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

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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 Ca^{2+}) 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 Ca^{2+} 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 Ca^{2+} 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 Ca^{2+}. Moreover, 10mM Ca^{2+} had a stronger effect on seedling growth than 20mM Ca^{2+}, suggesting that excessive Ca^{2+} combined with high salt concentrations imposes additional stress. Overall, these findings suggest that an optimal Ca^{2+} concentration of 10 mM contributes to seedling establishment during germination and growth. Appropriate application of exogenous Ca^{2+} 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 (Ca^{2+}) is essential in plants, positively affecting overall growth (Sacała *et al*., 2005). As a second messenger, Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} (Gul & Khan, 2006; Shaikh *et al*., 2007; Li *et al*., 2016). Ca^{2+} 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 Ca^{2+} under salt stress (Wang *et al*., 2014; Xu, 2014). Moreover, application of Ca^{2+} was found to cause an increase in the leaf water content and root activity in plants under salt stress (Yuan *et al*., 2014). Typically, $CaCl_2$ has a greater alleviating effect on salt-stress induced injury than Ca $(NO_3)_2$ (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 Ca^{2+} 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 ± 1ºC 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 μM $m^{-2}s^{-1}$ at 25 / 15°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. Germination (%) = (a / b) × 100 %

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

2. Recovery germination percentage (%) = (c / d) × 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. Water content = (FW - DW) / 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 20–25 / 15–20°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. RGR (%) = [ln (final DW) - ln (initial DW)] / duration of treatment (days).

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

5. Water content = (FW - DW) / 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 l. 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 Ca^{2+} and the control (p<0.05; Fig. 1A). Moreover, at a high salt concentration (200 mM), the germination percentage was higher at 10 mM Ca^{2+} than 0 mM Ca^{2+} (p<0.05; Fig. 1A). Meanwhile, at 20 mM Ca^{2+}, 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 Ca^{2+} 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 Ca^{2+} (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 Ca^{2+} 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 Ca^{2+} (Fig. 2B, C).

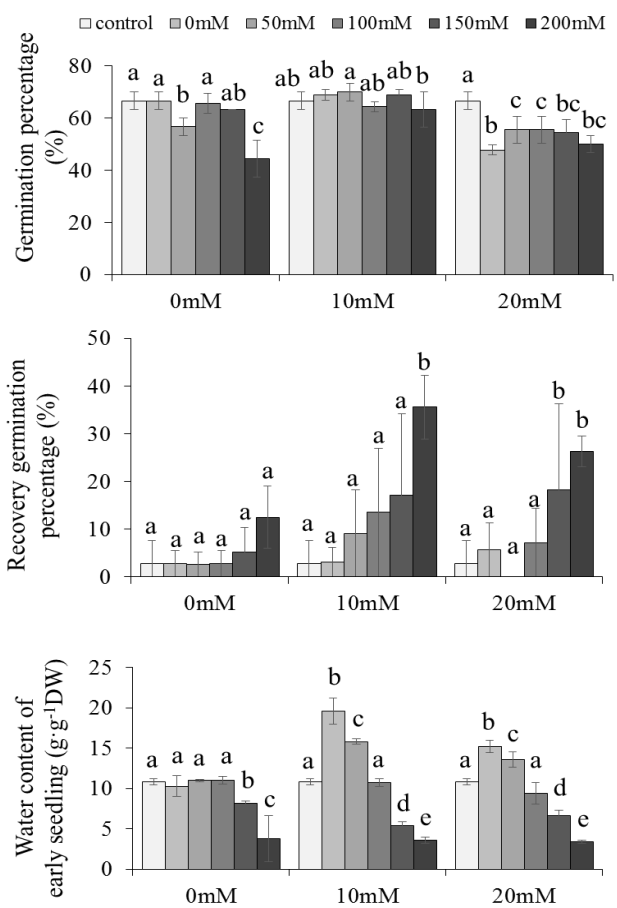

Fig. 1. Seed germination (A), recovery germination (B), and the water content of early seedlings (C) in *M. officinalis* plants grown under various levels of salt and calcium.
Values represent the mean ± S.D. Values in each treatment group followed by a different letter are significantly different (p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

