Physiological responses of rye seedlings to the combined stress of NaCl, ambrosia and freeze-thaw

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Abstract

Background: Crops are often affected by NaCl, ambrosia and freeze-thaw stress simultaneously during their growth, and many areas in Northeast China are facing such serious ecological stress problems. In this experiment, the physiological responses of rye seedlings to NaCl and ambrosia stress in a freeze-thaw environment were studied by artificial simulation technique. Malondialdehyde (MDA) content, soluble protein (SP) content, superoxide dismutase (SOD) activity, catalase (CAT) activity, net photosynthetic rate (P_n) and transpiration rate (T_r) were determined and analyzed.

Results: The results showed that: After stress treatment, MDA and SP contents of rye seedlings increased by 19.48%-88.96% and 22.54%-107.30%, SOD and CAT activities increased by 4.42%-26.60% and 23.31%-64.68% and P_n and T_r decreased by 40.00%-71.67% and 20.00%-80.00%.

Conclusion: In the face of stress, rye seedlings can reduce the damage caused by stress by increasing osmotic substance and antioxidant enzyme activity, so as to achieve the purpose of adapting to environment. The results revealed that combined NaCl, ambrosia and freeze-thaw stress had a significant superposition effect on plants compared with single NaCl, ambrosia and freeze-thaw stress.

1 Background

Soil freeze-thaw refers to the process of repeated freezing and thawing due to soil temperature changes, which is a common natural phenomenon in seasonal and permafrost regions and is commonly seen in northeast China, western mountainous areas and the Qinghai-Tibet Plateau [1]. The freeze-thaw stress will not only affect soil physical and chemical properties and biogeochemical cycles, but also affect the physiological and ecological processes of plants through habitat stress or soil environment changes, which may have an important impact on the productivity of vegetation ecosystem in areas where frozen soil is widely distributed [2]. The freeze-thaw stress affects plant physiological characteristics in many aspects, including affecting plant cell membrane, antioxidant enzyme activity, osmotic regulatory substances and photosynthesis [3]. In addition, overuse of fertilizers, poor irrigation practices and salinization due to the diversion of seawater into fresh water are among the most challenging constraints on plant growth and yield. Salinity seriously hinders plant growth by changing a variety of physiological and biochemical pathways, and causes secondary stress such as water shortage, nutritional imbalance and oxidative stress [4]. In the 1930s and 1940s, artemisia triphylla was introduced into China and spread rapidly, and has now spread among 15 provinces [5]. It has strong reproductive capacity, fast propagation speed, obvious growth advantages, broad ecological adaptability and strong allelopathy. Allelopathy refers to the indirect or direct harmful or beneficial effects on surrounding plants by releasing chemicals into the environment [6]. Studies have shown that ambrosia may release some allelopathic substances into the environment through volatilization, rain leaching and root secretion, which inhibit the seed germination and plant growth of other surrounding plants [7].

Rye (Secale cereale L.) is a gramineae, an annual or perennial herbaceous plant with strong cold and drought tolerance, mainly cultivated in northern mountainous areas or in colder regions [8]. In this study, Dongmu 70 was treated with NaCl and ambrosia stress. The effects of single and combined stress on malondialdehyde content, soluble protein content, superoxide dismutase activity, catalase activity, net photosynthetic rate and transpiration rate of rye seedlings under simulated freeze-thaw environment were analyzed to explore the change rules of various physiological indexes and explain the physiological response characteristics of rye seedlings under complex environment. It is helpful to understand and take effective measures to reduce the harm of NaCl, ambrosia and freeze-thaw on rye growth, and provide certain data reference and theoretical basis for plant cultivation.

2 Result

2.1 Changes in the soluble protein (SP) content of seedlings

It could be seen from Fig. 1 that soluble protein content showed an increasing trend under stress. Under non-freeze-thaw conditions, the soluble protein content in stress group was significantly increased, and NOO, OAO and NAO groups were increased respectively by 22.54%, 39.73% and 57.12%, compared with control group (OOO) ($P < 0.05$). During freeze-thaw period (T1-T3), the soluble protein content in freeze-thaw groups (OOF, NOF, OAF and NAF) was significantly increased by 71.51%, 68.41%, 82.42% and 107.30% respectively compared with control group (OOO) ($P < 0.05$). During unfreeze period (T4-T5), the soluble protein content gradually decreased with the increasing temperature. Compared with the single stress groups (NOO, OAO and OOF), the two kinds of stress compound groups (NAO, NOF and OAF) had more significant differences, and the three kinds of stress compound groups (NAF) had more significant differences compared with the two kinds of stress compound groups ($P < 0.05$). During the unfreeze period, the soluble protein content in the freeze-thaw group decreased, but there were significant differences in NaCl, ambrosia and freeze-thaw compound stress group compared with other groups ($P < 0.05$).

2.2 Changes in the malondialdehyde (MDA) content of seedlings

As shown in Fig. 2, the content of MDA in the stress group (NOO, OAO, NAO, OOF, NOF, OAF and NAF) was significantly different from that in the control group (OOO), increasing by 19.48%, 58.46%, 65.15%, 22.42%, 59.09%, 84.32% and 88.96%. Under the condition of non-freeze-thaw stress, the content of MDA in the stress group (NOO, OAO and NAO) was much higher than that in the control group (OOO), and the content of MDA in the compound stress group (NAO) and single ambrosia group (OAO) was much higher than that in the single NaCl stress group (NOO) ($P<0.05$). Under freeze-thaw stress condition, the content of MDA in the freeze-thaw group increased firstly and then decreased, and the MDA content under combined stress was significantly higher than that under single stress. It can also be seen that NaCl, ambrosia and freeze-thaw combined stress had significant difference with NaCl and freeze-thaw combined stress ($P<0.05$), but had no significant difference with ambrosia and freeze-thaw combined

stress (P>0.05). The results showed that the effects of compound stress on MDA content were greater than those of single stress, and the effects of ambrosia stress on MDA content were more significant than those of NaCl stress and freeze-thaw stress.

2.3 Changes in the superoxide dismutase (SOD) activity of seedlings

As shown in Fig. 3, under non-freeze-thaw conditions, the SOD activity in plant leaves increased significantly ($P < 0.05$), and NaCl stress group (NOO) was much higher than that of ambrosia stress group (OAO) and combined NaCl and ambrosia stress group (NAO) ($P < 0.05$). The SOD activity in freeze-thaw groups (OOF, NOF, OAF and NAF) was significantly increased ($P < 0.05$) compared with that in control group (OOO), by 13.65%, 26.60%, 25.85% and 24.06%, respectively. The SOD activity of NaCl, ambrosia and freeze-thaw combined stress group (NAF) was lower than that of NaCl and freeze-thaw combined stress group (NOF) and ambrosia and freeze-thaw combined stress group (OAF). The single freeze-thaw stress group (OOF) decreased significantly with the increase of temperature, while the combined stress group (NOF, OAF and NAF) did not change significantly with the increase of temperature ($P > 0.05$).

2.4 Changes in the catalase (CAT) activity of seedlings

As shown in Fig. 4, the CAT activity in wheat leaves of stress group was significantly increased compared with that of control group. In non-freeze-thaw stress group, NaCl stress group (NOO) and NaCl and ambrosia combined stress group (NAO) were significantly increased by 23.81% and 31.87%, compared with control group (OOO) ($P < 0.05$), but there was no significant difference in ambrosia single stress group ($P > 0.05$). The CAT activity in freeze-thaw stress groups (OOF, NOF, OAF and NAF) increased first and then decreased, and at the lowest temperature T3 increased by 37.32%, 56.26%, 46.59% and 64.68%. There were significant differences between the combined stress group and the single freeze-thaw stress group, and there were significant differences between NaCl, ambrosia and freeze-thaw combined stress group (NAF) and the other groups at the lowest temperature T3 ($P < 0.05$). In general, the effect of NaCl stress on CAT was significantly higher than that of ambrosia stress, and the effect of combined stress on CAT was significantly higher than that of single stress ($P < 0.05$). During thawing, CAT activity in leaves decreased, but the difference still existed.

