

Original Research

Resistance of Rye Seedlings to Drought and Freeze-Thaw Stress

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Abstract

The freeze-thaw (FT) and drought in early spring in Northeast China are two main ecological stresses to forage grasses. In this paper, *Secale cereale* L. grown under 2 d drought-stress (mild, moderate, and severe treat) with 5, 10 and 15% polyethylene glycol (PEG-6000) concentrations. The temperature gradient was set to 10, 5, 0, -5, 0, 5 and 10°C after FT treatment. The content of protein, soluble sugar and MDA increased with a trend of combined stress>FT>drought stress, indicating that the damage of rye under single FT stress was higher than that under the single drought stress. Under drought stress, FT could aggravate the damage to rye. At T7 (10°C) in the thawing stage, compared with the CK, the pattern of the decrease of RWC and chlorophyll and the increase of protein and MDA content was severe combined stress>FT>moderate combined stress>moderate drought stress. The results of this study showed that a proper drought treatment before FT stress could alleviate the physiological damage to the forage leaf.

Keywords: ecological stresses, PEG-6000, physiological response, seedlings

Introduction

Secale cereale L., as a common cold-season forage, is a perennial herb belonging to the rye widely planted in temperate regions due to its rapid growth and high yield [1, 2]. It can be planted in a cold temperate zone, temperate zone and subtropical zone, because of its

strong stress resistance and cold tolerance in spring [3, 4]. In China, it is mainly planted between the east of 107°E and 25°N-27°N, and the vernalization can be completed at the low temperature of -5~0°C [5, 6].

Plants can be threatened by various biological and abiotic stresses, including water, temperature, salinity and light. Plants living at high latitudes or altitudes often experience freeze-thaw(FT) cycles in winter and early spring and cold tolerance in winter is one of the limiting factors for plant survival [7]. Being exposed to sub-zero temperatures often results in injury

and death [8]. Several cell-level dysfunctions caused by cold stress include membrane damage, production of reactive oxygen species, protein denaturation and accumulation of toxic products [9, 10]. Kendall and McKersie [11] provided evidence of oxidative stress induced by FT such as accumulation of reactive oxygen species (ROS) which can damage protein and make it denatured [12, 13], and Min et al. [14] pointed out that MDA accumulated more in frozen samples at - 4.5°C than at - 4.0°C. Therefore, the study of frost resistance of plants under variable temperature conditions is helpful to elucidate the physiological adaptation mechanism of plants under seasonal variable temperature conditions.

Drought is the main abiotic stress in arid and semi-arid regions of the world, which seriously affects the yield and quality of crops [15-17]. Drought causes a series of physiological changes in plants. Plants adapt to drought stress through physiological adaptation and develop a wide range of physiological drought resistance mechanisms [18-21]. Plants exposed to drought stress lead to a decrease in relative water content [22, 23], Riedell [24] pointed out that the RWC of the leaves of plants not infected by aphids decreased significantly at the end of drought stress. The chlorophyll content of two species of spring wheat decreased after drought stress [25]. With the increase of drought stress, the chlorophyll content of two soybean varieties decreased [26]. Drought stress usually leads to the decrease of Soluble sugar (SS) content in *Rhodiola variegata* and *Casuarina equisetifolia* mediated by arbuscular mycorrhizal fungi [27-29]. PEG 6000 solution was used in the formulation of water stress. Yigit et al. [30] results showed increased water stress and reduce the percentage of germination in all species. PEG applications have been used in many species to identify their resistance to drought and it has given successful results. All studies conducted until now have proved that the increasing water stress decreases the germination proper. In addition, it has also been proven in the previously conducted studies that the tolerances of different origins to drought stress are different [31].

Plant physiological stress is a complex interaction between plants and the environment. Under natural conditions, the environmental impact of only a single factor without the interaction of complex stress factors will not occur [16]. Therefore, this research aims to study the changing pattern of RWC, protein, chlorophyll, SS and MDA contents in leaves of the seedlings under combined FT and drought stresses in high latitude, to reveal the physiological response characteristics of *Secale cereale* L., and to provide a reference for the range management.

