AcademicPres

Available online at www.notulaebotanicae.ro

Print ISSN 0255-965X; Electronic 1842-4309

Not Bot Horti Agrobo, 2012, 40(2): 58-66

Notulae Botanicae Horti Agrobotanici Cluj-Napoca

Mitigation of Salt Stress-Induced Inhibition of *Plantago crassifolia* Reproductive Development by Supplemental Calcium or Magnesium

Marius N. GRIGORE[1,2], Monica BOSCAIU[3], Josep LLINARES[4], Oscar VICENTE[1*]

[1]*Universitat Politècnica de València, Instituto de Biología Molecular y Celular de Plantas (UPV-CSIC), CPI, edificio 8E, Camino de Vera s/n, 46022 Valencia, Spain; ovicente@ibmcp.upv.es (*corresponding author)*

[2]*Permanent address: Alexandru Ioan Cuza University, Faculty of Biology, Carol I, 20 A, 700505 Iasi, Romania; mariusgrigorepsyche@yahoo.com*

[3]*Instituto Agroforestal Mediterráneo (UPV), CPI, edificio 8E, Camino de Vera s/n, 46022 Valencia, Spain; mobosnea@eaf.upv.es*

[4]*Universitat Politècnica de València, Instituto de Investigación para la Gestión Integral de Zonas Costeras (IGIC, UPV), Paranimf 1, 46730 Grau de Gandía, Valencia, Spain; jollipa@qim.upv.es*

Abstract

In *Plantago crassifolia*, a moderate halophyte characteristic of borders of salt marshes in the Mediterranean region, reproductive development is more sensitive to high soil salinity than vegetative growth. To investigate the possible role of calcium and magnesium salts in the responses of this species to salt stress, adult plants were submitted over a 2-month period to treatments with 300 mM NaCl-a concentration which affects, but does not completely inhibit seed formation in *P. crassifolia*-either alone or combined with low concentrations of $CaCl_2$ (10 mM) or $MgCl_2$ (20 mM). The NaCl treatment did not affect plant vegetative growth and had a stimulating effect on flowering. Yet almost half the spikes produced had aborted seeds, and the effect on seed number and quality-estimated by their mean weight and germination capacity-was obviously deleterious. Addition of calcium or magnesium chloride during the salt-stress treatment completely counteracted the negative effect of NaCl on the 'reproductive success' of the plants: the number, weight and germination frequency of the seeds were similar to that in the control, non-stressed plants. These results indicate that both divalent cations can suppress or mitigate the deleterious effects of salt stress. While this protective role is well established in the case of calcium, we provide here the first experimental evidence of a similar function for magnesium.

Keywords: $CaCl_2$, halophytes, $MgCl_2$, NaCl, salinity, stress tolerance

Introduction

Drought and salinity are two major environmental factors that reduce crop productivity and limit plant distribution in nature (Boyer, 1982; Bartels and Sunkar, 2005). Salt stress, mostly due to the presence of Na^+ and Cl^--by far the most abundant ions in saline soils-causes many adverse effects on plant growth, which are due to the low osmotic potential of the soil solution (osmotic stress), specific ion effects (ion toxicity), nutritional imbalance, or to a combination of these factors (Ashraf, 1994; Marschner, 1995). Elevated salt concentrations in the apoplast of cells impose a hyperosmotic shock by lowering the water potential, causing turgor reduction or loss, which limits cell expansion (Munns and Termaat, 1986; Zhu, 2002). In addition, sodium and chloride ions have inhibitory effects on many enzymes and some basic cellular processes, such as RNA processing or protein synthesis (Forment *et al.*, 2002; Niu *et al.,* 1995; Serrano *et al.,* 1999; Zhu *et al.,* 1998). These two primary components of salt stress negatively affect plant survival, growth and development (Botella *et al.,* 2005).

Secondary effects of NaCl stress include disturbance of K^+ uptake, membrane dysfunction, impairment of photosynthesis, generation of reactive oxygen species (ROS)-that is, of oxidative stress-and programmed cell death (Hasegawa *et al.,* 2000; Serrano *et al.,* 1999; Zhu, 2003). Potassium is an essential element for plants (Marschner, 1995) and Na^+ competes with K^+ for uptake into cells, by using the same membrane transporters, particularly when the external concentration of sodium is significantly higher than that of the nutrient (Niu *et al.,* 1995; Rodriguez-Navarro, 2000).

The divalent cation Ca^{2+}, which like potassium is also an essential mineral nutrient, has as well an important role in the mechanisms of plant response to salinity. Calcium is involved in a wide array of cellular processes, such as cell wall stabilisation, cell extension, secretory processes, maintenance of cell membrane integrity and control of membranes' permeability and selectivity, cation-anion balance and osmoregulation (Marschner, 1995), and is a messenger in abiotic stress signalling (Bartels and Sunkar, 2005; Xiong *et al.,* 2002). The protective effect of calcium against salt stress in plants, by counteracting some of the

deleterious effects of sodium, is well documented (Bressan *et al.*, 1998; Grattan and Grieve, 1999; Gul and Khan, 2008; Marschner, 1995; Öpik and Rolfe, 2005; Rengel, 1992). These vital roles of Ca^{2+} relate not only to the ionic relations in the plants but also to the physical soil conditions (Alam, 1999); in fact, calcium-in the form of gypsum treatments-has been applied in agriculture for decades to improve soil quality by reducing its salinity.

Concerning the other major divalent cation, Mg^{2+}, many of its physiological roles in plants have also been characterised (Marschner, 1995; Öpik and Rolfe, 2005). For example, magnesium ions are cofactors required for the activity of different enzymes, including enzymes involved in respiration and photosynthesis, or in the synthesis of DNA and RNA; Mg also forms part of the ring structure of the chlorophyll molecule. Apart from these general functions of magnesium, very little is known regarding its (possible) specific roles on the mechanisms of response of plants to high soil salinity and salt tolerance. However, there are some experimental data indirectly suggesting that Mg^{2+} could play a similar role to that of Ca^{2+}, protecting plant cells from the deleterious effects of NaCl.

