APPLICATION OF POTASSIUM FULVIC ACID IMPROVING TOLERANCE OF *SECALE CEREALE* L. SEEDLINGS TO CADMIUM STRESS AND FREEZE-THAW ENVIRONMENT

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Abstract. Freeze-thaw (FT) and cadmium (Cd) are the main ecological stress factors for plant growth in high-latitude regions. Potassium fulvic acid (FAK), a humic acid fertilizer, is widely used and can help plant resist abiotic stresses in farmland ecosystems. Through hydroponic experiments, this study investigated the physiological response characteristics of rye seedlings under FT and Cd compound stress and the potential protective effect of FAK on the rye seedlings. The results demonstrated that FT and Cd compound stress decreased the net photosynthetic rate (Pn) and transpiration rate (Tr) of rye seedlings, and FAK cannot relieve this decrease effectively. However, FAK enhanced the tolerance of rye seedlings to adverse stress, leading to significant increases in peroxidase (POD), superoxide dismutase (SOD), soluble protein (SP), and soluble sugar (SS), and a reduction in malondialdehyde (MDA) content. Therefore, it demonstrated that FAK has a protective effect on rye seedlings in FT and Cd compound stress, reducing the physiological damage to the seedlings. This study provides a theoretical basis for plants in northern high-latitude regions to avoid ecological disasters.

Keywords: *abiotic stresses, antioxidant enzyme, heavy metal, rye, physiological response*

Introduction

Most of the land in the northern hemisphere experiences seasonal changes in freeze-thaw (FT) environments annually (Rowlandson et al., 2018). FT refers to soil repeatedly freezing and thawing due to seasonal or diurnal temperature changes. This FT cycle frequently increases as global temperatures rise (Zhao et al., 2021). The northeast region of China is a typical FT ecological stress region. Frequent FT can damage vegetation root systems and directly impact local crop growth. However, most research currently focuses on the effects of the whole FT cycle, and there is a relative lack of research on the physiological and ecological effects of the partial FT process on plants.

Another important source of stress in China's northern region is cadmium (Cd). Cd is an essential trace element everywhere in the environment (Haider et al., 2021). Furthermore, Cd is a pollutant and can accumulate in the ecosystem. Cd affects the function of mitochondria by damaging the plant's oxidative-reductive regulation and

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stimulating the production of reactive oxygen species (ROS), which leads to the destruction of membrane lipids and disturbs the entire metabolism of the plant, resulting in slower plant growth and reduced yield (Haider et al., 2021; Hussain et al., 2021). Currently, most research on mitigating Cd toxicity in plants focuses on using growth-regulating substances, but the economic cost of these methods is high, and their widespread application is difficult.

High latitude regions are often confronted with ecological stresses, such as freeze-thaw and heavy metals, frequently occurring together and limiting crop growth. While many studies have examined the effects of single stress factors on plants, there is still a lack of understanding regarding the mechanisms underlying compound stress (Zandalinas and Mittler, 2022). To explore the mechanism behind this compound stress, we selected rye with good cold stress resistance as our research crop and evaluated the response mechanism of rye seedlings to adverse ecological stresses (Guo et al., 2022). Mitigating the damage caused by these stresses is crucial, especially as heavy metal toxicity can pose risks to human health through the food chain (Liao et al., 2023). We therefore attempted to identify a cost-effective approach to alleviate the damage caused by both stressors to plants. This study revealed that potassium fulvic acid, a natural organic acid fertilizer, possesses unique plant stress resistance functions (Lang et al., 2020). To evaluate its effectiveness in enhancing plant stress tolerance and physiological functions, we conducted hydroponic experiments that isolated the effects of single and compound stress on rye seedlings. We aimed to provide data support for effectively mitigating heavy metal pollution and crop cultivation in freeze-thaw regions and provide theoretical references for scientific management and avoidance of ecological disasters caused by compound stress, including agricultural ecosystems in high latitude and high-altitude areas.

