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ARTICLE

Foliar Application of γ-Polyglutamic Acid Enhances Chilling Tolerance in Pepper Seedlings by Orchestrating Root-to-Shoot Defense Responses

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ABSTRACT: Pepper (*Capsicum annuum* L.) is highly susceptible to chilling stress, which severely constrains its growth and productivity. Although the eco-friendly biostimulant γ-polyglutamic acid (γ-PGA) has shown promise in enhancing plant tolerance to abiotic stresses, its specific role and underlying mechanisms in alleviating chilling injury in pepper remain poorly understood. This study systematically investigated the physiological and molecular mechanisms by which foliar application of 100 mg·L^{-1} γ-PGA enhances chilling tolerance in pepper seedlings. Our results demonstrated that γ-PGA pretreatment significantly mitigated chilling-induced growth inhibition and promoted root development, evidenced by a 110.8% increase in the number of root tips. Analysis of photosynthetic performance revealed that γ-PGA effectively counteracted chilling-induced photosynthetic suppression, increasing the net photosynthetic rate (*Pn*) by 172.9% and the maximum photochemical efficiency of PSII (*Fv*/*Fm*) by 17.9%. Furthermore, γ-PGA treatment significantly reduced the accumulation of reactive oxygen species ($O_2^{\cdot-}$ and H_2O_2) and malondialdehyde (MDA) while promoting the synthesis of the osmoprotectant proline. This protective effect was associated with a strengthened antioxidant defense system; γ-PGA enhanced the activities of superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX). These changes were supported at the molecular level by the significant upregulation of their corresponding genes (*CaSOD*, *CaCAT*, and *CaAPX*), with *CaAPX* expression showing a striking 313.5% increase. In conclusion, foliar application of γ-PGA enhances chilling tolerance in pepper seedlings via a multi-faceted mechanism that includes improving root architecture, reinforcing the antioxidant defense system, facilitating osmotic adjustment, and protecting the photosynthetic apparatus. These findings provide a theoretical framework and practical support for using γ-PGA as an effective and sustainable biostimulant to improve pepper cultivation in environments prone to chilling stress.

KEYWORDS: Pepper; seedlings; γ-poly glutamic acid; chilling stress; antioxidant system; root morphology; photosynthesis

1 Introduction

Pepper (*Capsicum annuum* L.), an important economic crop native to the tropics, and its plants, particularly at the seedling stage, are highly sensitive to low-temperature stress [1]. China is a major global producer and consumer of peppers. Under cultivation systems such as solar greenhouses and early spring open-field cultivation, low temperature is an important environmental stress factor limiting pepper production, often leading to inhibited growth, reduced yield, and deteriorated quality [2,3]. Low-temperature stress causes excessive accumulation of reactive oxygen species (ROS) by disrupting key physiological processes such as photosynthesis, membrane system integrity, and redox homeostasis, ultimately resulting

in oxidative damage [4,5]. To mitigate chilling injury, researchers have pursued several agronomic strategies, including the development of cold-tolerant cultivars through selective breeding, the implementation of grafting techniques, and the application of exogenous protectants [2,6,7]. Among these approaches, the foliar application of bioactive compounds has attracted significant interest due to its operational simplicity, cost-effectiveness, and broad applicability. Various exogenous substances, such as salicylic acid, melatonin, and brassinosteroid, have been demonstrated to effectively enhance plant cold tolerance [5,8,9].

As an eco-friendly, non-toxic, and biodegradable polymer produced through microbial fermentation, poly-γ-glutamic acid (γ-PGA) has garnered significant attention as a potent agricultural biostimulant, primarily due to its ability to enhance plant resilience against abiotic stresses [10,11]. Studies have demonstrated, for example, that γ-PGA application enhances drought tolerance in maize by modulating photosynthesis and rhizosphere microbiology [12] and alleviated heavy metal toxicity (Cd and Pb) in cucumber by increasing antioxidant capacity [13]. In canola seedlings, γ-PGA has also been shown to stimulate proline biosynthesis, strengthening defenses against both salinity and chilling [14]. The fundamental mechanisms for this stress mitigation typically involves modulating key physiological responses, such as increasing the activity of antioxidant enzymes (e.g., superoxide dismutase (SOD), peroxidase (POD)), lowering malondialdehyde (MDA) content, and promoting the accumulation of osmolytes like proline to maintain cellular homeostasis [15,16].

The capacity of γ-PGA to protect plants against abiotic stresses is well-documented. Previous research has demonstrated that the exogenous application of γ-PGA promotes growth, increases yield, and mitigates heavy metal stress in pepper plants [17]. However, the role of γ-PGA in alleviating low-temperature stress in pepper, along with its underlying molecular mechanisms, remains largely unexplored. This study, therefore, investigates the physiological and molecular mechanisms by which γ-PGA enhances cold tolerance in pepper seedlings. We hypothesized that exogenous γ-PGA application mitigates chilling-induced damage by activating a multi-faceted defense response, which includes promoting root development, enhancing the capacity for ROS scavenging via the upregulation of antioxidant genes, and protecting the photosynthetic apparatus. To test this hypothesis, we investigated the effects of foliar-applied γ-PGA on root morphology, ROS accumulation, osmotic regulation, antioxidant enzyme activities, the expression of related genes (*CaSOD*, *CaCAT*, and *CaAPX*), and key photosynthetic parameters under controlled chilling stress. The findings are intended to provide a scientific basis for the application of γ-PGA as an effective strategy to improve cold resistance in pepper cultivation.

