

EFFECTS OF CALCIUM ON GERMINATION AND SEEDLING GROWTH IN *MELILOTUS OFFICINALIS* L. (FABACEAE) UNDER SALT STRESS

DA WEI ZHANG[†], THI SOAN VU[†], JUN HUANG, CHUN YU CHI, YI XING,
DONG DONG FU AND ZE NING YUAN*

¹College of life Sciences and Technology, Harbin Normal University, Harbin, 150025, China

*Corresponding author's email: xiaoyuan168ok@163.com

[†]These authors contributed equally to this paper.

Abstract

Melilotus officinalis L. (Fabaceae) is an important forage plant that has high contents of both protein and digestible fiber. In the present study, the effects of calcium chloride (0, 10 and 20 mM Ca²⁺) on *M. officinalis* germination and seedling growth under various levels of NaCl stress (0, 50, 100, 150 and 200 mM Na⁺) were investigated. The percentage germination was 66.67 % at salt levels ≤ 150 mM. Moreover, salt tolerance was greater in the seedlings than at germination level. An optimal Ca²⁺ concentration of 10 mM protected *M. officinalis* against salt stress, particularly in terms of germination and recovery germination under high salt conditions. In addition, 10 mM Ca²⁺ significantly enhanced the water content of early seedlings, as well as having a beneficial visual effect. The relative growth rate, biomass, and water content of underground parts and resource allocation of aboveground parts also increased significantly with addition of 10mM Ca²⁺. Moreover, 10mM Ca²⁺ had a stronger effect on seedling growth than 20mM Ca²⁺, suggesting that excessive Ca²⁺ combined with high salt concentrations imposes additional stress. Overall, these findings suggest that an optimal Ca²⁺ concentration of 10 mM contributes to seedling establishment during germination and growth. Appropriate application of exogenous Ca²⁺ could therefore help to improve salt tolerance in forage plants such as *M. officinalis* and those growing in saline-alkaline soil, so long as the optimal calcium level is applied.

Key words: Calcium chloride, Salt stress, Seed germination, Seedling growth, *Melilotus officinalis*.

Introduction

Soil salinization is increasing globally, having long-term effects on arable land (Boyer 1982). Widespread occurrence of saline and sodic soils has therefore resulted in numerous studies aimed at screening of enhanced tolerance in plants and improving our knowledge of salt stress. Tolerance to salt stress is defined as a plant's ability to grow and carry out its life cycle under high soluble salt concentrations (Parida, 2005). Understanding the mechanisms of salt tolerance is therefore crucial (Bartels & Sunkar, 2005). Germination and seedling growth are considered the crucial salt-sensitive period (Pujol *et al.*, 2000; Khan *et al.*, 2001). Investigations for seed germination under saline conditions suggest that salt stress negatively affects germination in most species (Mehrafarin *et al.*, 2011). Thus, seed germination on salt-containing substrate is a legitimate criterion for selection of salt-tolerant species (Sosa *et al.*, 2005).

Increasing salinity causes a reduction in the percentage of seed germination (Yuan & Shi, 2009; Xue *et al.*, 2012). However, seeds that fail to germinate can successfully do so if transferred from saline conditions to distilled water (Xue *et al.*, 2012). Salt exposure triggers a number of physiological changes; for example, inhibition of photosynthesis, the most fundamental of plant processes (Koyro 2006). Salt stress causes a decrease in leaf chlorophyll content, which in turn causes a decrease in net photosynthesis (Chookhampaeng, 2011). Moreover, increasing salt concentrations also cause a decrease in growth rate and water content (Yang *et al.*, 2009).

Calcium (Ca²⁺) is essential in plants, positively affecting overall growth (Sacała *et al.*, 2005). As a second messenger, Ca²⁺ causes a series of physiological and

ecological responses that protect plants from environmental stress (Li *et al.*, 2015). Previous studies have also shown sodium-induced Ca²⁺ deficiency in a number of plant species (Adcock *et al.*, 2001; Cramer, 2002). However, studies have also reported amelioration of the negative effects of salt on germination and seedling growth with the addition of supplemental Ca²⁺ (Gul & Khan, 2006; Shaikh *et al.*, 2007; Li *et al.*, 2016). Ca²⁺ therefore plays an important role in plant responses to salt (Rengel, 1992; Wang *et al.*, 2014). A significant increase in the biomass of *Malus xiaojinesis* as well as an increase in photosynthesis in sweet potato has also been observed with the addition of Ca²⁺ under salt stress (Wang *et al.*, 2014; Xu, 2014). Moreover, application of Ca²⁺ was found to cause an increase in the leaf water content and root activity in plants under salt stress (Yuan *et al.*, 2014). Typically, CaCl₂ has a greater alleviating effect on salt-stress induced injury than Ca (NO₃)₂ (Mao *et al.*, 2007).

Melilotus officinalis L. (Fabaceae), also known as yellow sweet clover, is an important forage plant with a long taproot that allows it to grow in semi-arid soil as well as a large digestible fiber content and high-quality protein (Guo *et al.*, 2012). The *M. officinalis* root system has also been shown to improve fertility in soil that is dry or semi-arid (Guo *et al.*, 2012). The fungicide properties of *M. officinalis* extracts have also been confirmed (Yin *et al.*, 2009). Furthermore, in a recent study on salt and alkaline stress during germination and seedling growth, it was revealed that *M. officinalis* showed moderate salt-alkali tolerance (VU *et al.*, 2015). Thus, based on its environmental and economic value, understanding the protective effect of Ca²⁺ on salt stress during the early stages of *M. officinalis* growth is important. However, at present,

little is known about the mechanisms of Ca^{2+} functions under salt stress. This study examined the effects of Ca^{2+} on germination and seedling growth in *M. officinalis* plants under salt stress. To do so, the germination percentage, recovery germination percentage, and water content of early seedlings, and growth rate, resource allocation, chlorophyll content, root activity, and water content of seedlings were determined. The findings provide a basis for further utilizations of *M. officinalis* in China.

Materials and Methods

Salt and Ca^{2+} treatment: The functions and mechanism of Ca^{2+} in *M. officinalis* under salt stress were examined using three Ca^{2+} treatments: 0, 10, and 20 mM Ca^{2+} (A, B, and C, respectively). Under each treatment, five concentrations of salt (NaCl) were applied: 0, 50, 100, 150, and 200 mM (A1–A5, B1–B5, and C1–C5, respectively) (Table 1).

