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MECHANISMS OF COMBINED EFFECTS OF SALT AND ALKALINE STRESSES ON SEED GERMINATION AND SEEDLINGS OF *MELILOTUS OFFICINALIS* (FABACEAE) IN NORTHEAST OF CHINA

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Abstract

In line with the salt-alkalinized soils found in the northeast of China, the conditions were simulated to investigate the mechanisms associated with this combination of stresses on *Melilotus officinalis*. The effects of salinity (NaCl: 0-300mM) in combination with alkali (pH: 7.1-9.8) on the seed germination and seedlings of *M. officinalis* were investigated. The results showed that germination was not inhibited completely by the salt-alkali conditions tested. The recovery germinations were significant higher than the control or had no significant differences with the control under the conditions of NaCl<200mM and pH=9.0, suggesting that non-germinated seeds may have a strategy to get through and resist the stress during germination stage. For the seedling growth, *M. officinalis* was capable of surviving at high pH (pH≤9.8) and the salinity (NaCl≤200mM) (seedling survival rate: 84.77±8.62%). The characteristic feature for combined salt-alkali stresses is the reciprocal enhancement between salt and alkali stresses. The combined action of salinity and pH should be considered when evaluating the effects of salt-alkali stresses. Correlation and regression analyses showed that salinity was the dominant stress factor, while pH was a secondary factor. From the physiological and ecological parameters, we suggested that *M. officinalis* is a salt-alkali tolerant species which can be used in vegetative restoration of saline soils in the northeast of China.

Key words: *Melilotus officinalis*, Salt-alkali stress, Germination, Seedling growth, Salt-alkali tolerance.

Introduction

The salinization of soil is an increasing environmental problem that has long-term effects on the arable land (Boyer 1982; Bray 1997). In nature, soil salinization and alkalinization frequently co-occur, so conditions in salt-alkali soil are very complex. Some salt-alkali soils have high salinity but low pH, while others have low salinity but high pH (Shi *et al*., 1998). In some areas, alkalization of the soil caused by $NaHCO_3$ and Na_2CO_3 may be a more severe problem than salinization caused by neutral salts such as NaCl and Na_2SO_4. Therefore, when saline soil contains CO_3^{2-} and/or HCO_3^-, it causes injury to plants due to salt stress as well as alkali stress (Shi & Wang, 2005). The existence of alkaline stress has been demonstrated previously (Campbell & Nishio, 2000; Hartung *et al*., 2002) and alkaline stress is more severe than saline stress (Shi & Wang, 2005; Yang *et al*., 2007). In China, salt-alkaline soils occupy a total of 99 million hectares (Tang *et al*., 2009) and over 70% of the grassland in northeastern China is now alkalinized (Guo *et al*., 2010). Still, salt-alkaline soils are regarded as important areas for crop production. Thus, the survival and growth of plants on saline and alkaline substrates are useful for making good use of salt-alkali soils and studies to this end will provide a basis for economical sustainable development.

Seed germination and seedling growth are the most critical life stages of plants and the organisms are more sensitive to environmental salt stress during these periods (Khan *et al*., 2001; Pujol *et al*., 2000). Therefore, seed germination in a saline substrate is a legitimate criterion that could be used for identifying plants that are tolerant of saline environments (Sosa *et al*., 2005). Salt tolerance is the ability of plants to grow and complete their life cycle on a substrate that contains high salt concentrations (Parida & Das, 2005). Previous studies have investigated seed germination of plants under high salinity using NaCl only as the stress agent (Shumway & Bertness, 1992; Gulzar & Khan, 2001; Yuan & Shi, 2009). Meanwhile, high levels of alkalinity can also limit seed germination (Guan *et al*., 2009) and germination rates decrease with increasing salt-alkali concentrations (Li *et al*., 2010a; Lin *et al*., 2012). Further studies have shown that non-germinated seeds can still germinate after being transferred from salinity or alkaline stress conditions to distilled water, but seeds under salt stress germinate better than those previously exposed to alkaline stress (Li *et al*., 2010b).

Salinity in conjunction with high pH can sharply reduce plant seedling survival rates. The interactive effects of salt and alkali stresses can also cause significant decreases in growth rate, water content, leaf chlorophyll content and root activity (Li *et al*., 2010c; Shi & Wang, 2005). On the contrary, membrane permeability increases with increasing salinity and pH (Li *et al*., 2010c). Further studies have demonstrated that the effects of alkali stress can be more severe than the effects of salt stress (Zhang & Mu, 2009).

As an important pasture forage plant in China, *Melilotus officinalis* (Fabaceae) has well-developed long taproot which can grow in semi-arid soils and this species contains high contents of digestible fiber and good quality protein (Guo *et al*., 2012). Furthermore, the root system of *M. officinalis* can help improve the fertility of dry and semi-arid soils (Guo *et al*., 2012). Moreover, the antiinflammatory properties of extracts from *M. officinalis* have been confirmed in animals (Pleşca-Manea

et al., 2002). A recent study focused on the NaCl stress on the seed germination of *M. officinalis* and the results have shown that salt stress resulted in a drop in germination percentage of *M. officinalis* (Süleyman *et al*., 2013).

