RESEARCH PAPER

Interactive effects of excess boron and salinity on histological and ultrastructural leaves of *Zea mays* amylacea from the Lluta Valley (Arica-Chile)

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Abstract

E.I. Bastías, M.B. González-Moro, and C. González-Murua. 2013. Interactive effects of excess boron and salinity on histological and ultrastructural leaves of *Zea mays* amylacea from the Lluta Valley (Arica-Chile). Cien. Inv. Agr. 40(3): 581-595. Maize is widely cultivated throughout the world, and more maize is produced each year than any other grain. Studies on the response of maize to salinity have typically focused on physiological aspects, but few studies have addressed the effects of salinity on the anatomical and ultrastructural characteristics of different plant organs. We analyzed the cell structure and changes in tissue organization in young leaves of *Zea mays* L. amylacea as a consequence of high salt and boron (B) levels. Plants were treated in nutrient solutions for 20 days with concentrations of 100 mM NaCl (Low salinity, L) or 430 mM NaCl (High salinity, H) in the case of saline treatments or with an excess of B supplied as boric acid to obtain 20 mg (334 μM) or 40 mg (668 μM) B kg^{-1}. The application of B under no salt and low salinity conditions did not result in ultrastructure changes in mesophyll cells (MC) or bundle sheath cells (BSC). The high salinity conditions the amylacea leaf cells showed some alterations in MC chloroplasts, which appeared swollen and rounded. The BSC chloroplasts lost their perpendicular disposition to the cell wall. Moreover, the absolute absence of plastoglobuli could indicate a greater resistance to oxidative damage.

Key words: Amylacea, amylacea maize, anatomical plant, boron, histological, salinity.

Introduction

High salt concentration in soil is one of the most important factors influencing the distribution of plants in certain natural habitats. The presence of salts is also the major constraint to food production because it limits crop yield and restricts the use of previously uncultivated land. Salt stress is a worldwide problem, especially acute in Northern and Central Asia, Australia and South America (Pessarakli and Szabolics, 1999). High salinity conditions are aggravated by the presence of B in soils and waters in arid and semiarid environments. High salt and B levels are found in the Lluta valley of northern Chile, where elevated

levels of B in soils and irrigation water limit the local agricultural production to a few crops. In addition, this region has an annual precipitation less than 1 mm (Novoa *et al*., 1989). Maize is classified as a salt-sensitive crop plant, whose growth and productivity are reduced when salinity is above 2.5 mS cm^{-1}, although the sensitivity depends on the cultivar (Cramer *et al*., 1994). *Zea mays* L. amylacea is a sweet maize variety well-adapted to the agro-ecological characteristics of the Lluta valley; it is the main crop grown in the region. This maize variety is used as a source of germplasm to improve salt and B tolerance in other maize cultivars (Bastías *et al*., 2004a, b).

Salt tolerance in non-halophytic plants is a complex trait (Shi *et al*., 2000). Many different physiological factors may contribute to salt tolerance, such as the generation of osmoprotectants in the cytoplasm, the provision of energy by ATPases for the export of Na$^+$ and Cl$^-$, or the presence of specific transport proteins for the movement of these ions into the vacuole or out of the cell into the apoplastic space (Bastías *et al*., 2010). Physiological mechanisms of tolerance to high levels of NaCl and B in amylacea maize have been studied previously, including the salt accumulation capacity of tissues, photosynthetic assimilation, water relations (Bastías *et al*., 2004b), root hydraulic conductance (Lo), abundance of aquaporins, and ATPase activity (Bastías *et al*., 2004a; Martínez-Ballesta *et al*., 2008). Amylacea maize behaves as a salt excluder that overcomes salt stress by the restriction of salt to root tissues, preventing Na$^+$ from being transported to leaves and accumulating in photosynthetic tissues. In contrast, B accumulation takes place in leaves and is enhanced under saline conditions. This cultivar did not exhibit symptoms of toxicity to B and salt during a 20-day treatment and was able to maintain relative water content in leaves. Moreover, the presence of B in part mitigated the negative effect of salinity in amylacea (Bastías *et al*., 2004a, b).

A number of studies have examined the morphological and structural changes induced by salts in higher plants (Hernandez *et al*., 2001; Yamane

et al., 2004). The structural changes caused by salinity occur at several levels of organization. At the whole-plant level, the anatomic-morphological changes that allow plants to tolerate salt effects are mainly directed towards maintaining water use efficiency and balancing the carbon gain (Beerling and Woodward, 1995). Studies on changes in leaf ultrastructure produced by salinity have mainly focused on ultrastructural changes in chloroplasts. Observed changes in rice and tomato leaves include swelling of the thylakoid membrane, accumulating starch grain, distorting of grana stacking and increasing in size and number of plastoglobuli (Sam *et al*., 2003/2004; Navarro *et al*., 2007). Changes in the cytoplasm were related to changes in the organelles, invaginations of the plasma membrane (Dekov *et al*., 2000) and swelling of mitochondrial cristae (Sam *et al*., 2003/2004). The effects of excess B on foliar structure have also been studied in citrus (Mandarin) (Papadakis *et al*., 2004a, b) and kiwi plants (Sotiropoulos *et al*., 2002). Damage to chloroplast morphology, alteration of the membrane systems, changes in grana stacking and increases of the plastoglobuli due to B excess were observed (Papadakis *et al*., 2004a, b).

Few studies have focused on the effect of salinity on the anatomical and ultrastructural characteristics of plant organs. We analyzed the cell structure and changes in the tissue organization of young leaves of amylacea resulting from high salt and B levels. Studying the structural properties and anatomical changes under conditions of excess salt and B can provide new insights on the underlying mechanisms of salt limitation and response to B because the physiological functions of tissues are linked to their structural properties.

