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Cold Hardiness and Physio-Biochemical Responses of Annual Branches in Five Early-Fruiting Walnut Varieties (*Juglans regia* L.) Under Simulated Low-Temperature Stress

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Abstract: As global climate change escalates, horticultural crops, especially walnuts, face increased vulnerability to frost damage. Cold hardiness—a crucial trait for survival—is influenced by complex physiological and biochemical mechanisms. This study assessed the cold hardiness of five walnut cultivars—'Xinxin 2', 'Wen 81', 'Wen 185', 'Zha 343', and 'Xinzaofeng'—under simulated low-temperature stress, focusing on differences in freezing tolerance. One-year branches were gradually cooled to temperatures as low as −30 °C. Key physiological metrics, including electrolyte leakage (EL) and regrowth (RG) potential, along with biochemical metrics like antioxidant enzyme activities and osmoregulatory compounds, were used to evaluate cold hardiness. A comprehensive cold resistance indicator, derived using the subordination function method, highlighted cultivar resilience. Results showed significant variation in cold tolerance, with 'Wen 185' and 'Wen 81' exhibiting superior resilience, while 'Xinxin 2' was the most susceptible. Logistic regression analysis of relative electrolyte conductivity (REC) data estimated the semi-lethal temperature (LT50), identifying 'Wen 81' as the most cold-tolerant cultivar (LT50 = −21.73 °C). Antioxidant enzymes and osmoregulatory compounds were crucial for maintaining cellular stability and recovery after freezing. These findings offer practical insights for breeding cold-resistant cultivars and strategies to mitigate frost damage.

Keywords: walnut varieties; cold hardiness; electrolyte leakage; antioxidant enzymes; low-temperature stress; regrowth potential; abiotic stress responses

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1. Introduction

The increasing frequency of temperature fluctuations and extreme frost events driven by climate change poses significant threats to horticultural crop production, particularly for cold-sensitive species like walnuts (*Juglans regia* L.) [1,2]. Walnuts are often cultivated in regions vulnerable to sudden, unpredictable frosts, which disrupt flowering, reduce yields, and adversely affect both local economies and global supply chains [3,4]. Addressing these challenges requires integrating climate-resilient traits into breeding programs to enable crops to withstand increasingly erratic temperature patterns [5,6]. Advances in understanding cold tolerance mechanisms have paved the way for targeted breeding strategies that select cultivars with enhanced cellular stability, antioxidant responses, and

recovery potential under low-temperature stress [7,8]. Studies on other nut crops, including almonds (*Prunus dulcis*), hazelnuts (*Corylus avellana*), pecans (*Carya illinoinensis*), and pistachios (*Pistacia vera* L.), have similarly highlighted the pivotal role of physiological and biochemical traits in determining cold tolerance [9–12]. For instance, research on pistachios has demonstrated the importance of antioxidant enzyme activity and specific physiological traits in mitigating frost damage [12,13]. Likewise, almonds, hazelnuts, and pecans rely on osmoregulatory substances, such as soluble sugar and proline, to bolster freezing resistance [14–16]. These findings underscore the universal relevance of such mechanisms across nut crops, providing valuable context for exploring cold tolerance in walnuts. This study investigates the physiological and biochemical responses of walnut cultivars to cold stress, with a focus on identifying traits that contribute to resilience against frost events. The findings provide practical insights for breeding programs aimed at developing cold-tolerant walnut varieties, which are essential for maintaining productivity and sustainability in the face of climate variability.

Vegetation in temperate zones must endure cold winters, sometimes facing extreme or prolonged low temperatures during dormancy [17,18]. While native temperate trees generally display resilience to freezing injuries, horticultural crops, bred for specific traits, are often more vulnerable [3,19]. Increasing frequent and severe frost events caused by global climate changes pose significant threats to the survival, yield, and quality of these crops [20–22]. Paradoxically, the risk of cold injury has risen alongside global temperature increases [23], particularly in high-altitude and high-latitude areas where fruit and nut trees are grown [24,25]. As a result, cold injury has become a pressing concern in regions like Central Asia and Europe, where walnut production is integral to local economies and livelihoods [20,26]. Understanding the varied responses of different horticultural cultivars to such cold events is essential for developing strategies to mitigate these impacts, especially in regions prone to extreme weather conditions.

Cold hardiness, defined as the ability to withstand injury from low and fluctuating temperatures [27,28], is a complex trait shaped by physiological and biochemical factors [29,30]. Plant resilience to freezing is often assessed using electrolyte leakage (EL) measurement, which provides immediate insights into cell membrane stability after freezing exposure [31,32]. However, since EL primarily captures direct cold-induced damage, it may not fully reflect a plant's long-term recovery capacity. Recovery and regrowth (RG) potential after freezing injury offer a more comprehensive measure of resilience and adaptability [33,34]. Integrating short-term damage indicators, such as electrolyte leakage, with long-term resilience metrics yields a more accurate assessment of a cultivar's overall cold hardiness [35].

Horticultural crops employ a diverse range of physiological and biochemical mechanisms to endure cold stress. These include the accumulation of osmoregulatory substances like proline, soluble sugars, and proteins, which stabilize cell membranes and proteins during freezing conditions [36,37]. Additionally, antioxidant defense systems mitigate oxidative damage caused by reactive oxygen species (ROS) generated during freezing stress. Key enzymes such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) play critical roles in neutralizing ROS and preserving cellular integrity [38,39]. Similar approaches have been successfully applied to other crops. For instance, studies on grapevines and peaches have demonstrated that the combination of electrolyte leakage measurements with antioxidant activity analyses provides a robust framework for assessing cold tolerance [40,41]. In olive trees, integrating biochemical indicators such as proline and soluble sugar content has helped identify frost-resistant cultivars [42]. These examples illustrate the broader applicability of combined physiological and biochemical metrics for understanding cold tolerance across diverse crop species. While these mechanisms are

well-documented across many fruit tree species, variation in cold tolerance among cultivars within the same species, such as walnuts, remains relatively unexplored. Exploring these intra-species differences is essential for selecting and breeding cultivars capable of withstanding the increasingly unpredictable climatic conditions associated with global warming [43].

In the arid regions of Central Asia, where specialty forestry and fruit industries are vital for both economic development and environmental sustainability [44–46], extreme low temperatures and frost events have seriously affected crop yield and quality [47,48]. Walnut is a key nut crop extensively cultivated in this region, with diverse cultivars adapted to local conditions [49,50]. Over time, long-term acclimation has produced locally specialized walnut types with enhanced cold resistance, providing valuable germplasm for breeding programs aimed at improving cold tolerance [51,52]. Despite the importance of cold-resistant walnut cultivars, detailed research on the variability in cold tolerance within walnut species remains limited. To address this gap, this study evaluates the cold tolerance of annual dormant branches from five early-fruiting walnut cultivars under simulated low-temperature stress. Specifically, we aim to (1) quantify the variability in cold tolerance among these cultivars, (2) assess their recovery and regrowth capacities following low-temperature exposure, and (3) investigate the role of key biochemical compounds in enhancing cold resistance. By integrating physiological damage metrics, antioxidant enzyme activities, and osmoregulatory compound levels, this research provides critical insights into the mechanisms of cold tolerance in walnuts. The findings will support breeding programs aimed at developing climate-resilient walnut cultivars and mitigating frost damage under increasing climate variability.

2. Materials and Methods

2.1. Orchards

The experimental site is located in the walnut plantation of Wensu County, Aksu Prefecture of Xinjiang, China (longitude 80°20′–80°25′ E, latitude 41°10′–41°15′ N), at an elevation of 1103.8 m. The area of the experimental orchard was 76 hectares. The region experiences a typical continental warm temperate arid climate, characterized by distinct seasonal variation and significant temperature differences between day and night. The average annual temperature is 10.2 °C, with recorded extremes of up to 39 °C in summer and as low as −24 °C in winter. The area receives an annual average precipitation of only 65.4 mm, with a frost-free period lasting up to 185 days per year. These environmental conditions create a challenging setting for walnut cultivation, particularly with regard to the trees' cold hardiness, making it an ideal location for studying the physiological responses of walnut branches to low-temperature stress.

2.2. Experimental Materials

One-year-old dormant branches from five walnut cultivars—'Xinxin 2', 'Wen 81', 'Wen 185', 'Zha 343', and 'Xinzaofeng'—were collected on 23–24 December 2023. These cultivars were selected for their prominence in early-fruiting walnut production in the region and their potential variability in cold tolerance. Among them, 'Xinxin 2', 'Win 185' and 'Xinzaofeng' are characterized by relatively small tree sizes, compact canopies, high productivity, and excellent quality, making them ideal for intensive orchard cultivation. In contrast, 'Wen 81' and 'Zha 343' exhibit vigorous growth, relatively open canopies, tall tree structures, strong adaptability, and medium-to-high but stable yields. The trees were 20 years old and pruned in an open-center shape, having undergone standard orchard management practices with no significant differences in overall health or growth conditions. From each cultivar, 25 one-year branches, each measuring 50 cm in length, were randomly

collected from 5 to 8 healthy trees on the middle part of the crown. Care was taken to ensure the samples were free from pests, diseases, and environmental damage. After collection, the branches were immediately transported to the Forestry Testing Center of the Xinjiang Academy of Forestry Sciences, where they underwent artificial low-temperature stress treatments.

To thoroughly assess cultivar resilience under cold stress, each metric was carefully selected to capture critical aspects of physiological response. Electrolyte leakage (EL) served as a rapid indicator of membrane integrity and immediate cold-induced cellular damage [53,54], while regrowth (RG) potential measured each cultivar's recovery capacity, reflecting adaptability to low-temperature conditions [55,56]. Additionally, evaluating antioxidant enzyme activity and osmoregulatory compounds provided deeper insights into the protective mechanisms that sustain cellular stability and functionality during freezing events [27].

2.3. Experimental Methods

2.3.1. Low-Temperature Stress Treatment

To simulate freezing conditions, the collected walnut branches were cleaned and sealed at both cut ends with plastic wrap to prevent moisture loss. The branches were then placed in a refrigerator (DW-40W380J, Haier Co., Ltd., Qingdao, China) at 2 °C, and the temperature was gradually lowered at a rate of 4 °C every 2 h until reaching −10 °C. From this point, stepwise cooling was applied, including treatments at −10 °C, −15 °C, −20 °C, −25 °C, and −30 °C. Each treatment was maintained for 48 h; after which, samples were taken. During the cooling process, a 10 cm segment from the tip of each branch was excised for regrowth and physiological testing. For the remaining branch samples, liquid nitrogen was used for rapid freezing to ensure the integrity of their physiological activity, followed by storage in an ultra-low-temperature freezer at −80 °C for subsequent osmoregulatory substances and enzyme activity measurements.

