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Short communication

Interactive effects of salt and alkali stresses on seed germination, germination recovery, and seedling growth of a halophyte *Spartina alterniflora* (Poaceae)

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Abstract

Soil salinization and alkalinization frequently co-occur in nature, but there is little information on the interactive effects of salt and alkali stresses on plants. Seed germination and early seedling growth are crucial stages for plant establishment. We investigated the interactive effects of salt and alkali stresses on seed germination, germination recovery and seedling growth of a halophyte *Spartina alterniflora*. Seed germination percentage was not significantly reduced at low salinity (≤200 mM) at pH 6.63–9.95, but decreased with increased salinity and pH. Ungerminated seeds germinated well after transfer to distilled water from treatment solutions, indicating that seeds can remain viable in high salt–alkaline habits. Shoot growth was stimulated at low salinity and pH, but decreased with increased salinity and pH. Radicle elongation decreased sharply with increased salinity and pH, and was significantly inhibited when pH≥9.0, indicating that the radicles are very sensitive to salt–alkaline stress. The deleterious effects of salinity or high pH alone were less than when combined. A reciprocal enhancement of salt and alkali stresses is a characteristic feature for salt–alkaline stress. Stepwise regression analysis indicates that salinity is the dominant factor, while pH and buffer capacity are secondary for salt–alkaline mixed stress.

Keywords: Germination recovery; Salt–alkaline mixed stress; Seed germination; Seedling growth; *Spartina alterniflora*

1. Introduction

Salt stress is a widespread environmental problem. Although great effort has been made on this problem, two vital aspects have been neglected: salt–alkaline mixed stress and complex salt stress. Saline soils contain multiple types of soluble salt components, and the compositions of soluble salts in saline soils are quite different among locations (Hardegree and Emmerich, 1990; Tobe et al., 2004). When a saline soil contains CO_3^{2-} and/or HCO_3^-, it causes injury to plants not only through salt stress but also through alkali stress. Because soil salinization and alkalinization frequently co-occur in nature, the conditions in natural salt–alkaline soil are very complex (Shi and Wang, 2005; Li et al., 2009). Therefore, the problem of salt–alkaline mixed stress should be recognized and investigated as thoroughly as that of neutral salt stress.

Seed germination is the critical stage in the life cycle of halophytes (Ungar, 1995; Song et al., 2005). Germination responses of halophytes to environmental parameters determine their distribution in saline environments (Tobe et al., 2000). Moreover, germination of halophytes in a saline substrate is also a legitimate criterion for selecting for tolerance in saline environments (Sosa et al., 2005). Halophytes vary in their tolerance to salinity during seed germination stage (Katembe et al., 1998; Song et al., 2008; Wang et al., 2008; Wei et al., 2008). Some studies indicate that seeds of halophytes can remain viable for an extended period of exposure to salt stress and germinate when conditions are favorable (Khan and Ungar,

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1997; Zia and Khan, 2004). In addition, high salinity can also completely inhibit seed germination at concentrations beyond the tolerance limits of the species (Khan et al., 2001).

Numerous studies have investigated seed germination of halophytes under salinity using only NaCl (Jaleel et al., 2007; Song et al., 2008; Wang et al., 2008; Wei et al., 2008). However, other chloride, sulfate and carbonate salts and their interactions play a significant role in affecting seed germination (Khan et al., 2002; Duan et al., 2004, 2007; Vicente et al., 2007). Previous studies find that alkaline salt stress and neutral salt stress are actually two distinct stresses (Shi and Yin, 1993). However, very few focus on seed germination response to salt–alkaline stress.

Spartina alterniflora is a rhizomatous perennial, native to the Atlantic and Gulf coast of North America (Bradley and Morris, 1991). It is an important cash halophyte and widely introduced to many countries because it can be widely utilized as a substantial source of bioactive material and fodder and for sewage treatment (Hu et al., 1998; Liu and Tian, 2002; Zhu and Qin, 2003; Zhu et al., 2007). It commonly dominates salt marshes and can survive and grow well at a wide range of salinity (NaCl) (Colmer et al., 1996; Vasquez et al., 2006).

