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A different role for hydrogen peroxide and the antioxidative system under short and long salt stress in *Brassica oleracea* roots

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Abstract

Salinity affects normal growth and development of plants depending on their capacity to overcome the induced stress. The present study was focused on the response and regulation of the antioxidant defence system in *Brassica oleracea* roots under short and long salt treatments. The function and the implications of hydrogen peroxide as a stressor or as a signalling molecule were also studied. Two different zones were analysed—the elongation and differentiation zone and the fully differentiated root zone—in order to broaden the knowledge of the different effects of salt stress in root. In general, an accumulation of hydrogen peroxide was observed in both zones at the highest (80 mM NaCl) concentration. A higher accumulation of hydrogen peroxide was observed in the stele of salt-treated roots. At the subcellular level, mitochondria accumulated hydrogen peroxide in salt-treated roots. The results confirm a drastic decrease in the antioxidant enzymes catalase, ascorbate peroxidase, and peroxidases under short salt treatments. However, catalase and peroxidase activities were recovered under long salt stress treatments. The two antioxidant molecules analysed, ascorbate and glutathione, showed a different trend during salt treatments. Ascorbate was progressively accumulated and its redox state maintained, but glutathione was highly accumulated at 24 h of salt treatment, but then its concentration and redox state progressively decreased. Concomitantly, the antioxidant enzymes involved in ascorbate and glutathione regeneration were modified under salt stress treatments. In conclusion, the increase in ascorbate levels and the maintenance of the redox state seem to be critical for root growth and development under salt stress.

Key words: Antioxidant, ascorbic acid, *Brassica oleracea*, glutathione, hydrogen peroxide, salt stress.

Introduction

Roots play a number of important roles during plant growth and development, and are typically the first part of the plant to encounter salinity. Roots have to cope with two types of stress—osmotic and salt toxicity. These in turn cause a reduction in water uptake, inhibition of root growth, and an induction of oxidative stress (Munns and Tester, 2008). It is known that oxidative stress results from the disruption of cellular homeostasis of reactive oxygen species (ROS) production. ROS accumulation induces oxidative damage of membrane lipids, nucleic acids, and proteins (Mittler, 2002). Therefore, a tight control of the steady-state concentration of ROS seems to

be necessary to avoid oxidative damage at subcellular levels, while simultaneously allowing ROS to perform useful functions as signal molecules under salt stress (Gomez *et al.*, 2004; Rubio *et al.*, 2009). Hydrogen peroxide (H₂O₂) is a versatile molecule that may be involved in several cell processes under normal and stress conditions (Quan *et al.*, 2008). Under stress conditions, H₂O₂ is produced and accumulates, leading to oxidative stress in plants. Increasing evidence indicates that hydrogen peroxide functions as a signalling molecule in plants. Therefore, the control of H₂O₂ concentration is critical for cell homeostasis.

The response of antioxidant systems to salt stress has been widely studied in leaves (Hernandez *et al.*, 2001; Mittova *et al.*, 2003; Gomez *et al.*, 2004; BenAmor *et al.*, 2006, among many others). In general, it is well accepted that plants with high levels of activity of the antioxidant systems, both constitutive and induced, have greater resistance to oxidative damage. However, data on the effects of salt stress in roots are scarce (Panda and Upadhyay, 2003; Bandooglu *et al.*, 2004; Mittova *et al.*, 2004; Tsai *et al.*, 2004; Kim *et al.*, 2005; de Azevedo Neto *et al.*, 2006; Cavalcanti *et al.*, 2007; Seckin *et al.*, 2009).

The antioxidative system includes antioxidant compounds such as carotenoids and ascorbate, glutathione, α -tocopherol, and several enzymes involved in the detoxification of ROS. These enzymes include superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR). SOD converts superoxide to H_2O_2 and molecular oxygen (Scandalios, 1993). Superoxide radicals are not toxic *per se* like other oxy-radical species, but they are a precursor of extremely reactive hydroxyl radicals which are generated in the presence of transition metals and ascorbate. SOD activity can be divided into Cu,Zn-SOD, Mn-SOD, or Fe-SOD isoforms depending on the metal present in the active site. APX is the most important POX in detoxifying H_2O_2 , catalysing the reduction of H_2O_2 to water (Foyer, 1996). APX, together with monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and GR, removes the H_2O_2 via the Foyer–Halliwell–Asada pathway (Foyer and Halliwell, 1976; Halliwell, 1987). CAT ($K_m=20\text{--}124$ mM, Yamaguchi *et al.*, 1986; Engel *et al.*, 2006) can also reduce H_2O_2 to water but it has lower affinity for H_2O_2 than APX ($K_m=20\text{--}74$ μ M, Mittler and Zilinskas, 1991; Ishikawa *et al.*, 1998). POXs are involved in several cellular processes.

On the other hand, antioxidant molecules such as ascorbate and glutathione are involved in many metabolic cell pathways (Noctor and Foyer, 1998). Ascorbate can react with ROS, such as ($O_2^{\cdot-}$), ($HO\cdot$) and (O_2), and can act as the substrate for the enzyme APX (Noctor and Foyer, 1998). Ascorbate is also the main reducing agent for transition metals in the cell wall and cytosol. Thus, ascorbate may also play the role of pro-oxidant in transition metal-rich environments. Reduced glutathione (GSH) acts as cell redox regulator and may act as a ROS scavenger. The balance between GSH and oxidized glutathione (GSSG) is critical for keeping a favourable redox status for the detoxification of H_2O_2 .

Root development is a polarized mechanism, where cell division and extension contribute to root growth. Cell division and root extension are produced in the root tip cells. It has been demonstrated that ROS production mediated by a plasma membrane NADPH oxidase regulates plant cell growth and that this process is controlled by the activation of plasma membrane Ca^{2+} - and K^+ -permeable channels in plant root cells (Foreman *et al.*, 2003). Furthermore, ascorbate and its redox state have also been reported in root growth (Pignocchi and Foyer, 2003;

Cordoba-Pedregosa *et al.*, 2007). Therefore, different effects of salinity might be expected, depending on the root zone analysed. The aim of this work was to evaluate the role of the antioxidative systems and the homeostasis of the redox state of the main cellular antioxidants—ascorbate and glutathione—in broccoli roots under short- and long-term salt stress in two different root zones. For this purpose, roots have been divided into two different regions. These regions represent the meristematic and not fully differentiated cells (zone I) and fully differentiated cells (zone II).

