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Effects of exogenous calcium on seed germination and physiological traits of alfalfa (*Medicago sativa*) seedlings

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Abstract

To enhance the cultivation and utility of alfalfa (*Medicago sativa*) in calcium-rich environments, we assessed the germination, growth, and physiological responses of seven alfalfa varieties—Crown, Dieter, PANGO, Gladiator, Victoria, WL525, and Magnum 801—under varying calcium chloride ($CaCl_2$) concentrations (0, 5, 25, and 50 mmol·L^{-1}). Germination indices, root and shoot growth, enzyme activities, and osmotic regulation parameters were analyzed to evaluate adaptive responses to calcium stress. Our results showed that alfalfa adapts to calcium stress by increasing root length, enhancing enzyme activities, regulating osmotic substance content, and reducing malondialdehyde levels, thereby striving to maintain stable dry matter content. However, the extent of these adaptive responses varied among the different varieties. Based on a comprehensive evaluation, the calcium adaptability of the varieties ranked in the following order: Gladiator > Victoria > Dieter > Magnum 801 > WL525 > Crown > PANGO. Notably, calcium concentrations of 5–25 mmol·L^{-1} were found to be optimal for germination, physiological regulation, and growth, whereas higher concentrations (50 mmol·L^{-1}) induced oxidative stress and impaired growth. This study highlights the role of exogenous calcium in enhancing physiological resilience and provides a robust framework for selecting calcium-tolerant alfalfa varieties suitable for cultivation in karst landscapes. These findings offer theoretical and practical insights for optimizing forage production in calcium-rich soils.

Keywords Alfalfa, Exogenous calcium stress, Physiology, Seed germination

Calcium is an essential macronutrient with critical roles in plant growth, development, and stress adaptation. It serves as a secondary messenger in cellular signaling, regulating key processes such as cell wall stabilization, enzymatic activity, and osmotic regulation [1, 2]. Under stress conditions, calcium ions (Ca^{2+}) mitigate damage by modulating photosynthesis and activating protective enzyme systems, enhancing resilience to salinity, drought, and oxidative stress [3, 4]. However, excessive calcium can induce ion toxicity, disrupt cellular homeostasis, and impair physiological and biochemical processes, ultimately inhibiting growth [5, 6]. These dual effects highlight the importance of defining optimal calcium concentrations for sustainable plant development under challenging environmental conditions. However, existing literature has predominantly centered on extreme calcium concentrations (both deficiency and excess) in mature plant systems, while comprehensive investigations into calcium threshold dynamics during the critical germination phase remain notably scarce. A particularly significant methodological limitation emerges in previous optimization attempts: researchers frequently relied on isolated evaluation metrics (e.g., germination percentage or biomass accumulation) while neglecting to account for the integrated mechanisms underlying multidimensional

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physiological responses. This could represent a significant factor contributing to the discrepancies in the conclusions across various studies.

Karst landscapes are widely distributed, particularly in southwest China, with Guizhou Province serving as a representative region. In Guizhou, karst areas constitute approximately 62% of the total land area [7]. These landscapes are characterized by carbonate rocks, primarily composed of soluble salts such as calcium carbonate and magnesium carbonate [8, 9]. The dissolution of calcium carbonate contributes to elevated calcium levels in karst soils, which can impair seed germination and seedling establishment, especially during early growth stages when plants are most vulnerable [10, 11]. Nevertheless, current research on plant calcium adaptation in karst regions predominantly centers on field observations under natural soil conditions, and lacks quantitative analysis of calcium concentration gradients under controlled experimental settings. This limitation constrains our comprehensive understanding of the mechanisms underlying calcium stress responses.

Alfalfa (*Medicago sativa*), often referred to as the "king of forage crops," is renowned for its high protein content, adaptability, and contributions to eco-circular agriculture. Its cultivation improves soil structure, mitigates salinization, and supports sustainable livestock production, particularly in marginal soils [12]. Previous research has demonstrated that low calcium concentrations can induce osmotic stress, while excessive calcium levels inhibit enzymatic activity and cause ion toxicity in plant seedlings [13]. However, the current research on alfalfa under calcium stress exhibits three significant limitations. Firstly, the majority of experiments have utilized hydroponic or sand culture systems, which do not adequately replicate the complex interactions between calcium and other elements in karst soil environments. Secondly, the focus has predominantly been on mature plants, with insufficient systematic evaluation of calcium sensitivity during the germination stage. Lastly, the genetic mechanisms underlying differences in calcium tolerance among various cultivars remain largely unexplored. These knowledge gaps hinder the development of precise calcium management strategies tailored to specific variety characteristics.

This study evaluates the germination, growth, and physiological responses of seven alfalfa varieties under varying concentrations of exogenous calcium. Specifically, it aims to (1) quantify germination and seedling performance under calcium stress, (2) elucidate the physiological mechanisms underlying calcium tolerance, and (3) identify optimal calcium concentrations and calcium-adaptable varieties for karst landscapes. Using a comprehensive evaluation framework that integrates principal component analysis and membership function methods [14, 15]. This study addresses the limitation of relying on a single evaluation metric, provides a robust theoretical foundation for optimizing alfalfa cultivation in calcium-rich environments.

Materials and methods

Plant materials

Seven alfalfa (*Medicago sativa*) varieties were used in this study: Crown, WL525, Dieter, PANGO, Victoria, Gladiator, and Magnum 801. Seeds were sourced from Guizhou Zhongzhiheng Ecological Technology Co., Ltd.

Experimental methods

Germination test

Calcium chloride ($CaCl_2$) solutions were prepared at concentrations of 0, 5, 25, and 50 mmol·L^{-1}. The 0 mmol·L^{-1} solution, treated with deionized water, served as the control (CK). Uniform, healthy seeds free from pests and diseases were selected and surface-sterilized using a 10% sodium hypochlorite (NaClO) solution for 15 min, followed by thorough rinsing with distilled water three to five times.

The sterilized seeds were evenly placed in Petri dishes lined with two layers of filter paper, with 50 seeds per dish. Each dish was treated with 8 mL of the respective $CaCl_2$ solution, and each treatment had three biological replicates. The dishes were incubated in a germination chamber maintained at 25 ± 2℃.

To maintain the appropriate solution concentration, water lost to evaporation was replenished daily by weighing and adding the required volume. Germination was defined as radicle emergence exceeding half the seed length. Germination counts were recorded daily, and the process was considered complete when no new seeds germinated for three consecutive days within a 10-day period [16].

On the final day of the experiment, the following indices were calculated: germination rate (GR), germination potential (GP), germination index (GI), and vigor index (VI), using the following formulas:

$$GR = N/M \tag{1}$$

where N represents the number of normally germinated seeds; M denotes the total number of seeds;

$$GP = K/M \tag{2}$$

where K refers to the number of normal germinating seeds at peak times, with M remaining the same as defined in Eq. 1.;

$$GI = \sum (Gt/Dt) \tag{3}$$

where Gt indicates the germination rate at time(t), and Dt represents the duration of the germination test;

$$VI = GI \times Sx \tag{4}$$

where Sx corresponds to the mean radicle length of the seed.

Seedling test

Fifteen healthy, uniformly growing seedlings from each treatment group were selected and transferred to new Petri dishes lined with two layers of filter paper for calcium stress treatment. Four calcium levels were established using $CaCl_2$ solutions at concentrations of 0, 5, 25, and 50 mmol·L^{-1}, with each treatment replicated three times.

The weighing method was used daily to replenish water lost to evaporation and maintain appropriate moisture levels. Seedling growth was observed and recorded daily. Any seedlings affected by mold or pests were promptly removed to prevent contamination.

After seven days of treatment, healthy leaves free of pests and diseases were collected, flash-frozen in liquid nitrogen, and stored at -80℃ for subsequent physiological measurements. On the final day, root length, bud length, dry weight, fresh weight, and relative water content were measured.

$$\text{Relative water content} = (\text{fresh weight} - \text{dry weight})/\text{fresh weight} \tag{5}$$

Measurement of physiological indexes

At the conclusion of the stress treatment, the physiological indexes of fresh leaves from different alfalfa varieties were measured. Peroxidase (POD) activity was assessed using the guaiacol method [17], superoxide dismutase (SOD) activity was measured using the WST-8 method, and catalase (CAT) activity was determined via the ultraviolet absorption method [18]. Proline (Pro) content was evaluated using the sulfosalicylic acid method [19], malondialdehyde (MDA) content was measured through the thiobarbituric acid method [20], and soluble sugar content was determined via anthrone colorimetry. The methodology primarily followed the procedures described by Huang et al. [21]. Each measurement included 3 biological replicates and 3 technical replicates to ensure accuracy and reliability.

