PHYSIOLOGICAL RESPONSES OF ALFALFA SEEDLINGS TO FREEZE-THAW, NaCl AND Na_2SO_4 STRESS

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(Received 23rd Dec 2019; accepted 23rd Mar 2020)

Abstract. NaCl and Na_2SO_4, two common neutral salts, often cause salt stress in saline-alkaline grassland in Northeast China. The purpose of this experiment is to reveal the effects of the combined stress of these two neutral salts and the freeze-thaw cycles of spring and autumn on the physiological and ecological characteristics of alfalfa seedlings. By measuring the soluble protein, soluble sugar, proline, malondialdehyde (MDA) content, Peroxidase activity (POD) and Superoxide dismutase (SOD) activity of alfalfa seedlings treated with two types of salt solution (NaCl and Na_2SO_4) in a freeze-thaw cycle, the physiological and ecological responses of alfalfa seedlings under combined stress were investigated, and the differences in the stress of plant seedlings between the two salt solutions were compared. Stress damage mainly resulted from low temperature, penetration and ionic toxicity. A series of tolerance mechanisms were observed in the plants to cope with the stress. Under the compound stress, plant damage was exacerbated. The highlight of this study is that two different types of salinity had ion specificity for the toxic effects of alfalfa, and the toxic effects of Cl^- were the main factors, not Na^+ or SO_4^{2-}. Alfalfa exposed to SO_4^{2-} salt could cope with salt stress relatively better.
Keywords: *alfalfa, freeze-thaw, tolerance mechanisms, salinity, combined stress*

Introduction

The northern part of China is cold in winter, and pasture survives winter difficultly. In the spring and autumn, pastures are often affected by cold currents, and are subjected to freeze-thaw cycles, resulting in poor growth and low yield, which reduce the utilization value of pasture. Therefore, temperature is one of the important limiting factors for the use value of pasture (Stushnoff and Junttila, 1986). At the same time, soil salinization in Northeast China is serious, and salt is one of the main limiting factors for improving crop growth and productivity (Flowers, 2004; Kanmani et al., 2017). The main consequences of plant exposed to salt stress are water deficits and excess ions, leading to several morphological and physiological changes (Greenway and Munns, 2003; Türkan and Demiral, 2009). High concentrations of ions in the external solution may be absorbed at a high rate, which may result in excessive accumulation in plant tissues. These ions may affect the membrane's ability to selectively permeate and interfere with the absorption of other ions, thereby altering the amount of a series of elements in the tissue (Hu and Schmidhalter, 2005). Therefore, in the face of soil salinization, breeding salt-tolerant varieties and direct use of saline-alkali land are more cost-effective solutions for improving and utilizing saline-alkali land.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1803_45574567

According to the survey, alfalfa (*Medicago sativa* L.) is one of the most common saline-alkali land forage in northeastern China and high latitudes. As a high-quality forage cultivated artificially, alfalfa has strong cold and salt resistance. It is a common plant for the improvement of saline-alkali land in Northeast China, and also a test material commonly used in the study of cold tolerance and salt-tolerance of plants. Alfalfa is sensitive to salt at seedling stage, and the seedlings have a consistent response to salt throughout the growth stage, and salt selection is most suitable at this stage (Al-Khatib et al., 1994, 1987).

At present, researches on the cold tolerance and salt tolerance of seedlings mainly focus on single factor stress conditions, such as salt tolerance physiology (Akandi et al., 2017), cold resistance and survival rate (Skinner and Bellinger, 2017), and drought resistance (Pompeiano et al., 2016), and those on the physiological laws of compound stress are rare. In the early spring and late autumn of the cold regions of Northeast China, freeze-thaw and salt stresses are the most common, and the salt types in the saline-alkaline grasslands in Northeast China are mainly alkaline salts, and some are neutral salts. The main components of neutral salts are sodium salts, including sodium chloride and sodium sulfate. Most studies on the salt tolerance of plant species are based on experiments where NaCl is the main salt, and the symptoms of injury are usually attributed to the toxicity of Na^+ and Cl^-. Relatively few studies (Nguyen et al., 2006; Renault et al., 2001; Rogers et al., 1998) focus on the effects of Na_2SO_4 on plant growth and physiology. Therefore, this study used Dongmu-1 alfalfa as the experimental object to study the changes of soluble protein, malondialdehyde (MDA), soluble sugar, proline content and SOD and POD activity in the alfalfa seedlings under the combined stress of freeze-thaw and salt (NaCl or Na_2SO_4). The physiological response mechanism of cold resistance and salt tolerance in alfalfa seedlings was revealed.

