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Root Plasma Membrane Lipid Changes in Relation to Water Transport in Pepper: a Response to NaCl and $CaCl_2$ Treatment

Claudia Silva[1,3], Francisco J. Aranda[2], Antonio Ortiz[2], Micaela Carvajal[1]*, Vicente Martínez[1], and José A. Teruel[2]

[1]Departamento de Nutrición y Vegetal. Centro de Edafología y Biología Aplicada del Segura-CSIC, Apdo. Correos 164, 30100 Espinardo, Murcia, Spain
[2]Departamento de Bioquímica y Biología Molecular A. Facultad de Veterinaria, Universidad de Murcia, 30100 Espinardo, Murcia, Spain
[3]Actual address: Facultad de Ciencias Agronómicas. Universidad de Tarapacá. Casilla 6-D, Arica, Chile

Seeds of *Capsicum annuum* were grown hydroponically in a nutrient medium with or without NaCl and with supplemented Ca^{2+}. Plasma membranes were isolated from roots using a two-phase aqueous polymer technique. The lipid composition (fatty acids, phospholipids and sterols) of the purified plasma membrane was determined. In the presence of NaCl, changes in lipid composition were shown, driving the membrane to a more rigid state. This was accomplished by an increase of (i) the saturation of fatty acids, (ii) the content of stearic acid versus palmitic acid, and (iii) the sterols concentration in the membrane. The changes in the phospholipid composition were also related to NaCl, which reverted when Ca^{2+} was also present in the nutrient solution. Furthermore, the alterations of plasma membrane lipid composition under salinity and calcium can be related to water transport properties of the membrane, but other physiological responses have to be taken into account.

Key words: calcium, lipid composition, pepper, plasma membrane, root hydraulic conductance, salt stress

The response of plants to salt excess is complex and involves changes in their morphology, physiology and metabolism. The root cell is the key site of plant interaction with salt in the surrounding medium. Previous work has demonstrated the controlling role of roots in salt sensitivity (Lacan and Durand 1995). It is generally accepted that the first deteriorating change during stress injury is an alteration in the structure and function of cell membranes. In many plants, alterations of lipids were observed as a result of water stress or salt treatment (Kuiper, 1985; Liljenberg, 1992). Changes in fatty acids, phospholipids and sterols may contribute, in the membrane, to the control of permeability and membrane functions (Schuler et al., 1990). Although in other reports it has been observed that osmotic stress induced alterations on the main phospholipids (PE and PC), but not the fatty acid composition (Norberg and Lijenberg 1992).

It is well-documented that calcium contributes to overall cell wall and membrane structural integrity. The association of calcium with the membrane components, especially phospholipids, is required to maintain membrane integrity and control membrane-associated functions (Hanson, 1984). Thus, calcium deficiency observed in plants in saline environments (Lynch and Laüchli, 1985) most likely impairs the membrane stability (Rengel, 1992), resulting in Na^+ accumulation and, eventually, cell death.

It has been shown that Na^+ displaces Ca^{2+} from membranes and alters K^+ and Ca^{2+} transport in plant roots (Cramer et al., 1985). Supplemented Ca^{2+} partially removes perturbations of selective ion transport, ionic balance and growth caused by salt stress. Under saline conditions, disorders may result from the effect on Ca^{2+} availability, competitive uptake, transport or partitioning within the plant (Grattan and Grieve, 1999; Cabañero et al., 2004). High NaCl concentrations have been shown to induce calcium deficiencies in different plants (Navarro et al., 2000; Kaya et al., 2002). In this way, it is known that external application of calcium can ameliorate the effects of high NaCl on diverse plant species (Cabañero et al., 2004). An adequate supply of calcium maintains membrane integrity and selectivity (Grattan and Grieve, 1999).

The response of plants to salinity involves water relations (Greenway and Munns, 1980). Root hydraulic conductance has been reported to be strongly reduced by salinity (Carvajal et al., 1998). Also, the effects of salinity seem to involve root aquaporins (Martínez-Ballesta et al., 2003), which leaded to suggest that there should be an effect of Ca^{+2} on aquaporins functionality involved in the amelioration of salinity effects (Carvajal et al., 2000).

The aim of the present study was to characterise the changes in root plasma membrane lipid composition in response to a saline growth medium, and if the alleviation effect observed on root hydraulic conductance when calcium is supplemented is related to these lipid changes.

