Physiological Effects of the Combined Stresses of Freeze-thaw, Acid Precipitation and Deicing Salt on Alfalfa Seedlings

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Abstract

Backgroud: Frequent phenomenon of freeze-thaw, together with widely used deicing salt and intense acid rain , often occurs in northeast China, causing damages to plants in defferent perspectives such as the permeability of biological membrane, osmotic adjustment, and photosynthetic system. Aiming to explore the resistant to freeze-thaw (F), acid rain (A) and deicing salt (D) of alfalfa, Medicago sativa CV. Dongmu-70 was used as experimental material and the contents of malondialdehyde (MDA), soluble protein, soluble sugar, proline and chlorophyll were tested.

Results: With the temperature decreased, the MDA content in seedlings of group under combined stress (A-D-F) increased and was significantly higher than that of group F by 69.48~136.40%. Osmotic substances like soluble sugar and proline, compared with group CK, were higher, while soluble protein content was lower. The chlorophyll contents in seedlings of treatment groups were lower than that of group CK, however, displayed a non-significant change during free-thaw cycle.

Conclusion: It can be observed that the injury of permeability of biological membrane and photosynthetic system inside alfalfa caused by stresses. Meanwhile, alfalfa maintain osmotic balance by adaptively increasing the potentials of osmotic substances like soluble sugar and proline. Furthermore, the influence of freeze-thaw and deicing salt stress is more significant, while the mixed stresses of acid rain with two factors mentioned above has little effect on plants.

Background

Freeze-thaw refers to a physical geological phenomenon of the soil layer freezing and melting during late winter and early spring in the northeast of China (1), which could do harms to plants (2). It has been reported that lawn-type plants would resist low-temperature damage by regulating the proline content (3). Additionally, considering the frequent and heavy snowfall in winter, deicing salt is widely used because of its low cost, regardless of its serious salt damage to green belt plants in the form of either runoff or splashes (4, 5). Besides, the extensive use of coal and oil, as well as the production of sulfur dioxide from coal burning during winter in northern China, can cause acid precipitation (6). Take it further, after North America and Europe, China is the third largest area subjected to acid rain in the world (7).

Alfalfa is characterized for its adaptability towards adversities like cold, heat, and drought and is widely cultivated in high latitudes in China (8), where acid deposition and deicing salt accompanied by freeze-thaw often occur together. A number of previous researches studied on the resistance of alfalfa under single factor (9, 10); however, in the actual environment, plants are often affected by multiple factors, hence studying physiological responses of alfalfa to compound stresses was a matter of urgency. In this experiment, *Medicago sativa* CV. Dongmu–70 was used as the experimental material to study physiological responses to combined stresses. Malondialdehyde (MDA) is an end-product of membrane lipid peroxidation and the content of MDA can indicate the extent of cell membrane damage (11). The soluble protein, soluble sugar and proline are osmotic adjustment substances that can promote cellular

bound water content (12). The chlorophyll content can reflect the photosynthetic intensity and the rate of material synthesis, showing the level of damage (13). The indexes above were investigated in order to explore the resistance of alfalfa to acid rain, deicing salt and freeze-thaw, expecting to provide a theoretical basis for improving the technology of cultivating to alleviate damages to plants.

Results

Changes in MDA content

Fig. 1 shows that the MDA content in seedlings of groups under combined stresses (A-F, D-F and A-D-F) was higher than that of the group under single freeze-thaw stress (F) by 7.87~62.60%, 63.40~120.96% and 69.48~136.40% [see Additional file 1], respectively. This indicates that combined stresses cause more intense stress conditions, resulting in an accumulation of MDA in the alfalfa plants. During the thawing period (8h~14h), the MDA content measured in seedlings of groups under either combined stresses or single freeze-thaw stress decreased. When the temperature rose to 10°C (14h), the content of MDA in seedlings of groups A-F, D-F and A-D-F decreased by 57.58%, 42.10% and 40.20% [see Additional file 1], respectively. It also can be observed from Fig. 1 that under freeze-thaw stress, the MDA content in seedlings of group A-D-F was significantly higher than that of group A-F ($P < 0.05$), while showed no significant difference compared with that of group D-F ($P > 0.05$). The above results indicated that the combined stresses had a more significant effect on the MDA content in seedlings than single freeze-thaw stress, and the deicing salt stress had a greater impact on MDA content than acid precipitation and freeze-thaw stress.

Changes in soluble protein content

According to Fig. 2, the soluble protein content in seedlings of the combined stresses groups showed a trend of increasing initially and then decreasing throughout the whole freeze-thaw cycle, while that of the single freeze-thaw stress group showed a fluctuant decreasing. When the temperature dropped to 0°C (6h), the soluble protein content in seedlings of groups under combined stresses reached its peak. At thawing stage (8h~14h), the soluble protein content in seedlings of groups under freeze-thaw stress was significantly lower than that of group CK ($P < 0.05$) [see Additional file 2], which may attribute to the adding of freeze-thaw stress. However, the content of soluble protein in seedlings of groups under compound stresses showed no significant difference compared with that of group under single freeze-thaw stress, indicating either acid precipitation stress or deicing salt stress had less impact on soluble protein content. Moreover, during this period, higher soluble protein content was measured in the freeze-thaw group than in the combined stresses groups, indicating that the combined stresses caused more damage to the plants.

