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Effects of Alkaline Stress on the Growth and Physiological Characteristics of Caucasian Clover

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ABSTRACT

Background: Saline-alkali soil seriously restricts plant growth. Caucasian clover (*Trifolium ambiguum* Bieb.) is a high-quality leguminous forage with strong adaptability and stress resistance. However, current research on Caucasian clover under saline-alkali stress remains limited. The purpose of this study is to use $NaHCO_3$ solution to carry out alkali stress on Caucasian clover plants and to understand the changes of plant growth and physiological characteristics under stress, which is of great significance for its popularization and cultivation in alkaline soil.

Methods: To study the effects of alkaline stress on the growth and physiological characteristics of Caucasian clover, plants were subjected to varying concentrations (0, 50, 100, 150, 200 mmol/L) of $NaHCO_3$ in pot experiments. Post-stress, parameters such as dry weight, root-to-shoot ratio, main root length, chlorophyll, malondialdehyde (MDA) contentand the activities of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) were measured.

Result: This study demonstrated that alkaline stress significantly affected the growth and physiological characteristics of Caucasian clover. With increasing alkaline stress, the aboveground and underground dry weights, as well as the main root length, decreased significantly. Photosynthesis was inhibited, with chlorophyll content increasing under low alkaline stress but decreasing sharply at higher concentrations. The activities of antioxidant enzymes (SOD, POD, CAT) increased significantly under low stress but gradually decreased under higher stress levels. Meanwhile, MDA content increased significantly under high alkaline stress. Correlation analysis showed a strong positive correlation between SOD activity and CAT activity, as well as between aboveground dry weight and main root length (P<0.01), while chlorophyll content was negatively correlated with MDA content (P<0.05).

Key words: Alkali stress, Antioxidant enzymes, Caucasian clover, Growth, Physiological.

INTRODUCTION

Soil salinization has become a global issue, representing one of the primary threats to soil degradation worldwide. Globally, approximately 8.31×10^8 hm^2 of soil are impacted by salinization. In China, around 9.2×10^6 hm^2 of farmland are affected by saline-alkaline soils (Daliakopoulos *et al*. 2016; Li *et al*., 2017). Saline-alkaline soils primarily include saline soils, caused by neutral salts such as NaCl and $NaSO_4$ and alkaline soils, caused by alkaline salts like $NaHCO_3$ and Na_2CO_3. In practice, these two types often coexist (Guo *et al*., 2015). Compared to saline soils, alkaline soils cause more severe and complex damage to plants, including ionic toxicity, osmotic stressand oxidative stress. Moreover, their high pH value causes the soil to become alkaline, further disrupting normal plant growth and physiological metabolism. Therefore, alkaline stress poses a greater and more complex threat than saline stress (Zhang and Mu, 2009; Li *et al*., 2010).

Poor water circulation in saline-alkaline soils leads to soil compaction, affecting the nutrient absorption process in plants, thus inhibiting growth and reducing yield. Studies have shown that both saline and alkaline stress significantly inhibit cotton growth (Guo *et al*., 2022). Under alkaline stress, plants undergo significant physiological changes. Ionic balance is disrupted as excessive sodium ions enter cells, affecting the absorption and transport of nutrients such as potassium and calcium (Maathuis and Amtmann, 1999). Photosynthesis is inhibited, leading to leaf chlorosis, a decline in photosynthetic capacity and reductions in both chlorophyll content and Rubisco activity (Lu *et al*., 2022). The integrity of the cell membrane is compromised, resulting in increased lipid peroxidation and elevated MDA levels (Guo *et al*., 2020). To cope with alkaline stress, plants activate antioxidant systems, with increased activity of antioxidant enzymes like SOD and POD (Gill and Tuteja, 2010).