Materials and methods

Plant material and growth conditions

Broccoli seeds (*Brassica oleracea* cv. Marathon) were pre-hydrated in aerated de-ionized water for 12 h and germinated in vermiculite, at 28 °C in an incubator, for 2 d. They were then transferred to a controlled-environment chamber with a 16 h light/8 h dark cycle and air temperatures of 25 °C and 20 °C, respectively. The relative humidity was 60% (day) and 80% (night), and photosynthetically active radiation (PAR) was 400 μ mol $m^{-2} s^{-1}$, provided by a combination of fluorescent tubes (Philips TLD 36 W/83, Germany and Sylvania F36 W/GRO, USA) and metal halide lamps (Osram HQL T 400 W, Germany). After 5 d, the seedlings were placed in 15.0 l containers with continuously aerated Hoagland (Hoagland and Arnon, 1938) nutrient solution: KNO_3 (14 mM), Ca ($(NO_3)_2 \cdot 4H_2O$ (7 mM), KH_2PO_4 (4 mM), $MgSO_4 \cdot 7H_2O$ (1 mM), H_3BO_3 (25 μ M), $MnSO_4 \cdot H_2O$ (2 μ M), $ZnSO_4 \cdot 7H_2O$ (2.0 μ M), $CuSO_4 \cdot 5H_2O$ (0.5 μ M), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.5 μ M), Fe-EDTA (20 μ M). The solution was completely replaced every week. After 21 d (when plants were 26 d old), plants were treated with 0, 40, and 80 mM NaCl, corresponding to electrical conductivities of 2, 6, and 10 dS cm^{-1} . A concentration of 40 mM NaCl was selected as the threshold because plant growth was not significantly affected at this level, while 80 mM NaCl induced a significant reduction of plant growth and production (DePascale *et al.*, 2005).

Determinations were made after 1, 7, and 14 d of saline treatments, when plants were 22, 29, and 36 d old. Roots were detached and washed with deionized water, cut into two zones, and immediately frozen in liquid nitrogen. Zone I comprised the apical region of the root (2 cm long), while zone II included the absorption region of the root that was sequentially cut (~8 cm long). Zone I can be considered as composed of cells undergoing differentiation and elongation, and zone II can be considered as being composed of mature cells.

Enzyme extraction

Frozen root samples were ground in a mortar with liquid nitrogen and extracted (1:2 w/v) in 50 mM K-phosphate buffer (pH 7.8), containing 0.5% (w/v) polyvinylpyrrolidone (PVP), 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 0.1 mM EDTA-Na, and 0.2% (v/v) Triton X-100. For APX activity, 20 mM ascorbate was added while EDTA-Na was omitted. All of the following operations were performed at 4 °C. The homogenate was centrifuged at 8000 rpm for 10 min. The supernatant fraction was filtered on Sephadex G-25 NAP columns (Amersham Pharmacia Biotch AB, Uppasala, Sweden), equilibrated with the same buffer used for the homogenization. The samples were concentrated in centrifugal filter devices (Amicon Ultra).

Enzyme assays

Total SOD activity was measured according to McCord and Fridovich (1969) by the ferricytochrome *c* method, using

xanthine/xanthine oxidase as the source of superoxide radicals. For the separation of SOD isoenzymes, a non-denaturing polyacrylamide gel electrophoresis (PAGE) was performed in 12% acrylamide gels using a Bio Rad mini protean II dual slab cell. SOD isoenzymes were located by the photochemical method of Weisiger and Fridovich (1973). Isoenzyme identification was performed by selective inhibition with potassium cyanide (KCN) or H_2O_2 (Olmos *et al.*, 1994).

The CAT activity was assayed by measuring the initial rate of H_2O_2 disappearance at 240 nm (Aebi, 1984). POX activity in broccoli roots was determined in assays containing 50 mM TRIS-acetate buffer (pH 5.0), 0.5 mM H_2O_2 , and 1.0 mM 4-methoxy- α -naphthol ($\epsilon_{595}=21\ 600\ \text{M}^{-1}\ \text{cm}^{-1}$). The reaction was initiated by the addition of enzyme. Controls were carried out in the absence of H_2O_2 and in the presence of 5.0 mM KCN (Barcelo, 1998).

APX activity was determined in a mixture containing 50 mM potassium phosphate (pH 7.0), 1.5 mM ascorbate, 1.0 mM H_2O_2 , and enzyme extract (Saher *et al.*, 2004). Activity was determined by following the H_2O_2 -dependent decomposition of ascorbate at 265 nm.

DHAR was determined as described by Saher *et al.* (2004). Total MDHAR activity was assayed at 25 °C by monitoring the decrease in the absorbance at 340 nm (Arrigoni *et al.*, 1981). Monodehydroascorbate (MDA) was generated by the ascorbate/ascorbate oxidase system. Total GR activity was determined by following the rate of NADPH oxidation, as measured by the decrease in the absorbance at 340 nm (Edwards *et al.*, 1990). The reaction rate was corrected for the small, non-enzymatic oxidation of NADPH by GSH. Total protein content was estimated according to Bradford (1976).

Lipid peroxidation

The level of lipid peroxides was determined as malondialdehyde (MDA) content by the thiobarbituric acid (TBA) reaction, as described by Saher *et al.* (2004). The homogenates were centrifuged at 10 000 g for 5 min, and 1.2 ml of 20% trichloroacetic acid (TCA) containing 0.5% (w/v) TBA was added to a 0.4 ml aliquot of the supernatant. The mixture was heated at 95 °C for 30 min and then quickly cooled on ice. The contents were centrifuged at 10 000 g for 15 min and the absorbance was measured at 532 nm. The concentration of MDA was calculated using an extinction coefficient of $155\ \text{mM}^{-1}\ \text{cm}^{-1}$.

Ascorbate and glutathione extractions

Root samples were ground in a mortar with liquid nitrogen and homogenized with 2 vols of cold 5% metaphosphoric acid (w/v) at 4 °C. The homogenate was centrifuged at 15 000 g for 10 min at 4 °C, and the supernatant was collected for analysis of ascorbate and glutathione.

GSH and GSSG measurements

The methods used for analysis of reduced and total glutathione employed the GR specificity, as described by Anderson *et al.* (1992). GSH was oxidized by DTNB (5,5'-dithio-bis-nitrobenzoic acid) to give GSSG and TNB (5-thio-2-nitrobenzene). GSSG was reduced to GSH by the action of GR and NADPH. GSSG was assayed from the sample after removal of GSH by 2-vinylpyridine and triethanolamine derivatizations. Changes in absorbance due to the rate of TNB formation were measured at 412 nm, and the contents were calculated using a standard curve. The amount of GSH was the difference between total glutathione and GSSG.

