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Effect of various salt–alkaline mixed stress conditions on sunflower seedlings and analysis of their stress factors

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Abstract

Sunflower seedlings were treated under 30 different conditions of alkalinity and salinity, which were established by mixing NaCl, $NaHCO_3$, Na_2SO_4, and Na_2CO_3, at various proportions. The treatments included a salt concentration range of 50–250 mmol and pH values from 7.12 to 10.72. Several physiological indices of seedlings stressed—including relative growth rate (RGR), leaf area, electrolyte leakage rate, proline content, citric acid content, and contents of Na^+ and K^+—were determined to analyze the characteristics of the stresses due to the salt–alkali mixes and their main stress factors.

The results showed that the physiological responses of sunflower closely correlated not only with salinity (the total concentration of stress salt) but also with the pH (or alkalinity) of the treatment solution. RGR, leaf area, and of K^+ content decreased with increasing salinity and pH. Electrolyte leakage rate, proline content, citric acid content, and Na^+ content increased with increasing salinity and pH. The deleterious effects of a high pH value or salinity alone were significantly less than those of high pH in combination with salinity. This result suggested that for a salt–alkali mix stress, a reciprocal enhancement between salt stress and alkali stress was a characteristic feature.

The buffer capacity of the treatment solution was taken as a stress factor in order to simplify the stress factor analysis. The results of the statistical analysis showed that for the stress factors of the salt–alkali mix stress, $[CO_3^{2-}]$ and $[HCO_3^-]$ could be fully represented by the buffer capacity; $[Na^+]$ could be fully represented by salinity; whereas $[SO_4^{2-}]$ was negligible. Therefore, four factors, salinity, buffer capacity, pH and $[Cl^-]$, could reflect all of stress factors. Perfect linear correlations were observed between all strain indices and the four stress factors. However, the effects of the four stress factors on the strain indices were significantly different in magnitude. Buffer capacity and salinity were dominant factors for all strain indices. Thus, it is reasonable to consider the sum of salinity plus buffer capacity as the strength value of salt–alkali mix stress. Furthermore, the relationships between different strain indices and various stress factors were shown to be different.

Keywords: Alkali stress; Buffer capacity; pH; Salinity; Salt stress; Salt–alkali mixed stress; Stress factor; Sunflower

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1. Introduction

Salinity stress is a widespread environmental problem. Although considerable effort has been devoted to solve this problem, two very important aspects have been neglected, i.e. salt–alkali stress and complex salt stress. Even though the world's land surface occupies about 13.2×10^9 ha, no more than 7×10^9 ha are potentially arable, and only 1.5×10^9 ha are currently cultivated. Of the cultivated area, about 0.34×10^9 ha (23%) are saline and another 0.56×10^9 ha (37%) are sodic (Tanji, 1990). Actually, the problem of soil alkalinization due to $NaHCO_3$ and Na_2CO_3, may be more severe than the problem of soil salinization caused by the neutral salts, such as NaCl and Na_2SO_4. For example, in the northeast of China, alkalinized grassland has reached more than 70% (Kawanabe and Zhu, 1991). Because soil salinization and alkalinization frequently co-occur, the conditions in the naturally salinized and alkalinized soil are very complex, the total salt contents and composition of salts and the proportion of neutral salts to alkaline salts may vary in different soils. Thus, the stresses imposed by these soil media on plants could be very complex and difficult to approach experimentally. Natural salt stresses are mostly mixed salts stresses, and most of them contain both neutral and alkaline salts. Therefore, the problems of alkaline stress and salt–alkali mixed stress ought to be recognized and investigated as thoroughly as salt stress.

To date, the research of salt stress still emphasizes NaCl as the main subject, but it is deeply developing towards various aspects such as Na^+ metabolism (Serrano et al., 1999), molecular biology of salt-resistance genes (Holmström et al., 2000; Huang et al., 2000; Quesada et al., 2002), and salt stress signal transduction (DeWald et al., 2001), and so on. However, there are only a few reports about stress by alkali. However, there have been some studies about calcareous soils (Brand et al., 2002; Nuttall et al., 2003), alkaline soil (Hartung et al., 2002; Yin and Shi, 1993), alkaline salt stress (Campbell and Nishio, 2000; El and Shaddad, 1996; Shi and Yin, 1992, 1993), and mixed salt stress (Shi et al., 1998). Furthermore, some reports clearly demonstrated the existence of alkali stress and showed that it is more severe than salt stress (Shi and Yin, 1993; Tang and Turner, 1999). In previous studies, it was found that alkali salt stress and neutral salt stress are actually two distinct kinds of stresses (Shi and Yin, 1993). Based on our results, alkaline salt stress is best called "alkali stress," while "salt stress" only includes the neutral salt stress.

The resistance to alkali stress of sunflower (*Helianthus annuus* L.) is stronger than that of other crops. Some sunflower breeds are able to grow on alkalinized soil. However, there are very few reports about sunflower resistance to salt stress or alkali stress (Liu and Baird, 2003). A cultivar of sunflower was selected as the material to investigate the features and acting factors of salt–alkali mixed stress.

The neutral salts NaCl and Na_2SO_4 and the alkaline salts $NaHCO_3$ and Na_2CO_3 are the main salt components in the extensive alkaline soil over much of northeast China (Ge and Li, 1990). Therefore, mixtures of the aforementioned salts, in various proportions, were used to simulate a range of mixed salt and alkaline conditions. Thirty kinds of the mixed salt and alkaline conditions with different salinities and pH values were obtained to investigate the effect of mixed salt and alkaline stresses on sunflower seedlings and to analyze the corresponding stress factors.

2. Materials and methods

2.1. Plant materials

H. annuus L. cv. Baikuiza 4 was provided by the Baicheng Academy of Agriculture Sciences, Jilin Province, China and was selected because of its tolerance to salt–alkaline conditions. Baikuiza 4 seeds were sown in 24 cm diameter plastic pots containing washed sand. Each pot contained six plants. All pots were placed outdoors avoiding rainfall. Seedlings were sufficiently watered with Hoagland nutrient solution every 2 days. Evaporated water was replenished with distilled water at other times.