2.3.2. Measurement of Electrical Conductivity (EC)

Electrical conductivity (EC) was used to assess cell membrane integrity, a key indicator of cold injury in plant tissues. Branches subjected to different temperature treatments were cut into 0.2 cm segments, avoiding buds, and 0.5 g of each segment was placed in a 15 mL flask containing distilled water. The samples were soaked for 12 h; after which, the initial electrical conductivity (EC_initial) of the leachate was measured with an LeiCi DDS-307 (ShengKe, Shanghai, China) conductivity meter. The samples were then boiled for 30 min and cooled, and the total electrical conductivity (EC_total) was measured again. The relative electrical conductivity (REC), which reflects the percentage of membrane damage, was calculated using the formula:

$$\mathrm{REC}(\%) = \left(\frac{\mathrm{EC}_{\mathrm{initial}}}{\mathrm{EC}_{\mathrm{total}}}\right) \times 100 \quad (1)$$

This method, based on Arora et al. (1992), was widely employed to estimate cold-induced damage in plant tissues due to its ability to directly quantify membrane permeability [53]. Each measurement was replicated three times for accuracy, and results were analyzed for significant differences among cultivars and treatment temperatures.

2.3.3. Rehydration and Regrowth (RG) Test

The upper 1.5 cm sections from five branches of each cultivar were cut and weighed to record the initial weight G1. The sample was then wrapped in aluminum foil and dried to a constant weight in an oven at 105 °C; after which, it was weighed again to obtain weight

G2. The moisture content of the branches (%) was calculated using the following formula: Moisture Content (%) = ((G1 − G2)/G1) × 100.

To assess the ability of branches to recover after freezing, we modified the method outlined by Wang et al. (2022) [35]. After the low-temperature treatments, 10 cm sections from the tips of five branches from each cultivar were soaked in water at room temperature for 12 h. The samples were then transferred to beakers containing deionized water and incubated in a growth chamber under controlled conditions (24 °C, 65% humidity, 1200 lx light intensity, 10 h photoperiod). The water in the beakers was changed every two days. The regrowth potential of the branches was monitored daily, and the number of buds that successfully germinated was recorded over a 30-day period. The germination rate (GR) was calculated as follows:

$$\text{Germination Rate}(\%) = \left(\frac{\text{Number of Buds Germinated}}{\text{Total Number of Buds}}\right) \times 100 \quad (2)$$

This regrowth test provided a long-term perspective on the resilience of branches, complementing the short-term damage assessment provided by the REC method.

2.3.4. Measurement of Osmoregulatory Substances

To better understand the physiological mechanisms underlying cold tolerance, the content of osmoregulatory substances, including soluble sugars (SSs), soluble proteins (SPs), and proline, was measured in branches subjected to different low temperatures. Approximately 1 g of fresh tissue was used for measuring osmoregulatory substances. The measurements were performed with three technical replicates for each biological replicate, ensuring the accuracy and reliability of the results. SSs were quantified using the phenol–sulfuric acid method [57]. SPs were determined by the Coomassie brilliant blue method [58], and proline content was measured using the method described by Bates et al. (1973) [59]. These compounds play critical roles in stabilizing cellular structures under freezing stress, helping the plant to maintain membrane integrity and protein function. Each measurement was repeated three times to ensure precision.

2.3.5. Measurement of Protective Enzyme System Activity

The activities of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), as well as the content of malondialdehyde (MDA), were measured to evaluate the role of these enzymes in mitigating oxidative stress during freezing. Approximately 1 g of fresh tissue was used for measuring enzyme activities. The measurements were performed with three technical replicates for each biological replicate, ensuring the accuracy and reliability of the results. Lipid peroxidation, as indicated by the malondialdehyde (MDA) content, was also measured using the thiobarbituric acid (TBA) reaction method [60]. The enzymatic assays for POD, SOD, and CAT were conducted using commercial kits supplied by Suzhou Ke Ming Biotechnology Co., Ltd., Suzhou, China [38], to ensure consistency and accuracy. This analysis helps in understanding how different walnut cultivars manage oxidative damage under low-temperature stress, which is crucial for determining their cold resistance potential.

2.4. Data Analysis

All experimental data were analyzed by one-way analysis of variance (ANOVA) or the Kruskal–Wallis test when data did not meet the assumption of normality. These tests were performed using the ggpubr and EasyStat packages in R 4.4.1 [61]. Post hoc tests (e.g., Tukey's HSD) were conducted as necessary to identify significant mean differences across treatments and cultivars. Results are presented as means ± standard error (SE), with significance levels set at $p < 0.05$. To assess relationships between cold tolerance traits,

Pearson correlation coefficients were calculated, offering insights into the interdependence of physiological and biochemical metrics. Dimensionality reduction was performed using principal component analysis (PCA) to visualize variability among cultivars and identify traits most strongly associated with cold tolerance. The PCA results were plotted using the ggbiplot package (version 0.55), allowing a clear representation of the trait-cultivar relationships. Additionally, ANOVA was conducted on the PC1 scores to examine the effects of low-temperature treatments on cold tolerance-related traits. These comprehensive statistical approaches provided a detailed understanding of the contributions of individual traits to cold tolerance across cultivars.

3. Results

3.1. Effect of Low Temperature on Electrical Conductivity (EC) and Relative Electrical Conductivity (REC)

Electrical conductivity (EC) measures the total ionic content in the solution, while relative electrical conductivity (REC) represents the ratio of conductivity from damaged cells to total conductivity after complete tissue lysis. The five walnut cultivars exhibited distinct trends in EC and REC under different low-temperature treatments (Table 1, Figure 1). As temperatures decreased from −10 °C to −30 °C, both EC and REC values progressively increased across all cultivars, indicating greater cell membrane permeability and associated damage due to freezing stress. The REC values for each cultivar increased as temperatures dropped, indicating immediate membrane damage due to cold stress. Cultivars 'Wen 81' and 'Wen 185' showed lower REC values at extreme temperatures, suggesting inherent cellular stability that supports cold tolerance. At −10 °C, REC values were relatively low, with 'Wen 81' exhibiting the lowest (~45%) and 'Xinxin 2' the highest (~75%), suggesting minimal membrane damage at this moderate cold level. However, as the temperature dropped further to −20 °C, differences between cultivars became more pronounced. 'Wen 81' maintained relatively lower REC values compared to 'Wen 185' and 'Zha 343', indicating stronger physiological adaptations to moderate cold stress, such as enhanced membrane stability or osmoprotectant mechanisms (Table 1, Figure 1).

At −30 °C, all cultivars displayed significantly higher REC values, indicating severe membrane damage. Nevertheless, 'Wen 81' and 'Wen 185' exhibited lower REC values compared to 'Xinxin 2' and 'Xinzaofeng', suggesting that these two cultivars maintain better membrane integrity under extreme cold conditions. This may be attributed to more efficient antioxidant defense and osmotic adjustment systems.

These results demonstrate significant differences in REC among cultivars, with 'Wen 81' and 'Wen 185' showing superior membrane stability under low-temperature stress, indicating their inherent cold tolerance mechanisms.

Table 1. The effect of low temperature on the electrical conductivities of annual branches of five walnut cultivars.

Variety	Electrical Conductivity ($\mu S \cdot cm^{-1} \cdot g^{-1} \cdot mL^{-1}$)				
	−10 °C	−15 °C	−20 °C	−25 °C	−30 °C
Xinxin 2	221 ± 2.64 a	233.33 ± 11.59 a	234.67 ± 4.16 c	253.67 ± 10.69 c	324.00 ± 12.49 b
Wen 81	141.33 ± 5.03 d	168.67 ± 3.21 c	187.00 ± 10.53 d	226.00 ± 11.53 d	263.00 ± 9.16 c
Wen 185	176.33 ± 8.96 bc	178.67 ± 4.16 bc	263.00 ± 9.16 b	343.67 ± 3.51 a	443.67 ± 11.02 a
Zha 343	166.00 ± 6.24 c	215.67 ± 20.79 a	281.67 ± 7.02 a	285.33 ± 11.23 b	352.33 ± 9.02 b
Xinzaofeng	187.00 ± 10.53 b	193.00 ± 3.00 b	260.33 ± 2.51 b	287.00 ± 9.85 b	341.33 ± 17.95 b

Note: Different lowercase letters in the same column indicate significant differences between varieties ($p < 0.05$).

