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BRIEF COMMUNICATION

Effects of the timing of calcium application on the alleviation of salt stress in the maize, tall fescue, and reed canarygrass seedlings

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Abstract

Calcium chloride (0.3 or 10 mM) was applied to the growth medium before, together with, or after sodium chloride application, and the effect of the timing of Ca application on the alleviation of salt stress was investigated. Seedlings of maize, tall fescue, and reed canarygrass were grown in medium with 0 and 200 mM NaCl for 5 d. Regardless of the plant species the maximum alleviation of NaCl stress was achieved when $CaCl_2$ and NaCl coexisted in the growth medium. The effects of Ca application were connected with the decrease in the Na content in the plant roots and shoots and increased ATPase activity in the roots.

Additional key words: ATPase, *Festuca arundinacea*, NaCl tolerance, *Phalaris arundinacea*, *Zea mays*.

Application of calcium (Ca) to the growth medium ameliorates salt stress in plants (Lahaya and Epstein 1969, El-Hamdaoui *et al*. 2003, Maeda *et al*. 2003, Sotiropoulos 2007). It has been reported that the alleviatory effect of Ca on salt stress might be caused by the reduction in sodium absorption and transfer from roots to the shoot (Cramer *et al*. 1989), the improvement in the electrostatic status in the cell wall of salt-treated plants (Stassart *et al*. 1981) and cell membranes (Lynch *et al*. 1987, Kinraide 1999, Murata *et al*. 2000, Yermiyahu *et al*. 1997), and the maintenance of pectic acid-binding Ca in the cell wall and the plasmalemma ATPase activity (Maeda *et al*. 2005). These reports showed the alleviatory effect of Ca application together with sodium chloride (NaCl) application to the growth medium. Since the actual saline fields already contained a large amount of salt, Ca would be applied to the soil immediately before or during the cultivation of the plant. However, there are few reports on the effect of the timing of Ca application to the growth medium on the alleviation of salt stress. In the present study, calcium in form of $CaCl_2$ was applied to the growth medium before, together with, or after NaCl application, and the effect of the timing of Ca application on the alleviation of salt stress in three gramineous species with different salt tolerance was investigated.

The seedlings of maize (*Zea mays* L.), tall fescue (*Festuca arundinacea* Schreb.), and reed canarygrass (*Phalaris arundinacea* L.) were used for the experiment. The seedlings were grown in a standard nutrient solution (1.3 mM NH_4NO_3, 0.16 mM $NH_4H_2PO_4$, 2 mM $Ca(NO_3)_2 . 4 H_2O$, 1 mM K_2SO_4, 2 mM $MgSO_4 . 7 H_2O$, 36 µM Fe-EDTA . 3 H_2O, 18 µM $MnSO_4 . 5 H_2O$, 46 µM H_3BO_4, 3 µM $ZnSO_4 . 7 H_2O$, 0.16 µM $CuSO_4 . 5 H_2O$, 5 nM $(NH_4)_6Mo_7O_{24} . 4 H_2O$) up to the fifth leaf stage with aeration. NaCl and $CaCl_2$ were then applied to the solution to adjust their concentrations to 200 mM NaCl and 0.3 or 10 mM $CaCl_2$.

Six treatments (A - F) were established: A - solution without NaCl and with 0.3 mM $CaCl_2$ (control), B - solution with 200 mM NaCl and 0.3 mM $CaCl_2$ (Na treatment), C - solution with 200 mM NaCl and 10 mM $CaCl_2$ (Na-Ca treatment), D - solution without NaCl and with 10 mM $CaCl_2$ for 3 d before being grown in the solution with 200 mM NaCl and 10 mM $CaCl_2$ (Ca pre-treatment), E - solution without NaCl and with 10 mM $CaCl_2$ for 3 d immediately before being grown in the solution with 200 mM NaCl and 0.3 mM $CaCl_2$ (Ca stop treatment), and F - solution without NaCl and with

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10 mM $CaCl_2$ for 3 d after being grown in the solution with 200 mM NaCl and 0.3 mM $CaCl_2$ (Ca post-treatment). Plants were grown in a growth chamber at 12-h photoperiod, irradiance of 50 µmol m^{-2} s^{-1}, temperature of 25°C and relative humidity 60 %. The solution was renewed daily to correct the NaCl and $CaCl_2$ concentrations. Five days after NaCl application (A - E treatments) or 3 d after $CaCl_2$ application (F treatment), the plants were harvested, and the dry mass (after 48 h at 80 °C) was measured. Then the lyophilized plants were wet-ashed, the Na content was determined with a flame spectrophotometer (*Shimadzu*, Kyoto, Japan).