Changes in soluble sugar content

It can be observed from Fig. 3 that the soluble sugar content of each test group was significantly higher than that of group CK during the freeze-thaw cycle. When the temperature decreased, the soluble sugar content in seedlings of all groups except CK increased and reached its peak at -5°C (8h). The results demonstrated that in the low-temperature environment, the soluble sugar content in the plants increased significantly, and the plants protected themselves by accumulating a large amount of soluble sugar. The highest soluble sugar content was measured in plants subjected to the combined stresses of freeze-thaw, deicing salt and acid precipitation. During the thawing period (8h~14h), the soluble sugar content in seedlings of all groups except CK showed a downward trend with the temperature increased. Notably, during the temperature rose from -5°C (8h) to 0°C (10h), the soluble sugar contents in seedlings of group F was significantly lower than that of group A-D-F by 17.13% (8h) and 14.79% (10h) [see Additional file 3] ($P < 0.05$), but the soluble sugar content in seedlings of groups A-F and D-F did not differ significantly from that of group F ($P > 0.05$). The findings indicated that the conditions resulting from the combination of the three stress factors caused the maximum accumulation of soluble sugar in plants.

Changes in proline content

As shown in Fig. 4, the proline content in seedlings of the test groups was higher than that of group CK throughout the whole freeze-thaw period, indicating that stresses resulted from acid rain and deicing salt caused an increase in proline content in plants. During the freezing period, the proline content in seedlings of groups F, A-F, D-F and A-D-F increased and peaked at -5°C (8h) with respectively 91.34%, 86.24%, 96.59% and 96.40% higher than those measured at 10°C (2h) [see Additional file 4]. During the thawing period (8h~14h), the proline content of groups F, A-F, D-F and A-D-F at -5°C (8h) decreased by 19.97%, 18.46%, 19.80% and 8.38% respectively, compared with those measured at 0°C (10h) [see Additional file 4]. Fig. 4 also showed that the proline content was significantly higher in group A-D-F than that in the group subjected to only freeze-thaw ($P < 0.05$). Besides, except CK, the proline content in seedlings of groups under acid rain stress was significantly higher than that of groups under non-acid-rain stress ($P < 0.05$), which indicated that freeze-thaw stress accompanied by acid rain stress resulted in more proline produced in plants to protect themselves.

Changes in chlorophyll content

During the freeze-thaw period, the chlorophyll content in seedlings of each experimental group exhibited an initial decrease followed by an increase (Fig. 5). At the freezing stage, the chlorophyll content in groups F, A-F, D-F and A-D-F showed a downward trend and reached the minimum value at -5°C (8h) that were 22.38%, 12.73%, 11.11% and 17.79% lower than those measured at 10°C (2h) [see Additional file 5], respectively. During the thawing period (8h~14h), compared with the chlorophyll content measured at

-5°C (8h), the contents measured at 10°C (14h) in seedlings of groups F, A-F, D-F and A-D-F were significantly increased by 42.32%, 25.60%, 25.77% and 20.65% ($P < 0.05$) [see Additional file 5]. However, there was no observed significant difference in the chlorophyll content among the experimental groups throughout the whole freeze-thaw period ($P > 0.05$).

Correlation analysis between indexes

Table 1 shows that under the freeze-thaw condition, MDA and proline were significantly positively correlated ($P < 0.01$), both of which was positively correlated with soluble sugar ($P < 0.05$). Chlorophyll was negatively correlated with MDA, proline and soluble sugar. However, there was no significant correlation between protein and the other indicators. The correlations between the indexes of the freeze-thaw + acid rain + deicing salt group were similar to those of the freeze-thaw group, but all correlations were highly significant in the former group ($P < 0.01$). These findings indicated that both proline and the soluble sugar content increased with the accumulation of MDA in plants under external stress, while the chlorophyll content decreased.

Discussion

Effects of combined stresses on the membrane system

MDA is one of the main products of membrane peroxidation in plants under adverse conditions and can strongly react with various components in the cell, causing cross-linking polymerization of macromolecules such as proteins and nucleic acids (14), leading to membrane structure damage and impaired physiological function (15, 16). It has been pointed out that the degree of membrane damage caused by complex stress environment of freeze-thaw, acid rain and deicing salt could be characterized by the changes of MDA content (17). In this experiment, the damage to the cell membrane caused by three stresses factors resulted in the most obviously increase in MDA content at -5℃, indicating that the combined stresses had severer membrane lipid peroxidation injury in the seedlings than single stress, which is consistent with published experimental results about the effects of low-temperature stress on plant physiological indexes (18, 19). Similar to the researches on *M. sativa* L. under salt stress (20), in this experiment, it has been pointed out that the salt stress aggravated the damage of the membrane and led to an increase of the MDA content.

Effect of combined stresses on osmotic adjustment substances

Protein is the material basis of life, the most abundant organic macromolecule in plan cells, the basic organic matter that constitutes cells, and the main component of enzymes that can participate in the chemical reaction of cells (21). It can be seen that the soluble protein contents in seedlings of groups under stresses were lower than the blank group when freeze-thaw stress treatment was to be carried out,

which could be explained by the reduction of activity of either protein synthetase or other plant enzymes in plants under adverse conditions, resulting in a decrease in soluble protein content. This result is similar to that presented by Mao and Xu, who reported that salt treatment leads to the decrease of protein content in plant seedlings (11). Soluble protein content in seedlings increased with the decrease of temperature in this experiment, attributing to the enhancement expression of related genes, which could effectively stimulate the accumulation of protective substances, reduce cell metabolism and resist low temperature (22). The reason why the soluble protein decreased in the period of temperature dropping to -5°C and rising to 10°C might be that the protein consumed when plants adapted to adverse conditions and maintained growth, similar to the results of Fleck et al., who investigated protein content in winter wheat leaves and their freeze resistance (23).

