



# Effects of *Tsukamurella tyrosinosolvens* P9 on growth, physiology and antioxidant enzyme of peanut under drought stress and after re-watering

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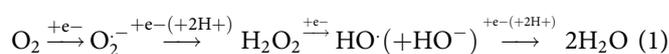
**Key words:** PGPR, Rare actinomycetes, Drought resistance, Biofertilizer

**Abstract: Background:** The plant-growth-promoting rhizobacterium *Tsukamurella tyrosinosolvens* is a rare strain of actinomycete, in order to recognize and expand the ecological functions of rare actinomycetes. **Methods:** In this experiment, we studied the effect of *Tsukamurella tyrosinosolvens* P9 on the drought resistance of peanut by inoculating peanut seedlings in pots and measuring the growth and physiological indicators of peanut under drought stress and re-watering conditions. **Results:** The results showed that during drought stress, the relative water content of the soil and leaves, chlorophyll content, and stomatal length, width, and aperture were significantly decreased while the levels of malondialdehyde (MDA), H<sub>2</sub>O<sub>2</sub> and stomatal density were significantly increased. Peanut growth was also inhibited. However, inoculation with the P9 strain significantly promoted the growth of peanut under drought stress as plant height, fresh weight, root length and root weight were significantly higher compared with the uninoculated drought stress group. In addition, in P9-inoculated plants, the water and chlorophyll contents were significantly higher and the activities of the antioxidant enzymes CAT and SOD were significantly increased (except during the six days of drought treatment). While the stomatal length, width, and aperture were improved, the levels of MDA and H<sub>2</sub>O<sub>2</sub> were significantly decreased. NBT staining showed that inoculation with P9 reduced O<sub>2</sub><sup>2-</sup> accumulation under stress. After re-watering, the physiological indexes of inoculated plants recovered more quickly and grew better. **Conclusions:** The results showed that *T. tyrosinosolvens* P9 enhanced drought resistance and improves peanut growth by increasing leaf water content, increasing photosynthesis, regulating stomatal closure, and improving antioxidant enzyme activity.

## Introduction

Peanut (*Arachis hypogaeae* L.) is a valuable food and oil crop, which is widely cultivated due to its high oil content and adaptability (Mingrou *et al.*, 2022). By 2019, the planting area of peanut in China was  $4.51 \times 10^6$  hectares, accounting for about 50% of oil crops (Wang *et al.*, 2022a, 2022b). Peanut mainly grows in arid and semi-arid areas; therefore, drought is an important environmental factor limiting its productivity, with annual production losses of up to 50% (Ding *et al.*, 2022; Li *et al.*, 2022a). Under drought stress, plants produce many reactive oxygen species (ROS), such as superoxide anion free radicals (O<sub>2</sub><sup>-</sup>), hydrogen peroxide

(H<sub>2</sub>O<sub>2</sub>), hydroxyl radicals (HO·), and singlet oxygen (<sup>1</sup>O<sub>2</sub>), which can react with proteins, lipids, and deoxyribonucleic acids (reaction (1)) (Collin, 2019). These interactions cause physiological disorders that inhibit plant



growth, such as electrolyte leakage and lipid peroxidation, and may even lead to plant death in severe drought conditions (Kaushal and Wani, 2016). Plant growth-promoting rhizobacteria (PGPR) have attracted attention here because they can improve plant drought tolerance; thus, screening and applying PGPRs may help improve dryland production.

PGPRs are a group of microorganisms that colonize the rhizospheres of plants, promoting plant growth and enhancing stress tolerance (Khan and Singh, 2021). PGPRs can enhance plant nutrient absorption, induce osmosis and cause antioxidant accumulation through the production of

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Received: 01 November 2022; Accepted: 31 January 2023;  
Published: 22 May 2023



plant hormones, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, and volatile compounds. They can cause changes in root morphology to promote plant hormone balance and restore plant growth and development under drought conditions (Vurukonda *et al.*, 2016; Kour *et al.*, 2019). For example, *Paenibacillus* sp., *Azospirillum* sp., *Rhizobium* sp., *Bacillus* sp., *Azotobacter* sp., *Klebsiella* sp., *Pseudomonas* sp., and *Serratia* sp. activated the antioxidant defense system and reduce oxidative damage, while promoting plant growth under stress (Abdelaal *et al.*, 2021). *Enterobacter* sp. and *Chryseobacterium* sp. could reduce the negative effect of ethylene on the growth and development of mung bean under stress by producing ACC deaminase to degrade the ethylene precursor ACC (Tittabutr *et al.*, 2013). Siderophores secreted by *Bacillus* sp. could improve the iron uptake of potato, which improved photosynthetic performance and tolerance to abiotic stress (Gururani *et al.*, 2013). In addition, *Bacillus*, *Pseudomonas*, *Enterobacter*, and *Moraxella* species stimulated cell growth and differentiation by synthesizing indoleacetic acid (IAA) to promote the development of lateral roots, branches, and tillers in wheat, which improved stress resistance and yield (Raheem *et al.*, 2018). Inoculation with PGPRs is helpful for improving plant drought resistance. However, few studies on PGPRs focus on actinomycetes (Bouskill *et al.*, 2013) that led us to explore the same.

The rare actinomycete *Tsukamurella tyrosinosolvens* P9 was isolated in our laboratory from the soil surrounding the rhizospheres of tea plants. We had reported for the first time that the strain possesses multiple growth-promoting properties, such as phosphorus solubilization and production of IAA, siderophores, and ACC deaminase. Moreover, it could significantly promote the growth of peanut seedlings, and alleviate salt stress (Zhang *et al.*, 2021; Xu *et al.*, 2022). However, whether P9 influences the growth of peanut under drought stress is unknown. In this study, we dynamically traced the effects of P9 inoculation on the growth and drought resistance of peanut seedlings under drought stress and after re-watering to analyze and elucidate the mechanism by which P9 improves the drought resistance of peanut. This lays a foundation for understanding and expanding the ecological function of rare actinomycetes and enhancing peanut drought resistance.

## Materials and Methods

### Experimental materials

The *Tsukamurella tyrosinosolvens* P9 strain was isolated in our laboratory and stored in the China Center for Type Culture Collection (Strain preservation number: CCTCC AA 2020052).

The peanut variety is the Silihong peanut, which is an early maturing local variety with a dark red seed coat.

The experimental soil was yellow soil and was collected in Yangniu Village (E106°39'11", N26°26'57"), Guizhou Province, China. Subsequent to sieving and sterilizing three times at 121°C for one hour, it was used in pot experiments.

### Preparation of P9 bacterial suspension and cultivation of peanut seedlings

LB liquid medium was inoculated with an appropriate amount of P9 strain (1%) and shaken at 150 rpm overnight at 30°C. The next day, the cultures were centrifuged at  $5000 \times g$  for 10 min, the supernatant was discarded, and the bacteria were rinsed twice with sterile water. The bacteria were collected and suspended in sterile water to prepare a  $10^8$  CFU/mL P9 suspension.

