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Antioxidant System Response in Hot Pepper Fruits (*Capsicum annuum* L.) under Saline Stress Conditions during Cold Storage

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ABSTRACT

A pot experiment was carried out with hot pepper (*Capsicum* spp. cv. 'Caballero') to investigate the antioxidant system response in fruits of this species under moderate saline conditions (12.5 and 25 mM NaCl) for 60 days and stored in the immature stage at 4°C for 90 days. After different storage periods, we counted the number of fruits and measured their length, diameter, and fresh weight, as well as superoxide dismutase (SOD) and catalase (CAT) activities, lipid peroxidation, ascorbic acid content, protein oxidation, chlorophyll *a* and *b* content, and carotenoids (violaxanthin, zeaxanthin, and β-carotene). The results showed no statistical changes in the number of fruits or in their morphological characteristics in the different treatments. However, the other parameters that were measured showed significant differences between salt treatments, storage periods, and interaction between both factors. The results suggested the involvement of the different SOD isoenzymes in the ripening process of fruits from the salt-treated plants. The 12.5 mM NaCl treatment was beneficial for the fruits, which presented higher levels of ascorbate (100 mg L^{-1}) than control fruits and for the long storage at 4°C where fruits showed lower oxidative stress parameters.

Keywords: ascorbate, carotenoids, catalase, cold storage, pepper, salinity, superoxide dismutase

Abbreviations: **APX**, ascorbate peroxidase; **ASC**, ascorbic acid; **BSA**, bovine serum albumin; **CAT**, catalase; **Chl**, chlorophyll; **CIBNOR**, Centro de Investigaciones Biológicas del Noroeste, S.C.; **CONACYT**, Consejo Nacional de Ciencia y Tecnología; **EC**, electric conductivity; **EDTA**, ethylenediaminetetraacetic; **HPLC**, high-performance liquid chromatography; **PVP**, acid polyvinyl-pyrrolidone; **ROS**, reactive oxygen species; **SOD**, superoxide dismutase; **TBARS**, thiobarbituric acid-reactive substances; **TCA**, tricloroacetic acid

INTRODUCTION

Salinity is one of the environmental stress issues with increasing relevance due to a remarkable profit decrease on several crops, particularly on arid and semi-arid regions, where soil salt content is naturally high and rainfall levels are insufficient for salt excess drainage (Boyer 1982; Serrano 1999; Zhu 2001). Salinity may represent osmotic stress on plants, as well as ionic toxicity along with oxidative stress induction, which causes interruption, restriction, or acceleration of the regular metabolic process, such as maturing and senescence always in a negative or an opposite sense (Walker *et al*. 1980; Lester 1999; Hernández *et al*. 2001; Bandeoğlu *et al*. 2004; Aktas *et al*. 2005). One of the most important horticultural species worldwide is the *Capsicum annuum* L. hot pepper, known in Mexico as *chile*. It is one of the most significant crops nationwide because of its high level of consumption in Mexican gastronomy, and indirectly due to its high antioxidant content. Although this particular species is slightly sensitive to salinity (Ogi and Izawa 1972; Kafkafi *et al*. 1982; Ayers and Wescot 1985), its optimal production requirements are commonly used on arid and semi-arid regions. However, since water is scarce and saline, agricultural production in these particular regions relies on adequate water supply. Consequently, aiming to assure long-term hot pepper production, salty water must be utilized on both arid and semi-arid regions for food production (Larrinaga-Mayoral 2001; Maggio *et al*. 2003; Ramírez-Serrano *et al*. 2008; Joseph and Jini 2011) even though it is known that oxidative stress is induced in plants exposed to salinity (Hernández *et al*. 1995, 2001; Gómez *et al*. 2004).

Fruit maturation and senescence have been considered as oxidative phenomena due to the presence of a strong oxidative damage, and in this type of situation, the antioxidant system is involved in level control of reactive oxygen species (ROS) (Jiménez *et al*. 2002; Martí *et al*. 2009; Camejo *et al*. 2010). Masia (1998) suggested that activity variation in two antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT) measured at regular intervals from the beginning of the harvest may be useful to identify some of the fruit maturing physiological aspects, providing a more rational criterion for the adequate harvest of each crop. Thus, understanding the ripening process of fresh fruits under saline conditions could be a first demand to improve fruit quality during its post-harvest storage. Despite the existence of studies concerning the effects of salinity on hot pepper (Walker *et al.* 1980; Larrinaga-Mayoral 2001; Maggio *et al*. 2003; Aktas *et al*. 2005), there is lack of information regarding the measurements of SOD and CAT activities as ripening biomarkers under this stress condition. We decided to select these biomarkers to assess physiological maturity changes in fruits, because they allow identifying the oxidative stress effect caused by NaCl in the development stages (green colour) and during storage when the fruit changes to the characteristic red colour of physiological maturity. Under such demand, this study is aimed to assess their response and determine the optimal post-harvest storage period for wide hot peppers. These measurements could be useful to set the threshold of salinity tolerance of this cultivation (Mittler and Blumwald 2010).

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Original Research Paper

MATERIALS AND METHODS

Reagents

All chemical reagents used in this work were purchased from Sigma-Aldrich® Corp. (St. Louis, MO, USA), unless specified otherwise.

Plant material

Hot pepper seeds (*C. annuum* L.) hybrid 'Caballero' (Sakata, SPP7502) were sown in germination containers and transplanted on 20 cm width, 30 cm height flowerpots with a commercial substrate "peat moss" type (Sunshine, Sun Gro Horticulture, Canada). Previously, the plants had a 10-day environmental adaptation period prior to saline treatment initiation by applying the triple fertilizer (Fertipron® 20-20-20 Probelte, S.A. Murcia, Spain N: P_2O_5: K_2O) with a dosage of 150 mg·L^{-1}. Plants were grown under greenhouse conditions at the Centro de Investigaciones Biológicas del Noroeste, S.C. (CIBNOR), located on the coastal area of El Comitán, on the southern region of the Baja California peninsula in Mexico.

Saline stress and storage conditions

Plants were grown in the presence of 12.5 and 25 mM of NaCl. Drinkable water with an electric conductivity (EC) of 1.3 dS m^1 was used for the NaCl solution preparation, which was considered as control (0 mM of NaCl). The treatments were applied daily for 60 days (until harvest of immature green fruits). The experiment was established following a randomized block with 10 repetitions.

