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# Physiological Evaluation of Drought Resistance in Five Alfalfa (*Medicago sativa* L.) Cultivars using PEG-induced Stress

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## ABSTRACT

**Background:** Screening drought-resistant alfalfa cultivars is crucial for sustainable forage production in semi-arid and arid regions. To investigate the effects of drought stress on the physiological characteristics of alfalfa (*M. sativa* L.) seedlings and to screen drought-resistant alfalfa varieties, this experiment used five alfalfa cultivars as materials and conducted germination tests under four polyethylene glycol (PEG)-6000 concentrations (0%, 5%, 10% and 20%).

**Methods:** The physiological indicators of the seedlings treated with PEG-6000, including free proline (Pro), soluble protein (SP), soluble sugar (SS), malondialdehyde (MDA), superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT), were measured and analyzed.

**Result:** The results showed that drought stress significantly affected the physiological characteristics of the five alfalfa cultivars. As the drought stress intensified, except for Pro and SP content, the five physiological indicators of WL363HQ showed an increasing trend; the changes in physiological indicators of WL168HQ, WL440HQ, Baimu 202 and Kehan under different stress concentrations exhibited significant variability. The comprehensive evaluation using the membership function method indicated that WL363HQ possessed superior drought resistance compared to the other cultivars. The findings of this study will provide theoretical basis for research on the drought resistance mechanisms of alfalfa, the breeding of new drought-resistant varieties and the cultivation of alfalfa cultivars in semi-arid and arid regions.

**Key words:** Alfalfa, Antioxidant enzymes, Drought stress, Membership function, Osmotic stress, Physiological indicators.

## INTRODUCTION

Alfalfa is perennial leguminous crop characterized by high biomass yield, strong adaptability, strong regenerative capacity and high nutritional value (Fan *et al*., 2025). It is acclaimed as the "King of Forage" (Sun *et al*., 2025). In China, the distribution of alfalfa is primarily in the northern regions, including Northwest, North and Northeast (Li and Liu, 2024). By the end of 2022, the preserved area of alfalfa is 27.15 million $hm^2$. The provinces with larger planting areas are Gansu, Shanxi, Xinjiang, Ningxia and Inner Mongolia, accounting for 91.2% of the total area (National Livestock Station, 2022).

Rainfall is scarce and water resources are limited in northern China, particularly in the northwest region (Zhang *et al*., 2022; Miao *et al*., 2024). Drought has led to reduced yields of alfalfa, causing economic losses and also restricting the improvement of its productivity and the expansion of its planting area (Liu *et al*., 2018; Varol *et al*., 2024). The growth, development and reproduction of plants require adequate supply of water (Seleiman *et al*., 2021). Approximately one-third of the global land is semi-arid or arid, while the majority of the other land also frequently experiences periodic climate droughts (Gebrechorkos *et al*., 2025). Among various abiotic stresses, drought is one of the most severe constraints that restrict the growth and development of plants. Depending on the duration and intensity, as well as the stage of plant development, drought can impact on the productivity of crops more than other environmental factors (Anjum *et al*., 2017). The mechanisms of drought resistance involve multiple aspects, including physiology, molecule and root structure, *etc* (Gupta *et al*., 2020). The physiological indicators include Pro, SS, SP, MDA, SOD, POD, CAT (Aili *et al*., 2023; Chen *et al*., 2024; Bai *et al*., 2025). Previous research have shown that with the intensification of drought stress, the values of the aforementioned physiological indices in alfalfa generally show an increasing trend (Wang *et al*., 2024; Sun *et al*., 2025). These indicators comprehensively reflect the physiological response and drought resistance capacity of alfalfa under drought conditions.

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The research on the drought resistance of alfalfa mainly focuses on the effects of single variety and limited stress concentration on the physiological indicators. The novelty of the present study lies in its comprehensive comparison of five distinct alfalfa cultivars subjected to a gradient of four PEG-6000 concentrations, simulating a broader range of drought intensities. This multi-cultivar, multi-stress-level approach enables a more robust evaluation of varietal differences in drought tolerance mechanisms.

