Effects of pH and calcium salt stress on the seed germination performance of three herbage species

Zhaoyi Wang[1], Sihui Tian[2], Jigao Wang[1], Honggang Shuai[1], Yaoyao Zhang[1], Yuefeng Wang[1], Baocheng Jin[1], and Xuechun Zhao[1]

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Abstract

Seed germination is critical for successful crop production, and the sensitivity to pH and salt stress depends on the plant's tolerance mechanisms. In view of the characteristics of calcium-rich and acidic soils in the karst areas of Guizhou Province, China, the effects of pH stress and calcium stress on the seed germination characteristics of three herbages were studied with the goal of exploring and revealing the mechanism of adaptation of the three herbages to an acidic soil environment and providing a theoretical basis for the selection and cultivation of acid-tolerant herbages in southwest China. In this study, six concentration gradients of CaCl2, including 0, 25 mmol/L, 50 mmol/L, 100 mmol/L, 150 mmol/L, and 200 mmol/L, and seven pH gradients, including 4.55, 5.35, 6.61, 7.03, 8.0, and 9.18 were established, respectively. The germination rate, germination potential and germination index of the seedlings were measured for each seed germination and seedling growth stage of orchardgrass, perennial ryegrass, and alfalfa, respectively. The results showed that when the concentration of salt stress began to change, the herbage seeds could adapt to salt stress at an appropriate pH condition. When only the pH value or CaCl2 concentration changes, the increase in pH and CaCl2 will inhibit the growth of shoots and roots. Weak acid can promote the growth of shoots and young roots, while alkaline conditions can inhibit their growth. The effect of a low concentration of CaCl2 was not apparent, while a high concentration of CaCl2 clearly inhibited the plants. The optimal pH and CaCl2 of the bud and root lengths changed after the interaction. In conclusion, there is a substantial difference between pH and calcium salt stress, and the interaction between pH and calcium salt concentration has a substantial influence on the salt and alkali tolerance of the three types of seeds.

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Abstract: Seed germination is critical for successful crop production, and the sensitivity to pH and salt stress depends on the plant's tolerance mechanisms. In view of the characteristics of calcium-rich and acidic soils in the karst areas of Guizhou Province, China, the effects of pH stress and calcium stress on the seed germination characteristics of three herbages were studied with the goal of exploring and revealing the mechanism of adaptation of the three herbages to an acidic soil environment and providing a theoretical basis for the selection and cultivation of acid-tolerant herbages in southwest China. In this study, six concentration gradients of $CaCl_2$, including 0, 25 mmol/L, 50 mmol/L, 100 mmol/L, 150 mmol/L, and 200 mmol/L,

and seven pH gradients, including 4.55, 5.35, 6.61, 7.03, 8.0, and 9.18 were established, respectively. The germination rate, germination potential and germination index of the seedlings were measured for each seed germination and seedling growth stage of orchardgrass (*Dactylis glomerata* L.), perennial ryegrass (*Lolium perenne* L.), and alfalfa (*Medicago sativa* L.), respectively. The results showed that under the interaction of pH and $CaCl_2$, the germination rate, germination potential and germination index of the three herbage seeds increased first and then decreased. When the concentration of salt stress began to change, the herbage seeds could adapt to salt stress at an appropriate pH condition. When only the pH value or $CaCl_2$ concentration changes, the increase in pH and $CaCl_2$ will inhibit the growth of shoots and roots. Weak acid can promote the growth of shoots and young roots, while alkaline conditions can inhibit their growth. The effect of a low concentration of $CaCl_2$ was not apparent, while a high concentration of $CaCl_2$ clearly inhibited the plants. The optimal pH and $CaCl_2$ of the bud and root lengths changed after the interaction. In conclusion, there is a substantial difference between pH and calcium salt stress, and the interaction between pH and calcium salt concentration has a substantial influence on the salt and alkali tolerance of the three types of seeds.**Keywords:** seed germination; pH; calcium; germination rate; germination potential; germination index