Fruit storage

Pepper fruits were collected from 45 days after transplanting in an immature green stage for analysing changes from commercial maturity to physiological maturity, respectively. Pepper samples were stored at 4°C. After day zero (control fruits), three fruits were selected randomly from each treatment at 15, 30, 45, 60, 75, and 90 days of storage. Each time, fruits were frozen in liquid nitrogen and stored at -80°C for enzymatic activity, ascorbic acid, pigment (chlorophyll and carotenoids), lipid peroxidation, and carbonyl protein analyses.

Morphological measurement

From control and salt-treated plants, we counted the number of pepper fruits per plant and measured their fresh weight, length, and diameter from the central region with a digital electronic vernier (Stanley, UPC22064, China).

Enzymatic extraction

1 g of frozen material was crushed with a mortar with 2 ml of a potassium phosphate buffer solution ($NaKP_i$) containing 0.1 mM of ethylenediaminetetraacetic acid (EDTA), 5 mM of cysteine, 0.2% (v/v) Triton X-100, 1% (p/v) polyvinyl-pyrrolidone (PVP40) molecular weight 40,000, and 0.1 mM phenyl-methyl-sulphonyl fluoride (PMSF); the mixture was ice-cooled for 30 min. Solid material was centrifuged at 1000 × *g* for 10 min, and the recovered supernatant was centrifuged again at 10,000 × *g* for 20 min. The volume obtained was divided into 1-mL aliquots for a total protein analysis, and for SOD and CAT enzymatic activity. The entire extraction procedure was performed at 4°C.

Total protein

Total protein content was determined by the Bradford method (1976), using bovine serum albumin (BSA) as standard.

Antioxidant enzymatic activities

Total SOD activity (E.C. 1.15.1.1) was measured following the ferricytochrome c reduction using xanthin/xanthin oxidase as the source of O_2^- radicals as described in McCord and Fridovich (1969). The method defines a unit of SOD as the amount of enzyme that causes 50% of maximum inhibition of NBT to blue formazan; the activity was expressed as SOD unit·mg $protein^{-1}$; the determination of each extract was performed in triplicate. SOD isoenzymes were separated in 12% acrylamide by PAGE with fresh made gels; SOD activity bands were detected in gels by the Photochemical NBT stain (Beauchamp and Fridovich 1971) with the same amount of protein in each line. The isoenzyme activity was quantified by recording gel transmittance on a Shimadzu CS-9000 densitometer (Gómez *et al*. 2004).

CAT activity (E.C. 1.11.1.6) was measured at 25°C in accordance to the method described by Aebi (1984) throughout the hydrogen peroxide (H_2O_2) disappearance in a buffer solution and following a change in absorbance at 240 nm in a spectrophotometer (Jenway, 6505, USA). The reaction mixture contained 10 mM hydrogen peroxide in a 50 mM potassium phosphate buffer solution pH 7.0 and 800 µl of fruit extract in a 1.2 mL total volume. CAT activity was expressed on mg protein basis. Each extract determination was performed in triplicate.

Ascorbic acid

The L-ascorbic acid content was measured using 10 g of pericarp tissue from the equatorial region of each fruit with the RQflex plus (Merck, Germany) reflectometer and with the L-ascorbic acid test (1169810001, Merck), where the reduction from yellow molybdophosphoric acid to blue phosphomolybdene acid was measured. The L-ascorbic acid was used as a positive control. Each determination was performed in triplicate, and concentration was expressed in mg L^{-1}.

Lipid peroxidation and protein oxidation

The extent of lipid peroxidation was estimated by determining the concentration of thiobarbituric acid-reactive substances (TBARS) (Cakmak and Horst 1991). Protein oxidation (carbonyl protein content) was measured by reaction with 2,4-dinitrophenylhydrazine, as described by Prasad (1996). Plant tissue (0.2 g) was homogenised with a potassium phosphate buffer 100 mM, pH 7.0 and 2.5 µg of each protease, leupeptin, pepstatin, and aprotinin inhibitors. The homogenised solution was filtered with a nylon mesh and centrifuged at 20,000 × *g* for 15 min. Protein concentration was determined in the supernatant with the method described by Bradford (1996). We took 400 µl of supernatant and added 100 µl of 20 mm DNPH in Eppendorf tubes. The tubes were incubated for 1 h and agitated for 15 min. Proteins were precipitated with 500 µl of 20% of trichloroacetic acid (TCA) and incubated for 10 min at room temperature. The precipitation rich in proteins was concentrated at 11,000 g for 3 min, and the supernatant was discharged. The precipitate was washed in triplicate with 1 ml of ethanol: ethyl acetate solution (1:1). Later, it was resuspended in 600 µl of guanidine HCl 6M and incubated for 15 min at 37°C. Absorbance was measured at 370 nm by guanidine, as well as the controls of HCl and DNPH. The content of carbonyl groups was calculated using a molar absorption coefficient of 22,000 M^{-1} cm^{-1}; the results were expressed per nmol·mg of protein.

Carotenoid pigments

Carotenoids were analysed by high-performance liquid chromatography (HPLC) according to the method described by Vidussi *et al*. (1996). Pericarp samples (0.5 g on fresh weight) were cut from the fruit equatorial region with a leather punch. Each sample was extracted with 2.0 mL of acetone under lightless conditions and on an ice bath. Samples were kept at -20°C in an acetone solution prior to the HPLC analysis. Before the injection, 500 µl of extract were mixed with 250 µl of 1 M ammonium acetate. The extract was injected in an HPLC system, equipped with a Millipore column, Waters Div. (Milford, MA, U.S.A.), µ-porasil 125 Å, 10 µm, 3.9 × 150 mm. The elution was performed at 1 ml·min^{-1} flow rate, using a binary gradient between solvent A (methanol: 0.5 N aqueous ammonium acetate, 70:30 v/v) and solvent B (methanol), which was programmed according to the following procedure (minutes; % solvent A, % solvent B): (0; 75, 25), (1; 50, 50), (15; 0, 100), (19; 75, 25). All the solvents used were purchased from

Table 1 Number of fruits, length, diameter and fresh weight of wide fruit peppers cv. Caballero of plants grown under salinity. Values represent the mean ± Standard Deviation (SD).

Treatment (mM de NaCl)	Number of harvested fruits	Fruit length (mm)	Fruit diameter (mm)	Fresh weight (g)
0	29 ± 8	63.4 ±5 .69	36.0 ± 2.43	21.1 ± 4.04
12.5	25 ± 5	71.5 ± 3.38	39.0 ± 2.31	25.4 ± 4.26
25	25 ± 5	68.8 ± 6.86	37.1 ± 2.79	23.0 ± 4.11

(n >3, mean ± SD values are shown).

Sigma-Aldrich® Corp., and all were HPLC grade. Pigment identification was performed in accordance to their retention periods and by comparison of the UV-Vis absorption spectrum, performed on line, with the one obtained from a library of established spectrum of reference crops from the pigment laboratory at CIBNOR facilities.

