Responses of Microstructure, Ultrastructure and Antioxidant Enzyme Activity to PEG-Induced Drought Stress in *Cyclocarya paliurus* Seedlings

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Abstract: Drought is one of the most important abiotic constraints on agricultural productivity, while global warming leads to the occurrence of more frequent drought events. *Cyclocarya paliurus* is a multiple-function tree species with medicinal value and timber production, but no information is available on its drought tolerance. In this hydroponic experiment, variations in leaf anatomical morphology, chloroplast ultrastructure, stomatal characteristics, and antioxidant enzyme activities were investigated under six levels of polyethylene glycol 6000 (PEG)-induced drought treatments to assess the drought adaption and physiological response of *C. paliurus* seedlings. The results showed that PEG-induced drought treatments reduced leaf epidermis, spongy tissue, leaf vein diameter, and spongy ratio, whereas the ratio of palisade tissue to spongy tissue, cell tense ratio, and vein protuberant degree all increased with enhancing the PEG6000 concentrations. Significant differences in stomatal width, stomatal aperture, and stomatal density existed among the treatments ($p < 0.01$). The stomatal aperture decreased significantly with the increase in PEG6000 concentrations, whereas the greatest stomatal density was observed in the 15% PEG6000 treatment. Compared with the control, higher drought stresses (20% and 25% PEG concentrations) caused damage at the cellular level and chloroplast lysis occurred. PEG6000 treatments also promoted the activities of SOD, POD, and CAT in *C. paliurus* seedlings, but this increase was insufficient to deal with the membrane lipid peroxidative damage under the high PEG concentrations. Correlation analysis indicated that in most cases there were significant relationships between leaf anatomical characteristics and antioxidant enzyme activities. Our results suggested that C. *paliurus* seedlings would not survive well when the PEG6000 concentration was over 15% (equal to soil water potential of −0.30 MPa).

Keywords: wheel wingnut; PEG concentration; leaf anatomy; stomata feature; cell morphology; antioxidant enzymes; drought tolerance

1. Introduction

Plants, like sessile organisms, are always influenced by environmental changes throughout their lifetimes, such as salt, waterlogging, drought, and cold, whereas drought is one of the most important abiotic constraints on agricultural productivity [1,2]. Moreover, global warming leads to changes in the Earth's water cycle, and drought events would occur more frequently and last for longer periods [3]. Water deficiency can damage plant organs, tissues, and cellular components in a variety of ways [4]. Arid environments not only reduce the carbon assimilation ability of trees, but also degrade the ability of forest seedlings to resist environmental stress, and even cause large-scale death of forests in severe cases [5,6]. Compared with adult trees, seedlings are more susceptible to the influence of hydraulic-driven mortality, which increases the risk of forest management failure. The hydraulic xylem cavitation caused by drought could increase the damage risk

and exert the reduction of leaf water potential and sap movement [7]. Under drought stress conditions, plants can modify both morphological and physiological traits to adopt the stress environments. As the main channel of regulating water vapor loss, plants can adjust their stomatal aperture to maintain or reduce the risk of hydraulic failure. Plants respond to water scarcity by first closing their stomata, reducing water loss and CO_2 fixation, and ultimately altering carbon metabolism [8]. When plants do not receive sufficient water, they accumulate large amounts of reactive oxygen species (ROS). However, the massive accumulation of ROS under water-deficient conditions has the potential to cause severe cellular damage, impair plant physiological and molecular activity, and accelerate cellular and chlorophyll breakdown [9].

Adaptive response in the morphological, physiological, biochemical, and molecular levels can help plants cope with soil drought, which determines their survival, occurrence, and distribution [10–12]. Many studies indicate that plants can limit water loss by controlling the level of stomata closure and rate of leaf gas exchange [13,14]. Meanwhile, dramatic changes in cell morphology and ultrastructure also occur under drought stress, which are typically reflected as changes in the quantity and size of chloroplasts, plastoglobuli, and starch grains [15,16]. Some research has reported that antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) have been linked to drought resistance [17–19]. Overall, plants protect themselves via morphological adaptation [20–22], osmotic regulation [23–25], and antioxidant enzyme activity [26,27], whereas the adaptive responses are species-specific [19,22,27].

Wheel wingnut (*Cyclocarya paliurus* (Batal.) Iljinskaja), the sole species in the genus, is a multifunctional tree that integrates medicinal function, timber production, and ornamental values, while it is naturally distributed in the subtropical mountains of China [28,29]. Because its leaves contain a variety of biologically active substances such as polysaccharides, triterpenoids, flavonoids, and phenolic compounds, *C. paliurus* possesses a variety of bioactivities, such as antihypertensive activity, hypoglycemic activity, enhancement of mental efficiency, and anticancer [30,31]. In 2013, China's National Health and Family Planning Commission designated *C. paliurus* leaves as a new food raw resource [32]. With the important pharmaceutical values, a considerable production of *C. paliurus* leaves are required for production and medical use. However, the existing *C. paliurus* resources are mainly distributed in natural forests and nature reserves, therefore, developing *C. paliurus* plantations is the best option for leaf production. Moreover, most current studies on *C. paliurus* were primarily focused on extraction of bioactive substances such as triterpenoids and flavonoids, pharmacological effects of leaf extracts, and the silvics of the species [31,33–36], while little is known regarding its drought-resistant capacity, especially its cytological and physiological responses to various drought events.

The objective of this study was to tentatively identify *C. paliurus* drought-resistant capacity by investigating its morphological, physiological, and cytological variations under various PEG-induced drought stresses during the seedling stage. We hypothesized that *C. paliurus* seedlings showed different morphological and physiological responses under drought stress mediated by different concentrations of PEG, and that there was a drought tolerance threshold. Once the maximum drought tolerance limit of the species was exceeded, the balanced plant–water relationship could not be maintained. The information provided by this study would be of great value for understanding the responses of microstructure, ultrastructure, and antioxidant enzyme activity to PEG-induced drought stress in *C. paliurus* seedlings and would contribute to establishing its optimal cropping strategies under the circumstance of climate change, especially in the selection of its planting sites and management practices.