Introduction

Seed germination and seedling growth are the most sensitive stages in plant life, representing the first contact with the environment, notably water and soil [1]. Rapid seed germination and stand establishment are critical factors that affect crop production under conditions of stress[2]. Salt stress, drought stress and alkaline stress can inhibit seed germination; yet there is a limited understanding of the potential interaction of these stresses, which often occur together in nature [3]. Guizhou Province lies in the heart of a karst region in southwest China, and its soil is acidic and rich in calcium salts [4,5]. Therefore, when seeds germinate in the soil, if the pH is too low, the phospholipid structure of the cell membrane will be destroyed; the permeability of the cell membrane will be changed, and the rate of degradation of stored substances in the seeds will be reduced. Simultaneously, the respiration intensity of seeds will be weakened, and the activity of hydrolases will be reduced, thus, inhibiting seed germination [6]. In contrast, higher pH values result in higher contents of OH^-, which will interfere with the absorption of some key anions, affect the membrane potential energy, and inhibit the metabolism of seed storage compounds and related proteolytic enzyme activity to affect the germination of seeds [7]. As an important element in plant growth and development, calcium is involved in cell division and differentiation and the degradation and synthesis of membrane phospholipids among others. It is the primary regulator of plant metabolism and development. Ca^{2+} controls a series of important physiological processes and enzyme activities related to seed germination in cells [8]. Salinity has negative effects on the survival of plants. Seed germination and early seedling establishment can be strongly inhibited under salt stress, leading to significant reductions in plant density and poor growth, such as plant stunting, smaller and fewer leaves per plant, and a lower yield [9]. Salinity often generates osmotic stress and ion toxicity, which, in turn, cause nutritional deficiency and oxidative stress, further contributing to the restriction of plant growth, wilting or even death. Thus, salt stress is a limiting factor for improving crop yield, and the enhancement of salt tolerance is therefore, an important focus of current crop breeding programs [10]. Research shows that alkaline soils (a high pH from 8.5 to 11) endanger crop production more than soils that contain excess salt [11], To date, the effects of salt stress on plants have been widely reported [12,13], Understanding how plants respond to pH and calcium salt stress is essential for improving the tolerance of plants. Most studies on the effects of acid soils on forage seed germination have focused on growth, nodule formation, nitrogen fixation, and mineral nutrient uptake. Little attention has been paid to the sole effects of H^+ toxicity or calcium on the germination and survival of seedlings of the Legumes and the Gramineae. The effects of solution pH and exogenous calcium on seed germination, seedling survival and the growth of herbage were reported in this study. In this context, this study aims to compare the response to pH and calcium salt stress of three forage plants commonly used in many countries as animal feed, including orchard grass (*Dactylis glomerata*), perennial ryegrass (*Lolium perenne*), and alfalfa (*Medicago sativa*). The physiology and biochemistry of seed germination and seedling growth were examined in an attempt to illuminate the effects of pH and calcium salt stress. In this context, under salt stress, the seed germination and seedling growth of *M. sativa* were

inhibited by inappropriate concentrations, which led to a decrease in the germination rate [14]. Previous studies have clearly shown that the seed germination rate is always high in salt-free conditions but decreases as the concentration of soil salt increases. However, the interaction of salinity and alkalinity (pH) on seed germination remains unclear [15].

Materials and Methods

Seeds of *D. glomerata*, *L. perenne*, and *M. sativa*were purchased from Zhong Zhi Heng Seed Company (Guiyang, China) in October 2018 and stored dry in cloth bags at room temperature for further use. Seed germination experiments were conducted at the laboratory of Department of Grassland Science (26°44' N and106deg65' E), Guizhou University (Guiyang, China).*2.1 Experimental Design*A seed germination experiment was conducted in March 2019 using a petri dish filter paper hydroponic method to assess the combined effect of salt stress and pH on seed germination based on the characteristics of rich calcium and acid soil in the karst area in Guizhou Province. Six $CaCl_2$ concentrations were established as follows: 0 (C_{CK}), 25 mmol/L (C_1), 50 mmol/L (C_2), 100 mmol/L (C_3), 150 mmol/L (C_4), and 200 mmol/L (C_5). There were six pH treatments, including 4.55 (pH_1), 5.35 (pH_2), 6.61 (pH_3), 7.03 (pH_4), 8.0 (pH_5), and 9.18 (pH_6), respectively. There were 36 treatments with three replicates (Table 1). Uniform and full-sized seeds of *D. glomerata*, *L. perenne*, and *M. sativa* were selected, sterilized with 0.5% $KMnO_4$ for 20 min, and washed three times with distilled water. They were then sterilized with ethanol 70% for 30 s, rinsed three times with distilled water and arranged neatly in petri dishes covered with two sheets of filter paper. Different concentrations of $CaCl_2$ and pH values were dripped on the seeds. Each per petri dish included 50 seeds, which were incubated at 25 degC in the dark. Each treatment was conducted in triplicate. The photoperiod was 12 h/day; the temperature was 25 +- 1 degC day and 14 +- 1 degC night, and the relative humidity was 80 +- 1%.**Table 1.** Design of the interaction between the concentration of $CaCl_2$ and pH on the seeds of three forage plants*D. glomerata*, *L. perenne*, and *M. sativa*

