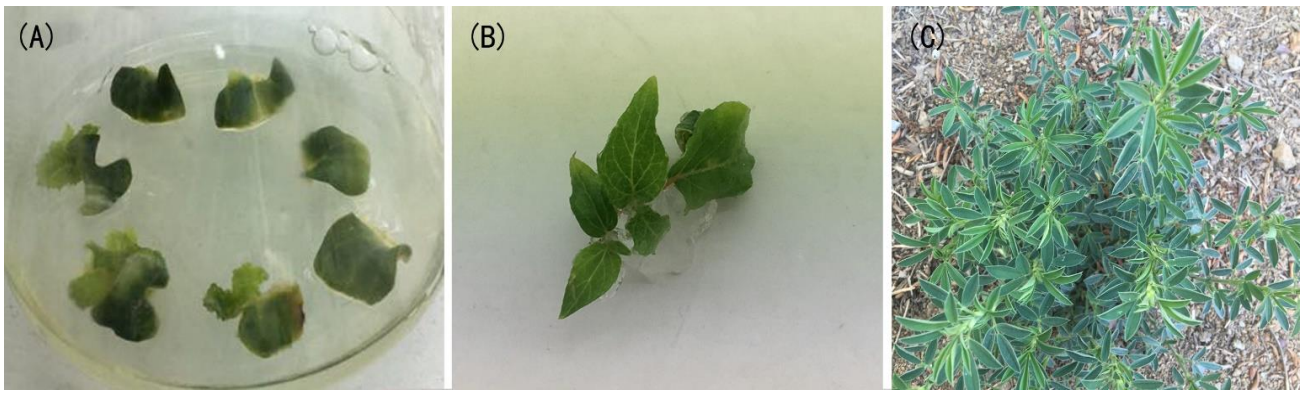

Fig. 2. Growth dynamic of salt tolerant callus-cultured alfalfa lines (Ms_1) from callus tissue transferred to sterile MS supplemented with half-natural seawater (A), through the formation of mutant lines (B), to transplanted plantlet of 3rd generation (C).

Plant materials and callus generation: Seeds of alfalfa Gongnong No.2 (*M. sativa* L. ssp. *varia* Gongnong No.2) were provided by Institute of Grassland Sciences, Jilin Academy of Agricultural Sciences, Gongzhuling Town, Changchun City, Jilin Province, China. Seeds were immersed in 70% ethanol for 1 min and rinsed by sterile water for three times. After surface-sterilized with 0.1% $HgCl_2$ for 20 min, seeds were rinsed using distilled water for five times. After germination on hormone-free MS (Flores, *et al.*, 1962) for 10 days, hypocotyls were excised into sections of 5 mm in length and transferred onto salinity-free MS medium supplemented with 9.05 μM of 2,4-D and 27 μM of 6 BA. Callus derived from the excised hypocotyls four weeks later when they were maintained on the same medium for the sub-culture at 21 days intervals. Totally, four sub-cultures were conducted in all under the condition at temperature of 26±1 ºC and illumination of 14 h per day with light intensity of 2000 lux supplied by four 200-W plant growth lamps (Oudi® Illumination™, Huzhou, Zhejiang, China).

Screening for salt tolerant lines: Four sub-cultures later, the 1/3 of all callus lines were sustained to be cultured on salinity-free MS medium supplemented with 9.05 μM of 2,4-D and 27 μM of 6 BA. These were labeled as the parent line (M_0). The left 2/3 of callus lines were treated with ethyl methyl sulfonate (EMS) at 25ºC for 24 h, rinsed with sterile water for 5 times, then transferred to another sterile MS supplemented with half-natural seawater obtained by concentration dilution ([NaCl] ~250 mM) (Fig. 2A). After four sub-cultures the line was labeled as 1st Mutant (Ms_1) (Fig. 2B), the left lines were continued to be sub-cultured for 24 times and transferred to salinity-free MS for the next 10 sub-cultures. Finally, the last lots of lines were labeled as 2nd Mutant (Ms_2). Throughout the whole sub-cultural process, condition was maintained to be the same one as described above.

All three callus-cultured lines (M_0, Ms_1, and Ms_2) were cut into sections of 1 cm in length for the screening of salt tolerance. For each line, 500 sections were randomly distributed into five replicated groups with 100 sections for one group. Two experiments were conducted to validate screening. In the first experiment, 500 sections from each line were cultured on sterile MS supplemented with half-natural seawater ([NaCl] ~250 mM). In the second experiment, survived lines from the 1st experiments were tested for survival at stepwise increased proportions of seawater concentration([NaCl] 300 mM, 350 mM, 420 mM, 450 mM, and 500 mM), until survival showed significant difference among left lines. Finally, two callus-cultured lines with contrasting salt-tolerances were selected to be confirmed through screening.

