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Mechanisms of Nitric Oxide in the Regulation of Chilling Stress Tolerance in *Camellia sinensis*

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Abstract: Tea [*Camellia sinensis* (L.)] plants are important economic crop in China. Chilling stress and freezing damages have seriously affected the quality of tea products that have been already regarded as the main restricting factors to industry's development. Nitric oxide (NO) plays a crucial role in resistance of abiotic stresses. An experiment was conducted in an artificial climate chamber to study the effect of NO on tea plants grown under chilling stress (−2 °C) for 0, 6, 24, 48, and 72 h. Foliar application of sodium nitroprusside (SNP) at a rate of 500 μmol·L^{-1} was used as NO donor. The experiment contained two factors: the first was the foliar application with SNP or distilled water, and the scond one was the chilling (−2 °C) exposure time (0, 6, 24, 48, and 72 h). The effects of NO on membrane lipid peroxidation, osmotic adjustment substances, and antioxidant activity under cold stress were studied. In addition, the gene expression of *CsICE1* and *CsCBF1* in respond to NO addition were also investigated using real-time polymerase chain reaction (RT-PCR). The results show that foliar addition of NO (500 μmol·L^{-1} of SNP) reduce the relative conductivity of tea leaves, inhibits the elevated malondialdehyde content, promotes the accumulation of proline, soluble protein and sugar, and increases the superoxide dismutase, catalase activities, thereby alleviates the damage of cold stress on tea leaves. The *CsICE1* expression in 500 μM SNP treatment was peaked at 24 h of low temperature stress, while it did not express at normal temperature. Therefore, the current study is considered a good scientific material in understanding how tea plants sense and defense the chilling stress and that plays an important role to improve the level of production and economic benefits. It is also provided significant theory bas to control chilling stress in tea plants.

Keywords: tea plants; chilling stress; tolerance; antioxidant enzymes; gene expression

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1. Introduction

Tea plant [*Camellia sinensis* (L.) O. Kuntze], a perennial evergreen woody plant, is warm-liked but cold-intolerant [1–3]. In the global trend of frequent extreme weather phenomena, low temperature, one of the abiotic stresses commonly encountered in tea cultivation, has become an important factor restricting the healthy development of tea industry [1]. Recent years, tea plantations in early spring were susceptible to low-temperature cold damage or "late spring coldness" hazards after the temperature warmed up, which caused the physiological metabolism disorders of tea plants, and in severe cases led to the decline in tea production and quality, which seriously affected the sustainable development

of China's tea industry [1–3]. Numerous studies had shown that the cold resistance of tea plants is closely related to the contents of malondialdehyde (MDA), soluble sugar, soluble protein, and the activities of superoxide dismutase (SOD) and catalase (CAT) in tea plants [2,3]. Tea plants with stronger cold resistance had higher contents of soluble sugar and soluble protein [4,5], and the activities of SOD and CAT are maintained at a higher level. Besides, the total activity of SOD isoenzymes and the number of spectral bands were significantly increased [5–7]. Therefore, it was of great significance to study the response mechanism of tea plants to low temperature stress and to explore the tolerance of tea plants under low temperature and chilling damage.

Nitric oxide (NO), as an important signal molecule in organisms, participates in plant stress response has become a research hotspot at present, due to the importance of signal regulation for plant resistance and defense [8]. Tian et al. [9] found that exogenous NO can induce stomata closure of wheat leaves to reduce water loss under the drought stress, and the activities of SOD and CAT in leaves can be improved while the content of MDA and H_2O_2 decreased. Zheng et al. [10] reported that exogenous NO promoted wheat seeds germination under high salt stress and resisted damages of mitochondrial oxidative to leaves, both the activities of SOD, CAT and the content of proline enhanced. Liu et al. [11] showed that the improvement of exogenous NO under low temperature stress is due to the protective enzyme activity in cucumber seedlings, thereby the plant's adaptability to low temperature stress enhanced. Chen et al. [12] showed that the germination rate of corn seeds was significantly improved as well as the relative water content, proline, and chlorophyll under the low temperature stress at different concentrations of sodium nitroprusside (SNP), on the other hand, MDA content was reduced. Regulation of exogenous NO has been widely studied on plants such as gramineae, legumes, vegetables, and woods under different abiotic stresses e.g., water, salinity, temperature, etc., however, there are few studies about physiological responses of tea plants mediated by NO under adversity stress, especially the effect of NO on physiological characteristics of tea plants under low temperature stress which has not been studied in depth. Accordingly, this study investigates the effects of cell membrane permeability, osmotic regulating substances, and cold-tolerant core genes expressions in tea leaves under low temperature stress. Besides, the study explore the impact of spraying SNP, an exogenous NO donor, on the seedling of tea plants. This research aimed to clarify the mitigation effect and the molecular mechanism of spraying SNP on tea plants under low temperature stress, and to provide a theoretical reference for further research on the ability improvement of tea seedlings to resist low temperature, chilling damage, and other abiotic stresses by exogenous NO.

