Article

Role of *Suillus placidus* in Improving the Drought Tolerance of Masson Pine (*Pinus massoniana* Lamb.) Seedlings

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Abstract: Masson pine is an important afforestation species in southern China, where seasonal drought is common. The present study focused on the effects of *Suillus placidus*, an ectomycorrhizal fungus, inoculation on the growth and physiological and biochemical performance of masson pine seedlings under four different watering treatments (well-watered, mild drought, moderate drought, and severe drought) to evaluate the symbiotic relationship between *S. placidus* and masson pine seedlings. Ectomycorrhizal-inoculated (ECM) and non-inoculated (NM) seedlings were grown in pots and maintained for 60 days using the weighing method. Results showed that seedlings' growth, dry weight, RWC, chlorophyll content, PSII efficiency, and photosynthesis decreased as drought stress intensified in both ECM and NM plants. This suggests that drought stress significantly limits the growth and photosynthetic performance of masson pine seedlings. Nevertheless, increased A_n/g_s and proline contents in both NM and ECM prevented oxidative damage caused by drought stress. In addition, increased peroxidase (POD) activity is an essential defense mechanism of ECM seedling under drought stress. Compared with NM, ECM seedlings showed faster growth, higher RWC, and photosynthetic performance, and lower lipid peroxidation in cell membranes under drought stress, as indicated by higher POD activity and lower proline and malondialdehyde (MDA). Our experiment found that *S. placidus* inoculation can enhance the drought resistance of masson pine seedlings by increasing antioxidant enzyme activity, water use efficiency, and proline content, thereby enhancing growth under water-deficiency conditions. *S. placidus* can be used to cultivate high-quality seedlings and improve their survival in regions that experience seasonal droughts.

Keywords: chlorophyll fluorescence; drought; ectomycorrhizal fungus; photosynthesis; *Pinus massoniana*

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1. Introduction

Drought stress is one of the factors limiting the survival, growth, and productivity of plants, and the frequency of droughts is expected to increase soon due to climate change [1]. Limitation in growth, stomatal closure, and decrease in photosynthetic rate are common problems observed after periods of drought [2]. Drought stress also results in low survival rates and poor early-stage growth of soil seedlings in reforestation practices [3,4]. Some plants alter their morphology and physiology to overcome drought stress [5], including changes in the mechanism of intracellular enzymatic reactive oxygen species (ROS) scavenging, contents of osmotic adjustment substances, and leaf morphology and leaf character for improved water efficiency [6,7]. Given the negative effects of drought on plants, it is necessary to devise suitable strategies that improve plant tolerance to drought stress.

Beneficial soil microorganisms, such as ectomycorrhizal fungi (ECMF), are extremely important because they contribute toward enhancing plant growth and drought resistance [8]. In ectomycorrhizal symbioses, host plants and fungi share a mutualistic rela-

tionship. Studies have reported that plants participating in ectomycorrhizal symbiosis have increased growth [9], higher endurance to biotic and abiotic stresses, including large potential for the phytoremediation of abandoned tailings pond [10], and improved tolerance to water-deficiency conditions [11]. However, owing to differences in the host plant and mycorrhizal fungal species [12], there are conflicting reports regarding the success of ectomycorrhizal symbiosis. As a result, the mechanisms underlying the effects of ectomycorrhizal symbioses on the growth and physiology of host plants under drought stress remain unclear. Sebastiana et al. [4] found that the inoculation of *Pisolithus tinctorius* did not increase plant water uptake or photosynthesis under low water content and that the effect of mycorrhizal fungi on osmotic adjustment and ROS production was insignificant. Lehto and Zwiazek [13] reviewed the decline of root hydraulic conductivity and water uptake in ectomycorrhizal plants. Chen et al. [14] also found that ECMF inoculation reduced the total dry weight of the host plant.

Masson pine (*Pinus massoniana* Lamb.) is one of the most widely distributed, native species in southern China, as well as an important industrial raw material due to its fast growth, high yield, and wide applications and distribution [15]. Masson pine often experiences uneven rainfall distribution and frequent seasonal droughts during afforestation and management in its concentrated distribution area, which seriously limits its growth and physiological performance. Masson pine is a typical ectomycorrhizal tree species and can form a symbiotic relationship with several ECMF, including *Lactarius deliciosus*, *Suillus luteus*, and *Cantharellus cibarius* [16]. Studies have focused on nutrient cycling, root system architecture, antioxidant activity, and water-deficiency responses in inoculated plants [17,18]. Nevertheless, studies have reported the sensitivity and acclimation of photosynthesis in masson pine, with particular focus on chlorophyll (Chl) *a* fluorescence, which is a quick method to estimate the photosynthetic response in plants under various environmental conditions [19], including water deficiency [20], cold hardiness [21], and toxic metals [22].