MATERIALS AND METHODS

Seeds of *Capsicum annuum* cv. California Wonder were surface-sterilised in 5% sodium hypochlorite solution for 5 min and then washed with de-ionised water, imbibed for 24 h and finally grown in vermiculite, at 30°C in darkness. When the cotyledons appeared, the seedlings were transferred to the controlled environment chamber, with 16 h

*Corresponding author; fax +34-968-396213
e-mail mcarvaja@cebas.csic.es

Abbreviations: DPH: diphenylhexatriene; PA: phosphatidic acid; PC: phosphatidylcholine; PLD: phospholipase D; PS: phosphatidylserine; PE: phosphatidylethanolamine.

light, 8 h dark cycle and air temperature of 25 and 20°C, respectively. The relative humidity (RH) was 60% (light period) and 80% (dark) and the photosynthetically active radiation (PAR) was 400 μmol m^{-2} s^{-1}, provided by a combination of fluorescent tubes (Philips TLD 36/83, Germany and Sylvania F36 W/GRO, USA) and metal halide lamps (Osram HQ1, T 400W, Germany). Nutrient solution was changed every 4 d and pH was checked and adjusted daily to 6.0-6.5.

Then, the seedlings were transplanted to half-strength Hoagland's solution. After 21 d of growth, they were separated in four fractions, one to be used as a (i) control, and the nutritive solution of the others was enriched with (ii) 100 mM NaCl, (iii) 10 mM $CaCl_2$ and (iv) 100 mM NaCl plus 10 mM $CaCl_2$ ($CaCl_2$ was added two days earlier than NaCl). The plants were subjected to these treatments for 4 d, and then the roots were harvested, weighed and used for plasma membrane isolation or for root hydraulic conductance measurements. The plasma membrane vesicles were obtained from at least three independent experiments.

Root hydraulic conductivity

Hydraulic conductance of pepper roots (L_0) was measured by natural exudation. This method was based on the volume of flow through detached root systems. The aerial part of the plant was removed, leaving a little part of the stem immediately under the leaves, which were sealed with silicone grease into plastic tubes. After 3 h (in the case of saline treatment), the exudate was collected using Pasteur pipettes and transferred to Eppendorf tubes. The sap was weighed and the roots were removed and weighed. Samples of sap (100 μl) were measured using an osmometer (Digital Osmometer, Roebling, Berlin, FRG). The osmotic potential difference between the xylem sap and the external solution $\Delta\Psi_\pi$, was calculated from their osmolarity values. The hydraulic conductance, L_0, which has the units, mg (g root FW)$^{-1}$ h^{-1} MPa^{-1}, was: $L_0 = J_v / \Delta\Psi_\pi$.

Where J_v, the sap-flow, was expressed in mg g^{-1} root fresh weight h^{-1}.

Plasma membrane vesicles isolation

Plasma membranes were isolated and purified by using a two-phase aqueous polymer technique (Larsson et al., 1987) with some minor modifications. Approximately 20 g of fibrous roots from 4-5 plants were washed in cold distilled water, finely chopped and vacuum-infiltrated with 35 mL of a solution containing 50 mM Hepes adjusted to pH 7.5 with NaOH, 1 mM dithiotreitol, 5 mM ascorbic acid, 0.06% (w/v) polyvinylpyrrolidone and 0.5 M sucrose. The buffer-saturated material was homogenised and filtered through a 250-μm mesh nylon cloth. The filtrate was centrifuged at 10,000g for 15 min and the supernatant further centrifuged at 100,000g for 30 min, to yield a microsomal pellet which was re-suspended in 2 mL of 0.33 M sucrose in 5 mM phosphate buffer at pH 7.8. The suspension was added to 6 g of an aqueous two-phase mixture, producing an 8-g system with a final composition of 6.1% (w/w) Dextran T-500, 6.1% (w/w) polyethyleneglycol 3350, 3 mM KCl, 0.33 M sucrose and 5 mM phosphate buffer at pH 7.8. This phase system was centrifuged at 4,000g for 3 min. The resulting plasma membrane fraction (upper phase) was purified using a bath procedure. Then, the purified upper phase was diluted with 0.33 M sucrose in 5 mM phosphate at pH 7.8 and centrifuged at 100,000g for 30 min. The pellet was re-suspended in 0.9 mL of 5 mM Mes-Tris (pH 6.5) plus 0.33 M sucrose and stored at –20°C. The protein concentration was assayed by the method of Bradford (Bradford, 1976), using bovine serum albumin as standard.

Lipid analysis of plasma membrane

Total lipid extraction from the plasma membrane fraction was performed by addition of boiling 2-propanol (1/1) followed by 0.75 mL of chloroform/methanol (2/1, v/v) to 0.5 mL of membrane vesicles, followed by vortexing. Thereafter, 0.5 mL of chloroform was added to the mixture, which was then vortexed and centrifuged at 10,000g for 6 min. The lower phase was collected, evaporated to dryness under a stream of dry nitrogen and stored at –20°C.

