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Research article

Dynamic responses of *Haloxylon ammodendron* to various degrees of simulated drought stress

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ABSTRACT

Haloxylon ammodendron, a C4 perennial, succulent and xero-halophytic shrub, is highly resistant to harsh environments, therefore, exploring the stress resistance mechanism will be beneficial for the use of xerophytes to prevent desertification. To determine osmotic adjustment (OA) and antioxidase functions under simulated drought stress, 8-week-old seedlings were treated with sorbitol solutions to maintain osmotic potentials (Ψs) at a control and −0.5 and −1.0 MPa. Under −0.5 MPa osmotic stress, *H. ammodendron* stably maintained the water content of assimilating branches, a result that was not significantly different from the result of the control group. Moreover, the Ψs decreased significantly, which helped plants absorb water efficiently from the environment, as *H. ammodendron* accumulated massive osmotic regulators in its assimilating branches to adjust shoot Ψs. Specifically, the contribution of Na^+ to shoot Ψs was up to 45%, and Na^+ became the main osmotic regulator of OA. During the treatments, the content and contribution of K^+ remained stable. However, the total contribution of three organic osmotic regulators (free proline, betaine and soluble sugar) was only 20%, and betaine was the main organic osmotic regulator, accounting for approximately 15% of the 20% contribution. Moreover, *H. ammodendron* seedlings presented strong antioxidases, especially when there was a high activity level of superoxide dismutase, and with an increase in treatment time and degree of osmotic stress, the activity of peroxidase and catalase increased significantly. Substantial accumulation of osmotic adjustment substances was an important strategy for *H. ammodendron* to cope with simulated drought stress, in particular, *H. ammodendron* absorbed much Na^+ and transported Na^+ into the assimilating branch for OA. The scavenging of reactive oxygen species by antioxidases was another adaptation strategy for *H. ammodendron* to adapt to simulated drought stress.

1. Introduction

Drought stress is a comprehensive threat, and the direct stress signal induced by drought is referred to as osmotic stress (Zhu, 2016; Nakashima et al., 2014). Plant growth is very sensitive to water and water deficits can cause serious damage to plants. In terms of plant morphology and structure, osmotic stress often results in plant wilting, as well as leaf shrinkage and yellowing. Persistent osmotic stress will cause changes in the structures and activities of enzymes, manifesting as a decrease in photosynthetic efficiency, which makes it impossible for plants to complete normal metabolism and growth, and even causes the death of plants (Sanders and Arndt, 2012; Chaves et al., 2009; Seki et al., 2007).

Osmotic adjustment (OA) is a vital adaptation strategy for plants to minimize the damage induced by drought stress at the cellular level (Blum, 2005). Plants decrease their osmotic potential (Ψs) by accumulating organic and inorganic solutes under drought stress (Blum, 2017; Ma et al., 2011; Raymond and Smirnoff, 2002). These compatible solutes normally consist of free proline, betaine, soluble sugars, and potassium ions (K^+) etc. (Kiani et al., 2007; Zeng et al., 2015; Farooq et al., 2009a, b; Behr et al., 2017), which help plants not only to maintain the water content, but also to protect enzymes from the damage induced by reactive oxygen species (ROS) (Farooq et al., 2009a, b). For instance, K^+ usually plays a necessary role in plant stress tolerance, and it is an activator of many enzymes and important component for maintaining cell Ψs in plant cells (Mengel and Arneke, 1982;

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Wang and Wu, 2017). In addition, free proline, betaine and soluble sugars are also important substances in OA. They can form hydrophilic colloid-like polymers, stabilize the structure and stability of macromolecules in cells and play a protective role when plants are subjected to osmotic stress (Yooyongwech et al. 2016, 2017; Liu et al., 2017). Generally, Na^+ is considered as a toxic ion in glycophytes (Deinlein et al., 2014).

Osmotic stress often generates massive ROS, i.e., H_2O_2, O_2^-, OH^- and malondialdehyde (MDA). Toxic levels of ROS cause severe damage to membrane permeability, DNA and proteins, and seriously disrupt the normal metabolism of plants (Mahajan and Tuteja, 2005; Kekec et al., 2013; Allen and Ort, 2001). In general, superoxide dismutase (SOD) is the first line of defense for plants (Misra and Gupta, 2006), and it can catalyze O_2^- into H_2O_2 and O_2, and then, H_2O_2 can be transformed to O_2 and H_2O under the catalysis of peroxidase (POD) and catalase (CAT) (Chaves et al., 2009; Liu et al., 2011). In terms of scavenging excess ROS, the antioxidase produced by plants powerfully can help to balance ROS levels in cells (Saxena et al., 2016).

Due to the long-term influence of harsh environments, desert plants have evolved unique morphological structures (such as leaf specialization) and strong resistance mechanisms during the course of evolution (Bechtold, 2018). In recent years, there has been increasing interest in exploring the resistance mechanisms of desert plants (Ashraf, 2010). *Haloxylon ammodendron*, a C4 perennial, succulent, and xero-halophytic shrub, has strong resistance to drought, salinity, high temperatures, barren conditions and other stresses (Fu, 1992; Tobe et al., 2000). *H. ammodendron* is often used as the pioneer species for sand fixation, and it plays vital roles in ecological restoration and maintenance of the structure and function of a desert ecosystem via sand fixation, water conservation and agriculture and pasture shelterbelts. *H. ammodendron* is widely distributed in the Gobi Desert region of Gansu, Inner Mongolia, Ningxia, Xinjiang and Qinghai provinces. Since 2017, in cooperation with the China Green Foundation and Ant Financial Services Group (Hangzhou, China), *H. ammodendron* has been widely cultivated in northwestern China and more than 10 million plants have been planted thus far. To date, the physiology of *H. ammodendron* under osmotic stress is still not well understood.

This study attempts to investigate the dynamic responses of OA and the antioxidases of *H. ammodendron* under simulated drought stress, and to analyze the role of these functions in the process of stress response. Therefore, sorbitol solution is used to simulate osmotic stress. The parameters consist of organic (proline, betaine, soluble sugars and soluble proteins) and inorganic (Na^+, K^+, Ca^{2+}) solutes, and the activities of antioxidase (SOD, POD, CAT) and water status are assessed.

2. Materials and methods

2.1. Plant growth conditions and treatments

Seeds of *Haloxylon ammodendron* were collected from wild plants in Alxa League Right Banner, Inner Mongolia Autonomous Region, China. (101.59° E, 39.22° N), and the annual average precipitation and temperature are 89 mm and 8.4 °C, respectively. After screening similar-sized and nutrient-rich *H. ammodendron* seeds, they were sterilized for 5 min with 75% ethanol (v/v), rinsed 5 times with distilled water and then placed on filter paper moistened with water for 12 h in the dark (25 ± 2 °C). After germination, seedlings with uniform growth were transplanted into plastic pots (5 × 5 × 5 cm; 5 seedling/pot) containing heat-sterilized vermiculite (the contents of Na^+ and K^+ in the vermiculite were $2.4\,\mu mol\,g^{-1}$ and $1.5\,\mu mol\,g^{-1}$, respectively) and 1/2 Hoagland nutrient solution (including 2 mM KNO_3, 0.5 mM KH_2PO_4, 0.5 mM $MgSO_4{\cdot}7H_2O$, 0.5 mM $Ca(NO_3)_2{\cdot}4H_2O$, 0.06 mM Fe-citrate·$3H_2O$, 50 μM H_3BO_3, 10 μM $MnCl_2{\cdot}4H_2O$, 1.6 μM $ZnSO_4{\cdot}7H_2O$, 0.6 μM $CuSO_4$ and 0.05 μM $Na_2MoO_4{\cdot}2H_2O$). The seedlings were grown in a greenhouse. The daily photoperiod, temperature, light intensity and air relative humidity were $16\,h\,d^{-1}$, 28 ± 2 °C/23 ± 2 °C (day/night), $1{,}200\,\mu mol\,m^{-2}\,s^{-1}$ and 50–70%, respectively. To maintain constant concentrations of nutrients, solutions were changed every 3 days.