The relevant physiological indicators including osmotic regulatory substances (Pro, SS, SP), membrane stability (MDA) and antioxidant systems (SOD, POD, CAT) were determined. Furthermore, the physiological responses and differences of five alfalfa varieties under simulated drought stress during the seed germination were compared. Our findings aim to provide valuable insights for selecting and breeding drought-resistant alfalfa varieties suitable for cultivation in water-limited environments.

## MATERIALS AND METHODS

### Plant materials

Five alfalfa samples including *M. sativa* L. cv. WL168HQ, *M. sativa* L. cv. WL363HQ, *M. sativa* L. cv. WL440HQ, *M. sativa* L. cv. Baimu 202 and *M. sativa* L. cv. Kehan were selected. The names and sources of tested materials are shown in Table 1.

### Experimental design

For each alfalfa variety, a certain amount of seeds with full particles and no pests were selected. All tesed seeds were sterilized by $H_2O_2$ for 15 minutes, washed by distilled water for 3 to 5 times and then, placed in petri dishes lined with double-layer filter paper. 50 seeds were placed in each dish and cultivated in light incubator for 7 days. The growth conditions in the incubator were maintained at a constant temperature of 25±1°C, with a 12/12 h light/dark photoperiod and relative humidity of 60%. The germination of seeds was observed and recorded regularly every day and distilled water was supplemented by weighing method to constant weight. After 7 days of cultivation, at least 0.5 g of well-growing germinating seedlings were selected from each dish for the determination of physiological indexes. The experiment set up control group (CK) and PEG-6000 stress treatment group, including mild stress (LD, 5% PEG-6000), moderate stress (MD, 10% PEG-6000) and severe stress (SD, 20% PEG-6000). Each of the four treatment groups for every cultivar was replicated three times (n=3).

### Determination of physiological indicators

The content of SP was determined using the BCA protein content kit. The content of SS was detected using the plant SS content detection kit. The content of Pro was determined using the PRO content kit. The content of MDA is determined by using the MDA content kit. The SOD activity was determined by the SOD-WST-8 method kit. CAT activity was detected using the CAT detection kit. POD activity was determined using POD kit. The measurements of the mentioned physiological indices were conducted from september 2023 to march 2024 by standard testing group Co., Ltd.. The comprehensive analysis of drought resistance was conducted by applying the fuzzy mathematics membership function method (Jia *et al*., 2025). The membership function values of the physiological indicators positively correlated with the drought resistance are calculated as follows:

$$F_{(ij)} = \frac{[X_{(ij)} - X_{(imin)}]}{[X_{(imax)} - X_{(imin)}]}$$

Where

$F_{(ij)}$ = Membership function value of the j index of i-alfalfa.

$X_{(ij)}$ = Represents the j index value of the i-alfalfa variety.

$X_{(imax)}$ and $X_{(imin)}$ = Maximum and minimum values of the j index of the i-alfalfa variety, respectively.

The membership function values of each physiological index of the i alfalfa variety are added up and the average value is calculated. The larger the average value, the stronger the drought resistance of the alfalfa variety.

### Statistical analysis

Excel 2010 software was conducted for data processing and SPSS Statistical Software (version 26.0; SPSS Institute Ltd, USA) was used for analysis of variance and significance. The results were expressed as "mean ± standard deviation". $P$ value <0.05 indicated significant difference, while $P$ value >0.05 indicated insignificant difference.

