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Full Length Research Paper

Germination responses of Alfalfa (*Medicago sativa* L.) seeds to various salt–alkaline mixed stress

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Salinization and alkalization in soil frequently co-occur in agriculture ecosystem, and directly affect plant production. Alfalfa (*Medicago sativa*) is important forage with good quality and high yield. Its production is markedly influenced by those attributes of soils, especially mixed salt-alkali stress. However, the knowledge on seeds germination linked to mixed salt-alkali stress received little attention, though seeds germination influence plant yields. In this study, we assessed that the effects of 30 mixed salt-alkali combinations (NaCl, Na_2SO_4, $NaHCO_3$, and Na_2CO_3; salinity 24 to 120 mmol/L and pH 7.03 to 10.69) on germination of alfalfa seeds. Our results showed that the interaction of salinity and alkalinity significantly affected germination rates of alfalfa seeds. The interactive effects between salinity and alkalinity resulted in changes in radicle length and plumule length of alfalfa seeds. The study also indicated some physiological responses (electrolyte leakage rate, proline content, soluble sugar, and Na^+/ K^+) of alfalfa seeds were affected by mixed salt-alkaline stress. The experimental results concluded that mixed salt-alkaline stress emphasizes the interaction between salt concentration and salt component, and differs from salt or alkali stress, and elucidated that seeds germination of plants may adapt to mixed salt-alkaline environment through modifying physiological mechanisms in plants.

Key words: *Medicago sativa* L., seed, germination, salinity, alkalinity, mixed salt-alkaline, stress.

INTRODUCTION

The existence of salt and alkali stresses has been clearly demonstrated by a number of reports, showing that they are an important factors in limiting agricultural productivity, and have cause severe problems in some areas (Shi and Sheng, 2005; Shi and Wang, 2005). There are 8.31×10^8 hm^2 areas in the world that is affected by salt or alkali stresses. Of this, area with salt-alkalinized soils is 4.34×10^8 hm^2 (Wang et al., 2008). Deng et al. (2006) reported that the grasslands affected by salt and alkali are estimated to amount to an area of 3.7×10^6 hm^2 in Songnen Plains in Northeast China, which accounts for approximately 70% of the natural grasslands.

Salt stress is one of most serious environmental factors limiting the growth of plants due to affecting plant physiological water deficit that leads to osmotic stress and an excess of sodium ions (Wang et al., 1997). In fact, soil salinization and alkalization frequently co-occur in natural ecosystem, and the area with mixed salinity and alkali is expanding (Kawanabe and Zhu, 1991). Furthermore, the problem of soil salt-alkalization may be more sever than the problem of soil salinization (Yang et al., 2008a, b, c). In general, salt stress refers to only neutral salt stress, but mixed salt-alkali stress is high salinity and low pH, or low salinity and high pH (Shi et al., 1998). Moreover, the salt-alkali stress can directly damage plant growth, alter the availability of nutrients, and disrupt the balance of ions and mineral nutrition (Shi and Zhao, 1997). However, relatively little attention has been given to the effect salt-alkali stress on plants.

Successful establishment of plants largely depends on successful germination of plant. Germination is a crucial stage in the life of plants and tends to be highly unpredictable over space and time. Seeds germination is affected by many biotic and abiotic factors, such as temperature, light, water, and so on (Al-Ahmadi and Kafi,

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Table 1. Salt composition and its molar ratio within treatments.

Treatment	Composition and ratios of salts			
	NaCl	Na_2SO_4	$NaHCO_3$	Na_2CO_3
A	2	1	0	0
B	1	1	1	0
C	12	9	8	1
D	8	9	12	1
E	12	1	8	9
F	0	0	2	1

2007; Pérez-García et al., 2007; Guma et al., 2009; Ruano et al., 2009). Also, salinity and alkalinity are the main limiting factors in germination of many species, because tolerance to salinity and alkalinity during germination is critical for the establishment of plants growing in salt-alkalinized soils (Unger, 1995).

The response of seeds germination to salinity has been reported to be more complex than plant growth because it depends on the availability of stored compounds. Numerous studies on the effect salinity on seeds germination focused on halophytes and non-halophytes (Tobe et al., 2000; El-Keblawy and Al-Rawai, 2005; Joshi et al., 2005). They responded to salt stress in similar way that lingeringly germinated (Almansouri et al., 2001; Khan and Gulzar, 2003; Khajeh-Hosseini et al., 2003). The inhibitory effect of salt stress on seeds germination is due to an osmotic effect and ion toxicity (Pujol et al., 2000; Bajji et al., 2002; Tobe et al., 2004), plant's nutrient requirements and uptake of nutrient cations (Drenovsky and Richards, 2003; Tuna et al., 2007). Although alkaline and salt may exert distinct effects of stress on alfalfa seedlings (Peng et al., 2008), the responses of plant seeds germination to salt-alkali stress are poorly documented.

Alfalfa is a perennial forage with high yield and have good quality (crude protein content can reach approximately 16 to 22%), and it is resistant to various stresses (Ehsanpour and Fatahian, 2003). The need for alfalfa in North China increases gradually due to increase in the number of livestock recently, although alfalfa has been widely planted in America, Canada, Australia, and other countries. Thus, we selected it (*M. sativa* cv. Gongnong No.1) as the material for determining the physiological adaptive mechanisms of germinating seeds to the mixed salt-alkali stress. Neutral salt (NaCl and Na_2SO_4) and alkali ($NaHCO_3$ and Na_2CO_3) are the main soil components in the Songnen Plains in Northeast China, and the concentration of salt in soils is more than 0.7%, and pH range is 8.5 to 10.0. Therefore, in this study, mixtures of four salts were used in various proportions to simulate a range of mixed salt-alkali conditions. The objective is to estimate the adaptive responses of alfalfa seeds, and to determine the physiological characteristics of its seeds, and to identify the mechanisms of plant tolerance against mixed salt-alkaline stress.

