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Comparison of adaptive strategies of alfalfa (*Medicago sativa* L.) to salt and alkali stresses

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Abstract

Alfalfa seedlings were stressed with salt or alkali. The growth, organic acids (OAs) and inorganic ions in stressed seedlings were measured to investigate the physiological adaptive mechanism, by which alfalfa tolerates alkali stress. For this purpose, we conducted some experiments under different salt and alkali stresses and then compared the results. The results showed that salt stress significantly stimulated alfalfa root growth. However, alkali stress decreased root dry weight by 20.83% compared with control treatment at 7 days (d). In root of seedlings subjected to alkali stress for 3 d, accumulation of Na^+, citrate and malate was strongly stimulated and raised by 763.29%, 217.47% and 298.00%, respectively, compared to control. However, in shoots, only when seedlings were subjected to alkali stress for 7d, the accumulation of contents increased sharply, indicating that response of root to alkali stress is more sensitive than that of shoot. Alkali stress greatly enhanced the Na^+ content, reduced the contents of inorganic anions, and induced deficit of negative charges. Under salt stress, Cl^- content heavily increased, and the contribution of OAs to osmotic adjustment was less than that of inorganic ions. Under alkali stress, alfalfa enhanced the synthesis of organic acids (OAs) (mainly citrate and malate) to compensate the shortage of inorganic anions. The OA metabolic regulation might play an important role in maintaining ion balance.

Keywords: Alfalfa, alkali stress, organic acid, osmotic adjustment, salt stress.
Abbreviations: OAs-organic acids; d-day; dry weight-DW.

Introduction

Soil salinization and alkalization is rapidly increasing on a global scale and currently affects more than 10% of arable lands (Boyer, 1982; Bray, 1997). Soil salinization and alkalization frequently co-occur in the nature under special conditions (Kawanabe and Zhu, 1991). However, to date, most reports have generally emphasized salt stress (Munns and Tester, 2008; Charkazi et al., 2010; Jemâa et al., 2011; Ibraheem et al., 2011). Simple alkali stress has been given more attention recently (Yang et al., 2007; Yang et al., 2008) but the mechanisms of alkali tolerance remains largely obscure. Soil alkalization frequently causes severe problems in some areas. For example, in northeast China, salt-alkalinized grassland covers >70% of land area, and is being expanded everyday (Kawanabe and Zhu, 1991; Läuchli and Lüttge, 2002). In previous studies, it has been suggested that salt stress can be defined 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 Yin, 1993; Yang et al., 2007). When 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 (Li et al., 2010a, b; Shi and Wang, 2005). The existence of alkali stress has been demonstrated clearly by a number of studies, which show the alkali stress be more severe than salt (Brand et al., 2002; Campbell and Nishio, 2000; El-Samad and Shaddad, 1996). Therefore, the problem of alkali stress should be recognized and investigated as thoroughly as salt stress. Alfalfa (*Medicago sativa* L.) is one of the most important forage crops which has high protein and highly digestible fibre contents, and is beneficial to restore alkalinized grasslands. Alfalfa is widely planted in America, Canada, Australia, and other countries (Deng et al., 2006). There are numerous reports on alfalfa response to salt stress (Ehsanpour and Fatahian, 2003; Wang and Han, 2009), heavy metals stress (Zhou et al., 2008), mixed salt-alkali stress (Peng et al., 2008), drought stress (Wang et al., 2009) etc. Previous studies confirmed that different plants differed in mechanisms of resistance to alkali stress, but there are no reports on mechanisms underlying physiological adaptation of Leguminosae to alkali stress. Salt stress in the soil generally involves osmotic stress and ion injury (Munns, 2002). Alkali stress encompasses the same stress factors but with the added influence of high-pH stress. Alkali stress is combination of salt stress and high pH stress. High-pH stress is the main reason of the injurious effect of alkali stress on plants, which is greater than salt stress injuries. The differential response of plants to salt stress and alkali stress are mainly due to high-pH stress. Therefore, comparing salt stress and alkali stress is important for understanding the high-pH or alkali tolerance. In this study, alfalfa seedlings were treated with either salt or alkali stress. The growth, inorganic ions and organic acids (OAs) were measured in stressed seedlings to investigate (1) the effects of salt and alkali stresses on alfalfa growth; (2) whether alfalfa has different adaptive strategies to different stresses; (3) whether the adaptive strategies of alfalfa differ in different organs.

