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Response of Caucasian Clover to Waterlogging Stress at Seedling Stage

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ABSTRACT

Background: Caucasian clover is a highly resilient leguminous forage species. It can endure prolonged periods in waterlogged settings. Examining the resilience of Caucasian clover under these circumstances is of considerable significance. In this work, the response of Caucasian clover to waterlogging stress during the seedling stage was investigated and the associated morphological changes and physiological mechanisms enabling its adaptation to inundated environments were analyzed.

Methods: The study was conducted in an artificial climate chamber from 2023 to 2024. Waterlogging stress was initiated after the clover seedlings had grown for 30 days and was maintained for 28 days. Plant growth was monitored at 7-day intervals and leaf and root samples were collected to assess physiological indices.

Result: The results demonstrated that after being subjected to waterlogging stress, Caucasian clover adapted morphologically by altering its root structure. Physiologically, Caucasian clover increased the activity of antioxidant enzymes such as peroxidase and catalase, which scavenged reactive oxygen species (ROS) produced by stress. The activity of the anaerobic respiratory enzyme alcohol dehydrogenase was also elevated, promoting ethanol fermentation and supplying a small amount of adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NAD^+) to the plant. The plant also increased the content of osmotic substances, including soluble sugars and proteins, to maintain cellular osmotic pressure, prevent cellular content leakage and protect plant cells.

Key words: Caucasian clover, Morphological differences, Physiological changes, Seedling stage, Waterlogging stress.

INTRODUCTION

Waterlogging poses a significant threat to most plants in agricultural and livestock production. Certain forage species exhibit high sensitivity to flooding catastrophes. Their survival rates diminish under excessive moisture conditions, leading to substantial yield reductions. In waterlogged conditions, the underground root system experiences prolonged oxygen deficiency, which impairs respiration. This restriction hinders the growth of the above-ground parts, disrupts the transport of essential substances within the plant, inhibits dry matter accumulation and ultimately impacts plant growth, development and yield. In recent years, increased rainfall due to global warming has exacerbated natural disasters like heavy rainfall and waterlogging. Therefore, selecting flood-tolerant varieties for planting in high-precipitation areas can reduce agricultural production losses and benefit regional livestock development (Heidrun *et al*., 2008).

Extended exposure to flooded environments induces considerable morphological and structural alterations in plants. Notably, there is a significant thickening of the basal stems, enlarged development of lenticels and the emergence of adventitious roots at the stem bases. Waterlogged conditions obstruct the formation of new leaves, diminish the size of leaf blades and precipitate a marked reduction in leaf area (Saikia *et al*., 2021). Stomatal closure, accelerated chlorophyll degradation, enhanced leaf senescence and increased yellowing diminish the leaves' capacity for light capture, thereby leading to a decline in photosynthetic activity and rapid wilting (Yan *et al*., 2018).

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The most severe impact of waterlogging is on the root system. The architecture of plant root systems is both flexible and dynamic (Amarapalli, 2022). In such conditions, roots become shallower, the growth of root hairs and primary roots ceases and in non-tolerant plant varieties, the roots progressively darken, decay and emit a foul odor, often culminating in plant mortality. Conversely, varieties that are resilient to flooding are capable of developing lateral and adventitious roots, thereby adapting to the inundated environment (Sharmin *et al*., 2024).

Under waterlogging stress, plants self-regulate through antioxidant enzyme systems, which are vital (Hou *et al*., 2024). Plants produce a large amount of reactive oxygen species (ROS) in conditions of excess water and the accumulation of excessive ROS damages plant cells, leading to injury or even death. The antioxidant enzyme system plays a crucial role in helping plants adapt to such