After the NaCl and $CaCl_2$ treatments, the content of pectic acid-binding Ca in the plant roots was calculated by determining the Ca content of the pectin fraction. The Ca content was determined with an atomic absorption spectrophotometer (*Shimadzu*). The fractionation of pectin was carried out by the method of Yamauchi *et al.* (1986). The fresh root tissues were homogenized in a mortar with distilled water at 0 °C. The homogenate was then centrifuged at 1 000 *g* for 30 min to precipitate cell wall materials, and the precipitate was washed three times with distilled water. The lyophilized cell wall was digested with 5 g dm^{-3} pectinase with shaking at 37 °C for 45 h. The reaction mixture was centrifuged at 1 000 *g* for 14 min, and the supernatant was regarded as the pectin fraction.

The separation of the plasmalemma from the plant root cells was carried out by the method of Gallagher and Leonard (1982). Thirty to 50 g fresh material was ground in 250 mM sucrose, 3 mM EDTA, 2.5 mM dithiotreitol (DTT), and 25 mM Tris-MES (pH 7.7) [4 cm^3(medium) g^{-1}(material)]. The filtered homogenate was centrifuged at 13 000 *g* for 15 min, followed by 80 000 *g* for 30 min. The precipitation obtained was suspended in 2 cm^3 of suspension buffer (250 mM sucrose, 1 mM DTT, 1 mM Tris-Mes, pH 7.2). The fraction rich in plasmalemma was obtained at the 0.34/0.45 g g^{-1} sucrose interfaces, when the fractions obtained by centrifuging at 13 000 to 80 000 *g* were centrifuged at 82 500 *g* for 2 h in 36-cm^3 gradients consisting of 28 cm^3 of 0.45 g g^{-1} and 8 cm^3 of 0.34 g g^{-1} sucrose in 1 mM Tris-MES (pH 7.2). The membranes were collected from the interface and diluted with a suspension buffer to a protein concentration of approximately 80 g dm^{-3}. The ATPase activity of the plasmalemma was measured in a 1-cm^3 volume containing the enzyme, 6 mM Tris-ATP, 60 mM Tris-MES (pH 6.5), 6 mM $MgSO_4$, and 100 mM KCl. The reaction was carried out for 10 min at 38 °C and terminated by the addition of 1 cm^3 of 500 g dm^{-3} TCA, and the amount of Pi released from the substrate was measured. The protein content was determined by the Bradford (1976) method with bovine serum albumin as a standard.

At 200 mM, dry masses were 82 % of control NaCl for maize, 72 % for tall fescue, 62 % for reed canarygrass, respectively (Table 1). Based on this result, the NaCl concentration for NaCl application was determined to be 200 mM since a clear difference was observed in the relative growth among the plant species (maize > tall fescue > reed canarygrass).

The growth in the Na-Ca treatment and Ca pre-treatment was higher than in the Na treatment. The rate of increase in the growth of reed canarygrass in the Na-Ca treatment and Ca pre-treatment in comparison with the Na treatment was the highest among all the three plant species. On the other hand, no significant difference was detected in the growth between the Na-Ca treatment and Ca pre-treatment. The growth in the Ca post-treatment was the lowest among all the treatments (Table 1). Therefore, regardless of the plant materials, the timing of Ca application to the growth medium was important for the alleviation of NaCl stress, and it was essential that $CaCl_2$ and NaCl should coexist in the growth medium.