MATERIALS AND METHODS

Plant material

Alfalfa (*Medicago sativa* L.) cv. Gongnong No.1 was chosen as the test organism for our research, and seeds were obtained from the Institute of Grassland Sciences, Jilin Academy of Agricultural Science in Gongzhuling Town, near Changchun City. Fully developed and average-sized seeds were selected in August 2005. This plant grows in Northeast China (44°40′ to 44°44′ N, 123°44′ to 123°47′ E) in a semi-arid, continental climate with a frost-free period of about 140 days. Mean annual temperature and precipitation are from 4.6˚C to 6.4˚C, and 280 to 400 mm during the recent decade. Ninety percent of the total precipitation is distributed from May to October. The soil in this area is mixed saline and alkaline (pH 8.5 to 10.0).

Design of simulated salt and alkaline conditions and stress treatment

To simulate salt-alkali conditions in natural ecosystem, two neutral salts (NaCl and Na_2SO_4) and two alkaline salts ($NaHCO_3$ and Na_2CO_3) were selected based on the salt components in the extent of salt–alkaline soil over Northeast China (Ge and Li, 1990). Other four groups were selected based on the tolerance of alfalfa against the varying ranges of salinity and pH in the soil in the Songnen Plains. Six treatment groups (labeled A to F) were set with gradually increasing alkalinity, and the salt composition of each groups are shown in Table 1. All treatment groups had a 2:1 molar ratio of monovalent salts (NaCl + $NaHCO_3$) to divalent salts (Na_2SO_4 + Na_2CO_3). That is, if the individual molar concentrations were the same, then the total ion concentrations were the same throughout the treatments. Within each group treatment, six different concentrations were utilized, namely 24, 48, 72, 96, 120 and144 mM, 36 salt-alkaline mixed stress treatments (labeled A_1 to F_6) with varying salinity of 24 to 144 mM and pH 7.03 to 10.69 (Table 2).

Alfalfa seeds were germinated in petri dishes (90 mm diameter) containing two layers of filter paper with simulated mixed salt-alkali saturated solution (10 ml), and the distilled water for the control. The petri dishes were washed with distilled water for three times and deionized water for one time, and then sterilized in oven with 120°C for 3 h. Alfalfa seeds were surface sterilized in 99.75% ethanol solution for 30 s, subsequently washed with distilled water and air-dried before being used in the germination experiments to avoid fungus attack. Each petri dish contained 50 seeds evenly placed on the filter paper. Mixed salt-alkali stress solutions of 36 groups (A_1 to F_6) were added in petri dishes with seeds and filter papers, and recorded the total weight. Germination tests were carried out in growth chambers (HPG-280BX) with 20°C and 12 h day and 12 h dark. Total weight was carried out every day for adding the losing distilled water. Each stress treatment was replicated 3 times.

Table 2. Main stress factors for different treatments.

Treatment groups	Main stress factors						
	pH value	Salinity(mM*)	$[Na^+]$(mM)	$[Cl^-]$(mM)	$[SO_4^{2-}]$(mM)	$[HCO_3^-]$(mM)	$[CO_3^{2-}]$(mM)
A_1	7.03	24	32	16	8	0	0
A_2	7.12	48	64	32	16	0	0
A_3	7.18	72	96	48	24	0	0
A_4	7.22	96	128	64	32	0	0
A_5	7.26	120	160	80	40	0	0
A_6	7.29	144	192	96	48	0	0
B_1	7.34	24	32	8	8	8	0
B_2	7.45	48	64	16	16	16	0
B_3	8.04	72	96	24	24	24	0
B_4	8.22	96	128	32	32	32	0
B_5	8.3	120	160	40	40	40	0
B_6	8.51	144	192	64	48	48	0
C_1	8.8	24	32	9.6	7.2	6.4	0.8
C_2	8.92	48	64	19.2	14.4	12.8	1.6
C_3	8.95	72	96	28.8	21.6	19.2	2.4
C_4	8.99	96	128	38.4	28.8	25.6	3.2
C_5	9.01	120	160	48	36	32	4
C_6	9.02	144	192	57.6	43.2	38.4	4.8
D_1	9.03	24	32	6.4	7.2	9.6	0.8
D_2	9.05	48	64	12.8	14.4	19.2	1.6
D_3	9.1	72	96	19.2	21.6	28.8	2.4
D_4	9.15	96	128	25.6	28.8	38.4	3.2
D_5	9.22	120	160	32	36	48	4
D_6	9.31	144	192	38.4	43.2	57.6	4.8
E_1	9.45	24	32	9.6	0.8	6.4	7.2
E_2	9.56	48	64	19.2	1.6	12.8	14.4
E_3	9.64	72	96	28.8	2.4	19.2	21.6
E_4	9.69	96	128	38.4	3.2	25.6	28.8
E_5	9.79	120	160	48	4	32	36
E_6	9.81	144	192	57.6	4.8	38.4	43.2
F_1	9.84	24	32	0	0	16	8
F_2	9.91	48	64	0	0	32	16
F_3	9.96	72	96	0	0	48	24
F_4	10.11	96	128	0	0	64	32
F_5	10.39	120	160	0	0	80	40
F_6	10.69	144	192	0	0	96	48

*mM = mmol/L.