Caucasian clover (*Trifolium ambiguum* Bieb.) is a perennial, high-quality leguminous forage originating from Eastern Europe and the Caucasus region. This forage species exhibits strong adaptability and resilience, with traits such as cold tolerance, drought tolerance and flood resistance (Liu *et al*., 2023). Research on the stress

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responses of Caucasian clover has primarily focused on low-temperature and water stress, with studies on alkaline stress being rare. Given the extensive saline-alkaline soils in China, which have great potential for development, selecting and breeding forage varieties with strong tolerance to saline-alkaline conditions is crucial. Therefore, the effects of alkaline stress on the growth and physiological characteristics of Caucasian clover are deeply investigated in this paper, aiming to reveal its response mechanisms to alkaline stress and provide a solid theoretical foundation for its promotion and cultivation in saline-alkaline soils.

MATERIALS AND METHODS

Cultivation of plant materials and stress treatment

The experiment was conducted from 2023 to 2024 in the artificial climate chamber of the Key Laboratory of Grassland Resources at Inner Mongolia Agricultural University, under controlled conditions of 25°C temperature, 45% relative humidityand a 16/8 h (light/dark) photoperiod. Caucasian clover was used as the experimental material, with seeds provided by Inner Mongolia Agricultural University. The seeds were germinated for 4 days in Petri dishes lined with two layers of moist filter paper, ensuring that the filter paper remained consistently wet throughout the germination period. On the 5th day, the seedlings were transplanted into nutrient pots filled with a mixture of vermiculite and volcanic rock (2:1, v/v). Four seedlings were transplanted into each pot, with the pot dimensions being 10 cm × 10 cm. Each treatment included 10 pots. During the growth period, the plants were regularly irrigated with Hoagland's full-nitrogen nutrient solution.

After 30 days of cultivation, plants with consistent growth were selected and subjected to alkaline stress using $NaHCO_3$ solutions. Five gradient concentrations were set: 0 mmol/L, 50 mmol/L, 100 mmol/L, 150 mmol/L and 200 mmol/L, with 0 mmol/L as the control. Each pot was watered with 150 mL of alkaline solution and daily water replenishment was done by weighing to ensure consistent alkaline solution concentration. On the 8th day post-stress, plants from each treatment were sampled to measure growth and physiological characteristics.

Measurement of growth characteristics

The plants were thoroughly cleaned, blanched in an oven at 105°C for 30 minutes, then dried to a constant weight at 65°C and weighed. The main root length was measured using a ruler.

$$\text{Root / shoot ratio} = \frac{\text{Underground dry weight}}{\text{Aboveground dry weight}}$$

Measurement of physiological characteristics

Chlorophyll content was determined using the 95% ethanol extraction method. The leaves from each treatment were cut into small pieces and placed in a mortar, where 95% ethanol was added. The mixture was ground into a homogeneous paste, then diluted with 95% ethanol to a final volume of 10 mL and stored in the dark for 24 hours until pigment extraction was complete. Absorbance was measured using a microplate reader at 665 nm and 649 nm wavelengths.

Chlorophyll a content (mg/g) =

$$(13.95\ A_{665}\text{-}6.88A_{649}) * V * N / W$$

Chlorophyll b content (mg/g) =

$$(24.96\ A_{649}\text{-}7.32\ A_{665}) * V * N / W$$

Chlorophyll (a + b) content (mg/g) =

Chlorophyll a content + Chlorophyll b content

A_{665} and A_{649} = Maximum absorbance peaks at the respective wavelengths.

V= Volume of the extraction solution.

N= Dilution factor.

W= Fresh weight of the leaves.

Collect plant roots to measure the following indicators. Superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and malondialdehyde (MDA) were measured by the detection kit provided by Solarbio.

Data processing and analysis

SPSS 27 statistical software was used for the least significant difference (LSD) multiple comparison tests and correlation analysis. Charts and graphs were created using Microsoft Excel 2019 and Origin 2021.