Experimental

Experimental Materials and Methods

Rye seeds (*Secale cereale* L. cv. *Dongmu 70*) were sterilized with 0.1% KMnO₄ for 2 hours, washed with

deionized water, evenly spread (25 grains per row x 20 rows) in a tray containing wet filter paper (20 cm x 30 cm), and cultured in a light incubator (MGC-450BP light incubator). The growth of seeds was observed every day. The seeds were ventilated properly. The temperature was 25°C during the day and 20°C at night. The light intensity (PPFD) was about 280 μmol m⁻² s⁻¹ (66%) for 12 h. Hoagland nutrient (including 4 mM Ca(NO₃)₂, 3.5 mM K₂SO₄, 1 mM NH₄H₂PO₄(MAP), 4.1 mM MgSO₄, 5×10⁻²mM Fe-Na EDTA, 5×10⁻²mM FeSO₄, 5×10⁻²mM H₃BO₃, 1×10⁻²mM Na₂B₄O₇, 1×10⁻²mM MnSO₄, 3×10⁻⁴mM CuSO₄, 1×10⁻³mM ZnSO₄, 2×10⁻⁴mM (NH₄)₂SO₄) was used every day to keep seeds wet. The simulation of the drought and FT stresses was carried out after 7 days of incubation.

PEG Drought Simulation Treatment

Using the above Hoagland nutrient solution to prepare PEG solution [32-34]. Four concentrations of PEG (5, 10, 15 and 20%) were set up in the pre-test to simulate drought treatment. The results showed that the lethal rate of plant leaves was about 70% under the 20% PEG concentration. Therefore, 5% (mild drought, D1), 10% (moderate drought, D2), 15% (severe drought, D3) were selected in this experiment. The seedlings with the same growth were treated with mild, moderate and severe drought for 2 d.

Freeze-Thaw Treatment

LT50 (semi-lethal temperature) indicates the resistance to FT stress, which results in 50% injury or plant death [14, 35]. Rye seedlings treated with different drought stress were subjected to FT stress. In the pre-test, the lethal rate of Rye was 50% when the temperature was -5°C. After 2 d of drought treatment, the seedlings were placed in the BPHJ-120A test box for FT stress treatment. During the freeze-thaw treatment, the seedlings have been under drought stress. The FT temperature gradually changed under programmed control for 14 h. The FT temperature was sampled every 2 h at 10, 5, 0, -5, 0, 5 and 10°C (T1-T7) [36, 37], and the non-freeze-thaw treatment (include CK, D1, D2, D3) was placed in a light incubator to simulate natural growth conditions (12 h day/night). The experiment was divided into eight treatment groups: CK-control group; D1-mild drought; D2-moderate drought; D3-severe drought; F-freeze and thaw; FD1-FT + mild drought; FD2-FT + moderate drought; FD3-FT + severe drought. At each temperature, eight treatment groups were randomly sampled with three replicates.

Measurement

Relative water content (RWC) of leaves was measured by saturated weighing method [38]. Took fresh leaves and weighed the fresh weight (Wf) (0.3 g

sample (about 10 seedlings) was taken); then immersed them in water for 24 h until water absorption was saturated. After taking them out, used filter paper to absorb surface water and weigh the fresh matter (W_f). Finally, put the samples into the oven that has been heated up to 105°C for 15 minutes, and then dried them at 80°C to constant weight and weighed the dry matter (W_d).

$$\text{RWC} = \frac{(W_f - W_d)}{(W_t - W_d)} \times 100\%$$

Soluble protein content was determined by Coomassie Brilliant Blue method [39-41]. Weighed 0.1 g fresh sample (0.1 g samples (3 to 5 seedlings) of the different treatments) and ground into homogenate with 5 ml distilled water. After centrifugation for 10 minutes at 3000r/min, the supernatant was reserved. After extracting 1.0 ml sample and fivefold dilution, taking 1.0 ml into the test tube. Each sample was repeated twice. 5 ml Coomassie Brilliant Blue G-250 solution was added to shake the sample. The absorbance was determined by colorimetry at 595 nm wavelength, and the protein content was determined by standard curve.

The content of chlorophyll (Chl) was determined by SPAD-502 Plus chlorophyll meter.

