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Regulation of Proline Metabolism in Soybean Leaves under Drought Stress at Seedling Phase

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ABSTRACT

Background: Soybean is the most important oil crop globally; however, droughts are expected to seriously affect the growth and development of soybean in the context of climate change.

Methods: In this study, polyethylene glycol (PEG) was used to simulate drought to explore the regulation of proline metabolism in soybean leaves under different degrees of drought stress at the seedling stage.

Result: The results showed that the activities of ornithine aminotransferase and Δ1-pyrroline-5-carboxylate synthetase increased while the activities of proline dehydrogenase decreased with an increase in drought stress. During the same number of treatment days, the higher the degree of drought stress, the greater the increase or decrease in enzyme activity. The content of leaf proline increased gradually with an extension in stress time at PEG concentrations of 5% and 10%, first increased and then decreased at PEG concentrations of 15% and 20% and the peak value appeared on the 7th and 9th day, respectively.

Key words: Drought, Ornithine aminotransferase, Proline dehydrogenase, Proline, Soybean, Δ1-pyrroline-5-carboxylate synthetase.

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INTRODUCTION

Drought is a severe weather condition that reduces crop yields. The most direct effects of drought on plants are wilting and yellowing leaves, plant height decrease and yield decrease. Soybean is the main oil crop in China and its production is mainly concentrated in northeast China, Huang-Huai River Basin and Yangtze River Basin. Two long-term climate studies indicate that the duration and severity of meteorological droughts will increase in the future in northeast China, where frequent droughts already occur (Leng *et al*., 2015; Spinoni *et al*., 2019). Therefore, it is necessary to explore the effects of varying drought severity and duration on soybean growth.

Osmotic regulation plays a crucial role in plant stress tolerance and drought-tolerant varieties tend to exhibit higher osmotic regulation than sensitive varieties (Blum, 2017). Not only is it involved in protein synthesis but it is also a good osmotic regulation substance, which functions to protect the cell membrane structure and maintain osmotic pressure in a variety of plants (Ashraf and Foolad, 2007). In addition, proline also has the function of scavenging hydroxyl free radicals, thus slowing down cell membrane peroxidation. Proline accumulation has been observed under various adverse conditions, such as drought ang high temperature stress (Mansour *et al*., 2017; Thounaojam *et al*., 2012).

Proline synthesis in plants mainly occurs in the cytoplasm and the synthesis pathway can be divided into the glutamate and ornithine pathways. Glutamic acid is catalyzed by Δ1-pyrroline-5-carboxylate synthetase (P5CS) to form glutamate-semialdehyde (GSA). Subsequently, GSA spontaneously converts to Δ1-pyrroline-5-carboxylate (P5C). Finally, Δ1-pryrroline-5-carboxylate synthetase (P5CR) reduces P5C to produce proline (Szabados *et al*., 2010; Quan *et al*., 2007). In addition, some scholars think that ornithine in plant mitochondria is transformed into P5C by ornithine aminotransferase (OAT), which can enter the cytoplasm through a transporter and then participate in the biosynthesis of proline. The breakdown of proline occurs in the mitochondria. During the process of proline decomposition, proline dehydrogenase (ProDH) participates in the first step reaction and catalyzes proline to produce P5C. Then, P5C is spontaneously converted to GSA and reduced to glutamate by P5C dehydrogenase (P5CDH).

To clarify the effects of drought on soybean at seedling phase, polyethylene glycol 6000 (PEG-6000) was used in this study to simulate different degrees of drought and the physiological changes of proline metabolism in two soybean cultivars with different tolerances for drought conditions were explored. This study provides a theoretical basis for soybean cultivation measures and drought resistance screening.