RESULTS AND DISCUSSION

Effects of akaline stress on growth characteristics of caucasian clover

Alkaline stress affected the biomass of the plants. As the stress increased, the aboveground dry weight, underground dry weightand main root length gradually decreased, while the root-to-shoot ratio first decreased, then increasedand decreased again (Table 1). Compared to the 0 mmol/L treatment, the aboveground dry weight decreased by 1.86%, 17.03%, 20.13% and 24.15% for the different alkaline treatments, while underground dry weight decreased by 11.82%, 17.73%, 26.60% and 37.45%. The main root length decreased by 1.63%, 11.44%, 15.80% and 17.98%. Under the 100 mmol/L alkaline treatment, the root-to-shoot ratio of the plants showed no significant difference compared to the 0 mmol/L treatment. However, under the 50 mmol/L, 150 mmol/L and 200 mmol/L treatments, the root-to-shoot ratio decreased by 10.21%, 8.01% and 17.50%, respectively. Yield reflects the growth status of the plant; when plants are subjected to alkaline stress, their growth and development are inhibited. Guo *et al*. (2024) found that under alkaline stress, the root length, fresh weight and dry weight of alfalfa were significantly reduced, which was consistent with the results of this study. In this study, it was found that after exposure to different concentrations of alkaline solutions, the aboveground dry weight, underground dry weightand main root length of Caucasian clover all showed a downward trend, indicating that both

aboveground and underground growth of the plants were inhibited under alkaline stress, leading to a reduction in biomass.

Effects of alkaline stress on chlorophyll and MDA content in caucasian clover

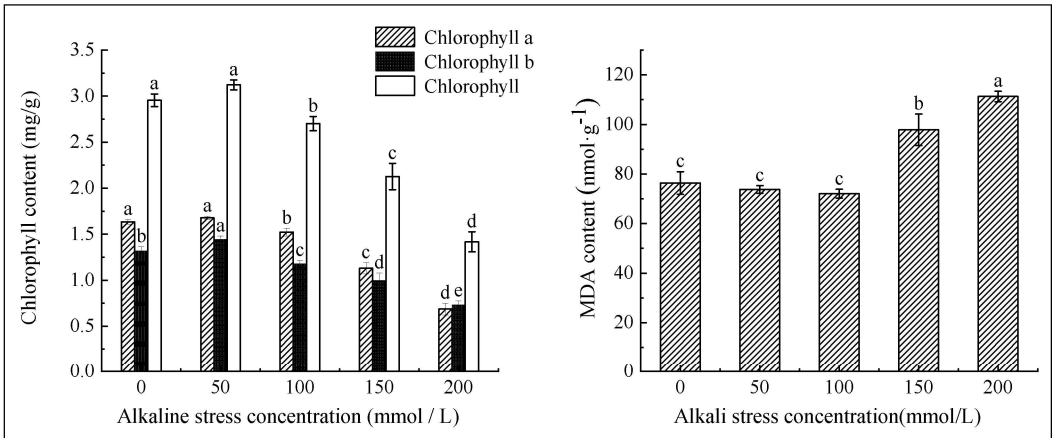
Chlorophyll content is an important reference indicator for plant alkali tolerance. With increasing alkaline stress concentration, the total chlorophyll, chlorophyll aand chlorophyll b contents in the leaves initially increased and then decreased (Fig 1A). When the concentration of alkaline solution reached 50 mmol/L, the total chlorophyll, chlorophyll aand chlorophyll b contents were highest, increasing by 2.60%, 9.46% and 5.66%, respectively, compared to the 0 mmol/L treatment and significantly higher than other treatment groups. Under the 200 mmol/L treatment, the total chlorophyll, chlorophyll aand chlorophyll b contents were the lowest, decreasing by 58.01%, 44.61% and 52.03%, respectively, compared to the 0 mmol/L treatment. Saline-alkaline stress can influence chlorophyll content to a certain extent (Chen *et al*., 2023). Han *et al*. (2020) found that chlorophyll content in alfalfa leaves initially increased and then decreased with increasing concentrations of alkaline treatment. In this study, the chlorophyll content in Caucasian clover leaves increased by 2.06% under 50 mmol/L alkaline stress compared to 0 mmol/L, but began to decrease at 100 mmol/L, with a more pronounced downward trend as stress concentration increased. Thus, low concentrations of alkaline stress can promote an increase in chlorophyll content to a certain extent, but once the stress level reaches a certain threshold, chloroplasts in plant leaves are damaged, affecting photosynthesis. Chlorophyll synthesis decreases, leading to a significant reduction in chlorophyll content as stress intensifies.