Materials and methods

Growth conditions and experimental design

The maize germplasm native to Northern Chile, *Zea mays* amylacea ("lluteño" local ecotype), was

germinated in a mixture of perlite and vermiculite (1:1; v/v). Seedlings were grown in plastic pots (2 L) with four plants per pot and irrigated every two or three days to maintain soil water at field capacity with Hoagland solution containing 20 mM NO_3-N/L (González-Moro *et al.*, 1997) adjusted to pH 5.5. The placement of maize pots was completely randomized. The plants were grown in a greenhouse with an average day/night temperature of 25/18 ºC and a relative humidity of 60/70%, respectively. Light intensity was set at 350 µmol m^{-2} s^{-1} and supplemented with warm-white lamps (Philips SON-T AGRO 400, Belgium), providing a 14 h photoperiod. Nutrient solutions were prepared using deionized water, and the pH, osmotic potential and conductivity were monitored weekly. During the first ten days after germination, plants were irrigated with the basic nutrient solution to maintain non-saline growing conditions. Subsequently, when plants showed a fully expanded third leaf, they were exposed to an excess of boron and salt for 20 days. The basic nutrient solution without the addition of extra salt (NaCl-0) or boron (B-0) was used as the control solution. This basic nutrient solution was supplemented in a factorial design with 100 mM NaCl (Low salinity, L) or 430 mM NaCl (High salinity, H) and with an excess of B supplied as boric acid to obtain 20 (334 µM) and 40 (668 µM) mg B kg^{-1} in the nutrient solution. Stress levels applied in this study were based on the reported concentrations of soil and irrigation water in different sectors the Lluta Valley. The harvesting of fresh plant material was performed between 10:00 and 11:00 A.M.

Preparation of tissue for anatomical studies

The third leaf of the maize plants was sampled and immediately cut into 2-3 mm^2 pieces and fixed in 5% glutaraldehyde in 0.05 M sodium cacodylate, pH 7.4 at room temperature for 48 h. Later they were vacuum-infiltrated in the same fixative several times until they sank. The segments were fixed at 4 ºC in sodium cacodylate

buffer for 3 h in total and washed three times (1 h each). Subsequently, samples were post-fixed in 1% osmium tetroxide in sodium cacodylate buffer for 2 h. The samples were dehydrated in an ascending series of acetone (30-100%) and propylene oxide. After dehydration, the samples were infiltrated with a series of araldite mixtures with propylene oxide (Durcupam ACM). Finally, tissues were embedded in 100% Araldite resin. Sections were cut with an ultramicrotome (Ultratome III, LKB Productor, and Control Unit Type 8802A). For light microscopy, thin sections (1 µm) were stained with toluidine blue (equal volumes of 1% basic toluidine blue and 1% Na tetraborate) and examined with a photo-microscope (Olympus BX 51). Ultrathin sections (<1 µm) were stained with uranyl acetate and lead citrate to be observed with a transmission electron microscope (TEM) (Philips 208C, operating at 80 kV). Microphotographs were taken from at least three sites in three different sections randomly, whose representative pictures are presented. Quantitative anatomical data were calculated using the leaf transverse section diagram according to Dengler *et al.* (1994).

Enzyme extraction and assays

Enzyme activities were determined in fully expanded third-position leaves from frozen material stored at -80 ºC. Extractions were performed at 4 ºC from plant tissue (0.1 g) ground in liquid nitrogen using a pre-chilled mortar and pestle. Plant material was homogenized with 100 mM Tris-HCl buffer (pH 7.2) containing 5 mM DTT, 10 mM $MgCl_2$, 1 mM EDTA*Na_2, 2 mM Cl_2Mn, 10% glycerol and 2% insoluble PVPP. The homogenate was centrifuged at 15000 g for 5 min at 4 ºC, and the resulting supernatant was used to determine phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) and NADP-malic enzyme (NADP-ME, EC 1.1.1.40). The extraction and assay for activity of PEPC was performed according to the method of Jiao and Chollet (1988). The enzymatic reaction for PEPC was measured at 30 ºC; the assay mixture contained 100 mM TRIS-HCl buffer

(pH 7.2) with 2 mM DTT, 5 mM $NaHCO_3$, 5 mM $KHCO_3$, 5 mM G6P, 2.5 mM PEP, 10000 units of malate dehydrogenase (MDH, EC 1.1.1.37), 0.2 mM NADH and the appropriate amount of the enzymatic extract in a total volume of 0.3 mL. PEPC activity was measured spectrophotometrically as the decrease in absorbance at 340 nm due to NADH and expressed as μmol NADH oxidized mg^{-1} protein h^{-1}. Extraction and activity measurement of NADP-malic enzyme was performed according to Kanai and Edwards (1973). The enzymatic reaction was measured at 30 ºC; the assay mixture contained 50 mM Tris-HCl buffer (pH 8.3), 5 mM L-malate, 0.5 mM NADP, 0.1 mM Na_2EDTA and 10 mM $MgCl_2$. The NADP-ME activity was determined spectrophotometrically, monitoring the increase in the absorbance at 340 nm due to NADPH that was expressed as μmol NADPH produced mg^{-1} protein h^{-1}.

Gel electrophoresis and protein blot analysis

Soluble proteins were extracted at 4 ºC from frozen powdered leaves in a buffer containing 50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM $MgCl_2$, 0.5% (w/v) PVPP, 0.1% (v/v), 2-mercaptoethanol, 20% (v/v) glycerol, 1 mM phenylmethylsulfonylfluoride (PMSF) and 4 mM leupeptin. Protein was separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). For analysis of leaf protein profiles, the percentage of polyacrylamide used in gels was 12% in the resolving gel and 4% in the stacking gel. Twenty micrograms of protein were loaded in each lane. After electrophoretic separation (150 mV for 45 min), the gel was stained with Coomassie Blue R-250 to quantify the amount of polypeptides. The apparent molecular weights of the polypeptide bands were estimated using prestained molecular weight markers for SDS-PAGE standards (Bio-Rad). The polypeptides were electrophoretically transferred to a nitrocellulose membrane (Amersham Hybond ECL, RPN 203D 0.45 μm pore size) overnight at low voltage. After blotting, the membrane was blocked with 5%

powdered non-fat dry milk in TBS containing 0.1% Tween 20 for 1 h at room temperature. The immunodetection was probed with anti-NADP-ME (1:1000) and anti-PEPC (1:1000) for 2 h at room temperature. Goat anti-rabbit IgG-alkaline phosphatase conjugate (Bio-Rad) at a dilution of 1:10000 was used as the secondary antibody for the detection of proteins. Scanning the density of the different protein bands and calculations was carried out with 1D-Manager (v. 2.0) software.