Free proline is an important osmotic regulator in plants. It has been reported that under stress conditions, the proline mass ratio in plant cells shall greatly increase, which reduces the cell osmotic potential and helps cells to absorb water, thus preventing protoplasm and protein molecules from dehydration (24). Hence, a change in the proline content can be used as an indicator of cold resistance of plants (3). The results of this experiment showed that the proline content of all groups was significantly increased in the low-temperature environment. This is consistent with the research results of Hare et al., who analyzed the cumulative effects of osmotic pressure under stress conditions and suggested that proline improved the stress resistance to low temperature (25). Li et al. not only found that the accumulation of osmotic adjustment substances like proline can decline the cellular water potential and maintain osmotic balance, but also demonstrated that, to some extent, the more anticold capability plants show, the more significant change in content of proline there will be (26). Acid rain can cause acute injuries of leaves and restrain the growth as well. Therefore, in this experiment, the proline content was deeper influenced in the A-D-F group than that in the other groups.

Soluble sugar plays an important role in the growth cycle of plants. It has been pointed out that under salt stress, plants can accumulate a large number of osmotic regulators such as soluble sugar and other inorganic salt ions (27), which could reduce the osmotic potential in cell, enhance water absorption and water holding capacity, maintaining cell growth and thus enhancing the ability to resist stress (28, 29). As a result, an upward trend of soluble sugar content in seedlings could be obtained in this study, indicating that plants can protect themselves under adverse conditions by accumulating soluble sugars (30). This may due to the increasing cell fluid concentration in plants subjected to low-temperature stress, lowing the freezing point and reducing the excessive dehydration of cells, and thus the protoplasm colloid was protected from cold coagulation, enhancing adaptability to low-temperature stress (31). It was also found that the soluble sugar content of the A-D-F group was much higher than that of the control group, owing to more soluble sugar activating the osmotic pressure regulation mechanism in plants to resist combined stresses (32).

Effect of combined stresses on chlorophyll

The pigment in leaves was designed to absorb light energy and provide energy in plants growth, thus the responses of chlorophyll content to adverse environment could show the ability of photosynthesis (25, 33). Consistent with the results of this experiment that the chlorophyll content in seedlings of group F reduced, Xin and Browse pointed out low temperature was one of the main factors that affects photosynthesis of herbage (34). However, the effects of other stress factors on chlorophyll and other indicators are also different. The study results of Zhou indicated that photosynthetic indicators such as chlorophyll a, b, and carotenoid content of *Populus euphratica* leaves under NaCl stress kept relatively stable level (35), similar to which, as a result, there was no significant difference among the groups A-F, D-F and A-D-F.

Conclusions

In this paper, alfalfa seedlings were used experimental material and the contents of malondialdehyde, soluble protein, soluble sugar, proline, and chlorophyll in plants were measured to disclose the responses of alfalfa to artificial acid rain, deicing salt and freeze-thaw. The accumulation of MDA content in the alfalfa showed that the cell membrane system would be damaged. Meanwhile, the increasing content of soluble sugar and proline to maintain osmotic balance, which could help to resist damage to plants in adversity. However, there was no regular change in chlorophyll content in plants under the combination of stress factors. In summary, the influence of freeze-thaw and deicing salt stress is more significant, while the mixed stresses of acid rain with two factors mentioned above has little effect on plants.

Abbreviations

MDA: malondialdehyde; TBA: thiobarbituric acid; TCA: trichloroacetic acid

Methods

Plant materials

About 1000 seeds of Dongmu-70 provided by the Life Sciences of Northeast Normal University of China were selected and soaked with 0.1% acidic $KMnO_4$ solution for 2 h at first, and rinsed with distilled water. Then seeds were arranged onto trays covered with two layers of filter papers and added with 100ml Hoagland nutrient solution. After the seeds germinated under dark condition at 20 °C for 24 h in the MGC-450BP light incubator (Shanghai Yiheng Scientific Instruments Co., Ltd), the full and similar sized seeds were picked and sprouted, then neatly spread on trays of 26 × 18 cm (length × width) with seeds (23 lines × 40 seeds per line) and were put in MGC-450BP light incubator (Shanghai Yiheng Scientific Instruments Co., Ltd) for germination, with 12-h light (16500 lux; 25 °C) and 12-h non-light (0 lux; 15 °C) for a week, 80 ml of nutrient solution was given every day, 40 mL each morning and evening.

Stress application

A deicing salt solution (0.1 M, 1000 ml) and an acid rain solution (pH=4.5 (sulfuric acid: nitric acid=3:1), 1000 ml) were prepared. Then the seedlings to be treated were evenly divided into eight groups recorded as A-D-F, A-F, D-F, A-D, A, D, F and CK (blank group) (Table 2). Both 7 ml of acid rain solution and 18 ml of deicing salt solution were added to groups A-D-F and A-D respectively. Both 7 ml of acid rain solution and 18 ml of distilled water were added to groups A and A-F respectively. Both 18 ml of deicing salt and 7 ml of distilled water were added to groups D and D-F respectively. 25ml of distilled water were added to F and CK respectively. After the reagents were added, all the test groups were placed in a light incubator whose light condition was set as 12-h light (16500 lux; 25 °C) and 12-h non-light (0 lux; 15 °C) for 2d and then were taken out for freeze-thaw treatment.