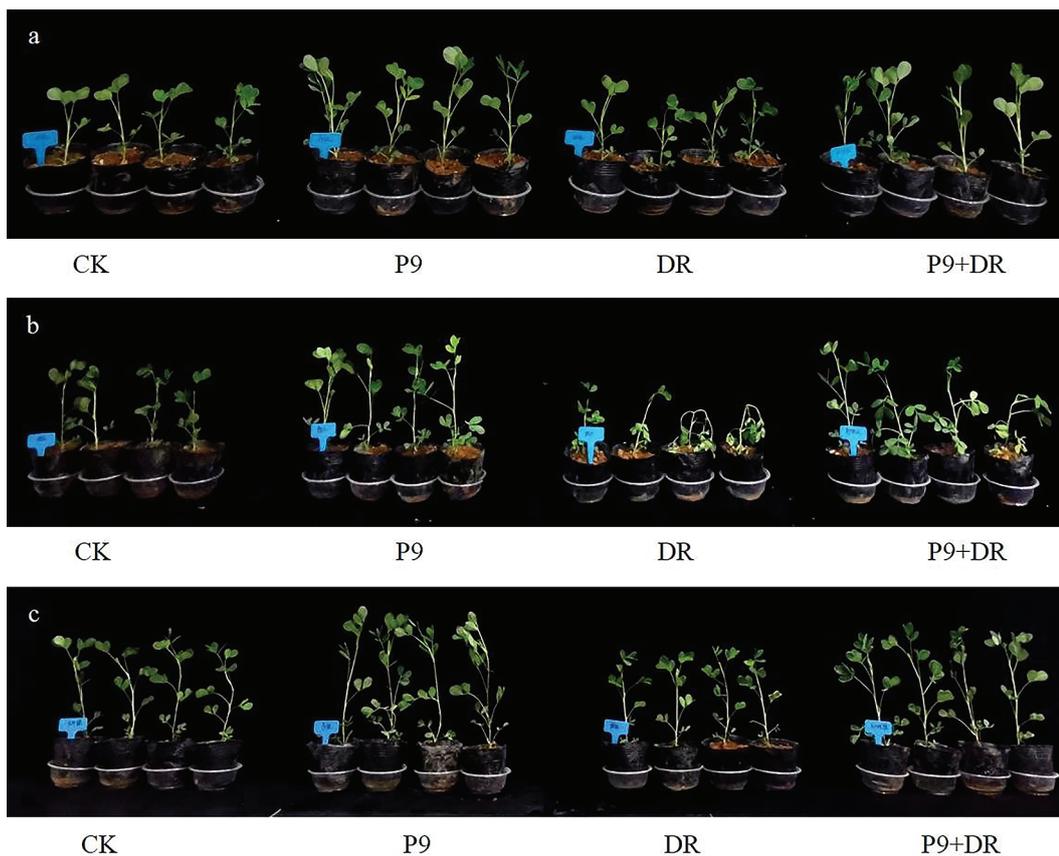
Peanut seeds were sterilized with 20% H<sub>2</sub>O<sub>2</sub> solution for 20 min and rinsed repeatedly with sterile water. After soaking in sterile water for 12 h, the seeds were placed in a Petri dish with a double layer of sterile moist filter paper and germinated in a light incubator (16/8 h photo-period) at 28°C for about 3 days. Germinated peanut seedlings were selected and planted in plastic pots containing 600 g sterile soil for routine management.

### Experimental design and treatments

The experiment setup was a pot experiment. Samples were divided into the control group (CK), inoculation group (P9), drought-stress group (DR), and inoculation+drought-stress group (P9+DR), with 24 replicates for each treatment. The inoculation treatment was performed when the first true leaves of peanut seedlings were fully expanded. P9 and P9+DR groups were irrigated with bacterial suspension (20 mL) every 3 days, whereas CK and DR groups were irrigated with the same volume of sterile water. After 30 days of irrigation, DR and P9+DR groups were subjected to drought stress without irrigation, and watering was resumed after 12 days of drought. CK and P9 groups continued to be watered or inoculated every 3 days. From the beginning of the drought treatment until 7 days after re-watering, we picked randomly 4 plants to measure the following indexes every 3 days, including the growth and physiological indexes, antioxidant enzyme activities, and the stomatal characteristics of leaves.

### Determination of plant growth indexes, physiological indexes and antioxidant enzyme activities

Peanut seedlings were collected, washed with sterile water, and dried with filter paper. Growth indicators, such as plant height, fresh weight, and root length and weight were measured. Physiological indexes assayed included chlorophyll content, proline content, malondialdehyde (MDA) content, leaf relative water content, soil relative water content, H<sub>2</sub>O<sub>2</sub> content, and ROS levels. The chlorophyll content was determined by the ethanol extraction colorimetric method (Wang *et al.*, 2009). The proline content was determined by the acid hydrate ninhydrin colorimetric method (Wang *et al.*, 2007). The MDA content was determined by the thiobarbituric acid colorimetric method (Song *et al.*, 2011). The relative water content of leaves was determined by the saturated water content method (Cohen *et al.*, 2009), and the relative water content of the soil was determined by the gravimetry method (Dobriyal *et al.*, 2012). ROS analysis was performed by nitro-blue tetrazolium (NBT) staining (Alexander *et al.*,



**FIGURE 1.** Effects of *Tsukamurella tyrosinosolvens* P9 inoculation on the growth of peanut under drought stress and after re-watering. (a) Drought stress 0 d; (b) Drought stress 12 d; (c) Re-watering 7 d. From left to right is CK (control peanut), P9 (P9-inoculated peanut), DR (drought-treated peanut), and P9+DR (P9-inoculated peanut under drought treatment), respectively. Each group had four replicates.

2020; Gowtham *et al.*, 2020). Antioxidant enzyme activities, including superoxide dismutase (SOD) activity, peroxidase (POD) activity, and catalase (CAT) activity, and the  $H_2O_2$  content were measured using relevant kits obtained from Comin Biotechnology Co., Ltd. (Suzhou, China). The determination of SOD activity was based on the NBT colorimetric method (García-Triana *et al.*, 2010), and POD activity was measured by the guaiacol colorimetric method (Markkola *et al.*, 2002). CAT activity was determined by the UV absorption method, and the  $H_2O_2$  content was measured by the titanium sulfate colorimetric method (Sima *et al.*, 2011).

#### Analysis of stomatal characteristics of peanut leaves

Peanut leaves were fixed and decolorized in anhydrous ethanol; the chlorophyll was then removed and transferred to a mixture of 95% ethanol and NaClO (6%–14%) in a 1:1 (v/v) ratio. After the leaves became transparent, they were transferred to clean water, placed on a glass slide with a few drops of distilled water, and covered with a coverslip (Sultana *et al.*, 2021). They were then observed and photographed using a BX53 microscope (Olympus Corp., Tokyo, Japan). Five random photographs were taken under a 40 objective to measure stomatal density (mean number of stomata/field-of-view area), length (length of guard cells), width (the width of guard cells), and aperture (width of the stomatal aperture) using cellSens Standard software (Olympus Corp.).