Statistical analysis

A variance analysis was performed for the morphological variables, fruit number, longitude, diameter, and weight, considering the salinity factor as the only variation source; as for the rest of the variables, a variance analysis was performed considering two factors or variation sources. The different saline treatments were considered as factor A, and the different periods (days) of fruit storage were considered as factor B. When significant differences and interactions among them were revealed among treatments for both cases, the Tukey's multiple media comparison test ($P \leq 0.05$) was used. The statistical analysis and the charts were performed with the Statistica program (StatSoft Inc. 2001).

RESULTS AND DISCUSSION

Morphological variables

The qualitative analysis revealed the harvested fruits showed adequate size, firmness, colour characteristics of such species, and variety in an immature green stage. Likewise, the fruit surface was plain and shiny with a total absence of any particular defect, such as fissure, decay, and sunburn. No significant differences were revealed among salt treatments concerning morphological variables as fruit number, longitude, diameter, and weight (**Table 1**). After the total storage period, control fruits showed the same green colour than at time zero, but fruits from salinity treatments presented a change in colour only after 75 days of storage: turning to orange in the 12.5 mM NaCl and to red in the 25 mM salt treatments. Relative to softening, fruits did not show symptoms of loss in their firmness until day 60. It was then patent that fruits ripened during storage in a different way depending on the conditions in which they developed, reaching the first symptoms of senescence at the end of the storage when plants were grown in the presence of NaCl.

Protein content

Protein content was generally higher in fruits grown in the presence of 12.5 mM NaCl (**Fig. 1**), which could be a response to mild saline stress. It has been described that exposure to mild stress can confer resistance to a second stress (crossed tolerance, Hernández-Saavedra and Ramírez-Serrano 2003, Senthil-Kumar et al. 2003). On the other hand, fruit storage produced a gradual decrease in protein content, but it was from 75 to 90 days where the decay was higher; another indication of the senescence process suffered by fruits as reported for peppers stored at 20ºC (Jiménez et al. 2003).

Chlorophyll *a* and *b* content

The chlorophyll (Chl) *a* content (**Fig. 2A**) revealed significant differences for salinity ($F_{2,42} = 16.36$ $p = 0.0000$), fruit storage periods ($F_{6,42} = 526.83$, $p = 0.0000$), and interaction between both factors ($F_{12,42} = 71.29$, $p = 0.0000$). Also, the

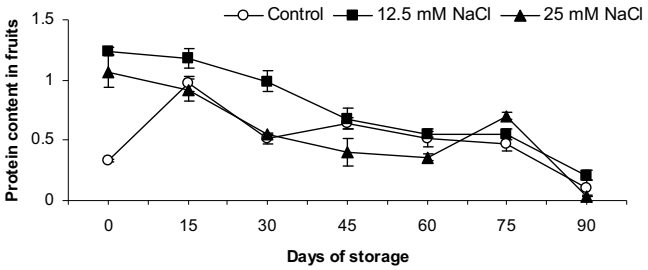

Fig. 1 Protein content (mg ml^{-1}) in pepper fruits of plants grown in the presence of NaCl and stored at 4ºC during different days. Values represent mean ± Standard Deviation (SD, n>3) and different letters indicate significant differences according to Tukey's *t*-test ($P < 0.05$).

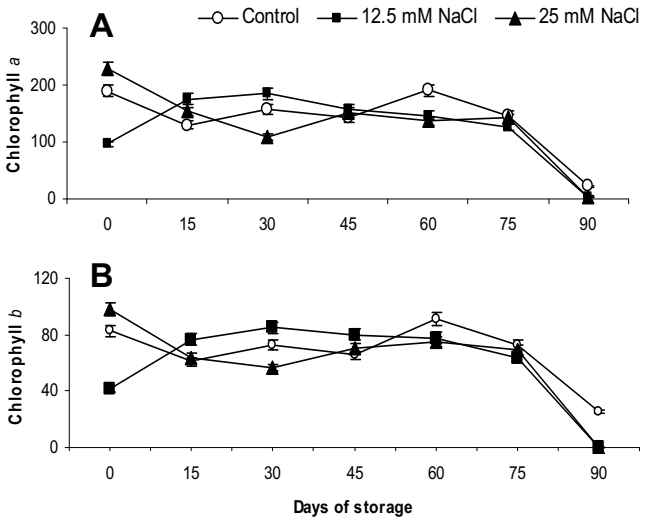

Fig. 2 Chl *a* (A) and chl *b* (B) content (mg g^{-1} fresh weight) in wide chile pepper fruits of plants grown in the presence of NaCl and stored at 4°C. Values represent mean ± SD (n>3) and different letters indicate significant differences according to Tukey's *t*-test ($P < 0.05$).

Chl *b* content (**Fig. 2B**) showed significant differences for salinity ($F_{2,42} = 23.67$ $p = 0.0000$), fruit storage periods ($F_{6,42} = 467.55$, $p = 0.0000$), and interactions between both ($F_{12,42} = 60.36$, $p = 0.0000$). The results also revealed the content of Chl *a* was two times higher than that of Chl *b* in the three treatments; however, the change pattern during storage was almost identical. In control fruits, Chl content varied from 180 to 120 mg.kg^{-1}or from 80 to 60 mg.kg^{-1} for Chl *a* and *b*, respectively, until day 75, when it started to drop at 90 days to around 20-30 mg.kg^{-1}. In the 12.5 mM NaCl treatment a Chl increase on both was observed from zero to 30 days of storage, decreasing significantly on day 45, until finally degrading on day 90. The increasing stage at the beginning could be related to fruit growth that might have been stimulated as a result to the moderate Na^+ y Cl^- ion increase, needing more cells to store them to maintain cellular homeostasis. Such Chl increase can be related with the values obtained in the morphological variables of the 12.5 mM NaCl treatment, since this treatment was the one producing the biggest fruits with the highest fresh weight. Regarding the 25 mM NaCl treatment, a significant increase in Chl was detected in the first 15-30 days of storage to slightly decrease till day 75. From this point on, Chl content