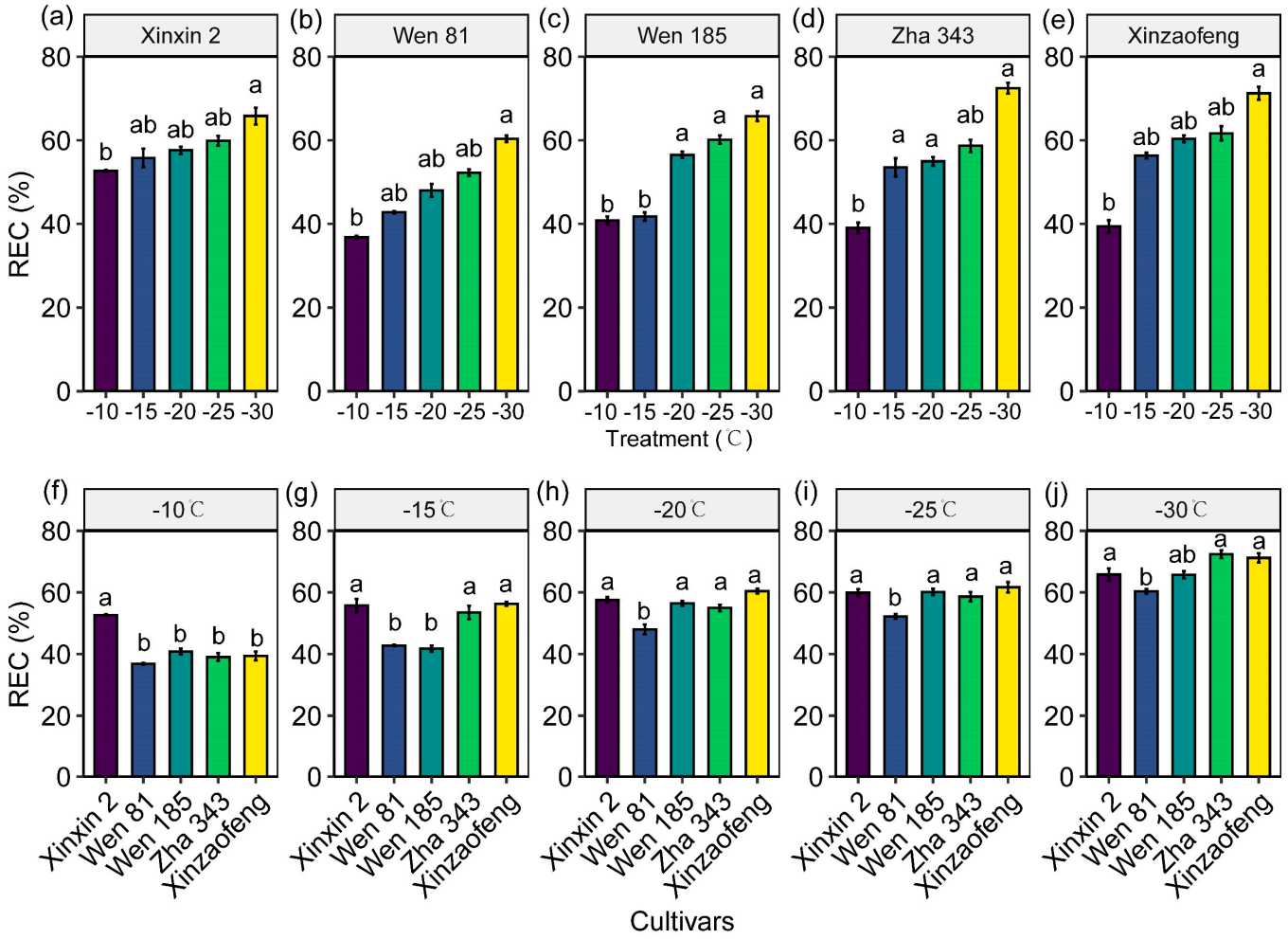

Figure 1. The progressive increase in REC values across the decrease temperatures from −10 °C to −30 °C of the same cultivars (**a**–**e**) or across the five walnut cultivars under the same low-temperature treatment ((**f**–**j**), $p < 0.05$). Different lowercase letters in the same column indicate significant differences among cultivars ($p < 0.05$).

3.2. Logistic Equation Analysis and LT50 Estimation

Logistic regression analysis of REC data estimated the semi-lethal temperature (LT50) for each cultivar, quantifying their cold tolerance levels (Table 2). The LT50 values ranged from −21.73 °C for 'Wen 81' to −6.51 °C for 'Xinxin 2', indicating substantial variability in cold hardiness among the cultivars. 'Wen 81', with the lowest LT50, exhibited the highest cold tolerance, followed by 'Wen 185', 'Zha 343', and 'Xinzaofeng'. In contrast, 'Xinxin 2' had the lowest cold tolerance, as indicated by its highest REC values and susceptibility to membrane damage.

Table 2. Logistics equation of the relative electrical conductivity and the LT50 of five walnut cultivars.

Cultivars	Logistics Equation	LT50/°C	R2	Sequence of Cold Resistance
Xinxin 2	$Y = 100/(1 + 1.18 \times 10^{0.0253}x)$	−6.51	0.77	5
Wen 81	$Y = 100/(1 + 2.72 \times 10^{0.0460}x)$	−21.73	0.97	1
Wen 185	$Y = 100/(1 + 2.70 \times 10^{0.0559}x)$	−17.76	0.92	2
Zha 343	$Y = 100/(1 + 2.65 \times 10^{0.0608}x)$	−16.00	0.87	3
Xinzaofeng	$Y = 100/(1 + 2.30 \times 10^{0.0580}x)$	−14.38	0.86	4

These findings are consistent with the trends observed in REC, where 'Wen 81' consistently showed lower values, suggesting superior cold resistance linked to improved cellular resilience and more robust protective mechanisms. In contrast, the high LT50 for 'Xinxin 2' confirms its lower tolerance to cold, as reflected in its greater membrane damage.

3.3. Impact of Low Temperatures on Bud Germination and Regrowth (RG) Potential

The regrowth potential, measured as the bud germination rate (GR), was significantly affected by low temperatures across all cultivars (Figure 2, Table 3). Following exposure to low temperatures, regrowth potential was significantly higher in 'Wen 81' and 'Wen 185', highlighting these cultivars' resilience and ability to recover from freezing stress. The RG data illustrate the long-term adaptability of each cultivar, complementing immediate REC measurements to provide a more complete picture of cold tolerance. At −10 °C and −15 °C, all cultivars maintained relatively high GRs, with 'Wen 81' achieving the highest (90.19 ± 4.69% at −10 °C and 85.37 ± 0.89% at −15 °C). This suggests that under moderate cold stress, 'Wen 81' retains strong regrowth potential, likely due to its ability to minimize membrane damage and maintain metabolic activity. However, as temperatures dropped to −20 °C, GRs sharply declined in all cultivars, particularly in 'Xinxin 2' and 'Zha 343', where values dropped to 47.51 ± 3.69% and 41.70 ± 2.90%, respectively. This decline is likely due to increased membrane damage and reduced recovery capacity, as reflected in their higher REC values.

Figure 2. Effect of low temperature on the bud germination of annual branches in five cultivars. Note: From left to right, from top to bottom, the varieties are 'Xinxin 2', 'Wen 81', 'Wen 185', 'Zha 343', and 'Xinzaofeng'; each panel indicates the step decreased temperature treatment from −10 to −30 °C.

At −25 °C, only 'Wen 81' and 'Wen 185' retained moderate GRs, further highlighting their resilience under extreme cold stress. Their ability to maintain some regrowth potential under such conditions indicates a more robust defense system, possibly involving higher soluble sugar content and antioxidant activity.

Table 3. The effect of low temperature on the bud germination rate (GR) of the annual branches in five walnut cultivars.

Cultivars	GR (%)				
	−10 °C	−15 °C	−20 °C	−25 °C	−30 °C
Xinxin 2	82.14 ± 3.05 aA	86.60 ± 4.38 abA	47.51 ± 3.69 aB	29.65 ± 3.70 aB	0.56 ± 2.31 aC
Wen 81	90.19 ± 4.69 aA	85.37 ± 0.89 aAB	66.76 ± 2.40 aAB	36.53 ± 5.16 aCB	1.50 ± 4.16 aC
Wen 185	88.26 ± 5.33 aA	88.59 ± 3.81 abA	68.91 ± 2.27 aAB	36.21 ± 4.45 aBC	0.31 ± 1.88 aCD
Zha 343	77.27 ± 1.16 aA	68.36 ± 3.22 abA	41.70 ± 2.90 bB	3.31 ± 1.60 bC	0.27 ± 1.98 aC
Xinzaofeng	73.41 ± 3.40 aA	65.95 ± 1.86 bAB	47.02 ± 1.45 bBC	7.20 ± 1.00 bCD	3.43 ± 4.85 aD

Note: Different lowercase letters in the same column indicate significant differences among varieties ($p < 0.05$), and uppercase letters indicate significant differences among temperature treatment ($p < 0.05$).

3.4. Variations in Antioxidant Enzyme Activities

The activities of antioxidant enzymes, including superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), along with the content of malondialdehyde (MDA), were measured to evaluate their role in mitigating oxidative stress during low-temperature exposure. These measurements are presented in Figures 3–6.

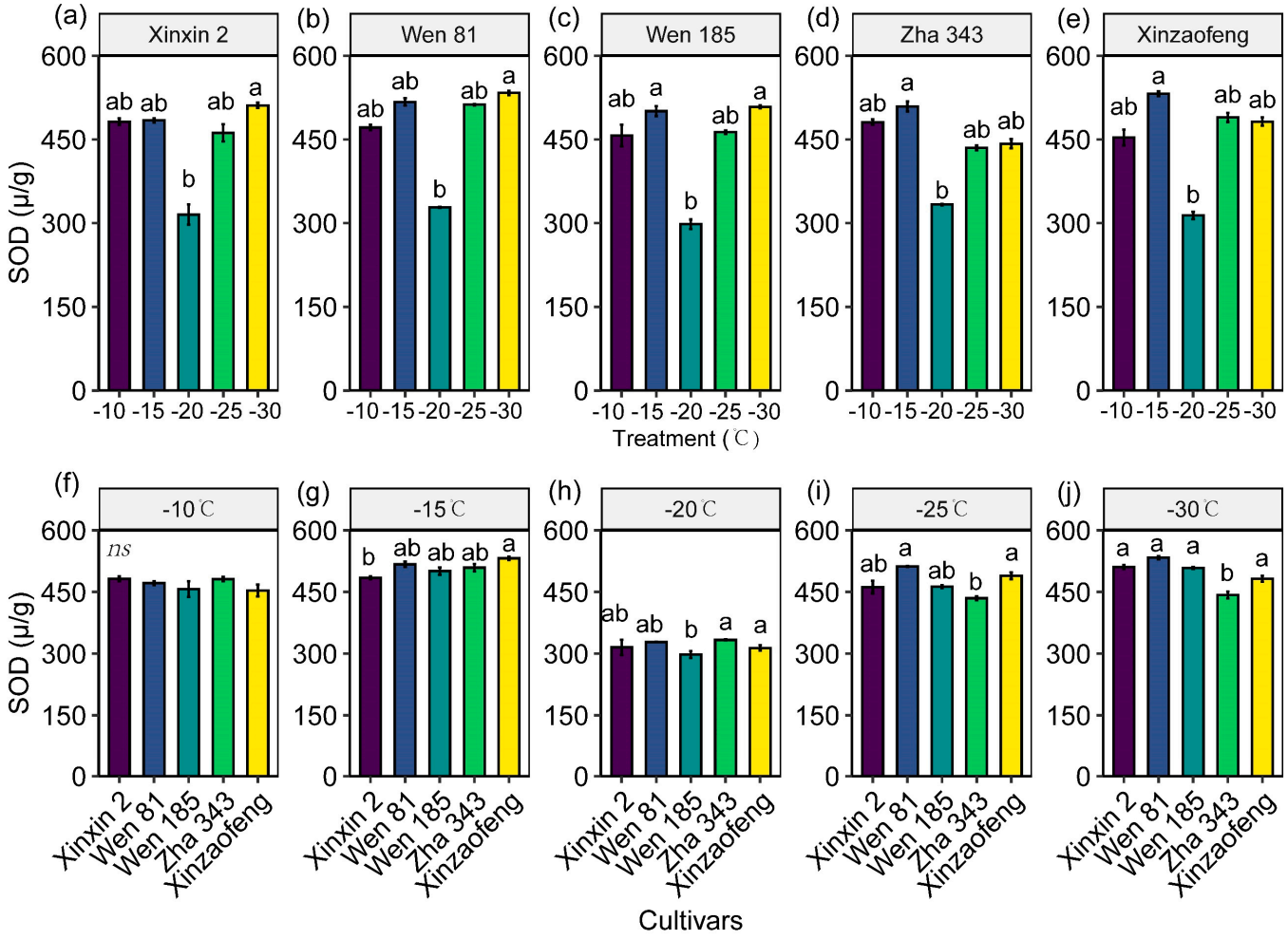

Figure 3. Changes in the superoxide dismutase (SOD) across the decrease temperatures from −10 °C to −30 °C of the same cultivars (**a**–**e**) or across the five walnut cultivars under the same low-temperature treatment ((**f**–**j**), $p < 0.05$). Different lowercase letters in the same column indicate significant differences among cultivars ($p < 0.05$). ns, not significant.