Materials and methods

Plant material and experimental conditions

Rye (*Secale cereale* L. cv. Dongmu 70) seeds were purchased from Beijing Zhengdao Seed Industry Co. Ltd. (Beijing, China). The seeds were immersed in a 0.1% $KMnO_4$ solution for 120 min, washed with deionized water, and then placed 200 seeds evenly on a cultivation dish. The cultivation dishes were divided into two groups, NFT (Non-freeze-thaw) and FT, and each group had four treatments (1 dish per treatment): CK, C, P, and C + P (*Table 1*). CK group was cultured with Hoagland nutrient solution, C group was cultured with 10 mg/L Cd^{2+} solution and Hoagland nutrient solution, P group was cultured with 0.5 g/L FAK solution and Hoagland nutrient solution, and C + P group was cultured with 10 mg/L Cd^{2+} solution, 0.5 g/L FAK solution, and Hoagland nutrient solution. Eight cultivation dishes (equal volumes of solution) were placed in MGC-450BP light incubator (Shanghai Yiheng Scientific Instruments Co., Ltd) (25 °C light for 12 h, 20 °C dark for 12 h) for germination. When the rye seedlings grew for 7 d (*Fig. 1B*), the FT group was placed in BPHJ-120A a test chamber with alternating high and low temperature (Shanghai Yiheng Scientific Instruments Co., Ltd) (*Fig. 1A*) for FT treatment while the NFT group was still cultured in the light incubator. Samples were then randomly collected by the five-spot-sampling. This study focused on the study of the FT process, and the FT group was sampled every 3 h with corresponding sampling temperatures of 10 °C (T1), 2.5 °C (T2), -5 °C (T3), 2.5 °C (T4), and 10 °C (T5). The NFT group was sampled at T5. Fresh samples were quickly fixed with liquid nitrogen and placed in a -80 °C ultra-low temperature refrigerator to determine physiological and biochemical characteristics. Due to the different measurement methods for each characteristics, we measured the fresh samples at the final time point (T5) for relative conductivity (RC) of NFT and FT groups. We also measured the fresh samples at T1, T2, T3, T4, and T5 for the FT and NFT groups for photosynthetic parameters.

Table 1. *Experimental design of treatments under NFT and FT environments*

	CK	**C**	**P**	**C + P**	**CK**	**C**	**P**	**C + P**
	NFT				**FT**			
FT					+	+	+	+
Cd		+		+		+		+
FAK			+	+			+	+

+ is for stress treatment; CK- control group; C- single cadmium stress; P- potassium fulvic acid; C + P - combined stress of cadmium and potassium fulvic acid

Measurement of osmolytes

Soluble protein (SP) content was determined using the Bradford method. Absorbance at 595 nm was recorded, and SP levels were calculated based on the standard curve (Bradford, 1976). Soluble sugar (SS) content was determined using the method of Wang et al. (2020). A sample (0.1 g) was homogenized in 10 mL distilled water. The enthrone reagent was added to the supernatant, and the mixture's absorbance was measured at 620 nm with a UV-6100 UV–visible spectrophotometer (Metash Co., Ltd).

Measurement of malondialdehyde (MDA) and relative conductivity (RC)

MDA is determined based on the thiobarbituric acid (TBA) method, and absorbance was detected at 450, 532, and 600 nm with a UV-6100 UV–visible spectrophotometer (Metash Co., Ltd), according to Shengxin et al. (2016). The relative conductivity was determined according to the method of Lu et al. (2022). Fresh leaf samples were washed and cut into strips about 1 cm. The leaves (0.1 g) were soaked in 20 ml of deionized water at room temperature (RT) for 12 h, and the initial conductivity was measured as R1. Then, the leaves were heated in boiling water for 30 min and cooled to room temperature. After shaking, the conductivity was measured as R2. The RC of seedlings is calculated by *Equation 1.*

$$RC = R1/R2 \times 100\% \qquad \text{(Eq.1)}$$

Measurement of antioxidant enzyme activities

The activity of superoxide dismutase (SOD) and peroxidase (POD) was determined using an enzyme kit from Nanjing Jiancheng Bioengineering Institute. For SOD, 0.1 g of fresh sample was taken and mixed with 10 ml of phosphate buffer (0.1 mol/L, pH 7–7.4) in an ice bag to make a homogenous slurry, which was then centrifuged at 3500 r/min for 10 min. 0.1 ml of the supernatant was taken. For POD, 0.1 g of sample was taken and mixed with 8 ml of phosphate buffer (0.1 mol/L, pH 7–7.3) in an ice bag to make a homogenous slurry, which was then centrifuged at 3500 r/min for 10 min. 0.1 ml of the supernatant was taken, and the activity of both enzymes was determined according to the kit's instructions.

Measurement of net photosynthetic rate (Pn) and transpiration rate (Tr)

Photosynthetic parameters were measured using a CIRAS-3 photosynthesis measurement instrument (Hansha Scientific Instruments Co., Ltd) (*Fig. 1C*). The CO_2 buffer bottle was placed outdoors in a ventilated area. The instrument's built-in LED light source was used for illumination, and the parameters were set to Accessory: PLC3 Universal Leaf Cuvette, 7 × 25 mm window, H_2O Reference: 80%, Light Source: LED 300 $umol \cdot m^{-2} s^{-1}$, RGBW Control: 4 Red, Green, Blue, and White parameters were set to 90, 0, 5, 5, respectively. The temperature was controlled at 25 ± 2 °C, and the leaf's Pn and Tr were measured.