As mentioned above, high Na^+ concentrations inhibit the activity of many different enzymes; in some cases (e.g., Albert *et al.*, 2000), it has been demonstrated that the molecular mechanism of inhibition involves the displacement by Na^+ of the Mg^{2+} cofactor from the enzyme's active centre. This suggests that an increase in the intracellular Mg^{2+} level (without reaching toxic concentrations) may counteract, at least partially, the effect of sodium on enzyme activities and confer some degree of halotolerance. In agreement with this idea, the detection in *Juncus acutus* of a progressive increment of Ca^{2+} and Mg^{2+} levels with increasing external NaCl concentrations (Boscaiu *et al.,* 2011) was interpreted as a salt-inducible defence mechanism which could contribute to protecting the plants from salt stress.

A few years back, we began a study on the responses to salt stress at different phases of the life cycle of *Plantago crassifolia*, a Mediterranean halophytic species (Boscaiu *et al.*, 2005; Vicente *et al.*, 2004). From this work it was concluded that the sensitivity of *P. crassifolia* to salt stress depended on its developmental stage, as had been previously reported for other species (Läuchli and Epstein, 1990; Johnson *et al.*, 1992): reproductive development was more sensitive to inhibition by NaCl than vegetative growth, but before induction of the reproductive phase older plants showed a higher level of salt tolerance than younger ones. We could establish conditions in which *P. crassifolia* plants, although still able to complete their life cycle, showed a clear reduction in 'reproductive success', as indicated by a lower number, mean weight and quality (i.e., germination capacity) of the seeds produced, compared to non-stressed controls (Boscaiu *et al.*, 2005).

In this paper, we describe experiments designed to confirm our working hypothesis that magnesium, as well as calcium, can mitigate the negative effects of salt stress. Specifically, we show that the partial inhibition of *Plantago crassifolia* reproductive development observed in the presence of 300 mM NaCl-a salt concentration that affects but does not completely block seed production-is released by the simultaneous addition of low concentrations of $CaCl_2$ or $MgCl_2$.

Material and methods

Plant material

The seeds of *Plantago crassifolia* were collected in summer 2009 from a salt marsh located in the La Albufera Natural Park (province of Valencia, E Spain). After a 6-month storage at room temperature, seeds were sown and germinated in seed trays containing a mixture of peat and vermiculite (3:1) in a growth chamber (Infraca), fitted with three 58 W Philips Master TL-D fluorescent lamps per shelf, providing a PAR of approximately 150 $\mu E\ m^{-2}\ s^{-1}$ during the light time of a 12-hour photoperiod. The temperature was kept at 25ºC in the light and 15ºC in the dark. After observing that young plants had grown 15 cm in length, they were transferred to individual plastic pots (12-cm diameter) with the same substrate. After 1 week, salt treatments started and were carried out by adding 150 mL of salt solutions (or distilled water for the control treatments) to pots once per week.

Saline treatments

The following six treatments were carried out over a 2-month period: (1) control (plants were watered with distilled water); (2) 300 mM NaCl; (3) 10 mM $CaCl_2$; (4) 20 mM $MgCl_2$; (5) 300 mM NaCl together with 10 mM $CaCl_2$; (6) 300 mM NaCl together with 20 mM $MgCl_2$. The 300 mM NaCl treatment was selected since it clearly affected the reproduction of the studied species, but flowering and seed production was not completely inhibited, as shown by Boscaiu *et al.* (2005). Treatment concentrations of 10 mM Ca and 20 mM Mg were chosen as optimal after performing previous trials at different concentrations (data not shown). Six different individuals from each treatment were selected to measure all the analysed parameters.

Soil analysis

Soil samples from three pots per treatment were collected at the end of the experiment, air-dried and then passed through a 2-mm sieve. Electrical conductivity and pH were measured in saturate soil paste extracts in a Crison Conductimeter Basic 30 and in a Crinson pH-meter Basic 20+, respectively (Schofield 1942; USSL Staff 1954).

Analysis of the reproductive traits of plants

Plant material was harvested after two months of treatment by separating the spikes produced by each plant. The vegetative part of plants was weighed on a precision bal-

ance and was then dried in an oven at 65°C until constant weight. The number of spikes corresponding to each plant, and the length of floral scapes and spikes, were recorded. After removing floral debris, the seeds formed in each spike were counted and weighed on a precision balance. The mean number and mean weight of seeds per spike were calculated for each treatment. Seeds were stored at room temperature for three months prior to testing their germination capacity.

Seed germination

Germination capacity was tested only in morphologically well-developed seeds, which were selected under a stereomicroscope, eliminating all aborted seeds. Four replicas of 20 seeds per treatment were placed in Petri dishes on cotton covered by two layers of filter paper moistened with distilled water. To avoid evaporation, dishes were sealed with parafilm and plates were incubated in the same chamber and under the same conditions described above. Upon radicle emergence, seeds were considered germinated and were removed from the Petri dishes. Germination counts were extended over a 30-day period.

Bivalent cation quantification

Ca^{2+} and Mg^{2+} levels were measured in the leaves of the same plants used for the growth experiments. For the total content of these cations, dry leaf material was milled and then 0.3 grams were digested in 8 ml of 65% (v/v) HNO_3 and 2 ml of 30% (v/v) H_2O_2 in a microwave digestor (Model: Ethos One, Milestone Microwave Laboratory Systems). In parallel, a second extraction method for the quantification of soluble Ca^{2+} and Mg^{2+} was carried out following the protocol proposed by Weimberg (1987), based on the extraction of the cations in boiling water. The quantification of both cations was performed by atomic absorption spectrometry (Model: Varian SpectrAA 220) for Ca^{2+} at 239.9 nm and for Mg^{2+} at 202.6 nm.