Materials and methods

Experimental materials

In this study, the indoor culture experiment method was adopted, and the test seedlings were Dongmu-1 alfalfa. The alfalfa seeds with uniform grain size and no pests were picked carefully, disinfected with 0.1% $KMnO_4$ for 10 min, rinsed with double distilled water, and then cultured in several culture dishes with a diameter of 100 mm. Two layers of filter paper were laid in each dish, and around 100 seeds were placed evenly place, immersed appropriate amount of water. The culture dishes were placed in a constant temperature incubator for growth and germination, and the light collection period was 12 h, with illumination time at 25 ℃ and non-lighting time at 15 ℃. During the germination period, appropriate amount of water was added three times a day. When the third cotyledon grew, the plants with the same growth were selected for stress treatment.

Experimental methods

The treatment solvents selected were NaCl and Na_2SO_4, and according to the growth condition of the seedling, the concentration of each salt was 100 mmol/L. After the treatment, the plants were cultured for two days. The salt-treated experimental groups and the control group (CK) were divided into two groups respectively. One was subjected to freeze-thaw stress, and the other was cultured at room temperature. The

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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temperature of the freeze-thaw cycle was controlled by an ultra-low temperature alternating test chamber, and the temperature was set to 10 ℃, 5 ℃, 0 ℃, -3 ℃ (Deng et al., 2005) have found that -3 ℃ is the lowest temperature for alfalfa seedlings to withstand low temperature), 0 ℃, 5 ℃ and 10 ℃, with an interval of two hours. Treated groups and control groups were sampled at each temperature. Three sets of parallel samples were taken from each group, and the relevant indicators were determined after sampling.

CK group is blank control group without salt solution and freeze-thaw cycle; FT group is only treated by a freeze-thaw cycle; FT + NaCl group is the one with NaCl solution and a freeze-thaw cycle; FT + Na_2SO_4 group is the one added Na_2SO_4 solution and subjected to a freeze-thaw cycle. T1-T7 correspond to 10 ℃, 5 ℃, 0 ℃, -3 ℃, 0 ℃, 5 ℃, 10 ℃, respectively.

Samples were grinded to homogenate with 5 ml distilled water (5 ml of 10% trichloroacetic acid solution used for MDA and soluble sugar content, and 5 ml of 3% sulfosalicylic acid solution used for proline content instead) in a mortar. After centrifugation for 10 min (3000 r/min), the supernatant was picked for measurement (protein: G250-Coomassie brilliant blue method; MDA and soluble sugar: thiobarbituric acid method; proline: acid ninhydrin colorimetry). The SOD and POD activity was determined by SOD (superoxide dismutase) and POD (peroxidase) kits (Nanjing Jiancheng Bioengineering Institute), respectively.

Data analysis

The experimental data were graphed with Microsoft (Redmond, USA) Excel, and statistical analysis was performed with SPSS 16.0 statistical software (IBM SPSS Statistics, Chicago, USA) using single factor variance analysis (one-way analysis of variance) and multiple comparisons with least significant difference (LSD). The significance level was at 0.05, the experiments were repeated five times, and all of the results are presented as mean ± SE.

Results and discussion

Change in osmotic adjustment substance content

Soluble protein, soluble sugar, and proline are common osmotic adjustment substances in plants, and their levels can reflect the degree of stress on plants.