2.2. Design of simulated salt and alkaline conditions

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). The four selected salts were mixed in various proportions ac-

Table 1
The salt composition and its molar ratio of various treatments

Treatment group	Salt composition and molar proportions			
	NaCl	Na_2SO_4	$NaHCO_3$	Na_2CO_3
A	1	1	0	0
B	1	2	1	0
C	1	9	9	1
D	1	1	1	1
E	9	1	1	9
F	1	1	9	9

cording to the tolerability of the Baikuiza 4 cultivar to the salt–alkaline stress and the varying ranges of salinity and pH in the soil. Six treatment groups (labeled as A–F) were set with gradually increasing alkalinity. The salt composition of the six treatment groups is shown in Table 1. All treatment groups had a 1:1 molar ratio of monovalent salts (NaCl + $NaHCO_3$) to divalent salts (Na_2SO_4 + Na_2CO_3); therefore, if the individual molar concentrations were the same then the total ion concentrations were the same throughout the treatments. Within each group, five concentration treatments were utilized, namely 50, 100, 150, 200 and 250 mmol L^{-1} totaling 30 salt–alkali mixed stress treatments (labeled as A1, . . ., F5) with varying salinity and pH.

2.3. Stress treatments

When the seedlings were 4 weeks old, they were subjected to stress treatments. Seedlings growing uniformly (in 96 pots) were selected, randomly divided into 32 sets, 3 pots per set. Each pot was considered as one replicate with three replicates per set. One set was used as a control; a second set was used for growth index determination at the beginning of treatment; and the remaining 30 portions were treated with various stress treatments. Control plants were maintained by watering with nutrient solution; plants under all the various stress treatments were watered with nutrient solution with added stress salt as the treatment solution. Stress treatments were performed around 4–5 p.m., by watering thoroughly treated plants with 1000 ml of treatment solution per pot, in three portions. The amount of evaporated water was determined by weight and replenished with distilled water daily.

2.4. Stain indices measurements

All plants were harvested carefully after 7 days of treatment, washed with tap water first, then with distilled water. Water remaining on the surface of the plants was blotted with filter paper. Roots and shoots were separated in each plant. Eighteen plants from each treatment (six per replicate) were sampled to determine strain indices.

2.4.1. Growth measurement

The fresh weights (fr. wt.) of shoot and root were weighed directly. The third leaf blade from each shoot bottom was taken to determine leaf area with an area meter (model 1671-VHA). The result of leaf area was expressed in cm^2 $piece^{-1}$. Relative growth rate (RGR) was determined as described in Kingsbury et al. (1984) using the following formula:

$$\mathrm{RGR} = \frac{\ln \text{biomass at end of treatment} \; - \ln \text{biomass at start of treatment}}{\text{duration of treatment (days)}}.$$

One gram of fresh leaf was taken from each pot to determine the rate of electrolyte leakage. Other fresh samples were oven-dried at 80 °C for 15 min, then vacuum-dried at 40 °C to constant weight, then the dry weight (dry wt.) was measured. The result of biomass was expressed in g dry wt.

2.4.2. Organic and inorganic solutes determinations

Dry samples were homogenized by powdering . Two hundred milligrams of dry shoot samples were taken to determine K^+ and Na^+ contents by flame photometry (Wang and Zhao, 1995), and 100 mg of dry shoot samples were used to measure proline content according to Zhu et al. (1983). The content of citric acid was determined by the pentabromoacetone method with modifications (Shi et al., 2002). The results of K^+ and Na^+ contents were expressed in mmol g^{-1} dry wt., proline in μmol g^{-1} dry wt., and citric acid in mmol g^{-1} dry wt.

2.4.3. Membrane permeability determination

Membrane permeability can be reflected by the rate of electrolyte leakage (REL). REL was determined as described by Lutts et al. (1996). Fresh leaf discs (1 g) were taken from each pot, and washed three times with deionized water to remove surface-adhered electrolytes. Leaf discs were divided equally and placed into two closed vials containing 20 ml of deionized water. One of the vials was incubated at 25 °C on a rotary shaker for 3 h, and then the electrical conductivity of the solution (EC_1) was determined with a conductivity gauge. The other vial was autoclaved at 120 °C for 20 min and electrical conductivity of the solution (EC_2) was determined after equilibration to 25 °C. REL can be defined as follows:

$$\mathrm{REL}\,(\%) = \frac{\mathrm{EC}_1}{\mathrm{EC}_2} \times 100.$$

2.5. Analysis of stress factors

It is commonly thought that salt stress involves both osmotic effects and specific ion effects, the former ones chiefly depending on salt concentration. For alkaline stress, besides these two kinds of effects, there seems to be a high-pH effect. Preliminary results (Shi et al., 1998) show that a special response of plants to alkaline stress is the adjustment of their internal and external pH and that the buffer capacity of the treatment solution is an important factor for changing pH. Therefore, the stress factors of a salt–alkali mixed stress should involve total salt concentration, various ion concentrations, pH values, and buffer capacity.

The total salt concentration and the concentration of each ion such as [Na^+], [Cl^-], [SO_4^{2-}], [HCO_3^-] and [CO_3^{2-}] in various treatment solutions were calculated based on the composition of the solutions. The pH values of various treatment solutions were determined with a digital pH meter. The buffer capacity was determined using the method of Shi et al. (1998) with buffer capacity 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.

2.6. Statistical data analysis

All data obtained were the average of three replicas. Statistical analysis on two-way variance analysis (ANOVA), correlation coefficient, and multivariate regression was performed using Microsoft Excel.

3. Results

3.1. Salinity and pH coverage with various treatment solutions

Fig. 1 shows that the pH values increase gradually from group A to group F. In addition, within the same treatment group, pH values increase with increasing total salt concentration. The range of pH values is greater among groups than within a group. For Na^+, the main toxic ion, the concentrations used were 75, 150, 225, 300, and 375 mmol, corresponding to the five salt concentrations in a treatment group. In sum, 30 salt–alkaline conditions with different salinity and pH values were established. The salinity coverage was from 50 to 250 mmol; [Na^+] coverage was from 75 to 375 mmol; pH coverage was from 7.12 to 10.72.