2 Materials and Methods

2.1 Plant Materials and Cultivation Conditions

The experimental material utilized in this study was Xiangla Bopi pepper (purchased from Qingxian Chunfeng Seed Co., Ltd., Hebei, China). The growing substrate consisted of a sterilized mixture of peat, perlite, and vermiculite (1:1:1, v/v/v). Individual seeds were sown in plastic pots (10 cm diameter, 12 cm height), each containing 120 g of the substrate, and then covered with a 1–2 cm layer of the same material. The pots were subsequently placed in a growth chamber (RDN-1000C, Ningbo Yanghui Instrument Co., Ltd., Zhejiang, China) at the Institute of Subtropical Agriculture, Fujian Academy of Agricultural Sciences (24°32′47.67″ N, 117°43′58.50″ E). Growth conditions were set to a 12 h/12 h light/dark photoperiod, a day/night temperature of 28/20°C, 70% relative humidity, and a maximum photosynthetic photon flux density (PPFD) of 480 μmol·m^{-2}·s^{-1}. Plants were irrigated weekly with half-strength Hoagland's nutrient solution.

2.2 Treatments

Uniformly grown pepper seedlings were selected at the six-leaf stage (approximately 48 days after sowing) and divided into four treatment groups. The treatments were as follows: (1) Control (CK): seedlings sprayed with sterile water and maintained under normal temperature conditions; (2) γ-PGA (CK+γ-PGA): seedlings sprayed with a 100 mg·L^{-1} γ-PGA solution under normal temperature conditions; (3) Low-temperature stress (L): seedlings sprayed with sterile water and subjected to low-temperature stress; and (4) Low-temperature stress + γ-PGA (L+γ-PGA): seedlings sprayed with a 100 mg·L^{-1} γ-PGA solution and subjected to low-temperature stress. The γ-PGA concentration was adopted from Gong et al. [17]. Each treatment was performed in triplicate, with 30 plants per replicate. Foliar sprays were applied daily at 9:00 a.m. for three consecutive days, ensuring complete coverage of the leaf surfaces without dripping (approximately 6 mL per plant). Immediately following the final application, seedlings in the L and L+γ-PGA groups were exposed to the low-temperature treatment. The stress conditions consisted of a 12 h/12 h (light/dark) photoperiod with a daytime temperature of 15°C, a nighttime temperature of 5°C, and 70% relative humidity. The third fully expanded leaves were randomly sampled at 0, 24, 48, 96, and 168 h after the initiation of stress. Samples were immediately snap-frozen in liquid nitrogen and stored at −80°C for subsequent analysis.

2.3 Photosynthetic Parameters

After 168 h of treatment, gas exchange parameters were measured on six randomly selected seedlings using a portable photosynthesis system (GFS-3000, Heinz Walz, Effeltrich, Germany). The measurements were conducted under the following conditions within the 4 cm^2 leaf chamber: a light intensity of 800 μmol·m^{-2}·s^{-1}, a CO_2 concentration of 400 μmol·mol^{-1}, and a flow rate of 750 μmol·s^{-1}. The recorded parameters included the net photosynthetic rate (*Pn*), transpiration rate (*Tr*), stomatal conductance (*Gs*), and intercellular CO_2 concentration (*Ci*). From these data, water use efficiency (*WUE*) was calculated as the ratio *Pn*/*Tr*. Following the gas exchange measurements, the maximum quantum yield of photosystem II (PSII; *Fv*/*Fm*) was determined on the same leaves. This was performed using a chlorophyll fluorometer (FluorPen FP-100/s, Photon Systems Instruments, Czech Republic) after the leaves were dark-adapted for 30 min.

2.4 Plant Growth Indexes and Root Characteristics

After 168 h of treatment, six seedlings were randomly selected from each group to assess plant height, stem diameter, fresh shoot and root weight. The roots of these seedlings were then scanned and analyzed using WinRHIZO software (Regents Instruments Inc., Quebec City, QC, Canada) to quantify key morphological traits, including total root length, surface area, volume, and the number of forks and tips.

2.5 ROS, MDA, and Proline

The accumulation of superoxide anion ($O_2^{\cdot-}$) was visualized at 168 h by staining with nitro blue tetrazolium (NBT), as described by Li, et al. [9]. To quantify $O_2^{\cdot-}$ and hydrogen peroxide (H_2O_2) levels, samples of the third functional pepper leaf were collected at 0, 24, 48, 96, and 168 h. The tissue was ground to a fine powder in liquid nitrogen and analyzed using commercial reagent kits (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) according to the manufacturer's protocol. The concentrations of $O_2^{\cdot-}$ and H_2O_2 were determined by measuring absorbance at 530 nm and 415 nm, respectively. MDA content was determined using the thiobarbituric acid (TBA) method, in which samples were homogenized in trichloroacetic acid (TCA) and reacted with TBA, and then absorbance was recorded

at 532 and 600 nm [18]. Proline content was quantified via the sulfosalicylic acid method [14]. Briefly, samples were extracted in sulfosalicylic acid, reacted with acid-ninhydrin, and extracted with toluene prior to absorbance measurement at 520 nm.

2.6 Antioxidative Enzymes

The activities of catalase (CAT), SOD, and ascorbate peroxidase (APX) were determined using commercial assay kits (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) according to the manufacturer's protocols. Specifically, CAT activity was assayed by monitoring the decomposition of H_2O_2 as a decrease in absorbance at 240 nm, one unit (U) was defined as the amount of enzyme decomposing 1 nmol of H_2O_2 per minute per gram of fresh weight (FW). SOD activity was quantified by its ability to inhibit the formation of blue formazan, measured at 560 nm, one unit of SOD was defined as the amount of enzyme causing 50% inhibition of NBT reduction per gram of FW. APX activity was determined from the rate of ascorbate (AsA) oxidation, which was followed as a decrease in absorbance at 290 nm, one unit was defined as the oxidation of 1 nmol of AsA per minute per gram of FW.