Experiment 1: Germination: Seeds were collected from *M. officinalis* growing in the middle of the Songnen Plain (46°04' N 125°93' E to 44°54' N 127°97' E), northeastern China, in September 2014 and kept at $4 \pm 1^\circ\text{C}$ until use. To break dormancy, the seeds underwent mechanical scarification consisting of vigorous rubbing to abrade the seed coat using 100 grit sandpaper (Süleyman *et al.*, 2013) followed by 10-min surface sterilization using 0.1% KMnO_4 then rinsing with distilled water. Average-size fully-developed seeds were then transferred to Petri dishes (9 cm in diameter) for germination experiment according to Vu *et al.*, with slightly modification (Vu *et al.*, 2015). Germination experiments were carried out in triplicate with 30 seeds per dish 3 replications. The dishes were incubated (Eyela Eyelatron FLI-301 NH, Rikakikai, Tokyo, Japan) under a light / dark photoperiod of 14 / 10 h at a light intensity of $100 \mu\text{M m}^{-2}\text{s}^{-1}$ at $25 / 15^\circ\text{C}$ (day / night). Germinating seeds were counted daily and fresh culture medium added periodically. The recovery germination detection was performed and germination index was calculated according to Mastafa *et al.*, (2012) and Vu *et al.*, (2015):

$$1. \text{ Germination (\%)} = (a / b) \times 100 \%$$

where *a* is the number of germinated seeds, and *b* is the total seeds number in the experiment.

$$2. \text{ Recovery germination percentage (\%)} = (c / d) \times 100 \%$$

where *c* is the seeds number showing germination recovery and *d* is the total seeds number.

The water content of early-stage seedlings was determined on day seven. All seedlings were collected then rinsed in distilled water. Remaining surface water was removed using filter paper then 10 seedlings per treatment were randomly selected for fresh weight measurements (FW). Dry weight (DW) was measured after drying the seedlings to a constant mass at a

temperature of 70°C . The following formula was then used to measure early seedling water contents (g/g DW) (Yang *et al.*, 2009):

$$3. \text{ Water content} = (\text{FW} - \text{DW}) / \text{DW}.$$

Experiment 2: Seedling experiments

Seedling growth: The seed germination conditions were as in Experiment 1. After 3 days, seedlings showing uniform germination were transferred to 20-cm deep plastic pots (25 plants per pot) with a diameter of 17 cm and filled with vermiculite. The pots were then placed outdoors at a day / night temperature of $20\text{--}25 / 15\text{--}20^\circ\text{C}$ and protection from the rain. The seedlings were watered daily using Hoagland's solution (Shi *et al.*, 1998).

Stress treatments: Stress treatments were carried out using 5-week-old seedlings. A total of 48 pots containing uniform-sized seedlings were divided randomly into 16 treatments (three pots per each treatment): 14 salt stress treatments, 1 control treatment (A1) and a final treatment to determine fresh and dry weights prior to treatment. Treatment solutions were increased daily in gradual increments of 50 mM (Na^+ concentration) up to the desired level. Control seedlings (A1) were watered with Hoagland's solution. Stress treatment was conducted at 07:00 a.m. by watering each pot with 500-mL treatment solution (three pots per treatment) once daily. Stress treatments lasted 4 weeks. Evaporated water was supplemented every day using distilled water (Yang *et al.*, 2009).

Physiological analysis

Growth characters, seedlings biomass and resource allocation: Plants were harvested 4 weeks after initiating treatment, rinsed with tap water then distilled water. The remaining water was removed using filter paper. Ten seedlings per treatment were randomly selected for the measurements of FW. Each plant was divided into aboveground and underground parts then dried at 70°C to a constant mass to determine the DW. Remaining fresh underground samples were used to measure root activity. Biomass was calculated using the mean aboveground and underground DW. Relative growth rates (RGR) were determined as follows (Kingsbury *et al.*, 1984):

$$4. \text{ RGR (\%)} = [\ln(\text{final DW}) - \ln(\text{initial DW})] / \text{duration of treatment (days)}.$$

The water content of the seedlings was calculated as follows (Yang *et al.*, 2009):

$$5. \text{ Water content} = (\text{FW} - \text{DW}) / \text{DW}.$$

Resource allocation of aboveground parts was calculated as the proportion of stem and leaf weights relative to the overall DW. Resource allocation of underground parts was measured as the proportion of root weights to the overall DW (Yuan *et al.*, 2011).

Table 1. Stress factors under each treatment.

Treatments Ca ²⁺ (mM)	NaCl (mM)				
	0	50	100	150	200
0 (A)	A1 (control)	A2	A3	A4	A5
10 (B)	B1	B2	B3	B4	B5
20 (C)	C1	C2	C3	C4	C5

Chlorophyll content, root activity, and water content: Chlorophyll content was calculated after 4-weeks stress treatment using a chlorophyll meter (SPAD 502; Konica Minolta, Tokyo, Japan), and expressed as digital readings known as “SPAD”. Chlorophyll contents were measured between 08:30 and 11:00 a.m using leaves from plants in each pot.

Activity of *M. officinalis* seedling roots was examined using a reduction assay with triphenyl tetrazolium chloride (TTC), with slight modifications (Comas *et al.*, 2000). TTC was used here to determine changes in root activity. Freshly prepared root samples were immersed in TTC solution at 37°C for 1.5 h (0.4% TTC in phosphate buffer, pH 7.0). To stop the reaction, sulfuric acid was added then ethyl acetate used to extract red product. Absorbance was then measured at 485 nm using a spectrophotometer. The percentage root activity under each treatment was calculated relative to the activity of control roots (Yang *et al.*, 2009).

Statistical analyses

Data were analyzed using SPSS software version 19.0 (IBM Corp., Armonk, NY, USA) and were indicated as means ± S.D. LSD (Least Significant Difference) was used to conduct pairwise comparisons of means ($p < 0.05$), and two-way ANOVA used to detect the significance of salinity and calcium as well as their interactive effect on the parameters. Pearson coefficients were also obtained along with multiple linear regressions.

Results

Seed germination characteristics of *M. officinalis*: The germination percentage decreased with increasing salt concentration from 0 to 200 mM (Fig. 1A); however, no significant differences were observed between 10 mM Ca²⁺ and the control ($p < 0.05$; Fig. 1A). Moreover, at a high salt concentration (200 mM), the germination percentage was higher at 10 mM Ca²⁺ than 0 mM Ca²⁺ ($p < 0.05$; Fig. 1A). Meanwhile, at 20 mM Ca²⁺, the germination percentage decreased significantly compared with the control ($p < 0.05$; Fig. 1A). A significant effect of salt, calcium and their interaction on the germination percentage was revealed using two-way ANOVA ($F = 51.31$, $F = 52.12$, and $F = 5.05$ ($p < 0.05$), respectively) (Table 2).

To examine the survival capacity of non-germinated seeds, seeds unable to germinate under salt stress were treated with distilled water to examine germination recovery. As shown in Fig. 1B, the addition of Ca²⁺ enhanced recovery germination, particularly at high salt concentrations. At 200 mM salt, the recovery germination percentage was significantly higher than the control at 10 and 20 mM Ca²⁺ ($p < 0.05$; Fig. 1B). Significant effects of salt and calcium, but not their interaction, on the recovery

germination percentage were also revealed using two-way ANOVA ($F = 14.66$, $F = 8.86$, and $F = 1.60$ ($p < 0.05$), respectively) (Table 2).