Table 1. Correlation coefficients between organic acids (OAs) and inorganic ions contents (represented as μmol g^{-1} DW) in root and shoot of alfalfa.

		Na^+	K^+	Cl^-	NO_3^-	$H_2PO_4^-$
Root	Citrate	0.88**	-0.47	-0.10	-0.27	-0.24
	Malate	0.83**	-0.31	-0.01	-0.01	-0.03
	Total OA	0.88**	-0.40	-0.03	-0.14	-0.13
Shoot	Citrate	0.86**	-0.51	-0.47	-0.29	-0.60
	Malate	0.93**	-0.77**	-0.32	-0.64**	-0.86
	Total OA	0.94**	-0.73**	-0.36	-0.57**	-0.82

*, **correlation was significant at 0.05 and 0.01 levels of probability. $r_{0.05}$=0.666, $r_{0.01}$=0.798, n=9.

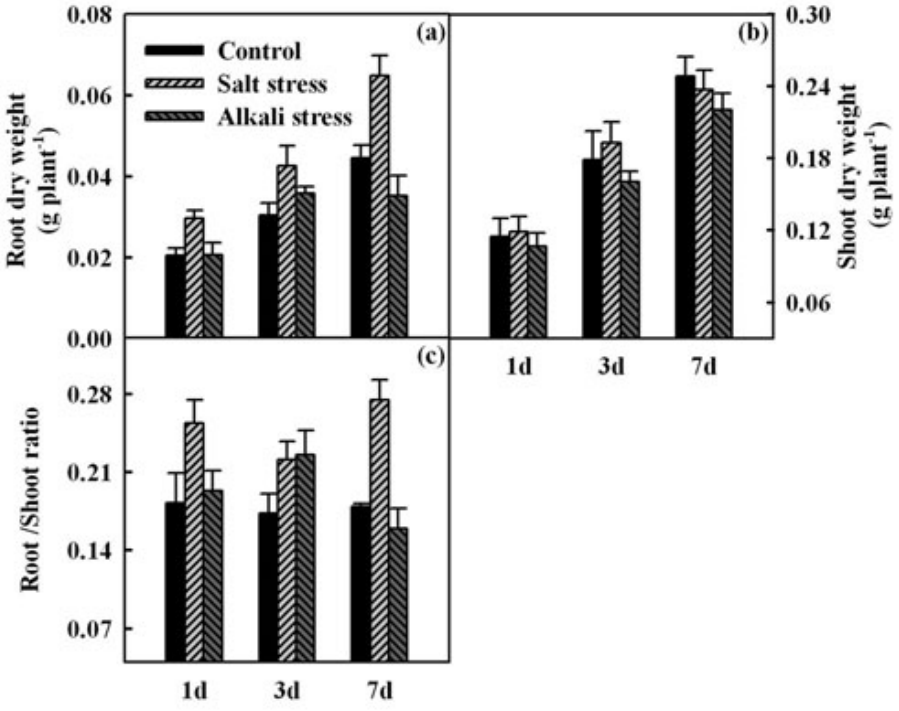

Fig 1. Effects of salt and alkali stresses on dry weight and root/shoot ratio in alfalfa seedlings. The 20-day-old seedlings were treated with 90 mM salt ($NaCl:Na_2SO_4$=9:1) and alkali ($NaHCO_3:Na_2CO_3$=9:1) stresses for 1, 3 and 7d. All data were represented by an average of three biological replicates and the standard errors (S.E.).

Results and Discussion

Growth

High salt stress generally inhibits growth of plants (Munns and Tester, 2008). Some reports clearly showed that both salt and alkali stresses limited wheat (Yang et al., 2008), and barley (Yang et al., 2009) shoot growth and growth of roots and shoots in sunflower (Liu et al., 2010). However, in the present study, salt stress significantly increased root dry weight (DW) and root-shoot ratio of alfalfa ($P < 0.01$; Fig. 1 a, c), especially at 7 d. Salt stress increased the root dry weight (DW) and root-shoot ratio by 45.83% and 53.18%, respectively. Nevertheless, salt stress had small effects on shoot DW ($P > 0.05$; Fig. 1b). On the other hand, alkali stress decreased DWs in both root and shoot by 20.83% and 11.19%, respectively, especially at 7 d ($P < 0.01$; Fig. 1a, b). This implies not only that salt and alkali stresses are distinct stresses, but also that the resistance of alfalfa to salt is stronger than alkali stress. A major salt tolerant alfalfa (*Medicago sativa* L.) cultivar in China, Gongnong 1, was tested in this study. The response of alfalfa growth to salt and alkali stress might be different in each cultivar (genotype) (Li et al., 2010c). Previous studies in other alfalfa cultivar have indicated that both salt and alkali stresses inhibited alfalfa shoot growth (Li et al., 2010c). However, we observed that both salt and alkali stresses only have a small effects on alfalfa shoot growth. But the root dry weight under alkali stress was much lower than that under salt stress (Fig. 1a, b). This indicated that response of alfalfa root to alkali stress was more sensitive than that of shoot, which was also supported by the results of Na^+, citrate, malate, and total OA.