MATERIALS AND METHODS

Trial design

The experiment was carried out under a glass awning on the campus of Northeast Agricultural University. Through previous experiments, the effects of drought stress on the activities of key enzymes in carbon metabolism and photosynthetic characteristics of the two soybean varieties were compared. Heinong84 (HN84) was determined to be a drought tolerant variety and Hefeng46 (HF46) was a medium drought tolerant variety (Song *et al*.,2022) and the test method was sand culture. A plastic pot with a height of 35 cm and an inner diameter of 30 cm with two holes punched at the bottom, was used in the test. After placing a gauze net on the bottom of the barrel, each barrel was loaded with 5 kg of washed river sand. Six soybean seeds of the same size and full grains were selected for sowing. Each pot contained three seedlings with the same growth trend. When true opposite leaves were fully unfolded, 500 mL nutrient solution was added to each pot every morning. When the seedlings grew to V3 phase according to Fehr, different concentrations of PEG were used to simulate different degrees of drought. The PEG concentration was set to 5%, 10%, 15% and 20% in treatment group and 0% in control group. The treatment solution (500 mL) was applied every morning for a total of 14 d at 8:00-9:00 a.m. On the 1^{st}, 3^{rd}, 5^{th}, 7^{th}, 9^{th}, 12^{th} and 14^{th} days of treatment, the penultimate and antepenultimate leaves were removed and wrapped in tinfoil. A total of 9 plants were collected in 3 pots each time and each pot of plants were considered as a biological replicate. The samples were stored in a refrigerator and transported back to the laboratory for analysis. The composition of nutrient solution is shown in Table 1.

Index determination

Proline content, P5CS activities, OAT activities and ProDH activities were determined according to the kit instructions (Suzhou Keming Biotechnology Co., LTD.). One technical replicate was performed for each sample.

P5CS activity

P5CS catalyzed the conversion of glutamic acid to glutamic acid-γ-semialdehyde and reduced $NADP^+$ to NADPH. The enzymatic activity of P5CS was determined by detecting the increase rate of NADPH at 340 nm.

OAT activity

OAT dehydrogenated ornithine and oxidized NADH in the presence of ornithine and α-ketoglutarate. The activity of OAT was determined by detecting the decrease of NADH at 340 nm.

ProDH activity

ProDH catalyzes the dehydrogenation of proline and the reduction of NAD^+ to NADH and ProDH activity was determined by measuring the rate of increase of NADH at 340 nm.

Proline content: Proline can be free in sulfosalicylic acid. After heating with acidic ninhydrin, the solution turns red and then the red substance is extracted with toluene. The content of proline was determined by absorbance at 520 nm.

Data statistics

Excel 2019 and Origin 2018 was selected for data arrangement and chart drawing. SPSS 25.0 was used for data analysis through one way ANOVA.

RESULTS AND DISCUSSION

Effect of drought on plant height of soybean

As shown in Fig 1, the growth of both soybean variety was slowed down by the drought at different concentrations of PEG significantly. Plant height was affected by PEG concentration in the order of 5, 10, 15, 20% from the smallest to the largest. In addition, the two varieties showed significant differences on the 14^{th} day of drought. The plant height of HN84 decreased by 8.64%, 10.47%, 21.21% and 25.62% at 5%, 10%, 15% and 20%PEG concentration, respectively, while that of HF46 decreased by 13.10%, 15.12%, 21.68% and 28.75%. The results of one-way ANOVA showed that there was no significant difference in plant height between the two cultivars under 15%PEG condition, but the plant height of HN84 was significantly higher than that of HF46 under 5%, 10%PEG and 20% PEG condition. On day 14 of the drought, the reason why there was no significant difference between the two varieties at 15% PEG may be because the plant height of HN84 at 15% PEG appeared significantly decreased compared with 5% and 10%, indicating that 15% PEG may be the critical point for HN84; while the plant height of HF46 at 20% PEG may have stopped growing due to excessive drought and HN84 may have further HN84 may have further activated some metabolic reactions in the body so that it could maintain growth. Therefore, HF46 was significantly lower than HN84 under 20% PEG.

Effects of drought stress on soybean Δ1-pyrroline-5-carboxylate synthetase

The changes in P5CS activity are shown in Table 2. Under different drought conditions, the activity of P5CS in both cultivars increased from day 1 to day 9, gradually stabilized

Table 1: Component concentration of nutrient solution.