SOD activity plays a crucial role in scavenging superoxide radicals generated during oxidative stress induced by low temperatures. As the treatment temperature decreased and the duration of exposure increased, the superoxide dismutase (SOD) activity in the one-year-old branches of the five walnut cultivars followed a complex pattern: an initial rise, followed by a decline, and then a subsequent increase, with the turning point occurring at −20 °C (Figure 3a–e). At −10 °C, no significant differences in SOD activity were observed among the cultivars. By −15 °C, however, 'Xinzaofeng' demonstrated the highest SOD activity (531.95 U/g), while 'Xinxin 2' recorded the lowest (484.33 U/g). At −20 °C, 'Zha 343' exhibited the peak SOD activity (333.4 U/g); whereas, 'Wen 185' showed the lowest value (297.96 U/g). At −25 °C, 'Wen 81' had the highest SOD activity (512.29 U/g), in contrast to 'Zha 343', which displayed the lowest (435.03 U/g). Finally, at −30 °C, 'Zha 343' continued to show the lowest SOD activity (442.51 U/g), which was significantly lower than that of the other four cultivars (Figure 3f–j).

POD is another important antioxidant enzyme that helps mitigate oxidative stress by decomposing hydrogen peroxide (H_2O_2) into water and oxygen. Figure 4a–e show that POD activity in the cultivars generally followed a pattern of increasing and then decreasing as temperatures dropped. 'Wen 81' exhibited the highest POD activity at −25 °C (304.22 U/g), and 'Wen 185' exhibited the highest POD activity at −20 °C (192.00 U/g), indicating an enhanced capacity to detoxify ROS at the specific temperature. 'Xinxin 2' showed minimal variation in POD activity across temperatures, reflecting limited adaptability to oxidative stress. This trend suggests that POD plays a significant role in cold tolerance, especially in cultivars such as 'Wen 81' and 'Wen 185' that showed strong enzyme activity in response to severe cold.

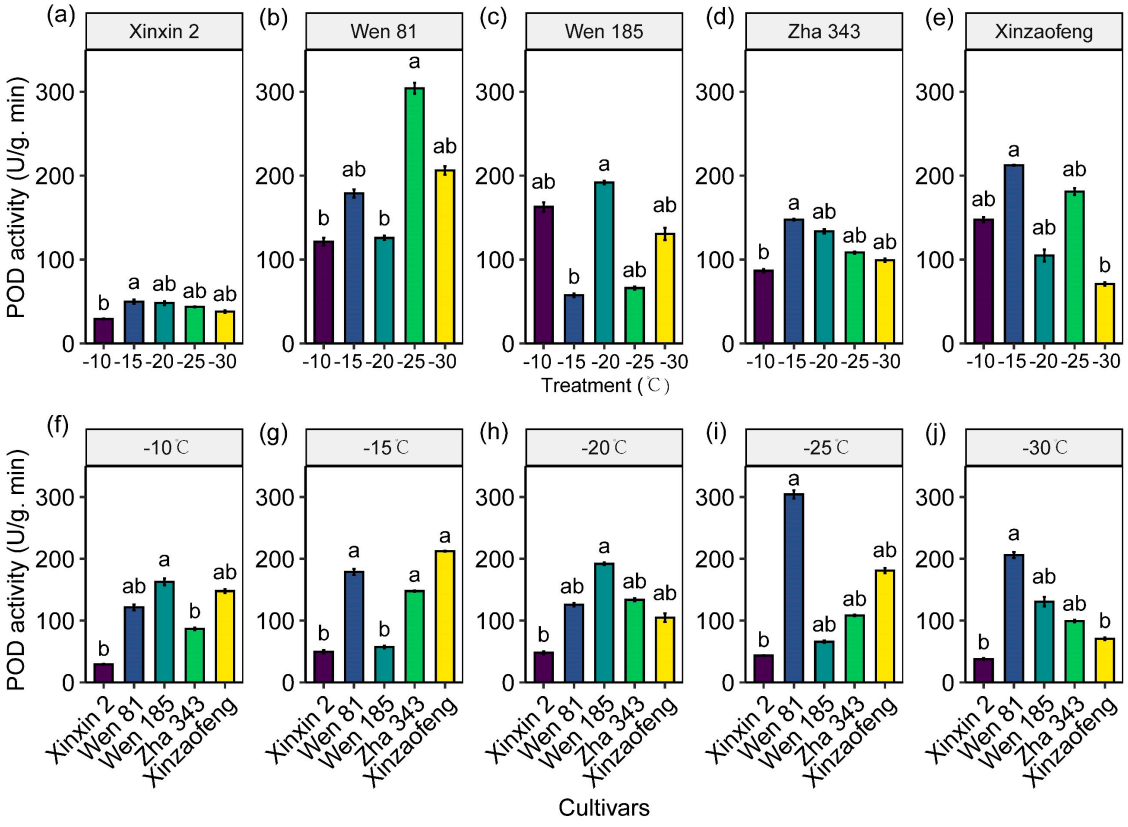

Figure 4. Changes in the enzymatic assays for peroxidase (POD) across the decrease temperatures from −10 °C to −30 °C of the same cultivars (**a**–**e**) or across the five walnut cultivars under the same low-temperature treatment ((**f**–**j**), $p < 0.05$). Different lowercase letters in the same column indicate significant differences among cultivars ($p < 0.05$).

CAT activity is essential for breaking down H_2O_2 and protecting cells from oxidative damage during cold stress. As shown in Figure 5, CAT activity in the five cultivars peaked at different temperatures. 'Wen 185' exhibited the highest CAT activity at −15 °C (124.12 U/g), while the lowest activity was observed at −30 °C, indicating that extreme cold conditions overwhelm CAT's protective function. 'Xinzaofeng' and 'Wen 81' also showed relatively high CAT activity at −20 °C, suggesting that these cultivars possess robust enzymatic defense systems at moderate cold levels. The fluctuations in CAT activity reflect the varying abilities of each cultivar to respond to increasing oxidative stress as temperatures decline.

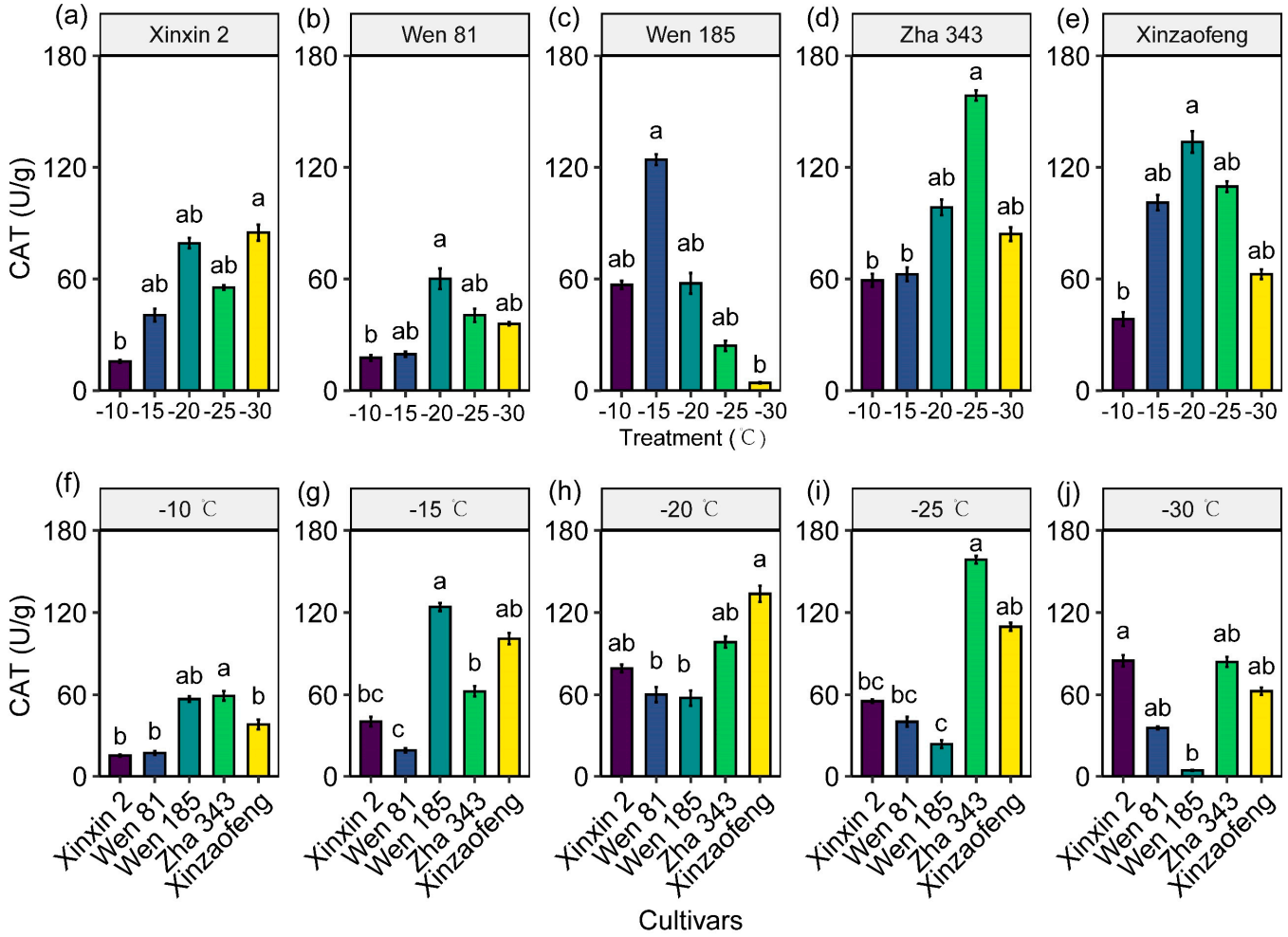

Figure 5. Changes in the catalase (CAT) content across the decrease temperatures from −10 °C to −30 °C of the same cultivars (**a**–**e**) or across the five walnut cultivars under the same low-temperature treatment ((**f**–**j**), $p < 0.05$). Different lowercase letters in the same column indicate significant differences among cultivars ($p < 0.05$).