pH	**$CaCl_2$ (mmol/L) 0(C_{ck})**	**$CaCl_2$ (mmol/L) 25(C_1)**	**$CaCl_2$ (mmol/L) 50(C_2)**	**$CaCl_2$ (mmol/L) 100(C_3)**	**$CaCl_2$ (mmol/L) 150(C_4)**	**C 2**
4.55(pH_1)	C_{ck} pH_1	C_1 pH_1	C_2 pH_1	C_3 pH_1	C_4 pH_1	C
5.35(pH_2)	C_{ck} pH_2	C_1 pH_2	C_2 pH_2	C_3 pH_2	C_4 pH_2	C
6.61(pH_3)	C_{ck} pH_3	C_1 pH_3	C_2 pH_3	C_3 pH_3	C_4 pH_3	C
7.03(pH_4)	C_{ck} pH_4	C_1 pH_4	C_2 pH_4	C_3 pH_4	C_4 pH_4	C
8.00(pH_5)	C_{ck} pH_5	C_1 pH_5	C_2 pH_5	C_3 pH_5	C_4 pH_5	C
9.18(pH_6)	C_{ck} pH_6	C_1 pH_6	C_2 pH_6	C_3 pH_6	C_4 pH_6	C

*2.2 Measurements and Data Compilation*The germination of the three seeds was observed every day, and the number of the seeds that had germinated were counted. The germination potential was measured after 7 days of germination, and the percentage of germination was calculated after 10 days. After counting the germination number on the tenth day, 10 germinated seeds were randomly selected with tweezers. The surface water was dried with filter paper, and the length of buds and roots were measured with a vernier caliper, and the germination rate, germination potential and germination index were determined. The number of seeds that germinated was recorded until no new seeds had germinated for three consecutive days. The seed germination vigor (GV), final germination rate (FGR) and germination index (GI) were calculated as described below:$GV = (n_t \ / \ N) \times 100\%$ 1 where n_t is the cumulative number of germinated seeds in 7 days, and N is the number of seeds used for the treatment.$FGR = (n \ / \ N) \times 100\%$ 2 where n is the number of seeds germinated in the treatment.GI = [?] ($Gt \ / \ Dt$) 3 where Gt is the number of germinated seeds at t days, andDt is the number of the corresponding germination days.*2.3 Statistical Analysis*Statistical analyses were conducted using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA). It primarily includes a one-way analysis of variance (ANOVA), two-way ANOVA, multiple comparative analysis and regression simulation among others. The graphics were generated using SigmaPlot 10.0 (SYSTAT, Chicago, IL, USA).

