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Responses of Antioxidant Enzymes, Osmoregulators and Their Related Genes to Drought Stress and Rehydration in *Auricularia fibrillifera*

Zhifen Yang, Feng Liu, Hao Guo, Yiqin Wang, Suqin Zhang, Guangdong Geng*
College of Agriculture, Guizhou University, Guiyang, Guizhou, China
*Corresponding author.

Abstract:

The antioxidases, osmoregulators and related gene responses to drought stress and rehydration were investigated to understand drought-tolerance mechanisms in *Auricularia* fibrillifera. Compared with in the sensitive cultivar HSQ, the three enzyme activities in resistant cultivar CSLZ significantly increased during drought stress. Drought stress generally increased the contents osmoregulators in both cultivars. The soluble sugar, soluble amino acid and proline contents in CSLZ exhibited significant increases compared with in HSQ under untreated control conditions. The enzyme activities and osmoprotectant contents in both cultivars decreased rapidly at 24 h after rehydration. These enzymes and osmoprotectants had key roles in responses to primary and severe drought stresses in CSLZ, respectively. Compared with in HSQ, the soluble sugar content in CSLZ showed the greatest increase under 50% and 60% of water loss conditions, followed by the superoxide dismutase activity and then the proline content. In total, 52,954 unigenes were obtained using the transcriptome analysis, which included many key differentially expressed genes involved in the responses to oxidative and osmotic stresses under drought conditions. The expression levels of these genes were generally consistent with the enzyme activities or osmoregulator contents under drought-stress and rehydration conditions, respectively. The defense-related pathways were also enriched under drought-stress conditions. Thus, these enzymes and osmoregulators may form a defense network to protect *Auricularia* against drought-related damage. Resistant CSLZ had stronger abilities to maintain higher enzyme activity levels and synthesize osmoprotectants, which confer drought resistance, than sensitive HSQ.

Keywords*: Antioxidant Enzymes, Auricularia, Drought Stress, Gene Repression Levels, Osmoregulators.*

I. INTRODUCTION

Auricularia, known as the wood ear, is widely used both as a food and medicine in East Asia, especially in China and Korea[1,2]. As an important edible mushroom in Chian[3], *Auricularia* has antioxidative[4], antiaging, antitumor[5], and antibacterial properties. The large-scale cultivation of *Auricul*aria usually occurs outdoors in China. In recent years, the drought incidence has increased

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worldwide, especially in arid and semi-arid areas, causing serious yield losses[6].

Upon exposure to drought, organisms manifest multiple changes, including external morphology, and internal solute and gene expression levels[7]. Reactive oxygen species (ROS) accumulate in the cells under drought-stress conditions, and this has a toxic effect on the cells, leading to irreparable functional disorders and even death[8], Superoxide dismutase (SOD) can convert the O^{2-} into H_2O_2, which will be further decomposed by catalase (CAT) and peroxidase (POD). The three antioxidases synergistically scavenge ROS to reduce the damage caused to the cells. The SOD, POD and CAT can remove excess ROS in organisms during drought stress. Osmotic adjustments are common drought-stress responses, which reduce the osmotic potential through the accumulation of osmotic substances. The soluble sugar, soluble protein, soluble amino acid (AA) and proline contents are closely correlated with different levels of water loss in *Auricularia auricula*[9].

Drought stress can trigger a wide variety of fungal responses, ranging from molecular processes to physiological metabolisms. Under drought-stress conditions, many genes are affected, which leads to a greater range of physiological and biochemical alterations, including changes in main biosynthetic, antioxidative and respiration pathways[10]. Transcriptome analyses of plants, such as rice[11], maize[12], Phormium tenax[13] and poplar[14], under drought-stress conditions have been reported.

For most terrestrial plants and fungi, drought can lead to serious damage or even death. However, the fruiting bodies of *Auricularia* become dry and hard, and enter dormancy during drought stress, which can protect them from drought-related damage. Once watered, dormancy is broken[15]. The unique viability of *Auricularia* is rare in the eukaryotic community under arid conditions. *Auricularia* is an ideal species to explore the adaptive mechanisms against drought stress owing to its rare drought tolerance and rehydration capability. Currently, there are limited reports on the physiological and molecular mechanisms of drought resistance in *Auricularia*; therefore, they remain largely unknown. Here, changes in antioxidant enzyme activities, osmoregulators and related gene expression levels under drought-stress and rehydration conditions were investigated in two *Auricularia fibrillifer*a cultivars. The objective was to understand the physiological, biochemical and molecular response patterns under drought-stress and rehydration conditions, and to clarify the physiological and molecular mechanisms involved in drought tolerance. The results will provide a valuable reference for *Auricularia* breeding and the cultivation of drought-resistant fungi.