The contents of SS and malondialdehyde (MDA) were determined by thiobarbituric acid method [39-41]. Weighed 0.5 g fresh sample (0.5 g sample (about 15 seedlings) selected randomly), added 5 ml 10% trichloroacetic acid (TCA), ground to homogenate, and centrifuged for 10 minutes at 4000 r/min. Absorbing

centrifugal supernatant 2 ml (CK plus 2 ml distilled water), adding 2 ml 0.6% thiobarbituric acid (TBA), put the mixture into boiling water bath for 15 minutes and cooled rapidly. With distilled water as blank sample, the extinction at 532, 600 and 450 nm wavelength was determined.

Statistical Analysis

All results with the experiment were expressed as a mean of 3 replicates. Microsoft Excel 2007 and SPSS 19.0 were used for data processing and one-way ANOVA. The minimum significant difference method was used to test the different significance at P = 0.05 significance level. Origin 8.0 software was used for mapping. All the results were presented by mean ±SE.

Results and Discussion

Effects of Freeze-Thaw and Drought Stress on RWC

RWC of rye decreased under either drought, FT or combined stress (Fig. 1). Compared with CK, RWC decreased as FD3>FD1>F>FD2>D3>D2>D1 in the frozen stage and T4 (Fig. 1). Compared with CK, RWC in D1, D2 and D3 groups decreased by 1.6, 6.5, 11.6%; F group by 17.7%, FD1, FD 2 and FD3 groups by 18.2, 15.0 and 23.0%, respectively (Fig. 1). At T7, compared with T1, the RWC of rye leaves treated by FT stress could not reach the level of T1, although the temperature at T7 was restored to the initial stage.

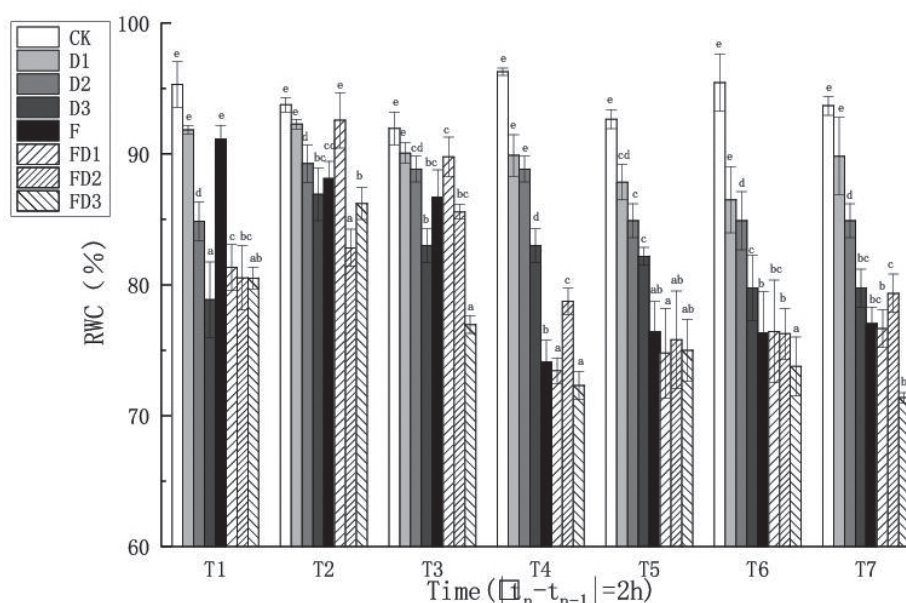


Fig. 1. Effects of freeze-thaw, mild (1), moderate (2) and severe (3) drought stress on RWC of rye seedlings. T1-T7 represents 2-14h respectively, corresponding to seven temperature gradients; D1, D2 and D3 represent mild, moderate and severe drought respectively; F represents Freeze-thaw. The different letters indicated the significant difference between different treatments at the same temperature (P<0.05). The data are expressed as mean (+standard error) (n = 3).