2. Materials and Methods

2.1. Plant Materials and Experimental Design

Half-sib family seeds of *C. paliurus* were collected in late October 2019 from a dominant tree in Jinzhongshan, Guangxi Autonomous Region (24°36′ N, 104°57′ E). After natural

drying and removing wings, the seeds were soaked with gibberellin, and stratificated according to the method of Fang et al. [28] in order to accelerate germination. The germinated seeds were transplanted into nonwoven bags (10 cm height and 8.5 cm diameter) for cultivation in April 2020. After one-year growth, *C. paliurus* seedlings were transported to the greenhouse in National Garden Experimental Demonstration Teaching Center of Nanjing Forestry University (31°59′ N, 119°18′ E), and then stem-cut was conducted at approximately 10 cm height for all the seedlings in mid-March 2021. When the sprouting seedlings reached about 10 cm height, vigorous *C. paliurus* seedlings with a similar size were chosen for the drought stress treatments. After clearing up the cultivation substrate with water, the seedlings were transferred to hydroponic boxes containing 30 L 1/2 Hoagland nutrient solution (pH 6.0) for pretreatment. The nutrient solution was completely renewed every 3 days, and oxygen pumps were used to ventilate for 2 h at each morning and afternoon.

After one-month pretreatment in hydroponic culture, the seedlings with mean height of 20 cm were put into 1/2 Hoagland nutrient solution with six concentrations of PEG6000 (m/v) for hydroponic culture studies. The six PEG6000 gradients were designed as 0%, 5%, 10%, 15%, 20%, and 25%, respectively, and arranged in a completely randomized block experiment. Each treatment contained three biological replications, and eight seedlings per replication. Totally, about 150 seedlings were used in the study. Meanwhile, a S20A environmental monitor was used to track the air temperature and humidity in the greenhouse during the experimental period (Xuzhou Farah Electronic Technology Co. Ltd., Xuzhou, China). The temperature in the greenhouse fluctuated between 14.2 and 36.9 °C, while the relative humidity of the air ranged from 30.8% to 98.3% during the experimental period.

Leaf sampling was performed at 0, 2, 6, 12, 24, and 48 h after different PEG6000 treatments. For each treatment, fully expanded leaves at middle and upper position of the seedlings were collected as mixed samples, then quickly preserved in the icebox and brought back to the laboratory. Some leaf samples were stored in the refrigerator at 4 °C for image observation and measurements of leaf anatomical structure, stomata morphology, and chloroplast ultrastructure. Another portion was kept at −80 °C for the detection of antioxidant enzymes.

2.2. Assessment of Leaf Anatomical Structure

Fully expanded leaves were collected from the same position of *C. paliurus* seedlings for each treatment after 24 h of different PEG6000 treatments; afterwards, samples were cut into about 5 mm × 5 mm small pieces and fixed in 70% FAA (formalin/acetic acid/70% alcohol ratio 0.5:0.5:9.0) for more than 24 h. Then, they were dehydrated with alcohol gradients and embedded in paraffin. About 4-μm-thick sections were cut with an RM2016 Pathology slicer (Shanghai Leica Instrument Co, Ltd., Shanghai, China), and stored at room temperature after dried. Fifteen representative samples were selected for taking pictures using a BX53 optical microscope (Olympus, Tokyo, Japan).

2.3. Transmission Electron Microscope (TEM) Observation of Chloroplast Ultrastructure

The leaf samples were prepared according to the method of Búfalo at al. [37], with a slight modification. In brief, the leaves sampled at 24 h after PEG treatments were cut into about 1 mm² small pieces, then were fixed in a 2.5% glutaraldehyde solution with a 0.1 M phosphate buffer (pH 7.4) at 4 °C for 2 h. The samples were post-fixed with a 1% osmium tetroxide aqueous solution in the same buffer for 7 h at 25 °C and then dehydrated in a graduated series of acetone and embedded in EMBed 812 resin. Ultra-thin sections were stained with uranyl acetate and lead citrate, and the samples were examined with a HT7800 transmission electron microscope (HITACHI, Tokyo, Japan) at 80.0 kV.

2.4. Scanning Electron Microscope (SEM) Observation of Stomatal Characteristics

Fresh *C. paliurus* leaves were collected at 24 h after the treatment and the collected samples were quickly put into 2.5% glutaraldehyde solution with 0.1 M phosphate buffer PB (pH 7.4) overnight at 4 °C. Then, the samples were dehydrated in ethanol series, critical-

point dried, mounted on metallic stubs, sputter-coated with gold [38], and eventually examined with an SU-8100 scanning electron microscope (HITACHI, Tokyo, Japan) at 3.0 kV.

2.5. Determination of Antioxidant Enzyme Activity

Leaf samples collected at 0, 2, 6, 12, 24, and 48 h after different PEG6000 treatments were used to analyze antioxidant enzyme activity, respectively. About 0.2 g leaf samples were weighed and placed in a pre-cooled mortar and ground into a homogenate with 0.05 mol/L pre-cooled phosphate buffer (pH 7.8) under ice bath conditions. Homogenized samples were centrifuged at 12,000× g for 15 min at 4 °C, and the supernatant was crude enzyme solution. The SOD activity was measured at 560 nm according to the method of Giannopolitis [39], POD activity was measured at 470 nm [40], and CAT activity was determined based on the method of Nakano [41].

2.6. Statistical Analysis

The main effect of the interaction between PEG6000 concentrations and treatment times was determined via the analysis of variance (ANOVA) using a general linear model, and Duncan's multiple range test was followed to further test the significance of the difference at $p < 0.05$. All data were presented in the form of mean ± standard deviation (SD). SPSS 25.0 (SPSS Inc, Chicago, IL, USA) was used to complete all statistical analysis processes.