2.5 Changes in the net photosynthetic rate (P_n) and transpiration rate (T_r) of seedlings

As shown in the Figs. 5 and 6, the net photosynthetic rate (P_n) and transpiration rate (T_r) of wheat decreased significantly under stress. Photosynthetic indexes in non-freeze-thaw group decreased significantly with the increase of stress ($P < 0.05$). The net photosynthetic rate of wheat was not significantly decreased under the single stress of NaCl, ambrosia and freeze-thaw ($P > 0.05$). In the case of two kinds of stress compound (NAO, NOF and NAF), compared with the control group (OOO), significantly decreased 40%, 71.67% and 70% ($P < 0.05$). At the lowest temperature T3, the P_n of wheat under NaCl, ambrosia and freeze-thaw combined stress was significantly different ($P < 0.05$), and even

decreased to negative value. With the increase of temperature, the freeze-thaw group recovered obviously, but it was still significantly different from the single stress group and the control group ($P < 0.05$). As for the transpiration rate, in the non-freeze-thaw stress group (NOO, OAO and NAO) the transpiration rate was significantly lower than that of control group (OOO) ($P < 0.05$). At the lowest temperature T3, the transpiration rates of freeze-thaw groups(OOF, NOF, OAF and NAF)were significantly different from those of the control group (OOO), which decreased by 20%, 70%, 70% and 80% ($P < 0.05$). There was no significant difference in transpiration rate between freeze-thaw groups with temperature increasing ($P > 0.05$).

2.6 Indicator correlation analysis

Correlation analysis has been done for reflecting the correlation between various physiological indicators (Table 1). Pearson correlation analysis showed that SP was positively correlated with CAT ($P < 0.01$), and positively correlated with SP ($P < 0.05$), and SOD was positively correlated with CAT ($P < 0.05$) under the freeze-thaw stress. There was a significant negative correlation between P_n and CAT in NaCl, ambrosia and freeze-thaw group ($P < 0.01$), and a positive correlation between MDA and SOD ($P < 0.05$).

Table 1
Pearson correlation analysis table between the physiological indexes of rye seedlings SP,MDA, SOD, CAT, P_n and T_r under freeze-thaw and NaCl + ambrosia + freeze-thaw environment.

		T_r	P_n	SP	MDA	CAT	SOD
Freeze-Thaw Group	T_r	1					
	P_n	0.919	1				
	SP	-0.889	-0.636	1			
	MDA	-0.695	-0.355	0.947	1		
	CAT	-0.895	-0.647	1**	0.943	1	
	SOD	-0.922	-0.694	0.997*	0.919	0.998*	1
		MDA	SP	T_r	P_n	CAT	SOD
NaCl + Ambrosia + Freeze-Thaw Group	MDA	1					
	SP	0.951	1				
	T_r	931	-0.962	1			
	P_n	0.979	-0.994	0.927	1		
	CAT	0.981	0.993	-0.924	-1**	1	
	SOD	0.998*	0.97	-0.867	-0.99	0.992	1
** indicates a significant correlation at the 0.01 level							
* indicates a significant correlation at the 0.05 level							

3 Discussion

3.1 Effect on the soluble protein (SP) content of seedlings

Under stress conditions, oxidative stress occurs due to the accumulation of reactive oxygen species in plants. Protein, as the executor of gene function, is directly involved in the response of plants to stress, and its content will change. Therefore, the soluble protein content in plant cells is one of the important indexes to measure the metabolism and physiological status of plants [9]. The soluble protein content of plants under NaCl stress and freeze-thaw stress significantly increased, which might be because the stress induced the increase of soluble protein to maintain low osmotic potential, increase the ability to absorb water and retain water, and enhance the ability to resist stress to reduce damage [10–11]. Although the mechanism of allelopathy effect of ambrosia on plant was not clear, the results showed that the

effect of ambrosia stress on soluble protein content was basically the same as NaCl stress and freeze-thaw stress. The soluble protein content of each freeze-thaw group reached the maximum value at the lowest temperature. As the temperature rose, the pressure was relieved and the soluble protein content decreased. It showed that the plant was triggered by stress to regulate itself, but returned to its normal state of activity when the stress was relieved [12]. The effects of several stresses on soluble proteins in plants were significantly increased under combined stress compared with single stress, indicating that the combined stress of NaCl, ambrosia and freeze-thaw further deepened the effects on plants.

3.2 Effect on the malondialdehyde (MDA) content of seedlings

Stress can induce intracellular reactive oxygen species (ROS), which can lead to membrane peroxidation and damage membrane structure [13]. Malondialdehyde (MDA) is produced when cell membrane is damaged by ROS, and its accumulation is an important indicator of the degree of lipid peroxidation in plant tissues [14]. According to the results, MDA content in barley increased under stress, which was a normal phenomenon [15]. Both NaCl and freeze-thaw stress increased MDA content in leaves [16–17]. This might be due to the balance of intracellular activated oxygen metabolism was destroyed under NaCl and freeze-thaw stress, resulting in the peroxidation of membrane lipids and the degradation of unsaturated fatty acids and producing MDA, which is the final product of lipid peroxidation [18]. Ambrosia stress had the same effect on MDA content as NaCl and freeze-thaw stress, and its effect was significantly higher than that of NaCl stress and freeze-thaw stress. However, the MDA content in leaves did not increase all the time, and increased to the highest at the lowest temperature (T3) and then decreased, which was also a process of self-recovery of plants. In addition, the MDA content in leaves under compound stress was significantly higher than that under single stress and increased with the increase of compound stress. The results showed that there was a synergistic relationship between the three kinds of stress. The higher the stress intensity was, the more MDA was accumulated and the more serious the cell membrane was damaged.

3.3 Effect on the superoxide dismutase (SOD) activity of seedlings

Superoxide dismutase (SOD) is an antioxidant metal enzyme that can catalyze the superoxide anion radical disproportionation into oxygen (O_2) and hydrogen peroxide (H_2O_2), which plays a crucial role in the balance between oxidation and anti-oxidation [19]. Superoxide dismutase and free radicals are usually in a state of dynamic equilibrium. The higher the SOD content increased, the stronger the antioxidant capacity increased. Conversely, the more antioxidant capacity decreased. However, when plants suffer from stress or pathological changes, a large number of ROS are produced, triggering a series of biochemical reactions, resulting in body damage. Superoxide dismutase (SOD), as one of the protective enzyme defense systems in plant cells, plays a role in preventing oxygen poisoning in plants, effectively protecting cells and the body itself, and enhancing the tolerance of plants under stress [20]. From the results, the increase of SOD content in plant leaves under NaCl stress and freeze-thaw stress promoted

plants to adapt to the stress environment. On the one hand, NaCl stress can change osmotic pressure of plant cells and damage biofilm to some extent. On the other hand, it can cause ion toxicity and produce a large number of ROS. The plant protective enzyme defense system reacts quickly to improve SOD activity, thereby clarifying ROS and alleviating membrane peroxidation, enhancing plant tolerance under stress [21]. With the decrease of temperature, the SOD content in leaves gradually increased, and at the lowest temperature (T3), it increased to the highest. The SOD content gradually decreased during thawing, indicating that SOD is very sensitive to environmental temperature changes and can quickly adapt to environmental temperature changes and reduce the damage to plants themselves. It also indicates that the changes brought by stress to plants are recoverable. The effect of ambrosia on SOD was similar to that of NaCl, and the effect was more significant. Superoxide dismutase content in NaCl and ambrosia combined stress groups (NAO) was significantly lower than that in single stress groups (NOO and OAO), and that in NaCl, ambrosia and freeze-thaw combined stress groups was lower than that in other complex groups (NOF and OAF). The results showed that the increase of SOD activity had a limited effect on the improvement of plant tolerance under stress. With the increasing stress level, ROS content in plants exceeded the threshold, and the antioxidant system of plants was destroyed, causing certain damage to plants, and SOD content in plants decreased. There was a significant difference between the effects of combined stress and single stress on SOD content in plants, and there was a synergistic complex relationship between NaCl, ambrosia and freeze-thaw stress.