After 8 weeks of growth, *H. ammodendron* seedlings were randomly divided into three groups, and treated with sorbitol solution (sorbitol dissolved in 1/2-strength Hoagland nutrient solution) to simulate different Ψs values of −1.0 MPa (severe treatment), −0.5 MPa (moderate treatment) and a control (1/2-strength Hoagland nutrient solution without sorbitol). Seedlings with similar conditions were randomly selected from each group at 0, 6, 24, 48 and 72 h after treatment. Each indicator included 6 replicates covering 15 seedlings. To minimize environmental influences, the pots were reassigned randomly every 3 days.

2.2. Determination of shoot water content

Shoot water content (g/g DW) = (FW - DW)/DW, where FW and DW represent the fresh and dry weight of the sample, respectively (Slama et al., 2007). DW was obtained by drying freshly assimilating branches in an oven at 80 °C for 48 h. The FW and DW were weighed by a 0.001 g precision electronic scale (SQP, Sartorius, Beijing, China).

2.3. Determination of shoot Ψs

The new assimilating branches of *H. ammodendron* seedlings were placed in a centrifuge tube and frozen in liquid nitrogen. After centrifugation, the supernatant fraction was collected to measure the tissue Ψs. By unfreezing slowly, the cell sap of the assimilating branches was obtained, and then, cell sap was measured by using a cryoscopic osmometer (Osmomat-030, Gonotec GmbH, Berlin, Germany) at 25 °C. The machine readings ($\mu mol{\cdot}g^{-1}$) were used to calculate shoot Ψs. Ψs (MPa) = -ic × R × T; where ic is the machine reading, $R = 8.314\,J\,K^{-1}{\cdot}mol^{-1}$ and T = 293.16 K (Wu et al., 2015).

2.4. Determination of shoot organic osmotic regulators (free proline, soluble sugars, betaine and soluble proteins)

Sulfosalicylic acid was used to extract proline (Gao et al., 2015; Trovato et al., 2008). Proline can react with an acidic ninhydrin solution and become red after heating, and then, once extracted by toluene, the absorbance can be measured at 520 nm by a spectrophotometer (UV-6100PCS, MAPADA Instrument Co., Ltd, Shanghai, China).

The soluble sugar content was determined by anthrone colorimetry (Leakey et al., 2009; Zhang et al., 2009). The soluble sugar of *H. ammodendron* was extracted according to the instructions of the soluble sugar assay kit (Comin Biotechnology, Suzhou, China), and the absorbance was recorded at 625 nm. Based on the glucose content in the standard curve, the corresponding soluble sugar content was calculated.

The betaine content was measured using the betaine assay kit specification (Comin biotechnology, Suzhou, China), and Reinecke's salt can produce precipitated material under strong acidic conditions that can be dissolved in acetone, and has a characteristic absorption peak at 525 nm (Kurepin et al., 2015). A spectrophotometer (UV-6100PCS, MAPADA Instrument Co, Ltd, Shanghai, China) was used to determine the absorbance at 525 nm.

The combination of protein and Coomassie brilliant blue (G-250) can become into blue compound complex under acidic solution conditions, and this complex has a characteristic absorption peak at 595 nm. The protein concentration is positively correlated with the color depth of the solution, and the protein content can be measured according to the standard curve (Runyon et al., 2015). This operation was carried out via a spectrophotometer (UV-6100PCS, MAPADA Instrument Co., Ltd, Shanghai, China) and soluble protein assay kit (Comin biotechnology, Suzhou, China).

2.5. Determination of shoot inorganic osmotic regulators (K^+, Na^+ and Ca^{2+})

The concentrations of inorganic ions (K^+, Na^+ and Ca^{2+}) were measured according to the method described by Wang et al. (2007) and Peterson et al. (2015). The weighed dry sample was crushed and transferred into a test tube, and soluble Na^+, K^+, and Ca^{2+} were extracted in deionized water in a 100 °C water bath for 2 h. Fast filter paper was used to gather the leach solution, and then, each ion analysis was performed using a flame photometer (Sherwood flame photometer-410, Cambridge, UK).

2.6. Calculation of the contributions of organic and inorganic osmotic regulators to Ψs

Based on shoot water content, the concentrations of inorganic solutes inorganic solutes (Na^+ and K^+) and organic solutes (betaine, proline and soluble sugar) were converted to osmolality, and then, the Ψs values of each solute (calculated Ψs (COP)) were calculated by the Van't Hoff formula as described by Guerrier (1996): $COP = -nRT$; where n is the number of solute molecules, $R = 8.314\ J\ K^{-1}\cdot mol^{-1}$ and $T = 293.16\ K$. The contributions of inorganic solutes and organic solutes to assimilating branch Ψs were computed as the formula contribution (%) = COP/Ψs × 100% (Guerrier, 1996; Ma et al., 2011).

2.7. Determination of relative plasma membrane permeability and MDA content

The 0.4 g fresh tissue sample with 20 mL distilled water was placed in a test tube and in a constant temperature shaker (IS-RDD3, Crystal, American) for 20 min at 25 °C, and S_1 was measured by a conductivity meter (EC-215, HANNA, Italy). Then, the test tube was placed into a water bath at 100 °C for 15 min, and the measured value S_2 was recorded after cooling to room temperature (25 °C) (Simon, 1974). The plant relative electrical conductivity (REC) was used to indicate the degree of damage. Plant plasma relative membrane permeability was calculated with the following formula: $REC\ (\%) = (S_1\text{-}S_{\text{distilled water}})/(S_2\text{-}S_{\text{distilled water}})$.

MDA can be a biomarker that reflects the degree of cell membrane peroxidation in plant cells. It was extracted using thiobarbituric acid (TBA) and measured by a spectrophotometric protocol (Bao et al., 2009). Absorbance was determined at 532 and 600 nm (UV-6100PCS, MAPADA Instrument Co., Ltd, Shanghai, China), and the difference in absorbance was used to calculate the MDA content.

2.8. Determination of activities of antioxidase (POD, SOD and CAT)

POD (EC 1.11.1.7), SOD (EC 1.15.1.1), and CAT (EC 1.11.1.6) were measured using the protocols proposed by Tuna et al. (2008), Alscher et al. (2002), and Liu et al. (2011), respectively, and the activities were defined as follows: one unit of POD activity was defined as a change of 0.01 at 470 nm in per minute per 1 g tissue per 1 mL reaction system; one unit of SOD activity was defined as the reduction in nitroblue tetrazolium (NBT) inhibited by 50% at 540 nm; one unit of CAT activity was defined as degradation of 1 nmol H_2O_2 per minute per 1 mg tissue. According to each specific assay kit (Comin biotechnology, Suzhou, China), 1.0 g of fresh sample with 10 mL of extraction solution was first mixed with homogenate at 4 °C and then centrifuged at 8,000 g for 10 min. The supernatant was used for the test of each enzyme's activity. Next, the readings of POD, SOD and CAT were recorded at 470 nm 560 nm and 240 nm, respectively. Finally, the obtained readings were used to calculate the activity of the enzymes, according to the methods described as the corresponding kit specification (Comin Biotechnology, Suzhou, China).