2.7 qRT-PCR Analysis

Total RNA was extracted from pepper leaf samples using the RNAprep Pure Plant Kit (Tiangen Biotech Co., Ltd., Beijing, China), and concentration and purity were assessed with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, first-strand cDNA was synthesized from the total RNA using the PrimeScriptTM 1st Strand cDNA Synthesis Kit (Takara Biotech Co., Ltd., Dalian, China), following the manufacturer's protocol. Gene-specific primers (Table 1) were designed using Oligo7 software (Molecular Biology Insights, Inc., Cascade, CO, USA). Target isoforms were selected for their known roles in stress physiology, including peroxisomal catalase (*CaCAT*) [19], mitochondrial manganese superoxide dismutase (*CaMnSOD*) [20], and peroxisomal ascorbate peroxidase (*SbpCaAPX*) [21]. Primer specificity was confirmed via agarose gel electrophoresis and sequencing of amplicons (Figs. S1 and S2). The primers were synthesized by BGI Tech Solutions Co., Ltd. (Shenzhen, China). qRT-PCR was performed on a LightCycler 480 system (Roche, Basel, Switzerland) using the SYBR® Green Pro Taq HS qPCR Kit (Accurate Biotechnology Co., Ltd., Hunan, China). Each 20 μL reaction was composed of 10 μL of 2× SYBR Green Pro Taq HS Premix, 0.4 μL of each primer (10 μM), 0.4 μL of ROX Reference Dye II, 2 μL of cDNA template, and 6.8 μL of nuclease-free water. The thermal cycling protocol consisted of an initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 15 s and a gene-specific annealing step for 30 s. The annealing temperatures were 52°C for *CaCAT*, 60°C for *CaSOD*, and 55°C for *CaAPX*. All analyses were performed in triplicate using biological replicates. Relative gene expression was quantified using the $2^{-\Delta\Delta Ct}$ method [22], with *CaActin* serving as the endogenous reference gene.

Table 1: Primer sequences of real-time quantitative PCR.

Gene Name	GenBank Accession No.	Primer Sequence (5′ to 3′)	Amplicon Size (bp)	Gene Targeted
CaCAT	NM_001324674.1	F: TTAACGCTCCCAAGTGTGCTCATC R: GGCAGGACGACAAGGATCAAACC	116	Catalase
CaSOD	NM_001324998.1	F: GTGAGCCTCCAAAGGGTTCTCTTG R: AAACCAAGCCACACCCAACCAG	127	Manganese superoxide dismutase
CaAPX	NM_001324587.1	F: TGTTGTTGCTGTTGAGGTCACTGG R: CATCTGGTAACCGCCCTTCCTTTG	98	Peroxisomal ascorbate peroxidase
CaActin	XM_016722297.2	F: GTCCTTCCATCGTCCACAGG R: GAAGGGCAAAGGTTCACAACA	135	*Capsicum annuum* actin

Note: F, forward primer; R, reverse primer.

2.8 Statistical Analysis

The data processing and analysis were conducted using Microsoft Excel 2013 (Microsoft, Redmond, WA, USA) and SPSS 22.0 (IBM, Armonk, NY, USA), with data visualized in Origin 2024 (OriginLab, Northampton, MA, USA). One-way ANOVA was used to identify significant differences, and Duncan's multiple range test was used to determine significant differences among the treatments at $p < 0.05$.

3 Results

3.1 Morphology and Biomass Analysis

As shown in Fig. 1, low temperature stress significantly inhibited the growth of pepper seedlings, whereas pre-spraying with γ-PGA effectively mitigated this damage. Under normal temperature conditions, γ-PGA application had no significant effect on most growth parameters compared to the CK treatment, with the sole exception of a 27.8% increase in the number of root tips. In contrast, exposure to low temperature stress substantially suppressed overall seedling growth. Compared with CK, L treatment caused significant reductions in the fresh weight of shoots (36.1%) and roots (44.8%). Above-ground growth was also stunted, with plant height, width, and stem diameter decreasing by 24.2%, 17.2%, and 16.0%, respectively. Furthermore, root architecture was severely compromised, as evidenced by decreases in total root length (32.8%), root surface area (61.1%), and the number of root tips (63.9%). Notably, the application of γ-PGA under low temperature stress promoted significant growth recovery. When compared to seedlings subjected to L treatment, the L+γ-PGA treatment increased shoot and root weight by 16.0% and 24.1%, respectively. Root parameters were also markedly improved, with total root length increasing by 31.2%, root surface area by 25.2%, and the number of root tips by a substantial 110.8%.