Screened seedling culture and treatment: Screened lines were cultured for continuous two generations, after which seeds of the third generation were sterilized with 0.5% sodium hypochlorite solution for 20 min, rinsed, and sown in germination trays filled with acid-washed sands (Fig. 2C). All trays were placed in the laboratory of Environment and Resources College, Dalian Nationalities University (39º02'N, 121º46'E). Seeds were germinated in the dark at 25±1ºC with 45~60% of relative humidity. When the intact root system emerged for each of 80% germinated seedlings, rooted seedlings were transplanted into plastic pots (top diameter × bottom diameter × height, 18 cm × 15 cm × 13 cm) filled with salinity-free sands. All seeds were washed by acid twice and rinsed by distilled water before used. Seedlings were cultured at temperature of 25/20ºC with a photoperiod of 11h/13h for day and night, respectively. Light was supplied by plant growth lamps (Oudi® Illumination™, Huzhou, Zhejiang, China) to maintain a photosynthetic photon flux density of 450 μmol m^{-2} s^{-1} measured at apical tip of seedlings. Nutrient solutions were supplied every one or two days depending on daily weather. Nutritional composition was adapted from Jiang *et al.* (2011). Briefly, mineral concentrations therein were: NH_4NO_3 4 mM, 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. In general, sand-cultured seedlings were watered with 400 ml of solution per pot per day.

The seawater treatment started 10 days after seedling transplanting. Seawater used for treatment was also collected from the same sea area (Fig. 1). A split block design was performed, wherein the main blocks were two screened lines (n=2, df=1) with contrasting salt-tolerances and the sub-blocks were different doses of diluted seawater treatment. Four seawater treatments were involved in all (n=4, df=3), which were differentiated by equivalent NaCl concentration. The maximum dose of treatment was set to employ half-natural seawater with equivalent 250 mM NaCl therein. The second maximum dose of treatment was set to employ the maximum level of NaCl concentration in soils of Songnen Plains of Northeast China (Peng *et al.*, 2008), which is 30% of

seawater concentration and equals 150 mM NaCl. The third dose of treatment was set to be moderate and proper for alfalfa growth with 10% of seawater concentration equaling 50 mM NaCl. Also involved was an untreated control. Ten pots were bulked as one replicate for one interactive treatment (lines × seawater doses), wherein six seedlings were planted in one pot. Totally, there were 80 pots and 480 alfalfa seedlings employed for physiological and biochemical analysis. Sixteen days after seawater treatment commencement, all seedlings were harvested and used for future determination.

Growth measures and physiological and biochemical analysis: Two seedlings from one pot were used for growth measures. Seedling height was measured for the length from root collar to the apical tip. Washed root morphologies were measured using Delta-T Scan® Software (Delta-T Devices Ltd., Cambridge, England) coupled to a flatbed scanner (HP ScanJet 4c, Hewlett-Packard Co., USA) with a transparency adapter (HP Transparency Adapter, Hewelett-Packard). Measured seedlings were cut into two parts of shoot and root, which were subsequently oven-dried at 70 ºC for 2 d. Dried samples were measured for dry weight. Oven-dried samples were powered using a mortar and pestle. Approximately 10 mg powder was digested in 1 M HCl over-night. Deposited samples were spun for 3 min at 11,000 rpm. The concentrations of Na^+ in the digested samples were determined using a flame photometer (6400A, Shandong, China).

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. Leaves were sampled and washed to clear leaf surface. Cleared 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 & Systeme GmbH, Wetzlar, Germany) with an associated camera (Leica DFC 420). For measuring stomatal density (SD), a grid of 1 mm^2 was randomly superimposed. Stomatal conductance (SC) was measured on the center of fully expanded leaves at an irradiance of 180 μmol m^{-2} s^{-1} with a diffusion porometer (Model VP-2000, Cayuga Development Research, Instruments, NYC, USA). Chlorophyll content was non-destructively assessed through the average of measures on light-green, green, and dark-green leaves with a chlorophyll meter (SPAD-502).