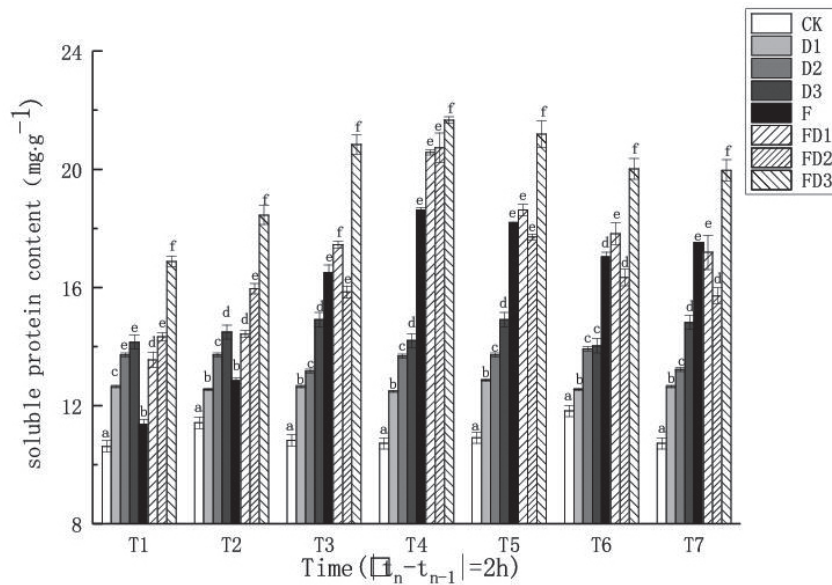


Fig. 2. Effects of freeze-thaw, mild (1), moderate (2) and severe (3) drought stress on RWC of rye seedlings. T1-T7 represents 2-14h respectively, corresponding to seven temperature gradients; D1, D2 and D3 represent mild, moderate and severe drought respectively; F represents Freeze-thaw. The different letters indicated the significant difference between different treatments at the same temperature ($P < 0.05$). The data are expressed as mean (+standard error) ($n = 3$).

Effects Of Freeze-Thaw And Drought Stress on Soluble Protein Content

The protein content of rye increased under drought, FT and combined stresses. The protein content of rye increased first and then decreased slowly under FT and combined stresses, and reached the maximum at T4 (Fig. 2). Compared with CK,

protein content in T4 stage increased with the trend of $FD3 > FD2 > FD1 > F > D3 > D2 > D1$ (Fig. 2). At T7 stage, compared with CK, the protein content of D1, D2 and D3 increased by 24.2, 19.1 and 33.1% respectively under single drought stress, and the protein content of F, FD1, FD2 and FD3 increased by 44.1, 40.5, 36.3 and 52.1% respectively (Fig. 2). At T7, compared with T1, regardless that the FT group regained its initial

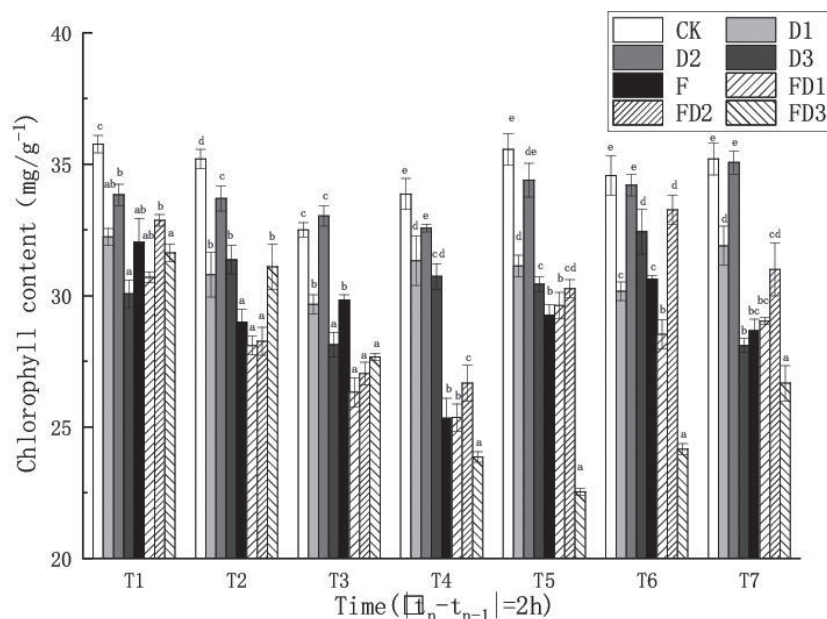


Fig. 3. Effects of freeze-thaw, mild (1), moderate (2) and severe (3) drought stress on RWC of rye seedlings. T1-T7 represents 2-14 h respectively, corresponding to seven temperature gradients; D1, D2 and D3 represent mild, moderate and severe drought respectively; F represents Freeze-thaw. The different letters indicated the significant difference between different treatments at the same temperature ($P < 0.05$). The data are expressed as mean (+standard error) ($n = 3$).

temperature, the protein content after FT stress was higher than that at T1, and the FD2 protein content had the least increase with a value of 9.6%.