MDA content, an indicator of lipid peroxidation and cellular damage, increased with decreasing temperature across all cultivars, as shown in Figure 6. At −10 °C, 'Zha 343' had the lowest MDA content (2.68 nmol/L), indicating minimal lipid peroxidation (Figure 6f). Conversely, 'Xinxin 2' had the highest MDA content at −10 °C (5.73 nmol/L), suggesting greater susceptibility to oxidative damage. As temperatures decreased further, MDA levels rose significantly (Figure 6a–e), with 'Wen 81' reaching the highest value at −30 °C (8.47 nmol/L). These results indicate that lower temperatures intensify lipid peroxidation, with cultivars such as 'Xinxin 2' being particularly vulnerable to oxidative damage. The enhanced activities of SOD and POD in 'Wen 81' and 'Wen 185' highlight their robust

antioxidant defense systems, which are critical for mitigating oxidative damage caused by freezing stress.

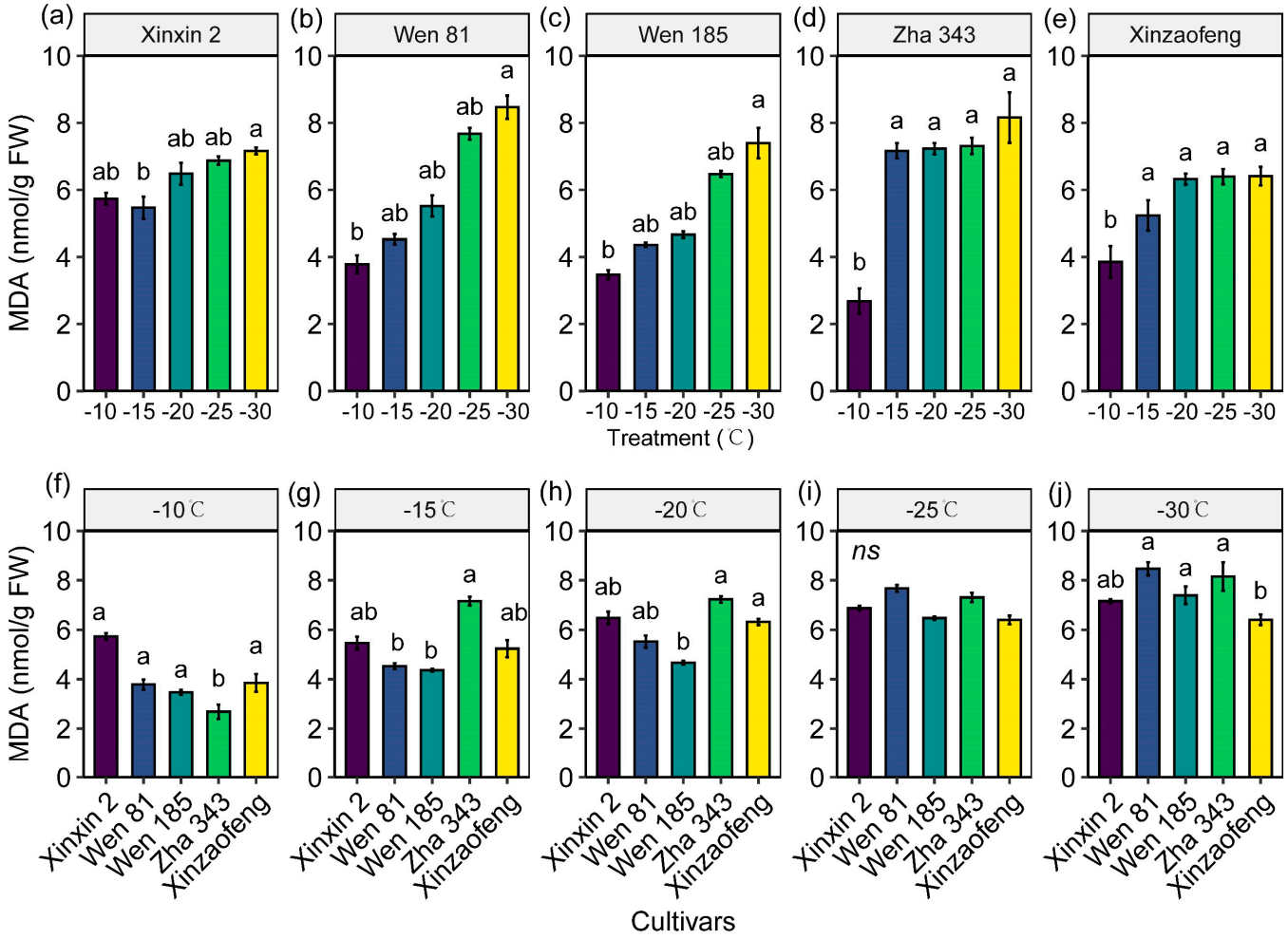

Figure 6. Changes in the malondialdehyde (MDA) content across the decrease temperatures from −10 °C to −30 °C of the same cultivars (**a**–**e**) or across the five walnut cultivars under the same low-temperature treatment ((**f**–**j**), $p < 0.05$). Different lowercase letters in the same column indicate significant differences among cultivars ($p < 0.05$). ns, not significant.

3.5. Accumulation of Osmoregulatory Substances

The content of osmoregulatory substances, including soluble sugars, proteins, and proline, increased as temperatures decreased across all cultivars (Figures 7–9). Soluble sugars are critical for osmotic adjustment and protecting cells from freezing-induced dehydration. Their accumulation under cold stress is essential for enhancing cold tolerance by supporting metabolic activity, ensuring energy supply, lowering the freezing point, retaining cellular water, and mitigating damage from water loss. However, in one-year-old branches of the five walnut cultivars, soluble sugar content did not significantly change with decreasing temperatures or prolonged exposure, although all cultivars followed a similar trend: 'Zha 343' > 'Xinzaofeng' > 'Wen 185' > 'Wen 81' > 'Xinxin 2' under each temperature treatment. These variations reflect the role of soluble sugars in stabilizing cell membranes and maintaining osmotic balance under low temperatures, particularly in 'Zha 343', which had the highest sugar content. At −15 °C, all five cultivars reached peak soluble sugar levels, with 'Zha 343' at 106.3 μg/g and 'Xinxin 2' at 54.0 μg/g. Between −10 °C and −15 °C, an increasing trend was observed, with 'Wen 81' showing the largest increase (17.36%) and 'Xinzaofeng' the smallest (3.67%).

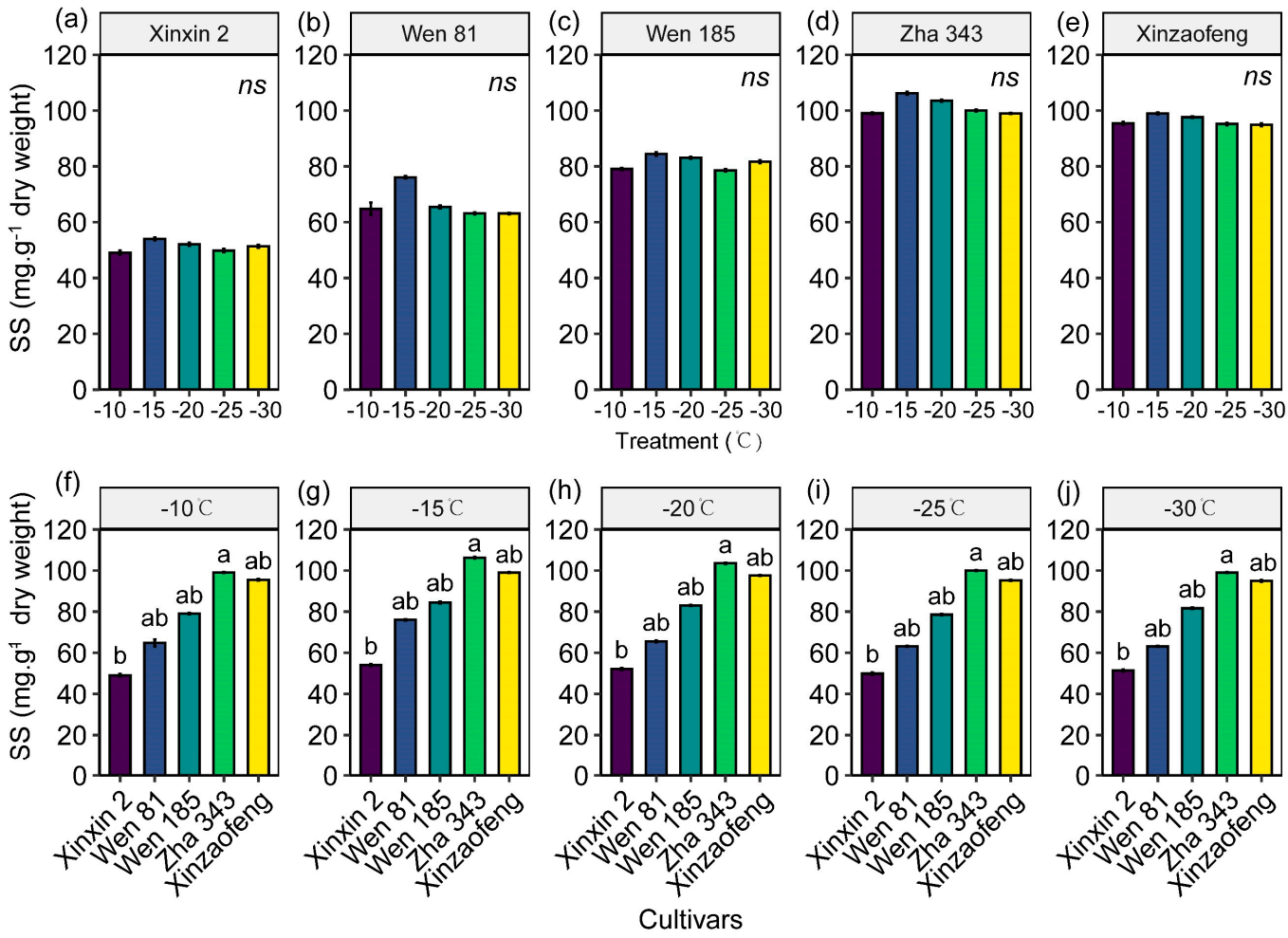

Figure 7. Changes in the soluble sugars (SSs) content across the decrease temperatures from −10 °C to −30 °C of the same cultivars (**a**–**e**) or across the five walnut cultivars under the same low-temperature treatment ((**f**–**j**), $p < 0.05$). Different lowercase letters in the same column indicate significant differences among cultivars ($p < 0.05$). ns, not significant.