The existing data on germination of *S. alterniflora* under salinity have been obtained using only NaCl (Mooring et al., 1971; Wijte and Gallagher, 1996a; Yuan and Shi, 2008; Elsey-Quirk et al., 2009). NaCl is generally the sole salinizing agent in salinity studies as it is usually the main component of the soluble salts mixture in saline soils. Nevertheless, the presence of HCO_3^- and CO_3^{2-} can elevate soil pH in some salt–alkaline habits of Tianjin, China, where *S. alterniflora* forms monodominant communities (Zhang, 2007). Most importantly, different ecotypes of the same species occurring in different habitats may have different tolerance to special environmental conditions (Bazzaz, 1973). However, little is known about its response to salt–alkaline mixed stress.

The aims of the present study were (1) to test the interactive effects of salt and alkali stresses on seed germination and germination recovery of *S. alterniflora*, (2) to test the interactive effects of salt and alkali stresses on early seedling growth of *S. alterniflora*, and (3) to analyze the features of salt–alkaline conditions.

2. Materials and methods

2.1. Seeds and field site description

S. alterniflora seeds were collected from a salt–alkaline habitat (117°45′E, 39°03′N) in Tianjin of China in November of 2007. Annual mean rainfall is around 622 mm and annual mean evaporation is around 1800 mm; annual mean temperature is 11.7 °C and mean temperatures of the coldest (January) and hottest (July) months are 3.5 and 26.2 °C, respectively (Yang, 2005).

Spikelets of *S. alterniflora* were separated from the spikes of the inflorescence by gently hand-shattering in order to capture only the seeds that were fully mature and ready to drop, and put in labeled closed plastic bags to reduce water loss. Using a light table, full spikelets containing caryopsis were selected at random. The caryopsis of *S. alterniflora* is recalcitrant and cannot tolerate drying to less than 40% dry weight either before or after