Statistic analysis

Data were analysed using SPSS, version 16. Significance of differences among treatments was tested by applying one-way ANOVA. Prior to the analysis of variance, the percentage data of seed germination were normalised by an arcsine transformation. When the ANOVA null hypothesis was rejected, post-hoc comparisons were performed using the Tukey test.

Results and discussion

Soil analysis

Salinity strongly affected the electrical conductivity (EC) of the substrate, which showed values correlated with the concentration of added salts. Thus, the high EC_{SE} induced by NaCl (more than 60 dS m^{-1}) increased even more with the additions of the other two salts, especially $MgCl_2$, which was applied at a higher concentration. In the absence of NaCl, a moderate EC was registered in the pots watered with $CaCl_2$ and $MgCl_2$ (Tab. 1). As expected, the pH decreased with increasing ion strength of the soil solution.

Tab. 1. pH and EC values of the plants substrates after 2-month treatments with different salts, as indicated

Treatment	pH	EC (dS m^{-1})
Control	7.31 ± 0.20	1.23 ± 0.06
300 mM NaCl	6.62 ± 0.18	62.5 ± 4.08
10 mM $CaCl_2$	6.14 ± 0.07	5.46 ± 0.87
20 mM $MgCl_2$	6.07 ± 0.12	12.54 ± 1.64
300 mM NaCl + 10 mM $CaCl_2$	6.49 ± 0.02	68.01 ± 4.17
300 mM NaCl + 20 mM $MgCl_2$	6.23 ± 0.04	74.30 ± 4.27

Effects of NaCl on vegetative growth and 'reproductive success' of Plantago crassifolia

Salinity did not affect the vegetative growth of *P. crassifolia*: as seen in Tab. 2, both fresh weight (FW) and dry weight (DW) varied only slightly among treatments. These data seemed to contradict previous results showing that *P. crassifolia* plants grown at the same NaCl concentration gave significantly lower FW and DW than those from control treatments (Vicente *et al.*, 2004). However, this apparent discrepancy can be easily explained by the fact that those earlier experiments were carried out on younger *Plantago* plants, whereas the plants used in the present study were at a more advanced developmental

Tab. 2. Effects of different salt treatments on *Plantago crassifolia* vegetative growth and reproductive development; the indicated parameters were measured after 2-month treatments (means ± SD, n = 6, where appropriated)

Parameters	Control	300 mM NaCl	10 mM $CaCl_2$	20 mM $MgCl_2$	300 mM NaCl + 10 mM $CaCl_2$	300 mM NaCl + 20 mM $MgCl_2$
FW (g)	11.38 ± 3.36	10.92 ± 2.51	10.24 ± 1.61	10.09 ± 1.45	11.19 ± 2.78	10.38 ± 1.82
DW(g)	1.33 ± 0.44	1.23 ± 0.30	1.34 ± 0.14	1.27 ± 0.09	1.31 ± 0.27	1.30 ± 0.17
Flowering plants (n°)	4	5	6	6	5	5
Total spikes (n°/treatment)	8	14	11	11	15	18
Mean spike number/plant	1.00 ± 1.00	2.00 ± 1.80	2.20 ± 0.84	2.20 ± 1.09	3.00 ± 1.38	3.00 ± 2.10
Mean scape length (cm)	23.41 ± 4.81	13.68 ± 3.56	15.20 ± 4.45	15.36 ± 5.38	12.87 ± 1.65	9.70 ± 1.44
Mean spike length (cm)	3.61 ± 0.16	2.87 ± 0.55	2.46 ± 0.92	2.72 ± 1.00	3.22 ± 0.57	2.60 ± 0.56
Spikes with aborted seeds (n°)	2	6	0	1	1	2

stage, as flowering already commenced two weeks after starting the treatments. We have previously shown that the effects of salt stress on *P. crassifolia* vegetative growth are largely dependent on the plant's age at the beginning of the treatment, older plants being more resistant to salt than younger ones (Vicente *et al.*, 2004).

On the other hand, NaCl clearly had an effect during the reproductive phase of the life cycle of *Plantago crassifolia*. Flowering seemed to be stimulated by the presence of salts, as observed by the number of plants producing flowers and by the number of spikes (as the total number per treatment and the mean number per plant), with higher values for those plants subjected to salinity. Whereas in non-treated controls the mean number of spikes per plant was one and the higher number was two, the mean number doubled in the 300 mM NaCl treatment. However, other measurements, such as the mean lengths of floral scapes and spikes-which were notably shorter in the salt-treated plants-clearly suggested that salt stress negatively affects reproductive development. Most important, of the total number of 14 spikes formed in the presence of 300 mM NaCl, six produced only aborted seeds (Tab. 2).

In an independent experiment, it was found that plants treated with 500 mM NaCl also produced a large number of spikes, but all seeds aborted (data not shown). Similarly, in our previous publication about the effect of salt stress on reproductive development in this species (Boscaiu *et al.*, 2005) we showed that the plants from treatments with a low saline concentration produced more flowers than the plants in controls; in this case, however, plants treated with high NaCl concentrations did not flower at all. Despite quantitative differences, which can be explained by the different developmental stage of the plants when they were subjected to the salt treatments-as mentioned above-these results indicate that in *P. crassifolia*, under the tested conditions, salt stress induces flowering but partially inhibits normal reproductive development.

To assess in more detail the effect of NaCl reducing the reproductive success of *P. crassifolia*, seed set and quality were determined. Plants flowering in the presence of 300 mM NaCl produced a significantly lower number of seeds per spike than the non-treated plants (Fig. 1). The quality of those seeds was worse than that in controls, as shown by their lower mean weight (Fig. 2) and reduced germination capacity (Fig. 3). The germination frequency of the seeds from this salt treatment was 81% *vs*. 97% in the control plants, but as mentioned in the Material and Methods section, only well-developed, apparently viable seeds were selected for the *in vitro* germination tests, eliminating the high percentage of seeds that appeared aborted upon microscopic observation.

