DOI: 10.1007/s10535-016-0626-2 BIOLOGIA PLANTARUM 60 (3): 543-554, 2016

Nitrogen deprivation induces cross-tolerance of *Poa annua* callus to salt stress

C.Z. ZHAO[1], P. LI[2], X.M. WANG[1], P. LI[1], X.Y. WANG[1], F. WANG[1], J.F. WANG[1], N. CHANG[1], and Y.R. BI[1]*

Ministry of Education Key Laboratory of Cell Activities and Stress Adaptations, School of Life Sciences, Lanzhou University, Lanzhou 730000, P.R. China[1]
North China Electric Power University, State Grid Corporation, Baoding, Hebei 071003, P.R. China[2]

Abstract

Alternative respiration pathway (AP) is an important pathway which can be induced by environment stresses in plants. In the present study, we show a new mechanism involving the AP in nitrogen deprivation-induced tolerance of *Poa annua* callus to salt stress. The AP capacity markedly increased under a 600 mM NaCl treatment or nitrogen deprivation pretreatment and reached a maximum under the nitrogen deprivation pretreatment combined with the NaCl treatment (–N+NaCl). Malondialdehyde (MDA) and H_2O_2 content and Na^+/K^+ ratio significantly increased under the 600 mM NaCl treatment but less under the –N+NaCl treatment. Moreover, both the nitrogen deprivation and the NaCl stress stimulated the plasma membrane (PM) H^+-ATPase activity and increased pyruvate content. The maximal stimulating effect was found under the –N+NaCl treatment. When the AP capacity was reduced by salicylhydroxamic acid (SHAM, an inhibitor of AP), content of MDA and H_2O_2 and Na^+/K^+ ratio dramatically increased, whereas PM H^+-ATPase activity decreased. Moreover, exogenous application of pyruvate produced a similar effect as the nitrogen deprivation pretreatment. The effects of SHAM on the *Poa annua* callus were counteracted by catalase (a H_2O_2 scavenger) and diphenylene iodonium (a plasma membrane NADPH oxidase inhibitor). Taken together, our results suggest that the nitrogen deprivation enhanced the capacity of AP by increasing pyruvate content, which in turn prevented the *Poa annua* callus from salt-induced oxidative damages and Na^+ over-uptake.

Additional key words: alternative pathway, catalase, H^+-ATPase, *in vitro* culture, malondialdehyde, oxidative stress, SHAM.

Introduction

High sodium (Na^+) content in soil has profound influences on plant distributions and significantly reduce plant productivity (Boyer 1982). Excessive salinity exerts adverse effects such as ion toxicity and osmotic stress (Zhu 2003). When plants are under salt stress, the primary effect is the accumulation of Na^+ ions leading to metabolic toxicity or osmotic imbalances. Efficient transport of excess Na^+ from the cytoplasm to vacuoles is the main mechanism for the adaptation of plants to salt stress (Niu *et al.* 1995, Morsomme and Boutry 2000).

Submitted 24 December 2014, *last revision* 22 October 2015, *accepted* 4 November 2015.

Abbreviations: AOX - alternative oxidase; AP - alternative respiration pathway; APX - ascorbate peroxidase; CAT - catalase; CP - cytochrome respiration pathway; c-PTIO - 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt; DPI - diphenylene iodonium; EL - electrolyte leakage; PM - plasma membrane; POD - peroxidase; ROS - reactive oxygen species; SHAM - salicylhydroxamic acid; SOD - superoxide dismutase; TBARS - thiobarbituric acid reactive substances; MDA - malondialdehyde; V_{alt} - capacity of alternative respiration pathway; V_{cyt} - capacity of cytochrome respiration pathway; V_{res} - capacity of residue respiration pathway.

Acknowledgements: This work was supported by the Major State Basic Research Development Program of China (973 Program; 2012CB026105), the National High Technology Research and Development Program (2007AA021401), the Foundation of Science and Technology Program of Lanzhou City (2015-3-53), the Foundation of Science and Technology Program of Gansu Province (1506RJZA209), the Scientific research project of Qinghai-Tibetan DC Interconnection Project in State Grid Corporation of China, and the Foundation of Science and Technology Program of Gansu Province (1208RJZA224). The first two authors contributed equally to this work.

* Corresponding author; e-mail: yrbi@lzu.edu.cn

This is typically achieved by transmembrane transporter proteins that exude Na^+ from the cytosol in exchange for H^+ (Na^+/H^+ antiporters). The Na^+/H^+ antiporter-mediated exudation is energy-dependent and is driven by proton-motive force generated by the plasma membrane (PM) H^+-ATPase (Serrano *et al.* 1999), the vacuolar membrane H^+-ATPase, and the H^+-pyrophosphatase (Rea and Sanders 1987, Rea and Poole 1993). It has been reported that PM H^+-ATPase antiporter expression was enhanced under salt stress in *Populus euphratica* (Wu *et al.* 2007) and PM H^+-ATPase activity is affected by salt treatment such as partial inhibition in tomato roots (Ballesteros *et al.* 1998), stimulation in *Arabidopsis* (Wang *et al.* 2010), and no effects in cotton roots (Hassidim *et al.* 1986). Thus, PM H^+-ATPase plays a key role in maintaining cellular Na^+/K^+ homeostasis. Regulation of expression and activity of PM H^+-ATPase represents an important regulatory mechanism for salt tolerance in plants (Li *et al.* 2011).

In addition to ion homeostasis, the overproduction of reactive oxygen species (ROS), such as hydrogen peroxide, superoxide radical, and hydroxyl radical (Borsani *et al.* 2001, Apel and Hirt 2004), may disturb cellular redox homeostasis leading to oxidative damages. A number of mechanisms exist by which some plant species can minimize the damaging effects of oxidative stress, including non-enzymatic and enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase. The importance of the ROS protection pathway in salt tolerance is evidenced by the observation that the activities of many of these enzymes increase along with salt stress (Liu *et al.* 2007, Wang *et al.* 2008, 2010). Additionally, genetic analyses have shown that alterations in the expression of these enzymes can improve or diminish salt tolerance. Many results suggest that antioxidant systems are an important mechanism for plant tolerance to many stress conditions. These antioxidant systems may be involved in cross-talk between different abiotic stress responses (Lin *et al.* 2010, Han *et al.* 2013).

In plant cells, the major sites for ROS production are chloroplasts and mitochondria (Bartoli *et al.* 2004, Asada 2006). Hydrogen peroxide, one of the ROS, is mainly produced in plants due to a PM-bound NADPH oxidase complex. Some chemicals inhibit this NADPH oxidase in mammals and also block the production of H_2O_2 in plants (Li *et al.* 2014). Plant mitochondria have an additional oxidase known as the alternative oxidase (AOX), an important protein in the alternative pathway (AP) which has been proposed to play a role in ROS metabolism. Alternative oxidase branches from the electron transport chain accepting electrons from the reduced ubiquinone and donating electrons directly to oxygen. Alternative oxidase shows an up-regulation in response to many abiotic stresses at the transcriptional level (Clifton *et al.* 2005). These stresses include excessive radiation, macroelement limitation, drought, salinity, and low temperature (Clifton *et al.* 2005, Fiorani *et al.* 2005, Sieger *et al.* 2005, Giraud *et al.* 2008, Wang *et al.* 2012). Furthermore, a link between AP capacity and ROS formation has been experimentally demonstrated where an altered *AOX* expression impacts on ROS formation during stress. For example in transgenic tobacco cell cultures with a reduced expression of *AOX*, a higher amount of ROS accumulates under phosphate deficiency than in wild type cells (Yip and Vanlerberghe 2001). In *Arabidopsis* lines over-expressing *AOX*, a lower ROS accumulation is observed during chilling stress, whereas lines with suppressed *AOX* expression have a higher ROS accumulation (Fiorani *et al.* 2005). More recently, *Arabidopsis aox1a* mutants have a high anthocyanin production, altered photosynthetic capacity, increased superoxide content, and reduced root growth under moderate irradiance and drought stress (Giraud *et al.* 2008). Altogether, these results support the role of AOX in alleviating ROS formation suggesting that AOX should also be considered as part of the plant oxidative stress responses. However to our knowledge, studies that have been carried out to elucidate the physiological roles of AOX in plant cross tolerance to environmental cues are still limiting.

Poa annua is one of the most widespread grass species worldwide (Chen *et al.* 2014) and plays very important roles in stabilizing sand dunes and in greening parks and playgrounds as well as in supplying food for animals. Many studies have reported that *Poa* shows a high tolerance to a high temperature, cold, drought, heavy metals, and high salinity (Duo *et al.* 2006, Peng *et al.* 2013, Lv *et al.* 2014, Puyang *et al.* 2015, Yu *et al.* 2015). In addition, it has adapted to diverse environments including extreme areas such as subarctic meadows (Heide 2001), the Antarctic Peninsula (Molina-Montenegro *et al.* 2012), and Mount Kilimanjaro (Hemp 2007). Moreover, there is almost no information available regarding physiological responses of *P. annua* under nitrogen deprivation and salt stress. The aim of this study is to demonstrate the physiological role of AOX in mediating nitrogen deprivation-induced salt tolerance in a *Poa annua* callus.

