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Physiological Response to Low Temperature of Four Genotypes of *Cyclocarya paliurus* and Their Preliminary Evaluation to Cold Resistance

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Abstract: *Cyclocarya paliurus* is a versatile tree species with immense potential for development, as it combines edible, medicinal, and ornamental functions. Low temperature is one of the important abiotic factors that affect plant survival and flourishing but their response mechanism to low temperature is not yet clear. In this study, we utilized annual shoots of four genotypes of *C. paliurus*, namely T2, W10, M31 and S12, as materials. The physiological responses of annual shoots of *C. paliurus* to low temperature stress were elaborated by determining and comparing indicators related to cold resistance, such as relative electric conductivity, semi-lethal low temperature, malondialdehyde, soluble sugar, soluble protein, proline, superoxide dismutase and peroxidase. The contents of malondialdehyde, proline, soluble protein and peroxidase activities were not only correlated with the treatment temperature but also related to the genotypes. Osmotic substance (soluble sugar, soluble protein and proline) contents and antioxidant enzyme activities (peroxidase and superoxide dismutase) of the four genotypes showed a trend of increasing and then decreasing with the five decreasing temperatures. Furthermore, a comprehensive evaluation of cold resistance was performed by using a combination of principal component analysis and membership function, with the cold resistance ranked as W10 > M31 > S12 > T2. Results from this study would provide some references for extending the plantation areas.

Keywords: annual shoot; electrolyte permeability; osmotic regulation; principal component analysis; membership function

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1. Introduction

Cyclocarya paliurus (Batal.) Iljinsk (CP), a deciduous tree belonging to the genus *Cyclocarya* (Juglandaceae), is widely distributed in the subtropical regions of China [1]. It has gained significant attention due to the pharmacological activity of its bioactive substances, particularly in hypoglycemia, hypolipidemia, and hypotension [2,3]. Animal experiments have preliminarily confirmed that trace elements such as Mg, Mn, Cu, and Zn are beneficial in lowering blood sugar in mice [4]. The research suggested that CP possesses benefits for treating hyperlipidemia [5]. CP polysaccharides were found to be one of its major functional constituents for treating type 2 diabetes by modulating gut microbiota and short-chain fatty acids [6,7]. Previous studies have analyzed the bioactive components in CP leaves in terms of separation technology, purification method and pharmacology [8–11]. However, few studies have been performed on CP cold resistance.

Low temperature is one of the abiotic stressors that restrict the geographical distribution, growth and development of plants across the world, the immobility of plants makes it impossible to escape from low temperature by migration [12]. To overcome cold stress, the plant has developed morphological, physiological and biochemical adaptations to maximize cold tolerance by regulating their metabolism [13]. Low temperatures can trigger the accumulation of reactive oxygen species and the elevation of malondialdehyde

content, which results from membrane lipid peroxidation in plants [14]. The increase in membrane lipid peroxidation under low temperature can further cause an increase in plasma membrane permeability, leading to electrolyte leakage and an increase in cytoplasmic conductivity. The process of plants responding to low temperature stress is complex, and there are various enzymatic and non-enzymatic substances that scavenge free radicals and reactive oxygen species in plants [15,16]. Under normal temperature, the increase in defensive protective enzyme is conducive to maintaining the balance between free radical production and scavenging in plants, thereby avoiding membrane lipid peroxidation. However, when the temperature is too low the plant's homeostasis is broken, resulting in protective enzymes being unable to scavenge free radicals in a timely manner [17]. Osmotic regulation is another way plants cope with low temperatures. The increase in proline (Pro), soluble sugar (SS) and soluble protein (SP) can effectively reduce the water loss from plant cells, balance the osmotic pressure inside and outside the cells, reduce the damage caused by low temperature, plants' adaptation to low temperatures [18–21]. Previous studies on apples [22], *Herba Rhodiola* [23], grapevines [24] and peaches [25] have shown that the plants' adaptation to low temperatures is associated with a variety of factors and is a complex and variable regulatory process. It is challenging to objectively compare the strength of plant cold resistance by applying a single morphological or physiological index. More scientific and comprehensive evaluation methods such as principal component analysis, cluster analysis, and membership function can be utilized.

The CP industry is thriving today and temperature is an important factor limiting the distribution of CP and hindering its industrial development, while little research has been done on the cold resistance of CP. Understanding how CP defends itself against cold stress is crucial not only for fundamental research but also for food security and sustainability of the CP industry. To study the physiological responses and analyze the physiological changes of four different CP genotypes under cold stress, we measured and calculated various parameters including relative electric conductivity (REC), semi-lethal temperature (LT_{50}), malondialdehyde (MDA), soluble sugar (SS), soluble protein (SP), proline (Pro), superoxide dismutase (SOD), and peroxidase (POD). Additionally, we employed correlation analysis, principal component analysis, and membership function analysis to comprehensively evaluate the cold resistance of different genotypes and select CP germplasm resources with strong cold resistance.

2. Materials and Methods

2.1. Plant Materials

In October 2014, single plants of dominant or subdominant trees from different places were selected for seed collection. After the process of stratification, strengthening and refining, we selected 8–15 plants of each genotype and planted them in the Baima base of Nanjing Forestry University in April 2017 (31.58° N, 119.15° E). The final planting spacing was 2 m × 3 m, and the management measures were consistent. On 21 February 2022, 4~8 plants were selected from four different genotypes (families) with the similar size of Tiantong 2# (T2), Wufeng 10# (W10), Muchuan 31# (M31) and Shiqian 12# (S12), respectively. Table 1 shows the detail information of their female parent. The annual shoots between 0.5 and 0.8 in diameter were collected, wrapped in parafilm and brought back to the laboratory.

Table 1. The basic information about the four genotypic female parents.

Genotypes	Elevation/m	Latitude North/°	Longitude East/°	Mean Annual Temperature/°C	Annual Precipitation/mm
T2	275	29.79	121.78	17.6	1531.9
W10	963.3	30.19	110.90	12.8	1400.0
M31	1230	28.97	103.78	17.3	1236.7
S12	1230	27.35	108.11	17.5	1816.4

The mean annual temperature and annual precipitation from the statistical yearbook.