Suillus is the dominant ECMF of *Pinus* species [23]. *S. placidus* is a member of the genus *Suillus*; this species has been found in masson pine plantation in our study area (Guizhou province). Rodríguez-Gutiérrez et al. [24] found that sympatric ECMF species are more efficient when they associate with *Pinus* species by increasing the growth and environmental adaptability of the host plant. Owing to the fact there has been little research on the resistance of *S. placidus* to drought in *Pinus* species, this study aimed to evaluate the influence of *S. placidus* colonization on the physiological performance of masson pine seedlings subjected to four watering treatments. We hypothesize that *S. placidus* inoculation can improve the drought tolerance of masson pine by increasing antioxidant enzyme activity, osmotic adjustment substance contents, leaf gas exchange, photosynthetic pigment contents, and PSII efficiency in masson pine seedlings. The results of this study can help increase the survival rate of masson pine under afforestation and provide important reference information for the development and use of local ECMF sources.

2. Materials and Methods

The experimental trials were conducted in a greenhouse at Guizhou University, in Guiyang, Guizhou, China.

2.1. Biological Material and Growth Conditions

S. placidus sporocarps were collected from under masson pine trees (*P. massoniana* Lamb.) at Qingyan forest farm (E 26°18', N 106°40'). The sporocarps were used to isolate the mycelium in pure cultures, as described by Brundrett et al. [25], and the sequences were deposited in NCBI under the accession number MT994624(ITS). The strain was first placed in petri dishes with Pachlewski medium (PACH) and subsequently in liquid PACH medium (without agar), as described by Pachlewski and Pachlewska [26]. The cultures were stored for 4 weeks under dark conditions at 25 °C, agitated at 80 r·min⁻¹, and used to inoculate masson pine seedlings.

Masson pine seeds were obtained from the same mother tree, sterilized using KMnO_4 (0.5%) for 30 min, and washed six times using sterile water. The seeds were then germinated in vermiculite wetted with distilled water at 25 °C for 28 days. After germination, the seedlings were transferred to plastic pots (20 cm × 16 cm × 20 cm) filled with a mixed medium (soil/vermiculite/perlite in a 4:1:1 v/v/v ratio; 4 kg per pot), which was sterilized at 121 °C for 2 h. The physicochemical properties of the mixed medium are as follows: soil organic matter 44.49 g·kg⁻¹, pH 4.65, total nitrogen 5.63 g·kg⁻¹, total phosphorus 0.15 g·kg⁻¹, and total potassium 8.84 g·kg⁻¹. The available nitrogen, phosphorus, and potassium levels were 157.50, 56.34, and 35.85 mg·kg⁻¹, respectively. Masson pine seedlings were simultaneously inoculated with *S. placidus* inoculum (50 mL per planting hole) during transplantation, ensuring that the inoculum was introduced at the root of the seedlings. Non-inoculated (NM) seedlings were treated with an equal amount of blank medium. The seedlings were watered once every 2 days in the greenhouse. One year after *S. placidus* inoculation, the inoculated (ECM) and NM masson pine seedlings were subjected to different watering treatments in the same greenhouse at Guizhou University. Mycorrhizal morphology is shown in Figure 1.

Figure 1. The mycorrhizal morphology of the symbiotic root system of *Suillus placidus* and *Pinus massoniana*.

2.2. Experimental Design

Eight treatments, including four watering treatments with two levels of inoculation, were tested. Each treatment had eight pots (3 seedlings per pot). All treatments were randomly performed.

Two factors were tested for all treatments: (1) whether the plants were inoculated with *S. placidus* or not, referred to as ECM or NM, respectively, and (2) whether water gradient was controlled by potted planting, which was maintained with the weighing method at 6 p.m. each day and by regulating soil moisture content via artificial irrigation. The watering treatments were well-watered (70%–80% of field moisture capacity), mild drought (60%–70% of field capacity), moderate drought (50%–60% of field capacity), and severe drought (35%–50% of field capacity) [27]. The experiment lasted for 60 days in the greenhouse at an average temperature of 24.7 °C and relative humidity of 77.9%.

Soil field moisture capacity was calculated using the method described by Zhou et al. [28]. Soil water content (SWC) was measured as follows [29]:

$$\text{SWC}(\%) = [(\text{fresh mass} - \text{dry mas}) / \text{dry mas}] \times 100$$

2.2.1. Estimation of Photosynthesis

Five masson pine seedlings from each treatment were randomly marked to measure the photosynthesis. Leaf gas exchange measurements were performed using an open infrared gas exchange analyzer system (Licor-6400XT, Li-Cor Inc, Lincoln, NE, USA) supplying photosynthetic photon flux density (PPFD) and with a red-blue light source (6400-02B, Li-Cor Inc, Lincoln, NE, USA). The completely mature needles were placed in the leaf chamber. The leaf instantaneous gas exchange parameters were CO_2 assimilation rate (A_n , $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), intercellular CO_2 concentration (C_i , $\mu\text{mol CO}_2 \text{ mol}^{-1}$), stomatal conductance (g_s , $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$), and transpiration rates (E , $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$), which were measured at a PPFD of 1200 $\mu\text{mol m}^{-2} \text{ s}^{-1}$, chamber CO_2 concentrations of