Fatty acids were methylated in 7 mL of methanol and sulphuric acid, 120/2.7 (v/v), at 70°C for 2 h, in N_2. Then, 5 mL of hexane were added and the organic fraction was separated after centrifugation; then, dehydrated Na_2SO_4 was added to remove any traces of water. Finally, the samples were concentrated under a stream of N_2. The methyl esters of the fatty acids were separated and quantified by gas-liquid chromatography, using a 10% DEGS column. The ramp temperature of the detector was from 140 to 195°C at 5°C min^{-1} and the injector temperature was 200°C. Pentadecanoic acid was included in all samples as standard.

Sterols were converted to their trimethylsilyl derivatives, by reaction with bis(trimethylsilyl)trifluoroacetamide (BTSA), for gas-liquid chromatography analysis as follows: 2 μL of β-cholestanol (4 mg mL^{-1}) (used as internal standard) were added to 0.25 mL of lipid fraction and incubated for 1 hour at 60°C with 50 μL of BTSA/dimethylformamide (1/20). The analysis was made in a Shimadzu Gas Chromatographer GC mini 3, using an OV-17 column (100-200 mesh). The detector temperature was kept constant at 275°C and the injector temperature was 280°C.

Phospholipids were separated by TLC on silica gel. First, it was developed with ethyl ether/hexane/formic acid 60/40/1 (v/v/v) to separate the non-polar lipids, which migrated with the solvent. Then, a second development was done in chloroform/methanol/water 65/25/4 (v/v/v), to resolve the phospholipids. They were identified by comparing the Rf with known standards and quantified by scraping off the spots and phosphate determination after the procedure previously described (Böttcher et al., 1961).

Fluidity

The fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) was measured on a spectrofluorimeter (Hitachi F4500), in a thermostatized holder and with continuous stirring. Plasma membrane vesicles (0.15 mg) were diluted in 1 mL of 40 mM Hepes/KOH (pH 7.0), 100 mM KCl, 5 mM $MgSO_4$, 0.1 mM EGTA and 1 μM DPH. This solution was kept at 25°C for 1 h to allow incorporation of DPH into the membrane and stabilization of its fluorescence

emission. The fluorescence polarization was measured at an excitation wavelength of 360 nm and an emission wavelength of 430 nm.

RESULTS

Hydraulic conductance, L_0, (Fig. 1) in plants treated with 10 mM $CaCl_2$ was not significantly different from that obtained in control plants. The addition of 100 mM NaCl for 3 d gave a reduction of around 98% with respect to the control values. However, when 100 mM NaCl was added to plants previously treated (2 d) with 10 mM $CaCl_2$, a lower reduction was observed (50%) when they were compared with the control. Similar results were observed when weight was measured (Fig. 2). Weight of whole plants was not affected by $CaCl_2$ treatment and was significantly reduced after the NaCl treatment, whilst values for plants which received $CaCl_2$ prior to NaCl did not differ significantly from the control.

Changes in the plasma membrane properties were investigated for plants of *Capsicum annuum* grown under saline conditions imposed by the presence of a high NaCl concentration in the nutrient solution. The role of calcium in ameliorating the negative effect of salinity on the plasma membrane was also investigated.

The content of fatty acids in the membrane was analyzed by GLC. The major component found was linoleic acid, followed by palmitic and oleic acids, and, to a lesser extent, stearic (Fig. 3). Linolenic acid appeared always below 3% with no significant changes with treatments. Oleic acid decreased in the presence of NaCl, but it increased in the presence of Ca^{2+} as palmitic acid decreased. In the presence of both NaCl and Ca^{2+}, oleic acid decreased and