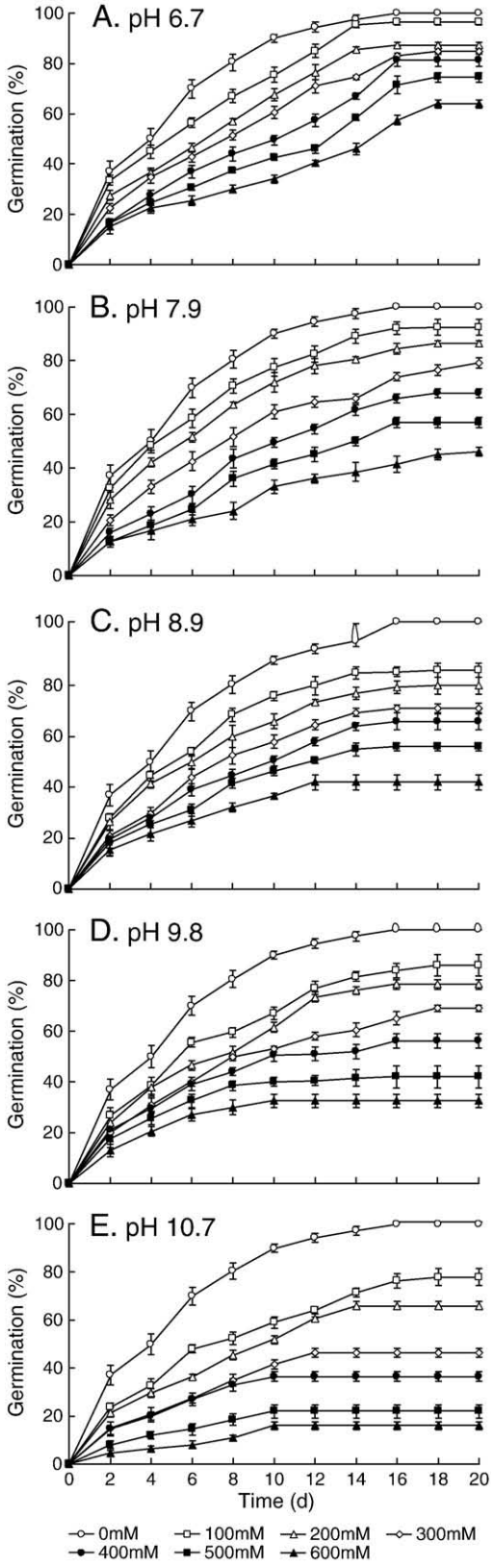

Fig. 1. Cumulative germination percentages (mean ± s.e., $n=4$) of *S. alterniflora* seeds incubated in each treatment group for 20 days.

Table 1
Two-way ANOVA of effects of salinity, pH, and their interactions on seed germination and seedling growth of *S. alterniflora*.

Source of variance	Initial germination (%)	Germination rate	Shoot length (cm)	Root length (cm)	Fresh weight (g plant^{-1})
Salinity	1061.26***	568.96***	215.81***	174.00***	88.07***
pH	762.18***	198.79***	127.34***	140.25***	52.01***
Salinity × pH	20.82***	4.86***	3.78***	23.69***	5.09***

Data represent *F*-values at 0.05 level.
*** $P<0.001$.

collection (Plyler and Carrick, 1993; Biber and Caldwell, 2008). The seeds were stored submerged in distilled water at about 4 °C, as the most efficient storage method for *S. alterniflora* seeds (Mooring et al., 1971; Wijte and Gallagher, 1996a, b; Yuan and Shi, 2008), in 500 ml closed brown glass bottles (about 5000 seeds per each bottle). The experiments were started within 1 week after seeds were collected.

2.2. Design of various salt–alkaline stresses

To simulate salt–alkaline conditions, two neutral salts (NaCl and Na_2SO_4) and two alkali salts ($NaHCO_3$ and Na_2CO_3) were mixed in various proportions described by Shi and Wang (2005) to establish different treatments. Treatments consisted of six levels of salinity (100, 200, 300, 400, 500, and 600 mM) in each of five pH levels: A (NaCl:Na_2SO_4:$NaHCO_3$: Na_2CO_3 = 1:1:0:0, pH 6.7±0.05), B (NaCl:Na_2SO_4:$NaHCO_3$:Na_2CO_3 = 1:2:1:0, pH 7.9±0.11), C (NaCl:Na_2SO_4:$NaHCO_3$:Na_2CO_3 = 1:9:9:1, pH 8.9±0.09), D (NaCl:Na_2SO_4:$NaHCO_3$:Na_2CO_3 = 1:1:1:1, pH 9.8±0.08), and E (NaCl:Na_2SO_4:$NaHCO_3$:Na_2CO_3 = 9:1:1:9, pH 10.7±0.07). In total, there were 30 stress treatments labeled as A1, …, A6, B1, …, E6, respectively.

2.3. Effect of various salt–alkaline stresses on seed germination

Healthy seeds of similar size and mass were selected. Seeds were surface sterilized in an aqueous solution of 0.1% $KMnO_4$ for 10 min to avoid fungus attack, and subsequently rinsed with distilled water before being used in seed germination experiments. Seed experiments were replicated four times with 50 seeds per treatment. Seeds were placed in 9-cm Petri dishes on three layers of Whatman No.1 filter paper (pH 7) moistened with 5 ml of distilled water or with treatment solutions. Dishes were placed in a germinator (LRH-250-GS II, China), and subjected to an alternating diurnal regime of 12 h of light at 25 °C and 12 h of dark at 15 °C for 20 days. This temperature regime was chosen to represent the mid-spring temperatures, when this species germinates. Germinated seeds were counted and removed every second day. Seed germination was defined as when the coleoptile elongated to 0.5 cm. To avoid changes in salinity, the original solution in each Petri dish was removed daily and 10 ml of new solution was added and removed again as completely as possible, and then 5 ml of new solution was added again.

