Photosynthesis, chlorophyll fluorescence, inorganic ion and organic acid accumulations of sunflower in responses to salt and salt-alkaline mixed stress

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Abstract

Sunflowers were treated with mixing proportions of NaCl, Na_2SO_4, $NaHCO_3$, and Na_2CO_3. Effects of salt and salt-alkaline mixed stress on growth, photosynthesis, chlorophyll fluorescence, and contents of inorganic ions and organic acids of sunflower were compared. The growth of sunflower decreased with increasing salinity. The contents of photosynthetic pigments did not decrease under salt stress, but their contents decreased sharply under salt-alkaline mixed stress. Net photosynthetic rates, stomatal conductance and intercellular CO_2 concentration decreased obviously, with greater reductions under salt-alkaline mixed stress than under salt one. Fluorescence parameters showed no significant differences under salt stress. However, maximal efficiency of PSII photochemistry, photochemical quenching coefficient, electron transport rate, and actual PSII efficiency significantly decreased but non-photochemical quenching increased substantially under salt-alkaline mixed stress. Under salt-alkaline mixed stress, sunflower leaves maintained a low Na^+- and high K^+ status; this may be an important feature of sunflower tolerance to salinity. Analysis of the mechanism of ion balance showed that K^+ but not Na^+ was the main inorganic cation in sunflower leaves. Our results indicated that the change in organic acid content was opposite to the change of Cl^-, and the contribution of organic acid to total charge in sunflower leaves under both stresses decreased with increasing salinity. This may be a special adaptive response to stresses for sunflower. Sunflower under stress conditions mainly accumulated inorganic ions instead of synthesizing organic compounds to decrease cell water potential in order to save energy consumption.

Additional keywords: salt stress; salt-alkaline mixed stress; chlorophyll fluorescence; photosynthesis; inorganic ions; organic acids.

Introduction

Soil salinity and alkalinity seriously afflict about 932 million ha of land globally, reducing productivity in about 100 million ha in Asia (Rao *et al.* 2008). The existence of alkali stress has been demonstrated clearly in a number of reports, which have shown it is more severe than salt stress (Campbell and Nishio 2000, Hartung *et al.* 2002, Shi and Sheng 2005, Shi and Wang 2005, Gao *et al.* 2008,Yang *et al.* 2007, 2008a,b,c). In previous studies, we defined salt stress as the stress of neutral salts (NaCl and Na_2SO_4); and alkali stress as the stress of alkaline salts ($NaHCO_3$ and Na_2CO_3) (Shi and Wang 2005, Shi and Sheng 2005). Salt stress generally involves osmotic and ionic stresses (Munns 2002). Comparison of alkali- with salt stress reveals an added high-pH effect of alkali stress. The absorptive function of the root is not only directly affected under alkali stress, but the high pH can also cause the solubility of some mineral elements to decline and even to precipitate, or make the ionic activity and free concentrations decrease (Shi and Zhao 1997, Li *et al.* 2009). High pH can result in loss of the normal physiological functions of the roots and the destruction of root cell structure (Yang *et al.* 2008a,b,c) and finally inhibit absorption of inorganic anions such as Cl^-, NO_3^-, and $H_2PO_4^-$, greatly affect the selective absorption of K^+–Na^+, and break the ionic balance and pH homeostasis in tissue (Yang *et al.* 2007, 2008b). Thus, plants in alkaline soil must cope with both physiological drought and ion toxicity, and also maintain intracellular ionic balance.

There are numerous reports on photosynthetic characteristics under salt stress (Qiu *et al*. 2003, Koyro 2006, Wei *et al*. 2006). Generally, photosynthesis is inhibited

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Abbreviations: DM – dry mass; ETR – electron transport rate; F_v/F_m – maximal efficiency of PSII photochemistry; FM – fresh mass; NPQ – non-photochemical quenching; OAs – organic acids; PSII – photosystem II; q_P – photochemical quenching coefficient; Φ_{PSII} – actual PSII efficiency.

by salt stress (Ma *et al*. 1997, Sultana *et al*. 1999, Qiu *et al*. 2003, Koyro 2006). Salt stress also affects photosynthetic components (Ma *et al*. 1997, Qiu *et al*. 2003) and chloroplast ultrastructure (Fidalgo *et al*. 2004). However, there are few reports on the effects of alkali stress on photosynthesis. Moreover, there has been increasing attention on the effects of salt on chlorophyll (Chl) fluorescence in both salt-sensitive and salt-tolerant species (Nieva *et al.* 1999, Morant-Manceau *et al.* 2004, Naidoo and Kift 2006, Sixto *et al.* 2006). However, to our knowledge, there have been few reports about the effects of alkali stress or salt-alkaline mixed stress on photosynthesis, especially on Chl fluorescence. Since photosystem II (PSII) is believed to play a key role in the response of photosynthesis to environmental perturbations (Baker 1991), the effects of salinity stress on PSII have been investigated extensively. However, the data on the effects of salinity stress on PSII photochemistry are conflicting. Some studies have shown that salt stress inhibits PSII activity (Everard *et al.* 1994, Netondo *et al.* 2004), whereas others have indicated no effect on PSII (Jimenez *et al.* 1997, Morant-Manceau *et al.* 2004).

The organic acids (OAs) have a potential role as metabolically-active solutes for osmotic adjustment, balance of cation excess, and pH homeostasis (López-Bucio *et al.* 2000). In recent years, reports have shown that some alkali-tolerant halophytes accumulate high concentrations of OAs in shoots under alkali stress (Shi *et al.* 2002, Yang *et al.* 2007, 2008b), but neither alkali-sensitive maize (Qu and Zhao 2004) nor the halophyte *Suaeda salsa* with weak alkali-tolerance (Qu and Zhao 2003) accumulated OAs in shoots. These reports clearly showed that the accumulation of OAs might have significant roles in coping with imbalance of charges and high pH stress.