2. Material and Methods

2.1. Experimental Materials

The biennial 'Bai Hao Zao' tea potted-seedlings were selected as the experimental material which is provided by Gaoqiao Base of Hunan Tea Research Institute, China. These tea seedlings were transplanted in polyethylene basins with diameter of 30 cm, where 3 tea seedlings in each basin were planted. The soil which used for the tea seedlings planting was a mixture of 70% tea garden soil and 30% organic fertilizer. The experiments were carried out in the Key Laboratory of Tea Science of the Ministry of Education, Hunan Agricultural University, China. The seedlings were placed in the greenhouse for 3 months and the greenhouse was controlled at 20–25 °C and relative humidity of 60–75%. $Na_2Fe(CN)_5$ with purity of 98.5% which was used in the experiment was purchased from Sangon Biotech Co., Ltd. (Shanghai, China). A stock solution of 100 mmol L^{-1} of $Na_2Fe(CN)_5$ was prepared and stored at 4 °C, diluting the liquor according to the required concentration when used.

2.2. Experimental Method

Experimental Design

The tea potted-seedlings were moved into an artificial intelligence climate incubator and were left for 3 days. The temperature in the incubator was controlled at 25 ± 1 °C, the

relative humidity was maintained at 60 ± 5%, and the photoperiod was 12 h d^{-1}. After 3 days, the seedlings were treated by foliar application with 500 μmol L^{-1} SNP or distilled water. The seedlings were sprayed once a day with 250 mL of the studied treatments and for 3 consecutive days until the water droplets on the front and back of the leaves were about to drop. After 3 days, the temperature in the incubator was set at −2 °C, the photoperiod at 12 h·d^{-1}, and the relative humidity at (60 ± 5)%. The seedlings were left in the incubator for 0, 6, 24, 48, and 72 h. The experiment contained two factors: the first was the foliar application with SNP or distilled water, and the scond one was the chilling (−2 °C) exposure time (0, 6, 24, 48, and 72 h). Each group consisted of 15 pots per treatment, three stumps per pot. The experiment was laid out in a randomized complete block design and repeated three time for each treatment. The first mature tea leaf was taken to measure the electrical conductivity, while one bud and two leaves were taken to measure the physiological indicators and fluorescence quantitative PCR analysis. Samples were put into the sealed bag immediately, frozen in liquid nitrogen rapidly, and stored in the refrigerator at −70 °C.

2.3. Measurement Items and Methods

Mature leaves of groups before and after treatment were washed with deionized water and wiped with clean gauze. After cutting, leaves were put into a small beaker filled with 20 mL deionized water and pumped for 15 min, sealed with plastic wrap and stood for 30 min, and the conductivity and initial conductivity of deionized water were measured. Then the beaker was put in boiling water bath for 15 min and coolled to room temperature. The boiling conductivity was measured and the relative conductivity was calculated [13]. The contents of proline was determined by ninhydrin colorimetry [14–16]. 0.5 g fresh tea leaves were grinded and extracted with 5 mL 3% sulfosalicylic acid solution, and then extracted in boiling water bath for 10 min. The absorbance value was measured by spectrophotometer at wavelength 520 nm. The soluble sugar contents were determined by anthranone colorimetric method [15]. 0.5 g fresh tea leaves were cut into pieces and put into 25 mL tube with stopper. 5 mL distilled water was added, the mixture was boiled in boiling water for 30 min, taken out, cooled, filtered, rinsed with distilled water several times, and scaled to 25 mL. 0.1 mL extract was taken, 0.9 mL distilled water and 5 mL anthrone reagent were added. After boiling water bath for 10 min and cooling, the absorbance at 620 nm was measured. The control treatment was 5 mL anthrone plus 1 mL water. The standard curve was drawn with 100 μg/mL sucrose standard solution, and the soluble sugar content of the sample was calculated. The reserve solution was prepared for determination of protein and malondialdehyde contents, SOD and CAT activities. 0.5 g tea tree leaves were accurately weighed in a pre-cooled mortar, and an appropriate amount of insoluble polyvinylpyrrolidone, quartz sand and 0.05 mol/L phosphoric acid buffer of pH 7.8 were added. After grinding and homogenizing, the leaves were transferred to a centrifugal tube and centrifuged at 10,000 r/min for 20 min. The supernatant was stored in a refrigerator for later use. The soluble protein contents were determined by coomassie brilliant blue G-250 staining method [14]. 5 mL coomash bright blue solution was added into 1.0 mL of the prepared supernatant and mixed thoroughly. After 2 min, the absorbance value was measured at 595 nm wavelength, the protein content was checked through the prepared standard curve. The contents of malondialdehyde was determined by thiobarbituric acid method [15]. 2 mL 0.6% thiobarbituric acid solution was added into 2 mL of the prepared supernatant (control treatment added 2 mL PBS), mixed well, reacted in boiling water bath for 15 min, and then centrifuged for 10 min after cooling. The absorbance of the supernatant was measured at 532 nm, 600 nm and 450 nm, and the content of malondialdehyde was calculated. The SOD activity was determined by nitrogen blue tetrazole (NBT) colorimetric method [17]; 1.5 mL 0.05 mol/LpH7.8 phosphoric acid buffer, 0.3 mL 130 mM methionine solution, 0.3 mL 750 μM azolotetrazole solution, 0.3 mL 100 μM edta-na2 solution, 0.3 mL 20 μM riboflavin solution and 0.5 mL distilled water were added into 0.1 mL prepared supernatant. For control, buffer was used instead of

enzyme solution. After mixing, one control tube was placed in the dark, and the other tubes were exposed to 4000 lx sunlight for 20 min. After the reaction, the absorbance of other tubes was measured at 560 nm wavelength, and SOD activity was calculated. while the CAT activity was determined by H_2O_2 ultraviolet absorption method [16]. 0.5 mL of 0.1 mol/L H_2O_2 solution and 2.5 mL of 0.1 mol/L phosphoric acid buffer with pH 7.0 were added into 0.1 mL prepared supernatant. After reaction, the absorbance was measured at 240 nm wavelength and CAT activity was calculated.