MDA is a breakdown product of lipid peroxidation in plant cell membranes and its content increases when plants are damaged. The higher the MDA content, the greater the degree of membrane peroxidation, indicating more severe damage to the plant. Therefore, MDA is often used as an indicator of the extent of stress on plants (Weismann *et al*., 2011). As the concentration of alkaline stress solution increased, MDA content in the roots of Caucasian clover first decreased and then increased (Fig 1B). No significant difference in MDA content was observed between the 50 mmol/L and 100 mmol/L treatments compared to the 0 mmol/L treatment. However, when the stress solution concentration reached 150 mmol/L, MDA content increased significantly, indicating severe damage to the cell membrane at this point. The MDA content was

Table 1: Effects of alkaline stress on aboveground dry weight, underground dry weight, root / shoot ratio and main root length of Caucasian clover (mean ± SD).

Alkaline concentration (mmmol/L)	Aboveground dry weight (g)	Underground dry weight (g)	Root / shoot ratio	Main root length (cm)
0	0.108±0.002a	0.068±0.003a	0.628±0.018a	12.23±0.47a
50	0.106±0.003a	0.060±0.004b	0.564±0.026b	12.03±0.50a
100	0.089±0.003b	0.056±0.002b	0.624±0.026a	10.83±0.35b
150	0.086±0.003bc	0.050±0.002c	0.578±0.037b	10.30±0.20bc
200	0.082±0.004c	0.042±0.002d	0.518±0.007c	10.03±0.23c

Note: Means ± SD in the same column followed by the same letters are not significantly different (P>0.05).



The same letters on the top of the bars are not significantly different (P>0.05). The same applies to the following figures.

Fig 1: Effects of different concentrations of $NaHCO_3$ on chlorophyll and MDA content of Caucasian clover.

highest under the 200 mmol/L treatment, being 45.71% higher than the 0 mmol/L treatment. Ahmad *et al*. (2013) reported that MDA content in different mulberry varieties increases over time with rising external $NaHCO_3$ levels, which is consistent with the findings of this study. Under the 50 mmol/L and 100 mmol/L alkaline treatments, the MDA content in Caucasian clover roots did not increase compared to the 0 mmol/L treatment, indicating that the degree of membrane oxidation was not high at this stage. However, the antioxidant enzyme system showed significant enhancement at these concentrations, suggesting that the plant could resist stress to some extent through self-regulation. When the stress solution concentration reached 150 mmol/L, MDA content increased significantly, while antioxidant enzyme activity began to decrease, indicating substantial damage to the plant at this stage.

Effects of alkaline stress on antioxidant enzymes activity in caucasian clover

When plants are subjected to stress, antioxidant enzymes in the plant eliminate superoxide anion radicals, alleviating the damage caused by stress (Zou *et al*., 2018). SOD, POD and CAT are important antioxidant enzymes that mitigate oxidative stress by increasing their activity to remove excess reactive oxygen species (Liu *et al*., 2022; Latha *et al*., 2024). The SOD activity in the roots of Caucasian clover showed significant changes under alkaline stress. As the concentration of the alkaline solution increased, SOD activity first increased and then decreased (Fig 2A). SOD activity was lowest under the 0 mmol/L treatment, with significant differences compared to other treatments. The SOD activity reached its maximum under the 100 mmol/L treatment, increasing by 98.6% compared to the 0 mmol/L treatment. Under the 150 mmol/L and 200 mmol/L treatments, SOD activity decreased slightly compared to the 100 mmol/L treatment but was still 62.1% and 15.94% higher, respectively, than the 0 mmol/L treatment. POD has a broad range of functions within plants, including the removal of H_2O_2 produced under stress and involvement in chlorophyll degradation in leaves. Under different concentrations of alkaline solution stress, POD activity first increased and then decreased (Fig 2B). Under the 100 mmol/L alkaline stress, POD activity reached its peak, showing the most significant difference compared to other treatments, being 82.31% of the 0 mmol/L control. Under the 50 mmol/L and 150 mmol/L treatments, POD activity also increased compared to the control, by 13.60% and 29.55%, respectively. There was no significant difference in POD activity between the 200 mmol/L treatment and the control.