environments. Peroxidase (POD) serves multiple roles in plants; it scavenges H_2O_2 produced due to stress, with its activity increasing in the early stages to provide protection. POD is involved in the degradation of chlorophyll in leaves and can serve as an indicator of plant senescence when its activity decreases. Catalase (CAT), which is widely distributed in plants, catalyzes the conversion of H_2O_2 to H_2O without requiring other energy sources. Its activity is associated with the plant's capacity to withstand unfavorable environments and increasing antioxidant enzyme activity enhances the plant's stress resistance (Wang *et al.*, 2019). Oxygen deficiency in the soil prevents normal respiration, causing plants to switch to anaerobic respiration in a flooded environment. Alcohol dehydrogenase (ADH), the key enzyme in the ethanol fermentation pathway of anaerobic respiration, reduces acetaldehyde to ethanol, providing some energy to temporarily mitigate the scarcity of energy supply due to oxygen deprivation (Borella *et al.*, 2019). Osmotic substances play a protective role when plants are subjected to stress. Plants actively accumulate these substances, including soluble proteins (SP) and soluble sugars (SS), to regulate cellular osmotic pressure, maintain water balance within cells and protect cell membrane structure (Kumari *et al*., 2015).

Caucasian clover (*Trifolium ambiguum* Bied.), also known as kura clover or honey clover, is a perennial leguminous forage grass. Native to the Caucasus Mountains, it is widely distributed across various altitudes and environments, including Crimea, eastern Turkey and northern Iran (Liu *et al.*, 2023). Caucasian clover plants are relatively low-growing, with a well-developed primary root and a robust underground tiller system. The dense tiller system stores large amounts of metabolic energy, enabling continuous growth in pastures. Adapted to the harsh conditions of its native range, Caucasian clover exhibits excellent stress tolerance, along with a long lifespan and high biomass (Jarvis *et al.*, 2008; Zhang *et al.*, 2022). In recent decades, research on Caucasian clover's stress tolerance has primarily focused on drought and cold resistance. However, its tolerance to waterlogging is also noteworthy. In coastal regions such as Canberra and New Zealand, Caucasian clover exhibits significant waterlogging tolerance, surviving up to six weeks under such conditions (Oram, 2005). Based on the above background, this study analyzed the morphological and physiological responses of Caucasian clover under waterlogging stress during the seedling period, providing references for the future promotion and selection of new varieties.

MATERIALS AND METHODS

Materials for testing and experimental design

The test material was the Caucasian clover variety named "*Trifolium ambiguum* Bieb. cv. Mengnong No.1", bred by Inner Mongolia Agricultural University. Clover seeds were sterilized in 75% ethanol for 30 seconds. The sterilized seeds were then uniformly arranged in Petri dishes lined with two layers of filter paper and placed in an incubation room at 25°C, where they were subjected to alternating dark and light conditions to promote germination. Healthy seedlings of 0.5-1 cm were selected and transplanted into pots containing nutrient soil at five days. The seedlings were allowed to grow in the pots for 30 days before being subjected to waterlogging. The plants were watered every 3 days with a 1/4 strength Hoagland nutrient solution during this period.

The waterlogging stress test simulated soil waterlogging using the double-pot method. The inner pot holds clover, while the outer pot acts as a water reservoir. This configuration enables the moisture in the inner pot to permeate the soil effectively. Both pots have a diameter of 16.8 cm. Nevertheless, the inner pot has a height of 12 cm, in contrast to the outer pot, which measures 14.5 cm in height, with two treatment levels: the control (CK) group and the waterlogging (WS) group. The CK group maintained the soil's relative water content at 70%-80% of the field's water-holding capacity, while the WS group controlled the water level to approximately 2 cm above the soil surface. A total of 60 pots were used, with each pot of seedlings watered to saturation before the test began. Soil moisture content was kept consistent across all pots. Once waterlogging stress treatment began, water levels were controlled continuously for 28 days. The CK group was watered every 3 days, while the WS group was rehydrated daily to maintain the water level at no more than 2 cm above the soil surface.

The experiment was carried out in the Key Laboratory of Grassland Resources, Ministry of Education of College of Grassland Science, Inner Mongolia Agricultural University from 2023 to 2024.