2.4. Fluorescence Quantitative PCR Analysis

The extraction of total RNA from tea fresh leaves was done by Tri-Reagent method. The total RNA was reverse transcribed into cDNA as fluorescent quantitative PCR templates, refering to the operation manual of Tiangen Biotech AceQTM qPCR SYBR Green Master Mix kit. Primer 5.0 was used to design primers (Table 1), which synthesized by Shanghai Bioengineering Co., Ltd., and GAPDH was used as internal reference gene. The reaction system was 10.0 μL AceQTM qPCR SYBR Green Master Mix, 0.4 μL in each 10 μmol/L upstream and downstream primers, 0.4 μL ROX Reference Dye II, 2.0 μL cDNA tamplates, added water to the final volume system of 20 μL.

Table 1. The pimer of *CsICE1* and *CsCBF1* genes expression analysis in tea plant.

Gene	Forward Primer (5′-3′)	Reverse Primer (5′-3′)
CsICE1	ATGTTTTGTAGCCGCAGAC	GCTTTGATTTGGTCAGGATG
CsCBF1	AGAAATCGGATGGCTTGTGT	TTGTCGTCTCAGTCGCAGTT
GAPDH	TTGGCATCGTTGAGGGTCT	CAGTGGGAACACGGAAAGC

PCR procedures were carried out by 3-step reaction for total 45 cycles: predenaturation at 95 °C for 15 min, denaturation at 95 °C for 10 s, annealing temperature at 58–60 °C, annealing time for 15 s–25 s and extension at 72 °C for 20 s. The $2^{-\Delta\Delta CT}$ method was applied to calculate the relative expression of genes.

2.5. Data Processing

Microsoft Excel 2010 Software was used for data processing, SPSS 17.0 Statistical Analysis Software was used for variance analysis and significant difference analysis, and the Duncan method was used for multiple comparisons.

3. Results

3.1. Effects of Exogenous NO and Low Temperature Stress on Relative Electrical Conductivity of Tea Leaves

As showed in Figure 1, the relative electrical conductivity of tea leaves was increased with chilling stress time. The relative electrical conductivity in the 500 μmol·L^{-1} SNP treatment group was significantly lower than the distilled water treatment ones, and both groups reached the peak value at 72 h treatment. The relative electrical conductivity of SNP treatment was significantly decreased by 22, 38, 46 and 37% at 6 h, 24 h, 48 h and 72 h, respectively, compared to water treatments. The ability to resist low temperature of tea leaves plasma membrane were improved in a short time after SNP treatment, meanwhile the increase rate of tea leaves relative conductivity were slowed down.

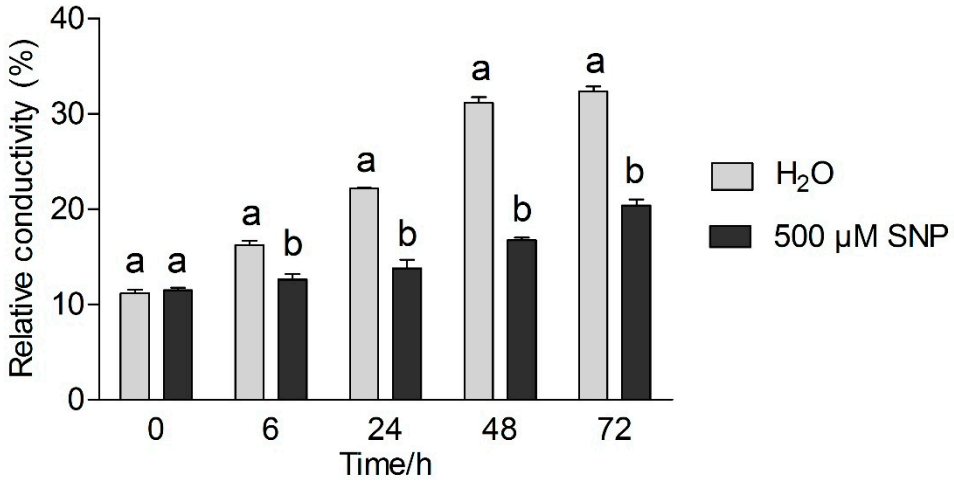

Figure 1. Effect of exogenous nitric oxide on electrolytic leakage in tea leaves under cold stress.

3.2. Effects of Exogenous NO and Low Temperature Stress on MDA and Proline Content in Tea Leaves

As it seen from Figure 2, malondialdehyde (MDA) was produced by low temperature, its content gradually increased with time extension of low temperature stress and were reached the peak value at 72 h in tea leaves. However, the MDA content in tea leaves could be significantly decreased by exogenous NO. MDA content in 500 μmol·L^{-1} SNP treatments was greatly reduced by 17, 22, 38, and 42%, respectively, at 6 h, 24 h, 48 h and 72 h, respectively, compared to distilled water treatments. There was a positive effect on the inhibition of MDA content being increased in tea leaves when SNP was sprayed under low temperature stress.