#### Statistical analysis

Data were analyzed using IBM SPSS Statistics 20.0. Duncan's method was used for the comparison of mean values. Excel was used for data processing and mapping. The least significant difference (LSD) test was used for multiple comparisons ( $p < 0.05$ ). Data were represented as means  $\pm$  standard errors.

#### Results

##### Effects of P9 inoculation on the growth of peanut under drought stress and after re-watering

The effects of *T. tyrosinosolvens* P9 strain inoculation on the growth of peanut seedlings under normal conditions, drought stress, and after re-watering were analyzed (Fig. 1; Table 1). Under normal water conditions, plant height and the fresh weight of inoculated peanuts were significantly higher than the controls ( $p < 0.05$ ). The root length and weight were also higher than the controls to different extents. Under drought stress, peanut growth was inhibited during extended drought. On day 12 of the drought condition, the plants showed obvious wilting due to severe water shortage; however, the growth indexes of P9+DR plants were significantly better than those of the DR group ( $p < 0.05$ ). Compared with DR treatment, the heights of P9+DR plants were 23.06%–59.97% greater, fresh weight increased by 26.38%–47.13%, root length improved by

TABLE 1

Effect of *Tsukamurella tyrosinosolvens* P9 inoculation on growth indexes of peanut under drought stress and after re-watering

Growth indexes	Treatment	Drought 0 d	Drought 3 d	Drought 6 d	Drought 9 d	Drought 12 d	Re-watering 7 d
Plant height (cm)	CK	10.44±0.21b	12.28±0.46c	14.75±0.53c	17.86±0.43b	20.35±0.04c	22.45±0.07b
	P9	13.40±0.30a	15.53±0.70ab	16.12±0.24b	21.96±0.65a	24.53±0.05a	26.67±0.17a
	DR	10.32±0.26b	13.62±0.82bc	15.00±0.34bc	16.87±0.16b	13.59±0.78d	17.16±0.21c
	P9+DR	13.48±0.27a	17.01±0.56a	19.05±0.38a	20.76±0.38a	21.74±0.30b	22.52±0.11b
Fresh weight (g)	CK	2.66±0.02b	3.43±0.07c	3.98±0.21bc	4.20±0.56ab	4.71±0.10b	5.00±0.06c
	P9	3.88±0.14a	4.82±0.22a	4.92±0.21a	5.08±0.43a	5.38±0.06a	6.18±0.03a
	DR	2.61±0.02b	3.26±0.09c	3.35±0.19c	3.56±0.16b	2.33±0.13d	3.67±0.09d
	P9+DR	3.84±0.05a	4.12±0.20b	4.59±0.35ab	4.86±0.05a	3.40±0.02c	5.40±0.05b
Root length (cm)	CK	8.19±0.67b	18.03±0.65c	18.34±0.33c	20.63±0.93c	20.71±0.61b	24.23±1.34c
	P9	10.37±0.26a	21.39±0.77b	22.43±0.96b	22.89±0.74ab	24.42±0.09a	31.82±0.42a
	DR	7.822±0.14b	20.79±.67b	22.07±0.43bc	21.29±0.61b	21.25±0.54b	27.46±0.97b
	P9+DR	9.18±0.41ab	29.03±0.51a	30.17±2.04a	25.10±0.78a	24.98±0.70a	30.26±0.62ab
Root weight (g)	CK	0.57±0.13b	0.64±0.00c	0.68±0.08b	0.70±0.10bc	0.81±0.07b	0.91±0.09c
	P9	1.01±0.08a	1.19±0.03a	1.22±0.08a	1.23±0.08a	1.27±0.02a	1.63±0.02a
	DR	0.60±0.03b	0.79±0.06b	0.81±0.03b	0.61±0.04c	0.55±0.02c	0.99±0.02c
	P9+DR	0.70±0.03b	1.14±0.06a	1.27±0.11a	0.91±0.06b	0.89±0.07b	1.28±0.13b

Note: Data represent means ± standard errors. For each sample, different lowercase letters in the same column represent significant differences between treatments at the same time points ( $p < 0.05$ ).

17.39%–39.63%, and root weight increased by 16.67%–61.82%. These observations suggest that P9 inoculation can promote nutritional growth and root development in peanut under drought stress. After re-watering, the growth status of the DR group peanut plants gradually recovered, while the recovery efficiency of P9+DR plants was better, and recovery occurred more quickly. Further, the plant height, fresh weight, and root weight of P9+DR plants were significantly greater than those of DR plants ( $p < 0.05$ ).

#### Effect of P9 inoculation on physiological indexes of peanut under drought stress and after re-watering

The physiological indexes of the plants were analyzed after different treatments (Figs. 2 and 3). Under normal water

conditions, P9 inoculation had no significant effect on the soil relative water and MDA contents of peanut seedlings; but, leaf relative water, chlorophyll, and proline contents were significantly higher than those of CK plants. The  $H_2O_2$  content was significantly lower than in CK plants ( $p < 0.05$ ). Under drought stress, while the soil water content decreased sharply, leaf water and chlorophyll contents decreased successively, and the contents of proline, MDA, and  $H_2O_2$  increased sharply with the extension of drought stress. The changes in the physiological indexes of inoculated plants showed different characteristics: the soil water, leaf water, and chlorophyll contents of the P9+DR group were significantly higher than those of the DR group while the proline, MDA, and  $H_2O_2$  contents were significantly lower