H_2O_2 determination

Root samples were homogenized in the extraction medium 0.1 M K-phosphate (pH 6.4) supplemented with 5 mM KCN. The H_2O_2 content in roots of broccoli was determined by the methodology

described by Cheeseman *et al.* (2006). Briefly, the assay mixture contained 250 μM ferrous ammonium sulphate, 100 μM sorbitol, 100 μM xylenol orange, and 1% ethanol in 25 mM H_2SO_4 . Changes in absorbance were determined by the difference in absorbance between 550 nm and 800 nm, and the contents were calculated using a standard curve.

Reduced (ASC) and oxidized (DHA) ascorbate measurement

The assay is based on the reduction of Fe^{3+} to Fe^{2+} by ascorbic acid in acidic solution. The Fe^{2+} forms complexes with bipyridyl, producing a pink colour that absorbs at 525 nm. DHA was reduced to ASC by pre-incubating the sample with dithiothreitol (DTT). The excess DTT was removed with *N*-ethylmaleimide, and the total ascorbate was determined. The amount of DHA was the difference between total ascorbate and the ASC. The contents were calculated using a standard curve.

Ion analysis

For the anion analysis, broccoli roots were dried, diluted, and injected into a Dionex-D-100 ion chromatograph. An ionpac AS 124-4 mm (10–32) column and AG 14 (4×50 mm) guard column were used. The flow rate was $1\ \text{ml}\ \text{m}^{-1}$, with 0.5 mM Na_2CO_3 and 0.5 mM NaHCO_3 as eluent. The anion concentration was measured with a conductivity detector and quantified with Chromleon/Peaknet 6.40 software by comparing peak areas with those of known standards. For cation analysis, an ICP plasma analyser (IRIS Intrepid II XDL, Thermo Electron Corporation) was used.

Histochemical detection of H_2O_2 and superoxide radicals (O_2^-) in broccoli roots

The histochemical detection of H_2O_2 in broccoli roots was performed using endogenous POX-dependent *in situ* histochemical staining, in which whole roots were vacuum-infiltrated with 0.1 mg ml^{-1} 3,3'-diaminobenzidine (DAB) in 50 mM TRIS-acetate buffer (pH 5.0) and incubated at 25 °C in the dark for 24 h. Controls were performed in the presence of 10 mM ascorbic acid (Hernández *et al.*, 2001). The histochemical detection of O_2^- was performed by infiltrating root quarters directly with 0.1 mg ml^{-1} nitroblue tetrazolium (NBT) in 25 mM K-HEPES buffer (pH 7.6) and incubating at 25 °C in the dark for 2 h (Hernández *et al.*, 2001). In both cases, roots were photographed directly using an Olympus SZX PT stereomicroscope.

Subcellular localization of H_2O_2

The histochemical method based on the generation of cerium perhydroxides as described by Olmos and Hellin (1997) was used for the subcellular location of H_2O_2 . Briefly, roots were pre-incubated in freshly prepared 5 mM CeCl_3 in 50 mM MOPS [3-(*N*-morpholino) propane sulphonic acid] at pH 7.0 for 30 min and then 5 mM CdCl_2 was added. After incubation, roots were fixed in a mixture of 2% (v/v) paraformaldehyde/0.5% (v/v) glutaraldehyde in 50 mM CAB (sodium cacodylate buffer), pH 7.0, for 1 h. After fixation, roots were washed twice for 10 min in CAB buffer and post-fixed for 1 h in 1% (v/v) osmium tetroxide in CAB. Roots were washed again in CAB (twice for 10 min), dehydrated in a graded ethanol series, and embedded in Spurr's resin. Blocks were sectioned on a Leica EM UC6 ultramicrotome and collected on copper grids, and some sections were stained with 2% uranyl acetate followed by 2.5% lead citrate, while others remained unstained, for better assessment of the ultrastructural localization of H_2O_2 . The root ultrastructure was observed with a Philips TECNAI 12 transmission electron microscope (FEI/Philips Electron Optics, Eindhoven, The Netherlands).

Confocal laser scanning microscopy

H₂O₂ production was monitored by confocal laser microscopy. Root samples were incubated for 30 min in fresh culture medium containing 10 µM DCFH-DA (2,7-dichlorofluorescein diacetate) and then washed three times with fresh medium without DCFH-DA to remove the excess fluorophore. Fluorescence images were obtained with a Nikon Eclipse TE2000 Confocal Laser Scanning Microscope C1si. Samples were excited with the 488 nm line of an argon laser and dye emission was collected at 520±10 nm. The DCF fluorescence was visualized in a single optical section of the root. All images were obtained at the same depth.

Labelling of glutathione (GSH+GSSG) was carried out with monochlorobimane (MCB) as described by Hartmann *et al.* (2003). Root samples were incubated for 30 min in fresh culture medium containing 100 µM MCB and then washed three times with fresh medium without MCB to remove the excess fluorophore. Sodium azide was freshly prepared and added to the dye solution at a final concentration of 5 mM to inhibit vacuolar sequestration of glutathione S-bimane (GSB) conjugate (Hartmann *et al.*, 2003). Fluorescence images were obtained with a Nikon Eclipse TE2000 Confocal Laser Scanning Microscope C1si. Samples were excited with the 405 nm line of an argon laser, and dye emission was collected at 520±20 nm. The fluorescent GSB conjugate was visualized in a single optical section of root.

Results*Nutrient analysis*

An increase in NaCl concentrations showed a uniform increase in Na⁺ ions and a decrease in K⁺ ions in both root zones, except at 24 h, when a significant increase of only Na⁺ was observed (Table 1). The Ca²⁺ concentration was unaltered at 24 h of 80 mM NaCl treatment in both root

zones. However, long-term treatments induce a significantly lower concentration of calcium in both root zones. Mg²⁺ concentrations were not significantly affected by the different salt treatments in both root zones.

Cl⁻ anions showed significantly higher concentrations in salt-treated plants. This concentration was parallel to Na⁺ accumulation (Table 1). SO₄²⁻ concentration was significantly increased at 24 h in both salt treatments in zone I but was highly reduced in zone II (Table 1). However, long-term salt treatments induced a significantly higher SO₄²⁻ concentration in both root zones (Table 1). PO₄³⁻ anion concentration was not significantly affected by the different salt treatments (Table 1).

H₂O₂ quantification and subcellular location

NaCl (40 mM and 80 mM) treatments for 24 h showed a significant increase of H₂O₂ in both root zones compared with control (Fig. 1A, B), with greater differences in zone II. However, H₂O₂ concentrations were only significantly higher with long-term salt treatments, 7 d and 14 d, in plants growing at 80 mM NaCl in both root zones (Fig. 1A, B).