## RESULTS AND DISCUSSION

### Effects of drought stress on osmotic adjustment substances of alfalfa seedlings

The responses of osmotic adjustment substances revealed clear, cultivar-specific strategies to drought stress (Table 2-4). A particularly notable pattern was observed in Pro content. Under MD stress, the Pro content of WL440HQ was significantly lower than that of CK ($P$<0.05), reducing by 54.59%. The Pro content of WL363HQ and WL440HQ was significantly lower than that of WL168HQ ($P$<0.05), with an average reduction of 54.28%. The most striking pattern in SP was displayed by Baimu 202, which showed a significant higher under MD stress. At this stress, the SP content of Baimu 202 was significantly higher than that of CK, LD and SD stress ($P$<0.05), with average increase of 33.80% and significantly higher than that of WL168HQ and WL363HQ ($P$<0.05), with average increase of 31.31%. Under LD stress, the SS content of WL168HQ was significantly higher than that of the other three varieties except Baimu 202 ($P$<0.05), with average increase of

**Table 1:** Name and origin of 5 alfalfa cultivars.

| Cultivars | Fall dormancy | Origin country |
|---|---|---|
| WL168HQ | 2.0 | America |
| WL363HQ | 5.0 | America |
| WL440HQ | 6.0 | America |
| Baimu 202 | 2.2 | America |
| Kehan | 3.0 | Canda |

32.99%. Under SD stress, the SS content of WL363HQ was significantly higher than that of CK ($P$<0.05), which increased by 27.07%.

Osmotic adjustment is the adaptive mechanism of plants against drought stress (Haghpanah *et al*., 2024; Thomas and Beena, 2024). The content of osmotic adjustment substances (Pro, SS and SP) in alfalfa was changed by drought stress. These substances can increase the solute concentration of plant cells, maintain a certain swelling pressure, stabilize cell osmosis and reduce the loss of water in cells (Feng *et al*., 2024; Zhao *et al*., 2024). Fifteen-day exposure to -1.2 MPa PEG-6000 significantly elevated SP, SS, MDA and CAT activity in both drought-tolerant Longzhong and drought-sensitive Gannong No. 3 seedlings (Zhang and Shi, 2018). The content of SP, SS, Pro increased in Qingshui (QS), Longdong (LD) and Gongnong NO.4 after medium PEG stress and severe PEG stress (Wang *et al*., 2024). In this experiment, the contents of Pro, SS and SP were different among five varieties. Under LD and MD stress, the Pro content of WL168HQ increased. The SS content of WL168HQ, WL363HQ and WL440HQ increased under all drought stress compared to CK. Under SD stress, the SP content of WL168HQ increased and the SS content of Kehan increased. The content of Pro and SS of Baimu 202 increased under LD stress. The SP content of Baimu 202 increased under MD stress.

**Effects of drought stress on membrane system of alfalfa seedlings**

The extent of membrane damage, indicated by MDA content, was clearly separated in five cultivars (Table 5). Under MD and SD stress, the MDA content of WL363HQ was significantly higher than that of CK ($P$<0.05), with average increase of 155.04%. Under different drought stress levels, the MAD content among the five varieties did not change significantly.

MDA is frequently used biomarker of lipid peroxidation, its content indicates the extent of membrane lipid peroxidation (Del Rio *et al*., 2005; Tsikas, 2017). Under drought stress, reactive oxygen species (ROS) can react with membrane lipid peroxidation and produce toxic substance MDA, which destroys cell structure and affects cell metabolic function (Ayala *et al*., 2014., Baroowa *et al*., 2017). MDA in drought-stressed plants rose markedly. In drought-stressed plants, salicylic acid (SA) application

**Table 2:** Effects of drought stress on Pro content of different alfalfa varieties.

| Cultivars | The Pro content under drought stress ($\mu g \cdot mg^{-1}$) | | | |
|---|---|---|---|---|
| | CK | LD | MD | SD |
| WL168HQ | 34.36±17.34Aa | 37.64±29.86Aa | 37.07±10.61Aa | 33.93±4.23Aa |
| WL363HQ | 32.20±14.57Aa | 23.60±14.38Aa | 16.42±6.48Ba | 31.99±13.44Aa |
| WL440HQ | 38.01±6.06Aa | 21.97±13.15Aab | 17.26±2.36Bb | 27.32±12.71Aab |
| Baimu202 | 35.70±20.08Aa | 36.04±22.28Aa | 21.82±15.25ABa | 21.33±11.93Aa |
| Kehan | 34.54±7.86Aa | 30.03±26.00Aa | 21.06±8.62ABa | 27.77±12.16Aa |

Note: CK: Control; LD: Mild drought; MD: Medium drought; SD: Severe drought.