Accumulation of inorganic ions

Low Na^+ and high K^+ are essential for the maintenance of a number of enzymatic processes in the cytoplasm of cells (Munns and Tester, 2008). Both salt and alkali stresses increased Na^+ contents and Na^+/K^+ ratios in roots and shoots (P

Table 2. Analysis of multiple linear regressions between organic acids (OAs) content and inorganic ions contents in shoots and roots of alfalfa. n=9; x_1=Na^+ content; x_2=K^+ content; x_3=Cl^- content; x_4=NO_3^- content; x_5=$H_2PO_4^-$ content; β_1-β_5: standardized regression coefficients corresponding to x_1-x_5. The lager β value indicates stronger effect of the contents of ions on organic acid accumulation.

	Regression equation	r^2	ANOVA test	β_1	β_2	β_3	β_4	β_5
Root	Citrate=32.69+0.11x_1-0.08x_2-0.32x_3+0.05x_4+0.55x_5	0.94	P=0.048	0.83	-0.70	-0.90	0.22	0.63
	Malate=48.74+0.12x_1-0.18x_2-0.59x_3+0.21x_4+1.23x_5	0.97	P=0.022	0.72	-1.26	-1.27	0.73	1.09
	Total OA=24.84+0.23x_1-0.25x_2-0.83x_3+0.25x_4+1.59x_5	0.95	P=0.036	0.80	-0.98	-1.03	0.50	0.81
Shoot	Citrate=121.29+0.11x_1+0.01x_2-0.10x_3+0.13x_4-0.02x_5	0.96	P=0.025	1.091	0.04	-0.27	0.33	-0.01
	Malate=134.45+0.16x_1+0.15x_2-0.24x_3-0.25x_4-0.74x_5	0.98	P=0.012	0.72	0.24	-0.28	-0.28	-0.26
	Total OA=294.80+0.27x_1+0.17x_2-0.31x_3-0.10x_4-0.97x_5	0.99	P=0.002	0.85	0.19	-0.25	-0.08	-0.24

<0.01; Figs. 2a, b, e, f), The Na^+ contents increased by 585.19% and 306.21% in roots and shoots under salt stress, and 763.29% and 1738.05% under alkali stress, respectively. The Na^+/K^+ ratios raised by 1043.54% and 362.56% under salt or alkali stress to the utmost degree, respectively. When seedlings were subjected to alkali stress for 3d, the Na^+ content in roots under alkali stress were much greater than that under salt stress (P < 0.01; Fig. 2a). However, in shoots, only when seedlings were subjected to alkali stress for 7d, Na^+ contents under alkali were higher than that under salt stress (P < 0.01; Fig. 2b). Salt stress had small effects on K^+ contents in roots and shoots, while alkali stress led to decrease of K^+ contents (P < 0.01; Fig. 2c, d), especially for 7 d, in which decreased by 91.73% and 27.73%, respectively. Under salt stress, the Cl^- contents in both roots and shoots increased with increment of stress duration (P < 0.01; Fig. 2g, h). Under alkali stress, the Cl^- contents in shoots were similar to control treatment, whereas Cl^- contents in roots were lower than that of control (P < 0.01; Fig. 2g, h). The effects of salt stress on the NO_3^-, and $H_2PO_4^-$ contents were small, but the alkali stress greatly reduced their contents (P < 0.01; Fig. 2i-l), by 88.97% and 63.21%, respectively. In summary, in alfalfa, both stresses induced the increase of Na^+ in shoots (Fig. 2b), whereas the increases under alkali were much greater than under salt stress. In addition, salt stress only had a minor effect on K^+ accumulation; however, alkali stress slightly reduced K^+ content in shoots, and strongly decreased the K^+ content in roots (Fig. 2c, d). These findings showed that alkali stress may greatly affect the transport of K^+ and Na^+ and disrupt the homeostasis of K^+–Na^+ in alfalfa. In the present study, alkali stress greatly enhanced the Na^+ contents in roots and shoots of alfalfa (Fig. 2a, b), reduced the contents of inorganic anions (Fig. 2g-l), and induced deficit of negative charges. Under alkali stress, the accumulation of organic acids may be a response to increased Na^+ (Fig. 2a, b) and/or the reductions of inorganic anions (Fig. 2g-l).