380 ppm, and a flow rate of $500 \text{ mmol m}^{-2} \text{ s}^{-1}$. The measurements were recorded in the morning (09:00–12:00) when photosynthetic rates are at their maximum. The ratio of A_n/g_s ($\mu \text{ mol CO}_2 \cdot \text{mol}^{-1} \text{ H}_2\text{O}$) was calculated to determine the efficiency of water use.

Chl *a* fluorescence was measured using the MONITORING-PAM Multi-Channel Chlorophyll Fluorometer (Heinz Walz GmbH, Effeltrich, Germany). After 40 min of dark adaptation, the slow induction kinetic curve was constructed. Ground state fluorescence intensity (F_0) was determined using a modulated light intensity of approximately $0.5 \mu \text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. Maximum fluorescence (F_m) was determined at a saturation pulse of 0.6 s (approximately $10,000 \mu \text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$). Rapid optical response curves (RLCs) were constructed with the actinic irradiances set at 0–1500 $\mu \text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, with a total of 11 gradients, and the relative electron transfer rate (ETR(II)) were obtained.

F_v/F_m , the maximum light energy conversion efficiency of PSII photochemistry, was determined using previously described equations [30]:

$$(F_m - F_0)/F_m$$

The electron transport rate through PSII (ETR(II)) was calculated as follows:

$$\text{PAR} \times 0.84 \times 0.5 \times Y(\text{II})$$

where 0.5 is the excitation energy divided between the two photosystems, and 0.84 is a common leaf absorbance for C3 plants [31].

The quantum efficiency of PSII $Y(\text{II})$ was determined per Genty et al. [32] as:

$$(F_m' - F)/F_m'$$

F_v'/F_m' , the photochemical quantum yield of open PSII centers in the light-adapted state, was determined as per Genty et al. [32] as:

$$(F_m' - F_o')/F_m'$$

qP, photochemical quenching, was determined as per van Kooten and Snel [33] as:

$$(F_m' - F)/(F_m' - F_o')$$

NPQ, non-photochemical quenching, also known as thermal dissipation efficiency, which is the ability to protect the light, was calculated according to Bilger and Bjorkman [34] as follows:

$$F_m/F_m' - 1$$

All indicators were registered using the WinControl-3 software (Heinz Walz, Effeltrich, Germany).

2.2.2. Growth and Water Status

The seedlings were harvested on the 60th day of watering treatment without damaging their root systems. The harvested seedlings were separated into roots, shoots, and needles. Once the fresh weights of the roots, shoots, and leaves were measured, the seedlings were then dried at 80 °C to their constant weight and dry weight (DW). Moreover, the collar diameter and plant height of seedlings were measured at harvest. Three seedlings were randomly selected from different pots to determine their biomass, stem diameter, and plant height (three replicates per treatment).

For each treatment, approximately 1 g of completely mature needles were sampled from three individual seedlings and weighed as fresh weight on the day of harvest.

$$\text{RWC} = [(\text{fresh weight} - \text{dry weight}) / (\text{turgid weight} - \text{dry weight})] \times 100\%$$

where dry weight was the weight after the needles were dried at 105 °C for 30 min and then at 80 °C to constant weight, and turgid weight was the weight after the needles were soaked in distilled water for 1 day [35].

2.2.3. Photosynthetic Pigment Content, Santioxidant Enzymes, and Osmotic Adjustment Substances

Approximately 0.1 g of fresh needles was extracted using 10 mL of 100% acetone. Photosynthetic pigment contents were measured using the method described by Dere et al. [36]. Chl a ($\text{mg}\cdot\text{g}^{-1}$), Chl b ($\text{mg}\cdot\text{g}^{-1}$), and carotenoid (Car, $\text{mg}\cdot\text{g}^{-1}$) contents were measured using a spectrophotometer (UV-2100, UNICO, Princeton, NJ, USA) and calculated using the following formulas:

$$\text{Chl a} = (11.75 A_{662} - 2.35 A_{645}) \times V / (1000 \times W)$$

$$\text{Chl b} = (18.61 A_{645} - 3.96 A_{662}) \times V / (1000 \times W)$$

$$\text{Chl (a + b)} = \text{Chl a} + \text{Chl b}$$

$$\text{Car} = (1000 A_{470} - 2.27 \text{Chl a} - 81.4 \text{Chl b}) / 227$$

where A_{662} , A_{645} , and A_{470} are the absorbances at 662, 645, and 470 nm, respectively; V (mL) is the extract volume, and W (g) is fresh needle weight.