Statistical analysis

The experimental data were analyzed by ANOVA and the differences were compared by employing the Duncan test with a significance of $P \leq 0.05$ using SPSS software, version 11/PC (SPSS 11.0, 2001). All experiments described were repeated three times independently with six replications each time.

Results

Effect of salinity and B excess on leaf anatomy

The effects of excess salinity and B on leaf structure was evaluated by light microscopy performed on leaf thin sections of control versus stressed plants. Leaves of *Zea mays* amylacea exhibited typical Kranz-type anatomy (Figure 1A). Bundle sheath cells (BSC) appeared as large chlorophyllous cells in a single cell layer around the vascular bundles (veins), forming a compact sheath of isodiametric cells in the form of a crown. Mesophyll cells (MC) were radially arranged around the BSC. Under light microscopy, the presence of chloroplasts was easily recognized in the MC and BSC. The chloroplasts in the BSC showed their characteristic centrifugal position. The upper and lower epidermis were a monostratified layer of cells delimited towards the outside by a thick and smooth cellular wall. The application of excess B under non-saline conditions did not affect the organization of foliar tissue. Thus, as

observed in the leaf cross-section of plants grown with extra B (20 and 40 mg kg⁻¹) (Figure 1B), the Kranz anatomy and the large vascular bundles were clearly maintained and did not differ from the control plants. The application of the low salinity treatment (100 mM NaCl) for 20 days in the presence or absence of B did not cause any alterations in the organization of the foliar tissue or in the structure of the small, intermediate or large vascular bundles, as observed in the leaf cross-section (data not shown). Under conditions of high salinity, regardless of the presence or absence of B, slight changes were observed in the upper and lower epidermis (Figure 1C). Only high salinity induced the formation of irregular, dorsiventrally deformed and compressed cell walls in epidermis cells. In addition, greater intercellular space and more distorted spaces between cell walls of MC in the leaf tissue were observed (Figure 1C).

Morphometric parameters were estimated for the foliar tissue of amylacea plants under different B and salt treatments (Table 1). Leaf thickness, the distance between the adaxial and abaxial surfaces, decreased significantly with the increase in NaCl concentration in the nutrient solution. Under non-saline conditions, the presence of boron significantly increased

leaf thickness, up to 40%. Similarly, under saline conditions, mainly under high salinity, a significant recovery of leaf thickness was observed when extra B was applied, with an increase of more than 30%. The diameters of the vascular bundles and the distance and the number of MCs between two adjacent vascular bundles did not show statistically significant variation between the different treatments. The number of MCs adjacent to vascular bundles showed a statistically significant decrease under high salinity independent of B. As a result, an average of approximately 14 MCs were arranged around the vascular bundle in the control and low salinity-treated leaves, whereas an average of 10 MC occurred around the vascular bundles in leaves of plants treated with the high salinity solution (430 mM). However, no changes were observed in the diameter of the vascular bundle sheath or in the distance between two proximate vascular bundles. The mesophyll was formed by a one-cell layer between the upper epidermis and lower epidermis in the space between two vascular bundles. The number of MC lodged between two vascular bundles was also maintained at 4-5. Chloroplast number in BSC was substantially reduced (45%) under saline treatments although no significant difference in chloroplast number in MC between treatments was found (Table 1).
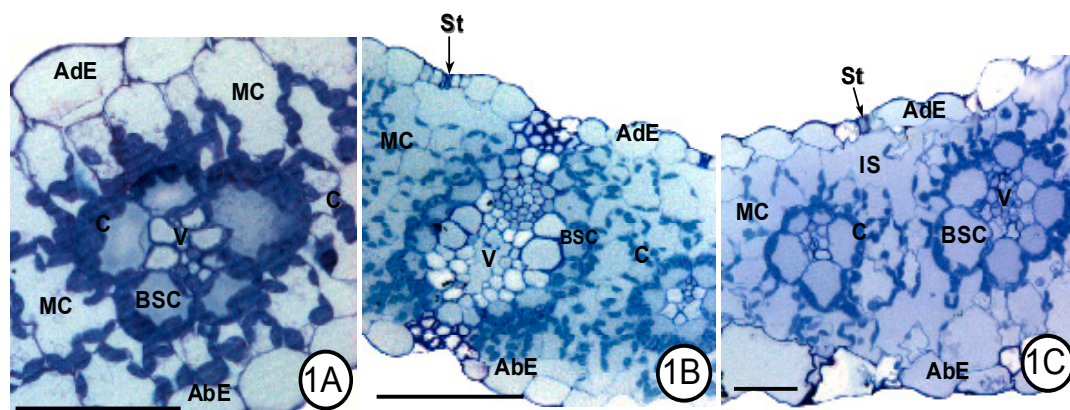
Figure 1. Light micrographs of cross sections of leaves in control plants (1A), 20 mg B kg⁻¹ (1B) and 430 mM NaCl (1C) of *Zea mays* L. amylacea. A-C: general view, leaf cell organization with typical Kranz anatomy. Bars represent 50 μm. Labels: AbE, abaxial epidermis cell; AdE, adaxial epidermis cell; BSC, bundle sheath cell; C, chloroplasts; MC, mesophyll cell; V, vascular bundle; St, stomata; IS, intercellular space.