Result

*3.1 Effects of pH on herbage seed germination***Figure 1.** Germination parameters of *D. glomerata*,*L. perenne*, and *M. sativa* seeds under different pH values. Capital letters indicate differences between species. Lowercase letters indicate the difference between different treatments. With the increase in pH value, the germination rate, germination potential and germination index of the seeds of *D. glomerata*,*L. perenne*, and *M. sativa* first increased and then decreased. The germination rate, germination potential and germination index of *M. sativa* reached their maximum values when the pH value was 6.61, 5.35, and 6.61, which was 83.33%, 76.0%, and 4.17, respectively. The germination rate, germination potential and germination index of *D. glomerata* and *L. perenne* were the highest when pH was 6.61. Among them, the germination rate of *D. glomerata* and *L. perenne* were 26.67% and 52.67%; the germination potential was 12.67% and 25.33%, and the germination index was 1.33 and 2.63, respectively (Figure 1).**Figure 2.** The lengths of seed buds and roots of *D. glomerata*, *L. perenne*, and *M. sativa* seeds treated with different pH values. Capital letters indicate differences between species. Lowercase letters indicate the difference between different treatments. The bud and root lengths of *D. glomerata*, *L. perenne*, and*M. sativa* seeds all presented an "M" trend with increasing pH values. The bud length and root length of the three types of seeds reached their maximum value when the pH was 5.35 (Figure 2). The bud lengths of *D. glomerata*, *L. perenne*, and *M. sativa*were 3.32 cm, 6.10 cm, and 1.42 cm, respectively, while the root lengths were 4.22 cm, 5.03 cm, and 4.55 cm, respectively.*3.2 Effects of $CaCl_2$ on the germination of herbage seeds*The germination rate, germination potential, and germination index of*D. glomerata*, *L. perenne*, and *M. sativa* seeds all increased first and then decreased as the concentration of $CaCl_2$ increased (Figure 3). The germination rate, germination potential, and germination index of *D. glomerata* were the highest when the $CaCl_2$ concentration was 25 mmol/L, which resulted in values of 38.0%, 20.0%, and 1.90, respectively. The germination rate, germination potential, and germination index of*L. perenne* were the highest when the $CaCl_2$concentration was 100 mmol/L, 50 mmol/L, and 100 mmol/L, which were 68.67%, 52.67%, and 3.43, respectively. The germination rate, germination potential, and germination index of *M. sativa* were their highest when the $CaCl_2$ concentration was 25 mmol/L, 0 mmol/L, and 25 mmol/L, which were 90.67%, 83.33%, and 4.53, respectively.**Figure 3.** Germination parameters of *D. glomerata*,*L. perenne*, and *M. sativa* seeds under different $CaCl_2$ concentrations (mmol/L). Capital letters indicate differences between species. Lowercase letters indicate the difference between different treatments. Similar to the differential responses for pH values, the bud lengths of the *M. sativa* and *L. perenne* seeds presented an "M" trend with increasing concentrations of $CaCl_2$, while the seeds of *D. glomerata* presented an "W" trend. The bud lengths of *M. sativa* and *L. perenne* seeds were the highest when the $CaCl_2$ was 25 mmol/L, while the bud lengths of*D. glomerata* seeds reached their maximum when there was no $CaCl_2$ in the treatment. The root lengths of the *M. sativa* and *D. glomerata* seeds presented an "W" trend with increasing $CaCl_2$ concentrations similar to the bud length of *D. glomerata*, while the *L. perenne* seeds presented an "M" trend. The bud lengths of *D. glomerata*,*L. perenne*, and *M. sativa* seeds were the highest when the concentration of $CaCl_2$ was 50 mmol/L, 0 mmol/L, and 25 mmol/L, respectively (Figure 4).**Figure 4.** Seed bud and root lengths of *D. glomerata*,*L. perenne*, and *M. sativa* seeds under different concentrations of $CaCl_2$ (mmol/L). Capital letters indicate differences between species. Lowercase letters indicate the difference between different treatments.*3.3 Interactive effects of pH and $CaCl_2$ on the germination of herbage seeds*The germination rates all showed *M. sativa* >*L. perenne* > *D. glomerata*. The seed germination rate of the three herbage species all increased first and then decreased with the increase in pH values at the same concentration of $CaCl_2$. The maximum seed germination rate was mostly at pH 6.61. In contrast, except for *D. glomerata* at pH 5.3, at the same pH value, the rate of germination of the three herbage species increased first and then decreased with the increase in $CaCl_2$ concentration, and the seed germination rate was mostly the highest when the $CaCl_2$ concentration was 50 mmol/L or 20 mmol/L. The maximum value of the germination rate of*D. glomerata, L. perenne. and M. sativa* was 40.67%, 58.0 %, and 88.0%, respectively, when the concentration of $CaCl_2$ was 25 mmol/L, 50 mmol/L, and 25 mmol/L, respectively, and the pH values were all 6.61, respectively. When the pH was 4.55, and the concentration of $CaCl_2$ was 150 mmol/L and 200 mmol/L, the seeds of *D. glomerata* did not germinate (Figure 5).**Figure 5.** Germination rate of *D. glomerata*, *L. perenne*, and *M. sativa* seeds under the interaction of pH and $CaCl_2$. Bar represents the mean +- standard deviation (n = 3). Letters represent the level of significance based on a one-way analysis of variance (ANOVA) with a post hoc Duncan's test (P [?] 0.05). The seed germination