The Na contents of the roots and shoots in the Na treatment remarkably increased, and the increase was the highest in reed canarygrass, followed by tall fescue and maize. The Na contents of the roots and shoots in the Ca-Na treatment and Ca pre-treatment markedly decreased. Regardless of the plant species, the Na contents in the Ca stop treatment increased to the same level as those in the Na treatment. While the Na content of maize in the Ca post-treatment was the same as that in the Na treatment, the Na contents in tall fescue and reed canarygrass in the Ca post treatment markedly increased and were the highest among all the treatments (Table 1).

Pectic acid-binding Ca was detected only in the control, Ca-Na treatment and Ca pre-treatment for each plant species (Table 1). The ATPase activity markedly decreased in the Na treatment, and the decrease was the highest in reed canarygrass. On the contrary, the ATPase activity was less decreased in the Ca-Na treatment and Ca pre-treatment. Although the ATPase activity in the Ca stop treatment was similar as that in the Na treatment, the values in the Ca post-treatment of tall fescue and reed canarygrass were considerably lower than those in the Na treatment.

The maximum alleviatory effect of $CaCl_2$ application on NaCl stress was achieved when $CaCl_2$ and NaCl coexisted in the growth medium (Table 1), and the Na contents of the roots and shoots in the Ca-Na treatment and Ca pre-treatment were significantly lower than those in the Na treatment (Table 1). In addition, no alleviatory effect of $CaCl_2$ application was observed in Ca stop treatment and Ca post-treatment (Table 1). Sodium ions reach the xylem through apoplasmic and symplasmic pathways and are transported to the shoot in an intact plant (Pitman 1977). The salt tolerance of gramineous species was determined by the different Na concentrations in the leaves (Aslam *et al.* 1993), and it was suggested that the regulation of Na uptake by roots and the low Na content in leaves result in considerable salt tolerance (Shachtman and Munns 1992). Hence, it

Table. 1. Effects of the timing of treatment with NaCl and $CaCl_2$ on the growth, Na contents, root pectic acid-binding Ca contents (Binding Ca), and root ATPase activities (ATPase) of maize, tall fescue, and reed canarygrass. Results were means ± SD, n = 6. For treatments A - F see text, n.d. - not detected.

Plant species	Treatment	Dry mass [g plant^{-1}]	Shoot Na content [mg g^{-1}(d.m.)]	Root Na content [mg g^{-1}(d.m.)]	Binding Ca [mg g^{-1}(d.m.)]	ATPase [nkat mg^{-1}(protein)]
Maize	A	2.51 ± 0.05	1.01 ± 0.51	8.51 ± 2.12	1.21 ± 0.03	6.02 ± 0.50
	B	2.06 ± 0.03	15.1 ± 1.20	15.3 ± 1.21	n.d.	3.81 ± 0.52
	C	2.26 ± 0.03	3.52 ± 1.10	9.24 ± 1.18	0.49 ± 0.03	9.10 ± 0.52
	D	2.30 ± 0.03	3.51 ± 1.10	9.14 ± 2.12	0.48 ± 0.03	9.51 ± 0.54
	E	2.04 ± 0.03	13.0 ± 1.13	13.3 ± 2.31	n.d.	4.21 ± 0.53
	F	2.04 ± 0.04	15.5 ± 2.01	14.2 ± 2.31	n.d.	4.20 ± 0.51
Tall fescue	A	2.31 ± 0.03	4.02 ± 0.51	8.51 ± 2.11	0.92 ± 0.03	5.72 ± 0.54
	B	1.67 ± 0.04	18.3 ± 1.10	18.3 ± 2.42	n.d.	3.01 ± 0.52
	C	1.97 ± 0.03	4.52 ± 2.02	9.51 ± 1.41	0.48 ± 0.04	8.04 ± 1.02
	D	2.00 ± 0.03	4.51 ± 1.03	9.53 ± 2.43	0.52 ± 0.03	8.51 ± 1.07
	E	1.67 ± 0.04	18.3 ± 2.01	18.4 ± 1.28	n.d.	3.07 ± 0.52
	F	1.53 ± 0.04	30.2 ± 2.03	23.4 ± 2.31	n.d.	1.01 ± 0.51
Reed canarygrass	A	2.10 ± 0.02	2.03 ± 0.51	5.34 ± 1.05	0.88 ± 0.02	5.07 ± 1.07
	B	1.30 ± 0.03	34.3 ± 2.12	28.4 ± 2.11	n.d.	2.02 ± 0.53
	C	1.80 ± 0.04	11.1 ± 1.31	15.3 ± 2.32	0.51 ± 0.04	8.21 ± 0.73
	D	1.83 ± 0.04	11.2 ± 1.14	13.4 ± 2.41	0.53 ± 0.04	8.31 ± 1.02
	E	1.29 ± 0.03	34.2 ± 1.07	28.2 ± 2.37	n.d.	2.02 ± 0.33
	F	1.29 ± 0.03	34.2 ± 1.07	28.2 ± 2.37	n.d.	0.62 ± 0.31

