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Exogenous Activation of the Ethylene Signaling Pathway Enhances the Freezing Tolerance of Young Tea Shoots by Regulating the Plant's Antioxidant System

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Abstract: Tea plants (*Camellia sinensis* (L.) O. Kuntze) frequently suffer severe damage as a result of freezing temperatures in early spring, which severely affect tea quality and tea production in China. Emerging evidence has demonstrated that the ethylene signaling pathway plays an important role in tea plants' freezing responses. However, how ethylene modulates the response to freezing in sprouting tea shoots is not clear. This study verified that the measurement of relative electrolyte leakage in young shoots after 1 h at −5 °C is a rapid way to evaluate their freezing tolerance in the laboratory. Further exploration of the mechanism involved in increasing tea-shoot freezing tolerance by monitoring changes in the transcription of ethylene-related genes and cold signaling-related genes, and the physiological and biochemical changes after the application of ethephon (2-chloroethylphosphonic acid, an ethylene release reagent), revealed that exogenous ethephon significantly increased the freezing tolerance of tea shoots within 3 days of treatment, while concomitantly altering the expression of the ethylene signaling pathway-related genes (i.e., *CsETR1*, *CsETR2*, and *CsEBF1*). Moreover, antioxidant enzyme activities, including superoxide dismutase, catalase, and peroxidase, were uniformly upregulated, which might constitute a major physiological change induced by ethylene signaling and may be responsible for the observed increase in freezing resistance. Nevertheless, soluble sugars and starch, trehalose metabolism, and cold signaling-related genes did not appear relevant to the freezing tolerance increase following ethephon application. This study demonstrated that the freezing tolerance of sprouting tea shoots can be rapidly increased by the exogenous activation of the ethylene signaling pathway and upregulation of the plant's antioxidant system.

Keywords: ethylene; cold spell; tea shoots; antioxidant activity; freezing tolerance; gene regulation; freezing injury

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1. Introduction

Low temperatures represent a form of environmental stress that causes alterations in a plant's morphological structure, along with its physiological, biochemical, and molecular processes, such as a reduction in cell water content, loss of membrane integrity and increased membrane permeability, and the accumulation of reactive oxygen species (ROS) [1–3]. To alleviate such damage, plants have evolved effective adaptation mechanisms [4]. In particular, cold acclimation (CA) is a key biological process by which plants acquire freezing tolerance [5]. The C-repeat/dehydration responsive-element binding

factor (ICE-CBF/DREBs) transcriptional cascade is a core signaling module for initiating cold-response processes that are regulated by multiple signals, such as plant hormones and calcium levels [5,6]. At the physiological and biochemical levels, the accumulation of sucrose and increased concentrations of certain molecules, such as hydrophilic and late embryogenesis abundant (LEA) polypeptides, in CA enhances cell membrane stability in plants to protect them against freezing stress [7]. Generally, plants can acquire freezing resistance through previous exposure to chilling. However, as extreme weather events are occurring with increasing frequency, de-acclimated plants or newly generated tissues without the prior chilling exposure necessary to obtain this freezing resistance capacity are particularly susceptible to a sudden temperature decrease in early spring (such as a freezing cold spell). Such abnormal freezing stress usually causes severe yield losses, owing to the abortion of flower buds in peach and pear trees, or frostbite of the vegetative organs of vegetables and tea plants [2].

Tea (*Camellia sinensis* (L.) O. Kuntze), a perennial woody cash plant species, is native to southeast China and has become adapted to growing in warm environments [8]. Its young shoots are processed into tea, the most widely consumed non-alcohol beverage in the world [9–11]. Young tea leaves and buds tend to be more sensitive to low temperatures. Frost damage of the new shoots of tea plants in spring generally occurs when the daily average air temperature is below 4 °C and the lowest air temperature is near or below 0 °C [12]. Meteorological data reveals that cold spells occur less than once every two years on average, with the sudden freezing cold shock in early spring destroying the sprouting shoots of tea plants in most productive regions, adversely affecting both tea production and quality in China and causing huge economic losses [6,8]. Accordingly, the selection and cultivation of freezing-tolerant tea varieties is an important approach to coping with low winter temperatures. However, no resistant tea cultivar has yet been developed that can withstand cold-spell damage. Hence, the identification of an exogenous substance that can help plants rapidly increase their freezing tolerance without undergoing CA should prove an effective way to alleviate spring freezing-induced damage. Recently, the application of exogenous calcium has been reported to improve the freezing hardiness of tea plants [13]. Changes in active oxygen-scavenging ability and sugar metabolism are the main mechanisms used to enhance the freezing resistance of tea plants [1,6]. Additionally, Li et al. reported that treatment with melatonin increased the freezing tolerance of tea plants [14]. Similarly, secondary metabolites also improved freezing resistance in tea plants. For example, Wang et al. reported that EGCG could induce freezing tolerance in tea plants via the CsICE-CsCBF-CsCOR pathway [15]. However, the above studies used mature leaves as research objects; thus, it remains unknown whether the conditioning treatments used in such studies can effectively improve shoot resistance to freezing. Our previous analysis of the expression and metabolic spectrum of tea shoots after simulated freezing showed that ethylene and calcium ions were the main signaling pathways in tea shoots [12]. Furthermore, little is known regarding the effect of ethylene on the freezing resistance of tea. The substitution of the ethylene signal with the application of an exogenous compound may be a novel method to improve the freezing resistance of tea shoots.

Although ethylene has an irreplaceable status in the regulation of cold stress, the question of whether it plays positive or negative regulatory roles remains controversial [16]. Thus, the positive regulatory role of ethylene treatment during the post-harvest cold storage of fruit has been extensively investigated. For instance, Lafuente et al. reported that the chilling-induced production of ethylene is involved in the reduction of chilling damage in Fortune mandarin (*Citrus clementina* Hort. Ex Tanaka × *Citrus reticulata* Blanco) fruits; nonetheless, ethylene treatment did not ameliorate the symptoms of chilling damage when these fruits were subsequently exposed to temperatures of 2 °C [17]. In turn, ethylene reduced chilling injury (CI), concomitant with the activation of phenylalanine ammonia-lyase (PAL) [17]. Consistent with this finding, the treatment of citrus fruit with ethylene reduced chilling damage during cold storage (1.5 °C) by promoting PAL and catalase (CAT) activities [18]. Similarly, postharvest propylene (a functional ethylene analog)