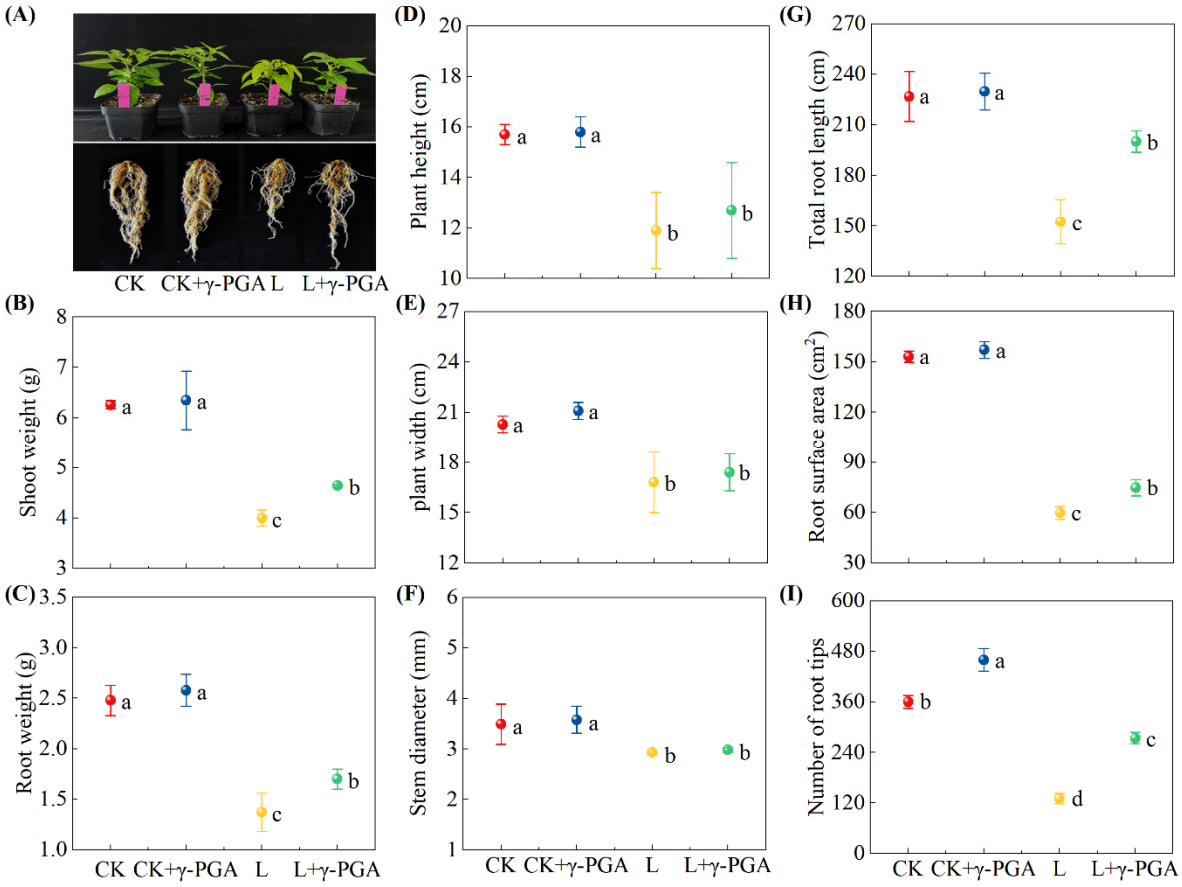

Figure 1: The effects of γ-PGA on pepper seedlings under low-temperature stress. (**A**) Representative morphology of seedlings after 168 h of treatment. (**B**–**I**) Quantitative analysis of key growth parameters: (**B**) shoot fresh weight, (**C**) root fresh weight, (**D**) plant height, (**E**) plant width, (**F**) stem diameter, (**G**) root length, (**H**) root surface area, and (**I**) number of root tips. Treatments are abbreviated as follows: CK (normal temperature), CK+γ-PGA (normal temperature + 100 mg·L-1 γ-PGA), L (low temperature), and L+γ-PGA (low temperature + 100 mg·L-1 γ-PGA). Different letters indicate statistically significant differences among treatments as determined by Duncan's multiple range test ($p < 0.05$).

3.2 Photosynthetic Capacity

Under normal growth conditions, while pretreatment with γ-PGA for 168 h did not significantly alter leaf *Gs*, *Ci*, *WUE*, or *Fv*/*Fm* in pepper seedlings relative to the CK group, it did enhance *Pn* and *Tr* by 4.8% and 4.4%, respectively (Fig. 2). The imposition of low-temperature stress significantly impaired photosynthetic performance. Relative to unstressed controls, stressed seedlings exhibited sharp declines in *Pn* (68.9%), *Tr* (49.7%), *Gs* (50.8%), *WUE* (38.0%), and *Fv*/*Fm* (22.2%), which occurred concurrently with an 8.4% rise in *Ci*. However, these detrimental impacts were effectively mitigated by L+γ-PGA pretreatment. Specifically, compared to seedlings under stress alone, those pretreated with L+γ-PGA displayed significant improvements in *Pn* (by 172.9%), *Tr* (by 47.3%), *Gs* (by 48.5%), *WUE* (by 85.2%), and *Fv*/*Fm* (by 17.9%), along with a significant 10.5% decrease in *Ci*.