II. MATERIALS AND METHODS

2.1 Materials and Culture Conditions

Drought-resistant cultivar (CSLZ) and drought-sensitive cultivar (HSQ) of *Auricularia* fibrillifera were used for this study. The culture medium for *Auricularia* consisted of 83% sawdust, 14% wheat bran, 1% bean bran, 1% gypsum and 1% lime. In total, 1 kg of mixed substrate was placed in heat-resistant

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polyethylene bags and then autoclaved at 121℃ for 1.5 h. The sterilized medium was inoculated with 0.5% grain spawn and maintained at 25℃ in the dark. When the culture medium was fully colonized by mycelia, the polyethylene bags were removed, and each cultivar was placed in a culture room (25 ± 1℃, 15-h naturally scattered light/9-h dark) at Guizhou University (26°26′N, 106º38′E and 1,120 m altitude), which is situated in Guizhou Province, Southwest China. They were sprayed regularly with water ~8 times per day, 15 ml water per bag of substrate. When the diameters of the fruit bodies reached 2 to 3 cm, 40 fruit bodies of a uniform size were collected as the first samples (T1 stage, no drought stress). Then, drought-stress treatments were started, and the fruit bodies on the substrate dehydrated naturally. When the water loss percentages of fruit bodies were 30% (T2 stage) and 60% (T3 stage), the second and third samples were selected, respectively. Afterward, the fruit bodies were rewatered. The fourth and fifth samplings were performed at 1 h (R1 stage, 50% of water loss) and 24 h (R2 stage, no water loss) after rehydration. Regularly watered fruit bodies on the substrate were used as parallel controls. All the samples were immediately frozen in liquid nitrogen after sampling and stored at −80℃. A randomized complete block design with three biological replications was employed.

2.2 Analysis of Physiological and Biochemical Parameters

The physiological and biochemical parameters were measured using the fruit bodies of *Auricularia*. The fruit bodies were collected and ground in extraction solutions in an ice bath and were centrifuged at 8,000 × g at 4℃ for 10 min. The supernatants were used as the extracts. The SOD (EC 1.15.1.1, Cas No.: BC0170), POD (EC 1.11.1.7, Cas No.: BC0090) and CAT (EC 1.11.1.6, Cas No.: BC0200) activities, as well as the soluble sugar (Cas No.: BC0035), soluble protein (Cas No.: PC0020), soluble AA (not including proline and hydroxyproline, Cas No.: BC1570) and proline (Cas No.: BC0290) contents were measured in accordance with the assay kits' instructions (Solarbio Co., Beijing, China). Supernatants used to measure the soluble AA and proline contents were prepared as above with a modification, being centrifuged at 10,000 × g.

2.3 Transcriptome Analysis

Drought-resistant CSLZ was selected for the further transcriptome analysis. Total RNA from the fruit bodies at T3 and R1 stages was extracted using TRIzol reagent (Thermo Fisher Scientific, MA, USA) following the manufacturer's protocol and then treated with TaKaRa RNase-free DNase I for 30 min. The total RNA quantity and quality were checked using a NanoDrop 1000 spectrophotometer. A total of 20 μg RNA was used for cDNA library construction and transcriptome sequencing (BGISEQ-500) at Beijing Genome Institute. The unigene annotation was performed by searching for homologous sequences against the NCBI Nr database using BLASTx software (E-value < 1×10^{-5}). The transcript expression levels are expressed as fragments per kilobase per million mapped fragments (FPKMs).

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2.4 Data Analysis

Statistical software (SPSS 20.0, IBM Inc.) and graphics software (Origin 2017, OriginLab Inc.) were used for the data analysis and Figure construction, respectively. Duncan's multiple range test was performed to determine significant differences between means at a significance level of $p < 0.05$.