Lipid peroxidation was evaluated by measuring MDA content using a modified thiobarbituric acid (TBA) method (Glenn *et al.,* 2014). Approximately 0.1 g of dried leaf tissues were ground in 10 ml of 10% trichloroacetic acid (TCA) using a mortar and pestle. The homogenate was centrifuged at 10,000 rpm for 20 min. The reaction mixture containing 2 ml of extract and 2 ml of TBA was heated at 95ºC for 30 min, quickly cooled on ice, and then centrifuged again at 10,000 rpm for 20 min. The absorbances at 450,532 nm and 600 nm were determined using an ultraviolet spectrophotometer (UV-2550, Shimadzu, Japan).

Proline content was determined with fresh leaf material (500 mg), which was extracted with 5 ml of 3% sulfosalicylic acid at 100ºC for 10 min with shaking. The extracts were filtered through glass wool and analyzed for proline content using the acid ninhydrin method. Briefly, 2 ml of the aqueous extract was mixed with 2 ml of glacial acetic acid and 2 ml of acid ninhydrin reagent (1.25 g of ninhydrin, 30 ml of glacial acetic acid and 20 ml of 6 M orthophosphoric acid) and heated at 100ºC for 30 min. After cooling, the reaction mix was partitioned against toluene 4 mL) and the absorbance of the organic phase was determined at 520 nm. The resulting values were compared with a standard curve constructed using known amounts of proline (Sigma, St Louis, MO, USA).

Root Na^+ flux measures using NMT: Net Na^+ flux was measured by YoungerUSA (Xuyue, Beijing, China) NMT Service Center using a non-invasive micro-test technique (NMT-YG-100, YoungerUSA LLC, Amherst, MA01002, USA) with ASET 2.0 (Sciencewares, Falmouth, MA02540, USA) and iFluxes 1.0 (YoungerUSA, LLC, Amherst, MA010002, USA) software. After backfilling, electrode tips were filled with commercially available ionophore cocktails. 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). Immediately after seawater addition, root segments with apices of 1 to 2 cm were sampled by excising and used for steady-state measurements of net Na^+ 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 Na^+ 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 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 (Fig. 3).

Na^+ 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 (Na^+ was 0.5 mM in the measuring buffer). The Na^+ 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 Na^+ ions in alfalfa roots were usually in the range of several minutes. Therefore, Na^+ fluxes were recorded for 8 to 10 min at each point, which is long enough to ensure the absence of oscillations.

Statistical analysis: The callus survival rate (%*CSR*) was calculated as:

$$\%\ CSR = \text{number of survived callus sections} / 100$$

where number of all callus sections was 100 for all screening experiments. The Na^+ ion flux rate was calculated using Fick's law of diffusion:

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

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.

Parameter of seedling growth and physiological and biochemical parameters were analyzed for their responses to effects of the callus-cultured lines (L) and seawater treatments (T) using a two-way ANOVA analysis with the GLM procedure in SAS software (SAS Institute Inc., NC, USA). When ANOVA indicated the significant interaction of L × T, results were arranged following all component treatments (t=8, n=10) according to LSD test at the 0.05 significance-level; when no interaction was found, main effects of L and T were analyzed separately (n=20). Due to the null effect of T, net Na^+ flux results were only compared between lines (t=2, n=40) for each investigating point. Due to the possibly scattered result points throughout the NMT measurement, overall NMT results between the two lines were compared with box-whisker patterns, which was calculated and graphed by Sigmaplot V12.0 (© LEADTOOLS, © DUNDAS SOFTWARE LTD., © wpcubed GmbH, Germanry).

Results

Screening for the salt-tolerant lines: In the first screening experiment, a half day time after callus culture, *CSR* of sections from the M_0 lines declined to be almost 50%; then 1 d later, the *CSR* declined to be only less than 20%, while the *CSR* for Ms_1 and Ms_2 was calculated to be 74% and 56%, respectively, which were higher than that of M_0 lines (P=0.0034). However, difference between Ms_1 and Ms_2 was not statistically different. After one more sub-culture, all M_0 line sections died out, but CSR for Ms_1 and Ms_2 did not show any statistical changes. Therefore, salinity concentration in the half-natural seawater was confirmed to be the critical level for the differentiation between survivals of M_0 and Ms_1 or Ms_2.

In the second experiment, Ms_1 and Ms_2 were firstly compared for *CSR* under the 60% seawater. Although the CSR for Ms_1 was almost higher by a half fold than Ms_2, difference failed to pass statistical significance (Data not shown). However, under the 70% seawater treatment, *CSR* for Ms_1 was 26.57%, while that for Ms_2 was only 15.04%, the difference between which passed significance test (p<0.0001).