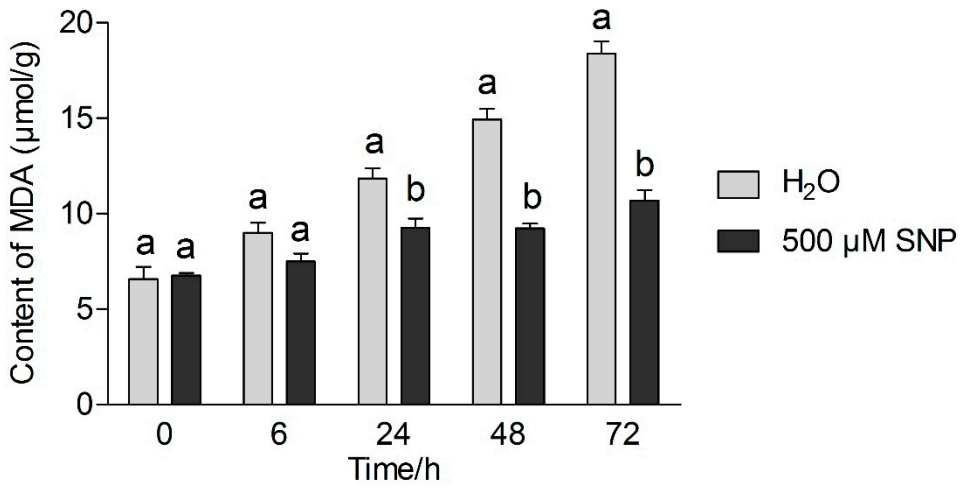

Figure 2. Effect of exogenous nitric oxide on MDA content in tea leaves under cold stress. Means with different letters have significant differences according to Dunkin' test $p < 0.05$.

Figure 3 shows the proline content in tea leaves. The proline results showed an upward trend with the time extension of low temperature stress. The proline content of tea leaves sprayed with SNP under low temperature stress was higher than that of water treatments, and reached the peak value at 72 h. The proline content of 500 μmol·L^{-1} SNP treatments was significantly increased by 6, 37, 14, and 16% at 6 h, 24 h, 48 h and 72 h, respectively, compared to water treatments. The results illustrated the significant effect 500 μmol·L^{-1} SNP on increasing proline content of tea leaves under low temperature stress.

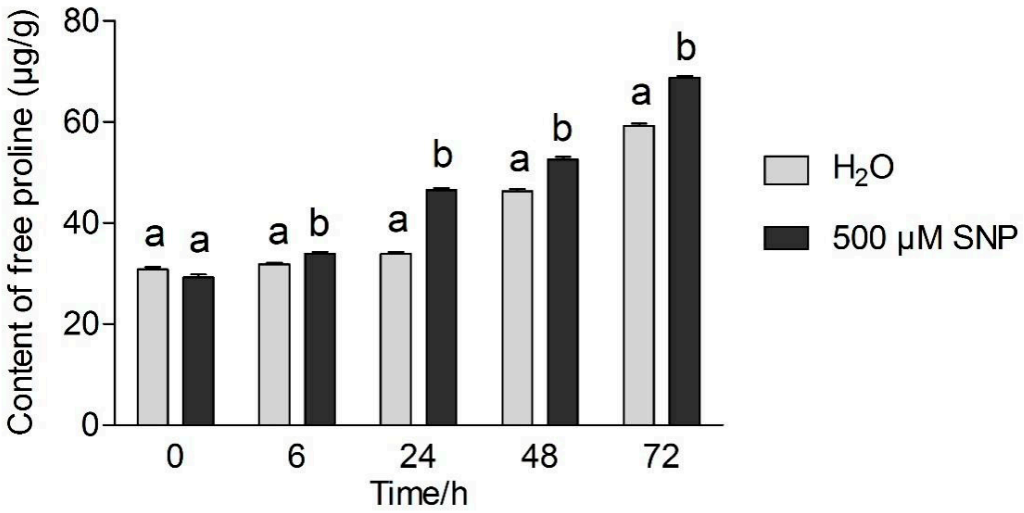

Figure 3. Effect of exogenous nitric oxide on proline content in tea leaves under cold stress.

3.3. Effects of Exogenous NO and Low Temperature Stress on Soluble Protein and Sugar Content in Tea Leaves

As illustrated in Figure 4, the soluble protein content of tea leaves showed an upward trend during the low temperature stress process. The soluble protein content of sprayed with SNP was significantly higher than that of water treatments ($p < 0.05$), and reached the highest value at 72 h. The soluble protein content of 500 μmol·L^{-1} SNP was significantly increased by 15, 9, 6, and 4% at 6 h, 24 h, 48 h and 72 h, respectively, compared to water treatments.