Table 1. Salt and boron effect on leaf morphological and anatomic parameters of maize plants (*Zea mays* L. amylacea). Treatments: 0 mM NaCl (control), 100 mM NaCl, 430 mM NaCl, 0 ppm B (B0), 20 ppm B (B20) and 40 ppm B (B40). Values represent the mean ± SE (n = 10). Values with the same letter are not significantly different according to LSD at a level P≤0.05. 20 mg of protein was loaded in each track. Values are means of three plants.

| Treatments		Leaf thickness (mm)	BSC diameter (mm)	BSC separation (mm)	MC adjacent to vascular bundles	BSC chloroplast number	MC chloroplast number
NaCl (0 mM)	B0	300.00 ± 5.23 cd	134.28 ± 16.10 a	251.43 ± 21.05 a	15.50 ± 1.23 b	6.27 ± 0.29 b	4.97 ± 0.32 ab
	B20	508.56 ± 13.28 a	142.85 ± 6.40 a	252.85 ± 14.20 a	12.00 ± 0.89 d	7.55 ± 0.07 ab	4.80 ± 0.42 ab
	B40	342.84 ± 11.28 b	132.85 ± 3.70 a	224.43 ± 6.13 a	16.00 ± 1.30 a	8.51 ± 0.47 a	5.66 ± 0.44 a
NaCl (100 mM)	B0	274.67 ± 6.49 d	157.14 ± 6.40 a	210.00 ± 21.80 a	15.00 ± 0.78 b	6.20 ± 0.54 b	5.40 ± 0.57 a
	B20	314.59 ± 16.67 bc	131.42 ± 11.93 a	250.00 ± 6.40 a	12.00 ± 0.83 d	6.23 ± 0.46 b	4.10 ± 0.20 ab
	B40	320.56 ± 13.98 bc	142.85 ± 9.06 a	231.43 ± 7.14 a	14.00 ± 0.92 c	4.85 ± 0.54 c	4.30 ± 0.41 ab
NaCl (430 mM)	B0	218.99 ± 2.63 e	157.14 ± 18.85 a	235.71 ± 3.70 a	10.00 ± 0.91 e	3.50 ± 0.28 c	4.80 ± 0.59 ab
	B20	292.46 ± 23.99 cd	167.14 ± 8.06 a	247.14 ± 3.37 a	11.00 ± 0.97 e	3.62 ± 0.27 c	3.40 ± 0.23 b
	B40	287.50 ± 4.66 cd	171.43 ± 16.94 a	242.85 ± 5.26 a	10.00 ± 0.85 e	4.50 ± 0.43 c	4.00 ± 0.31 ab

Effect of excess salinity and B on leaf ultrastructure

The presence of large IS between MC was characteristic in the leaf tissue (Figure 2A). Amylacea maize showed a thin primary cell wall in photosynthetic MC and some other cell types. Between the primary walls of two adjacent MC, the middle lamella held adjoining cells together because of the presence of pectin substances between them. When the three adjacent cells were joined, the cellular maize was visible. This cellular maize is normally formed by pectin-enriched polysaccharides. As cells age, the pectin material in the cellular maize is degraded, thus leading to the appearance of large intercellular spaces.

Mesophyll cells in general showed a lower number of chloroplasts per cell (Figure 1A; Table 1) and smaller chloroplasts (Figures 2A) compared to BSC. MC chloroplasts contained abundant grana thylakoids, a character indicative of the presence of PSII in this cell type to provide reducing power, and showed absence of starch grains (Figure 2A). The BSC chloroplasts were larger and more abundant than MC chloroplasts and were arranged in an ordered way in a centrifugal position within BSC, according to the typical disposition shown by NADP-ME subtype C_4 species, such as maize (Figure 1). The leaf ultrastructure of control plants is shown in Figure 2. In amylacea, the BSC chloroplasts accumulated large starch grains (S) and contained few or no grana (Figure 2B). In addition, the BSC showed the typical large central vacuole (Figure 2A).

The application of the low salinity (100 mM NaCl) (Figure 2B) solution did not result in ultrastructural changes in MC or BSC. Alteration in the conformation of the cell wall was not observed; the structure of the primary wall and middle lamella was maintained, similar to what was observed in control leaf cells (Figure 2A).

The most remarkable changes were observed in MC under high salinity conditions either in presence or absence of B (Figures 2C-2D). First, the leaf cells of amylacea maize in high salinity conditions showed a tendency to increase the thickness of the cell wall and an altered shape and disposition of the plasma membrane (Figure 2C). The MC plasmalemma in salt-stressed plants was partly detached from the cell wall at various positions, indicating plasmolysis, which was not observed in the control plants (Figure 2A). This change led to the formation of small vesicles in the
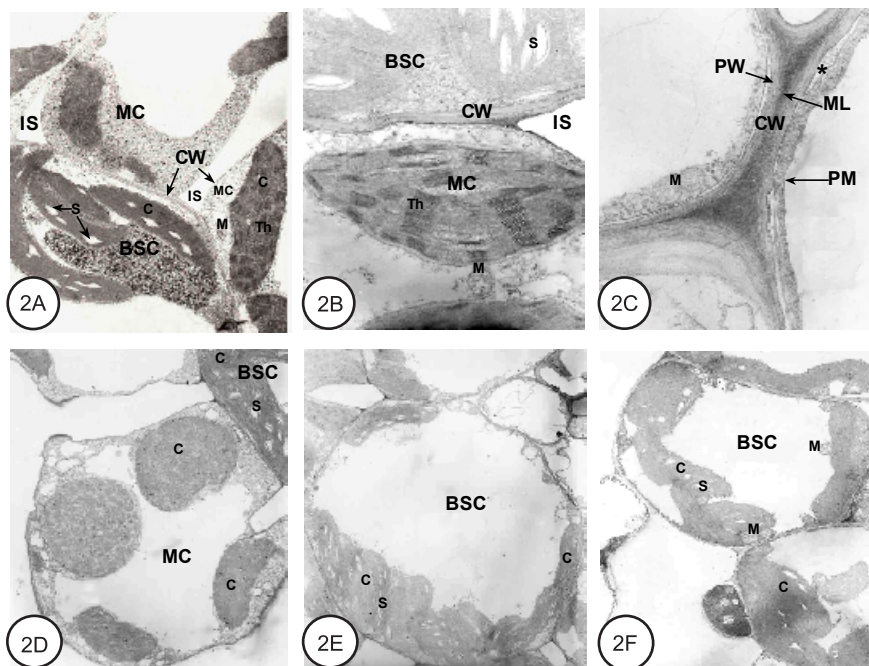
Figure 2. Transmission electron micrographs of leaves in control plant of *Zea mays* L. amylacea, mesophyll and bundle sheath cell (x 2,000) (2A), chloroplast of mesophyll and bundle sheath cell of salt-treated plant (100 mM NaCl, x10,000) (2B), plasma membrane and cell wall of salt-treated plant (430 mM NaCl, x16,000) (2C), chloroplast of mesophyll cell of salt-treated plant (430 mM NaCl x 2,500) (2D), chloroplast of bundle sheath cell of salt-treated plant (430 mM NaCl x 2,500) (2E), chloroplast of bundle sheath cell of salt-treated plant (430 mM NaCl+B40 x 3,200) (2F). Labels: BSC, bundle sheath Cell; C, chloroplasts; CW, cell wall; MC, mesophyll cell; ML, middle lamella; M, mitochondria; PM, plasma membrane; PW, primary cellular wall; IS, intercellular space; Th, thylakoid; S, starch.