Sunflower is a salt-tolerant crop, and some varieties can be planted in saline areas. In spite of that, little attention was paid to physiological mechanisms of sunflower to salt- and alkali stresses (Shi and Sheng 2005). In this paper, various stress conditions were established firstly, and then seeds were sown in order to be close to natural conditions. The effects of salt or salt-alkaline mixed stress on some physiological traits such as photosynthesis, Chl fluorescence, and contents of inorganic ions and OAs in leaves of sunflower were investigated to elucidate the mechanism by which plants adapt to high pH stress.

Materials and methods

Plant materials: *Helianthus annuus* L. cv. Baikuiza 6 was selected due to its tolerance to salt-alkaline conditions, with seeds provided by the Sunflower Institute, Jilin Province, China.

Design of simulated salt- and alkaline conditions: The study included two treatment groups, namely salt stress (S) and salt-alkaline mixed stress (MSA). Two stress intensities, 50 and 100 mM, were applied for each treatment group. Group S contained only neutral salts and group MSA contained neutral and alkaline salts. In the present study, according to the salt components in the extant salt-alkaline soil of northeast China (Ge and Li 1990), the actual performance of cv. Baikuiza 6 in the field production of salt-alkalinized areas and results of Shi and Sheng (2005), two alkaline salts were mixed in a 9:1 molar ratio ($NaHCO_3$:Na_2CO_3) for alkali stress. Two neutral salts were mixed in a 9:1 molar ratio (NaCl:Na_2SO_4) to ensure the same total ion concentration when total salinity was the same. The concentration of alkaline salts of MSA was 45 mM, with added neutral salts to obtain the total concentrations. The salt compositions of the two treatment groups are shown in Table 1.

Stress treatments: Fifteen 24-cm diameter plastic pots were filled with washed sand and then divided into five sets and three pots per set, with each pot as one replicate; therefore there were three replicates per set. One set was a control, and the others were the stress treatments. Before sowing, the control pots were thoroughly watered with 1 l of nutrient solution per pot, in three portions, and the treated pots were watered with the nutrient solution containing the corresponding salts. Both stresses (S and MSA) shared one control (0 mM). Then the seeds of cv. Baikuiza 6 were sown in pots. After sowing, the pots were watered using the method above at approximately 17:00–18:00 h every 3 d. Each pot finally contained six plants. The experiment was conducted during May–June, with all pots placed outdoors and protected from rainfall.

Table 1. Concentrations [mM] of various salts ($NaHCO_3$, Na_2CO_3, NaCl, and Na_2SO_4) in treatments. Data of pH are means of three replicates.

		$NaHCO_3$	Na_2CO_3	NaCl	Na_2SO_4	Total salinity	pH
Control		0	0	0	0	0	6.76
Treatment groups	S	0	0	45	5	50	6.79
		0	0	90	10	100	6.78
	MSA	40.5	4.5	4.5	0.5	50	8.21
		40.5	4.5	49.5	5.5	100	8.23

Net photosynthetic rate (P_N), stomatal conductance (g_s) and intercellular CO_2 concentration (C_i) of leaves were determined during 8:30–10:30 h on fully-expanded first blades, using a portable open-flow gas exchange system *LI-6400* (*LI-COR Biosciences*, Lincoln, USA) at 30 d after sowing. The respective results were expressed as μmol(CO_2) m^{-2} s^{-1}, mol(H_2O) m^{-2} s^{-1}, and μmol(CO_2) mol^{-1}. The photosynthetically active radiation was 1 mmol m^{-2} s^{-1} (saturation light). The ambient CO_2 concentration was 360 ± 10 μmol mol^{-1}, the air temperature, and humidity were about 24°C and 50%. Measurements were repeated five times for each blade, for five blades per pot, and the averages were recorded. The water use efficiency (WUE) was calculated as the ratio of P_N/E (Nieva *et al.* 1999).

Chl *a*, Chl *b*, and carotenoids (Car) were extracted with acetone and spectrophotometeric determinations were done at 440, 645, and 663 nm for each of the three samples. The calculations used the methods of Zhu (1993), and were expressed in mg g^{-1} fresh mass (FM).

Chl fluorescence was measured using a *LI-6400* system with the *6400-40* Leaf Chamber fluorometer. Seedlings were kept in the darkness for 30 min before recording the fluorescence of blades which is the same ones with photosynthetic indices. The minimal fluorescence level (F_o) was determined by a modulated light, which was sufficiently low (<1 μmol m^{-2} s^{-1}) not to induce any significant variable fluorescence. The maximal fluorescence level (F_m) was determined by a 0.8-s saturation pulse at 4,200 μmol m^{-2} s^{-1} on dark-adapted leaves (30 min). In the light-adapted leaf, the intensity of the saturation pulses to determine the maximal fluorescence (F_m') was 6,000 μmol m^{-2} s^{-1}, for 0.8 s, whereas the 'actinic light' was 200 μmol m^{-2} s^{-1}. Measurements of the quantum yield of electron transfer of PSII (Φ_{PSII}) were obtained by application of a saturation light pulse (6,000 μmol m^{-2} s^{-1} with pulse duration of 0.8 s) under ambient irradiation. The fluorescence parameters were calculated according to Schreiber *et al.* (1986).