In view of these results, H₂O₂ was studied using different techniques to locate the tissue distribution and subcellular location of H₂O₂ production. To avoid a greater body of data, these experiments have been developed at 80 mM of NaCl and 14 d of salt treatment (Figs 2, 3) when greater differences were observed.

DAB was used to localize the hydrogen peroxide as a dark brown precipitate and analyse the tissue distribution of

Table 1. Effect of increasing NaCl concentrations on the content of cations and anions in zones I and II of *Brassica oleraceae* roots

Days of treatment		Cations (mmol g DW ⁻¹)				Anions (mM)			
		Na ⁺	K ⁺	Ca ²⁺	Mg ²⁺	Cl ⁻	SO ₄ ²⁻	PO ₄ ³⁻	NO ₃ ⁻
Zone I									
1 d	0	0.06d	1.66b	0.09b,c	8.9e	8.9e	0.06d	62.3a	20.4a,b
	40	1.35c	1.22b	0.12b,c	21.7d	21.7d	0.12c	61.3a	20.8a,b
	80	3.54a,b	0.98b	0.14b,c	72.0a	72.0a	0.20b	66.4a	11.4a,b
7 d	0	0.06d	2.16a	0.22a	8.7e	8.7e	0.05d	47.9b	22.3a,b
	40	2.69b	0.89b	0.11b,c	37.6c	37.6c	0.15c	46.2b	22.6a
	80	3.21a,b	0.55b	0.12b,c	55.0b	55.0b	0.23b	48.2b	21.4a,b
14 d	0	0.06d	4.22a	0.16a,b	3.5f	3.5f	0.14c	16.7c	22.9a
	40	2.49b,c	1.30b	0.08b,c	18.2d	18.2d	0.35a	17.6c	21.1a,b
	80	4.39a	0.92b	0.08c	38.5c	38.5c	0.34a	19.6c	22.0a,b
Zone II									
1 d	0	0.06d	1.41b,c,d	0.14a,b,c	15.9d,e	15.9d,e	0.18c	68.5a	32.3a
	40	1.70b,c	1.39b,c,d	0.15a,b,c	16.8d,e	16.8d,e	0.03d	71.6a	29.3b
	80	3.35a	1.38b,c,d	0.14a,b,c	57.2b	57.2b	0.03d	45.3b	25.1c
7 d	0	0.09d	1.58b,c	0.22a	11.7e	11.7e	0.14c	50.2b	24.2c
	40	2.60a,b	0.81d,e	0.13b,c	42.6c	42.6c	0.10c	48.7b	33.8a
	80	3.56a	0.47e	0.15a,b,c	89.7a	89.7a	0.35b	48.2b	33.5a
14 d	0	0.06c,d	1.98a,b	0.17a,b	3.1f	3.1f	0.28b	28.9c	32.8a
	40	3.59a	1.06c,d,e	0.08b,c	23.6d	23.6d	0.52a	25.4c,d	28.7b
	80	3.65a	0.74e	0.07c	42.9c	42.9c	0.84a	22.6d	25.8c

Values represent the means ±SD of five different samples. Means within a column without a common letter are significantly different by Tukey's test (P <0.05)

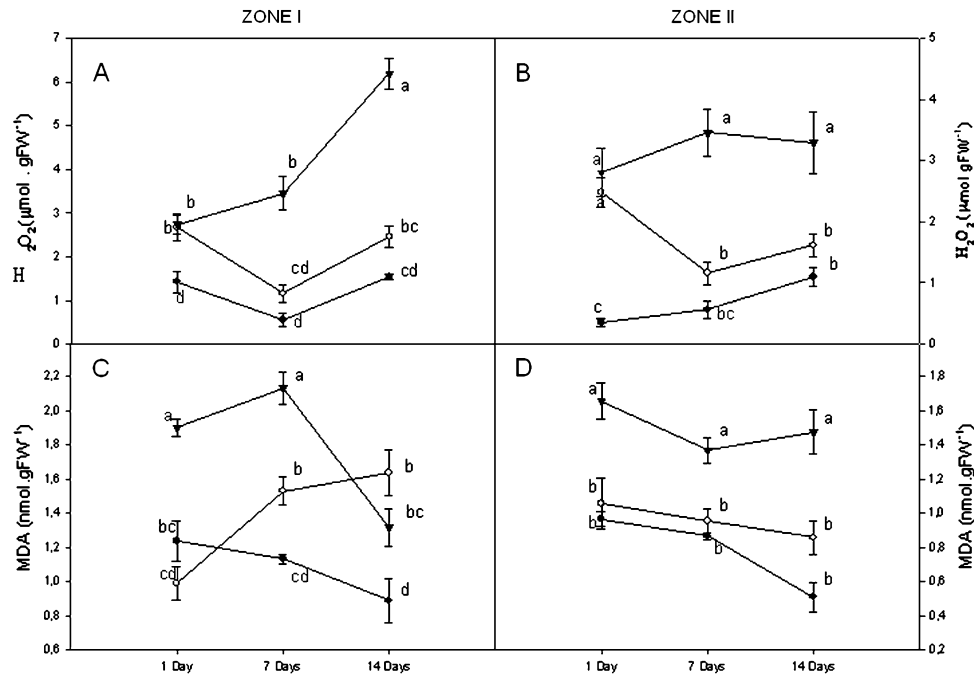


Fig. 1. Time course of hydrogen peroxide and MDA contents in zone I (A and C) and zone II (B and D) of *Brassica oleracea* roots grown under control conditions (inverted triangles), and with 40 mM NaCl (open circles) and 80 mM NaCl (filled circles). Values represent the means \pm SD of five different samples. Significant differences ($P < 0.05$) between days and treatments are indicated by different letters according to Tukey's test.

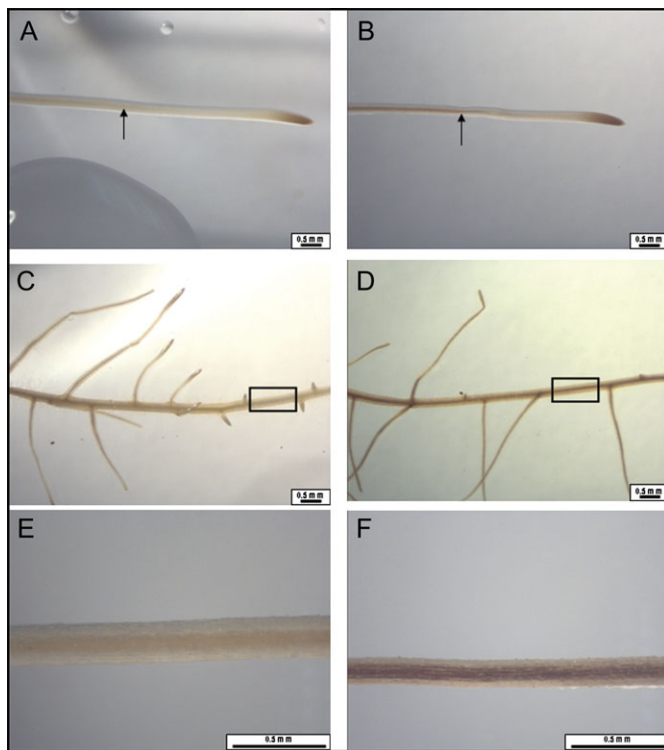


Fig. 2. Hydrogen peroxide location in root tissues using DAB. Zone I (A and B, arrows indicate the beginning of the staining in the stele) and zone II (C and D; E and F are magnifications of the boxed area in C and D) of *Brassica oleracea* roots grown under control conditions (A, C, and E) and with 80 mM NaCl (B, D, and F) during 14 d.