Material and methods

Plants and treatments: Embryogenic calli were obtained from mature seeds of *P. annua* as described by Li *et al.* (2008). The callus was maintained in a controlled growth chamber in darkness, a temperature of 25 °C, and an air humidity of 70 % for four months. For experiments, about 0.5 g of the callus was sub-cultured in 30 cm^3 of

Gamborg B_5 solid medium. After 7 d, the callus was transferred to 30 cm^3 of a B_5 liquid medium on a rotary shaker at 110 rpm for various treatments. A nitrogen-depleted B_5 liquid medium was formed by removing a nitrogen source from the B_5 base medium. Details are as follows: $MgSO_4 . 7 H_2O$ (0.25 g dm^{-3}), $CaCl_2 . 2 H_2O$ (0.15 g dm^{-3}), $NaH_2PO_4 . 2 H_2O$ (0.17 g dm^{-3}), K_2SO_4 (2.15 g dm^{-3}). In recovery experiments, we added 0, 0.5, 1, 5, 10, 20, and 30 mM NH_4NO_3 as -N, 0.5N, 1N, 5N, 10N, 20N, and 30N, respectively, and in order to prevent osmotic stress resulted from the above treatments, the same concentrations of K^+ as K_2SO_4 were supplied to the normal B5 medium (Watanabe *et al.* 2010). Sodium chloride at different concentrations was added to the medium to induce salt stress. An inhibitor of the AP (1 mM salicylhydroxamic acid, SHAM), a ROS scavenger (50 U cm^{-3} CAT), an inhibitor of PM NADPH oxidase (20 µM diphenylene iodonium, DPI), inhibitors of ethylene synthesis (10 µM $AgNO_3$ or 50 µM amino-oxyacetic acid, and an NO scavenger (200 µM 2-(4-carboxy-phenyl)-4,4,5,5-tetramethylimid-azoline-1-oxyl-3-oxide potassium salt, c-PTIO) were added to the medium for various treatments. The pH of the medium was adjusted to 5.8 with KOH. The callus was collected at the indicated time points and washed for 2 min with distilled water and then collected onto filter paper by vacuum aspiration. The samples were immediately used for the following assays.

Lipid peroxidation assay: Malondialdehyde (MDA) content was measured as the indicator of lipid peroxidation following the method of Liu *et al.* (2007) with some modifications. About 0.5 g of the callus was homogenized in 10 % (m/v) trichloroacetic acid at 4 °C. After centrifugation at 3 000 *g* for 15 min, the supernatant was collected and incubated with an equal volume of 0.5 % (m/v) thiobarbituric acid at 100 °C for 30 min. The absorbance of the supernatant was measured at 532 nm with readings at 600 nm subtracted to reduce a non-specific interference (*Unico UV-2000*, *Jinpeng*, Shanghai, China). The amount of MDA was calculated using a coefficient of absorbance (ε) of 155 mM^{-1} cm^{-1}.

Electrolyte leakage assay: Electrolyte leakage was determined according to the method described by Sairam and Srivastava (2002). The callus was collected and washed three times with de-ionized water in order to remove surface-adhered electrolytes. The washed samples were then placed in test tubes and immersed in 10 cm^3 of de-ionized water at 25 °C for 3 h. After the incubation, the conductivity of the bathing solution was measured (C_1), and the conductivity of the de-ionized water was also measured (C_0). The callus was then heated in boiling water for 1 h and the total conductivity was measured in the bathing solution (C_2). Relative electrolyte leakage [%] = $(C_1 - C_0) / (C_2 - C_0) \times 100$].

Determination of antioxidant enzyme activities: The callus was homogenized in a 50 mM phosphate buffer (pH 7.8) containing 1 mM Na_2EDTA and 2 % (m/v) polyvinylpyrrolidone. The homogenate was centrifuged at 15 000 *g* and 4 °C for 30 min, and the supernatant was used for the following enzyme activity assays.

Superoxide dismutase activity (SOD, EC 1.15.1.1) was assayed by the nitroblue tetrazolium (NBT) method of Prochazkova *et al.* (2001). One unit of SOD was defined as the amount of enzyme required to cause a 50 % inhibition of the reduction of NBT as monitored at 560 nm. Catalase activity (CAT, EC 1.11.1.6) was assayed as described by Aebi (1982). The activity was determined as decrease in absorbance at 240 nm (coefficient of absorbance, ε = 39.4 mM^{-1} cm^{-1}) for 1 min following the decomposition of H_2O_2. Ascorbate peroxidase activity (APX, EC 1.11.1.11) was measured according to Janda *et al.* (1999) by monitoring the rate of ascorbate oxidation at 290 nm (ε = 2.8 mM^{-1} cm^{-1}). Peroxidase activity (POD, EC1.11.1.7) was measured according to Adam *et al.* (1995) by monitoring the rate of guaiacol oxidation at 470 nm (ε = 26.6 mM^{-1} cm^{-1}). All the enzyme activities were measured at 25 °C in 3 cm^3 of reaction mixtures. Measurements were made with a spectrophotometer *Unico UV-2000* with no lag period.

Measurement of O_2 consumption: Respiration rates were measured according to the method described by Vanlerberghe *et al.* (1997) and Parsons *et al.* (1999). Oxygen consumption was measured using a Clarke-type oxygen electrode (*Hansatech*, King's Lynn, Norfolk, UK) in a reaction medium containing a 50 mM phosphate buffer (pH 5.7) at 25 °C. Air-saturated, temperature-equilibrated water was used to calibrate the electrode at 25 °C. Potassium cyanide (3 mM) and SHAM (2 mM) were used to inhibit cytochrome *c* oxidase and AOX, respectively. To avoid oxygen-limiting conditions inside the cuvette, all measurements were terminated before O_2 reached about 50 to 60 % of air saturation levels. The capacity of the AP (V_{alt}) was calculated as difference between respiration in the presence of 3 mM KCN and residual respiration (V_{res}). The capacity of CP (V_{cyt}) was calculated as difference between respiration in the presence of 2 mM SHAM and residual respiration (V_{res}). Residual respiration was determined as respiration in the presence of 3 mM KCN and 2 mM SHAM.

Determination of H_2O_2 content: Hydrogen peroxide content was determined according to Veljovic-Jovanovic *et al.* (2002). The callus (0.2 g) was grounded in 2 cm^3 of 1 M $HClO_4$ in the presence of 5 % (m/v) insoluble polyvinylpyrrolidone. The homogenate was centrifuged at 12 000 *g* for 10 min and the supernatant was neutralized with 5 M K_2CO_3 to pH 5.6 in the presence of 0.1 cm^3 of 0.3 M phosphate buffer (pH 5.6). The solution was centrifuged at 12 000 *g* for 1 min and the sample was incubated with 1 U of ascorbate oxidase (*Sigma*, St. Louis, USA) for 10 min to oxidize ascorbate prior to assay. The reaction mixture consisted of 0.1 M phosphate buffer (pH 6.5), 3.3 mM dimethylamine borane (*Sigma*, St. Louis, USA), 0.07 mM 3-methyl-2-benzothiazolinonehydrazone hydrochloride hydrate (*Sigma*) and 0.3 U of peroxidase (*Sigma*). The reaction was initiated by the addition of 0.2 cm^3 of the sample. An absorbance change at 590 nm was monitored at 25 °C.

Plasma membrane H$^+$-ATPase activity determination: Assay of ATP was performed as described by Qiu and Su (1998). The membrane protein (10 - 15 mg) was added to 0.5 cm^3 of a reaction medium containing 25 mM Hepes-Tris (pH 6.5), 3 mM ATP, 50 mM KCl, 1 mM Na_3MoO_4, and 0.015 % (m/v) trichloroacetic acid. The H$^+$-ATPase activity was determined by measuring the released P_i and calculated by subtracting the residual H$^+$-ATPase activity (in the presence of Na_3VO_4) from the total H$^+$-ATPase activity (in the absence of Na_3VO_4).

Measurement of Na$^+$ and K$^+$ content was performed using a scanning electron microscope (*Phillips Electronics NV*, Eindhoven, The Netherlands) fitted with a *Kevex* energy dispersive X-ray detector (*Kenex*, Valencia, CA, USA) as described by Zhao *et al.* (2004). Briefly, the callus was rinsed five times with de-ionized water and then dried at 50 °C for 48 h. The dried callus was crushed with a mortar and pestle, and used for analysis. At least three spots per sample were examined. The content of Na$^+$ or K$^+$ was expressed as Na$^+$ or K$^+$ atomic number percentage of all elements measured (calcium, potassium, sodium, magnesium, phosphorus, sulfur, silicon, and chlorine) in the callus.