Based on its potential beneficial environmental and economic value, further knowledge is required on seed germination and seedling growth under combined salt and alkali stresses. However, there has not been carried out research on the effect of combined salt and alkali stresses on *M. officinalis.* To elucidate the effects of the combined stresses on *M. officinalis* and based on the salt components found in salt-alkaline soils, two neutral (NaCl and Na_2SO_4) and alkali ($NaHCO_3$ and Na_2CO_3) salts were selected for investigation. Under salt-alkali stress conditions, germination parameters including germination percentage, germination index and recovery germination were assessed, and growth parameters including survival rate, growth rate, water content, chlorophyll content, root activity and membrane permeability were also determined. The main objectives of this present study are: (1) to evaluate the mechanisms of the effects of salt and alkali stresses on seed germination and seedling growth of *M. officinalis*; (2) to investigate the features of salt-alkali stress in this species. These investigations will help provide a scientific basis for the development and utilization of *M. officinalis* to improve exploitation of salt-alkali soils.

Materials and Methods

Design of the simulated salt and alkali conditions: To simulate different natural salt-alkaline conditions, two neutral salts (NaCl and Na_2SO_4) and two alkali salts ($NaHCO_3$ and Na_2CO_3) were mixed in various proportions (Shi & Wang, 2005) (Table 1). Each treatment consisted of six levels of salinity (50, 100, 150, 200, 250 and 300 mM) at each of four pH levels: A (pH 7.3 ± 0.17), B (pH 8.0 ± 0.23), C (pH 9.0 ± 0.35), and D (pH 9.8 ± 0.19). pH levels in each treatment group were determined using a digital pH meter. In total, there were 24 stress treatments, and these were coded fromA1 to D6 (Table 2).

Experiment 1: Seed germination: Seeds of *M. officinalis* were collected from the midlands of the Songnen Plain (125°93' E, 46°04' N–127°97' E, 44°54' N) in the northeast of China in middle September 2012. The seeds were kept in a refrigerator at 4 ±1°C until used. For breaking dormancy induced by hard seed coat, seeds were exposed to mechanical scarification which consisted in a vigorous rubbing of the seed with 100 grit sandpaper to abrade the seed coat (Süleyman *et al*., 2013). Then seeds were surface sterilized with 0.1% $KMnO_4$ for 10 min and subsequently rinsed with distilled water. Seeds were placed in 9-cm Petri dishes on two layers of filter paper moistened with 5 mL of distilled water (control) or with one of the treatment solutions. The germination experiments were replicated three times using 30 seeds per dish. These dishes were placed in an incubator (Eyela Eyelatron FLI-301 NH, Japan) operating a 10/14 h (night/day) photoperiod at 100 μM $m^{-2}s^{-1}$ and a 15/25°C (night/day) thermo-period. Germinated seeds were counted every day and fresh culture medium was added periodically. The germination tests lasted 7 days, and then non-germinated seeds were transferred to distilled water to investigate the germination recovery. After 2 days, the recovered germinated seeds were counted. Germination and recovery germination percentages were calculated according to Mostafa Ahmadizadeh *et al*. (2012), while germination index was calculated according to Yuan & Shi (2009).

Table l. The salt composition and molar ratios of the various treatments.

Treatment group	Salt composition and molar proportions			
	NaCl	Na_2SO_4	$NaHCO_3$	Na_2CO_3
A (pH 7.3)	1	1	0	0
B (pH 8.0)	1	2	1	0
C (pH 9.0)	1	1	1	1
D (pH 9.8)	9	1	1	9

Table 2. Stress factors of the various treatments.

Treatment	Stress factors	
	Salinity (mM)	pH
Control	0	7.05
A1	50	7.15
B1	50	7.63
C1	50	8.47
D1	50	9.52
A2	100	7.22
B2	100	7.77
C2	100	8.63
D2	100	9.70
A3	150	7.31
B3	150	7.85
C3	150	8.84
D3	150	9.83
A4	200	7.45
B4	200	8.08
C4	200	9.11
D4	200	9.89
A5	250	7.52
B5	250	8.13
C5	250	9.22
D5	250	9.94
A6	300	7.57
B6	300	8.23
C6	300	9.35
D6	300	10.08

1. Germination percentage = $(a/b) \times 100\%$

where a represents germinated seeds total, and b represents the number of seeds for germination.

2. Recovery germination percentage = $c/d \times 100\%$

where c represents the number of germinated seeds in the recovery experiment, d represents the total number of seeds for the recovery germination experiment.

3. Germination index = $G_1/D_1 + G_2/D_2 + \ldots + G_7/D_7$

where $D_1, D_2, \ldots, D_7$ are the first, second, and subsequent days until the 7th day of seed germination. $G_1, G_2, \ldots, G_7$ are the number of germinated seeds on $D_1, D_2, \ldots, D_7$, respectively.