Germination rate (GR) was determined using a modified Timson's index described as follows:

$$GR = \Sigma G / t$$

where G is the percentage of seed germination at 2-d intervals and t is the total germination period (Khan and Ungar, 1997). The greater the value, the more rapid the germination. The maximum value that can be obtained for the modified Timson's Index is 50.

Ungerminated seeds in each treatment were rinsed three times with 10 ml of distilled water and then placed in new Petri dishes with the former filter paper moistened with 5 ml of distilled water, and incubated for an additional 20 days for

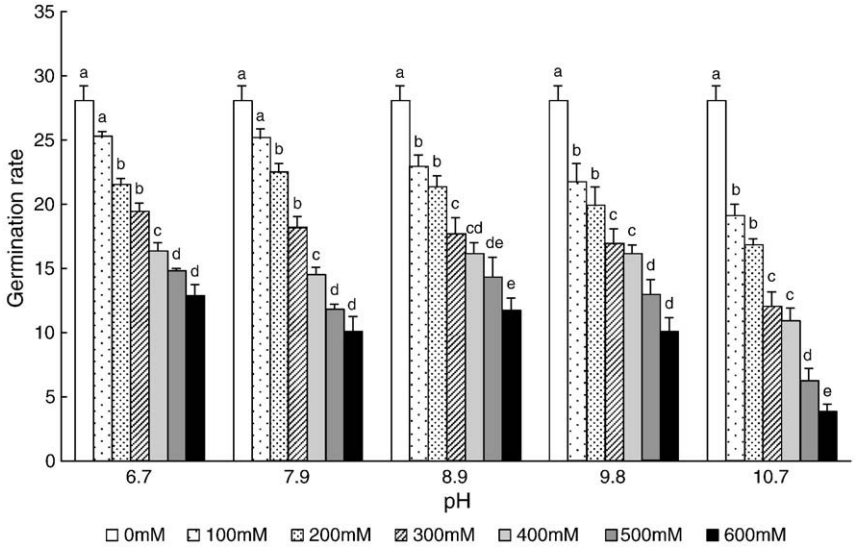

Fig. 2. Mean germination rates (mean±s.e., $n=4$) of *S. alterniflora* seeds incubated in each treatment group. Values at each treatment group having a different small letter are significantly different from each other ($P<0.05$).

germination recovery. Initial germination percentage (G_I), recovery percentage (RP) and final germination percentage (G_F) were determined by the following formulae, respectively:

$$G_I(\%) = (b / c) \times 100$$

$$RP(\%) = [(a-b) / (c-b)] \times 100$$

$$G_F(\%) = (a / c) \times 100$$

where a is the sum of the number of seeds germinated in salt solutions plus those that recovered to germinate in distilled water, b is the total number of seeds germinated in treatment solutions, and c is the total number of seeds tested.

2.4. Effect of various salt–alkaline stresses on seedling growth

Seeds were incubated initially in distilled water. When the coleoptile had just emerged (<0.5 cm), 20 of these young seedlings were transferred into Petri dishes containing treatment solutions. Seedling incubation was terminated after 20 days. Shoot length, radicle length and fresh weight of seedlings were recorded.

2.5. Data analysis

Germination data were transformed (arcsine) before statistical analysis to ensure homogeneity of variance. A two-way ANOVA was used to test the significance of main effects (salinity and pH) and their interaction on seed germination and seeding growth. All data were expressed as mean±s.e. Tukey's HSD test and paired two-tailed tests were performed for multiple comparisons to determine significant ($P<0.05$) differences between individual treatments.

3. Results

Germination percentage of *S. alterniflora* was highest in distilled water. Germination percentages were not markedly reduced at low salinity (≤200 mM) at pH 6.63–9.95, but were significantly inhibited by high salinity and pH (Fig. 1). A two-way ANOVA showed that seed germination was significantly affected by salinity ($F=1061.26$, $P<0.001$), pH ($F=762.18$, $P<0.001$) and their interaction ($F=20.82$, $P<0.001$; Table 1).