CAT is an essential protective enzyme in the plant antioxidant system, responsible for removing H_2O_2 generated during metabolism to protect plant cells from damage (Guo and Shi, 2024). As shown in Fig 2C, CAT activity in the roots initially increased and then decreased with rising concentrations of alkaline stress solution. Significant differences in CAT activity were observed between all treatments and the 0 mmol/L control, with increases of 209.08%, 281.81%, 172.72% and 72.73%, respectively. CAT activity was highest under the 100 mmol/L treatment, with significant differences compared to other treatments. Under the 150 mmol/L treatment, CAT activity began to decline but did not significantly differ from the 50 mmol/L treatment. Studies have shown that as the concentration of alkaline stress increases, the antioxidant enzyme activity in barley seedlings' roots first increases and then decreases. Additionally, alkaline-tolerant barley materials exhibit higher antioxidant enzyme activity than alkaline-sensitive materials (Jin *et al*., 2023). Liang *et al*. (2022) conducted stress experiments on *Cyperus esculentus* using mixed saline-alkaline solutions of different concentrations and found that under saline-alkaline stress, *Cyperus esculentus* could eliminate oxygen free radicals by increasing SOD and POD activities in the leaves, thereby reducing the damage caused by the stress. Similar results were observed in this study; measurements of SOD, POD and CAT activities in the roots of Caucasian clover revealed that their activities initially increased and then decreased with rising stress solution concentrations. Antioxidant enzyme activities peaked at 100 mmol/L, followed by a decline, with SOD, POD and CAT activities increasing by

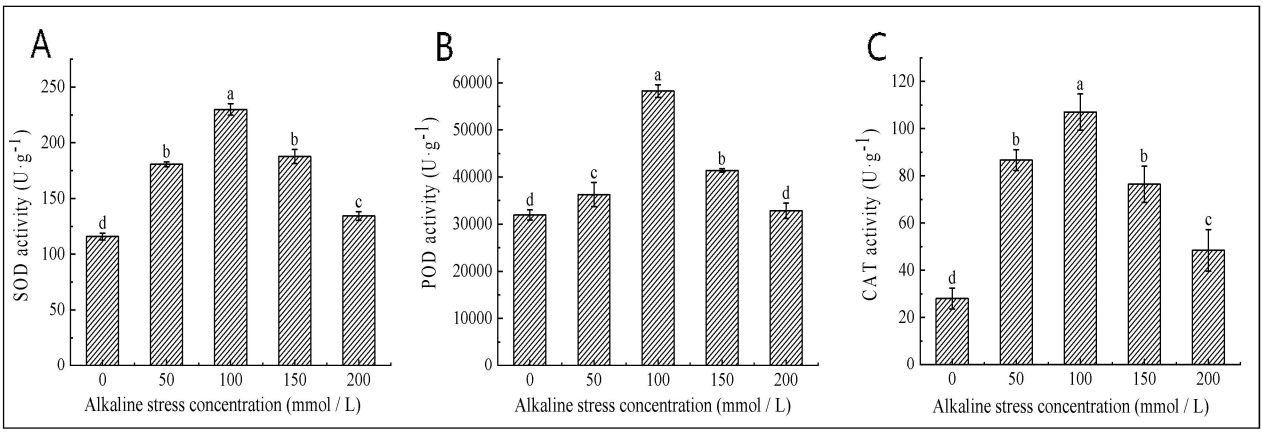


Fig 2: Effects of different concentrations of $NaHCO_3$ on the activity of antioxidant enzymes in Caucasian clover.