The water content of early-stage seedlings was decreased significantly at salt concentrations ≥ 150 mM ($p < 0.05$; Fig. 1C). However, the addition of Ca²⁺ significantly enhanced the water content of primary seedlings at 0, 50 and 100 mM. Significant effects of salt, calcium and their interaction on the water content of early-stage seedlings were observed using two-way ANOVA ($F = 374.95$, $F = 30.72$, and $F = 31.91$, ($p < 0.05$), respectively) (Table 2). Moreover, radicle elongation of early-stage seedlings decreased with increasing salinity, and was strongly inhibited at high salt concentrations (Fig. 2A). However, the radicle length of early-stage seedlings was greatly improved by the addition of Ca²⁺ (Fig. 2B, C).

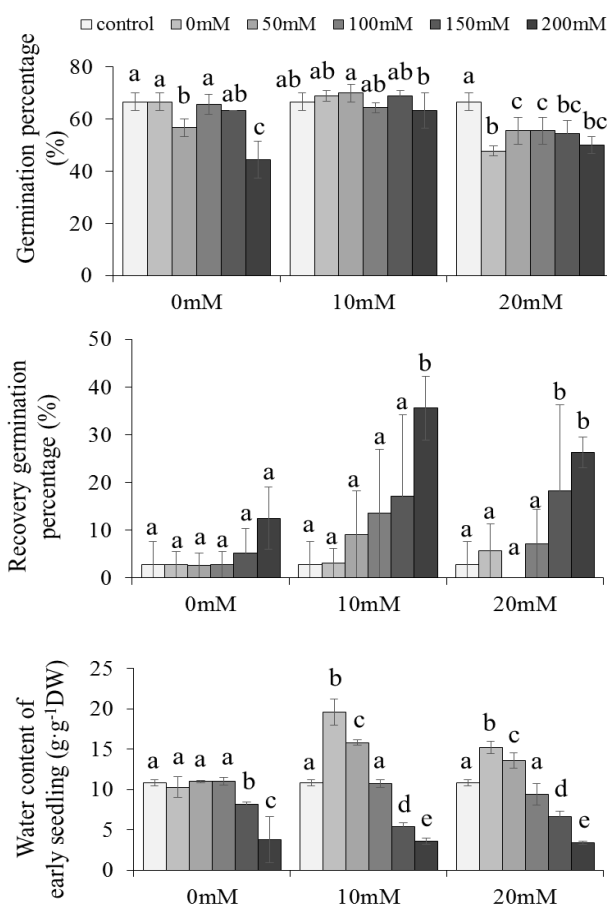


Fig. 1. Seed germination (A), recovery germination (B), and the water content of early seedlings (C) in *M. officinalis* plants grown under various levels of salt and calcium. Values represent the mean ± S.D. Values in each treatment group followed by a different letter are significantly different ($p < 0.05$) (The same as in the figure 3 and 4).

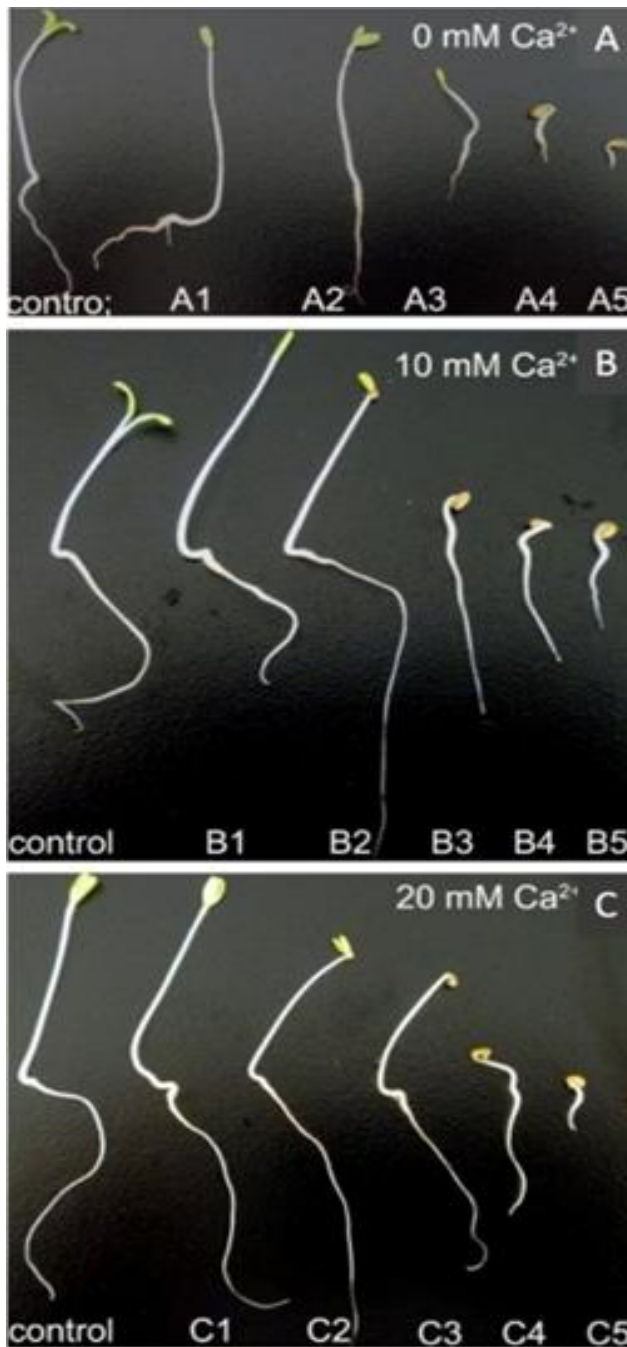


Fig. 2. Effect of calcium chloride on radicle elongation in early-stage seedlings of *M. officinalis* under saline conditions.

Growth characters, seedling biomass, and resource allocation: Without the addition of Ca²⁺, the RGR of aboveground parts was decreased significantly with salt concentrations exceeding 50 mM ($p < 0.05$; Fig. 3A). However, at 10 mM Ca²⁺, the RGR of aboveground tissues was not significantly different from the control at a 0 to 100 mM Na⁺ (Fig. 3A). Significant effects of salt and calcium, but not their interaction, on the RGRs of aboveground tissue were revealed with two-way ANOVA ($F = 22.10$, $F = 6.51$, and $F = 0.71$ ($p < 0.05$), respectively) (Table 2). At 10 mM Ca²⁺, the RGR of underground parts was not significantly different from that of control plants under increasing salinity ($p < 0.05$; Fig. 3B). Meanwhile, at 20 mM Ca²⁺, the RGR of underground parts was decreased significantly with salt concentrations exceeding

100 mM (Fig. 3B). Two-way ANOVA revealed a significant effect of salt on underground RGR ($F = 7.32$, $p < 0.05$) (Table 2).

