Introduction

Drought is a major factor limiting plant growth and crop productivity [1], and is expected to increase by the end of the 21st century due to projected temperature rise [2]. Drought stress ranges from mild to severe can accelerate leaf senescence and induce osmotic stress [3], eventually lead to death. In fact, even short-term drought stress can lead to significant annual losses in crop yields and hinder sustainable agriculture [4].

Drought stress induces the production of reactive oxygen species (ROS) [5], which causes cellular oxidative damage [6]. Plants have evolved complex ROS scavenging mechanisms, including non-enzyme (such as ascorbic acid (ASC), glutathione (GSH) and flavonoids) and enzyme systems (superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), peroxidase (POD) and glutathione reductase (GR)) [7]. Specifically, the ascorbic acid-glutathione (AsA-GSH) cycle is considered to be the most critical participant...
in the antioxidant metabolic system that scavenges abnormal ROS [8]. Proline (Pro) as a protective agent plays a key role in protecting cell redox potential and scavenging free radicals under stress [9]. Drought resistance strategies include the use of plant biological stimuli or plant hormones to improve the adaptability of plants against environmental stress [10]. The effectiveness of these drought resistance methods can be examined from morphological and physiological characteristics, including the ability to maintain canopy, root growth and Pro accumulation [11].

In addition to using plant biological stimuli or plant hormones, beneficial microorganisms in the soil, such as mycorrhizal fungus and endophytes, are promising ways to promote plant growth under water deficit conditions [12, 13]. Endophytic fungal-plant symbiosis can increase water stress and salt stress tolerance [14-16]. Presently, there is still an active discussion regarding the function of endophytic fungus is suitable for sustainable resources as natural antioxidants [17-19].

Rice is one of the most important staple foods for more than half of the world’s population [20]. However, drought stress largely limits rice productivity [21]. Although plant drought tolerance has been profusely addressed in the literature, the application of endophytic fungus has rarely been attempted under PEG stress. The present study aimed to elucidate the mechanisms underlying increased tolerance to drought stress in rice conferred by endophyte EF0801 through regulating AsA-GSH cycle. It also provides new ideas and a basis for the study of plant drought resistance.

Materials and Methods

Cultivation and Treatment of Materials

Endophytic fungus EF0801 is congeneric to Sordariomycetes sp, with 99% similarity [16], was isolated from the leaves of Suaeda salsa in saline-alkali land in China, and its PEG tolerance was screened [13]. EF0801 was transferred into a sterilized conical flask containing 125 mL potato dextrose broth medium and cultured in a constant temperature oscillator at 24±1°C and 125 rpm for 10 d. The fermentation broth was used to infect rice seedlings.

The rice seeds were sterilized in 1% sodium hypochlorite solution for 20 min, rinsed and germinated. Following transferring seeds (100 seeds) on a plastic beaker (700 mL) containing sterilized Hoagland nutrient solution and cultured in the artificial climate chamber (16 h/8 h light/dark, light intensity 10000 lux, 28°C/26°C day/night, and relative humidity 80%). Four days seedlings were divided into E+ group (inoculated with 5% fermentation broth) and E- group (non-inoculated). Seedlings of each group were cultured in the nutrient solution containing 0, 5, 10, 15 or 20% PEG. The endophytic fungus was colonized in rice roots, and the inoculation degree was determined by Liu and Chen [22]. EF0801 colonized more than 90% in E+ group seedlings, while no colonized in E- group seedlings. Each group was supplemented Hoagland solution every day. Each treatment was repeated three times. Physiological and biochemical indexes of seedlings were measured after 6 days of treatment.

Determination of Growth Indexes and Biomass

Plant height, root length and fresh weight (FW) of ten seedlings were measured, and they were oven-dried at 100°C until reached a constant weight and dry weight (DW) was determined.

Determination of Proline Content

Pro content was determined according to the method of Zhang and Qu [23]. 0.5 g fresh leaves were homogenized in 5 mL 5-sulfosalicylic acid (3%) and heated at 100°C for 10 min. After centrifugation at 3000 ×g for 15 min, 2 mL supernatant was added with 2 mL acetic acid, 4 mL acidic ninhydrin and 2 mL distilled water, boiled for 1 h. Then 4 mL toluene was added after the ice bath cooling for 30 s. After separating red toluene by oscillation, the absorbance of the colored solutions was measured at 520 nm.