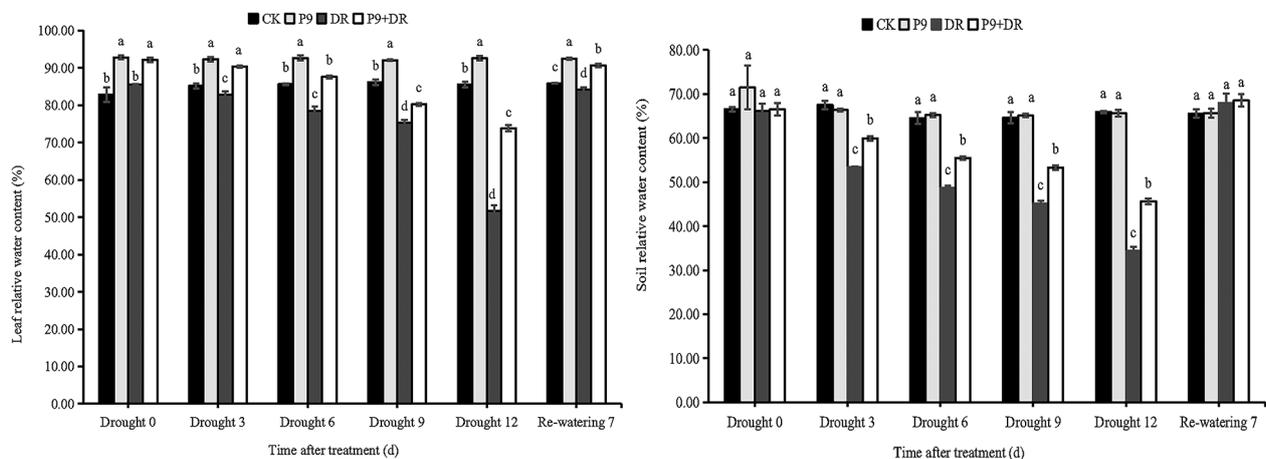
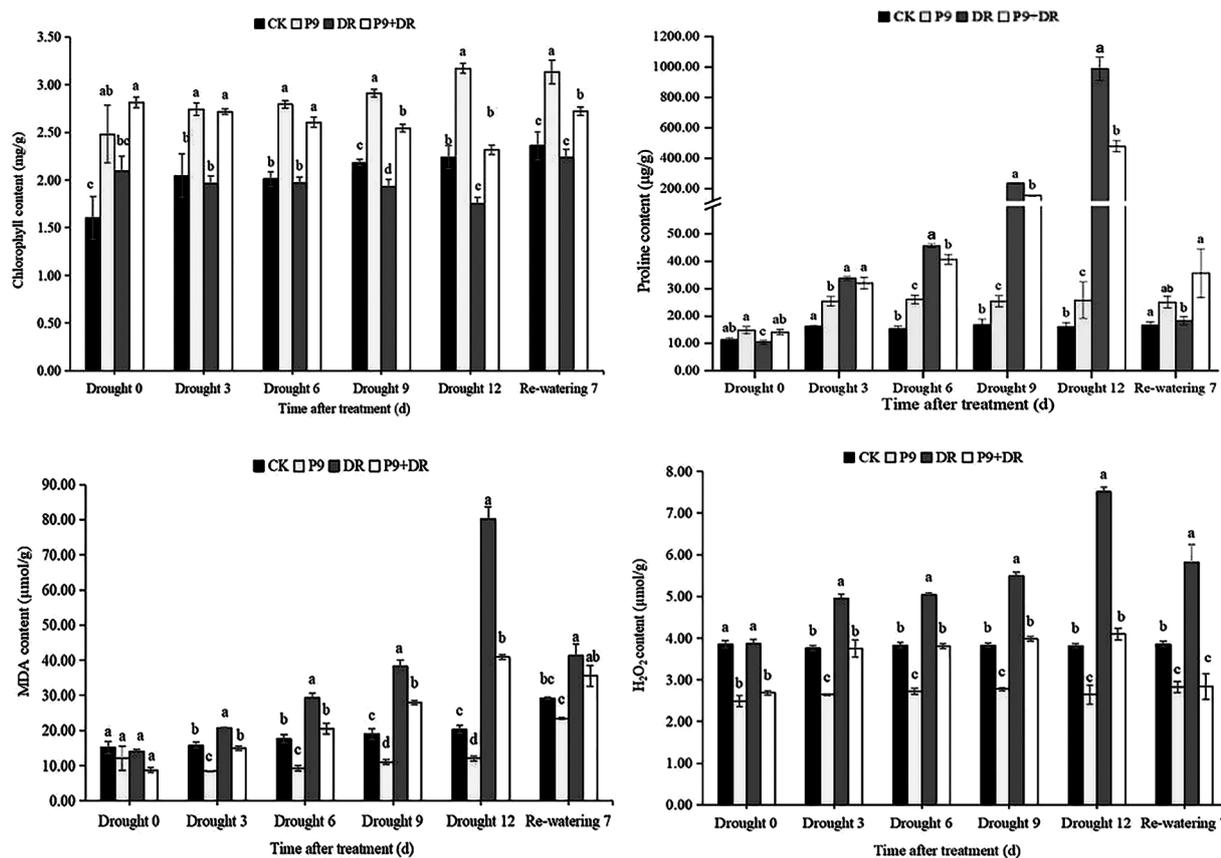


FIGURE 2. Effect of *Tsukamurella tyrosinosolvens* P9 inoculation on the relative water content of peanut leaves and soil under drought stress and after re-watering. For each sample, different lowercase letters after the numbers in the same column represent significant differences between treatments at the same time points ( $p < 0.05$ ). Values are given as means ± S.E.



**FIGURE 3.** Effect of *Tsukamurella tyrosinosolvens* P9 inoculation on physiological indexes of peanut under drought stress and after re-watering. For each sample, different lowercase letters after the numbers in the same column represent significant differences between treatments at the same time points ( $p < 0.05$ ). Values are given as means  $\pm$  S.E.

than those of the DR group ( $p < 0.05$ ) However, the proline content was not significantly different from that of the DR group after 3 days of drought. To elaborate, during 0–12 days of drought, compared with the DR group, the soil water content of the P9+DR group increased from 0.48%–32.77%, the leaf water content increased by 6.56%–42.97%, the chlorophyll content increased by 31.61%–38.78%. However, the MDA content decreased by 26.83%–49.03%, and the  $H_2O_2$  content decreased by 24.24%–45.41%. It showed that P9 inoculation reduced the loss of water from plants and soil under stress, and alleviated the damage to seedlings induced by drought stress. After 7 days of re-watering, the soil water content returned to normal, leaf water and chlorophyll contents increased while the contents of proline, MDA, and  $H_2O_2$  decreased significantly. However, the leaf water content, chlorophyll content, and proline content of P9+DR plants were higher, while MDA and  $H_2O_2$  contents were lower than the DR group. Therefore, inoculation with P9 could reverse the effects of drought stress on peanut more quickly after re-watering.