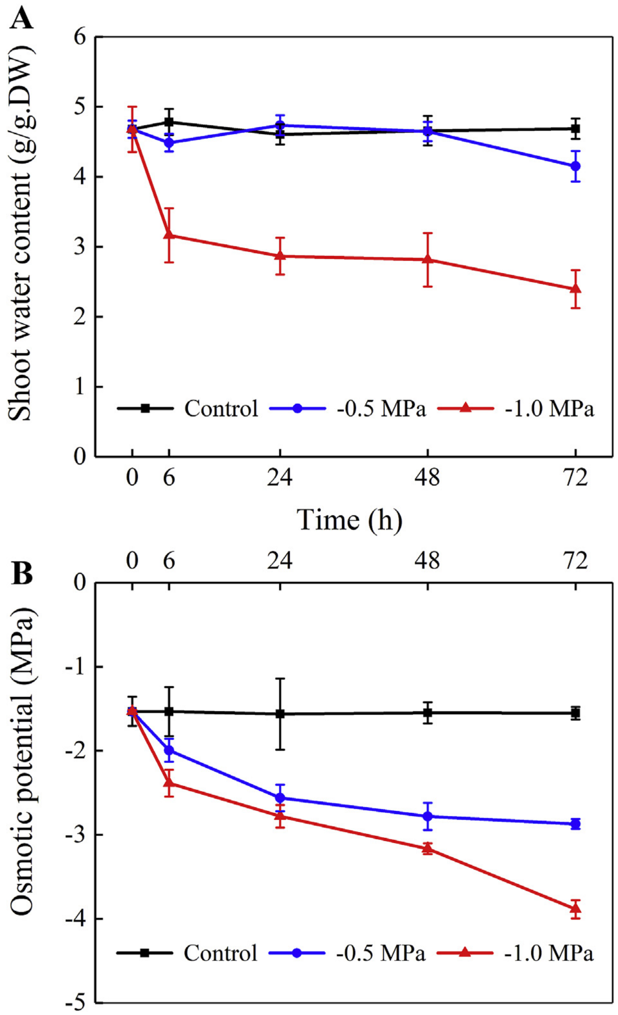

Fig. 1. Dynamic changes of shoot water content and shoot osmotic potential in *Haloxylon ammodendron* in response to various osmotic stresses. **(A)** Shoot water content, **(B)** Osmotic potential. Values and bars represent means and SEs ($n = 6$), respectively. Lines in different colors indicate different treatments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.9. Data analysis

The data were analyzed based on a one-way analysis of variance (ANOVA) by using SPSS 16.0 (SPSS Inc, Chicago, IL, USA), and all values were reported as the means ± standard error (SE). Origin 2017 (OriginLab Inc, Northampton, USA) was used to construct the figures.

3. Results

3.1. Dynamic changes in shoot water content and Ψs in Haloxylon ammodendron in response to osmotic stresses

Shoot water content of the control group maintained a stable level (Fig. 1A). There was only a slight fluctuation among the moderate treatment group (−0.5 MPa), and no significant difference was observed between moderate osmotic stress (−0.5 MPa) and the control group. Under severe osmotic stress, shoot water content of *H. ammodendron* seedlings showed a remarkable reduction with the elongation of treatment time. After 6 h of treatment, compared with that in the control, shoot water content under −1.0 MPa osmotic stress significantly decreased by 33.8%, and compared with that under −0.5 MPa osmotic stress, the content reduced by 29.4%. After 72 h of treatment, compared with that in the control, shoot water content under −1.0 MPa osmotic stress decreased by 48.9%, and compared with that under −0.5 MPa osmotic pressure, the content decreased by 42.3%. Compared with that at 0 h, the water content after 72 h of treatment under −1.0 MPa osmotic stress significantly decreased by 95.3%, while it decreased by only 12.7% under −0.5 MPa osmotic stress.

During the osmotic stress treatment, the Ψs of the control group remained stable (Fig. 1B). The Ψs gradually decreased with sustained stress under −1.0 MPa and −0.5 MPa. At 72 h of treatment, the Ψs under the severe osmotic stress (−1.0 MPa) group reduced significantly, and the potential was 1.35 and 2.5 times higher than that under −0.5 MPa and control, respectively, and the Ψs of the moderate osmotic stress (−0.5 MPa) group was 0.5 times higher than the control group. After 72 h of treatment, the Ψs of assimilating branches decreased by 153.9% (−1.0 MPa) and 87.3% (−0.5 MPa).

3.2. Dynamic changes in the contents of shoot organic osmotic regulators and their contribution to Ψs in H. ammodendron in response to osmotic stresses

The content of free proline remained consistent during the treatment time (Fig. 2A). With continued treatment, the severe and moderate osmotic groups demonstrated similar trends of increase. Compared with that in the control group and the −0.5 MPa group, the free proline content in the −1.0 MPa osmotic stress group significantly increased by 17.9%, and 13.5%, respectively. Moreover, compared with that at 0 h, the free proline content at the end of osmotic stress (72 h) increased by only 38.0% (control) and 16.0% (−0.5 MPa).

The betaine content clearly and rapidly accumulated during the beginning stage (0–24 h) under −1.0 MPa osmotic stress (Fig. 2B); however, the increase rate dropped from 24 to 72 h. The betaine content under −0.5 MPa grew slowly from 0 to 24 h, but there was a significant increase from 24 to 72 h.

The content of soluble sugars continuously accumulated and exhibited an increasing trend (Fig. 2C), and there was only a slight difference between the treatment groups (−0.5 and −1.0 MPa). From the beginning of the treatment to 72 h, the soluble sugar contents were 2.3 and 1.8 times higher in the treatment groups (−1.0 and −0.5 MPa, respectively) than in the control group.

The content of soluble proteins showed a slow increase in the treatment groups (−0.5 and −1.0 MPa) for 0–48 h (Fig. 2D). However, compared with that under −1.0 MPa osmotic stress, the soluble protein content under −0.5 MPa osmotic stress was higher from 48 to 72 h. Up to 72 h, compared with that in the control, the soluble sugar content under osmotic stress (−0.5 MPa and −1.0 MPa) significantly increased by 16.9% and 23.7%, respectively. Protein is a manifestation of the activities of life. As shown in Fig. 2D, the soluble protein content under moderate osmotic stress continued to increase throughout the treatment, but during the later period of osmotic stress (48–72 h), the soluble protein content decreased under −1.0 MPa osmotic stress, presumably because the extent of this severe osmotic stress exceeded the tolerance of *H. ammodendron* seedlings at that time, causing hydrolysis of plant cell proteins. At the same time, *in vivo* RNA transcription and translation were affected by osmotic stress, and the amount of protein synthesis, as well as soluble protein content, began to decrease.

In this study, three kinds of organic osmotic regulators (betaine, soluble sugar and free proline) were involved (Fig. 3). We found that in each treatment group (including the control group), with the osmotic stress treatment, the contribution of betaine to Ψs was always significantly higher than the contributions of soluble sugar and proline. Under moderate osmotic stress (Fig. 3B, −0.5 MPa), the contribution of betaine to the Ψs decreased (0–24 h) until it reached a steady state (24–72 h). However, under severe osmotic stress (Fig. 3C, −1.0 MPa), the contribution of betaine to the Ψs remained stable at approximately 13% from 6 h to 24 h, and then declined (24–72 h). Betaine, as a common osmotic regulator in higher plants, exhibited a sustained trend with increasing treatment time and had a larger contribution to the Ψs than the other two osmotic regulators; however, in terms of OA, the contribution of betaine showed a decrease. Soluble sugar is also a common osmotic regulator, showing a similar trend as betaine, but its contribution was less than 3%. The sum of the contributions of free proline and betaine were less than 4%, and the total contributions of organic osmotic regulators were approximately 20%. Proline is often