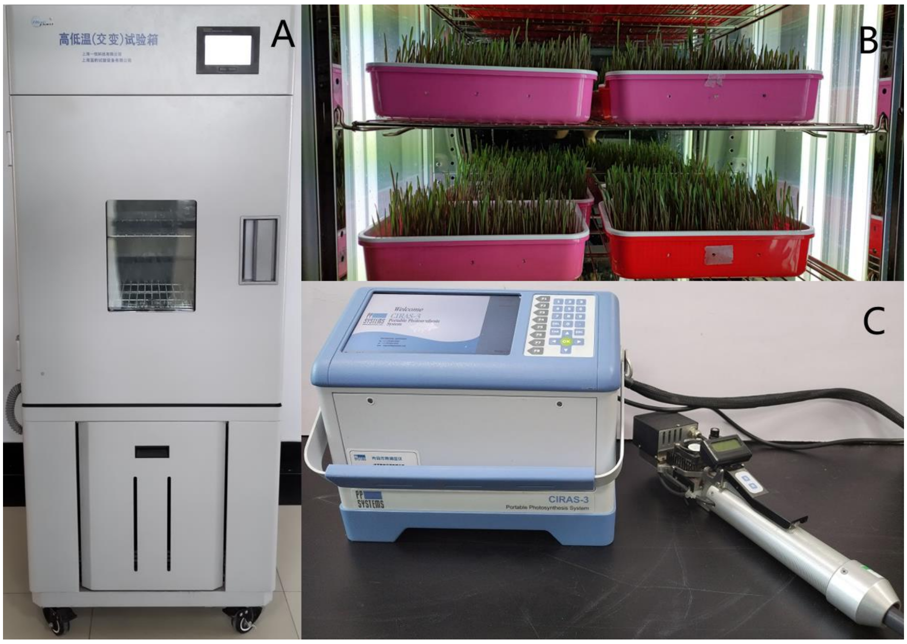

Figure 1. *Photos of experimental culture and equipment. A: BPHJ-120A a test chamber with alternating high and low temperature (Shanghai Yiheng Scientific Instruments Co., Ltd). B: 7-day rye seedlings in light incubator. C: CIRAS-3 photosynthesis measurement instrument (Hansha Scientific Instruments Co., Ltd)*

Statistical analysis

All non-photosynthetic physiological characteristics were three parallel samples, and photosynthetic physiological characteristics were six parallel samples. The data were expressed as mean ± standard error (SE) (n = 3), which was statistically performed with R 3.3.1 statistical software (R Foundation for Statistical Computing, Vienna, Austria) for one-way analysis of variance (ANOVA), and Duncan model was used to determine the significance between treatments. Pearson correlation coefficient was used to describe the correlation between variables.

Change in relative conductivity (RC)

As shown in *Figure 2*, the RC for FT was more significant than that for NFT. Among the NFT groups, the RC was in the following order: P > C > CK > C + P. Under FT, the RC for C was the highest, increasing by 25.3%, 38.0%, and 35.6% compared to CK, P, and C + P.

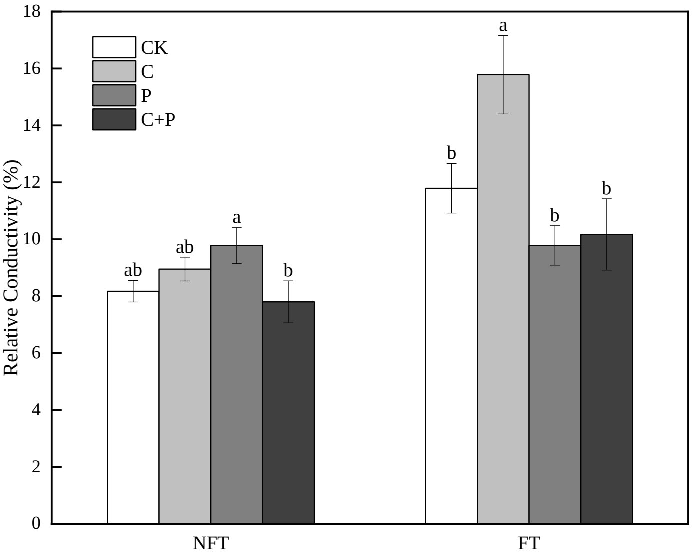

Figure 2. *The changes in RC of rye seedlings under different treatment groups. The lowercase letters indicated the significant difference between different treatments at FT or NFT. (P < 0.05). The data are expressed as mean ± standard error) (n = 3)*