Principal component analysis and membership comprehensive evaluation

The original data for each index were standardized using SPSS software, followed by principal component analysis (PCA) to reduce the dimensionality and simplify the data for each index [14, 15]. Based on the formula, the factor weight (W_j) and membership function value ($\mu(X_j)$) for the seven alfalfa varieties were calculated individually. Subsequently, the calcium adaptability measure (D) was computed using these factor weights and membership function values, allowing for a comprehensive evaluation of the adaptability of the seven alfalfa varieties to calcium stress. The calculation formula is as follows:

$$W_j = P_j / \sum P_j \tag{6}$$

When the calcium adaptability indicator is positively correlated with calcium resistance,

$$\mu(X_j) = (X_j - X_{\min})/(X_{\max} - X_{\min}) \tag{7}$$

When the calcium adaptability indicator is negatively correlated with calcium resistance,

$$\mu(X_j) = 1 - [(X_j - X_{\min})/(X_{\max} - X_{\min})] \tag{8}$$

$$D = \sum\nolimits_{j=1}^{n} [\mu(X_j) \cdot W_j] \tag{9}$$

where j = 1,2,3,... n, X_j represents the value of the j-th comprehensive index (principal component), P_j represents the contribution rate corresponding to the j-th principal component of each variety, $X_{\min}$ and $X_{\max}$ represent the minimum and maximum value of the j-th comprehensive index, respectively.

Data analysis

Data organization was performed using Microsoft Excel, and SPSS was employed for variance analysis and multiple comparisons. Graphs were generated using Origin software. All data are presented as mean ± standard error (SE).

Results

Effects of exogenous calcium on seed germination of alfalfa

The germination performance of alfalfa varieties varied under different concentrations of calcium chloride ($CaCl_2$) (Fig. 1). While the germination rate (GR) and germination potential (GP) exhibited minimal variation across treatments, significant differences were observed in the germination index (GI) and vigor index (VI) ($P < 0.05$). For most varieties, moderate calcium concentrations (5–25 mmol·L^{-1}) enhanced germination metrics, whereas a high concentration (50 mmol·L^{-1}) led to marked declines ($P < 0.05$). Among the varieties, at CK, the germination rates and germination potentials of Gladiator, Dieter, Victoria, WL525, and Magnum 801 showed no significant differences, while those of Crown and PANGO were lower, with significant differences compared to the former five varieties ($P < 0.05$). When the calcium concentration increased to 50 mmol·L^{-1}, Magnum 801's GR and GP were significantly different

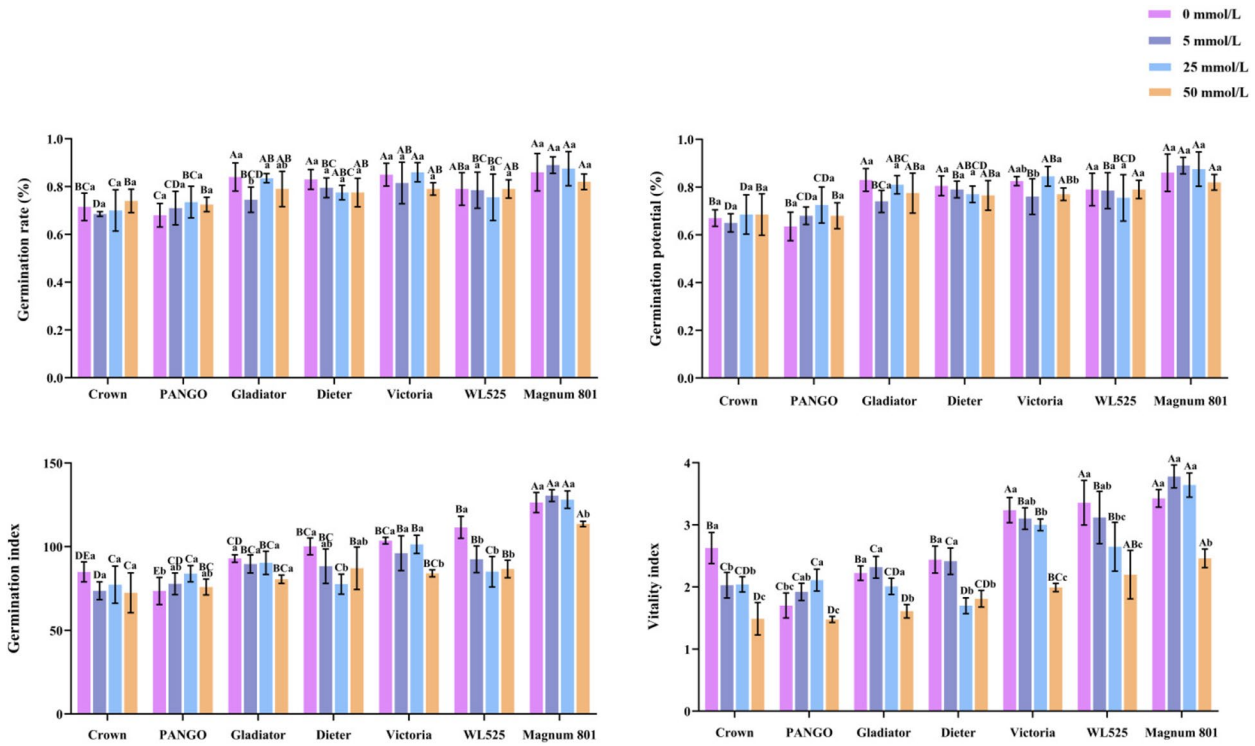

Fig. 1 Germination rate (GR), germination potential (GP), germination index (GI), and vigor index (VI) of different alfalfa varieties under varying calcium stress concentrations. Error bars represent standard error; different letters indicate significant differences at $P < 0.05$. Lowercase letters denote significant differences among different calcium concentrations within a variety, while uppercase letters signify significant differences between varieties at a given concentration

from those of Crown and PANGO ($P < 0.05$), but no differences were observed among the other varieties.

In terms of the germination index, all varieties except PANGO and Magnum 801 showed a decreasing trend with increasing stress concentration. Among all the varieties, Magnum 801 exhibited the highest germination index across all stress treatments, with a significant difference between the control (CK) and the 50 mmol·L^{-1} treatment ($P < 0.05$). The germination index of WL525 decreased rapidly with increasing calcium concentration.

The vigor index reflects the overall vitality and quality of seeds, serving as a quantitative measure of germination potential and rate, and providing insights into seed vigor and germination power [22]. As calcium concentration increased, the vigor index of PANGO, Gladiator, and Magnum 801 initially increased and then decreased. Gladiator and Magnum 801 exhibited peak values at 5 mmol·L^{-1}, while PANGO reached its maximum at 25 mmol·L^{-1}. In contrast, the vigor index of Crown, Victoria, and WL525 exhibited a gradual decline. Compared with the control, the vigor index of all seven alfalfa varieties significantly decreased at the 50 mmol·L^{-1} $CaCl_2$ concentration ($P < 0.01$). At a calcium concentration of 5 mmol·L^{-1}, Magnum 801 differed significantly from the other six varieties ($P < 0.05$). Meanwhile, Victoria and WL525 showed significant differences from the other four varieties ($P < 0.05$), and Magnum 801 had the highest VI among all varieties under this concentration stress. When the calcium concentration increased to 50 mmol·L^{-1}, Magnum 801 still had a significant difference from the other five varieties except WL525 ($P < 0.05$), and its VI remained the highest at this concentration.

Effects of exogenous calcium on the growth characteristics of alfalfa seedlings

As the concentration of calcium increased, significant differences ($P < 0.05$) were observed in the root length of five alfalfa varieties, excluding Victoria and Magnum 801. Among the seven varieties, Magnum 801 exhibited the longest average root length (22.35 mm), while Crown showed the shortest average root length (12.44 mm) (Table 1). Regarding bud length, Gladiator and WL525 initially increased and then decreased with increasing calcium concentration, with the longest bud length observed under 5 mmol·L^{-1} $CaCl_2$ stress, significantly higher than the control ($P < 0.05$). In contrast, the bud length of other varieties decreased progressively. At

Table 1 Effects of calcium stress on the growth characteristics of alfalfa seedlings