III. RESULTS

3.1 Effects of Drought Stress and Rehydration on SOD Activity

The SOD activity levels of two cultivars were significantly ($p < 0.05$) higher than those of the controls at the T2, T3 and R1 stages (Fig 1.). With the drought stress increased, the SOD activity in CSLZ also increased rapidly, reaching 5.90-fold that of the control at the T3 stage. After rehydration, the SOD activity of CSLZ decreased quickly and returned to the control level at the R2 stage. The SOD activity change in HSQ was similar to that in CSLZ; however, it was the highest at the R1 stage, reaching 1.70-fold that of the control. The SOD activity in CSLZ was more sensitive to drought stress than in HSQ, because its peak value was (3.60-fold) higher and earlier than that of HSQ. The SOD activity peaks in two cultivars occurred at the T3 and R1 stages, indicating that the fruit bodies might remove excessive superoxide anions by synthesizing a large amount of SOD to reduce the drought damage.

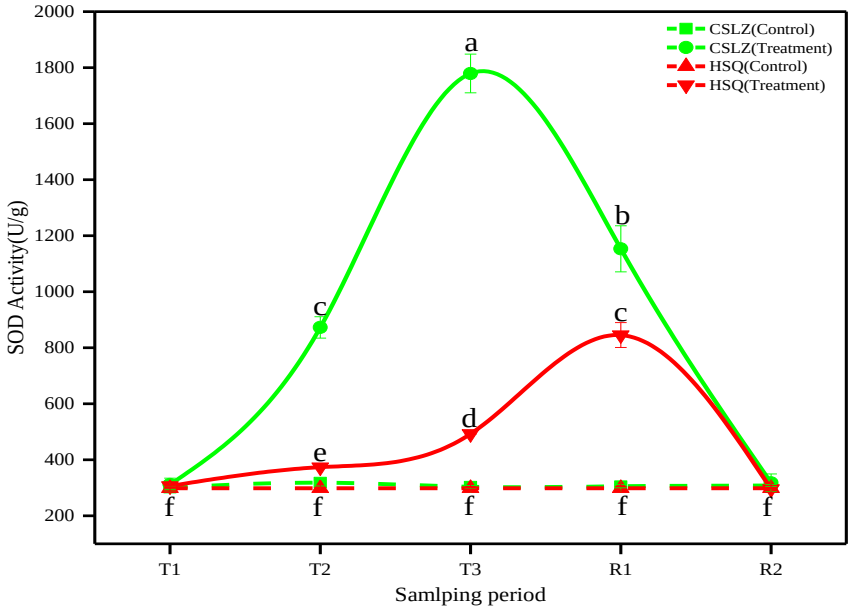

Fig 1: Effects of drought stress and rehydration on the SOD activity levels in two cultivars of *Auricularia*. Bars show means ± SDs (n = 3). Values with different letters are significantly different at $p < 0.05$.

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3.2 Effects of Drought Stress and Rehydration on POD Activity

The POD activity levels of two cultivars exhibited significant ($p < 0.05$) increases at the T2, T3 and R1 stages, compared with the controls (Fig 2.). When compared with HSQ, the POD activity of CSLZ increased significantly at both the T2 and T3 stages. The POD activity in CSLZ increased quickly at the T2 stage, peaked at the T3 stage, and then decreased rapidly after rehydration, indicating that its response was more sensitive and rapid to drought stress and rehydration than that of HSQ.