Determination of pyruvate content: Pyruvate content was analyzed according to the method of Millar *et al.* (1998) with some modifications. The callus (1 g) was snap frozen in liquid nitrogen, ground to a fine powder, thoroughly mixed with 5 cm^3 of 2 M perchloric acid in 10 mM phosphate buffer (pH 3.5), and kept on ice for 10 min. The extracts were then centrifuged at 10 000 g for 10 min. The supernatant was then filtered and the pH was adjusted to 7.2 with KOH. After removal of precipitates, aliquots were assayed for lactate dehydrogenase (10 units)-dependent NADH oxidation at 340 nm in a solution of 0.2 mM NADH in 0.5 M triethanolamine with 0.05 M EDTA (pH 7.5)

Western blot analysis: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Li *et al.* (2014). The protein extracts were prepared from the callus. Protein content was determined using a *Thermo Scientific Evolution 60S*, (New York, USA) spectrophotometer. The same amounts of the total protein were loaded and separated on an 11.5 % (m/v) acrylamide gel containing 6 M urea. After electrophoresis, the separated proteins were transferred onto a polyvinylidene difluoride membrane. The membrane was blocked with 5 % (m/v) non-fat milk in *Tween 20* and 10 mM Tris-HCl (pH 8.0) for 90 min and then in 150 mM NaCl. An alternative oxidase (AOX1 and AOX2) antibody (*AS04054*, *Agrisera*, Stockholm, Sweden) was added and incubated with the membrane overnight. After washing, an alkaline phosphatase-conjugated secondary antibody was added and incubated for visualization according to the manufacturer's instruction. Protein content was determined by the Bradford (1976) method.

Statistical analysis: Each experiment was repeated at least three times. Values are expressed as means ± SEs. All comparisons were performed using one-way analysis of variance and Duncan's multiple range tests for independent samples. In all cases, the confidence coefficient was set at $\alpha = 0.01$ or $\alpha = 0.05$.

Results

Since electrolyte leakage (EL) and MDA content are considered as indicators of stress-induced cell damages, we used them to assess the extent of the salt stress in *P. annua* callus. The EL and the MDA content in the *P. annua* callus increased as the NaCl concentration increased (Fig. 1*A*,*B*), but no changes in EL and the MDA content was observed at 200 mM NaCl. At 400 mM NaCl, the EL and the MDA content only slightly increased, however, they increased markedly at 600 mM NaCl. Under the 800 mM NaCl treatment, the cells were severely damaged, as the EL was close to that of dead cells.

The capacity of total respiration, $V_{\rm alt}$, and $V_{\rm cyt}$ were measured in the *P. annua* callus under 0 - 800 mM NaCl. The results show that the total respiration increased in the presence of NaCl up to 600 mM and then decreased at 800 mM. Concomitantly, $V_{\rm alt}$ increased in the 600 mM NaCl treatment, whereas $V_{\rm cyt}$ remained almost unchanged (Fig. 1*C*). Therefore, we used 600 mM NaCl in the following experiments.

To investigate the possible effects of nitrogen deprivation on salt-induced oxidative stress, the *P. annua* callus was first pretreated in the -NB$_5$ liquid medium for 3 d. After that, the callus was treated with 600 mM NaCl in the normal B$_5$ liquid medium for 48 h (Fig. 2). The results show that the -N pretreatment markedly alleviated the increase of EL and MDA content under the salt stress. Moreover, the -N+NaCl treatment exhibited a higher $V_{\rm alt}$ capacity than the -N pretreatment or the NaCl treatment alone (Fig. 2C). Salicylhydroxamic acid (an inhibitor of the AP) eliminated the effect of -N on EL and MDA. In addition, the AP capacity measurement and the Western blot analysis demonstrated that the AP capacity and AOX protein content increased in the callus under the salt stress, and the highest accumulation was observed in the -N+NaCl treatment. Salicylhydroxamic acidapplication diminished the effect of -N on AP capacity and AOX protein content under the salt stress (Fig. 2*D*).

The callus was treated with 0, 0.5, 1, 5, 10, 20, and 30 mM NH_4NO_3 (hereafter referred to as -N, 0.5N, 1N, 5N, 10N, 20N, and 30N) in the B_5 liquid medium without any other forms of nitrogen. Compared to 30N, the AP capacity was significantly induced by -N, 0.5N, 1N and

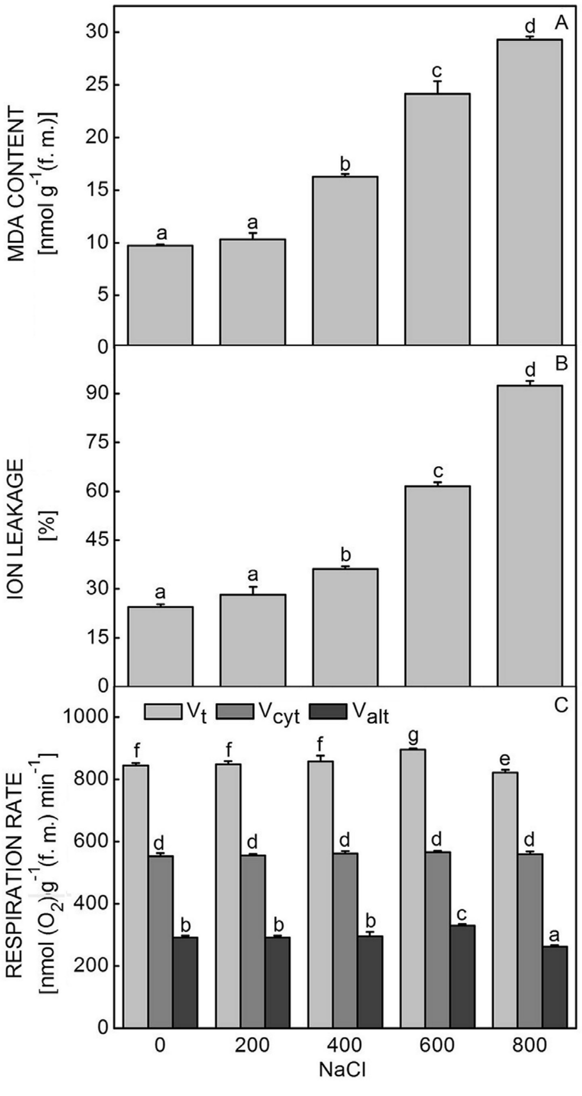

Fig. 1. The effects of NaCl (0 - 800 mM) on malondialdehyde (MDA) content (*A*), ion leakage (*B*), and respiration rate (*C*) in a *Poa annua* callus. The 7-d-old callus was exposed to a B5 liquid medium containing different concentrations of NaCl for 48 h. Means ± SEs from three independent experiments. The *bars* with different letters indicate significant differences at $P < 0.05$.

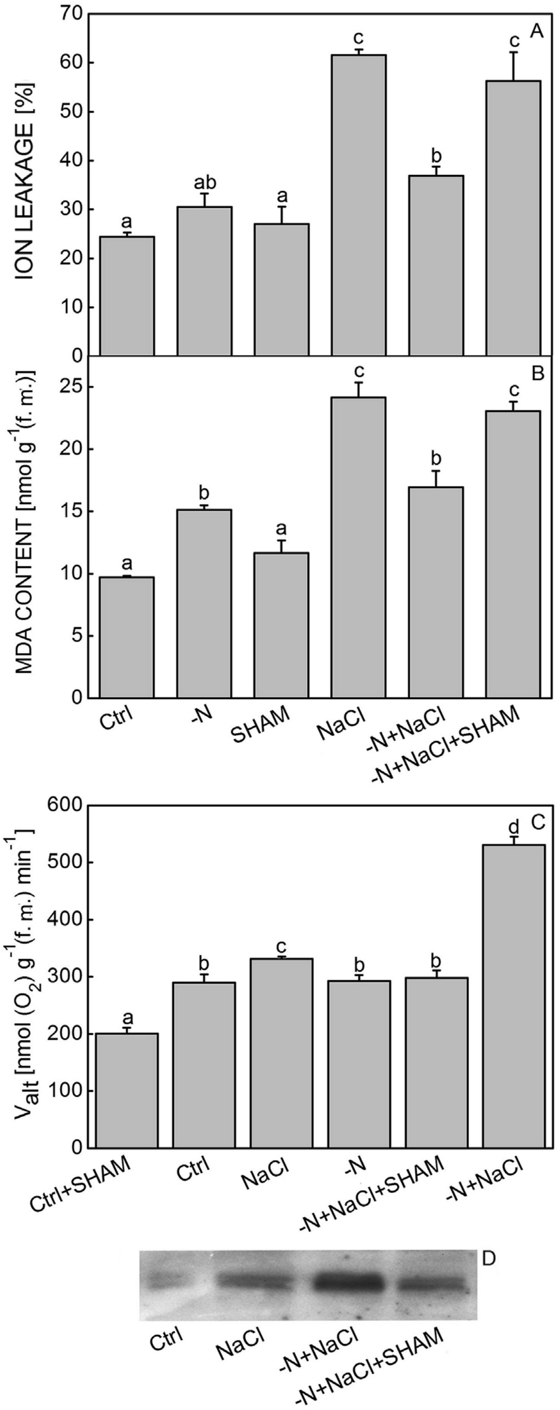

Fig. 2. The effects of N deprivation (-N) and/or SHAM pretreatment on ion leakage (*A*), MDA content (*B*), alternative respiration (V_{alt}) (*C*), and AOX expression (*D*) in a *P. annua* callus under a salt stress. The callus was cultured on a B5 solid medium for 7 d, then exposed to an N-depleted B5 liquid medium and 1 mM SHAM alone or both for 3 d. After that, the callus was re-cultured in a fresh B5 liquid medium (-N) with or without 600 mM NaCl for 48 h as -N+NaCl, -N+NaCl+SHAM, -N, SHAM+Ctrl, and control (Ctrl) treatments, respectively. Means ± SEs from three independent experiments. The *bars* with different letters indicate significant differences at $P < 0.05$.