Effects Of Freeze-Thaw and Drought Stress on Chlorophyll Content

Except for group D2 of T3, compared with CK, the chlorophyll (Chl) content of rye decreased under drought, FT and combined stresses, decreased first and then gradually recovered under combined stresses (Fig. 3). In T4 stage, compared with CK, Chl decreased as FD3>F, FD1>FD2>D3>D1>D2 (Fig. 3). The Chl content of F, FD1 and FD2 reached the minimum at T4, while FD3 reached the minimum at T5. In T7 stage, compared with CK, Chl content decreased by 11.3, 2.1 and 27.3% respectively under drought stress, while D2 decreased insignificantly. Chl contents of F, FD1, FD2 and FD 3 decreased by 26.3, 22.6, 17.8 and 35.7% respectively (Fig. 3). Compared with T1, the Chl content in FT group decreased after FT stress, regardless of the temperature at T7 returned to the initial stage, which was similar to that in RWC.

Effects Of Freeze-Thaw and Drought Stress on Soluble Sugar Content

Compared with CK, SS content increased except T1 (Fig. 4). Compared with CK, the increase of SS content at T4 showed a trend of FD3>FD2>FD1> F>D3>D2>D1 (Fig. 4). The SS content of FD2 reached the maximum at T4, while both F and FD3 reached the maximum at T5. Compared with CK, SS content increased by 1.35,

4.4 and 22.2% under drought stress ($p<0.05$), and increased by 30.1, 40.1, 40.6 and 50.1% under FT and combined stress, respectively ($p<0.05$). At T7, although the temperature was restored to the initial stage, the SS content increased after FT stress, and the increase in FD2 group was the least, which was similar to the change of protein.

Effects Of Freeze-Thaw And Drought Stress on MDA Content

Compared with CK, MDA content of rye increased under drought, FT and combined stress, and MDA content increased first and then decreased under the combined stress (Fig. 5). At T4, compared with CK, the increase of MDA content tended to be FD3>FD2>FD1>F>D3>D2>D1, and reached the maximum at T4 in FD3 group (Fig. 5). Compared with CK, MDA content increased by 21.0, 14.8 and 29.5% under drought stress at T7 (Fig. 5). At T7, although the temperature of T7 returned to the initial stage, MDA content of F, FD1 and FD3 still increased while FD2 decreased after FT treatment.

Correlation Analysis

Pearson correlation analysis of five indexes of rye seedlings showed that under the single FT stress and combined stress, there were positive relationships between either SS and MDA, or SS and MDA ($p<0.05$); there were negative relationships among RWC and protein content, SS and MDA, protein content and Cl, Chl and SS and Chl and MDA ($p<0.05$) (Table 1, 2).