Dry mass, inorganic ions, and organic acids (OAs): All plants were harvested in the morning, 31 d after sowing and were first washed with tap water, followed by distilled water. Roots, stems and leaves were separated, and FM determined per pot. Then the samples were oven-dried at 80°C for 15 min, vacuum-dried at 40°C to a constant mass and dry mass (DM) recorded. Dry samples of plant material (100 mg) were treated with 10 ml of deionized water at 100°C for 1 h, and the extract used to determine the contents of free inorganic ions and OAs. The contents of NO_3^-, Cl^-, SO_4^{2-} and oxalic acid were determined by ion chromatography (*DX-300* ion chromatographic system; *AS4A-SC* ion-exchange column, *CD M-II* electrical conductivity detector, mobile phase: $Na_2CO_3/NaHCO_3$ = 1.7/1.8 mM; *Dionex*, Sunnyvale, USA). Other OAs were also determined by ion chromatography (*DX-300* ion chromatographic system; *ICE-AS6* ion-exclusion column, *CDM-II* electrical conductivity detector, *AMMS-ICE II* suppressor, mobile phase: 0.4 mM heptafluorobutyric acid; *Dionex*). A flame photometer (*6420 Flame Photometer, Gaomi Sophisticated Analytical Instrument Factory*, Gaomi, China) was used to determine K^+ and Na^+ contents.

Statistical analysis: All experiments were based on three replicated measurements. Data were analyzed by one-way analysis of variance (*ANOVA*) using the statistical software *SPSS 14.0* (*SPSS*, Chicago, IL, USA). The treatment mean values were compared by post hoc least significant difference (LSD) test. Statistical LSD tests were performed among 0 mM, 50 mM (S), 100 mM (S), 50 mM (MSA), and 100 mM (MSA). The term significant indicates differences for which $P \leq 0.05$.

Results

Growth and photosynthetic pigments: Under both stresses, with increasing salinity the total plant DM decreased and the extent of reductions under salt- and salt-alkaline mixed stress (F = 102.6, P<0.05) were greater than those under salt stress (F = 77.70, P<0.05) (Fig. 1*A*). Under salt stress, the contents of Chl *a* , Chl *b*, and Car did not change significantly with increasing salinity; under salt-alkaline mixed stress, their contents decreased sharply with increasing salinity (F = 24.17, P<0.05; F = 51.72, P<0.05; F = 22.27, P<0.05) (Fig. 1*B*,*C*,*E*, respectively). Chl *a*/*b* only sharply increased at 100 mM under salt-alkaline stress (F = 8.27, P<0.05) but not salt stress (Fig. 1*D*).

P_N, g_s, and C_i decreased obviously, and the extent of reductions under salt-alkaline mixed stress (F = 12499.86, P<0.05; F = 6476.20, P<0.05; F = 297.53, P<0.05) were greater than those under salt stress (F = 344.53, P<0.05; F = 893.25, P<0.05; F = 330.86, P<0.05). However, the trend of WUE was opposite (Fig. 1*F–I*) (salt stress: F = 671.25, P<0.05; salt-alkaline mixed stress: F = 45.77, P<0.05).

Chl fluorescence: F_v/F_m (F = 31.36, P<0.05), q_P (F = 7.97, P<0.05), ETR (F = 10.44, P<0.05), Φ_{PSII} (F = 10.44, P<0.05) significantly decreased but NPQ (F = 41.44, P<0.05) increased substantially under salt-alkaline mixed stress. However, they showed no significant differences under salt stress (Fig. 1*J–N*).

Inorganic cations: The content of K^+ in leaves increased under salt-alkaline mixed stress (F = 12.77, P<0.05), but there was no significant change of K^+ under salt stress (Fig. 2*A*). The Na^+ content in leaves increased sharply