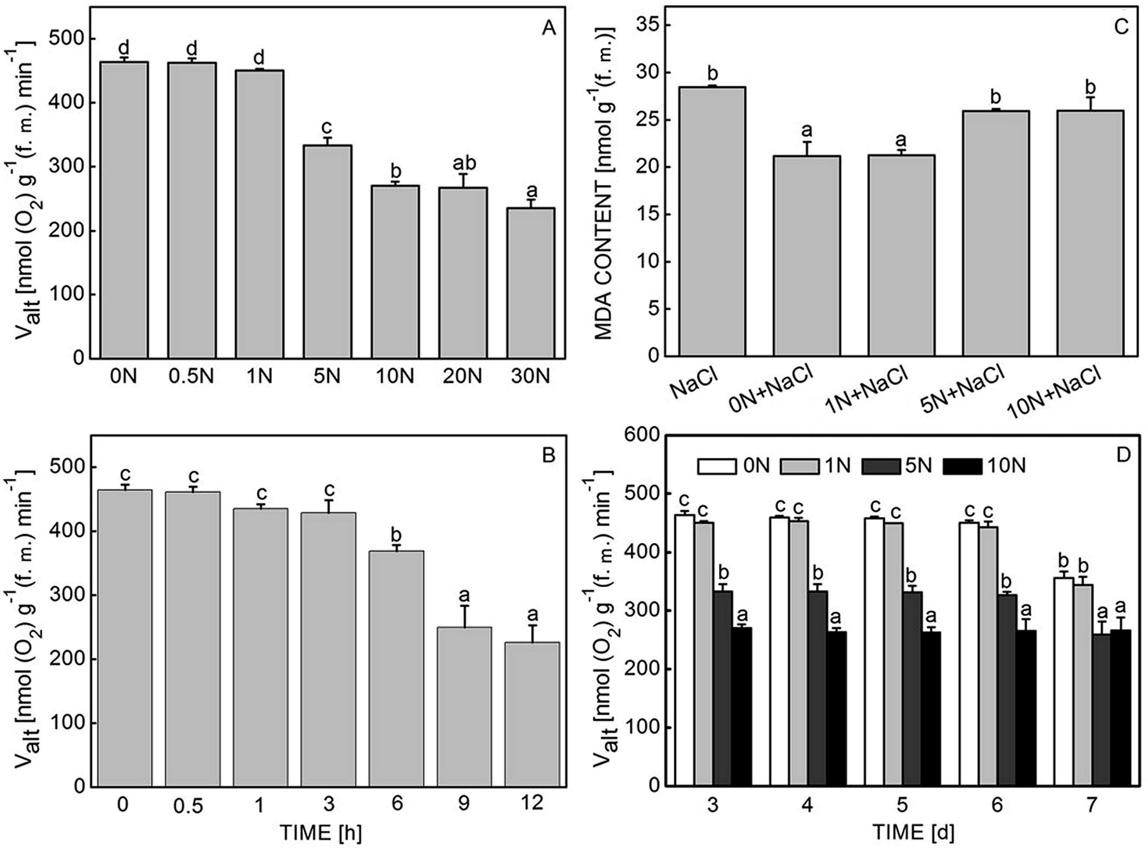

Fig. 3. The capacity of alternative respiration pathway (V_{alt}) and MDA content in a *P. annua* callus under different treatments. *A* - The callus was treated with different concentrations of N for 3 d. *B* - The callus was treated with –N for 3 d and then fed with 30 mM NH_4NO_3 for different times. *C* - The MDA content in the callus pre-treated with -N for 3 d, followed by exposure to -N, 1N, and 5N for 9 h and 600 mM NaCl for 48 h. *D* - The V_{alt} in the callus with different concentrations of N for 3 to 7 d. -N, 0.5N, 1N, 5N, 10N, 20N, and 30N mean 0, 0.5, 1, 5, 10, 20, and 30 mM NH_4NO_3, respectively. Means ± SEs from three independent experiments. The *bars* with different letters indicate significant differences at $P < 0.05$.

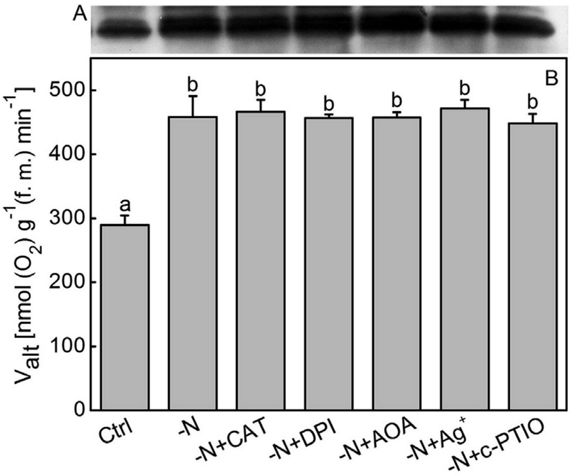

Fig. 4. Changes of AOX (*A*) and V_{alt} (*B*) in a *P. annua* callus under different treatments. The 7-d-old callus was exposed to -N, 50 U cm^{-3} CAT) 20 μM DPI, 50 μM aminooxyacetic acid (AOA), 10 μM $AgNO_3$ (Ag^+), and 200 μM c-PTIO for 3 d. Ctrl - control. Means ± SEs from three independent experiments. *Bars* with different letters indicate significant differences at $P < 0.05$.

slightly induced by 10N and 20N (Fig. 3). The inductive effect of 5N on the AP was lower than the shortage of N (-N, 0.5N, and 1N) but higher than the normal N (30N). Moreover, after treatment with -N for 3 d, the callus was fed with 30 mM NH_4NO_3 for different times to test the capacity of the AP. The results show that V_{alt} exhibited a gradual decline with extended feeding time and restored to the 30 N levels after 9 h of feeding (Fig. 3*B*).

To further confirm that the -N pretreatment-induced AP enhancement contributed to salt tolerance in the *P. annua* callus, to the callus treated with -N for 3 d, 1, 5, and 10 mM NH_4NO_3 were added. After 9 h, the callus was re-cultured for 48 h in the normal B_5 liquid medium containing 600 mM NaCl. the results indicate that both the -N and 1N pretreatments significantly reduced and the 5N and 10N slightly reduced MDA content in the subsequent salt stress (Fig. 3*C*). In addition, the inductive effect of -N on the AP reached a peak on the sixth day of the -N treatment and then declined. These results suggest that the AP might play a key role in -N induced tolerance to the salt stress in the *P. annua* callus.

To better understand the mechanism of the -N induced AP under the NaCl stress, we examined the effects of ethylene, NO, and H_2O_2 on AP capacity under the -N conditions by using the respective inhibitors. The AP capacity and the content of AOX in the *P. annua* callus increased in all the treatments compared to the control, but almost no differences in enhancement among the different treatments were observed. In addition, AOX activity was also stimulated by several organic acids, especially pyruvate, an allosteric activator of AOX (Wang *et al.* 2010). Interestingly, NaCl and the -N alone markedly increased pyruvate content in the *P. annua* callus, and the highest pyruvate content was found under the -N+NaCl treatment (Fig. 5*A*). Further, we investigated the effect of exogenous pyruvate on AP capacity in the *P. annua* callus, and the AP capacity was significantly enhanced by 10 mM pyruvate. To confirm that the -N-induced salt tolerance in the *P. annua* callus was dependent on AP enhancement, 10 mM pyruvate was used in the following experiments. The treatment with 10 mM pyruvate in the absence of NaCl had no significant effects on MDA content in the callus. However, the application of pyruvate attenuated the effect of NaCl on MDA in the callus. In contrast, the addition of 1 mM SHAM reversed the protective effect of pyruvate on MDA under the salt stress (Fig. 5*C*). These results indicate that the increased pyruvate content might contribute to AP induction under the -N conditions, and the AP enhanced the tolerance of the *P. annua* callus to the salt stress.