Varieties	Concentration of $CaCl_2$(mmol·L^{-1})	Root length (RL) (mm)	Bud length (BL) (mm)	Dry weight (DW) (g·$plant^{-1}$)	Relative water content (RWC) (%)
Crown	0	10.37 ± 1.64Db	18.37 ± 2.08ABa	0.0015 ± 0.0001ABa	94.72 ± 0.23BCa
	5	15.03 ± 3.801CDa	16.65 ± 3.22CDab	0.0015 ± 0.0001Aa	94.45 ± 0.45BCa
	25	13.43 ± 1.68Bab	13.96 ± 1.60Bb	0.0017 ± 0.0001ABCa	94.04 ± 0.31Aa
	50	10.94 ± 2.47Bab	14.25 ± 1.83BCb	0.0018 ± 0.0002Aa	92.43 ± 0.95Ab
PANGO	0	13.64 ± 2.12BCDa	17.37 ± 0.62BCa	0.0012 ± 0.0001BCb	95.65 ± 1.07ABa
	5	14.92 ± 2.39CDa	15.46 ± 1.97 Da	0.0012 ± 0.0001Ab	95.58 ± 0.99Aa
	25	13.86 ± 1.02Ba	15.79 ± 0.71Ba	0.0016 ± 0.0001BCDa	94.61 ± 0.17Aa
	50	10.12 ± 1.13Bb	12.93 ± 2.45CDb	0.0018 ± 0.0001ABa	92.75 ± 1.01Ab
Gladiator	0	16.07 ± 2.75BCDab	18.71 ± 1.25ABb	0.0012 ± 0.0001BCb	95.69 ± 0.61ABa
	5	17.39 ± 2.53BCa	22.18 ± 0.64Ba	0.0035 ± 0.0022Aa	95.28 ± 0.88ABa
	25	15.17 ± 2.30Bab	16.59 ± 1.96Bc	0.0014 ± 0.0001CDb	94.00 ± 2.23Aa
	50	12.57 ± 2.13ABb	17.14 ± 1.05Abc	0.0014 ± 0.0002ABb	93.48 ± 1.80Aa
Dieter	0	17.34 ± 3.53BCab	20.56 ± 0.39Aa	0.0011 ± 0.0000Cb	95.86 ± 0.20Aa
	5	20.46 ± 1.78ABa	20.83 ± 0.68Aa	0.0011 ± 0.0000Aab	95.70 ± 0.43Aab
	25	15.99 ± 3.39Bbc	21.00 ± 2.01Aa	0.0012 ± 0.0001 Da	95.09 ± 0.49Ab
	50	12.81 ± 1.60ABc	16.83 ± 1.24ABb	0.0012 ± 0.0001Bab	93.48 ± 0.48Ac
Victoria	0	12.61 ± 2.44CDa	18.11 ± 1.64Ba	0.0012 ± 0.0002BCb	95.56 ± 0.91ABa
	5	13.29 ± 0.93 Da	18.67 ± 2.37Ca	0.0016 ± 0.0000Aab	95.01 ± 0.13ABab
	25	12.18 ± 3.90Ba	18.31 ± 2.73Ba	0.0018 ± 0.0001ABa	94.20 ± 0.46Ab
	50	12.09 ± 1.47Ba	14.82 ± 0.63BCb	0.0017 ± 0.0000ABab	93.20 ± 0.38Ac
WL525	0	19.43 ± 6.56ABab	14.96 ± 0.85Cb	0.0017 ± 0.0001Aa	94.23 ± 0.18Ca
	5	22.68 ± 2.20Aa	18.21 ± 0.77Ca	0.0019 ± 0.0001Aa	93.90 ± 0.52Ca
	25	15.11 ± 0.99Bbc	16.68 ± 1.01Bab	0.0020 ± 0.0002Aa	93.94 ± 0.56Aa
	50	12.59 ± 2.66ABc	10.20 ± 2.72Dc	0.0019 ± 0.0001Aa	93.04 ± 0.78Ab
Magnum 801	0	23.24 ± 4.37Aa	18.23 ± 0.65Ba	0.0015 ± 0.0001ABa	94.55 ± 0.59Cab
	5	20.74 ± 2.90ABa	17.84 ± 1.82CDa	0.0016 ± 0.0001Aa	94.84 ± 0.75ABCa
	25	23.90 ± 5.50Aa	17.08 ± 1.54Ba	0.0018 ± 0.0001ABa	94.21 ± 0.25Aab
	50	21.51 ± 14.09Aa	13.86 ± 1.42BCb	0.0015 ± 0.0003ABa	93.55 ± 1.07Ab

Note: Values are means ± standard error. Different letters within the same column and variety indicate significant differences at $P < 0.05$. Lowercase letters denote significant differences among different calcium concentrations within a variety, while uppercase letters signify significant differences between varieties at a given concentration

the same calcium concentration, significant differences in root and bud lengths were observed among varieties ($P < 0.05$). For instance, under 25 mmol·L^{-1} calcium stress, the root length of Magnum 801 and the bud length of Dieter were significantly different from those of the other 6 varieties ($P < 0.05$). Under 50 mmol·L^{-1} calcium stress, the root length of Magnum 801 was significantly different from that of Crown, PANGO, and Victoria ($P < 0.05$).

No significant differences were found in the dry weight of Crown, WL525, and Magnum 801 across different calcium concentrations. Among all varieties, WL525 had the highest average dry weight (0.0019 g·$plant^{-1}$. With increasing calcium concentration, the relative water content (RWC) of alfalfa varieties (except Gladiator) consistently decreased. Compared with the control, Dieter and Victoria showed significant reductions in RWC under 25 mmol·L^{-1} $CaCl_2$ stress ($P < 0.01$), while Crown, PANGO, and WL525 exhibited significant decreases under 50 mmol·L^{-1} $CaCl_2$ stress ($P < 0.05$). Notably, at this stress concentration, no significant differences were detected among all varieties.

Effects of exogenous calcium on physiological characteristics of alfalfa seedlings

Effects on protective enzyme activity and malondialdehyde content

Exogenous calcium significantly affected the malondialdehyde (MDA) content in alfalfa seedlings ($P < 0.05$) (Fig. 2). The MDA content in Crown, PANGO, Gladiator,

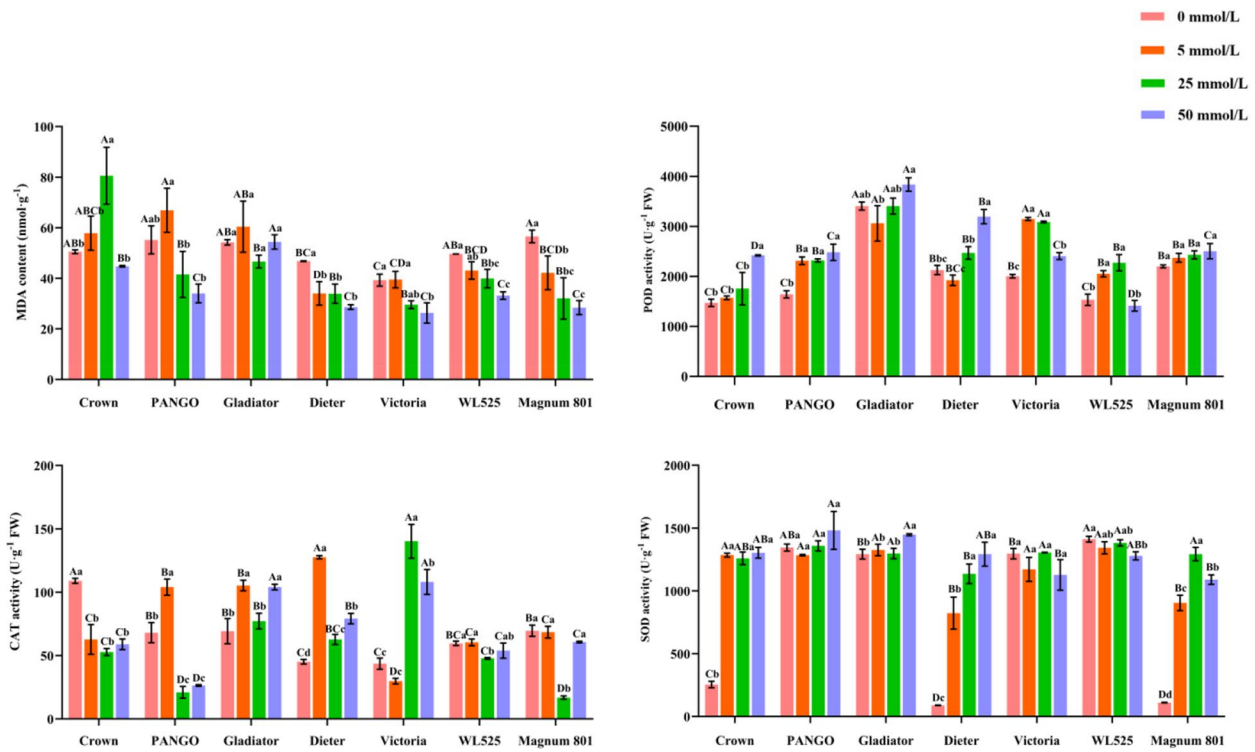

Fig. 2 Effects of exogenous calcium on the activity of protective enzymes and malondialdehyde (MDA) content in alfalfa seedlings. Error bars represent standard error; different letters indicate significant differences at $P < 0.05$. Lowercase letters denote significant differences among different calcium concentrations within a variety, while uppercase letters signify significant differences between varieties at a given concentration

and Victoria first increased and then decreased as the calcium stress concentration increased. In contrast, the MDA content in Dieter, WL525, and Magnum 801 generally decreased with increasing calcium stress concentration. Notably, at a calcium concentration of 50 mmol·L^{-1}, the MDA content in Dieter, Victoria, WL525, and Magnum 801 was significantly lower than that of the control group ($P < 0.05$), indicating reduced membrane lipid peroxidation in these seedlings under 50 mmol·L^{-1} $CaCl_2$ stress [23]. At 25 mmol·L^{-1} calcium concentration, Crown exhibited the highest MDA content, significantly differing from all other varieties ($P < 0.05$), while no significant differences existed among the remaining six. When increased to 50 mmol·L^{-1}, Gladiator showed the maximal MDA level, demonstrating significant differences compared with Crown and other varieties ($P < 0.05$).