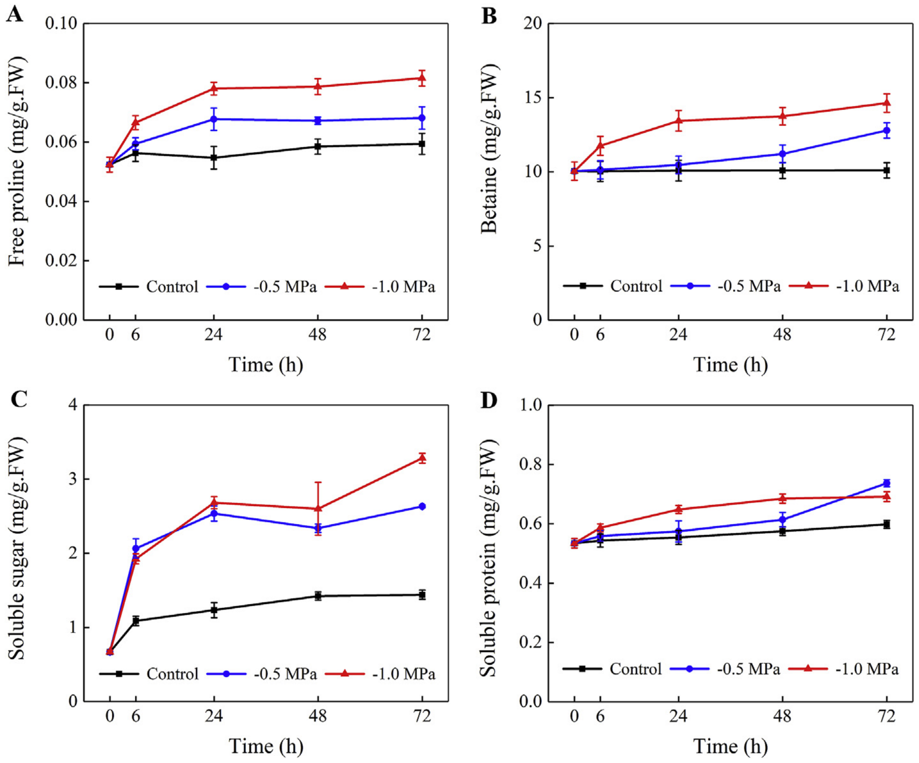

Fig. 2. Dynamic changes of the contents of shoot organic osmotic regulators (free proline, betaine, soluble sugar and soluble protein) in *H. ammodendron* in response to various osmotic stresses. **(A)** Free proline, **(B)** Betaine, **(C)** Soluble sugar, (d) Soluble protein. Values and bars represent means and SEs (n = 6), respectively. Lines in different colors indicate different treatments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

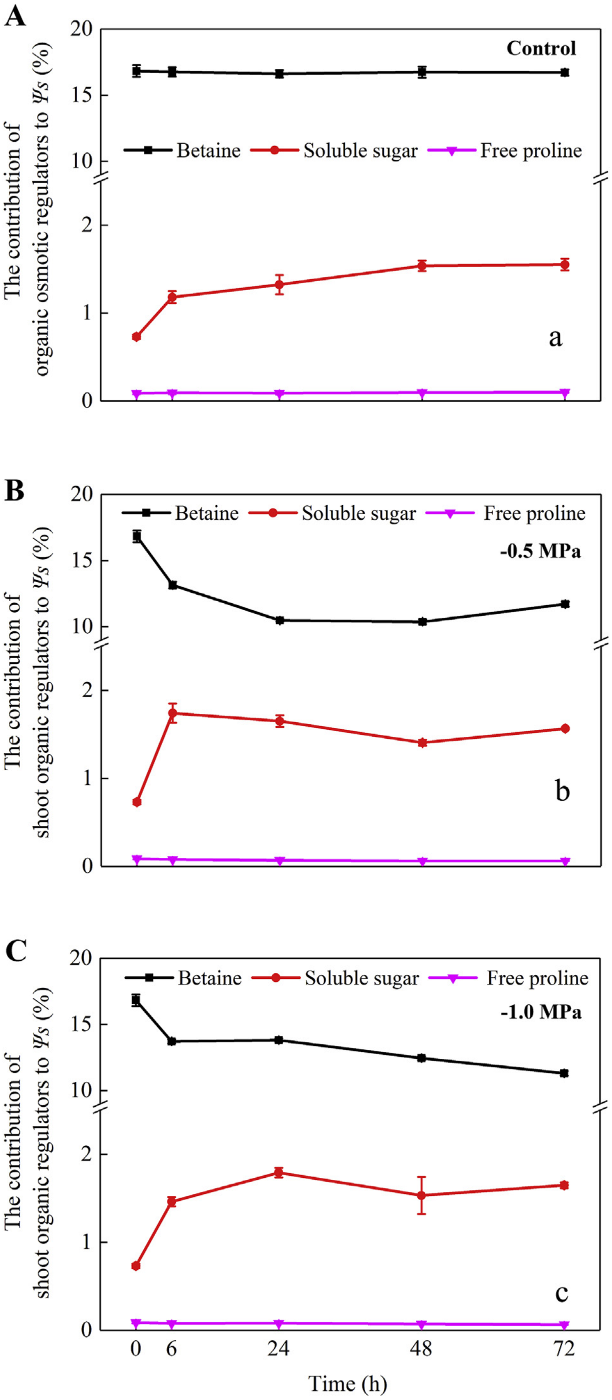

Fig. 3. Dynamic changes of the contribution of shoot organic osmotic regulators (free proline, betaine and soluble sugar) to *Ψs* in *H. ammodendron* in response to various osmotic stresses. **(A)** Control group, **(B)** −0.5 MPa treatment group, **(C)** −1.0 MPa treatment group. Values and bars represent means and SEs (n = 6), respectively. Lines in different colors indicate different organic osmotic regulators. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

regarded as an important organic osmotic regulatory substance (Yaish, 2015). In this study, the addition of osmotic stress (Fig. 3B and C, −0.5 and −1.0 MPa, respectively) significantly increased the content of free proline in the early stage of osmotic stress (0–6 h), but the concentration of free proline under osmotic stress was less than 0.5%.

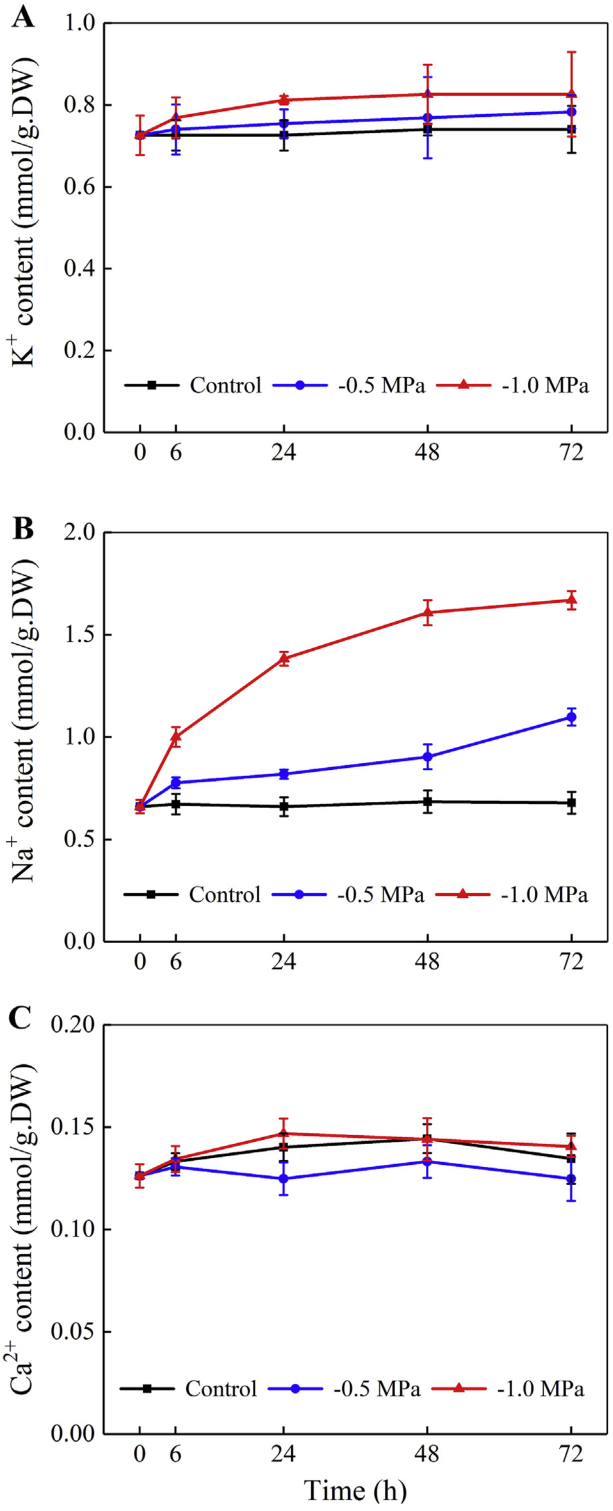

Fig. 4. Dynamic changes of the contents of shoot inorganic osmotic regulators (K^+, Na^+ and Ca^{2+}) in *H. ammodendron* in response to various osmotic stresses. **(A)** K^+ content, **(B)** Na^+ content, **(C)** Ca^{2+} content. Values and bars represent means and SEs (n = 6), respectively. Lines in different colors indicate different treatments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