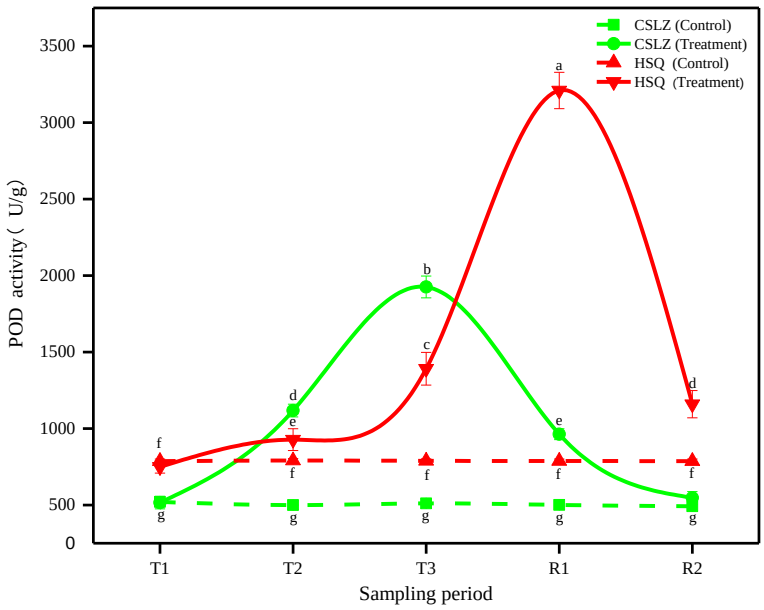

Fig 2: Effects of drought stress and rehydration on the POD activity levels in two cultivars of *Auricularia*. Bars show means ± SDs (n = 3). Values with different letters are significantly different at $p < 0.05$.

3.3 Effects of Drought Stress and Rehydration on CAT Activity

The CAT enzyme is involved in removing ROS. The CAT activity levels of two cultivars significantly ($p < 0.05$) increased compared with the controls under drought-stress and rehydration conditions (Fig 3.). The CAT activity in CSLZ increased during drought stress and peaked at the R1 stage, reaching 5.20-fold that of the control. The CAT activity in HSQ peaked at the T3 stage. The peak value was 4.30-fold greater than that of the control. The peak CAT activities of both cultivars appeared between 50% and 60% of water loss, indicating that a high CAT activity removed the ROS, reducing the injury level caused by drought stress.

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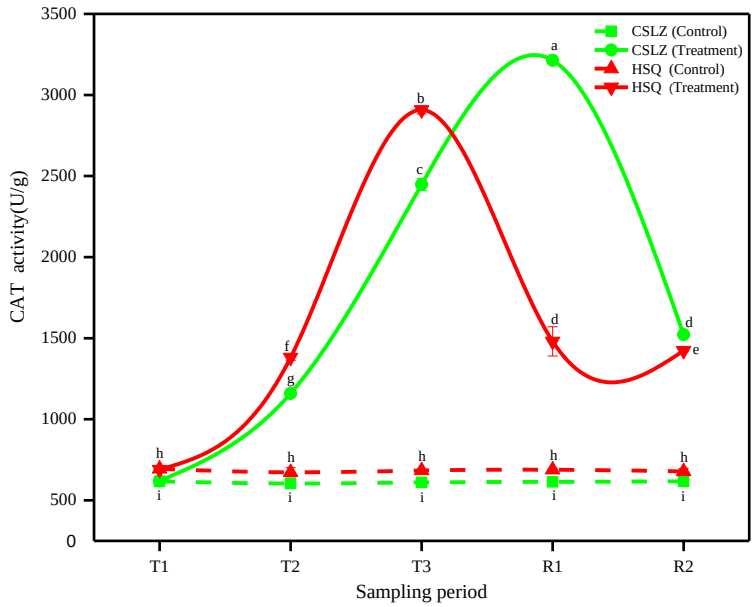

Fig 3: Effects of drought stress and rehydration on the CAT activity levels in two cultivars of *Auricularia*. Bars show means ± SDs (n = 3). Values with different letters are significantly different at $p < 0.05$

3.4 Effects of Drought Stress and Rehydration on the Osmoregulators

Soluble Sugars:The soluble sugar contents of the two cultivars were significantly ($p < 0.05$) higher than those of the controls under drought-stress conditions (Fig 4A.). The level in CSLZ showed a significant increase, except at the T2 stage, compared with in HSQ. The contents in CSLZ under control conditions was significantly ($p < 0.05$) higher than in HSQ at every stage. The content of CSLZ increased during drought stress and peaked at the T3 stage, reaching 5.90-fold that of the control. The soluble sugar content decreased quickly after rehydration. CSLZ produced more soluble sugars than HSQ at the T3 and R1 stages.