Different capital letters within the same column indicate significant difference between different varieties with the same treatment at the 0.05 level;different lowercase letters within the same row indicate significant difference between different treatment at the 0.05 level. This is applicable for the following tables as well.

**Table 3:** Effects of drought stress on SP content of different alfalfa varieties.

| Cultivars | The SP content under drought stress ($mg \cdot g^{-1}$) | | | |
|---|---|---|---|---|
| | CK | LD | MD | SD |
| WL168HQ | 14.05±2.21Aa | 11.63±2.47Aa | 11.20±2.53Ba | 14.25±3.88Aa |
| WL363HQ | 15.14±2.19Aa | 12.42±3.62Aa | 12.54±0.78Ba | 12.00±2.12Aa |
| WL440HQ | 12.87±1.91Aa | 14.59±4.86Aa | 14.56±3.78ABa | 12.48±3.39Aa |
| Baimu202 | 13.17±3.23Ab | 10.62±0.64Ab | 17.28±0.60Aa | 11.81±0.81Ab |
| Kehan | 14.33±0.18Aa | 14.37±4.70Aa | 15.03±2.21ABa | 12.32±1.96Aa |

**Table 4:** Effects of drought stress on SS content of different alfalfa varieties.

| Cultivars | The SS content under drought stress ($mg \cdot g^{-1}$) | | | |
|---|---|---|---|---|
| | CK | LD | MD | SD |
| WL168HQ | 1.79±0.14Aa | 2.92±0.77Aa | 2.50±0.77Aa | 2.00±0.29Aa |
| WL363HQ | 1.81±0.06Ab | 1.97±0.40Bab | 2.48±0.45Aab | 2.30±0.12Aa |
| WL440HQ | 1.95±0.41Aa | 1.98±0.46Ba | 2.12±0.84Aa | 2.48±0.73Aa |
| Baimu202 | 2.41±1.03Aa | 2.64±0.16ABa | 1.83±0.29Aa | 2.22±0.42Aa |
| Kehan | 2.18±0.16Aa | 1.92±0.39Ba | 1.86±0.37Aa | 2.55±0.83Aa |

markedly reduced MDA content from day 7, reaching in the lowest levels by day 14. In contrast, well-irrigated SA-treated plants maintained a stable MDA concentration (17 nmol·g$^{-1}$ fresh weight) (González-Villagra *et al*., 2022). Three alfalfa cultivars, including Longzhong (drought-tolerant), Longdong (moderately tolerant) and Gannong No. 3 (sensitive), were compared after 12 d of PEG-6000 stress at physio-biochemical levels. Prolonged stress markedly triggered a pronounced rise in MDA accumulation (Zhang *et al*., 2019). This study showed that the MDA content of WL363HQ and Baimu 202 increased with the increase of drought stress.

**Effect of drought stress on enzyme activity of alfalfa seedlings**

The content of SOD, POD, CAT in five alfalfa varieties changed greatly (Table 6-8). Under MD stress, the SOD content of Baimu 202 was 6.80 U·mg$^{-1}$, which was significantly lower than that of WL168HQ (12.19 U·mg$^{-1}$, $P$<0.05) and decreased by 44.22%. The POD content of WL168HQ under MD stress was the highest, which was significantly higher than that under SD stress ($P$<0.05) and increased by 64.94%. The CAT content of Baimu 202 (856.75 U·mg$^{-1}$) under LD stress was significantly higher than that under MD and SD stress ($P$ < 0.05), with an average increase of 40.67% and significantly higher than that in WL440HQ (411.41 U·mg$^{-1}$), increased by 51.98%.