Methods of measurement of indicators

During the waterlogging stress period, photos were taken to document the growth conditions of the plants. Roots and leaves from five pots of Caucasian clover seedlings were randomly selected and sampled on the 0^{th}, 7^{th}, 14^{th}, 21^{st} and 28^{th} days of the stress treatment to determine physiological indices.

Data processing and analysis

The data were organized and analyzed using Excel 2019 and SPSS 27, with plots generated in Origin 2021. A t-test was used to compare the physiological data under different treatments, with a significance level set at $p<0.05$.

RESULTS AND DISCUSSION

Effects of waterlogging stress on the morphology of Caucasian clover

When plants are subjected to stress, changes in external morphology are often more apparent. Clover in WS group continued to grow under waterlogging conditions, however,

the color of leaves gradually turned yellow as waterlogging persisted. By 28 days, the leaf color in the WS group was noticeably different from that in CK group, although no significant difference was observed in the overall morphology of the above-ground parts. When plants are subjected to waterlogging stress, their growth morphology changes significantly. The chlorophyll content in the leaves gradually decreases due to environmental impacts during waterlogging. This decrease in chlorophyll directly leads to a change in leaf color, gradually shifting from green to yellow (Fig 1).

In this study, the color of Caucasian clover leaves waterlogged for 28 days began from green to yellow compared to those not waterlogged. This lightening may be due to the production of free radicals and subsequent damage to the cell membrane system caused by waterlogging stress, leading to the decomposition of chlorophyll. These findings are consistent with the results obtained by Yin *et al.* (2022) regarding the effects of waterlogging stress on myrtle leaves.

Root morphology of Caucasian clover after 28 days of waterlogging stress. The main root length in the WS group was significantly shorter than in the CK group, with more lateral roots observed in the WS group and fewer in the CK group. The root morphology of the two groups differed significantly (Fig 2).

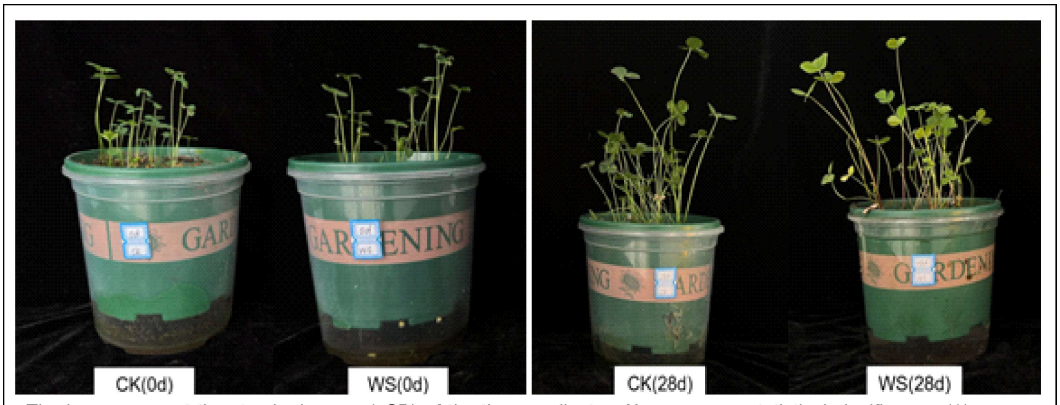
The root system is highly sensitive to soil water content. In this study, Caucasian clover slowed the growth of its main root, increased the number of lateral roots and formed adventitious roots to enhance contact with the soil after 28 days of waterlogging stress. Zhang (2007) found that different plants exhibit distinct morphological changes in their root systems under flooded conditions. The aeration tissues of wild soybean, red clover and white clover develop further to facilitate gas exchange in flooded environments under waterlogging stress. In contrast, mung bean adopts a different strategy under waterlogging by developing a large number of new lateral roots. These newly developed lateral roots not only increase the contact area between the root system and the soil, allowing for greater absorption of oxygen and nutrients, but also partially replace the function of the original root system, thereby helping mung bean maintain normal physiological activities under flooded conditions. Xiong (2020) found in her study on red clover that waterlogging-tolerant varieties maintained normal physiological activities under flooded conditions by increasing the number of lateral roots and promoting root growth near the soil surface. The findings of Zhang and Xiong are consistent with the results of this experiment.