3.4 Effect on the catalase (CAT) activity of seedlings

Catalase (CAT), superoxide dismutase (SOD) and peroxidase (POD) together form the antioxidant enzyme system, which is one of the key enzymes in biological system defense. The main function is to decompose H_2O_2 into H_2O and O_2, while H_2O_2 is a harmful reactive oxygen species that can be reduced to various harmful free radicals to oxidize various components in cells, resulting in cell lesions. Therefore, CAT as a scavenger of H_2O_2 plays a very important role in biological organism [22]. When the organism is under stress, the receptor material on the cell membrane receives and transmits stress signals, and the intracellular ROS content increases rapidly. The ROS scavenging system in the organism regulates the intracellular ROS balance through antioxidant enzymes. As the main reactive oxygen species, H_2O_2 will cause oxidative damage and programmed cell death when the concentration is too high. Catalase is the main pathway of decomposition of H_2O_2 and plays an important role in biological stress resistant [23]. The results showed that CAT activity in wheat leaves under stress treatment was significantly higher than that in control group. The possible reason is that H_20_2 accumulated to a certain level as a signal molecule can stimulate the production of more antioxidant substances to enhance plant tolerance, which is also a coordinated adaptation of plants to environmental stress [24]. A large number of studies have shown that CAT can be induced to express when plants are stressed by various environmental conditions such as salt and low temperature, but CAT activity may also be inhibited when the stress is too high [25]. Under freeze-thaw conditions, CAT activity in plant leaves increased first and then decreased, which indicated that the body's antioxidant enzymes were activated to cope with environmental changes. When the body adapt to the environment, the level of antioxidant enzymes will recover. On the whole, the effects

of ambrosia stress on plant CAT enzyme activity were significantly less than NaCl stress and freeze-thaw stress. Compound stress had a more significant effect on CAT activity in plants, and CAT activity in compound stress groups (NAO, NOF, OAF and NAF) was significantly higher than that in other groups, indicating that NaCl, ambrosia and freeze-thaw stress had a synergistic effect.

3.5 Effect on the net photosynthetic rate (P_n) and transpiration rate (T_r) of seedlings

The main function of leaves is photosynthesis, and stress affects the morphological structure of leaves, and then affects their functions. The study on photosynthetic characteristics of plants has become one of the most common methods for identification and evaluation of plant stress tolerance, which is of great significance in production practice. As an important physiological process susceptible to environmental effects, photosynthesis is extremely sensitive to temperature changes [26–27]. Studies have shown that low temperature reduces the activity of plant photosynthase and photosynthetic electron transfer rate, and reduces the ability of plants to use light energy [28]. The NaCl stress inhibits the photosynthetic process of plant leaves through stomatal and non-stomatal constraints, resulting in lower photosynthetic indexes. Transpiration is a way for plants to exchange materials, removing excess heat from the body and maintaining the stability of the conditions required for plant physiological responses [29]. In terms of photosynthetic physiological characteristics, plants can adjust stomata, water use efficiency and chlorophyll fluorescence parameters of leaves by reasonably coordinating the relationship between carbon assimilation and water consumption, so as to enhance plant adaptation to stress [30]. According to the results, the transpiration rate and net photosynthetic rate of plant leaves in the stress treatment group decreased significantly, indicating that the T_r and P_n of wheat were reduced by all three kinds of stress, and did not return to the original state when the temperature rose to 10℃ again. This may be due to the fact that freeze-thaw stress reduces the activity of photosynthase in leaves and damages the chloroplast of wheat seedlings, thus reducing the photosynthetic rate of wheat [31]. Stress can significantly reduce the transpiration rate of plants, resulting in limited gas exchange required for photosynthesis, thus reducing the net photosynthetic rate of plants. The mechanism of the inhibition of artemisin on plants is not clear, but the results show that its effect on plant photosynthetic characteristics is similar to NaCl and freeze-thaw stress, limiting plant photosynthesis. From the point of view of combined stress and single stress, the P_n and T_r of leaves in the combined stress group were significantly lower than that in the single stress group. Even under the combined stress of NaCl, artemisin and freeze-thaw, P_n showed negative values, indicating that the respiration rate was lower than the photosynthetic rate and plants could not grow during this period. In this state for a long time, plants will die. Compound stress further deepened the effects on plants, and there was a synergistic stress relationship among NaCl, artemisin and freeze-thaw stress.

3.6 Principal component analysis(PCA)

As shown in Fig. 7, principal component analysis showed that stress treatment had significant ($P \leq 0.05$) effects on seedling indexes (SP, MAD, SOD, CAT, P_n and T_r) of rye. The total proportion of the two principal

components was 85.623%, the variance explanation rate of PC1 was 74.477%, and the characteristic root was 4.496. The variance explanation rate of PC2 was 11.146%, and the characteristic root was 0.669. The results showed that principal component analysis was effective. The load coefficients of the six indexes on PC1 were all very high, indicating that they can be well explained by PC1. The soluble protein content, malondialdehyde content, superoxide dismutase activity and catalase activity (SP, MDA, SOD and CAT) had significant positive effects on PC1, while the net photosynthetic rate and transpiration rate (P_n and T_r) had significant negative effects on PC1. MDA, SP and P_n had significant positive effects on PC2, while SOD had significant negative effects on PC2. The effects of CAT and T_r on PC2 were not obvious. The scores of PC1 were higher under artemisin stress (OAO), artemisin and freeze-thaw stress (OAF), NaCl, artemisin and freeze-thaw stress (NAF), and the scores of PC2 were higher under freeze-thaw stress (OOF), NaCl stress (NOO), NaCl and freeze-thaw stress (NOF). In conclusion, NaCl and freeze-thaw stress have a great relationship with MDA, P_n, SOD and SP. The relationship between artemisin stress and each index was close, and the explanation rate of PC1 was higher, so the effect of artemisin stress was significant.

4 Conclusion

In this study, under NaCl, artemisin and freeze-thaw stress, MDA and soluble protein contents of rye seedlings increased, SOD and CAT activities increased, and P_n and T_r decreased. The accumulation of MDA content indicates that cell membrane is damaged, and the increase of SP maintains osmotic potential and enhances the ability of plants to resist stress. The increase of SOD and CAT activities can enhance the antioxidant capacity, which is beneficial to the protection of the body. However, P_n and T_r, as photosynthetic indexes, are easily affected by the environment, and the photosynthetic physiological characteristics of plants will decrease significantly under external stress. The results showed that single NaCl, artemisin and freeze-thaw stress were not so serious, and the effects of combined NaCl, artemisin and freeze-thaw stress on plants were significantly superimposed. Therefore, in complex practical environment, more attention should be paid to plant growth under multiple adverse conditions rather than single stress.