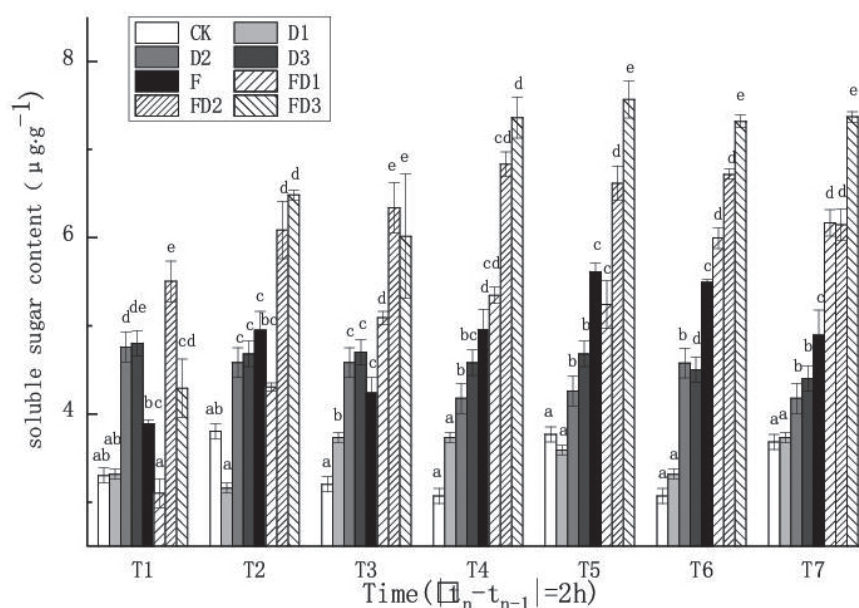


Fig. 4. Effects of freeze-thaw, mild (1), moderate (2) and severe (3) drought stress on RWC of rye seedlings. T1-T7 represents 2-14 h respectively, corresponding to seven temperature gradients; D1, D2 and D3 represent mild, moderate and severe drought respectively; F represents Freeze-thaw. The different letters indicated the significant difference between different treatments at the same temperature ($P<0.05$). The data are expressed as mean (+standard error) ($n = 3$).

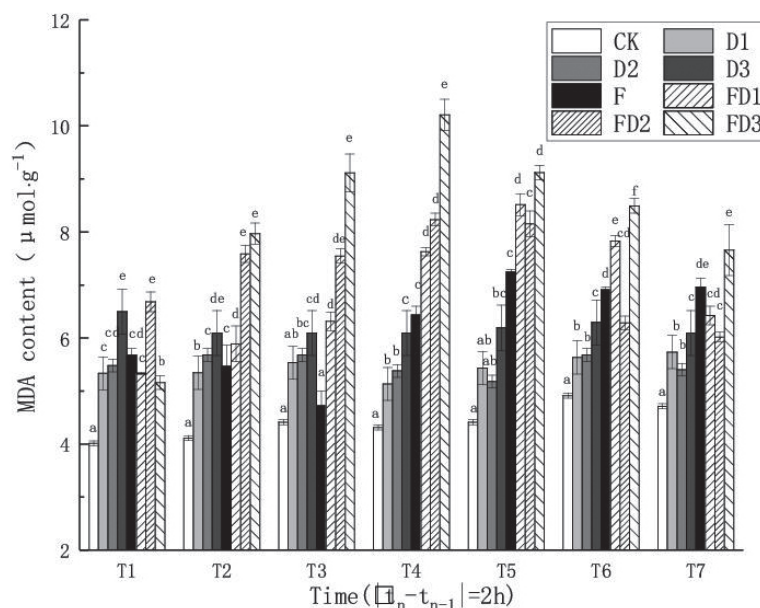


Fig. 5. Effects of freeze-thaw, mild (1), moderate (2) and severe (3) drought stress on RWC of rye seedlings. T1-T7 represents 2-14h respectively, corresponding to seven temperature gradients; D1, D2 and D3 represent mild, moderate and severe drought respectively; F represents Freeze-thaw. The different letters indicated the significant difference between different treatments at the same temperature ($P < 0.05$). The data are expressed as mean (+standard error) ($n = 3$).

RWC was positively correlated with Chl under single FT stress ($p < 0.01$, $r = 0.554$) (Table 1), except the combined stress (Table 2); Chl and SS were not correlated under the single FT stress (Table 1), but negatively correlated under the combined one ($p < 0.01$, $r = -0.447$) (Table 2).

Discussion

RWC is an important index reflecting leaf water stress and affecting plant water relationships [42], showing the degree of water deficit of plants under stress [43]. Drought stress further reduces the RWC of wheat and rice [44]. Flexas et al. [45] concluded that the

Table 1. Pearson correlation analysis between the indices when the temperature returns to the initial stage under single freeze-thaw stress.

	RWC	Protein	Chl	SS	MDA
RWC	1	-0.870**	0.554**	-0.792**	-0.853**
Protein		1	-0.596**	0.821**	0.907**
Chl			1	-0.244	-0.692**
SS				1	0.710**
MDA					1

*Correlation is significant at the 0.05 level. **Correlation is significant at the 0.01 level, $n = 3$.

Table 2. Pearson correlation analysis between the indices when the temperature returns to its initial stage under combined stress.