Sampling and measurements

After the seeds were germinated and grown for 8 days in different stress treatments, germinated seeds were counted, with emergence of the radicle considered as germination. Lengths of plumule and radicle of 10 seeds randomly selected in each petri dish were determined. All seeds were harvested after the final treatment. The seeds samples were oven-dried at 100°C for 10 min, then dried at 80°C to constant weight and the dry weights (DW) were recorded.

Membrane permeability of seeds can be reflected by the electrolyte leakage rate (ELR). ELR was determined as described by Lutts et al. (1996). 10 seeds were placed in the test-tube with 5 ml deionized water, and the electrical conductivity of the solution was determined using a conductivity gauge (a_1). The seeds were dipped in the deionized water at 25°C for 5 h, and the electrical conductivity of the solution (a_2) was measured. Sequentially, the test-tube with alfalfa seeds and solution was placed in boiling water (100°C) for 60 min, and the electrical conductivity of the solution (a_3) was determined after equilibration to 25°C. ELR can be defined as follows:

$$ELR = (a_2 - a_1) \times 100 / (a_3 - a_1)$$

Table 3. Two-way ANOVA of the effects of salinity (Salt concentration) and pH (Salt component) on germination rate, radicle length, plumule length, electrolyte leakage rate, proline content, soluble sugar content, Na^+ content, K^+ content, Cl^- content, SO_4^{2-} content, Na^+/K^+ in the alfalfa.

		Germination rate		Radicle length		Plumule length		Electrolyte leakage rate		Proline content		Soluble sugar content	
	df	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Salinity	6	369.261	0.001	108.681	0.001	64.102	0.000	624.62	0.001	58.64	0.001	44.18	0.000
pH	5	197.566	0.001	59.032	0.000	75.947	0.000	49.032	0.001	16.864	0.001	26.106	0.000
Salinity × pH	30	17.366	0.001	4.323	0.000	7.66	0.000	7.207	0.001	1.264	0.229	3.663	0.000

		Na^+ content		K^+ content		Cl^- content		SO_4^{2-} content		Na^+/K^+	
		F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Salinity	6	322.749	0.001	38.502	0.001	49.556	0.001	63.166	0.001	198.42	0.001
pH	5	281.49	0.001	40.525	0.001	213.67	0.001	12.175	0.001	85.834	0.001
Salinity × pH	30	135.055	0.001	3.619	0.001	25.387	0.001	7.693	0.001	17.384	0.001

The seeds with germination were homogenized by powdering in order to determine proline contents, and soluble sugars, and inorganic ion contents. Proline contents and soluble sugars were measured using ninhydrin and phenol method respectively (Zhu, 1993). Dry samples of 100 mg of alfalfa seeds were treated with 10 ml deionized water at 100℃ for 20 min, and the extract used to determine free inorganic ion contents. The SO_4^{2-} and Cl^- contents were determined by ion chromatography (DX-300 ion chromatographic system, AS4A-SC chromatographic column, CDM-II electrical conductivity detector, mobile phase: $Na_2CO_3/NaHCO_3$ = 1.7/1.8 mM, DIONEX, USA). An atomic absorption spectrophotometer (TAS-990, Purkinje General, Beijing) was used to determine contents of Na^+ and K^+. The results of proline contents and soluble sugars were expressed in mmol/L, Na^+ and K^+, SO_4^{2-} and Cl^- in mg/g.

Statistical data analysis

All statistical tests were performed with the statistical program SPSS 13.0. Transformation of variables was not required in any case. Two-way ANOVA was used to analyze the effects of salt (salt concentration) and alkaline (salt composition) stresses, and the interaction between them, on alfalfa seed germination rate, growth indexes, that is, lengths of plumule and radicle, solutes contents, and ion contents (Na^+, K^+, Cl^- and SO_4^{2-}).

RESULTS

Germination rates of alfalfa seeds

Salinity and alkalinity significantly affected alfalfa seeds germination rate, and furthermore, the salt composition (alkali stress) and salt concentration (salt stress) have significant interactive effects on the seeds germination rates (Table 3). As a whole, germination rates in the alfalfa seeds in mixed salt-alkaline stress treatments was lower than that of the control, with the exception of group A_1, A_2 in low salt concentration (Figure 1). The alfalfa seeds germination rates of all treatment groups decreased with increased salinity and alkalinity respectively. Moreover, the germination rates of alfalfa seeds sharply decreased in group E and F. Meanwhile, high salinity with high alkalinity led to ungerminated seeds, in groups D, E, and F, (Figure 1).