Because of the salt component, salinity and pH values in the 30 simulated salt–alkaline conditions are similar to the conditions in natural salt–alkaline soil. These simulated salt–alkaline conditions reproduced

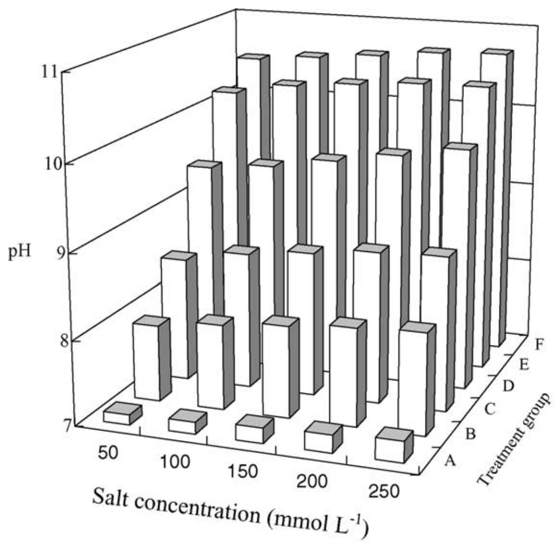

Fig. 1. Salinity and pH of various treatments. The values of pH are means of three replicates. (A) $NaCl{:}Na_2SO_4{:}NaHCO_3{:}Na_2CO_3$ = 1:1:0:0, pH 7.12–7.25; (B) $NaCl{:}Na_2SO_4{:}NaHCO_3{:}Na_2CO_3$ = 1:2:1:0, pH 7.91–8.20; (C) $NaCl{:}Na_2SO_4{:}NaHCO_3{:}Na_2CO_3$ = 1:9:9:1, pH 8.47–8.83; (D) $NaCl{:}Na_2SO_4{:}NaHCO_3{:}Na_2CO_3$ = 1:1:1:1, pH 9.41–9.88; (E) $NaCl{:}Na_2SO_4{:}NaHCO_3{:}Na_2CO_3$ = 9:1:1:9, pH 10.18–10.46; (F) $NaCl{:}Na_2SO_4{:}NaHCO_3{:}Na_2CO_3$ = 1:1:9:9, pH 10.47–10.72.

Table 2
Result of two-way variance analysis (ANOVA) of salinity (S) and treatment group (T) for the strain indexes selected

Dependent variable	Independent variable	
	S	T
RGR (day^{-1})	41.94***	22.55***
Leaf area (cm^2 $piece^{-1}$)	44.80***	25.06***
Electrolyte leakage rate (%)	13.64***	32.65***
Proline content (μmol g^{-1} dry wt.)	9.593**	4.495*
Citric acid content (mmol g^{-1} dry wt.)	31.56***	16.81***
Na^+ content (mmol g^{-1} dry wt.)	43.26***	20.22***
K^+ content (mmol g^{-1} dry wt.)	36.84***	46.79***

Numbers represent F at 5% level.
* $P < 0.05$.
** $P < 0.001$.
*** $P < 0.0001$.

the complex natural salt–alkaline conditions and can be useful in the development of an applicable method to approach the study of complex salt–alkaline stress conditions in nature.

3.2. Strains in sunflower seedlings under various salt and alkali mixed stresses

Among the many physiological indices of plant responses to salt or alkaline stress, seven were selected in order to analyze the mechanism of salt–alkali mixed stress. High salinity coupled with high pH caused all the stressed plants in treatment groups F4 and F5 to die. Therefore, the strain index data were obtained from the survivor plants in the remaining 28 treatments.

A two-way variance analysis (ANOVA) of salinity and treatment group for the data of strain indices selected was performed and the results are shown in Table 2.

3.2.1. Growth of sunflower seedlings under various salt–alkali mixed stresses

RGR and leaf area were selected as growth parameters. RGR is an ideal index for evaluating seedling growth, and leaf area closely relates to photosynthetic production on which growth depends. Both growth parameters of sunflower seedlings decreased with increasing salinity and alkalinity except for treatment group A1 (Fig. 2). RGR and leaf area of A1 are greater than the control, which indicates that the growth of sunflower seedlings was not inhibited but stimulated under low salinity and alkalinity. The growth of sunflower seedlings was inhibited by high alkalinity and this effect tended to be more serious with increasing salinity (Fig. 2), for example, the RGR of D1 (salinity = 50 mmol, pH 9.41) was 83% relative to the control, but the RGR of D5 (salinity = 250 mmol, pH 9.88) was −8.03%. On the other hand, the inhibition effects on growth by salinity were increased with increasing alkalinity (Fig. 2), for example, at the same salinity, the RGR of A5 (pH 7.25) was 51.8% relative to the control, whereas E5 (pH 10.46) was −12.2%. The results of a two-way analysis of variance (ANOVA) showed that the effects of salinity and treatment group on RGR and leaf area were significant (Table 2).

3.2.2. Electrolyte leakage rate in the leaves of sunflower under various salt–alkali mixed stresses

Leaf electrolyte leakage rate is a good strain index as it reflects the degree of plant injury by salt or alkali stresses. Generally, plasma membranes are injured more seriously with intensifying stress, leading to an increase in the electrolyte leakage rate. As it happens with growth parameters, electrolyte leakage rate increased with rising salinity and alkalinity (Fig. 3). These results indicated that increasing salinity may have caused more serious injury on membranes, and furthermore, given the same salinity values, the injury is more serious with increasing alkalinity. The results of a two-way ANOVA showed that the effects of salinity and alkalinity on electrolyte leakage rate were significant ($P < 0.0001$), and the effect of treatment group (alkalinity) was greater than that of salinity (Table 2).

3.2.3. Solute contents in sunflower seedlings under various salt and alkali mixed stresses

In general, accumulation of inorganic and organic solutes in the cell is one of the main physiological responses of plant to salt or salt–alkaline stress. The accumulated solutes function as osmolytes, pH adjustment agents and other roles. Most plants stressed by NaCl absorb Na^+ abundantly, whereas their K^+ contents decrease. The contents of four important solutes, proline, citric acid, Na^+, and K^+, clearly changed in the response of sunflower seedlings to salt–alkaline stress. Therefore, these four solutes were selected for investigation.