Effect of waterlogging stress on the POD and CAT activity of Caucasian clover

The POD activity in the leaves of the WS group increased by 56.28%, 83.54% and 117.98% at 7, 14 and 21 days, respectively, compared to the CK group after the waterlogging treatment. The POD activity in the WS group reached its peak at 21 days, showing the most significant difference compared to the CK group. POD activity in the WS group showed a slight decrease but remained 69.11% higher than that of the CK group by 28 days. Overall, the POD activity in the WS group's leaves exhibited an increasing trend followed by a slight decrease (Fig 3a).

The POD activity in the roots of the WS group reached its maximum at 7 days, which was 111.96% higher than that of the CK group, a significant difference. It decreased at 14 and 21 days but remained 44.98% and 46.41% higher than CK, respectively. There was a slight increase at 28 days, with POD activity being 45.83% higher than CK. The overall trend of POD activity in the roots of the WS group showed an initial increase, followed by a decrease and then a slight increase over time (Fig 3b).

The CAT activity in the leaves of the WS group showed an increasing trend at 7 and 14 days. At 7 days, the CAT activity in the WS group increased by 90.91% compared to the CK group. By 14 days, CAT activity reached its maximum, showing a 200.00% increase compared to the CK group, a more significant difference. CAT activity in the



The bars represent the standard errors (±SD) of the three replicates. Ns means no statistical significance, *** means $p<0.001$, ** means $p<0.01$, * means $p<0.05$.

Fig 1: Morphological alterations and leaf color variations of Caucasian clover in response to waterlogging stress from zero to 28 days.

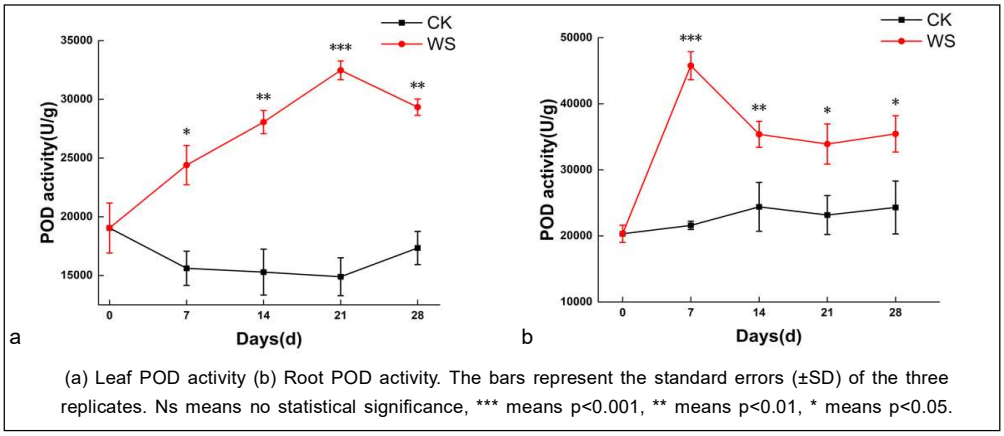
WS group showed a decreasing trend, but it remained higher than that of the CK group after 14 days. CAT activity in the WS group was 33.33% and 15.39% higher than the CK group at 21 and 28 days, respectively. Overall, CAT activity in the WS group's leaves exhibited an increasing trend followed by a decrease (Fig 4a).