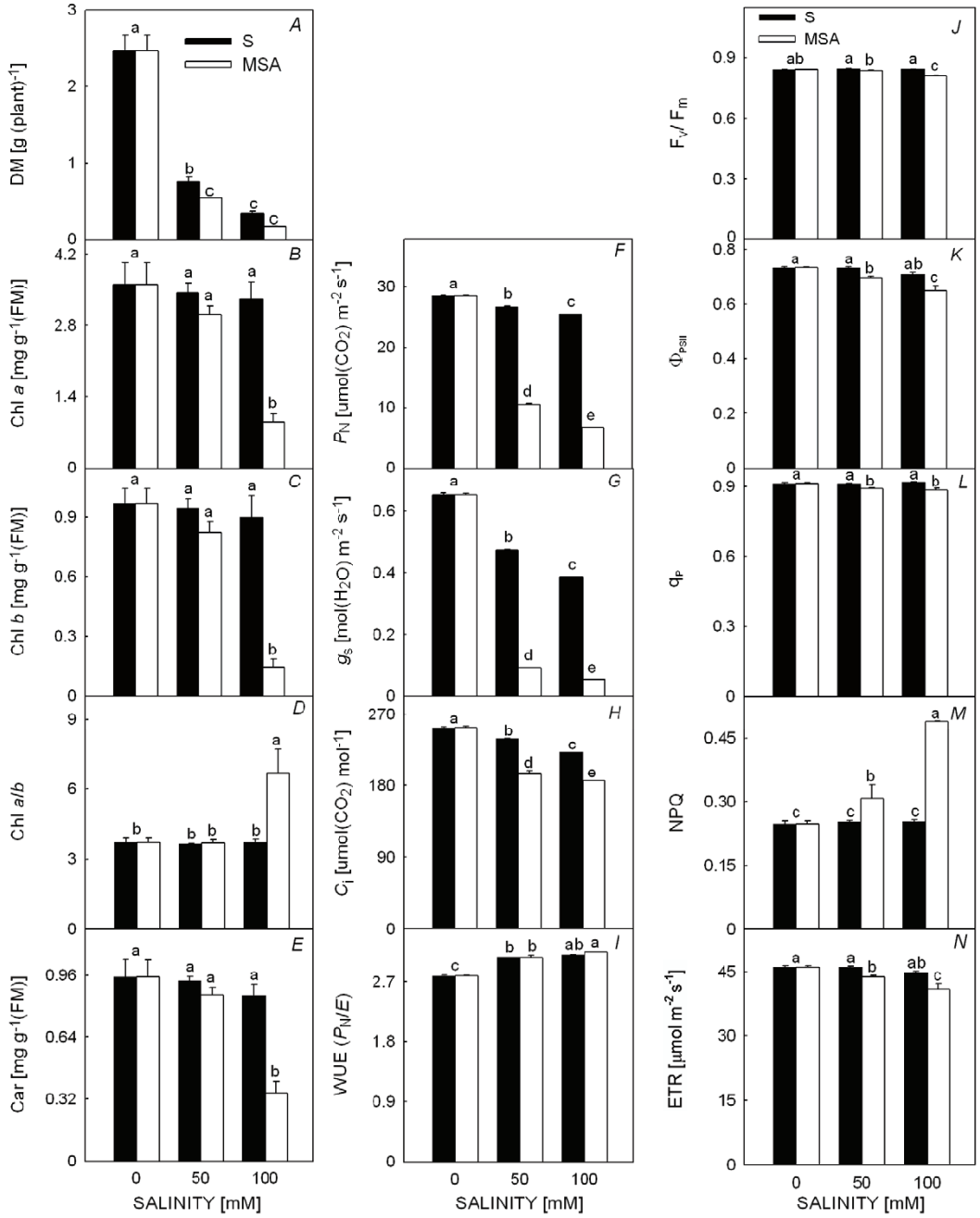

Fig. 1. Effects of salt (S) and salt-alkaline mixed (MSA) stress on *A*: total plant dry mass (DM), *B*: chlorophyll (Chl) *a*, *C*: Chl *b*, *D*: Chl *a*/*b*, *E*: carotenoid (Car), *F*: net photosynthetic rate (P_N), *G*: stomatal conductance (g_s), *H*: internal CO_2 concentration (C_i), *I*: water use efficiency (WUE), *J*: F_v/F_m, *K*: Φ_{PSII}, *L*: q_P, *M*: NPQ, *N*: ETR in sunflower leaves. S: 50 or 100 mM ($NaCl:Na_2SO_4$=9:1); MSA: 45 mM ($NaHCO_3$:Na_2CO_3=9:1) + 5 or 55 mM ($NaCl:Na_2SO_4$=9:1). Means followed by different letters in the same curve are significantly different at $P \leq 0.05$, according to least significant difference (LSD) test.

under salt-alkaline mixed stress (F = 31458.6, P<0.05). Under 50 mM of salt stress the Na^+ content was the same as controls, but Na^+ content increased sharply at 100 mM salinity (F = 4530.6, P<0.05) (Fig. 2*B*). There was no change of Na^+/K^+ at lower salinity, but it increased sharply at higher salinity (Fig. 2*C*) (salt stress: F = 800.96, P<0.05; salt-alkaline mixed stress: F = 379.81, P<0.05).

Inorganic anions: The Cl^- content in leaves increased sharply under salt stress (F = 98.80, P<0.05). The Cl^- content was the same as controls at 50 mM, but increased sharply at 100 mM salinity under salt-alkaline mixed stress (Fig. 2*D*) (F = 270.51, P<0.05). The NO_3^- contents in leaves decreased under both stresses (Fig. 2*E*) (salt stress: F = 826.74, P<0.05; salt-alkaline mixed stress:

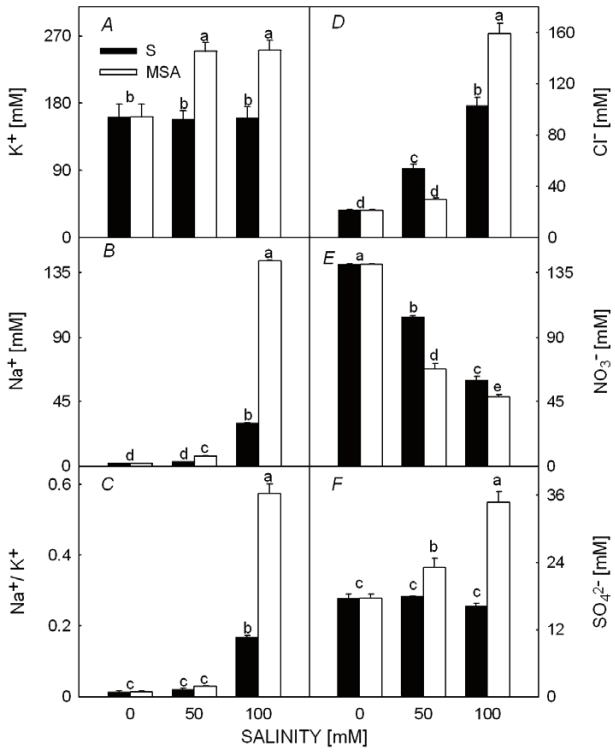

Fig. 2. Effects of salt- and salt-alkaline mixed stresses on K^+ (*A*), Na^+ (*B*), Na^+/K^+ (*C*), Cl^- (*D*), NO_3^- (*E*), SO_4^{2-} (*F*) in sunflower leaves. Legend refers to Fig. 1.

$F = 442.04$, $P<0.05$). The SO_4^{2-} content in leaves increased under salt-alkaline mixed stress ($F = 31.26$, $P<0.05$), but there was no significant change under salt stress (Fig. 2*F*).