The relationship between the contents of the four solutes in the shoots of sunflower and versus salinity and

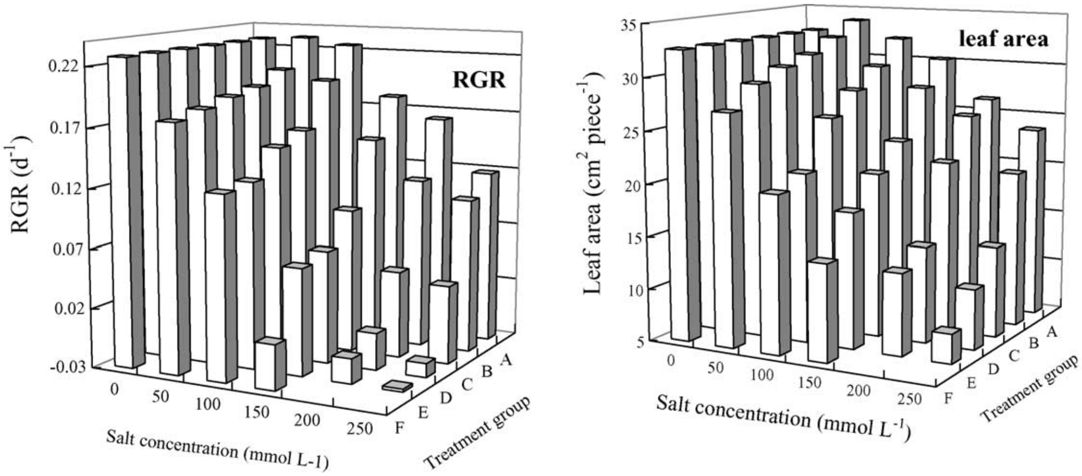

Fig. 2. Effects of various salt and alkali mixed stresses on the RGR and leaf area of sunflower seedlings. Four-week-old seedlings were stressed with mixed salts for 7 days. The values are means of three replicates. (A) $NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 1:1:0:0, pH 7.12–7.25; (B) $NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 1:2:1:0, pH 7.91–8.20; (C) $NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 1:9:9:1, pH 8.47–8.83; (D) $NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 1:1:1:1, pH 9.41–9.88; (E) $NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 9:1:1:9, pH 10.18–10.46; (F) $NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 1:1:9:9, pH 10.47–10.72.

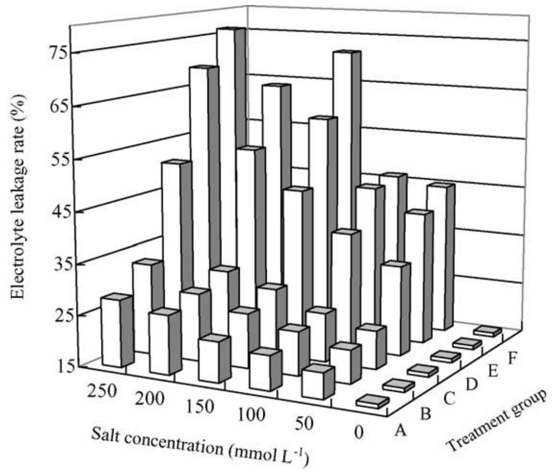

Fig. 3. Effects of various mixed salt and alkali stresses on electrolyte leakage rate in the leaves of sunflower. Four-week-old seedlings were stressed with mixed salts for 7 days. The values are means of three replicates. (A) $NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 1:1:0:0, pH 7.12–7.25; (B) $NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 1:2:1:0, pH 7.91–8.20; (C) $NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 1:9:9:1, pH 8.47–8.83); (D) $NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 1:1:1:1, pH 9.41–9.88; (E) $NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 9:1:1:9, pH 10.18–10.46; (F) $NaCl:Na_2SO_4:NaHCO_3:Na_2CO_3$ = 1:1:9:9, pH 10.47–10.72.

alkalinity was shown in Fig. 4. The results of two-way ANOVA (Table 2) showed that the effects of salinity and alkalinity on the contents of the four solutes were all significant, with P values below 0.0001, except for proline content.

Proline contents increased with rising salinity; the degree of increase also tended to be higher with upsurges in salinity. Similarly, the extent of proline accumulation also increased with increasing alkalinity. Furthermore, the proline content increased most steeply when both salinity and alkalinity were high (salinity $\geq$150 mmol, pH > 8.8). These results demonstrate that alkali stress also can cause heavy accumulation of proline and that the physiological functions of the proline accumulated in sunflower under salt and alkali mixed stress may not be just behave as an osmolyte and protectant but may also have other roles related to alkali stress.

The citrate contents of all groups increased when salinity was augmented; concomitantly, the extent of citrate content increase tended to be higher with increasing alkalinity. As it occurs with proline, citrate content increased sharply when both salinity and alkalinity were high.

The Na^+ contents of the all treatment groups increased with incrementing salinity, this basically was in agreement with the results from previous salt stress experiments (Shi and Yin, 1992, 1993) (Fig. 4). The degree of Na^+ increase tended to be higher with increasing alkalinity. Conversely, the K^+ content of the six groups decreased with increasing salinity, and the extent of decrease diminished with increasing alkalinity (Fig. 4).

3.3. Analysis of the acting factors of salt–alkali mixed stresses

3.3.1. Data of stress factors for various mixed salt–alkali stress treatments

All plants in the F4 and F5 groups died after stress treatment surely because the stress strengths were over their tolerability. The data of stress factors for the other 28 treatments can be seen in Table 3.