treatment reportedly alleviated CI in banana fruits (*Musa* sp. cv. Williams, Cavendish sub-group AAA) and enhanced PAL activity [19]. Furthermore, the positive effect of the ethylene signal on cold tolerance has also been observed in grape (*Vitis vinifera*) and apple (*Malus* × *domestica*) seedlings, during which study, low-temperature treatment increased ethylene production, whereas the application of 1-aminocyclopropane-1-carboxylate (ACC) enhanced the seedlings' cold tolerance [20,21]. In contrast, ethylene plays a negative role in the regulation of cold tolerance in *Medicago truncatula* [22] and *Arabidopsis thaliana* [23], as both plant species are hypersensitive to freezing, following treatment with ethylene or ACC. These results indicate that the ethylene signaling machinery is differentially regulated in different plant species. Moreover, during fruit ripening or exposure to abiotic stress, ethylene production and mitochondria respiration are connected through the involvement of alternative oxidase and plant uncoupling mitochondrial protein; in addition, plasma membrane NADPH oxidases are involved in ethylene and other hormone signaling mediation related to plant stress tolerance by generating ROS [24]. Nevertheless, whether ethylene signaling regulates the freezing resistance of tea shoots, along with the involvement of ethylene signaling in the regulation of the tea plant's antioxidant system, remains to be elucidated.

Here, we investigated the effects of ethylene signaling on the freezing tolerance of tea sprouting shoots by promoting and inhibiting ethylene synthesis and perception. We observed that the freezing resistance of tea plants could be correlated with high ethephon (2-chloroethylphosphonic acid, an ethylene release reagent) levels. Moreover, the ethephon-induced increase in freezing tolerance was associated with the activation of antioxidant enzymes. These results help us to further clarify the mechanism underlying the ethylene-induced freezing tolerance of tea plants and provide a means to anticipate the threat to tea production and quality posed by spring cold spells.

2. Materials and Methods

2.1. Plant Materials

Experimental plant materials included 10-year-old potted plants of the tea cultivars 'Zhongcha 108 (ZC108)' and 'LongJing 43 (LJ43)' grown at the Tea Research Institute of the Chinese Academy of Agricultural Sciences (TRICAAS, 30°18′ N, 120°10′ E) in Hangzhou, China. Partially overwintered tea plants at the budding stage were moved into a growth chamber under a 13 h light/11 h dark photoperiod (LED light, 4000~5000 lux, Ningbo Pulangte Instrument, Ningbo, China) at 24 °C (day) and 20 °C (night), with 70% constant humidity. Another group of tea plants continued to sprout naturally in an outdoor environment. Sprouting tea plants (ZC108) at the one-bud and two-leaf stage, taken from both the growth-chamber and natural-condition groups, were used for the optimization of conditions for electrical conductivity detection. Moreover, tea plants (LJ43) in the growth chamber were treated with different reagents when they reached the one-bud and one-leaf stages; then, the young shoots were sampled for the detection of physiological and molecular indicators at different time points following treatment.

2.2. Exogenous Spraying Treatments and Sampling

For the optimization of conditions for the electrical conductivity measurement assay, young shoots (one bud and two leaves) were sampled for freezing treatment and relative electrolyte leakage (REL) measurement. Four biological replications were included.

For the experiment involving exogenous spraying, 0.005% Tween treatment was used as a negative control, and 10 mM ethephon, 10 mM ACC, 0.1 mM AVG, 1 mM phosgene ($CoCl_2$), and 1 mM silver nitrate ($AgNO_3$) in 0.005% Tween at a volume of 0.5 L were sprayed on the leaves of each group of tea plants until they were dripping. The plants were sprayed once at the same time of day; young shoots were sampled at the same time of day for REL measurement at 3 d following treatment. To further explore the role of ethephon in freezing resistance enhancement and the related physiological and molecular changes, a reduced dose of ethephon treatment at 1 mM was administered, and a detailed timetable

(1, 3, 5, and 7 days after treatment) for sampling was carried out. Five biological replications were performed for REL measurement, along with three biological replications for enzyme activity, sugar content, and gene expression analysis. REL measurements were performed immediately in the laboratory once sampling was completed; samples for physiological and molecular analyses were frozen in liquid nitrogen and then stored at −80 °C.

2.3. Relative Electrolyte Leakage Assay and ACC Measurement

To optimize the conditions for the REL assay, at least three young shoots were placed in a 50-mL centrifuge tube. Then, 15 mL of ultrapure water was added and the tube was shaken at 200 rpm at 25 °C for 2 h. Electrical conductivity was measured and the value was recorded as *a*. An equal number of young shoots was placed in 50-mL centrifuge tubes and treated at 0, −2, and −5 °C for 1 h. Then, 15 mL of ultra-pure water was added, and the buds were shaken at 200 rpm for 2 h at 25 °C. Conductivity was measured and the value was recorded as *b*. After the determination of *a*, the same sample was boiled in a water bath for 15 min and cooled to 25 °C, and the measured value was marked as *d*. After the determination of *b*, the same sample was boiled in a water bath for 15 min and cooled to 25 °C, then the determined value was marked as *c*. REL was determined using the formula REL = $(b - a)/(c - a) \times 100\%$. The same assay method was performed for other samples once the optimal freezing temperature was determined.

For the content determination of ACC, 0.1 g of ground samples, mixed with an extra 1 mL of water, were kept overnight. After centrifuging these samples, 0.2 mL measures of supernatants were put into 2 mL Eppendorf tubes; an amino acid standard solution was also added into another empty 2 mL Eppendorf tube. To each tube, 20 µL of norleucine internal standard solution, 100 µL of triethylamine acetonitrile solution, and 100 µL of phenyl isothiocyanate acetonitrile solution were added. After keeping the mixture at 25 °C for 1 h, 400 µL n-hexane was added to each tube, and the mixture was incubated for 10 min. The solutions in the lower layer were filtered through a 0.45 µm membrane and then used for ACC analysis via a high-performance liquid chromatography (HPLC) method (RIGOL L3000, Beijing, China). Chromatographic separation was performed on a C18 (Sepax Technologies, Newark, DE, USA) analytical column (250 × 4.6 mm; 5-µm particle size) at 40 °C. The column flow rate was maintained at 1 mL/min. Sodium acetate (0.76%) in 93:7 water/acetonitrile was used as mobile phase A, and 80% acetonitrile was used as mobile phase B. Norleucine was used as an internal standard.