The trend of CAT activity in the roots of the WS group was similar to that in the leaves, with an increasing trend observed at 7 and 14 days, showing increases of 65.22% and 208.33% compared to the CK group, respectively. The difference was more significant compared to the CK group. The activity showed a decreasing trend but remained 266.67% and 92.86% higher than in the CK group at 21 and 28 days. Overall, CAT activity in the roots of the WS group also exhibited an increasing trend followed by a decrease (Fig 4b).

Antioxidant enzymes are crucial for plant metabolism and play a significant role in helping plants survive under adverse conditions. Plants produce large amounts of reactive oxygen species (ROS) and free radicals in waterlogged conditions, which exacerbate the peroxidation of cell membranes. POD and CAT are key antioxidant enzymes that eliminate ROS and free radicals from the plant. Li *et al*. (2014) found that POD activity first increased and then decreased in the roots of dogbane under waterlogging conditions. Similarly, Su *et al*. (2022) observed a trend of increasing and then decreasing CAT and POD

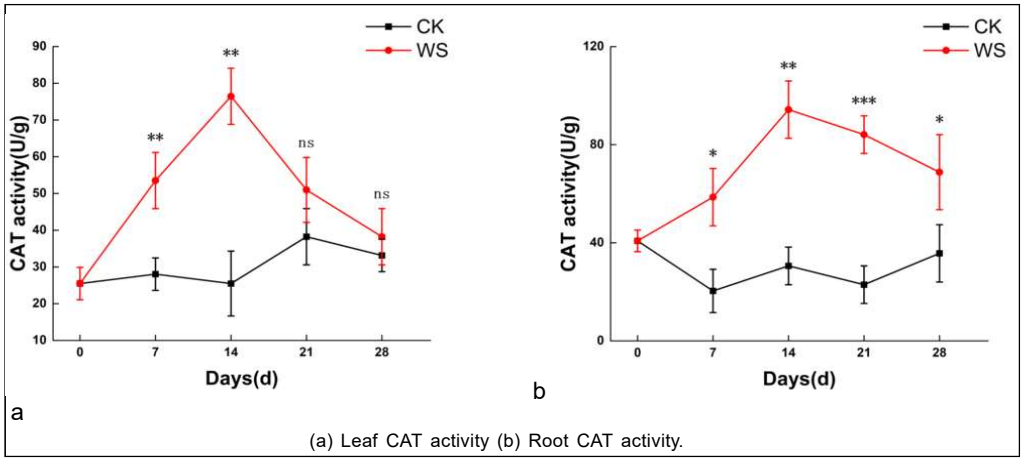


Fig 2: Morphological alterations in the roots of caucasian clover following 28 days of waterlogging stress.



(a) Leaf POD activity (b) Root POD activity. The bars represent the standard errors (±SD) of the three replicates. Ns means no statistical significance, *** means p<0.001, ** means p<0.01, * means p<0.05.

Fig 3: POD activity of Caucasian clover leaves and roots.



(a) Leaf CAT activity (b) Root CAT activity.

Fig 4: CAT activity of Caucasian clover leaves and roots.

activities in three types of mangroves under stress, consistent with the results of this experiment. In this experiment, POD and CAT activities increased in both leaves and roots during the early stages of waterlogging, indicating that antioxidant enzymes act promptly under stress to scavenge ROS, reduce oxidative damage and help the plant adapt more quickly to the hypoxic environment. The slight decrease in activity at the later stage may be due to the plant experiencing intensified injury, leading to excessive production of H_2O_2 that surpasses the catalytic capacity of enzymes like POD and CAT. The increase in POD activity in the root system may be attributed to the plant's continuous efforts to adapt to the flooded environment by mobilizing POD activity to meet its survival needs.

Effect of waterlogging stress on ADH activity in Caucasian clover

The ADH activity in the leaves of the WS group reached its maximum at 7 days, increasing by 466.67% compared to the CK group, a significant difference. ADH activity in the leaves showed a decreasing trend at 14, 21 and 28 days, but ADH activity was still 266.70% and 255.35% higher than in the CK group at 14 and 21 days, with a significant difference. The decrease in ADH activity was more pronounced on day 28. Although it remained 18.11% higher than CK, the difference was not statistically significant. Overall, the ADH activity in the WS group's leaves exhibited an initial increase followed by a decrease (Fig 5a).