3. Results

3.1. Leaf Characteristics

Leaf morphology and anatomy of *C. paliurus* seedlings were obviously affected by PEG-induced drought stress (Figure 1). After 24 h of the PEG treatments, both leaf color and shape were changed compared with control (0% PEG6000). For instance, the leaf edge displayed obvious crimp at the treatment of 10% PEG6000 concentration, while obvious partial leaf epidermis was seriously damaged at high PEG6000 concentrations. Especially, the leaves of *C. paliurus* seedlings treated with 20% PEG6000 showed distortion and deformation, whereas the leaves treated with 25% PEG6000 almost collapsed and the tissue was indistinguishable (Figure 1).
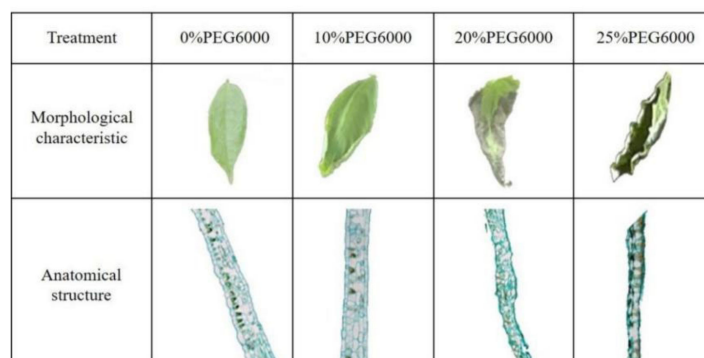
Figure 1. Morphological and anatomical characteristics of *C. paliurus* leaves sampled at 24 h after treatments with different concentrations of PEG6000.

Significant differences in the anatomical characteristics of *C. paliurus* leaves were observed among the various PEG6000 treatments (Table 1). PEG-induced drought stress significantly reduced the thickness of the upper epidermis, sponge tissue, leaf thickness, the diameter of leaf vein, and spongy ratio ($p < 0.05$), whereas the P/S, cell tense ratio, and vein protuberant degree all rose with the increase in PEG6000 concentrations (Table 1). Compared with the control (no PEG6000 addition), leaf thickness decreased by 13.8, 19.8, 30.4, and 47.3% in the treatments of 5%, 10%, 15%, and 20% PEG6000 concentrations,

respectively, but the ratio of palisade tissue to spongy tissue increased by 23.4, 34.3, 45.3, and 62.5% (Table 1).

Table 1. Variations in anatomical characteristics of *C. paliurus* leaves sampled at 24 h after treatments of different PEG6000 concentrations (mean $\pm$ SD, n = 4).

Anatomical Structure Index	PEG6000 Concentrations				
	0%	**5%**	**10%**	**15%**	**20%**
UE/μm	11.25 ± 0.63 a	10.35 ± 0.14 ab	10.04 ± 0.41 bc	9.35 ± 0.36 c	5.66 ± 0.74 d
LE/μm	10.43 ± 0.92 a	8.93 ± 1.42 a	8.80 ± 0.26 a	8.60 ± 1.10 a	6.17 ± 1.19 b
PT/μm	19.18 ± 0.97 a	18.40 ± 0.55 a	17.48 ± 1.21 b	15.09 ± 1.26 b	12.93 ± 1.90 b
ST/μm	30.58 ± 2.22 a	23.89 ± 0.88 b	21.15 ± 1.05 c	16.68 ± 1.48 d	12.88 ± 0.63 e
P/S	0.64 ± 0.07 c	0.79 ± 0.04 bc	0.86 ± 0.11 ab	0.93 ± 0.13 ab	1.04 ± 0.15 a
LT/μm	71.43 ± 2.79 a	61.57 ± 0.41 b	57.26 ± 0.55 c	49.71 ± 2.13 d	37.64 ± 1.42 e
VD/μm	465.07 ± 10.93 a	407.59 ± 13.23 b	373.99 ± 4.61 c	334.87 ± 10.89 d	273.55 ± 12.85 e
CTR/%	0.27 ± 0.02 b	0.30 ± 0.01 ab	0.31 ± 0.02 ab	0.30 ± 0.02 ab	0.34 ± 0.04 a
SR/%	0.43 ± 0.02 a	0.39 ± 0.02 ab	0.37 ± 0.02 bc	0.34 ± 0.03 c	0.34 ± 0.02 c
VPD	6.52 ± 0.33 b	6.62 ± 0.18 b	6.53 ± 0.14 b	6.74 ± 0.08 b	7.28 ± 0.47 a

UE: upper epidermis; LE: lower epidermis; PT: palisade tissue; ST: spongy tissue; P/S: palisade tissue/spongy tissue; LT: leaf thickness; VD: vein diameter; CTR: cell tense ratio; SR: spongy ratio; VPD: vein protuberant degree. The results of ANOVA analysis showed that different letters indicate significant differences among the treatments for the same index ($p < 0.05$). Note: The *C. paliurus* leaves were severely damaged in 25% PEG6000 concentration after treatment of 24 h, and anatomical morphological parameters could not be distinguished.

3.2. Stomata Feature

PEG-induced drought stress influenced the stomata feature of *C. paliurus* leaves (Figures 2 and 3), but the stomata only appeared in the lower epidermis. As shown in Figure 2, the treatments with higher PEG concentrations resulted in near closure of stomata.
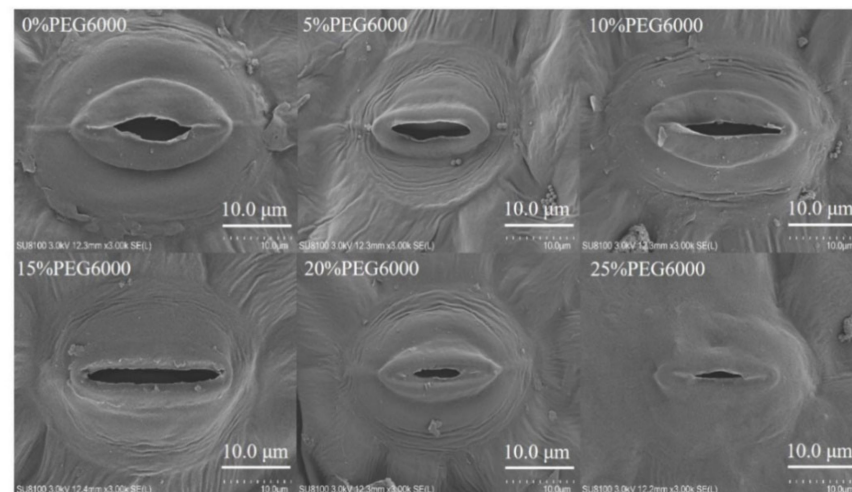
Figure 2. Stomata characteristics of *C. paliurus* leaves sampled at 24 h after different PEG-induced drought treatments. Scales: 10 μm.