Experiment 2: Seedling experiments

Plant growth: For plant growth, fully developed seeds of *M. officinalis* were selected to germinate. First, seeds were exposed to mechanical scarification which consisted in a vigorous rubbing of the seed with 100 grit sandpaper to abrade the seed coat (Süleyman *et al*., 2013). Then seeds were surface sterilized with 0.1% $KMnO_4$ for 10 min and subsequently rinsed with distilled water. Seeds were placed in 9-cm Petri dishes on two layers of filter paper moistened with 5 mL of distilled water. These dishes were placed in an incubator (Eyela Eyelatron FLI-301 NH, Japan) operating a 10/14 h (night/day) photoperiod at 100 μM $m^{-2}s^{-1}$ and a 15/25°C (night/day) thermo-period for germination. Three days after germination, uniform seedlings were transferred in plastic pots (17 cm diameter × 20 cm depth) filled with vermiculite. Each pot contained 25 plants. All pots were placed outdoors (day temperature 23–28°C; night temperature 18–23°C) and protected from the rain. Seedlings were carefully watered every day with Hoagland nutrient solution (Shi *et al*., 1998).

Stress treatment: Stress treatments were applied to 5-week-old seedlings. Seventy-eight pots of uniform seedlings were selected and randomly divided into 26 treatment groups (3 replicate pots per treatment). There were 24 stress treatments, one control and one set aside for determining fresh and dry weights at the beginning of the stress treatments. Salt levels were achieved by the addition of the necessary salt to the nutrient solution. Salinity was gradually increased by 50 mM (Na^+ concentration) increments daily until the final concentrations were achieved. During this period, the control was watered with nutrient solution only. Stress treatments were performed once a day at 7:00 am by watering thoroughly with 500 mL of treatment solution per pot in three equal doses. The amount of evaporated water was replenished daily with distilled water (Shi & Wang, 2005; Yang *et al*., 2009). The stress treatments lasted for 14 days in total.

Physiological determinations

Survival and growth: All plants were harvested carefully after 2 weeks of stress treatment, and rinsed first with tap water and then with distilled water. Water remaining on the surfaces of the plants was blotted with filter paper. The number of live and dead plants in each pot was counted and survival rate was expressed as a percentage. Ten seedlings were selected at random for determining the above-ground and below-ground fresh weights (FW). Then, these samples were dried to constant mass at 70°C to determine dry weights (DW). The remainder of the fresh samples was taken to measure various physiological indices. Relative growth rate (RGR) was determined by the following formula: RGR = [ln (final DW) − ln (initial DW)] / duration of treatment (days) (Kingsbury *et al*., 1984). Water content was calculated using the following formula: (FW - DW) / DW, and then expressed as g/g DW (Yang *et al*., 2007).

Leaf chlorophyll, root activity, and membrane permeability: Leaf chlorophyll content was determined using a Minolta chlorophyll meter (SPAD 502, Konica Minolta, Inc., Tokyo, Japan) at the end of the stress period. The digital reading of the SPAD-502, normally known as the "SPAD" value, is used widely as an indicator of leaf chlorophyll content. Measurements were taken on the leaves of all live plants in each pot from 08:30 to 11:00 am.

Root activity was determined using the triphenyl tetrazolium chloride (TTC) reduction assay with slight modifications (Comas *et al*., 2000), as TTC is a sensitive indicator of changes in cellular metabolism. The fresh root was immersed in TTC solution for 1.5 hours at 37°C (0.4% TTC in pH 7.0 phosphate buffer) and then sulfuric acid was added to stop the reaction. Then, the red product in each root was extracted using ethyl acetate. The absorbance was measured using a spectrophotometer at 485 nm. Relative root activity of the various treatments was expressed as a percentage relative to the activity of the control roots.

Membrane permeability can be assessed by measuring the electrolyte leakage rate (ELR). ELR was measured using the methods of Lutts *et al*. (1996) with some slight modifications. Fresh roots (0.1 g) were taken from each pot and cut into pieces of 3 mm in length. These pieces were washed three times with deionized water to remove surface-adhered electrolytes and placed in vials containing 15 mL of deionized water. Roots in the vials were deflated for 10 min and sinked for 20 min, and then the initial electrical conductivity (EC1) was measured using a DDS-307 conductivity meter at 25°C (Leici Company, Shanghai, China). The vial was transferred to a waterbath at 100°C for 30 min to release all of the electrolytes, before cooling to 25°C for measuring the electrical conductivity (EC2). ELR can be defined as follows:

$$ELR(\%) = EC1/EC2 \times 100.$$

Statistical analysis: All data were analyzed using the SPSS 19.0 statistical software program and expressed as means ± S.D. The least significant difference (LSD) test was performed for multiple comparisons to determine significant differences ($p<0.05$) between individual treatments. Two-way ANOVA was performed to test the significance of the main effects (salinity and pH group) and their interaction on physiological indices. Pearson coefficients and multiple linear regressions were performed in SPSS 19.0 (IBM Corp., in Armonk, NY, USA).

Results

Seed germination: The germination percentage and germination index of *M. officinalis* was the greatest in the distilled water treatment (Table 3). Salinity in combination with alkaline stress caused significant declines in germination percentages in all treatment groups, except for groups A1 and B1. The germination indices also decreased significantly under salt-alkali stresses. When salinity was greater than 150 mM, germination percentages and indices decreased sharply, regardless of pH (Table 3). A two-way ANOVA showed that germination percentage was affected significantly by salt ($F = 190.23$, $p<0.05$), pH ($F = 151.64$, $p<0.05$) and

their interaction ($F = 7.10$, $p<0.05$), while germination index was also affected significantly by salt ($F = 243.03$, $p<0.05$), pH ($F = 22.00$, $p<0.05$) and their interaction ($F = 2.89$, $p<0.05$) (Table 4).