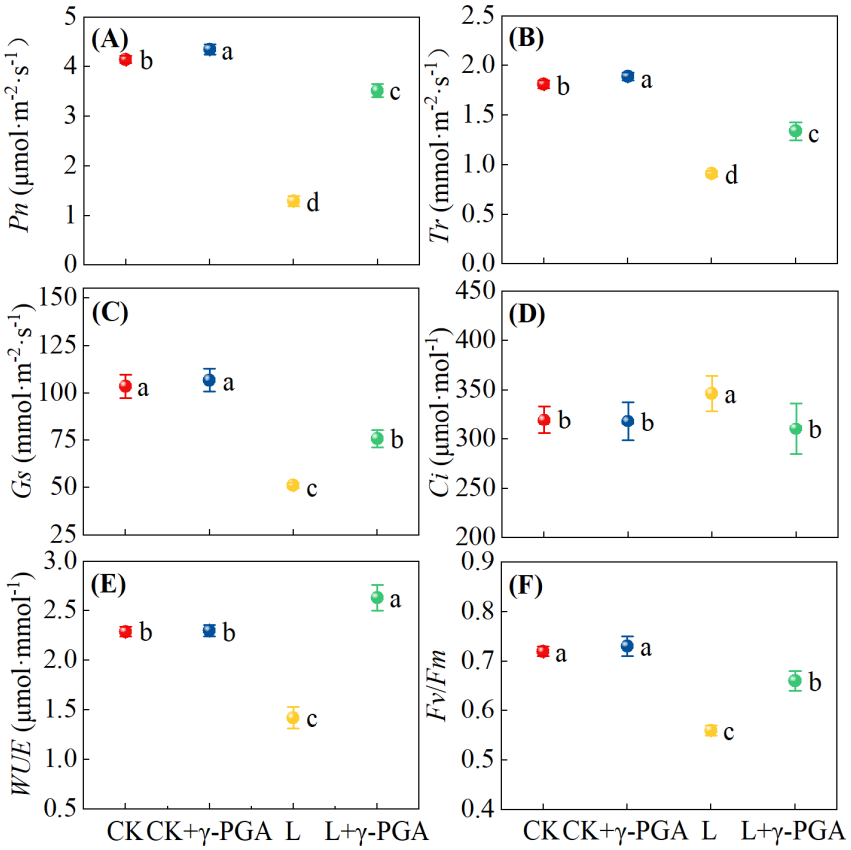

Figure 2: The effect of γ-PGA on gas exchange and chlorophyll fluorescence in pepper seedlings under low-temperature stress. The parameters, measured after 168 h of exposure, were: (**A**) net photosynthetic rate (*Pn*), (**B**) transpiration rate (*Tr*), (**C**) stomatal conductance (*Gs*), (**D**) intercellular CO_2 concentration (*Ci*), (**E**) water use efficiency (*WUE*; *Pn*/*Tr*), and (**F**) the maximum photochemical efficiency of photosystem II (*Fv*/*Fm*). Treatments are abbreviated as follows: CK (normal temperature), CK+γ-PGA (normal temperature + 100 mg·L^{-1} γ-PGA), L (low temperature), and L+γ-PGA (low temperature + 100 mg·L^{-1} γ-PGA). Different letters indicate significant differences among treatments as determined by Duncan's multiple range test ($p < 0.05$).

3.3 ROS Accumulation

Histochemical analysis using NBT staining showed that pepper leaves subjected to 168 h of low-temperature stress displayed the largest area and greatest intensity of blue precipitate, a visual indicator of $O_2^{\cdot-}$ production (Fig. 3). This staining was markedly reduced in leaves pretreated with γ-PGA. To verify these observations, we quantified ROS levels. Low-temperature stress alone (L treatment) caused a continuous increase in both $O_2^{\cdot-}$ and H_2O_2 concentrations, which peaked at 168 h, reaching levels 4.4 and 3.6 times higher than those of the CK treatment, respectively. However, pretreatment with γ-PGA significantly counteracted this stress-induced accumulation. Compared with L treatment, L+γ-PGA reduced $O_2^{\cdot-}$ content by 45.9%, 54.3%, 47.7%, and 43.3% at 24, 48, 96, and 168 h, respectively. Similarly, H_2O_2 content decreased by 43.7%, 55.6%, 57.4%, and 49.0% at the same respective time points. Therefore, γ-PGA pretreatment is an effective strategy for inhibiting ROS accumulation in pepper leaves under conditions of low-temperature stress.

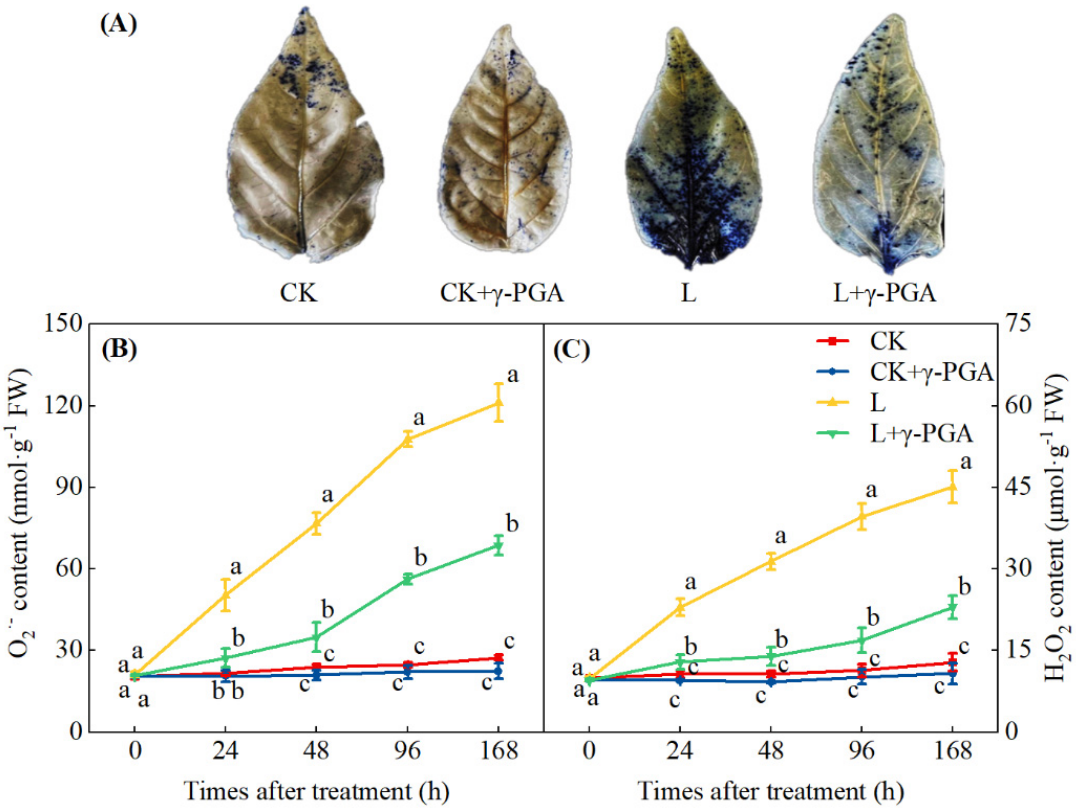

Figure 3: The effect of γ-PGA on the accumulation of ROS in pepper seedlings under low-temperature stress. (**A**) Representative images of nitro-blue tetrazolium (NBT) staining in leaves after 168 h of treatment; (**B**) superoxide anion ($O_2^{\cdot-}$) content; (**C**) hydrogen peroxide (H_2O_2) content. Treatments are abbreviated as follows: CK (normal temperature), CK+γ-PGA (normal temperature + 100 mg·L^{-1} γ-PGA), L (low temperature), and L+γ-PGA (low temperature + 100 mg·L^{-1} γ-PGA). Different letters indicate significant differences among treatments ($p < 0.05$), as determined by Duncan's multiple range test.