Inorganic salts	Concentration (mg/L)	Inorganic salts	Concentration (mg/L)
$MgSO_4$	240.00	$CuSO_4\cdot 5H_2O$	0.08
KH_2PO_4	136.00	$ZnSO_4\cdot 7H_2O$	0.22
NH_4SO_4	235.8	$MnCl_2\cdot 4H_2O$	4.90
$CaCl_2$	220.00	H_3BO_3	2.86
$Na_2MoO_4\cdot H_2O$	0.03	Fe-EDTA*	-

Note: *5.57g $FeSO_4\cdot 7H_2O$ and 7.45 g Na_2EDTA were dissolved to 1 L, respectively. 1 mL reserve solution were added to nutrient solution per liter.

after day 9 and reached the maximum on day 14. Under the same drought days, the activity of P5CS of the two cultivars increased significantly with the aggravation of drought. It was noted that the P5CS activity of HN84 was slightly higher than that of HF46 at 5%, 15% and 20% PEG conditions, however the P5CS activity of HN84 was significantly higher than that of HF46 at 10% PEG concentration.

In this study, P5CS activity showed an increasing trend under all drought levels. Deng *et al*. (2016) study on *Jatropha curcas* seeds reached a similar conclusion. Adamipour *et al*. (2020) study on roses showed that excessive metabolite proline could cause negative feedback regulation and reduce P5CS activity. However, the results of this experiment showed that P5CS activity did not decrease even under severe drought conditions. This suggests that such a feedback regulation may not be widespread among plants. In addition, the different responses of the two cultivars to 10% PEG concentration indicated that HN84 was more adaptable to drought than HF46.

Effects of drought stress on ornithine aminotransferase in soybeans

The changes in OAT activity are shown in Table 3. The OAT activity of both cultivars increased gradually with an increase in the duration and degree of stress. For the same stress durations, the OAT activity of HN84 was lower than that of HF46. On the first day of stress, HN84 only responded significantly to 20% PEG concentration, while the OAT activity of HF46 increased significantly under 10%, 15% and 20% PEG conditions. On the third day of stress, compared with the control group, HN84 had no significant difference under 5% PEG, while the OAT activity of HN46 significantly increased under all PEG conditions. After that, the OAT activities of the two varieties were significantly higher than those of the control.

The OAT, as a key enzyme in the ornithine pathway of proline synthesis, has different effects on drought stress resistance in different plants. Zhang *et al*. (2017) showed that there was no significant difference in OAT activity in peanut leaves under drought conditions. Zhao *et al*. (2011) study on rice seedlings showed that OAT activity in leaves increased slightly in the early stage of drought, although it decreased significantly thereafter. In contrast, the OAT activity in the leaves of the two soybean cultivars in this study increased with the duration of drought stress. The OAT activity of HN46 significantly increased on days 1-3 under drought stress and was higher in each treatment compared with those for HN84. The OAT of the medium drought-

Table 2: Effects of drought on ∆1-pyrroline-5-carboxylate synthetase activity of HN84 and HF46.

Variety	Treatment	P5CS activity(nmol/min/g)						
		1 d	3 d	5 d	7 d	9 d	12 d	14 d
HN84	0%PEG	8.63±0.53a	8.21±0.54a	7.93±0.35a	7.99±0.48a	8.35±0.34a	8.35±0.43a	8.35±0.62a
	5%PEG	13.73±0.61b	15.26±0.98c	19.92±0.52e	24.01±0.35fg	24.50±0.1fg	27.38±0.1h	30.50±0.1j
	10%PEG	15.80±0.55c	17.24±0.68d	25.46±0.32g	32.64±0.08k	43.75±0.33m	43.92±0.1m	44.92±0.09m
	15%PEG	19.18±0.51e	24.05±0.98fg	30.67±0.53j	40.77±0.43l	46.49±0.34n	49.54±0.45o	50.42±0.29op
	20%PEG	23.69±0.74f	28.82±0.6i	40.88±0.41l	44.94±0.31m	51.54±0.33p	53.61±0.11q	54.73±0.59q
HF65	0%PEG	8.15±0.16a	8.05±0.42a	8.12±0.46a	8.06±0.64a	8.35±0.22a	8.56±0.14a	8.07±0.33a
	5%PEG	9.61±0.41bc	10.52±0.30cd	15.94±0.17f	19.60±0.03g	22.18±0.31h	23.51±0.72i	24.50±0.64i
	10%PEG	11.00±0.16d	13.57±0.28e	16.08±0.11f	21.37±0.35h	28.68±0.65j	29.55±0.24j	29.83±0.18jk
	15%PEG	12.73±0.43e	22.24±0.15h	30.15±0.31kl	38.59±0.45n	44.29±0.56p	45.92±0.57q	48.61±0.31r
	20%PEG	15.19±0.36f	29.30±0.39jk	37.17±0.19m	43.13±0.3o	47.61±0.3r	50.77±0.44s	50.77±0.51s