Germination rate of *S. alterniflora* seeds was given by a modified Timson's index (Khan and Ungar, 1997). Germination rate of *S. alterniflora* seeds decreased significantly with increased salinity and pH (Fig. 2). A two-way ANOVA showed that germination rate was significantly affected by salinity ($F=568.96$, $P<0.001$), pH ($F=198.79$, $P<0.001$) and their interaction ($F=4.86$, $P<0.001$; Table 1).

When seeds were transferred to distilled water after 20 days from the treatment solutions, ungerminated seeds of *S. alterniflora* did germinate well (Table 2). Final germination percentages of *S. alterniflora* seeds in each treatment were similar to controls (Table 2). Final ungerminated seeds were presumed dead, since there was no evidence of dormancy in the controls.

Table 2
Germination percentages of *S. alterniflora* seeds incubated in each treatment group.

Salinity (mM)		Seed germination (%, mean±s.e., $n=4$)		
		Initial $(b/c)\times100$	Recovery* $[(a-b)/(c-b)]\times100$	Final** $(a/c)\times100$
0 (control)		100±0[a]	0±0[a]	100±0[a]
pH 6.7	100	96.5±1.0[a]	0±0[a]	96.5±1.0[a]
	200	87.5±1.0[a]	39.9±8.1[b]	92.5±1.0[a]
	300	85.0±1.2[a]	26.8±2.1[b]	89.0±1.2[a]
	400	81.5±2.5[a]	29.7±10.0[b]	87.0±2.6[a]
	500	74.5±1.9[b]	46.8±6.1[c]	86.5±1.0[a]
	600	64.0±1.6[c]	57.0±3.7[d]	84.5±1.9[a]
pH 7.9	100	92.5±3.0[a]	35.8±12.6[b]	95.0±2.6[a]
	200	86.5±1.0[a]	55.4±3.6[c]	94.0±0.0[a]
	300	79.0±2.0[a]	38.4±8.5[b]	87.0±2.6[a]
	400	68.0±1.6[b]	56.4±2.9[c]	86.0±1.6[a]
	500	57.0±2.0[c]	66.4±6.2[d]	85.5±3.0[a]
	600	46.0±1.6[d]	64.8±3.9[d]	81.0±2.0[a]
pH 8.9	100	86.0±2.8[a]	71.8±10.0[b]	96.0±1.6[a]
	200	80.0±3.3[a]	60.6±8.26[b]	92.0±2.8[a]
	300	71.0±3.5[b]	65.6±2.4[b]	90.0±1.6[a]
	400	66.0±3.3[b]	65.2±7.9[b]	88.0±3.7[a]
	500	56.0±1.6[c]	71.7±4.6[b]	87.5±2.5[a]
	600	42.0±2.8[d]	63.1±6.2[b]	78.5±4.1[a]
pH 9.8	100	86.0±4.3[a]	71.8±19.6[a]	96.0±2.8[a]
	200	78.5±1.9[a]	53.4±13.5[b]	90.0±2.8[a]
	300	69.0±1.2[b]	54.7±6.6[b]	86.0±1.6[a]
	400	56.0±2.8[c]	66.0±5.0[b]	85.0±2.6[a]
	500	42.0±4.3[d]	72.3±3.2[b]	84.0±1.6[a]
	600	32.5±2.5[e]	70.5±3.3[b]	80.0±2.8[a]
pH 10.7	100	78.0±3.7[b]	68.3±10.5[a]	93.0±2.6[a]
	200	66.0±1.6[c]	76.8±10.5[a]	92.0±4.0[a]
	300	46.5±1.9[d]	73.9±5.3[a]	86.0±3.3[a]
	400	36.5±1.9[e]	78.0±3.7[a]	85.5±2.8[a]
	500	22.0±2.8[f]	80.9±7.2[a]	85.0±6.0[a]
	600	16.0±1.6[g]	69.1±3.8[a]	74.0±3.7[b]

*Recovery germination was recorded as $[(a-b)/(c-b)]\times100$, where a is the sum of the number of seeds germinated in the salt–alkaline solutions plus the number that recovered to germinate in distilled water, b is the number of seeds germinated in the initial salt–alkaline solutions, and c is the total number of seeds tested. **Final germination was recorded as $(a/c)\times100$. Values at each treatment group followed by different lowercase letters are significantly different ($P<0.05$).