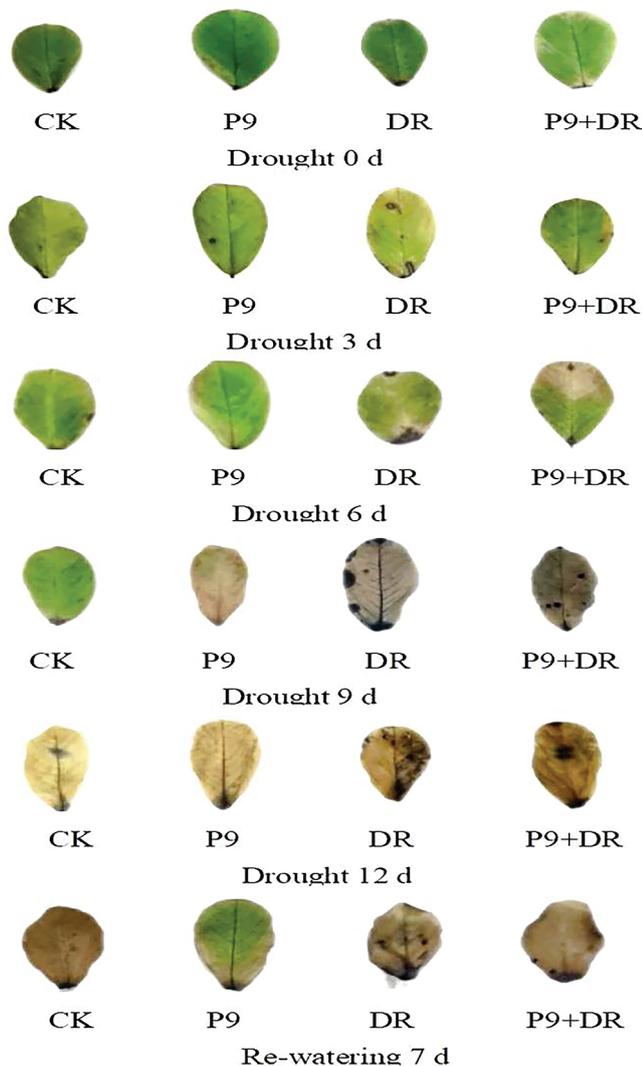
*Effects of P9 inoculation on ROS accumulation in peanut leaves under drought stress and after re-watering*

Oxygen free radicals can convert NBT into a blue or brown precipitate; thus, it can be used to visually locate and observe the sites where  $O^{2-}$  is produced and accumulated (Alexander *et al.*, 2020). The results of the NBT staining

showed that  $O^{2-}$  production increased significantly under drought stress, and the brown deposition in leaves became more obvious with the extension of drought which decreased after re-watering (Fig. 4). Compared with drought stress alone, the accumulation of  $O^{2-}$  in inoculated plants was significantly lower, also indicating that the P9 inoculation alleviated the oxidative damage caused by drought stress, which was consistent with the results of the  $H_2O_2$  content determination.

*Effects of P9 inoculation on antioxidant enzyme activities in peanut under drought stress and after re-watering*

As shown in Table 2, under normal water conditions, there was no significant difference in the enzyme activities of SOD, CAT, and POD between inoculated and uninoculated plants. Under drought stress, while antioxidant enzyme activities increased, with the prolongation of drought stress, the changes in the activities of the three enzymes differed. The activities of SOD and POD increased gradually, with a significant change in POD activity, while CAT activity increased first and then decreased. During days 3–12 of drought stress, the CAT activity of the P9+DR group was significantly higher than that of the DR group. The SOD activity was also significantly higher, except after 6 d of drought stress. However, the POD activity changed only slightly and was only significantly higher than the DR group after day 9 of drought treatment. After re-watering, the



**FIGURE 4.** Effects of *Tsukamurella tyrosinosolvens* P9 inoculation on ROS accumulation in peanut leaves during different drought stress times and after re-watering. *In vivo* localization of ROS in peanut leaves as revealed by nitro-blue tetrazolium (NBT) staining. From left to right are CK (control peanut), P9 (P9-inoculated peanut), DR (drought-treated peanut), and P9+DR (P9-inoculated peanut under drought treatment), respectively.

activities of SOD, POD, and CAT in the P9+DR and DR groups returned to normal levels, and there was no significant difference between the groups.

#### *Effect of P9 inoculation on the stomatal status of peanut under drought stress and after re-watering*

The stomatal status of peanut leaves was analyzed under normal conditions, drought stress, and post-drought re-watering (Table 3). Under normal conditions, while stomatal density, length, width, and aperture were constant, these indexes were significantly higher in the P9 group than in the CK group ( $p < 0.05$ ). Under drought stress, stomatal length, width, and aperture decreased, whereas stomatal density increased, and the changes were more pronounced with longer drought stress. However, the stomatal length, width, and aperture of P9+DR plants were significantly higher than those of the DR group ( $p < 0.05$ ). Compared

with the DR group, stomatal length, width, and aperture were increased by 11.81%–21.90%, 13.60%–28.75%, and 18.36%–39.17%, respectively, in the P9+DR group during drought treatment. The effect of stress on stomatal density was slightly different. In the early stages (0–3 d) of drought stress, the stomatal densities of inoculated plants were significantly higher than those of the DR group, while in the late stages of drought (9–12 d), the stomatal density of the P9+DR group was significantly lower than that of the DR group ( $p < 0.05$ ). After 7 days of re-watering, the stomatal status gradually returned to its unstressed status, and the stomatal length, width, and aperture of P9+DR plants were significantly higher than those of the uninoculated group ( $p < 0.05$ ). The stomatal density did not differ significantly between the two treatments.

#### **Discussion**

In this study, under normal water conditions, P9 inoculation in peanut plants improved their growth indexes, the relative water content, chlorophyll content, proline content. While the stomatal indexes were significantly higher than in the CK group; the contents of MDA and  $H_2O_2$  of the inoculated plants were significantly lower than in the CK group ( $p < 0.05$ ). The results showed that inoculation with *T. tyrosinosolvens* P9 significantly improved the physiological indexes of peanut, reduced the level of stress, and promoted seedling growth. Another study showed that inoculation with *Bacillus pumilus* and *B. mycoides* could improve photosynthesis, water use efficiency, and the antioxidant enzyme activity of runner bean plants and promote plant growth (Stefan et al., 2013). In another report, inoculation with *B. velezensis* could improve the stomatal aperture and density of pepper, resulting in a greater  $CO_2$  absorption rate and enhanced photosynthesis (Sevirasari et al., 2022). Similar results were obtained for *Klebsiella variicola*-, *Raoultella planticola*-, and *Pseudomonas fluorescens*-inoculated maize; *B. subtilis*-, *P. fluorescens*-, and *P. putida*-inoculated mint; and *B. amyloliquefaciens*-inoculated wheat (del Rosario Cappellari et al., 2015; Gou et al., 2015; Danish and Zafar-ul-Hye, 2019).