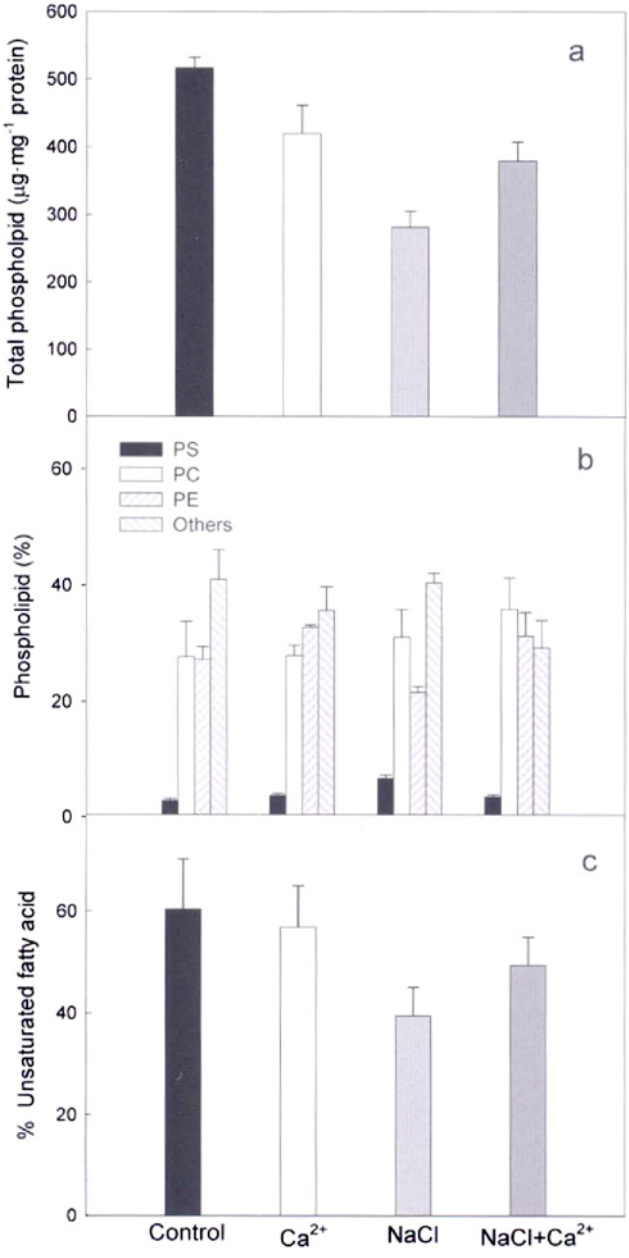

Figure 1. Root hydraulic conductance of plants grown in different conditions: Control, 10 mM $CaCl_2$, 100 mM NaCl and 100 mM NaCl plus 10 mM $CaCl_2$. Data are means (n = 5 ±S.E.)

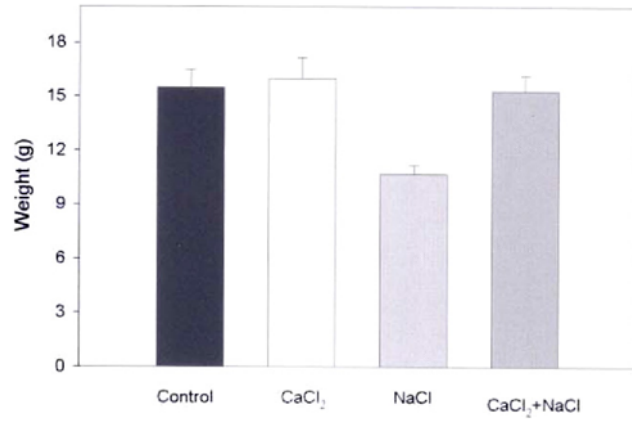

Figure 2. Fresh weight of plants grown in different conditions: Control, 10 mM $CaCl_2$, 100 mM NaCl and 100 mM NaCl plus 10 mM $CaCl_2$. Data are means (n = 5 ±S.E.)

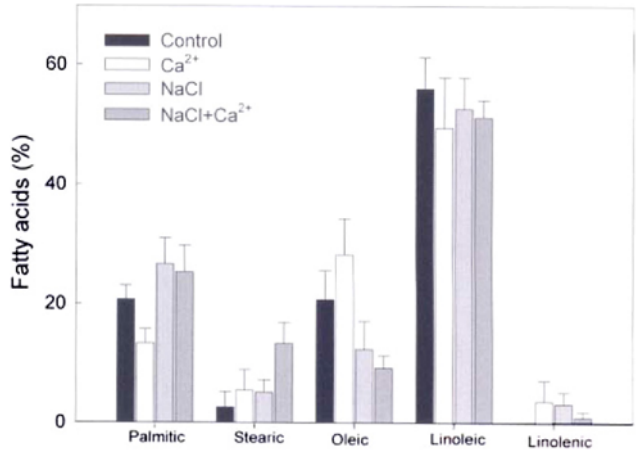

Figure 3. Total fatty acids of plasma membrane vesicles from roots of plants grown in different conditions: Control, 10 mM $CaCl_2$, 100 mM NaCl and 100 mM NaCl plus 10 mM $CaCl_2$. Data are means (±S.E.) of at least three independent membrane preparations.

stearic acid increased. The analysis of fatty acids was also carried out on whole root tissue with no significant differences between the samples (data not shown).