space between the cell wall and the plasmalemma (indicated by an asterisk). The marked alteration of plasma membrane integrity was observed as a wavy and diffuse aspect in MCs. Second, some alterations were observed in MC chloroplasts; certain chloroplasts appeared swollen, with a rounded and voluminous aspect (Figure 2D). In addition, a disorganization of the internal system of thylakoid membranes was evident in the distortion of grana stacking and the swelling of stroma in some chloroplasts. However, other chloroplasts showed no alterations in the internal membrane system and thus were similar to the control. The BSC chloroplasts also showed certain disorganization, losing their perpendicular disposition to the cell wall and becoming oriented parallel to the cell wall (Figure 2E); however, other chloro-

plasts maintained their normal spatial disposition perpendicular to the cell wall. Additionally, an irregular pattern was observed for the presence of starch grains; some BSC chloroplasts showed few starch grains, while others maintained a normal abundance. Additionally, in BSC chloroplasts a certain dilation of the internal system of thylakoidal membranes was evident, where the separation of stromatic lamellae occurred (Figure 2F). Apart from these changes, structural damage in mitochondria was not observed in either MC or BSC, although swelling took place to some extent (Figure 2F). Plastoglobuli were not observed in amylacea maize, at least in the 3rd fully expanded leaf, under control, saline or B treatments (Figure 2).

*Enzyme activities*

The activities of the two enzymes involved in carboxylation and decarboxylation in the C4 cycle, PEPC and NADP-ME, were affected little by salinity levels or presumed toxic B levels (Figure 3), and their activities remained relatively constant. The activity of PEPC (Figure 3a) was very similar among the different treatments, with values between 40-50 μmol NADH mg^{-1} prot h^{-1} and a slight tendency to increase with salinity. The activity of NADP-ME also increased from 55 μmol NADPH mg^{-1} prot h^{-1} in the control to 67.01 μmol NADPH mg^{-1} prot h^{-1} under low salt conditions but decreased with higher salt (Figure 3b). The immunoblotting of PEPC and NADP-ME revealed a major protein with a molecular weight of 90 kDa, which corresponded to PEPC and a band whose molecular mass was 48.5 KDa, corresponding to NADP-ME (Figure 4).

**Discussion**

Boron effect

The application of excess B in non-saline conditions did not cause visual symptoms or alteration of the anatomy of foliar tissue of amylacea maize. Amylacea did not present any symptoms of toxicity in the presence of B, which makes sense because B behaved as a mobile element inside the plant, and its accumulation in the roots was very low (< 200 ppm B). The typical Kranz anatomy of maize was observed under normal and extra B levels. Moreover, there was a tendency towards better photosynthetic performance as indicated by the CO_2 assimilation and stomatal conductance (Bastías *et al.*, 2004b) when B was present, which may be explained by an increase in the photosynthetic capacity of mesophyll tissue as the number of MCs surrounding each vascular
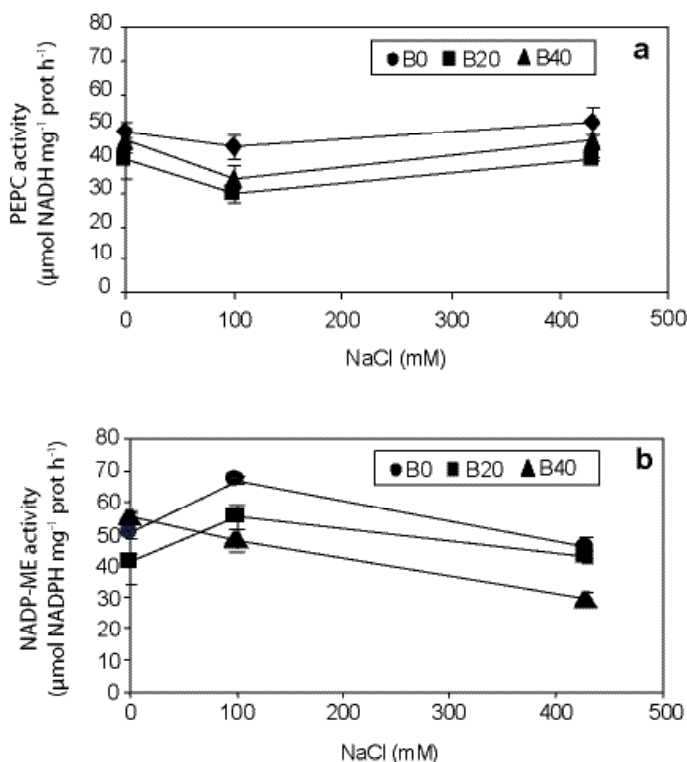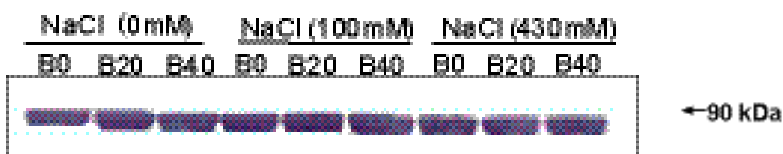