Growth index of alfalfa seeds

The interaction between salt and alkali stresses has significant effects on radicle length and plumule length (Table 2). Radicle length decreased along the salinity and alkalinity gradient, but this pattern was not found within groups A and D treatment (Figure 2a). Within A group, A_2 was higher than other group treatments, while radicle length in low-alkalinity treatments (A and B treatment) increased slightly from 0 to 24 mmol/L salinity, and thendecreased within D treatment,. Within group C and D alkalinity treatments, plumule lengths slowly decreased along the salinity gradient, while sharply decrease in groups E and F. At same salinity level, increase of alkalinity (pH) induced decreases of plumule length and radicle length (Figure 2). Meanwhile, radicle and plumule were not present in high salinity (72 to 144 mmol/L) and high alkalinity

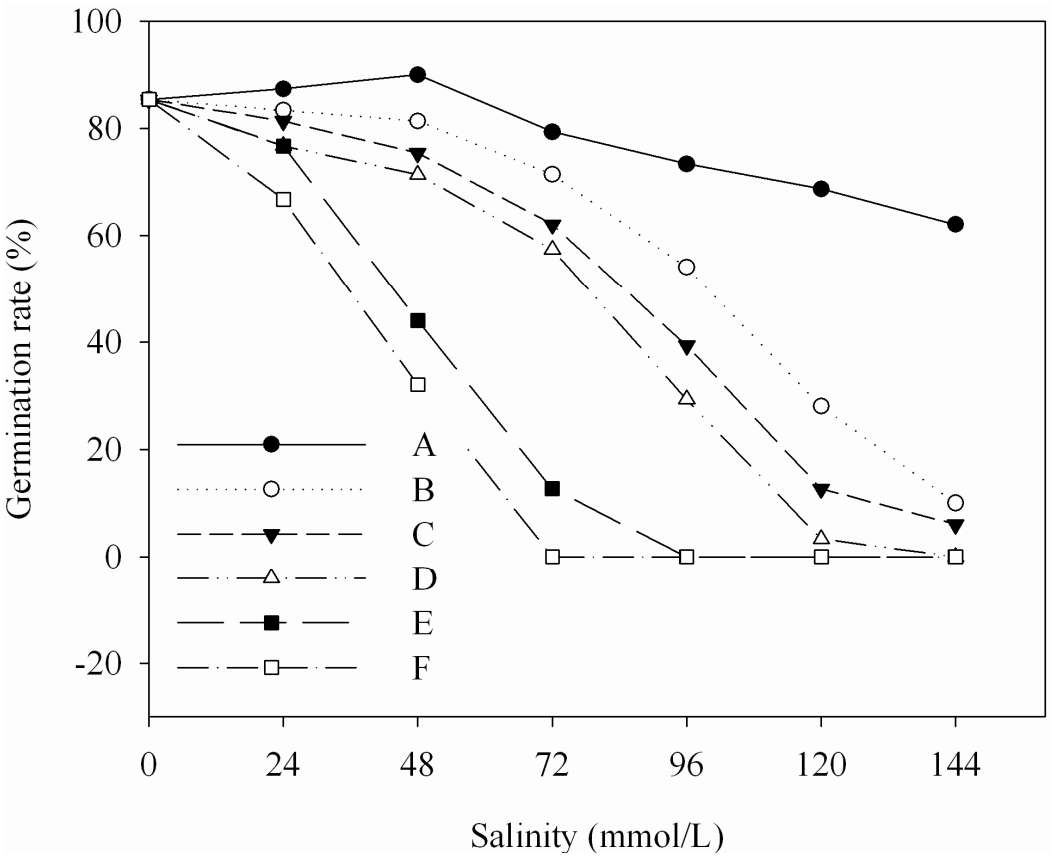

Figure 1. Effects of various salt-alkaline mixed stresses on alfalfa germination. The values are means of three replicates. A ($NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 2:1:0:0; pH 7.03 to 7.29); B ($NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 1:1:1:0; pH 7.34 to 8.51); C ($NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 12:9:8:1; pH 8.80 to 9.02); D ($NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 8:9:12:1; pH 9.03 to 9.31); E ($NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 12:1:8:9; pH 9.45 to 9.81); F ($NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 0:0:2:1; pH 9.84 to 10.69).

(groups D, E, and F) due to ungerminating seeds (Figure 2).

Alfalfa seeds electrolyte leakage rate (ELR)

Electrolyte leakage rate (ELR) is considered as a good physiological index that reflects the degree of plant injury, because the plasma membranes of cells are seriously injured by intensifying stress, leading to an increase in the ELR. The interactions between salt and alkali stresses affect the ELR of alfalfa seeds. Our results indicated that the ELR of alfalfa seeds gradually increased with increasing salinity and alkalinity, this was attributed to the damage seeds cell membranes resulting from mixed salt-alkali stress. The seeds ELR in the same alkalinity treatment groups increased along the salinity gradient, but no difference were observed among these alkali stresses under the same salinity conditions (Figure 3).

Ion contents in the alfalfa seeds

With increasing salinity, Na^+ content, and Na^+/K^+ increased. K^+ content increased slightly, and then decreased in groups A and B treatments, but decreased dramatically in the other four groups treatments. Within the same salinity treatment, Na^+ and Na^+/ K^+ increased, and K^+ decreased along the alkalinity gradient (Figures 4a, b, e). However, Na^+/ K^+ in group D were significantly higher than that in groups A to C when the salinity was 96 and 120 mmol/L. This implies that salinity and alkalinity exhibited cooperative effects at higher salt and alkali levels. The contents of Cl^- and SO_4^{2-} might be related to the ion concentrations of treatment solutions. Cl^- content of group A was significantly higher than other treatment groups (B to F) at each salinity levels. Along increasing salinity gradient, Cl^- content increased within group A treatment, whereas it increased, and then decreased or remained horizontal line within other group treatments (Figure 5c). SO_4^{2-} contents of A and B treatments group