Alteration of flowering time under adverse environmental conditions is a common phenomenon, observed in many plant species (Wada and Takeno, 2010; Yaish *et al.*, 2011). Stress can induce both, early or delayed flowering, depending on the particular plant species, but also on the type and the intensity of the stress conditions. These different outputs probably correspond to different strategies that can be used by plants to cope with stress: either they invest their resources (energy, nutrients, metabolic precursors) in defence reactions against stress, delaying reproductive development-which would also require those resources-or they escape stress using a strategy of 'survival through the next generation', by induction of early flowering and reproduction to produce seeds (e.g., Blanvillain *et al*., 2011), as seems to be the case in our experiments. Nevertheless, since salt stress imposes additional energy requirements on plants, fewer resources are available for development of reproductive structures, male and female gametophytes and, after fertilisation, for embryo and seed development (Cheesman, 1988). This could explain why the number of seeds and especially their viability, is severely affected in the plants treated with 300 mM NaCl.

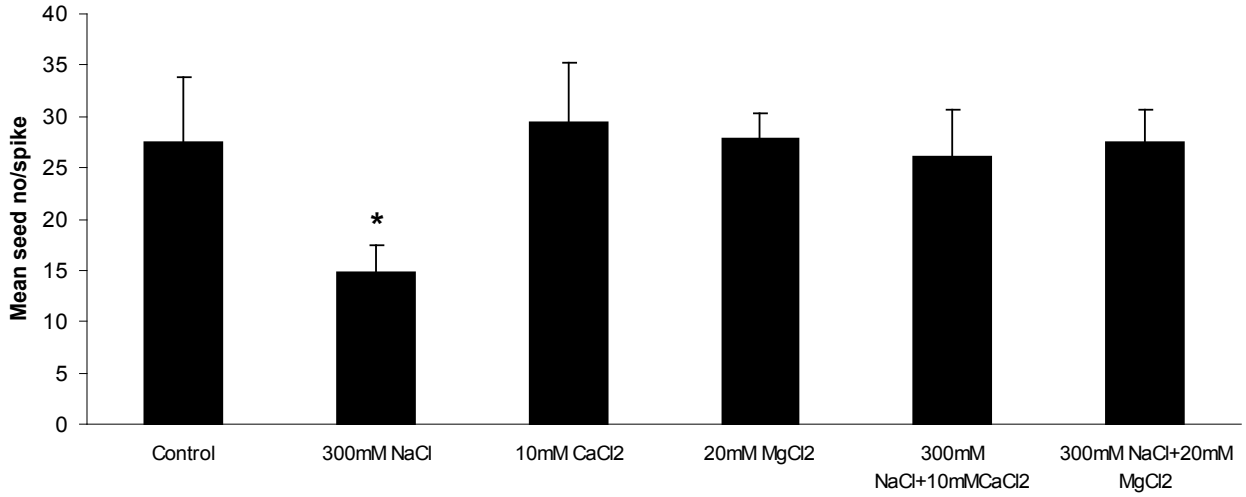

Fig. 1. Number of seeds produced per spike in *Plantago crassifolia* plants, after the indicated salt treatments (mean ± SD; n = number of spikes formed for each treatment, see Tab. 2). The asterisk indicates statistically significant differences

62

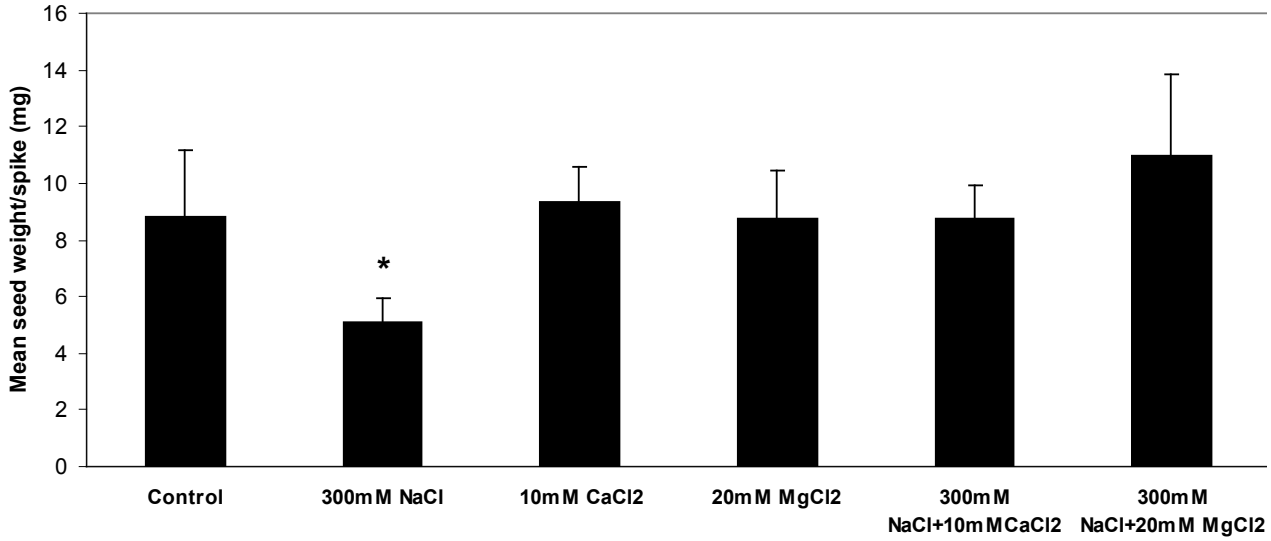

Fig. 2. Weight of seeds produced per spike in *Plantago crassifolia* plants, after the indicated salt treatments (mean ± SD; n = number of spikes formed for each treatment, see Tab. 2). The asterisk indicates statistically significant differences