Note: Different letters indicate significant differences at the 5% level by one-way ANOVA.

Table 3: Effects of drought on ornithine aminotransferase activity of HN84 and HF46.

Variety	Treatment	OAT activity (μmol/min/g)						
		1d	3d	5d	7d	9d	12d	14d
HN84	0%PEG	43.56±0.28a	43.60±0.19a	43.50±0.93a	43.47±0.67a	43.52±0.38a	43.53±1.11a	43.65±0.28a
	5%PEG	43.64±0.34a	43.88±0.54a	46.54±0.30b	48.68±0.45c	52.59±0.37d	56.15±0.51fg	58.40±0.64h
	10%PEG	44.78±0.43ab	46.31±0.13b	50.78±0.26cd	52.90±0.38de	56.76±0.66fgh	61.62±0.48ij	65.52±0.42kl
	15%PEG	43.08±0.39a	50.10±0.66c	54.78±0.22ef	55.99±0.52f	60.77±0.98i	63.57±0.73jk	68.86±0.57m
	20%PEG	46.25±0.61b	54.94±0.22ef	58.20±0.63gh	62.14±0.90ij	64.40±0.49kl	66.56±0.39l	70.23±0.60m
HF46	0%PEG	44.50±0.26a	44.48±0.60a	44.58±0.14a	44.56±0.47a	44.25±0.34a	44.62±0.33a	44.44±0.62a
	5%PEG	46.13±0.42ab	48.38±0.50b	50.96±0.36c	56.81±0.63e	58.39±0.54e	62.54±0.78f	64.89±0.46gh
	10%PEG	47.06±0.35b	48.37±0.50b	52.96±0.18cd	58.24±0.64e	62.36±0.52f	66.69±0.32hi	70.11±0.59j
	15%PEG	47.47±0.82b	52.70±0.80cd	56.39±0.54e	63.43±0.53fg	68.52±0.77ij	74.68±0.27l	76.03±0.48lm
	20%PEG	47.92±0.78b	54.28±0.77d	56.50±0.69e	66.34±0.52h	72.51±0.46k	75.55±0.18lm	77.55±0.66m

Note: Different letters indicate significant differences at the 5% level by one-way ANOVA.

tolerant varieties can adapt to drought conditions earlier. An increase in OAT activity under drought conditions was indeed observed in this study. We hypothesized that the effect of OAT may be somewhat different in different plants.

Effects of drought stress on proline dehydrogenase in soybeans

The changes in ProDH activity (Table 4) for the two cultivars were consistent and both decreased with an extension of drought stress duration and the differences were significant. On the first day of drought, the ProDH activities of HN84 in 5% and 15%PEG treatments were significantly lower than 10% and 20% PEG treatments, while that of 10% and 20% PEG treatments were significantly lower than control. Compared with the control, the ProDH activities of HF46 in all treatments decreased significantly, but there was no significant difference between treatments. From the third day of drought, ProDH activity of each treatment of the two varieties decreased significantly with the aggravation of drought degree under the same days.