3.4 MDA and Proline Content

Exposure to low temperature stress progressively increased MDA and proline levels in pepper seedling leaves, which peaked at 168 h at concentrations 4.4 and 3.9 times higher than the CK, respectively (Fig. 4). However, pretreatment with γ-PGA significantly mitigated these effects. Compared to the L, L+γ-PGA treatment reduced MDA accumulation by 30.6%, 36.5%, 32.8%, and 22.6% at 24, 48, 96, and 168 h, respectively. Conversely, it enhanced proline accumulation by 39.8%, 74.2%, and 40.3% at 48, 96, and 168 h. These findings demonstrate that γ-PGA enhances chilling tolerance in pepper seedlings by reducing lipid peroxidation and promoting the synthesis of protective osmolytes.

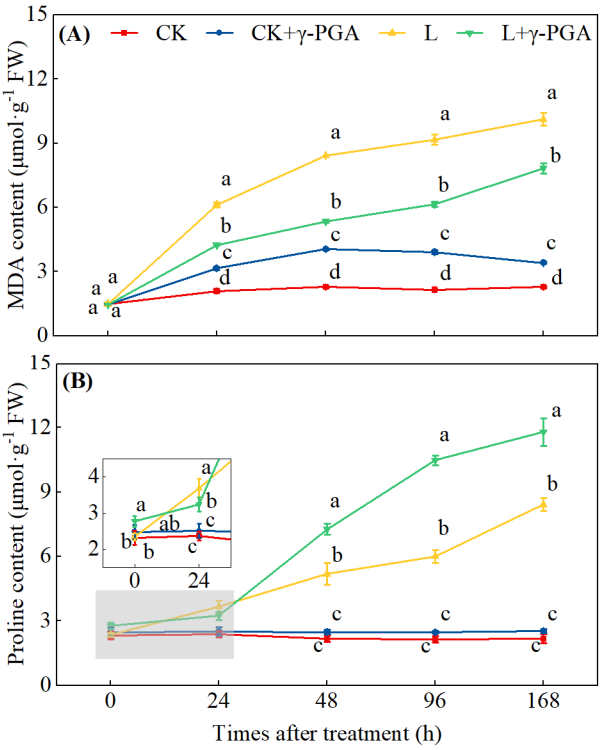

Figure 4: The effect of γ-PGA on (A) MDA and (B) proline content in pepper leaves under low-temperature stress. Treatments are abbreviated as follows: CK (normal temperature), CK+γ-PGA (normal temperature + 100 mg·L^{-1} γ-PGA), L (low temperature), and L+γ-PGA (low temperature + 100 mg·L^{-1} γ-PGA). Different letters indicate significant differences among treatments ($p < 0.05$) according to Duncan's multiple range test.

3.5 Antioxidant Enzyme Activity and the Relative Expression of Related Genes

The effect of γ-PGA on the antioxidant defense system in pepper seedling leaves under normal and low-temperature stress conditions was evaluated by measuring the activities of CAT, SOD, and APX and the expression of their encoding genes (*CaCAT*, *CaSOD*, and *CaAPX*) (Fig. 5). Under normal conditions, γ-PGA pretreatment significantly increased antioxidant capacity compared to the CK. At 168 h, this was evidenced by 49.3%, 10.0%, and 14.8% increases in CAT, SOD, and APX activities, respectively, which were supported by the significant upregulation of their corresponding genes. Conversely, low-temperature stress alone triggered a transient and ultimately insufficient antioxidant response. The activities and expression of all three enzymes initially rose, with CAT/*CaCAT* peaking at 48 h and SOD/*CaSOD* and APX/*CaAPX* peaking earlier at 24 h. However, prolonged stress (168 h) led to a significant decline in SOD and APX activities (by 23.1% and 15.3%, respectively) and the downregulation of their genes relative to the CK treatment. Crucially, γ-PGA pretreatment counteracted this decline. The L+γ-PGA treatment group maintained significantly higher enzyme activities and gene expression than the L treatment group, indicating a more stable antioxidant defense under stress. For example, at 168 h, CAT and SOD activities were 43.7% and 51.6% higher, respectively. The protective effect on APX was particularly strong, with its activity and *CaAPX* gene expression at 168 h increasing by 109.8% and 313.5%, respectively, over the L treatment. Collectively, these findings demonstrate that γ-PGA enhances low-temperature tolerance in pepper seedlings by strengthening the antioxidant defense system at both the enzymatic and transcriptional levels.