Figure 3. Effect of salinity and excess of B on PEPC activity (a) and NADP-ME activity (b) in leaf extracts of *Zea mays* L. amylacea after 20 days of treatment with NaCl (0 mM; 100 mM; 430 mM) and B (B0, control; B20, 20 mg kg^{-1}; B40, 40 mg kg^{-1}). Values represent the mean ± SE of three independent experiments.

a. Western NADP- ME
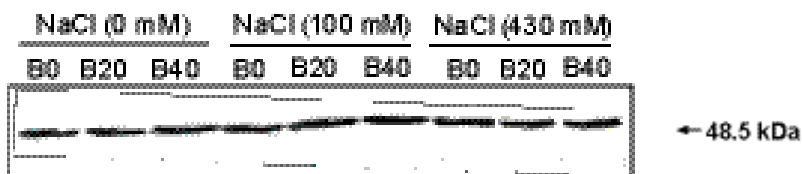
b. Western PEPCase

Figure 4. Effect of salinity and excess of B on NADP-ME (a) and PEPC (b) polypeptides in leaves of *Zea mays* L. amylacea using Western blot analysis after 20 days of treatment with NaCl and B. (a) the band of 48.5 kDa corresponds to NADP-ME protein and (b) the band of 90 kDa corresponds to PEPC. The relative amount of NADP-ME and PEPC were calculated following densitometry scanning of the Western blot using the value measured for the amount of NADP-ME and PEPC protein present in the plant control as a maximum

bundle as well as the number of chloroplasts in MC and BSC (increased slightly) (Table 1).

An ameliorative effect of B on some leaf biomass parameters has also been observed in tobacco plants (Ruiz *et al*., 2001). Nevertheless, in peach, kiwi, tangerine (Clementina) and orange (Navelina) plants, a reduction in the thickness of the cross-sections of the leaf was observed due to the reduction in the thickness of the spongy parenchyma, whose cells showed a disarranged and shrunken appearance, and to the damage in palisade parenchyma and disorganization of mesophyll cells, accompanied by large conspicuous intercellular spaces (Sotiropoulos *et a*l., 2002; Papadakis *et al*., 2004a, b). The transmission electron microscopy images of amylacea did not reveal remarkable changes in cell structure (Figure 2F). In contrast, considerable effects were reported at the level of the machinery involved in photosynthesis in orange plants (Navelina) and mandarin plants (Clementina) in conditions of excess B. At lower B concentrations, 8 to 16 times less than applied in our study, excess B resulted in a significant decrease in the width, length and relative volume of the chloroplasts of mesophyll cells (Papadakis

et al., 2004a, b). At the ultrastructural level, the chloroplast membrane system in the amylacea ecotype did not show any alteration of its aspect or organization (data not shown) in contrast to the results reported in orange and mandarin plants. In these citrus species, B excess did cause a dilation of the thylakoidal membrane system, decrease in thylakoid volume and, in some cases, their partial destruction (Papadakis *et al*., 2004a, b). The linking of B to some molecules of the cell wall (Bonilla *et al*., 2010) would reduce the movement of B into the cell, avoiding toxic B accumulation in the protoplasm and organelles. Thus, the beneficial effect of B on leaf area (Bastías *et al*., 2004b) was manifested in a way in cell structural components because the leaf cross-section area increased (Table 1). The absence of changes in leaf ultrastructure confirmed that cellular structure was well maintained in amylacea maize cells under excess B, at least up to 40 ppm B.

Salt effects

Amylacea maize behaves as a salt excluder, overcoming salt stress by the restriction of salt

to root tissues and preventing Na^+ from being transported to leaves and accumulating in photosynthetic tissues (Bastías *et al*., 2004a, b). According to previous results (Bastías *et al*., 2004b), this cultivar was able to accumulate Na^+ in root tissues up to 4.5%, while in leaves, this value was 4 times lower, which led a reduction in dry weight between 25-31% and 33-53% for roots and leaves, respectively.

In contrast to salt localization, B was distributed to a greater extent in leaves, and its transport to aerial parts was enhanced under salt conditions (Bastías *et al*., 2004b). Thus, the highest B content (850 ppm) was reached in 100 mM NaCl+40 mg B kg^{-1}-treated leaves, while the highest salt level accumulated in leaf tissue occurred when amylacea plants were grown under 430 mM NaCl, either in the absence or presence of 10 ppm B. Despite the lower Na^+ accumulation in leaves compared to roots, the growth of the leaves was more affected than that of the roots. In fact, leaf area diminished in the presence of salts (Bastías *et al*., 2004b), and leaf thickness decreased in plants grown at high salt levels (Table 1). The size of histological components such as bundle sheath, vascular bundle and bundle sheath-inter separation remained practically unchanged, and the number of MC adjacent to vascular bundles was reduced approximately 28%, which we attribute to the decrease in leaf thickness mainly because of the decrease in photosynthetic mesophyll parenchyma. Additionally, the application of boron prevented the decrease in leaf thickness observed under saline conditions (Table 1) and maintained a relatively good organization of Kranz anatomy (Figure 1B) even when salt accumulation reached 1% in the tissue (Bastías *et al*., 2004b).