potential experiments all also showed that *M. sativa* > *L. perenne* > *D. glomerata*. The germination rate of the *D. glomerata, L. perenne*seeds both increased first and then decreased with the increase in pH value at the same $CaCl_2$ concentration. The seeds generally were the most likely to germinate at pH 6.61. In contrast, the germination of *D. glomerata* and *L. perenne* seeds increased first and then decreased with the increase in $CaCl_2$concentration at the same pH value. The seeds of *D. glomerata* and*L. perenne* germinated at their highest rates when the $CaCl_2$ concentration was 25 mmol/L and 50 mmol/L, respectively. The maximum value of the germination potential of *D. glomerata, L. perenne* and *M. sativa* seeds was 15.33%, 35.33%, and 84.0% when the $CaCl_2$ concentration was 25 mmol/L, 50 mmol/L, and 25 mmol/L, respectively, and the pH values were all 6.61, 5.35, and 5.35, respectively. The seeds of *D. glomerata* did not germinate when the pH was 4.55 or 5.35 and the $CaCl_2$concentration was 150 mmol/L and 200 mmol/L or when the pH was 8.0 or 9.18 and the $CaCl_2$ concentration was 200 mmol/L (Figure 6).**Figure 6.** Germination potential of *D. glomerata*,*L. perenne*, and *M. sativa* seeds under the interaction of pH and $CaCl_2$. The bar represents the mean +- standard deviation (n=3). Letters represents the level of significance according to a one-way analysis of variance (ANOVA) with a post hoc Duncan's test (P [?] 0.05). The germination index all also showed *M. sativa* >*L. perenne* > *D. glomerata*. The seed germination index of the three herbage species all increased first and then decreased with the increase in pH value at the same $CaCl_2$ concentration. The seeds germinated at the highest rate when the pH was 6.61. In contrast, except for *D. glomerata*at pH 5.35, the seed germination index of the three herbage species increased first and then decreased with the increase in $CaCl_2$ concentration at the same pH value. The seeds primarily germinated at their highest rate when the $CaCl_2$ concentration was 50 mmol/L or 25 mmol/L. The maximum value of the germination index of *D. glomerata, L. perenne and M. sativa* was 2.03, 2.90, and 4.40 when the $CaCl_2$concentration was 25, 50, and 25 mmol/L, respectively, and pH value was 5.35, 6.61, and 6.61, respectively. The seeds of *D. glomerata* did not germinate when the $CaCl_2$ concentration was 150 mmol/L and 200 mmol/L at pH 4.55 or when the $CaCl_2$concentration was 200 mmol/L at pH 5.35 (Figure 7).**Figure 7.** Germination index of *D. glomerata*, *L. perenne*, and *M. sativa* seeds under the interaction of pH and $CaCl_2$. The bar represents the mean +- standard deviation (n = 3). Letters represent the level of significance based on a one-way analysis of variance (ANOVA) with a post hoc Duncan's test (P [?] 0.05).**Table 2.** Results (F-values) of an ANOVA of the pH and $CaCl_2$ and their interactions on the germination rate, germination potential, and germination index of *D. glomerata*,*L. perenne*, and *M. sativa*

Species	**Germination rate (%) pH**	**Germination rate (%) $CaCl_2$**	**Germination rate (%) pH×$CaCl_2$**	**Germination potentia pH**
D. glomerata	43.13^{**}	114.34^{**}	7.40^{**}	24.47^{**}
L. perenne	11.51^{**}	18.28^{**}	1.39	4.48^{**}
M. sativa	162.16^{**}	42.83^{**}	3.11^{**}	131.07^{**}

ANOVA, analysis of variance. *P¡0.05. **P¡0.01. A two-factor interaction analysis showed that the pH values and $CaCl_2$ concentrations both had significant effects on the germination rate, germination potential and germination index of*D. glomerata*, *L. perenne* and *M. sativa*. The treatments of pH and $CaCl_2$ had a significant interaction on the germination rate, germination potential, and germination index of*D. glomerata* and *M. sativa*. There was an interaction between pH and $CaCl_2$ treatment on the germination potential of *L. perenne* (Table 2).**Table 3.** Bud lengths of *D. glomerata*, *L. perenne*, and *M. sativa* seedlings under the interaction of pH and $CaCl_2$

Species		**$CaCl_2$ (mmol/L)**	**$CaCl_2$ (mmol/L)**	**$CaCl_2$ (mmol/L)**	**$CaCl_2$ (mmol/L)**	**$CaCl_2$ (mm**
		C_{ck}	**C_1**	**C_2**	**C_3**	**C_4**
D. glomerata	pH_1	3.02±0.55Bab	3.17±0.45Bbc	3.47±0.61Bb	4.20±0.33Aab	3.29±0.13Bbc
	pH_2	3.32±0.52Da	3.92±0.24Ca	5.22±0.28Ac	4.62±0.50Ba	4.24±0.02CD
	pH_3	2.84±0.03Dab	3.56±0.19Cab	3.93±0.19Bb	4.39±0.03Aab	4.36±0.06Aa
	pH_4	2.63±0.41Babc	2.97±0.32Bcd	3.89±0.47Ab	4.19±0.48Aab	4.05±0.33Aa