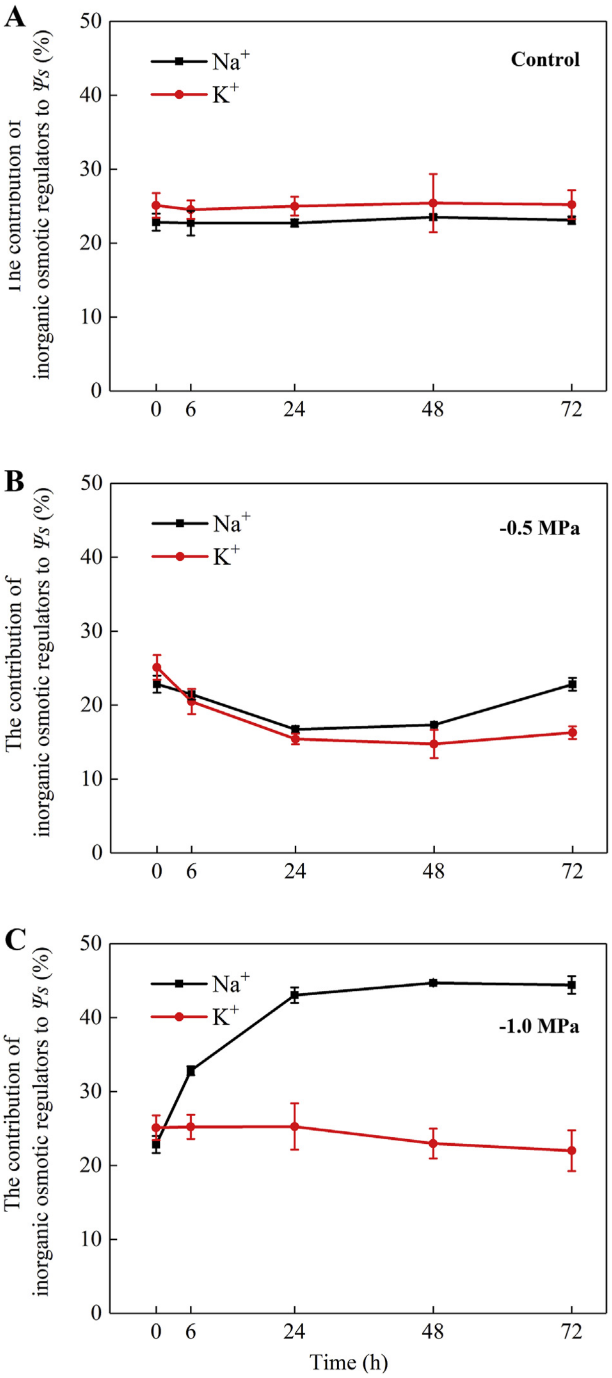

Fig. 5. Dynamic changes of the contribution of shoot inorganic osmotic regulators to *Ψs* in *H. ammodendron* in response to various osmotic stresses. **(A)** Control group, **(B)** −0.5 MPa treatment group, **(C)** −1.0 MPa treatment group. Values and bars represent means and SEs (n = 6), respectively. Lines in different colors indicate different ions. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.3. Dynamic changes in the contents of shoot inorganic osmotic regulators and their contribution to *Ψs* in *H. ammodendron* in response to osmotic stresses

The K$^+$ content showed no significant change overall (Fig. 4A). Under both −0.5 and −1.0 MPa osmotic stresses, there was only a slight increase in K$^+$ content. Even at 72 h, the accumulation of K$^+$ content was only 10.4% higher than the K$^+$ content of the control seedlings under −1.0 MPa. The Na$^+$ content of the control group remained stable within the observation time (Fig. 4B), and it increased as the osmotic stress time continued in the treatment groups (−0.5 and −1.0 MPa osmotic stress). In particular, Na$^+$ content increased rapidly under the −1.0 MPa osmotic stress treatment. Compared with that at 0 h, the Na$^+$ content after 72 h of treatment significantly increased by 145.9% (−0.5 MPa) and 61.8% (−1.0 MPa). The content of Ca^{2+} among each group showed a similar trend of change (Fig. 4C). Compared with that in the control, the Ca^{2+} content under −0.5 MPa was lower, whereas the Ca^{2+} content was higher under −1.0 MPa osmotic stress, increasing at the early stage (0–24 h) but then decreasing.

As it was showed Fig. 5, the contribution of Na$^+$ was significantly higher than of the contribution of K$^+$ to Ψs with the elongation of treatment time. Under the condition of moderate osmotic stress (Fig. 5B, −0.5 MPa), the contribution of Na$^+$ to the Ψs decreased in the early stage (0–24 h) and increased later (24–72 h), while the contribution of K$^+$ decreased (0–24 h) and maintained a steady level (24–72 h). The contribution of Na$^+$ to the Ψs under severe osmotic stress (Fig. 5C, −1.0 MPa) showed a sharp increasing trend (0–24 h) and then remained stable (24–72 h), while K$^+$ always maintained a stable contribution to Ψs overall.

3.4. Dynamic changes in shoot relative plasma membrane permeability and MDA content in H. ammodendron in response to osmotic stresses

Relative plasma membrane permeability is an indicator of intracellular ionic leakage, so the REC was used to indicate the degree of damage (Fig. 6A). The REC increased slowly in −0.5 MPa treatment group but did not change substantially. However, under −1.0 MPa osmotic stress group, the increase in REC was slight in the initial stage (0–24 h) and then increased rapidly. When the duration of osmotic stress was as long as 24 h, the REC was 1.9 and 1.4 times higher under the −1.0 MPa osmotic stress group than under the control and −0.5 MPa groups, respectively. At the end of the treatment time (72 h), REC was 2.8 and 2.2 times higher under the −1.0 MPa osmotic stress group than under the control and −0.5 MPa groups, respectively. The MDA content increased more rapidly under the −1.0 MPa osmotic stress group than under the control and −0.5 MPa treatment groups (Fig. 6B). The MDA content was also 1.9 and 1.5 times greater under the −1.0 MPA group than under the control and −0.5 MPa treatment groups, respectively, while 72 h after the treatments, it was 3.7 and 1.5 times higher, respectively.

3.5. Dynamic changes in the activities of antioxidases (SOD, POD and CAT) in H. ammodendron in response to osmotic stresses

The activity of SOD in the treatment groups (−0.5 MPa and −1.0 MPa) showed similar increasing trends during 0–24 h (Fig. 7A). The SOD activity remained stable after 24 h when under the −0.5 MPa osmotic stress group, while it increased under the −1.0 MPa osmotic stress group. The activities of SOD under the treatment (−0.5 MPa and −1.0 MPa) groups were 35.1% and 16.0% higher, respectively, than the activity under the control group.

Under −1.0 MPa osmotic stress, the activity of POD increased continuously (Fig. 7B), especially during 0–6 h, it showed the fasted increase. When under the −0.5 MPa osmotic stress treatment, the POD activity significantly increased for only 24–48 h after treatment, but it remained stable during other treatments.