The non-germinated seeds were placed in distilled water for recovery germination to examine their survival capacity. When pH was 7.3, all non-germinated seeds recovered and germinated, regardless of salinity; meanwhile, when pH was greater than 8.0 and salinity was greater than 100 mM, further increases in either salinity or pH inhibited the recovery germination of non-germinated seeds (Table 3). A two-way ANOVA showed that recovery germination percentage was not affected by salt ($F = 1.53$, $p<0.05$), but was affected significantly by pH ($F = 38.65$, $p<0.05$) and their interaction ($F = 1.90$, $p<0.05$) (Table 4).

Survival and growth: Survival rates of *M. officinalis* did not differ significantly from the controls in all group A treatments and B1-B5, C1-C4 and D1-D3 treatments (Table 3). When salinity was greater than 250 mM and pH was greater than 9.0, survival rates of seedlings decreased sharply. Therefore, physiological data were obtained from surviving plants in the remaining 21 treatments. A two-way ANOVA showed that seedling survival rate was affected significantly by salt ($F = 187.89$, $p<0.05$), pH ($F = 121.30$, $p<0.05$) and their interaction ($F = 40.68$, $p<0.05$) (Table 4).

RGR of aboveground and underground *M. officinalis* seedlings decreased significantly with increasing salinity and pH (Fig. 1A, B). A two-way ANOVA showed that RGR of aboveground material was affected significantly by salinity ($F = 7.85$, $p<0.05$) and pH ($F = 7.16$, $p<0.05$), but was not affected by their interaction ($F = 1.15$, $P=0.35$). RGR of underground parts of the plant was affected significantly by salinity ($F=22.92$, $p<0.05$) and pH ($F=18.85$, $p<0.05$), but not by their interaction ($F = 1.59$, $P=0.14$) (Table 4).

Water content of aboveground material was unaffected at low salinity (≤100 mM), regardless of the pH (Fig. 1C). A two-way ANOVA showed that the water content of the aboveground tissues was affected significantly by salt ($F = 15.41$, $p<0.05$), pH ($F = 4.47$, $p<0.05$) and their interaction ($F = 3.13$, $p<0.05$) (Table 4). No significant difference was detected in water content of underground tissues when salinity was less than 200 mM and pH was lower than 8.0 (Fig. 1D). A two-way ANOVA showed that water content of the underground was affected significantly by salt concentration ($F = 11.23$, $p<0.05$) (Table 4).

Table 3. Seed germination, germination index, recovery germination, and seedling survival rate of *Melilotus officinalis* under various salt and alkali stresses.

Salinity (mM)	Seed germination (%)	Germination index	Recovery germination (%)	Seedling survival rate (%)
0 (pH 7.1)	70.00 ± 5.77a	34.36 ± 5.3a	4.18 ± 2.22a	100% ± 0a
pH 7.3				
50	63.33 ± 6.67a	29.29 ± 4.60b	2.78 ± 4.81a	100% ± 0a
100	51.11 ± 1.92b	22.17 ± 0.61c	11.89 ± 5.70ac	100% ± 0a
150	47.78 ± 1.92b	12.66 ± 0.64d	25.21 ± 6.03b	100% ± 0a
200	41.11 ± 1.92c	10.50 ± 1.89de	24.09 ± 5.21b	100% ± 0a
250	35.56 ± 1.93cd	7.57 ± 1.46e	21.47 ± 5.16bc	100% ± 0a
300	32.22 ± 1.92d	5.39 ± 1.82e	20.72 ± 4.09bc	100% ± 0a
pH 8.0				
50	63.33 ± 5.77a	28.35 ± 2.96b	2.56 ± 4.44a	100%+0a
100	42.22 ± 8.39b	17.11 ± 3.82c	1.59 ± 2.75a	98.15% + 3.21a
150	17.78 ± 1.92c	8.31 ± 0.96d	1.28 ± 2.22a	100% ± 0a
200	12.22 ± 1.92cd	5.39 ± 0.50d	1.23 ± 2.14a	100% ± 0a
250	11.11 ± 1.92cd	4.94 ± 1.01d	0a	100% ± 0a
300	8.89 ± 3.85d	4.09 ± 1.53d	0a	44.17%± 1.36b
pH 9.0				
50	37.78 ± 9.62b	15.40 ± 2.05b	1.45 ± 2.51a	98.04% ± 3.39a
100	26.67 ± 3.34c	11.01 ± 2.43c	1.15 ± 1.99a	94.44% ± 9.62a
150	14.44 ± 5.09d	6.30 ± 2.04d	1.11 ± 1.92a	92.70 ± 3.40a
200	8.89 ± 1.92de	4.48 ± 0.42de	0a	92.66 ± 3.83a
250	7.78 ± 1.92de	3.39 ± 0.74de	0a	1.75 ± 3.03b
300	4.44 ± 1.93e	1.76 ± 0.50e	0a	3.41 ± 3.05b
pH 9.8				
50	60.00 ± 5.77b	27.84 ± 1.32b	1.75 ± 3.04a	100% ± 0a
100	38.89 ± 5.09c	16.38 ± 2.48c	1.28 ± 2.22a	100% ± 0a
150	12.22 ± 5.09d	5.71 ± 1.88d	0a	98.48 ± 2.63a
200	6.67 ± 3.34de	3.57 ± 1.29de	0a	84.77 ± 8.62b
250	5.56 ± 1.93de	2.03 ± 0.73de	0a	4.02 ± 0.83c
300	2.22 ± 1.92e	0.69 ± 0.65e	0a	1.85 ± 3.21c