ANOVA results showed that significant differences in stomatal width, stomatal aperture, and stomatal density existed among the treatments ($p < 0.01$), whereas no significant difference was detected in stomatal length (Figure 3). Compared with the control, the stomatal aperture in the PEG6000 treatments of 5%, 10%, 15%, 20%, and 25% concentrations decreased by 38.2%, 49.1%, 53.9%, 67.5%, and 76.6%, respectively. However, the mean greatest stomatal density was observed in the 15% PEG6000 treatment, reaching 107.78 No. mm^{-2}.
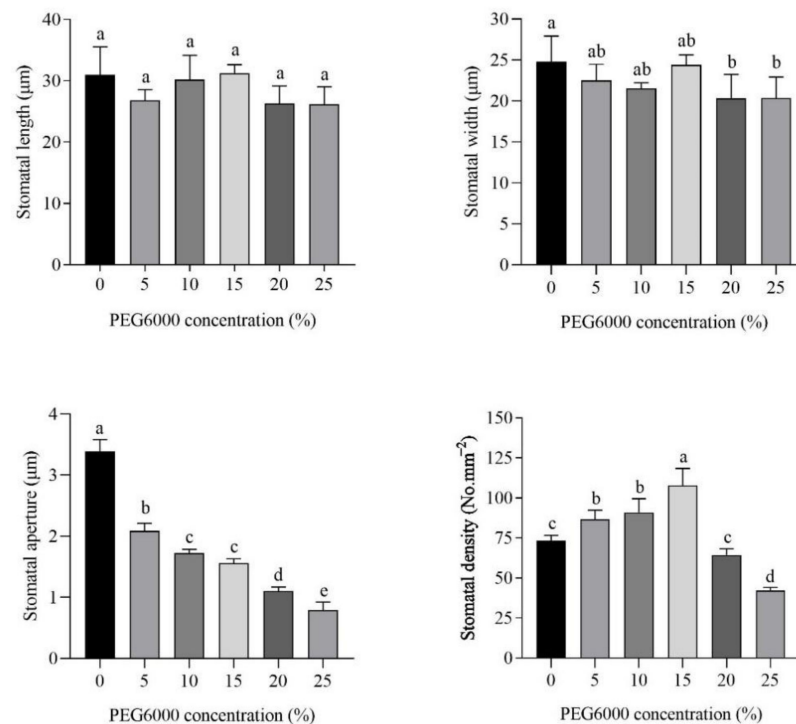
Figure 3. Variations in mean stomatal structure of *C. paliurus* leaves sampled at 24 h after treatments of different PEG concentrations.

3.3. Chloroplast Ultrastructure

Figure 4 showed that chloroplasts were mainly distributed in palisade mesophyll cells. In the CK (0% PEG6000), palisade parenchyma cells showed regular contours and chloroplasts arranged closely to the cell wall (Figure 4A). However, the PEG-induced drought stress resulted in varying degrees of cell deformation, and ultrastructural damage (chloroplasts, starch grains, and thylakoids) (Figure 4B–F).
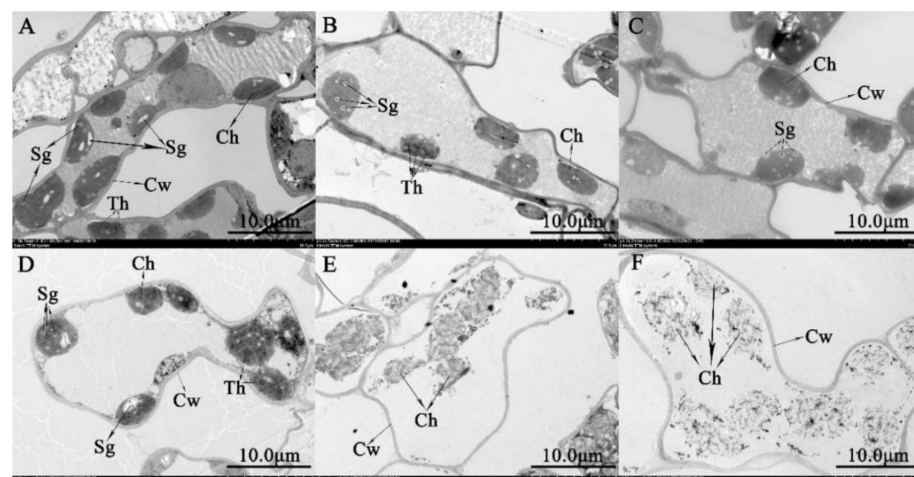
Figure 4. Transmission electron micrographs of *C. paliurus* leaves subjected to different PEG concentrations. Scale bars: 10 μm. (**A**) 0% PEG6000; (**B**) 5% PEG6000; (**C**) 10% PEG6000; (**D**) 15% PEG6000; (**E**) 20% PEG6000; (**F**) 25% PEG6000. Abbreviations: Ch: chloroplast; Sg: starch grain; Cw: cell wall; Th: thylakoid.