Under drought stress, plants activate its antioxidant enzyme defense system to cope with oxidative stress. Studies have shown that the activities of antioxidant enzymes such as CAT, SOD and POD are increased, so as to eliminate ROS accumulated due to drought stress, thus maintaining the redox balance of cells and protecting cell membrane systems and biological macromolecules from oxidative damage (Kapoor *et al*., 2020; Rao *et al*., 2025; Srabon *et al*., 2025). This adaptive response is one of the important physiological mechanisms for alfalfa to cope with drought stress. Under drought stress, the activities of CAT, SOD and POD of Zhongmu No.3 increased to varying degrees, thus eliminating ROS in the body (Zhang *et al*., 2024). The activities of POD, CAT and SOD of 12 alfalfa seedling exposed to PEG-6000 exhibited initial up-regulation, followed by a gradual decline that varied with varieties and stress levels (Li *et al*., 2016). In this study, with the aggravation of drought stress, the SOD of WL363HQ and WL440HQ increased, while that of Baimu 202 decreased. Under LD stress, SOD of Kehan increased. Under MD and SD stress, SOD of WL168HQ increased. Under different degrees of drought stress, the POD activities of WL363HQ and Kehan showed upward trend. CAT activity of WL168HQ, WL363HQ and Kehan was higher than that of CK under drought stress. Under LD stress, Baimu 202 had the highest CAT activity. CAT activity of WL440HQ was higher under MD and SD stress.

**Table 5:** Effects of drought stress on MDA content of different alfalfa varieties.

| Cultivars | The MDA content under drought stress (mg·g$^{-1}$) | | | |
|---|---|---|---|---|
| | CK | LD | MD | SD |
| WL168HQ | 15.53±2.35Aa | 10.20±3.53Aa | 14.04±4.99Aa | 14.18±9.32Aa |
| WL363HQ | 5.56±2.74Cb | 14.86±4.29Aa | 9.98±4.35Aab | 13.50±2.09Aa |
| WL440HQ | 15.64±1.78Aa | 16.39±9.15Aa | 9.82±7.83Aa | 11.81±3.39Aa |
| Baimu 202 | 8.85±1.93BCa | 15.11±6.07Aa | 11.00±2.44Aa | 9.67±3.04Aa |
| Kehan | 13.63±4.43ABa | 12.49±12.60Aa | 8.46±4.37Aa | 8.00±1.19Aa |

**Table 6:** Effects of drought stress on SOD content of different alfalfa varieties.

| Cultivars | Content of SOD under drought stress (U·mg$^{-1}$) | | | |
|---|---|---|---|---|
| | CK | LD | MD | SD |
| WL168HQ | 9.88±0.54Aa | 9.11±2.24Aa | 12.19±3.11Aa | 10.35±1.73Aa |
| WL363HQ | 5.02±0.65Aa | 7.46±1.48Aa | 8.96±1.87ABA | 6.92±4.13Aa |
| WL440HQ | 6.40±3.63Aa | 10.12±6.46Aa | 8.48±1.98ABA | 8.99±1.36Aa |
| Baimu 202 | 8.86±4.83Aa | 6.08±1.24Aa | 6.80±2.36Ba | 7.37±0.45Aa |
| Kehan | 9.07±1.08Aa | 11.28±4.99Aa | 8.42±2.41ABA | 8.77±2.71Aa |