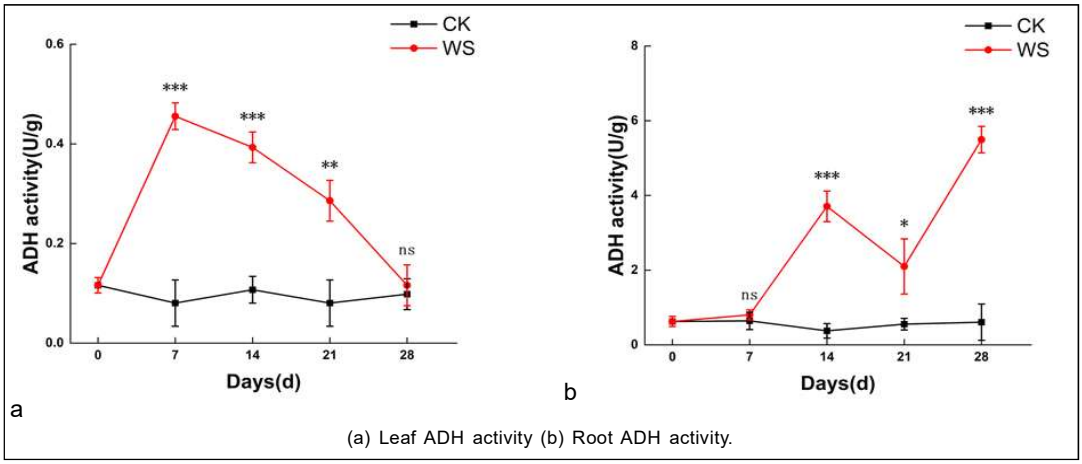
In the root system, the ADH activity in the WS group rose by 25% at 7 days compared to the CK group, with no significant difference. At 14 days, ADH activity in the WS group surged by 888.01% compared to CK, showing a significant difference. ADH activity decreased but remained 278.95% higher than CK on day 21. The WS group's ADH activity increased again, with a significant difference from CK by 28 days. The CK group also showed an increase, measuring 804.36% higher than CK. Overall, the ADH activity in the WS group displayed an increasing-decreasing-increasing pattern in the root system (Fig 5b).

Under waterlogging conditions, aerobic respiration is blocked, forcing the plant to switch to anaerobic respiration. Ethanol fermentation produces some ATP and NAD^+, providing limited energy for the plant to maintain growth in a low-oxygen environment during this process. ADH is the key enzyme in the ethanol fermentation pathway. Gao *et al.* (2023) found that waterlogging stress significantly increased ADH activity and promoted ethanol fermentation in kiwifruit, which differs from the results of this experiment. In the study of Wei *et al.* (2013) ADH also showed a similar trend of increasing and then decreasing, aligning with the results of this experiment. In this experiment, ADH activity in leaves initially increased and then decreased. In roots, ADH activity did not change significantly in the early stage but increased after 7 days, decreased slightly at 21 days-remaining higher than CK-and increased again in the later stage. The increase in activity suggests that Caucasian clover enhanced the efficiency of ethanol fermentation to produce energy for growth under waterlogging stress, indicating greater tolerance to waterlogging. The decrease in activity was likely due to metabolic disorders in the plant following the stress, with ADH activity increasing again after self-regulation.

Effect of waterlogging stress on SS and SP content of Caucasian clover

The SS content in the leaves of the WS group increased slowly at 7, 14 and 21 days compared to 0 days, showing increases of 45.32%, 33.18% and 26.38%, respectively, compared to the CK group, with significant differences. At 28 days, the SS content in the leaves of the WS group increased dramatically, by 132.68% compared to the CK group, with a highly significant difference. The SS content in the leaves of the WS group consistently showed an increasing trend (Fig 6a).