As the calcium stress concentration increased, peroxidase (POD) activity in Victoria and WL525 seedlings increased initially and then decreased. In contrast, POD activity in Crown, PANGO, Gladiator, and Dieter seedlings steadily increased, peaking at 50 mmol·L^{-1} $CaCl_2$ stress. No significant differences in POD activity were observed in Magnum 801 seedlings across different treatments. Under the stress of 0, 25, 50 mmol·L^{-1} $CaCl_2$ solution, Gladiator exhibited the highest POD activity.

Catalase (CAT) activity in alfalfa seedlings was significantly influenced by exogenous calcium ($P < 0.05$). The CAT activity in PANGO, Gladiator, Dieter, and WL525 seedlings was highest at a 5 mmol·L^{-1} calcium concentration, while Victoria seedlings exhibited peak CAT activity at 25 mmol·L^{-1}. Exogenous calcium also significantly affected superoxide dismutase (SOD) activity in all alfalfa varieties except PANGO and Victoria ($P < 0.05$). The SOD activity in Magnum 801 seedlings increased initially and then decreased, while Dieter seedlings showed a continuous increase in SOD activity. At 25 mmol·L^{-1} exogenous calcium, there were significant differences in SOD activity between Dieter and all varieties except Crown ($P < 0.05$), and there were significant differences in CAT activity between Victoria and all the other varieties at this concentration ($P < 0.05$). When the concentration of exogenous calcium was 50 mmol·L^{-1}, the SOD activity of Victoria and Magnum 801 was significantly different from that of Gladiator and PANGO, and the CAT activity of Victoria and Gladiator was also significantly different from that of the other 5 varieties at this concentration ($P < 0.05$).

Effects on proline and soluble sugar contents

As the concentration of exogenous calcium increased (Fig. 3), the proline (Pro) content in the seedlings of

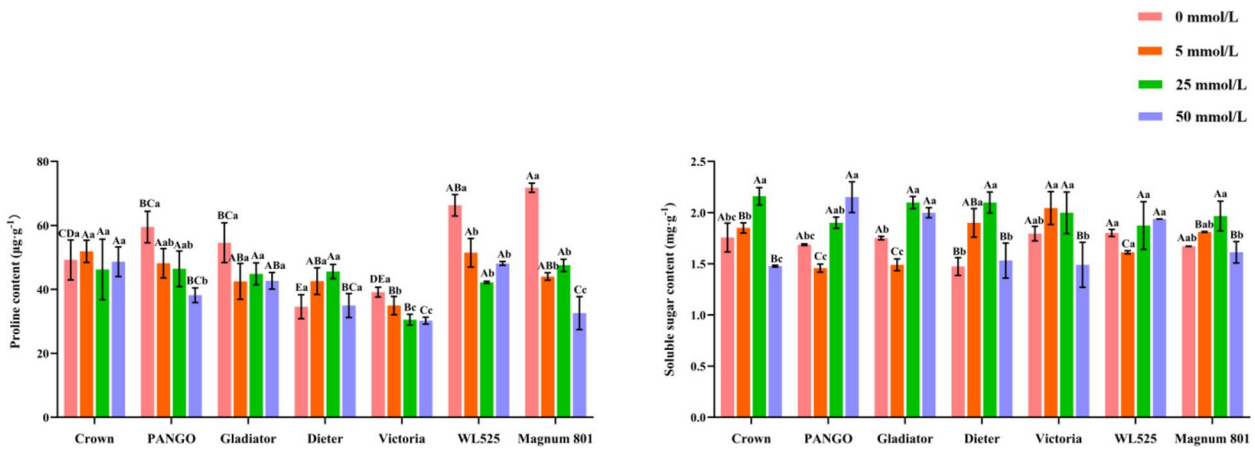

Fig. 3 Effects of exogenous calcium on proline (Pro) and soluble sugar (SS) contents in alfalfa seedlings. Error bars represent standard error; different letters indicate significant differences at $P < 0.05$. Lowercase letters denote significant differences among different calcium concentrations within a variety, while uppercase letters signify significant differences between varieties at a given concentration

PANGO, Victoria, WL525, and Magnum 801 generally decreased. Among these, the Pro content in all varieties except WL525 decreased to its lowest value under 50 mmol·L^{-1} $CaCl_2$ stress. No significant differences in Pro content were observed among Crown, Gladiator, and Dieter at different concentrations. For most varieties, except Crown and Dieter, the highest Pro content was observed in the control group. At the same calcium concentration, significant differences in Pro content were observed among different varieties. Specifically, at a calcium concentration of 25 mmol·L^{-1}, the Pro content of Victoria differed significantly from that of all other varieties ($P < 0.05$). Moreover, at a calcium concentration of 5 mmol·L^{-1}, the Pro content of Victoria showed significant differences compared to that of Crown, PANGO, and WL525 ($P < 0.05$).

Exogenous calcium significantly affected the soluble sugar (SS) content in alfalfa seedlings (except WL525) ($P < 0.05$). With the increase in $CaCl_2$ concentration, the SS content of all varieties (except PANGO, Gladiator, and WL525) initially increased and then decreased, reaching varying peak values. At 25 mmol·L^{-1} $CaCl_2$, the SS content in Crown, Gladiator, Dieter, and Magnum 801 seedlings reached its peak. It is worthy of note that at this concentration, no significant differences were observed among all varieties. The SS content in PANGO increased gradually, reaching a peak of 2.15 mg·g^{-1} at 50 mmol·L^{-1} $CaCl_2$. However, at 50 mmol·L^{-1} $CaCl_2$, the SS content in Crown, Victoria, and Magnum 801 seedlings was the lowest. At this concentration, significant differences were observed between PANGO, Gladiator, WL525 and the other four varieties ($P < 0.05$).

Correlation analysis between seed germination, seedling growth, and physiological indicators under calcium stress

Correlation analysis revealed significant relationships among germination metrics, growth parameters, and physiological traits (Fig. 4). Root length (RL) showed a significant positive correlation with germination potential (GP) and germination index (GI) but was significantly negatively correlated with superoxide dismutase (SOD) activity, indicating a trade-off between growth and oxidative stress mitigation. Bud length (BL) was significantly positively correlated with peroxidase (POD) activity, relative water content (RWC), and catalase (CAT) activity, while exhibiting a significant negative correlation with proline (Pro) content, suggesting their collective role in maintaining water balance and shoot development.

Additionally, Pro content was negatively correlated with CAT activity and RWC, indicating its compensatory role in osmotic adjustment when antioxidant enzyme activity is insufficient. These relationships provide insights into the interplay between growth, enzymatic defense, and osmotic regulation under calcium stress.

Principal component analysis and comprehensive evaluation of calcium stress resistance in different alfalfa varieties

Principal component analysis (PCA) is a multivariate statistical technique that reduces multiple variables to a smaller number while preserving as much of the original information as possible. PCA results indicated that the cumulative contribution rate of the first four principal components to the germination, growth, and physiological indicators of calcium adaptability in alfalfa was 93.013%. The eigenvalues for these components were