2.4. Measurement of Enzyme Activity

Total protein content was determined using a bicinchoninic acid assay (*BCA*) kit (Suzhou Comin Biotechnology, Suzhou, China), following the manufacturer's instructions. Briefly, 1 g of sample was added to 10 mL of enzyme extraction buffer. Then, the absorbance at 562 nm was quantified using a spectrophotometer.

The activities of SOD, POD, and CAT were estimated using the corresponding reagent kits (Superoxide Dismutase (SOD) Test Kit (WST-8 assay/microdilution assay), Plant Peroxidase (POD) Assay Kit (Guaiacol Colorimetry) (Suzhou Comin Biotechnology, Suzhou, China), and Catalase (CAT) Detection Kit (ultraviolet absorption) (Suzhou Comin Biotechnology, Suzhou, China), following the manufacturer's instructions. Samples of approximately 0.1 g, previously ground with 1 mL of extraction buffer in an ice bath, were poured into 2 mL tubes. The tubes were centrifuged at 8000× *g* for 10 min at 4 °C and the supernatants were used for the assay.

2.5. Measurement of Sugar Contents

For the starch content assay, the plant starch content assay kit (microdilution assay) (Suzhou Comin Biotechnology, Suzhou, China) was applied following the manufacturer's instructions. Briefly, samples of 0.1 g, ground with 0.1 mL of reagent I from the kit, were transferred to Eppendorf tubes and placed in an 80 °C water bath for 30 min, then centrifuged at 3000× *g* for 5 min at 25 °C. The precipitates were resuspended with 0.5 mL of distilled water

and incubated in a water bath at 95 °C for 15 min. After cooling the samples to 25 °C, 0.35 mL of reagent II was added to each tube and the tube was kept for 15 min at 25 °C. Subsequently, the samples were mixed with an extra 0.85 mL of distilled water and centrifuged at 3000× *g* for 10 min at 25 °C. The supernatants were used for starch measurement.

For soluble sugar content determination, the 0.1 g samples, ground with 0.1 mL of distilled water, were poured into centrifuge tubes and then placed in a water bath at 95 °C for 10 min. The tubes were centrifuged at 8000× *g* for 10 min at 25 °C after cooling to room temperature. The supernatants were used for soluble sugar measurement. Soluble sugar content was determined, using the Plant Soluble Sugar Content Assay Kit (microdilution assay) (Suzhou Comin Biotechnology, Suzhou, China) according to the manufacturer's instructions.

For the trehalose content assay, the 0.1 g samples, ground with 0.1 mL of extraction buffer under an ice bath, were poured into 2 mL tubes. After keeping the tubes for 45 min at room temperature, they were centrifuged at 8000× *g* for 10 min at 25 °C and the supernatants were used for the trehalose assay. Trehalose sugar content was determined using the Plant Soluble Sugar Content Assay Kit (microdilution assay) (Suzhou Comin Biotechnology, Suzhou, China), according to the manufacturer's instructions.

2.6. RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted from 0.1 g tissue samples using the RNAprep Pure Plant Plus Kit (Tiangen, Beijing, China). The total RNA (1 μg) was treated with RNase-free DNase I (Takara, Otsu, Japan) to remove the residual genomic DNA. In turn, cDNA was synthesized using a PrimerScript RT reagent kit (Takara, Japan), according to the manufacturer's protocol. cDNA products (20-fold dilution) were used as the template, then quantitative real-time PCR (qRT-PCR) was performed using the SYBR Green I Master Mix on a LightCycler 480 Real-time PCR system (Roche, Madison, WI, USA). *CsPTB* was used as a reliable internal reference [25]. The primer sequences used for qRT-PCR are listed in Table 1. The reaction setup, program, and data analysis used for qRT-PCR were as previously described by Hao et al. [25].

Table 1. Primer sequences for gene expression profiles.

Gene	Primer	Primer Sequence (5′-3′)	Product Size (bp)
CsETR1	*qRT-CsETR1-F*	CACGTACTGGGCTAGAGCTTCAACTTTC	192
	qRT-CsETR1-R	CAACCACCTCTCCGGGCATGTATTTTC	
CsETR2	*qRT-CsETR2-F*	TCTCTTGGCTCGGCTGTCTCTTCGTT	154
	qRT-CsETR2-R	CATCTTCATCGCCACTTGCTGTCA	
CsERS1	*qRT-CsERS1-F*	CCGAAATAATGGTGGTGCTGGTCTGG	166
	qRT-CsERS1-R	CTGCATTGTTGGTTCATTTGGGCTATTG	
CsEIN2	*qRT-CsEIN2-F*	GGAGAGGGCTGTGTTTGGAGAGTGGA	199
	qRT-CsEIN2-R	GCAGGCAGAAGCAGGGGGTCATT	
CsEIN3	*qRT-CsEIN3-F*	ATGGTGGCCTCAATTGGGTCTTCC	198
	qRT-CsEIN3-R	GTGGCACTCTCCTTCGCTGTCATCTTAT	
CsEBF1	*qRT-CsEBF1-F*	GACCTCTGCCAGTGTCCTTC	191
	qRT-CsEBF1-R	CCCTGATCCCCAACATGAGG	
CsNADPHoxidase-2	*qRT-CsNADPHoxidase-2-F*	TGGGAAAGCAAGTGAGTGACAATAGC	169
	qRT-CsNADPHoxidase-2-R	TAAGCAGAGAAAGACCAAACCAAGAGTG	
CsAPX-6	*qRT-CsAPX-6-F*	TTGTTGCATTATCCGGGGCT	151
	qRT-CsAPX-6-R	GCACGATCTGAAGGAAGACCA	
CsGST-3	*qRT-CsGST-3-F*	GAAGTTGTTGGGACATTGGGC	186
	qRT-CsGST-3-R	GACTCGCAAATGGGCTTTCC	
CsGST-4	*qRT-CsGST-4-F*	AGCTTCTAGGTGCATCGCC	165
	qRT-CsGST-4-R	CACTATGGATGAGAACCGGCA	
CsGPX	*qRT-CsGPX-F*	GCTCCCTTTTGCAGTGGTTT	161
	qRT-CsGPX-R	ATTGTGCATGCGGTTTCCTG	
CsGR	*qRT-CsGR-F*	ATGGTGAAGAAATAAGGGCTGATGC	152
	qRT-CsGR-R	TATGCTTGGTATGTTTGTGCGAGAGT	
CsCu-ZnSOD	*qRT-CsCu-ZnSOD-F*	TGGGATTGTTGGTTTCTCGGT	214
	qRT-CsCu-ZnSOD-R	GGCAAAGCTGATGCTCAACC	
CsPTB1	*qRT-CsPTB1-F*	TGACCAAGCACACTCCACACTATCG	107
	qRT-CsPTB1-R	TGCCCCCTTATCATCATCCACAA	