The CAT and POD activities under treatment (−0.5 MPa and −1.0 MPa) showed a similar trend (Fig. 7C), and there was no significant increase at the primary stage (0–6 h). Compared with those in the control, the activities of CAT under −0.5 MPa and −1.0 MPa osmotic stress were 4.3 and 2.3 times higher, respectively, at the primary stage (0–6 h) than at 0 h.

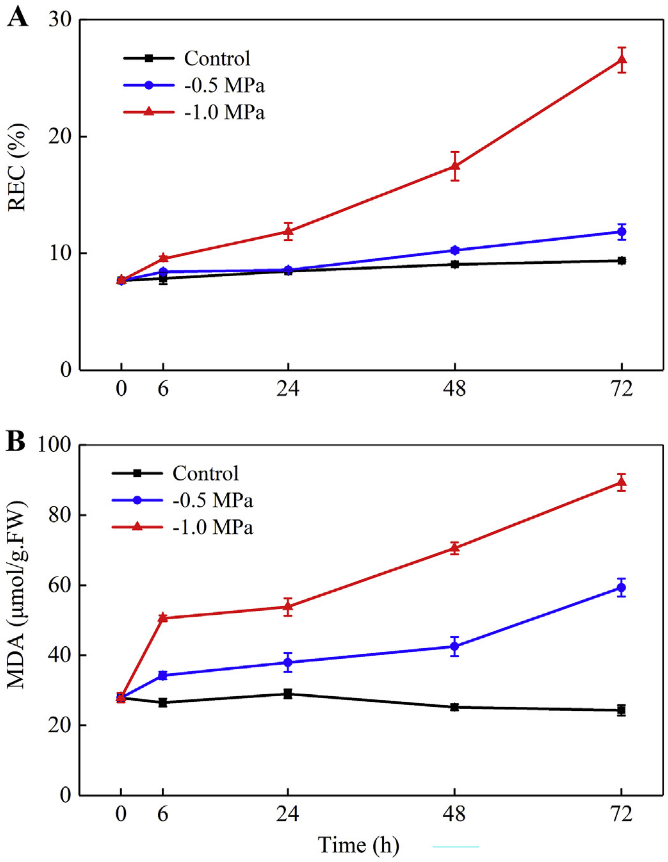

Fig. 6. Dynamic changes of shoot relative electric conductivity (REC) and malondialdehyde (MDA) content in *H. ammodendron* in response to various osmotic stresses. **(A)** REC, **(B)** MDA. Values and bars represent means and SEs ($n = 6$), respectively. Lines in different colors indicate different treatments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

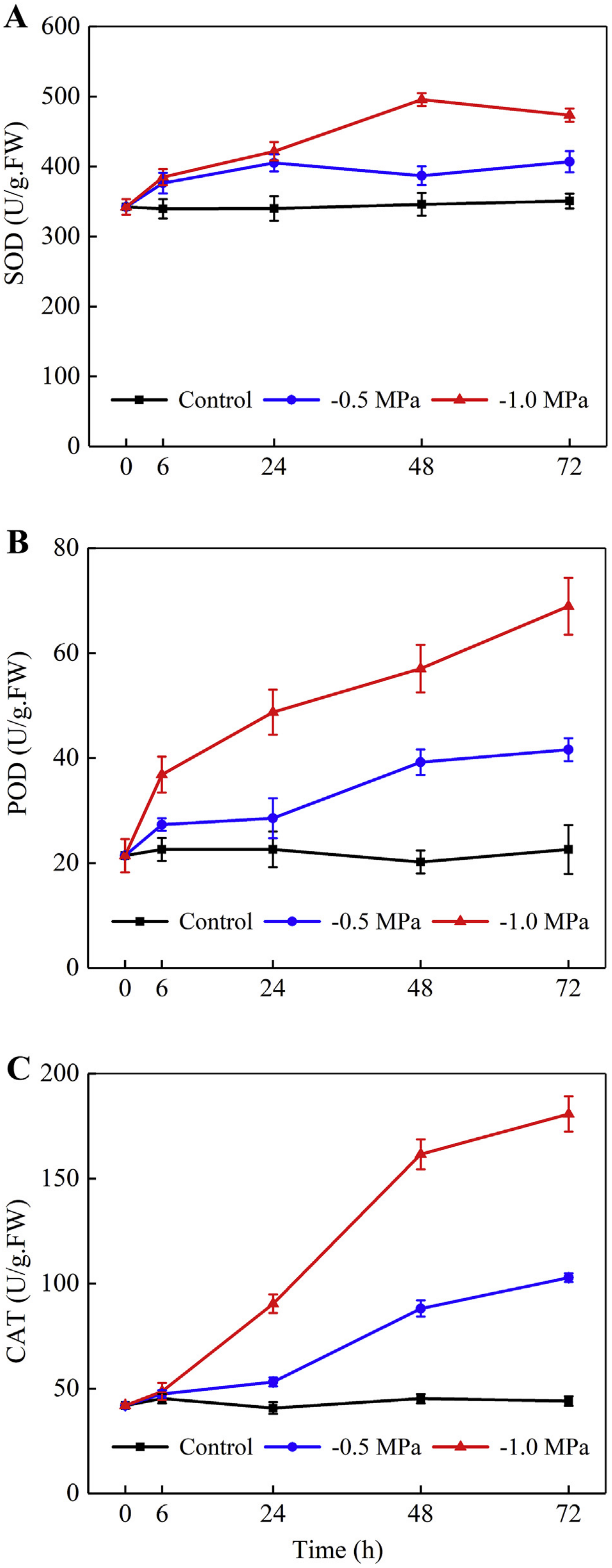

Fig. 7. Dynamic changes of the activities of antioxidases (SOD, POD and CAT) in *H. ammodendron* in response to various osmotic stresses. **(A)** SOD activity, **(B)** POD activity, **(C)** CAT activity. Values and bars represent means and SEs ($n = 6$), respectively. Lines in different colors indicate different treatments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4. Discussions

4.1. Reducing the osmotic potential to maintain stable shoot water content is an important strategy for H. ammodendron to adapt to osmotic stress

OA is perhaps one of the major strategies for plants to cope with drought and salt stress (Barcia et al., 2014; Serraj and Sinclair, 2002; Silva et al., 2015). In general, most plants tend to wilt permanently due to persistent osmotic stress. In our study, both under moderate (−0.5 MPa) and severe osmotic stress (−1.0 MPa), the Ψs of all treatments decreased significantly, but shoot water content remained stable under −0.5 MPa (Fig. 1). Under severe osmotic stress (−1.0 MPa), there was a significant decrease in only the Ψs for 0–6 h after treatment. In addition, 6 h after treatment, shoot Ψs remained at a stable level. Under osmotic stress conditions, *H. ammodendron* actively accumulated a large amount of soluble substances to reduce its own Ψs, and then, its water absorption ability was improved. This approach effectively helps plants survive under osmotic stress (Flowers, 2004; Gouiaa et al., 2012; Shabala and Cuin, 2008; Zeng et al., 2015).

4.2. Contribution of organic osmotic regulators to Ψs decreased gradually in H. ammodendron during osmotic stress

Accumulating soluble substances can be divided into organic substances (in small molecules) and inorganic ions. Organic solutes are important substances for plants in osmotic stress regulation (Raymond and Smirnoff, 2002). In response to drought or salt stress, proline accumulation usually occurs in the cytoplasm, and the accumulation of proline in the cytoplasm plays an important role in cytoplasmic OA (Leigh et al., 1981; Ketchum et al., 1991). Most crops respond to stress by accumulating betaine (Ashraf and Foolad, 2007; Mansour, 2000; Yang et al., 2003), which protects thylakoid membranes function in chloroplasts under stress (Robinson and Jones, 1986). Soluble sugars participate in maintaining plant water content and osmotic regulation in plants facing drought stress (Xu et al., 2007), and soluble sugars also act in membrane protection and scavenging of radicals (Krasensky and Jonak, 2012).