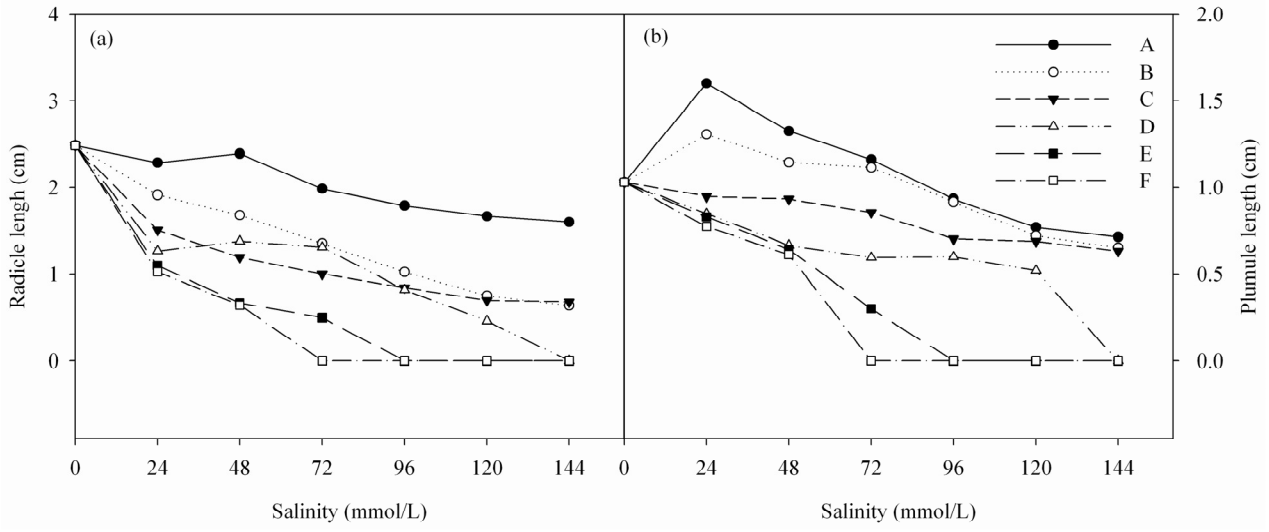

Figure 2. Effects of various salt-alkaline mixed stresses on radicle length (a), plumule length (b) of germinated alfalfa. The values are means of three replicates. A ($NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 2:1:0:0; pH 7.03 to 7.29); B ($NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 1:1:1:0; pH 7.34-8.51); C ($NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 12:9:8:1; pH 8.80-9.02); D ($NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 8:9:12:1; pH 9.03 to 9.31); E ($NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 12:1:8:9; pH 9.45 to 9.81);F ($NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 0:0:2:1; pH 9.84 to 10.69).

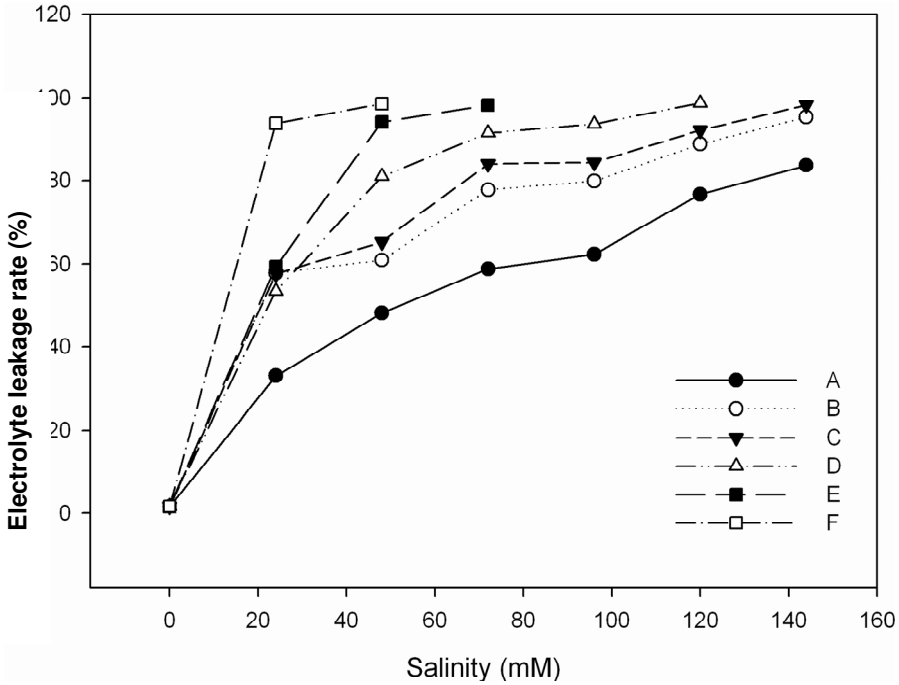

Figure 3. Effects of various salt-alkaline mixed stresses on Electrolyte leakage rate of germinated alfalfa. The values are means of three replicates. A ($NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 2:1:0:0; pH 7.03 to 7.29); B ($NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 1:1:1:0; pH 7.34 to 8.51); C ($NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 12:9:8:1; pH 8.80 to 9.02); D ($NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 8:9:12:1; pH 9.03 to 9.31); E ($NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 12:1:8:9; pH 9.45 to 9.81); F ($NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 0:0:2:1; pH 9.84 to 10.69).