Proline accumulation, which is critical for osmotic adjustment and protecting cellular integrity under freezing conditions, was highest in 'Wen 81' at −20 °C, with a proline content significantly higher than that of the other cultivars (Figure 8). Under normal growth conditions, proline content in plants is relatively low, but in response to cold, drought, diseases, and environmental pollution, plants accumulate large amounts of proline. The accumulation of proline is closely related to the plant's resistance to adverse environmental stresses. The results in Figure 8 show that the proline content in one-year-old branches of the five walnut cultivars generally exhibited a pattern of first increasing and then decreasing as treatment temperature decreased and duration increased, reaching a maximum at −25 °C. 'Xinxin 2', 'Wen 185', and 'Zha 343' all reached their highest proline content at −25 °C. Between −25 °C and −30 °C, the proline content of all five cultivars showed a decreasing trend. Across the five low-temperature treatments, 'Wen 81' and 'Xinzaofeng' had relatively low proline levels, with their changes significantly different from those of the other three cultivars. 'Xinxin 2' and 'Zha 343' showed a substantial increase in proline content between −20 °C and −25 °C, significantly higher than at other temperatures, while 'Wen 185' showed a significant increase in proline content between −10 °C and −15 °C. At −25 °C, 'Xinxin 2' had the highest proline content (54.90 μg/g), while 'Xinzaofeng' had the lowest (5.60 μg/g).

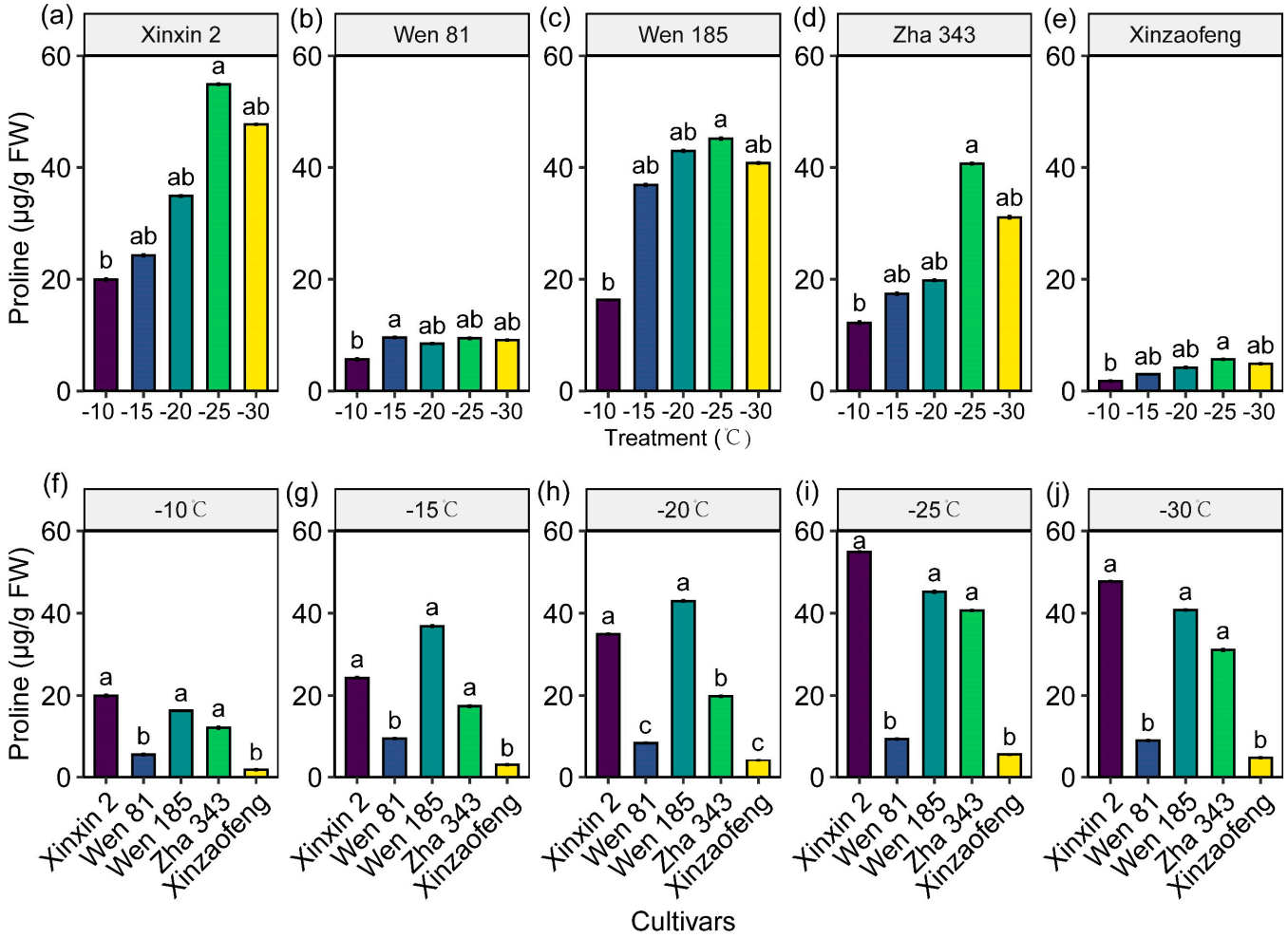

Figure 8. Changes in the proline content across the decrease temperatures from −10 °C to −30 °C of the same cultivars (**a**–**e**) or across the five walnut cultivars under the same low-temperature treatment ((**f**–**j**), $p < 0.05$). Different lowercase letters in the same column indicate significant differences among cultivars ($p < 0.05$).

Soluble proteins, as hydrophilic colloidal substances, play a crucial role in enhancing water retention capacity in cells and reducing ice crystal formation, thereby mitigating cellular damage under low temperatures. The results in Figure 9 show that the soluble protein content in the one-year-old branches of the five walnut cultivars generally followed a pattern of first increasing, then decreasing, and then increasing again as the treatment temperature decreased and duration increased. At −10 °C, 'Xinxin 2' had the highest soluble protein content (4.12 mg/g), while 'Xinzaofeng' had the lowest (2.34 mg/g). Across the five low-temperature treatments, the soluble protein content was highest at −10 °C, indicating that soluble proteins were produced by all five walnut cultivars in response to low-temperature stress to counteract damage. At −15 °C, 'Xinxin 2' and 'Xinzaofeng' had significantly higher soluble protein content compared to the other three cultivars. By −30 °C, 'Xinxin 2' exhibited the highest soluble protein content (2.34 mg/g), while 'Wen 185' had the lowest (1.34 mg/g), suggesting that 'Xinxin 2' had better cold resistance than 'Wen 185' under extreme cold conditions.

These findings highlight the significant variability in cold tolerance among the studied cultivars. Specifically, 'Wen 81' and 'Wen 185' exhibited superior performance across key metrics, including lower REC values, enhanced antioxidant enzyme activities, and greater bud regrowth rates. Conversely, 'Xinxin 2' displayed the highest susceptibility to freezing

stress. These results underscore the critical role of antioxidant defenses and membrane stability in cold tolerance.

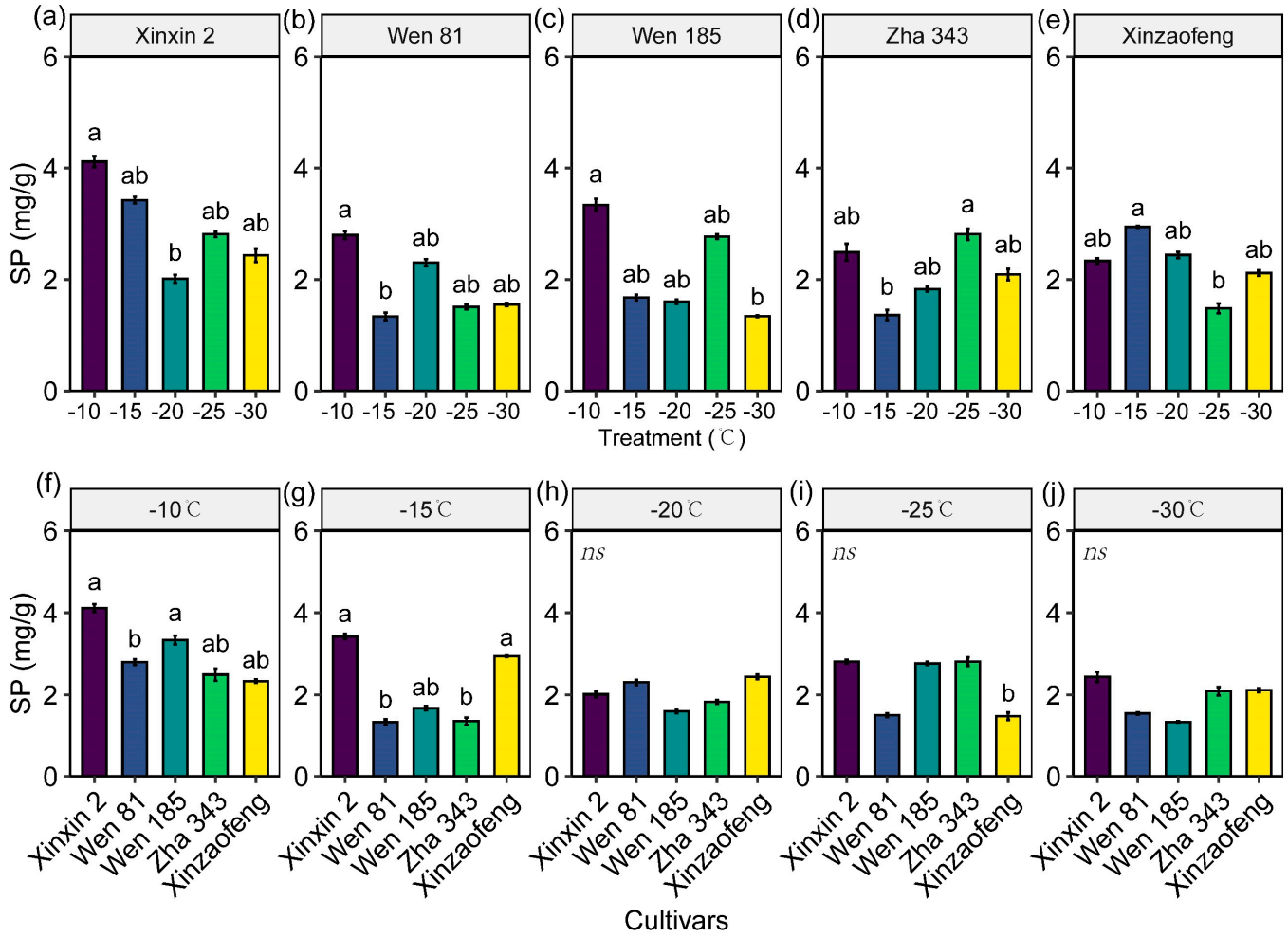

Figure 9. Changes in the soluble protein (SP) content across the decrease temperatures from −10 °C to −30 °C of the same cultivars (**a**–**e**) or across the five walnut cultivars under the same low-temperature treatment ((**f**–**j**), $p < 0.05$). Different lowercase letters in the same column indicate significant differences among cultivars ($p < 0.05$). ns, not significant.

