Response of antioxidant system to drought stress and re-watering in *Alfalfa* during branching

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**Abstract.** This paper aimed to reveal the response mechanism of active oxygen metabolism and antioxidant enzyme activities in *Alfalfa* under drought stress and re-watering, and the pot experiment was used, to explore the changes of H₂O₂, O₂⁻, electrolyte leakage conductivity and MDA, SOD, POD, CAT activity in Golden Empress (tolerant cultivar) and Sanditi (non-tolerant cultivar) under drought stress and re-watering during branching stage. Three water gradients were set up: CK (Maximum field capacity of 75%±5%), T1 (Maximum field capacity of 45%±5%), T2 (Maximum field capacity of 35%±5%) to compare, and the drought rehydration was also studied. Results: the results indicated that H₂O₂ content, O₂⁻ production rate, relative conductivity and MDA content were higher than the control, and the increase extent of Golden Empress was higher than the Sanditi under drought stress and after re-watering the recovery capability of Golden Empress was also higher than the Sanditi. After 7 days of re-watering, all indexes were restored to the control level, indicating that the re-watering have compensation effect after drought. After drought stress, to weaken the damage of active oxygen Golden Empress was mainly by increasing the activity of POD and SOD, but Sanditi was mainly through the POD and CAT activity increased to effectively remove ROS. Under drought stress, active oxygen in leaves of Alfalfa increased, and thus the membrane system was damaged which lead to the increase of MDA content and relative electric conductivity. Plants play a defensive role by increasing the activity of antioxidant enzymes and scavenging reactive oxygen species. After re-watering, the stress effect was reduced, and the physiological indexes of plants were restored to the control level. In general, tolerant cultivar has stronger antioxidant properties under drought and re-watering.

1. **Introduction**

Drought, as a worldwide problem restricting agricultural production, has been concerned by the world in recent years (Xing et al, 2016). Drought has become a major limiting factor for crop yield and quality because of climate warming, water scarcity, environmental degradation and many other reasons. Under drought stress, the balance of ROS generation and removal was destroyed, plant cells will produce large amounts of reactive oxygen species such as singlet oxygen (¹⁰O₂), superoxide anion (the O₂⁻), hydrogen peroxide (H₂O₂), hydroxyl radical (·OH). These reactive oxygen species can lead to a series of changes in physiological metabolism, such as membrane lipid peroxidation, toxicity of other cell components, and inhibition of enzyme activity that are detrimental to plant growth and development (Nxele et al, 2017). For drought, re-watering can cause changes in plant physiological mechanisms, such as restoring physiological metabolic activity and physiological damage,
compensating for physiological functions that are reduced by drought stress. Huang et al (2014) studies show that water stress made the poinsettia cell extravasation conductivity and H$_2$O$_2$ content increased significantly, drought resistant cultivars were restored to the level before stress after re-watering, the drought sensitive varieties were not restored. Yang et al (2013) research on Crown vetch showed that O$_2^\cdot$ increased with the degree of drought stress and after re-watering restored to the control level. Under drought stress, a large amount of reactive oxygen species accumulated and caused injury. The protective enzymes and antioxidants in plants can scavenge reactive oxygen species (Tian et al, 2015). Some scholars believed that drought leads to the decrease of SOD, CAT and POD activity and unsaturated fatty acid peroxidation in cell membranes induced by free radicals (Li et al 2016; Yan et al 2009). In order to keep the plant physiological balance, to protect them against the damage of reactive oxygen, plants positively regulate their physiological function under water stress and re-watering. In the long term evolution, plants formed a relatively perfect and complex antioxidant protection system to effectively resist water stress. Antioxidant enzymes can either direct or indirectly eliminate reactive oxygen species in cells, which protect cells from oxidative damage, and have a detoxifying effect on plants at the same time.

Alfalfa (Medicago sativa L.) has many advantages such as strong resistance, wide distribution, good quality, high economic value, known as "the king of grass", and it is the largest cultivated pasture in China. Therefore, under the background of implementing some ecological environment, such as returning farmland to grassland, it is of great significance to study the drought resistance mechanism of Alfalfa in order to improve the water use and preserve the ecological environment (Han et al, 2005). This study used two kind of alfalfa as experimental material, investigating the effects of drought and re-watering on Alfalfa antioxidant capacity during the branching stage, in order to study the molecular mechanism of crop adaptation to drought stress.

2. Materials and methods

2.1. Experimental design.
Alfalfa varieties: Golden Empress (tolerant cultivar) and Sanditi (non-tolerant cultivar), were used as experimental material. This study was conducted by pot experiment at the Agronomy College at Shenyang Agricultural University, under the outdoor natural conditions. The bottom diameter of the bucket is 28cm, and the height is 30cm. The soil was brown soil, and the maximum field water holding capacity was 25%. After soil drying, sieving, mixing with fertilizer (base fertilizer were urea 0.15g/kg soil, diammonium phosphate 0.10g/kg soil and potassium sulfate 0.15g/kg soil), each pot hold 15 kilograms of mixed soil. During irrigation, a PVC tube with a diameter of 2cm and a length of 35cm was inserted into each bucket. Each side of each tube was provided with 6 small holes for layering irrigation, and the spacing between the two adjacent holes was 5-6cm, and the part inserted into the soil was wrapped with two layers of gauze to prevent the soil from blocking the small holes. Set three moisture gradients:
- Control (CK): maximum field capacity of 75%±5%;
- Moderate stress (T1): maximum field capacity of 45% ± 5%;
- Severe stress (T2): maximum field capacity of 35% ± 5%.