The distribution of salt to mesophyll cells as consequence of being dragged by the transpirational stream would likely restrict damage mainly to epidermis and mesophyll cells, with minor consequences for BSC. At a minimum, this movement is what can be deduced by the enlarged intercellular spaces between in mesophyll and the compressed cell walls in epidermis cells. Many studies have suggested that reduced cell turgor potential and cell wall elasticity could lead to the formation of small cells and intercellular space area in salt-treated plants (Suarez *et al*., 1998). Changes observed in MC included a more irregular, smaller and less prismatic shape, which confirmed that the leaf thickness reduction under conditions of salinity was due to a reduction in cell size (Figure 1C). The bundle sheath showed tightly packed cells around the vascular sheath. An adaptive role has been suggested for the bundle sheath under water stress because it stores water to buffer transpiration surges (Barhoumi *et al*., 2007). As suggested for *Aeluropus littoralis*, a halophytic salt excreter grown at similar salt levels to amylacea in this study, the osmotic stress induced by salinity has no significant effect on the volume or shape of maize BSC. Although drastic overall changes in the foliar anatomy were not observed, salinity reduced the photosynthetic parenchyma tissue involved in primary carbon assimilation, the mesophyll tissue, by approximately 30% (Table 1) with respect to bundle sheath cell tissue, the tissue involved in photosynthetic carbon reduction. Both the reduction of MC number (Table 1) and MC size indicated a limitation of cell growth, likely due to the effect of osmotic stress on cell division and expansion (Hsiao *et al*., 1985). In addition, considering that leaf thickness was reduced (Table 1), the decrease observed in the CO_2 assimilation rate (Bastías *et al*., 2004b) would not be due to a limitation in mesophyll conductance, opposite to what has been described in other species where leaf thickness increased with salinity (Burssens *et al*., 2000). Even when leaves accumulated high levels of salts in leaf tissues, stomata did not present any damage (Figure 1C) and seemed to be functional in avoiding water loss and maintaining water status (Bastías *et al*. 2004a,b). Nevertheless, the reduction in mesophyll photosynthetic capacity due to a reduction in the number and size of mesophyll cells will influence the depletion of photosynthetic capacity. The loss of membrane integrity, confirmed by an increase in its permeability in a previous study (Bastías

et al., 2004b), would be assumed to be located preferentially also in mesophyll cells. This damage manifested in MC tissue will likely require adjustments in BSC photosynthetic capacity to maintain a balance, as far as possible, between both types of C4 photosynthetic cells. The size of BSC was maintained as well as the number of BSCs forming the vascular sheath, but the high depletion (50%) of the number of chloroplasts in BSC (Figure 1C; Table 1) will tend to compensate the decrease in mesophyll parenchyma photosynthetic capacity. This photosynthetic cooperation between cell types was also manifested in the maintenance of the two key enzyme activities involved in carboxylation and decarboxylation of the C_4 cycle when expressed on a protein basis, that is, PEPC located in MC and NADP-ME restricted to BSC, respectively (Figures 3 and 4).

The ultrastructural analysis of amylacea maize leaf by TEM showed alterations only under high salinity levels (430 mM NaCl), either in the absence or presence of B (Figure 2C, 2D and 2E). Changes were observed at the level of the cell wall, plasma membrane and chloroplast structure in both BSC and MC, and in some cases, changes in chloroplast disposition and organization of thylakoidal membranes occurred. As an example, according to Lal and Edwards (1996), under water stress conditions, maize presented disorganization of the Kranz anatomy, collapse of mesophyll cells, change in the centrifugal position of the chloroplasts of the BSC towards a centripetal position, collapse of the bulliform cells and loss of intercellular spaces.

Biochemical or ultrastructural modifications/ alterations, produced as a consequence of the different polysaccharide composition of the matrix and the synthesis of cell wall proteins, were also observed previously under saline conditions in some species (Yang and Yen, 2002). A thicker cell wall could help to preserve the pressure potential of the cell in an osmotic medium (Singh *et al*., 1989). According to previous data recorded for amylacea, plants exposed to high salt levels

showed a rigidification of the cell wall because the modulus of elasticity increased under saline conditions of 430 mM NaCl, but the increase was reversed by the addition of extra B (Bastías *et al*., 2004b). Many studies also suggested that reduced cell turgor potential and cell wall elasticity lead to the formation of small cells and intercellular spaces in salt-treated plants (Suarez *et al*., 1998).

The invaginations of the plasma membrane have been described in response to saline and water stress (Serraj *et al*., 1995; Niu *et al*., 1996). In amylacea maize, the plasma membrane presented small cristae-like invaginations and was detached from the cell wall in several regions (Figure 2C), indicating an incipient plasmolysis that may be caused by excess ions or osmotic stress. This alteration of the plasma membrane is in accordance with the increase in membrane permeability observed in this maize under high salinity conditions of 430 mM NaCl (Bastías *et al*., 2004b). Nevertheless, the structural damages observed by TEM in amylacea maize were less than those described in other maize and rice plants under saline conditions (Pareek *et al*., 1997). Recent evidence has demonstrated that the presence of apparent invaginations in the plasma membrane originated as a result of endosomal vesicles called plasmalemosomas. This presence of located multivesicular bodies could correspond to a situation of relocalization of tonoplast aquaporins because they exhibit PIP-type aquaporin that would help the cell to maintain an osmotic balance between the cytoplasm and the vacuole under stress conditions. Alternatively, multivesicular bodies could mediate the specific solute uptake toward the vacuole to avoid direct contact between ions and cytoplasmic components (Kirch *et al*., 2000; Vera-Estrella *et al*., 2004).