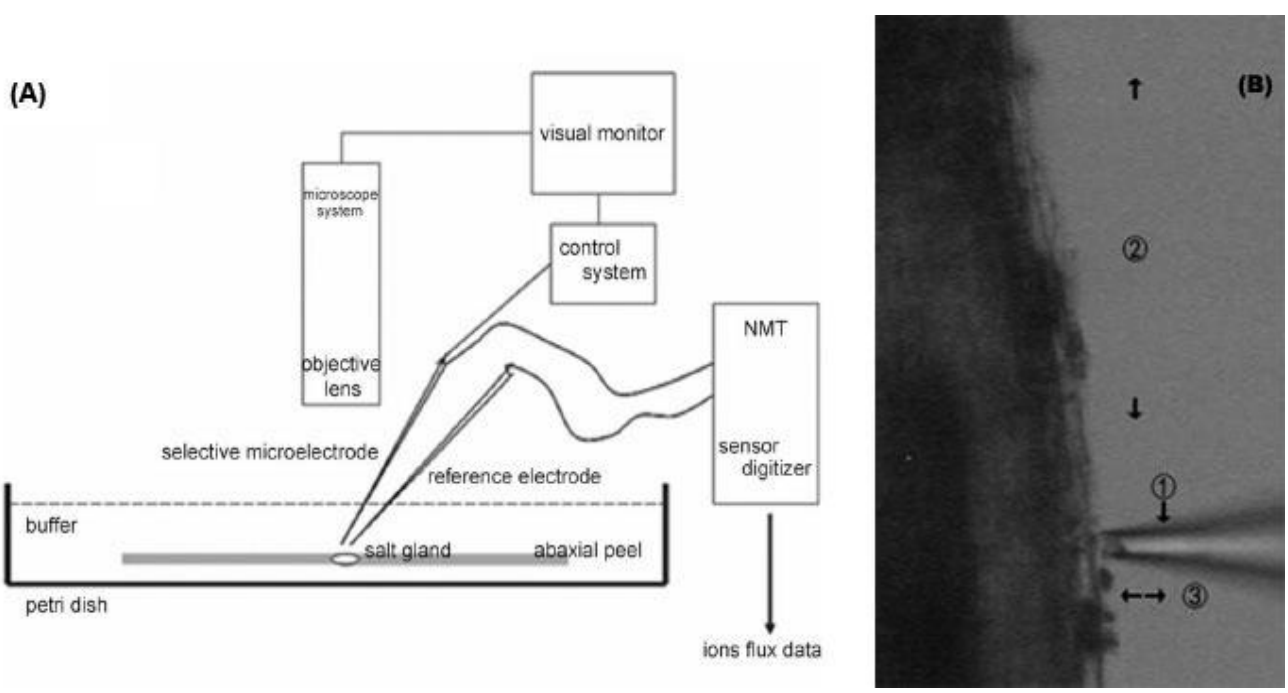

Fig. 3. Schematic diagram of NMT (non-invasive micro-test technology) system for measuring Na^+ flux in roots of callus-cultured alfalfa lines exposed to seawater treatment. (A) The diagram showing the system used to measure selective ion flux from using a non-invasive ion-selective electrode. (B) A micrograph of electrodes (①) (locating at 500 μm from the root apex ②) movements between the two measuring positions (③) on the root surface.

Table 1. *F* values from ANOVA analysis of different callus-cultured alfalfal lines (C), four-leveled seawater treatment (T), and their interaction (C×T) on tested parameters.

Source of variation	*df*	Tested parameters		
		—Seedling growth—		
		Height	Root length	Shoot biomass
C	1	**215.24*** **	**45.99*** **	**112.66*** **
T	3	**298.61*** **	**117.33*** **	**153.11*** **
C×T	3	2.44	**6.14** **	**4.77** **
		—Functional anatomy—		
		Chlorophyll content	Stomatal density	Stomatal conductance
C	1	**4.79***	**95.19*** **	**32.47*** **
T	3	**506.80*** **	**276.66*** **	**950.05*** **
C×T	3	0.52	**9.77*** **	0.46
		—Physiology and biochemistry—		
		Foliar Na^+ content	MDA content	Proline content
C	1	**3.60***	**146.85*** **	**85.76*** **
T	3	2.10	**191.44*** **	**272.65*** **
C×T	3	1.80	**29.49*** **	**10.57*** **

Height, root length, and shoot biomass: No interactive effects of callus-cultured lines and seawater treatment were detected on seedling height (Table 1). Seedling height of the Ms_1 line was measured to be 14.57±1.16 cm, which was greater by 3.67% than the M_0 line (14.06±1.16 cm) (p<0.0001). Both root length and shoot biomass was greatest in the M_0-10% treatment, followed by the controlled M_0 line (Fig. 4). Root length and shoot biomass in the Ms_1 line declined by 4% and 3.8% than in the M_0 line, respectively (Both p<0.0001, data not shown).