5 Experimental Materials And Methods

5.1 Plant culture

About 8000 full grain rye seeds of DongMu 70 provided by Beijing Zhengdao Seed Industry Co., Ltd, China were selected. Soaked and disinfected with 0.1% $KMnO_4$ solution for 2 hours, then rinsed with distilled water. Placed the seeds evenly on 8 trays (20 rows×45 per row) of 26 × 19 cm (length×width) with a density of about 2/cm^2. The tray was placed in the incubator (MGC-450BP) for about 9 days, and the culture conditions were set as 12 hours of light (culture temperature of 25℃, light intensity of 16500 LUX) and 12 hours of darkness (culture temperature of 15℃, light intensity of 0 LUX) to simulate the

alternating environment of day and night. A daily ration of Hoagland nutrient solution was provided during incubation to ensure adequate hydration.

5.2 Stress treatment and sampling

The 8 culture plates were randomly named into 8 treatment groups (as shown in Table 2). They were blank control group (OOO), single NaCl stress group (NOO), single ambrosia stress group (OAO), NaCl and ambrosia combined stress group (NAO), single freeze-thaw stress group (OOF), NaCl and freeze-thaw combined stress group (NOF), ambrosia and freeze-thaw combined stress group (OAF), and NaCl ambrosia and freeze-thaw combined stress group (NAF). The ambrosia powder was dissolved in distilled water to obtain 0.1g/mL ambrosia mother liquor, and then diluted into 0.02g/mL ambrosia solution with Hoagland nutrient solution and added into the tray. The NaCl powder was added into Hoagland nutrient solution to prepare NaCl stress solution with concentration of 50mmol/L and was added into the ambrosia stress solution to a concentration of 50mmol/L to obtain the compound stress solution of NaCl and ambrosia.

When the seedlings were mature, the freeze-thaw group was treated with freeze-thaw stress, and the non-freeze-thaw group continued to be cultured in the incubator. The wheat in freeze-thaw treatment group (**F) was placed in an alternating incubator (BPHJ-120A) for freeze-thaw stress treatment with a cycle of 14h. The freeze-thaw stress decreased uniformly from room temperature of 15℃ to -5℃ and then rose to 10℃ (temperature change rate was 0.5℃/12min). At 2h, 5h, 8h, 11h and 14h after freeze-thaw, samples were taken from 8 treatment groups, with 5 samples per time. They were denoted as T1, T2, T3, T4 and T5, corresponding to temperatures of 10℃, 2.5℃, -5℃, 2.5℃ and 10℃ respectively. Fresh plant leaves were selected for the determination of net photosynthetic rate and transpiration rate. And for the determination of MDA, soluble protein, CAT and SOD, fresh leaves were wrapped in tin foil and fixed in liquid nitrogen for 45-50s before being stored in ultra-low temperature refrigerator, which was convenient to be taken at any time.

Table 2
Experimental design of groups under NaCl(N),ambrosia(A) and freeze-thaw(F) stress

	OOO	NOO	OAO	NAO	OOF	NOF	OAF	NAF
NaCl	-	□	-	□	-	□	-	□
Ambrosia	-	-	□	□	-	-	□	□
Freeze-thaw	-	-	-	-	□	□	□	□
□ add stress, - no stress								

5.3 Measurement indicators and measurement methods

5.3.1 Determination of soluble protein (SP) content

The soluble protein content in plant leaves was determined by the Coomassie brilliant blue method [32]. Weighed the shredded leaves 0.1g and placed them in a mortar, added 5ml distilled water to grind to the homogeneous slurry, then centrifuged them at a rate of 3000r/min for 10min in a centrifuge (TDL-40B, Anting Scientific Instrument Factory, Shanghai). Took 1mL supernatant, added 4ml distilled water to dilute to 5 times, mixed well and took 1ml into test tube. Each sample was taken twice. 5mL Coomassie brilliant blue solution was added, shaken well, after 2min, zeroing with distilled water, and the absorbance of the solution was determined by a UV-6100 UV-VIS spectrophotometer (Metash Co., Ltd) at 595nm wavelength. Leaf soluble protein concentration was calculated through the standard curve of protein solution, and then the soluble protein content was calculated according to formula (1).

Soluble protein content (mg/g) = C×VT / (Vs×Fw×1000) (1)

Where:

C: the standard curve value;

VT: total volume of extract (mL);

WF: fresh weight of sample (g);

VS: sample amount for determination (mL).

5.3.2 Determination of malondialdehyde (MDA) content

The content of malondialdehyde in plant leaves was determined by thiobarbituric acid colorimetry [33]. Weighed 0.5g seedling leaves, added 5ml of 10% trichloroacetic acid solution (TCA) and ground until homogenized. Centrifuged (TDL-40B, Anting Scientific Instrument Factory, Shanghai) at a speed of 4000r/min for 10min, then absorbed 2mL of supernatant and mixed it with 2ml 0.6% TBA solution in 15mL test tube. The mixture was then reacted in boiling water bath for 15min, cooled rapidly and centrifuged again. Distilled water was used as blank zeroing, and the absorbance of supernatant was measured at 532nm, 600nm and 450nm. The MDA content in leaves was calculated according to formulas (2) and (3).

MDA concentration (μmol/L) = 6.45 (D532-D600) -0.56×D450 (2)

MDA content (μmol/g) = cMDA×VT/FW (3)

Where:

D450, D532 and D600 are the absorbance of supernatant at 450nm, 532nm and 600nm respectively;

cMDA is MDA concentration (μmol/L);

FW is fresh weight of plant leaf (g);

VT is the volume of extracted liquid (mL).

5.3.3 Determination of superoxide dismutase (SOD) activity

SOD activity measurement: Weighed 0.2g sample and added 1.8 mL phosphate buffer solution (0.1mol/L, PH = 7.0 ~ 7.4) as homogenizing medium. Ground in ice water bath until homogenized, centrifuged at 4000r/min for 10min. The SOD activity was determined by referring to the kit of Nanjing Jiancheng Institute of Biological Engineering.

5.3.4 Determination of catalase (CAT) activity

CAT activity measurement: Accurately weighed tissue weight and added 9 times the volume of normal saline according to the weight (g): volume (mL) = 1:9 radio (0.2g of rye leaves, corresponding to 1.8mL of normal saline). 10% tissue homogenate was prepared under the ice bath condition, and centrifuged at 2500r/min for 10min. The CAT activity was determined according to the kit of Nanjing Jiancheng Institute of Biological Engineering.

5.3.5 Determination of net photosynthetic rate (P_n) and transpiration rate (T_r)

CIRAS-3 portable photosynthetic system was used to measure the net photosynthetic rate (P_n) and transpiration rate of rye leaves. When the net photosynthetic rate (P_n) was stable at 1 decimal place, the count was started. During the measurement, each group randomly selected 10 rye seedlings with healthy growth, consistent growth and uniform light. The temperature of the samples was fixed at 17.5 ± 0.7°C, and the vapor pressure deficit (VPD) was fixed at 1.9 ± 0.2 mB, and photosynthetically active radiation (PAR) was about 1200 μmol/ (m2s).

5.4 Statistical Analysis

Data were analyzed using R 3.3.1 statistical software (R Core Team, 2019) for one-way analysis of variance (ANOVA). All results were displayed in bars, drawn using Origin 8.0 software. Different letters in the figure indicated that different treatment groups have significant differences at the same time. The experiment was repeated 5 times, and the data were expressed as mean ± standard error (SE) (n = 5).