2.2. Low Temperature Treatment

The annual shoots were first cut into 15-cm long cuttings. After washing the surface of the annual shoots and wiping them dry, the materials were divided into 5 groups of 3 cuttings in each group, and three replicates were performed in each group. According to the temperature conditions in the area where CP was to be planted, the shoots were exposed to the temperature of 4 °C (as control), −4 °C, −8 °C, −16 °C and −20 °C for 12 h, respectively, followed by 4 °C for 12 h.

2.3. Measurements of Physiological Parameters

Electrolyte permeability was measured by electrical conductivity [26]. The shoots were cut into 2 mm pieces, weight 0.5 g and placed in a tube containing 20 mL of deionized water. Samples were immersed and vacuumed for 30 min, and then the conductivity of solution was measured at room temperature (R_1). Samples were then boiled in a water-bath for 20 min and cooled to room temperature to measure the final electrical conductivity (R_2). The REC was calculated as follows,

$$REC = (R_1/R_2) \times 100\%$$

REC was fitted in the logistic regression equation, $y = 100/(1 + ae^{-bt})$, where, y represents the REC, a and b are the function parameters [27].

MDA content was extracted and determination by thiobarbituric acid method [28]. Soluble sugars and starch content were quantified by anthrone colorimetric method [29]. SP content was analyzed by the Coomassie brilliant blue G-250 [30]. Pro content was determined by ninhydrin colorimetry method [31]. SOD was determined using nitroblue tetrazolium chloride [32] and POD was measured using guaiacol method [33].

2.4. Evaluation of Cold Resistance

Principal component analysis method was used to transform individual indicators into several composite indicators, and the comprehensive analysis was combined with the membership function. The composite score formula was the following,

$$\mu_i = \frac{F_i - F_{imin}}{F_{imax} - F_{imin}}$$

$$W_i = \frac{P_i}{\sum_{i=1}^{n} P_i}$$

$$D = \sum_{i=1}^{n} (\mu_i \times W_i)$$

μ_i represents the membership function value of i, F_i means the principal component score value, F_{imin} is the principal component minimum score and F_{imax} is the maximum one of i, W_i means the principal component weight of i, P_i is the principal component contribution rate of i, and D represents the composite score.

2.5. Statistical Analyses

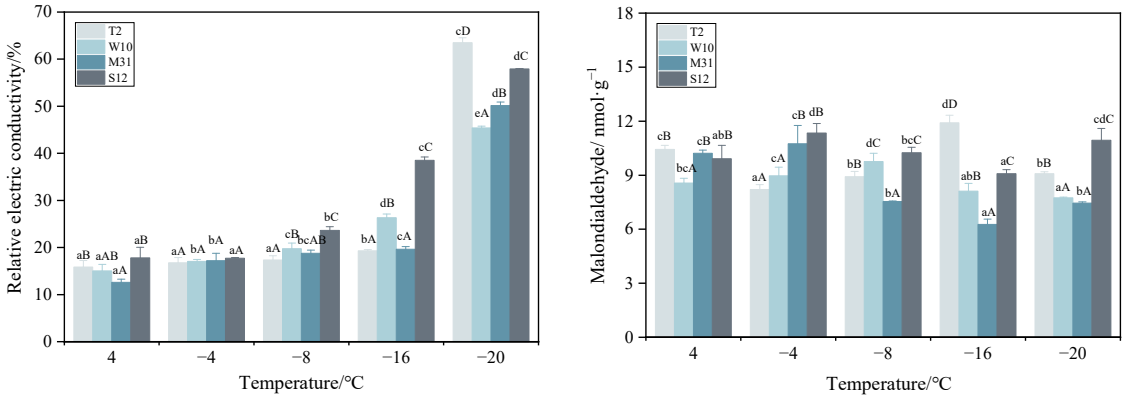
Data were presented as mean ± standard deviation (SD) and analyzed using two-way analysis of variance (ANOVA), followed by Duncan's tests ($p < 0.05$) in SPSS 26.0 (IBM Corp. in Armonk, NY, USA). Principal component analysis and membership function analysis were also performed using SPSS 26.0 (IBM Corp. in Armonk, NY, USA).

3. Results

3.1. Variation in Cell Membrane Damage

The two-way ANOVA showed that temperature, genotype, and the interaction between the two had a significant effect on REC (Supplementary data Table S1). Figure 1a

illustrated the REC values of four CP genotypes after exposure to low temperature. As the temperature decreased, the REC values increased for all four genotypes. At −20 °C treatment, the REC values peaked with 63.46%, 45.38%, 50.15% and 57.92% for T2, W10, M31, and S12, respectively. Except for the −4 °C treatment, the REC values at 4 °C, −8 °C, −16 °C and −20 °C were significantly different for the four genotypes.



(**a**) (**b**)

Figure 1. Variations in relative electric conductivity (**a**) and malondialdehyde content (**b**) in the four genotypes of *Cyclocarya paliurus* under cold stress. Significant differences between temperature treatments ($p < 0.05$) were indicated by different lowercase letters, and significant differences between genotypes ($p < 0.05$) were indicated by different capital letters.

Table 2 presents the data on logistic functions and LT_{50}s of the four genotypes. The results indicated that the fitting equations had high reliability as the fitting degrees R^2 of all functions ranged from 0.703 to 0.85, with the p value less than 0.05. The LT_{50} ranged from −20.194 °C to −29.015 °C, with W10 having the lowest value (−29.015 °C), followed by M31 (−25.787 °C) and T2 (−21.291 °C), while S12 had the highest value (−20.194 °C).

Table 2. Logistic function and semi-lethal temperature (LT_{50}) for the four genotypes.