The biomass of aboveground parts decreased significantly with salt levels exceeding 50 mM, and Ca²⁺ had no effect ($p < 0.05$; Fig. 3C). In contrast, Ca²⁺ enhanced the biomass of underground parts, especially at 10 mM Ca²⁺ ($p < 0.05$; Fig. 3D). Significant effects of salt and calcium on aboveground biomass was found using two-way ANOVA ($F = 29.22$ and $F = 6.16$, ($p < 0.05$), respectively) (Table 2). Meanwhile, only salt had a significant effect on the underground biomass ($F = 6.49$, $p < 0.05$) (Table 2).

Resource allocation of aboveground and underground parts was unaffected at 10 mM Ca²⁺ with increasing salinity ($p < 0.05$; Fig. 3E, F). Significant effects of salt and calcium on resource allocation of aboveground parts was observed using two-way ANOVA ($F = 12.35$ and $F = 7.88$ ($p < 0.05$), respectively) (Table 2). Resource allocation of underground parts was significantly affected by salt only ($F = 26.83$, $p < 0.05$) (Table 2).

Chlorophyll content, root activity, and water content:

No significant differences in leaf chlorophyll contents were detected at 0 and 10 mM Ca²⁺ ($p < 0.05$; Fig. 4A). Meanwhile, a significant effect of salt was revealed by using two-way ANOVA ($F = 4.69$, $p < 0.05$) (Table 2). Without Ca²⁺, no significant differences in relative root activity were observed compared with the control ($p < 0.05$; Fig. 4C). Positive effects were observed with addition of 10 mM Ca²⁺ at 0 mM NaCl only. Both salt and calcium caused a decrease in relative root activity ($p < 0.05$; Fig. 4C). Significant effects of salt, calcium and their interaction on relative root activity were demonstrated by using two-way ANOVA ($F = 4.34$, $F = 12.16$ and $F = 4.82$ ($p < 0.05$), respectively) (Table 2).

Without Ca²⁺, no significant differences were observed in the water content of aboveground compared with control plants at salt levels of less than 150 mM ($p < 0.05$; Fig. 4B). Salt and calcium treatment did not affect the water content aboveground according to two-way ANOVA (Table 2). With the addition of 10 mM Ca²⁺, underground water content was also unaffected by salt stress ($p < 0.05$; Fig. 4D). Furthermore, the underground water content was higher at 10 mM Ca²⁺ than at 0 and 20 mM Ca²⁺ under salt concentrations not exceeding 150 mM (Fig. 4D). No significant effects of salt or calcium treatment on the water content of underground tissues were found using two-way ANOVA (Table 2).

Correlations and multiple linear regressions: Table 1 shows the various stress factors. Significant correlations were observed between salt and all parameters, except for relative root activity, the water content of aboveground and underground parts, and resource allocation to aboveground and underground parts. Moreover, significant correlations were also observed between calcium and four parameters of germination percentage, RGR aboveground, relative root activity, and water content aboveground ($p < 0.05$; Table 3). Correlations between salt and each stress parameter were greater than those between calcium and each parameter, except for relative root activity and the water content of aboveground parts (Table 3).

Table 2. Two-way ANOVA showing the effects of salt (S), calcium (Ca), and the interactive effect on germination and seedling growth in *M. officinalis*.

Physiological parameter	Source of variation (F)		
	S	Ca	S × Ca
Germination percent (%)	51.31*	52.12*	5.05*
Recovery germination percentage (%)	14.66*	8.86*	1.60
Water content of primary seedling (g/g DW)	374.95*	30.72*	31.91*
RGR aboveground (%)	22.10*	6.51*	0.71
RGR underground (%)	7.32*	0.46	0.21
Biomass aboveground (g DW)	29.22*	6.16*	0.49
Biomass underground (g DW)	6.49*	0.36	0.27
Resource allocation aboveground (g/g DW)	12.35*	7.88*	3.57
Resource allocation underground (g/g DW)	26.83*	10.23	4.29
Chlorophyll content (SPAD)	4.69*	0.78	0.70
Relative root activity (%)	4.34*	12.16*	4.82*
Water content aboveground (g/g DW)	0.26	2.30	0.97
Water content underground (g/g DW)	0.95	1.77	0.52

F-values: 0.05

Chlorophyll content, root activity and relative root activity were determined as described in the Material and Methods

Table 3. Correlation coefficients between the physiological indices and two stress factors.

Physiological parameter	Stress NaCl	Factor
		CaCl ₂
Germination percentage (%)	-0.58*	-0.39*
Recovery germination percentage (%)	0.56*	0.21
Water content of primary seedling (g/g DW)	-0.89*	0.07
RGR aboveground (%)	-0.76*	-0.28*
RGR underground (%)	-0.55*	-0.11
Biomass aboveground (g DW)	-0.79**	-0.23
Biomass underground (g DW)	-0.49**	-0.11
Resource allocation aboveground (g/g DW)	-0.51	-0.16
Resource allocation underground (g/g DW)	0.49	0.37
Chlorophyll content (SPAD)	-0.56*	0.14
Relative root activity (%)	-0.07	-0.46*
Water content aboveground (g/g DW)	-0.09	-0.32*
Water content underground (g/g DW)	-0.18	0

* Significant difference at 0.05 and ** at 0.01 (n = 16)

Chlorophyll content, root activity and relative root activity were determined as described in the Material and Methods

Table 4. Multiple linear regression analyses of the physiological indices and two stress factors.

Y	Model	β ₁	β ₂	R ²
Germination percentage (%)	Y=68.93-0.09X ₁ -0.55X ₂	-0.58	-0.39	0.49
Recovery germination percentage (%)	Y=-2.69+0.10X ₁ +0.32X ₂	0.65	0.23	0.48
Water content of primary seedling (g/g DW)	Y=15.37-0.06X ₁ +0.04X ₂	-0.89	0.07	0.80
RGR aboveground (%)	Y=5.72-0.01X ₁ -0.04X ₂	-0.76	-0.28	0.66
RGR underground (%)	Y=7.47-0.01X ₁ -0.01X ₂	-0.57	-0.14	0.31
Biomass aboveground (g DW)	Y=0.88-0.09X ₁ -0.05X ₂	-0.79	-0.23	0.67
Biomass underground (g DW)	Y=0.47-0.03X ₁ -0.01X ₂	-0.49	-0.11	0.25
Resource allocation aboveground (g/g DW)	Y=30.67-0.03X ₁ -0.08X ₂	-0.51	-0.16	0.68
Resource allocation underground (g/g DW)	Y=18.36+0.02X ₁ +0.04X ₂	0.49	0.37	0.47
Chlorophyll content (SPAD)	Y=46.57-0.03X ₁ +0.07X ₂	-0.56	0.14	0.33
Relative root activity (%)	Y=108.84-0.04X ₁ -2.03X ₂	-0.07	-0.46	0.21
Water content aboveground (g/g DW)	Y=3.61-0.02X ₂	-0.08	-0.37	0.11
Water content underground (g/g DW)	Y=6.04-0.01X ₁	-0.21	0	0.03