2.7. Statistical Analysis and Graphing

To visualize the changing trends of enzyme activity and sugar content, the detected result at a given time point was divided by the value of day 0. A one-way ANOVA, followed by Tukey's HSD comparisons, was used for an analysis of the statistical differences. The *t*-test function in Microsoft Excel XP was used for the analysis of statistical differences, where $p < 0.05$ was considered significant and $p < 0.01$ was considered highly significant. Values shown are the means ± SE. All graphs were generated using GraphPad Prism 8 (https://www.graphpad.com/, accessed on 1 October 2022).

3. Results

3.1. Determination of REL in Tea Shoots and the Effects of Ethylene Signaling Regulators

REL is an important index that is commonly used to characterize and compare the freezing resistance of plants grown under different conditions. The morphological structure and freezing resistance of tea shoots differ with their developmental state. Therefore, it is necessary to optimize the conductivity measurement conditions. In this study, one bud and two leaves were used for the measurement of REL. For young shoots that germinated under natural conditions, compared with the control (normal condition), those receiving the −2 °C and −5 °C treatments exhibited a 3.86% and 80.22% increase in REL, respectively. Similarly, for young shoots that germinated in a growth chamber, the −2 °C and −5 °C treatments increased the REL by 0.1% and 47.46%, respectively. Thus, the freezing resistance of tea shoots could be effectively identified by measuring REL, following low-temperature treatment at −5 °C for 1 h.

To determine the effect of ethylene on the freezing tolerance of tea shoots, two ethylene biosynthesis inhibitors (AVG and $CoCl_2$), an ethylene action inhibitor ($AgNO_3$), an ethylene release agent (ethephon) [26], and the precursor of ethylene biosynthesis (ACC) were used to manipulate either ethylene levels or ethylene signaling. The ratio of REL (−5 °C) in the presence of molecules, vs. REL (−5 °C) with no molecules, is shown in Figure 1B. The ratio decreased by 2.97, 1.97, 1.72, and 1.00 times upon ethephon, ACC, AVG, and $CoCl_2$ treatment, respectively, whereas it increased 1.58 times upon $AgNO_3$ treatment. Among these, ethephon treatment with enhanced ethylene signaling effectively reduced the REL and enhanced the freezing resistance of freezing-treated tea shoots, whereas $AgNO_3$, a competitive inhibitor of the ethylene signal receptor, significantly reduced the freezing resistance of the shoots. In contrast, neither exogenous ACC-, $CoCL_2$-, nor AVG-treated plants showed any significant effects.

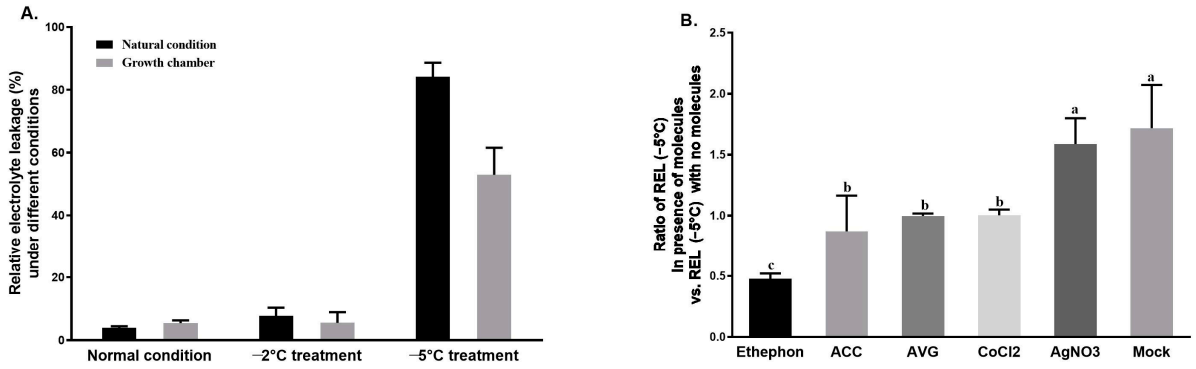

Figure 1. Relative electrolyte leakage (REL) detection. (**A**) REL of tea shoots under different treatments. (**B**) Effects of ethylene enhancer (ethephon, ACC) or inhibitor (AVG, $CoCl_2$, or $AgNO_3$) treatment on the REL (−5 °C) of tea shoots. Error bars indicate the standard error of the mean (SEM) for five independent experiments. Bars with different letters indicate significant differences ($p < 0.05$), as assessed by a one-way ANOVA, followed by Tukey's HSD comparisons.

3.2. Time Course of Ethephon Application and the Enhancement of Freezing Resistance

To further clarify the mechanism whereby ethephon promotes the freezing tolerance of sprouting tea shoots, plants at the one-bud and one-leaf stage were treated with ethephon, after which the young shoots were collected at different time points after treatment to measure the REL. Under freezing stress, in comparison to the 0.005% Tween-treated control samples, REL levels were reduced by 14.10% at 1 d after ethephon treatment (Figure 2A) and were reduced by 12.22% at 3 d after ethephon treatment. Under freezing stress, compared to 0.005% Tween treatment samples, tea plants that were sprayed with ethephon had a lower REL on days 1 and 3. These results indicated that ethephon treatment significantly enhanced the freezing resistance of tea plants in the first 3 days, after which the effect gradually declined (Figure 2A). Moreover, the content of ACC, an ethylene synthesis precursor, was significantly higher than that of the control plants after the application of ethephon for 1 to 7 days (Figure 2B). These findings indicate that ethephon application enhanced the freezing resistance of shoots by increasing ACC content and provided the potential for a rapid response to spring freezing injury to tea plants during the production season.