The phospholipid concentration in the plasma membrane was also determined. A decrease of the total amount of phospholipid was found with all the treatments assayed. However, the decrease was higher in the case of NaCl treatment (Fig. 4a). The individual phospholipids were quantified (Fig. 4b). The amount of fatty acid measured was expressed in PC and PE, as they were the major phospholipids found; PS was also distinguish and the rest were combined since they were more difficult to assign. Although, the fact that part of the amoung of other phospholipids was PA as a consequence of PLD activity is likely, the amount of PS, PC and PE were similar to other species (Norberg and Lijenberg, 1991). Differences in phospholipid composition can be seen in all treatments. However, the most striking effects observed were the increase of PS in the presence of NaCl which was reverted when NaCl was added with Ca^{+2}, and the decrease of PE also in the presence of NaCl. Also, an additional supply of Ca^{2+} recovered completely the PE content. When the fatty acids content (Fig. 4c) of all phospholipids is grouped into saturated and unsaturated fatty acids, a clear pattern is observed. It is noticeable that the degree of instauration was decreased in the presence of NaCl and partially restored with additional supply of Ca^{2+}.

The analysis of the fatty acids present in each phospholipid is shown in Table 1. In all phospholipids studied, the predominant saturated fatty acid in control samples was palmitic acid. In PS and PE, the predominant unsaturated fatty acid was linoleic acid. The effect of NaCl in PS was to decrease palmitic acid and increase stearic acid percentage. In the presence of Ca^{2+}, the same amount of both saturated acids was found, but linoleic acid decreased. In the samples from plants grown with NaCl and/or Ca^{2+}, oleic acid was increased in PC and PS. In PC, the predominant unsaturated fatty acids in control plants were linoleic acid and oleic acid. In the treatments containing Ca^{2+}, palmitic acid was replaced by stearic acid. Oleic acid was decreased in the presence of NaCl, linoleic acid being the predominant unsaturated fatty acid. In PE, the predominant unsaturated fatty acid was linoleic acid, whilst in the presence of NaCl, linoleic acid was decreased and palmitic acid was increased. For the rest of the phospholipids, there was an increase in the amount of palmitic acid in all treatments. In our sam-

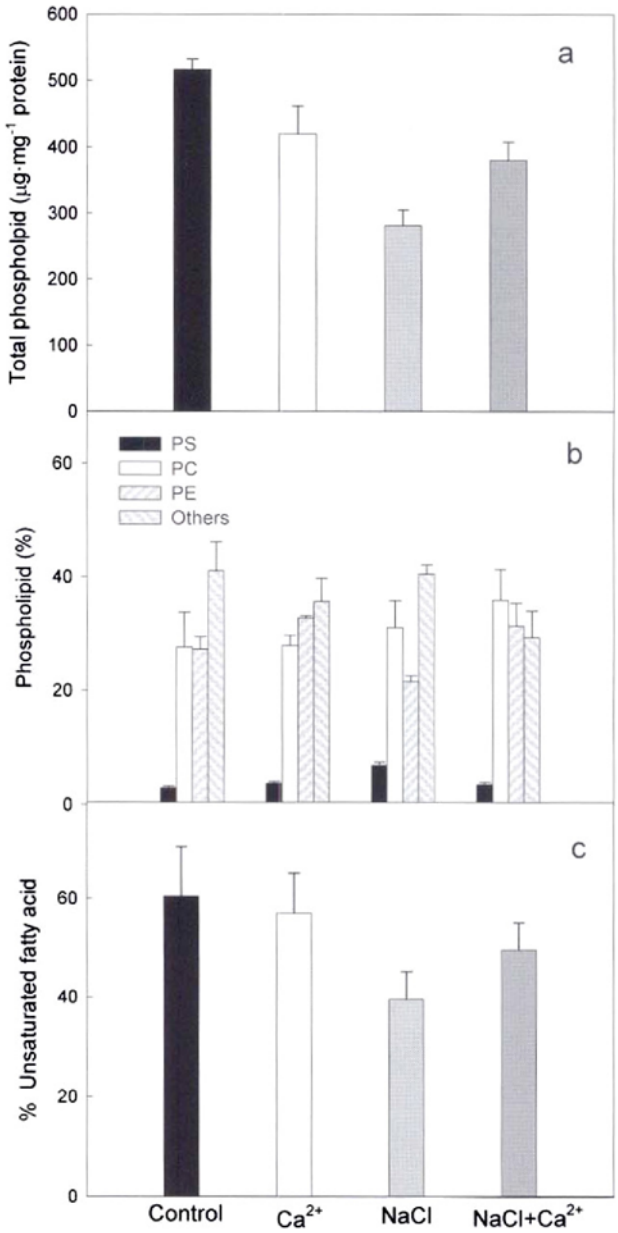

Figure 4. Phospholipids composition. (a) Total phospholipids content in plasma membrane vesicles, as measured from inorganic phosphate after extensive hydrolysis of the lipid extract. The concentration of phospholipids is referred to the protein concentration in plasma membranes. (b) Main phospholipids content present in plasma membrane, separated by thin layer chromatography: PS, PC, PE and others. (c) Degree of unsaturation, as % of unsaturated fatty acids in the phospholipids fraction, for ealch treatment. Data are means (±S.E.) of at least three independent membrane preparations.