X₁ = Salinity; X₂ = Calcium. β₁, β₂: standardized regression coefficients corresponding to X₁ and X₂. The greater the absolute β value, the stronger the effect of the stress factor. R²: square of the total correlation coefficients (n = 16; p<0.001)

Chlorophyll content, root activity and relative root activity were determined as described in the Material and Methods

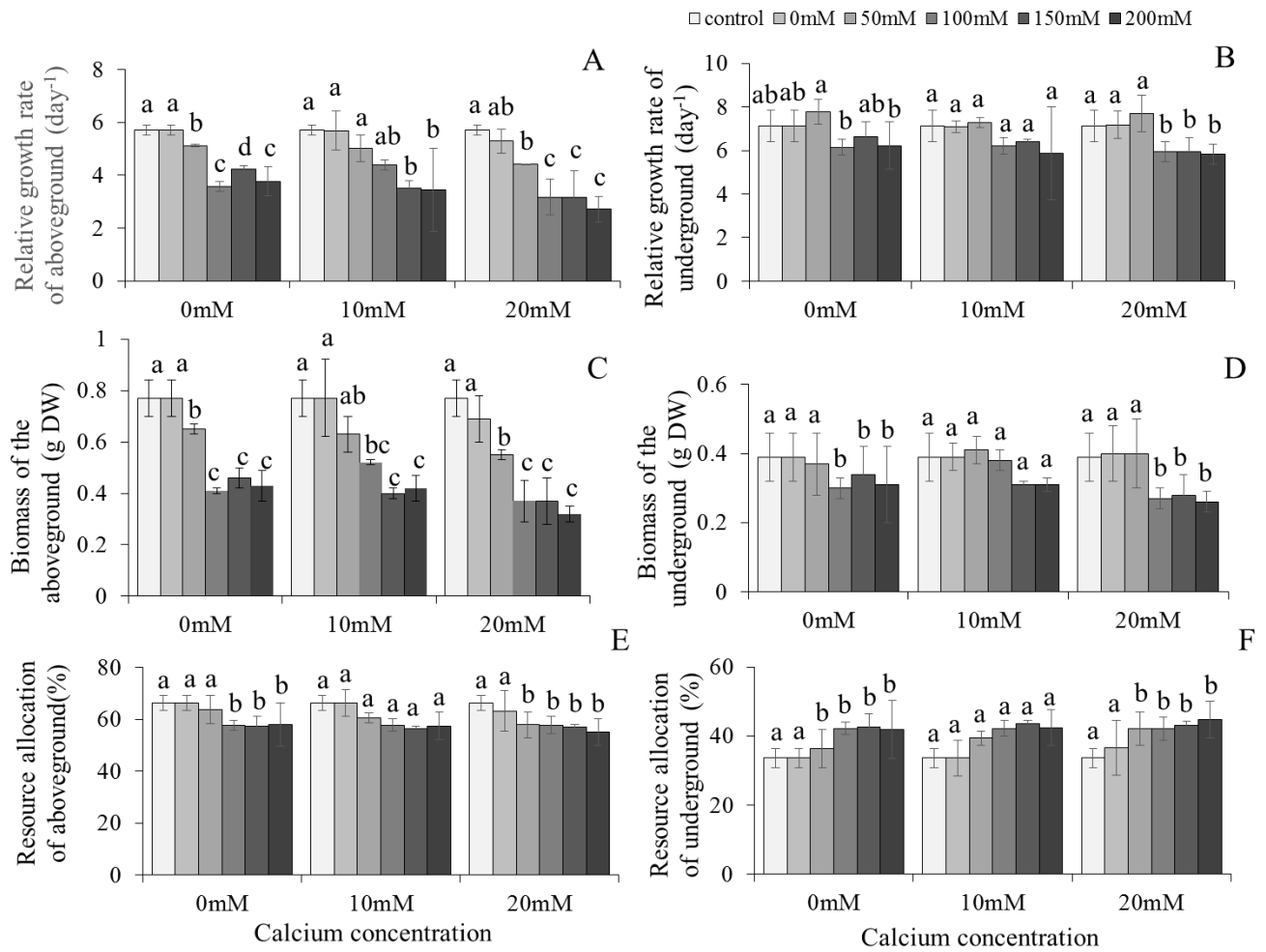


Fig. 3. Relative growth rates of aboveground (A) and underground parts (B), the biomass of aboveground (C) and underground parts (D), and resource allocation of aboveground (E) and underground parts (F) of *M. officinalis* grown under various levels of salt and calcium.

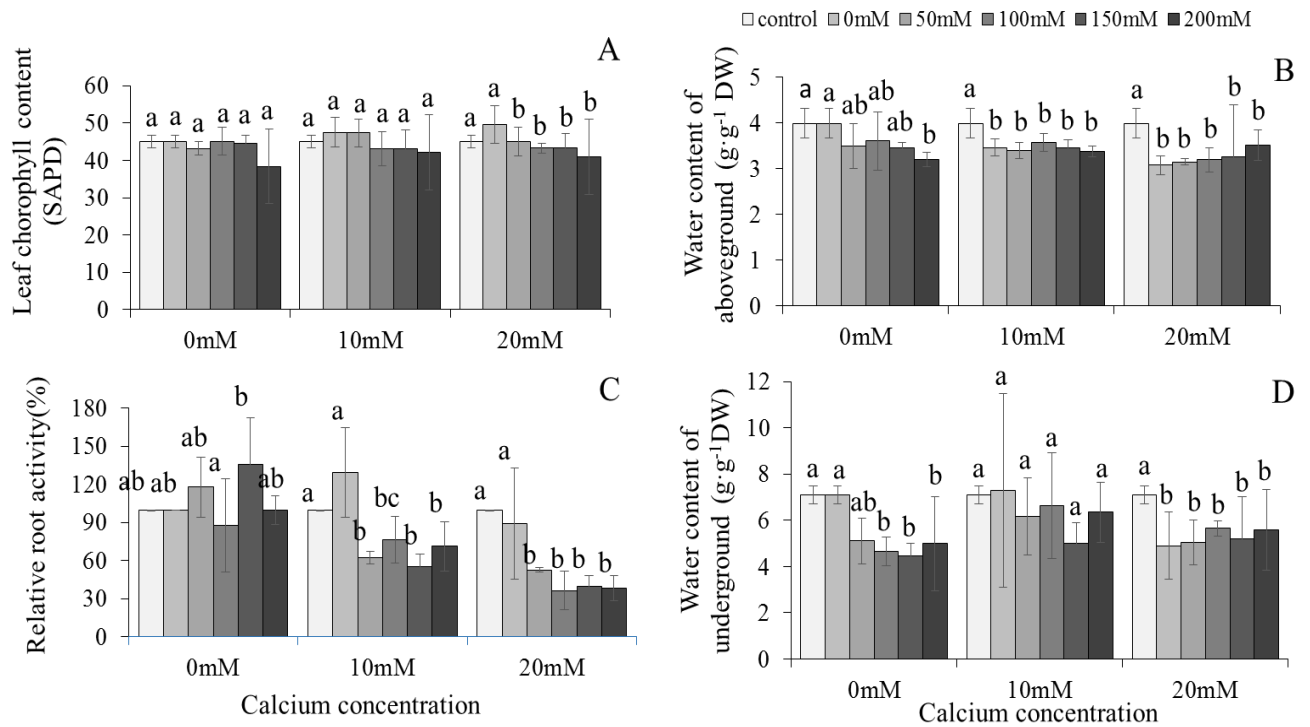


Fig. 4. Leaf chlorophyll content (A), water content of aboveground parts (B), relative root activity (C), and the water content of underground parts (D) of *M. officinalis* grown under various levels of salt and calcium.