Genotype	Logistic Equation	R^2	p Value	LT_{50}/°C
T2	$y = 100/(1 + 5.185e^{0.083t})$	0.703	0.038	−21.291
W10	$y = 100/(1 + 5.490e^{0.059t})$	0.822	0.034	−29.015
M31	$y = 100/(1 + 6.252\ e^{0.071t})$	0.827	0.032	−25.787
S12	$y = 100/(1 + 4.740\ e^{0.077t})$	0.850	0.026	−20.194

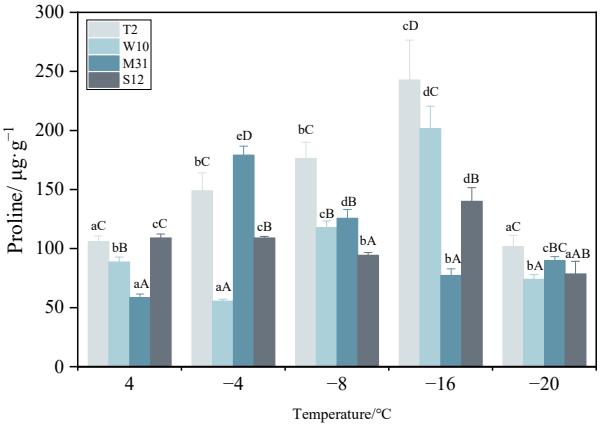
Temperature, genotype, and the interaction of the two all had significant effects on MDA (Supplementary data Table S2). Figure 1b revealed the MDA content variations in the four genotypes under low temperature treatment. The MDA content of each genotype differed significantly at the five low temperatures. M31 and S12 had the highest MDA content at −4 °C, W10 reached maximum MDA content at −8 °C, and T2 had the highest MDA content at −16 °C. Furthermore, the MDA content varied significantly among the four genotypes at the same temperature.

3.2. Variation in Osmoregulation Substances

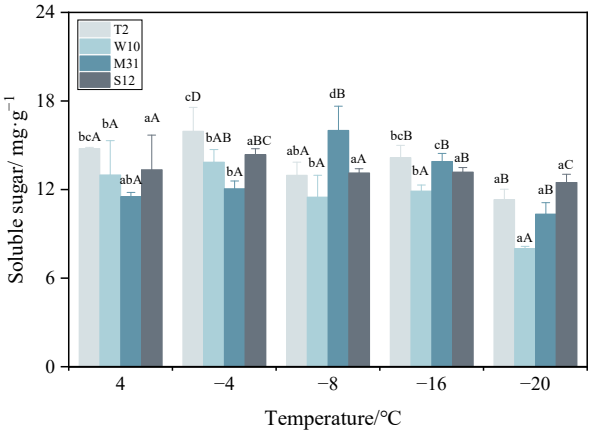
The results of two-way ANOVA showed that temperature, genotype, and the interactive effect of the two were significant on the content of osmolytes that included Pro, SS, and SP (Supplementary data Tables S3–S5).

It had shown that the Pro content was not only related to the temperature treatment but also to the genotype (Figure 2a). T2, W10 and S12 all peaked at −16 °C with 242.88 $\mu g \cdot g^{-1}$, 201.67 $\mu g \cdot g^{-1}$ and 140.06 $\mu g \cdot g^{-1}$, respectively, while M31 peaked at −4 °C

with 179.3 μg·g^{-1}. Additionally, the Pro content of T2 was higher than that of S12, W10 and M31 under all five low temperature treatments.



(**a**)



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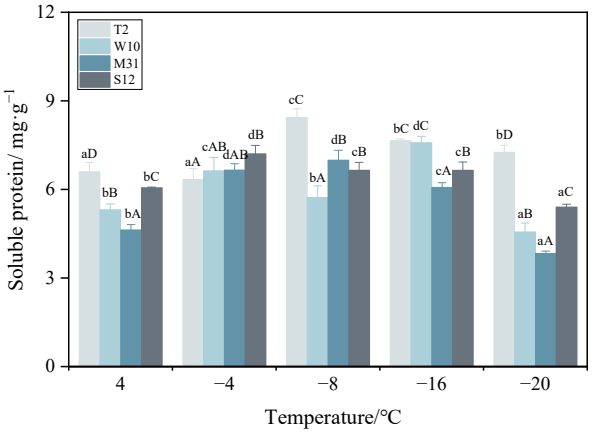

(**c**)

Figure 2. Variations in osmotic substances in four genotypes of *Cyclocarya paliurus* under cold stress. (**a**) content of Pro; (**b**) content of SS; (**c**) content of SP. Significant differences between temperature treatments ($p < 0.05$) were indicated by different lowercase letters, and significant differences between genotypes ($p < 0.05$) were indicated by different capital letters.

The SS content of the four genotypes ranged from 8.00 mg·g^{-1} to 16.01 mg·g^{-1} under 5 low temperature treatments (Figure 2b). ANOVA showed that there were significant differences in SS contents among the five temperature treatments. Moreover, when the temperature treatments were −4 °C, −8 °C, −16 °C and −20 °C, there were significant differences in SS contents among the four genotypes. T2, W10, and S12 reached their peaks at −4 °C with 15.96 mg·g^{-1}, 13.86 mg·g^{-1} and 14.37 mg·g^{-1}, respectively, while M31 achieved its maximum value 16.01 mg·g^{-1} at −8 °C.

An analysis of variance indicated that there were significant differences in SP content among the genotypes under the same low temperature, and a significant difference was also observed in the SP content within the same genotype under different temperature treatments (Figure 2c). S12 attained the highest value of 7.21 mg·g^{-1} under −4 °C low temperature treatment, T2 and M31 peaked at 8.44 mg·g^{-1} and 6.99 mg·g^{-1}, respectively, under −8 °C treatment, and W10 reached a maximum value of 7.58 mg·g^{-1} when the low temperature treatment was −16 °C.

3.3. Variation in Antioxidant Enzymes

According to ANOVA results, SOD activity was not affected by the interaction between temperature and genotype, although it exhibited noteworthy differences among the same genotype at different low temperatures (Figure 3a, Supplementary data Table S6). Further, there were significant differences in SOD among the four genotypes when the temperature treatments were −4 °C and −20 °C, but no significant differences were observed among the genotypes when treated at 4 °C, −8 °C and −16 °C. The maximum SOD activity of W10 and S12 was at −8 °C with 476.58 U·(g·min)$^{-1}$ and 504.22 U·(g·min)$^{-1}$, respectively. Thereafter, their enzymatic activities decreased with lower temperature treatments. The highest value of 530.21 U·(g·min)$^{-1}$ of M31 occurred at the temperature of −16 °C, while T2 showed the maximum SOD activity at −20 °C with 525.42 U·(g·min)$^{-1}$.