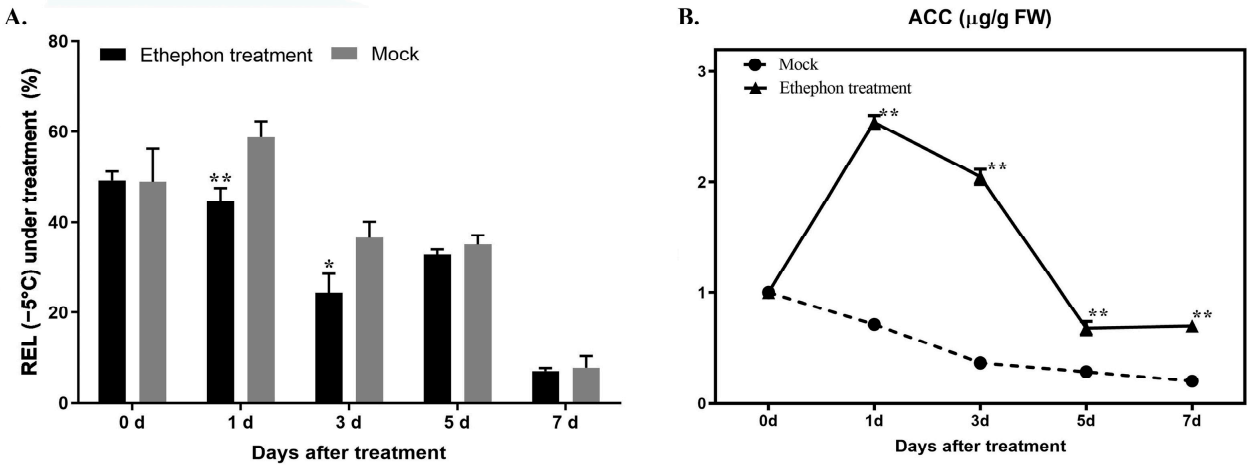

Figure 2. Relative electrolyte leakage (REL) detection and the ACC content measurement of tea shoots under ethephon treatment. (**A**) REL assessment (−5 °C). Data are the means ± SE of five biological replicates. Mock: 0.005% Tween treatment. (**B**) Changes in ACC content following ethephon treatment. Data are the means ± SE of three biological replicates. * Indicates significant differences ($p < 0.05$); ** indicates highly significant differences ($p < 0.01$), as determined using the *t*-test. FW: Fresh weight.

3.3. Changes in Antioxidant Enzyme Activity after Ethephon Application

To test the physiological change in tea shoots following ethephon application, the samples' antioxidant enzyme activities and metabolites were analyzed. SOD can scavenge the superoxide radicals induced by oxidative stress, mainly by converting O_2^- to O_2 and H_2O_2 [23]; then, removal is completed via peroxidase POD or CAT [4]. In our study, all the antioxidant enzyme activities of SOD, POD, and CAT were significantly higher than those of the control plants at 1 d following ethephon treatment (Figure 3). The enzyme activities of SOD, POD, and CAT increased by about 39.1%, 33.0%, and 34.9%, respectively. The increases in SOD enzyme activities continued during the days following ethephon treatment; however, the POD level showed no significant change at 3–7 d following ethephon treatment. These results suggest that ethephon treatment could activate the antioxidant system of the tea plants.

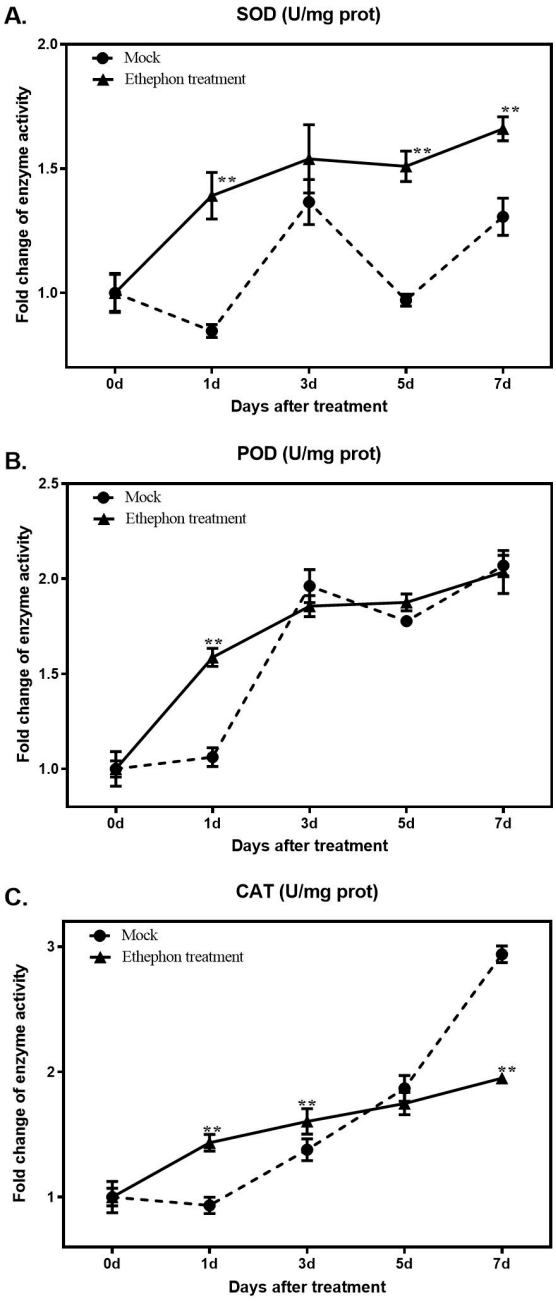

Figure 3. Antioxidant enzyme activity detection in tea shoots following ethephon treatment. (**A**) SOD activity; (**B**) POD activity; (**C**) CAT activity; data represent the means ± SE of three biological replicates. ** indicates highly significant differences ($p < 0.01$), as determined using the *t*-test. FW: Fresh weight.

However, soluble sugar, which maintains cell membrane integrity, showed a significant decrease after ethephon treatment, compared to control plants; in contrast, starch content was significantly higher in treated plants than in the control plants. This might be related to the developmental stage of the tea plants. In turn, the trehalose content showed no significant change upon ethephon treatment (Figure 4). Together, these findings indicate that whereas the mechanism of freezing resistance enhancement in tea plants occurred mainly by increasing the soluble sugar content, the ethylene signaling pathway rapidly promoted freezing resistance in tea shoots, mainly through a rapid increase in antioxidant enzyme activities and the enhancement of ROS-scavenging ability.