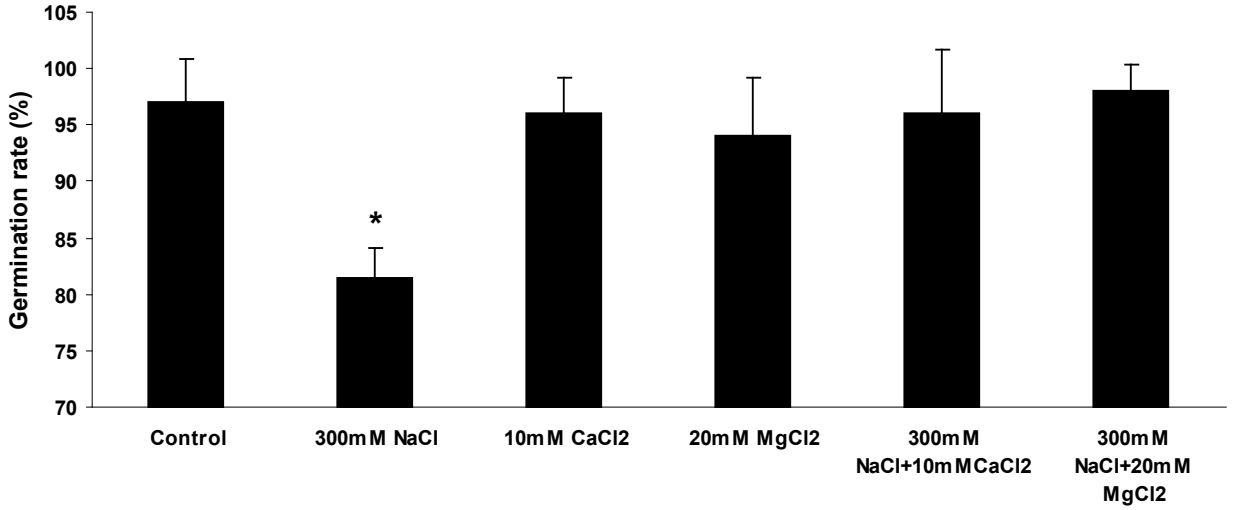

Fig. 3. Germination capacity (%) of seeds formed in *Plantago crassifolia* plants, after the indicated salt treatments (mean ± SD; n = 4). Only morphologically well-developed seeds, selected under a stereomicroscope, were used for the *in vitro* germination tests. The asterisk indicates statistically significant differences

Effects of $CaCl_2$ and $MgCl_2$ on vegetative and reproductive development

Both, $CaCl_2$ and $MgCl_2$ are toxic to plants at sufficiently high levels, so low concentrations of the salts-10 and 20 mM, respectively-were chosen for these experiments, after checking that they had no effect on *P. crassifolia* vegetative growth. Concerning reproductive development, both cations showed a similar behaviour. As for NaCl, they appeared to stimulate flowering, inducing the formation of more spikes than in the control, considering both, total number (11 *vs*. 8) and mean spike number per plant (2,2 *vs*. 1,0); the plants treated with Ca^{2+} or Mg^{2+} also produced shorter scapes and spikes (Tab. 2). However, contrary to the effect of NaCl, $CaCl_2$ and $MgCl_2$ at the tested concentrations did not affect the quantity or the quality of the produced seeds: the mean number and mean weight of seeds per spike, and the germination frequency did not differ significantly from those registered in the controls (Figs. 1-3).

Ca^{2+} and Mg^{2+} alleviate the effects of salt stress on Plantago crassifolia reproductive development

To investigate the possible effects of Ca^{2+} and Mg^{2+} under salt stress conditions, two batches of the plants were watered with solutions containing 300 mM NaCl plus 10 mM $CaCl_2$ or plus 20 mM $MgCl_2$. These combined treatments, as expected, did not affect vegetative growth of *P. crassifolia* plants, and clearly stimulated flowering (Tab. 2). A partially additive effect of the mono and divalent cations could be observed, since the total number of spikes and the mean number of spikes per plant were higher in the combined treatments with NaCl and $CaCl_2$ (or $MgCl_2$)