Organic acids: Citrate, malate, glycolate, formate, lactate, acetate, succinate and oxalate were detected in sunflower leaves. Stresses simulated OAs to accumulate significantly and under both stresses, the concentrations of OAs were the highest at 50 mM salinity. However, OA concentrations were higher under salt-alkaline mixed stress ($F = 156.79$, $P<0.05$) than salt stress ($F = 43.57$, $P<0.05$) (Fig. 3*I*). Citrate ($F = 1085.9$, $P<0.05$), malate ($F = 66.0$, $P<0.05$) and oxalate ($F = 52.64$, $P<0.05$) clearly accumulated under salt-alkaline mixed stress, but did not accumulate under salt stress and even decreased slightly ($F = 39.32$, $P<0.05$; $F = 5.20$, $P<0.05$; $F = 4.03$, $P>0.05$) (Fig. 3*A,B,H*). Glycolate ($F = 15.15$, $P<0.05$) and acetate ($F = 52.96$, $P<0.05$) clearly decreased under salt-alkaline mixed stress; however, at 50 mM of salt stress ($F = 46.20$, $P<0.05$; $F = 39.54$, $P<0.05$), their concentrations clearly increased (Fig. 3*C,F*). With increasing salinity, formate accumulated significantly under salt stress ($F = 30.56$, $P<0.05$), but under salt-alkaline mixed stress ($F = 61.17$, $P<0.05$), it accumulated only at 50 mM (Fig. 3*D*). Under both stresses, the concentrations of succinate decreased at 100 mM, with the decrease under salt stress ($F = 28.90$, $P<0.05$) greater than under salt-alkaline mixed stress ($F = 2.37$, $P>0.05$) (Fig. 3*G*). The concentration of lactate was higher under salt stress ($F = 39.54$, $P<0.05$) than for controls, but under salt-alkaline mixed stress ($F = 5.12$, $P>0.05$), the lactate concentration was higher than for controls only at 50 mM salinity (Fig. 3*E*).

Discussion

Growth: Salt- and alkali stresses greatly inhibited sunflower growth. The injury from salt-alkaline mixed stress was greater than that from salt stress at the same salinity (Fig. 1*A*), consistently with previous reports (Shi and Sheng 2005, Shi and Wang 2005, Yang *et al.* 2007, 2008a,b). The different extents of injury caused by salt- and salt-alkaline mixed stresses might be due to different mechanisms of action. The deleterious effects of salt stress are commonly thought to result from low water potentials and ion toxicities (Munns 2002). However, alkali stress exerts the same stress factors as salt stress but with the added influence of high-pH stress. Plant survival under alkali stress, therefore, depends not only on its ability to cope with water stress and ion toxicity, but also on its resistance to high pH. Therefore, plants need to expend more material and energy to adapt to alkali stress.

P_N*, *g_s* and *C_i: P_N of a plant usually decreases with increasing stress (Sultana *et al.* 1999, Koyro 2006, Wei *et al.* 2006), consistently with our results that P_N of sunflower was markedly lower under salt-alkaline mixed stress than salt stress (Fig. 1*F*). The clear reduction of P_N under salt-alkaline mixed stress was not only related to both destruction of photosynthetic capacity and decreasing g_s (Fig. 1*F,G*) (Yang *et al*. 2009), but was also closely related to an imbalance of mineral elements in plant cells. The g_s of sunflower in the present study decreased under both stresses and g_s is often closely correlated with the change in environmental water potential (Sultana *et al.* 1999, Koyro 2006, Maricle *et al.* 2007). Under both stresses, environmental water potential decreased with increased stress intensity. However, the reduction of g_s under salt-alkaline mixed stress was greater than under salt stress (Fig. 1*G*). The phenomenon showed that the change of g_s of sunflower might be a response to the decreased environmental water potential. High pH caused by alkalized condition may stimulate sunflower roots to generate the physical or chemical signal to affect stomatal opening and closing and gas exchange. The decrease of g_s might cause the obvious decrease of C_i under stress (Fig. 1*H*). Furthermore, the increased WUE under both stresses might be an adaptive response to the decreased water content (Yang *et al.* 2008c).

Table 2. Percentage of the contribution of various free ions of total amount of ions in sunflower leaves under salt- and salt-alkaline stresses. OA – organic acid; S1 – 50 mM ($NaCl:Na_2SO_4 = 9:1$); S2 – 100 mM ($NaCl:Na_2SO_4 = 9:1$); MSA1 – 45 mM ($NaHCO_3:Na_2CO_3 = 9:1$) + 5 mM ($NaCl:Na_2SO_4 = 9:1$); MSA2 – 45 mM ($NaHCO_3:Na_2CO_3 = 9:1$) + 55 mM ($NaCl:Na_2SO_4 = 9:1$).

	Na^+ [%]	K^+ [%]	Cl^- [%]	SO_4^{2-} [%]	NO_3^- [%]	OA [%]
Control	0.57	40.02	5.30	4.36	34.90	14.85
S1	0.81	38.13	13.00	4.32	25.14	18.61
S2	6.97	37.02	23.83	3.76	13.89	14.52
MSA1	1.49	50.91	6.02	4.71	13.91	22.96
MSA2	19.70	34.54	21.94	4.78	6.66	12.38