confirmed that the alleviatory effect of Ca application was caused by the control of Na invasion into these plant roots, and that Ca treatment prior to and simultaneous with NaCl-treatment is effective.

We focused on the changes in the pectic acid-binding Ca of the cell walls and on those in the activity of plasmalemma ATPase, because Ca plays important roles for ion uptake in cell surface as mentioned below. The pectic acid-binding Ca exists in the middle lamella of the cell wall and maintains the cell wall structure (Clarkson and Hanson 1980). It was reported that Ca application to the medium had an effect on cell wall synthesis (Eklund and Eliasson 1990) and on the maintenance of cell wall structure together with boron (Matoh and Kobayashi 1998). It was reported that the high content of pectic acid-binding Ca on the cell wall contributed to the alleviatory effect of Ca on the growth inhibition through the decrease in apoplasmic Na invasion and translocation (Maeda *et al.* 2005).

In the present study, a high content of pectic acid-binding Ca was observed only in the Ca-Na treatment and Ca pre-treatment in which the alleviatory effect on NaCl stress was observed (Table 1). This result indicates that pectic acid-binding Ca may contribute to the alleviation of NaCl stress through the control of Na invasion into the plants' roots. On the other hand, it was reported that Ca application to the growth medium increased the plasmalemma ATPase activity (Qui and Su 1998), and that a high ATPase activity induced a high ability of Na exclusion (MacRobbie 1977). Based on these findings and the results that the ATPase activity significantly increased in the Ca-Na treatment and Ca pre-treatment (Table 1), it is suggested that the increase in ATPase activity appeared to contribute to the alleviation of NaCl stress through the improvement in Na exclusion ability. On the contrary, the results that Ca application in the Ca stop treatment did not affect the salt tolerance indicate that intracellular Ca may have little effect on the alleviation, and that exogenous Ca in the growth medium contributes to the alleviatory effect of $CaCl_2$ application on NaCl stress through the maintenance of pectic acid-binding Ca on the cell wall and the ATPase activity.

Among the three gramineous species used in the present study, reed canarygrass showed the highest rate of increase in the relative growth in the Ca-Na treatment and Ca pre-treatment to the level of relative growth observed in the Na treatment (Table 1). This result was caused by the lowest relative growth of reed canarygrass in the Na treatment for the same values in the Ca-Na treatment and Ca pre-treatment among the three species. Regardless of the plant species, the alleviatory effect of Ca application was caused by the control of Na invasion into the plant that was caused by the high pectic acid-binding Ca content and ATPase activity of the roots (Table 1). Therefore, it might be considered that there was no clear difference in the alleviatory effect of Ca application among the gramineous species with different levels of salt tolerance.

In conclusion, it was essential that $CaCl_2$ coexisted with NaCl in the growth medium in order to alleviate the NaCl stress in the plant, and no alleviatory effect of Ca application was observed in Ca stop treatment and Ca post-treatment. The alleviatory effects of Ca application on NaCl stress was caused by the decrease in the Na content of the plant roots and shoots. Further, it is suggested that this decrease was related to the high pectic acid-binding Ca content and the ATPase activity of the roots.

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