Change in soluble protein (SP)

From *Figure 3*, it can be seen that SP content under FT was generally higher than NFT. In FT, the SP content of P and C + P was relatively low. During the freezing stage (T1-T3), as the temperature decreased, the FT groups showed a decreasing trend, with C and C + P reaching their minimum values of 16.8 and 14.5 mg/g, respectively, at T3. During the thawing stage (T3-T5), the FT groups generally showed an upward trend as the temperature increased.

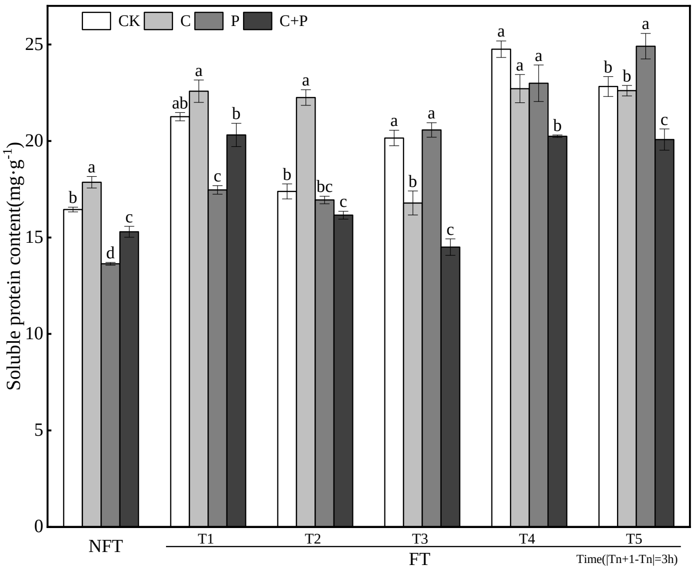

Figure 3. *The changes in SP of rye seedlings under different treatment groups. T1-T5 represents five sampling times; the lowercase letters indicated the significant difference between different treatments at the same sampling time (P < 0.05). The data are expressed as mean ± standard error (n = 3)*

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Change in soluble sugar (SS)

In *Figure 4*, the SS content of all treatments in FT was generally higher than that in NFT, with the Cd stress of FT having the highest content among all treatments. At T3, the P of FT reached its minimum and decreased by 16.95% compared to the P of NFT. At T5, the trend in FT was consistent with that in NFT. C + P of FT showed a decreasing trend from T1 to T5, except at T3, and was lower than Cd stress of FT at all times except T3.

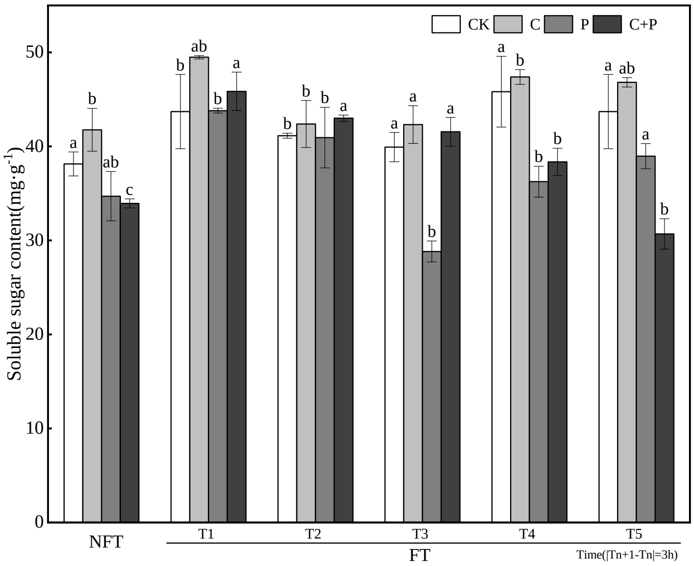

***Figure 4.** The changes in SS of rye seedlings under different treatment groups. T1-T5 represents five sampling times; the lowercase letters indicated the significant difference between different treatments at the same sampling time (P < 0.05). The data are expressed as mean ± standard error (n = 3)*

Change in malondialdehyde (MDA)

As shown in *Figure 5*, the MDA content in each treatment under FT was generally higher than under NFT. In FT, the trend in CK was not very clear, while P and C showed a trend of first decreasing and then increasing, and both reached their minimum at the lowest temperature. C + P showed a decreasing trend; at T5, it decreased by 47.9% compared to T1. During the temperature-rising stage at T5, C, P, and C + P decreased compared to CK by 17.6%, 9.3%, and 32.4%, respectively.