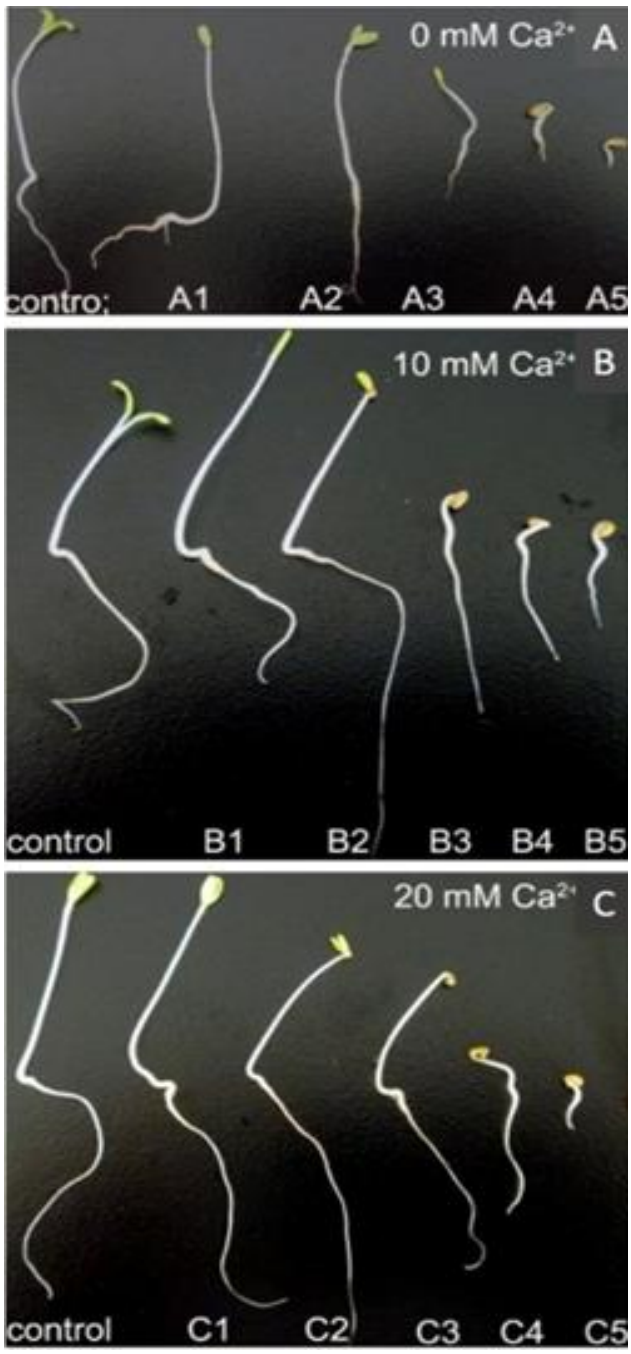

Fig. 2. Effect of calcium chloride on radicle elongation in early-stage seedlings of *M. officinalis* under saline conditions.

Growth characters, seedling biomass, and resource allocation: Without the addition of Ca^{2+}, the RGR of aboveground parts was decreased significantly with salt concentrations exceeding 50 mM ($p<0.05$; Fig. 3A). However, at 10 mM Ca^{2+}, 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 Ca^{2+}, 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 Ca^{2+}, 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 Ca^{2+} had no effect ($p<0.05$; Fig. 3C). In contrast, Ca^{2+} enhanced the biomass of underground parts, especially at 10 mM Ca^{2+} ($p<0.05$; 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 Ca^{2+} 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 Ca^{2+} ($p<0.05$; Fig. 4A). Meanwhile, a significant effect of salt was revealed by using two-way ANOVA ($F = 4.69$, $p<0.05$) (Table 2). Without Ca^{2+}, 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 Ca^{2+} 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 Ca^{2+}, 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 Ca^{2+}, underground water content was also unaffected by salt stress ($p<0.05$; Fig. 4D). Furthermore, the underground water content was higher at 10 mM Ca^{2+} than at 0 and 20 mM Ca^{2+} 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, expect 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
		CaCl2
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	β1	β2	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

X_1 = Salinity; X_2 = Calcium. β1, β2: standardized regression coefficients corresponding to X_1 and X_2. The greater the absolute β value, the stronger the effect of the stress factor. R^2: 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