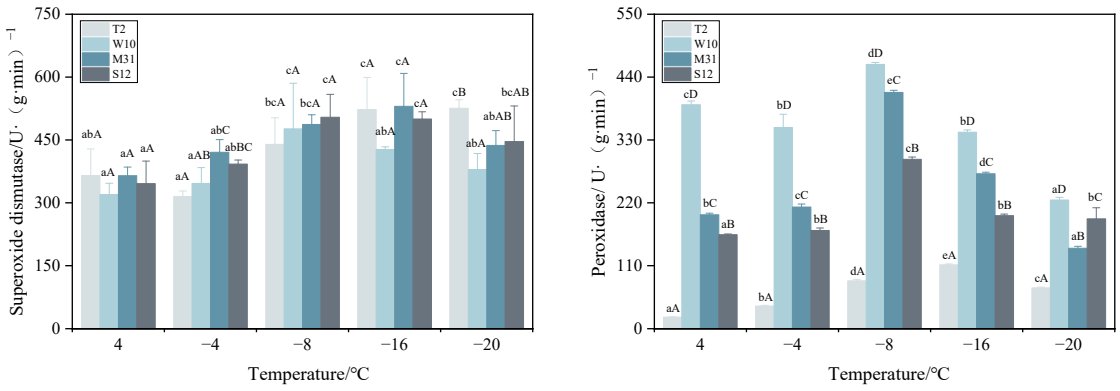

Figure 3. Variations in superoxide dismutase activity (**a**) and peroxidase activity (**b**) in the four genotypes of *Cyclocarya paliurus* under cold stress. Significant differences between temperature treatments ($p < 0.05$) were indicated by different lowercase letters, and significant differences between genotypes ($p < 0.05$) were indicated by different capital letters.

The POD activities of T2, W10, M31 and S12 differed significantly under the five low temperature treatments (Figure 3b). Only T2 had the maximum POD activity at −16 °C, while W10, M31 and S12 peaked at −8 °C. Additionally, there was a significant difference in POD between genotypes. W10 had the highest POD enzyme activity under different low temperature treatments, followed by M31 and S12, while the lowest was T2. Besides, the interaction between temperature and genotype had a significant effect on POD activity (Supplementary data Table S7).

3.4. Correlations of LT_{50} to Physiological Parameters

It can be seen from Figure 4 that the LT_{50} values has significant correlations with MDA, POD, SP and SS, and the correlation coefficients reached 0.47, −0.69, 0.36 and 0.37, respectively. REC is significantly correlated with SOD, SP, and SS with correlation coefficients of 0.32, −0.27, and −0.48, respectively. SP shows significant positive correlations with SOD, Pro, and SS, corresponding to correlation coefficients of 0.26, 0.62, and 0.44. The correlation analysis results of between LT_{50} and physiological indices, including LT_{50}, REC, MDA, POD, SOD, SP, Pro and SS, indicated that the correlation coefficients between the indicators are small, despite a certain association. Therefore, it is not feasible to evaluate the resistance under low temperature using a single indicator.

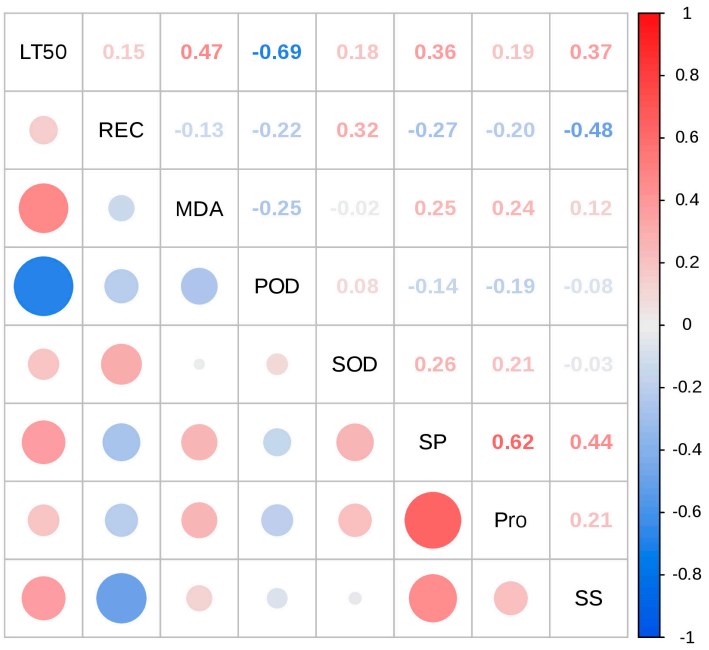

Figure 4. The correlation analysis between semi-lethal temperature (LT_{50}) and physiological indices. The size of the circle represents the magnitude of the correlation coefficient. Red indicates a positive correlation, while blue indicates a negative correlation.

3.5. Assessment of Cold-Resistance

To assess the cold resistance of different CP genotypes, principal component analysis was performed on the seven relevant indexes. The cumulative variance contribution of the four principal components is 84.6% (Table 3), containing most of the index information. Thus, it is possible to convert the seven physiological indexes into four comprehensive indexes to evaluate the cold resistance of different CP genotypes. With a contribution of 27.327%, the eigenvalue of principal component 1 is 1.913, which mostly contains the information of SOD (X_4), SP (X_5) and Pro (X_6). Principal component 2 has an eigenvalue of 1.830 with 26.144% contributing rate, mainly reflects the information of REC (X_1) and SS (X_7). The eigenvalue of principal component 3 is 1.120 with a contribution of 16.00%, primarily reveals the information of POD (X_3). With an eigenvalue of 1.059, principal component 4 has a contribution ratio with 15.123% and mainly represents the information of MDA (X_2).

Table 3. Principal component load and eigen values.