Organic acids

Tartrate, citrate, malate, formate, lactate, oxalate were detected in roots and shoots of alfalfa. Of these OAs, citrate and malate were the dominant OAs in roots and shoots; while only trace amounts of tartrate, formate, lactate and oxalate were detected (Fig. 3). The tartrate content raised sharply under alkali stress by increasing of stress time; however, there was no or only a trace of tartrate detected under salt stress and control treatments (P < 0.01; Fig. 3a, b). Salt stress did not elevate citrate, malate and total OA contents in roots and shoots. However, when seedlings were subjected to alkali stress for 3d, alkali stress strongly stimulated accumulations of citrate (P < 0.01; Fig. 3c, d), malate (P < 0.01; Fig. 3e, f) and total OA (P < 0.01; Fig. 3m, n) in roots, and after 3d their contents in roots decreased. In shoots, only when seedlings were subjected to alkali stress for 7d, citrate (P < 0.01; Fig. 3c, d), malate (P < 0.01; Fig. 3e, f) and total OA (P < 0.01; Fig. 3m, n) contents increased sharply. Salt and alkali stresses slightly affected the accumulation of formate and lactate (Fig. 3g-j). Under salt stress and control treatment the oxalate content changes were not significant, but the contents decreased sharply under alkali stress in roots with increment of stress time, while they increased in shoots (Fig. 3k, l). Furthermore, the responses of Na^+ and citrate, malate, and total OA to both stresses were similar. Correlation analysis showed that there were negative correlations between OAs contents and inorganic anions. On the other hand, the citrate, malate and total OA content were positively correlated with Na^+ content (Table 1). A multiple linear regression analysis was performed for each OA (Table 2). The P values were lower than 0.05, indicating a high linear correlation between each OA and the five ions. The analysis indicated that Na^+ content was a dominant factor that affected OA accumulation in shoots. Under alkali stress, alfalfa enhanced the synthesis of OA, to compensate the shortage of inorganic anions. The OA metabolic regulation might play an important role in maintaining ion balance as well. Another interesting phenomenon is a quicker accumulation of Na^+ and OAs in roots than those in shoots, during adaptation of alfalfa to alkali stress (Figs. 2 and 3). When seedlings were subjected to alkali for 3d, the stress strongly stimulated accumulations of Na^+, citrate and malate in roots; however, in shoots, only when seedlings were subjected to alkali stress for 7d, their contents increased sharply (Figs. 2 and 3). This suggests that response of root to alkali stress was more sensitive than that of shoot. Plant root was the primary organ perceiving and responding Na^+ stress and high-pH stress caused by alkali using signaling systems involved in stress tolerance. pH adjustment outside roots might be the most vital mechanism, by which alfalfa resisted to the alkali stress and might probably be an important future research direction for plant alkali-stress physiology.

Osmotic adjustment

At 7d, both salt and alkali stresses increased proline contents in roots and shoots, in which increases under alkali were much greater than salt stress (P < 0.01; Fig. 3o, p). The accumulated proline under salt stress is usually considered as an organic-compatible osmolyte and a protecting agent for the activity of intracellular macromolecules (Tang, 1989). The proline accumulation is closely related to osmotic adjustments (Shi, 1995). In this study, the contribution of proline to osmotic adjustment of alfalfa was insignificant (Tables 3 and 4). In addition, the mechanisms governing osmotic adjustment under both stresses were different. In this study, the percentage contributions of Na^+ and OAs (mainly citrate and malate in roots and shoots) to osmotic adjustment were significantly higher under alkali than salt stress, while percentage contribution of K^+ and Cl^- was lower under alkali stress than salt (Tables 3 and 4).