As can be seen from *Figure 1*, the three treatment groups exhibited substantially the same trend throughout the freeze-thaw cycle. At the initial stage of temperature decrease, the protein content increased slightly, mainly due to the small temperature stress, and the plant stress response rapidly regulated the metabolism and increased protein content. At the lowest temperature (-3 ℃), the degree of stress reached the maximum and the protein content went up significantly, indicating that alfalfa seedlings were most sensitive at -3 ℃. The low temperature caused plants to produce a stress-resistant reaction, and the related genes were overexpressed (Guy, 1990; Hannah et al., 2005; Hahn and Walbot, 1989; Seppänen et al., 1998), and cold-resistant proteins were synthesized, resulting in an increase in the total amount of protein. This is consistent with the conclusion of (Weiser, 1970) who proposed that low temperature could induce plant protein production. When the temperature gradually rose, the soluble protein content of the three treatment groups decreased (Bian et al., 2018). However, the

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difference was that the FT group and the FT + Na_2SO_4 group decreased significantly, while the FT + NaCl group decreased slowly, indicating that as the temperature rose, the low temperature stress on the plants was gradually reduced, and the excess soluble proteins were catabolized progressively. The addition of salt could destroy plant cells, causing cells to rupture in high salinity solutions, which is an irreversible process. Cl^- had strong destructive power to cells, and even if the temperature rose, a large amount of soluble proteins could not be metabolized, while the destructive power of SO_4^{2-} was relatively weaker, and soluble proteins in FT + Na_2SO_4 group were partially metabolized.

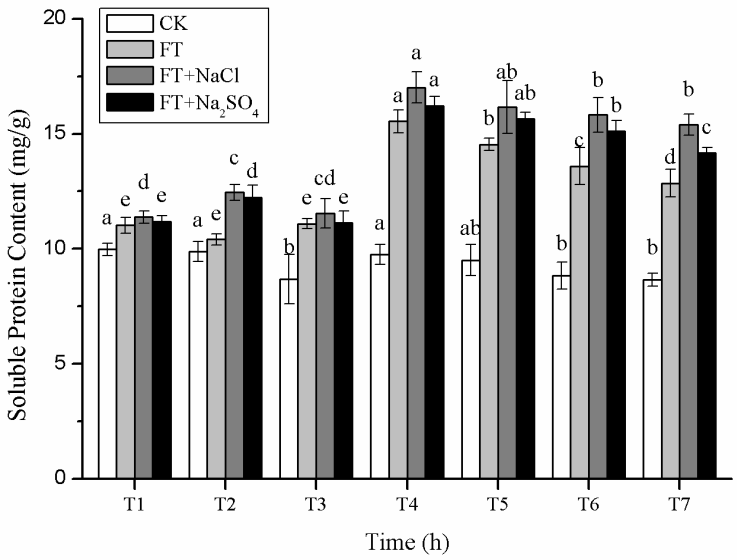

Figure 1. *Effect of freezing-thawing cycle and two neutral salts on the content of soluble protein*

Studies have shown that freeze-thaw cycles can change the accumulation of lipids and carbohydrates in plants (Skinner et al., 2014). A large amount of ions would be accumulated in the salt-treated plants and are sequestered in the vacuole, and non-absorbable compounds, such as sugar (Briens and Larher, 2010; Matoh and Matsushita, 2010) and leaf proline (Pagter et al., 2009), may act as compatible organic solutes to balance cytoplasmic osmotic pressure (James et al., 2002; Volkmar et al., 1998). As can be seen from *Figures 2* and *3,* as the temperature descended, the soluble sugar and proline contents gradually ascended, reaching the highest at the lowest temperature (-3 ℃) (Bao et al., 2017). Cooling allowed the seedlings to actively accumulate soluble sugar and proline, maintain the osmotic potential inside and outside the cells, ensure the normal structure of the cell membrane, reduce the freezing point of the cells, increase the hydration of the cells, giving rise to enhancement of water retention capacity and avoidance of probiotic damage to dehydration at low temperature. The soluble sugar and proline contents decreased slightly as the temperature went up in the late stage of the experiment, but they were still high. There was almost no significant difference between the groups ($P > 0.05$). The cell thawing process was slow, and sustained low temperature damage resulted in accumulation of soluble sugar and proline used to reduce coercive damage in plants. Moreover, the changes of the index of each group were roughly as follows: FT + NaCl group > FT + Na_2SO_4 group > FT group > CK