Determination of Oxidative Status and Electrical Conductivity

MDA content was determined by following the method of Heath and Packer [24]. Fresh leaves (0.5 g) were homogenized in the ice bath with 4 mL 0.1% trichloroacetic acid (TCA). 1 mL supernatant was added to 4 mL of 0.5% thiobarbituric acid (TBA) in 20% TCA. The mixture was incubated at 100°C for 30 min and immediately cooled to stop the reaction. Finally, the mixture was determined by the spectrophotometer at 532 nm.

The determination of H_{2}O_{2} content was according to Gao [25]. Fresh leaves (0.5 g) were homogenized in precooled acetone (3 mL) at 4°C and centrifuged at 12000 ×g at 4°C for 15 min. After 1 mL supernatant was added with 0.1 mL sulfuric acid (5%) and 0.2 mL concentrated ammonia water and centrifuged at 4000 ×g for 10 min. The precipitate was washed three times with acetone, and then dissolved with 4 mL sulfuric acid (2 M), then dissolved with 4 mL sulfuric acid (2 M). The mixture was determined by the spectrophotometer at 520 nm.

The determination of O_{2}^{-} content was according to the method of Wang and Luo [26], with slight modifications. Fresh leaves (0.5 g) were homogenized in 4°C pre-cooling phosphate buffer (5 mL, pH 7.8) and centrifuged at 4°C (12000 ×g) for 15 min. 1 mL supernatant was added with 10 mM hydrochloric acid amine (1 mL) and incubated at 30°C for 1 h. Following, 17 mM p-aminobenzenesulfonic acid (1 mL) and 7 mM α-lactam (1 mL) were added and the mixed liquor was
incubated at 30°C for 20 min. The change of absorbance at 530 nm by spectrophotometer.

Electric conductivity (EC) was determined by referring to the method of Dionisio-Sese and Tobita [27]. Fresh leaves (0.1 g) were immersed in deionized water at 30°C for 2 h, the initial conductivity (EC1) was measured using a conductivity meter. The tubes were incubated at 121°C for 20 min and the final EC (EC2) was measured: EC = EC2-EC1.

Determination of Antioxidant Enzyme Activities

Fresh leaves (0.3 g) were homogenized in 3 mL 50 mM phosphate buffer solution containing 0.1 mM EDTA, 4% (w/v) polyvinylpyrrolidone (PvP-40) and TritonX-100 (pH 7.5) in ice bath, then centrifuged at 10000×g (4°C, 30 min). The supernatant was used to analyze the activities of GR and APX.

GR activity was measured according to Ma and cheng [28]. The supernatant (0.1 mL) was added into 3 mL reaction solution (0.1 mM Tris-HCl (pH 8.0), 0.2 mM NADPH, 1 mM glutathione oxide (GSSG)). The changes in absorbance of the mixture were measured at 340 nm. 1 unit GR activity (U) is 1 μmol NADPH oxidized per minute.

APX activity was determined using the methods of Nakano and Asada [29]. The supernatant (0.05 mL) was added to 3 mL reaction solution (containing 50 mM Hepes-NaOH, 1.0 mM H2O2, and 0.5 mM ASC), and the changes in absorbance of the mixture were measured at 290 nm. 1 unit APX activity (U) is 1 μmol ASA oxidized per minute.

Determination of Antioxidant Content

Fresh leaves (0.4 g) were homogenized in a pre-cooling bowl with 4 mL 5% (w/v) TCA and quartz sand, and then centrifuged at 10000×g for 20 min at 4°C, the supernatant was collected for the analysis of TASC, ASC, TGSH and GSH.

TASC content and ASC content were determined as described by Lu [30] and Wu [31].

The supernatant (1 mL) was added to 0.25 mL 0.1 M phosphate buffer (pH 7.7) and 0.25 mL 2 mM dithiothreitol, respectively. After reaction at room temperature for 10 min, 0.4 mL TCA solution (10%), 0.4 mL phosphoric acid (44%), 0.4 mL 2,2-bipyridine (4%) and 0.2 mL FeCl3 (3%) were added, fully shaken, and then kept at 37°C for 1 h. The absorbance was measured at 525 nm to determine the content of TASC.