Drought affects plant growth and metabolism. Generally, when the soil moisture is 55%–60%, 45%–50%, and 35%–40%, plants suffer from mild, moderate, and severe water stress, respectively, which can lead to plant growth arrest and even death (Yuan et al., 2016). PGPRs were shown to improve the stress resistance of plants. For example, inoculation with *P. putida* increased the total root length, stem length, and biomass of sunflower seedlings under water stress (Sandhya et al., 2009). Inoculation with *B. licheniformis* increased the dry weights of maize roots and stems under stress (Akhtar et al., 2020) and *Burkholderia seminalis* inoculation increased the biomasses of tomato and pepper under stress (Tallapragada et al., 2016). On the basis of phylogenetic profiling, the dominant phylum of drought-adaptive bacteria has been observed to be Proteobacteria with 31 species, of which 16 species belonged to the genus *Pseudomonas* followed by *Enterobacter*, *Serratia*, *Acinetobacter*,

TABLE 2

Effects of *Tsukamurella tyrosinosolvens* P9 inoculation on antioxidant enzyme activities in peanut under drought stress and after re-watering

Antioxidant enzyme activity	Treatment	Drought 0 d	Drought 3 d	Drought 6 d	Drought 9 d	Drought 12 d	Re-watering 7 d
SOD (U/g)	CK	1828.86±171.74a	1640.97±45.15c	1636.49±82.02b	1660.05±59.71c	1649.56±73.62c	1637.37±65.48a
	P9	1849.14±132.73a	1742.66±69.62bc	1751.87±72.26b	1769.36±59.41c	1770.46±47.23c	1750.09±49.84a
	DR	1643.11±54.78a	1843.93±28.99b	2008.87±10.01a	2078.87±46.22b	2195.53±10.48b	1674.21±106.48a
	P9+DR	1741.92±66.04a	2039.59±21.92a	2168.71±32.18a	2331.86±36.97a	2467.34±50.10a	1778.16±53.31a
CAT (nmol/g)	CK	703.54±133.67a	807.05±33.70c	833.71±27.60c	816.31±21.87c	814.73±15.72b	807.27±39.10b
	P9	886.37±9.91a	892.02±14.33c	894.28±19.99c	938.35±9.03ab	906.49±21.66a	919.14±42.92ab
	DR	807.27±28.90a	1037.57±65.20b	1162.09±52.57b	925.24±22.16bc	675.51±14.61c	850.44±64.74ab
	P9+DR	874.85±31.71a	1182.66±12.14a	1360.52±41.51a	1047.51±61.22a	896.09±33.08a	983.55±6.90a
POD (U/g)	CK	9848.00±396.73b	11432.67±372.37b	11323.33±222.85c	12138.00±244.85c	12147.33±444.20b	12154.67±25.10a
	P9	12376.00±221.38a	12284.67±506.83ab	12158.67±513.40bc	12730.67±40.42c	12864.00±444.14b	12700.00±323.26a
	DR	10578.00±293.48ab	12416.67±226.32ab	12940.67±182.66ab	13409.33±97.64b	24396.00±2498.06a	12208.67±136.69a
	P9+DR	12220.67±218.68a	12945.33±211.24a	13464.00±121.50a	15184.67±276.03a	22470.67±952.72a	11956.00±1023.66a

Note: SOD, CAT and POD respectively represent superoxide dismutase, peroxidase and catalase. Values are given as means±S.E. for each sample; different lowercase letters after numbers in the same column represent significant differences between treatments at the same time points ( $p < 0.05$ ).

TABLE 3

Effects of *Tsukamurella tyrosinosolvens* P9 inoculation on the stomatal status of peanut under drought stress and after re-watering

Stomatal status	Treatment	Drought 0 d	Drought 3 d	Drought 6 d	Drought 9 d	Drought 12 d	Re-watering 7 d
Stomatal density/ (/mm <sup>2</sup> )	CK	59.23±3.58b	61.75±0.28d	64.56±1.01c	62.60±0.74d	60.35±2.19d	64.28±3.09b
	P9	74.95±1.29a	74.39±1.01b	74.67±0.28b	76.07±1.56c	76.35±0.56c	74.11±1.75a
	DR	63.44±1.01b	70.74±0.49c	79.44±0.28a	88.14±0.74a	102.74±0.84a	71.86±2.02a
	P9+DR	75.51±0.74a	76.63±0.00a	78.60±0.28a	83.09±0.28b	93.19±1.22b	76.63±2.12a
Stomatal length (µm)	CK	23.91±0.17c	24.75±0.33c	23.92±0.23c	23.97±0.14c	23.81±0.43b	24.08±0.47b
	P9	26.77±0.54b	28.55±0.19a	28.56±0.32a	29.68±0.95a	28.84±0.17a	28.43±0.17a
	DR	24.11±0.24c	23.65±0.07d	22.78±0.35d	21.05±0.21d	20.66±0.25c	23.96±0.36b
	P9+DR	28.26±0.03a	27.36±0.36b	26.82±0.18b	25.66±0.28b	23.10±0.57b	27.76±0.10a
Stomatal width (µm)	CK	14.92±0.15b	15.02±0.26b	15.03±0.14c	14.77±0.41b	15.59±0.38ab	15.35±0.22bc
	P9	17.37±0.15a	17.28±0.03a	18.23±0.57a	16.74±0.53a	17.11±0.46a	18.23±0.83a
	DR	15.44±0.22b	14.09±0.24c	13.88±0.10d	12.56±0.14c	11.06±0.59c	14.00±0.23c
	P9+DR	17.54±0.14a	17.53±0.13a	16.33±0.17b	15.39±0.39b	14.24±0.63b	16.46±0.31b
Stomatal aperture (µm)	CK	5.08±0.04b	5.07±0.04b	5.11±0.05c	5.12±0.07b	5.24±0.10b	5.14±0.06c
	P9	6.11±0.02a	6.01±0.03a	6.18±0.07a	5.86±0.23a	6.00±0.06a	6.25±0.19a
	DR	5.12±0.02b	4.77±0.06c	4.58±0.10d	4.29±0.02c	3.14±0.23d	4.79±0.12c
	P9+DR	6.06±0.06a	5.99±0.04a	5.61±0.07b	5.27±0.10b	4.37±0.15c	5.74±0.08b

Note: Values are given as means ± S.E. for each sample; different lowercase letters after numbers in the same column represent significant differences between treatments at the same time points ( $p < 0.05$ ).

*Burkholderia*, etc. The second dominant phylum included *Firmicutes*, of which most belonged to the genus *Bacillus*. Another phylum reported included Actinobacteria of which most members belonged to the genus *Streptomyces* (Kour and Yadav, 2022).

It was reported that *Streptomyces* sp. inoculation improved the growth and drought resistance of plants such as chickpea and tomato (Sreevidya et al., 2016; Koçak, 2019). Several studies have shown that the ecological distribution of actinomycetes is negatively correlated with soil moisture, and they tend to be dominant in drought

environments because they are strongly drought-tolerant (Khamna et al., 2010). In this study, we found that the growth of peanut was inhibited when the period of drought was extended, however, seedlings inoculated with *T. tyrosinosolvens* P9 fared significantly better than the DR group. The plant height, fresh weight, and root length and weight were all significantly higher in these inoculated plants ( $p < 0.05$ ). The colonization by PGPRs is a key factor influencing their growth-promoting function in plants (Mohanram and Kumar, 2019). We found that *T. tyrosinosolvens* P9 (a member of actinobacteria) could stably

colonize the root tips of peanut seedlings, accumulate in root hairs, migrate to stem tissues to then exert their growth-promoting effects on peanut (Li *et al.*, 2022b). All these results provided a theoretical basis for the application of this strain to improve the drought resistance of peanut.