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group, indicating that compound stress was severer than single stress, and the increased osmotic adjustment substance was both used to maintain the osmotic potential of the cells and to resist the damage caused by low temperature. Therefore, the content of osmotic adjustment substances in the complex stress group was significantly higher than that in the freeze-thaw group. However, if the increase of osmotic adjustment substances was only related to the external environmental salt concentration, it was independent of the salinity type. It should have shown that the FT + Na_2SO_4 group had more osmotic adjustment substances, since Na_2SO_4 in the equimolar two salt solutions should contain a higher concentration of Na^+. Therefore, the difference between the two salt-treated groups was caused by the difference between Cl^- and SO_4^{2-} ions, and the alfalfa seedlings were more sensitive to Cl^-.

Changes in biofilm damage

MDA is a product of membrane lipid peroxidation in plant cells. Studies (Bailly et al., 1996; Greenway and Munns, 2003) have shown that degree of could be reflected in the content of MDA. It can be seen from *Figure 4* that there was no significant change in MDA content in the CK group ($P > 0.05$), and the MDA changes in the freeze-thaw group and the two compound stress groups were the same. With the decrease of temperature, the MDA content increased significantly, and the three groups all peaked at T4 (-3 ℃), 18.30 μmol·L^{-1}, 23.75 μmol·L^{-1}, 21.55 μmol·L^{-1}, respectively. Compared with T1, T2 and T3, there were significant differences ($P > 0.05$), indicating that the lower the temperature was, the higher the MDA content produced in the alfalfa seedlings. With the increase of temperature, the MDA content of the three treatment groups decreased slightly but remained high. There was almost no significant difference between the groups ($P > 0.05$). This may be due to the membrane lipid peroxidation caused by low temperature and resulted in the accumulation of MDA. The continuous low temperature caused the cells to thaw slowly, and although the temperature gradually went up, plants were still subjected to cold stress, showing a slight decline and followed a trend to be stable.

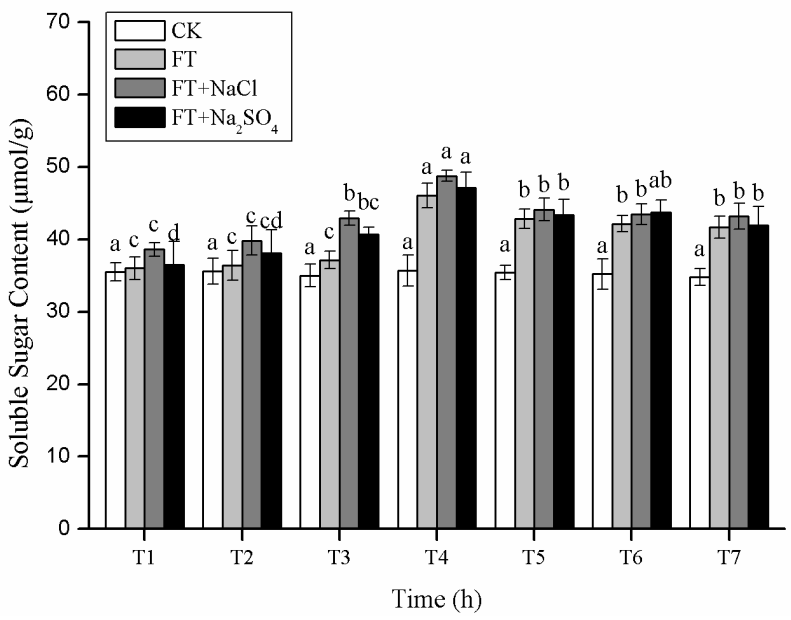

Figure 2. *Effect of freezing-thawing cycle and two neutral salts on the content of soluble sugar*