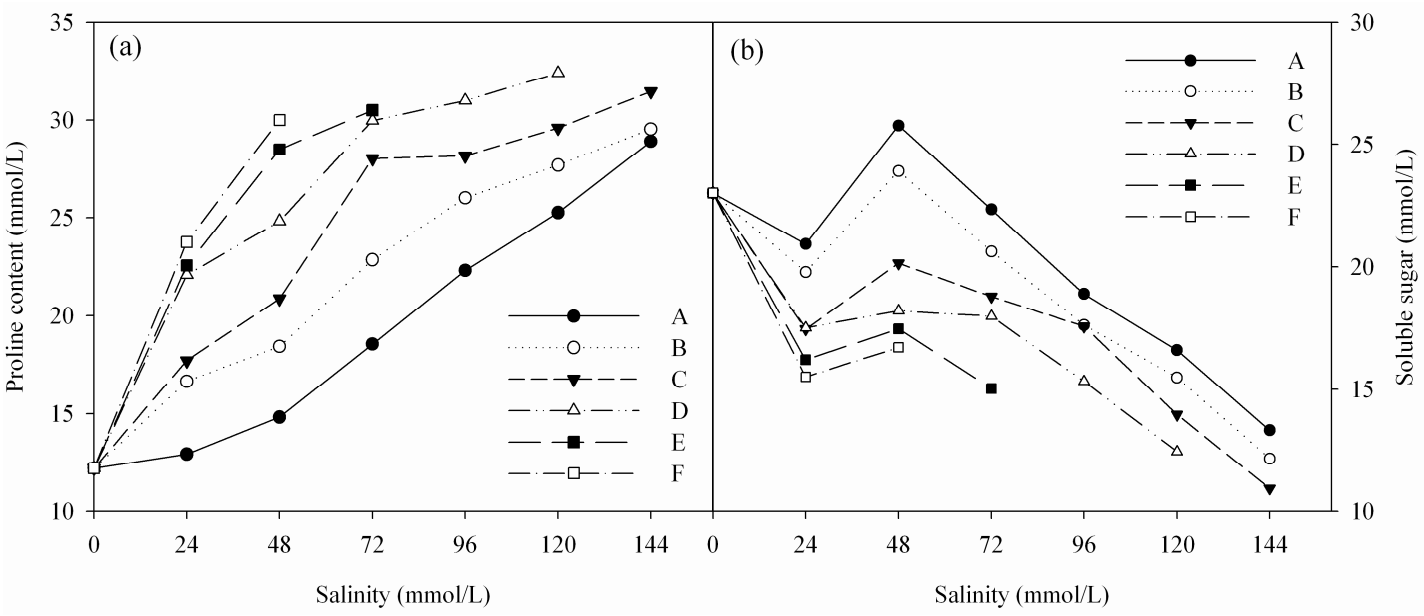

Figure 4. Effects of various salt–alkaline mixed stresses on the proline contents (a) and soluble sugar contents (b) in germinated alfalfa. The values are means of three replicates.A ($NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 2:1:0:0; pH 7.03 to 7.29);B ($NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 1:1:1:0; pH 7.34 to 8.51);C ($NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 12:9:8:1; pH 8.80 to 9.02);D ($NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 8:9:12:1; pH 9.03 to 9.31); E ($NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 12:1:8:9; pH 9.45 to 9.81);F ($NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 0:0:2:1; pH 9.84 to 10.69).

were higher than other treatment groups (C to F) at high salinity concentration. SO_4^{2-} contents increased, and then decreased within groups A to E, but within F treatment SO_4^{2-} contents obviously decreased SO_4^{2-} contents (Figure 5d).

Solutes contents in the alfalfa seeds

Solutes, that is, proline and soluble sugars are sensitive physiological indexes of plants responding to some stresses. Salinity and alkalinity treatments had significant effects on solutes contents in alfalfa seeds, and there was significant interaction between salinity and alkalinity on soluble sugar content, but proline content was significantly affected by interaction between salinity and alkalinity (Table 3). The proline content increased with increasing salinity within groups A to F, and the rate of increase along the salinity gradient also tended to be more rapid, especially for the high alkalinity treatments (groups D to F). The proline content in the same alkalinity treatment groups increased with increasing salinity, and no difference was observed among these alkali stresses under the high salinity conditions (group D to F). The results showed that mixed salt-alkali stress can cause heavy accumulation of proline (Figure 5a). Soluble sugars of groups A to C increased from 24 to 48 mM, and then decreased, and concomitantly, the extent of decreasing soluble sugars tends to get higher with increasing salinity within the same alkalinity treatment (Figure 5b). Soluble sugars content remain horizontal line from 24 to 72 mmol/L salinity, and then decreased in group C.

DISCUSSION

Seeds germination

Germination is one of the most critical periods in the life cycle of plant. Under salt stress, a low water potential is a determining factor inhibiting seeds germination (Debez et al., 2004). However, our results indicated that high pH significantly affected seeds germination (Figure 1). We observed that the interaction between increasing salinity and pH also decreased the alfalfa germination rates (Figure 1). In addition, alfalfa germination was stimulated at low salt stress, and maximal germination rates were at 48 mM of A group (Figure 1). However, this did not occur under salt–alkaline mixed stress (B to F groups), implying not only differences of salt and salt–alkaline mixed stresses, but also more tolerance of alfalfa to salt stress (A group) than salt–alkaline mixed stress (B to F groups). The different extents of injury caused by salt (A group) and salt–alkaline mixed (B to F groups) stress might be due to different mechanisms of actions. Salt–alkaline mixed stress exerts not only salt stress, but also the added influence of high-pH stress, and so with