Shoot length of *S. alterniflora* seedlings decreased with increased salinity and pH, except treatment for treatments A1 (salinity 100 mM, pH 6.63) and B1 (salinity 100, pH 7.68) (Fig. 3A). A two-way ANOVA indicated that shoot length was significantly affected by salinity ($F=215.81$, $P<0.001$), pH ($F=127.34$, $P<0.001$) and their interaction ($F=3.78$, $P<0.001$; Table 1).

Radicle length of *S. alterniflora* seedlings under various salt–alkaline mixed stresses was significantly less than of controls. Radicle growth of *S. alterniflora* was completely inhibited when pH≥9.0 (Fig. 3B). A two-way ANOVA indicated that radicle length was significantly affected by salinity ($F=174.00$, $P<0.001$), pH ($F=140.25$, $P<0.001$) and their interaction ($F=23.69$, $P<0.001$; Table 1).

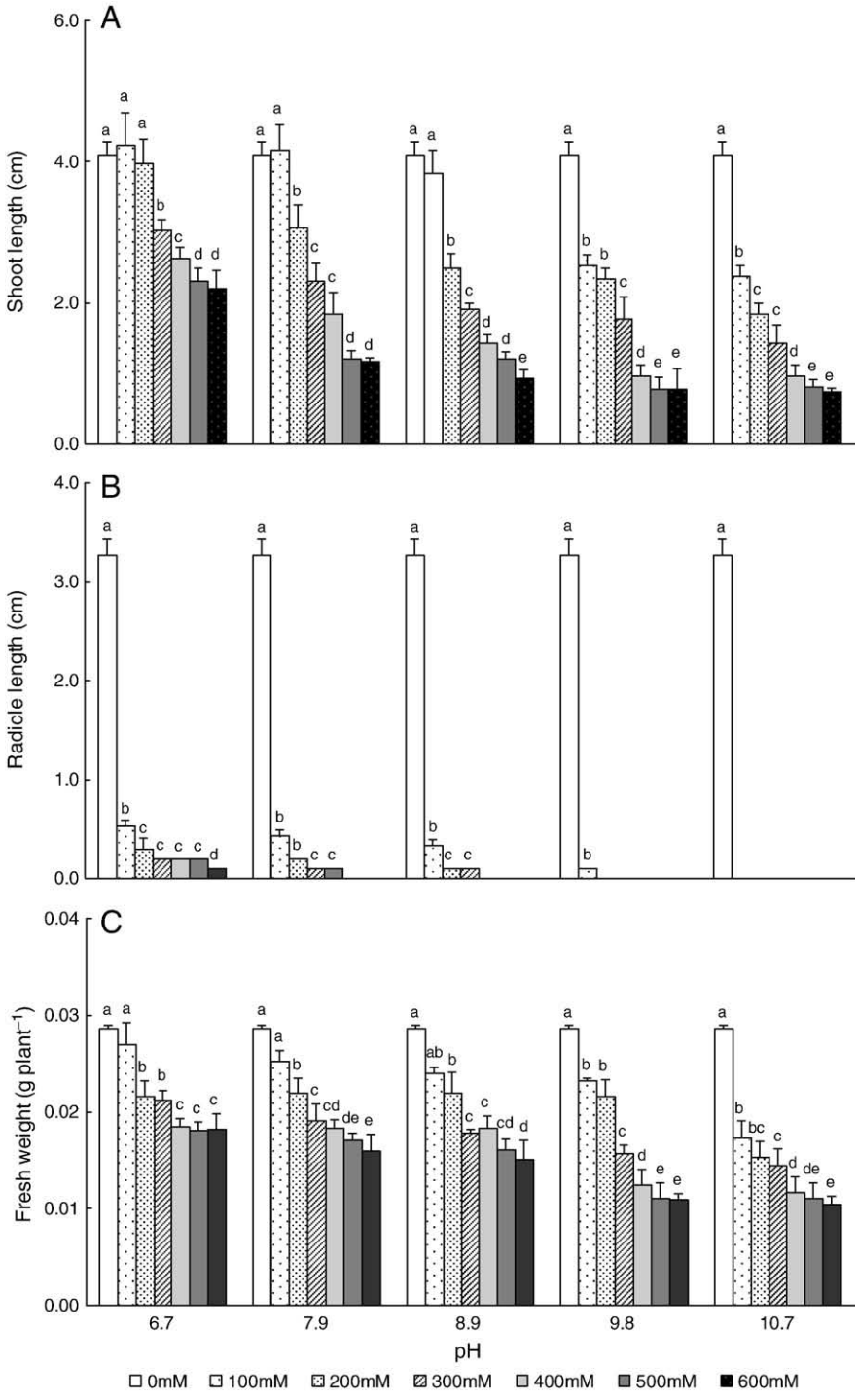

Fig. 3. Shoot length (A), radicle length (B), and fresh weight (C) of *S. alterniflora* seedlings in each treatment group. Values are given as mean±s.e ($n=4$). Values at each treatment group having a different small letter are significantly different from each other ($P<0.05$).

Fresh weight of *S. alterniflora* seedlings decreased with increased salinity and pH (Fig. 3C). A two-way ANOVA showed that fresh weight was significantly affected by salinity ($F=88.07$, $P<0.001$), pH ($F=52.01$, $P<0.001$) and their interaction ($F=5.09$, $P<0.001$; Table 1).