Chloroplast structure is closely related to photosynthesis, and PEG6000 treatments significantly affected the structure of chloroplasts (Figure 5). In the CK (0% PEG6000), the

shape of chloroplast was oval, and the thylakoid structure was well ordered (Figure 5A), while in the 5% and 10% PEG treatments, the quantity of starch grain obviously increased, and the leaf structure was relatively intact (Figure 5B,C). However, in the 15% PEG6000 treatment, slight interior disintegration in the chloroplast occurred, and dense black dots appeared on the edge of the chloroplast (Figure 5D). Especially, in the treatments with high PEG concentrations (20% and 25%), the chloroplast was severely damaged, the internal structure could no longer be distinguished (Figure 5E), which eventually lead to chloroplast deformation (Figure 5F).
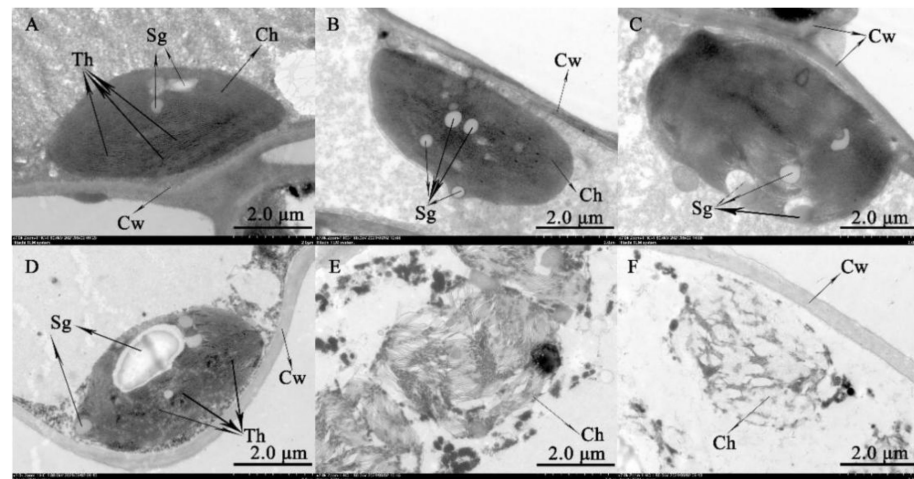

Figure 5. Transmission electron micrograph of *C. paliurus* leaves sampled at 24 h after treatments of different PEG concentrations. Scale bars: 2 μm. (**A**) 0% PEG6000; (**B**) 5% PEG6000; (**C**) 10% PEG6000; (**D**) 15% PEG6000; (**E**) 20% PEG6000; (**F**) 25% PEG6000. Abbreviations: Ch: chloroplast; Sg: starch grain; Cw: cell wall; Th: thylakoid.

3.4. Antioxidant Enzyme Activity

Two-way ANOVA indicated that antioxidant enzyme activities in *C. paliurus* leaves were significantly affected by PEG-induced drought stress, treatment times, and their interactions (Table S1, $p < 0.001$). The SOD activity showed an increasing trend with enhancing the PEG concentrations at 2 h of treatments and reached a maximum of 627.05 $U \cdot g^{-1} \cdot min$ FW at the treatment time of 12 h under the 20% PEG6000 concentration (Figure 6A). However, at 48 h of the treatment, the SOD activity in each treatment decreased significantly, and the SOD activity in 25% PEG6000 concentration was only 122.77 $U \cdot g^{-1} \cdot min$ FW, which is 21.34% lower than control.

Similarly, the increasing tendencies of POD and CAT activities were observed for all PEG-induced drought treatments before 12 h of the treatment (Figure 6B,C). However, the highest activities of POD and CAT were detected in the treatments of 15% PEG6000 concentration at 24 h and 25% PEG6000 concentration at 12 h, respectively, reaching 132.73 and 63.04 $U \cdot g^{-1} \cdot min$ FW. Moreover, it is noticed that after 24 h of PEG treatment, antioxidant enzyme activities measured in the treatments with higher PEG additions (e.g., 20% and 25% PEG6000 concentrations) all significantly reduced (Figure 6, $p < 0.05$). Compared with the values at 24 h, the SOD, POD, and CAT in higher PEG addition treatments at the treatment time of 48 h decreased by 76.13%, 7.12%, and 9.57%, respectively.
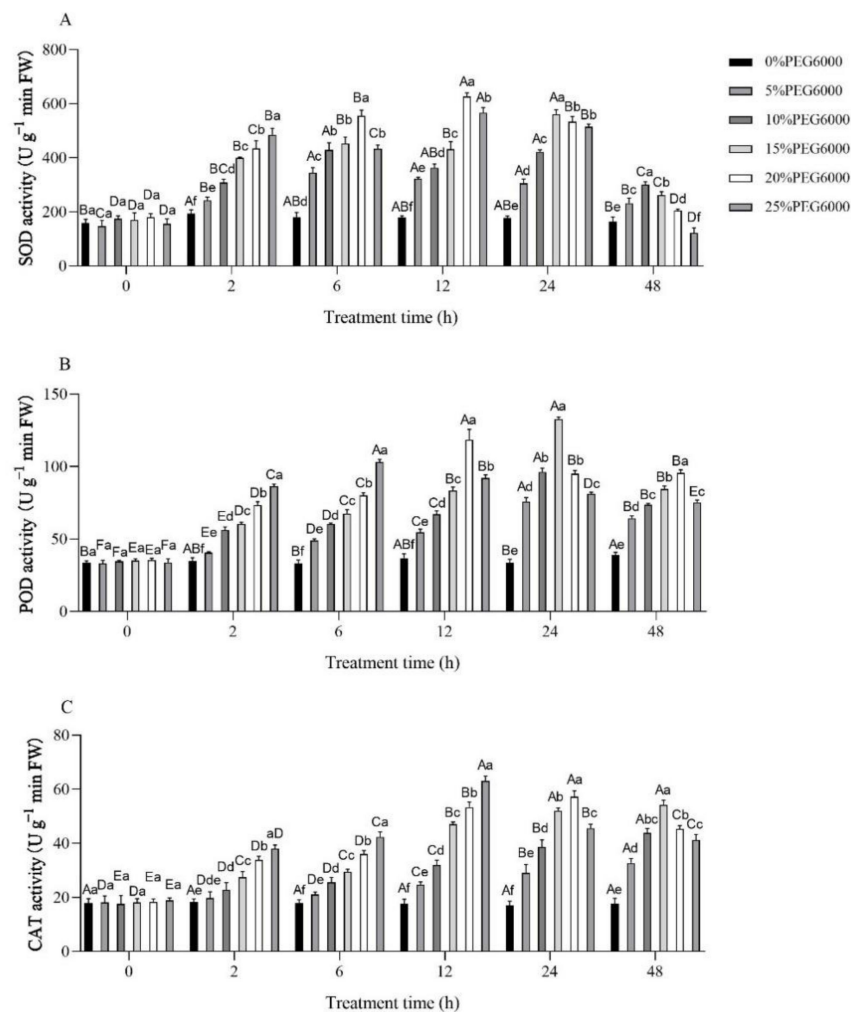
Figure 6. Variations in antioxidant enzyme activities of SOD (**A**), POD (**B**) and CAT (**C**) in *C. paliurus* leaves treated with different PEG6000 concentrations at various treatment times. SOD: superoxide dismutase; POD: peroxidase; CAT: catalase. Different capital letters indicate that the significant difference at various treatment times for the same PEG6000 concentration ($p < 0.05$), while different small letters indicate the significant difference among different PEG6000 concentrations at the same treatment time ($p < 0.05$).