The activities of the antioxidant enzymes and contents of osmotic adjustment substances were analyzed according to the instructions provided by the kits (Comin, Keming Biotechnology Co. Ltd., Suzhou, China); the needle weight of each index was about 0.1 g FW. Nitroblue tetrazolium and guaiacol were used to measure superoxide dismutase (SOD) activity [37] and guaiacol was used to measure peroxidase (POD) activity [38]. SOD and POD activities were expressed as $\text{U}\cdot\text{g}^{-1}$ FW. The percentage of inhibition of 50% of activity in the xanthine oxidase coupling reaction system was defined as one unit of SOD activity, while absorbances were read at 560 nm. One unit of POD activity is defined as an increase in 0.005 of record absorbance in a 1 mL reaction system per gram fresh weight per min, and absorbances were read at 470 nm. Catalase (CAT) can degrade H_2O_2 [39], CAT activity was expressed as $\text{nmol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ FW and was measured at 240 nm. One unit of CAT activity is defined as the degradation of 1 nmol H_2O_2 in the reaction system per gram fresh weight per min. Malondialdehyde (MDA) can react with thiobarbituric acid; the content of MDA was expressed as $\text{nmol}\cdot\text{g}^{-1}$ FW. Absorbance was measured at 532 nm and 600 nm [40]. Proline was extracted using sulfosalicylic acid (SA) [41], and proline content was expressed as $\mu\text{g}\cdot\text{g}^{-1}$ FW. Absorbance was measured at 520 nm. The content of soluble sugars was measured via anthrone colorimetry [42], and soluble protein content was determined using the bicinchoninic acid (BCA) assay [43]. The contents of soluble sugars and soluble protein were expressed as $\text{mg}\cdot\text{g}^{-1}$ FW. Absorbance was measured at 620 and 562 nm, respectively. All parameters were measured using a microplate reader (Spark 10M, Tecan, Grödig, Austria) in three replicates per treatment. Detailed experimental procedures can be found in the Supplementary Materials.

2.2.4. Statistical Analysis

Data are represented as the mean $\pm$ standard error of three replicates. Data were subjected to one-way ANOVA (multiple comparisons with Tukey's test, $p < 0.05$) and two-way ANOVA (the significance of effects of ECMF, drought treatments, and their interactions on the parameters related to the study of NM and ECM seedlings, $p \leq 0.05$, 0.01 and 0.001) with SPSS 18.0. Data used for ANOVA were tested for normality and homogeneity. The correlation between growth and photosynthetic physiological indices was evaluated using Pearson's correlation coefficient at $p \leq 0.05$. Origin Pro 8.5 was used to draw figures. The effect of watering treatments on each physiological and biochemical index was collectively evaluated via principal component analysis (PCA), using the statistical program R3.6.3.

3. Results

3.1. Growth and Water Status

Drought reduced the height, stem diameter, and DW of both ECM and NM seedlings, while the degree of reduction increased with increasing drought stress (Table 1). However, the stem height and DW of ECM seedlings were higher than those of NM seedlings at all watering treatments.

Table 1. Effect of drought stress and ectomycorrhizal fungi (ECMF) inoculation on the growth and water status of masson pine seedlings.

Parameter	Drought	Growth of Height (cm)	Growth of Stem Diameter (cm)	Dry Weight (g)	RWC (%)
NM	well watered	5.13 ± 0.19 ^a	1.54 ± 0.02 ^{ab}	4.62 ± 0.11 ^c	0.85 ± 0.02 ^{ab}
	mild drought	4.13 ± 0.07 ^b	1.37 ± 0.02 ^c	5.07 ± 0.94 ^{bc}	0.76 ± 0.03 ^{abc}
	moderate drought	3.33 ± 0.12 ^c	0.74 ± 0.03 ^d	2.89 ± 0.24 ^c	0.74 ± 0.05 ^{bc}
	severe drought	1.93 ± 0.07 ^d	0.5 ± 0.01 ^e	2.02 ± 0.04 ^c	0.65 ± 0.04 ^c
ECM	well watered	5.63 ± 0.15 ^a	1.65 ± 0.01 ^a	8.05 ± 0.65 ^{ab}	0.90 ± 0.00 ^a
	mild drought	4.27 ± 0.12 ^b	1.47 ± 0.05 ^{bc}	10.81 ± 1.18 ^a	0.86 ± 0.01 ^{ab}
	moderate drought	3.40 ± 0.1 ^c	0.77 ± 0.03 ^d	4.99 ± 0.72 ^{bc}	0.83 ± 0.02 ^{ab}
	severe drought	2.13 ± 0.09 ^d	0.52 ± 0.02 ^e	3.02 ± 0.51 ^c	0.80 ± 0.03 ^{abc}
	Drought	***	***	***	*
	ECM	*	**	***	*
	D *ECM	n.s.	n.s.	*	n.s.