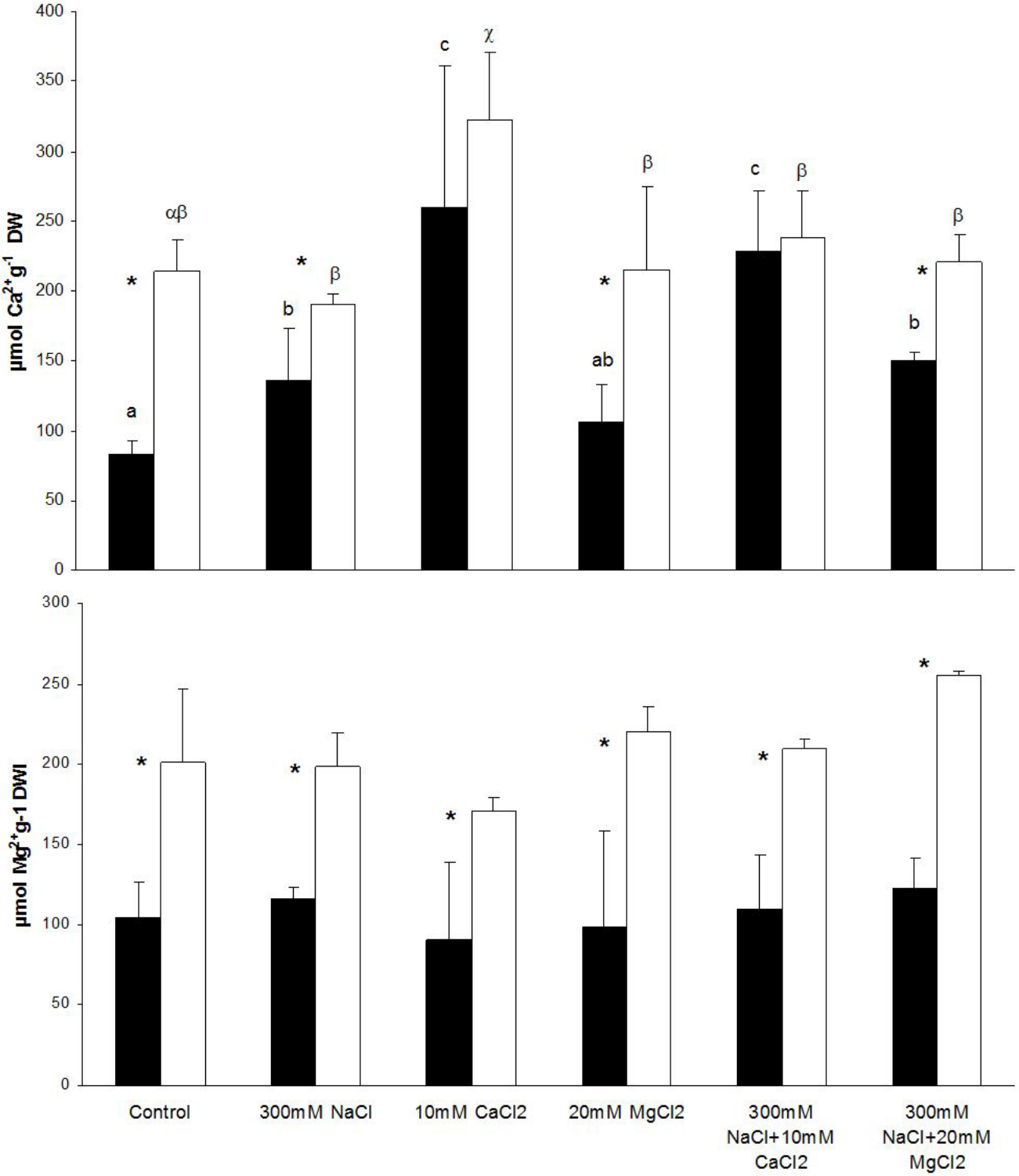

Fig. 4. Calcium (upper panel) and magnesium (lower panel) contents, expressed in µmol g^{-1} DW, in *Plantago crassifolia* plants, after the indicated salt treatments (mean ± SD; n = 6). White bars correspond to total cation contents, extracted by acid hydrolysis of the plant material, and black bars to the soluble fraction, extracted in boiling water. The asterisk indicates statistically significant differences between the values obtained by the two extraction methods. Different lower case letters, Latin for the soluble fraction and Greek for total cation contents, indicate significant differences between treatments

than for the plants treated with either salt alone (Tab. 2). However, the reduction of seed set and seed viability, which was detected in the presence of NaCl, was not observed when sodium chloride was supplemented with calcium or magnesium chloride: the mean number of seeds per spike (Fig. 1), the mean weight of seeds (Fig. 2) and the germination rate (Fig. 3) were similar to the values measured for the control plants not subjected to salt stress. In

fact, according to the Tukey test, for all these parameters the only statistically significant differences were observed for the treatment with NaCl. Therefore, according to our working hypothesis, Mg^{2+}, as well as Ca^{2+}, can counteract the inhibitory effects of high soil salinity on the reproductive development of *P. crassifolia*, at least under the specific experimental setup used in this study.

As mentioned in the Introduction, it is well known that Ca^{2+} can alleviate the adverse effects of salinity on many plant species (Agboola *et al.,* 1998; Ebert *et al.,* 2002; Marschner, 1995; Munns, 2002; Rengel, 1992), and there are many reports demonstrating this protective role of calcium in different systems and under varied experimental conditions. For example, it has been shown that addition of Ca^{2+} to root media containing NaCl favours plant growth in both halophytic (Colmer *et al.*, 1996) and non-halophytic species (Cramer *et al.,* 1986; Kinraide, 1999; LaHaye and Epstein, 1996; Suhayda *et al.,* 1992). Calcium also reduced the toxic effects of Na^{+} and Mg^{2+} on the germination of *Kalidum capsicum* (Tobe *et al.,* 2002) and *Hordeum vulgare* (Bliss *et al.*, 1986). Another study found that calcium significantly improved the germination in the presence of NaCl of seeds from different halophytes-*Arthrocnemum indicum, A. macrostachyum, Desmostachya bipinnata, Halopyrum mucronatum* and *Urochondra setulosa*- growing naturally in salt flats located in coastal regions from Karachi to Gadani (Gul and Khan, 2006). In several genotypes of barley, wheatgrass, maize, tomato, sorghum, cotton and bean, supplemental Ca^{2+} favourably increased fresh weight of the plants in the presence of NaCl (Cramer, 2002). In sorghum, an increase of the calcium concentration in the nutrient solution, from 1 to 10 mM, prevented the shortening of the leaves' growth zone which was observed in the presence of 100 mM NaCl (Bernstein *et al.,* 1993).

There are also a few published data suggesting an indirect role of Mg^{2+} in the mechanisms of plant defence against salt stress. For example, foliar application of magnesium was found to increase the total chlorophyll content in NaCl-treated strawberry plants (Yildirim *et al.,* 2009), but this is probably due to the role of the cation in building the chlorophyll molecule structure. On the other hand, since magnesium is very important in plant nutrition (Marschner, 1995) and salinity dramatically affects the mineral equilibrium in the soil solution, magnesium should be expected to play some positive role in the response of plants to high salinity, at least by improving plant nutrition. In any case, to the best of our knowledge, results directly demonstrating that $MgCl_2$ can counteract the deleterious effects of salt (NaCl) stress in plants have not been previously reported.