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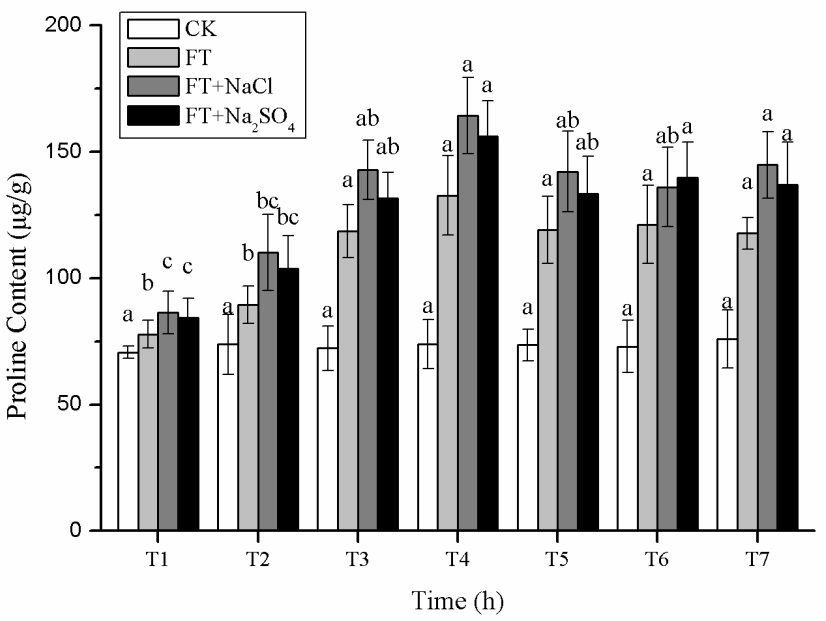

Figure 3. *Effect of freezing-thawing cycle and two neutral salts on the content of proline*

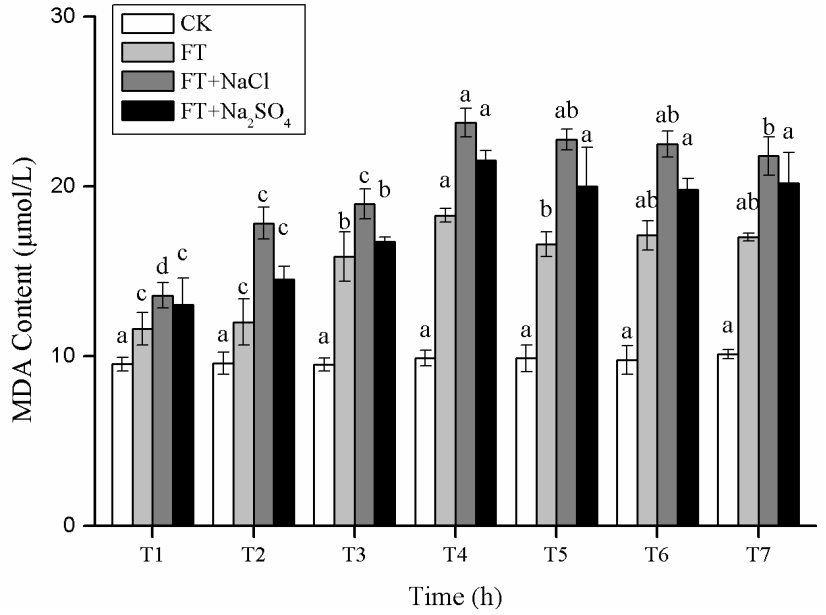

Figure 4. *Effect of freezing-thawing cycle and two neutral salts on the content of MDA*

In the longitudinal comparison, the MDA content of the three treatment groups was higher than that of the CK group, indicating that the alfalfa seedlings started to be subjected to cold stress at 10 ℃. The combined stress of freeze-thaw and NaCl resulted in the highest MDA content in the seedlings, 4.28-13.84% more than that in the FT + Na_2SO_4 group, and the MDA content in the FT group was the lowest, indicating that the alfalfa seedlings were subjected to salt stress after the salt solution treatment. Research showed that plants responded to salinity in a two-stage response (Munns, 2005). The first stage was due to the osmotic pressure caused by the salt outside the

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plants, and the second stage was due to the toxic effect of the salt, which exceeded the ability of the cells to divide the salt in the vacuole. Salinity exacerbated cell membrane lipid peroxidation and accumulated more MDA. Cl^- and SO_4^{2-} showed differences in plant stress, and the damage caused by Cl^- was stronger than by SO_4^{2-}, probably because alfalfa had effective mechanisms to exclude Na and S and part Cl ions from leaves. The remaining not excluded Cl ions produced toxic effects, destroyed cell structure and inhibited plant growth. The decrease in growth may also be due to an increase in the metabolic cost of excluding, separating, or increasing the synthesis of the permeate, reducing the amount of organic compound available for growth (Lissner and Schierup, 1997; Mccree, 1986).