ECM: inoculated with *Suillus placidus*; NM: uninoculated; Values (means ± SE) followed by different letters among treatments indicate significant differences at the 5% level; n.s. not significant * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

The needle RWC of both ECM and NM seedlings gradually declined with increasing drought stress, and the RWC of ECM seedlings was higher than that of NM seedlings under all watering treatments.

3.2. Physiological Measurements

3.2.1. Gas Exchange

A_n , g_s , C_i , and E of both NM and ECM seedlings gradually declined due to increased drought stress, whereas ECM treatment resulted in improved photosynthesis and ameliorated the decrease induced by drought stress compared with NM. Notably, A_n of ECM was significantly higher than that of NM under all watering treatments (Figure 2 and Table S1).

In contrast, the A_n/g_s of both NM and ECM gradually increased with increasing water deficiency. Compared with NM, ECM treatment reduced A_n/g_s under all watering treatments; however, the difference was not significant under the same watering treatments and the increasing range of A_n/g_s of ECM was higher than that of NM when compared with well-watered treatment.

3.2.2. Chl Fluorescence

Figure 3 shows that the F_v/F_m , $Y(II)$, F_v'/F_m' , qP , and NPQ of NM seedlings gradually decreased with increasing water deficiency and significantly decreased under severe drought treatment (except NPQ). In ECM seedlings, water deficiency decreased NPQ and qP , while F_v/F_m , $Y(II)$, and F_v'/F_m' slightly increased under mild drought, but decreased under moderate and severe drought conditions.

S. placidus inoculation had no significant effects on PSII function when compared with that in NM seedlings, whereas the F_v/F_m , $Y(II)$, F_v'/F_m' , and qP of ECM seedlings were slightly higher than those of NM.

Figure 4 presents the light response curves of $ETR(II)$ of ECM and NM seedlings, which gradually decreased with increasing drought stress. Compared with NM seedlings, *S. placidus* inoculation resulted in increased $ETR(II)$ under all watering treatments.

Figure 2. Effects of drought and ECMF on photosynthesis. Values (means $\pm$ SE) followed by different letters among treatments indicate significant differences ($p \leq 0.05$). Values with the same letter are non-significantly different for each treatment ($p > 0.05$). ECMF: ectomycorrhizal fungi.

Figure 3. Effects of drought and ECMF on PSII activity. Values (means $\pm$ SE) followed by different letters among treatments indicate significant differences ($p \leq 0.05$); values with the same letter are non-significantly different for each treatment ($p > 0.05$). NPQ showed no significant differences among different treatments, so it was not shown.

Figure 4. Effects of drought and ECMF on relative electron transfer rate ETR (II).

3.3. Biochemical Measurements

3.3.1. Photosynthetic Pigment Contents

A drought had no significant effect on the levels of Chl b, Car, and Chl a/b in the needles of NM seedlings but significantly decreased the levels of Chl a and Chl total under moderate and severe drought conditions. In the needles of ECM, except for a significant increase at mild drought and decrease at the severe drought of Chl a, drought stress showed no significant effect on other photosynthetic pigment contents in ECM seedlings.

The effect of ECMF inoculation on photosynthetic pigment contents varied with watering treatments, except that the Chl a content under moderate condition, and Chl a/b ratio under mild drought, moderate and severe drought conditions in ECM seedlings were higher than those in NM seedlings, the pigment contents of ECM were lower than NM at each watering treatment (Figure 5).

Figure 5. Effects of *S. placidus* and drought stress on photosynthetic pigments. Values (means ± SE) followed by different letters among treatments indicate significant differences ($p \leq 0.05$). Values with the same letter are non-significantly different for each treatment ($p > 0.05$). ECMF: ectomycorrhizal fungi.

3.3.2. Osmotic Adjustment Substances

Proline and MDA levels in the needles of both ECM and NM seedlings significantly increased with increased drought stress (Figure 6 and Table S1). The inoculation of ECMF significantly decreased the levels of proline and MDA under drought stress as compared to NM, except the proline level at mild drought.

Figure 6. The effect of *S. placidus* and drought stress on osmotic adjustment substances. Values (means $\pm$ SE) followed by different letters among treatments indicate significant differences ($p \leq 0.05$); values with the same letter are non-significantly different for each treatment ($p > 0.05$). Soluble sugar and soluble protein showed no significant differences among different treatments, so they were not shown.

3.3.3. Antioxidant Enzyme Activity

Drought stress significantly increased the SOD activity of NM, whereas a strong reduction of SOD in ECM was observed, which started under moderate drought (Figure 7 and Table S1). Drought stress significantly increased POD activity and reached its maximum value under moderate drought in both ECM and NM seedlings. Compared to well-watered, mild drought stress insignificantly increased CAT activity of NM but significantly decreased under severe drought stress. Drought significantly decreased the CAT activity of ECM.