Multiple linear regressions showed that R² values for the water content of primary seedlings, RGR, biomass and resource allocation of aboveground parts were > 0.66 ($p < 0.001$), indicating a relatively high linear correlation. All remaining parameters exhibited weak correlations with both factors (Table 4).

Discussion

Seed germination: It is well established that exposure to NaCl can cause nutritional disruption in plants, particularly in terms of calcium deficiency (Rengel 1992). It is therefore possible that the deleterious effects of salt stress could potentially be ameliorated by the addition of Ca²⁺ to saline media. In line with this, previous studies investigated whether adding Ca²⁺ to salinized nutrient solution could reduce the negative effects of NaCl (Rengel 1992; Elphick *et al.*, 2001; Wang *et al.*, 2016). Our results revealed an increase in germination percentage with the addition of 10 mM Ca²⁺ (Fig. 1A). That is, the negative effects of high salt stress on the germination of *M. officinalis* seeds were ameliorated by the addition of Ca²⁺ (10 mM), in agreement with previous studies (Cachorro *et al.*, 1994; Wang *et al.*, 2016). However, at 20 mM Ca²⁺, the germination percentage was lower than that of the control, suggesting that excessive Ca²⁺ can also impose stress on the plant (Fig. 1A). Moreover, the highest germination percentage observed was 66.67 %, with integrative characteristics of seedling growth showing good vigor at salt levels ≤ 150 mM (Fig. 1A). These findings suggest that *M. officinalis* possesses moderate tolerance to salt stress.

Recovery germination reflects the ability of seeds to remain dormant under saline conditions (Ungar 1996). Most salt-tolerant seeds maintain viability during extended periods of exposure to hypersaline conditions, germinating when conditions improve (Khan & Ungar 1997). In the present study, salt stress did not completely eliminate the ability of *M. officinalis* seeds to germinate and seed viability was maintained. Thus, salt stress only temporarily inhibited germination. Furthermore, the greater the salt-induced inhibition of seed germination, the better the germination recovery rate when the stress was removed (Fig. 1B). We suggest, therefore, that non-germinated *M. officinalis* seeds possess a strategy that allows them to survive under high salt conditions. Moreover, the addition of Ca²⁺ significantly increased the recovery germination percentage under conditions of high salt stress; notably, a Ca²⁺ concentration of 10 mM (Fig. 1B). These results suggest that supplemental Ca²⁺ can protect *M. officinalis* against the effects of salt stress during seed germination, particularly at higher salt concentrations. Significant retardation of root elongation can also occur under high salt conditions during germination (Qu *et al.*, 2008). In the present study, radicle elongation was sensitive to salt stress at NaCl concentrations exceeding 100 mM (Fig. 2A). However, addition of Ca²⁺ helped improve radicle elongation (Fig. 2B, C), suggesting that optimal Ca²⁺ can aid seedling establishment during germination and early growth.

The osmotic pressure in saline soil solution resulting from the high salt concentration exceeds that in plant cells, thereby reducing the ability of plants to uptake water (Munns *et al.*, 2006). Water content therefore has a potential effect on plant growth (Moorea *et al.*, 2008). One of the important functions of supplemental Ca²⁺ is to prevent the inhibition of root hydraulic conductivity (Azaizeh *et al.*, 1992), which could subsequently enhance the water content. In the present study, supplemental Ca²⁺ significantly increased the water content of early *M. officinalis* seedlings at salt concentrations ranging from 0 to 100 mM; most notably, at 10 mM Ca²⁺ (Fig. 1C). Moreover, during the seedling growth stage with supplemental Ca²⁺ (10 mM) effectively prevented the reduction in water content of underground parts during salt stress (Fig. 4D), similar to a previous study (Azaizeh & Steudle 1991). Adjustment of roots to the saline medium results in an increase in water in the plant cells (Rengel 1992); therefore, we speculate that supplemental Ca²⁺ prevents the inhibition of hydraulic conductance in roots of *M. officinalis*, thereby helping the plant absorb more water from the soil.

Growth characteristics: Ca²⁺ is an important secondary messenger in plants (Kader & Lindberg, 2010), stimulating growth in the presence of salt. Growth of salt-stressed maize was partially restored with supplemental Ca²⁺ (Azaizeh *et al.*, 1992), while in the present study, a positive effect of 10 mM supplemental Ca²⁺ on the RGR was observed at salt concentrations of less than 100 mM (Fig. 3B). These findings suggest that *M. officinalis* shows innate salt tolerance during growth, and thus, the addition of Ca²⁺ (10 mM) only slightly promoted the RGR. Overall, therefore, supplemental Ca²⁺ (10 mM) improved *M. officinalis* growth at salt concentrations not exceeding 150 mM (Fig. 3A, B), particularly in terms of underground growth, which is consistent with previous reports (Hou *et al.*, 2015; Yang *et al.*, 2017). Thus, supplemental addition of 10 mM Ca²⁺ was beneficial in helping underground parts to accumulate material for growth (Fig. 3D, F), thereby reducing the negative effects of salt stress. However, 20 mM Ca²⁺ imposed stress on plant growth under high salt concentrations (Fig. 3A, B, C, D, E). These results suggest that the mitigating effects of exogenous Ca²⁺ are therefore limited. An appropriate Ca²⁺ level could help promote growth and alleviate salt stress damage, even though the alleviating effects were not as obvious under high salt stress and high Ca²⁺ levels, which often had an inhibitory effect (Murillo-Amador *et al.*, 2006).