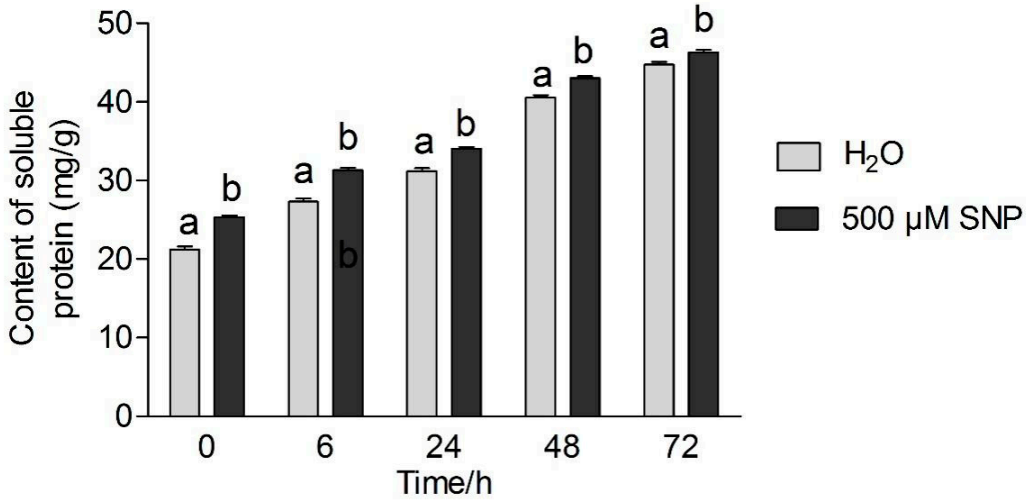

Figure 4. Effect of exogenous nitric oxide on soluble protein content in tea leaves under cold stress.

As it seen from Figure 5, the soluble sugar content of tea leaves was increased under low temperature stress, and was reached the highest value at 72 h. The soluble sugar content of SNP-treated tea leaves was higher than that of water treatments. The soluble sugar content of 500 μmol·L^{-1} SNP was significantly increased by 13, 50, 46, and 44% at 6 h, 24 h, 48 h and 72 h, respectively, compared to water treatments.

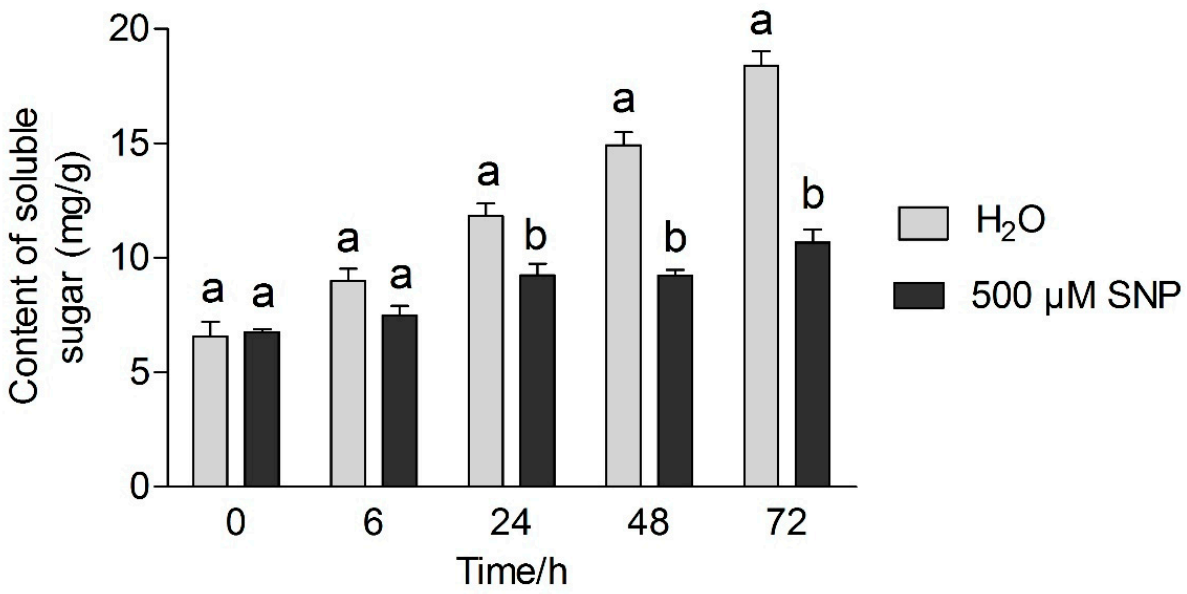

Figure 5. Effect of exogenous nitric oxide on soluble sugar content in tea leaves under cold stress.

3.4. Effects of Exogenous NO and Low Temperature Stress on Antioxidant Enzymes Activity in Tea Leaves

It could be seen from Figure 6 that the catalase (CAT) activity of tea leaves showed a trend of first increased then decreased under low temperature stress. CAT activity changed little at 0–6 h, and significantly increased at 6 h–24 h, after 24 h later the activity decreased, furthermore, the CAT activity of SNP-treated tea leaves was higher than that of water treatments. During the entire low temperature stress process, SNP increased CAT significantly by 19, 47, 27, and 31% at 6 h, 24 h, 48 h, 72 h, respectively, compared with water treatments.