Under drought stress, an imbalance between ROS production and clearance occurs in plants, which leads to an excessive accumulation of ROS. The free radical oxygen could convert unsaturated fatty acids into lipid peroxidation products, such as MDA, which further damage the structure and function of the cell membrane. Therefore, MDA is an important indicator of the degree of damage to membrane lipids and stability caused by ROS (Das and Roychoudhury, 2014). H<sub>2</sub>O<sub>2</sub> is a common ROS and its accumulation can lead to cell membrane damage (Ahmed *et al.*, 2009). In this study, the contents of MDA and H<sub>2</sub>O<sub>2</sub> were significantly increased under drought stress. However, P9 inoculation significantly reduced both MDA and H<sub>2</sub>O<sub>2</sub> contents ( $p < 0.05$ ). NBT staining also clearly indicated more obvious brown depositions and the accumulation of O<sup>2-</sup> was even greater in the leaves of the DR group. This suggests that the P9 strain can alleviate oxidative damage caused by drought stress. Batool *et al.* (2020) also found that inoculation with *B. subtilis* could enhance the ability to scavenge ROS and reduce the accumulation of ROS and MDA that reduced membrane damage, and enhanced drought resistance.

PGPRs can improve the stress resistance of plants, which is closely related to their ability to regulate plant physiological changes. Soil water deficiency is an important factor limiting plant growth. PGPRs can effectively adhere to soil and improve soil water status. Additionally, IAA-producing PGPR strains can stimulate root development, and increase water absorption (Sandhya *et al.*, 2009; Marasco *et al.*, 2013; Luo *et al.*, 2019). Kubi *et al.* (2021) reported that plant root inoculation with IAA-secreting *P. psychrotolerans* promoted the growth of *Zea mays*. As a major plant hormone, IAA was involved in plant cell division and differentiation, germination, and most importantly root growth (Etesami and Beattie, 2017). In this report, the root length and root weight of the P9+DR group were significantly higher than those of the DR group. Hence, the inoculation of the P9 strain obviously improved the root development and water uptake under drought-stress conditions. This improvement may be related to the ability of the microbe to secrete IAA (37.26 g/mL) (Zhang *et al.*, 2021). Marulanda *et al.* (2006) also reported that plants treated with *Bacillus thuringiensis* had better root biomass, including much more root branch numbers and root surface areas, and could absorb more water from the soil.

Leaf water content is an important indicator of the degree of water deficiency, and higher relative water content indicates that a plant is more adaptable to environmental stress (Moshelion *et al.*, 2015). During drought stress, inoculation with *P. putida* improved the water status of chickpea leaves and alleviated membrane damage and oxidative stress (Tiwari *et al.*, 2016). In this study, when drought was extended, the water contents of the soil and leaves decreased significantly, but these indexes were significantly higher in the P9 inoculated plants ( $p < 0.05$ ). Obviously, PGPR

improves the drought resistance of plants by delaying plant dehydration, increasing their water content, and maintaining proper cellular water status (Khan *et al.*, 2020). Similar results have also been reported for *Bacillus* sp., *Pseudomonas* sp. and *Enterobacter* sp. (Niu *et al.*, 2018; He *et al.*, 2021). Stomatal regulation is the initial response of plants to drought stress; in particular, plants reduce water loss by regulating stomatal characteristics, including stomatal morphology (stomatal size and density) and stomatal movement (stomatal aperture) (McAdam and Brodrigg, 2012; Daszkowska-Golec and Szarejko, 2013). Plants grown under water deficit conditions have a lower stomata conductance to facilitate the conservation of water (Hayat *et al.*, 2010). A decline in leaf water content will lead to a decrease in stomatal aperture and even closure (Casson and Hetherington, 2010). Under drought stress, the stomatal density of peanut leaves significantly increased, while stomatal length, width, and aperture all significantly decreased ( $p < 0.05$ ). However, the stomatal density of P9-inoculated plants was higher at the beginning of the stress period but lower than the DR group by the end of the stress period (9–12 d). The stomatal length, width, and aperture were significantly higher than the DR group ( $p < 0.05$ ). This is likely due to the following hypothesis: under drought conditions, the leaf area shrinks due to an obvious decrease in leaf water content. Thus, the stomatal density increases due to an increase in stomata number per unit area.

The leaf size has been negatively correlated with stomatal density (Doheny-Adams *et al.*, 2012; Liu *et al.*, 2012). However, peanut adapts to drought by reducing stomatal length, width, and aperture to maintain lower transpiration rates and reduce water loss. A similar response mechanism is found in vegetable crops, such as eggplant, corn, and wheat (Fu *et al.*, 2013; Zhao *et al.*, 2015; Wang *et al.*, 2016), as well as *Leymus chinensis*, *Arabidopsis thaliana*, and cattail (Xu and Zhou, 2008; Doheny-Adams *et al.*, 2012; Cruz *et al.*, 2019). These results all show that more, smaller stomata are a response to water stress. Furthermore, higher ROS levels could lead to increased ABA accumulation, which leads to stomatal closure (Pirasteh-Anosheh *et al.*, 2016). These results are similar to those of the present study, which showed that ROS accumulation was higher in uninoculated plants, and therefore leaf stomatal size and aperture were significantly decreased. In addition, the leaf water contents of inoculated plants were still 73.83%–80.24% after 9–12 d of stress, and peanut plants do not need to reduce transpiration by closing or shrinking the stomata. Hence, inoculation with PGPR could increase the leaf area by increasing soil and leaf water contents during drought stress, which promoted stomatal opening and relieved the inhibition of photosynthesis (Liu *et al.*, 2019a; Bashir and Naz, 2020; Suryanti and Umami, 2020). Accordingly, although the chlorophyll content of peanut significantly decreased under drought stress: with its content in the P9 +DR group being significantly higher than those of the DR group ( $p < 0.05$ ). Stress can lead to the degradation of chlorophyll and a decrease in plant photosynthetic capacity (Kawamitsu *et al.*, 2000). However, inoculation with *Acinetobacter* sp., *Serratia marcescens*, and *Pseudomonas* sp.

reduced the damage to photosynthetic organs, increased the chlorophyll content and the photosynthetic rate of plants to compensate for the adverse effect of drought (Abbasi *et al.*, 2013; Liu *et al.*, 2019b; Khan and Singh, 2021). After re-watering, the stomatal status of the plants gradually returned to normal and bacterial inoculation promoted the rapid recovery of the density, size, and aperture of stomata and the photosynthetic function in peanut. Similar results have been reported in elderberry inoculated with *Acinetobacter calcoaceticus* and sorghum inoculated with *Bacillus* sp. (Liu *et al.*, 2019b; Santana *et al.*, 2020).