The supernatant (1 mL) was mixed with 0.5 mL phosphate buffer (0.1 M, pH 7.7). After 30 s, the operation was performed according to the above TASC content method to determine the content of ASC.

TGSH and GSH content was determined as described by Ellman [32].

TGSH content was determined. The supernatant (1 mL) was mixed with 0.5 mL phosphate buffer (0.1 M, pH 7.7), and then 1 mL NADPH (0.15 mM) and 1 U GR were added. After the reaction at room temperature for 2 min, 0.6 mL DTNB (0.6 mM) was added immediately, and then the mixture was kept in water bath at 30°C for 5 min. The absorbance was measured at 412 nm.

GSH content was determined. The supernatant (1 mL) was mixed with 2.5 mL phosphate buffer (0.1 mM, pH 7.7), and then 0.6 mL DTNB (0.6 mM) was added immediately. The mixture was kept in water bath at 30°C for 5 min. Following, the absorbance was measured at 412 nm.

Statistical Analysis

All data were analyzed by SPSS 25.0 software package. Significantly difference between treatments was conducted using LSD multiple comparison (p<0.05). Two-factor analysis of variance (ANOVE) was used to analyze the difference between the experimental treatments.

Results

Growth and Biomass in Rice Seedlings

With the increasing PEG concentrations, plant height and root length were significantly reduced in both of E- group and E+ group (Fig. 1a, b, c). Plant height of E+ group was significantly higher than that of E- group, however, root length of E+ group was shorter than that of E- group at 0, 5 and 10% PEG concentrations.

The aboveground dry weight of E- group and E+ group significantly decreased with the increasing PEG concentrations (Fig. 1d). However, the aboveground dry weight of E+ group was higher than that of E- group under 5% and 15% PEG stress. The underground dry weight of E- group and E+ group first increased and then decreased with the increasing PEG concentrations, and no significant difference was observed between E- group and E+ group (Fig. 1e).

Oxidative Stress Indicators in Rice Leaves

With the increasing PEG concentrations, EC significantly increased in E- group, but first increased and then decreased in E+ group (Fig. 2a). Under 15% and 20% PEG stress, EC of E+ group was significantly lower than that of E- group. Correspondingly, MDA content of E- group and E+ group increased with the increasing PEG concentrations (Fig. 2b). There was a significant difference between E+ group and E- group under PEG stress.

H2O2 and O2- are ROS produced by cellular metabolism under stress. H2O2 and O2- content in E- group firstly decreased and then increased with the increasing PEG concentrations, but those of E+ group (except for 20% PEG) showed no significant change.
Nevertheless, \( H_2O_2 \) content under 0, 5 and 10% PEG treatment and \( O_2^- \) content under 0%, 15 and 20% PEG treatment of E+ group were significantly lower than that of E- group.

Proline content in E+ group significantly increased with the increasing PEG concentrations, while there was no significant change in E- group (Fig. 2e). Pro content of E+ group was significantly higher than that of E- group under PEG stress.

Antioxidant Capacity of Rice Leaves

GR activity of E- group decreased with the increasing PEG concentrations, but there was no significant difference, while that of E+ firstly increased and then decreased (Fig. 3a). Under 5% and 10% PEG stress, GR activity of E+ group was significantly higher than that of E- group. APX activity of E- group and E+ group was significantly decreased with the increasing PEG concentrations (Fig. 3b), but there was no significant difference between the E+ group and the E- group under PEG stress.

In E- group, TASC content firstly decreased and then unchanged with the increasing PEG concentrations, but ASC content significantly decreased (Fig. 3c, d). In E+ group, TASC content gradually increased, however, ASC content firstly increased and then decreased. TASC content under 5-20% PEG stress and ASC content under 5% and 10% PEG stress of E+ group were significantly higher than those of E- group.

TGSH and GSH content of E- group and E+ group significantly decreased with the increasing PEG concentrations (Fig. 3e, f). Under PEG stress, TGSH and GSH content of E+ group was significantly higher than those of E- group.

GR and APX activities as well as TASC, ASC, TGSH and GSH contents of E+ group were higher than those of E- group (Fig. 3g). In each treatment, GSH content of rice seedlings was the lowest but TASC content was the highest.