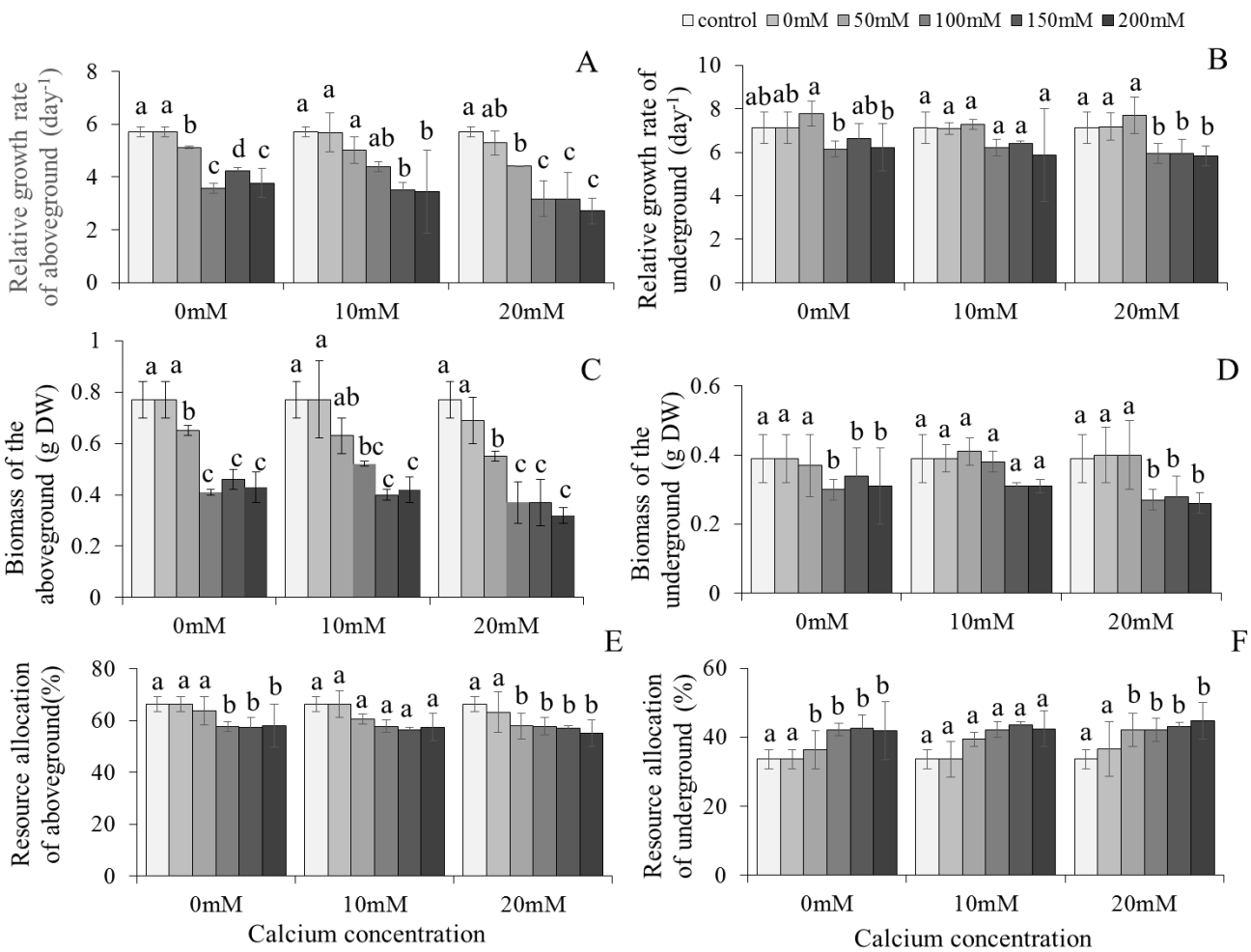

Fig. 3. Relative growth rates of aboveground (A) and underground parts (B), the biomass of aboveground (C) and underground parts (D), and resource allocation of aboveground (E) and underground parts (F) of *M. officinalis* grown under various levels of salt and calcium.