3.6. Correlation Analysis and PCA

A correlation analysis was conducted to identify relationships between various cold resistance indices, including REC, GR, antioxidant enzyme activities, and osmoregulatory substance content (Figure 10). The analysis revealed a strong negative correlation between EC, REC, MDA, and regrowth potential (GR), indicating that increased cell membrane damage is associated with reduced regrowth potential and weaker antioxidant defenses.

In the principal component analyses (PCAs), the PC1 and PC2 axes accounted for 37.4% and 19.5% of the total variance, respectively, for the 10 measured plant traits under low-temperature treatments (Figure 11a). PC1 was primarily associated with traits reflecting cellular stability and damage degree, such as EC, MDA, REC, and GR. PC2 highlighted biochemical responses, including antioxidant enzyme activities and osmotic adjustments. Significant differences among the low-temperature treatments were visible in the principal components of the cold hardiness traits (Figure 11b). Together, these principal components provide a comprehensive framework for understanding the physiological and biochemical factors driving cold tolerance variability among the studied walnut cultivars. When analyzing the low-temperature treatment results for the 10 plant traits among cultivars,

the PC1 and PC2 axes of the PCAs accounted for 37.4% and 19.5% of the total variance, respectively (Figure 11c). Variation between the results of the 10 traits from the PCA under low-temperature treatment showed no significant variation among cultivars (Figure 11d). Principal component analysis (PCA) of cold resistance traits further reinforced the influence of temperature treatment on hardiness-related parameters. However, it cannot completely distinguish the cold hardiness among the five cultivars. The PCA underscores the importance of cellular stability and antioxidant activity as key contributors to cold tolerance variability among the studied cultivars.

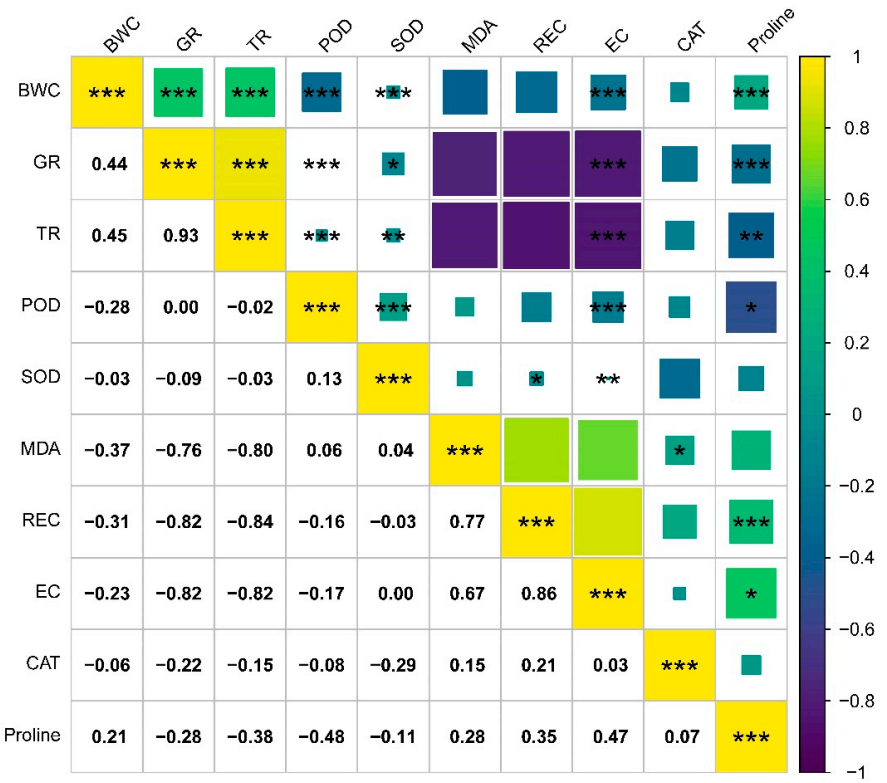

Figure 10. Correlation analysis of cold resistance indices of five different walnut cultivars. Note: *** indicated significant differences at $p < 0.001$, ** indicated significant differences at $p < 0.01$, and * indicated significant differences at $p < 0.05$.

3.7. Evaluating Cold Resistance Using Mean Membership Function

The survival mechanism of plants in response to cold challenges is highly complex, and single physiological indices are often insufficient to determine their cold resistance capabilities. Therefore, a subordination function method (SF) was used to calculate the average membership function values of various physiological indices under multiple low-temperature conditions as a comprehensive indicator of cold resistance. In this study, subordination function values for 11 physiological indices measured in five walnut cultivars under different low-temperature conditions were calculated (Table 4). The results show that 'Wen 185' exhibited the highest cold resistance, with a membership function value of 0.592, while 'Wen 81' exhibited the lowest, with a value of only 0.400. Based on the mean membership function values, the mean membership function value of the five walnut cultivars is as follows: 'Wen 185' > 'Zha 343' > 'Xinxin 2' > 'Xinzaofeng' > 'Wen 81'.

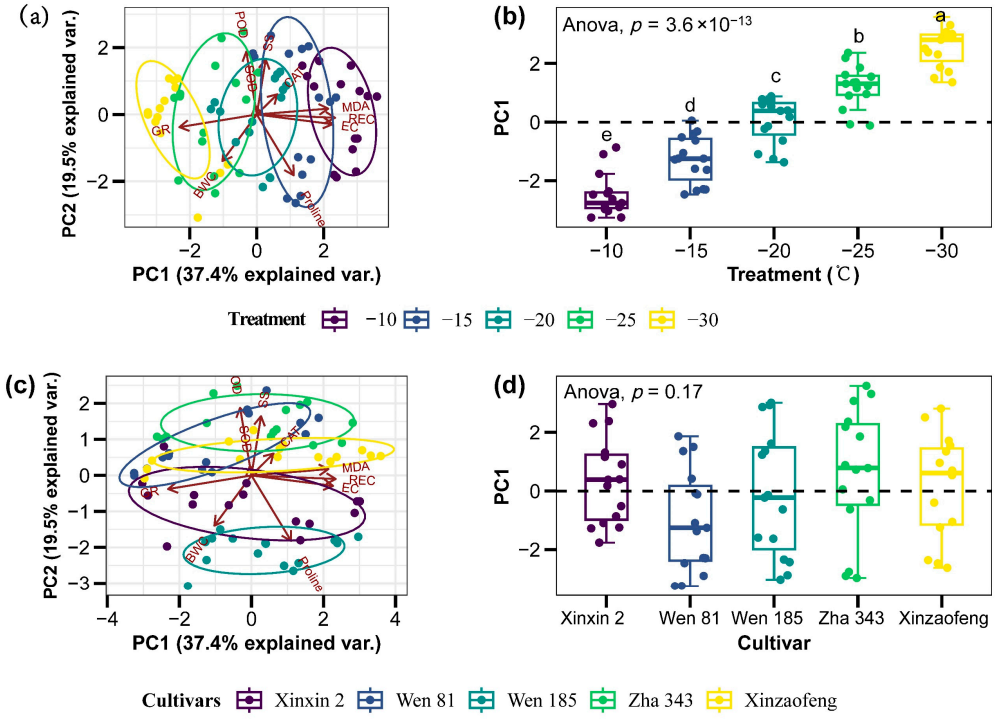

Figure 11. Principal component analysis of 10 traits among five low-temperature treatments (**a**), and ANOVA showing the differences among temperature treatments (**b**). Principal component analysis of 10 traits among five cultivars under low-temperature treatment (**c**), and ANOVA showing the differences among five cultivars under low-temperature treatment (**d**) along PC1. Horizontal lines within boxes indicate the median based on measurements (dots) within a region. Boxes indicate the upper (75%) and lower (25%) quartiles. Whiskers represent the ranges of the minimum and maximum values. Different lowercase letters in the same column indicate significant differences among cultivars ($p < 0.05$).

Table 4. Subordination values of cold resistance indicators of shoots from five different walnut cultivars.

Cultivars	Xinxin 2	Wen 81	Wen 185	Zha 343	Xinzaofeng
EC	0.235	0.000	1.000	0.504	0.518
REC	0.814	0.000	0.842	0.683	1.000
BWC	1.000	0.000	0.813	0.442	0.300
GR	0.687	0.917	1.000	0.000	0.000
SP	1.000	0.019	0.967	1.000	0.000
SS	0.000	0.265	0.573	1.000	0.905
CAT	0.233	0.122	0.000	1.000	0.637
POD	0.000	1.000	0.086	0.249	0.528
SOD	0.348	1.000	0.362	0.000	0.706
MDA	0.375	1.000	0.061	0.718	0.000
Proline	1.000	0.076	0.803	0.712	0.000
Mean membership function value	0.518	0.400	0.592	0.573	0.418
Cold resistance level	MR	LR	HR	HR	LR

4. Discussion

Our study identified significant variability in cold hardiness among five early-fruiting walnut cultivars, reflecting a complex interplay of physiological and biochemical factors crucial for their survival under low-temperature stress. The TR, cultivars, and their interactions had significant effects on most of the cold-tolerant traits (Table A1). Notably, 'Wen

185' emerged as the most cold-tolerant cultivar, demonstrating the effectiveness of using a combination of traditional and innovative assessment methods.

4.1. Discrepancies in Cold Hardiness Assessments

The observed differences in cold tolerance rankings across the methods can be explained by the distinct physiological aspects that each method targets. Measurements of EC and REC reflect immediate membrane damage due to cold stress, while antioxidant enzyme activities, such as those of SOD and CAT, provide insights into the plant's ability to neutralize oxidative stress and recover from damage [62,63]. Additionally, the bud germination rate (GR) offers a comprehensive view of the plant's recovery potential, integrating short-term damage with long-term resilience mechanisms [63].

LT50 and REC provide valuable insights into the immediate cellular response to freezing temperatures [64]. 'Wen 81' consistently exhibited the highest cellular integrity, reflecting its superior cold tolerance, closely followed by 'Wen 185' (Figure 1 and Table 1). These results align with previous studies, showing that the LT50 values for walnut cultivars fall within a similar range, further confirming the presence of genetic variability in cold hardiness [51]. In contrast, the regrowth tests, which assess the plant's recovery capacity after cold exposure [65], emphasize the resilience of 'Wen 81' and 'Wen 185' (Figure 2 and Table 3). These tests offer valuable insights into not just survival but also the potential for regrowth, highlighting adaptability to cold stress.

However, when broader physiological and biochemical data are integrated through the average membership function values [35], 'Wen 185' ranked highest (Table 4), suggesting that more holistic assessments may uncover aspects of cold tolerance not evident from single-parameter tests. This underscores the importance of using a multifaceted approach when evaluating cold responses, combining immediate damage indicators with measures of recovery potential [66,67].