Ca^{2+} and Mg^{2+} levels in plants

The two cations were extracted from plant material by two different methods, as described in the 'Material and Methods' section. With acid digestion, total Ca and Mg was theoretically recovered, including the fraction associated with cell walls, membranes, etc., whereas by extraction in boiling water only the soluble fraction was quantified (Fig. 4). As expected, the differences between total and soluble cation levels were generally significant, although the patterns of variation were similar. Addition of $CaCl_2$ to pots resulted in a significant increase of total and soluble Ca^{2+} in the plants, as compared to other treatments-including NaCl-stressed and control plants-but the increment was relatively smaller in those plants treated simultaneously with NaCl (Fig. 4, upper panel). This can be explained by competition between Ca and Na uptake, since high Na concentration inhibits Ca absorption by plants (Alam, 1999). Plant processes such as growth, photosynthesis, mineral nutrition, water and ion transport are affected by these Na-Ca interactions (Cramer, 2002). Interestingly, the differences in Mg levels among treatments were not statistically significant (Fig. 4, lower panel). This could be explained, in part, by a mathematical artefact due to the relatively high variability of Mg contents in individual plants and the correspondingly high SD values. However, it could also indicate that magnesium levels are tightly regulated in the cells. Nevertheless, mean Mg values were slightly higher in the salt-stressed plants supplemented with $MgCl_2$ than in those treated with NaCl alone, and these small increase was probably sufficient to prevent the salt-induced inhibition of reproductive development in *Plantago crassifolia*.

Conclusions

Treatment of adult *Plantago crassifolia* plants with 300 mM NaCl, previous to the reproductive phase of development, did not affect vegetative growth but stimulated flowering and decreased the 'reproductive success' of the plants, as shown by the reduction in the number and quality-lower mean weight, lower germination capacity-of the produced seeds, compared with the control, non salt-stressed plants. Addition of low concentrations of $CaCl_2$ or $MgCl_2$ to the plants simultaneously with the NaCl treatments did not affect vegetative growth or flowering, but completely prevented the NaCl-dependent inhibition of reproductive development: the number, mean weight and germination rate of the seeds were the same as for the control plants. These results demonstrate that both divalent cations have a protective role against the effects of salt stress in plants. Whereas this fact is well established in the case of Ca, the results presented here, to our knowledge, constitute the first experimental demonstration of a similar role for Mg.

Acknowledgements

Work in the UPV laboratories was funded by a grant to O.V. from the Spanish Ministry of Science and Innovation (Project CGL2008-00438/BOS), with contribution from the European Regional Development Fund. M.-

N.G. acknowledges the support provided by the Romanian POSDRU/89/1.5/S/49944 project 'Developing the innovation capacity and improving the impact of research through post-doctoral programmes', and by COST Action FA0901: 'Putting Halophytes to work-From Genes to Ecosystems' for his stay in Valencia in the frame of a Short Term Scientific Mission.

References

Agboola DA (1998). Effect of saline solution and salt stress on seed germination of some tropical forest tree species. Tropical Biol 45:324-331.

Alam SM (1999). Nutrient uptake by plants under stress conditions, p. 285-313. In: Pessarakli M (Ed.). Handbook of plant and crop stress, 2nd ed. Marcel Dekker, Inc., New York, Basel.

Albert A, Yenush L, Gil-Mascarell MR, Rodríguez PL, Patel S, Martínez-Ripoll M, Blundell TL, Serrano R (2000). X-ray structure of yeast Hal2p, a major target of lithium and sodium toxicity, and identification of framework interactions determining cation sensitivity. J Mol Biol 295:927-938.

Ashraf M (1994). Breeding for salinity tolerance in plants. Crit Rev Plant Sci 13:17-42.

Bartels D, Sunkar R (2005). Drought and salt tolerance in plants. Crit Rev Plant Sci 24:23-58.

Bernstein L, Läuchli A, Silk WK (1993). Kinematics and dynamics of sorghum (*Sorghum bicolor* L.) leaf development at various Na/Ca salinities. I. Elongation growth. Plant Physiol 103:1107-1114.

Blanvillain R, Wei S, Wei P, Kim JH, Ow DW (2011). Stress tolerance to stress escape in plants: role of the OXS2 zinc-finger transcription factor family. EMBO J 30:3812-3822.

Bliss RD, Platt-Aloia KA, Thompson WW (1986). The inhibitory effect of NCl on barley germination. Plant Cell Environ 9:727-733.

Boscaiu M, Ballesteros G, Naranjo MA, Vicente O, Boira H (2011). Responses to salt stress in *Juncus acutus* and *J. maritimus* during seed germination and vegetative plant growth. Plant Biosyst 145:770-777.

Boscaiu M, Estrelles E, Soriano P, Vicente O (2005). Effects of salt stress on the reproductive biology of the halophyte *Plantago crassifolia*. Biol Plant 49:141-143.

Botella MA, Rosado A, Bressan RA, Hasegawa PM (2005). Plant adaptive responses to salinity stress, p. 37-70. In: Jenks MA, Hasegawa PM (Eds.). Plant abiotic stress. Blackwell Publishing, Oxford.

Boyer JS (1982). Plant productivity and environment. Science 218:443-448.

Bressan RA, Hasegawa PM, Pardo JM (1998). Plants use calcium to resolve salt stress. Trends Plant Sci 3:411-412.

Cheesman JM (1988). Mechanisms of salinity tolerance in plants. J Plant Physiol 87:547-550.

Colmer TD, Fan TWM, Highasi RM, Läuchli A (1996). Interactive effects of Ca^{2+} and NaCl salinity on the ionic relations and proline accumulation in the primary root tip of *Sorghum bicolor*. Physiol Plant 97:421-424.