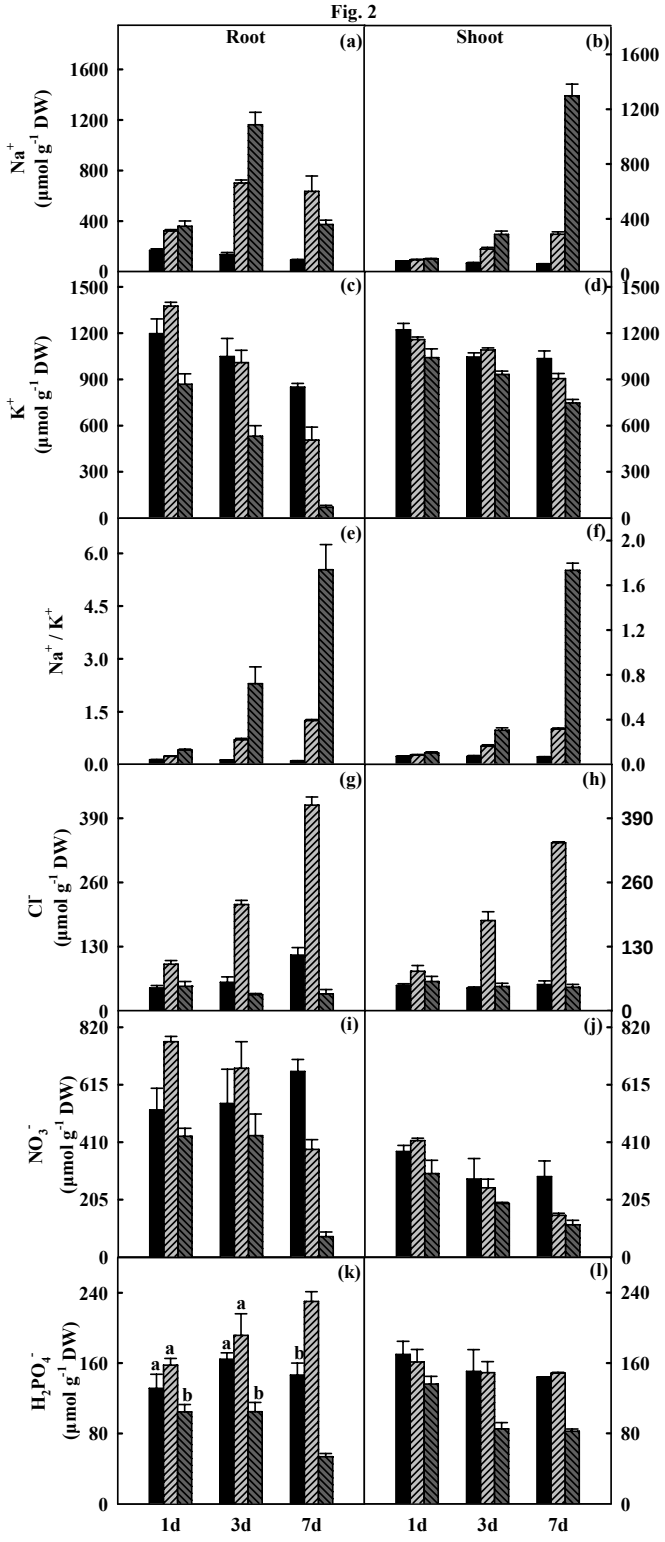

Fig 2. Effects of salt and alkali stresses on the contents of inorganic ions in alfalfa seedlings. The 20-day-old seedlings were treated with 90 mM salt (NaCl:Na_2SO_4=9:1) and alkali ($NaHCO_3$:Na_2CO_3=9:1) stresses for 1, 3 and 7d. All data were represented by an average of three biological replicates and the standard errors (S.E.).