	RWC	Protein	Chl	SS	MDA
RWC	1	-0.707**	0.331	-0.547**	-0.599**
Protein		1	-0.675**	0.667**	0.667**
Chl			1	-0.447*	-0.513**
SS				1	0.817**
MDA					1

RWC of soybean and tobacco decreased under water stress. The results showed that the RWC of rye leaves decreased under drought stress (Fig. 1). At this time, the water deficit of leaves reduced and with the increase of drought stress, RWC gradually decreased (Fig. 1). The degree of leaf water deficit gradually deepened. According to the research of Cui et al. (2016), the RWC of leaves decreases gradually with the prolongation of drought duration, i.e. the deepening of drought degree. RWC decrease first and then increased gradually under FT stress, which may be due to the dehydration of water considering that the frozen cells in plant cells may lead to a significant decrease in RWC when the temperature was below 0°C. When the temperature rose, the cells rehydrated and RWC gradually increased [46]. At T4 and T7, RWC decreased dramatically under the combined stress than under the single drought one, possibly because the change of leaf temperature may be an important factor controlling leaf water status [44]. At T4 and T7, the decrease of RWC showed a trend of severe combined stress > mild combined stress > FT stress > moderate combined stress, which indicated that mild drought and severe drought treatment aggravated the decline of RWC under FT stress, and moderate drought treatment alleviated the impact of freeze-thaw on RWC. Compared with T1, although the temperature of T7 returned to the initial stage, the physiological damage to seedlings caused by FT stress was severe.

Soluble proteins play an important role in the osmotic regulation of plant cells. When plants are exposed to environmental stress, they can induce the production of multiple reactive oxygen species (ROS) [47]. Excessive accumulation of ROS may directly damage the proteins [48]. The results showed that the protein content increased under drought stress [49]. Under adversity conditions, the balance of ROS production and removal will be disrupted, causing plant damage. There are many studies on the effects of drought stress on osmotic adjustment substances. It is generally believed that when plants lack water, the soluble protein content will increase to a certain extent, thereby increasing the drought adaptability of the plant [50]. With the deepening of drought stress, the protein content increased gradually, indicating that the more serious the drought was, the more damage to the seedlings. Balestrasse et al. [32] showed that cold treatment could significantly increase protein content. The increase of protein content during the freeze-thaw period in the lower temperature stage and the decrease in the higher temperature stage may be due to the production of injury-related proteins induced by freeze to reduce the damage to seedlings. At the thawing stage, seedlings were favorable for recovery from freezing, and the content of injury-related proteins decreases [46]. Combined stress increase the protein content, however, at the stage of temperature recovery, the FD2 protein content was lower than that of F group, suggesting that reasonable control of water before FT stress may accelerate the decrease of soluble protein content, which

is conducive to the recovery of physiological properties of rye. Compared with T1, the effect of FT stress on protein was irreversible, although the temperature of T7 stage was restored to the initial stage.

In photosynthesis, chlorophyll is the basic factor and power of plant growth and biomass production. The stability of chlorophyll during adversity allows cells to maintain functional chloroplasts so that photosynthesis can be restored after plants recover from adversity [51]. Chlorophyll content in leaves is an important trait affecting seedling growth rate under cold conditions [52]. Low temperature and drought stress can damage chloroplast matrix [53], ROS damage [54], and ultimately reduce photosynthesis. Chl content was lower under low temperature and drought stress [55]. The results showed that the Chl content of rye decreased under drought stress, and the Chl content of rye further decreased under moderate drought stress. Under FT stress, Chl decreased with the decrease of temperature, showing a trend of decreasing first and increasing slowly. At T7, the Chl content under single severe drought stress (D3) was lower than that under single FT stress (F), which indicated that the damage of severe drought to rye was greater than that under FT stress. The combined stress accelerated the decline of Chl, however, at T7, the content of Chl tended to increase under F stress compared with either mild or moderate drought treatment, indicating that a moderate drought treatment before FT stress was helpful to improve the resistance of rye.