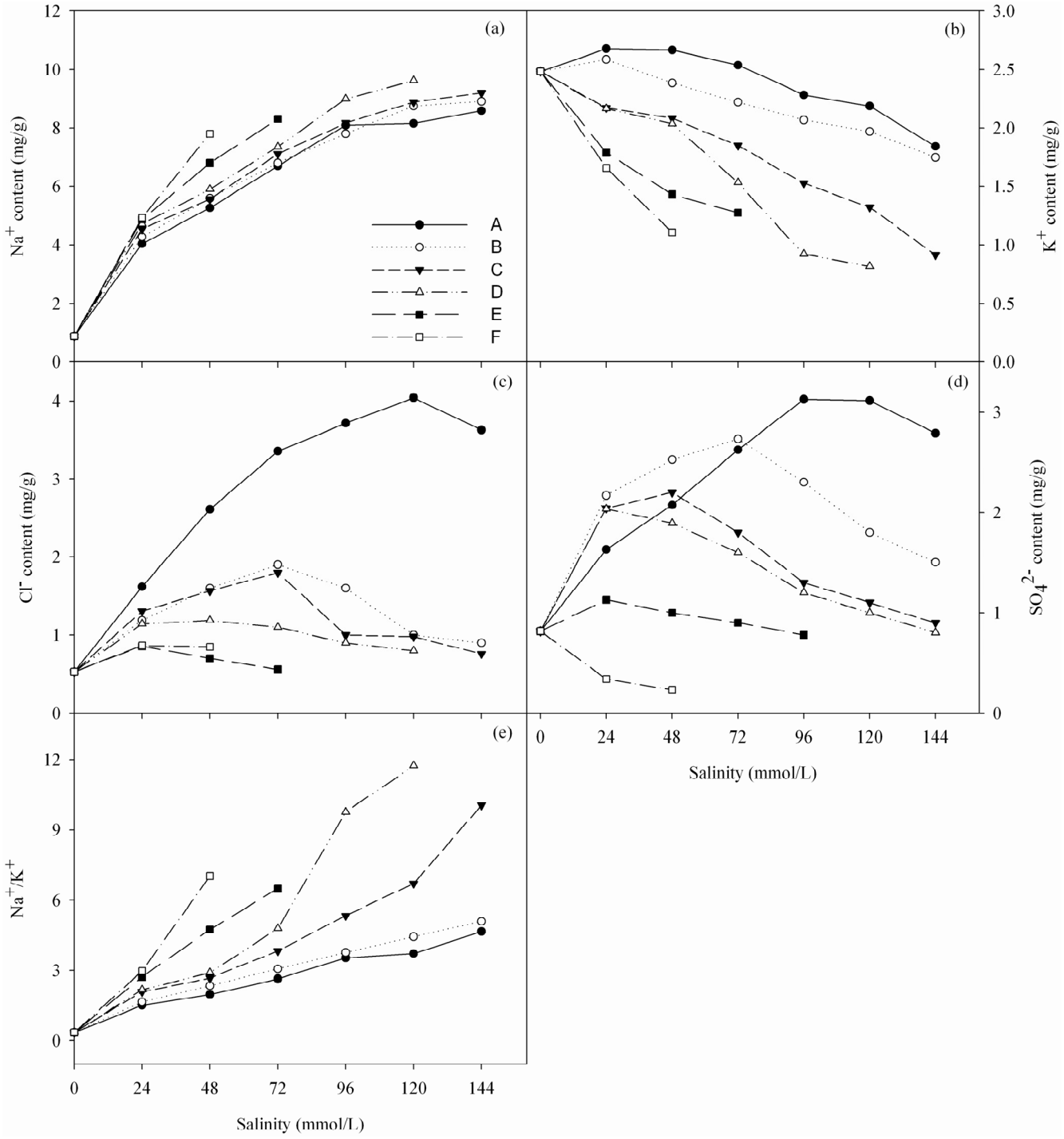

Figure 5. Effects of various salt–alkaline mixed stresses on ions contents in germinated alfalfa seed. (a) Na^+; (b) K^+; (c) Cl^-; (d) SO_4^{2-}; (e) Na^+/K^+. The values are means of three replicates. A ($NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 2:1:0:0; pH 7.03 to 7.29); B ($NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 1:1:1:0; pH 7.34 to 8.51); C ($NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 12:9:8:1; pH 8.80 to 9.02);D ($NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 8:9:12:1; pH 9.03-9.31); E ($NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 12:1:8:9; pH 9.45 to 9.81); F ($NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 0:0:2:1; pH 9.84 to 10.69).

greater harmful effects. Our results showed that germination was not inhibited by high pH at low salinity, but was inhibited at higher salinity, which implied a function of pH adjustment in seeds and allowed

germination at low salinity. Under higher salinity, high pH may decompose the seeds structure and even result in the death, the reason for it might be complex and deserving of further research. Ungerminated seeds caused by salt stress or salt–alkaline mixed stress may be in a state of dormancy to escape from the rigorous environment (Gulzar and Khan, 2001). When rain falls and decreases salinity in soil, alfalfa seeds would be able to germinate again, which might be a strategy for a plant to live in the soil with high salinity or pH.