Change in activities of SOD

In *Figure 6*, in NFT, the difference between CK and C was not significant, while P had the highest SOD activity, and C + P had the second highest. The SOD activity in FT was lower than that in NFT. From T1 to T2, SOD showed an upward trend as the temperature decreased. At the lowest temperature (T3), CK and P reached their minimums, and C was slightly higher than at T2. As the temperature increased at T4, except for C + P, the SOD activity of the other treatments increased by 32.3%, 3.0%, and 46.4%, respectively, compared to T3. At T5, P increased significantly, while the other treatments decreased significantly.

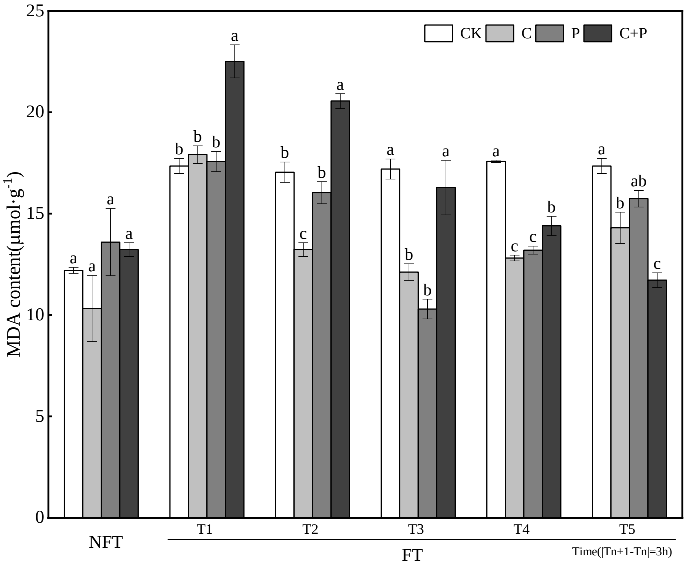

Figure 5. *The changes in MDA of rye seedlings under different treatment groups. T1-T5 represents five sampling times; the lowercase letters indicated the significant difference between different treatments at the same sampling time (P < 0.05). The data are expressed as mean ± standard error (n = 3)*

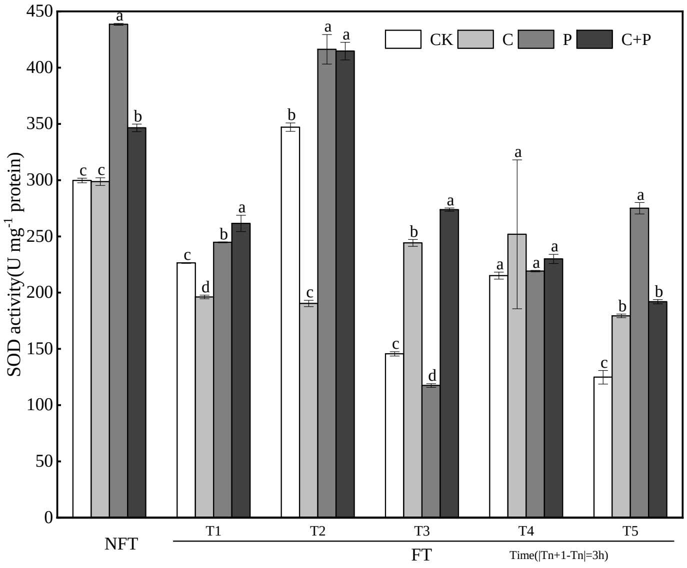

Figure 6. *The changes in SOD of rye seedlings under different treatment groups. T1-T5 represents five sampling times; the lowercase letters indicated the significant difference between different treatments at the same sampling time (P < 0.05). The data are expressed as mean ± standard error (n = 3)*

Change in activities of POD

In *Figure 7*, CK had the lowest POD activity in the NFT group, followed by C, while P had the highest. The POD activity in the FT group was lower than that in the NFT group. At T2 of FT group, C was lower than CK and decreased by 26.7% compared to CK ($P < 0.05$). C + P had a higher POD activity than CK throughout the freezing and thawing process, with relatively stable changes and a peak at T3. During the freezing stage (T1-T3) in the FT group, the POD activity generally increased with decreasing temperature, while P showed a decreasing trend. During the thawing stage (T3-T5), the POD activity showed a decreasing trend, and P reached its minimum at T4, which was significantly different from the other treatments ($P < 0.05$).