4. Discussion

4.1. Relationships between Leaf Morphological Indexes and Antioxidant Enzyme Activities

Plants have evolved multiple mechanisms to adapt to the changes in their surrounding environment. For instance, plants can adapt to environmental stress (such as water scarcity) by anatomical changes [42,43], while the protective enzymes protect the cell membrane system, reduce the leaf damage caused by cell membrane lipid peroxidation, and delay the damage of mesophyll cell function, aging, and decomposition of biological macro-molecules [44]. Our results showed that the leaves of *C. paliurus* seedlings responded differently to drought stress levels. Overall, PEG-induced drought treatments had a strong negative impact on morphological and physiological indexes of *C. paliurus* leaves, especially under higher PEG concentrations (Table 1 and Figure 5). Correlation analysis indicated that there were significant relationships between leaf anatomical characteristics and antioxidant enzyme activities, except for the correlations of POD to LE, UE, CTR, and VPD as well as the relationship between SOD and VPD (Table 2). Based on the values of correlation coefficients (Table 2), it seems that CAT is more correlated to leaf anatomical characteristics than SOD and POD, indicating that the functioning of CAT activity is indispensable for protecting

plants against stress. Furthermore, it is found that all three antioxidant enzyme activities were negatively correlated with ST, LT, VD, and SR ($p < 0.01$), but positively correlated with P/S ($p < 0.01$), CTR, and VPD (Table 2), suggesting that the seedlings with a higher ratio of P/S (palisade to sponge tissue) would have better adaptability to drought stress, in agreement with the results from rapeseed [45] and olive [46]. It is generally believed that leaf epidermal thickness increases with water deficiency [47,48]; however, in our study leaf thickness of all PEG treatments decreased (Table 1). The possible reason is that the treatment period was too short to recover the water lost and cell organelles damaged in the leaves treated with the PEG.

Table 2. Pearson correlation coefficients of antioxidant enzyme activities to anatomical structure indexes and stomatal parameters of *C. paliurus* leaves.

Enzyme Activity	Anatomical Structure Index (*n* = 15)										Stomatal Parameters (*n* = 18)			
	LE	UE	PT	ST	P/S	LT	VD	CTR	SR	VPD	SL	SW	SA	SD
SOD	−0.696 **	−0.634 *	−0.816 **	−0.943 **	0.767 **	−0.899 **	−0.919 **	0.574 *	−0.881 **	0.481	−0.160	−0.327	−0.903 **	−0.035
POD	−0.417	−0.454	−0.617 *	−0.806 **	0.643 **	−0.708 **	−0.744 **	0.432	−0.821 **	0.252	0.077	−0.091	−0.667 **	0.454
CAT	−0.810 **	−0.733 **	−0.864 **	−0.972 **	0.814 **	−0.958 **	−0.969 **	0.652 **	−0.872 **	0.579 *	−0.223	−0.356	−0.871 **	−0.059

** indicates significance at $p < 0.01$, * indicates significance at $p < 0.05$. SOD: superoxide dismutase; POD: peroxidase; CAT: catalase; UE: upper epidermis; LE: lower epidermis; PT: palisade tissue; ST: spongy tissue; P/S: palisade tissue/spongy tissue; LT: leaf thickness; VD: vein diameter; CTR: cell tense ratio; SR: spongy ratio; VPD: vein protuberant degree; SL: stomatal length; SW: stomatal width; SA: stomatal aperture; SD: stomatal density.

Stoma is generally considered as a key switch for controlling CO_2 and water transport [49], and its opening and closure affect photosynthesis, transpiration, and respiration of plants [50,51]. However, drought is thought to be a major factor affecting stomatal parameters [52], while plants can maintain high antioxidant enzyme activity to protect cells from oxidative damage in adverse water environmental conditions [53]. Our results showed that the three antioxidant enzymes (SOD, POD, and CAT) were not significantly correlated with stomatal length, stomatal width, and stomatal density ($p > 0.05$), but had a significantly negative correlation with stomatal opening ($p < 0.01$, Table 3), indicating that stomatal opening may be the most effective regulation mechanism to deal with water loss caused by drought stress in *C. paliurus*. The results from this study confirmed that the important ways for plants to reduce water loss is to control stomatal conductance by adjusting stomatal opening for reducing transpiration and gas exchange rates [54,55]. However, some other studies also indicated that plants can change the density, distribution, and size of stomata to adjust their morphology in the process of long-term water loss [49,56,57].

Table 3. Correlation analysis of PEG concentrations with spongy tissue, leaf thickness, leaf vein diameter, and gas exchange capacity (GEC) per leaf area in *C. paliurus* leaves.

Index	Equation	Correlation Coefficient	Ratios of PEG Treatments to CK (%)	
			15%PEG	20%PEG
Spongy tissue (μm)	$y = 30.55 \times e^{-4.177x}$	$R^2 = 0.9563$	46.6↓	56.7↓
Leaf thickness (μm)	$y = 73.18 \times e^{-2.991x}$	$R^2 = 0.9419$	34.6↓	43.7↓
Leaf vein diameter (μm)	$y = 491.1 \times e^{-3.19x}$	$R^2 = 0.9303$	34.6↓	44.2↓
GEC per leaf area (μm. mm^{-2})	$y = 295.35 \times e^{-7.32x}$	$R^2 = 0.7955$	60.2↓	72.4↓

Gas exchange capacity per leaf area = Stomatal density × Stomatal aperture; CK: no PEG6000 addition.