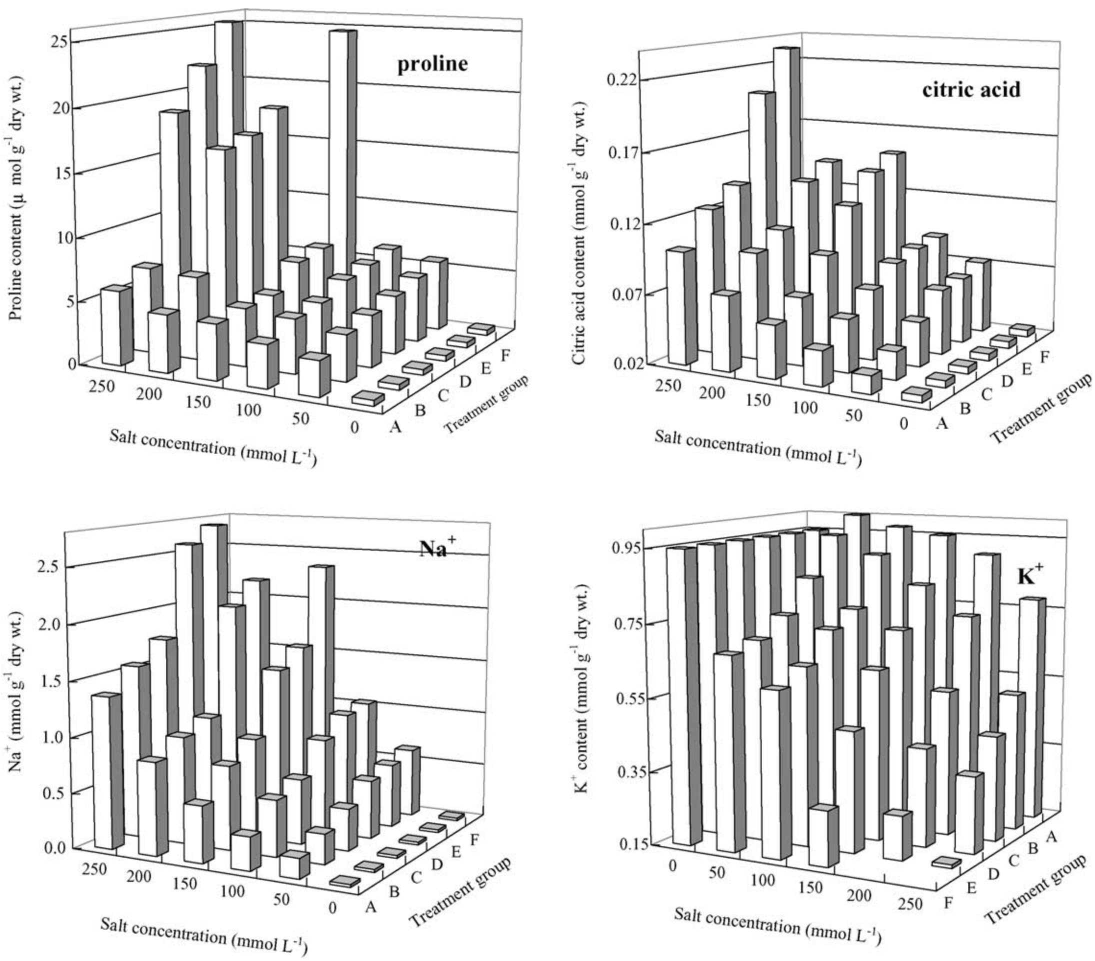

Fig. 4. Effects of various salt and alkali mixed stresses on the contents of proline, citric acid, Na^+, K^+ in the shoots of sunflower. Four-week-old seedlings were stressed with mixed salts for 7 days. The values are means of three replicates. (A) NaCl:Na_2SO_4:$NaHCO_3$:Na_2CO_3 = 1:1:0:0, pH 7.12–7.25; (B) NaCl:Na_2SO_4:$NaHCO_3$:Na_2CO_3 = 1:2:1:0, pH 7.91–8.20; (C) NaCl:Na_2SO_4:$NaHCO_3$:Na_2CO_3 = 1:9:9:1, pH 8.47–8.83; (D) NaCl:Na_2SO_4:$NaHCO_3$:Na_2CO_3 = 1:1:1:1, pH 9.41–9.88; (E) NaCl:Na_2SO_4:$NaHCO_3$:Na_2CO_3 = 9:1:1:9, pH 10.18–10.46; (F) NaCl:Na_2SO_4:$NaHCO_3$:Na_2CO_3 = 1:1:9:9, pH 10.47–10.72.

Table 3
Stress factors for various treatments

Treatment	Salinity ($mmol\,L^{-1}$)	Buffer capacity ($[H^+]$, mmol)	pH	$[Cl^-]$ ($mmol\,L^{-1}$)	$[SO_4^{2-}]$ ($mmol\,L^{-1}$)	$[CO_3^{2-}]$ ($mmol\,L^{-1}$)	$[HCO_3^-]$ ($mmol\,L^{-1}$)
A1	50	0.01	7.12	25	25	0	0
B1	50	8	7.91	12.5	25	0	12.5
C1	50	19.8	8.47	2.5	22.5	2.5	22.5
D1	50	28.9	9.41	12.5	12.5	12.5	12.5
E1	50	43.9	10.2	22.5	2.5	22.5	2.5
F1	50	50.2	10.5	2.5	2.5	22.5	22.5
A2	100	0.02	7.14	50	50	0	0
B2	100	17.2	8.01	25	50	0	25
C2	100	38.7	8.62	5	45	5	45
D2	100	57.8	9.49	25	25	25	25
E2	100	82.2	10.3	45	5	45	5
F2	100	106.4	10.5	5	5	45	45
A3	150	0.03	7.18	75	75	0	0
B3	150	26.8	8.09	37.5	75	0	37.5
C3	150	58.1	8.71	7.5	67.5	7.5	67.5
D3	150	86.8	9.62	37.5	37.5	37.5	37.5
E3	150	123.4	10.4	67.5	7.5	67.5	7.5
F3	150	159.6	10.6	7.5	7.5	67.5	67.5
A4	200	0.04	7.21	100	100	0	0
B4	200	33.5	8.16	50	100	0	50
C4	200	76	8.8	10	90	10	90
D4	200	113.1	9.75	50	50	50	50
E4	200	167	10.5	90	10	90	10
A5	250	0.05	7.25	125	125	0	0
B5	250	38.6	8.2	62.5	125	0	62.5
C5	250	97.3	8.83	12.5	112.5	12.5	112.5
D5	250	151	9.88	62.5	62.5	62.5	62.5
E5	250	196.5	10.5	112.5	12.5	112.5	12.5

Buffer capacity and pH data were measured experimentally and the mean of three replicates is reported; other values were calculated.

3.3.2. Correlativity between stress factors and strain indices

Correlation coefficients between stress factors and strain indices were calculated in order to study their relationship and the results are shown in Table 4. All the correlation coefficients between five stress factors (pH, buffer capacity, salinity, $[Na^+]$, and $[CO_3^{2-}]$), and seven strain indices were statistically significant. Buffer capacity showed the highest total correlation strength among stress factors followed by $[CO_3^{2-}]$, salinity, and $[Na^+]$. For the seven strain indices, their factor with the highest correlation was buffer capacity. Thus, alkaline salts have a profound effect on strain.