POD and SOD activities in ECM seedlings were significantly higher than those in NM for each watering treatment, while CAT activity was lower under mild and moderate drought conditions.

3.4. Correlation between Growth and Physiological, Biochemical, and Physiological Indexes

The Pearson correlation analysis shows that almost all parameters showed significant correlation with each other, except soluble sugar. As the most direct indicator of plant stress resistance, growth parameters had a significantly positive correlation with RWC, CAT activity, Chl a, Chl a, Chl (a + b), gas exchange measurements (A_n , C_i , E , and g_s), and chlorophyll fluorescence characteristics (F_v/F_m , $Y(II)$, qP , and F_v'/F_m'), but had extreme significant negative correlation with An/g_s , POD activity, proline and MDA contents (Table S2).

Figure 7. The effect of *S. placidus* and drought stress on antioxidant enzyme activities. Values (means $\pm$ SE) followed by different letters among treatments indicate significant differences ($p < 0.05$). Values with the same letter are non-significantly different for each treatment ($p > 0.05$). ECMF: ectomycorrhizal fungi.

3.5. Principal Component Analysis (PCA)

PCA analysis shows the effects of different watering treatments on various physiological indexes in NM (Figure 8a) and ECM (Figure 8b) seedlings. In NM seedlings, watering treatments and physiological parameters were loaded into two major principal components (PC1 and PC2), explaining 78.0% of the total variance. NM under well-watered and mild drought showed high gas exchange measurements, chlorophyll fluorescence characteristics, and contents of Chl a, Chl b, and Chl (a + b), and thus, located in the negative side of PC1 and both negative and positive sides of PC2. NM under moderate and severe drought showed high contents of proline and MDA, and high A_n/g_s , located in the positive side of PC1 and both negative and positive sides of PC2. As is shown in Figure 8a, it can be concluded that photosynthesis-related indexes were highly positively correlated with well-watered and mild drought, while osmotic regulatory substances are involved in regulating severe drought stress in NM seedlings. In ECM seedlings, watering treatments and physiological parameters were loaded into PC1 and PC2, explaining 76.69% of the total variance. ECM seedlings under well-watered and mild drought showed high gas exchange measurements (A_n , C_i , E , and g_s), chlorophyll fluorescence characteristics (except for NPQ), and SOD activity, and located on the negative side of both PC1 and PC2. ECM under moderate and severe drought showed high contents of proline and MDA and high A_n/g_s and POD activity, being located in the positive side of PC1 and both negative and positive sides of PC2. The results showed that photosynthesis in ECM was positively correlated with sufficient water, while antioxidant enzyme system and osmotic regulatory substances were mainly involved in the regulation of severe drought stress.

4. Discussion

4.1. Physiological Response

Photosynthesis and growth are the primary processes affected by drought stress [44]. In our study, E , g_s , C_i , and A_n of both ECM and NM seedlings simultaneously decreased with increasing water deficiency, indicating that under drought stress, seedling photosynthesis was limited by stomatal factors [45]. g_s is an important channel for gas exchange between plants and the environment, stomata closure in response to ABA signals results in low C_i ; thus, CO_2 concentrations decrease at the carboxylation site of ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase [46], resulting in decreased A_n and down-regulation of photosynthesis. This is a practical control mechanism for preventing water deficits from damaging the photosynthetic apparatus [47]. In addition, the decrease in photosynthesis under drought stress is responsible for the decrease in plant growth and dry

weight, because it provides the basis for plant growth [48]. Moreover, the decrease in g_s was much higher than the decrease in A_n under water-deficiency conditions in our study, indicating that the decrease in the RWC of NM and ECM seedlings was reduced mainly through water-saving strategies, which were achieved through stomatal closure [49,50].

The A_n of NM seedlings was significantly lower than for ECM seedlings under all watering treatments, suggesting that *S. placidus* inoculations can improve plant photosynthesis and may be beneficial for increasing dry weight (Table S2). Liu et al. [51] and Gong et al. [52] also found that inoculation of mycorrhizal fungi could improve the net photosynthetic rate and C assimilation capacity of host plants under drought conditions. Zhu et al. [53] showed that inoculation of mycorrhizal fungi can improve stomatal conductance and transpiration rate of host plants under drought stress so that more external CO_2 can enter the plant, thereby providing more raw materials for photosynthesis and increasing the net photosynthetic rate (A_n) of host plants and resistance to adversity stress in host plants. Compared with NM, the A_n/g_s of ECM was lower under drought stress. In contrast, the increased rates in ECM were higher than those in NM. This may be because the drought-induced reduction in the g_s of ECM was higher than that of NM, whereas the decrease in the A_n of ECM was lower than that of NM.