H₂O₂. Mainly zone I showed the staining in the root tip. Salt-treated roots showed a darker staining in the root tip compared with control (Fig. 2A, B) and the staining was also observed to be dense in the newly formed xylem 4–5 mm from the tip (Fig. 2A, B, see arrows). Similarly, salt treatment induced more staining in zone II (Fig. 2C, D). With higher magnification, this staining was mainly located in the stele of the root (Fig. 2E, F). The greater staining observed in the stele and elongation zone of both control and salt-treated roots may be due to a higher permeability of these tissues for water flow and, consequently, there is greater DAB transport.

For *in vivo* analysis of H₂O₂ production, we used a fluorochrome (DCFH-DA) that reacts with H₂O₂ and produces fluorescence that can be located by laser confocal microscopy. Zone I of the control showed very low fluorescence compared with salt-treated roots (Fig. 3A, B). This fluorescence seems to be located in the cytoplasm and apoplast of the root tip cells. Similarly, zone II of control roots showed a very low fluorescence compared with salt-treated roots (Fig. 3E, F).

Finally, at the subcellular level, H₂O₂ production was located using a precipitation technique through the reaction of H₂O₂ with cerium chloride (Olmos *et al.*, 2003). The main differences at the subcellular level were observed in the mitochondria. Salt treatment induces the accumulation of H₂O₂ in mitochondria of both root zones (Fig. 3D, H) compared with the control (Fig. 3C, G). This H₂O₂ seems to be mainly located in the mitochondrial cristae and mitochondrial external membrane (Fig. 3D, H).

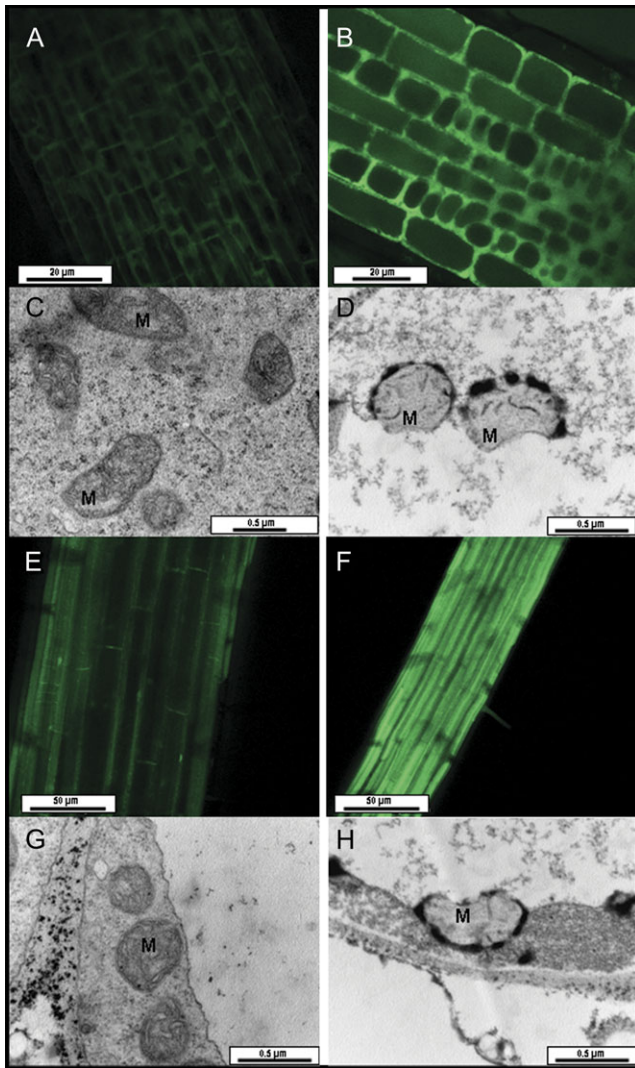


Fig. 3. Hydrogen peroxide was located using two different techniques: *in vivo* labelling of hydrogen peroxide using DCFH-DA, located by laser confocal microscopy (A, B, E, and F), and the cerium chloride precipitation technique, located by transmission electron microscopy (C, D, G, and H). Zone I (A, B, C, and D) and zone II (E, F, G, and H) of *Brassica oleracea* roots grown under control conditions (A, C, E, and G) and with 80 mM NaCl (B, D, F, and H) during 14 d.

Lipid peroxidation

The damage by NaCl to cellular membranes due to lipid peroxidation was estimated from MDA concentrations and the results showed that MDA was significantly higher with increased NaCl concentrations. Zone I seems to be more affected by both salt treatments. Short-term salt treatments induce accumulation of only MDA at 80 mM NaCl in both root zones. However, zone I showed a significantly higher accumulation of MDA in both long-term salt treatments at 40 mM and 80 mM NaCl (Fig. 1C) while zone II only showed a significantly higher accumulation of MDA at 80 mM NaCl (Fig. 1D).

Antioxidant enzymatic activity

Superoxide dismutase: Total SOD activity was unaltered by both salt treatments at 24 h in both root zones (Fig. 4A, B). However, total SOD activity was highly induced in zone I at 7 d and 14 d in both salt treatments. Interestingly, total SOD activity was highly reduced in zone II at 7 d in both salt treatments but was unaltered at 14 d (Fig. 4A, B).

The isozyme composition of SODs was determined on native gels stained for SOD activity. Two Cu,Zn-SODs, one Mn-SOD, and one Fe-SOD were identified in root samples (Fig. 5). Fe-SOD, Mn-SOD, and Cu,Zn-SOD II activities can be observed in all samples. However, Cu,Zn-SOD I can be observed only in zone I at 7 d. In general, Fe-SOD seems to be the main isozyme. The analysis of the activities of the different isozymes is well correlated with the total activity. In zone I at 7 d, Fe-SOD is induced at 80 mM NaCl, and Cu,Zn-SOD I and Mn-SOD are similarly induced (Fig. 5).