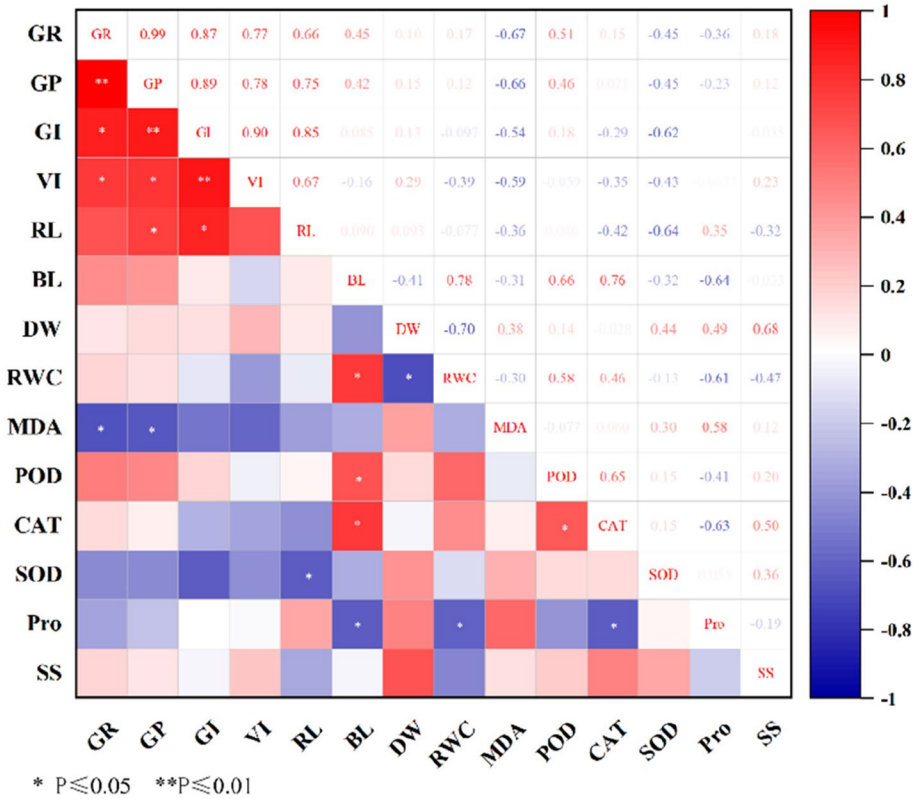

Fig. 4 Correlation heatmap between alfalfa seed germination, seedling growth, and physiological indicators under calcium stress. GR, germination rate; GP, germination potential; GI, germination index; VI, vigor index; RL, root length; BL, bud length; DW, dry weight; RWC, relative water content; MDA, malondialdehyde; POD, peroxidase; CAT, catalase; SOD, superoxide dismutase; Pro, proline; SS, soluble sugar. *$P < 0.05$; **$P < 0.01$

5.297, 3.943, 2.567, and 1.215, respectively, demonstrating their ability to effectively represent the original data (Fig. 5, Table 2).

As shown in Table 2, the first principal component was primarily influenced by germination rate (GR), germination potential (GP), and germination index (GI), reflecting the seed germination performance of alfalfa under calcium stress. The second principal component was primarily driven by relative water content (RWC) and bud length (BL), serving as indicators of alfalfa's growth and water retention under stress. The third principal component was dominated by soluble sugar content (SS) and dry weight (DW), representing the accumulation of substances in alfalfa under calcium stress. The fourth principal component was defined by malondialdehyde (MDA), proline (Pro), and peroxidase (POD) activity, which are associated with cell membrane lipid peroxidation and subsequent repair processes in alfalfa under stress conditions. These four principal components collectively serve as comprehensive indicators for evaluating the calcium adaptability of alfalfa varieties.

Based on the factor weights (W_j) and membership function values ($\mu(X_j)$), the calcium adaptability index (D) was calculated to comprehensively evaluate the adaptability of seven alfalfa varieties to calcium stress. Membership function values ($\mu(X_j)$) are the projected values of the sample on each principal component, reflecting the performance of that component. The D-value is the comprehensive score obtained by the sum of the scores of multiple principal components weighted by the variance contribution rate, and the 7 varieties are sorted according to the D-value. As shown in Table 2, the contribution rates of the four principal components were 37.834%, 28.163%, 18.335%, and 8.682%, respectively. Using the weight calculation formula (Formula 6), the weights of the four principal components were determined to be 0.4068, 0.3028, 0.1971, and 0.0933, respectively. The membership function method was then applied, with the first four principal components serving as evaluation indicators. The membership function values for each principal component were calculated using Formulas 7, 8, and 9, and the results were ranked to compare the calcium stress resistance of the alfalfa varieties. According to the results in Table 3, the alfalfa varieties were ranked in terms of calcium adaptability as follows: Gladiator > Victoria > Dieter > Magnum 801 > WL525 > Crown > PANGO. These rankings align with observed physiological and growth responses, validating the robustness of the comprehensive evaluation framework.

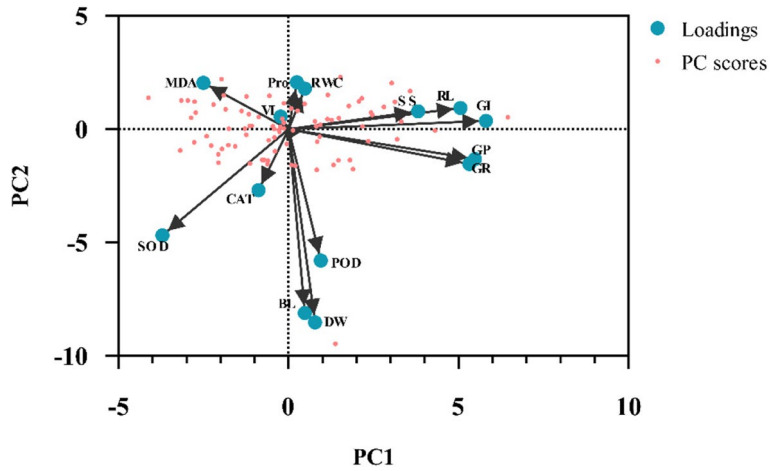

Fig. 5 Principal component analysis (PCA) of growth and physiological characteristics of alfalfa under calcium stress. Parameters analyzed include: GR, germination rate; GP, germination potential; GI, germination index; VI, vigor index; RL, root length; BL, bud length; DW, dry weight; RWC, relative water content; MDA, malondialdehyde; POD, peroxidase; CAT, catalase; SOD, superoxide dismutase; Pro, proline; SS, soluble sugar

Table 2 Eigenvectors and contribution rates of principal components in calcium stress experiments

Indices	Principal component 1	Principal component 2	Principal component 3	Principal component 4
germination rate	0.08	0.00	0.06	0.01
germination potential	0.08	-0.01	0.05	0.06
germination index	0.07	-0.05	0.00	0.05
vitality index	0.06	-0.07	0.04	-0.21
root length	0.06	-0.06	-0.05	0.28
bud length	0.04	0.1	0.00	0.17
dry weight	-0.01	-0.07	0.2	0.18
relative water content	0.02	0.11	-0.09	0.14
MDA	-0.06	-0.02	0.05	0.4
POD	0.03	0.08	0.11	0.33
CAT	0.00	0.1	0.12	0.05
SOD	-0.05	0.01	0.12	-0.07
Pro	-0.03	-0.1	-0.02	0.39
soluble sugar	0.00	0.00	0.23	-0.2
Eigenvalues	5.297	3.943	2.567	1.215
Contribution rate (%)	37.834	28.163	18.335	8.682
Cumulative contribution rate (%)	37.834	65.997	84.331	93.013
weights	0.4068	0.3028	0.1971	0.0933

Discussion

Calcium is an essential element for plant growth, playing a pivotal role in seed germination, development, and various physiological processes. In karst environments, where soils are naturally rich in calcium, this element significantly influences seed germination and seedling development [24].

Effects of exogenous calcium on seed germination in alfalfa

The effects of calcium on seed germination in alfalfa vary across different varieties, with multiple factors influencing the germination capacity of seeds [25]. Key indicators such as germination rate (GR), germination potential (GP), germination index (GI), and vigor index (VI) are commonly used to assess seed germination ability [26].

Table 3 Membership function values and comprehensive evaluation scores of different alfalfa varieties

Varieties	Membership function value ($\mu(X_j)$)				Comprehensive Valuation (D)	sort
	Principal component 1	Principal component 2	Principal component 3	Principal component 4		
Crown	0.0000	0.3664	0.4946	0.4580	0.2512	6
PANGO	0.0535	0.5392	0.0545	0.5689	0.2488	7
Gladiator	0.4353	0.8272	1.0000	1.0000	0.7180	1
Dieter	0.6388	1.0000	0.0000	0.4988	0.6092	3
Victoria	0.6481	0.8014	0.8097	0.0000	0.6659	2
WL525	0.3973	0.0000	0.6770	0.3790	0.3304	5
Magnum 801	1.0000	0.1004	0.2636	0.6981	0.5543	4

Under varying calcium concentration stress, the GR and GP of the tested alfalfa varieties were only marginally affected. Notably, with the exception of Gladiator's GR and Victoria's GP, no significant differences were observed between the calcium treatments and the control for these two parameters. However, both the GI and VI were significantly impacted by calcium stress. These results suggest that the GI and VI are more sensitive to calcium stress than the GR and GP, a finding consistent with studies on other plants such as pepper, spinach, and sorghum [27–29].

In this study, when the calcium concentration was increased to 50 mmol·L^{-1}, a significant decrease in the GI of Victoria, WL525, and Magnum 801 was observed compared to their respective controls. Additionally, the VI of all seven tested varieties was significantly reduced relative to the control. These findings indicate that osmotic stress and ion toxicity induced by high calcium concentrations (50 mmol·L^{-1}) led to damage in alfalfa seed germination, disrupting physiological and metabolic processes and ultimately reducing germination capacity [30]. Interestingly, at 25 mmol·L^{-1} calcium, the GI of PANGO was significantly higher than the control, consistent with findings by Weng et al. [31], who observed that moderate calcium concentrations can enhance seed germination in mulberry seedlings. Conversely, the GI of Crown and Gladiator did not differ significantly under varying calcium concentrations. However, both varieties exhibited lower germination and vigor indices compared to Victoria, WL525, and Magnum 801, indicating varietal differences in response to calcium stress.