Chlorophyll content, root activity, and water content: Chlorophyll content is directly related to photosynthesis and is therefore an important physiological index. Salt stress can reduce the chlorophyll content in saline environments (Chookhampaeng 2011). Here, no significant decrease in leaf chlorophyll content was observed both without Ca²⁺ and under 10 mM Ca²⁺, suggesting that salt and optimal calcium concentrations did not inhibit light absorption. However, 20 Ca²⁺ treatment combined with 100-200 mM salt caused a decrease in leaf chlorophyll content compared with the control, suggesting that 20 mM Ca²⁺ imposed stress on

photosynthesis. Photosynthesis can affect the growth rate, and can also act as growth rate-regulated feedback (Cramer, 2002). Moreover, root activity was not inhibited under high salt stress, inconsistent with previous studies (Yang *et al.*, 2009; Zhang & Mu, 2009). Although little is known about the effects of Ca^{2+} on root activity, our results suggest that the addition of Ca^{2+} irregularly enhances root activity, unlike indices such as germination, seedling growth and water content. Triphenyltetrazolium chloride (TTC) is transformed into triphenyltetrazolium formazan (TTF: red insoluble compound) in the roots; thus, investigation is now needed to determine whether Ca^{2+} can interfere with this transformation. A greater understanding of the effects of supplemental Ca^{2+} in terms of root activity is therefore required.

Combined salt-calcium effects: This study aimed to understand the effect of salt and calcium during plant growth, subsequently constructing a model showing these effects. Regression analyses of eight parameters revealed that the effect of salt stress was dominant. Moreover, regression analysis showed the differing effects of salt and calcium on seven parameters. These differences likely reflect the physiological mechanism of the plant responses to salt and calcium.

Conclusions

In conclusion, *M. officinalis* is a moderate salt tolerant species. This tolerance is related to the ability of seeds to germinate and seedlings to grow at NaCl concentrations of 150 mM or lower. Physiological indices measured during salt stress partially improved with supplemental Ca^{2+} , thereby improving overall salt tolerance. Overall, Ca^{2+} exerted a greater positive effect on germination than on seedling growth, while the optimal concentration for enhancing salt tolerance and promoting seedling growth in *M. officinalis* was found to be 10 mM.

Acknowledgements

This work was supported by the Natural Science Foundation of Heilongjiang Province, China (grant number C201306), the Pre-research of Science and Technology Development Project by Harbin Normal University (grant number 12XYG-03), the Science and Technology Project by Heilongjiang Province Education Department (grant number 12541248), and the National Natural Science Foundation of China (grant number 31400444). We thank Dr. Jingmei Lu for her help with collection of seeds of *M. officinalis*. We also thank the native English speaking scientists of Elixigen Company (Huntington Beach, California).

References

- Adcock, K.G., J.W. Gartrell and R.F. Brennan. 2001. Calcium deficiency of wheat grown in acidic sandy soil from Southwestern Australia. *J. Plant Nutr.*, 24: 1217-1227.
- Azaizeh, H. and E. Steudle. 1991. Effects of salinity on water transport of excised maize (*Zea mays* L.) roots. *Plant Physiol.*, 97(3): 1136-1145.
- Azaizeh, H., B. Gunse and E. Steudle. 1992. Effects of NaCl and CaCl_2 on water transport across root cells of maize (*Zea mays* L.) Seedlings. *Plant Physiol.*, 99(3): 886-894.
- Bartels, D. and R. Sunkar. 2005. Drought and salt tolerance in plants. *Crit. Rev. Plant Sci.*, 24: 23-58.
- Boyer, J.S. 1982. Plant productivity and environment. *Science*, 218: 443-448.
- Cachorro, P., A. Ortiz and A. Cerda. 1994. Implications of calcium nutrition on the response of *Phaseolus vulgaris* L. to salinity. *Plant Soil*, 159: 205-212.
- Chookhampaeng, S. 2011. The Effect of salt stress on growth, chlorophyll content proline content and antioxidative enzymes of Pepper (*Capsicum annum* L.) seedling. *Eur. J. Sci. Res.*, 49: 103-109.
- Comas, L., D.M. Eissenstat and A.N. Lakso. 2000. Assessing root death and root system dynamics in a study of grape canopy pruning. *New. Phy.*, 147: 171-178.
- Cramer, G.R. 2002. Sodium-calcium interactions under salinity stress. In: Läuchli A. and U. Lüttge (Eds). *Salinity: Environ. Plant. Mol.* Kluwer Academic Publishers, The Netherlands, pp. 205-227.
- Elphick, C.H., D. Sanders and F.J.M. Maathuis. 2001. Critical role of divalent cations and Na^+ efflux in *Arabidopsis thaliana* salt tolerance. *Plant. Cell. Environ.*, 24(7): 733-740.
- Gul, B. and M.A. Khan. 2006. Role of calcium in alleviating salinity effects in coastal halophytes. In: *Ecophy. High Sal. Tol. Plants*. Springer. Printed in the Netherlands, pp. 107-114.
- Guo, Z.B., P.Z. Wang, C.A. Liu, F. Liu and F.M. Li. 2012. Effect of introducing biennial legume species, *Melilotus officinalis*, on plant community in the early stage of vegetation succession and soil nutrients in the semiarid loess plateau of China. *Acta. Bot. Bor-Occ. Sin.*, 32: 0787-0794.
- Hou, L.L., J.L. Zhang, F. Guo, Sh. Yang, J.J. Meng, Y. Geng, X.G. Li and Sh. B. Wan. 2015. Effects of exogenous Ca^{2+} on peanut plant characters under salt stress. *Shandong Agr. Sci.*, 47(10): 25-28.
- Kader, M.A. and S. Lindberg. 2010. Cytosolic calcium and pH signaling in plants under salinity stress. *Plant Sig. Behav.*, 5(3): 233-238.
- Khan, M.A. and I.A. Ungar. 1997. Effects of thermoperiod on recovery of seed germination of halophytes from saline conditions. *Amer. J. Bot.*, 84: 279-283.
- Khan, M.A., B. Gul and D.J. Weber. 2001. Seed germination in relation to salinity and temperature in *Sarcobatus vermiculatus*. *Bio. Planta.*, 45: 133-135.
- Kingsbury, R.W., E. Epstein and R.W. Pearcy. 1984. Physiological responses to salinity in selected lines of wheat. *Plant. Physiol.*, 74(2): 417-423.
- Koyro, H.W. 2006. 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.
- Li, N., H.L. Zhang, X.F. Li and Ch. Liu. 2015. Function of Ca^{2+} in salt stress in plants. *Chi. Bull. Life Sci.*, 27(4): 504-508.
- Li, Y. T., M.M. Chen, Zh. M. Wang, G.M. Liu, L.L. Wang and D.X. Liu. 2016. Effects of exogenous calcium on the seed germination in *Phragmites australis* under NaCl stress. *Acta. Agr. Bor-Sin.*, 31: 270-275.
- Mao, G.L., G.Q. Zheng, Y.C. Zhang and X. Ch. Zhou. 2007. Effects of different calcium salt treatment on salt tolerance of *Lycium barbarum* L callus under NaCl stress. *Agr. Res. Arid. Areas.*, 25(1): 131-134.
- Mehrafarin, A., H.N. Badi, G.Z. Noormohammadi, S.E. Rezazadeh and A. Qaderi. 2011. Effects of environmental factors and methanol on germination and emergence of Persian Fenugreek (*Trigonella foenum-graecum* L.). *Afri. J. Agri. Res.*, 6: 4631-4641.