In this study, under normal conditions, there was a higher content of betaine in *H. ammodendron,* and its content was 100 and 5 times higher than the contents of free proline and soluble sugar, respectively. During the process, the content of betaine increased significantly, as well as free proline and soluble sugar; however, the contribution of organic osmotic regulators to Ψs decreased slightly with treatment time (24–72 h) under osmotic stress, probably because the synthesis rate of organic osmotic regulators was limited by specific physiological and biochemical processes. In addition, shoot Ψs decreased faster.

Previous studies of glycophytes under drought or salt stress showed that these soluble substances play necessary adaptive functions in terms of mediating osmotic regulation and defending subcellular structures (Ashraf and Foolad, 2007). However, not every higher plant accumulates enough betaine or free proline to avoid the adverse influences of abiotic stress. In *H. ammodendron*, the sum of the contributions of free proline and betaine was less than 4%, and the total contributions of organic osmotic regulators were approximately 20%. Thus, these substances were not the main osmotic substances for OA in *H. ammodendron.*

4.3. Contribution of inorganic osmotic regulators to Ψs increased significantly in H. ammodendron during osmotic stress

K^+ is involved in many physiological and biochemical processes in plants, such as in the promotion of plant photosynthesis, protein synthesis and osmotic regulation (Zhu, 2003; Gierth and Mäser, 2007). In some halophytes growing in arid desert areas, a large accumulation of Na^+ has been determined to have a beneficial role, but the importance and contribution of Na^+ to OA have been seldom studied (Slama et al., 2007; Ma et al., 2011). In our study, the accumulation of Na^+ in *H. ammodendron* seedlings increased significantly with osmotic stress. Similar to results in other studies, some plants can accumulate a large amount of Na^+ to regulate plant cell water potential (Ma et al., 2014; Zhang et al., 2014; Wang et al., 2008; Yue et al., 2012; Zhang et al., 2013). Under moderate osmotic stress (−0.5 MPa), the Na^+ content increased relatively slowly, which may have been due to the minimal degree of osmotic stress, and there was no large absorption of Na^+. Under severe osmotic stress (−1.0 MPa), the Na^+ content increased significantly in the early stage (0–24 h), which indicated that *H. ammodendron* rapidly initiated Na^+ uptake under severe osmotic stress (−1.0 MPa). However, the accumulation of Na^+ under severe osmotic stress (−1.0 MPa) did not always increase rapidly (0–24 h), and there was also a very slow increase phase (48–72 h). These results indicated that to be able to adapt, *H. ammodendron* can control the absorption of Na^+ within a certain limit; therefore, plant cells can prevent the accumulation of excess Na^+, and plants can avoid poisoning from excess Na^+. On the other hand, the Na^+/H^+ antiporter can store excess Na^+ in the vacuole so that the Na^+ can be used to control the cell Ψs indirectly (Ma et al., 2011), given the large amount of previously accumulated Na^+. These results reflected the sensitivity and importance of Na^+ accumulation in *H. ammodendron* in terms of osmotic stress resistance.

In general, an increase in Na^+ is usually accompanied by a gradual loss of K^+ when plants are subjected to salt stress (Wu et al., 2018). Because of ROS accumulation, the guard cell outward-rectifying K^+ channels (KOR/GORK) and ROS-activated nonselective cation channels (NSCC) channels are activated, resulting in more K^+ efflux and a disruption of Na^+/K^+ ratio homeostasis (Dasgan et al., 2002; Hauser and Horie, 2010; Wu et al., 2018). K^+ in *H. ammodendron* increased slightly after treatment, and Na^+ always increased. Therefore, the stability of K^+ in *H. ammodendron* seedlings may be an important factor for coping with osmotic stress. *H. ammodendron* had a stronger selective absorptive capacity for Na^+ over K^+ as occurs in other salt-accumulating halophytes (Yue et al., 2012; Ma et al., 2011). Therefore, we speculated that Na^+ could be an effective and efficient regulator of OA in response to water deficits in *H. ammodendron*.

We also combined two inorganic (Na^+ and K^+) and three organic substances (free proline, betaine and soluble sugar) to analyze the contribution of OA substance to Ψs (Fig. S1). The results indicated that the contribution of inorganic solutes was at least twice that of organic solutes under each treatment. In particular, at the end (24–72 h) of severe osmotic stress (−1.0 MPa), the contribution of inorganic solutes to Ψs was greater than 60%, and this contribution played a leading role in osmotic regulation because it resulted in a more direct and rapid absorption of Na^+, K^+ and other inorganic ions from the external environment. Compared with organic OA, inorganic ion OA was more efficient in maintaining the osmotic pressure of the cell and was the main factor in allowing *H. ammodendron* to address the low Ψs from the environment.

Ca^{2+} is a necessary macroelement for plant metabolism (López-Marqués et al., 2018). In this study, the Ca^{2+} concentration was generally constant because of the high Ca^{2+} concentration-retreat feedback mechanism in plant cells that allows the cells to avoid the toxic effects of prolonged high Ca^{2+} concentrations (Rengel and Zhang, 2003). Thus, the concentration of Ca^{2+} increased only for a short period under −1.0 MPa osmotic stress. On the other hand, the fluctuation in Ca^{2+} content is related to stomatal closure (Bao et al., 2009; Conn et al., 2011; Hirschi, 2004; Rienmüller et al., 2010; Stael et al., 2011). In this study, the Ca^{2+} content showed little fluctuation, without a significant increase or decrease. Thus, plants likely needed a long time to close their stomata, so the fluctuation of Ca^{2+} content also reflected its important role in stomatal regulation under osmotic stress.

4.4. Increasing antioxidase activity is another important strategy for H. ammodendron during osmotic stress

The stability of the cell membrane system is the basis of plant resistance to stress to a certain extent (Cona et al., 2006; Gill and Tuteja, 2010; Gupta and Huang, 2014); however, excessive membrane lipid peroxidation will destroy the structure and function of the cytomembrane, and seriously disrupt the normal physiological functions of plant cells (Csiszár et al., 2012; Puniran-Hartley et al., 2014). MDA is the most important product of membrane lipid peroxidation, and a certain amount of MDA can induce antioxidant enzyme activity in plant cells, eliminating membrane lipid peroxidation. However, excessive MDA is one kind of threat to plants (Han et al., 2014). In our study, both relative plasma membrane permeability (REC) and MDA served as indicators that represented the severity of damage, high REC was indicative of severe leakage of intracellular ions, and high levels of MDA indicated harmful substance over accumulated.

In the study of the physiology of plant stress resistance, changes in antioxidase enzyme activities are often used as important indicators of the ability of plants to resist osmotic stress (Wang et al., 2008; Rao et al., 1996). In the present study, three main antioxidases (SOD, POD and CAT) were selected to serve as the osmotic stress resistance indexes. SOD is the main antioxidant enzyme in plants and the first line of defense (Misra and Gupta, 2006). During the treatments, SOD activity was always significantly higher than POD and CAT activity. In addition, under moderate osmotic stress (−0.5 MPa), from 24 to 48 h, SOD activity decreased, while CAT and POD activity increased rapidly. This scenario may have occurred because O_2^- in the plant cells was transformed to H_2O_2 and O_2 under the catalysis of SOD, and then, H_2O_2 was