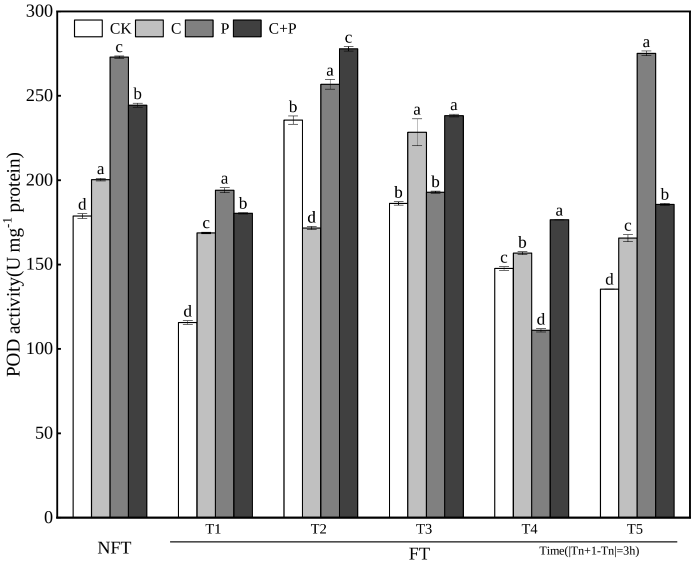

Figure 7. *The changes in POD of rye seedlings under different treatment groups. T1-T5 represents five sampling times; the lowercase letters indicated the significant difference between different treatments at the same sampling time (P < 0.05). The data are expressed as mean ± standard error (n = 3)*

Change in photosynthetic parameters

In the experiment (*Fig. 8*), the Pn and Tr of the FT group were lower than those of the NFT group. Compared to CK, C, P, and C + P treatments, the values of Pn in FT + CK, FT + C, FT + P, and FT + C + P decreased by 65.8%, 70.4%, 76.9%, and 64.3%, respectively, at T1. Similarly, Tr was reduced by 62.0%, 63.6%, 73.4%, and 69.4% in the same treatments, indicating that FT was dominant in inhibiting Pn and Tr. The significant reductions observed at other sampling times also suggest the predominant effect of FT in controlling Pn and Tr. As the FT process continued, photosynthetic parameters in the FT and NFT groups showed significantly decreasing trends. The treatments containing potassium fulvic acid (P, C + P, FT + P, FT + C + P) had higher Pn and Tr values than CK and C. At T3, Pn values of rye seedlings in FT + CK, FT + P, FT + C, and FT + C + P decreased by 76.2%, 54.1%, 74.8%, and 68.9%, respectively, compared to CK. At T5, Pn of FT + P increased by 29.7% compared to FT + CK, Pn of FT + C + P increased by 14.4%, and the Pn of FT + C decreased by 29.4%. In the experiment, the Pn of the leaves of each treatment continued to decline with the change of temperature, and Pn did not increase with the increase of temperature. At the same time, at T3, Tr of FT + CK, FT + P, FT + C, and FT + C + P decreased by 62.1%, 54.2%, 74.1%, and 55.6%, respectively, compared to CK. Compared to FT + CK, Tr of FT + C decreased by 31.7%, and Tr of FT + P and FT + C + P increased by 17.2% and 14.6%, respectively.

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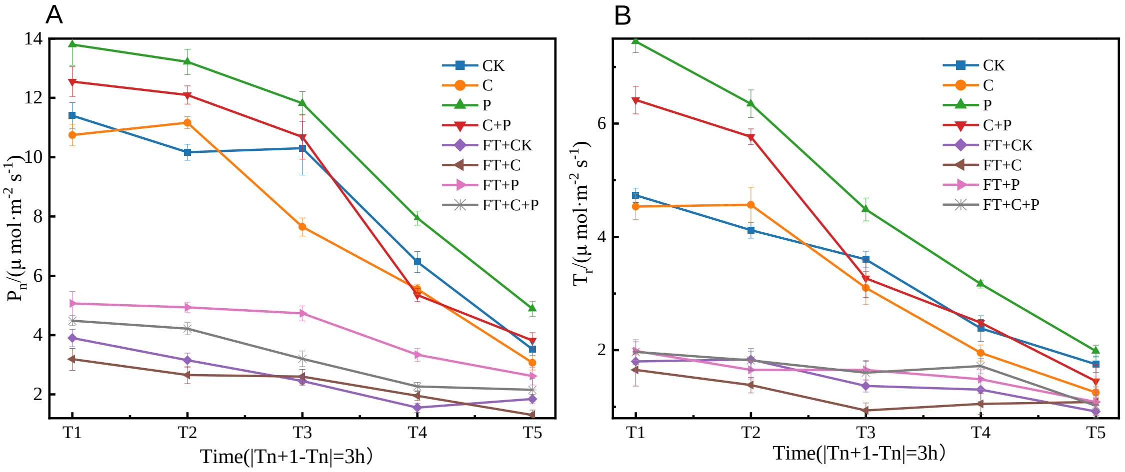