The correlativity between $[SO_4^{2-}]$ and the various strain indices was the lowest among all the stress factors and none of its correlation coefficients reached a level of significance. Therefore, the effect of $[SO_4^{2-}]$ on strain indices could be overlooked. Two of the seven correlation coefficients between $[Cl^-]$ and the seven strain indices were statistically significant and therefore the impact of $[Cl^-]$ should not be neglected.

For the correlativity among various stress factors not listed in Table 4, there was a full positive correlativity between salinity and $[Na^+]$ ($r = 1$), and the correlation coefficients of salinity and $[Na^+]$ with the seven strain indices were the same. Therefore, salinity could effectively represent $[Na^+]$. Buffer capacity was dependent on $[CO_3^{2-}]$ and $[HCO_3^-]$. If the expression $2[CO_3^{2-}] + [HCO_3^-]$ is used to represent the total strength of alkaline salt, the correlation coefficient between it and buffer capacity is estimated as 0.9943. Thus, $[CO_3^{2-}]$ and $[HCO_3^-]$ could be fully represented by the buffer capacity.

Table 4
Correlation coefficients between stress factors and strain indices

Stress factor	Strain index							
	RGR	Leaf area	Citrate content	Na^+ content	K^+ content	Proline content	Electrolyte leakage rate	Mean absolute value
pH	−0.5699**	−0.6332**	0.5733**	0.5607**	−0.7666**	0.5181**	0.8219**	0.6348
Salinity	−0.7481**	−0.7265**	0.7398**	0.7507**	−0.5564**	0.6110**	0.4169*	0.6499
Buffer capacity	−0.8869**	−0.9133**	0.8825**	0.8817**	−0.9229**	0.8530**	0.9543**	0.8992
$[Na^+]$	−0.7481**	−0.7265**	0.7398**	0.7507**	−0.5564**	0.6110**	0.4169*	0.6499
$[Cl^-]$	−0.3166 (NS)	−0.3403 (NS)	0.4284*	0.4416*	−0.1801 (NS)	0.2112 (NS)	0.2353 (NS)	0.3076
$[SO_4^{2-}]$	−0.0745 (NS)	−0.0171 (NS)	0.0276 (NS)	0.0387 (NS)	−0.1467 (NS)	−0.0321 (NS)	−0.3633 (NS)	0.1000
$[HCO_3^-]$	−0.5138**	−0.4607*	0.3734 (NS)	0.3709 (NS)	−0.4470*	0.4749*	0.2176 (NS)	0.4083
$[CO_3^{2-}]$	−0.7551**	−0.8034**	0.8051**	0.8036**	−0.8181**	0.7347**	0.9345**	0.8078

NS = not significant. $r_{0.05} = 0.374$, $r_{0.01} = 0.487$, $n = 28$.
* Correlation significant at 0.05 level of probability.
** Correlation significant at 0.01 level of probability.

3.3.3. *Multivariate regression analysis between main stress factors and various strain indices*

According to above analysis data, $[SO_4^{2-}]$ could be neglected, osmosis and $[Na^+]$ could be represented by salinity, and $[HCO_3^-]$ and $[CO_3^{2-}]$ could be represented by buffer capacity. Thus, the four main stress factors, buffer capacity, salinity, pH, and $[Cl^-]$, might be enough to represent all the stress factors involved.

These four main stress factors were taken as independent variables where x_1 = buffer capacity, x_2 = salinity, x_3 = pH, and $x_4 = [Cl^-]$; and the strain indices were taken as dependent variables, with Y = RGR and so on. Multivariance regression analysis was performed for each strain index using the formula $Y = a + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4$. The importance of each stress factor was compared according to their standardized regression coefficients (b') and the significance of regression was estimated by the square of total correlation coefficient (R^2).

The results of regression are shown in Table 5. It showed that the R^2 values were larger than 0.9, except for proline (0.8437), indicating a high linear correlativity between each one of the strain indices and the four stress factors. The importance of the four factors was clearly shown by comparing the values of b' (Table 5). Among the absolute values of the four b', those of buffer capacity (b'_1) were the highest for all of the strain indices, except leaf area, seemingly indicating that buffer capacity was a dominant factor for all strain indexes. Furthermore, the absolute values of b'_2 were greater than those of b'_3 and b'_4, except for electrolyte leakage rate and proline; therefore, salinity was another dominant factor besides buffer capacity. Moreover, the significances of pH and $[Cl^-]$ were determined by analyzing b'_3 and b'_4. pH was an important factor for electrolyte leakage rate, proline, and K^+ content, but was less important and could be neglected for other strain indices. The factor $[Cl^-]$ was negligible for all stress indexes. In sum, buffer capacity and salinity were both dominant factors, pH was less important, and $[Cl^-]$ was the least important one.

4. Discussion

4.1. *Simulation of mixed salt–alkali conditions*

Four salts (NaCl, $NaHCO_3$, Na_2SO_4, and Na_2CO_3) were mixed at various proportions to simulate com-

Table 5
Results of multiple regression between each strain index and four factors

Y	Regression parameters									
	b_1	b_2	b_3	b_4	a	b'_1	b'_2	b'_3	b'_4	R^2
RGR	−0.37800	−0.27054	−1.36200	0.12075	121.17	−0.62562	−0.55823	−0.04809	0.12075	0.9609
Leaf area	−0.06551	−0.05525	−1.31436	0.00859	46.895	−0.51033	−0.53658	−0.21844	0.00023	0.9802
Electrolyte leakage rate	0.20189	0.00477	4.11760	0.08235	−14.636	0.67954	0.02002;	0.29567	0.17087	0.9401
Proline content	0.13763	0.02234	−2.42375	−0.03315	20.021	1.12403	0.22745	−0.42231	−0.16690	0.8437
Citrate content	5.08×10^{-4}	2.86×10^{-4}	3.93×10^{-5}	1.12×10^{-4}	− 0.0147	0.60422	0.42831	0.10071	0.08294	0.9432
Na^+ content	0.00818	0.00431	0.03675	0.00188	− 0.4208	0.63816	0.41934	0.06115	0.09058	0.9518
K^+ content	−0.00181	−0.00142	−0.04584	0.00052	1.6453	−0.44576	−0.43598	−0.39846	0.07955	0.9616