Salt stress can affect the ultrastructure of chloroplasts and destroy their membrane systems (Flowers and Yeo, 1995). Several studies have also reported alterations in chloroplast ultrastructure in halophytes and glycophytes affected by salinity (Salama *et al*., 1994). The effects

of salt stress were reported to cause swelling of thylakoids, distortion of stroma lamella and grana lamella in chloroplasts and even the dissolution of the chloroplast envelope (Mitsuya *et al*., 2000; Yamane *et al*., 2004; Sam *et al*., 2003/2004). The present study showed that the thylakoids in amylacea under high salinity were able to maintain their integrity. However, the alteration in chloroplast shape did not lead to severe damage of their internal organization or a drastic distortion of the thylakoidal membrane system (Figures 2D, and 2E). Some mesophyll chloroplasts did have a swollen aspect (Figure 2D), likely due to an osmotic imbalance between stroma and cytoplasm as a consequence of salt accumulation (Rahman *et al*., 2000). These alterations in shape or organization are likely due to the increase in the ionic composition of the stroma matrix. A distortion of grana stacking and stroma swelling were more notable in MC chloroplasts than in BSC, which was logically expected to happen given the greater damage in mesophyll cells. Chloroplast membrane permeability to NaCl has been described as low because this ion is excluded from chloroplasts when its level is low in order to maintain relatively constant ionic concentration, but chloroplasts can accumulate Na^+ when Na^+ levels are high. The response of Na^+ exclusion in chloroplasts appears to be an important prerequisite for maintaining photosynthetic capacity under severe salinity conditions and for developing an optimal degree of tolerance to this stress (Flowers and Yeo, 1995). Therefore, the loss of grana stacking in some MC chloroplasts could be related to the increase in the Na^+ concentration in chloroplast stroma (Utrillas and Alegre, 1997), and this osmotic imbalance between stroma and cytoplasm will cause that chloroplasts take this globular, spherical aspect (Figure 2D). The maintenance of the organization of thylakoidal structure under high salinity would explain a relatively high photosynthetic capacity of this maize (Bastias *et al*., 2004a, b). The photosynthetic assimilation rate showed a decrease of only 22%, apparently with no structural damage to

the photochemical apparatus or its functioning, as reflected by the maintenance of fluorescence parameters (Bastías *et al*., 2004b). Starch grains are typically restricted to BSC chloroplasts in C_4 plants (Leegood, 1985). It has been reported that starch accumulation is a generalized effect in salt-stressed plants (Barhoumi *et al*., 2007). In amylacea maize plants under high salt conditions, some BSC showed a starch grain accumulation (Figures 2D and 2E). An increase in starch grains in chloroplasts under saline conditions may be attributed to either damage to the sucrose-phosphate synthase present in the cytosol, directing the triose phosphate pathway towards starch synthesis, or to a toxic effect of salts on the enzymes involved in starch degradation (Rahman *et al*., 2000). It is important to note the total absence of plastoglobuli in chloroplasts in control and salt-boron-treated plants of amylacea maize (Figure 2), these lipid droplets were found in the cytoplasm of other plants treated with salt or exposed to water stress and may be a result of loose lipids in the cell membrane (Rahman *et al*., 2000). This characteristic indicates that salt-boron tolerance in amylacea maize could be partly explained by the absence of plastoglobuli in chloroplasts maintaining membrane integrity, potentially indicating a greater resistance to oxidative damage. The activities of C_4 photosynthetic enzymes resulted in a slight increase in PEPCase activities and a decrease in NADP-ME activities due to salinity (Figure 3), whereas almost no visible damage in the structure of MC and BSC chloroplasts (Figure 2) as has been previously reported by Omoto *et al*. (2012) in maize cv Honey Bantam as adaptation responses to salinity. The immunoblotting of PEPC and NADP-ME revealed no drastic changes in its expression, confirming the degree of tolerance of both stresses of amylacea (Figure 4).

In conclusion, the plants did not exhibit severe symptoms of toxicity to either NaCl or boron. This study shows that in amylacea maize, the MC and BSC in leaves had different responses to salinity, but there was very little change in the anatomi-

cal parameters and cell ultrastructure, including the absolute absence the plastoglobuli. The anatomical characteristics of the leaf confirmed the positive effect of B on the growth of amylacea under saline conditions. These properties may be responsible for their ability to withstand this stressful environment.

Resumen

E.I. Bastías, M.B. González-Moro y C. González-Murua. 2013. Efectos interactivos del exceso de boro y salinidad en la histología y ultraestructura de hojas de *Zea mays* amylacea del valle de Lluta (Arica-Chile). Cien. Inv. Agr. 40(3): 581-595. El maíz se cultiva en todo el mundo, se produce un mayor peso del maíz cada año, más que cualquier otro grano. Las respuestas de maíz a la salinidad se han centrado en aspectos fisiológicos, pero pocos estudios se han enfocado a los efectos de la salinidad sobre las características anatómicas y ultraestructurales de los diferentes órganos de la planta. Se analizó la estructura de la célula y la alteración en la organización del tejido en las hojas jóvenes de *Zea mays* L. amylacea como consecuencia de la alta concentración de los niveles de salinidad y boro (B). Las concentraciones fueron de 100 mM de NaCl (baja salinidad, L) o 430 mM de NaCl (de alta salinidad, H) en el caso de los tratamientos de solución salina, o con un exceso de B suministrado como ácido bórico para obtener 20 (334 μM) y 40 (668 μM) B mg kg^{-1} en la solución de nutritiva durante 20 días. La aplicación de B en condiciones no salinas y de baja salinidad no provocaron en cambios ultraestructurales en las células del mesófilo (CM) o células de la vaina del haz (CVH). En condiciones de alta salinidad las células de las hojas de amylacea mostraron algunas alteraciones en los cloroplastos de CM, mostrando un aspecto hinchado y redondeado. Los cloroplastos de CVH pierden su disposición perpendicular a la pared celular. Por otra parte, la ausencia absoluta plastoglobulina en las células, podría indicar una mayor resistencia al daño oxidativo.

Palabras clave: Amylacea, maíz amiláceo, anatomía de la planta, histología, salinidad.

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