Drought treatment was carried out in branching stage, 3 replicates per treatment, and soil relative water content was measured by weighing method and soil moisture meter. Add the loss of moisture at 18:00 every day. After 7 days of drought treatment, the water was recovered until the alfalfa was mature. Sampling was carried out on the same day, third days, fifth days and seventh days after re-watering. The standard of re-watering was consistent with the control group. In case of rainy days, all the samples were moved into the vinyl house. In addition to the water supply differences, other indicators are consistent.

2.2. Index determination.
The relative conductivity was measured by DDS-11A type conductivity meter; the content of
malondialdehyde (MDA) was determined by thiobarbituric acid method; the rate of superoxide anion radical (\( \text{O}_2^- \)) production was determined by hydroxylamine method; hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) content was measured by spectrophotometer; superoxide dismutase (SOD) was determined by nitrogen tetrazolium four (NBT) method; peroxidase (POD) activity was measured by guaiacol assay; the activity of catalase (CAT) was determined by UV spectrophotometry (Zou, 2000).

2.3. Statistics.
All statistical analyses were performed using Microsoft Excel 2010 and SPSS 22.0. One-way ANOVA analysis was applied to the same cultivar under different water treatments. Duncan multiple difference method was used to compare the differences. The significant difference among varieties was analysed by independent variance t test.

3. Result and analysis

3.1. Effect on the accumulation of activate oxygen
As shown in Figure 1, after drought stress for 7 days, the \( \text{H}_2\text{O}_2 \) content of the two varieties increased with the increase of stress level and the difference was significant (\( P<0.05 \)). The re-watering for 3 days, compared with CK, there was no significant difference between each treatment in Golden Empress, but two treatments of Sanditi were still significantly higher than the control. The re-watering for 7 days, the change of \( \text{H}_2\text{O}_2 \) content of the two varieties was \( T1 < CK < T2 \), which indicated after re-watering 7 days, two varieties \( \text{H}_2\text{O}_2 \) content under moderate water stress have been restored to the normal level, while severe stress has not been restored.

Under drought stress, \( \text{O}_2^- \) production rate of Golden Empress and Sanditi increased with the increase of stress level (Figure 1) and the difference between CK and T2 treatment was significant, but no significant difference between varieties. The re-watering for 3 days, the difference between Golden Empress treatments was not significant, while the Suntory T2 treatment was still significant difference with CK. At the 5th day after re-watering, all treatments of two varieties have returned to the control level, but under severe stress Sanditi had significant difference with Golden Empress.

![Figure 1](image1.png)

Figure 1 Variation of superoxide anion-producing rate and hydrogen peroxide content in alfalfa leaves under drought stress and re-watering. G: Golden Empress, S: Sanditi, 0: the 7 days of drought, 3: the 3 days of re-watering, 5: the 5 days of re-watering, 7: the 7 days of re-watering. The bar indicates mean ± SD (\( n=3 \)). Columns with different capital letters are statistically different (\( P<0.05 \)). “*” indicates the statistically different (\( P<0.05 \)) between two varieties.

3.2. Effect on membrane lipid peroxidation
Drought stress caused the relative conductivity of Golden Empress and Sanditi (\( P<0.05 \)) increased significantly (Figure 2). Compared with CK, with the increase of stress intensity, the difference between each treatment of Golden Empress was significant (\( P<0.05 \)), but no significant difference between T1 and T2 treatment of Sanditi. After 5 days of re-watering, all treatments of two varieties recovered to some extent. Compared with the stress for 7 days, Golden Empress two treatments were reduced by 19.2% and 37.9%; Sanditi decreased 14.8% and 14.9% respectively. The re-watering for 7
days, compared with CK, two varieties of all treatments have been restored to the normal level and Golden Empress appeared overcompensation. During rehydration, Sanditi relative conductivity was always higher than that of Golden Empress, and reached a significant level (P<0.05).

In branching period, drought stress and re-watering resulted in larger fluctuation of MDA content in two varieties, and the change of the same moisture gradient increased first and then decreased (Figure 2). Under drought stress, with the increase of stress level, MDA content of Golden Empress and Sanditi increased, and there was significant (P<0.05) difference between various treatments, but no significant (P>0.05) difference between varieties. With re-watering for 3 days compared to 5 days, two varieties MDA content decreased gradually, Golden Empress decreased 48.4% and 38.2%, and Sanditi dropped 53.6% and 52.8%, respectively, which Golden Empress had significant (P<0.05) differences between different treatments, but the difference was not significant (P>0.05) in Sanditi. After 7 days of re-watering, the two varieties recovered to the control level.