Chloroplasts, one of the organelles, are most sensitive to abiotic stress in leaf tissue, and are also the main part of ROS production [58]. Our result showed that no obvious damage occurred in the chloroplasts of the CK, 5% PEG6000 and 10% PEG6000 treatments, while only the increase in starch grain quantity and its size were observed in the chloroplasts (Figures 4 and 5), in agreement with the result from *Brassica napus* [45]. However, the high concentrations of PEG6000 (20% and 25%) caused severe decomposition of chloroplasts, and the starch grains were reduced, in accordance with the decrease in antioxidant enzyme activities (Figure 6). The decrease in antioxidant enzyme activity could explain the reason for chloroplast damage, because the lower enzyme activity is not enough to resist cell oxidative damage caused by ROS accumulation. In addition, it was found that

chloroplasts have self-digestion under the PEG6000-induced drought stress (Figure 6), confirming that autophagy can regulate plant ROS to respond to osmotic stress and improve resistance [59,60].

4.2. Dynamics in Antioxidant Enzyme Activity with Experiencing Drought Periods

To overcome the deleterious impact of drought, antioxidant defense mechanisms are evolved in plants to detoxify reactive oxygen species and maintain protein and amino acid stabilization [61,62]. For example, in response to the harmful effects of drought, plants can enhance antioxidant systems such as SOD, POD, and CAT to scavenge the excessive ROS and maintain the cellular redox balance in stress environments [63,64]. The results from this study showed that a rapid upsurge in the activities of SOD, POD, and CAT was observed in all *C. paliurus* seedlings at the first six hours after treatment with the PEG (Figure 6), indicating that the redox defense ability is quickly improved to cope with drought stress when compared with no-PEG treatment [65]. However, the activities of SOD, POD, and CAT in the seedlings treated with higher PEG concentrations (20% and 25%) almost decreased with prolonging the treatment times, which might be related to the damage of chloroplast structure and ROS scavenging capability (Figures 5 and 6). Regression analysis indicated that a polynomial model can best describe the relationships between the activities of SOD, POD, and CAT with treatment times (Figure 7). Although the activities of SOD, POD, and CAT in the PEG treatments showed similar variations with treatment times, an obvious difference existed in correlation indexes for different PEG treatments and as well as for various antioxidant enzymes, with R^2 ranging from 0.25 to 0.96. For example, correlation indexes between SOD and treatment times increased with enhancing the PEG concentrations (Figure 7A), whereas correlation indexes between POD and treatment times decreased (Figure 7B), indicating that the response of the three antioxidant enzymes to the drought stress is different even if a certain synergistic effect among the three enzymes may exist.

4.3. Preliminary Assessment on Drought-Resistance of C. paliurus Seedlings

For most plants, the growth during seedling stage is more sensitive to environmental changes, whereas the seedlings were proposed to better assess the stress resistance of plants [66]. Many studies have shown that plants may exhibit "drought tolerance" when undergoing adaptive changes in anatomical structures, stomatal features, and antioxidant enzyme activity in the leaves [67–69]. In order to preliminarily evaluate the critical range of drought tolerance of *C. paliurus* seedlings, regression analyses between PEG concentrations and key anatomical characteristics, as well as gas exchange capacity (defined as the product of stomatal density and stomatal aperture per leaf area) and three antioxidant enzymes in leaf samples of the 24 h treatment were further performed (Table 3 and Figure 8). Figure 8 showed that POD activity sharply decreased when the PEG concentrations were over 15%, while the activities of SOD and CAT started to reduce at the 20% PEG treatment. Meanwhile, from the *k* values in the equations it was found that gas exchange capacity (stomatal feature) in the leaves was more sensitive to PEG-induced drought stress than the anatomical indexes (Table 3). Compared with CK (no PEG6000 addition), gas exchange capacities in the treatments with 15% and 20% PEG6000 addition reduced by 60.2% and 72.4%, respectively. Moreover, chloroplast deformation was observed in the treatments of 20% and 25% PEG concentrations (Figure 5). Therefore, we infer that threshold of *C. paliurus* seedlings to PEG-induced drought stress might be about 15% PEG, which is equal to soil water potential (water osmotic pressure) of −0.30 MPa.
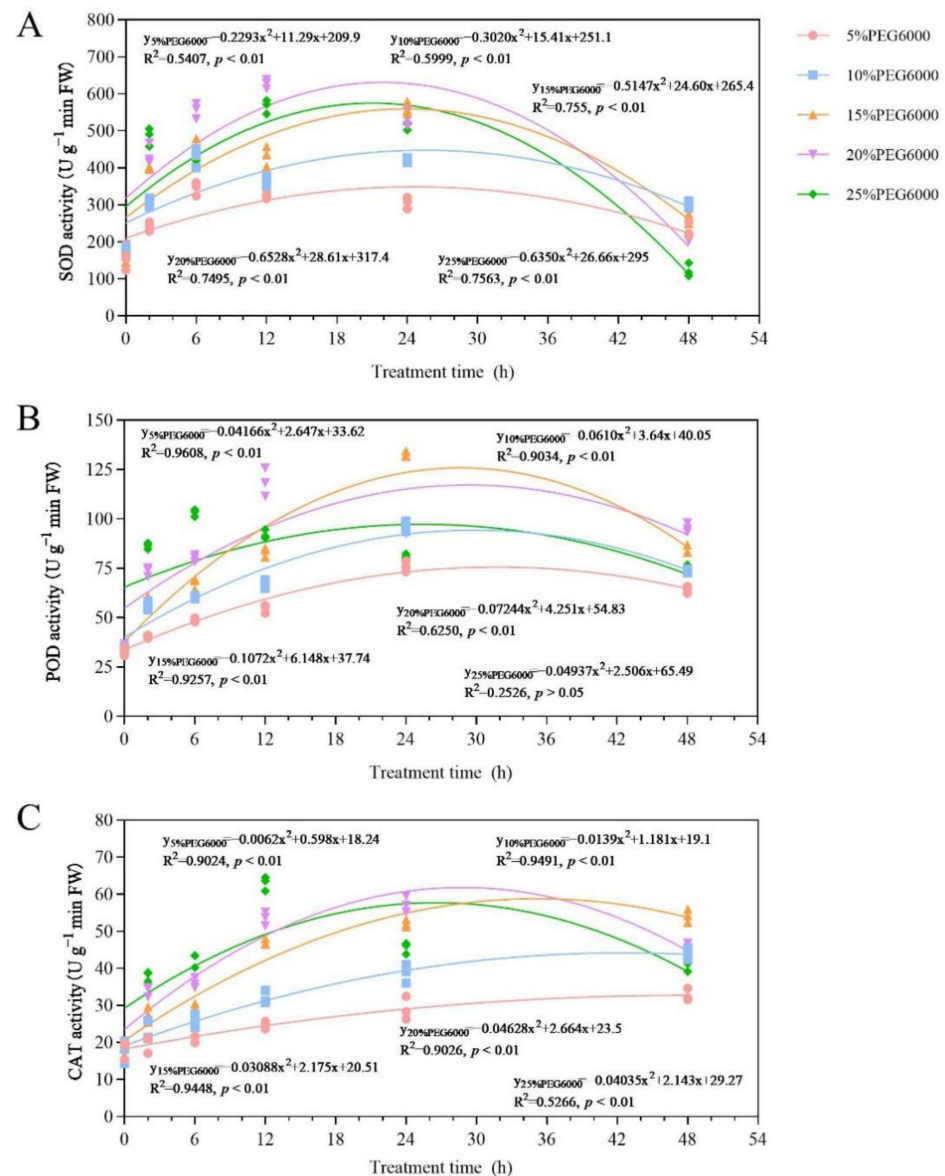
Figure 7. The relationship between treatment times and the activities of SOD (**A**), POD (**B**), and CAT (**C**) at different PEG concentrations. SOD: superoxide dismutase; POD: peroxidase; CAT: catalase.