**Table 7:** Effects of drought stress on POD content of different alfalfa varieties.

| Cultivars | POD content under drought stress (U·mg$^{-1}$) | | | |
|---|---|---|---|---|
| | CK | LD | MD | SD |
| WL168HQ | 2191.45±728.55Aab | 2471.49±1149.34Aab | 3980.31±1601.50Aa | 1395.55±649.69Ab |
| WL363HQ | 2050.63±1440.62Aa | 3909.55±753.43Aa | 3133.97±1818.25Aa | 3815.66±2042.30Aa |
| WL440HQ | 2803.74±1321.19Aa | 2292.11±720.18Aa | 2240.66±1526.20Aa | 2723.34±1293.63Aa |
| Baimu 202 | 3946.55±2194.00Aa | 2773.49±2178.08Aa | 2022.67±318.49Aa | 2348.85±1866.61Aa |
| Kehan | 2228.90±711.54Aa | 2821.94±3211.07Aa | 2419.04±1063.46Aa | 2739.52±696.15Aa |

**Table 8:** Effects of drought stress on CAT content of different alfalfa varieties.

| Cultivars | CAT content under drought stress (U·mg$^{-1}$) | | | |
|---|---|---|---|---|
| | CK | LD | MD | SD |
| WL168HQ | 540.94±35.58ABA | 711.98±210.29ABA | 560.78±182.15Aa | 580.90±177.11Aa |
| WL363HQ | 451.15±114.19ABA | 576.95±99.47ABA | 564.73±32.27Aa | 584.13±286.00Aa |
| WL440HQ | 539.69±58.28ABA | 411.41±145.85Ba | 552.78±248.65Aa | 587.94±198.76Aa |
| Baimu 202 | 637.73±177.33Ab | 856.75±86.04Aa | 471.27±70.33Ab | 545.38±19.04Ab |
| Kehan | 429.30±71.40Ba | 531.53±389.37ABA | 448.71±67.50Aa | 690.82±220.86Aa |

**Table 9:** Comprehensive evaluation indices of alfalfa cultivars.

| Cultivars | Pro | SP | SS | MDA | SOD | POD | CAT | D value | Ranking |
|---|---|---|---|---|---|---|---|---|---|
| WL168HQ | 0.491 | 0.519 | 0.454 | 0.617 | 0.413 | 0.431 | 0.337 | 0.466 | 4 |
| WL363HQ | 0.610 | 0.326 | 0.493 | 0.582 | 0.525 | 0.633 | 0.740 | 0.559 | 1 |
| WL440HQ | 0.428 | 0.543 | 0.344 | 0.547 | 0.564 | 0.487 | 0.632 | 0.506 | 3 |
| Baimu 202 | 0.503 | 0.390 | 0.549 | 0.369 | 0.431 | 0.390 | 0.406 | 0.434 | 5 |
| Kehan | 0.541 | 0.625 | 0.425 | 0.470 | 0.337 | 0.803 | 0.366 | 0.509 | 2 |

### Evaluation of drought resistance ability of five alfalfa varieties

This study found that the changes of osmotic adjustment substances, membrane system and antioxidant enzyme activities of five alfalfa varieties under different drought stress degrees, which indicated that the adaptation of alfalfa to drought stress was affected by varieties and stress degrees. The drought resistance of five alfalfa varieties was evaluated by membership function method (Table 9). The order of drought resistance of five alfalfa varieties was WL363HQ > Kehan > WL440HQ > WL168HQ > Baimu 202. WL363HQ cultivar serves as an excellent germplasm resource for breeding programs aimed at improving drought tolerance.

### Practical implications

While this study clarifies the physiological basis of drought resistance, it provides deeper investigation of physiological data with molecular analyses. The findings of this study have direct applications for alfalfa breeding and management in water-limited environments.

## CONCLUSION

The effects of drought stress on physiological indexes of alfalfa seedlings including Pro, SP, SS, MAD, SOD, POD and CAT were studied. The results showed that under a certain concentration of drought stress, the above physiological indexes of some alfalfa varieties showed an upward trend, so as to enhance the adaptation to drought stress. There are differences in drought resistance among five alfalfa varieties and WL363HQ has the strongest drought resistance. Baimu 202 had the weakest drought resistance.

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### Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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