Discussion

Drought stress adversely affects the physiological and biochemical processes of plants and ultimately reduces crop yield [33]. Mild drought improved root growth [34], our result showed that underground dry weight increased in rice seedlings treated with inoculated and uninoculated endophytic fungus in both of E- group and E+ group under 5% PEG concentration. Shoots and roots became shorter at other PEG concentrations, suggesting that drought stress inhibited the shoot and root growth [35]. Similar results were found in this study. The endophytic fungus can benefit host plants by evolutionary adaptation...
Fig. 2. Effects of endophyte on EC a), MDA content b), \( \text{H}_2\text{O}_2 \) content c), \( \text{O}_2^- \) content d) and Pro content e) of rice seedlings exposed to PEG stress for 6 days. The data are means ± SD of three replicates. Different letters show significant differences with \( P<0.05 \).

Fig. 3. Effects of endophyte on GR activity a), APX activity b), TASC content c), ASC content d), TGSH content e) and GSH content f) and heatmap g) of rice seedlings exposed to PEG stress for 6 days. The data are means ± SD of three replicates. Different letters show significant differences with \( P<0.05 \).
and actively regulate the growth and development of the host [36]. Many studies have shown endophytic fungus can improve plant drought resistance and promote plant growth [37, 38]. In the present study, we further demonstrated that endophytic infection increased plant height and aboveground dry weight. The increases in growth properties and biomass accumulation may be attributed to improved carbon assimilation due to enhanced photosynthetic capacity of E+ group. Remarkably, shorter roots were observed in E+ group than E- group under drought stress. Extracorporeal hyphae of endorhizal fungus can increase the absorption range of plant roots, improve the expression of the aquaporin gene in the plasma membrane of host plants and enhance the tolerance of plants to drought [39]. Hence, we suggested that endophytic fungal infection may actively regulate the growth and development of the host under drought stress, so further growth of roots was not required.

Drought stress can cause oxidative damage in plants due to excessive accumulation of ROS [40], which result in increases of EC in grapes [41] and MDA in rice [42]. The findings of this study are consistent with the above findings. Interestingly, we found that EC and MDA, H2O2, as well as O2− content of E+ group were lower than those of E- group. Amjad et al. [43] also showed that EC and the contents of MDA and H2O2 in endophyte-infected wheat under drought stress were lower than those in endophyte-uninfected. Hence, these results suggested that endophytic fungus can mitigate drought stress-induced oxidative damage. Pro is a protective agent which can eliminate ROS in vivo, protect membrane structure from oxidative damage and enhance the hydration between proteins to improve plant drought resistance [44]. Sun et al. [45] showed that endophyte increase the expression level of Pro synthesis-related genes and reduce the transcription level of Pro metabolism-related genes, thus increase Pro content of rice seedlings under salt stress. The present study also found that endophyte increased Pro content under PEG stress.

Plants can increase antioxidant contents to reduce ROS [33], such as ASC and GSH, also increase endogenous enzyme activities to resist oxidative stress, such as APX and GR [46, 47], but some other studies found stress reduced the APX activity of cassava [48] and the expression level of GR gene in wheat [49]. The present study found that the contents of TASC, ASC, TGSH and GSH in E+ group decreased with the increasing PEG concentrations. Endophytic fungus can increase the contents of GSH and ASC and GR activity in rice under Cd stress [50] and regulate AsA-GSH cycle to alleviate oxidative stress in citrus under drought stress [51]. This result is also confirmed by our research showing higher activities of GR and APX and the contents of TASC, ASC, TGSH and GSH in E+ group compared with E- group under PEG stress. Endophytes alleviate the negative effects of stress on their hosts by stimulating and expressing stress response genes, transcription factors, and producing antioxidants and antioxidant enzymes, etc [52]. Therefore, it is necessary to further study the molecular mechanisms and their utilization of endophytes in the mitigation of various environmental stresses on plant.

**Conclusions**

Drought stress increased EC and MDA content, resulting in plasma membrane damage and inhibiting plant growth. The endophytic fungus used in this study can inhibit oxidative stress by increasing the contents of antioxidants and the activities of antioxidant enzymes. Proline as an osmotic regulator and membrane protector improved plant resistance. These findings indicated that endophytic fungus could be operated to improve drought resistance of rice seedlings in an ecologically friendly manner.

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**Conflict of Interest**

The authors have declared that no conflict of interest exists.

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