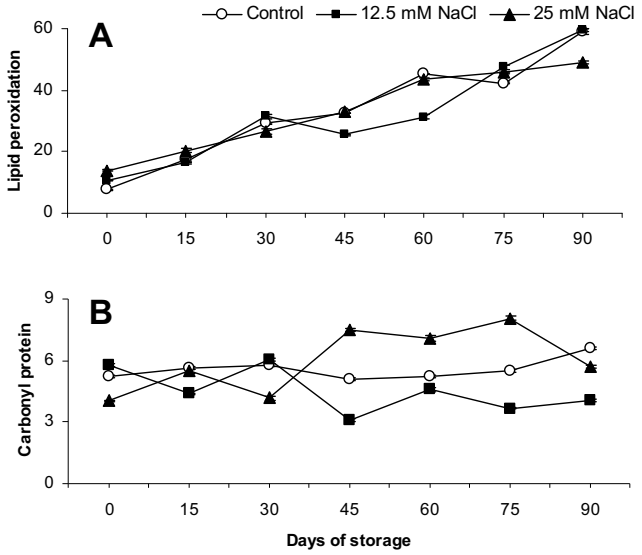

Fig. 3 Lipid peroxidation (nmol MDA g^{-1} fresh weight) (A) and carbonyl protein (nmol mg^{-1} protein) (B) content in wide chile pepper fruits of plants grown in the presence of NaCl and stored at 4°C. Values represent mean ± SD (n>3) and different letters indicate significant differences according to Tukey's *t*-test ($P < 0.05$).

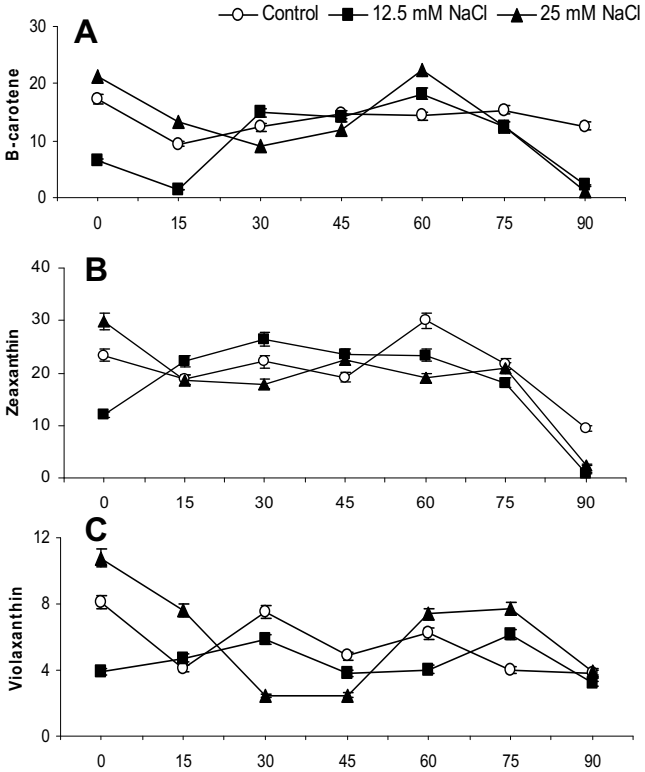

Fig. 4 Violaxanthin (A), zeaxanthin (B), and beta-carotene (C) content (mg kg^{-1} fresh weight) in wide chile pepper fruits of plants grown in the presence of NaCl and stored at 4°C. Values represent mean ± SD (n>3) and different letters indicate significant differences according to Tukey's *t*-test ($P < 0.05$).

sharply decreased to zero, where Chl *a* and *b* had been completely degraded. This loss was patent in the fruits from the salt treatments that presented orange to red coloration. Chl degradation is one of the visible symptoms of pepper ripening (Jiménez *et al*. 2003) and it is known that ROS are involved in the transformation of chloroplast into chromoplasts (Bouvier *et al*. 1998). Such transition happened only in the salt treated peppers or at least faster than in control fruits, where after 75 days of storage changes were more pronounced.

Carotenoid content

The change in coloration observed in peppers stored after a saline treatment was also due to carotenoid synthesis. The analyses of carotenoid violaxhanthin (**Fig. 3A**), zeaxanthin (**Fig. 3B**), and β-carotene (**Fig. 3C**) in peppers revealed significant differences for salinity ($F_{2,42}$ = 184.92, 35.62, 148.83 p = 0.0000 for violaxanthin, zeaxanthin and β-carotene, respectively), for the fruit storage period ($F_{6,42}$ = 376.28, 385.79, 204.15, p = 0.0000 for violaxanthin, zeaxanthin, and β-carotene, respectively), as well as for the interaction between both factors ($F_{12,42}$ = 151.58, 71.50, 157.22, p = 0.0000 for violaxanthin, zeaxanthin, and β-carotene, respectively). The identified carotenoid composition was similar to that reported in other *C. annuum* varieties (Deli *et al*. 1992, 1996; Collera-Zúñiga *et al*. 2005), where zeaxanthin, violaxanthin, and β-carotene were more abundant in this study. Zeaxanthin and violaxanthin were significantly lower in salt treated fruits after 90 days of storage than in control fruits. These results were consistent with other studies quantifying hot pepper carotenoids as a maturing function (Davies *et al*. 1970; Mínguez-Mosquera and Hornero-Méndez 1994; Markus *et al*. 1999). Multiple observations suggest that oxidative stress regulates carotenoid biosynthesis during the chloroplast to chromoplast transition in plants (Bouvier *et al*. 1998). It is also mentioned that a low antioxidant enzyme regulation induces carotenogenic gene expression. This information suggests a crossing communication among antioxidant enzymes and plastid carotenogenesis, which can be inferred by the bond between catalase and carotenoid biogenesis, as shown in initial studies on plants and mushrooms. Chl *a* and *b* decrease is a result derived from the organoleptic maturing change of senescence or fruit aging, along with the 'increase' or evident visual presence of carotenoid pigments, turning coloration changes (greenish/orange and greenish/reddish) evident after 90 postharvest days in the saline treatments.

Lipid peroxidation and protein oxidation

In order to examine the possible oxidative stress in peppers grown in salinity conditions and stored at 4ºC, the extent of lipid peroxidation and protein oxidation was analysed in the fruits. The lipid peroxidation activity (**Fig. 4A**) showed significant differences for salinity ($F_{2,42}$ = 41.9, p = 0.0000), for fruit storage periods ($F_{6,42}$ = 8358.0, p = 0.0000), as well as for the interaction between both factors ($F_{12,42}$ = 223.5, p = 0.0000). This peroxidation increased significantly on the three saline treatments, which was six times higher after 90 days although the control fruits had generally higher levels of peroxidation than salt-treated fruits. Such data indicated that oxidative damage on cellular membranes increased during fruit maturation, which was revealed by deterioration signs such as fruit firmness loss after 60 storage days although fruits from the 12.5 mM NaCl treatment showed lower levels at the end of the storage period.