Ascorbate peroxidase: This enzyme catalyses the reduction of H_2O_2 using ASC as co-factor. This activity progressively decreased with all salt treatments in both root zones (Fig. 4C, D).

Catalase: In response to short-term salt treatment, CAT activity decreased proportionally to salt concentration in zone I (Fig. 4E) but was only affected by 80 mM NaCl in zone II (Fig. 4F). However, after 7 d of salt treatment CAT activity was unaltered by salt treatments in both root zones, compared with control (Fig. 4E, F).

Peroxidase: This activity was highly decreased by salt treatments at 24 h in both root zones (Fig. 4G, H). However, after 7 d of salt treatment, POX activity was higher in salt-treated roots in zone I. This effect was only observed in zone II at 80 mM NaCl (Fig. 4H).

The isozyme analysis of POXs by isoelectrofocusing revealed the presence of at least eight different isozymes, four basic (B1, pI=9.0; B2, pI=8.4; B3, pI=7.5; and B4, pI=7.4) and four acidic (A1, pI=4; A2, pI=4.7; A3, pI=4.85; and A4, pI=5.8) (Fig. 6). The most abundant isozymes were B4 and A1. The basic B4 isozyme was inhibited by salt treatments in zones I and II at 24 h. However, it was highly induced at 7 d by salt treatments in both root zones (Fig. 6). The acidic A1 isozyme was slightly affected by salt treatments, since a significant decrease of this isozyme was only observed at 24 h of 80 mM NaCl treatment (Fig. 6).

Ascorbate–glutathione cycle enzymes

Monodehydroascorbate reductase: Total activity was unaltered by salt treatments in both root zones during the different days analysed (Fig. 7A, B).

Dehydroascorbate reductase: Total activity decreased strongly with both salt treatments at 24 h in zone I and II (Fig. 7C, D). After 7 d of salt treatments, DHAR activity was recovered in

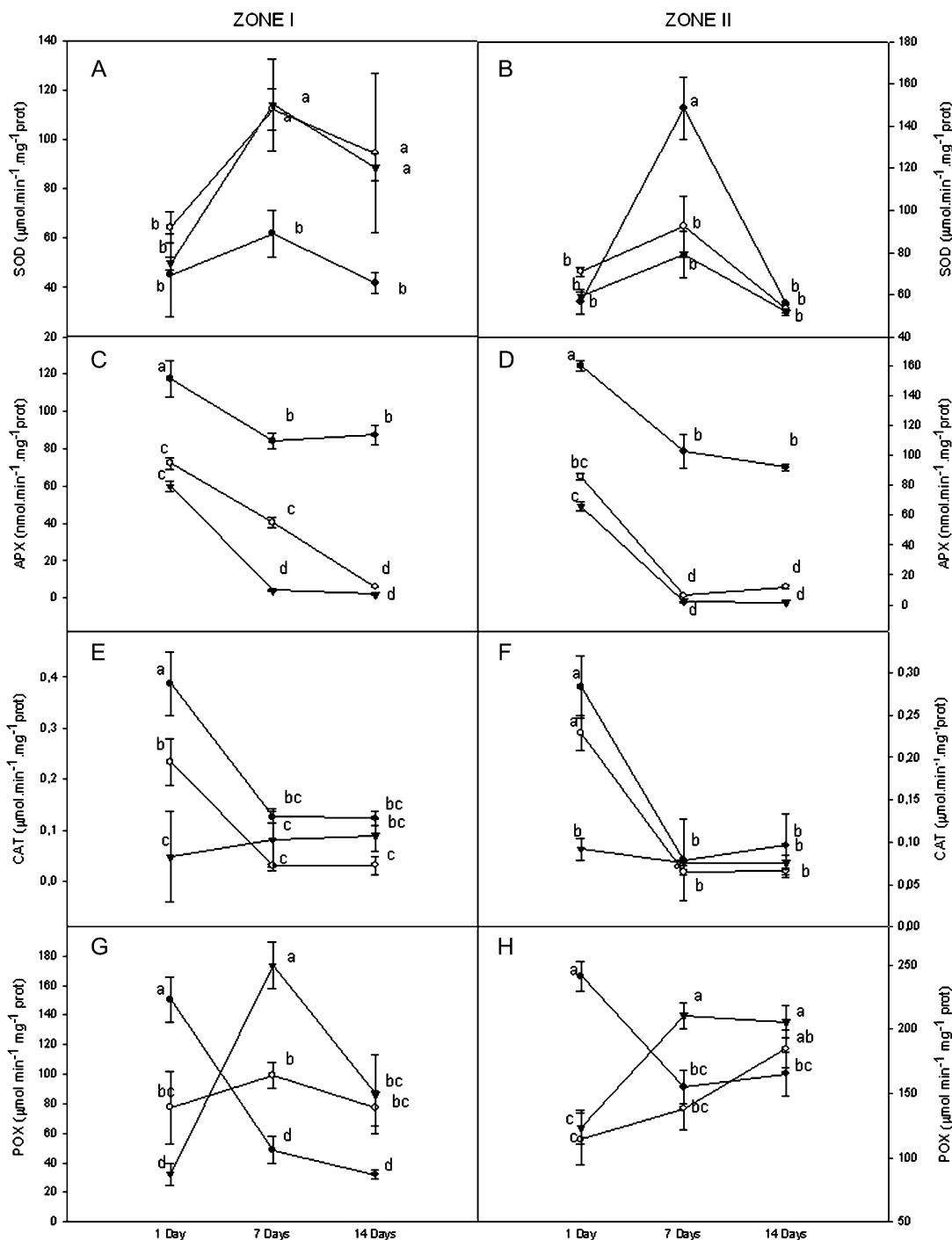


Fig. 4. Time course of SOD, APX, CAT, and POX enzyme activities in zone I (A, C, E, and G) and zone II (B, D, F and H) of *Brassica oleracea* roots grown under control conditions (inverted triangles), and with 40 mM NaCl (open circles) and 80 mM NaCl (filled circles). Values represent the means \pm SD of five different samples. Significant differences ($P < 0.05$) between days and treatments are indicated by different letters according to Tukey's test.

all salt treatments, showing no differences between the two root zones.

Glutathione reductase: Total activity was greatly reduced by salt treatments in both root zones over the whole period analysed (Fig. 7E, F) but zone II showed a greater reduction of GR activity at 80 mM during the first 7 d of salt treatments (Fig. 7F).