Soluble Proteins: Soluble proteins maintain the osmotic potential at a low level to defend against water stress in cells. The soluble protein contents of the two cultivars were significantly ($p < 0.05$) enhanced compared with those of the controls under drought-stress and rehydration conditions (Fig 4B.). CSLZ showed a significant (1.70-fold) increase at the T3 stage compared with HSQ. The soluble protein content of CSLZ increased during drought stress, peaking at the T3 stage, reaching 3.90-fold that of the control. The contents began to decrease after rehydration. For HSQ, the soluble protein content rose rapidly from the T1 to T2 stage, remained stable at the T3 and T4 stages, and then decreased. The soluble protein contents were maintained higher levels than other parameters after rehydration.

The AA: The AA contents of the two cultivars were significantly ($p < 0.05$) higher than those of the controls at the T3 stage (Fig 4C.). The AA content of CSLZ significantly increased compared with in HSQ

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at the T3 and R1 stages. The AA content was significantly ($p < 0.05$) higher in CSLZ than in HSQ under untreated control conditions. As the drought stress increased, the AA content in CSLZ increased. It rapidly returned to the control level at the R2 stage after rehydration. However, the AA content in HSQ increased rapidly from the T1 stage to T2 stage and then decreased. Thus, CSLZ appeared to respond more strongly to drought stress than HSQ.

Proline: The proline contents of two cultivars significantly ($p < 0.05$) increase, except at the R2 stages, compared with the controls (Fig 4D.). CSLZ increased significantly more than HSQ under both drought-stress and rehydration conditions. Under control conditions, the proline content in CSLZ was significantly higher than in HSQ at each stage. The change in the proline content was similar to that of the soluble sugar content. As the drought stress increased, the proline content in CSLZ also increased, resulting in a strong osmotic adjustment ability and drought resistance.

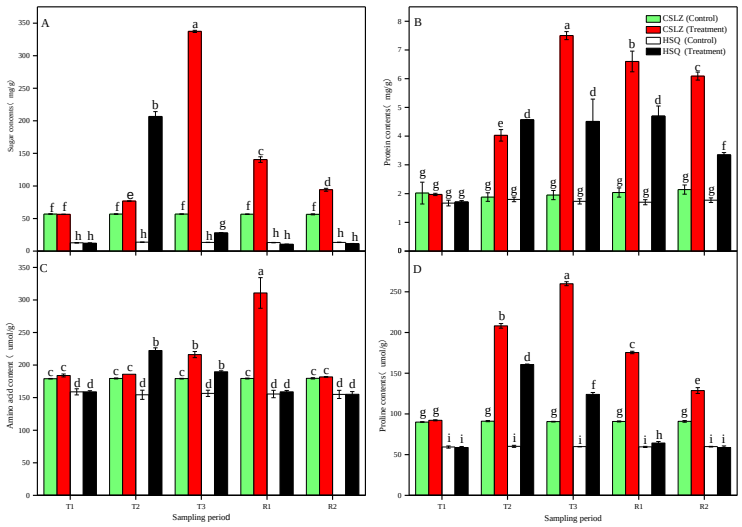

Fig 4: Effects of drought stress and rehydration on the soluble sugar (A), soluble protein (B), soluble amino acid (C) and proline (D) contents in two cultivars of *Auricularia*. Bars show means ± SDs (n = 3). Values with different letters are significantly different at $p < 0.05$.

3.5 Correlations between Parameters

The POD activity level was not correlated with the CAT activity level, nor the sugar, protein, AA and proline contents (TABLE I). There were positive correlations among all the other parameters. These results demonstrated that these antioxidant enzymes and osmoregulators work together to form a defense network that protects *Auricularia* against drought stress.

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TABLE I. Correlations among physiological parameters in *Auricularia* under drought-stress and rehydration conditions.

Index	SOD	POD	CAT	Soluble sugures	Soluble proteins	AA
POD	0.483**					
CAT	0.564**	0.225				
Soluble sugars	0.695**	0.025	0.369*			
Soluble proteins	0.717**	0.302	0.743**	0.691**		
AA	0.480**	-0.174	0.674**	0.535**	0.583**	
Proline	0.754**	-0.009	0.468**	0.844**	0.698**	0.572**

*, ** Significant correlations at 5% and 1% levels, respectively (n = 30).