Chlorophyll content and stomatal performance: Neither chlorophyll nor SC responded to the interaction of callus-cultured lines and seawater treatment (Table 1). Compared to the M_0 line, chlorophyll content was greater by 6.25% (p<0.0001) while SC declined by 5.45% (p<0.0001) in the Ms_1 line (data not shown). Generally, SD in the Ms_1 line was about 160 mm^{-2} (Fig. 5A), while that in the M_0 line was more than 180 mm^{-2} (Fig. 5B) (p<0.0001). The highest SD was found in the controlled M_0 line, followed by the controlled Ms_1 line (Fig. 5C). The lowest SD was found in the Ms_1 and M_0 lines treated by 50% seawater.

The interaction of callus-cultured lines and seawater treatment did not have any significant effect on foliar Na^+ content (Table 1). Instead, significant difference occurred between the main effects of callus-cultured lines. Compared to the M_0 line, foliar Na^+ content declined by 46% in the Ms_1 line (p=0.0238) (30.63±41.53 mg g^{-1} and 16.64±5.62 mg g^{-1}, respectively). Both MDA and proline contents showed a general increase with the proportion of seawater, and both were highest in the callus-cultured lines treated with 50% seawater (Fig. 6A, B). Compared to the M_0 line, MDA content declined by 15.6% but proline content increased by 14.8% in the Ms_1 line (Both p<0.0001, data not shown).

Root Na^+ flux measured through NMT: From the seawater treatment commencement on, net Na^+ flux showed higher rate in the Ms_1 line than in the Ms_0 line (Fig. 7A). All net Na^+ flux rates in the Ms_1 line were positive, indicating absolute ion effluxes. Net Na^+ flux rate in the M_0 line was measured to be positive in the first 30 s, after which the frequency of negative values of net Na^+ flux rate increased until the end of 80 s. This suggested the crescent Na^+ influx into roots of the M_0 line. Box scatted dots indicated that the 1st percentile values of the Ms_1 line was comparable with the 3rd percentile ones of the M_0 line (Fig. 7B). Mean Na^+ flux rate in the Ms_1 and M_0 lines were calculated to be 826.83±186.71 and 261.68±152.18 pmol cm^{-2} s^{-1}, respectively.

Discussion

Selecting salt-tolerant alfalfa from callus-cultured lines: Alfalfa is a valuable forage crop which is grown in areas of limited rainfall, high temperature and saline threated. Therefore, the screening work on callus-cultured lines with the aim to enhance salt tolerance in alfalfa had started as early as 1990s. On the basis of salt-selected suspension-cultures of alfalfa, Lu *et al.* (2013) underlined that the degree of salt tolerance of the cell lines was highly dependent on the concentration of Ca^{2+} in the growth medium. Subsequently, Peng *et al.* (2008) continued to research ion characteristics and found that salt-tolerant plants were able to maintain lower Na^+/K^+ ratios than the unselected plants, but Ca^{2+} only gave some amelioration of NaCl toxicity when multiple ion factors, e.g. Na^+, K^+, and Cl^-, were involved to be considered simultaneously. Although these studies contributed to limited information for seawater irrigation to alfalfa, the technical manipulations and the considerations of ion characteristics supplied useful basement. Beside the NaCl compounds, seawater also contains several macro- and micro-elements. Therefore, our results of screening in the present study are of great applicative meaning. Survival rate of callus-cultured lines of Ms_1 in the present of study was comparable with former studies (Peng *et al.*, 2008). This illustrates that the method adapted in our study was available for alfalfa.