Effects of exogenous calcium on growth characteristics of alfalfa seedlings

A well-developed root system enhances the nutrient absorption and material transport capacities of plants under abiotic stress, thereby promoting growth in challenging environmental conditions [32]. At 5 mmol·L^{-1} $CaCl_2$, a significant increase in root length was observed in Crown, and a significant increase in bud length was noted in Gladiator and WL525 compared to the control. In contrast, at 50 mmol·L^{-1} $CaCl_2$, root and bud lengths decreased in all seven varieties compared to the control. These results suggest that moderate exogenous calcium (5 mmol·L^{-1}) can promote root and bud growth in certain alfalfa varieties. However, when the calcium concentration exceeds a threshold, growth is inhibited, which is consistent with findings from Zhang et al. [33] and highlights the varietal differences in the adaptability of alfalfa to calcium stress.

The effect of calcium concentration on the dry weight of alfalfa was relatively minor, although significant differences were observed in the dry weight of PANGO, Gladiator, Dieter, and Victoria compared to the control. Notably, PANGO exhibited a significant increase in dry weight at both 25 mmol·L^{-1} and 50 mmol·L^{-1} $CaCl_2$, while Gladiator showed a significant increase at 5 mmol·L^{-1} $CaCl_2$, and Dieter and Victoria displayed significant increases at 25 mmol·L^{-1} $CaCl_2$. These findings indicate that certain concentrations of calcium can help alfalfa maintain high or stable dry weight under calcium stress. This could be attributed to the plant's ability to adjust its growth pattern, resource allocation, and physiological regulation to sustain growth and vitality under stress [34].

A decrease in plant relative water content (RWC) is a clear indicator of water deficiency, and a reduction in root water absorption capacity can lead to decreased RWC in plants [35]. In this study, all alfalfa varieties except Gladiator showed a significant decrease in RWC at 50 mmol·L^{-1} $CaCl_2$ compared to the control, with a general trend of reduction observed across the varieties.

Effects of exogenous calcium on physiological characteristics of alfalfa seedlings

Calcium ions play a crucial role in maintaining and regulating ion balance, enabling plants to adapt to adverse

environmental conditions. However, when calcium ion concentrations exceed optimal levels, calcium stress can occur [36]. Calcium stress is known to induce the production of reactive oxygen species (ROS) in plant cells, leading to the peroxidation of unsaturated fatty acids in cell membrane lipids. This results in the accumulation of malondialdehyde (MDA), a byproduct of lipid peroxidation. The level of MDA is often used as an indicator of plant resilience, with higher MDA content signifying greater damage to plants [37–39].

Appropriate calcium treatments can reduce the accumulation of ROS and MDA, thereby lowering membrane permeability and enhancing plant adaptability to calcium stress [40]. In the present study, the MDA content in six out of the seven alfalfa varieties tested showed significant changes in response to increasing calcium stress. At a concentration of 5 mmol·L^{-1} $CaCl_2$, MDA levels increased in PANGO, Gladiator, and Victoria seedlings. At 25 mmol·L^{-1} $CaCl_2$, the MDA content increased in Crown seedlings. However, when calcium concentrations exceeded these levels, the MDA content decreased, possibly due to the regulation of ROS and MDA by high calcium concentrations. It is also possible that other physiological processes, such as the upregulation of protective enzymes, contributed to the reduction in MDA levels.

Superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) are crucial components of the reactive oxygen species scavenging system, and their activity levels are indicative of plant stress resistance [41]. SOD plays an essential role in mitigating oxidative stress by converting superoxide radicals (O_2^-) into hydrogen peroxide (H_2O_2) and oxygen (O_2), thus reducing the potential damage caused by ROS [42]. POD, a primary antioxidant enzyme in plant cells, helps mitigate stress-induced damage by enhancing its activity under abiotic stress [43, 44]. CAT, which exhibits high enzymatic activity, is effective in decomposing H_2O_2, although it has a relatively weak affinity for it [45]. The increased activities of SOD, POD, and CAT reflect the protective role of calcium in maintaining plant health.

In this study, the POD and SOD activities of Crown and Dieter seedlings showed a consistent upward trend throughout the stress period, indicating that their protective enzyme systems were relatively intact. Conversely, after reaching their peak, POD and SOD activities in Victoria and WL525 seedlings gradually declined as the calcium stress concentration increased. High-vigor seeds exhibit enhanced antioxidant enzyme activity that effectively scavenges reactive oxygen species (ROS), thus maintaining cellular membrane integrity [46]. In cultivars Victoria and WL525, the observed post-stress declines in GI and VI demonstrated strong synchrony with decreasing POD and SOD activities. This coordinated response highlights the critical relationship between antioxidant capacity and oxidative balance, mechanistically linking the dynamic equilibrium of enzymatic antioxidants and ROS levels to seed germination performance. This decline suggests that the protective enzyme systems in these seedlings were compromised, leading to an imbalance in the production and removal of peroxide compounds, which ultimately interfered with normal growth. Except for WL525 seedlings, different concentrations of $CaCl_2$ significantly affected CAT activity in all alfalfa varieties. CAT activity initially increased with increasing calcium concentration but began to decrease once a peak concentration was surpassed. This pattern suggests that calcium concentrations between 5 and 25 mmol·L^{-1} enhanced the metabolism of the seven alfalfa varieties, improving their adaptability to external stress. However, concentrations above this range impaired normal plant growth.

Proline is a key component of plant proteins and serves as an osmoregulatory substance within the plant cytoplasm, especially under stress conditions [47]. When plants experience stress, water balance within the plant is disrupted; soluble sugars help stabilize cell membranes and improve cellular water retention capacity [48]. Both soluble sugar and proline levels increase under stress, thereby enhancing plant stress resistance [49].

Previous research demonstrates that moderate calcium stress induces concurrent elevation of soluble sugar content and antioxidant enzyme activity, acting synergistically to mitigate membrane lipid peroxidation damage [50]. Notably, elevated calcium concentrations appear to disrupt enzymatic functionality in glucose metabolism and suppress sugar biosynthesis, potentially via impaired photosynthetic efficiency. In this experiment, all varieties except WL525 and Magnum 801 exhibited significant changes in soluble sugar content in response to increasing calcium stress. The soluble sugar content generally followed a pattern of initial increase followed by a decrease, which may be attributed to the plant's attempt to maintain osmotic stability in response to environmental stress. This ensures the stability of internal osmotic potential and supports normal metabolic and growth processes. However, when calcium concentrations exceed a threshold, the osmotic balance is disrupted, leading to a decrease in soluble sugar content. Similarly, significant changes in proline content were observed in Victoria, WL525, and Magnum 801 seedlings, with proline levels decreasing as calcium stress concentration increased. This suggests that as calcium concentrations increase, damage intensifies, resulting in a reduced rate of osmoregulatory substance synthesis.

Evaluation of calcium adaptability of different alfalfa varieties and optimal calcium concentration screening

The tolerance of plants to abiotic stress is a complex trait, and to avoid the one-sidedness of single-indicator evaluations, more scholars are using comprehensive evaluation methods combining principal component analysis (PCA) and membership function methods. For example, in the evaluation of disease resistance in sorghum seedlings [51], the selection of suitable tree species in dry and dusty mining areas in Northwest China [24, 52], and the assessment of drought resistance in Gleditsia sinensis seedlings [53], comprehensive evaluation methods have been used to scientifically evaluate differences among plant varieties. In this study, calcium stress had different effects on 14 indicators of alfalfa seed germination, seedling growth, and physiology, and there were significant or highly significant correlations among these indicators. To avoid the limitations of using a single indicator for evaluation, we also used a comprehensive evaluation method combining PCA and membership functions.

Among the seven alfalfa varieties, Gladiator, Victoria, Dieter, and Magnum 801 had composite evaluation values (D) greater than 0.5, indicating strong adaptability to calcium. Composite evaluation values between 0.2 and 0.3 were observed for Crown and PANGO, showing weak adaptability to calcium.

In this study, appropriate calcium concentrations promoted alfalfa seed germination, seedling growth, and physiological regulation, whereas non-optimal calcium concentrations inhibited these processes. The comprehensive evaluation ranked the calcium concentrations in terms of effectiveness as follows: $5 > 25 > 0 > 50$ mmol·L^{-1} (Table 4). The comprehensive evaluation values for 5 and 25 mmol·L^{-1} $CaCl_2$ were higher than that of the control, suggesting these concentrations positively influenced alfalfa growth. In contrast, the comprehensive evaluation value for the 50 mmol·L^{-1} $CaCl_2$ treatment decreased by 60.9% compared to the control, indicating that high calcium concentrations were toxic to alfalfa, inhibiting its growth. This highlights the importance of determining an optimal range of calcium concentrations, providing theoretical support for promoting and utilizing alfalfa in calcium-rich areas.