Index	PC1	PC2	PC3	PC4
REC (X_1)	0.020	−0.881	−0.260	−0.179
MDA (X_2)	0.138	0.050	−0.134	0.946
POD (X_3)	−0.067	0.061	0.968	−0.140
SOD (X_4)	0.748	−0.475	0.202	−0.153
SP (X_5)	0.811	0.387	−0.130	0.107
Pro (X_6)	0.753	0.192	−0.131	0.259
SS (X_7)	0.324	0.798	−0.152	−0.096
Eigen value	1.913	1.830	1.120	1.059
Variance contribution rate%	27.327	26.144	16.001	15.123
Cumulative variance contribution rate%	27.327	53.471	69.472	84.595

The following functional formula was used to obtain the scores of four principal components, and the results are shown in Table 4.

$$F_1 = 0.014X_1 + 0.100X_2 - 0.048X_3 + 0.541X_4 + 0.586X_5 + 0.544X_6 + 0.234X_7$$
$$F_2 = -0.651X_1 + 0.037X_2 + 0.045X_3 - 0.035X_4 + 0.286X_5 + 0.142X_6 + 0.590X_7$$
$$F_3 = -0.246X_1 - 0.127X_2 + 0.915X_3 + 0.191X_4 - 0.123X_5 - 0.124X_6 - 0.144X_7$$
$$F_4 = -0.174X_1 + 0.919X_2 - 0.136X_3 - 0.149X_4 + 0.104X_5 + 0.252X_6 - 0.093X_7$$

The score of every genotype F was calculated through the score function of the four principal components. Subsequently, the value μ of each principal component was calculated by membership function. The overall evaluation value D was calculated by considering their respective weights, and the value of D indicated the level of cold resistance. A higher D value corresponded to stronger cold resistance. From Table 4, the genotype W10 had the maximum D value (0.585), while T2 had the minimum D value (0.336), and the ranking of cold resistance of the four different genotypes was W10 > M31 > S12 > T2.

Table 4. The score of the comprehensive evaluation.

Genotype	F1	F2	F3	F4	μ1	μ2	μ3	μ4	D
T2	1.138	0.561	−1.389	0.649	0.359	0.296	0.074	0.641	0.336
W10	−0.840	−0.215	1.132	−0.478	0.712	0.464	0.737	0.401	0.585
M31	−0.413	−0.236	0.498	−0.680	0.636	0.469	0.570	0.358	0.522
S12	0.115	−0.110	−0.241	0.509	0.542	0.442	0.376	0.611	0.492
Weight	-	-	-	-	0.323	0.309	0.189	0.179	-

4. Discussion

The cell membrane system is the primary site of low temperature injury to plants. REC reflects changes in membrane permeability, the colder the temperature, the greater the degree of cellular damage, which is consistent with the previous studies [34]. In M31, W10 and S12, the significant differences were observed just at −4 °C and −8 °C, while REC did not differ significantly until the temperature reached −16 °C, which just indicates that RECs of different genotypes do not respond to low temperatures to the same extent. From Figure 1a, it can be seen that the REC of T2 was higher than that of W10 and M31 at 4 °C. The REC of T2 was the lowest under −4 °C, −8 °C and −16 °C. However, when the temperature was −20 °C, T2's REC was the highest among the four genotypes, which also indicates that it is not scientific to use a single REC as an evaluation of cold resistance [16,26]. LT_{50} is a crucial turning point since it marks the onset of irreversible damage to plant tissues. When the temperature falls below LT_{50}, plant cell tissues rapidly freeze, leading to changes in physiological metabolism and irreversible damage to plants. Thus, the cold resistance of plants is stronger with lower LT_{50} [35]. Judging from the LT_{50}, W10 had the strongest cold resistance among the four CP genotypes, followed by M31 and T2, and S12 had the weakest cold resistance. As the product of membrane lipid peroxidation, MDA can indicate

the degree of membrane lipid peroxidation to some extent. The MDA content differed among the four genotypes and could be influenced not only by ROS but also by antioxidant protective enzymes [36]. When the temperature was −20 °C, the rate of ROS production was far greater than the removal capacity of SOD and POD, and the excess of ROS finally led to the accumulation of MDA content [37,38].

Previous study has proposed Pro as an effective cell membrane protector of cell membranes [39]. The present study found that the content of Pro exhibited an increasing and then decreasing trend in response to low temperature, suggesting that Pro responded to protect the annual shoots of CP. However, when the temperature was too low the regulatory and protective mechanisms of Pro were broken, leading to a decrease in its levels, consistent with the findings of Soloklui [40]. Li has reported that the content of SP and SS increased and then decreased during the low temperature [41,42], and changes in the levels of SP and SS after low temperature treatment measured in this study exhibited the same pattern. It is possible that the cells are stimulated by cold stimulation, which may promote the formation of new proteins [21], while the low temperature contributes to the gradual accumulation of SS content, jointly improves the protective effect on the cell membrane. However, when the temperature continues to decline, excessive accumulation of ROS cannot be cleared timely resulting in the absence or alteration of bases [43]. Osmoregulatory function is impaired, causing the SP and SS content to gradually decrease.

SOD and POD are important protective enzymes in the antioxidant system that effectively scavenge free radicals and enhance the ability of plants to resist low temperatures. Figure 3a shows the SOD activities of the different genotypes increased at −8 °C and −16 °C, suggesting that the plants were protecting themselves [44,45]. The POD and SOD enzyme activities of the annual shoots of the four genotypes of CP indicated that the enzymatic activities responded to low temperature and enhanced the ability to scavenge ROS [15]. However, when the temperature was −20 °C, the high concentration of ROS may lead to the destruction of enzyme protein molecules and a decrease in content [46].