The three main stress factors (salinity, buffer capacity, and pH) of various salt and alkali stresses are shown in Table 3. The results of stepwise regression analysis between each germination or growth index and the three main stress factors are shown in Table 4. There was a high linear correlation between each index and the three main stress factors ($P<0.001$). The effects of the three main stress factors on the five indices differed in magnitude (Table 4). Among the absolute values of the three regression coefficients, those of salinity (β_1) were the highest for all the

Table 3
Data on stress factors of various treatments.

Treatment	Stress factors		
	Salinity (mM)	Buffer capacity *	pH
A1	100	0.02	6.63
B1	100	17.15	7.68
C1	100	36.10	8.70
D1	100	52.80	9.70
E1	100	74.20	10.60
A2	200	0.04	6.68
B2	200	33.40	7.83
C2	200	71.60	8.78
D2	200	112.00	9.79
E2	200	166.10	10.63
A3	300	0.06	6.70
B3	300	50.25	7.88
C3	300	125.50	8.82
D3	300	160.40	9.87
E3	300	221.00	10.67
A4	400	0.080	6.72
B4	400	65.50	7.92
C4	400	138.60	8.90
D4	400	220.40	9.88
E4	400	332.00	10.68
A5	500	0.10	6.75
B5	500	85.25	7.98
C5	500	168.00	8.92
D5	500	271.00	9.90
E5	500	379.00	10.78
A6	600	0.12	6.80
B6	600	102.00	8.05
C6	600	206.20	9.00
D6	600	316.70	9.95
E6	600	444.00	10.80

* Buffer capacity was defined as the millimolar amount of H^+ needed to drop the pH of 1 L of treatment solution to the same pH as the control by titration with HCl. Treatments consisted of six levels of salinity (100, 200, 300, 400, 500, and 600 mM) in each of the five pH levels: A (pH 6.7±0.05), B (pH 7.9±0.11), C (pH 8.9±0.09), D (pH 9.8±0.08), and E (pH 10.7±0.07). In total, there were 30 stress treatments labeled as A1, …, A6, B1, …, E6, respectively.

Table 4
Results of stepwise regression between each index and the three main stress factors.