Values represent means ± S.D. Values at each treatment group followed by different letters are significantly different ($p<0.05$)

Table 4. Two–way ANOVA of effects of salinity (S), treatment group (T), and their interactions on seed germination and seedling growth of *Melilotus officinalis*.

Physiological parameters	Source of variation (F)		
	S	T	S×T
Germination percentage (%)	190.23*	151.64*	7.10*
Germination index	243.03*	22.00*	2.89*
Recovery germination percentage (%)	1.53	38.65*	1.90*
Seedlings survival rate (%)	187.89*	121.30*	40.68*
RGR of aboveground (%)	7.85*	7.16*	1.15
RGR of underground (%)	22.92*	18.85*	1.59
Water content of aboveground (g/gDW)	15.41*	4.47*	3.13*
Water content of underground (g/gDW)	11.23*	2.13	0.68
Chlorophyll content of leaf (SPAD)	6.02*	2.56	1.18
Relative root activity (%)	33.53*	26.89*	6.97*
Electrolyte leakage rate (ELR)	12.25*	11.26*	1.53

Data represent *F*-values at 0.05 level

Chlorophyll content of leaf was determined using a Minolta chlorophyll meter (SPAD-502), and the digital reading was expressed as the "SPAD" to reflect the leaf chlorophyll content. Root activity was determined using the reduction of triphenyl tatrazolium chloride (TTC). Relative root activity of various treatments was expressed as a percentage relative to the control