Table 5 provides a comprehensive summary of the physiological and biochemical traits associated with cold tolerance across the five walnut cultivars. Notably, 'Wen 81' and 'Wen 185' demonstrated superior cold tolerance, characterized by low REC values (60.36% and 65.74%, respectively), high regrowth potential (36.53% and 36.21%), and elevated antioxidant enzyme activities, such as SOD and POD. These traits underscore their robust cellular stability and efficient defense mechanisms against oxidative damage. In contrast, 'Xinxin 2' exhibited the highest REC value (65.80%) and the lowest LT50 (−6.51 °C), indicating significant membrane damage and poor adaptability under freezing stress. The lower antioxidant enzyme activities and osmoregulatory compound levels in 'Xinxin 2' further reflect its susceptibility to oxidative stress and dehydration. The mean membership function values further validate these observations, ranking 'Wen 185' as the most cold-resistant cultivar (0.592), followed by 'Zha 343' (0.573), while 'Wen 81' scored lower due to its reduced osmoregulatory responses. This analysis highlights the importance of integrating physiological and biochemical metrics to comprehensively evaluate cold tolerance across walnut cultivars.

Previous studies have shown that different methods of assessing cold tolerance often lead to variations in cultivar rankings due to the multidimensional nature of cold stress response [62,63]. For instance, plants may show significant membrane damage under initial cold stress, but their strong antioxidant defense systems enable them to recover more effectively over time [68,69]. This is consistent with our findings, where 'Wen 81' demonstrated strong recovery potential despite some initial membrane damage, likely due to its robust antioxidant enzyme activity and efficient osmoprotectant accumulation (Table 5).

Table 5. Synthesized findings linking cultivars to specific traits contributing to cold tolerance.

Trait Cultivars	Physiological Metrics			Biochemical Metrics			Comprehensive Indicator
	REC (%)	LT50 (°C)	GR (%)	SOD (mg/g)	POD (μg·min)	SS (mg/g)	Average Membership Function Values
Xinxin 2	65.80	−6.51	29.65	510.90	37.97	51.41	0.518
Wen 81	60.36	−21.73	36.53	533.33	206.22	63.10	0.40
Wen 185	65.74	−17.76	36.21	508.41	130.67	81.72	0.592
Zha 343	72.47	−16.00	3.31	442.51	99.22	98.99	0.573
Xinzaofeng	71.23	−14.38	7.20	482.11	70.89	94.98	0.418

Our study's methodological approach, combining electrolyte leakage measurements with regrowth assessments, provides a comprehensive view of the cultivars' resilience to cold stress [34,35,66]. Such dual assessments are critical for breeding and selecting walnut varieties that are better suited for regions prone to cold conditions.

4.2. The Protective Role of the Antioxidant System and Osmoregulatory Substances in Cold Injury

The variability in cold hardiness among cultivars can also be linked to the activity of antioxidant enzymes such as SOD, POD, and CAT, which play critical roles in mitigating oxidative stress under cold conditions. The greater resilience observed in 'Wen 81' and 'Wen 185' can be attributed to their enhanced antioxidant defenses, which help maintain cellular integrity under stress [27,70]. This is supported by findings that higher antioxidant enzyme activity is correlated with improved cold tolerance, as observed in various studies on walnut and other species [26,30].

The enhanced activity of antioxidant enzymes in 'Wen 81' and 'Wen 185' likely played a pivotal role in minimizing oxidative damage (Figures 3 and 4), as reflected in their relatively lower REC values compared to other cultivars (Figure 2). The ability to efficiently scavenge reactive oxygen species (ROS) is crucial for maintaining membrane stability during cold stress [71]. Consequently, cultivars with higher antioxidant enzyme activities are better equipped to mitigate oxidative damage, which supports their overall recovery potential, as seen in their higher bud germination rates (Table 3).

The higher antioxidant enzyme activities observed in the walnut cultivars play a vital role in scavenging reactive oxygen species (ROS) and protecting cellular integrity under freezing stress. Similar trends have been documented in other nut crops, such as pistachios (*Pistacia vera*), where diverse antioxidant enzymes, including ascorbate peroxidase (APX), polyphenol oxidase (PPO), catalase (CAT), and guaiacol peroxidase (GPX), contribute to mitigating oxidative damage under cold stress [12]. However, the precise roles of these antioxidant enzymes in freezing tolerance require further investigation in other nut crops to better understand their mechanisms and broader applicability.

In addition to the antioxidant system, osmoregulatory substances play a crucial role in enhancing cold tolerance. Compounds such as soluble sugars (SSs), proline, and soluble proteins (SPs) contribute to osmotic adjustment and membrane stabilization during freezing stress [36,37]. In this study, walnut cultivars subjected to lower temperature treatments exhibited significantly higher levels of SSs, proline, and SPs (Figures 7–9), which are known to protect cellular structures by preventing dehydration and stabilizing cellular membranes, and maintained protein functionality under extreme conditions. Similar patterns have been reported in other nut crops, such as almonds, hazelnuts, and pecans, where elevated levels of soluble sugars and proline have been strongly correlated with enhanced freezing resistance [14–16]. These findings highlight that osmoregulatory substances, together with antioxidant enzyme activities, form a dual defense mechanism critical for cold hardiness in walnut cultivars. This synergy not only reduces cellular damage during freezing stress

but also supports post-stress recovery, making it a key area for further exploration in cold tolerance research across nut crops.

4.3. Climate Change and Breeding Implications

As climate change leads to more frequent and severe cold events [15], the need to enhance the cold hardiness of walnut cultivars is becoming increasingly urgent [22]. Our findings demonstrate that antioxidant enzyme activity and osmoregulatory responses are critical in boosting cold tolerance among these cultivars. This insight is crucial for breeding programs aimed at developing varieties that can withstand cold extremes, especially in regions prone to frost [72,73]. Cultivars such as 'Wen 81' and 'Wen 185' have shown remarkable cold resilience, positioning them as prime candidates for further breeding and cultivation in areas experiencing heightened cold event frequencies and intensities. As climate conditions continue to evolve, selecting cultivars with robust regrowth potential and cellular stability post-freezing is vital for ensuring stable yields and enhancing the resilience of walnut production systems [74]. Our research underscores the importance of targeted breeding strategies that not only enhance innate cold tolerance but also improve cultivars' recovery capabilities after cold exposure. Future research should focus on genetic analyses to develop walnut varieties with advanced cold tolerance traits, thereby bolstering the industry's resilience to changing climatic conditions [75,76].

The notable variability in cold tolerance observed among the cultivars studied presents significant opportunities for breeding programs to hone these traits [77]. Given the anticipated increase in frost occurrences as projected by climate models [17], cultivating resilient cultivars like 'Wen 185'—known for its superior cold hardiness—is crucial for maintaining stable walnut production. This study advocates for the integration of both traditional and molecular breeding techniques to refine and enhance cold tolerance traits [78].

4.4. Limitations and Future Directions

While this study provides valuable insights into the physiological and biochemical mechanisms underlying cold tolerance in walnut cultivars, certain limitations should be acknowledged. First, the controlled freezing experiments, while effective in isolating key physiological responses, do not fully replicate the complexity of natural environments [32,79]. Factors such as fluctuating day–night temperatures, soil moisture variability, wind exposure, and interactions with pests or diseases were not accounted for in this study [80,81]. These environmental variables can significantly influence cold tolerance responses and may lead to variability in results when applied to field conditions [82,83].

Future studies should prioritize validating these findings under natural orchard settings across diverse geographic regions and multiple growing seasons. Such efforts would provide a more comprehensive understanding of how environmental heterogeneity impacts cultivar performance [84,85]. Additionally, integrating long-term field trials with genomic and transcriptomic analyses could uncover the genetic and molecular mechanisms driving cold tolerance, facilitating the identification of reliable genetic markers [76,86].

Expanding the scope of this research to include a broader range of cultivars, particularly those adapted to different climatic zones, will enhance the generalizability of the findings. These studies will also contribute to breeding programs by identifying cold-resistant genotypes with superior adaptability to extreme climatic conditions. As climate change continues to intensify frost events, developing walnut cultivars with enhanced recovery potential and cellular stability will be critical for ensuring sustainable production in vulnerable regions.

5. Conclusions

This study offers a comprehensive assessment of the cold hardiness of selected walnut cultivars, highlighting significant variability in their physiological and biochemical responses under simulated low-temperature stress. The findings emphasize the crucial roles of antioxidant enzyme activities and osmoregulatory compounds in enhancing cold tolerance, offering valuable insights for breeding programs focused on developing climate-resilient walnut cultivars.

However, this research was conducted under controlled conditions, and future studies should validate these findings across diverse environmental contexts to better understand the interactions between cultivars and natural field conditions. Expanding the scope of research to include more walnut cultivars, particularly those grown in different geographic regions, will provide a broader perspective on genetic variability and adaptability to frost events.

Additionally, future investigations should focus on identifying genetic markers linked to cold tolerance. Integrating molecular and transcriptomic analyses with physiological assessments could uncover the underlying genetic mechanisms driving resilience, thereby accelerating the development of cold-tolerant cultivars. Such efforts will not only enhance our understanding of the complex traits governing cold hardiness but also support the sustainable production of walnuts in regions increasingly affected by climate variability and extreme weather events.

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Conflicts of Interest: The authors declare no conflicts of interest.

Appendix A

Table A1. Two-way ANOVA for TR and cultivars on cold-tolerant traits of annual branches.

Traits	TR (*df* = 4)		Cultivars (*df* = 4)		TR × Cultivars (*df* = 16)	
	F	***p***	***F***	***p***	***F***	***p***
EC	697.43	0.000 ***	152.63	0.000 ***	33.31	0.000 ***
REC	286.824	0.000 ***	56.901	0.000 ***	9.683	0.000 ***
GR	593.856	0.000 ***	37.273	0.000 ***	4.144	0.000 ***
BWC	4.878	0.002 **	5.560	0.000 ***	0.162	NS
SOD	401.642	0.000 ***	10.264	0.000 ***	6.757	0.000 ***
POD	69.83	0.000 ***	1075.64	0.000 ***	231.78	0.000 ***
MDA	183.60	0.000 ***	23.49	0.000 ***	14.48	0.000 ***
CAT	163.5	0.000 ***	274.9	0.000 ***	97.4	0.000 ***
Proline	15,407	0.000 ***	60,338	0.000 ***	2574	0.000 ***
SS	166.36	0.000 ***	9249.63	0.000 ***	14.97	0.000 ***
SP	168.64	0.000 ***	145.61	0.000 ***	65.78	0.000 ***

Note: *** indicated significant differences at $p < 0.001$, ** indicated significant differences at $p < 0.05$, and NS indicated no significant differences.

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