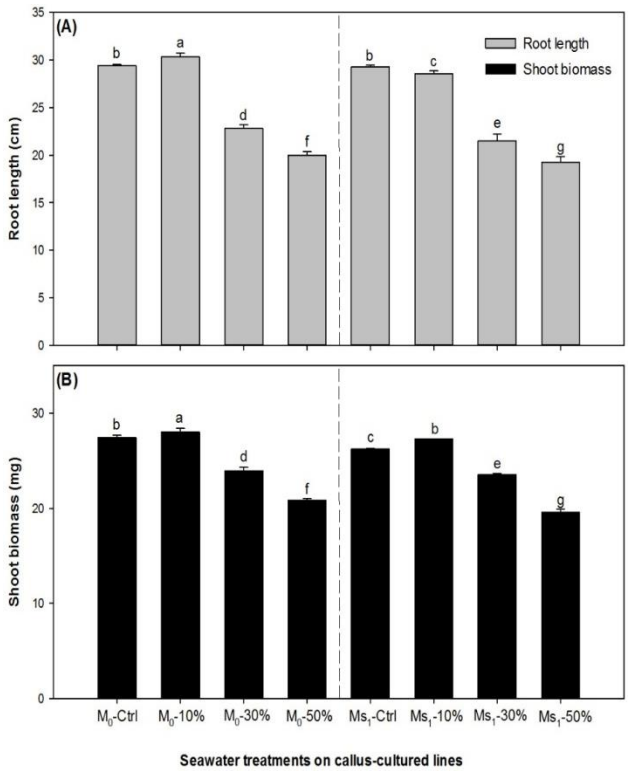

Fig. 4. Root length (A) and shoot biomass (B) of two callus-cultured alfalfa lines (M_0 and Ms_1) subjected to the control (ctrl) and seawater treatments at concentrations of 10%, 30%, and 50%. Different letters indicate significant differences among treatment according to LSD test at 0.05 level.

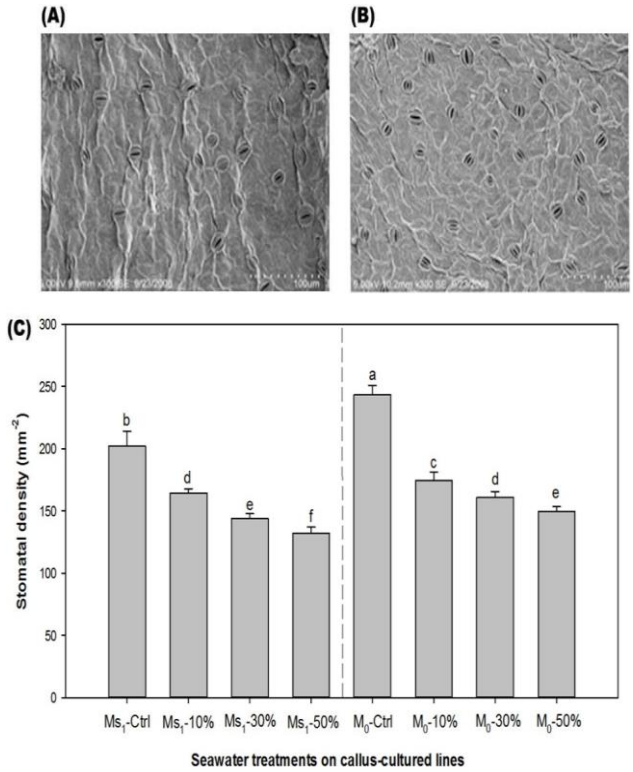

Fig. 5. The micro-photographs of typical stomatal densities of callus-cultured alfalfa lines of M_0 (A) and Ms_1 (B) subjected to the control (ctrl) and seawater treatments at concentrations of 10%, 30%, and 50% (C). Different letters indicate significant differences among treatments according to LSD test at 0.05 level.

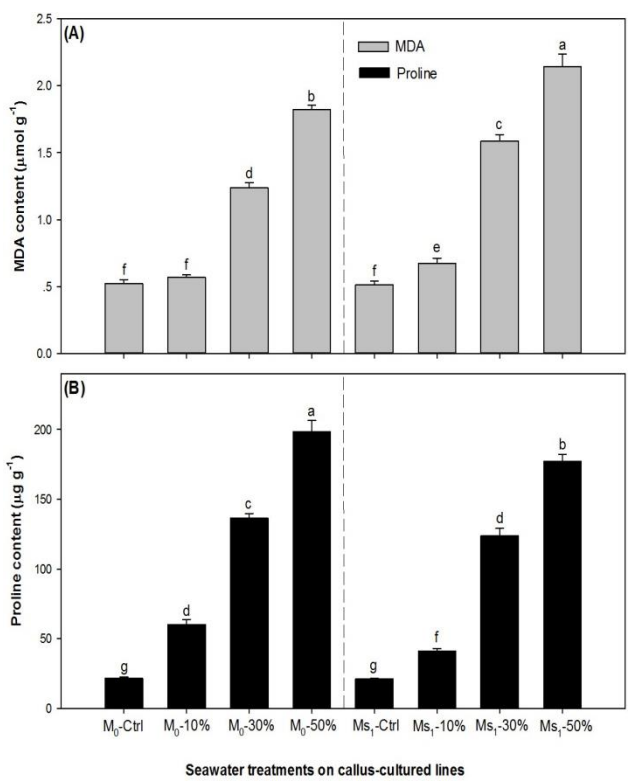

Fig. 6. MDA (A) and proline (B) contents of two callus-cultured alfalfa lines (M_0 and Ms_1) subjected to the control (ctrl) and seawater treatments at concentrations of 10%, 30%, and 50%. Different letters indicate significant differences among treatments according to LSD test at 0.05 level.