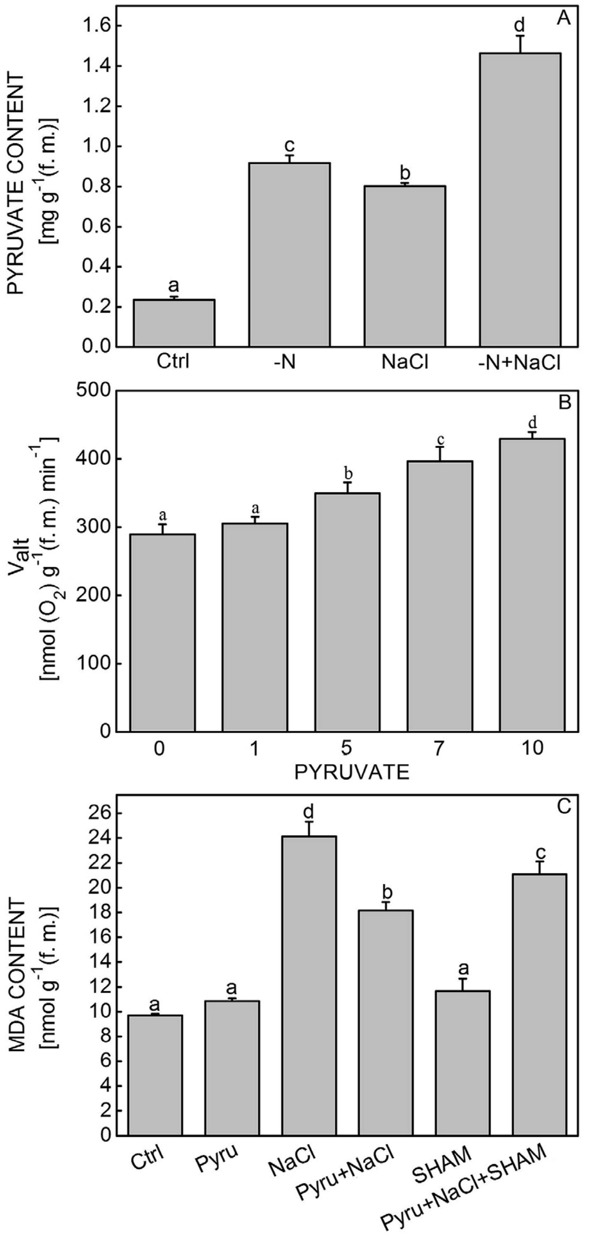

Fig. 5. The effect of N deprivation pretreatment (-N) on pyruvate (Pyru) content (*A*), the effect of exogenous pyruvate on V_{alt} (*B*), and MDA content (*C*) in a *P. annua* callus under different treatments. The callus in Fig. 5*A* was treated as described in Fig. 2. The callus in Fig. 5*B* was treated with 0 to 10 mM pyruvate for 3 d. The callus in Fig. 5*C* was treated as described in Fig. 2 except for 10 mM pyruvate in the place of -N. Ctrl - control. Means ± SEs from three independent experiments. The *bars* with different letters indicate significant differences at $P < 0.05$.

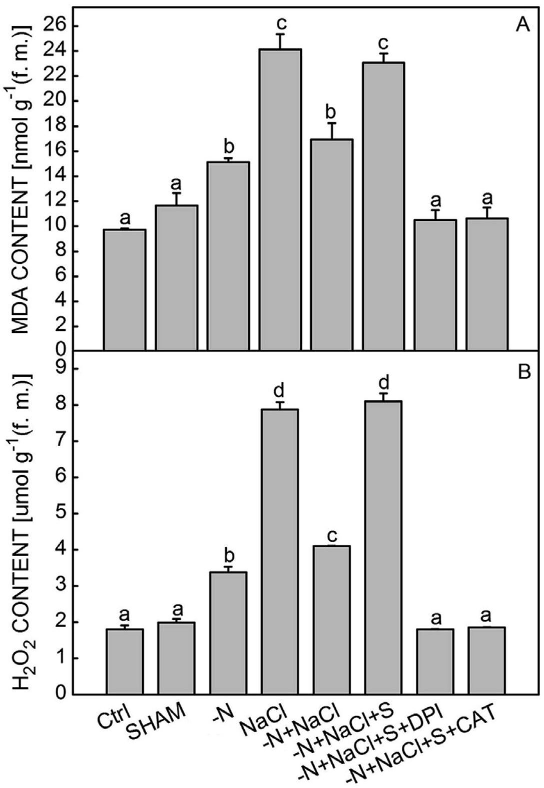

Fig. 6. The changes of MDA content (*A*) and H_2O_2 content (*B*) in a *P. annua* callus under different treatments. The callus was pre-treated with -N+SHAM for 3 d, then exposed to 600 mM NaCl and 50 U cm^{-3} CAT or 20 μM DPI or SHAM (S). Means ± SEs from three independent experiments. The *bars* with different letters indicate significant differences at $P < 0.05$.

We further examined the activities of antioxidant enzymes under the different treatments. The activities of CAT, SOD, APX and POD in the *P. annua* callus were significantly higher under the -N+NaCl treatment than the -N conditions or NaCl treatment alone (Fig. 8). The salt stress alone caused a significant increase in content of H_2O_2 and MDA, but the values decreased under the -N+NaCl treatment. The effect of the -N+NaCl treatment on H_2O_2 and MDA in the *P. annua* callus was reversed by the SHAM pre-application. The application of an H_2O_2 inhibitor DPI or CAT also decreased production of H_2O_2 and MDA content under the -N+NaCl+SHAM treatment (Fig.6*A*,*B*).

To further investigate the mechanism of -N induced salt tolerance in the *P. annua* callus, Na^+ and K^+ content were determined under the different treatments. After the 600 mM NaCl treatment for 48 h, the intracellular Na^+ content dramatically increased (415.37 %) compared to the control, whereas the K^+ content decreased by 70.90 % resulting in a dramatic increase of Na^+/K^+ ratio. However, the combined -N and NaCl treatment resulted in a remarkable decrease in Na^+ content (63.75 % of that at the NaCl treatment) and an increase in K^+ content (118.06 %), which ultimately led to a significant decrease of Na^+/K^+ ratio compared to the salt treatment alone, but it was still higher than that in the control. Further, the application of SHAM caused non-functionality of the AP under the -N+NaCl treatment and increased Na^+ content and decreased K^+ content leading to a marked increase of Na^+/K^+ ratio compared to the NaCl treatment alone. In addition, under 0 mM NaCl, the pretreatment with SHAM only slightly increased Na^+ content and kept K^+ content unchanged (Fig. 7*A*,*B*,*C*).

The PM H^+-ATPase activity significantly increased under the -N or NaCl treatment in comparison with the control. The combined -N and NaCl treatment further increased its activity, whereas SHAM eliminated the effect of the -N+NaCl treatment on activity of PM H^+-ATPase or even lowered it under the NaCl stress alone (Fig. 7*D*).

Discussion

Salinity is a major abiotic stress factor that disrupts ionic homeostasis and imposes ionic toxicity to plants (Zhu 2003). The occurrence of salt stress is accompanied by nutrition deprivation in plants grown in the desert and sand dune region. A recent study provides a novel evidence for “cross tolerance” and “stress memory” in different plant responses to different stresses (Walter *et al.* 2012, Munné-Bosch and Alegre 2013). This study demonstrates for the first time that nitrogen deprivation increased salt-stress tolerance in the *P. annua* callus through a mechanism that involved pyruvate and subsequent induction of the AP. Evidence provided indicates that the -N induced antioxidant system was also necessary for salt tolerance.

It is widely accepted that ROS, as signals, can activate expression of many stress-responsive genes (Foyer and Noctor 2009, Boubakri *et al.* 2013) and regulate cellular antioxidant capacity through increasing antioxidant enzyme activities and biosynthesis of non-enzymatic antioxidants such as ascorbic acid and glutathione (Jiang *et al.* 2012). A higher antioxidant capacity can feedback control intracellular ROS content to a stable state (Burke *et al.* 1985, Gill and Tuteja 2010, Hasanuzzaman and Fujita 2011, Bose *et al.* 2014). Application of scavengers or inhibitors of ROS can decrease resistance of plants to environmental stresses (Li *et al.* 2011, Zhu *et al.* 2013, Wang *et al.* 2013, 2014). However, excessive ROS cause damage to plant cells and their accumulation must be controlled (Foyer and Noctor 2005). The results of the present study show that the activities of SOD, CAT, APX, and POD in the *P. annua* callus were significantly up-regulated under the -N conditions, and the highest activities of these enzymes were found under the -N+NaCl treatment. Many stress factors, including excess of zinc or selenium, can significantly up-regulate antioxidant enzyme activities (Chen *et al.* 2013, Ding *et al.* 2014, Saidi *et al.* 2014). The content of H_2O_2 decreased in the *P. annua* callus treated with -N+NaCl compared to the NaCl treatment alone (Fig. 6*B*). Therefore, we propose that increasing ROS-scavenging enzymes should be a very important mechanism in -N induced salt tolerance in the *P. annua* callus.

Numerous studies have indicated the involvement of AOX in response to salt stress and macroelement deficiency in different species (Smith *et al.* 2009, Wang *et al.* 2010, Watanabe *et al.* 2010). An increase of AOX has been linked to ability to keep a ubiquitin pool in a more oxidized state and thus to minimize formation of harmful ROS *via* a non-specific interaction of reduced electron carriers with molecular oxygen (Maxwell *et al.* 1999). The capacity of AP in callus treated with -N+NaCl was significantly higher than that in callus treated with NaCl or -N alone, and AP capacity in the stressed *P. annua* callus was higher than in the unstressed callus (Fig. 2), suggesting a synergistic effect of the NaCl and -N on AP induction.

It was postulated that H_2O_2, nitric oxide, and ethylene might be secondary messengers in the signal transduction pathway to induce the AP (Wang *et al.* 2010, 2012). Our results show that the exogenous application of the inhibitors H_2O_2, NO, and ethylene did not diminish the -N induced AP enhancement in the *P. annua* callus suggesting that these signal messengers might not function in AP induction under the -N conditions. In addition, AP capacity is also influenced by the presence of pyruvate, an allosteric activator of AOX (Vanlerberghe and McIntosh 1997). Hu *et al*. (2006) reported that pyruvate content significantly increases in tolerant roots of cucumber but not in sensitive leaves under chilling stress. Kumar *et al* (2007) also found that plants grown at a high altitude exhibit a greater AP activity compared to plants grown at a low altitude implying that pyruvate content increases with an increase in altitude. In this study, a significant increase in pyruvate content was found in the callus with the NaCl or -N treatment, and the highest content was observed under the -N+NaCl treatment. These results suggest that the -N induced AP enhancement might result from pyruvate accumulation and thus enhanced subsequent salt tolerance.