The protein carbonyl measurement (**Fig. 4B**) also revealed significant differences for salinity ($F_{2,42}$ = 1984.3 p = 0.0000), for the fruit storage period ($F_{6,42}$ = 101.9, p = 0.0000), as well as for the interaction between both factors ($F_{12,42}$ = 916.9, p = 0.0000). The carbonyl group formation in the control fruits was maintained between 5-6 nmol.mg^{-1} of protein until day 75, finally increasing at the end of the storage period. The protein carbonyl content in the 12.5 mM NaCl treatment was significantly lower with regards to the one in control fruits. The highest protein carbonyl values in the 25 mM NaCl treatment were evident in the 75, 60, and 45 days of post-harvest storage periods, respectively (**Table 2**). Ion excess can provoke the formation of ROS causing oxidative stress that can be evaluated by analysing lipid peroxidation and protein oxidation (Gómez *et al*. 2004). Also, ripening of fruits has been described as an oxidative phenomenon with increased levels of lipid and pro-

Table 2 Carbonylated proteins in fruit peppers cv. 'Caballero' of plants grown under salinity and stored at 4ºC.

Treatment (mM de NaCl)	Time (days of storage)	CO-protein (nmol.mg^{-1} protein)
0	0	5.202 ± 0.065 i*
0	15	5.621 ± 0.071 fgh
0	30	5.751 ± 0.063 fg
0	45	5.075 ± 0.050 i
0	60	5.208 ± 0.058 i
0	75	5.514 ± 0.077 gh
0	90	6.585 ± 0.069 d
12.5	0	5.778 ± 0.066 f
12.5	15	4.370 ± 0.035 jk
12.5	30	6.064 ± 0.055 e
12.5	45	3.103 ± 0.044 n
12.5	60	4.568 ± 0.073 j
12.5	75	3.671 ± 0.052 m
12.5	90	4.080 ± 0.069 l
25	0	4.033 ± 0.035 l
25	15	5.468 ± 0.085 h
25	30	4.179 ± 0.157 kl
25	45	7.489 ± 0.050 b
25	60	7.058 ± 0.160 c
25	75	8.076 ± 0.082 a
25	90	5.689 ± 0.087 fgh

(n >3, mean ± SD values are shown).
* Different letters within a column indicate significant differences according to Tukey's t-test ($P < 0.05$).

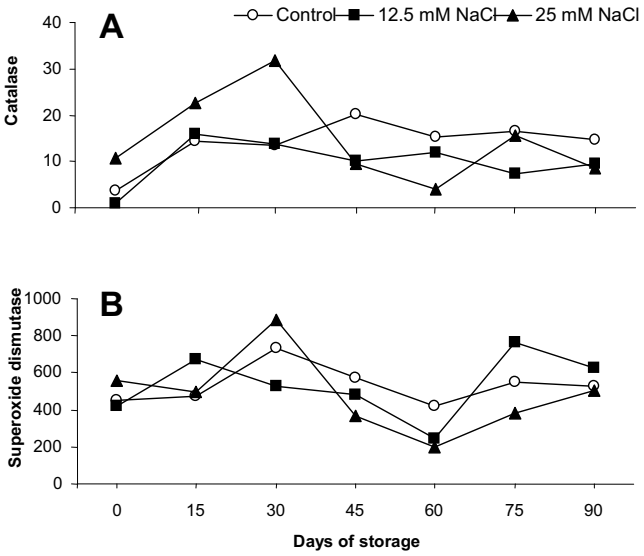

Fig. 5 Superoxide dismutase (SOD) (A) and catalase (CAT) (B) activities (U mg^{-1} protein) in wide chile pepper fruits of plants grown in the presence of NaCl and stored at 4°C during different days. Values represent mean ± SD (n>3) and different letters indicate significant differences according to Tukey's t-test ($P < 0.05$).

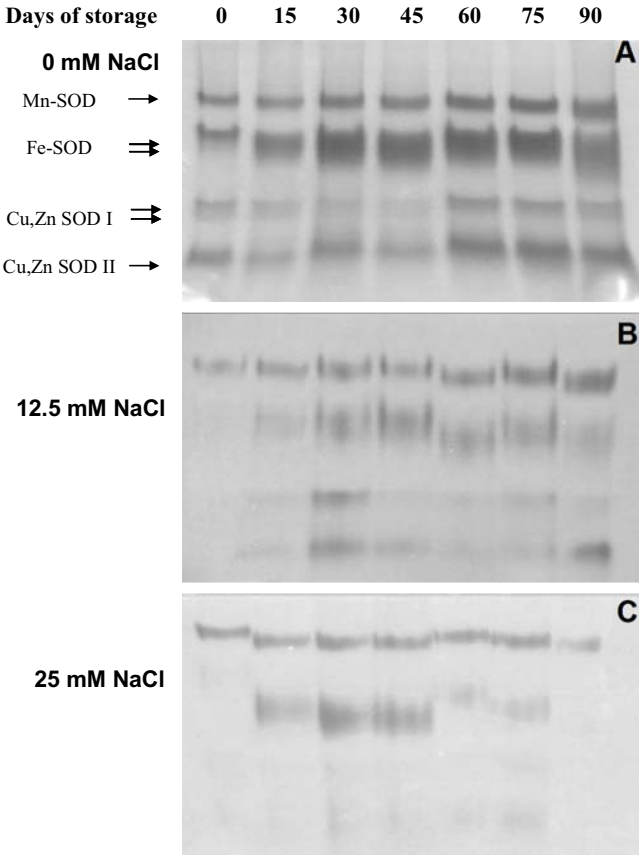

Fig. 6 SOD isoenzymes after PAGE in wide chile pepper fruits of plants grown in the presence of NaCl and stored at 4°C.

tein oxidations (Jiménez *et al*. 2002). This study revealed that proteins were more susceptible to oxidative damage than lipids during storage of salt-treated pepper fruits and that salinity modified the oxidation pattern depending on salt concentration, where fruits from the 12.5 mM NaCl treatment were less affected by the oxidative process occurring during ripening and senescence than fruits grown in the presence of 25 mM NaCl.