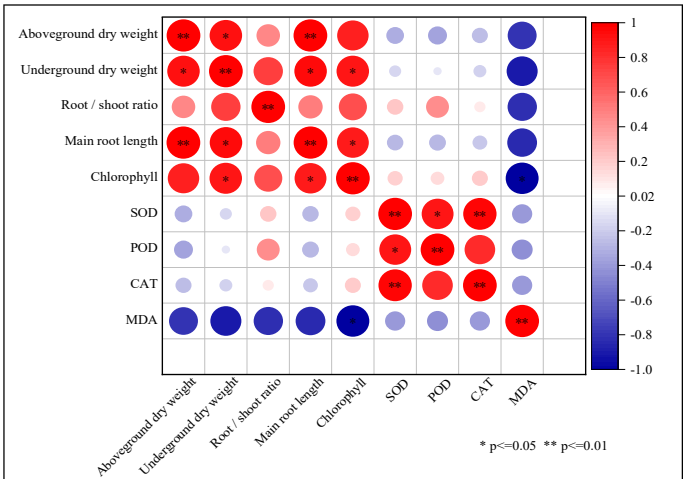
98.6%, 82.31% and 281.81%, respectively, compared to the 0 mmol/L treatment. From the experimental results, it can be seen that alkaline stress triggers the plant's self-defense mechanism, where the antioxidant enzyme system increases its activity to counteract stress-induced damage. However, when the stress solution concentration reached a certain threshold, the damage caused by the stress exceeded the plant's self-regulation capacity, leading to a decrease in antioxidant enzyme activity.

Correlation analysis

Correlation analysis was performed on the indicators under alkaline stress, revealing certain correlations between them (Fig 3). Under stress, SOD activity was highly positively correlated with CAT activity and aboveground dry weight was highly positively correlated with main root length (P<0.01). Aboveground dry weight was significantly positively correlated with underground dry weight, underground dry weight with main root length, chlorophyll content with underground dry weight, main root length with chlorophyll contentand POD activity with SOD activity (P<0.05). Chlorophyll content was significantly negatively correlated with MDA content (P<0.05). Hou *et al*. (2024) reported that SOD activity in soybean leaves is highly positively correlated with POD, CAT and APX activities (P<0.01). This study conducted a correlation analysis of various physiological parameters under alkaline stress. The results revealed a significant relationship between SOD activity and the activities of POD and CAT, indicating that the antioxidant enzymes work synergistically to mitigate stress effects. MDA content was significantly negatively correlated with chlorophyll content, as high concentrations of alkaline solution caused significant damage to cell membranes, leading to increased MDA content and decreased chlorophyll content. This result is consistent with the findings of She *et al*. (2021).

Principal component analysis

Principal component analysis (PCA) is a multivariate statistical analysis method that converts multiple related variables into a smaller set of uncorrelated comprehensive variables, known as principal components. In studies of plant stress responses, multiple indicators related to plant growth and physiological status may be measured. PCA consolidated these indicators into a few major components, simplifying the data structure and revealing the key information within the data (Cicevan *et al*., 2016). PCA was conducted on nine indicators under alkaline stress in Caucasian clover, including aboveground dry weight, underground dry weight, chlorophyll contentand SOD activity. The results showed that three principal components were extracted based on eigenvalues greater than 1 and a cumulative contribution rate greater than 85%, with a cumulative contribution rate of 96.71%, indicating that these three principal components encompass most of the information related to the alkaline tolerance of Caucasian clover (Table 2). The first principal component had an eigenvalue of 4.82 and a contribution rate of 53.60%, with main contributing indicators being aboveground dry weight, underground dry weight, main root length, chlorophyll contentand MDA. The second principal component had an eigenvalue of 3.16 and a contribution rate of 35.10%, with the main contributing indicators being SOD, POD and CAT. The third principal component had an eigenvalue of 0.72 and a contribution rate of 8.01%, with the main contributing indicator being the root-to-shoot ratio. The indicators of aboveground dry weight, underground dry weight, main root length, chlorophyll content and MDA are key evaluation criteria for alkaline tolerance. The PCA biplot results showed that the five treatments were well distinguished with no overlap between them. SOD, POD, CAT, root-to-shoot ratioand chlorophyll content clustered in the first quadrant, MDA clustered in the third quadrant and