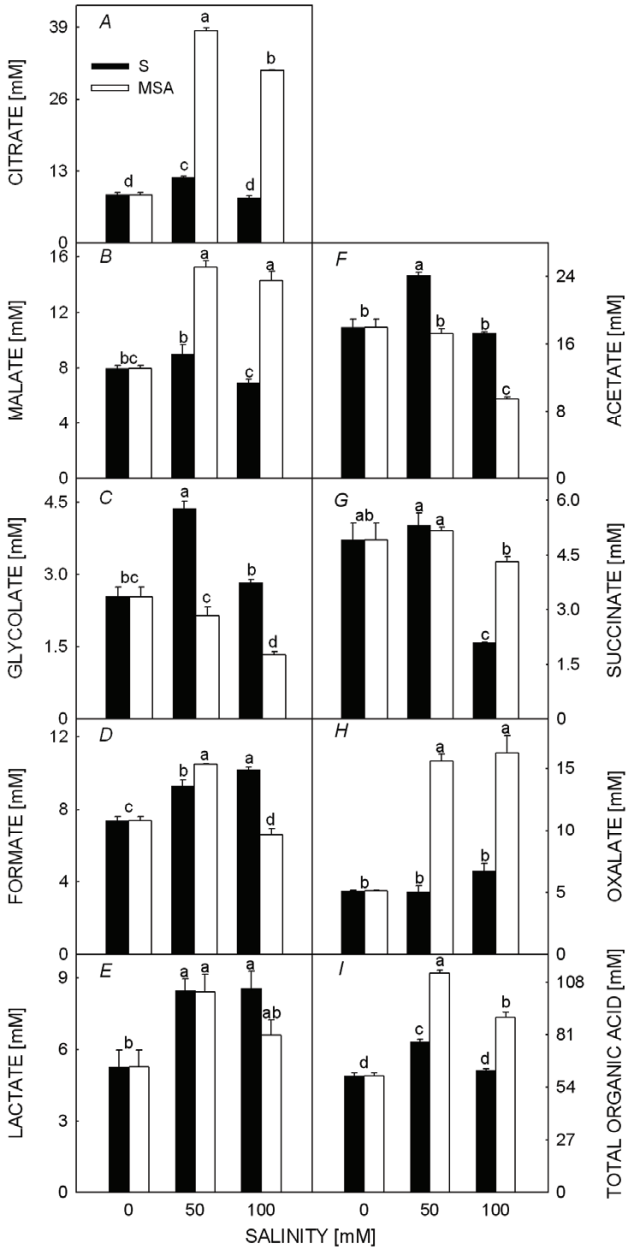

Fig. 3. Effects of salt and salt-alkaline mixed stresses on citrate (*A*), malate (*B*), glycolate (*C*) formate (*D*), lactate (*E*), acetate (*F*), succinate (*G*), oxalate (*H*) and total organic acid (*I*) in sunflower leaves. Legend refers to Fig. 1.

Pigments: The accumulation of Chl and Car were not inhibited obviously under salt stress (Fig. 1*B,C,E*). But under salt-alkaline mixed stress, the contents of Chl and Car decreased sharply. This might be ascribed to high pH and the existence of HCO_3^- and CO_3^{2-} causing the solubility of metal ions to sharply decline and even to precipitate (Li *et al.* 2009); in such a case, plants can not sufficiently absorb metal ions (Mg^{2+} and Fe^{2+}) to synthesize Chl. Alternatively, alkaline salts might increase the activity of the Chl-degrading enzyme chlorophyllase (Reddy and Vora 1986). The decrease in photosynthetic pigments may be one reason for the reduction of photosynthesis under salt-alkaline mixed stress.

Chl fluorescence: Stresses not only directly lead to photosynthetic machinery injury, but also affect photosynthetic electron transport and RuBP carboxylase/oxygenase (Rubisco) activity (Delfine *et al.* 1998, Luo *et al.* 2000, Sayed 2003). It is helpful to identify the affected parts of photosynthetic machinery through analyzing the changes of Chl fluorescence. There were no large differences in F_v/F_m, Φ_{PSII}, q_P, and ETR, as well as NPQ between control and salt-stressed plants in our experiment. The results indicated that salt stress did not induce increased susceptibility of PSII to photoinhibition. Under salt-alkaline mixed stress, the decreases of F_v/F_m, Φ_{PSII}, q_P and ETR were accompanied with the increase in NPQ. An increase in NPQ has been thought to be an energy dissipation mechanism that protects the photosynthetic apparatus against excess light (Demmig-Adams and Adams 1992). The reason may be that high pH caused a series of damages, such as destroying of photosynthetic machinery and primary electron acceptors, reducing the fluorescence yield, weakening PSII activity and reducing the photochemical reaction, so that plants are exposed to photoinhibition and photoprotection.

Inorganic ions accumulation: The results showed that K^+ was the main inorganic osmolyte of sunflower under both stresses. This differs in other plants such as *Kochia sieversiana* (Yang *et al*. 2007), in which Na^+ concentrations are obviously higher than K^+ ones under salt- and alkali stresses. This result reflects a specific adaptability of sunflower under long-term stress. The plants accumulated a large amount of K^+ instead of Na^+; this not only

Table 3. Percentage contribution of eight organic acids to total moles of organic acid in sunflower leaves under salt- and salt-alkaline mixed stresses. Legend refers to Table 2.

	Citrate [%]	Malate [%]	Glycolate [%]	Formate [%]	Lactate [%]	Acetate [%]	Succinate [%]	Oxalate [%]
Control	14.57	13.26	4.24	12.35	8.80	30.05	8.20	8.53
S1	15.35	11.59	5.66	11.99	10.94	31.13	6.87	6.47
S2	13.07	11.02	4.51	16.24	13.64	27.57	3.33	10.63
MSA1	34.03	13.54	1.90	9.32	7.47	15.32	4.59	13.83
MSA2	34.64	15.87	1.47	7.29	7.32	10.54	4.80	18.06

reduces the water potential to achieve osmotic adjustment, but also reduces Na^+ toxicity (Munns 2002).

Under salt stress, plants accumulate cations such as Na^+ and K^+ (Khan *et al.* 2000, Parida and Das 2005), and simultaneously accumulate inorganic anions such as Cl^- (Ghoulam *et al.* 2002, Santa-Cruz *et al.* 2002), NO_3^- and SO_4^{2-} or synthesized organic anions (Sagi *et al.*1997) to neutralize massive cations and maintain stable intracellular pH (Yang *et al.* 2007). Interestingly, our results showed that the change of OAs was opposite to the change of Cl^-, and the contribution of OAs to total charge in sunflower leaves under both stresses decreased with increasing salinity (Table 2). This was very different from our previous studies (Yang *et al.* 2007, 2008b) and may be another adaptive response to stress. Sunflower in stress conditions accumulated many inorganic ions instead of synthesizing organic compounds to decrease cell water potential, possibly to save energy consumption.