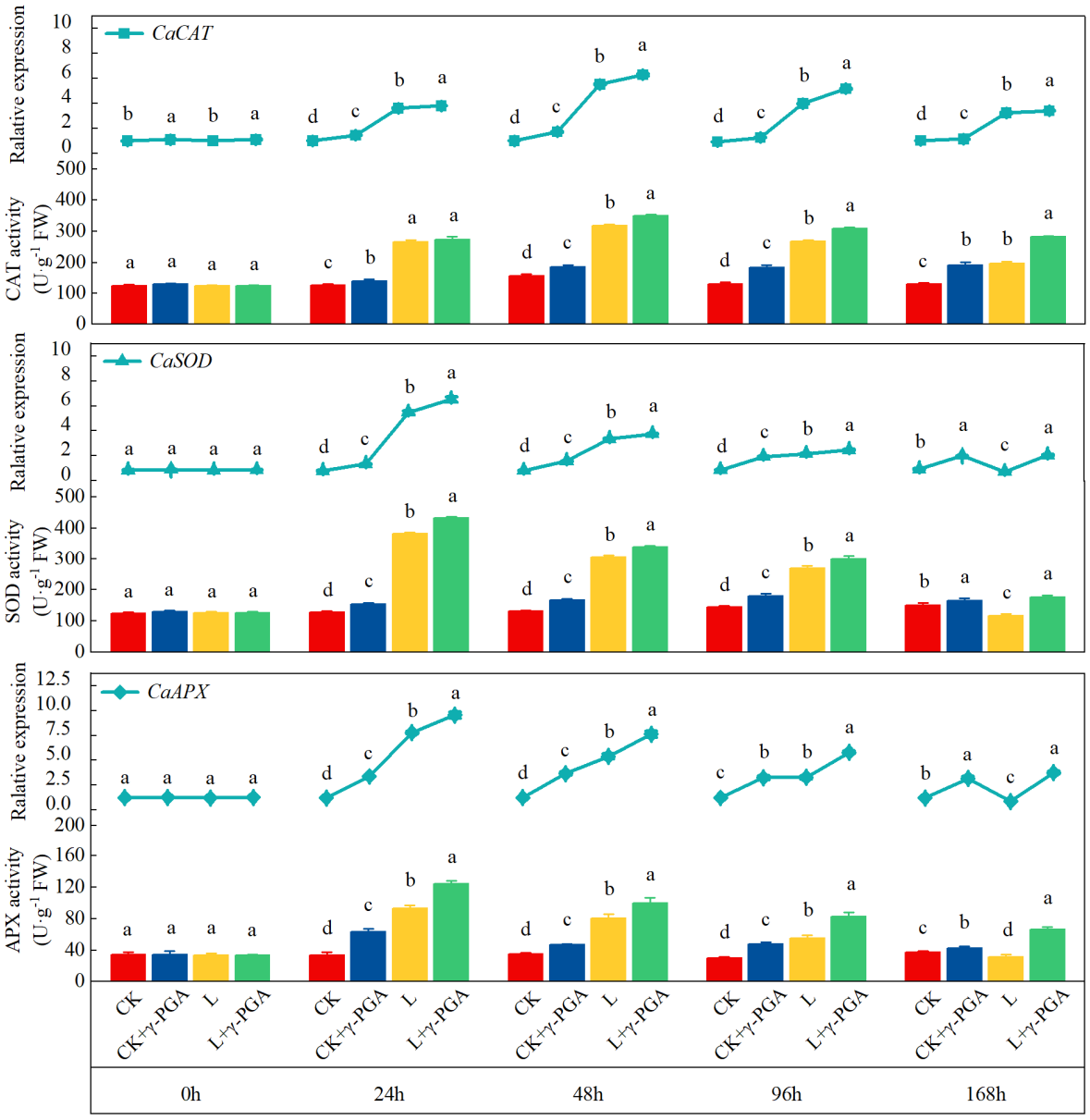

Figure 5: The effect of γ-PGA on antioxidant enzymes and related genes expression of pepper leaves low-temperature stress. Treatments are abbreviated as follows: CK (normal temperature), CK+γ-PGA (normal temperature + 100 mg·L^{-1} γ-PGA), L (low temperature), and L+γ-PGA (low temperature + 100 mg·L^{-1} γ-PGA). Different letters indicate significant differences among treatments ($p < 0.05$) according to Duncan's multiple range test.

4 Discussion

4.1 γ-PGA Application Preserves Root System Architecture and Function under Chilling Stress

Chilling stress is known to impair root development, thereby limiting water and nutrient uptake and inhibiting shoot growth [23]. In the present study, γ-PGA pretreatment mitigated the chilling-induced reductions in root biomass, total length, surface area, and number of root tips (Fig. 1C,G–I). Notably, the most pronounced effect was a 110.8% increase in the number of root tips compared to the untreated, low-temperature control. This finding is significant because the root tip meristem is a primary site for

stress perception and hormonal signal transduction [24]. Consequently, a more robust root system is better equipped to sustain water and mineral nutrient uptake under stress, thereby alleviating the inhibition of shoot growth, an effect supported by the concurrent increase in shoot fresh weight (Fig. 1B).

The mechanisms by which foliar-applied γ-PGA promotes root development are likely multifaceted, involving both long-distance signal transduction and regulation of the rhizosphere microenvironment. One potential pathway involves the translocation of γ-PGA itself, or of systemic signaling molecules induced by its application, from the leaves to the roots, where they can directly regulate physiological processes. Alternatively, the effect may be mediated by the rhizosphere. Previous research has shown that γ-PGA can modulate the soil microbial community, enriching for beneficial plant growth-promoting bacteria (PGPB) that in turn enhance root activity and stress tolerance [16,25]. Furthermore, γ-PGA may influence root development by modulating endogenous plant hormone networks, particularly those involving auxin and cytokinin, which are pivotal for lateral root formation and meristem maintenance [15,26].

4.2 γ-PGA Application Alleviates Photosynthetic Inhibition under Chilling Stress

Under chilling stress, photosynthesis is typically suppressed due to combined damage to PSII and repression of Calvin cycle enzymes, resulting in diminished carbon fixation and elevated ROS accumulation [27]. Consistent with these effects, we observed that chilling stress significantly decreased the *Pn*, *Fv*/*Fm*, and *Gs* (Fig. 2). Notably, the *Ci* increased despite the reductions in *Gs* and *Pn*. This paradoxical rise in *Ci* indicates that non-stomatal factors, such as inhibition of Rubisco activity or constraints in ATP synthesis, constituted the primary limitation to photosynthesis, rather than restricted CO_2 diffusion [28]. Pretreatment with γ-PGA effectively alleviated this photosynthetic inhibition. The amelioration was marked by a pronounced increase in Pn (by 172.9%) and Fv/Fm (by 17.9%), accompanied by a decrease in *Ci*. These responses collectively suggest that γ-PGA targeted both photochemical and biochemical constraints. The recovery of *Fv*/*Fm* implies improved PSII stability and reduced photoinhibition, likely through protection of thylakoid membrane integrity [29]. Furthermore, γ-PGA pretreatment enhanced *Gs* and *Tr*, indicating a positive effect on stomatal function. This improvement may be mediated through modulation of guard cell osmoregulation or altered sensitivity to abscisic acid (ABA). The significant increase in intrinsic *WUE* further highlights an optimized balance between CO_2 uptake and water loss, which is critical for stress adaptation.