	Model	R^2	ANOVA test	β_1	β_2	β_3
Germination percentage (%)	$Y=95.7-0.09X_1$	0.51	$P<0.001$	−0.71		
Germination rate	$Y=37.8-0.03X_1-1.4X_3$	0.91	$P<0.001$	−0.85		−0.39
Shoot length (cm)	$Y=8.8-0.005X_1+0.002X_2-0.6X_3$	0.93	$P<0.001$	−0.86	0.29	−0.80
Root length (cm)	$Y=1.5-7.8E-4X_1+0.001X_2-0.1X_3$	0.86	$P<0.001$	−0.95	0.98	−1.41
Fresh weight (g plant^{-1})	$Y=0.04-1.6E-5X_1-6.4E-6X_2-0.001X_3$	0.88	$P<0.001$	−0.61	−0.18	−0.45

X_1=Salinity; X_2=Buffer capacity; X_3=pH. β_1–β_3: standardize regression coefficients corresponding X_1–X_3. The greater the absolute β value, the stronger effect of the stress factor on germination or growth index. R^2: the square of total correlation coefficient. n=30.

indices. The results indicated that salinity was a dominant factor, and that pH and buffer capacity were secondary.

4. Discussion

Seed germination of *S. alterniflora* was highest in distilled water and there was no indication of dormancy. Similar observations have been made by Mooring et al. (1971) and Elsey-Quirk et al. (2009) in the USA for *S. aterniflora* seeds from North Carolina and Southwest Louisiana, respectively. In both those studies, no stratification was necessary to facilitate germination. However, other studies have demonstrated that seed germination of *S. alterniflora* was high following stratification (Wijte and Gallagher, 1996a). It is presently not understood why *S. alterniflora* seeds from different provenances should show this difference. Germination of *S. alterniflora* seeds was not inhibited at low salinity (≤200 mM) at pH 6.63–9.95, but was only 16% under 600 mM salinity (pH 10.80; Table 2). Generally, the difference between germination of seeds at low salinities and that in deionized water was not significant, but they gradually decreased with further increases in salinity (Khan and Ungar, 1997; Katembe et al., 1998).

Seeds of many halophytes under high salinity conditions that inhibit germination will recover and germinate after transfer to distilled water (Song et al., 2006; Qu et al., 2008). However, seed germination of some species is permanently inhibited by high salinity (Khan et al., 2001). In our results, ungerminated seeds of *S. alterniflora* germinated well after transfer to distilled water from the treatment solutions. Final germination percentages in each treatment were similar to controls (Table 2). Therefore, seeds of *S. alterniflora* are well adapted to salt–alkaline habitats via a high capacity for germination recovery.

Although seedlings of halophytes can develop in solutions with low concentrations of salts, radicle growth may be greatly retarded by high salinity (Malcolm et al., 2003; Qu et al., 2008). In the present study, radicle growth of *S. alterniflora* decreased sharply with increased salinity and pH (Fig. 3B). This indicates that radicles of *S. alterniflora* are very sensitive to salinity and pH.

The effects of the combined salt and alkali stresses were an important contribution of this study. The deleterious effects of salinity or high pH alone were significantly less than those of combined salinity and high pH. There was an interactive effective effect of salt and alkali stresses on the germination and early seedling growth. A reciprocal enhancement between salt stress and alkali stress is characteristic of salt–alkaline mixed stress (Shi et al., 1998; Shi and Wang, 2005; Peng et al., 2008).

It is very difficult to objectively estimate the effects on plant growth of natural salt-alkalinized soil, especially for soil with high pH. Salt concentration, i.e. [Na^+], or specific conductance might be used to represent the strength of salt stress (Tanji, 1990) whereas buffer capacity or pH might be used to represent alkali stress strength (Shi et al., 1998); but none of these indices completely reflect mixed salt–alkaline stress strength. According to our results, synthetic conditions which combine salinity, pH and buffer capacity of salt–alkaline soil may constitute a new model to solve this problem (Table 4).

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