Organic acid accumulation: In the present study, sunflower accumulated three main OAs (citrate, malate, and oxalate) in leaves under salt-alkaline mixed stress (Table 3). Compared to results of our previous studies, the mechanism by which OAs metabolism was modulated in sunflower was significantly different from that in halophytes such as *Kochia sieversiana* (Yang *et al.* 2007) and *Suaeda glauca* (Yang *et al.* 2008b). In both the latter two species, the accumulated oxalate was approximately 90% of total OAs, under both alkali- and salt stresses. These data indicated that the modulation of OAs metabolism may play a different role in different plant species. The metabolic regulation of OAs under alkali stress may involve one or more key enzymes. These key enzymes may be known enzymes of basal metabolic pathways such as the tricarboxylic acid cycle, glyoxylate cycle, glycolysis or other pathways. Enzyme activity can be regulated at the level of synthesis (transcription, translation, and modification of new polypeptides), or after synthesis by the action of activators and inhibitors.

In conclusion, changes in OAs metabolism are an important *in vivo* adaptive response of halophytes to alkali stress, and should constitute an important future direction for research into the physiology of plant alkali stress.

References

Baker, N.R.: A possible role of photosystem II in environmental perturbations of photosynthesis. – Physiol. Plant. **81**: 563-570, 1991.

Campbell, S.A., Nishio, J.N.: Iron deficiency studies of sugar beet using an improved sodium bicarbonate-buffered hydroponics growth system. – J. Plant Nutr. **23**: 741–757, 2000.

Delfine, S., Alvino, A., Zacchini, M., Loreto, F.: Consequences of salt stress on conductance to CO2 diffusion, Rubisco characteristics and anatomy of spinach leaves. Aust. – J. Plant Physiol. **25**: 395-402, 1998.

Demmig-Adams, B., Adams, W.W., III: Photoprotection and other responses of plants to high light stress. – Annu. Rev. Plant Physiol. Plant Mol. Biol **43**: 599-626, 1992.

Everard, J.D., Gucci, R., Kann, S.C., Flore, J.A., Loescher, W.H.: Gas-exchange and carbon partitioning in the leaves of celery (*Apium graveolens* L.) at various levels of root-zone salinity. – Plant Physiol. **106**: 281-292, 1994.

Fidalgo, F., Santos, A., Santos, I., Salema, R.: Effects of long-term salt stress on antioxidant defence systems, leaf water relations and chloroplast ultrastructure of potato plants. – Assoc. Appl. Biol. **145**: 185-192, 2004.

Gao, C.Q., Wang, Y.C., Liu, G.F., Yang, C.P., Jiang, J., Li, H.Y.: Expression profiling of salinity-alkali stress responses by large-scale expressed sequence tag analysis in *Tamarix hispida*. – Plant Mol. Biol. **66**: 245-258, 2008.

Ge, Y., Li, J.D.: A preliminary study on the effects of halophytes on salt accumulation and desalination in the soil of Songnen Plain, northeast China. – Acta Pratacu. Sin. **1**: 70-76, 1990.

Ghoulam, C., Foursy, A., Fares, K.: Effects of salt stress on growth, inorganic ions and proline accumulation in relation to osmotic adjustment in five sugar beet cultivars. – Environ. Exp. Bot. **47**: 39-50, 2002.

Hartung, W., Leport, L., Ratcliffe, R.G., Sauter, A., Duda, R., Turner, N.C.: Abscisic acid concentration, root pH and anatomy do not explain growth differences of chickpea (*Cicer arietinum* L.) and lupin (*Lupinus angustifolius* L.) on acid and alkaline soils. – Plant Soil **240**: 191-199, 2002.

Jimenez, M.S., Gonzalez-Rodriguez, A.M., Morales, D., Cid, M.C., Socorro, A.R., Caballero, M.: Evaluation of chlorophyll fluorescence as a tool for salt stress detection in roses. – Photosynthetica **33**: 291-301, 1997.

Khan, M.A., Ungar, I. A., Showalter, A. M.: The effect of salinity on the growth, water status, and ion content of a leaf succulent perennial halophyte, *Suaeda fruticosa* (L.) Forssk. – J. Arid. Environ. **45**: 73-84, 2000.

Koyro, H.W.: Effect of salinity on growth, photosynthesis, water relations and solute composition of the potential cash crop halophyte *Plantago coronopus* (L.). – Environ. Exp. Bot. **56**: 136-146, 2006.

Li, C.Y., Fang, B., Yang, C.W., Shi, D.C., Wang, D.L.: Effects of various salt–alkaline mixed stresses on the state of mineral elements in nutrient solutions and the growth of alkali resistant halophyte *Chloris virgata*. – J. Plant Nutr. **32**: 1137-1147, 2009.

López-Bucio, J., Nieto-Jacobo, M.F., Ramírez-Rodríguez, V., Herrera-Estrella, L.: Organic acid metabolism in plants: from adaptive physiology to transgenic varieties for cultivation in extreme soils. – Plant Sci. **160**: 1-13, 2000.

Luo, J, Zhang, M.Q., Lv, J.L., Lin, Y.S.: Effects of water stress on the chlorophyll a fluorescence induction kinetics of sugarcane genotypes. – J. Fujian Agr. Univ. **29**: 18-22, 2000.

Ma, H.C., Fung, L., Wang, S.S, Altman, A., Hüttermann, A.: Photosynthetic response of *Populus euphratica* to salt stress. – Forest Ecol. Manag. **93**: 55-61, 1997.

Maricle, B.R., Lee, R.W., Hellquist, C.E., Kiirats, O., Edwards, G.E.: Effects of salinity on chlorophyll fluorescence and CO2 fixation in C4 estuarine grasses. – Photosynthetica **45**: 433-400, 2007.

Morant-Manceau, A., Pradier, E., Tremblin, G.: Osmotic adjustment, gas exchanges and chlorophyll fluorescence of a hexaploid triticale and its parental species under salt stress. – J. Plant Physiol. **161**: 25-33, 2004.

Munns, R.: Comparative physiology of salt and water stress. – Plant Cell Environ. **25**: 239-250, 2002.

Naidoo, G., Kift, J.: Responses of the saltmarsh rush *Juncus kraussii* to salinity and waterlogging. – Aquat. Bot. **84**: 217-225, 2006.