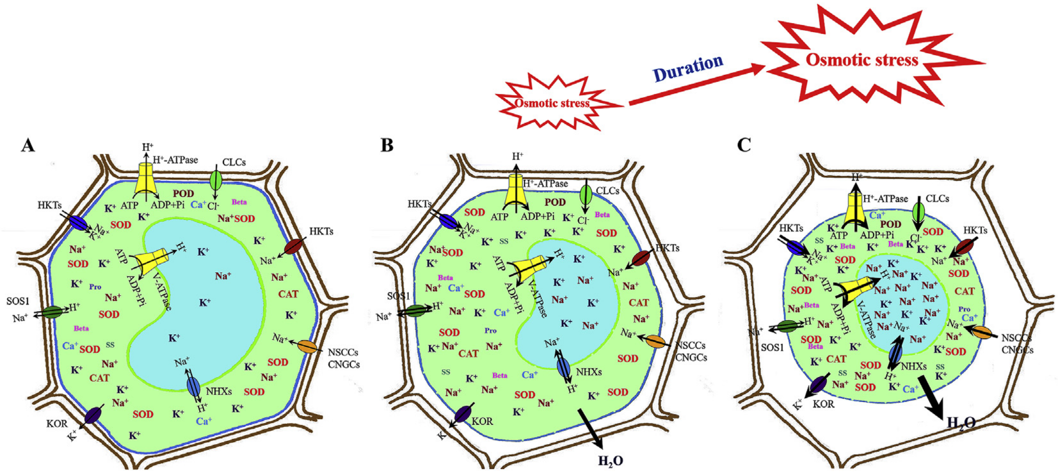

Fig. 8. Schematic model of osmotic regulators and antioxidases in shoot cells of *H. ammodendron* in response to various degrees of simulated drought stress. **(A)** Under control condition, water content remained stable, the shape of vacuoles is normal, and plasma membrane clinged to the cell wall. **(B)** Under moderate osmotic stress condition, Na^+ was transported into cytosol probably through high-affinity K^+ transporters (HKTs), non-selective cation channels (NSCCs) and Cyclic Nucleotide-Gated Channels (CNGCs). Plasma membrane Na^+/H^+ antiporter (salt overly sensitive 1, SOS1) and vacuole Na^+/H^+ antiporter (NHXs) began to function to reduce Na^+ toxicity in cytosol. K^+ was transported into cytosol probably through HKTs and out of the cell by K^+ outward-rectifying channels (KORs). Cl^- was probably transported by voltage-gated chloride channels (CLCs). MDA increased and the membrane system was damaged (REC increased). The organic osmotic regulators including betaine, free proline and soluble sugar and the activities of antioxidant enzymes (SOD, POD and CAT) increased obviously. Slight plasmolysis occurred due to mild cell water loss. **(C)** Under severe osmotic stress condition or after long term of moderate osmotic stress condition, more Na^+ was absorbed and vacuole Na^+/H^+ antiporter (NHXs) continue to transport Na^+ into vacuoles to reduce Ψs. MDA increased continuously and the membrane system was damaged seriously (REC increased) especially after long term of severe osmotic stress. The organic osmotic regulators including betaine, free proline and soluble sugar and the activities of antioxidant enzymes (SOD, POD and CAT) increased dramatically. Serious plasmolysis occurred due to largely cell water loss.

transformed to O_2 and H_2O under the catalysis of POD and CAT (Chaves et al., 2009; Liu et al., 2011). This effect may also be a remedial in *H. ammodendron*. The activities of POD and CAT showed a similar increase trend during the osmotic stress. The POD enzyme was more sensitive to osmotic stress than the other enzymes because it increased more rapidly (1.2 times under −0.5 MPa and 1.6 times under −1.0 MPa) than the other two enzymes at the beginning of the osmotic stress treatments (0–6 h).

REC and MDA contents both increased with treatment time and showed a similar trend in both the moderate (−0.5 MPa) and severe (−1.0 MPa) osmotic treatment groups. Under moderate osmotic stress (−0.5 MPa), REC and MDA increased slowly during the pretreatment time (0–48 h), and there was a clear increase during only the later stage (48–72 h), indicating that under control of the antioxidant enzyme system, *H. ammodendron* seedlings were not significantly affected by MDA within a certain period of time (0–48 h) and that REC increased slowly; thus, plant cells were less damaged. However, when under severe osmotic stress (−1.0 MPa), MDA and REC increased more significantly, especially at the end of the treatment (48–72 h). Therefore, the duration of osmotic stress could be regarded as another stress factor, that is, the increase in stress time results in an increase in stress level. In addition, osmotic stress of *H. ammodendron* seedlings was divided into three stages: adjustment period, adaptation period and incontrollable period. During the adjustment period (0–24 h under −0.5 MPa and 0–6 h under −1.0 MPa), seedlings were less affected by osmotic stress, and the activities of antioxidase enzymes were low. During the adaptation period (24–48 h under −0.5 MPa and 6–48 h under −1.0 MPa) under moderate osmotic stress (−0.5 MPa), the amount of the 3 protective enzymes increased significantly. Under severe osmotic stress (−1.0 MPa), there was a slight decrease in the activity of SOD, and the activities of CAT and POD increased sharply to balance the ROS metabolism system; however, the MDA content began to accumulate. During the incontrollable period (48–72 h), under moderate osmotic stress (−0.5 MPa), the amount of all protective enzymes increased. However, under severe osmotic stress (−1.0 MPa), SOD activity decreased, and POD and CAT activities increased.

H. ammodendron seedlings had a strong antioxidant system that synergistically contained SOD, POD and CAT; moreover, under normal conditions, *H. ammodendron* seedlings even contained high activity levels of SOD, which may be an important adaptation mechanism produced by plants over their long-term evolution. The antioxidant enzyme system in *H. ammodendron* showed different response mechanisms between stress degree and duration. Consequently, *H. ammodendron* seedlings had strong resistance to the product of membrane lipid peroxidation due to the high activity of antioxidant enzymes and their dynamic complementarity during osmotic stress. These factors are important in the survival of *H. ammodendron* in arid and semi desert areas.

In the present study, under moderate osmotic stress condition, Na^+ was transported into cytosol probably through high-affinity K^+ transporters (HKTs), non-selective cation channels (NSCCs) and Cyclic Nucleotide-Gated Channels (CNGCs) (Maathuis et al., 2014; Apse and Blumwald, 2007). Plasma membrane Na^+/H^+ antiporter (salt overly sensitive 1, SOS1) and vacuole Na^+/H^+ antiporter (NHXs) began to function to reduce Na^+ toxicity in cytosol (Zhang et al., 2013; Plett and Møller, 2010). K^+ was transported into cytosol probably through HKTs and out of the cell by K^+ outward-rectifying channels (KORs) (Sun et al., 2009; Chen et al., 2007). Cl^- was probably transported by voltage-gated chloride channels (CLCs) (Bergsdorf et al., 2009). MDA increased and the membrane system was damaged (REC increased). The organic osmotic regulators including betaine, free proline and soluble sugar and the activities of antioxidant enzymes (SOD, POD and CAT) increased obviously. Slight plasmolysis occurred due to mild cell water loss. Under severe osmotic stress condition or after long term of moderate osmotic stress condition, more Na^+ was absorbed and vacuole Na^+/H^+ antiporter (NHXs) continue to transport Na^+ into vacuoles to reduce Ψs (Zhang et al., 2013). MDA increased continuously and the membrane system was damaged seriously (REC increased) especially after long term of severe osmotic stress. The organic osmotic regulators including betaine, free proline and soluble sugar and the activities of antioxidant enzymes (SOD, POD and CAT) increased dramatically. Serious plasmolysis occurred due to largely cell water loss (Fig. 8).

5. Conclusions

In conclusion, substantial accumulation of osmotic adjustment substances is an important strategy for *H. ammodendron* to cope with simulated drought stress. In particular, *H. ammodendron* absorbed a large amount of Na^+ and transported Na^+ into the assimilating branch for OA. At the same time, K^+ remained stable. The contribution of inorganic solutes (Na^+ and K^+) accounted for approximately 40–70% of the OA, and among them, Na^+ was the main OA substance for *H. ammodendron* in terms of adapting to simulated drought stress. In addition, maintaining a higher level of antioxidase to scavenge ROS is another important strategy for *H. ammodendron* to adapt to simulated drought stress.

Author contributions

J.-L. Z. conceived the project. X.-P. L. and L. Z. prepared the plant samples and conducted the physiological experiments. X.-P. L. and H.-J. G wrote the manuscript. Y.-P. W., K.-Z. S., and Q. Z. gave supports for data analysis and revised the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.03.019.

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