A-D-F, D-F, A-F and F were subjected to the freeze-thaw treatment, the seedlings were put into BPHJ-120A high-low temperature test chamber (Shanghai Yiheng Scientific Instruments Co., Ltd) to carry out a freeze-thaw cycle for a period of 14h, with the constant temperature curve set at 10, 5, 0, -5, 0, 5 and 10 °C (2h~14h). The initial setting temperature of the BPHJ-120A high-low temperature test chamber (Shanghai Yiheng Scientific Instruments Co., Ltd) was 15 °C which was close to room temperature at night. The temperature dropped steadily to -5 °C at a speed of 0.5 °C every 12 min (about 0.04 °C /min), and then the temperature rose from -5 to 10 °C at a speed of 0.5 °C every 12 min (about 0.04 °C /min). The groups A-D, D, A, and CK were still maintained in the MGC-450BP light incubator (Shanghai Yiheng Scientific Instruments Co., Ltd) whose light condition was unchanged. At each temperature, 9-d samples were taken from each treatment group at random according to the required amount of the measurement, to be specific, 0.5 g for measuring malondialdehyde (MDA) and soluble sugar content, 0.1 g for soluble protein content, 0.5 g for proline content.

Biochemical Characterization

MDA and soluble sugar content were measured with thiobarbituric acid (TBA) chromatometry method (36). Leaf samples (0.5 g) were ground into homogenate with 5 ml 10% trichloroacetic acid (TCA) solution. After the mix centrifuged (centrifuge: TDL-40B, Shanghai, Anting Scientific Instrument Factory) at 4000 rpm for 10 min, 2 mL of centrifugal supernatant was absorbed into tubes and mixed with 2 ml 0.6% TBA solution. The mixture was bathed in a boiling water for 15 min and then cooled to room temperature quickly. Absorbance under 450, 532 and 600 nm was measured with a UV-6100 UV–visible spectrophotometer (Metash Co. Ltd).

The content of soluble protein was determined by Coomassie brilliant blue method (24). Taking leaves (0.1 g) ground into homogenate with deionizer water (5 ml), which was centrifuged (centrifuge: TDL-40B, Shanghai, Anting Scientific Instrument Factory) for 10 min at 3000 rpm. 1 ml supernatant was absorbed into a test tube and diluted 5 times by deionizer water (4 ml). 1 ml of the diluted solution was mixed with 5 ml Coomassie brilliant blue G-250 (Shanghai Huishi biochemical reagent Co., Ltd). The absorbance was measured at 595 nm after 2 min with a UV-6100 UV–visible spectrophotometer (Metash Co. Ltd), and the protein content was determined by standard curve (25).

The proline content was determined by acid ninhydrin colorimetric method (37). Taking 0.5g of the sample and grinding with 5ml 3% sulfosalicylic acid solution into a homogenate. After being extracted by boiling water bath for 10min and cooled to the room temperature, the mixture was centrifuged (centrifuge: TDL-40B, Shanghai, Anting Scientific Instrument Factory) at 4000 rpm for 10min. 2ml of supernatant was absorbed and added with 2ml of ice-cold acetic acid and 3 ml color reagent (2.5% acidic ninhydrin). The mixture was bathed into a boiling water for 40 min. 5 ml toluene was added for extraction, and the absorbance was measured at a wavelength of 520 nm.

Chlorophyll content was directly measured on the leaves of plant seedlings with SPAD-502Plus chlorophyll meter, one leaf each time for three repeats (Konica Minolta Holdings, Inc.) (38).

Data processing

Statistical analysis was performed with SPSS 16.0 statistical software (IBM SPSS Statistics, Chicago, USA) using one-way analysis of variance (ANOVA) and multiple comparisons were used on the basis of results from tests of significance with least significant difference method (LSD). The significance level was at 0.05, all the experimental data were plotted with Origin 8.0 software (Electronic Arts Inc.). The experiments were repeated three times, and all of the results are presented as mean ± SE.

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and materials: All data generated or analyzed during this study are included in this published article and its supplementary information files.

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Contributions

GZ Bao, WY Tang and QR An designed the experiments; QR An and YX Liu performed most of the experiments; N Zhao performed part of the experiments; JQ Tian and SN Zhu analyzed the data; and WY Tang, N Zhao and JQ Tian wrote the manuscript. All authors agree with the manuscript contents and with its submission. All authors read and approved the final manuscript.

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Tables

Table 1 Pearson correlation analysis under freeze-thaw (F) and combined (A-D-F) stresses

		MDA	protein	chlorophyll	proline	soluble sugar
Freeze-Thaw	MDA	1.000				
	protein	0.090	1.000			
Group	chlorophyll	-0.867*	-0.348	1.000		
	proline	0.887**	0.270	-0.904**	1.000	
	soluble sugar	0.781*	0.016	-0.761*	0.855*	1.000
Freeze-Thaw	MDA	1.000				
+Acid Rain	protein	-0.153	1.000			
	chlorophyll	-0.904**	0.296	1.000		
+deicing salt	proline	0.946**	-0.291	-0.936**	1.000	
Group	soluble sugar	0.895**	-0.261	-0.947**	0.921**	1.000

** indicates a significant correlation at the 0.01 level;

* indicates a significant correlation at the 0.05 level.