Chlorophyll fluorescence plays an important role in the absorption, transfer, dissipation, and distribution of light energy during photosynthesis. The effect of any environmental factor on photosynthesis can be reflected by chlorophyll fluorescence dynamics in leaves [54]. The change in F_v/F_m can be used to identify plant tolerance to drought. $Y(II)$ can represent the change in net photosynthetic rate to some extent. F_v'/F_m' reflects the excitation energy capturing efficiency of the open PSII reaction center under light. The F_v/F_m , $Y(II)$, and F_v'/F_m' of NM significantly decreased under moderate stress, whereas that of ECM significantly decreased under severe stress, suggesting that PSII was damaged due to water deficiency [55] and that inoculated seedlings suffered less damage than uninoculated seedlings. Compared with NM seedlings, ECM seedlings had slightly higher F_v/F_m , indicating higher efficiency of the seedlings at containing light energy in the pigment bed of PSII [56]. Likewise, $Y(II)$ and F_v'/F_m' were insignificantly higher in ECM, further indicating that ECM can improve the photosynthetic tolerance of *P. massoniana* to drought. These results are similar to those of Baker [57].

The ETR (II) of both ECM and NM seedlings gradually decreased with increasing water deficiency, indicating that drought stress damaged the PSII reaction center and reduced the electron transfer rate, and blocked the synthesis of NADPH and ATP, resulting in a decrease in the assimilation capacity of photosynthetic carbon (i.e., a decrease in A_n) [58]. The decrease in CO_2 assimilation, in turn, results in the over-reduction of the components of the electron transport chain, and hence, the ability of the needle to dissipate excess light energy is limited [59].

The light energy absorbed by photosynthetic pigments is mainly dissipated by chlorophyll fluorescence and non-photochemical pathways, except for photochemical reactions [60]. qP and NPQ are two forms of chlorophyll fluorescence dissipation, both of which are closely related to electron transport activity [61]. Drought stress could result in excess excitation energy, and the NPQ process is known as the main mechanism of energy dissipation and is responsible for protecting photochemical components from potential damage [62]. However, we found that the NPQ of both ECM and NM gradually decreased under water-deficiency conditions, suggesting that thermal dissipation was affected in masson pine seedlings, i.e., masson pine seedlings dissipated excess energy through other methods. This conclusion is supported by the findings of Yi et al. [63], who found that zeaxanthin (Z) (which correlates with the development of NPQ) content significantly decreased under water deficiency. Takahashi et al. [64] also reported that thermal dissipation was not the main way to protect plant PSII from stress conditions. qP reflects the level of photosynthetic activity of plants, and the decrease in qP in both ECM and NM seedlings showed that less number of PSII reaction opening centers open in proportion and electronically participate in CO_2 fixation [65]. Compared with NM seedlings, the higher ETR (II) of

ECM seedlings indicates that *S. placidus* inoculation could maintain the stability of the CO₂ assimilation process and photochemical reaction process (i.e., higher A_n in ECM than in NM seedlings), and higher qP in ECM seedling also showed that ECM treatment could improve photosynthesis under drought stress. This is one of the reasons for higher growth under mycorrhizal treatment (Table S2).

4.2. Biochemical Response

Drought stress results in water loss and damages the cell membrane integrity of chloroplasts, which further affects chlorophyll biosynthesis and promotes its decomposition. Similarly, the RWC of both ECM and NM needles showed a large decrease under moderate and severe drought conditions, as did chlorophyll content, which is consistent with the results of Pan et al. [66]. Sanchez et al. [67] also reported that water loss results in a decrease in total chlorophyll content, which was also the case in this study. However, there was no significant difference in pigment content between NM and ECM seedlings, indicating that *S. placidus* inoculation has no significant effect on pigment content, which is similar to the results of Zhu et al. [53].

In addition, chlorophyll is the light energy capture molecule of photosynthesis and the main carrier of photosynthesis. In general, a significant positive correlation between chlorophyll content and photosynthesis has been observed [68,69], similar results can be found in our study (Table S2). This may suggest the higher chlorophyll b content under all watering treatment and higher chlorophyll a and chlorophyll (a + b) under well-watered and mild drought conditions in NM seedlings should be associated with an increased photosynthesis rate. However, the photosynthetic rate of NM seedlings was lower than that of ECM under all watering treatments. This may be because the relationship between chlorophyll content and photosynthetic efficiency is complex and controlled by many factors [70]. This result also suggests that chlorophyll content cannot be used as an indicator to directly evaluate the photosynthetic efficiency of plants in our study. This is likely because chlorophyll often absorbs more light energy than what the plants need for photosynthesis under stress conditions [71]. Guo et al. [72] also found that chlorophyll content and quantum yield are independent factors.