The SS content in the root system of the WS group increased at 7 and 14 days, by 153.46% and 294.24% higher than the CK group, respectively, with significant differences. The SS content in the WS group showed a decreasing trend but remained 144.31% higher than the



(a) Leaf ADH activity (b) Root ADH activity.

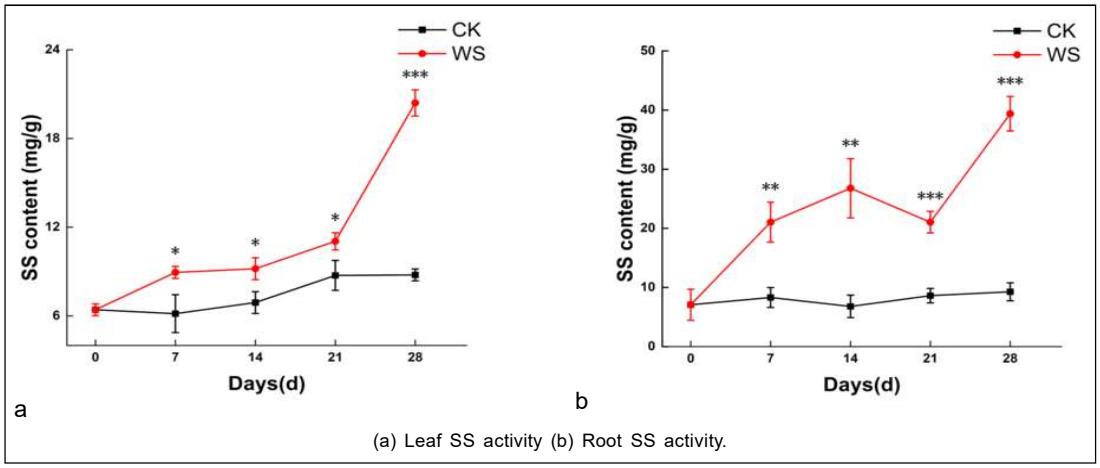
Fig 5: ADH activity of Caucasian clover leaves and roots.

CK group on day 21. The SS content in the WS group increased again compared to the CK group, with a larger increase of 324.89%, showing a highly significant difference at 28 days. The SS content in the WS group's root system showed an overall trend of first increasing, then decreasing and then increasing again (Fig 6b).

The SP content in the leaves of the WS group increased by 17.20% compared to the CK group at 7 days, then slightly decreased at 14 days, but remained 16.80% higher than the CK group, with the decrease not being significant.The SP content in the WS group rose again, being 16.87% higher than in the CK group when the treatment was on day 21. The differences between the WS group and the CK group were significant at 7, 14 and 21 days. At 28 days, the SP content in the WS group began to decrease, being 2.21% lower than in the CK group, though the difference was not significant. Overall, the SP content in the WS group's leaves exhibited a trend of increasing, decreasing, increasing and then decreasing again (Fig 7a).