Species		**$CaCl_2$ (mmol/L)**	**$CaCl_2$ (mmol/L)**	**$CaCl_2$ (mmol/L)**	**$CaCl_2$ (mmol/L)**	**$CaCl_2$ (mm**
	pH_5	2.39±0.30Bbc	2.59±0.13Bd	3.68±0.16Ab	3.89±0.37Aab	3.55±0.26Ab
	pH_6	1.95±0.26Cc	2.56±0.34Bd	3.62±0.29Ab	3.69±0.44Ab	3.12±0.22AB
L. perenne	pH_1	5.20±0.37Ab	4.78±0.48Aab	3.72±0.23Bb	3.20±0.63Bb	2.25±0.19Cd
	pH_2	5.36±0.12Ab	4.91±0.07Bab	3.75±0.18Cb	3.24±0.38Dab	2.86±0.02Ec
	pH_3	6.10±0.51Aa	5.32±0.36Ba	4.32±0.23Ca	3.99±0.01Ca	3.76±0.17Cab
	pH_4	4.94±0.53Abc	4.66±0.16Ab	4.53±0.01ABa	3.89±0.62Bab	3.87±0.31Ba
	pH_5	4.49±0.03Ac	3.70±0.36Bc	3.53±0.37Bbc	3.31±0.12Bab	3.42±0.28Bb
	pH_6	4.40±0.07Ac	3.62±0.02Bc	3.21±0.12Cc	2.39±0.03Dc	2.06±0.06Ed
M. sativa	pH_1	0.97±0.21BCcd	1.30±0.30ABa	1.47±0.25Aabc	1.65±0.34Aab	1.26±0.15AB
	pH_2	1.07±0.12Dbc	1.35±0.02Ca	1.62±0.17Bab	1.81±0.07Aab	1.41±0.13Cab
	pH_3	1.22±0.02Cab	1.41±0.12BCa	1.70±0.25ABa	1.94±0.23Aa	1.62±0.33AB
	pH_4	1.42±0.09ABa	1.57±0.15ABa	1.34±0.11Bbc	1.67±0.24ABab	1.72±0.04Aa
	pH_5	0.98±0.08Dcd	1.41±0.04BCa	1.28±0.14Cbc	1.64±0.12Aab	1.65±0.01Aa
	pH_6	0.82±0.11Dd	1.01±0.07CDb	1.20±0.08BCc	1.53±0.11Ab	1.56±0.26Aab

Capital letters indicate differences between species. Lowercase letters indicate the difference between different treatments. The increase in bud lengths was *L. perenne* >*D. glomerata* > *M. sativa*. The bud lengths of the three herbage species all increased first and then decreased with the increase in pH value at the same $CaCl_2$concentration. The bud lengths of *L. perenne* and *M. sativa*were the highest at pH values of 6.61 or 7.03, and the bud length of*D. glomerata* was the highest at pH 5.35. In contrast, the bud lengths of *D. glomerata* and *M. sativa* increased first and then decreased at the same pH value, while the bud length of *L. perenne* decreased with the increase in $CaCl_2$concentration. The bud lengths of *D. glomerata* and *L. perenne* reached their maximum when the $CaCl_2$concentration was 100 mmol/L and 0 mmol/L, and the bud length of*M. sativa* reached its maximum when the $CaCl_2$concentration was 100 mmol/L or 150 mmol/L. The bud lengths of *D. glomerata, L. perenne and M. sativa* were the highest at 5.22, 6.10, and 1.94 when the $CaCl_2$ concentration was 50 mmol/L, 0 mmol/L, and 100 mmol/L, respectively, and pH value was 5.35, 6.61, and 6.61, respectively (Table 3).**Table 4.** Root lengths of *D. glomerata*, *L. perenne*, and *M. sativa* seedlings under the interaction of pH and $CaCl_2$

Species		**$CaCl_2$ (mmol/L)**	**$CaCl_2$ (mmol/L)**	**$CaCl_2$ (mmol/L)**	**$CaCl_2$ (mmol/L)**	**$CaCl_2$ (mm**
		C_{ck}	**C_1**	**C_2**	**C_3**	**C_4**
D. glomerata	pH_1	1.65±0.23Cb	1.73±0.19Cc	1.63±0.18Cb	3.03±0.23Ba	4.07±0.43Ac
	pH_2	1.92±0.17Cab	1.99±0.15Cbc	2.77±0.29Ba	3.14±0.16Ba	5.28±0.42Ab
	pH_3	2.16±0.21Ca	2.28±0.22Cab	2.88±0.26Ba	3.31±0.32Ba	6.22±0.49Aa
	pH_4	1.33±0.12Dc	2.44±0.27Ca	2.51±0.22Ca	3.52±0.33Ba	6.72±0.81Aa
	pH_5	0.54±0.08Ed	1.01±0.09Dd	1.82±0.16Cb	3.05±0.25Ba	4.59±0.33Abc
	pH_6	0.32±0.04Dd	0.95±0.13Cd	1.62±0.11Bb	2.23±0.21Ab	2.38±0.21Ad
L. perenne	pH_1	3.79±0.36Ab	3.14±0.50Bc	3.13±0.25Bab	2.09±0.18Cc	1.42±0.14De
	pH_2	5.03±0.44Aa	3.36±0.48Bbc	3.18±0.12Ba	2.37±0.07Cb	1.74±0.04Dd
	pH_3	5.07±0.41Aa	3.62±0.37Babc	3.42±0.14Ba	2.33±0.18Cbc	1.93±0.08CD
	pH_4	5.46±0.61Aa	4.25±0.34Ba	2.85±0.12CDbc	2.93±0.18Ca	2.33±0.12Da
	pH_5	4.87±0.33Aa	4.04±0.45Bab	2.75±0.19Cc	2.14±0.12Dbc	2.13±0.13Db
	pH_6	4.64±0.43Aa	4.02±0.31Bab	2.62±0.13Cc	1.11±0.07Dd	0.71±0.05DE
M. sativa	pH_1	3.23±0.27Cb	3.82±0.44Bb	5.38±0.49Aa	0.65±0.13Dd	0.30±0.05De
	pH_2	4.55±0.35Ba	4.79±0.36Ba	5.41±0.59Aa	0.73±0.11Cd	0.47±0.06Ce
	pH_3	1.66±0.14Dc	3.01±0.22Ccd	4.15±0.63Bb	6.95±0.90Aa	4.08±0.31Ba
	pH_4	1.27±0.09Dd	3.48±0.28Bbc	3.73±0.38Bb	5.65±0.33Ab	3.55±0.33Bb
	pH_5	0.80±0.11Ee	2.77±0.2Cd	3.36±0.36Bb	5.01±0.48Ab	2.88±0.25BC