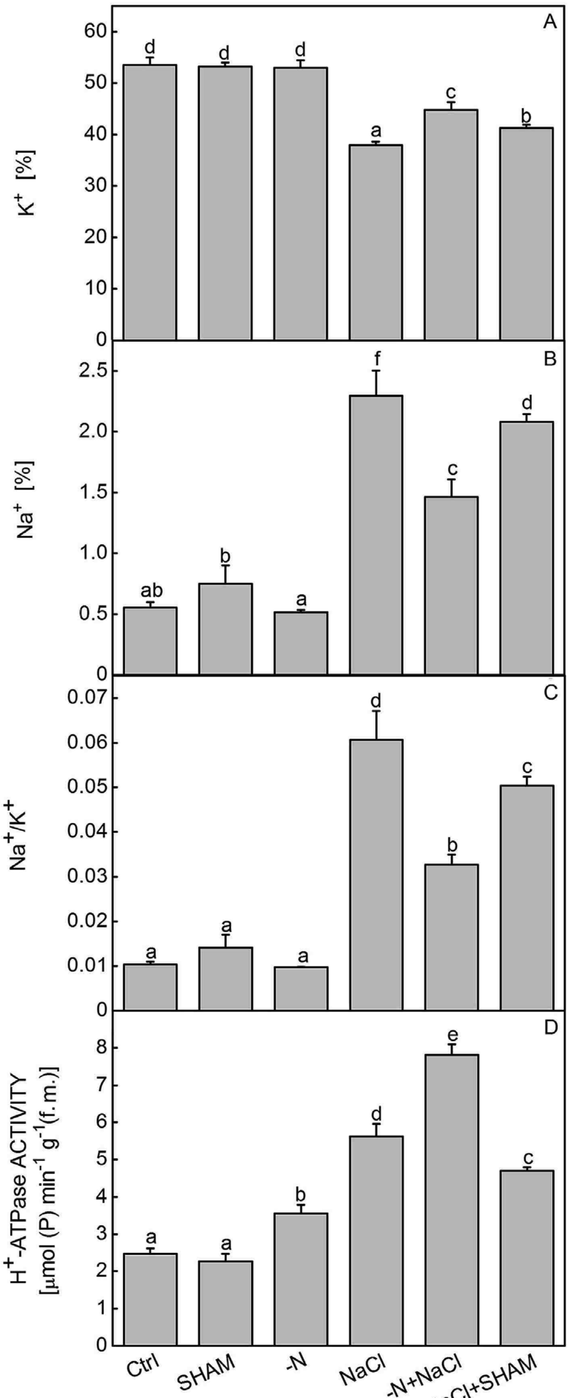

Fig. 7. The effects of the alternative respiration pathway on K$^+$ (*A*) and Na$^+$ (*B*) percentages of all elements measured, on Na$^+$/K$^+$ ratio (*C*), and PM H$^+$-ATPase activity (*D*) under different treatments in *P. annua* callus. The callus was treated as in Fig. 2. Means ± SEs from three independent experiments. The *bars* with different letters indicate significant difference at $P < 0.05$.

Intracellular ion homeostasis is essential for plants to counteract salt stress (Neill *et al*. 2002). Maintenance of ion homeostasis, particularly the Na$^+$/K$^+$ ratio, is of critical importance for plants to survive under salt stress (Sun *et al*. 2010). The presence of sufficient K$^+$ in the cytoplasm is essential for activating enzymes, controlling membrane potentials, and exerting osmotic adjustment (Zhu 2003). It has been reported that AOX-OE lines show a novel response of altered ionic homeostasis (a lower leaf Na$^+$ content and a higher root Na$^+$ content) and an improved salinity tolerance compared to wild type plants (Munns and Tester 2008). Our study also demonstrates that the -N+NaCl treatment increased K$^+$ content but decreased Na$^+$ content and Na$^+$/K$^+$ ratio in the *P. annua* callus under the salt stress (Fig. 7). These observations suggest that the -N alleviated an NaCl-induced injury by maintaining ion homeostasis in the *P. annua* callus. The application of SHAM together with the -N eliminated the effect of the -N on element ratio (Fig. 7). These findings suggest that inhibition of the AP can block the effect of the -N on ion homeostasis in the *P. annua* callus under the salt stress.

Based on the above discussion, it is interesting to question what links Na$^+$ transport and the AP under salinity. Our data and previous results show that increased AOX expression results in a lower ROS and Na$^+$ content but a higher K$^+$ content (Smith *et al*. 2009). The link between enhanced ROS accumulation and lowered Na$^+$ under salt treatment has been previously documented in *Arabidopsis* (Smith *et al*. 2009, Wang *et al*. 2010). Foyer and Noctor (2005) suggested that ROS are able to act as signalling molecules. Zhu *et al* (2007) suggested that ROS might interfere with the selectivity of transporter proteins or disturb cytoplasmic pH. Natrium transport affects cytoplasmic pH which must be re-adjusted, and ROS may significantly disrupt the ability of cells to do the re-adjustment. Recent researches also showed that increased or decreased ROS content increase the stability of *Salt Overly Sensitive 1* mRNA (Chung *et al*. 2008) and activity of PM H$^+$-ATPase. In addition, application of H_2S induces H_2O_2 accumulation *via* regulating glucose-6-phosphate dehydrogenase and PM NADPH oxidase. Accumulation of H_2O_2 in turn stimulates PM H$^+$-ATPase activity and PM Na$^+$/H$^+$ antiporter protein expression, which finally results in regulated ion homeostasis in *Arabidopsis* tolerance to salt

(Li *et al.* 2014). These results imply that complex signalling networks exist in plant tolerance to salt stress and these force us to understand whether AOX and ROS share the same glucose-6-phosphate dehydrogenase pathway in the *P. annua* callus under -N-induced salt tolerance.

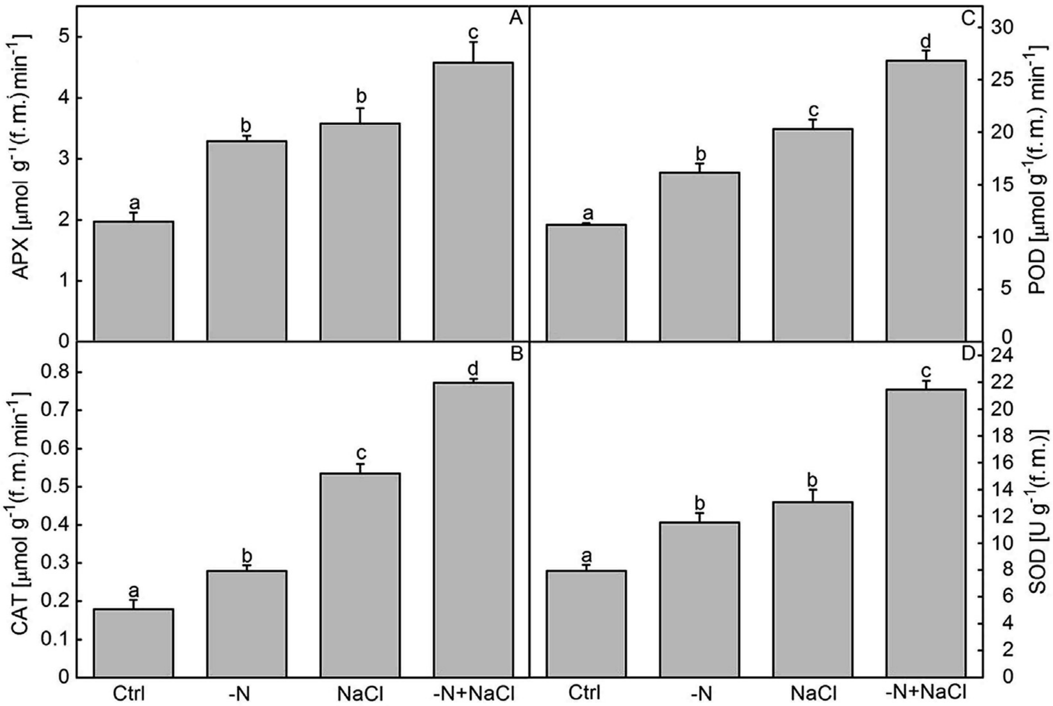

Fig. 8. The changes of antioxidant enzyme activities (*A* - APX; *B* - CAT; *C* - POD; *D* - SOD) in a *P. annua* control callus (Ctrl) and a callus treated using media -N, NaCl, or -N+NaCl for 3 d. Means ± SEs from three independent experiments. The *bars* with different letters indicate significant differences at $P < 0.05$.

References

Adam, A.L., Bestwick, C.S., Barna, B., Mansfield, J.W.: Enzymes regulating the accumulation of active oxygen species during the hypersensitive reaction of bean to *Pseudomonas syringae* pv. *phaseolicola*. - Planta **197**: 240-249, 1995.

Aebi, H.: Catalase *in vitro*. - Methods Enzymol. **105**: 121-126, 1982.

Apel, K., Hirt, H.: Reactive oxygen species: metabolism, oxidative stress, and signal transduction. - Annu. Rev. Plant Biol. **55**: 373-399, 2004.