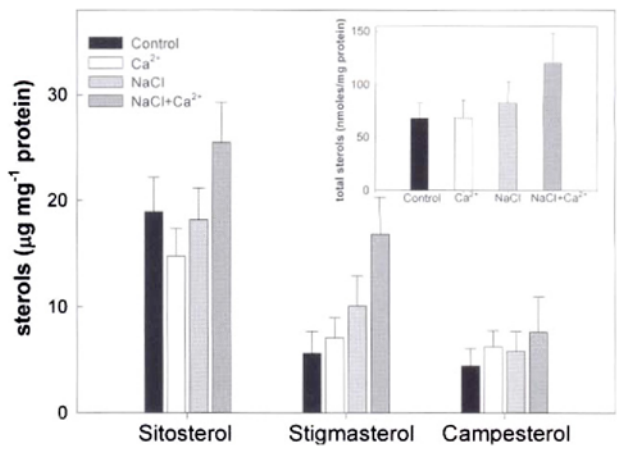

Figure 5. Sterols content in plasma membrane vesicles from roots of plants grown in different saline conditions: Control, 10 mM $CaCl_2$, 100 mM NaCl and 100 mM NaCl plus 10 mM $CaCl_2$. Sterol concentration is referred to the protein concentration in plasma membranes. In the insert, the total sterol content for each treatment is shown. Data are means (±S.E.) of at least three independent membrane preparations.

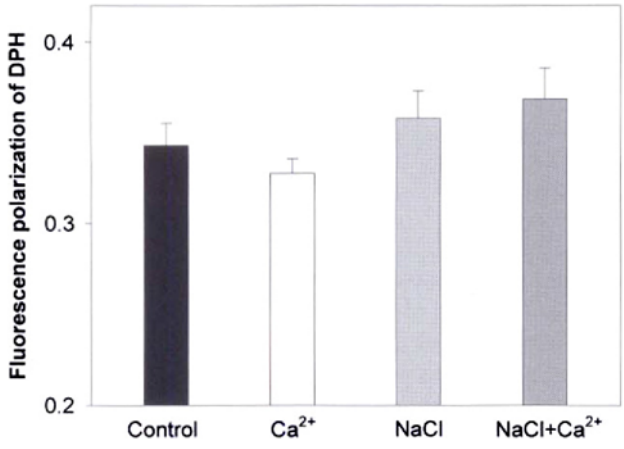

Figure 6. DPH polarization. Plasma membrane fluidity for each treatment. The plasma membrane fluidity was estimated by the fluorescence polarization of the fluorescent dye DPH. Data are means (±S.E.) of at least three independent membrane preparations.

ples, the content of linolenic acid was not significant in most cases and was not quantified (data not shown).

In all treatments, the predominant sterol in the plasma membrane preparations was sitosterol, followed by stigmasterol and with a lesser amount of campesterol (Fig. 5). In the treatments with NaCl, an important increase in stigmasterol content was seen. In the sample treated only with Ca^{2+}, there was a decrease of sitosterol. When NaCl was added with $CaCl_2$, the total amount of sterol is increased. No significant differences were observed for campesterol.

The total amount of sterols was also affected by the salt treatments (Fig. 5 insert). The presence of NaCl promoted an enrichment of sterols in the plasma membrane.

To test whether the differences in lipid composition induced changes in plasma membrane fluidity, steady-state fluorescence polarization of DPH was determined (Fig. 6). Sodium ions induced a slight increase in the polarization of DPH inserted in the plasma membrane vesicles; indicating that DPH molecules are in a more rigid environment in the membrane bilayer, allowing a lower degree of molecular motion. This is directly related to a decrease of the membrane fluidity.

DISCUSSION

In the experiments described in this paper, a strong reduction of L_0 in roots pepper plants after 4 d of NaCl supply has been observed. Reductions in root hydraulic conductance, L_0, of salinized plants have been shown in several reports (Evlagon et al., 1990; Carvajal et al., 1998), suggesting that there must have been a toxic effect of NaCl on water pass through the membranes. In the experiments of this paper $CaCl_2$ was able to partially compensate for the toxic effect of NaCl on pepper root hydraulic conductance (Fig. 1). This beneficial effect of calcium on the development of plants grown under saline conditions has been reported previously (Cramer et al., 1988) and we suggested that the reduction of L_0 was related to aquaporins functionality (Carvajal et al., 2000). Therefore, calcium may act by stabilizing the plasma membrane and so by aquaporins.