Note: $Y = a + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4$, where x_1 = buffer capacity, x_2 = salinity, x_3 = pH, x_4 = [Cl^-], b'_1–b'_4 = standardized regression coefficients corresponding x_1–x_4 and R^2 = the square of total correlation coefficient.

plex salt–alkaline conditions. As a result, 30 kinds of salt–alkaline conditions with different salinity and pH values were established. The results showed that the simulated 30 treatment conditions evenly covered various salt–alkaline conditions in a range of total salt concentration from 50 to 250 mM and pH from 7.12 to 10.72. The stressing conditions and interference factors are very complex and unrestrainable in natural salt–alkaline soils, and this greatly limits investigations on complex salt–alkaline stress. However, through the methods used in this work we successfully reproduced the complex salt–alkaline conditions under artificial conditions and made the research of complex salt–alkaline stress possible. These salt–alkaline conditions created by mixing four salts were different from the previous works of salt stress (Cheeseman, 1988) and alkali stress (Shi and Yin, 1992, 1993) which involved both salinity and alkalinity and produced a new salt–alkali mixed stress. By utilizing this new method, research on plant salt stress could be further expanded approached in a closer way to natural soil conditions.

4.2. *Responses of sunflower seedlings to salt–alkali mixed stress*

Baikuiza 4, the sunflower cultivar used in this work, is relatively tolerant to salt; as a consequence, the growth parameters of plants treated with low salt concentration solutions were better than the control (A1 in Fig. 2). However, both RGR and leaf area decreased with increasing salinity and alkalinity under all other stress conditions (Fig. 2), especially at high salinity and high alkalinity, treatments in which growth was inhibited so intensely that all the plants died (F4 and F5). It is generally considered that salt stress inhibits plant growth by water deficiency and ion toxicity among other factors (de Lacerda et al., 2003; Marcum, 1999; Ghoulam et al., 2002; Soussi et al., 1998), but in salt-tolerant species, plant growth is only moderately inhibited, or even stimulated, by salt stress (Cramer et al., 1986; Marcum, 1999). The results of the plant set A1 confirmed that the sunflower variety used was a relatively salt-tolerant one.

Changes in RGR are a consequence of salt stress effects in intact plants. It can be seen in Fig. 2 that the growth inhibition effect of alkaline salt stress was stronger than that of neutral salt stress at the same salt concentration (cf. A3 and F3 in Fig. 2). This was in agreement with the results of wheat growing in calcareous soil (Nuttall et al., 2003), onion (Sharma et al., 2001), eucalyptus (James et al., 2002), pea (El and Shaddad, 1996), among others. Therefore, our results indicated that high pH and ion imbalance around rhizosphere caused by alkaline salt (Campbell and Nishio, 2000; Shi et al., 1998) were also main factors in inhibiting plant growth, and this might be related to the effects of high pH around the roots on the transport of ABA (Degenhardt et al., 2000). However, the effects of high pH were closely related to salt concentration, i.e. the effects of high pH were clearly enhanced with increasing salinity (cf. D1–D5 in Fig. 2). Therefore, the peculiar—and evident—feature of a salt–alkali mixed stress is their reciprocal enhancement of its individual components (salinity and alkalinity).

The permeability of the plasma membrane is an evident index that reflects the degree of stress-induced injury to plants (Hong and Lin, 1996; Surjus and Durand, 1996). Electrolyte leakage rate data (Fig. 3) showed that the permeability of the cell membrane of sunflower seedlings was not only increased with rising salinity but also with rising alkalinity. Thus, from the stress-caused injury on plasma membrane, it can be seen that a reciprocal enhancement between salt stress and alkali stress was an evident feature of the salt–alkaline mixed stress.

The primary physiological response of plants to salt or osmosis stress is to undergo osmotic adjustments by two processes: accumulation of ions in the vacuole and synthesis of compatible solutes in the cytosol. The changes of solutes in the shoots of sunflower seedlings under salt–alkali mixed stress (Fig. 4) corresponded to plant responses to salt stress, namely, increases of Na^+, proline, and citric acid contents and a decrease of K^+ content with increasing stress. Nevertheless, salt–alkaline mixed stress was distinct from salt stress in some particular aspects:

First, proline accumulated under salt or drought stress is usually considered as an organic-compatible osmolyte and a protecting agent for the activity of intracellular macromolecule (Tang, 1989); proline accumulation is closely related to osmotic effects. In this experiment, it is evident that proline content increased not only with rising salinity, but also when alkalinity increased at the same salt concentration (Fig. 4). This suggests that the induction of proline synthesis is related not only to changes in water potential and [Na^+], but also to high pH. Our results indicate that the physiological function of accumulated proline, in addition to being and osmolyte and protecting agent, could be an involvement in injury due to alkaline stress, an aspect that ought to be investigated further.

Second, it has been found recently that citric acid accumulation is related to aluminum toxicity (Li et al., 2000), phosphorus deficiency (Neumann et al., 1999), and several others adverse conditions. However, the accumulation of citric acid under salt stress depends on the chemical properties of the stress-inducing salt. Citric acid accumulation is moderate or absent under neutral salt stress (Francoise et al., 1991; Shi et al., 2002), but it is heavy when the stress is due to alkaline salts (Shi et al., 2002). The results of our experiments (Fig. 4) proved again that alkali stress clearly affects on organic acid metabolism in plants. The content of citric acid in the shoots of stressed sunflower seedlings increased with increasing salinity and alkalinity (Fig. 4). Citric acid is a small organic molecule that certainly may function as an osmolyte. However, previous work has also shown that the distribution of citric acid accumulated under alkaline stress in plants was very different from that of proline, i.e. while of proline accumulation occurred mainly in the younger tissue of alkali stressed plants, citric acid accumulated mainly in older tissue and was associated with the distribution of Na^+ (Shi et al., 2002). This suggested that the function of the accumulated citric acid was different from proline and mainly related to pH adjustment in the cell. This is still unclear and further investigation is required.

Third, the metabolism of Na^+ and K^+ is an important component of salt stress (Cheeseman, 1988). Usually, Na^+ increases and K^+ decreases in plants stressed by salt (de Lacerda et al., 2003). The results of salt–alkaline mixed stress in this experiment, though, showed that [Na^+] increased and [K^+] decreased not only with increasing salinity but also with rising alkalinity (cf. Fig. 4), a phenomenon perhaps related to plasma membrane being destroyed more severely by alkaline stress. These changes in [Na^+] and [K^+] are a reflection of a reciprocal enhancement between salt stress and alkali stress, which was the peculiar feature of salt–alkali mixed stress. Moreover, [Na^+] and [K^+] changes also reflected the effects of salt–alkali mixed stress on the metabolism of Na^+ and K^+ and indicate that the physiological responses of plant to salt–alkali mixed stress were more complex than that of salt stress alone. This is an aspect that should be researched more deeply.