Asada, K.: Production and scavenging of reactive oxygen species in chloroplasts and their functions. - Plant Physiol. **141**: 391-396, 2006.

Ballesteros, E., Kerkeb, B., Donaire, J.P., Belver, A.: Effects of salt stress on H^+-ATPase activity plasma membrane-enriched vesicles isolated from sunflower roots. - Plant Sci. **134**: 181-190, 1998.

Bartoli, C.G., Gomez, F., Martinez, D.E., Guiamet, J.J.: Mitochondria are the main target for oxidative damage in leaves of wheat (*Triticum aestivum L.*). - J. exp. bot. **55**: 1663-1669, 2004.

Borsani, O., Valpuesta, V., Botella, M.A.: Evidence for a role of salicylic acid in the oxidative damage generated by NaCl and osmotic stress in *Arabidopsis* seedlings. - Plant Physiol. **126**: 1024-1030, 2001.

Bose, J., Rodrigo-Moreno, A., Shabala, S.: ROS homeostasis in halophytes in the context of salinity stress tolerance. - J. exp. Bot. **64**: 1241-1257, 2014.

Boubakri, H., Wahab, M.A., Chong, J., Gertz, C., Gandoura, S., Mliki, A., Bertsch, C., Soustre-Gacougnolle I.: Methionine elicits H_2O_2 generation and defense gene expression in grapevine and reduces *Plasmopara viticola* infection. - J. Plant Physiol. **170**: 1561-1568, 2013.

Boyer, J.S.: Plant productivity and environment. - Science **218**: 443-448, 1982.

Bradford, M.M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. - Anal. Biochem. **72**: 248-254, 1976.

Burke, J.J., Gamble, P.E., Hatfield, J.L., Quisenberry, J.E.: Plant morphological and biochemical responses to field water deficits. I. Responses of glutathione reductase activity and paraquat sensitivity. - Plant Physiol. **79**: 415-419, 1985.

Chen, J., Wang, W.H., Wu, F.H., You, C.Y., Liu, T.W., Dong, X.J., He, J.X., Zheng, H.L.: Hydrogen sulfide alleviates aluminum toxicity in barley seedlings. - Plant Soil **362**: 301-318, 2013.

Chen, S., McElroy, J.S., Flessner, M.L., Dane, F.: Utilizing next-generation sequencing to study homeologous polymorphisms and herbicide-resistance-endowing mutations in

Poa annua acetolactate synthase genes. - Pest Manage. Sci. **71**: 1141-1148, 2014.

Chung, J.S., Zhu, J.K., Bressan, R.A., Hasegawa, P.M., Shi, H.: Reactive oxygen species mediate Na^+-induced SOS1 mRNA stability in *Arabidopsis*. - Plant J. **53**: 554-565, 2008.

Clifton, R., Lister, R., Parker, K.L., Sappl, P.G., Elhafez, D., Millar, A.H., Day, D.A., Whelan, J.: Stress-induced co-expression of alternative respiratory chain components in *Arabidopsis thaliana*. - Plant mol. Biol. **58**: 193-212, 2005.

Ding, Y.Z., Feng, R.W., Wang, R.G., Guo, J.K., Zheng, X.Q.: A dual effect of Se on Cd toxicity: evidence from plant growth, root morphology and responses of the antioxidative systems of paddy rice. - Plant Soil **375**: 289-301, 2014.

Duo, L.A., Gao, Y. B., Zhao, S. L.: Growth responses of *Poa pratensis* to the stress of four heavy metals. - Acta bot. boreal.-occident. sin. **26**: 0183-0187, 2006.

Fiorani, F., Umbach, A.L., Siedow, J.N.: The alternative oxidase of plant mitochondria is involved in the acclimation of shoot growth at low temperature. A study of *Arabidopsis* AOX1a transgenic plants. - Plant Physiol. **139**: 1795-1805, 2005.

Foyer, C.H., Noctor, G.: Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. - Plant Cell **17**: 1866-1875, 2005.

Foyer, C.H., Noctor, G.: Redox regulation in photosynthetic organisms: signaling, acclimation, and practical implications. - Antioxid. Redox Signal. **11**: 861-905, 2009.

Gill, S.S., Tuteja, N.: Reactive oxygen species and antioxidant mechinery in abiotic stress tolerance in crop plants. - Plant Physiol. Biochem. **48**: 909-930, 2010.

Giraud, E., Ho, LH., Clifton, R., Carroll, A., Estavillo, G., Tan, Y.F., Howell, K.A., Ivanova, A., Pogson, B.J., Millar, A.H., Whelan, J.: The absence of alternative oxidase1a in *Arabidopsis* results in acute sensitivity to combined light and drought stress. - Plant Physiol. **147**: 595-610, 2008.

Han, R.M., Lefevre, I., Albacete, A., Perez-Alfocea, F., Barba-Espin, G., Diaz-Vivancos, P., Quinet, M., Ruan, C.J., Hernandez, J.A., Cantero-Navarro, E., Lutts, S.: Antioxidant enzyme activities and hormonal status in response to Cd stress in the wetland halophyte *Kosteletzkya virginica* under saline conditions. - Physiol. Plant. **147**: 352-368, 2013.

Hasanuzzaman, M., Fujita, M.: Selenium pretreatment upregulates the antioxidant defense and methylglyoxal detoxification system and confers enhanced tolerance to drought stress in rapeseed seedlings. - Biol. trace Elements Res. **143**: 1758-1776, 2011.

Hassidim, M., Braun, Y., Lener, H.R., Reihold, L.: Studies on H^+ translocating ATPase in plants of varying resistance to salinity. - Plant Physiol. **81**: 1057-1061, 1986.

Heide, O.M.: Flowering responses of contrasting ecotypes of *Poa annua* and their putative ancestors *Poa infirma* and *Poa supina*. - Ann. Bot. **87**:795-804, 2001.

Hemp, A.: Introduced plants on Kilimanjaro: tourism and its impact. - Plant Ecol. **197**: 17-29, 2007.

Hu, W.H., Shi, K., Song, X.S., Xia, X.J., Zhou, Y.H., Yu, J.Q.: Different effects of chilling on respiration in leaves and roots of cucumber (*Cucumis sativus*). - Plant Physiol. Biochem. **44**: 837-843, 2006.

Janda, T., Szalai, G., Tari, I., Paldi, E.: Hydroponic treatment with salicylic acid decreases the effects of chilling injury in maize (*Zea mays* L.) plants. - Planta **208**: 175-180, 1999.

Jiang, Y.P., Cheng, F., Zhou, Y.H., Xia, X.J., Mao, W.H., Shi, K., Chen, Z.X., Yu, J.Q.: Cellular glutathione redox homeostasis plays an important role in the brassinosteroid-induced increase in CO_2 assimilation in *Cucumis sativus*. - New Phytol. **194**: 932-943, 2012.

Kumar, N., Vyas, D., Kumar, S.: Plants at high altitude exhibit higher component of alternative respiration. - J. Plant Physiol. **164**: 31-38, 2007.

Li, J.S., Chen, G.L., Wang, X.M., Zhang, Y.L., Jia, H.L., Bi, Y.R: Glucose-6-phosphate dehydrogenase-dependent hydrogen peroxide production is involved in the regulation of plasma membrane H^+-ATPase and Na^+/H^+ antiporter protein in salt-stressed callus from *Carex moorcroftii*. - Physiol. Plant. **141**: 239-250, 2011.

Li, J.S., Jia, H.L., Wang, J., Cao, Q.H., Wen, Z.C.: Hydrogen sulfide is involved in maintaining ion homeostasis via regulating plasma membrane Na^+/H^+ antiporter system in the hydrogen peroxide-dependent manner in salt-stress *Arabidopsis thaliana* root. - Protoplasma **251**: 899-912, 2014.

Li, J.S., Wang, X.M., Bi, Y.R.: Tissure culture and rapid propagation of *Carex moorcroftii*. - Plant Physiol. Commun. **44**: 516-517, 2008.

Lin, Y.L., Chao, Y.Y., Kao, C.H.: Exposure of rice seedlings to heat shock protects against subsequent Cd-induced decrease in glutamine synthetase activity and increase in specific protease activity in leaves. - J. Plant Physiol. **167**: 1061-1065, 2010.

Liu, Y.G., Wu, R.R., Wan, Q., Xie, G.Q., Bi YR.: Glucose-6-phosphate dehydrogenase plays a pivotal role in nitric oxide-involved defense against oxidative stress under salt stress in red kidney bean roots. - Plant. Cell physiol. **48**: 511-522, 2007.

Lv, Y. W., He, J. Y., Bai, X. M., Dong, Qin., Lei, Y. W.: Evaluation of physiological responses and resistances of nine wild *Poa* to low temperature. - Acta argestia sin. **22**: 326-333, 2014.

Maxwell, D.P., Wang, Y., McIntosh, L.: The alternative oxidase lowers mitochondrial reactive oxygen production in plant cells. - Proc. nat. Acad. Sci. USA **96**: 8271-8276, 1999.

Millar, A.H., Atkin, O.K., Menz, R.I., Henry, B., Farquhar, G., Day, D.A.: Analysis of respiratory chain regulation in roots of soybean seedlings. - Plant Physiol. **117**: 1083-1093, 1998.