Changes in antioxidant enzyme activity

Salinity-induced increased antioxidant activity is associated with plant salt tolerance (Bose et al., 2014). It can be seen from *Figures 5* and *6* that the trend of SOD and POD activity in the treated groups was consistent, and the change rule was first raised and then maintained at a higher level during the whole freeze-thaw cycle. As the temperature decreased, the SOD activity gradually increased, and the maximum value appeared at T4 (-3 ℃), then decreased slightly, but was still very high. POD activity increased first, and the maximum value appeared at T4 (-3 ℃), then the decrease was not obvious, and there was no significant difference between the groups ($P > 0.05$). The activity of SOD and POD increased significantly with temperature, indicating that membrane lipid peroxidation occurred in plants under low temperature and salt stress, and a large amount of superoxide anion radicals were produced. ROS was rapidly produced in plants in the form of H_2O_2 and O_2^- (Zang et al., 2015). The production of ROS led to the activation of programmed cell death, and the cells underwent various stages of apoptosis, such as externalization of phosphatidylserine, DNA laddering, loss of plasma membrane integrity (Swapnil et al., 2017). The stress resistance of plants induced the activity of SOD and POD. SOD could convert superoxide anion into H_2O_2, whereas POD could decompose H_2O_2 and synergize with SOD to scavenge reactive oxygen species in order to reduce cell membrane damage. In the late stage of freeze-thaw cycle (temperature rose phase), the plants were exposed to sustained low temperature, SOD and POD activity was still high, mainly because the long-term cold environment caused the active oxygen free radicals to continue to accumulate, although the temperature was in the recovery phase, plants were still severely stressed. SOD is an important component of the active oxygen scavenging system in plants (Zhu et al., 2007). When plants responded to external environmental stress, POD and SOD acted synergistically to protect the body from external stress, and the activity changes of the two were significantly correlated (Qi et al., 2003).

The three treated groups showed the strongest POD and SOD activity in the FT + NaCl group, the second in the FT + Na_2SO_4 group, and the weakest in the FT group, indicating that compound stress could lead to increased cell membrane lipid peroxidation, and different types of salt solutions had different effects on plant cells. Compared with Na^+ (Bhivare et al., 1988; Chavan and Karadge, 1980) and SO_4^{2-}, plants were more sensitive to Cl^-, which may give rise to enhancement of cell membrane lipid peroxidation, and ROS was produced enough to increase the antioxidant activity of plants. In addition, studies (Cameron and Pakrasi, 2010) have shown that sulfate in cells could promote the synthesis of glutathione, which had been shown to be critical for many processes in plants and promoted plant growth.

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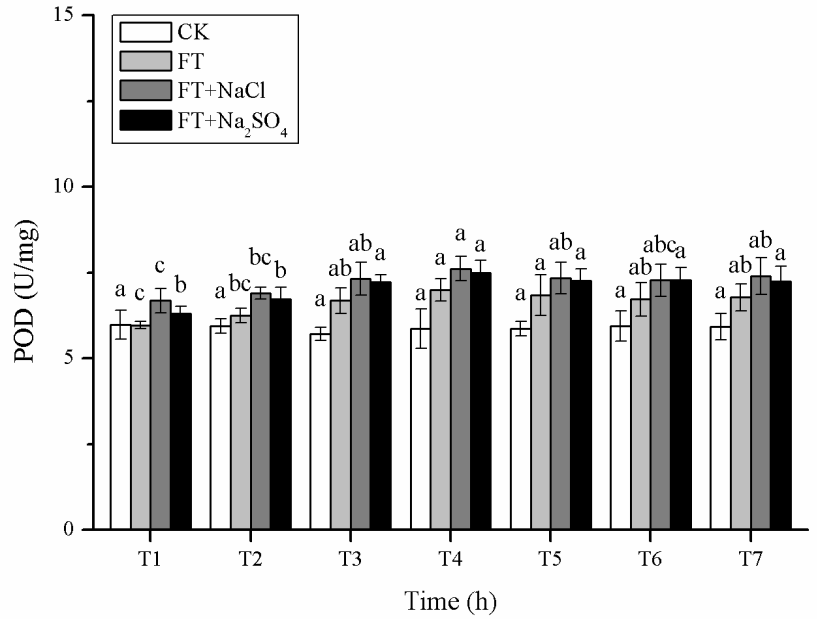