The cold hardiness of plant is connected to its geographical source, including the natural group's location, the annual precipitation, the mean annual temperature, the minimum annual temperature, and other variables [47,48]. The plantation and natural forests of CP are mostly concentrated in subtropical areas, and its leaf yield is difficult to fulfill the demand of food and pharmaceutical industries due to the restricted suitable planting area [49–51]. To meet the market requirements, it is necessary to study the mechanism of cold tolerance of CP and expand the planting area of CP to the north. As shown in Table 1, the mean annual temperatures of the T2, M31 and S12 origins are 17.6, 17.3 and 17.5, respectively, while the W10 origin has a mean annual temperature of 12.8 °C, which is the lowest of the four. The T2 origin had the lowest elevation at 275 m, while the elevation of W2 is 963.3 m, which is much higher than T2 and slightly lower than M31 and S12. In terms of location, W10 is the northernmost, S12 is located at the southmost, T2 lies at the easternmost while M31 is the westernmost. A preliminary assessment of cold resistance revealed that W10 had the most potent cold resistance, which may be closely linked to its origin with the lowest mean annual temperature and farthest northern location. The cold resistance of T2 was the weakest, which may be closely related to the lowest elevation of its origin [52]. However, it remains to be studied in more detail which factors of origin, such as mean annual temperature, latitude, longitude, altitude and others, have the greatest impact on CP cold resistance.

5. Conclusions

The contents of MDA, Pro, SP and POD activities were not only correlated with the treatment temperature but also related to the genotypes. Osmotic substance (SS, SP and Pro) contents and antioxidant enzyme activities (POD and SOD) of the four genotypes showed a trend of increasing and then decreasing with the five decreasing temperatures. LT_{50} was significantly correlated with MDA, POD, SP and SS. Furthermore, the combined evaluation of the membership function and principal component analysis revealed that

the four genotypes were ranked W10 > M31 > S12 > T2 in terms of cold resistance. W10 and M31 have better cold resistance and are suited to plant in areas not lower than −25 °C, while T2 and S12 have weaker cold resistance and are suitable for introduction to regions with temperatures not lower than −20 °C.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f14081680/s1, Table S1: The tests of between-subjects effects for dependent variable REC; Table S2: The tests of between-subjects effects for dependent variable MDA; Table S3: The tests of between-subjects effects for dependent variable Pro; Table S4: The tests of between-subjects effects for dependent variable SS; Table S5: The tests of between-subjects effects for dependent variable SP; Table S6: The tests of between-subjects effects for dependent variable SOD; Table S7: The tests of between-subjects effects for dependent variable POD.

Author Contributions: Z.Z.: Investigation, data curation, visualization, writing—original draft preparation and funding acquisition. Y.G.: investigation and formal analysis. Q.M.: investigation and data curation. J.W.: conceptualization, writing—review and editing and supervision. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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

References

1. Fang, S.Z. A review on the development history and the resource silviculture of *Cyclocarya paliurus* industry. *J. Nanjing For. Uni.* **2022**, *46*, 115–126.
2. Li, Q.; Hu, J.; Xie, J.; Nie, S.; Xie, M.Y. Isolation, structure, and bioactivities of polysaccharides from *Cyclocarya paliurus* (Batal.) Iljinskaja. *Ann. N. Y. Acad. Sci.* **2017**, *1398*, 20–29. [CrossRef] [PubMed]
3. Kakar, M.U.; Naveed, M.; Saeed, M.; Zhao, S.; Rasheed, M.; Firdoos, S.; Manzoor, R.; Deng, Y.; Dai, R. A review on structure, extraction, and biological activities of polysaccharides isolated from *Cyclocarya paliurus* (Batalin) Iljinskaja. *Int. J. Biol. Macromol.* **2020**, *156*, 420–429. [CrossRef] [PubMed]
4. Xie, M.Y.; Li, L.; Nie, S.P.; Wang, X.R.; Lee, F.S. Determination of speciation of elements related to blood sugar in bioactive extracts from *Cyclocarya paliurus* leaves by FIA-ICP-MS. *Eur. Food Res. Technol.* **2006**, *223*, 202–209. [CrossRef]
5. Ma, Y.; Jiang, C.; Yao, N.; Li, Y.; Wang, Q.; Fang, S.; Shang, X.; Zhao, C.C.; Ni, Y.; Zhang, J.; et al. Antihyperlipidemic effect of *Cyclocarya paliurus* (Batal.) Iljinskaja extract and inhibition of apolipoprotein B48 overproduction in hyperlipidemic mice. *J. Ethnopharmacol.* **2015**, *166*, 286–296. [CrossRef] [PubMed]
6. Li, Q.; Hu, J.; Nie, Q.; Chang, X.; Fang, Q.; Xie, J.; Li, H.; Nie, S. Hypoglycemic mechanism of polysaccharide from *Cyclocarya paliurus* leaves in type 2 diabetic rats by gut microbiota and host metabolism alteration. *Sci. China Life Sci.* **2021**, *64*, 117–132. [CrossRef] [PubMed]
7. Yao, Y.; Yan, L.; Chen, H.; Wu, N.; Wang, W.; Wang, D. *Cyclocarya paliurus* polysaccharides alleviate type 2 diabetic symptoms by modulating gut microbiota and short-chain fatty acids. *Phytomedicine* **2020**, *77*, 153268. [CrossRef]
8. Shen, Y.; Peng, Y.; Zhu, X.; Li, H.; Zhang, L.; Kong, F.; Wang, J.; Yu, D. The phytochemicals and health benefits of *Cyclocarya paliurus* (Batalin) Iljinskaja. *Front. Nutr.* **2023**, *10*, 1158158. [CrossRef]
9. Zhao, J.; Wang, Z.; Xu, D.; Sun, X. Advances on Cyclocarya paliurus polyphenols: Extraction, structures, bioactivities and future perspectives. *Food Chem.* **2022**, *396*, 133667. [CrossRef]
10. Chen, Z.; Jian, Y.; Wu, Q.; Wu, J.; Sheng, W.; Jiang, S.; Shehla, N.; Aman, S.; Wang, W. *Cyclocarya paliurus* (Batalin) Iljinskaja: Botany, ethnopharmacology, phytochemistry and pharmacology. *J. Ethnopharmacol.* **2022**, *285*, 114912. [CrossRef]
11. Liu, W.; Wu, Y.; Hu, Y.; Qin, S.; Guo, X.; Wang, M.; Wu, L.; Liu, T. Effects of *Cyclocarya paliurus* aqueous and ethanol extracts on glucolipid metabolism and the underlying mechanisms: A meta-analysis and systematic review. *Front. Nutr.* **2020**, *7*, 605605. [CrossRef] [PubMed]
12. Theocharis, A.; Clément, C.; Barka, E.A. Physiological and molecular changes in plants grown at low temperatures. *Planta* **2012**, *235*, 1091–1105. [CrossRef] [PubMed]
13. Ritonga, F.N.; Chen, S. Physiological and molecular mechanism involved in cold stress tolerance in plants. *Plants* **2020**, *9*, 560. [CrossRef] [PubMed]