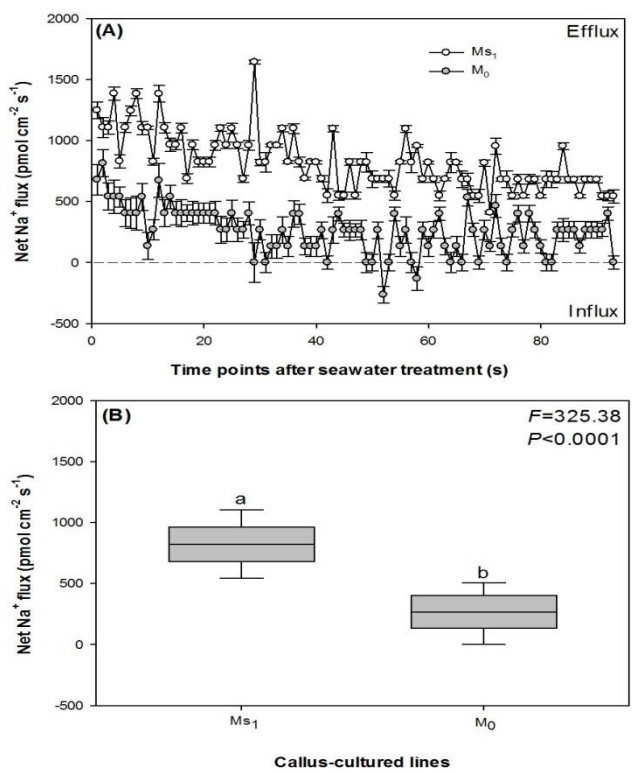

Fig. 7. Net Na^+ flux rates of callus-cultured lines (A) (adapted from Feng *et al.* [2014]) at each time point from the commencement of seawater treatment, and general comparison of net Na^+ fluxes of Box-whisker between the two callus-cultured lines (B). Different letters indicate significant differences of means between treatments according to LSD test at 0.05 level..

In the term of improvement of salt-tolerance of alfalfa, more recent work turned to focus on screening for critical expressing genes (Rahman *et al.*, 2015), salt-responsive proteins (Rahman *et al.*, 2015), inoculating rhizobial strains (Tang *et al.*, 2013), antioxidant enzyme enriched cultivars (Ashrafi *et al.*, 2015), and salt-tolerant half-sib families (Turhan, *et al.*, 2014). These studies strengthened research on mechanism impacting salt tolerance in alfalfa, but rarely conducted seawater irrigation for alfalfa. Therefore, the present study aimed to identify the physiological mechanism of advances in salt resistance of callus-generated mutant compared to parent plants.

Seedling growth and stomatal performance in response to seawater: In spite of insignificant effects of interactive callus-cultured lines and seawater treatment, our seedling height in the Ms_1 line in one-month time nearly caught the top level of ones from one half-sib family (Wang *et al.*, 2014) and other callus-culture studies ((Turhan, *et al.*, 2014) but lower than transgenic ones (Tang *et al.*, 2013). Also, seedling height was greater in the Ms_1 line than in the M_0 line, although both root length and shoot biomass showed tiny decline in the M_0 line relative to the Ms_1 line treated with no or 10% seawater (Fig. 4). These results demonstrated that seawater-screened alfalfa mutant tended to invest more to shoot morphology establishment rather than shoot dry mass accumulation or root morphology. The overall shoot biomass in the Ms_1 line seemed to be less than other alfalfa genotypes but at the similar NaCl level (Yang *et al.*, 2014).

Any reductions in the rate of photosynthesis by salinity could also be due to lower stomatal conductance (Tang *et al.*, 2013). Therefore, salt-stress caused reduction of SC can be contributed to the depressed photosynthesis. The lower SC in the Ms_1 line than the M_0 line may indicate that mutant seedlings engaged salt tolerance at the cost of decreased photosynthesis and relevant SC with water transpiration. These reductions were related to the trait of SD, which was found to be much lower in the Ms_1 line than in the M_0 line (Fig. 5). However, seedlings in the Ms_1 line had greater amount of chlorophyll. These results together demonstrated that the Ms_1 line adapted the strategy of enhanced salt-tolerance as declined foliar water potential but reinforced chlorophyll content. These traits were also typical for salt tolerance in other species, such as rice (Rahman *et al.*, 2015) and poplar (Abbruzzese *et al.*, 2009).