Table 2 Experimental design of groups under acid rain (a), deicing salt (D) and freeze-thaw (F) stress

	A-D-F	A-F	D-F	F	A-D	A	D	CK
Acid rain	+	+	-	-	+	+	-	-
Deicing salt	+	-	+	-	+	-	+	-
Freeze-thaw	+	+	+	+	-	-	-	-

+ add stress, - no stress

Figures

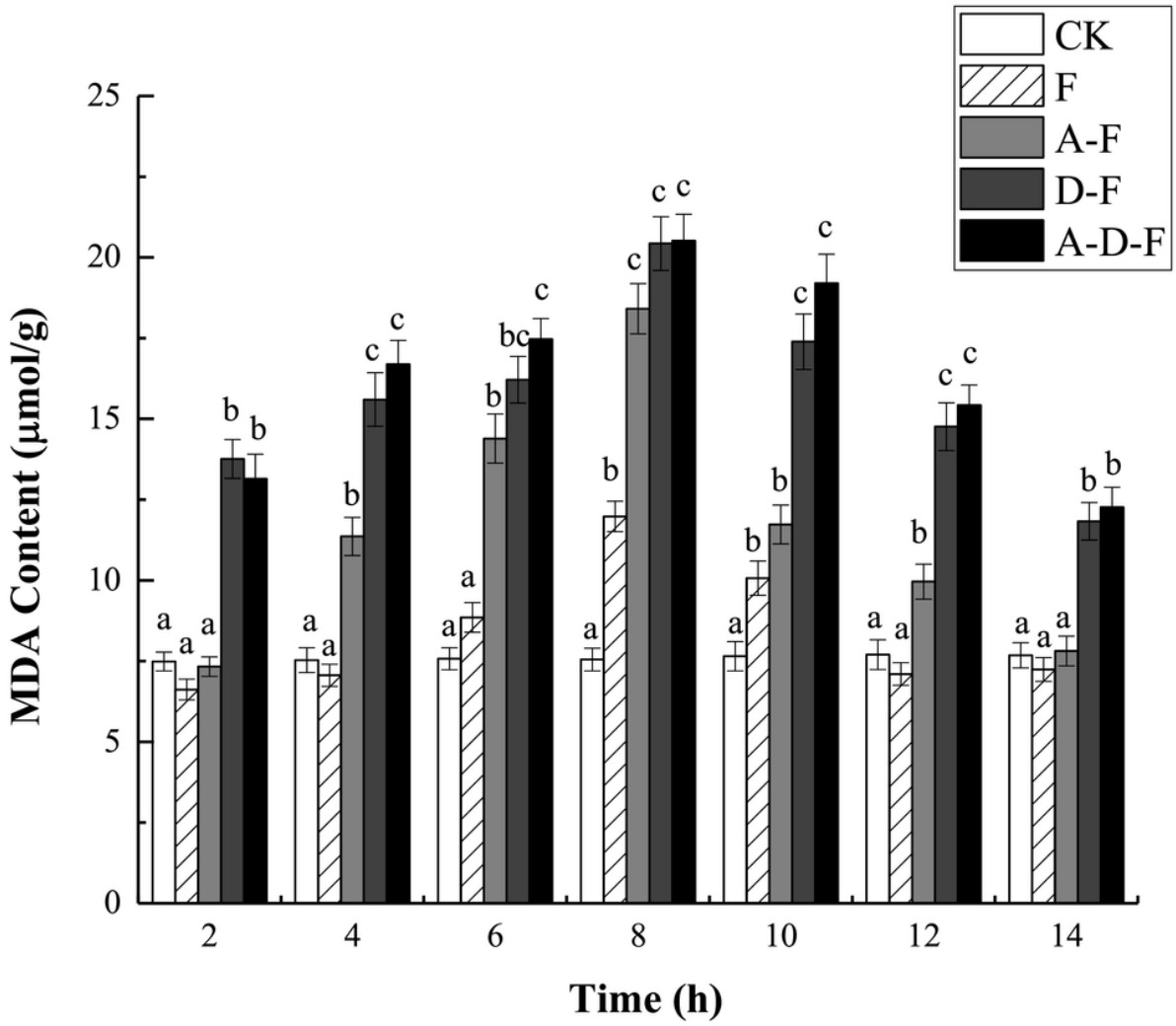

Figure 1

Combined effects of freeze-thaw, deicing salt and acid rain on the MDA content in alfalfa seedlings (mean ± SE, n = 3). 2 ~ 14h represent different temperature corresponding to 10, 5, 0, -5, 0, 5, and 10 ℃, respectively. CK represents the blank group. The letters A, D and F represent acid rain treatment, deicing salt treatment and freeze-thaw treatment, respectively. The different letters indicate significant differences among the various treatments ($P < 0.05$).