To reduce the toxic effect of drought, plants eliminate the excess accumulated ROS by regulating antioxidant enzymes and non-enzymatic antioxidants. The enzymatic antioxidant defense system mainly includes SOD, POD, and CAT:  $O_2^-$  produced under stress can be converted to  $H_2O_2$  and  $O_2$  by SOD, while CAT and POD convert  $H_2O_2$  to  $H_2O$ , thus reducing ROS damage to plant cells (Sharma *et al.*, 2012). In this study, the activities of SOD and POD increased, especially that of POD, under drought stress, while the activity of CAT initially increased and then decreased. Plants often resist oxidative damage by increasing the activity of antioxidant enzymes under stress (Jin *et al.*, 2015). For example, the CAT activity of ramie increased first and then decreased during prolonged stress, while the activities of SOD and POD increased gradually. This indicates that the changes in enzyme activity were closely related to the intensity and duration of stress (Huang *et al.*, 2013). As the large amount of  $H_2O_2$  produced by drought stress inhibits CAT activity, its activity decreased during the later stage of drought. However, the increase in SOD and POD activities, especially POD, may compensate for such decreased CAT activity to some extent (Pan *et al.*, 2006). However, the antioxidant enzyme activity of inoculated peanut was higher than that of the DR group, which indicated that strain P9 was able to activate the antioxidant enzyme system of peanut. Therefore, inoculation with strain P9 significantly reduced the accumulation of  $O_2^-$  and  $H_2O_2$  during drought stress by increasing the activities of CAT, POD, and SOD and enhancing the drought resistance of peanut. A study by Gupta *et al.* (2015) found increased CAT and SOD activities in leaves of *Helianthus annuus* seedlings inoculated with *Azotobacter chroococcum* and *Bacillus polymyxa* under water deficit conditions. Further, similar results also have been reported in other studies (Ghorbanpour *et al.*, 2013; Sarma and Saikia, 2014; Gusain *et al.*, 2015; Zhang *et al.*, 2020).

Osmotic regulation is another important protective mechanism against stress in plants. As a common osmotic regulator, a large amount of proline is essential to reduce oxidative stress and reduce damage to the photosystem and chlorophyll during stress (Hayat *et al.*, 2012; Shan *et al.*, 2015). In this study, under drought stress, the proline content of peanut significantly increased, and the increase in proline content was lower in the late stage of stress in the P9+DR group compared to the DR group. The increment in proline by PGPR under drought has been reported in cucumber, potato plants, and great millet to improve their survival and drought tolerance (Vanderlinde *et al.*, 2010; Wang *et al.*, 2012; Gururani *et al.*, 2013; Kour *et al.*, 2020).

However, there are different results in some reports. For example, Naveed *et al.* (2014) found that the proline content of wheat increased with worsening drought, whereas inoculation with *Burkholderia phytofirmans* reduced proline accumulation. Proline accumulation also decreased in plants inoculated with *Klebsiella* sp., *Enterobacter ludwigii*, and *Flavobacterium* sp. (Gontia-Mishra *et al.*, 2016). However, other studies showed that proline accumulation is a symptom of stress injury instead of serving as an indicator of stress tolerance. The accumulation level is usually positively correlated with the degree of stress in cells (Monreal *et al.*, 2007). In cultivars with weak drought resistance, proline accumulation is higher under stress (Sundaresan and Sudhakaran, 1995; Silvente *et al.*, 2012). Therefore, in our work, the accumulation of proline was reduced in inoculated peanuts in the late drought period, explaining why strain P9 improved the stress due to water shortage and alleviated photosynthetic damage.

After re-watering, the soil water content returned to the normal level in the DR group, but the leaf water content remained lower, and the contents of MDA and  $H_2O_2$  were still significantly higher than those of the CK plants. Thus, although the growth conditions of the peanut plants gradually recovered, the plants did not completely recover from the stress and had some degree of oxidative damage. As for the P9+DR group, after re-watering, while the water content, chlorophyll content, and proline content were significantly higher than the DR group, the  $H_2O_2$  content was significantly lower than the latter ( $p < 0.05$ ), and the growth of peanut plants improved. Bresson *et al.* (2014) also found that inoculation with *Phyllobacterium brassicacearum* not only maintained the water content of *A. thaliana* leaves under stress but also aided plant recovery after re-watering. Similar studies have been reported in elderbush inoculated with *A. calcoaceticus* and wheat inoculated with *Alternaria alternata* (Liu *et al.*, 2019b; Qiang *et al.*, 2019). Therefore, strain P9 conferred drought tolerance to the re-watered plants, which was beneficial to photosynthesis and biomass accumulation under stress and after re-watering.

## Conclusion

In this paper, the effects of the PGPR strain *T. tyrosinosolvens* P9 on peanut growth and drought resistance were investigated. During drought stress, the water content of the soil and leaves and the chlorophyll content decreased with the length of the drought period. Although the effects of drought were alleviated in the plants by increasing their proline content and antioxidant enzyme activities, the MDA and  $H_2O_2$  contents increased sharply with the extension of the drought, and the growth of peanut plants was inhibited. Compared with the DR group, inoculation with the P9 strain significantly increased the water contents of the soil and leaves; chlorophyll content, and the length, width, and aperture of the stomata ( $p < 0.05$ ). The stomatal density decreased at the later stage of drought stress; the activities of CAT and SOD (except for 6 d of stress) were significantly increased, and the contents of MDA and  $H_2O_2$  in inoculated plants were significantly lower than those in the DR group ( $p < 0.05$ ). Accordingly, during drought, the growth indexes

of inoculated seedlings were significantly better than those of uninoculated plants ( $p < 0.05$ ). In addition, inoculated peanut plants resumed growing faster after the drought stress. Therefore, *Tsukamurella tyrosinosolvens* P9 inoculation could reduce the effects of stress, improve peanut drought resistance, promote seedling growth by improving plant water status, photosynthetic performance, and stomatal status, while activating the antioxidant enzyme system, reducing membrane lipid peroxidation and cell damage caused by stress. Our results provide a theoretical foundation for the further application of various PGPR strains and a new approach to improving drought resistance in agricultural plants.

**Acknowledgement:** We thank Candace Webb, Ph.D., from Liwen Bianji (Edanz) ([www.liwenbianji.cn](http://www.liwenbianji.cn)) for editing the English text of the draft of this manuscript.

**Funding Statement:** This work was supported by the National Natural Science Foundation of China under Grant 31760030 and 32060028, and the Science and Technology Planning Project of Guizhou Province under Grant [2022]009.

**Author Contributions:** Lizhen Han is the corresponding author while Changmei Long, Tingting Yang, and Yujie Han are Lizhen Han's graduate students. Changmei Long designed the experiment and wrote the manuscript. The three students performed all experiments. All authors read and approved the final manuscript.

**Availability of Data and Materials:** All data generated or analyzed during this study are included in this published article.

**Ethics Approval:** Not applicable.

**Conflicts of Interest:** The authors declare that they have no conflicts of interest to report regarding the present study.

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