Antioxidant enzymatic activities

Catalase (CAT) was analysed in the fruits (**Fig. 5A**), revealing significant differences for salinity ($F_{2,42}$ = 217.22, p = 0.0000), for the fruit storage period ($F_{6,42}$ = 313.48, p = 0.0000), as well as for the interaction between both factors ($F_{12,42}$ = 159.62, p = 0.0000). The CAT activity on the 25 mM NaCl saline treatment increased after 30 storage days; nevertheless, it immediately decreased on an equal proportion until 90-day storage. A very similar behaviour was observed for the total SOD activity (**Fig. 5B**), which showed significant differences for salinity factors ($F_{2,42}$ = 29.85, p = 0.0000), fruit storage periods ($F_{6,42}$ = 251.87, p = 0.0000), as well as for the interaction between both factors ($F_{12,42}$ = 91.33, p = 0.0000). This activity increased in control fruits with storage till 30 days and then decreased till 60 days followed by a slight increase at the end of the analysed storage period. Salt treatments followed a similar pattern but with changes being more pronounced. The SOD isoenzyme analysis in control fruits (**Fig. 6A**) revealed the presence of at least 6 isoforms identified as one Mn-SOD, two Fe-SODs, and three Cu,Zn-SODs. During storage, Mn-SOD and Fe-SOD isoenzymes in control plants increased after 30 and 15 days, respectively; while Cu,Zn-SODs increased at 60 days, with differences among the three isoenzymes, showing Cu,Zn-SODII as the highest activities. The treatment with NaCl provoked a loss in activity of some of the isoenzymes, only Mn-SOD band appears in fruits before storage (**Fig. 6B** and **C**). However, when fruits were stored at 4ºC, some of the bands appear again as Fe-SOD with the highest activity at 30 and 45 days, and some of the Cu,Zn-SODs, mainly in the 12.5 mM NaCl treated fruits. In relation to Mn-SOD, in the fruits grown in the presence of 12.5 mM NaCl, its activity increased during the 90-day storage period, which was similar to the control treatment; while the Fe-SOD activity increased after 15 to 45 days to decrease later, unlike the control treatment. In the 25 mM NaCl treatment, Mn-SOD remained constant through the storage period, Fe-SODs decreased from 45 to 90 days while Cu,Zn-SODs were undetectable in fruits stored at 4°C.

The results reported by Masia (1998) demonstrated that both SOD and CAT activities were sensitive to low temperatures. Also related to pepper fruit ripening, changes in SOD activity have been described by Imahori *et al*. (2000) who reported an increase in SOD activity from the fruit greenish stage to the greenish/yellowish stage; however, in

a previous work in our laboratory, Jimenez *et al*. (2003) reported that the SOD activity showed a decrease at the end of the maturing stage when pepper fruits matured during storage at 20°C, with a previous increase in the activity at the beginning of the storage. The results of this study showed that SOD activity varied during ripening and senescence of pepper fruits, and the isoenzyme pattern analysis revealed changes happening in the different organelles: mitochondrial Mn-SOD was found to be maintained all over the storage period while Cu,Zn-SODs were the most affected in the 25 mM NaCl treatment. The absence of important changes in the Mn-SOD has been also observed during the ripening of peach fruits (Camejo *et al*. 2010), which reveals an efficient enzymatic antioxidant system to scavenge $O_2^{-\bullet}$ generated at mitochondrial and/or at peroxisomal level. The observed decrease in SOD activity between 30 and 60 days of storage mainly due to a loss in Fe-SOD and Cu,Zn-SOD II could provoke an increase in $O_2^{-\bullet}$ in the chloroplasts. It might be related with increases in carotenoid synthesis and other non-enzymatic antioxidant as ascorbic acid in the developing fruit that modify the state of the physiological maturity of the wide hot pepper fruit tissues; a fact added to the additional stress generated by low temperatures applied during the post-harvest storage period. Previous studies performed on apple fruit stored at low temperatures mentioned a decrease in SOD activity (Du and Bramlage 1994; Masia 1998). The total SOD activity and the different SOD isoenzyme patterns found in our study could reflect SOD sensitivity to low temperatures on wide hot pepper fruit *cv*. Caballero, which can be considered sensitive to coldness and, in accordance to the ISO 6659:1981 norm, it must be kept at 8°C refrigeration storage from three to five weeks. It has been recommended that storage at 4°C is possible for a period no longer than three weeks, since longer exposition periods can cause damage for cooling. However, neither damage for cooling, nor necrosis was observed in this study, even when fruits were kept at 4°C for 90 days. Concerning hot pepper cultivations sensitive to cooling, H_2O_2 can inactivate SOD enzymes irreversibly (Bowler *et al*. 1992). Such decrease on SOD activity could be related with increasing gene expression levels of the alternative oxidase enzyme (AOX), which directly reduces the radical superoxide to a water molecule. This result has been demonstrated when hot pepper fruits were kept at low temperatures (Purvis 2002; Fung *et al*. 2004).

Ascorbic acid

The ascorbic acid (ASC) analysis (**Fig. 7**) results revealed significant differences for salinity ($F_{2,42}$ = 29.12, p = 0.0000), for the fruit storage periods ($F_{6,42}$ = 304.61, p = 0.0000), as well as for the interaction between both factors ($F_{12,42}$ = 63.78, p = 0.0000). The results showed that ASC remained within the range reported on different types of hot peppers (from 46 to 243 mg.100 g^{-1} fresh weight) (Howard *et al*. 1994, 2000; Lee *et al*. 1995; Kader and Lee 2000; Jiménez *et al*. 2003). In control fruits, ASC significantly increased after 60 days, confirming previous studies reporting increases in hot pepper during maturing process (Howard *et al*. 1994, 2000; Osuna-García *et al*. 1998). Ascorbate content and activities of the ascorbate-glutathione (ASC-GSH) cycle enzymes have been reported to increase during maturation of sweet pepper (Imahori *et al*. 2000; Jiménez *et al*. 2002, 2003). ASC could be used as a substrate by ascorbate peroxidase (APX) at the end of the storage as described in fruits stored at 20°C (Jiménez *et al*. 2003), aiming to detoxify the H_2O_2 produced by the increased SOD in this condition.

In the salt-treated plants, ASC in fruits from the 12.5 mM of NaCl treatment was higher than that in control fruits, and decreased during the first 30 days; it recovered its initial level by day 45 and decreased again, but it reached values higher than control fruits at the end of the storage period. In the 25 mM NaCl treatment, the ASC pattern was

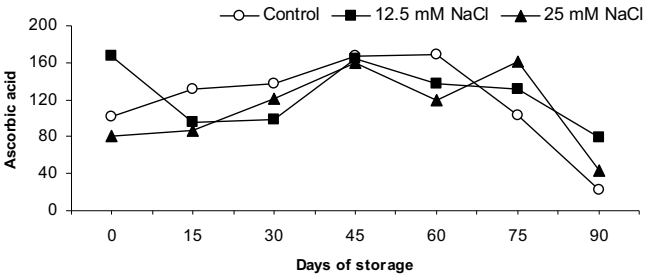

Fig. 7 Ascorbic acid content (mg L^{-1}) in wide chile pepper fruits of plants grown in the presence of NaCl and stored at 4°C. Values represent mean ± SD (n > 3) and different letters indicate significant differences according to Tukey's *t*-test ($P < 0.05$).