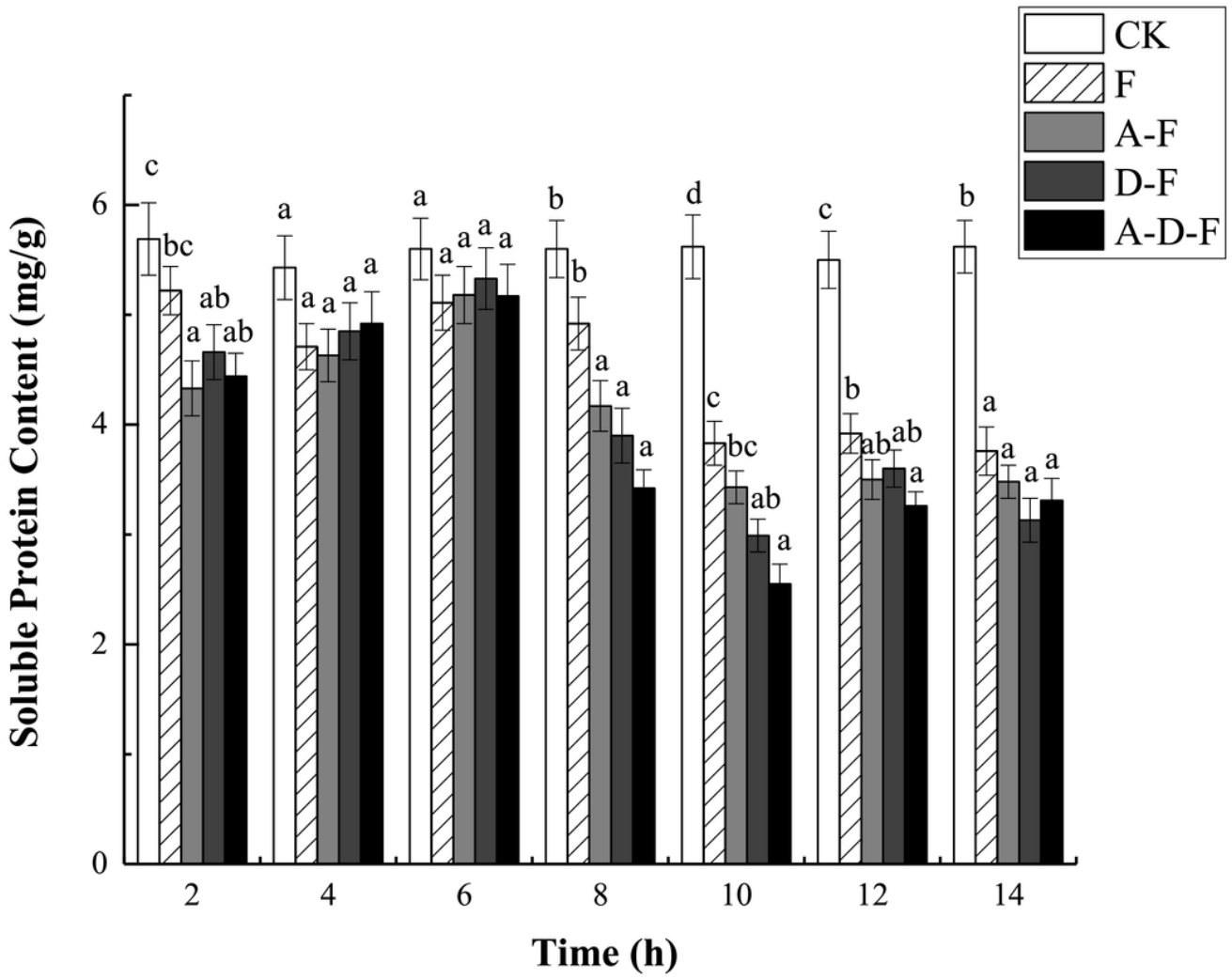

Figure 2

Combined effects of freeze-thaw, deicing salt and acid rain on the soluble protein content in alfalfa seedlings (mean ± SE, n = 3). 2 ~ 14h represent different temperature corresponding to 10, 5, 0, -5, 0, 5, and 10 ℃, respectively. CK represents blank group. The letters A, D and F represent acid rain treatment, deicing salt treatment and freeze-thaw treatment, respectively. The different letters indicate significant differences among the various treatments ($P < 0.05$).