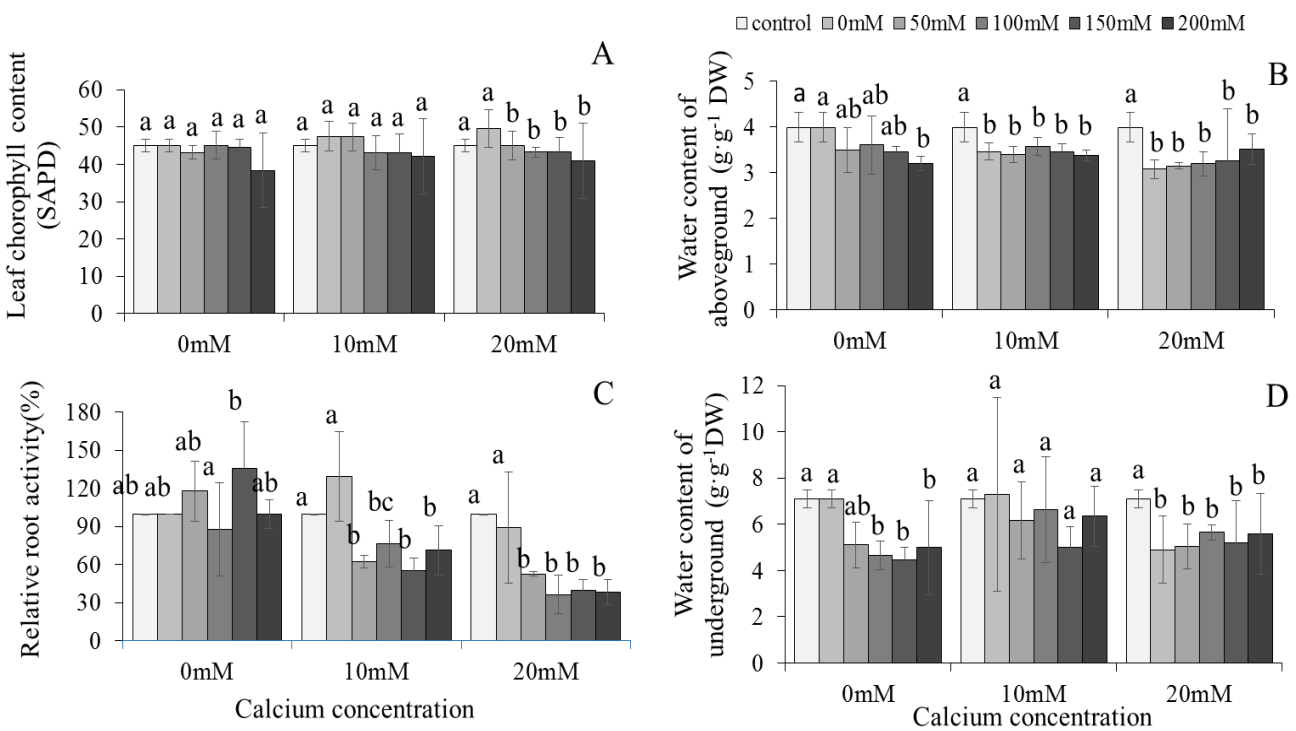

Fig. 4. Leaf chlorophyll content (A), water content of aboveground parts (B), relative root activity (C), and the water content of underground parts (D) of *M. officinalis* grown under various levels of salt and calcium.

Multiple linear regressions showed that R^2 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 Ca^{2+} to saline media. In line with this, previous studies investigated whether adding Ca^{2+} 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 Ca^{2+} (Fig. 1A). That is, the negative effects of high salt stress on the germination of *M. officinalis* seeds were ameliorated by the addition of Ca^{2+} (10 mM), in agreement with previous studies (Cachorro *et al*., 1994; Wang *et al*., 2016). However, at 20 mM Ca^{2+}, the germination percentage was lower than that of the control, suggesting that excessive Ca^{2+} 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 Ca^{2+} significantly increased the recovery germination percentage under conditions of high salt stress; notably, a Ca^{2+} concentration of 10 mM (Fig. 1B). These results suggest that supplemental Ca^{2+} 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 Ca^{2+} helped improve radicle elongation (Fig. 2B, C), suggesting that optimal Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} significantly increased the water content of early *M. officinalis* seedlings at salt concentrations ranging from 0 to 100 mM; most notably, at 10 mM Ca^{2+} (Fig. 1C). Moreover, during the seedling growth stage with supplemental Ca^{2+} (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 Ca^{2+} prevents the inhibition of hydraulic conductance in roots of *M. officinalis*, thereby helping the plant absorb more water from the soil.

Growth characteristics: Ca^{2+} 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 Ca^{2+} (Azaizeh *et al*., 1992), while in the present study, a positive effect of 10 mM supplemental Ca^{2+} 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 Ca^{2+} (10 mM) only slightly promoted the RGR. Overall, therefore, supplemental Ca^{2+} (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 Ca^{2+} 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 Ca^{2+} imposed stress on plant growth under high salt concentrations (Fig. 3A, B, C, D, E). These results suggest that the mitigating effects of exogenous Ca^{2+} are therefore limited. An appropriate Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} and under 10 mM Ca^{2+}, suggesting that salt and optimal calcium concentrations did not inhibit light absorption. However, 20 Ca^{2+} treatment combined with 100-200 mM salt caused a decrease in leaf chlorophyll content compared with the control, suggesting that 20 mM Ca^{2+} 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.

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