Table 1. Percentage of fatty acids in the phospholipids of the plasma membrane vesicles. Data represent means of three independent membrane preparations. The error was in all cases below 22.3%. n.d., not detected.

Fatty acid		Phospholipids			
		PC	PE	PS	Others
Palmitic	control	44.8	40.4	54.8	46.8
	Ca^{2+}	n.d.	49.4	33.1	85.9
	NaCl	72.5	73.9	3.1	82.9
	NaCl+Ca^{2+}	n.d.	82.9	38.7	74.0
Stearic	control	9.5	8.3	6.88	12.4
	Ca^{2+}	35.4	5.8	33.0	2.1
	NaCl	6.1	7.1	43.3	4.4
	NaCl+Ca^{2-}	19.9	4.3	2	2.7
Oleic	control	22.8	13.4	7.83	15.3
	Ca^{2-}	34.7	6.6	22.8	2.6
	NaCl	5.1	7.1	22.2	7.8
	NaCl+Ca^{2+}	40.1	7.9	12.4	6.6
Linoleic	control	22.9	37.9	30.4	20.2
	Ca^{2+}	29.9	38.3	10.2	9.4
	NaCl	16.2	12.0	31.4	4.9
	NaCl+Ca^{2+}	40.0	5.0	46.9	17.0

Short-term experiments performed by adding supplemental calcium to the saline growth media only had ameliorative effects under NaCl stress (Azaizeh and Steudle, 1991; Carvajal et al., 2000; Cabañero et al., 2004). However, other results indicated that the addition of extra calcium could not completely restore growth after salt stress was already imposed, although plants maintained normal root growth rates when high calcium concentrations were present in the growth solution before the addition of NaCl (Cramer et al., 1986; Ortiz et al., 1994). Therefore, in our experiments, the extra-calcium treatment was added 2 days before the NaCl-treatment was applied. But a complete restoration was observed after calcium application together with NaCl. This effect was probably due to the fact that the reduction of growth obtained in saline plants was not very high as a consequence of the short term effect of the salinity treatments.

The fatty acids composition of phospholipids changed under salinity conditions (Table 1): In some cases (NaCl+Ca^{2+} treatment) unsaturated fatty acids, such as oleic acid in PC, were partially replaced by saturated ones, such as palmitic acid. As a result, the degree of saturation in the membrane became higher, the lipid order increased and the fluidity of the membrane decreased, contributing to the stabilization of the membrane. And in other cases, stearic acid decreases to palmitic acid (e.g. NaCl treatment), producing a more rigid membrane, since the melting point of stearic acid is higher than that of palmitic acid (53°C and 41°C, respectively). Opposite results have been reported previously (Cachorro et al., 1993), indicating that an increase of the level of saturation could be related to salt resistance. This indicate that in our plants, the resistance mechanism was not related to the fatty acid composition, but gives to cal-

cium (directly or indirectly) a role in this process. Also, the higher rigidity that instauration confers, is also manifested when the ability of a molecule to move inside the membrane is observed. The fluorescence polarization of a molecule was slightly enhanced in membranes subjected to salt-stress conditions (Fig. 4). However, no effect of Ca was observed.

It has been reported that phospholipid composition influences the dynamics and structural properties of the membranes including water surrounding molecules (Sum, 2005). Also, the water molecules at the interface with phospholipid bilayer were influenced by headgroups of the phospholipid species (Bhide and Berkowitz, 2005) which modulate the transbilayer flux of water (Milhaud, 2004). On the other hand, it has been reported that aquaporin activity was modulated by phospholipids (Zampighi et al., 2003). Therefore, the phospolipid composition might influence water pass through membrane in the two different routes: *via* lipid bilayer and *via* aquaporins. We have shown that calcium restores some of the properties of the membrane from plants treated with NaCl. The most significant is an important increase of PS content, accompanied by an decrease in PE content (Fig. 4b), which could be an important key in the modulation of water transport through plasma membrane.