Netondo, G.W., Onyango, J.C., Beck, E.: Sorghum and salinity: II. Gas exchange and chlorophyll fluorescence of sorghum under salt stress. – Crop Sci. **44**: 806-811, 2004.

Nieva, F.J.J., Castellanos, E.M., Figueroa, M.E., Gil, F.: Gas exchange and chlorophyll florescence of C_3 and C_4 saltmarsh species. – Photosynthetica **36**: 397-406, 1999.

Parida, A.K., Das, A.B.: Salt tolerance and salinity effects on plants: a review. – Ecotoxicol. Environ. Saf. **60**: 324-349, 2005.

Qiu, N.W., Lu, Q.T., Lu, C.M.: Photosynthesis, photosystem II efficiency and the xanthophyll cycle in the salt-adapted halophyte *Atriplex centralasiatica*. – New Phytol. **159**: 479-486, 2003.

Qu, Y.G., Zhao, K.F.: Comparison of the stress effects of NaCl and Na_2CO_3 on *Suaeda salsa*. – Chin. J. Plant Physiol. Mol. Biol. **29**: 387-394, 2003.

Qu, Y.G., Zhao, K.F.: Comparative studies on growth and physiological reaction of *Zea mays* under NaCl and Na_2CO_3 stress. – Chin. Acta Agron. Sin. **30**: 334-341, 2004.

Rao, P.S., Mishra, B., Gupta, S.R., Rathore A.: Reproductive stage tolerance to salinity and alkalinity stresses in rice genotypes. – Plant Breeding **127**: 256-261, 2008.

Reddy, M.P., Vora, A.B.: Changes in pigment composition, Hill reaction activity and saccharides metabolism in bajra (*Pennisetum typhoides* S&H) leaves under NaCl salinity. – Photosynthetica **20**: 50-55, 1986.

Sagi, M., Dovrat, A., Kipnis, T., Lips, H.: Ionic balance, biomass production, and organic nitrogen as affected by salinity and nitrogen source in annual ryegrass. – J. Plant Nutr. **20**: 1291-1316, 1997.

Santa-Cruz, A., Martinez-Rodriguez, M. M., Perez-Alfocea, F., Romero-Aranda, R., Bolarin, M.C.: The rootstock effect on the tomato salinity response depends on the shoot genotype. – Plant Sci. **162**: 825-831, 2002.

Sayed, O.H.: Chlorophyll fluorescence as a tool in cereal crop research. – Photosynthetica **41**: 321-330, 2003.

Schreiber, U., Schliwa, U., Bilger, W.: Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer. – Photosynth. Res. **10**: 51-62, 1986.

Shi, D.C., Sheng, Y.M.: Effect of various salt–alkaline mixed stress conditions on sunflower seedlings and analysis of their stress factors. – Environ. Exp. Bot. **54**: 8-21, 2005.

Shi, D.C., Wang, D.L.: Effects of various salt-alkali mixed stresses on *Aneurolepidium chinense* (Trin.) Kitag. – Plant Soil **271**: 15-26, 2005.

Shi, D.C., Yin, S.J., Yang, G.H., Zhao, K.F.: Citric acid accumulation in an alkali-tolerant plant *Puccinellia tenuiflora* under alkaline stress. – Acta Bot. Sin. **44**: 537-540, 2002.

Shi, D.C., Zhao, K.F.: Effects of NaCl and Na_2CO_3 on growth of Puccinellia tenuiflora and on present state of mineral elements in nutrient solution. – Acta Pratacu. Sin. **6**: 51-61, 1997.

Sixto, H., Aranda, I., Grau, J.M.: Assessment of salt tolerance in *Populus alba* clones using chlorophyll fluorescence. – Photosynthetica **44**: 169-173, 2006.

Sultana, N., Ikeda, T., Itoh R.: Effect of NaCl salinity on photosynthesis and dry matter accumulation in developing rice grains. – Environ. Exp. Bot. **42**: 211-220, 1999.

Wei, Y., Xu, X., Tao, H., Wang, P.: Growth performance and physiological response in the halophyte *Lycium barbarum* grown at salt-affected soil. – Ann. Appl. Biol. **149**: 263-269, 2006.

Yang, C.W., Chong, J.N., Kim, C.M., Li, C.Y., Shi, D.C., Wang, D.L.: Osmotic adjustment and ion balance traits of an alkali resistant halophyte *Kochia sieversiana* during adaptation to salt and alkali conditions. – Plant Soil **294**: 263-276, 2007.

Yang, C.W., Jianaer, A.H., Li, C.Y., Shi, D.C., Wang, D.L.: Comparison of the effects of salt-stress and alkali-stress on the photosynthesis and energy storage of an alkali-resistant halophyte *Chloris virgata*. – Photosynthetica **46**: 273-278, 2008a.

Yang, C.W., Shi, D.C., Wang, D.L.: Comparative effects of salt stress and alkali stress on growth, osmotic adjustment and ionic balance of an alkali-resistant halophyte *Suaeda glauca* (Bge.). – Plant Growth Regul. **56**: 179-190, 2008b.

Yang, C.W., Wang, P., Li, C.Y., Shi, D.C., Wang, D.L.: Comparison of effects of salt and alkali stresses on the growth and photosynthesis of wheat. – Photosynthetica **46**: 107-114, 2008c.

Yang, C.W., Xu, H.H., Wang, L.L., Liu, J., Shi, D.C., Wang, D.L.: Comparative effects of salt-stress and alkali-stress on the growth, photosynthesis, solute accumulation, and ion balance of barley plants. – Photosynthetica **47**: 79-86, 2009.

Zhu, G. L.: Carotenoid and chlorophyll determine. – In: Zhu, G.L. (ed.): Laboratory Manual of Plant Physiology. Pp. 51-54. Beijing University Press, Beijing 1993.