Table 4 Selection of optimal calcium concentration for tested alfalfa varieties

	Membership function			
	L1	L2	L3	L4
germination rate	1	0.167	0.833	0
germination potential	0.731	0.038	1	0
germination index	1	0.520	0.467	0
vitality index	1	0.944	0.688	0
root length	0.628	1	0.534	0
bud length	0.697	1	0.722	0
dry weight	1	0.898	0.571	0
relative water content	1	0.920	0.532	0
MDA	0	0.081	1	0.160
POD	1	0.388	0.238	0
CAT	0	0.473	0.701	1
SOD	0.327	1	0	0.519
Pro	0	0.726	0.693	1
soluble sugar	0	1	0.6	0.6
Comprehansive evaluation	0.599	0.654	0.613	0.234

L1: 0 mmol·L^{-1} $CaCl_2$; L2: 5 mmol·L^{-1} $CaCl_2$; L3: 25 mmol·L^{-1} $CaCl_2$; L4: 50 mmol·L^{-1} $CaCl_2$

Conclusion

This study investigated the effects of varying concentrations of calcium stress on the germination and physiological growth of alfalfa (*Medicago sativa*) seeds, providing valuable data for the introduction and breeding of alfalfa in calcium-rich areas such as karst landscapes. The results demonstrated that calcium significantly influences alfalfa seed germination, seedling growth, and physiological characteristics. To mitigate the effects of calcium stress, alfalfa seedlings adapt to changes in calcium-rich environments through osmotic regulation and enhanced enzymatic activity. Therefore, when cultivating alfalfa in calcium-rich karst regions, it is essential to analyze the soil calcium content at the planting site. When the soil calcium content exceeds the critical stress threshold (50 mmol·L^{-1}), calcium stress will have adverse effects on seed germination and seedling growth. Conversely, if the calcium concentration ranges from 5 to 25 mmol·L^{-1}, it will be conducive to germination, physiological regulation, and growth. Future molecular and transcriptomic studies are needed to elucidate the genetic mechanisms of calcium tolerance in alfalfa. This can be achieved by exploring the dynamic changes in gene expression under calcium stress, identifying key transcription factors closely associated with calcium tolerance, and comparing the transcriptomic differences among alfalfa varieties with varying degrees of calcium tolerance. The findings from these studies will provide a solid foundation for breeding alfalfa varieties with enhanced stress resistance.

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Authors' contributions

L.Z. supervised the experiments. J.P., J.Z., C.L. and S.L. performed the experiments. J.P. analyzed the data. J.P. wrote the manuscript. All the authors read and approved the final manuscript.

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Data availability
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
There are no ethical issues involved in this paper.

Consent for publication
The manuscript is approved by all authors for publication.

Competing interests
The authors declare no competing interests.

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References

1. Zhao L, Wang P, Chen C, Song G, Tang H. Effects of exogenous calcium on growth and physiological characteristics of prickly lily seedlings in karst mountains under drought stress. J Nuclear Agricult. 2017;31(10):2039–46.
2. Liu C, Huang Y, Liang Y. Adaptive Mechanism Exploration of Camellia limonia in Karst High Calcium Environment by Integrative Analysis of Metabolomics and Metagenomics. Tropical Plant Biol. 2022;15:22–39. https://doi.org/10.1007/s12042-021-09308-0.
3. Wu J, Wang R, Liu X, et al. Calcium dynamics during the growth of Agaricus bisporus: implications for mushroom development and nutrition. Chem Biol Technol Agric. 2023;10:99. https://doi.org/10.1186/s40538-023-00471-y.
4. Tan H, Sun W, Zhao L, et al. Exogenous Application of Sorbitol-Chelated Calcium Enhances Salt Tolerance in Rapeseed (Brassica napus L.). J Soil Sci Plant Nutr. 2024;24(3):4196–210. https://doi.org/10.1007/s42729-024-01820-y.
5. Borghesi E, Carmassi G, Uguccioni MC, Vernieri P, Malorgio F. EFFECTS OF CALCIUM AND SALINITY STRESS ON QUALITY OF LETTUCE IN SOILLESS CULTURE. J Plant Nutr. 2013;36(5):677–90. https://doi.org/10.1080/01904167.2012.721909.
6. Ren H, Zhang Y, Zhong M, et al. Calcium signaling-mediated transcriptional reprogramming during abiotic stress response in plants. Theor Appl Genet. 2023;136:210. https://doi.org/10.1007/s00122-023-04455-2.
7. Yang L, Zhao C, Jiao S, et al. Reconstructing spatial pattern of historical cropland in karst areas of Guizhou. Southwest China Sci Rep. 2022;12:22391. https://doi.org/10.1038/s41598-022-26793-9.
8. Wang P, Ding L, Li F, Liao J, Wang M. Herbivore camping reshapes the taxonomy, function and network of pasture soil microbial communities. PeerJ. 2022;10:e14314. https://doi.org/10.7717/peerj.14314.
9. Ding L, Tian L, Jingyi L, Zhang Y, Wang M, Wang P*. Grazing lowers soil multifunctionality but boosts soil microbial network complexity and stability in a subtropical grassland of China. Front Microbiol. 2023;13:1027097. https://doi.org/10.3389/fmicb.2022.1027097.
10. Meng W, Ran J, Dai Q, et al. Morphological and physiological adaptation characteristics of lithophytic bryophytes to karst high calcium environment. BMC Plant Biol. 2023;23:160. https://doi.org/10.1186/s12870-022-03980-4.
11. Ding L, Wang P*. Afforestation suppresses soil nitrogen availability and soil multifunctionality in a subtropical grassland. Sci Total Environ. 2021;761:143663. https://doi.org/10.1016/j.scitotenv.2020.143663.
12. Su K, Mu L, Zhou T, et al. Intercropped alfalfa and spring wheat reduces soil alkali-salinity in the arid area of northwestern China. Plant Soil. 2024;499:275–92. https://doi.org/10.1007/s11104-022-05846-y.
13. Dong L, Hou Z, Li H, Li Z, Fang C, Kong L, Li Y, Du H, Li T, Wang L, He M, Zhao X, Cheng Q, Kong F, Liu B. Agronomical selection on loss-of-function of GIGANTEA simultaneously facilitates soybean salt tolerance and early maturity. J Integr Plant Biol. 2022;64(10):1866–82. https://doi.org/10.1111/jipb.13332.
14. Filho EGA, Braga LN, Silva LMA, et al. Physiological changes for drought resistance in different species of Phyllanthus. Sci Rep. 2018;8:15141. https://doi.org/10.1038/s41598-018-33496-7.
15. Sivakumar J, Prashanth JEP, Rajesh N, et al. Principal component analysis approach for comprehensive screening of salt stress-tolerant tomato germplasm at the seedling stage. J Biosci. 2020;45:141. https://doi.org/10.1007/s12038-020-00111-9.
16. Cui X, Guo Z, Mao Y, et al. Evaluation on salt tolerance of 33 alfalfa varieties during germination. J Domestic Anim Ecol. 2022;43(02):55–65.
17. Yang Z, Yong-Sheng L, Xiu-Feng G. A new method for accurate determination of peroxidase activity based on fluorescence decrease of guaiacol. Chin J Anal Chem. 2015;43(7):1040–6.
18. Kadhum MA, Hadwan MH. A precise and simple method for measuring catalase activity in biological samples. Chem Pap. 2021;75:1669–78. https://doi.org/10.1007/s11696-020-01401-0.
19. Sun XW, Liu YF. Comparison of determination of proline content with toluene extraction and without extraction — sulfosalicylic acid method. J Hebei Normal Univ Sci Technol. 2006;(04):26-28+38.
20. Senthilkumar M, Amaresan N, Sankaranarayanan A. Estimation of Malondialdehyde (MDA) by Thiobarbituric Acid (TBA) Assay. In: Plant-Microbe Interactions. Springer Protocols Handbooks. New York: Humana; 2021. p. 103–105. https://doi.org/10.1007/978-1-0716-1080-0_25.
21. Huang L, Ding L, Wang W, et al. Effects of simulated drought stress on the growth and physiological and biochemical parameters of Paspalum wettsteinii. Acta Physiol Plant. 2023;45:82. https://doi.org/10.1007/s11738-023-03556-1.
22. Wang C, Wu B, Jiang K. Allelopathic effects of Canada goldenrod leaf extracts on the seed germination and seedling growth of lettuce reinforced under salt stress. Ecotoxicology. 2019;28:103–16. https://doi.org/10.1007/s10646-018-2004-7.
23. Bjornson M, Dandekar A, Dehesh K. Determinants of timing and amplitude in the plant general stress response. J Integr Plant Biol. 2016;58(2):119–26. https://doi.org/10.1111/jipb.12373.
24. Zhu XC, Ma MG, Tateno R, et al. Effects of vegetation restoration on soil carbon dynamics in Karst and non-karst regions in Southwest China: a synthesis of multi-source data. Plant Soil. 2022;475:45–59. https://doi.org/10.1007/s11104-021-05220-4.
25. Gao Y, Zhao X, Liu X, Liu C, Zhang K, Zhang X, Zhou J, Dong G, Wang Y, Huang J, Yang Z, Zhou Y, Yao Y. OsRAV1 Regulates Seed Vigor and Salt Tolerance During Germination in Rice. Rice (New York, NY). 2024;17(1):56. https://doi.org/10.1186/s12284-024-00734-8.
26. Gobade A, Arathi S, Gijare S, et al. Evaluating salt tolerance in soybean core collection: germination response under salinity stress. Genet Resour Crop Evol. 2024. https://doi.org/10.1007/s10722-024-02081-5.
27. AL-Quraan NA, Samarah NH, AL-Fawaz AM. The physiological effect of GABA priming on pepper (Capsicum annuum L.) during seed germination under salt stress. Plant Growth Regul. 2024;104(2):953–74. https://doi.org/10.1007/s10725-024-01207-0.
28. Ali Naghizadeh A, Zarandi MM, Khoshroo SM, et al. Investigating the Effect of Green Silver Nanoparticles on Seed Germination and Physiological Parameters of Spinach (Spinacia oleracea L.) under Salt Stress. Russ J Plant Physiol. 2024;71:102. https://doi.org/10.1134/S102144372460586X.
29. Xing Y, Chen X, Zhang M, et al. $CaCl_2$ priming promotes sorghum seed germination under salt stress by activating sugar metabolism. Plant Growth Regul. 2023;101:385–97. https://doi.org/10.1007/s10725-023-01025-w.
30. Fan R, Su X, Guo Y, et al. Cotton seedling drought tolerance is improved via salt preconditioning. Protoplasma. 2021;258:263–77. https://doi.org/10.1007/s00709-020-01561-6.
31. Weng X, Li H, Zhou Y, et al. Exogenous Calcium can Promote Nutrient Uptake and Stoichiometric Balance of Mulberry Seedlings Under Salt Stress. J Soil Sci Plant Nutr. 2024. https://doi.org/10.1007/s42729-024-02063-7.
32. Zhou J, Huang L, Wang P, et al. Root morphology and transcriptomic analyses of the response to heat stress in Paspalum wettsteinii hack. Genet Resour Crop Evol. 2024;71:3219–39. https://doi.org/10.1007/s10722-023-01828-w.
33. Zhang Y, Qiao D, Zhang Z, et al. Calcium signal regulated carbohydrate metabolism in wheat seedlings under salinity stress. Physiol Mol Biol Plants. 2024;30:123–36. https://doi.org/10.1007/s12298-024-01413-0.