It is well-known that PS binds calcium with a stoichiometry of 2:1 in the outer surface of the membrane, with a millimolar dissociation constant (Hauser et al., 1976; Holwerda et al., 1981). In plant cells, it has been shown that PS was asymmetrically distributed among both membrane leaflets (O´Brien et al., 1997). In animal and fungal plasma membranes, PS was normally distributed asymmetrically and was, in general, associated with the cytosolic face (Devaux, 1991; Schroit and Zwaal, 1991). Other authors (Takeda and Kasamo, 2001) have been described that PS was also located in the inner leaflet of the plant plasma membrane in the same way as in mammalian cells (Zachowski, 1993). When calcium is present, it is partially bound to PS in the membrane, contributing to the charge stabilization of the membrane; however, a decrease of calcium can perturb the biophysical properties of the membrane and producing its destabilization. It has been shown that sodium binding to PS produces dehydration of the membrane, and, finally, disruption of the membrane integrity (Ekerdt and Papahadjopoulos, 1982). Therefore, it is feasible that one response of the root cells to high salinity concentrations would be the increase of the PS level in the membrane, to achieve more calcium binding to the surface of the membrane, thus contributing to its stabilization. However, the distribution of PS in our membranes should be further investigated.

On the one hand, it has been pointed out that calcium induces higher levels of decarboxylase activity, probably by inducing the synthesis of the enzyme (Hawrot and Kennedy, 1978). On the other hand, the PE methyltransferase activity is enhanced by the presence of calcium, with a $K_{0.5} < 0.5$ μM (Kowluru et al., 1985). Finally, the exchange reaction due to the PS synthase activity is calcium-dependent with a K_m of 0.9 mM (Dennis and Kennedy, 1970). Therefore, an increase of calcium concentration could lead to the conversion of PS to PE and PC, and PE to PC, producing a decrease of PS content and a consequent increment of PE concentration. The maintenance of PE and PC by calcium has been reported in apple fruit (Picchioni et al., 1998) suggesting that Ca^{2+} prevents lipid catabolism (Bonza et al., 2001). It has been also reported that the ratio of PC/PE, the major phospholipids in plants, takes importance from the point of view of acclimation to water stress, what support our results (Berglund et al., 2004)

Sterols serve crucial functions to control the fluidity and permeability of membranes as well as being precursors for additional metabolites, such as growth regulators. An important chemical change induced by saline conditions involves the composition of sterols in the plasma membrane. We found that the major sterols in our samples were sitosterol, stigmasterol and campesterol, in this order.

The total sterols content in the membrane increased (Fig. 5 insert), the principal changes being found in stigmasterol and sitosterol, when the growing medium was supplemented by both sodium and calcium (Fig. 5), the relative content of stigmasterol being greatly increased. However, upon sodium treatment, stigmasterol increased. This is feasible, since stigmasterol is formed from sitosterol by the activity of a sterol Δ^{22}-desaturase, which belongs to a P450 family and regulates the balance between the two sterols. In seed tissue, it has been shown that the conversion of sitosterol to stigmasterol is a critical slow step (Holmberg et al., 2002). Besides, it has been reported that, under salt-stress conditions (1 M NaCl, 90 min), the expression of sterol Ä^{22}-desaturase is induced up to 4-fold in *Saccharomyces cerevisae*, indicating an activation of sitosterol-to-stigmasterol transformation and conversion of campesterol into brassicasterol (Yale and Bohnert, 2001).

It is surprising that Ca^{2+} does not alter the total amount of sterols but when it is supplemented with Na^+, the total sterols in the PM increases (Fig. 5, insert). The first committed step in the biosynthesis of sterols is the conversion of cycloarterol into Δ^5-C24-alkyl sterols, catalysed by an S-adenosylmethionine-dependent sterol-C24-methyltransferase type 1. It has been proposed that C24 methylation of cycloartenol is a major site of regulation in the sterol biosynthesis pathway (Nes, 2000). In contrast to animals, higher plants contain mixtures of 24-alkyl-Δ^5-sterols, of which campesterol is the principal 24-methyl sterol, and sitosterol and stigmasterol are the predominant 24-ethyl sterols (Schaller et al., 1998). High salt concentrations in the growing medium lead to an increase in total sterol content, as a plant response to the salt (Fig. 5 insert). An increase in the sterol content of a membrane in the liquid-crystalline phase facilitates hydrophobic interactions among the acyl chains, introducing a higher order in the membrane and, consequently, a more rigid membrane (Demel and De Kruijff, 1976). Sitosterol was found to be the sterol most efficient in reducing water permeability in soybean PC bilayers (Schuler et al., 1991). These results show that sitosterol and stigmasterol, the two major sterols in our samples, probably could play different roles in regulating plant membrane properties.

All these results point to a response of the cell in order to protect itself against the aggression of high salt content, by driving the membrane to a more rigid state and probably by changing transport properties of the membrane. Thus, the fact that Ca^{2+} ameliorated the effect of NaCl could be

explained by changes in the plasma membrane phospholipid composition (including instauration of fatty acids). However, a role of Ca^{2+} as modulator of metabolic pathways has also to be taken into account in the physiological response of plants to salinity.

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