* Indicates significant correlation at the 0.05 level; ** indicates significant correlation at the 0.01 level.

Fig 3: Correlation analysis of each index under alkali treatment.

Table 2: Principal component analysis of each index under alkali treatment.

Index	PC1	PC2	PC3
Eigen value	4.82	3.16	0.72
Percentage of variance (%)	53.60	35.10	8.01
Cumulative (%)	53.60	88.70	96.71
Aboveground dry weight	0.40	-0.21	-0.27
Underground dry weight	0.44	-0.11	0.18
Root / shoot ratio	0.33	0.14	0.74
Main root length	0.40	-0.18	-0.26
Chlorophyll	0.44	0.06	-0.16
SOD	0.03	0.55	-0.17
POD	0.05	0.53	0.26
CAT	0.03	0.52	-0.39
MDA	-0.42	-0.17	0.10

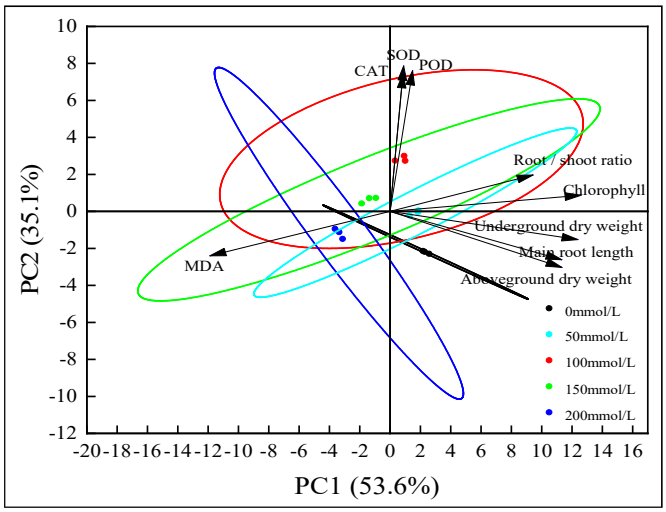


Fig 4: Biplot of principal component analysis of each index under alkali treatment.

aboveground dry weight, underground dry weight and main root length clustered in the fourth quadrant (Fig 4).

Conclusion

Alkaline stress significantly inhibited the growth of Caucasian clover, leading to a reduction in biomass and photosynthesis was suppressed under certain concentrations of alkaline stress. Damage to the cell membrane resulted in a significant increase in MDA content under high-concentration stress. Correlation analysis revealed a strong association between SOD activity and CAT activity and a high correlation between aboveground dry weight and main root length, while chlorophyll content was negatively correlated with MDA content. Principal component analysis further confirmed the importance of aboveground dry weight, underground dry weight, main root length, chlorophyll contentand MDA as key indicators for evaluating the alkali tolerance of Caucasian clover. These indicators can serve as valuable references for screening and breeding alkali-tolerant forage varieties. Caucasian clover mitigates damage from alkali stress by enhancing antioxidant enzyme activity and demonstrates a certain level of tolerance to low concentrations of alkali stress (50~100 mmol/L).

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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.

Informed consent

All animal procedures for experiments were approved by the Committee of Experimental Animal care and handling

techniques were approved by the University of Animal Care Committee.

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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