34. Wang K, Nan L, Guo Q. Changes in root endogenous hormone levels and rhizosphere fungi diversity in alfalfa under drought stress. Plant Growth Regul. 2024;102:313–24. https://doi.org/10.1007/s10725-023-01062-5.
35. Cao X, Sun L, Wang W, et al. Exogenous calcium application mediates K^+ and Na^+ homeostasis of different salt-tolerant rapeseed varieties under $NaHCO_3$ stress. Plant Growth Regul. 2024;102:367–78. https://doi.org/10.1007/s10725-023-01066-1.
36. Lu QH, Wang YQ, Yang HB. Effect of exogenous calcium on physiological characteristics of salt tolerance in Tartary buckwheat. Biologia. 2021;76:3621–30. https://doi.org/10.1007/s11756-021-00904-9.
37. Fang X, Mo J, Zhou H, et al. Comparative transcriptome analysis of gene responses of salt-tolerant and salt-sensitive rice cultivars to salt stress. Sci Rep. 2023;13:19065. https://doi.org/10.1038/s41598-023-46389-1.
38. Ahmad F, Kamal A, Singh A, et al. Salicylic Acid Modulates Antioxidant System, Defense Metabolites, and Expression of Salt Transporter Genes in Pisum sativum Under Salinity Stress. J Plant Growth Regul. 2022;41:1905–18. https://doi.org/10.1007/s00344-020-10271-5.
39. Sadeghi A, Razmjoo J, Karimmojeni H, Baldwin T. C Differential responses of Hollyhock (Alcea rosea L.) varieties to salt stress in relation to physiological and biochemical parameters. Sci Rep. 2024;14(1):8105. https://doi.org/10.1038/s41598-024-58537-2.
40. Kolupaev YE, Shakhov IV, Kokorev AI, et al. The Role of Reactive Oxygen Species and Calcium Ions in Implementing the Stress-Protective Effect of γ-Aminobutyric Acid on Wheat Seedlings Under Heat Stress Conditions. Cytol Genet. 2024;58:81–91. https://doi.org/10.3103/S0095452724020063.
41. Kirova E, Moskova I, Geneva M, et al. Antioxidant potential of tolerant and susceptible wheat varieties under drought and recovery. Cereal Res Commun. 2022;50:841–9. https://doi.org/10.1007/s42976-021-00222-5.
42. Gokce A, Sekmen Cetinel AH, Turkan I. Involvement of GLR-mediated nitric oxide effects on ROS metabolism in Arabidopsis plants under salt stress. J Plant Res. 2024;137(3):485–503. https://doi.org/10.1007/s10265-024-01528-1.
43. Meena NL, Kumari A, Maheshwari C, Bhardwaj R, Dhaka AS, Hasan M. Antioxidant Defense Mechanism and High-Temperature Stress Tolerance in Plants. In: Shahid, M., Gaur, R. (eds) Molecular Dynamics of Plant Stress and its Management. Singapore: Springer; 2024;193-210. https://doi.org/10.1007/978-981-97-1699-9_8.
44. Zhu Z, Dai Y, Yu G, Zhang X, Chen Q, Kou X, Mehareb EM, Raza G, Zhang B, Wang B, Wang K, Han J. Dynamic physiological and transcriptomic changes reveal memory effects of salt stress in maize. BMC Genomics. 2023;24(1):726. https://doi.org/10.1186/s12864-023-09845-w.
45. Kutlu I, Gulmezoglu N. Suitable Humic Acid Application Methods to Maintain Physiological and Enzymatic Properties of Bean Plants Under Salt Stress. Gesunde Pflanzen. 2023;75:1075–86. https://doi.org/10.1007/s10343-022-00766-4.
46. Wei Y, Zhang H, Fan J, Cai Q, Zhang Z, Wang J, Zhang M, Yan F, Jiang J, Xie H, Luo X, Wei L, Lin Y, He W, Qu M, Zhang X, Zhu Y, Xie H, Zhang J. Multiplex-genome-editing based rapid directional improvement of complex traits in rice. Plant Biotechnol J. 2024;22(9):2624–8. https://doi.org/10.1111/pbi.14375.
47. Zulfiqar F, Ashraf M. Proline Alleviates Abiotic Stress Induced Oxidative Stress in Plants. J Plant Growth Regul. 2023;42:4629–51. https://doi.org/10.1007/s00344-022-10839-3.
48. Zarbakhsh S, Shahsavar AR. Exogenous γ-aminobutyric acid improves the photosynthesis efficiency, soluble sugar contents, and mineral nutrients in pomegranate plants exposed to drought, salinity, and drought-salinity stresses. BMC Plant Biol. 2023;23(1):543. https://doi.org/10.1186/s12870-023-04568-2.
49. Bisht D, Mishra S, Bihani SC, et al. Salt Stress Tolerance and Calcium Signalling Components: Where We Stand and How Far We can Go? J Plant Growth Regul. 2024. https://doi.org/10.1007/s00344-024-11235-9.
50. Liu G, Lan J, Liu Y, Zhu J, Zheng C. Effects of Exogenous Calcium on Seedling Physiological Characteristics and Endogenous Hormone of Eucalyptus urophylla seedlings under Low Temperature Stress. J Northwest Forest Univ. 2017;32(6):101–6.
51. Chen S, Zhao Z, Liu X, Li K, Arif M, Zhang B, Dong L, Wang R, Ren M, Xie X. Response and disease resistance evaluation of sorghum seedlings under anthracnose stress. Sci Rep. 2024;14(1):21978. https://doi.org/10.1038/s41598-024-70088-0.
52. Zhu X, Cao B, Zhao S, et al. Selection and evaluation of suitable tree species in dry and dusty mining areas of Northwest China. J For Res. 2022;33:1817–28. https://doi.org/10.1007/s11676-022-01477-2.
53. Liu F, Zhao Y, Wang X, Wang B, Xiao F, He K. Physiological response and drought resistance evaluation of Gleditsia sinensis seedlings under drought-rehydration state. Sci Rep. 2023;13(1):19963. https://doi.org/10.1038/s41598-023-45394-8.

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