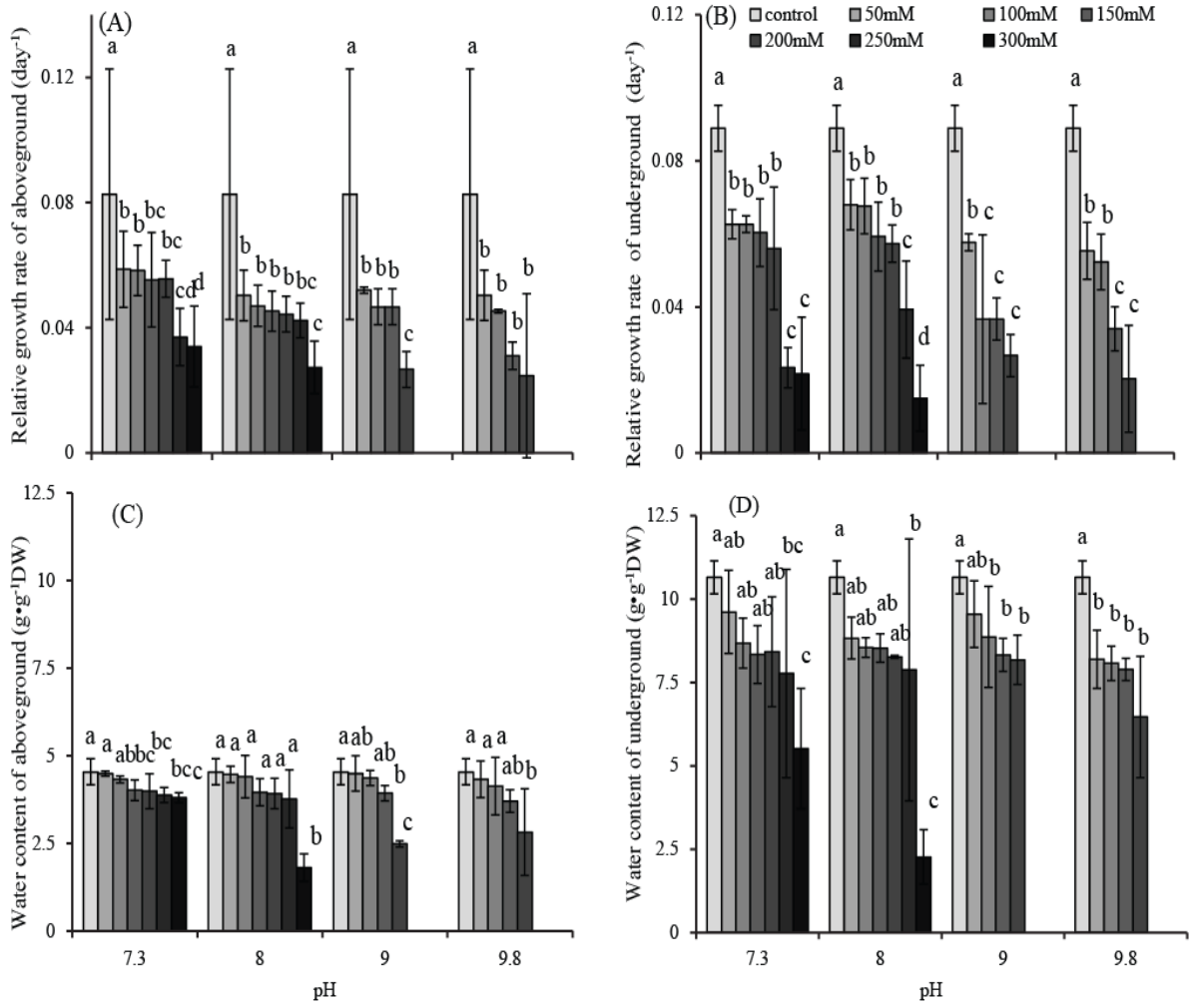

Fig. 1. RGR of aboveground (A), RGR of underground (B), water content of aboveground (C), and water content of underground (D) of *Melilotus officinalis* seedlings under various salt and alkali stresses. Values represent means ± S.D. Values at each treatment group followed by different letters are significantly different (p<0.05).

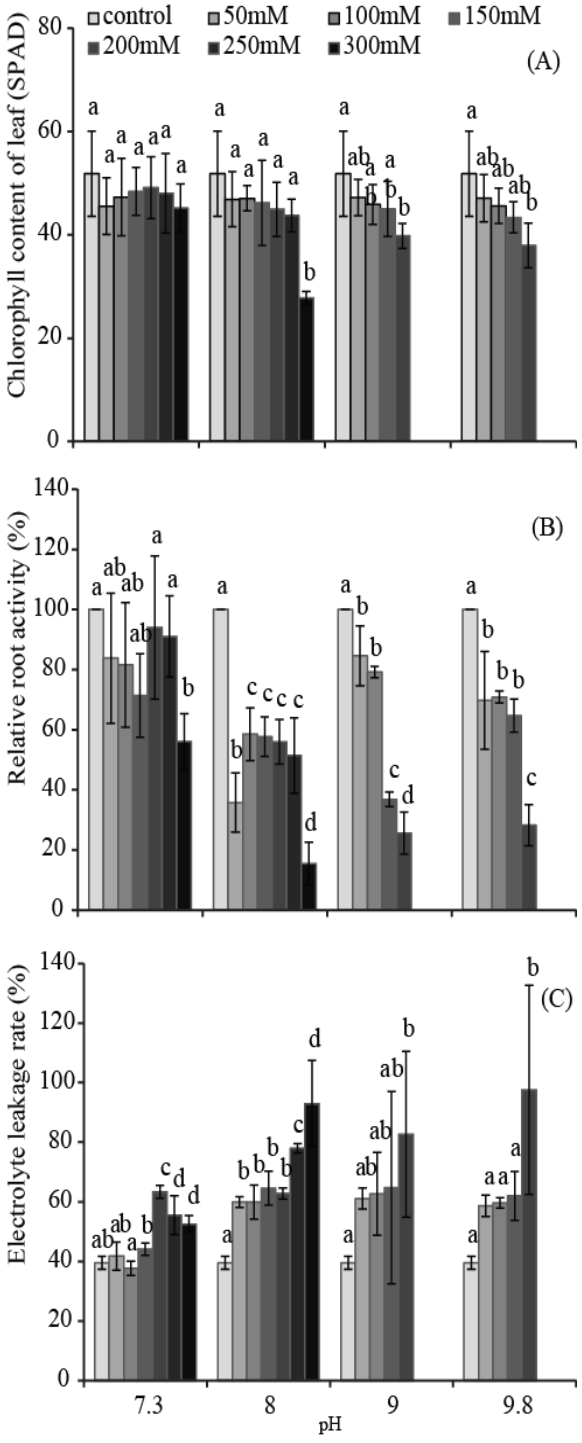

Fig. 2. Leaf chlorophyll content (A),relative root activity (B), and electrolyte leakage rate (C) of *Melilotus officinalis* seedlings under various salt and alkali stresses. Values represent means ± S.D. Values at each treatment group followed by different letters are significantly different (p<0.05). Chlorophyll content of leaf was determined using a Minolta chlorophyll meter (SPAD-502), and the digital reading was expressed as the "SPAD" to reflect the leaf chlorophyll content. Root activity was determined using the reduction of triphenyl tatrazolium chloride (TTC). Relative root activity of various treatments was expressed as a percentage relative to the control.

Leaf chlorophyll content, root activity, and membrane permeability: Leaf chlorophyll contents of *M. officinalis* seedlings did not decrease significantly in treatment groups A and B, except for B6. No significant difference was detected between stressed and control plants when salinity was lower than 150 mM in treatments C and D, regardless of pH (Fig. 2A). A two-way ANOVA showed that chlorophyll content was affected significantly by salinity ($F = 6.02$, $p<0.05$) (Table 4).

Relative root activity of *M. officinalis* seedlings was not decreased significantly in treatment group A, except for A6, but in the other groups activity decreased with increasing salinity and pH (Fig. 2B). A two-way ANOVA showed that relative root activity was affected significantly by salinity ($F = 33.53$, $p<0.05$), pH ($F = 26.890$, $p<0.05$) and their interaction ($F = 6.97$, $p<0.05$) (Table 4).