Molina-Montenegro, M.A., Carrasco-Urra, F., Rodrigo, C., Convey, P., Valladares, F., Gianoli, E.: Occurrence of the non-native annual bluegrass on the Antarctic mainland and its negative effects on native plants. - Conserv. Biol. **26**: 717-723, 2012.

Morsomme, P., Boutry, M.: The plant plasma membrane H(+)-ATPase: structure, function and regulation. - Biochim. biophys. Acta **1465**: 1-16, 2000.

Munné-Bosch, S., Alegre, L.: Cross-stress tolerance and stress "memory" in plants: an integrated view. - Environ. exp. Bot. **94**: 1-2, 2013.

Munns, R., Tester, M.: Mechanisms of salinity tolerance. - Annu. Rev. Plant Biol. **59**: 651-681, 2008.

Neill, S.J., Desikan, R., Clarke, A., Hurst, R.D., Hancock, J.T.: Hydrogen peroxide and nitric oxide as signalling molecules in plants. - J. exp. Bot. **53**: 1237-1242, 2002.

Niu, X., Bressan, R.A., Hasegawa, P.M., Pardo, J.M.: Ion homeostasis in NaCl stress environments. - Plant Physiol. **109**: 735-742, 1995.

Parsons, H.L., Yip, J.Y.H., Vanlerberghe, G.C.: Increased respiratory restriction during phosphate-limited growth in transgenic tobacco cells lacking alternative oxidase. - Plant Physiol. **121**: 1309-1320, 1999.

Peng, Y., Huang, B. R., Xu, L.X., Ll, Z.: Heat stress effects on osmotic potential, membrane fatty acid composition and lipid peroxidation content of two Kentucky Bluegrass cultivars differing in drought tolerance. - Acta Horticult. sin. **40**: 971-980, 2013.

Procházková, D., Sairam, R.K., Srivastava, G.C., Singh, D.V.: Oxidative stress and antioxidant activity as the basis of senescence in maize leaves. - Plant Sci. **161**: 765-771, 2001.

Puyang, X. H., An, M. Y., Han, L. B., Zhang, X. B.: Protective effect of spermidine on salt stress induced oxidative damage in two Kentucky bluegrass (*Poa pratensis* L.) cultivars. - Ecotoxic. Environ. Safety **117**: 96-106, 2015.

Qiu, Q.S., Su, X.F.: The influence of extracellular-side Ca^{2+} on the activity of the plasma membrane H^+-ATPase from wheat roots. - Aust. J. Plant Physiol. **25**: 923-928, 1998.

Rea, P.A., Poole, R.J., Vacuolar H^+-translocating pyrophosphatase. - Annu. Rev. Plant Physiol. **44**: 157-180, 1993.

Rea, P.A., Sander, D.: Tonoplast energization: two H^+ pumps, one membrane. - Physiol. Plant. **71**: 131-141, 1987.

Saidi, I., Chtourou, Y., Djebali, W.: Selenium alleviates cadmium toxicity by preventing oxidative stress in sunflower (*Helianthus annuus*) seedlings. - J. Plant Physiol. **171**: 85-91, 2014.

Serrano, R., Mulet, J.M., Rios, G.: A glimpse of the mechanisms of ion homeostasis during salt stress. - Plant Sci. **162**: 897-904, 1999.

Sairam, P.K, Srivastava, G.C.: Changes in antioxidant activity in subcellular fraction of tolerant and susceptible wheat genotypes in response to long term salt stress. - Plant Sci. **162**: 897-904, 2002.

Sieger, S.M., Kristensen, B.K., Robson, C.A., Amirsadeghi, S., Eng, E.W., Abdel-Mesih, A., Moller, I.M., Vanlerberghe, G.C.: The role of alternative oxidase in modulating carbon use efficiency and growth during macronutrient stress in tobacco cells. - J. exp. Bot. **56**: 1499-1515, 2005.

Smith, C.A., Melino, V.J., Sweetman, C., Soole, K.L.: Manipulation of alternative oxidase can influence salt tolerance in *Arabidopsis thaliana*. - Physiol. Plant. **137**: 459-472, 2009.

Sun, J., Wang, M.J., Ding, M.Q., Deng, S.R., Liu, M.Q., Lu, C.F., Zhou, X.Y., Shen, X., Zheng, X.J., Zhang, Z.K., Song, J., Hu, Z.M., Xu, Y., Chen, S.L.: H_2O_2 and cytosolic Ca^{2+} signals triggered by the PM H-coupled transport system mediate K^+/Na^+ homeostasis in NaCl-stressed *Populus euphratica* cells. - Plant. Cell Environ. **33**: 943-958, 2010.

Umbach, A.L., Fiorani, F., Siedow, J.N.: Characterization of transformed *Arabidopsis* with altered alternative oxidase levels and analysis of effects on reactive oxygen species in tissue. - Plant Physiol. **139**: 1806-1820, 2005.

Vanlerberghe, G.C., McIntosh, L.: Alternative oxidase: from gene to function. - Annu. Rev. Plant Physiol.. **48**: 703-734, 1997.

Veljovic-Jovanovic, S., Noctor, G., Foyer, C.H.: Are leaf hydrogen peroxide concentrations commonly overestimated? The potential influence of artefactual interference by tissue phenolics and ascorbate. - Plant Physiol. Biochem. **40**: 501-507, 2002.

Walter. J., Jentschh, A., Beierkuhnlein, C., Kreyling, J.: Ecological stress memory and cross stress tolerance in plants in the face of climate extremes. - Environ. exp. Bot. **94**: 3-8, 2012.

Wang, H.H., Huang, J.J., Liang, X.L., Bi, Y.R.: Involvement of hydrogen peroxide, calcium, and ethylene in the induction of the alternative pathway in chilling-stressed *Arabidopsis* callus. - Planta **235**: 53-67, 2012.

Wang, H,H,, Liang, X,L., Huang, J.J., Zhang, D.K., Lu, H.X., Liu, Z.J., Bi, Y.R.: Involvement of ethylene and hydrogen peroxide in induction of alternative respiratory pathway in salt-treated *Arabidopsis* calluses. - Plant. Cell Physiol. **51**: 1754-1765, 2010.

Wang, L., Guo, Y.J., Jia, L.X., Chu, H.Y., Zhou, S., Chen, K.M., Wu, D., Zhao, L.Q.: Hydrogen peroxide acts upstream of nitric oxide in the heat shock pathway in *Arabidopsis* seedlings. - Plant Physiol. **164**: 2184-2196, 2014.

Wang, X.M., Hou, C., Liu, J., He, W.L., Nan, W.B., Gong, H.L., Bi, Y.R.: Hydrogen peroxide is involved in the regulation of rice (*Oryza sativa* L.) tolerance to salt stress. - Acta Physiol. Plant. **35**: 891-900. 2013.

Wang, X.M., Ma, Y.Y., Huang, C.H., Wan, Q., Li, J.S., Bi, Y.R.: Glucose-6-phosphate dehydrogenase plays a central role in modulating reduced glutathione levels in reed callus under salt stress. - Planta **227**: 611-623, 2008.

Watanabe, C.K., Hachiya, T., Takahara, K., Kawai-Yamada, M., Uchimiya, H., Uesono, Y., Terashima, I., Noguchi, K.: Effects of *AOX1a* deficiency on plant growth, gene expression of respiratory components and metabolic profile under low-nitrogen stress in *Arabidopsis thaliana*. - Plant Cell Physiol. **51**: 810-822, 2010.

Wu, Y.X., Ding, N., Zhao, X., Zhao, M., Chang, Z.Q., Liu, J.Q., Zhang, L.X.: Molecular characterization of PeSOS1 the putative Na^+/H^+ antioporter of *Populus euphratica*. - Plant mol. Biol. **65**: 1-11, 2007.

Yip, J.Y., Vanlerberghe, G,C.: Mitochondrial alternative oxidase acts to dampen the generation of active oxygen species during a period of rapid respiration induced to support a high rate of nutrient uptake. - Physiol. Plant. **112**: 327-333, 2001.

Yu, L., Ma, H. L.: The endogenous hormone level and drought adaptability of four Kentucky bluegrass species in Gansu. - China J. Desert Res. **35**: 0182-0188, 2015.

Zhao, L.Q., Zhang, F., Guo, J.K., Yang, Y.L., Li, B.B., Zhang, L.X.: Nitric oxide functions as a signal in salt resistance in the calluses from two ecotypes of reed. - Plant Physiol. **134**: 849-857, 2004.

Zhu, J., Fu, X., Koo, Y.D., Zhu, J.K., Jenney, F.E., Jr., Adams, M.W., Zhu. Y., Shi, H., Yun, D.J., Hasegawa, P.M., Bressan, R.A.: An enhancer mutant of *Arabidopsis salt overly sensitive 3* mediates both ion homeostasis and the oxidative stress response. - Mol. cell. Biol. **27**: 5214-5224, 2007.

Zhu, J.K.: Regulation of ion homeostasis under salt stress. - Cur. Opin. Plant Biol. **6**: 441-445, 2003.

Zhu, Y., Zuo, M.X., Liang, Y.L., Jiang, M.Y., Zhang, J.H., Scheller, H.V., Tan, M.P., Zhang, A.Y.: MAP65-1a positively regulates H_2O_2 amplification and enhances brassinosteroid-induced antioxidant defence in maize. - J. exp. Bot. **64**: 3787-3802, 2013.