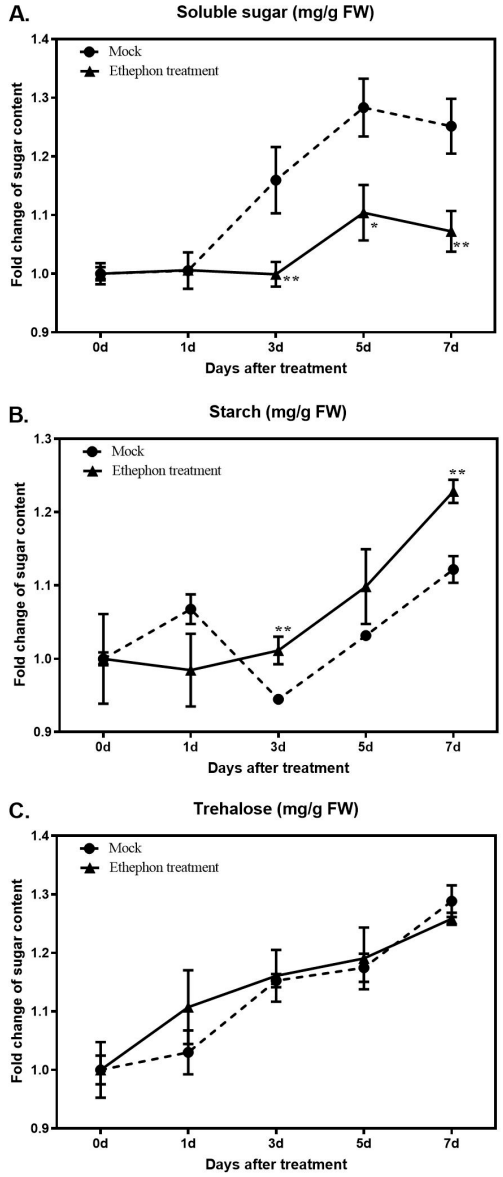

Figure 4. Sugar content changes in tea shoots following ethephon treatment. (**A**) Soluble sugar content; (**B**) starch content; (**C**) trehalose content. FW: Fresh weight. Data represent the means ± SE of three biological replicates.* Indicates significant differences ($p < 0.05$); ** indicates highly significant differences ($p < 0.01$), as determined using the *t*-test.

3.4. Analysis of Gene Expression Related to Ethylene Signalling and Antioxidant Activities

Following ethephon treatment, the expression levels of *CsETR1*, *CsETR2*, *CsEIN2*, *CsEIL3*, and other genes involved in ethylene signal transduction were observed to differ significantly (Figure 5). Compared with that of the control, at 1 h following treatment, the expression of *CsETR1* and *CsEBF1* exhibited maximum differences, being down-regulated 0.48 times ($p < 0.01$) and 0.70 times ($p < 0.05$), respectively. The *CsETR2* and *CsEIN2* transcript levels reached maximum differences at 1 and 5 d, respectively (fold-increase of 1.49 times ($p < 0.05$) and 1.21 times ($p < 0.05$)). Conversely, *CsERS1* did not exhibit any significant difference between ethephon treatment samples and the control samples (Figure 5). These results showed that the increase in the freezing resistance of the sprouting shoots induced by ethephon treatment was mainly achieved by stimulating the ethylene signaling pathway. Nonetheless, the way in which ethylene signaling further increased freezing tolerance warrants further study.

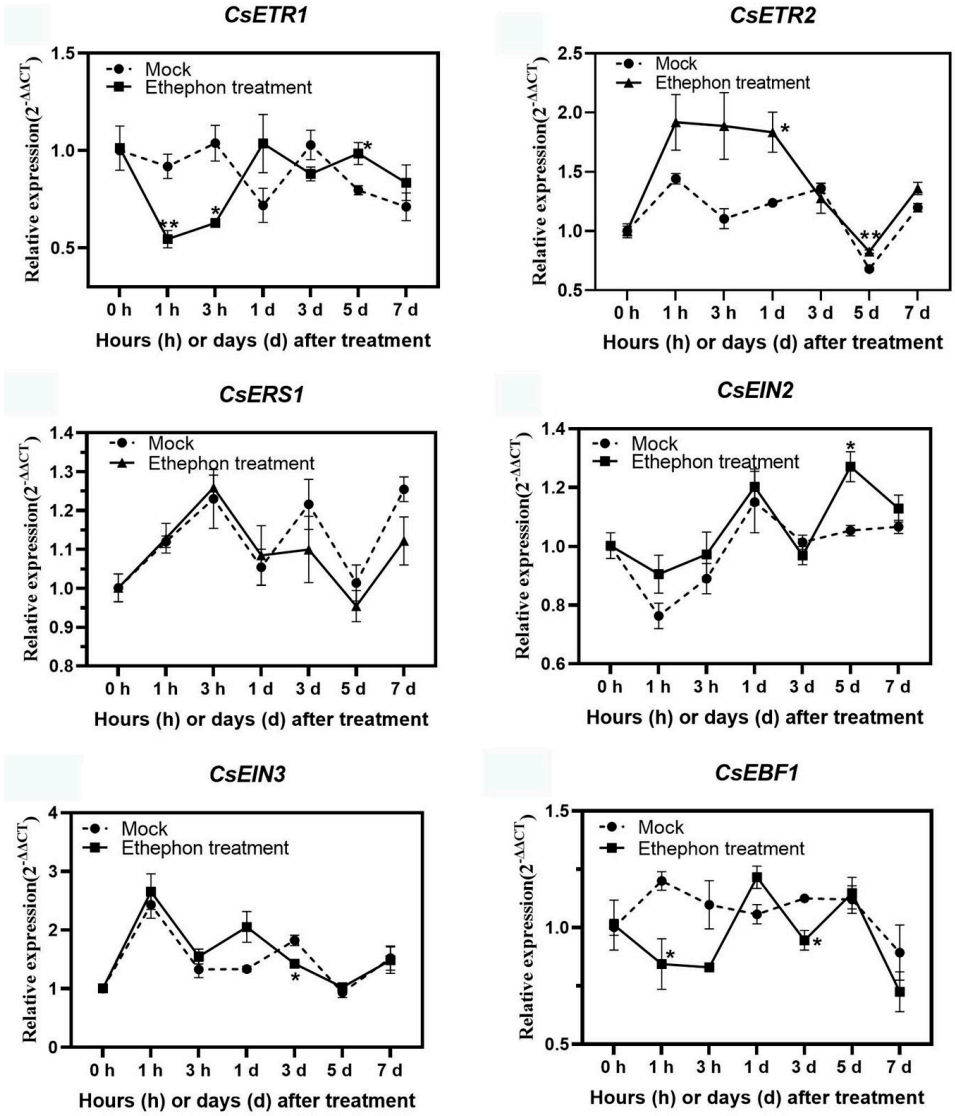

Figure 5. Expression patterns of ethylene signaling pathway-related genes following ethephon treatment at different times. The solid line represents treatment with ethephon; the dotted line represents the negative control (sprayed with water). Error bars indicate the standard error of the mean (SEM) of three independent experiments. * Indicates significant differences ($p < 0.05$); ** indicates highly significant differences ($p < 0.01$), as determined using the *t*-test.