The soluble sugar in plant leaves, as a major osmotic regulator, could promote vitrification (a phenomenon in which intracellular water hardens like glass during freezing or cold stress without ice crystallization), thus avoiding damage caused by crystallization when water withdraws [56, 57]. Plants respond to water shortage and adapt to water shortage because of the accumulation of osmotic regulators, which are related to stress tolerance [58]. It is reported that under natural conditions, the SS content increases when plants are exposed to low temperature, and decreases with temperature rises. Cold acclimation can induce combined accumulation to protect cells from frostbite [10] and proteins from freezing and dehydration [59]. The results showed that SS content in leaves of rye seedlings increased under different stress treatments, and increased with the increase of drought stress. At the T4 and T7 stages, the increase of SS content under FT stress was greater than that under the drought one, indicating that the damage degree of rye under the single FT stress was greater than that under the single drought one, and rye accumulated more combined to resist the stress. Under drought stress, SS content increased more efficiently under FT stress than that without it.

The SS content of FD2 reached the maximum at T4 and decreased gradually at the thawing stage, while both F and FD3 reached the maximum at T5 stage, indicating that the damage to rye by freezing and thawing was still accumulating despite of the increase

of temperature, and moderate drought treatment was helping to alleviate the damage caused by freezing and thawing and improve the freezing resistance of rye. Compared with T1, the effect of FT stress on rye was irreversible, although the temperature at T7 returned to the initial stage.

MDA is the final product of lipid peroxidation in the plant cell membrane. The content of MDA can reflect the degree of plant damage. The results showed that MDA content increased under the drought stress, which was consistent with the results of Talaat et al. [60]. Freeze-thaw stress leads to excessive accumulation of ROS, which leads to oxidative stress [14, 61], and oxidative damage is also expressed on cell membranes through an accumulation of MDA [14]. Rye accumulated more MDA at T4 than at T3, which was similar to the results of Min et al. [14] that the plants accumulated more MDA at - 4.5°C than at - 4°C. At T3-T7, MDA content increased first and then decreased under FT stress, probably because the damage of leaves was induced by frostbite, and then recovered after temperature compensation [62], so the MDA content decreased after temperature rose. At T7, MDA content increased with a pattern of severe combined stress>FT stress>moderate combined stress>moderate drought stress, probably because the moderate drought stress promoted the improvement of FT tolerance of rye [59, 63], so MDA content was lower.

Pearson correlation analysis showed that under the single FT and the combined stress, the protein was positively correlated with SS and MDA, and SS was also positively correlated with MDA, indicating that under the single FT and the combined stress, cell membrane lipid peroxidation leads to an increase in MDA content, and rye accumulated more combined to protect cells and protein from frostbite under low temperature. RWC was negatively correlated with protein, SS and MDA, in addition, protein and Chl as well as Chl and SS, MDA had the same correlation, which indicated that low temperature caused water freezing in leaf cells, which lead to water stress, so RWC decreased. What's more, membrane lipid peroxidation resulted in cell function damage and chlorophyll content decrease.

Conclusions

In our research, drought and FT stress can lead to oxidative stress, i.e. the accumulation of reactive oxygen species, which can lead to oxidative stress in proteins, membrane lipids and other cellular components. The results showed that drought stress and FT stress could decrease the relative water content and chlorophyll content of leaves, at this moment, the water retention capacity of the leaves reduced, and increase the content of protein, SS and MDA to adjust the cell turgor pressure and osmotic potential to alleviate the damage of rye seedlings under drought stress. When the temperature dropped to the lowest point, the damage

degree of rye under single FT stress was higher than that under single drought one, and FT stress could aggravate the damage of rye under drought stress. When the temperature rose to 10°C, the relative water content and chlorophyll content of rye leaves treated by FT stress were lower than those of T1, and the contents of protein, SS and MDA (except FD2 group) were higher than those of T1. It showed that although the temperature returned to the initial stage, the damage of FT stress to rye was irreversible. The damage caused by drought and FT on seedlings showed a trend of severe combined stress>FT stress>moderate combined stress>moderate drought stress, which indicated that the moderate drought treatment under FT conditions might help alleviate the physiological damage of FT on leaves. In the range management of high latitude and high-altitude areas, appropriate water control of forage grass in the early stage of spring and autumn freezing and thawing can effectively alleviate the damage caused by large fluctuations of temperature to plants by improving their physiological adaptation mechanism, avoiding a large number of deaths when plants are exposed to variable temperatures, and improving the survival rate.

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Conflict of Interest

The authors declare no conflict of interest.

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