14. Hasanuzzaman, M.; Bhuyan, M.B.; Zulfiqar, F.; Raza, A.; Mohsin, S.M.; Al Mahmud, J.; Fujita, M.; Fotopoulos, V. Reactive oxygen species and antioxidant defense in plants under abiotic stress: Revisiting the crucial role of a universal defense regulator. *Antioxidants* **2020**, *9*, 681. [CrossRef] [PubMed]
15. Georgieva, K.; Mihailova, G.; Gigova, L.; Dagnon, S.; Simova-Stoilova, L.; Velitchkova, M. The role of antioxidant defense in freezing tolerance of resurrection plant *Haberlea rhodopensis*. *Physiol. Mol. Biol. Plants* **2021**, *27*, 1119–1133. [CrossRef]
16. Punia Bangar, S.; Trif, M.; Ozogul, F.; Kumar, M.; Chaudhary, V.; Vukic, M.; Tomar, M.; Changan, S. Recent developments in cold plasma-based enzyme activity (browning, cell wall degradation, and antioxidant) in fruits and vegetables. *Compr. Rev. Food. Sci. Food Saf.* **2022**, *21*, 1958–1978. [CrossRef] [PubMed]
17. Arakawa, K.; Kasuga, J.; Takata, N. Mechanism of Overwintering in Trees. In *Survival Strategies in Extreme Cold and Desiccation. Advances in Experimental Medicine and Biology*, 2nd ed.; Iwaya-Inoue, M., Sakurai, M., Uemura, M., Eds.; Springer: Singapore, 2018; Volume 1081, pp. 129–147.
18. Ghosh, U.K.; Islam, M.N.; Siddiqui, M.N.; Cao, X.; Khan, M.A.R. Proline, a multifaceted signalling molecule in plant responses to abiotic stress: Understanding the physiological mechanisms. *Plant Biol.* **2022**, *24*, 227–239. [CrossRef] [PubMed]
19. Seydel, C.; Kitashova, A.; Fürtauer, L.; Nägele, T. Temperature-induced dynamics of plant carbohydrate metabolism. *Physiol. Plant.* **2022**, *174*, e13602. [CrossRef]
20. Janmohammadi, M.; Zolla, L.; Rinalducci, S. Low temperature tolerance in plants: Changes at the protein level. *Phytochemistry* **2015**, *117*, 76–89. [CrossRef]
21. Kang, C.H.; Lee, Y.M.; Park, J.H.; Nawkar, G.M.; Oh, H.T.; Kim, M.G.; Lee, S.I.; Kim, W.Y.; Yun, D.J.; Lee, S.Y. Ribosomal P3 protein AtP3B of Arabidopsis acts as both protein and RNA chaperone to increase tolerance of heat and cold stresses. *Plant Cell Environ.* **2016**, *39*, 1631–1642. [CrossRef]
22. Hu, Y.; Wang, Y.X.; Zhu, Y.F.; Chen, B.H. Multivariate statistical analyses and predictive model of cold resistance associated with eleven crabapples and Fuji apple. *Cryoletters* **2018**, *39*, 235–244.
23. He, S.; Zhao, K.; Ma, L.; Yang, J.; Chang, Y.; Ashraf, M.A. Comparison of cold resistance physiological and biochemical features of four *Herba Rhodiola* seedlings under low temperature. *Saudi J. Biol. Sci.* **2016**, *23*, 198–204. [CrossRef]
24. Jiang, H.Y.; Gao, Y.H.; Wang, W.T.; He, B.J. Evaluation of cold resistance in grapevines via photosynthetic characteristics, carbohydrate metabolism and gene expression levels. *Acta Physiol. Plant.* **2016**, *38*, 251. [CrossRef]
25. Duk, J.Y.; Hwang, J.Y.; Chung, S.W.; Oh, H.D.; Yun, S.K.; Lee, H.J. Changes in cold hardiness and carbohydrate content in peach (*Prunus* persica) trunk bark and wood tissues during cold acclimation and deacclimation. *Sci. Hortic.* **2017**, *219*, 45–52.
26. Zhou, W.; Leul, M. Uniconazole-induced alleviation of freezing injury in relation to changes in hormonal balance, enzyme activities and lipid peroxidation in winter rape. *Plant Growth Regul.* **1998**, *26*, 41–47. [CrossRef]
27. Lu, Y.; Hu, Y.; Li, P. Consistency of electrical and physiological properties of tea leaves on indicating critical cold temperature. *Biosyst. Eng.* **2017**, *159*, 89–96. [CrossRef]
28. Zhao, S.; Xu, C.; Zou, Q.; Meng, Q. Improvement of method for measurement of malondialdehyde in plant tissues. *Plant Physiol. Comm.* **1994**, *30*, 207–210.
29. Liu, M.; Gao, H.; Gao, Y.; Xue, X. Study on the physiological and biochemical changes of *Phoebe sheareri* seed during its dormancy breaking. *J. Nanjing For. Uni.* **2023**, *47*, 9–17.
30. Shiba, K.S.; Kanamori, K.; Harada, T.; Nakao, M.; Nakajima, K.; Kodaira, T.; Nakagawa, H. A cause of discrepancy between values for urinary protein as assayed by the Coomassie Brilliant Blue G-250 method and the sulfosalicylic acid method. *Clin. Chem.* **1985**, *31*, 1215–1218. [CrossRef] [PubMed]
31. Ábrahám, E.; Hourton-Cabassa, C.; Erdei, L.; Szabados, L. Methods for determination of proline in plants. In *Plant Stress Tolerance. Methods in Molecular Biology*, 2nd ed.; Sunkar, R., Ed.; Humana Press: New Jersey, NJ, USA, 2010; Volume 639, pp. 317–331.
32. Giannopolitis, C.N.; Ries, S.K. Superoxide dismutases: I. occurrence in higher plants. *Plant Physiol.* **1977**, *59*, 309–314. [CrossRef] [PubMed]
33. Amako, K.; Chen, G.X.; Asada, K. Separate assays specific for ascorbate peroxidase and guaiacol peroxidase and for the chloroplastic and cytosolic isozymes of ascorbate peroxidase in plants. *Plant Cell Physiol.* **1994**, *35*, 497–504.
34. Zhang, R.; Liu, B.; Xin, G.; Zhang, X.; Li, J.; Wang, Y. Evaluation of cold tolerance of seven walnut varieties. *Cryoletters* **2022**, *43*, 74–82. [CrossRef]
35. Wang, Y.; Ya, H.; Chen, B.; Zhu, Y.; Dawuda, M.; Svetla, S. Physiological mechanisms of resistance to cold stress associated with 10 elite apple rootstocks. *J. Integr. Agric.* **2018**, *17*, 857–866. [CrossRef]
36. Morales, M.; Munné-Bosch, S. Malondialdehyde: Facts and artifacts. *Plant Physiol.* **2019**, *180*, 1246–1250. [CrossRef] [PubMed]
37. Gu, K.; Hou, S.; Chen, J.; Guo, J.; Wang, F.; He, C.; Zou, C.M.; Xie, X. The physiological response of different tobacco varieties to chilling stress during the vigorous growing period. *Sci. Rep.* **2021**, *11*, 22136. [CrossRef] [PubMed]
38. Gall, H.L.; Philippe, F.; Domon, J.M.; Gillet, F.; Pelloux, J.; Rayon, C. Cell wall metabolism in response to abiotic stress. *Plants* **2015**, *4*, 112–166. [CrossRef]
39. Aslamarz, A.A.; Vahdati, K.; Hassani, D.; Rahemi, M.; Mohammadi, N.; Leslie, C. Cold hardiness and its relationship with proline content in Persian walnut. *Eur. J. Hortic. Sci.* **2011**, *76*, 84–90.
40. Soloklui, A.A.G.; Ershadi, A.; Fallahi, E. Evaluation of cold hardiness in seven Iranian commercial pomegranate (*Punica granatum* L.) cultivars. *Hortscience* **2012**, *47*, 1821–1825. [CrossRef]