similar although lower than control fruits, except at the end of storage where it was found to be higher. Not all salinity effects are negative; it seems that a mild NaCl treatment, as the one used in our work, should be beneficial in terms of increase in ascorbic acid in control pepper fruits, and that NaCl resulted in a better nutritional quality if pepper fruits have to be stored at 4°C for a long period. Salinity can then cause a few favourable effects on yield crop, quality, and disease resistance (Larrinaga-Mayoral *et al*. 2001). It was demonstrated on a study performed with spinach where yield crop initially increased with low to moderate salinity applications (Osawa 1963). Sugar content increased in carrots, while starch content in potatoes decreased as salinity levels increased (Bernstein 1995). It has been shown, throughout this study, that proper sodium chloride application to a 25.0 mM concentration becomes potentially useful as an effective treatment for wide hot pepper, considering facilitating Chl degradation and accelerating coloration changes, and increasing carotenoid content, without affecting fruit growth and its organoleptic properties. This could decrease fruit maturing period in the plant and, also, reduce maintenance cost in greenhouse cultivations.

CONCLUSIONS

The results revealed that superior SOD and CAT activity levels in the control treatment fruit and their direct relationship with low levels of lipid peroxidation, protein oxidation, Chl degradation, as well as with a higher carotenoid content become evident at a lower oxidative stress degree. As a result the wide hot pepper fruit shows a longer maturing period and thus a 'characteristic' but out of phase senescence (modified in time) during storage at 4°C.

Therefore, SOD activity levels in the saline treatments, compared to the basal treatment, allowed differentiating the maturing stage in fruits subjected to moderate salinity concentrations even under additional stress conditions. The utilization of maturing biomarkers in saline stress conditions can help to determine the salinity threshold on cultivations with a higher demand, or to select the most competent for arid and semiarid regions. In addition, they can help to determine when the fruit irrigated with salty waters should be harvested, aiming to avoid oxidative stress increase generated in the plant and to prevent the damaging effects caused by salinity in the fruit.

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REFERENCES

Aebi H (1984) Catalase *in vitro*. *Methods in Enzymology* **105**, 121-126

Aktas H, Karni L, Dong-Chil C, Turhan E, Bar-Tal A, Alón B (2005) The supression of salinity-associated oxygen radicals production in pepper (*Capsicum annuum*) fruit, by manganese, zinc and calcium relation to its sensitivity to blossom-end rot. *Physiologia Plantarum* **123**, 67-74

Ayers R, Westcot W (1985) Water quality for agriculture. Irrigation and drainage paper No. 29, FAO, Rome

Bandeoğlu E, Eyidoğan F, Yücel M, Öktem HA (2004) Antioxidant responses of shoots and roots of lentil to NaCl salinity stress. *Plant Growth Regulation* (Vol 42), Kluwer Academic Publishers, Amsterdam, the Netherlands, pp 69-77

Beauchamp C, Fridovich I (1971) Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. *Analytical Biochemistry* **44**, 276-287

Bernstein L (1975) Effects of salinity and sodicity on plant growth. *Annual Review of Phytopathology* **13**, 295-312

Bouvier F, Backhaus RA, Camara B (1998) Induction and control of chromoplast-specific carotenoid genes by oxidative stress. *The Journal of Biological Chemistry* **273**, 30651-30659

Bowler C, van Montagu M, Inzé D (1992) Superoxide dismutase and stress tolerance. *Annual Review of Plant Physiology and Plant Molecular Biology* **43**, 83-116

Boyer JS (1992) Plant productivity and environment. *Science* **218**, 443-448

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248-254

Cakmak I, Horst WJ (1991) Effect of aluminium on lipid peroxidation, superoxide dismutase, catalase and peroxidase activities in root tips of soybean (*Glycine max*). *Physiologia Plantarum* **83**, 463-468

Camejo D, Martí MC, Román P, Ortiz A, Jiménez A (2010) Antioxidant system and protein pattern in peach fruits at two maturation stages. *Journal of Agricultural and Food Chemistry* **58**, 11140-11147

Collera-Zúñiga, O, García FJ, Meléndez RG (2005) Comparative study of carotenoid composition in three Mexican varieties of *Capsicum annuum* L. *Food Chemistry* **90**, 109-114

Davies BH, Matthews S, Kirk JTO (1970) The nature and biosyntesis of the carotenoids of different colour varieties of *Caspicum annuum*. *Phytochemistry* **9**, 797-805

Deli J, Matus Z, Szaboles J (1992) Carotenoid composition in the fruits of black paprika (*Capsicum annuum* variety *longum nigrum*) during ripening. *Journal of Agricultural Food Chemistry* **40**, 2072-2076

Deli J, Matus Z, Toth G (1996) Carotenoid composition in the fruits of *Capsicum annuum* cv. Szentesi kosszarvú during ripening. *Journal of Agricultural Food Chemistry* **44**, 711-716

Du Z, Bramlage WJ (1994) Superoxide dismutase activities in senescing apple fruit (*Malus domestica* Borkh.). *Journal of Food Science* **59**, 581-584

Fung RWM, Wang CY, Smith DL, Gross KC, Tian M (2004) MeSA and MeJA increase steady-state transcript levels of alternative oxidase and resistance against chilling injury in sweet peppers (*Capsicum annuum* L.). *Plant Science* **166**, 711-719

Gómez JM, Jiménez A, Olmos E, Sevilla F (2004) Location and effects of long-term NaCl stress on superoxide dismutase and ascorbate peroxidase isoenzymes of pea (*Pisum sativum* cv Puget) chloroplasts. *Journal of Experimental Botany* **55**, 119-130

Hernández JA, Olmos E, Corpas FJ, Sevilla F, del Río LA (1995) Salt induced oxidative stress in chloroplasts of pea plants. *Plant Science* **105**, 151-167

Hernández JA, Ferrer MA, Jiménez A, Ros A, Sevilla F (2001) Antioxidant systems and $O_2^{\cdot-}/H_2O_2$ production in the apoplast of pea leaves. Its relation with salt-induced necrotic lesions in minor veins. *Plant Physiology* **127**, 817-831

Hernández-Saavedra NY, Ramírez-Serrano R (2004) Differential expression and function of copper-zinc superoxide dismutases under metal stress in the marine yeast *Debaryomyces hansenii*. *Journal of Marine Biotechnology* **6**, S1-S5

Howard LR, Smith RT, Wagner AB, Villalon B, Burns EE (1994) Provitamin A and ascorbic acid content of fresh peppers cultivars (*Capsicum annuum*) and processed jalapenos. *Journal of Food Science* **59**, 362-365