ELR of *M. officinalis* seedlings were not increased significantly at low salinity (≤100 mM) in treatment groups A, C and D (Fig. 2C). However, in treatment group B, ELR of seedlings increased significantly when salinity was greater than 50 mM. Two-way ANOVA showed that ELR of *M. officinalis* seedling roots was affected significantly by salt ($F = 12.25$, $p<0.05$) and pH ($F = 11.26$, $p<0.05$) (Table 4).

Correlation and multiple linear regressions: Stress factors of various salt and alkali stresses are shown in Table 2. The correlations between salinity and all parameters were significant, except for recovery germination percentage (p<0.01, Table 5), while correlations between pH and four parameters were significant (p<0.01, Table 5). The correlations between salinity and all stress parameters were greater than those between pH and all parameters, except for recovery germination percentage (Table 5).

Multiple linear regressions showed that the R^2 values for germination percentage, germination index, and RGR of above- and under-ground parts of the seedlings were larger than 0.61 (p<0.001), suggesting a relative high linear correlation between these parameters and the two stress factors. The remaining parameters exhibited weak correlation with the two stress factors (Table 6). In addition, the effects of the two stress factors on all parameters were different in magnitude (Table 6). In summary, salinity was the dominant factor, while pH was a secondary stress factor.

Discussion

Seed germination: Seed germination is a crucial stage in the life cycle of plants. The germination conditions had a considerable effect on the percentage germination (Hu *et al*., 2009). Under salt stress, delayed germination is a major factor inhibiting seed germination (Chen *et al*., 2012). Our results indicate that salt-alkali stresses significantly affect seed germination of *M. officinalis*, especially under combined high salinity and high pH conditions. The results also show that the magnitude of germination inhibition by alkaline stress on *M. officinalis* was greater than for saline stress (Table 3). In the present study, the seeds which did not germinate under low pH (7.3) conditions could subsequently recover and germinate, but only a few seeds could do this following initial high alkali and salt stresses. According to these results, we propose that non-germinated seeds may be a

strategy allowing *M. officinalis* to survive in high salinity soils. When soil salinity is high, the seeds may be in a state of dormancy to escape this unfavorable environment, but when it rains and soil salinity decreases the seeds are then able to germinate. Seeds which do not re-germinate have lost their viability and were permanently inhibited by the initial stress (Khan *et al*., 2001). This was mainly the case for seeds exposed to combined high salt and high pH stresses, which not only aggravates the effects of osmotic stress and ionic toxicity on the seeds but also decomposes seed structure and even destroys the embryo.

Survival and growth: Environmental factors controlled seed germination as well as seedling growth (Hu *et al*., 2013). In general, severe salt stress inhibits plant growth and even leads to plant death (Munns & Tester, 2008). However, *M. officinalis* is capable of surviving under relatively low pH (≤ 8.0) regardless of salinity or under moderate salinity (≤ 200 mM) regardless of alkalinity. The results confirm that *M. officinalis* is a salt-alkali tolerant species. Meanwhile, high salinity coupled with high pH caused the plants in groups C5, C6, D5 and D6 to die (Table 3). The effects of high pH were enhanced by increasing salinity. The growth inhibition caused by alkali stress was stronger than that resulting from salt stress (Fig. 1A, B), which is consistent with previous reports (Yang *et al*., 2007; Zhang & Mu, 2009). RGR of above- and under-ground were not affected by the interaction of salinity and pH, suggesting that the two stress factors influence the growth of *M. officinalis* independently rather than interactively. The results demonstrate that the effects of salinity, pH, and their interaction on parameter indices of *M. officinalis* are different. Therefore, further study will be focused on the causal effects of salinity, pH, and their interaction on plant growth. Combined salt-alkali stresses also induced reductions in water content of above- and under-ground tissues, especially under high salinity and high pH conditions (Fig. 1C, D). It is evident that a feature of combined salt-alkaline stress is the reciprocal enhancement of the individual components of salinity and alkalinity stress. Moreover, high salinity in combination with high pH led to a decrease in root activity and an increase in membrane permeability, which could damage root functions and cause inhibition of plant growth. Within the normal physiological adaptability of *M. officinalis*, its RGR decrease was likely due to reductions in leaf chlorophyll content, water content, and root activity under high combined salt-alkali stresses.

Table 5. Correlation coefficients between physiological indices and the two factors.

Physiological parameters	Stress factors	
	Salinity (mM)	pH
Germination percentage (%)	-0.77**	-0.54*
Germination index	-0.89**	-0.16
Recovery germination percentage (%)	0.12	-0.61**
Seedlings survival rate (%)	-0.60**	-0.41**
RGR of aboveground (%)	-0.61**	-0.45**
RGR of underground (%)	-0.73**	-0.34**
Water content of aboveground (g/gDW)	-0.61**	-0.20
Water content of underground (g/gDW)	-0.63**	-0.11
Chlorophyll content of leaf (SPAD)	-0.49**	-0.11
Relative root activity (%)	-0.56**	-0.17
Electrolyte leakage rate (ELR)	-0.55**	-0.27*

Asterisks indicate a significant (* at 0.05 level, ** at 0.01 level). n = 21

Chlorophyll content of leaf was determined using a Minolta chlorophyll meter (SPAD-502), and the digital reading was expressed as the "SPAD" to reflect the leaf chlorophyll content. Root activity was determined using the reduction of triphenyl tatrazolium chloride (TTC). Relative root activity of various treatments was expressed as a percentage relative to the control

Table 6. Multiple linear regressions between each physiological parameter and the two stress factors.