Cramer GR (2002). Sodium-calcium interactions under salinity stress, p. 205-227. In: Läuchli A, Lüttge U (Eds.). Salinity: Environment-Plants-Molecules. Kluwer Academic Publishers, New York.

Cramer GR, Läuchli A, Epstein E (1986). Effect of NaCl and $CaCl_2$ on ion activities in complex nutrient solution and root growth of cotton. Plant Physiol 81:792-797.

Ebert G, Eberele J, Ali-Dinar H, Ludders P (2002). Ameliorating effects of Ca $(NO_3)_2$ on growth, mineral uptake and photosynthesis of NaCl-stresses guava seedlings (*Psidium guajava* L.). Sci Hort 93:125-135.

Forment J, Naranjo MA, Roldán M, Serrano R, Vicente O (2002) Expression of *Arabidopsis* SR-like splicing proteins confers salt tolerance to yeast and transgenic plants. Plant J 30:511-519.

Grattan SR, Grieve CM (1999). Mineral nutrient acquisition and response by plants grown in saline environments, p. 203-229. In: Pessarakli M (Ed.). Handbook of plant and crop stress. 2nd ed. Marcel Dekker, Inc., New York.

Gul B, Khan A (2006). Role of calcium in alleviating salinity effects in coastal halophytes, p. 107-114. In: Khan MA, Weber DJ (Eds.). Ecophysiology of high salinity tolerant plants. Springer, Dordrecht.

Hasegawa PM, Bressan RA, Zhu JK, Bohnert HJ (2000). Plant cellular and molecular responses to high salinity. Annu Rev Plant Physiol Plant Mol Biol 51:463-499.

Johnson DW, Smith SE, Dobrenz AK (1992). Genetic and phenotypic relationships in response to NaCl at different developmental stages in alfalfa. Theor Appl Genet 83:833-838.

Kinraide TB (1999). Interactions among Ca^{2+}, Na^{+} and K^{+} in salinity toxicity: quantitative resolution of multiple toxic and ameliorative effects. J Exp Bot 50:1495-1505.

LaHaye PA, Epstein E (1969). Salt toleration by plants: enhancement with calcium. Science 166:395-396.

Läuchli A, Epstein E (1990). Plant responses to saline and sodic conditions, p.113-137. In: Tanji, K.K. (Ed.). Agricultural Salinity Assessment and Management. Am Soc Civil Eng, New York.

Marschner H (1995). Mineral nutrition of higher plants, 2nd ed. Academic Press, London, 889 p.

Munns R (2002). Comparative physiology of salt and water stress. Plant Cell Environ 25:239-250.

Munns R, Termaat A (1986). Whole-plant responses to salinity. Austral J Plant Physiol 13:143-160.

Niu X, Bressan RA, Hasegawa PM, Pardo JM (1995). Ion homeostasis in NaCl stress environments. Plant Physiol 109:735-742.

Öpik H, Rolfe S (2005). The physiology of flowering plants, 4th ed. Cambridge University Press, Cambridge, 392p.

Rengel Z (1992). The role of calcium in salt toxicity. Plant Cell Environ 15:625-632.

Rodriguez-Navarro A (2000). Potassium transport in fungi and plants. Biochim Biophys Acta 1469:1-30.

Schofield CS (1942). United States National Resources Planning Board. The Pecos river joint investigation: Reports of Participating Agencies. US Government Printing Office, Washington, DC, 407 p.

Serrano R, Mulet JM, Rios G, Marquez JA, de Larriona IF, Leube MP, Mendizabal I, Pascual-Ahuir A, Proft MRR, Montesions C (1999). A glimpse of the mechanism of ion homeostasis during salt stress. J Exp Bot 50:1023-1036.

Suhayda CG, Redmann RE, Harvey BL, Cipywnyk AL (1992). Comparative response of cultivated and wild barley species to salinity stress and calcium supply. Crop Sci 32:154-163.

Tobe K, Li X, Omasa K (2002). Effect of sodium, magnesium and calcium salts on seed germination and radicle survival of a halophyte, *Kalidium capsicum* (*Chenopodiaceae*). Australian J Bot 50:163-169.

USSL Staff (1954). Diagnosis and improvement of saline and alkali soil. US Salinity Laboratory. USDA. Agriculture Handbook 60, U.S. Government Printing Office, Washington, D.C., 160 p.

Vicente O, Boscaiu M, Naranjo MA, Estrelles E, Bellés JM, Soriano P (2004). Responses to salt stress in the halophyte *Plantago crassifolia* (*Plantaginaceae)*. J Arid Environ 58:463-481.

Wada KC, Takeno K (2010). Stress-induced flowering. Plant Signaling and Behavior 5:944-947.

Weimberg R (1987). Solute adjustments in leaves of two species of wheat at two different stages of growth in response to salinity. Physiol Plant 70:381-388.

Xiong L, Schumaker KS, Zhu JK (2002). Cell signaling during cold, drought, and salt stress. Plant Cell 14 (suppl):S165-S183.

Yaish MW, Colasanti J, Rothstein SJ (2011). The role of epigenetic processes in controlling flowering time in plants exposed to stress. J Exp Bot 62:3727-3735.

Yildirim E, Karlidag H, Turan M (2009). Mitigation of salt stress in strawberry by foliar K, Ca and Mg nutrient supply. Plant Soil Environ 55:213-221.

Zhu JK (2002). Salt and drought stress signal transduction in plants. Annu Rev Plant Physiol Plant Mol Biol 53:247-273.

Zhu JK (2003). Regulation of ion homeostasis under salt stress. Curr Opinion Plant Biol 6:441-445.

Zhu JK, Liu J, Xiong L (1998). Genetic analysis of salt tolerance in *Arabidopsis.* Evidence for a critical role of potassium nutrition. Plant Cell 10:1181-1191.