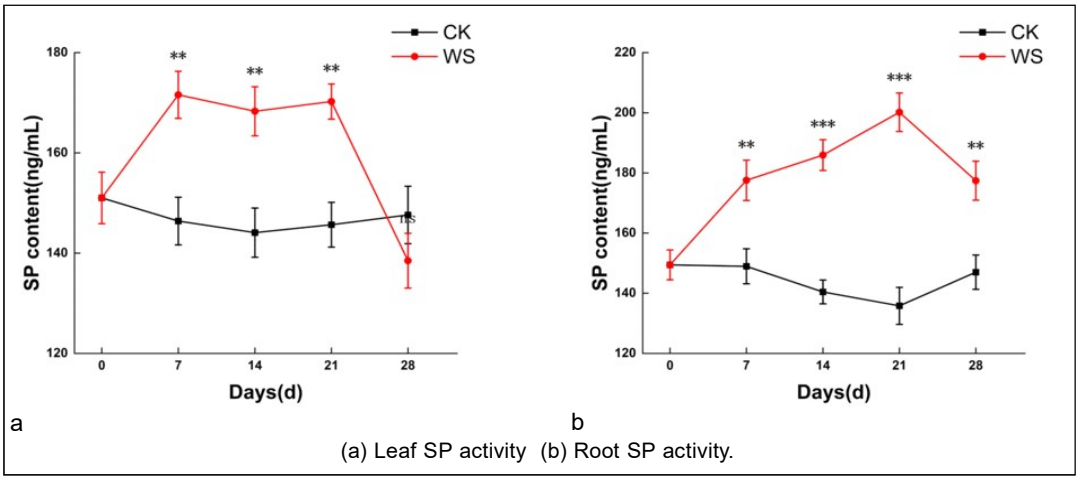
The SP content in the root system of the WS group showed an increasing trend at 7, 14 and 21 days, being 19.19%, 32.40% and 47.38% higher than that of the CK group, respectively. The SP content in the root system of the WS group reached its maximum, showing the most significant difference compared to the CK group on day 21. And the SP content began to decline at 28 days. Overall, the SP content in the WS group's root system showed a trend of increasing and then decreasing (Fig 7b).

Plants protect themselves by accumulating osmoregulatory substances when subjected to stress. The osmoregulatory substances SS and SP play a pivotal role in maintaining stable turgor pressure in plants, which is essential for sustaining normal growth. Feng *et al.* (2022) suggested that wheat can resist waterlogging stress by accumulating SS. Zhou *et al.* (2019) found that poplar could protect itself by significantly increasing both SS and SP under waterlogging stress, consistent with the results of this experiment. SS content in leaves increased as waterlogging time extended, with a significant rise in the late stage of stress in this experiment. In roots, the SS content demonstrated an initial increase in the early stage, a notable shift in the late stage and a slight decline at 21 days. However, it remained higher



(a) Leaf SS activity (b) Root SS activity.

Fig 6: SS activity of Caucasian clover leaves and roots.



(a) Leaf SP activity (b) Root SP activity.

Fig 7: SP activity of Caucasian clover leaves and roots.

than that of the CK group. The rise in SS content may be due to stress-induced conversion of large sugar molecules into smaller, soluble sugars like sucrose, providing energy for plant survival under adverse conditions and thereby maintaining the Waterlogging tolerance of clover. The trend of SP content in the leaves and roots of Caucasian clover was similar, showing an initial increase followed by a decrease. The increase in SP content was closely related to the activity of various protective enzymes, which prevented protein degradation, thereby leading to an increase in SP content. The subsequent decrease in SP content was likely due to cellular damage, which may have been related to the reduced activity of protective enzymes.

CONCLUSION

Waterlogging stress hampered the growth of Caucasian clover's main roots, reducing their length significantly compared to the control. However, the stress encouraged the development of lateral roots. These lateral roots replaced the original root system, expanding the root-soil contact area. This adaptation allowed the plant to absorb more oxygen, helping it endure adverse conditions and sustain growth and metabolic activities despite Waterlogging.

Waterlogging stress significantly increased the activities of POD, CAT and ADH enzymes in the leaves and roots of Caucasian clover. Higher antioxidant and anaerobic respiratory enzyme activities help the plant eliminate free radicals, provide a small amount of energy to sustain growth and protect against damage caused by reactive oxygen species to plant cell membranes. The SS and SP contents rise to maintain cellular osmotic balance and protect various enzymes, enabling the plant to resist the hypoxic environment induced by waterlogging stress.

In summary, when Caucasian clover is subjected to waterlogging stress, it adapts morphologically by increasing the number of lateral roots and physiologically by rapidly enhancing the activities of antioxidant and anaerobic respiratory enzymes, as well as increasing the content of osmotic regulating substances. These adaptations reduce the damage caused by waterlogging stress and enhance the plant's waterlogging tolerance.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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