Many studies indicated there are significant differences in drought tolerance among the plant species (genotypes), and the adaptive responses are species-specific [19,22,27]. It was reported that drought-tolerant species showed a better response in extreme environments than drought-sensitive plants due to their antioxidant enzyme activities [67–70]. For instance, Guo et al. reported that the perennial ryegrass (*Lolium perenne*) could recover from the three drought intensities simulated by polyethylene glycol-6000 (−0.09 MPa, light; −0.29 MPa, medium; −0.58 MPa, heavy) [70], showing a high drought tolerance. Unfortunately, as we did not conduct the recovery experiment in the present study it is hard to accurately judge drought tolerance of *C. paliurus* seedlings. However, we can make a preliminary judgment that *C. paliurus* belongs to a medium drought-tolerant tree species based on the result from the present study, even if more detailed investigations are required in the future.
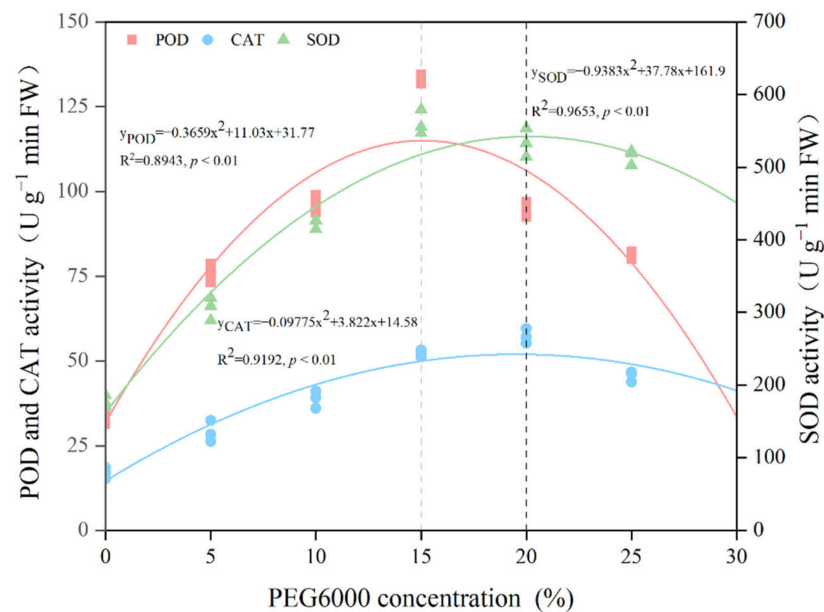
Figure 8. The relationships between PEG concentrations and antioxidant enzyme activities in the leaves sampled at 24 h of treatment. SOD: superoxide dismutase; POD: peroxidase; CAT: catalase. The grey dashed line indicates maximum POD enzyme activity at 15%PEG concentration. The black dashed line indicates that SOD and CAT have reached the maximum enzyme activity at 20%PEG concentration.

5. Conclusions

PEG-induced drought stress significantly affected anatomical morphology, chloroplast ultrastructure, stomatal characteristics, and antioxidant enzyme activities in the leaves of *C. paliurus* seedlings. With increasing the PEG concentrations, *C. paliurus* seedlings mainly showed adaptation to drought stress via increasing the ratio of palisade tissue to spongy tissue, cell tense ratio, and vein protuberant degree in leaf anatomical morphology, reducing gas exchange capacity in stomatal features, and upregulating antioxidant enzymes activities in ROS defenses. Moreover, chloroplast deformation was observed in the treatments of high PEG concentrations (20% and 25%). Correlation analysis indicated that in most cases there were significant relationships between leaf anatomical characteristics and antioxidant enzyme activities. Based on the results from this study, we suggest that *C. paliurus* seedlings have a certain drought tolerance but would not survive well when the PEG6000 concentration was over 15% (equal to soil water potential of -0.30 MPa). The finding from the present study would provide a preliminary basis for assessing drought tolerance of *C. paliurus* seedlings, whereas investigation of transcriptional regulation and metabolic networks at the molecular level are still required. Moreover, more detailed investigation both in the pot experiment and field trial are needed to accurately evaluate drought tolerance of *C. paliurus*, based on the critical water threshold assessed in this study.

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