Numerous studies (Kaur *et al*., 2017; Kaur *et al*., 2018) have showed that the activity of ProDH, a key enzyme in the proline decomposition pathway, decreases in multiple crops under drought conditions. The decreasing ProDH activity reduces the decomposition of proline and ensures the accumulation of proline in plants. In the present study, the ProDH activity of HN84 decreased less than that of HF46. However, the ProDH activity of HN84 was much less than that of HF46 under normal conditions. Therefore, compared with HF46, HN84 still showed lower ProDH activity. In addition, drought tolerant variety HN84 had earlier response time to drought than medium drought-tolerant variety HF46. This feature is consistent with previous research (Wu *et al*. 2022).

Effects of drought stress on proline content in soybeans

The changes in proline content under drought stress are shown in Table 5. The proline content of both varieties showed a continuous increase under 5% and 10% PEG stress. The proline content of the two varieties showed a tendency to increase first and then decrease under 15% and 20% PEG stress. During the 15% PEG treatment, the peak proline content in the two varieties appeared on day 9, at 18.79 and 18.19, for HN84 and HF46, respectively. Under 20% PEG treatment, the peak of HN84 appeared on day 9, while that of HF46 appeared on day 7, with peaks of 25.48 and 21.14, respectively. The maximum increase in HN84 compared with the control was 642.86% and that in HF46 was 365.64%.

Table 4: Effects of drought on proline dehydrogenase activity of HN84 and HF46.

Variety	Treatment	ProDH activity (nmol/min/g FW)						
		1d	3d	5d	7d	9d	12d	14d
HN84	0%PEG	46.07±0.06a	46.24±0.16a	46.18±0.08a	46.29±0.2a	46.25±0.17a	45.82±0.32a	46.11±0.12ab
	5%PEG	43.64±0.22d	43.71±0.14d	42.88±0.06f	41.86±0.12g	41.04±h0.23	39.86±0.32ij	37.55±0.3kl
	10%PEG	45.12±0.07bc	42.21±0.1ef	41.2±0.18gh	38.22±0.23k	37.05±0.19lm	36.49±0.36m	35.33±0.39n
	15%PEG	43.92±0.04d	41.54±0.23fgh	39.28±0.2j	37.05±0.25lm	35.74±0.3n	32.92±0.3p	31.13±0.33q
	20%PEG	44.93±0.54c	40.32±0.23i	40.25±0.28i	34.24±0.16o	33.26±0.4p	31.52±0.16q	28.06±0.36r
HF46	0%PEG	55.03±0.17a	55.2±0.11a	55.75±0.16a	55.43±0.16a	55.66±0.18a	55.79±0.19a	55.84±0.13a
	5%PEG	54.02±0.08b	52.04±0.12d	50.00±0.22e	48.40±0.29f	44.10±0.55i	42.75±0.36j	39.38±0.27l
	10%PEG	54.18±0.11b	52.67±0.28cd	49.62±0.3e	46.50±0.33g	42.75±0.25j	41.18±0.16k	37.4±0.43n
	15%PEG	53.36±0.13bc	50.40±0.68e	48.29±0.23f	44.97±0.13h	40.68±0.36k	39.04±0.31lm	36.13±0.2o
	20%PEG	53.35±0.17bc	48.44±0.25f	45.99±0.4g	42.41±0.56j	38.35±0.31m	35.74±0.17o	33.01±0.35p

Table 5: Effects of drought on proline content of HN84 and HF46.