Beyond direct effects on photosynthetic machinery, the observed recovery may also be linked to γ-PGA-driven improvements in nutrient metabolism. As a metal chelator, γ-PGA could increase the bioavailability of Mg^{2+}, an essential cofactor for chlorophyll biosynthesis and Rubisco activation [30]. Intriguingly, transcriptomic evidence from other species indicates that γ-PGA can elevate chlorophyll content and photosynthetic activity without upregulating related biosynthetic genes, pointing to post-transcriptional or post-translational regulation of chlorophyll homeostasis [31]. A similar mechanism may be active in pepper, thereby supporting Calvin cycle function and enhancing carbon assimilation under chilling stress.

4.3 γ-PGA Application Enhances Chilling Tolerance through Antioxidant Activation and Osmotic Adjustment

Low-temperature exposure induced significant oxidative stress, evidenced by the accumulation of $O_2^{\cdot-}$, H_2O_2, and the membrane damage marker MDA [3]. However, pretreatment with γ-PGA markedly attenuated this accumulation (Figs. 3 and 4). Concurrently, γ-PGA stimulated the production of proline, a key osmoprotectant involved in stabilizing cellular structures and scavenging ROS [32]. These results suggest

that γ-PGA confers chilling tolerance through a dual mechanism: the direct mitigation of oxidative damage and the enhancement of osmotic adjustment. The protective action of γ-PGA appears to be mediated by the robust activation of the enzymatic antioxidant system. While prolonged chilling stress alone led to a decline in SOD and APX activities, γ-PGA pretreatment not only reversed this trend but significantly elevated the activities of CAT, SOD, and APX (Fig. 5). This response was supported at the transcriptional level by the upregulation of their corresponding genes (*CaCAT*, *CaSOD*, and *CaAPX*), with *CaAPX* expression showing a remarkable 313.5% increase. This suggests that γ-PGA bolsters antioxidant capacity by modulating gene expression, ensuring a sustained defense under stress. However, it is noteworthy that while γ-PGA pretreatment significantly reduced ROS accumulation under low temperatures, ROS levels remained elevated compared to the normal temperature control. This indicates that γ-PGA primarily functions to mitigate rather than completely eliminate oxidative stress, as persistent low temperatures continue to induce ROS production by inhibiting the photosynthetic electron transport chain and disrupting membrane integrity [4]. Drawing on prior research, we hypothesize that plants recognize γ-PGA as a biogenic elicitor. As a microbial exopolysaccharide, γ-PGA may be perceived by plant pattern recognition receptors (PRRs), triggering signaling cascades involving Ca^{2+}, MAP kinases, and hormonal cross-talk [33,34]. This hypothesis is supported by the observed upregulation of antioxidant genes in non-stressed plants treated with γ-PGA. These signaling events likely activate transcription factors (e.g., NAC, WRKY) that bind to the promoters of antioxidant genes, preparing the plant for an impending stress [15]. The substantial enhancement of APX is particularly significant, as its role in scavenging chloroplastic H_2O_2 is critical for protecting the photosynthetic apparatus from photo-oxidative damage, which aligns with the observed recovery of photosynthetic function [4,35]. It is important to acknowledge that the expression of single gene isoforms, as measured by qRT-PCR, reflects only one layer of regulation within complex multigene families. Total enzyme activity (e.g., SOD, APX, CAT) represents the integrated result of the expression of multiple family members and subsequent post-translational modifications [19]. This study focused on mitochondrial *CaMnSOD*, peroxisomal *CaAPX*, and peroxisomal *CaCAT*, given their previously identified roles in mediating low-temperature stress responses in pepper [5,36,37]. The positive correlation between the transcript abundance of these isoforms and total enzyme activity (Fig. 5) indicates that they are major contributors to the overall antioxidant capacity. Therefore, our data provide compelling evidence that γ-PGA enhances chilling tolerance, at least in part, by promoting the transcriptional activation of critical components within the broader antioxidant network.

4.4 Agricultural Implications and Future Perspectives

This study demonstrates that the foliar application of 100 $mg{\cdot}L^{-1}$ γ-PGA is a practical and effective strategy for enhancing chilling tolerance in pepper seedlings. For early spring cultivation and protected agriculture in temperate zones, where productivity is often constrained by suboptimal temperatures, this approach is particularly advantageous [38]. Furthermore, because γ-PGA is produced via microbial fermentation, it is a cost-effective treatment, which supports its agronomic feasibility [39]. The observed systemic effect, in which foliar application also improved root traits, underscores the potential value of γ-PGA in integrated nutrient and stress management programs.

To deepen the understanding of γ-PGA's mechanisms and advance its practical use, future research should focus on the following directions:

(1) Elucidation of molecular mechanisms: Identifying potential receptors for γ-PGA within plant tissues and elucidating downstream signaling transduction pathways, such as Ca^{2+} influx and protein kinase activation, would clarify the molecular basis of γ-PGA-induced cold tolerance.

(2) Analysis of in planta translocation and persistence: Employing isotopic or fluorescent labeling techniques to trace the absorption, transport, distribution, and persistence of γ-PGA in plants.
(3) Validation under field conditions: Conducting field trials across diverse ecological environments to evaluate the practical efficacy of γ-PGA on crop productivity and to optimize agronomic parameters including concentration, timing, and method of application.
(4) Carrier function and synergistic effects: Future studies should leverage the properties of γ-PGA as a biodegradable encapsulation material [40,41] to deliver complementary cryoprotectants, such as melatonin and brassinolide [9,42]. This strategy aims to develop efficient, broadly adaptable composite formulations that offer synergistic enhancement or sustained-release effects.

5 Conclusions

This study reveal that foliar-applied γ-PGA enhances chilling tolerance in pepper seedlings through a multi-faceted defense strategy. It promotes root development, strengthens the antioxidant system at enzymatic and transcriptional levels, increases osmotic adjustment via proline accumulation, and protects the photosynthetic machinery from chilling-induced damage. This study provides novel insights into the role of γ-PGA in alleviating low-temperature stress in a solanaceous vegetable crop and supports its potential as an effective, sustainable biostimulant for improving crop resilience in suboptimal temperature environments.

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