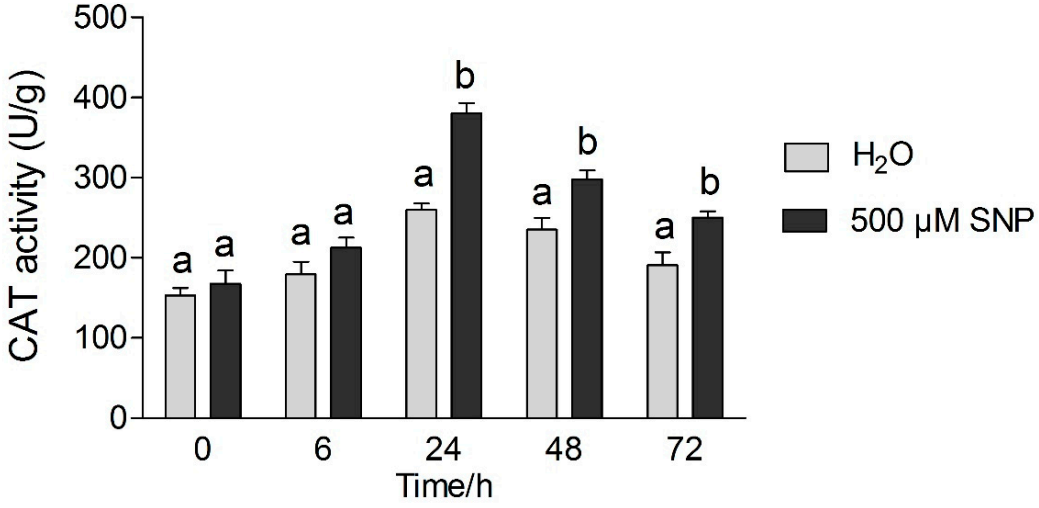

Figure 6. Effect of exogenous nitric oxide on catalase (CAT) activity in tea leaves under cold stress.

Figure 7 illustrated that the superoxide dismutase (SOD) activity of tea leaves showed a trend of first increased then decreased during the low temperature stress process, and the SOD activity reached the peak value at 24 h. SNP increased SOD activity significantly by 12, 28, 20, and 7% at 6 h, 24 h, 48 h, 72 h, respectively, compared with water treatments.

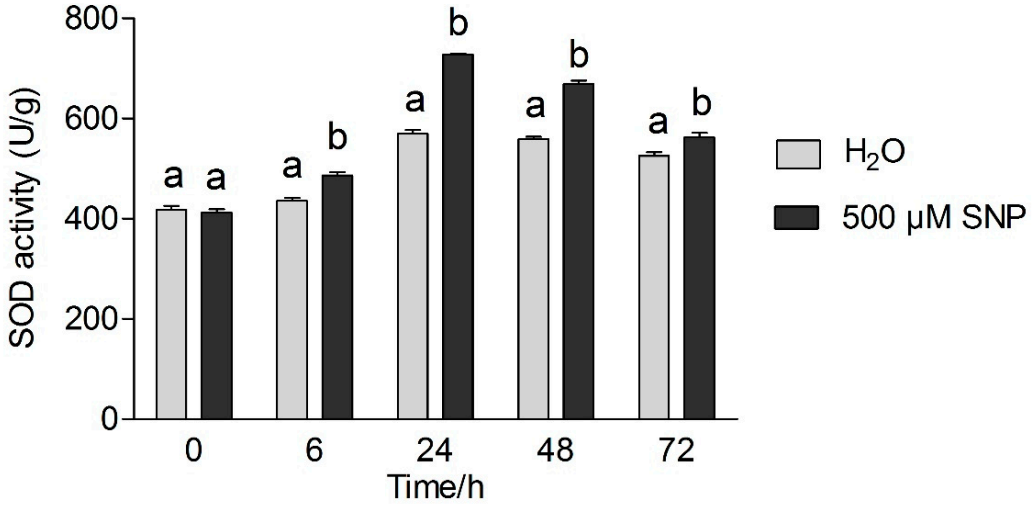

Figure 7. Effect of exogenous nitric oxide on superoxide dismutase (SOD) activity in tea leaves under cold stress.

3.5. Effects of Exogenous NO and Low Temperature Stress on CsICE1 and CsCBF1 Gene Expression in Tea Leaves

As demonstrated in Figure 8, the expression of *CsICE1* gene in tea leaves increased first and then decreased under low temperature stress, moreover, the expression of *CsICE1* gene treated by SNP was always evidently higher than that in pure water treatment ($p < 0.05$). In the process of low temperature stress, the expression of *CsICE1* gene in tea leaves of water treatment group did not change much, it did a slight up-regulation of gene expression at the 6 h, then the expression decreased again at 24 h and was close to the level of 0 h, the minimum value appeared at 48 h, yet it rebound to express at 72 h. *CsICE1* gene in tea plant leaves of SNP group responded quickly to low temperature, it up-regulated the expression at 6 h, which was 3.9 times that of water treatment group; the expression reached its peak value at 24 h, which was 6.4 times that of water treatment group; however, it began to decrease after 24 h, additionally, it was 6.0 times that of water treatment group at 48 h, but it changed slowly during 48 h–72 h and remained at a certain level of expression, furthermore, SNP was 1.96 times that of water treatment group at 72 h.

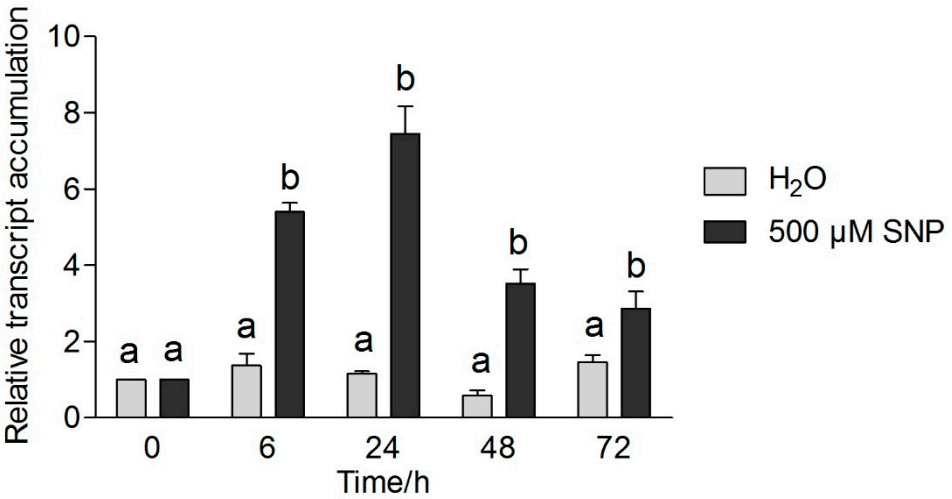

Figure 8. Expression of the *CsICE1* genes under cold stress.