Figure 8. *The changes in Pn and Tr of rye seedlings under different treatment groups. Error bars show standard error (SE) of the three replicates*

Correlation analysis

Pearson correlation analysis was performed on the physiological characteristics of rye seedlings in the FT environment (*Fig. 9*). The results showed that the two antioxidant enzymes (POD and SOD) were significantly negatively correlated with the SP content, with correlation coefficients of -0.88 and -0.59 ($P < 0.01$), respectively. POD and SOD were significantly positively correlated, with a correlation coefficient of 0.66 ($P < 0.01$). SP, MDA, SS, Pn, and Tr were negatively correlated with RC but were not significant. Pn and Tr were significantly positively correlated, with a correlation coefficient of 0.77 ($P < 0.01$). SS and MDA were significantly positively correlated, with a correlation coefficient of 0.57 ($P < 0.01$).

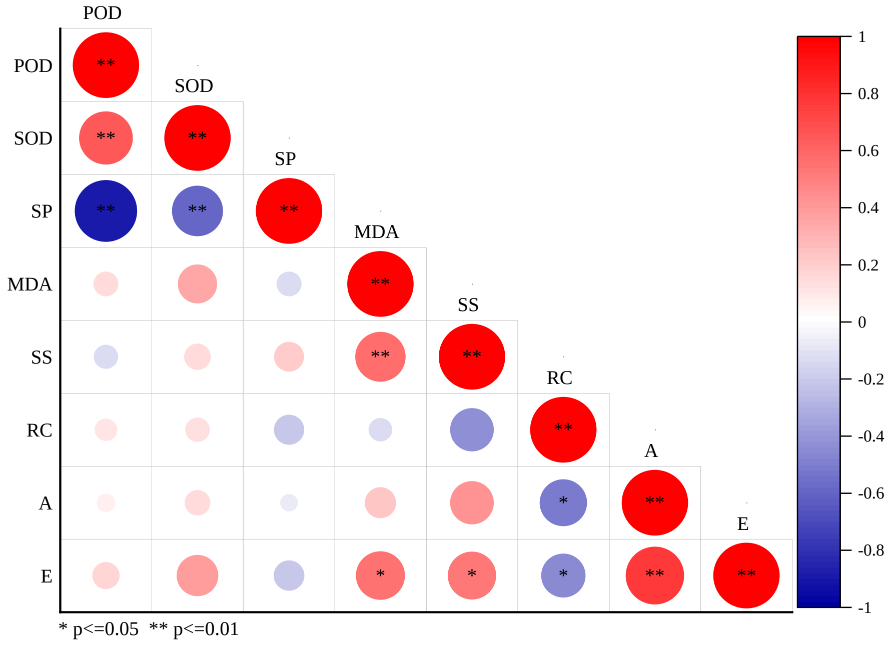

Figure 9. *Pearson correlation analysis between POD, SOD, SP, MDA, SS, RC, Pn, and Tr in rye seedlings under FT environment (**P < 0.01, *P < 0.05)*

Discussion

Effects of compound stress on osmoregulation

Plants tend to increase the osmotic potential at the cellular level by synthesizing and accumulating osmolytes, such as SS and SP, to overcome harmful effects under abiotic stress (Ozturk et al., 2021). They have been shown to change the SS and SP concentrations to adapt to environmental changes. In a previous report, low-temperature stress caused a decrease followed by an increase in pepper seedlings' SP and SS content (Jing et al., 2022), which is consistent with our research. Also, rye seedlings are more sensitive to SP changes than SS. Under Cd stress, the SP and SS content increased compared to CK, which was also confirmed in a study on the toxicity of Cd^{2+} to wheat seedlings by Xiao et al. (2019). The increased SP and SS content may provide more energy support based on sugar metabolism for rye seedlings in response to Cd stress (Gu et al., 2022). The SP and SS content of adding FAK in the FT environment showed a trend of decreasing first and then increasing, which is speculated to be due to the ability of P treatment to improve permeability and membrane stability to enhance plant freezing tolerance (Zhang et al., 2021). However, the overall SP and SS content of P were lower, indicating that FAK can effectively inhibit Cd toxicity (Li et al., 2022). FAK and Cd may have compensatory effects under FT environment that need further study.