- Moorea, J.P., M. Vicré-Gibouin, J.M. Farrant and A. Driouich. 2008. Adaptations of higher plant cell walls to water loss: drought vs desiccation. *Physiol. Plant*, 134(2): 237-245.
- Mostafa, A., V. Mostafa, Z. Mohammad and Sh. Hossein. 2012. Recovery response of durum wheat landraces in drought stress under laboratory and greenhouse conditions. *Cur. Res. J. Bio. Sci.*, 4(1): 65-70.
- Munns, R., R.A. James and A. Läuchli. 2006. Approaches to increasing the salt tolerance of wheat and other cereals. *J. Exp. Bot.*, 57(5): 1025-1043.
- Murillo-Amador, B., H.G. Jones, C. Kaya, R.L. Aguilera, J.L. García-Hernández, E. Troyo-Diéguez, N.Y. ávila-Serranod and E. Rueda-Puentee. 2006. Effects of foliar application of calcium nitrate on growth and physiological attributes of cowpea (*Vigna unguiculata* L. Walp.) grown under salt stress. *Environ. Exp. Bot.*, 58: 188-196.
- Parida, A.K. and A.B. Das. 2005. Salt tolerance and salinity effects on plants: a review. *Ecotox. Environ. Safe*, 60: 324-349.
- Pujol, J.A., J.F. Calvo and L. Ramirez-Diaz. 2000. Recovery of germination from different osmotic conditions by four halophytes from southeastern Spain. *Ann. Bot.*, 85: 279-286.
- Qu, X.X., Z.Y. Huang, J.M. Baskin and C.C. Baskin. 2008. Effect of temperature, light and salinity on seed germination and radicle growth of the geographically widespread halophyte shrub *Halocnemum strobilaceum*. *Ann. Bot.*, 101: 293-299.
- Rengel, Z. 1992. The role of calcium in salt toxicity. *Plant Cell Environ.*, 15(6): 625-632.
- Sacała, E., A. Biegun, A. Demczuk and E. Grzys. 2005. Effect of NaCl and supplemental calcium on growth parameters and nitrate reductase activity in Maize. *Acta Soc. Bot. Pol.*, 74: 119-123.
- Shaikh, F., B. Gul, W.Q. Li, X.J. Liu and M.A. Khan. 2007. Effect of calcium and light on the germination of *Urochondra setulosa* under different salts. *J. Zhejiang. Uni. Sci. B.*, 8(1): 20-26.
- Shi, D. Ch., Y.M. Sheng and K.F. Zhao. 1998. Stress effects of mixed salts with various salinities on the seedlings of *Aneurolepidium Chinense*. *Acta Bot. Sin.*, 40: 1136-1142.
- Sosa, L., A. Allanes, H. Reinoso, R. Mariana and V. Luna. 2005. Osmotic and specific ion effects on the germination of *Prosopis strobilifera*. *Ann. Bot.*, 96: 261-267.
- Süleyman, A.V.C.I., K.A.Y.A. Mehmet Demir, İ.L.E.R.İ. Onur and İ. Özaşik. 2013. Effect of dormancy breaking treatments and salinity on germination of *Melilotus alba* and *M. officinalis*. *App. Sci. Res.*, 9: 1293-1297.
- Ungar, I.A. 1996. Effect of salinity on seed germination, growth, and ion accumulation of *Atriplex patula* (Chenopodiaceae). *Amer. J. Bot.*, 83(5): 604-607.
- Vu, T.S., D.W. Zhang, W.H. Xiao, Ch.Y. Chi, Y. Xing, D.D. Fu and Z.N. Yuan. 2015. Mechanisms of combined effects of salt and alkaline stresses on seed germination and seedlings of *melilotus officinalis* (fabaceae) in northeast of china. *Pak. J. Bot.*, 47(5): 1603-1611.
- Wang, G., Q. Xiao, Y.J. Yi, H.B. Yang, Y.X. Shi and J.Y. Liu. 2014. Alleviatory function of exogenous calcium in responses of sweet potato (*Ipomoea batatas* Lam.) seedlings to NaCl stress. *Plant Physiol. J.*, 50(3): 338-346.
- Wang, Z.J., J. Wang, X.Y. Jiao, Y.S. Chen, Zh. D. Xu, Z.L. Ou, X. Wu and Z.A. He. 2016. Effects of salt stress and exogenous calcium on seed germination of *Rhus chinensis*. *Gen. App. Bio.*, 35 (3): 706-714.
- Xu, Ch. Sh. 2014. Effects of calcium on biomass and antioxidant systems in seedlings of *Malus xiaojinensis* under salt stress. *Plant. Physio. J.*, 50 (6): 817-822.
- Xue, J.G., X.G. Wang, X.G. Du, P. Sh.Mao, T.J. Zhang, L. Zhao and J.G. Han. 2012. Influence of salinity and temperature on the germination of *Haloxylon ammodendron* and *Ceratoides arborescens*. *J. Anim. Vet. Adv.*, 11: 3244-3249.
- Yang, C.W., H.H. Xu, L.L. Wang, J. Liu, D.C. Shi and D.L. Wang. 2009. Comparative effects of salt-stress and alkali-stress on the growth, photosynthesis, solute accumulation, and ion balance of barley plants. *Photosynthetica*, 47(1): 79-86.
- Yang, Sh., L.L. Hou, F. Guo, J.L. Zhang, Y. Geng, J.J. Meng, X.G. Li and Sh. B. Wan. 2017. Effects of exogenous Ca²⁺ on growth and development, physiology and yield of peanut under salt stress. *Chi. J. App. Eco.*, 28(3): 894-900.
- Yin, Sh. W., Q.X. Wang and Q. Li. 2009. Antifungal activity of twenty common plant extracts against two plant fungi. *Nat. Pro. Res. Dev.*, 21: 306-311.
- Yuan, X.T., W. Liu, Y.N. Xuan, Y.Y. Zhang and Y.Q. Yan. 2014. Physiological effects of exogenous Ca²⁺ on *Nitraria tangutorum* under salt stress. *Plant. Physiol. J.*, 50(1): 88-94.
- Yuan, Z.N. and F. Ch. Shi. 2009. Ecological adaptation strategies in alien species: effects of salinity, temperature and photoperiod on *Spartina alterniflora* Loisel., seed germination. *Pol. J. Eco.*, 57: 677-684.
- Yuan, Z.N., J.M. Lu, J.Y. Chen and S.Z. Jiang. 2011. Patterns of resource allocation in different habitats of *Kalimeris intergrifolia* in Northeast China. *Spa. J. Agri. Res.*, 9(4):1224-1232.
- Zhang, J.T. and Ch. Sh. Mu. 2009. Effects of saline and alkaline stresses on the germination, growth, photosynthesis, ionic balance and anti-oxidant system in an alkali-tolerant leguminous forage *Lathyrus quinquenervius*. *Soil Sci. Plant Nutr.*, 55: 685-697.

(Received for publication 3 January 2018)