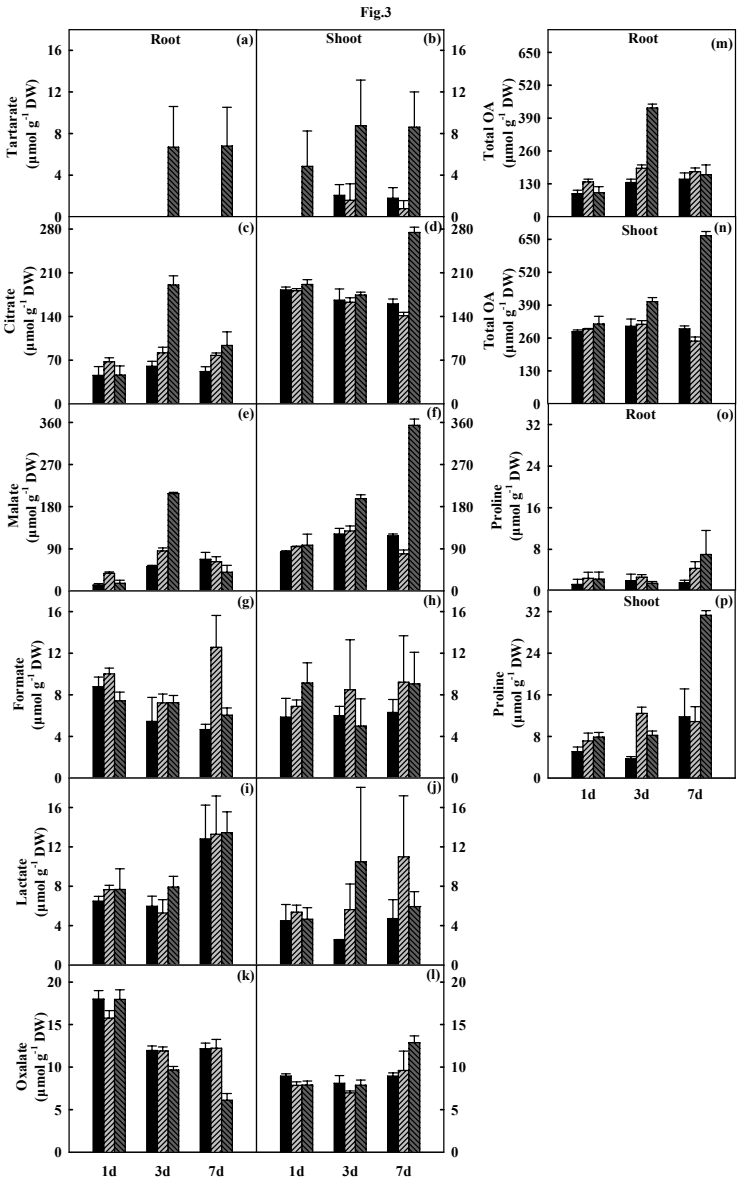

Fig 3. Effects of salt and alkali stresses on the contents of organic acids (OAs) and proline in alfalfa seedlings. The 20-day-old seedlings were treated with 90 mM salt (NaCl:Na_2SO_4=9:1) and alkali ($NaHCO_3$:Na_2CO_3=9:1) stresses for 1, 3 and 7d. All data were represented by an average of three biological replicates and the standard errors (S.E.).

Materials and methods

Plant materials

A major alfalfa (*Medicago sativa* L.) cultivar in China, Gongnong 1, was chosen as a test genotype. Seeds were sown in 17 cm diameter plastic pots containing 2.5 kg of washed sand. Each pot contained 9 seedlings, which were sufficiently watered with Hoagland nutrient solution everyday. Evaporation was compensated by distilled water when required. All pots were placed outdoors and sheltered from rain.

Table 3. Contributions of various solutes to total measured solutes in roots of alfalfa under salt and alkali stresses

Time	Na^+ (%)	K^+ (%)	Cl^- (%)	NO_3^- (%)	$H_2PO_4^-$ (%)	OA (%)	Proline (%)
Control							
1d	7.76	55.42	2.13	24.32	6.08	4.23	0.06
3d	6.44	50.12	2.75	26.25	7.87	6.49	0.09
7d	4.60	42.21	5.60	32.87	7.27	7.38	0.08
Salt stress							
1d	11.23	48.13	3.29	26.90	5.52	4.84	0.08
3d	23.50	33.79	7.21	22.58	6.41	6.42	0.09
7d	26.99	21.47	17.70	16.32	9.77	7.57	0.18
Alkali stress							
1d	18.81	45.44	2.59	22.58	5.48	4.98	0.12
3d	43.10	19.72	1.21	16.08	3.89	15.95	0.05
7d	48.01	9.05	4.40	9.39	6.93	21.33	0.90

[a]Percent contribution of a given solute to total solutes was calculated according to means of triplicate samples.