41. Li, H.; Luo, W.; Ji, R.; Xu, Y.; Xu, G.; Qiu, S.; Tang, H. A comparative proteomic study of cold responses in potato leaves. *Heliyon* **2021**, *7*, e06002. [CrossRef]
42. Li, Z.; Li, X.; He, F. Non-structural carbohydrates contributed to cold tolerance and regeneration of *Medicago sativa* L. *Planta* **2023**, *257*, 116. [CrossRef]
43. Soualiou, S.; Duan, F.; Li, X.; Zhou, W. Crop production under cold stress: An understanding of plant responses, acclimation processes, and management strategies. *Plant Physiol. Biochem.* **2022**, *190*, 47–61. [CrossRef] [PubMed]
44. Raseetha, S.; Oey, I.; Burritt, D.J.; Heenan, S.; Hamid, N. Evolution of antioxidant enzymes activity and volatile release during storage of processed broccoli (*Brassica oleracea* L. italica). *LWT-Food Sci. Technol.* **2013**, *54*, 216–223. [CrossRef]
45. Wang, J.; Germinara, G.S.; Feng, Z.; Luo, S.; Yang, S.; Xu, S.; Li, C.; Cao, Y. Comparative effects of heat and cold stress on physiological enzymes in *Sitophilus oryzae* and *Lasioderma serricorne*. *J. Stored Prod. Res.* **2022**, *96*, 101949. [CrossRef]
46. Xu, H.; Li, L.; Mao, N.; Gan, Z.; Xue, S.; Li, X.; Zhang, B.; Liu, G.M.; Wu, X. Physiological response of *Kobresia pygmaea* to temperature changes on the Qinghai-Tibet Plateau. *BMC Plant Biol.* **2022**, *22*, 1–14.
47. Willi, Y.; Van Buskirk, J. A review on trade-offs at the warm and cold ends of geographical distributions. *Philos. Trans. R. Soc. B* **2022**, *377*, 20210022. [CrossRef]
48. Solé-Medina, A.; Robledo-Arnuncio, J.J.; Ramírez-Valiente, J.A. Multi-trait genetic variation in resource-use strategies and phenotypic plasticity correlates with local climate across the range of a Mediterranean oak (*Quercus faginea*). *N. Phytol.* **2022**, *234*, 462–478. [CrossRef] [PubMed]
49. Huang, Z.; Xu, Z.; Sun, C. Study on seasonal dynamics of seed rain and characteristics of soil seed banks in *Cyclocarya paliurus*. *J. Nanjing For. Uni.* **2022**, *47*, 18–26.
50. Qin, J.; Yue, X.; Shang, X.; Fang, S. Response of nitrogen use efficiency, N-assimilating enzymes and growth in *Cyclocarya paliurus* seedlings to different nitrogen nutrition. *J. Plant Nutr.* **2023**, *46*, 3547–3556. [CrossRef]
51. Li, C.; Wan, Y.; Shang, X.; Fang, S. Integration of transcriptomic and metabolomic analysis unveils the response mechanism of sugar metabolism in *Cyclocarya paliurus* seedlings subjected to PEG-induced drought stress. *Plant Physiol. Biochem.* **2023**, *201*, 107856. [CrossRef]
52. Lu, W.X.; Zhang, B.H.; Zhang, Y.Y.; Yang, S.C. Differentiation of cold tolerance in an artificial population of a mangrove species, *Kandelia obovata*, is associated with geographic origins. *Front. Plant Sci.* **2022**, *12*, 3451. [CrossRef]