Abbreviations

MDA: Malondialdehyde

SP: soluble protein

SOD: superoxide dismutase

CAT: catalase

P_n: net photosynthetic rate

T_r: transpiration rate

PCA: Principal component analysis

Declarations

Ethics approval and consent to participate

The research did not involve human or animal participants. All methods in the study were performed in accordance with relevant institutional /national/international guidelines.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

When writing statement, WZ designed research. WZ, JX, GD, JL and ZW conducted experiments. WZ and GD wrote the main manuscript and JX, JL and ZW prepared figures 1-7.All authors reviewed the manuscript.

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Figures

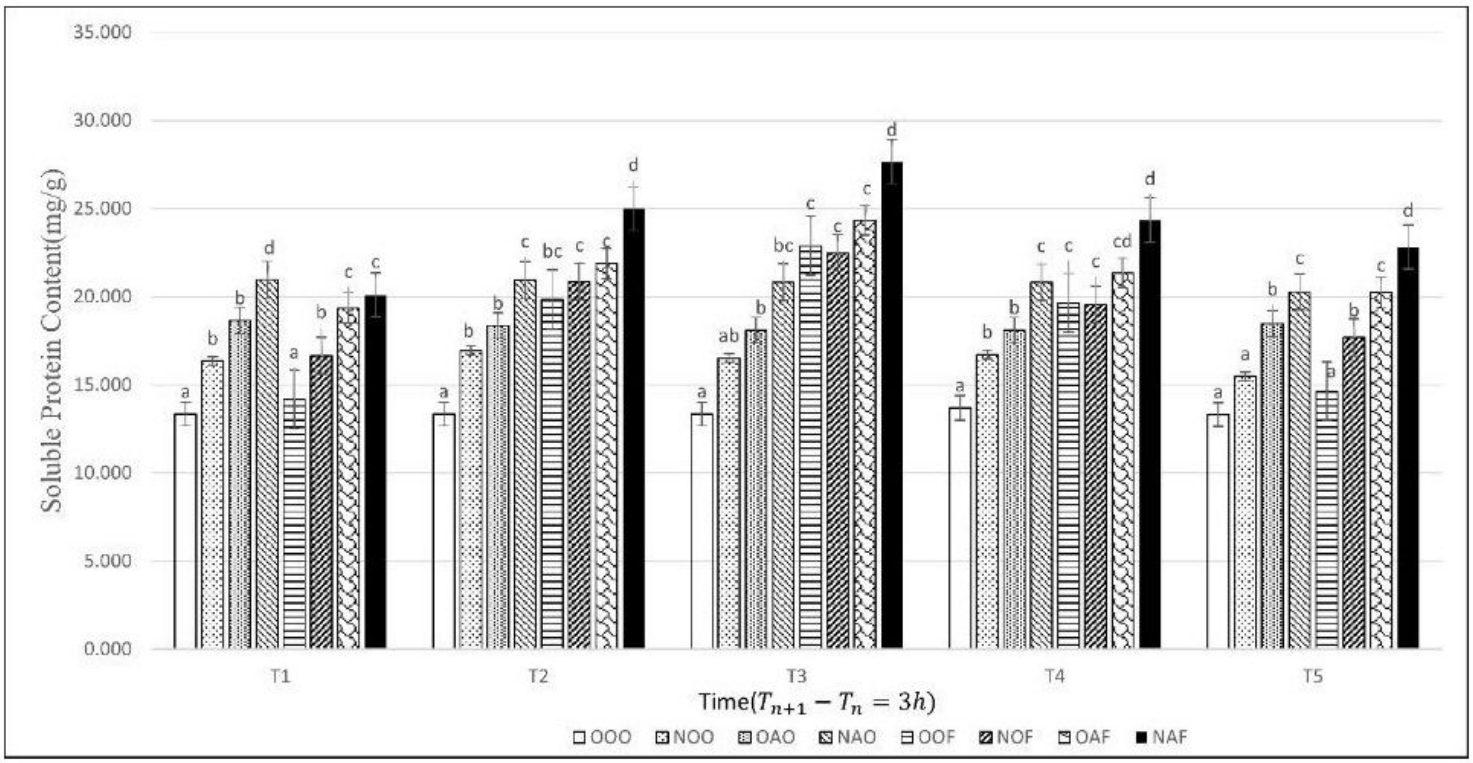

Figure 1

Changes of soluble protein (SP) content of rye seedling leaves under different treatment conditions. (T1-T5 refers to the 2nd, 5th, 8th, 11th and 14th sampling points in freezing-thawing cycle, corresponding temperatures are 10, 2.5, -5, 2.5 and 10℃ respectively. N: NaCl stress, A: artemisin stress, F: freeze-thaw stress, O: no stress. Different letters indicate the difference between different treatment groups at the same time (P<0.05).)