The expression variation of genes related to ROS metabolism following ethephon treatment was further detected. Notably, the gene for NADPH oxidase, which is engaged in ROS production, was significantly suppressed at 1 and 3 d following ethephon treatment, with the expression fold change being 9.55 times less than that of the control. In contrast, the expression levels of *CsAPX-6*, *CsGST-3*, and *CsGST-4*, encoding proteins engaged in ROS-scavenging, were upregulated following ethephon treatment. In particular, the upregulation of *CsAPX-6* was observed at 7 d following ethephon treatment and showed a maximum increase (1.02-fold) at 1 d. The expression of *CsGST-3* and *CsGST-4* was rapidly stimulated and reached its maximum increase at 7 d (13.40-fold) following ethylene treatment. Nevertheless, the *CsGPX*, *CsGR*, and *Cu-ZnSO*$_4$ genes did not show significant variation following ethephon treatment. These results suggested that ethephon reduced ROS production by suppressing NADPH-oxidase expression while increasing ROS-scavenging-related gene expression (Figure 6).

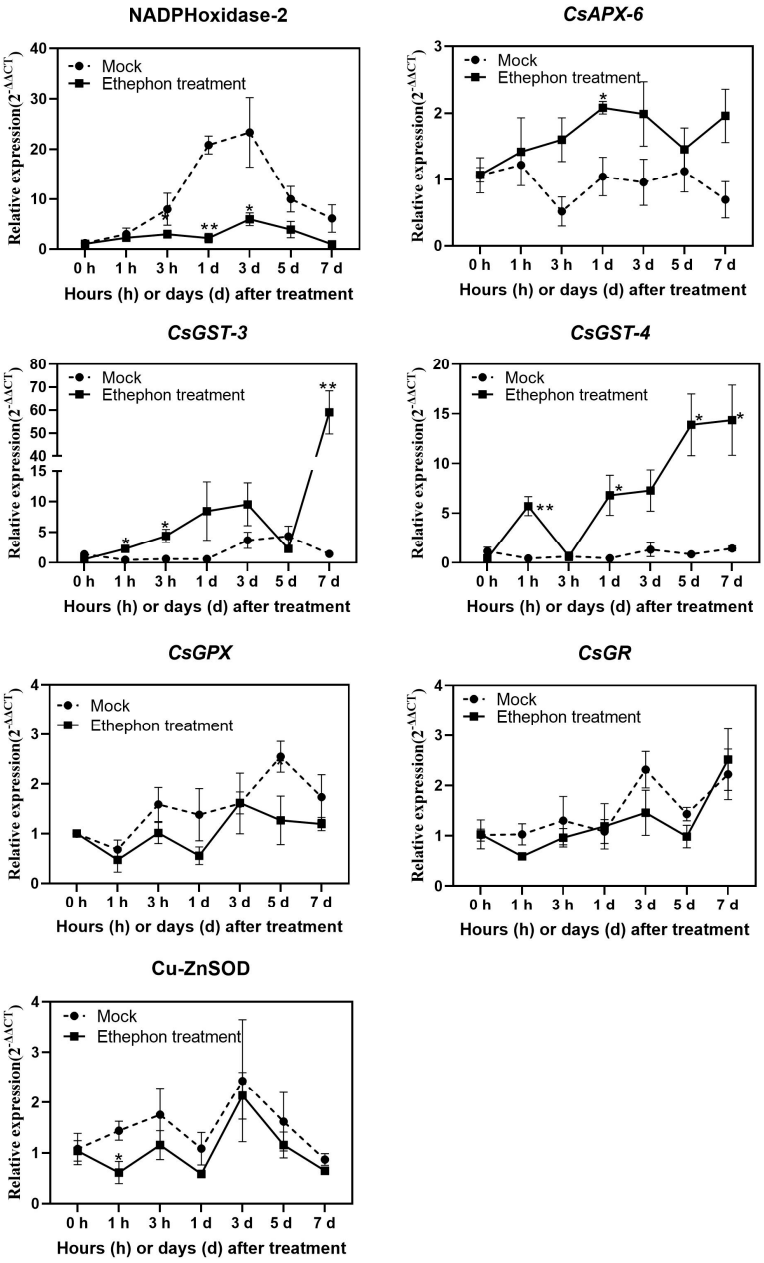

Figure 6. Expression analysis of ROS metabolism-related genes following ethephon treatment at different times. The solid line represents treatment with ethephon; the dotted line represents the negative control, sprayed with water. The error bars indicate the standard error of the mean (SEM) from three independent experiments. * Indicates significant differences ($p < 0.05$); ** indicates highly significant differences ($p < 0.01$), as determined using the *t*-test.

4. Discussion

An increasing frequency of cold spells in late spring, occurring due to climate change, is severely affecting tea production in China and other countries. Therefore, it is urgent to elucidate the common cold spell-responsive mechanisms across sprouting tea shoots and find a method to rapidly improve the freezing resistance of tea. In our previous studies, plant hormones and sugar metabolism were found to play a central role in the tea plants' response to winter cold [6,27]. Furthermore, during a cold spell, the role of the ethylene signaling pathway in response to freezing stress was highlighted [6]. During the past few decades, increasing evidence suggests that ethylene has crucial regulatory effects on plant growth and development, and on plant responses to environmental stress factors [15,20,28].