Species		$CaCl_2$ (mmol/L)	$CaCl_2$ (mmol/L)	$CaCl_2$ (mmol/L)	$CaCl_2$ (mmol/L)	$CaCl_2$ (mm
	pH_6	0.00±0.00Df	1.09±0.09Ce	1.57±0.17Bc	4.02±0.45Ac	1.34±0.18BC

Capital letters indicate differences between species. Lowercase letters indicate the difference between different treatments. The increase in root lengths was *M. sativa* >*L. perenne* > *D. glomerata*, which was similar to the bud length. The root lengths of the three herbage species almost increased first and then decreased with the increase in pH value at the same $CaCl_2$ concentration. When the pH was 6.61 or 7.03, the root lengths of *D. glomerata* and *L. perenne* reached their maximum, and the root length of *M. sativa* reached its maximum when the pH was 5.35 or 6.61. In contrast, the root lengths of*D. glomerata* and *M. sativa* increased first and then decreased, while the root length of *L. perenne* decreased as the concentration of $CaCl_2$ increased. *L. perenne* had the longest roots in the absence of calcium, while those of *D. glomerata* were the longest when the $CaCl_2$ concentration was 150 mmol/L and 0 mmol/L. *M. sativa* had the longest roots when the $CaCl_2$ concentration was 50 mmol/L or 100 mmol/L.*D. glomerata, L. perenne*, *and M. sativa* had the longest roots of 6.72, 5.46, and 6.95 cm when the concentration of $CaCl_2$ was 150 mmol/L, 0 mmol/L, and 100 mmol/L, respectively, and the pH values were 7.03, 7.03, and 6.61, respectively (Table 4).**Table 5.** Results (F-values) of an ANOVA with pH and $CaCl_2$, and their interactions on bud lengths and root lengths in *D. glomerata*, *L. perenne*, and *M. sativa*

Species	Bud length (cm) pH	Bud length (cm) $CaCl_2$	Bud length (cm) pH×$CaCl_2$	Root length (cm) pH	Root length (cm) $CaCl_2$
D. glomerata	3.78^{**}	7.06^{**}	2.31^{**}	16.10^{**}	38.07^{**}
L. perenne	3.57^{**}	3.16^{*}	4.86^{**}	3.54^{**}	1.88
M. sativa	17.15^{**}	20.26^{**}	7.45^{**}	6.33^{**}	12.19^{**}

ANOVA, analysis of variance. $^{*}P$¡0.05. $^{**}P$¡0.01. A two-factor interaction analysis showed that both the pH and $CaCl_2$ concentration had significant effects on the bud lengths and root lengths of *D. glomerata*, *L. perenne*, and*M. sativa* except for $CaCl_2$ on *L. perenne*. The treatments of pH and $CaCl_2$ all had significant interaction on the bud lengths and root lengths of *D. glomerata*and *M. sativa* (Table 5).