The expression of *CsCBF1* gene in tea leaves under low temperature stress was shown in Figure 9, where the expression of *CsCBF1* gene in tea leaves was almost undetectable at 0 h, yet the gene expression increased rapidly when the tea leaves under low temperature

stress, and then the expression gradually declined. The expression of *CsCBF1* gene treated by SNP was gradually increased and then decreased, and was always significantly higher than that of water treatment ($p < 0.05$). The expression of *CsCBF1* gene in tea leaves was up-regulated at 6 h during the low temperature stress process, it reached the peak value at 24 h and began to decline at 48 h, furthermore decreased at its trough value at 72 h. *CsCBF1* gene of SNP responded quickly to low temperature, and its expression was up-regulated at 6 h, which was 1.1 times that of water treatment group; the expression reached its peak value at 24 h, which was 2.01 times that of water treatment group. The expression of *CsCBF1* gene decreased after 24 h, and was 1.95 times that of water treatment group at 48 h, however, the expression changed slowly from 48 h to 72 h, and was 1.05 times that of water treatment group at 72 h.

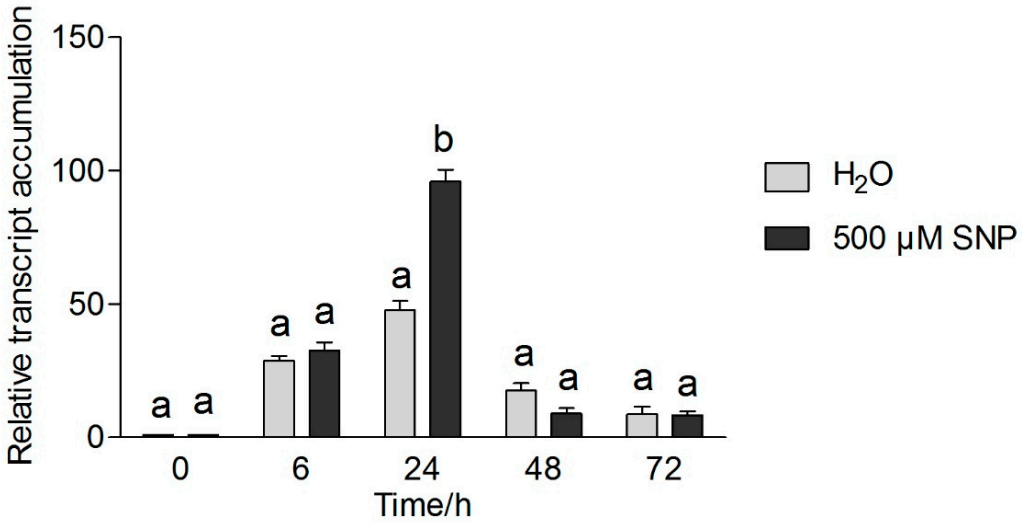

Figure 9. Expression of the *CsCBF1* genes under cold stress.

4. Discussion

The plant needs the ideal conditions for growth, including temperatures [18–23]. Cell membrane is the main part of plants that suffers from the damage at low temperature, since the permeability of cell membrane increased and the electrolyte of intracellular extravasated due to the low temperature, which could lead to cell damage. Studies had shown that the relative permeability of plasma membrane in plant seedlings could be significantly reduced and the accumulation of malondialdehyde (MDA) considerably inhibited under low temperature stress with an appropriate sodium nitroprusside (SNP) concentration, thus the adaptability of plant seedlings to low temperature stress could be improved [24,25]. This study revealed that the accumulation of MDA content was drastically restrained while the relative electrical conductivity was declined in tea leaves by exogenous nitric oxide (NO), which were consistent with the findings of Xu et al. [24] and Fan et al. [26]. The reason might be that low temperature promoted the accumulation of H_2O_2 and O^{2-}, which in turn stimulated the production of MDA, and caused damage to the cell membrane structure of tea leaves. As a signal molecule, NO reduced the accumulation of MDA, the membrane peroxide, and to some extent, the structure of cytomembrane membrane in tea leaves under low temperature stress was protected as well as the damage caused by peroxidation of cytomembrane membrane was alleviated.

Adversity stress can directly or indirectly cause changes of intracellular osmotic potential which may bring about cytoplasmic dehydration, whereas plants adapt to adversity by accumulating osmotic substances in a short period of time for osmoregulation. Soluble protein, soluble sugar, and proline are the essential osmoregulation substances in plants, which can eliminate toxic substances produced by low temperature stress, thereby reducing ice crystals formation caused by water loss [27]. In this study, the content of proline, soluble protein, and soluble sugar in tea leaves were noticeably enhanced by exogenous NO, which