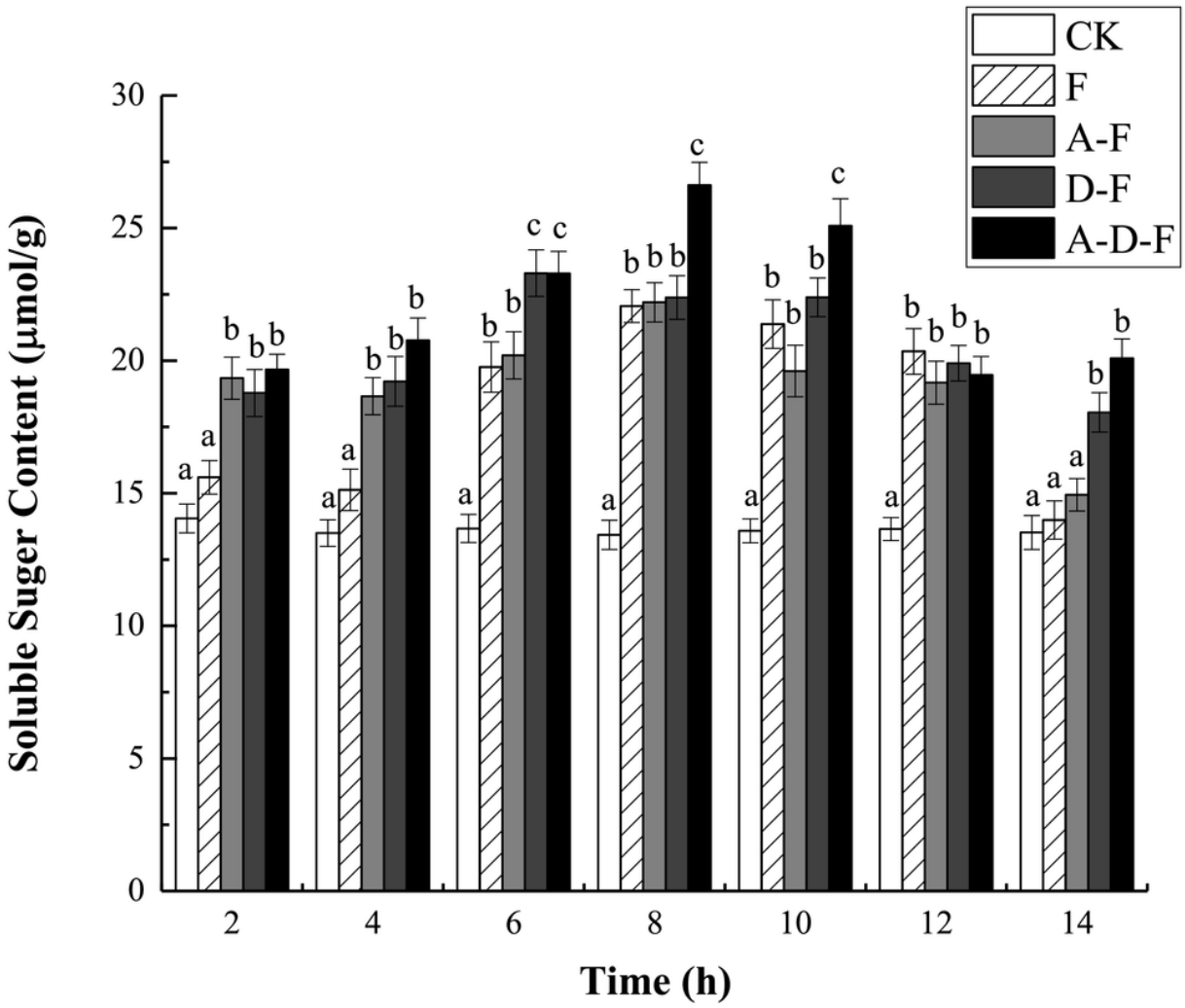

Figure 3

Combined effects of freeze-thaw, deicing salt and acid rain on the soluble sugar content in alfalfa seedlings (mean ± SE, n = 3). 2 ~ 14h represent different temperature corresponding to 10, 5, 0, -5, 0, 5, and 10°C. CK represents blank group. The letters A, D and F represent acid rain treatment, deicing salt treatment and freeze-thaw treatment, respectively. The different letters indicate significant differences among the various treatments ($P < 0.05$).