Table 4. Percent[a] contributions of various solutes to total measured solutes in shoots of alfalfa under salt and alkali stresses

Time	Na^+ (%)	K^+ (%)	Cl^- (%)	NO_3^- (%)	$H_2PO_4^-$ (%)	OA (%)	Proline (%)
Control							
1d	4.11	55.54	2.32	17.12	7.72	12.96	0.23
3d	3.99	54.83	2.40	14.60	7.90	16.09	0.19
7d	3.72	54.51	2.79	15.14	7.58	15.64	0.62
Salt stress							
1d	4.58	52.20	3.60	18.72	7.26	13.33	0.32
3d	8.21	50.19	8.40	11.35	6.85	14.43	0.57
7d	13.74	43.33	16.32	7.12	7.11	11.87	0.52
Alkali stress							
1d	5.46	53.00	3.00	15.15	6.93	16.05	0.40
3d	14.62	47.67	2.50	9.79	4.36	20.65	0.42
7d	43.45	25.02	1.59	3.87	2.76	22.26	1.05

[a]Percent contribution of a given solute to total solutes was calculated according to means of triplicate samples.

Temperatures during the experiment were 24–28°C during the day and 17–20°C over night.

Design of simulated saline and alkaline conditions

Two neutral salts, NaCl and Na_2SO_4 were mixed in a molar ration of 9:1, for the salt stress treatment. For the alkali stress treatment, two alkaline salts, $NaHCO_3$ and Na_2CO_3, were also mixed in a molar ration of 9:1. The applied stress intensity was 90 mM, and the pH values were 6.96 under salt stress and 9.21 under alkali stress.

Stress treatments

When the seedlings were 20 days old, 27 pots with uniformly growing seedlings were selected and randomly divided into 9 sets, as 3 pots per set. Each pot was considered as a single replicate. Therefore, there were three replicates per set. Three sets were used as control (one set per time point). Three sets were treated with salt stress (one set per time point) and the remaining three sets were treated with alkali stress (one set per time point). The seedlings were treated with 90 mM salt (NaCl:Na_2SO_4=9:1) and alkali ($NaHCO_3$:Na_2CO_3=9:1). Stress treatments were performed with the application of nutrient solutions containing the appropriate stress salts. Control plants were watered with nutrient solution.

Measurement of physiological indices

Plants were harvested after treatment for 1, 3 and 7 days (d) respectively, and were washed with distilled water. Roots and shoots were separated and oven dried at 80°C for 10 min, and vacuum-dried at 40°C to a constant weight. Dry samples of plant material were treated with 10 ml of deionized water at 100°C for 1h, and the extracts used to determine the contents of free inorganic ions and organic acids (OAs) (Yang et al., 2010). Cl^-, NO_3^-, $H_2PO_4^-$, SO_4^{2-} and oxalate were determined by ion chromatography (DX-300ion chromatographic system, AS4A-SC chromatographic column, mobile phase: Na_2CO_3/$NaHCO_3$ = 1.7/1.8mM; DIONEX, Sunnyvale, USA). The other OAs (tartrate, citrate, malate, formate, lactate, oxalate, glycollate, acetate and succinate) were also determined by ion chromatography (DX-300 ion chromatographic system; ICE-AS6 ion-exclusion column, mobile phase: 0.4mM heptafluorobutyric acid; DIONEX, Sunnyvale, USA). An atomic absorption spectrophotometer (TAS-990, Purkinje General, Beijing, China) was used to determine the concentrations of Na^+ and K^+. The contents of proline were measured using ninhydrin method (Zhu et al., 1983).

Statistical analysis

All experiments were based on three biological replications. All data were represented by an average of the three biological replicates and the standard errors (S.E.). Data were analyzed by one-way analysis of variance (ANOVA) using the statistical software SPSS 14.0 (SPSS Inc., Chicago, USA), with significance tested at $P < 0.01$.

Conclusion

In conclusion, salt stress significantly stimulated alfalfa root growth; but alkali stress decreased root dry weight. In roots, alkali stress strongly stimulated when seedlings were subjected to alkali stress for 3d. However, accumulations of Na^+, citrate and malate in shoots stimulated and increased only when seedlings were subjected to alkali stress for 7d, indicating that response of root to alkali stress was more sensitive than that of shoot. Alkali stress greatly increased Na^+ content but reduced the contents of inorganic anions and induced deficit of negative charge. Moreover, alkali stress enhanced the synthesis of OA (mainly citrate and malate) to compensate the shortage of inorganic anions. The OA metabolic regulation might play an important role in maintaining ion balance. In both root and shoot, salt stress strongly induced Na^+ and Cl^- while the contribution of OAs to osmotic adjustment was less than that of inorganic ions.

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