was consistent with the results of [28–31]. It could be speculated that low temperature stress led to destruction of cells structure, however, NO directly acted on the components of cell wall through apoplast to promoted the transport of intracellular water from roots to leave, thereby developed the stability of cell membranes and reduced electrolyte extravasation. NO is a signal transduction molecule involved in plant physiological processes, which plays a vital role in plant growth and development, and its signal system can induce and activate plant antioxidant systems. Studies have shown that the activity of CAT, POD, SOD and other enzymes in some crops could be substantially advanced by NO, in the meantime, the survival rate of crops was improved under low temperature stress [29,32], which were in line with the results of this experiment. The results suggest that NO might had a high affinity with iron-containing CAT and other related enzymes, which could regulate the activity of heme iron-containing enzymes and inhibit the activity of target enzymes such as non-heme iron-containing aconitase [33]. The raise of SOD activity might be owing to the induction of SOD coding genes expression by NO, which acted as a signal molecule under low temperature stress [34]. A proper concentration of SNP could increase the activity of antioxidant enzymes such as SOD and CAT, effectively remove the active oxygen accumulated in plants caused by low temperature stress, improve the tolerance ability to low temperature, and reduce the damage of low temperature stress to plants.

When plants exposed to low temperature stress, not only a series of physiological and biochemical changes will be caused, but also the signal transduction pathway activated, the expression of cold resistance genes induced as well. The current study found that the expression of *CsICE1* gene in tea leaves could be induced by exogenous NO under low temperature stress, which made the gene expression presented a dynamic growth from weak to strong, and then then the intensity became weak, but the changes were not significant in the expression of *CsICE1* gene in tea leaves without sprayed by SNP during the whole process of low temperature stress. These conclusions were identical with the findings of Chinnusamy et al. [35] and Chang et al. [36], that is, the expression of the *ICE1* gene in plant tissues would always maintain a certain level regardless of any changes in the external environment. The reason might be the expression of *ICE1* gene was constitutive [37], this gene could have advanced the cold resistance of plants through regulating the over expression of CBF/DREB gene and then CBF inducing the expression of cold-related functional genes. The results showed that the expression of *CsCBF1* gene in tea leaves was almost undetectable at 0 h, however, it was quickly expressed after low temperature stress, furthermore, the expression of *CsCBF1* gene reached its peak at 24 h and then began to decrease with the lapse of time, the outcomes in this research were consistent with previous studies. The findings of Wang et al. [38], Chang et al. [36] showed that there was no expression of *CsCBF1* gene when its at room temperature, yet the expression level improved promptly after low temperature stress, and later the transcription level of *CsCBF1* gene gradually decreased. These suggested that *CsCBF1* gene was involved in the early process of low temperature signal transduction in tea plants and readjusted the adaptation mechanism to low temperature stress in its body. These might be because the expression of *CsCBF1* gene was inducible, and *CsICE1* was the upstream regulatory gene of *CsCBF1*, in addition, the activity of the *ICE1* protein was inactivated when the plant was under normal growth conditions, thus it could not bind to the *CEF* gene promoter effectively. Accordingly, *CsCBF1* gene was not expressed at room temperature; when the *ICE* protein activity was being activated by low temperature stress, it could be bind to the *CBF* gene promoter effectively and the entire low temperature stress pathway as was as the expression of *CsCBF1* gene were being activated [35], therefore, the expression of *CsCBF1* gene promoted quickly after the tea leaves were subjected to low temperature stress.

The expression of *CsICE1* and *CsCBF1* genes could be enhanced at the transcription level when they were under low temperature stress and they could be further regulated by the exogenous NO. Based on these conclusions, we putted forward the following possible mechanisms: the first is assumes that the low temperature stress could induce a large amount of reactive oxygen species (ROS), which had a high biological activity.

In the meantime, the expression of multiple antioxidant enzyme-related coding genes in cells might be induced by NO, an important signal molecule, which might be possible to achieve the ability to eliminatig ROS [39] and blocking the harmful effects of ROS on plants [40–42]. The second mechanism suggests that the protein conformation might be transformed by NO through the interaction of S-nitrosylation and heme iron due to the strong oxidizing properties of NO and its high affinity for heme iron and non-heme iron. The changes gave rise to the diffusion of NO into cell nucleus and the activation or inactivation of transcription factors, therefore caused the activity of transcription factors altered directly. The signal cascade system, including the formation of SA, the synthesis of cGMP, Ca^{2+} flow and reversible protein phosphorylation/dephosphorylation, could be activated by the reaction of NO, which might be also activated the MAPK cascade kinase signaling pathway [36]. Based on these signal cascade systems to regulate the activity of transcription factor, the gene expression and the transcription activity would be changed and affected [39].

5. Conclusions

In this study, the results show that concentration of 500 μmol·L^{-1} of sodium nitroprusside (SNP) treatment could reduce the relative conductivity of tea leaves, inhibit the elevated malondialdehyde content, promote the accumulation of proline, soluble protein and sugar, and increase the superoxide dismutase, catalase activities, thereby alleviate the damage of cold stress on tea leaves. The CsICE1 expression in 500 μM SNP treatment was peaked at 24 h under low temperature stress, CsCBF1 did not express at normal temperature, the expression level after 6 h of chilling stress increased rapidly then decreased after 24 h and reached the peak. Therefore, understanding that how tea plants sense and defense the chilling stress plays an important role to improving the level of production and economic benefits of tea plants.

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Abbreviations

CBF	C-repeat binding factors
cDNA	Complementary deoxyribonucleic acid
cDNA-AFLP	Cdna amplified fragment length polymorphism
cGMP	Cyclic guanosine 5′-monophosphate
ICE1	Inducer of CBF expression 1
qRT-PCR	quantitative real-time polymerase chain reaction

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