Effects of compound stress on cell membrane lipids

RC is an important parameter that reflects cell membrane permeability and can indicate the degree of damage to the cell membrane (Zhang et al., 2018). In this study, the values of RC increased in stressed rye seedlings under FT environment, similar to the results observed in a study of tomato seedlings by Lu et al. (2022). We also found that the RC value was highest under FT and Cd compound stress, which may be ascribed to a synergistic effect of FT and Cd compound stress, thus exacerbating the damage to the plant cell membrane. MDA is considered the main product of lipid membrane peroxidation, and the content of MDA reflects the damage to the structures of cell membranes (Nizar et al., 2021). The MDA content significantly increased under FT environment, showing that FT increases the degree of lipid peroxidation in rye seedlings (Yildiztugay et al., 2017). The combined FT and Cd treatment showed a trend of decreasing first and then increasing, which is consistent with the observations of Gu et al. (2022) and demonstrates that Cd stress disrupts the function and integrity of the cell membrane. Compared to the control, the MDA and RC content was lower in P treatment of FT, suggesting that FAK may induce an antioxidant response to remove ROS, alleviate lipid peroxidation and oxidative damage, and maintain membrane stability.

Effect of compound stress on photosynthetic parameters

Photosynthesis is an important characteristic for evaluating plant growth and stress resistance and reflects the vitality of plants. Pn and Tr were significantly reduced under the FT environment. When the temperature increased, the photosynthesis of rye seedlings was still suppressed, similar to the observations in a study of *Lumnitzera littorea* (Jack) (Hao et al., 2022). Stomatal and non-stomatal factors are the two main factors that limit plant photosynthesis. Based on the observed decreases in Pn and Tr, we infer that the decreased photosynthesis in rye seedlings is mainly due to non-stomatal factors, as the FT damaged the photosynthetic organs, leading to a decrease in CO_2 and a reduction in photosynthesis efficiency. In addition, Cd stress under FT environment further damaged the chloroplast structure and photosynthetic pigments, exacerbating the damage to the plant photosynthetic system, as confirmed in a study of lettuce by Tang et al. (2022). We also found that FAK can improve the photosynthetic efficiency of leaves through stomatal regulation and alleviate the damage caused by Cd stress (Fang et al., 2020). However, its effects on FT environment were insignificant, and the addition of FAK cannot effectively mitigate the severe FT damage.

Effect of compound stress on antioxidant enzymes

Various abiotic stresses, such as FT and Cd stress, can lead to the overproduction of ROS in plants, which are highly reactive and toxic, and ultimately result in oxidative stress (Gill Tuteja, 2010). SOD and POD are key components of the plant antioxidant enzyme system that help limit ROS concentration and maintain their stable levels within the cell. Generally, SOD and POD activity in plants increases as temperatures decrease (Wang et al., 2021). However, our study found that FT decreased or maintained antioxidant enzyme activity in the rye at a lower level, suggesting that this is because the FT exceeds the limits that plants can withstand (Sorensen et al., 2018). Compared to NFT environment, Cd stress resulted in higher POD activity under FT environment, suggesting that FT causes more severe damage to rye. However, SOD activity tended to decrease in FT and Cd compound stress, possibly due to the interaction of FT and Cd compound stress (Wang et al., 2021) and Cd increasing ROS accumulation and reducing antioxidant enzyme activity under low temperatures (Liu et al., 2022). Compared to the other treatments in the FT group, adding FAK resulted in higher POD and SOD activity, indicating that rye seedlings treated with FAK better-protected biomolecules from oxidative damage (Qu et al., 2022). However, in FT and Cd compound stress, the increase in both enzyme activities was significant; FT and Cd compound stress causes double damage to plants. FAK could only partially alleviate this damage rather than effectively inhibit it. In conclusion, FT and Cd compound stress synergistically affect plants, worsening oxidative damage, whereas FAK has an antagonistic effect on both stresses.

Conclusion

In summary, Cd cause irreversible damage to the photosynthetic system of rye seedlings. FT and Cd compound stress further exacerbates the physiological damage to rye, inhibiting the permeability regulation function of rye seedlings and decreasing the activity of antioxidant enzymes. FAK can alleviate Cd toxicity and oxidative damage and improve the tolerance of rye seedlings in FT and Cd compound stress. The results demonstrate that FAK can mitigate detrimental plant stress, contributing to sustainable agricultural production. However, the mechanisms of its effects require further investigation.

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