Variety	Treatment	Proline content (μg/gFW)						
		1d	3d	5d	7d	9d	12d	14d
HN84	0%PEG	3.31±0.11a	3.47±0.23a	3.52±0.09a	3.43±0.28a	3.43±0.14a	3.51±0.21a	3.57±0.10a
	5%PEG	3.18±0.16a	5.19±0.26b	5.76±0.47b	6.72±0.36c	7.31±0.24cd	8.92±0.16e	9.82±0.31f
	10%PEG	3.71±0.12a	7.62±0.34cd	7.42±0.09cd	7.37±0.53cd	8.85±0.23e	9.80±0.45f	10.68±0.23fg
	15%PEG	3.87±0.11a	10.04±0.37f	12.54±0.51h	14.94±0.30i	16.06±0.20j	8.73±0.39e	7.80±0.27d
	20%PEG	5.40±0.12b	11.39±0.22g	17.79±0.34k	25.48±0.54l	20.87±0.24m	10.06±0.40f	9.96±0.08f
HF46	0%PEG	4.02±0.47a	4.42±0.27a	4.57±0.19a	4.54±0.23a	4.61±0.15a	4.68±0.09a	4.56±0.15a
	5%PEG	4.63±0.25a	6.07±0.41b	6.44±0.15bc	6.78±0.16bc	7.26±0.13cd	8.57±0.07e	9.86±0.51f
	10%PEG	4.58±0.32a	6.94±0.27bc	7.28±0.18cd	8.17±0.18de	8.35±0.06e	9.74±0.37f	11.24±0.12g
	15%PEG	4.43±0.35a	8.54±0.15e	12.09±0.49g	14.92±0.45h	24.43±0.29k	15.03±0.49h	8.48±0.12e
	20%PEG	4.41±0.30a	9.71±0.57f	14.11±0.40h	21.14±0.60j	18.19±0.34i	14.16±0.41h	8.14±0.44de

Note: Different letters indicate significant differences at the 5% level by one-way ANOVA.

To resist the adverse effects of drought, numerous crops accumulate proline. Schafleitner *et al*. (2006); Ghaffari *et al*. (2021) showed that drought significantly increased the proline content in chickpea and potato leaves. It was also found that soybean leaf proline content increased significantly under different drought conditions. However, in this experiment, the trend of proline content under low and high stress was clearly different. The continuous increase in proline content under 5% and 10% PEG stress may have been dependent on the increased activity of P5CS and OAT for proline synthesis and the decreased activity of ProDH for proline decomposition.

A decrease in proline content during drought has also been reported previously. Zhu *et al*. (2016) reported that under drought conditions, the activity of P5CS in alfalfa decreased, thus reducing proline synthesis. This result could be because of the negative feedback regulation of P5CS activity caused by excessive proline. In addition, abscisic acid (ABA) is also believed to regulate the change in proline content and the two substances often display the same trends. He *et al*. (2018) observed overexpressed ABA receptor gene *ZmPYL* in *Arabidopsis thaliana*, with significantly enhanced proline content. Stewart (1980) showed that this promotion was achieved by stimulating an increase in glutamate content, a proline synthetic substrate. The activity of P5CS did not decrease in the present study, so it was speculated that the decrease in proline content at 15% and 20% PEG stress conditions may have been caused by the decrease in ABA content in the two cultivars under long-term drought stress.

Comparison of each index between the two varieties on the 14th day

As shown in Fig 2, on day 14 of drought stress, the P5CS activity of HN84 was increased by 265.27%, 437.96%, 503.83% and 555.45% at 5%, 10%, 15% and 20%PEG

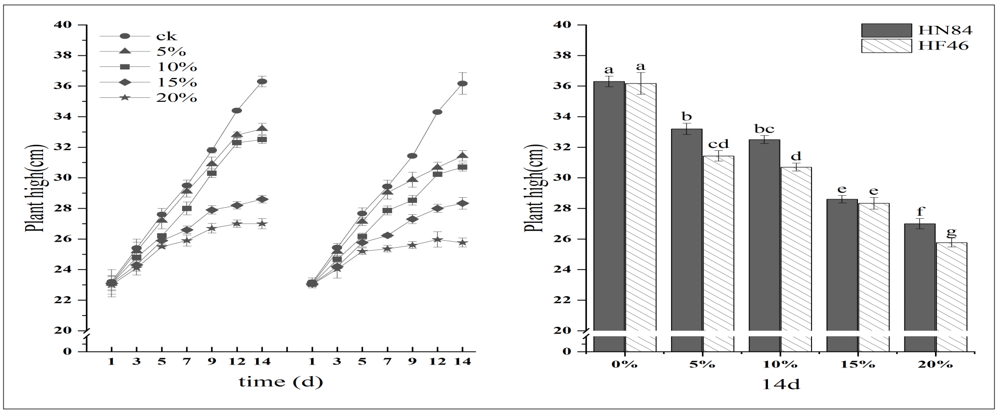

Fig 1: Effects of drought on plant high of HN84 and HF46.