Figure 5. *Effect of freezing-thawing cycle and two neutral salts on the content of POD*

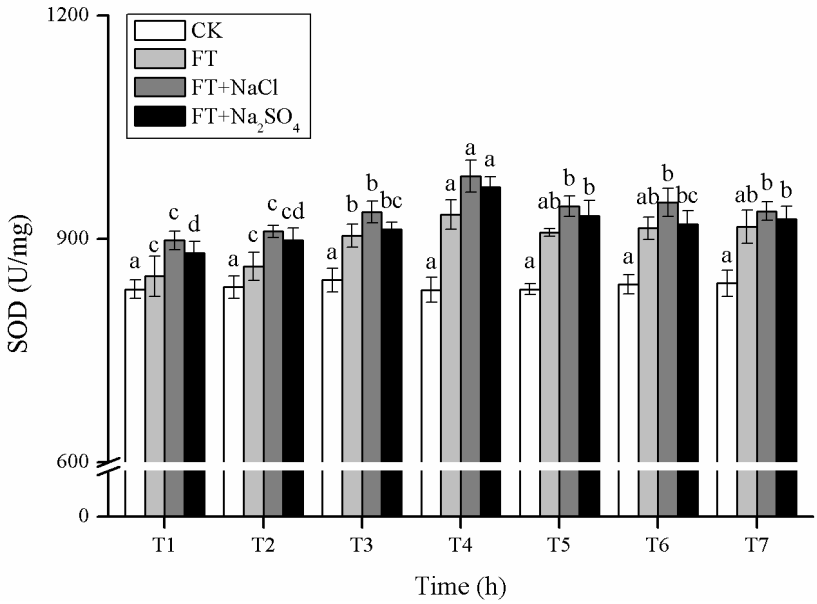

Figure 6. *Effect of freezing-thawing cycle and two neutral salts on the content of SOD*

Conclusion

Freeze-thaw cycles and salinity have a negative impact on the growth of alfalfa seedlings and have certain regularity, which could be proved by many related studies as well (Lissner and Schierup, 1997; Lissner et al., 1999; Matsushita and Matoh, 1991; Vasquez et al., 2006). Our research suggested that this might be due to temperature, penetration and ion specificity. Alfalfa showed certain tolerance to low temperature and salinity, which was characterized by overexpression of related genes to synthesize soluble protein, huge production of soluble sugar, proline and other solutes regulating

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osmotic potential, the accumulation of MDA by cell membrane lipid peroxidation, and the enhancement of antioxidant enzyme activity to scavenge ROS. In a freeze-thaw cycle, each indicator and temperature had a certain degree of negative correlation. Compared with single freeze-thaw stress, compound stress had stronger ability to inhibit plant growth, mainly due to the penetration and toxicity of salt ions. For different types of salinity, the plant response was different, showing pronounced ion specificity. Equimolar NaCl was more stressful than Na_2SO_4 on alfalfa seedlings, and the stress was mainly from the toxic effect of Cl^- rather than Na^+ or SO_4^{2-}. This may be due to the fact that alfalfa was more sensitive to Cl^- and had Na^+, SO_4^{2-} and partial Cl^- efflux mechanisms. Cl^- might affect a variety of important chemical actions in cells, and cells underwent normal metabolic activities, thereby inhibiting plant growth. It can provide a reference for scientific management of either farmland or lawn of alfalfa. However, whether the adaptation mechanisms of short-term stress were consistent with those of long-term one, it needs to be further investigated.

Acknowledgments. This work was supported by the [National Natural Science Foundation of China] under Grant [31772669].

Conflict of interests. The authors declare no conflict of interests.

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