3.6 Expression of Genes Involved in Responses to Oxidative and Osmotic Stresses

In total, 52954 unigenes were obtained using the transcriptome analysis. The differentially expressed genes (DEGs) related to antioxidant enzymes or osmotic regulator were investigated under drought-stress (T3 stage) and rehydration (R1 stage) conditions. Five DEGs related to SOD activity were found at the T3 stage (Table II). The increase in the SOD activity might result from the up-regulation of CL3433.Contig3_All, Unigene2440_All and Unigene2441_All. Among them, the former contributed to the SOD1 activity, while the latter two regulated the SOD activity. There were 34 DEGs involved in the regulation of the POD activity. The top three DEGs significantly induced were CL3509.Contig1_All, CL2147.Contig3_All and Unigene21950_All at the T3 stage, while the DEGs CL2147.Contig3_All, Unigene1770_All and Unigene4883_All were down-regulated at the R1 stage. Most DEGs (75%) related to the CAT activity were up-regulated.

A number of DEGs were involved in the soluble sugar accumulation. The number of down-regulated DEGs was 1.80-fold that of up-regulated DEGs at the T3 stage. After rehydration, the number of down-regulated DEGs were reduced by 51.10% compared with that at the T3 stage, indicating that the accumulation of soluble sugar under drought-stress conditions was greatly influenced by the down-regulated DEGs. Protein biosynthesis occurs mainly in the ribosome and involves transcription and translation, which are synergistically regulated by many genes. The number of down-regulated DEGs was

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2.50-fold that of up-regulated DEGs at the T3 stage. After rehydration, the number of down-regulated DEGs decreased sharply, which indicated that the accumulation of soluble proteins was largely owing to the down-regulation of these DEGs under drought-stress conditions. The DEGs encoding 20S proteasome subunit beta 4, S-phase kinase-associated protein 1, ubiquitin-conjugating enzyme E2W and 20S proteasome subunit alpha 5 were significantly down-regulated to inhibit protein hydrolysis at the T3 stage. In the AA catabolic pathway, some DEGs encoding 3-hydroxy acid dehydrogenase, malonic semialdehyde reductase, 3-oxoacyl-[acyl-carrier protein] reductase and beta-glucosidase were significantly down-regulated at the R1 stage, indicating that they might be key genes that inhibit amino acid catabolism. In the AA synthetic pathway, there were 114 and 72 DEGs expressed at the T3 and R1 stages, respectively. The DEGs CL3334.Contig1_All, Unigene8964_All, Unigene3257_All and Unigene11810_All were significantly up-regulated at both stages, and they might be the key genes required for the AA synthesis. The down-regulated DEGs were predominantly found in the proline metabolic and biosynthetic pathways. The DEGs encoding proline iminopeptidase (CL3211.Contig2_All, CL857.Contig4_All and CL857.Contig5_All) in the proline biosynthetic pathway were down-regulated at both stages, and they might be the key genes required for the proline synthesis.

Table II. The number of DEGs related to antioxidant enzymes or osmoregulators in *Auricularia* under drought-stress and rehydration conditions.

Antioxidant enzymes /osmoregulators	The number of involved DEGs at the T3 stage		The number of involved DEGs at the R1 stage	
	Up-regulation	Down-regulation	Up-regulation	Down-regulation
SOD	3	2		
POD	5	18	2	9
CAT	5		4	3
Soluble sugers	483	857	486	419
Soluble proteins	557	1393	517	609
The AA	194	365	189	169
Proline	1	12	1	7
Total	1248	2647	1199	1216

DEGs: differentially expressed genes.

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IV. DISCUSSION

Drought has always been regarded as an urgent global environmental problem that seriously restricts agricultural development[16]. An increase in drought stress did not weaken the SOD activity levels in three pilithic mosses, indicating that they have the ability to eliminate O^{2-}[17]. The SOD, POD and CAT activities in the leaves and petals of marigold increase under drought-stress conditions[18]. In this experiment, the SOD, POD and CAT activity levels of two *Auricularia* cultivars were significantly greater than those of the controls under drought-stress conditions (Fig 1–3.). These results were in agreement with those of previous reports[17-18]. The activity peaks of the three enzymes in the two cultivars occurred at the T3 and R1 stages, indicating that the fruit bodies might synthesize large amounts of antioxidant enzymes to reduce the drought-related damage by removing excessive ROS. Earlier reports also indicated that drought stress increased antioxidant enzymes activity in plants[19]. Similar results were obtained in this work (Fig 1–3.). When compared with sensitive HSQ, the SOD, POD and CAT activities in resistant CSLZ increased significantly during drought stress. After rehydration, the activity levels of the three enzymes in both cultivars decreased quickly at the R2 stage. Thus, *Auricularia* synthesized large amounts of antioxidant enzymes under drought-stress conditions, which contributed to drought resistance. The resistant CSLZ had a stronger ROS scavenging capability than the sensitive HSQ.