Ethylene occurrence in response to freezing has also been documented in several plant species, although whether ethylene plays positive or negative regulatory roles may differ among species. Earlier studies demonstrated that cold treatment altered the release of ethylene [29] in plants such as rice, maize, and cucumber. Ethylene level is reportedly lower following CA in *Medicago truncatula* seedlings, while inhibiting its biosynthesis-enhanced freezing tolerance [22]. Similarly, Shi et al. observed that ethylene played a negative regulatory role in *Arabidopsis*, and that ACC- and AVG-treated plants showed lower and higher freezing tolerances, respectively [23]. In contrast, Wang et al. reported the enhancement of freezing hardiness in the ACC-treated roots of apple seedlings [21]. Furthermore, CA can induce ethylene production by inducing the expression of *NtACS1/3* and *NtACO1* [30], whereas the inhibition of ethylene biosynthesis by antisense *SlACS2* reduced the chilling tolerance of tomato fruit [31]. In the present study, ethephon- and Ag^+-treated tea shoots showed increased and decreased freezing hardiness, respectively, which is consistent with earlier studies on apple and tomato plants [21,32].

Ethylene biosynthesis and signaling enhancers or inhibitors serve as effective tools to investigate the action and internal mechanism of ethylene with regard to altering plants' freezing tolerance. Notably, not all treatments using ethephon and ACC as enhancers and AVG, $CoCl_2$, and $AgNO_3$ as inhibitors had an effect on tea-shoot freezing tolerance. Ethephon has a hormonal mechanism of action and its effects are dependent on ethylene release [25]. Ethephon can spontaneously release ethylene without relying on ACC as a substrate. In the present study, ethephon, but not ACC, exhibited a freezing tolerance-enhancement effect in tea shoots within a short time period following surface spraying of the leaves. AVG and $CoCl_2$ are inhibitors of ethylene synthesis, whereas $AgNO_3$ is an inhibitor of ethylene signaling; these also showed varied effects regarding freezing tolerance induction or suppression in tea shoots. ACC, the immediate precursor of ethylene in ethylene synthesis, is a non-proteinogenic amino acid produced by ACC synthase (ACS). ACC is widely used to induce ethylene responses. However, because of the waxy layer on the surface of tea leaves, it is unclear whether ACC can effectively enter tea leaves via spraying. Moreover, recent research demonstrates that ACC exhibits ethylene-independent signaling during *Arabidopsis thaliana* reproduction [31]. Such considerations may underlie the different effects seen from ACC and ethephon treatments in the present study; nevertheless, the underlying mechanism remains to be elucidated. Therefore, this study mainly focused on the role and possible mechanism of ethephon in enhancing the freezing resistance of tea plants.

In addition to the ethylene biosynthesis pathway, multiple ethylene signaling regulators are involved in the plant's response to freezing stress. In previous studies, overexpression of the tomato ethylene response factor TERF2/LeERF2 induced ethylene production and increased freezing tolerance with the induction of COR genes and a reduction in ROS production and accumulation; moreover, the transgenic plants demonstrated the accumulation of osmolytes and the formation of chloroplasts [30,33]. ERF108 has also been shown to act in freezing resistance by regulating the synthesis of raffinose through binding to the GCC box in the promoter of *PtrRafS*. Furthermore, plants overexpressing the *PtrERF108* genes showed lower MDA and H_2O_2 levels and greater freezing tolerance upon exposure to a -4 °C freezing treatment [34]. In a recent study, lower MDA content and ROS accumulation and higher antioxidant enzyme activities were observed in *CdERF1*-overexpressing *Arabidopsis* plants. In addition, the genes *CBF2*, *pEARLI1* (encoding a lipid transfer protein), *PER71* (encoding a peroxidase), and *LTP* (encoding a lipid transfer protein) were activated in transgenic plants [35]. In our study, the expression of genes that regulate the ethylene signaling pathway, including *CsETR1*, *CsETR1*, *CsEIN2*, and *CsEIL3*, differed significantly following ethephon treatment. Such differences suggest that ethylene induces freezing tolerance by activating the ethylene signaling pathway.

Cell membrane damage, which can be measured by the relative extent of electrolyte leakage, commonly occurs in plants exposed to freezing stress [12]. Our results showed that the increase in the ethylene signaling pathway was related to CI reduction, exhibited

as a decrease in REL. As important osmotic substances in the process of CA, soluble sugars reportedly act as membrane stabilizers and might act as antioxidant substances in response to freezing stress. The production of ROS scavengers induced by soluble sugars presumably serves to protect the cells from oxidative stress [27,36]. Our results showed that the contents of, e.g., soluble sugar, starch, and trehalose in young shoots did not show significant changes under ethephon treatment, which finding was inconsistent with the observation of the degradation of starch and the increase in soluble sugar during CA [27]. Hence, the enhancement of chilling tolerance induced by ethylene signaling would not be triggered by sugar metabolism in tea plants. The existence of other compatible metabolites related to chilling tolerance, such as small peptides and amino acids, remains to be determined. ROS plays a dual role in plant biology and is closely related to the plant stress response [37]. In apple plants, the application of exogenous ACC significantly promoted antioxidant enzyme activities and accumulated H_2O_2 to a lower extent, accompanied by ethylene production [21]. Therefore, high antioxidant activities from enzymes such as SOD, CAT, and POD in ethephon-treated tea plants might serve to scavenge free radicals and protect plant cells from oxidative damage.

Plants under freezing stress over-accumulate ROS, which leads to oxidative damage [1]. Two major sources of ROS are currently recognized, derived from the mitochondrial respiratory chain and via NADPH oxidases. In the present study, the NADPH oxidase gene was upregulated under chilling conditions, whereas ethephon application significantly decreased its expression. In addition, the expression of genes related to the clearance of ROS was activated under ethephon treatment. These results were corroborated by our physiological data, such as the significant enhancement of antioxidant enzyme activity following ethephon treatment. These results indicated that ethephon treatment might act to improve freezing tolerance by enhancing antioxidant enzyme activities through the activation of the ROS signaling pathway. To some extent, our study revealed the mechanism of ethylene signaling for improving freezing resistance in tea shoots. However, further research is warranted to elucidate more details on the regulatory pathways. Nonetheless, this study provided an effective method to rapidly improve the freezing resistance of tea shoots and minimize the threat that increasingly frequent spring cold spells pose to tea productivity.

5. Conclusions

Exogenous ethylene application played a positive role in the response to a cold spell found in the sprouting shoots of tea plants. Freezing tolerance of tea shoots can be enhanced by alleviating cell injury, increasing antioxidant activities, and altering ethylene signaling pathway-related gene expression. Therefore, our study provides a sound theoretical basis for the development of a practical measure that tea farmers may use to effectively protect their tea plantations from freezing injury by enabling tea plants to respond rapidly to an early spring cold spell.

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