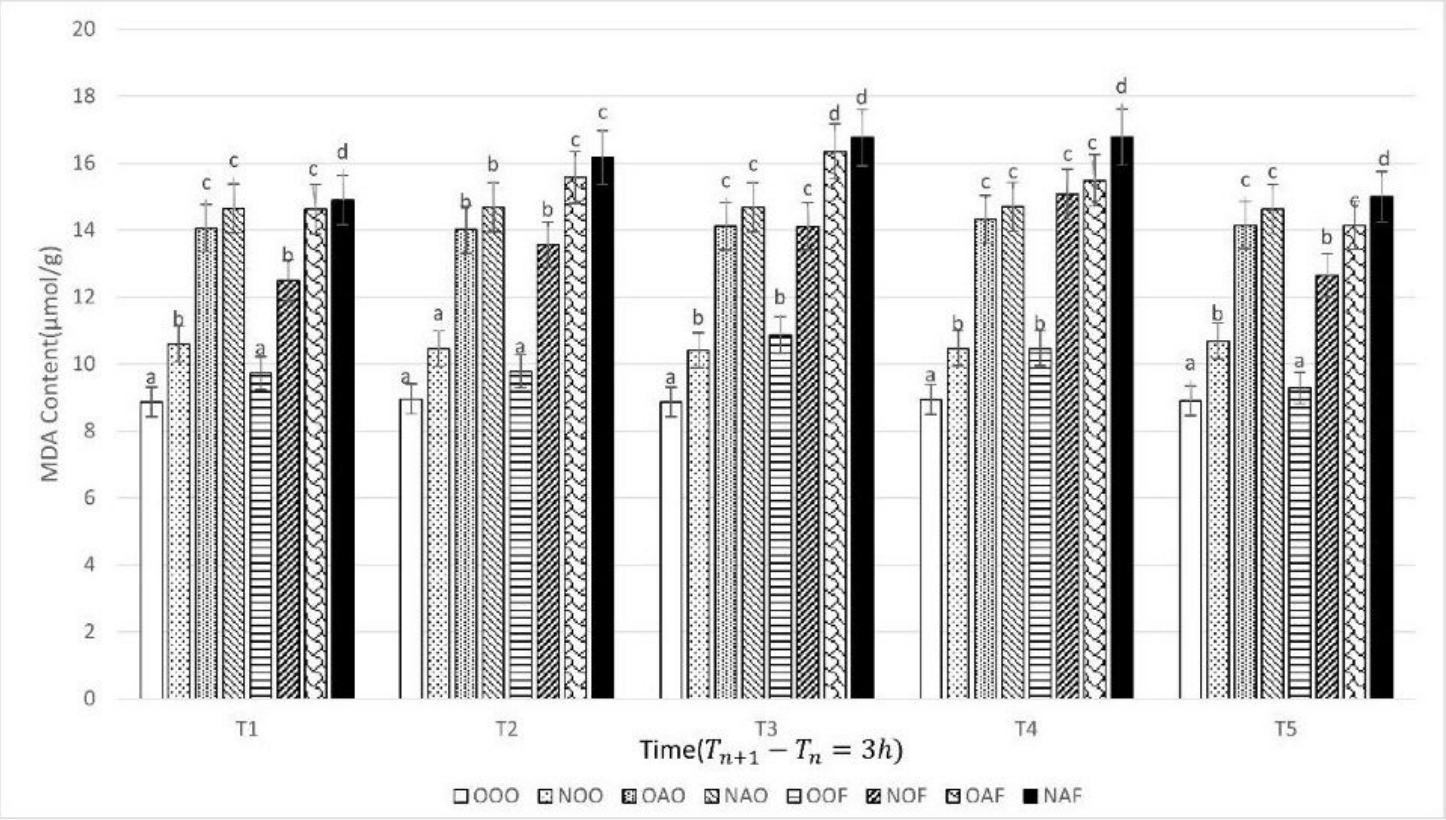

Figure 2

Changes of malondialdehyde (MDA) content of rye seedling leaves under different treatment conditions. (T1-T5 refers to the 2nd, 5th, 8th, 11th and 14th sampling points in freezing-thawing cycle, corresponding temperatures are 10, 2.5, -5, 2.5 and 10℃ respectively. N: NaCl stress, A: artemisin stress, F: freeze-thaw stress, O: no stress. Different letters indicate the difference between different treatment groups at the same time ($P<0.05$).)

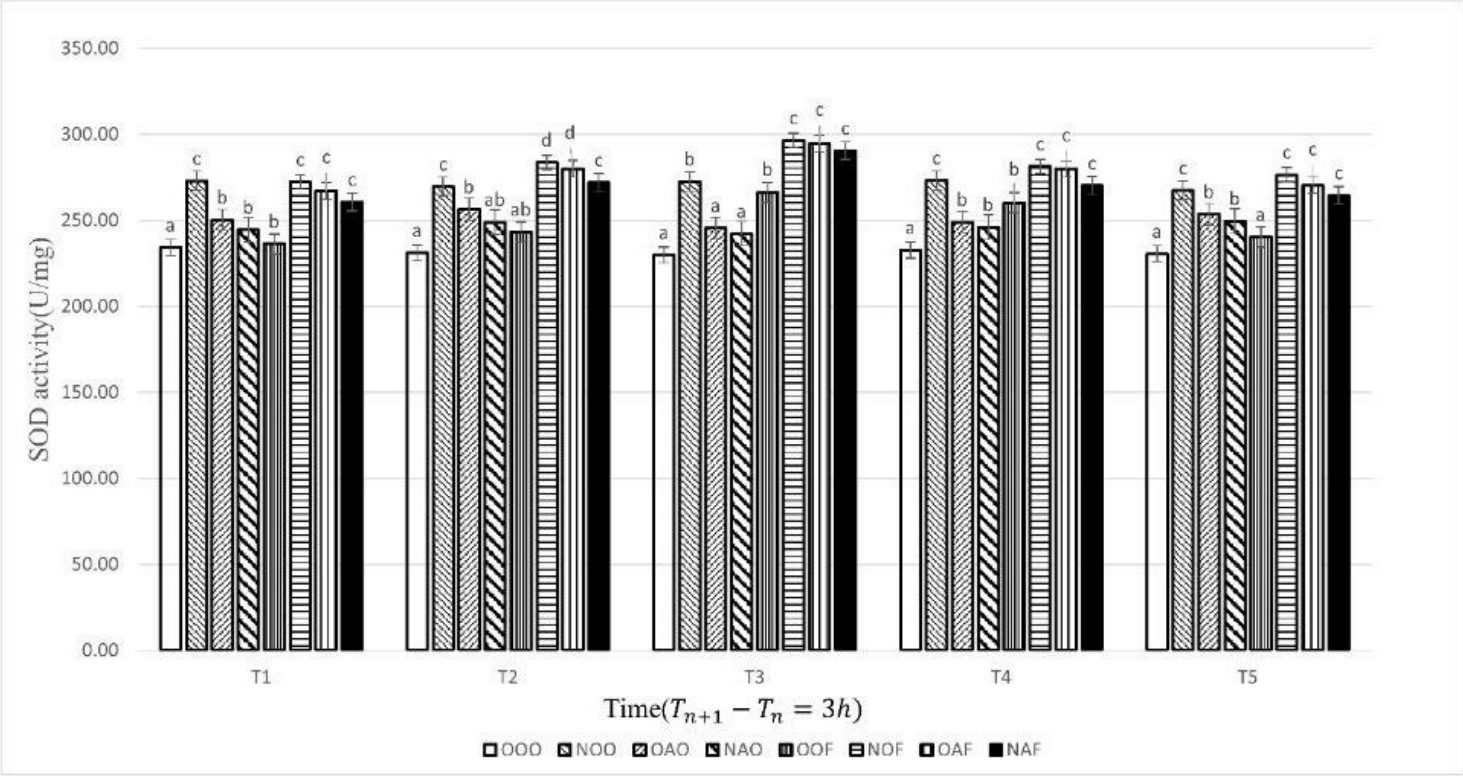

Figure 3

Changes of superoxide dismutase (SOD) activity of rye seedling leaves under different treatment conditions. (T1-T5 refers to the 2nd, 5th, 8th, 11th and 14th sampling points in freezing-thawing cycle, corresponding temperatures are 10, 2.5, -5, 2.5 and 10°C respectively. N: NaCl stress, A: artemisin stress, F: freeze-thaw stress, O: no stress. Different letters indicate the difference between different treatment groups at the same time (P<0.05).)