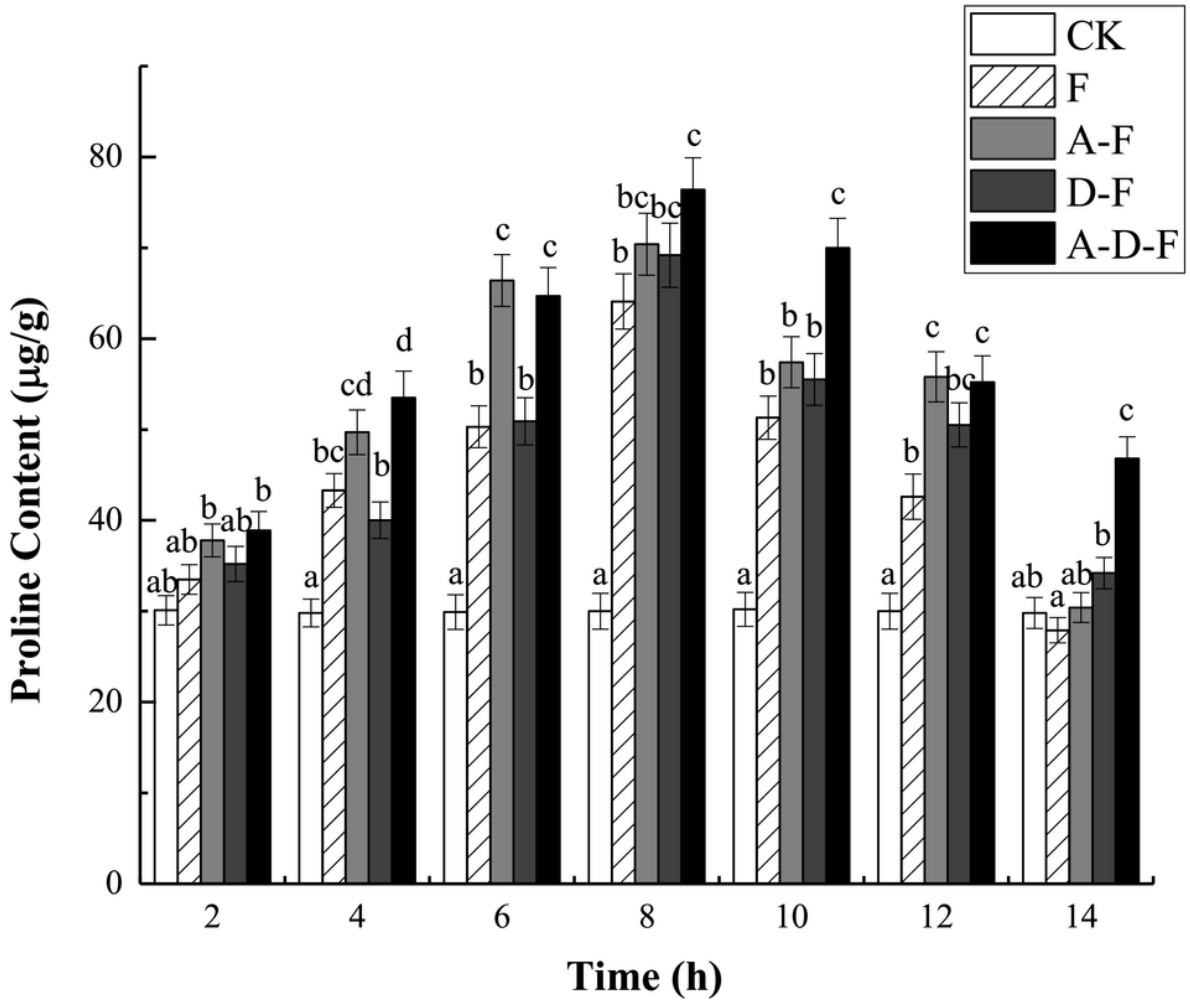

Figure 4

Combined effects of freeze-thaw, deicing salt and acid rain on the proline content in alfalfa seedlings (mean ± SE, n = 3). 2 ~ 14h represent different temperature corresponding to 10, 5, 0, -5, 0, 5, and 10°C. CK represents blank group. The letters A, D and F represent acid rain treatment, deicing salt treatment and freeze-thaw treatment, respectively. The different letters indicate significant differences among the various treatments ($P < 0.05$).

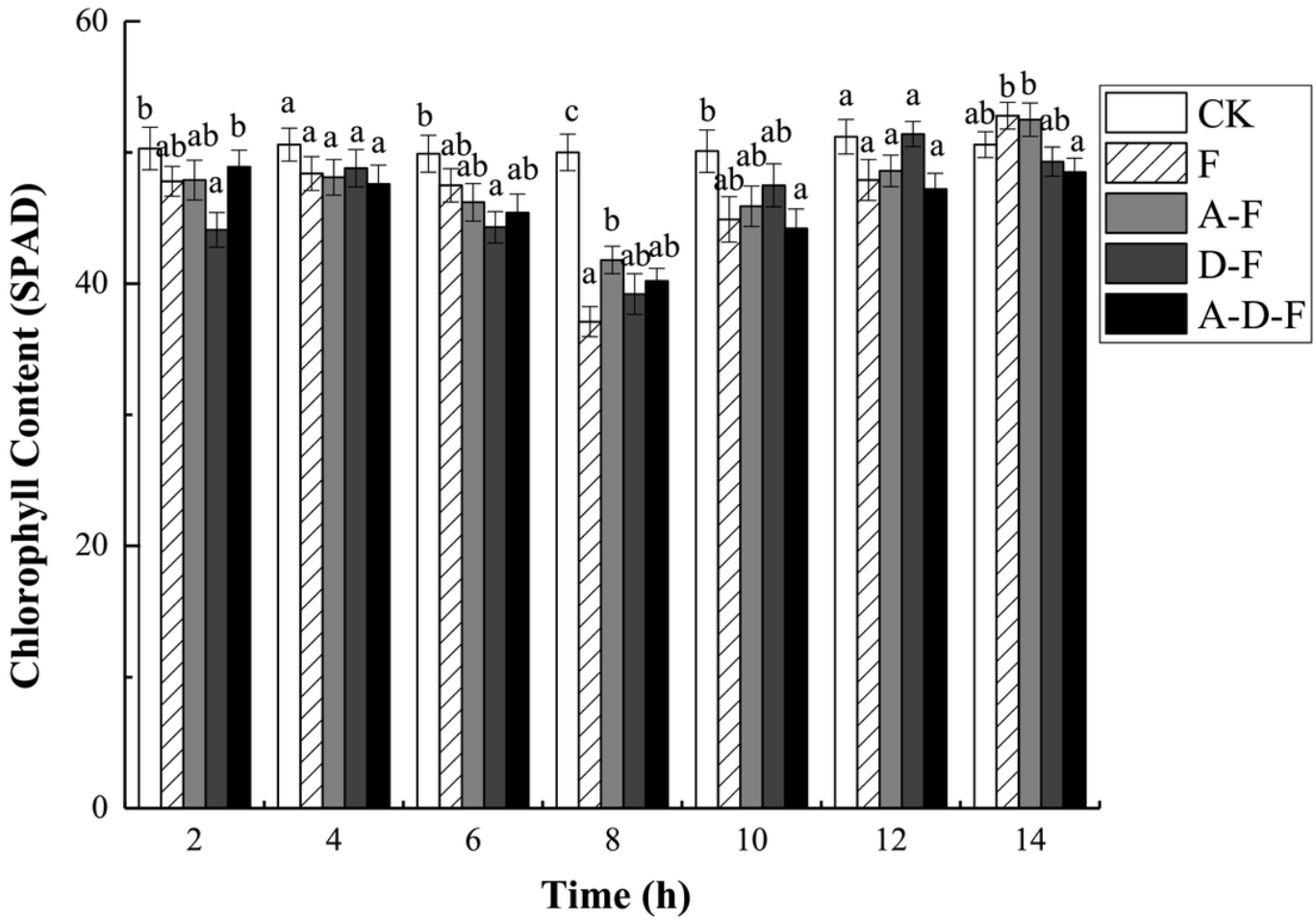

Figure 5

Combined effects of freeze-thaw, deicing salt and acid rain on the chlorophyll content in alfalfa seedlings (mean ± SE, n = 3). 2 ~ 14h represent different temperature corresponding to 10, 5, 0, -5, 0, 5, and 10°C. CK represents blank group. The letters A, D and F represent acid rain treatment, deicing salt treatment and freeze-thaw treatment, respectively. The different letters indicate significant differences among the various treatments ($P < 0.05$).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile2.xls
- Additionalfile5.xls
- Additionalfile4.xls

- Additionalfile3.xls
- Additionalfile1.xls