Antioxidant metabolites

ASC and DHA: Roots accumulated significantly higher levels of ASC and DHA at 80 mM NaCl, except zone I at 14 d when no significant differences were observed between salt treatments and control (Table 2). Total ascorbate showed a similar trend to the reduced ascorbate (Table 2). The ASC/DHA ratio was unaltered in both root zones at

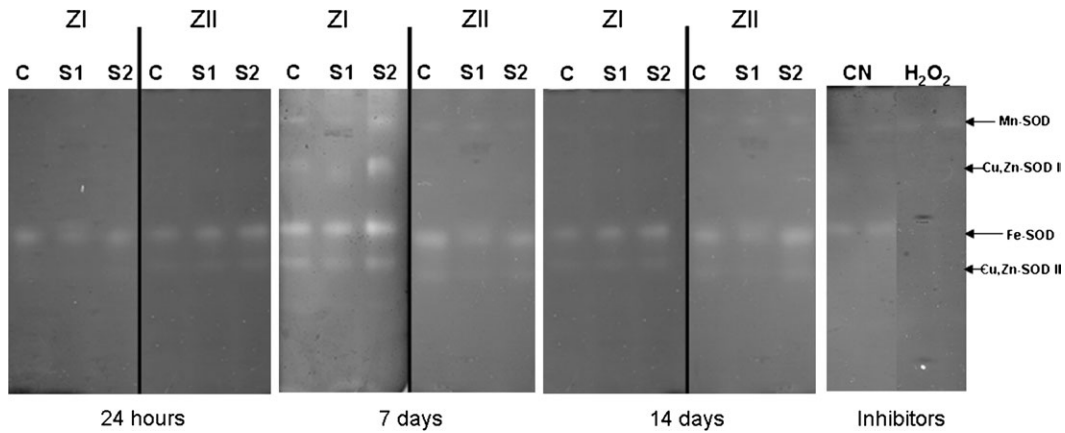


Fig. 5. SOD isoforms detected in native gel. The protein concentration loaded in each well was the same. C, control; CN, cyanide; S1, 40 mM NaCl; S2, 80 mM NaCl; ZI, zone I; ZII, zone II.

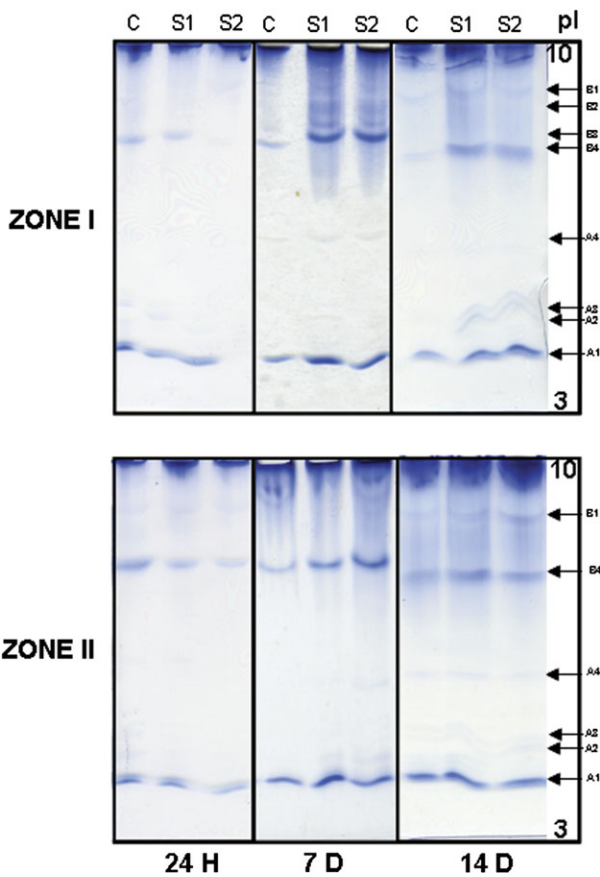


Fig. 6. Peroxidase isoforms detected by isoelectrofocusing gel electrophoresis (pH 3–10). The protein concentration loaded in each well was the same. C, control; S1, 40 mM NaCl; S2, 80 mM NaCl.

24 h of salt treatments (Table 2). After 7 d of salt treatments, this ratio was significantly increased in both root zones. However, at 14 d, the ASC/DHA ratio was slightly reduced in zone I at 80 mM NaCl but zone II showed a higher ratio at 80 mM NaCl compared with the control and 40 mM NaCl (Table 2).

GSH and GSSG: Both root zones accumulated a much higher concentration of GSH at 80 mM NaCl during the first 24 h compared with the control and with 40 mM NaCl (Table 2). After 7 d of salt treatments, both root zones showed an unaltered glutathione concentration in control and 40 mM NaCl but it was significantly reduced at 80 mM NaCl (Table 2). After 14 d of salt treatment, the glutathione concentration was highly reduced in both root zones (Table 2). Total glutathione showed a similar trend to GSH at 24 h of salt treatments (Table 2). However, after 7 d of salt treatment, the total glutathione concentration was unaltered in both root zones. After 14 d of salt treatment, the total glutathione concentration was significantly reduced in both root zones (Table 2). The GSSG concentration was significantly higher at 80 mM NaCl compared with the control and with 40 mM NaCl in both root zones at 24 h and 7 d of salt treatment. However, after 14 d no significant differences were observed between salt treatments and control (Table 2). The GSH/GSSG ratio was significantly affected by the salt treatments (Table 2). After 24 h of salt treatment, the GSH/GSSG ratio was only significantly reduced in zone I. After 7 d and 14 d of salt treatments, the GSH/GSSG ratio was significantly and progressively reduced in both root zones compared with the control (Table 2).

Subcellular location of total glutathione

The results presented here showed a high increment of glutathione during the first 24 h at 80 mM NaCl but not at 40 mM NaCl. To confirm this result, the subcellular location of total glutathione was developed using MCB, and fluorescence was located by laser confocal microscopy. Zone I showed a much higher fluorescence in the root tip of salt-treated roots (Fig. 8B) compared with the control (Fig. 8A). At a higher magnification, this higher fluorescence seems to be located in the nuclei of salt-treated roots (Fig. 8D) compared with the control (Fig. 8C). Similarly, zone II of salt-treated roots showed a higher fluorescence in the cytoplasm of the cells (Fig. 8F) compared with the control (Fig. 8E).