Seeds growth

Some reports clearly demonstrated that the destructive effects of alkali ($NaHCO_3$ and Na_2CO_3) stress on plants were greater than salt (NaCl and Na_2SO_4) stress (Yang et al., 2008a, b). The injurious effects caused by salt stress are commonly thought to be a result of low water potentials and ion toxicities (Al-Khateeb, 2006; Song et al., 2008). Under salt–alkaline mixed stress, in addition to the above factors, plants also have to deal with stress caused by high pH and ion interactions. The high pH environment surrounding the roots could directly cause the precipitation of metal ions and phosphorus, destroying the nutrient supply and ion balance around the roots (Yang et al., 2008c). Moreover, high pH may lead to lack of protons, the destruction or inhibition of transmembrane electrochemical-potential gradients in root cells, and the loss of normal physiological root functions such as ion absorption (Shi and Wang, 2005; Guan et al., 2009). In this study, we also observed that both increased salinity and alkalinity (high-pH) induced the decrease of plumule and radicle lengths of alfalfa seed (Figure 2a and b) and the increase of electrolyte leakage rate (Figure 3), showing that high-pH may harm membrane system of seed and limit mitosis of plumule and radicle cells.

Seeds osmotic adjustment

Na^+ is the main toxic ion in salinized soil. Low Na^+ and high K^+ in the cytoplasm are essential for the maintenance of a number of enzymatic processes (Munns and Tester, 2008). Na^+ enters plant cells through the high-affinity K^+ transporter (HKT) and through non-selective cation channels (Zhu, 2003). The similarity in size of the hydrated ionic radii of Na^+ and K^+ makes them difficult to discriminate, and this is the basis of Na^+ toxicity (Blumwald, 2000). Under salt stress, Na^+ competes with K^+ for uptake into roots (Munns and Tester, 2008). However, our results showed that both increasing salinity and high pH induced the increases in Na^+ content and the decreases in the K^+ content in alfalfa seeds (Figure 4a, b and Table 3). We propose that this was not a response to osmotic stress, but a specific response to high-pH stress. This implied that the high-pH of salt–alkaline mixed stress might interfere with control of Na^+ and K^+ uptake in the radicle and increase intracellular Na^+ to a toxic level, and disrupt the ionic balance or pH homeostasis in tissue. The increased level of Na^+ caused by high pH might also be related to decreased exclusion of Na^+. It is well known that many plant species have a Na^+ exclusion mechanism that depends on a Na^+/H^+ antiporter, such as salt overly sensitive 1 (SOS1), which exchanges cytoplasmic Na^+ for external H^+ (Munns and Tester, 2008; Zhu, 2003). The exchange activity relies on the transmembrane proton gradient, which is established by H^+-ATPase (Zhu, 2003). Under strong alkali stress, the lack of external protons might weaken the exchange activity of the Na^+/H^+ antiporter in the radicle plasma membrane (Munns and Tester, 2008), and possibly reduce exclusion of Na^+ into the rhizosphere and enhance accumulation of Na^+ in alfalfa seeds.

Ionic imbalance in plants is caused mainly by the influx of excess Na^+ (Munns and Tester, 2008). Plants in saline conditions usually accumulate inorganic anions or organic anions to neutralize the high concentrations of cations and maintain a stable intracellular pH (Yang et al., 2008b). Our results also showed that alfalfa seed also might accumulate Cl^- and SO_4^{2-} to maintain ionic balance in seed. In addition, contents of Cl^- and SO_4^{2-} might be related to the ion concentrations of treatment solutions (Figure 4c and d). In general, the accumulation of proline, a main organic osmolyte, relates closely with osmotic stress intensity. The results revealed that proline content increased not only with increasing salinity, but also when alkalinity increased at the same salt concentration (Figure 5a). Proline accumulation in seeds may be a response to Na^+ influx (Figures 4a and 5a), it may distribute in the cytoplasm to balance the osmotic pressure from vacuoles and protect biomacromolecules. It is known that proline accumulation plays as much of a positive role in the mixed salt-alkali tolerance of many plants as it contributes to membrane stability (Ashraf and Harris, 2004). The role of the soluble sugars might be significantly different from proline, during adapting of alfalfa seed to salt–alkaline mixed stress (Figure 5a), and high pH that may interfere with seed sugar metabolism, this should be further investigated.

Synergistic effect of salt stress and alkali stress

The salinization of soil is a widespread environmental problem. Although considerable efforts have been devoted to solve this problem, mixed salt-alkali stress has been neglected. Our results indicated that salt-alkali mixed stress was more sensitive than salt stress due to the stress caused by mixed salt-alkali stress has the contribution of not only salt or alkali individually, but also

synergistic effects of salt and alkali stresses. For example, the destruction caused by the combined effect of salt and alkali on germination rate was more severe than that of caused by neutral salt, particularly at high salt concentrations (Figure 1).

The mixed salt-alkali stress differs from only salt or alkali stress because there is a significant interaction between salt concentration and salt components. Alfalfa can tolerate the stress of high- alkaline and low salt stress, and it can germinate within the treatment of high salt concentration and low salt component. However, the synergistic effects of high salt and alkali lead to the ungerminated seeds. Therefore, the destruction of mixed high salt-alkali is more severe than that of only salt or alkali. Overall, alfalfa is a tolerant plant of salt-alkali. Reducing the salt concentration or pH could alleviate the damage resulting from mixed salt-alkali stress, particularly for barren land with a high pH such as that in northeast China. Nevertheless, the interaction between salt and alkali stress in natural soil is more complex than that in pot experiments, and it therefore needs further investigation.

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