Howard LR, Talcott ST, Brenes CH, Villalon B (2000) Changes in phytochemical and antioxidant activity of selected pepper cultivars (*Capsicum* species) as influenced by maturity. *Journal of Agricultural Food Chemistry* **48**, 1713-1720

Imahori Y, Kanetsune Y, Ueda Y, Chachin K (2000) Changes in hydrogen peroxide content and antioxidative enzyme activities during the maturation of sweet pepper (*Capsicum annuum* L.) fruit. *Journal of the Japanese Society for Horticultural Science* **69**, 690-695

Jiménez A, Gomez JM, Navarro E, Sevilla F (2002) Changes in the antioxidative systems in mitochondria during ripening of pepper fruits. *Plant Physiology and Biochemistry* **40**, 515-520

Jiménez A, Romojaro F, Gómez JM, Llanos MR, Sevilla F (2003) Antioxidant systems and their relationship with the response of pepper fruits to storage at 20°C. *Journal of Agriculture Food Chemistry* **51**, 6293-6299

Joseph B, Jini D (2011) Development of salt stress-tolerant plants by gene manipulation of antioxidant enzymes. *Asian Journal of Agricultural Research,* **5**, 17-27

Kader AA, Lee SK (2000) Preharvest and postharvest factors influencing vitamin C content of horticultural crops. *Postharvest Biology and Technology* **20**, 207-220

Kafkafi U, Valoras N, Letey J (1982) Chloride interaction with nitrate and phosphate nutrition in tomato (*Lycopersicum esculentum* L.). *Journal of Plant Nutrition* **5**, 1369-1385

Larrinaga-Mayoral JA (2001) Efecto de NaCl sobre la fisiología, crecimiento y producción en plantas de chile (*Capsicum annuum* L.). PhD thesis, Centro de Investigaciones Biológicas del Noroeste, La Paz, B.C.S. Mexico, 142 pp

Lee Y, Howard LR, Villalón B (1995) Flavonoids and antioxidant activity of fresh pepper (*Capsicum annuum*) cultivars. *Journal of Food Science* **60**, 473-476

Lester DR (1999) Gibberellin 2-oxidation and the *SLN* gene of *Pisum sativum*. *Plant Journal* **19**, 65-73

Maggio A, De Pascale S, Ruggiero C, Barbieri G (2003) Physiological responses of pepper to salinity and drought. *Journal of the American Society for Horticutural Science* **128**, 48-54

Markus F, Daood HG, Kapitany J, Biacs PA (1999) Change in the carotenoid and antioxidant content of spice red pepper (paprika) as a function of ripening and some technological factors. *Journal of Agricultural Food Chemistry* **47**, 100-107

Martí MC, Camejo D, Olmos E, Sandalio LM, Fernández-García N, Jiménez A, Sevilla F (2009) Characterization and changes in the antioxidant system of chloroplasts and chromoplasts isolated from green and mature pepper fruits. *Plant Biology* **11**, 613-624

Masia A (1998) Superoxide dismutase and catalase activities in apple fruit during ripening and post-harvest and with special reference to ethylene. *Physiologia Plantarum* **104**, 668-672

McCord JM, Fridovich I (1969) Superoxide dismutase: An enzymic function for erythrocuprein. *Journal of Biological Chemistry* **244**, 6049-6055

Mínguez-Mosquera MI, Hornero-Méndez D (1994) Comparative study of the effect of paprika processing on the carotenoids in peppers (*Capsicum annuum* L.) of the "Bola" and "Agridulce" varieties. *Journal of Agricultural and Food Chemistry* **42**, 1555-1560

Mittler R, Blumwald E (2010) Genetic engineering for modern agriculture: Challenges and perspectives. *The Annual Review of Plant Biology* **61**, 443-462

Ogi Y, Izawa G (1972) Quantitative changes of free amino acids and amides in barley plants during ammonia and nitrate assimilation. *Plant Cell Physiology* **13**, 249-259

Osawa T (1963) Studies on salt tolerance of vegetable crops with special reference to osmotic effects specific ion effects. *Journal of the Japanese Society for Horticultural Science* **32**, 221-223

Osuna-García JA, Wall MM, Waddell CA (1998) Endogenous levels of tocopherols and ascorbic acid during fruit ripening of new Mexican-type chile (*Capsicum annumm* L.). *Journal of Agricultural and Food Chemistry* **46,** 5093-5096

Prasad TK, Anderson MD, Martin BA (1994) Evidence for chilling-induced oxidative stress in maize seedlings and regulatory role for hydrogen peroxide. *Plant Cell* **6**, 65-74

Prasad TK (1996) Mechanisms of chilling-induced oxidative stress injury and tolerance: Changes in antioxidant system, oxidation of proteins and lipids and protease activities. *The Plant Journal* **10**, 1017-1026

Purvis A (2002) Regulation and role of the alternative oxidase in chilling injury of green bell pepper (*Capsicum annuum*). *Acta Horticlturae* **553**, 289-292

Ramírez-Serrano R, Larrinaga-Mayoral JA, Murillo-Amador B, Hernández-Saavedra NY, Fujiyama H (2008) Respuesta antioxidante enzimática en frutos de chile ancho (*Capsicum annuum* L.) bajo condiciones de estrés salino. *Interciencia* **33**, 377-383

Senthil-Kumar M, Srikanthbabu V, Raju BM, Ganeshkumar, Shivaprakash N, Udayakumar M (2003) Screening of inbred lines to develop a thermotolerant sunflower hybrid using the temperature induction response (TIR) technique: A novel approach by exploiting residual variability. *Journal of Experimental Botany* **54**, 2569-2578

Serrano R (1999) A glimpse of the mechanisms of ion homeostasis during salt stress. *Journal of Experimental Botany* **50**, 1023-1036

StatSoft Inc. (2001) STATISTICA. System reference. StatSoft, Inc., Tulsa, Oklahoma, USA, 1098 pp

Vidussi F, Claustré H, Bustillos J, Cailliau C, Marty J (1996) Determination of chlorophylls and carotenoids of marine phytoplankton: Separation of chlorophyll *a* from divinyl-chlorophyll *a* and zeaxanthin from lutein. *Journal of Plankton Research* **18**, 237-282

Walker RR, Hawker JS, Torokfalvy E (1980) Effect of NaCl on growth, ion composition and ascorbic acid concentrations of *Capsicum* fruit. *Scientia Horticulturae* **12**, 211-220

Zhu JK (2001) Plant salt tolerance. *Trends in Plant Science* **6**, 66-71