Na^+ accumulation and MDA and proline contents: One of the typical symptoms of toxicity by salt stress to plant is the excessive accumulation of Na^+ in shoot organs, which controls or reduces the uptake of other nutrients, such as K^+, Ca^{2+}, and Mn^{2+} (Sultana *et al.*, 2001). Also, over-loading of Na^+ in foliar organs would also result in the loss of equilibrium of K^+/Na^+ causing the damage of membrane (Rahman *et al.*, 2015). Therefore, the ability to inhibit Na^+ accumulation in shoots is usually performed to screen for the salt tolerant genotypes (Tang *et al.*, 2013). Additionally, Sultana *et al.* (2001) also reported the salt tolerant rice cultivars of rice in response to seawater irrigation. In the present study, the Ms_1 line showed much less Na^+ content in shoot organs than the M_0 line, which is an important trait of salt tolerance to prevent over-loading of Na^+. This may result from two possibilities: detained Na^+ accumulation or controlled Na^+ influx in roots. The more possible explanation needs the demonstration of root ion characteristics.

When polyunsaturated fatty acids in the cell membrane are undergoing peroxidation MDA is produced (Tang *et al.*, 2013). Oxidative stress-induced membrane damage and cell membrane stability have been used as efficient criteria to assess the degree of salt tolerance of plants (Peng *et al.* 2008). In our study, foliar MDA content in both callus-cultured lines were induced to rise with the increase of seawater proportion (Fig. 6A). These trends concur with the general response to exogenous salt stress, implying the damage level of Na^+ accumulation on foliar membrane cells. However, our results also revealed that MDA content was lower in the Ms_1 line than the M_0 line, suggesting a less damaged performance of cell membrane in the mutant seedlings.

Proline functions as a compatible osmolyte to protect the frame and structure of protein (Bertrand *et al.*, 2015). Rapid adjustment of proline concentration is evaluated to be a functional mechanism for salt tolerance in alfalfa (Flowers, 2004). As results about MDA, proline content in both lines rose with the increase of seawater proportion (Fig. 6B), suggesting the sensitive self-mediation of the callus-cultured lines. The increased proline content in the Ms_1 line than in the M_0 line indicated a better ability to mediate osmotic balancing to protection of sub-cellular structures, possibly through activating enzymes and increasing cellular osmolarity (turgor pressure) that provided the turgor necessary for cell expansion under stress conditions （Ahmed *et al.*, 2010).

Net Na^+ flux: Under salinity conditions, intracellular Na^+ to K^+ homeostasis is crucial for cell metabolism and is considered to be a strategy commonly used by tolerant plants (Flowers, 2004). To maintain an optimal Na^+/K^+ balance in cytosol, plants remove excess Na^+ through Na^+ extrusion to the external environment and/or compartmentalization into the vacuoles, along with retention of physiological K^+ concentration in the cytoplasm (Flowers, 2004). Active Na^+ extrusion from the cytosol is typically carried out by transmemebrane transport proteins such as PM-located Na^+/H^+ antiporters. As described above, one of the capacities of roots to show salt tolerant plasticity is to either restrict Na^+ uptake or exclude it from the cytosol. Traditional measuring techniques were generally limited for routine estimation of Na^+ transport in roots. However, with the invention and popularization of NMT, measures on Na^+ extrusion became fast, convenient, and cost-effective (Jayakannan & Rengel, 2011). Since then, the measure on root Na^+ flux using NMT has been employed for several times to identify plant salt tolerance through micro-flux monitoring. In these studies, the dynamic of Na^+ flux was usually measured in the term of minutes, and results were generally robust to show the difference between influx

and efflux. However, Na^+ flux at a shorter term of seconds is scarce. Additionally, to our knowledge, our study is the first one to measure root Na^+ flux with NMT in response to seawater treatment.

Net Na^+ flux showed absolute extrusions in the Ms_1 line throughout the first 1.5 min after NMT measure commencement, while the net Na^+ influx occurred from the 30-s on in the M_0 line (Fig. 7A). Results were also confirmed by comparison between the overall averages (Fig. 7B). These results clearly indicate a critical trait of salt tolerance of the Ms_1 line to be Na^+ extrusion from root cell. This agrees with the second surmise we put forward about the salt tolerant strategy of stimulating Na^+ efflux and explained less Na^+ accumulation in shoot organs.

Acknowledgments

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