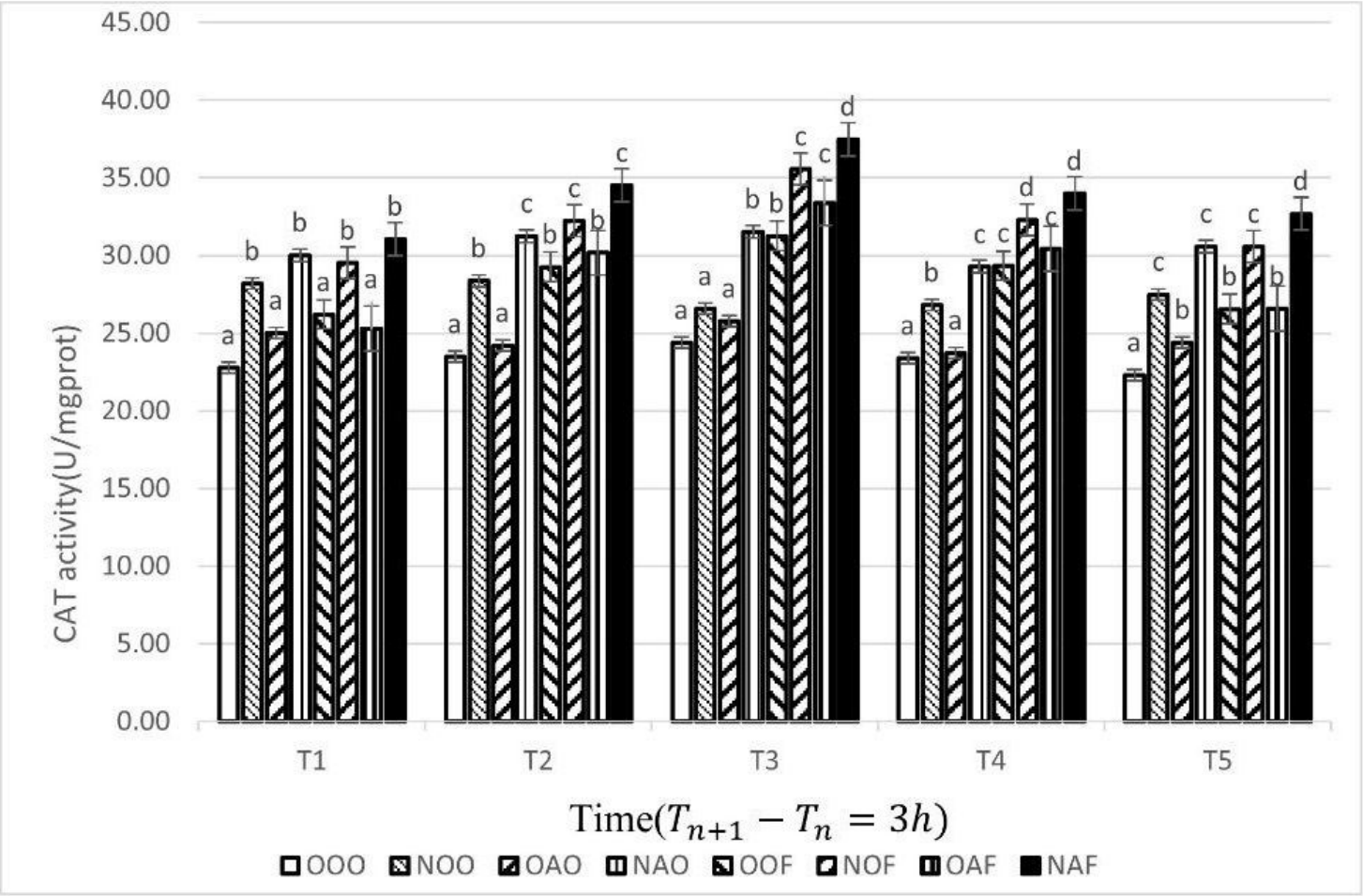

Figure 4

Changes of catalase (CAT) activity of rye seedling leaves under different treatment conditions. (T1-T5 refers to the 2nd, 5th, 8th, 11th and 14th sampling points in freezing-thawing cycle, corresponding temperatures are 10, 2.5, -5, 2.5 and 10°C respectively. N: NaCl stress, A: artemisin stress, F: freeze-thaw stress, O: no stress. Different letters indicate the difference between different treatment groups at the same time ($P<0.05$).)

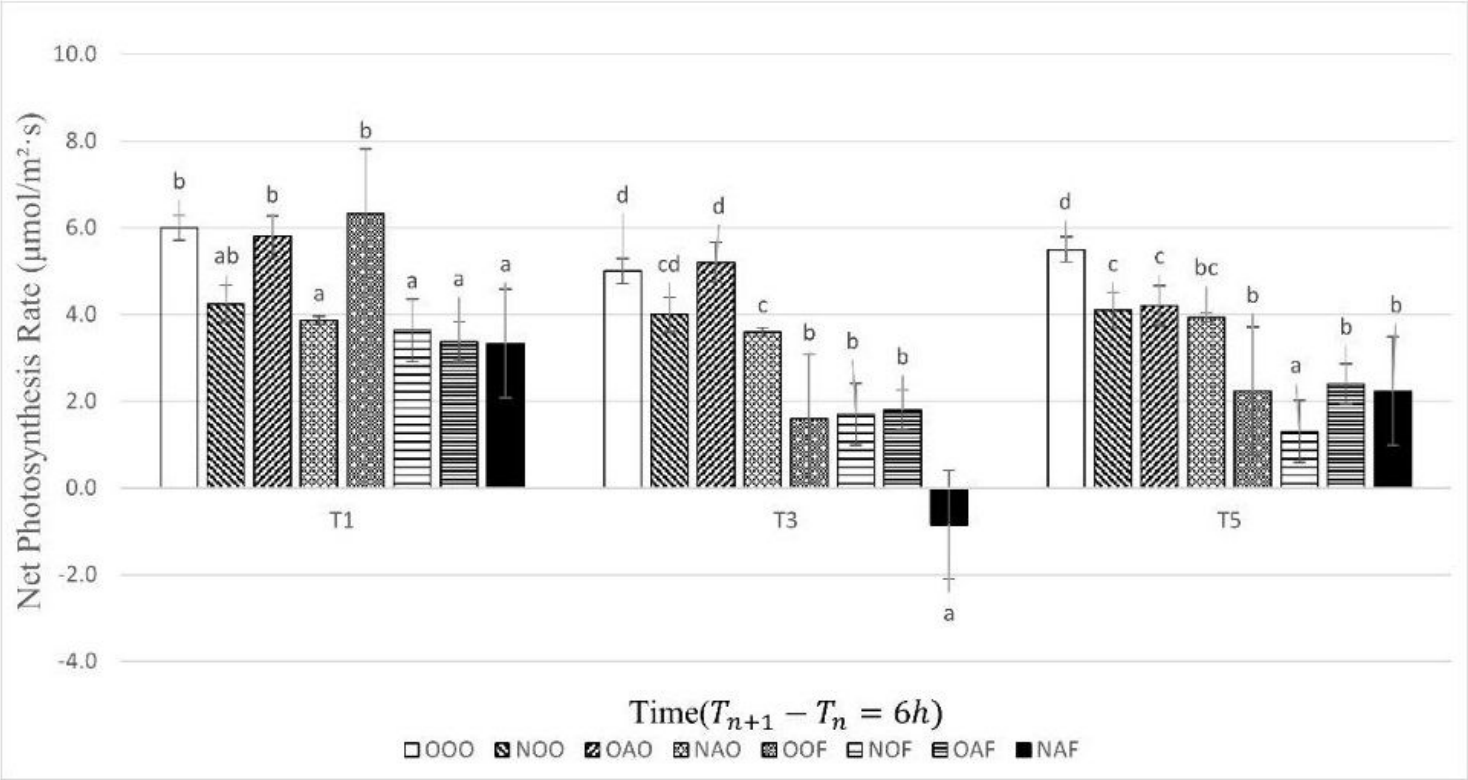

Figure 5

Changes of net photosynthetic rate (P_n) of rye seedling leaves under different treatment conditions. (T1-T5 refers to the 2nd, 5th, 8th, 11th and 14th sampling points in freezing-thawing cycle, corresponding temperatures are 10, 2.5, -5, 2.5 and 10°C respectively. N: NaCl stress, A: artemisin stress, F: freeze-thaw stress, O: no stress. Different letters indicate the difference between different treatment groups at the same time ($P<0.05$).)