Discussions

*4.1 Effect of pH value on seed germination of herbage*The soil pH depends on the location, bedrock, climate and vegetation type [16]. The response of seed germination to pH change has been a concern for a long time [17]. The effect of pH value on seed germination has become an important indicator to assess whether a species can adapt to changes in soil pH [18,19]. Different species vary in their pH values and thresholds for germination; some species have a wider pH range and a higher germination rate [20]. Other species only germinate at certain pH values. The response of *M. sativa* to soil acidity during germination has also been studied. It can grow well and produce large yields even in highly acidic soils (pH < 4.0) [21]. The results showed that the germination rates of *M. sativa*, *D. glomerata* and *L. perenne* were significantly inhibited at pH 4.55 and 7.03 to 9.18. Low and high hydrogen (H+) concentrations inhibit the utilization of other metal ions by herbages [22]. However, slightly acidic conditions tend to be favorable. Studies have shown that slightly acidic conditions are beneficial to the germination of several types of feed crops[23]. Seeds placed on filter paper in petri dishes germinated at pH 3 to 8 without any statistically significant differences. This is also the reason why the buds and young roots in this experiment can promote their growth at weakly acidic conditions. In addition, higher pH values can affect germination by inhibiting proteolytic enzymes involved in the metabolism of seed storage compounds [24]. Hydroxyl ions (OH^-) may also interfere with the uptake of essential anions [25]. Therefore, the osmotic pressure of the membrane is affected, leading to the inhibition of enzyme activity, and, in turn, the change in membrane potential inhibits seed germination, buds and root cell elongation.*4.2 Effect of $CaCl_2$ concentration on the seed germination of herbage*Karst areas in the Guizhou Province are rich in calcium; the CaO weighted average content is 25.27%-55.63%

[26]. Such high levels of calcium have become an important limiting factor for the germination of forage seeds. Salt stress can lead to ionic stress, osmotic stress, and secondary stresses, particularly oxidative stress, in plants [27]. The stress of salt on seed germination primarily includes osmotic stress, toxic effects, ion absorption imbalances and alkaline stress [28], However, as an important element for plant growth and development, calcium controls a series of important physiological processes and enzyme activities related to seed germination [29,30]. In this study, the germination of *M. sativa* seeds did not respond significantly to the increase in $CaCl_2$ concentration, while the *D. glomerata* and *L. perenne* seeds germinated significantly more poorly under these conditions. Overall, there was no apparent effect of $CaCl_2$ on the buds and young roots at low concentrations, while the seeds were significantly inhibited at high concentrations. These results indicate that *D. glomerata* and*L. perenne* seeds are likely to be more sensitive to $CaCl_2$ than *M. sativa* seeds under salt-stressed or non-stressed conditions. They also showed that the *M. sativa*seeds had a higher tolerance to salt during germination than those of the forage crops red clover (*Trifolium pratense* L.) and white clover (*T. repens* L.) in comparison with their salt tolerance [31]. Although plant seedlings can grow in solutions with low salt concentrations, high salinity may substantially inhibit root elongation, particularly in the young roots of *M. sativa* [32]. High salinity is commonly owing to high concentrations of Na^+ and Cl^- in the soil solution, resulting in hyperosmotic and hyperionic conditions, respectively, which impede the plant from absorbing water and nutrients from the soil [33]. Ca is a vital element for cell division, the maintenance of structure and building cell walls, and it plays an important role in imbibition during seed germination. Ca^{2+} can mitigate the adverse effect of salinity during seed germination [2], which confirms the observed result of $CaCl_2$ treatment in salt-stressed seeds. Higher salinity results in a greater reduction in the germination rate and increases the time to germination [34].*4.3 Effects of different pH values and $CaCl_2$concentrations on the germination of forage seeds*In this study, through a two-factor variance analysis of seed germination, pH and $CaCl_2$ concentration, we found that the pH had a more significant effect on the seed germination rate of*M. sativa*, *D. glomerata* and *L. perenne* compared with $CaCl_2$, while the combined treatment of pH and $CaCl_2$ significantly reduced the seed germination of*D. glomerata* and *L. perenne*. Studies have shown that, owing to the high pH, a low concentration of alkali stress also strongly inhibited seedling growth, and the detrimental effect was much more marked than that of salt stress [35]. Alternatively, this study found that under the interaction of calcium salt stress and acid-base stress, the pH requirement of *L. perenne* decreased, and the $CaCl_2$ increased, indicating that both are involved in the regulation of seed germination. Thus, the results also indicate that seed germination and seedling stages have different physiological responses to the salt and alkali stresses. The specific molecular mechanisms merit further research. To manage salt stress, plants have developed a series of strategies that are regulated by changes in gene and protein expression, which change in specific metabolic and signaling pathways. Our results clearly showed that the acid, alkali and salt tolerance of the three herbage seeds had a substantial influence on each other. When the salt stress concentration began to change, the herbage seeds could adapt to salt stress at a suitable pH condition. pH stress and calcium salt stress are actually two different types of stress, and the salt-alkali tolerance of the three herbage seeds is largely affected by the interaction of salt and pH.

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