Drought also results in ROS accumulation in plants, which may result in oxidative damage and limited plant growth, ultimately leading to cell death [73]. MDA accumulation is an important indicator of lipid peroxidation in cell membranes [74], and the MDA content gradually increased in response to increasing water deficit, indicating that plant cell membranes were damaged, particularly under severe drought conditions, thereby limiting plant growth [73]. Osmotic adjustment substances and antioxidant enzymes quench superoxide anions and prevent ROS from damaging plant cell membranes under stress [75,76]. Our results showed that drought stress induced the activation of POD and increased proline content in NM and ECM seedlings; indicating that they play key roles in drought tolerance and reducing MDA accumulation caused by drought stress (Table S2). Yin et al. [77] suggested that antioxidant enzymatic activity and osmotic adjustment substances are not always activated under stress conditions, which is consistent with our results because CAT decreased under moderate and severe drought and soluble protein and soluble sugar contents were not significantly changed under drought stress in both NM and ECM seedlings. Compared with NM seedlings, higher POD and SOD activities and lower MDA and proline contents were observed in ECM seedlings under drought conditions, demonstrating lower oxidative damage and its greater capacity for drought defense [78]. This result may explain the better performance of ECM seedlings under all watering treatments (i.e., increase in growth index). Zhang et al. [79] and Gholinezhad et al. [80] also reported that the enhancement of water stress tolerance caused by mycorrhizal fungal colonization was related to the increased antioxidant activity of the mycorrhiza.

4.3. PCAs

PCA evaluated the effects of water status and various physiological and biochemical responses in NM and ECM seedlings under four watering treatments. There was a distinct separation between well-watered, mild, moderate, and severe drought conditions (Figure 8). In addition, Person's correlation coefficient and PCA revealed close relationships among the various indicators, showing the involvement of different mechanisms in NM and ECM seedlings in response to water deficiency.

In NM seedlings, well-watered and mild drought were positively related to photosynthesis-related indexes (except C_{ar}). High accumulation of MDA and proline contents and high A_n/g_s were found under moderate and severe drought conditions, which were negatively associated with other traits. This observation suggests that NM seedlings can improve water use efficiency and accumulate osmotic adjustment substances to facilitate water absorption and resist drought stress [81]. Similar results have been found by Gholinezhad et al. [80]. Among them, RWC exhibited a negative relationship with proline, which was consistent with the results of Abboud et al. [59]. This may relate to an adaptive mechanism to reduce water loss and preserve cell membrane integrity [82].

Similarly, the physiological and biochemical effects of the four watering treatments in ECM were similar to those in NM. However, beyond that, under moderate and severe drought conditions, ECM not only improved osmotic adjustment substance contents and water use efficiency but also increased POD activity, suggesting that the overproduction of POD could be an adaptive response of ECM seedlings to water deficiency. This may be due to its key role in scavenging active oxygen caused by water shortage. Therefore, except for osmotic adjustment substance (proline), antioxidase activity (POD) is one of the crucial elements of defense mechanism under drought stress in *S. placidus* inoculation seedlings. These results agree with those of Tani et al. [83] and Langeroodi et al. [84], who reported that proline and POD are related to the decrease in oxidative damage caused by drought stress.

In addition, as the main scavenging mechanism of ROS, POD and SOD were highly positively correlated and showed no close correlation with drought in NM seedlings. However, POD and SOD in ECM seedlings were negatively correlated and were closely related to drought. Those results suggest that *S. placidus* colonization appears to affect these functions of SOD and POD. A similar result was also reported in the studies of Chen et al. [85] and Yin et al. [86]. Though POD and SOD activities increased in response to water deficiency in NM seedlings, the increase was not able to detoxify drought-induced ROS, which can be demonstrated by higher MDA content under drought conditions [87]. While the higher SOD activity in ECM may contribute to the better growth under well-watered and mild drought, higher POD activity enhanced the effectiveness of the ROS scavenging system.

5. Conclusions

We explored the effect of *S. placidus* inoculation on the drought resistance of masson pine for the first time. Water deficiency harmed the growth, RWC, Chl pigments, and photosynthetic process of both NM and ECM seedlings. Compared to NM seedlings, ECM seedlings showed higher water status, growth and C assimilation capacity, and lower oxidative damage (lower MDA content). *S. placidus* inoculation reduced drought-induced oxidative damage by increasing antioxidant defense systems (POD activity), water use efficiency, and accumulation of osmotic adjustment substances (proline). The results from this study demonstrate that *S. placidus* inoculation could improve the growth and drought resistance of masson pine seedlings, and *S. placidus*-*P. massoniana* symbiont could be a candidate for increasing the survival rate and growth of seedlings after afforestation.

Supplementary Materials: The following are available online at <https://www.mdpi.com/1999-4907/12/3/332/s1>, Table S1. Effect of drought stress and ectomycorrhizal fungi (ECMF) inoculation on various indicators of masson pine seedlings; Table S2. Correlation between growth index and antioxidant enzyme activity and photosynthetic physiological index.

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