Figure 2 Variation of relative conductivity and MDA content in branching alfalfa leaves under drought stress and re-watering

3.3. Effect on the activity of protective enzymes in leaves

SOD is the first line of defence for plants to scavenge reactive oxygen species (ROS). As shown in Figure 3, the effects of drought on Golden Empress and Sanditi SOD activity: with the increase of stress intensity, SOD activity first increased and then decreased, between the same species and two

Figure 3 Variation of SOD, POD, CAT activity in branching alfalfa leaves under drought stress and re-watering
varieties of each treatment showed no significant (P>0.05) difference. After 3 days and 5 days re-watering, Golden Empress SOD activity had a certain degree recovery, and at the third days of re-watering SOD activity was the strongest. In the whole process Sanditi and the control had no significant (P>0.05) difference, indicated that changing the SOD activity was not the main measure of Sanditi to resist drought stress.

After 7 days of drought stress, POD activity of Golden Empress leaves increased 5.4% and 92.8% respectively, Sanditi leaves POD activity were decreased by 11% and 16.5%. After 3 days re-watering, compared with CK, the POD activity of T2 treatment had increased by 57%, Sanditi each treatment had no significant difference with CK. After 5 days re-watering, Golden Empress and Sanditi treatments were recovered to the control level. The results showed that the two alfalfa POD activity had obvious difference under drought stress and re-watering.

Compared with CK, after 7 days of drought stress, CAT activity of two cultivars were higher than that of control, the increase amplitude of Sanditi was significantly higher than that of Golden Empress, reached a significant level (P<0.05). The re-watering for 3 days, Golden Empress and Sanditi CAT activity in leaves were higher than CK, but the difference was not significant (P>0.05). It can be seen from Figure 6, after re-watering, POD of two alfalfa cultivars compared with the CK showed no significant (P>0.05) difference.

4. Discussion

4.1. Effect of drought stress and re-watering on activate oxygen metabolism of alfalfa
Drought stress increased the content of reactive oxygen species (H$_2$O$_2$ content and O$_2$$^•$ production rate) in alfalfa leaves, but recovered to control level after re-watering. The drought will destroy the structure and function of plant cell membrane system and membrane system is the first victim’s position (Sun et al, 2008). Under drought stress, compared with the control, the relative conductivity and MDA content of two varieties increased, indicating that drought stress caused damage to alfalfa. Compared with T1 treatment, T2 treatment of Golden Empress had just returned to the control level after the 7th days re-watering, and T2 treatment of Sanditi returned to the control level after the 5th days re-watering, illustrating that under severe stress, membrane lipid peroxidation in plants was more serious, so that the cell membrane system was damaged deeper. After re-watering, the cell membrane system gradually recovered to the level of CK, indicating that re-watering could compensate for the physiological functions of crops due to water stress (Tian et al 2015).

4.2. Effect of drought stress and re-watering on antioxidant defence system of alfalfa
After stress was released, the response of higher plants to re-watering was rapid growth in a short time to compensate for the losses caused by drought stress. This compensation effect is an adaptation to environmental change. Generally speaking, under drought stress, antioxidant enzyme activity in plants is positively related to the antioxidant capacity of plants. After drought stress, a large amount of reactive oxygen species (such as H$_2$O$_2$ content and O$_2$$^•$ ) are produced in the plant, which promoted the activity of antioxidant enzymes in the body. Under mild or moderate water stress, SOD activity showed an upward trend, and there was a downward trend under severe or long-term stress. At the same time, drought resistant varieties can maintain higher SOD activity, which is similar to the results of Ge et al (2005). Drought resistant varieties not only can maintain higher SOD activity to scavenge O$_2$$^•$ in the rehydration process, and POD, CAT and SOD corresponding synergy, to further clear the H$_2$O$_2$, to the maximum extent to avoid damage of active oxygen on cell membrane. In the branching stage of re-watering after 7 days, the POD activity recovery ability of Golden Empress was higher than that of Sanditi, which was similar with changes of CAT. In general, drought resistant varieties have stronger antioxidant capacity during drought and re-watering (Wang et al, 2012).

5. Conclusion
(1) After drought stress, H$_2$O$_2$ content, O$_2$$^•$ production rate, relative conductivity and MDA content of
two alfalfa varieties leaves increased with the increase of stress degree. After 3 days re-watering, the stress effect still existed, but with the extension of rehydration time, the stress effect was alleviated.

(2) Under drought stress and re-watering, \( \text{H}_2\text{O}_2 \) content and \( \text{O}_2^* \) production rate in Golden Empress leaves were lower than that of Sanditi, indicated that Golden Empress had stronger drought resistance than Sanditi.

(3) Under drought stress, antioxidant enzymes activities in alfalfa increased so that the antioxidant mechanism of two kinds of alfalfa was different, to reduce the damage of active oxygen Golden Empress mainly by increasing POD and SOD activities, Sanditi was mainly through the POD and CAT activities increased to effectively remove ROS.

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