4.3. Salt stress and alkali stress synergism

According to our results, we concluded that the mixed salt–alkaline stress not only caused stress due to both salt and alkali, but, additionally, it displayed an interaction between salt stress and alkali stress. If total salt concentration (salinity) is taken as a measure of the strength of the salt stress and pH (from group A to group F) is taken as the strength of the alkali stress, a synergism between salinity and pH can be found (Figs. 2–4). The stress to sunflower seedlings due to salinity as a singular factor (cf. groups A1–A5) or high pH as a singular factor (groups E1 and F1 approached the cases) was smaller than that of salinity coupled with high pH (cf. D5 and E5).

Usually, neutral salt stress or salt stress in general involves osmotic effects, which depend on salt concentration, and specific ion effects (Cramer et al., 1986; Cheeseman, 1988). On the other hand, alkaline salt stress or alkali stress alone, in addition to osmotic and specific ion effects, also includes high-pH effects (Shi and Yin, 1993). The mechanism of salinity tolerance in plants mainly involves two processes: one, osmosis adjustment, includes ion accumulation and synthesis of compatible solutes, among other phenomena; the other, ion toxic avoidance, includes specific ion metabolism and compartmentation of toxic ions (Cheeseman, 1988), etc. Although the mechanism of tolerance to alkalinity in plants is still unclear, a pH adjustment process is possibly involved (Shi and Yin, 1993), additionally to the above two processes. The osmosis effects and ion toxic effects depend on stress salt concentration, while pH effects depend on buffer capacity, which in turn is closely related to both the alkalinity and concentration of the stress salt. In other words, the higher the alkalinity and the concentration the greater the buffer capacity, and it is more difficult for plants to adjust. Perhaps this is the cause of the synergism between salt stress and alkaline stress. Nevertheless, the phenomenon of salinity enhancing the harm of high pH is complex and is related to the mechanism of alkaline stress resistance in sunflower, and deserves further investigation.

Our results proved once more the relaxing action of neutralization to the alkali stress (Shi, 1995; Yan et al., 2000): lowering the soil's salinity or pH based on its salt components can reduce salt–alkaline soil's harm to the plant. Especially for barren land with high pH, the recovery of vegetation is possible to be achieved by decreasing its pH.

4.4. Salinity and buffer capacity were the dominant factors of mixed salt and alkali stress

4.4.1. Action factors of mixed salt and alkali stress

One of the factors that causes the complexity of natural salt and alkali mixed environmental conditions is that the components, proportions, and contents of contained salts are so varied. Different salts have different chemical properties and their actions on plant are also different. The effective actions of mixed salts, especially neutral salts mixed with alkaline salts, are much more complex than those of a singular neutral salt (Liu and Zhu, 1997) or a singular alkaline salt (El and Shaddad, 1996; Shi and Yin, 1993; Shi et al., 2002). In general, the stress factors of the neutral salt NaCl are mainly the ion effects of Na^+ as a dominant injuring ion and the osmotic effects of low water potential caused by high salt concentration (Cheeseman, 1988; Cramer et al., 1986), but for the alkaline salt Na_2CO_3 there is an added stress factor, namely high pH (Shi and Yin, 1993; Shi et al., 1998, 2002). However, when various neutral salt and alkalic salt are mixed together, the effects of the mixed salts are more than just a simple combination of the separate effects from the two kinds of salts due to the interactions between different ions and so on. Thus, the effects of salt and alkali mixed stress should be experimentally analyzed in depth.

The novel concept of buffer capacity is key in simplifying stress factor analysis of the 30 simulated salt–alkaline conditions. Buffer capacity could represent the concentration and proportion not only of the two alkaline salts but also of the carbonate and hydrocarbonate anions. According to above correlation analysis data, $[SO_4{}^{2-}]$ could be neglected, osmosis and $[Na^+]$ could be represented by salinity, and $[HCO_3{}^-]$ and $[CO_3{}^{2-}]$ could be represented by buffer capacity. Thus, the four factors, buffer capacity, salinity, pH and $[Cl^-]$, could basically represent all the effectors of mixed salt and alkali stress (Table 4). The results of statistical analysis (Tables 4 and 5) proved convincingly that the introduction of the concept of buffer capacity is correct and significant in bringing to light the mechanism of alkali stress on plant.

4.4.2. Relationship between various stress factor and strain index

The magnitudes of different stress factors on several strain indices were found to be different because of the varying accruing mechanisms in plants. From the regression analysis results of seven strain indices, shown in Table 5, it was evident that both buffer capacity and salinity were dominant and indispensable factors. The significance of buffer capacity was much greater than salinity for six of the seven indices, and only slightly smaller for one index (leaf area). Thus, buffer capacity was a very important strength index of mixed salt–alkaline stress, whereas $[Cl^-]$ and pH were less significant and even negligible in some cases. The results of regression also showed that the degrees of impact of different stress factors on several strain indices were not equal and that the difference might be related to both the physiological mechanisms of the plant's responses to stress and the physiological processes associated with strain development.

4.4.3. Ideal strength index of mixed salt–alkaline stress

It was very important to determine proper strength indices in researching stress. Salt concentration, $[Na^+]$, 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 alkaline stress strength (Shi et al., 1998); but for mixed salt–alkaline stress, none of these indices could completely reflect the stress strength. According to the results in Table 5, it was reasonable to consider salinity plus buffer capacity as the strength index of mixed salt–alkaline stress. After comparing the results of individually taking salinity, pH, or buffer capacity as the strength of stress (Shi et al., 1998), it was evident that using salinity plus buffer capacity as the strength index of mixed salt–alkaline stress was a more reasonable approach.

It is very difficult to objectively estimate the potential degree of injury to plant for a salt–alkalinized soil, especially for soils with high pH values. According to this conclusion, studies using synthetic conditions combining salinity and buffer capacity of soil should constitute a new model to solve this problem.

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