Y	Model	β_1	β_2	R^2
Germination percentage (%)	$Y=83.20–0.17X_1–7.75X_2$	-0.70	-0.43	0.83
Germination index	$Y=34.10–0.11X_1–1.71X_2$	-0.89	-0.16	0.82
Recovery germination rate (%)	$Y=19.27+0.02X_1–4.87X_2$	0.21	-0.64	0.42
Seedlings survival rate (%)	$Y=150.64–0.22X_1–9.80X_2$	-0.55	-0.33	0.46
RGR of aboveground (%)	$Y=0.08–0.006X_2$	-0.64	-0.45	0.61
RGR of underground (%)	$Y=0.10–0.007X_2$	-0.75	-0.39	0.68
Water content of aboveground (g/gDW)	$Y=5.28–0.006X_1–0.16X_2$	-0.63	-0.24	0.43
Water content of underground (g/gDW)	$Y=11.16–0.02X_1–0.27X_2$	-0.64	-0.16	0.42
Chlorophyll content of leaf (SPAD)	$Y=54.58–0.04X_1–1.51X_2$	-0.55	-0.24	0.30
Relative root activity (%)	$Y=109.25–0.18X_1–7.83X_2$	-0.64	-0.33	0.42
Electrolyte leakage rate (ELR)	$Y=24.63+0.13X_1+7.37X_2$	0.65	0.43	0.48

X_1 = Salinity; X_2 = pH. β_1, β_2: standardize regression coefficients corresponding X_1 and X_2. The greater the absolute β value, the stronger effect of the stress factor on physiological parameter. R^2: square of total correlation coefficient. n = 21. (p<0.001)

Chlorophyll content of leaf was determined using a Minolta chlorophyll meter (SPAD-502), and the digital reading was expressed as the "SPAD" to reflect the leaf chlorophyll content. Root activity was determined using the reduction of triphenyl tatrazolium chloride (TTC). Relative root activity of various treatments was expressed as a percentage relative to the control

Leaf chlorophyll content, root activity, and membrane permeability: Leaf chlorophyll content is an important physiological index in plants as it is related directly to photosynthesis. In this present study, no significant decreases in leaf chlorophyll content were observed in all group A treatments and in B1-B5, C1-C3 and D1-D3 treatments (Fig. 2A). These results indicate that high salinity in combination with low pH or low salinity in combination with high pH might not inhibit light absorption in *M. officinalis* seedlings. However, high salinity (≥200 mM) in conjunction with high pH (≥9.0) caused a significant decrease in leaf chlorophyll content. It is possible that the high salinity and high pH conditions inhibited chlorophyll synthesis (Shi & Zhao, 1997).

The TTC assay is useful for determining viability and for estimating metabolic or respiratory condition of various types of tissue (Hofstra *et al*., 1981). TTC is a sensitive indicator of changes in cellular metabolism, and this technique was used in the present study to indicate changes in root metabolism. Our results presented that the root activity of *M. officinalis* seedlings was not reduced significantly by low pH (7.3) and high salinity (250 mM), which demonstrates that root metabolism was unaffected (Fig. 2B). However, root activity declined significantly when pH was greater than 8.0, regardless of salinity, implying that the inhibiting action of alkaline stress on root metabolism is greater than for saline stress.

Membrane permeability reflects the degree of stress injury to plants (Lin & Wu, 1996; Surjus & Durand, 1996) and ELR is an ideal physiological index as it provides a measure of membrane permeability (Shi & Wang, 2005). As stress intensifies, generally there is increased injury to the plasma membranes and thus an increase in ELR. In the present study, the ELR of *M. officinalis* seedlings increased with increasing salinity and pH, which demonstrates that membrane permeability of *M. officinalis* seedlings declined not only with increasing salinity but also with increases in pH. These results indicate that salt-alkali stresses may damage the membrane system, and this may explain why the RGR of *M. officinalis* declined under high salt-alkali stress. Subsequently, a reciprocal enhancement between salt stress and alkali stress is an evident feature of combined salt-alkaline stress via the induction of injury to the plasma membrane.

The character of combined salt-alkaline stresses: In the present study, we took an objective estimate of the effects on plant growth caused by conditions in salt-alkalinized soil and constructed models to elucidate the nature of salt-alkali stresses. From regression analyses of ten parameters, it is evident that salinity is the dominant stress factor and this is in agreement with previous reports (Li *et al*., 2010b; Shi & Wang, 2005). The regression analyses showed that impact of salt or alkali stresses differed in magnitude on the ten physiological parameters. We speculate that these differences might be related to the physiological mechanism of the plant's response to stress, as well as the physiological processes associated with the response development.

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