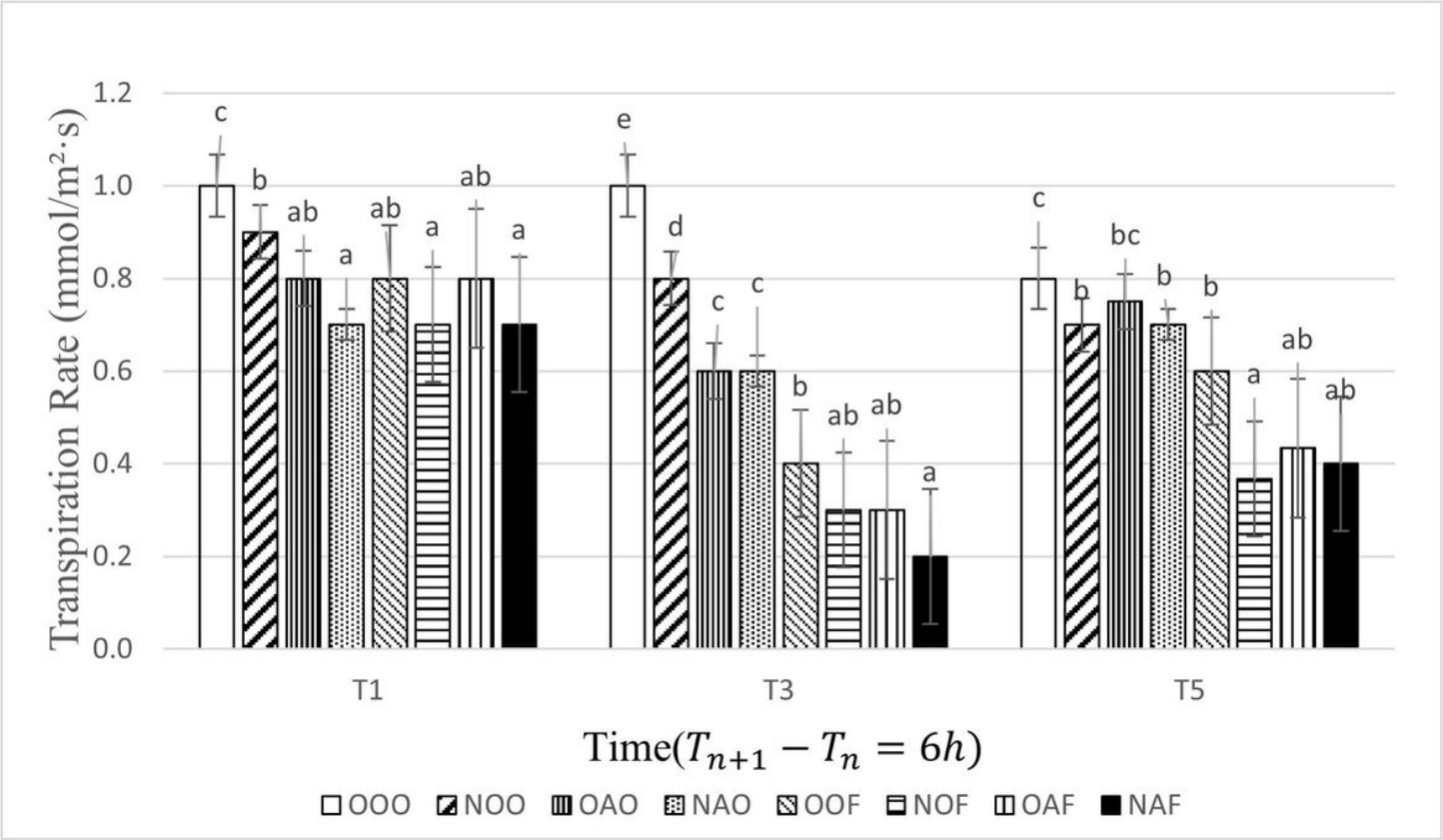

Figure 6

Changes of transpiration rate (T_r) of rye seedling leaves under different treatment conditions. (T1-T5 refers to the 2nd, 5th, 8th, 11th and 14th sampling points in freezing-thawing cycle, corresponding temperatures are 10, 2.5, -5, 2.5 and 10°C respectively. N: NaCl stress, A: artemisin stress, F: freeze-thaw stress, O: no stress. Different letters indicate the difference between different treatment groups at the same time ($P<0.05$).)

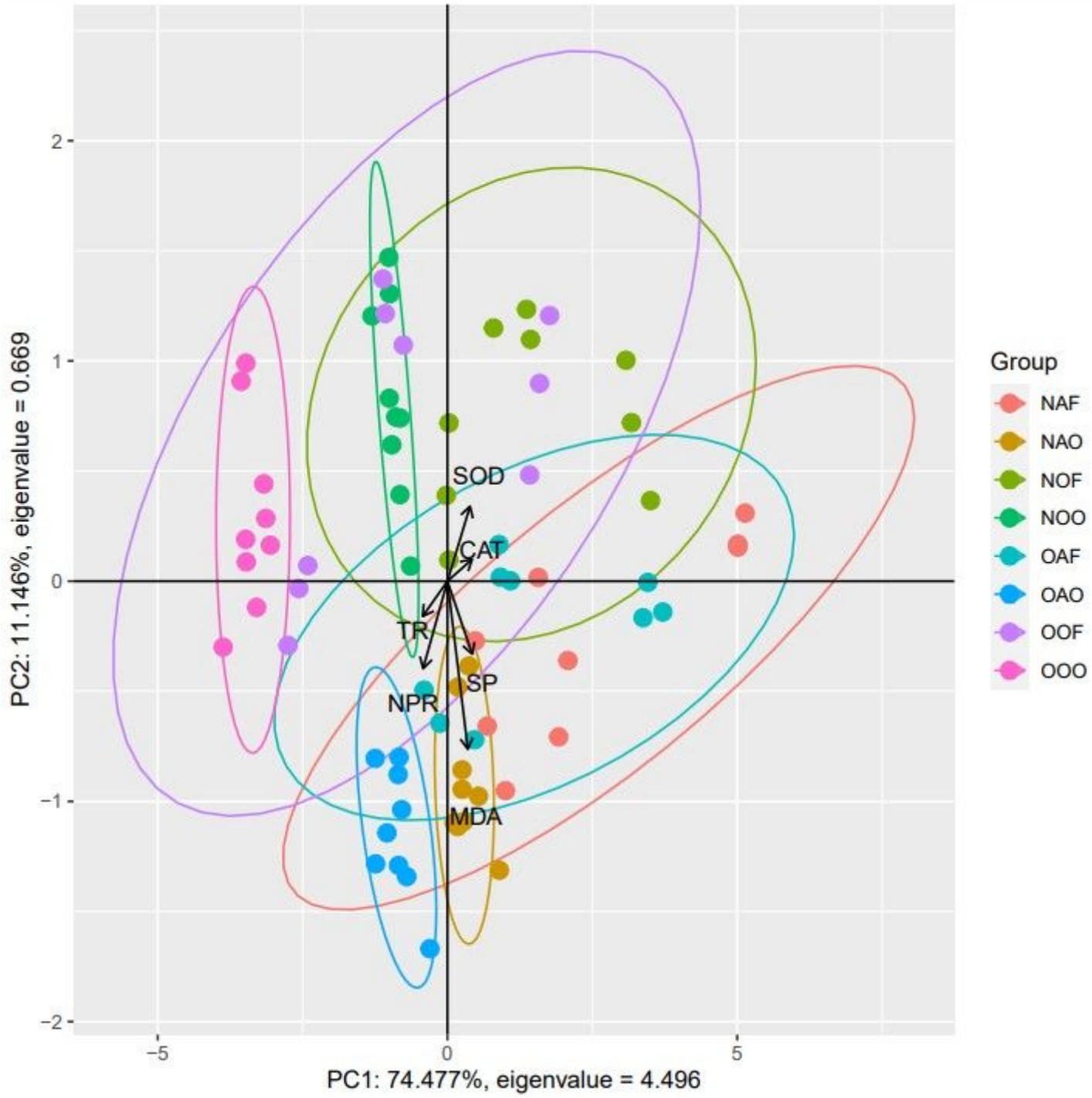

Figure 7

Principal component analysis (PCA) of six indexes of rye seedling leaves under different treatment conditions. It was used to analyze the malondialdehyde content (MDA), soluble protein content (SP), superoxide dismutase activity（SOD）, catalase activity (CAT), net photosynthetic rate (P_n) and transpiration rate (T_r) of rye seedlings under different treatment conditions. (N: NaCl stress, A: artemisin stress, F: freeze-thaw stress, O: no stress,)