Osmotic adjustment aids in stabilizing cell membranes and enables plants to tolerate oxidative damage[1, 20]. The proline content of common bean (*Phaseolus vulgari*s L.) significantly increases under drought-stress conditions[21]. Increases in the AA pools occur in a variety of plant species as a response to water stress[22]. Here, we found that drought stress generally increased the soluble sugar, soluble protein, soluble AA and proline contents in the two *Auricularia* cultivars (Fig 4.). The results are in accordance with those of Khan M et al[23]. and Isabella et al[21]. The contents decreased at the R2 stage after rehydration. In addition, the osmoprotectant contents in CSLZ increased more sharply and was maintained longer than in HSQ at the T3 to R1 stages. The soluble sugar, soluble AA and proline contents in CSLZ exhibited significant increases compared with in HSQ under the untreated control conditions. There was a similar report in rapeseed by Khan et al[23]. In this experiment, the soluble sugar content in CSLZ significantly increased 10.1-fold and 11.5-fold over the levels in HSQ at the T3 and R1 stages, respectively. This is in accordance with the results of Ma et al[9]. Compared with in HSQ, the soluble sugar content in CSLZ increased the most at the T3 and R1 stages, followed by SOD activity and the proline content. The CSLZ/HSQ ratio for osmoprotectants increased more than the antioxidant enzyme ratios at the T3 and R1 stages (serious drought stresses). However, they demonstrated just the opposite (osmoprotectants increased less than the antioxidant enzymes) trends at the T2 stage (primary drought stress). These findings suggested that the osmoprotectants played key roles in *Auricularia* responses to severe drought stress and that CSLZ synthesized more osmoprotectants and maintained increased levels longer than HSQ.

During abiotic stress responses, it is necessary for plants to increase antioxidant system-related gene expression levels, scavenge unnecessary ROS and maintain the cellular redox balance[23]. The expression levels of CAT, SOD and POD genes increases along with drought severity in muskmelon (*Cucumis melo*

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L.), and the up-regulation of these genes is higher in drought-tolerant SC-15 than in drought-susceptible EC-564755[24]. The relatively high mRNA levels of SOD, CAT and ascorbate-glutathione cyclase confirm their correlation to ROS clearance[24]. In addition, the expression levels of the key genes encoding osmotic adjustment substance synthases are also induced by drought stress[25]. In the present work, using a transcriptome analysis, we obtained 52,954 unigenes, which included many key DEGs involved in the responses to oxidative and osmotic stresses under drought conditions. The FPKMs of the defense-related genes were basically consistent with the enzyme activities or osmotic substance contents at the T3 and R1 stages, respectively. The result is also supported by previous studies[24]. Additionally, the defense-related pathways, including ko04146 (peroxisome), ko01230 and ko00330 (the AA biosynthesis and metabolism), ko00500 (starch and sucrose metabolism) and ko04141 (protein processing) were enriched under drought stress conditions. Some new DEGs were expressed after recovery compared with during salt stress. Thus, different genes may be involved in the responses to drought stress and rehydration, and they might be involved in specific metabolic pathways and mechanisms.

V. CONCLUSIONS

In conclusion, the levels of antioxidant enzymes and osmotic adjustment substances in the two cultivars were generally enhanced under drought-stress conditions, forming a defense network to protect *Auricularia* against drought-related damage. Resistant CSLZ had strong abilities to maintain higher enzyme activity levels and synthesize more osmoregulation substances, which are closely related to drought resistance, than sensitive HSQ. The physiological and antioxidase responses correlated with the repressed levels of related genes as assessed by a transcriptome analysis. These findings provide new insights into drought-resistance mechanisms and useful information for the cultivation and breeding of drought-resistant *Auricularia*.

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