Note: Different letters indicate significant differences at the 5% level by one-way ANOVA. The error line represents the standard error.

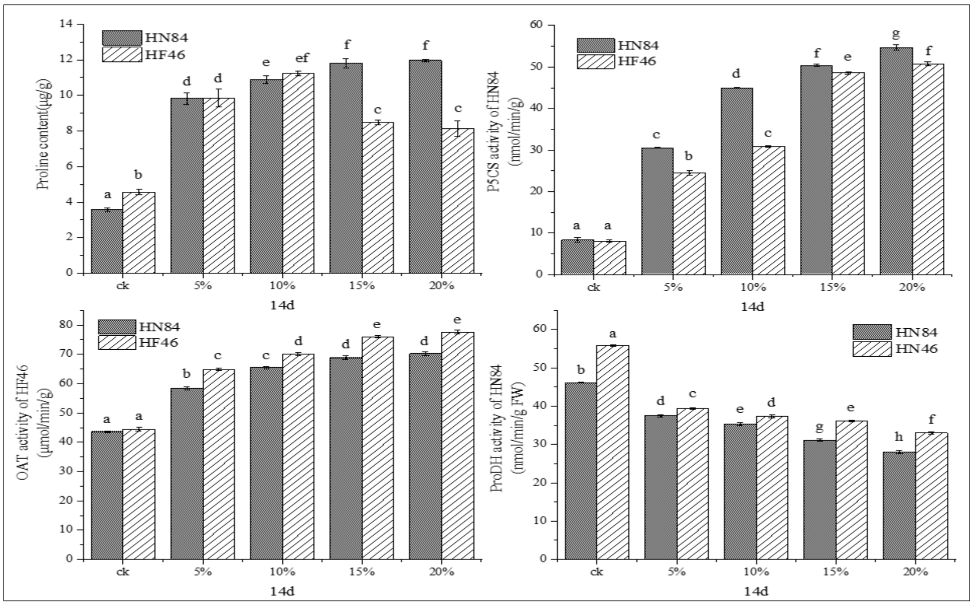

Fig 2: Comparison of each index between the two varieties on the 14th day.

Note: Different letters indicate significant differences at the 5% level by one-way ANOVA. The error line represents the standard error.

concentrations, respectively. The P5CS activity of HF46 was increased by 203.59%, 282.03%, 502.35% and 529.12%, respectively. Compared with the control, the OAT activity of HN84 in each treatment increased by 33.79%, 50.10%, 57.75%, 60.89%, respectively and that of HF46 increased by 46.02%, 57.76%, 71.08% and 74.50%. Under the same stress, the P5CS activity of HN84 was significantly higher than that of HF46, but the activity of OAT was significantly lower than that of HF46.

On day 14 of stress, the ProDH activity of HN84 treatments decreased by 18.56%, 23.38, 32.49%, 39.21% compared with the control group, respectively. HF46 decreased by 29.48%, 33.02%, 35.30% and 40.88%, respectively. The activity of HN84 was significantly lower than that of HF46.

Analysis of the proline content of the two varieties showed that there was no significant difference in the proline content of HN84 and HF46 under the conditions of 5% and 10%PEG concentration, but the proline content of HN84 was significantly higher than that of HF46 under the conditions of 15% and 20%PEG concentration. HN84 has higher P5CS activity and lower ProDH activity, while HF46 has higher OAT activity. According to the proline content of the two varieties, the following assumptions were made: under mild stress, the medium drought-tolerant variety could rely on OAT to offset the lower levels of P5CS and ProDH to ensure the proline content; Under severe stress, the effect of P5CS and ProDH was amplified in drought-tolerant cultivars, while the effect of OAT was weakened relatively, so that drought-tolerant cultivars could accumulate more proline.

CONCLUSION

In this study, the results showed that the P5CS and OAT activity of HN84 and HF46 increased while the ProDH activity decreased. The proline content of the two cultivars also increased in different degrees. The proline content of the two cultivars increased continuously under mild drought (5%, 10%) degrees and showed a single peak curve change under severe stress (15%, 20%) degrees.

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