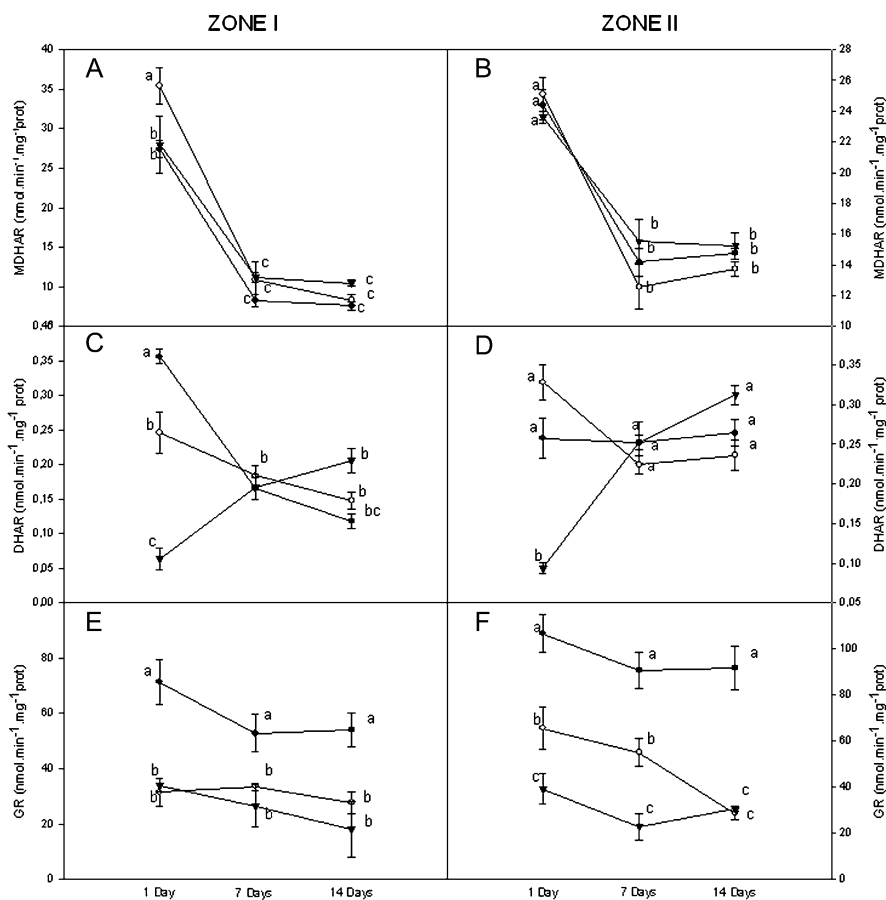


Fig. 7. Time course of MDHAR, DHAR, and GR enzyme activities in zone I (A, C, and E) and zone II (B, D, and F) of *Brassica oleracea* roots grown under control conditions (inverted triangles), and with 40 mM NaCl (open circles) and 80 mM NaCl (filled circles). Values represent the means \pm SD of five different samples. Significant differences ($P < 0.05$) between days and treatments are indicated by different letters according to Tukey's test.

Discussion

The effects of salt stress on plants can be mainly classified as two different factors, osmotic stress induced by the high saline concentration in the culture medium and the toxic effect of sodium accumulation in the cells. These two effects occur in two sequential phases. First, a rapid response to the increase of external osmotic pressure and a parallel Na^+ influx that causes depolarization, which, in turn, induces K^+ loss from root cells, take place during the first minutes. Secondly, a slower response takes place due to accumulation and redistribution of Na^+ in root cells (after several days). These effects are dependent on the salt concentrations (Munns and Tester, 2008). These authors consider that the threshold level is ~ 40 mM NaCl for the majority of the species, probably due to the osmotic effect of the salt outside of the roots. Therefore, salt tolerance to higher concentrations of NaCl will be controlled by several factors (Munns and Tester, 2008). Of these, the effective control of the oxidative damage induced by both effects, osmotic and toxic, might be critical for plant tolerance to high saline concentrations.

Brassica oleracea is considered to be a moderately salt-tolerant species (Ashraf *et al.*, 2001). It has recently been

observed that broccoli root presents a phi cell layer surrounding the endodermis. Phi cell layers and the endodermis act as a partial apoplastic barrier under salt stress, controlling the passage of sodium and chloride to the stele in *B. oleracea* roots (Fernandez-Garcia *et al.*, 2009). However, this implies an accumulation of Na^+ in cortical and phi cell layers. Therefore, a mechanism of cell compartmentalization of Na^+ and the plant defence system against ROS accumulation can be useful to prevent the negative effect of oxidative stress induced by salinity.

Cation balance is altered in salt-treated roots

The present results confirm a rapid accumulation of sodium and chloride ions in roots which was proportional to the external concentration of NaCl. The increase in Na^+ content and decrease in K^+ ion uptake disturb the ionic imbalance as observed in most species exposed to salt stress (Munns and Tester, 2008). Loss of K^+ is harmful for cell physiology and biochemistry, and could be considered as the main reason for salt toxicity (Shabala *et al.*, 2006). Non-selective cation channels (NSCCs) are considered to be the major pathway for Na^+ influx into root cells (Demidchik and Tester 2002; Demidchik and Maathuis, 2007).

Table 2. Effect of increasing NaCl concentrations on ascorbate (reduced ascorbate, dehydroascorbate, total ascorbate, and its ratio) and glutathione (reduced glutathione, oxidized glutathione, total glutathione, and its ratio) content in zones I and II of *Brassica oleracea* roots

Days of treatment		$\mu\text{mol g FW}^{-1}$				nmol g FW^{-1}			
		ASC	DHA	Total ascorbate	ASC/DHA	GSH	GSSG	Total glutathione	GSH/GSSG
Zone I									
1 d	0	2.0b,c	1.0c	3.0b,c	2.0b,c	13.5b	1.9e	17.3c	7.1a
	40	2.3b,c	1.6b	3.9b	1.5c	13.2b,c	2.9d,e	19.0b	4.6b
	80	3.5a	1.6ab	5.1a	2.1b,c	25.3a	5.5b,c	36.a	4.7b
7 d	0	1.4c	0.7d	2.1c	2.0b,c	13.b,c	4.9b,c	22.8b	2.7b
	40	2.0b,c	0.7d	2.7c	2.8b	9.8c,d	6.7a,b	23.2b	1.4c
	80	3.3a,b	0.6d	3.9b	5.5a	7.3d,f	8.2a	23.7b	0.9c
14 d	0	3.9a	1.6b	5.5a	2.5b	10.4c,d	5.3b,c	21.0b	2.0c
	40	3.9a	1.8a	5.7a	2.2b	4.2e,f	4.1c,d	12.4d	1.0c
	80	3.2a,b	1.9a	5.1a	1.7c	2.8e	5.8b,c	14.4c,d	0.5d
Zone II									
1 d	0	1.3f	0.5c	1.8c	2.5f	15.5b	3.0d	21.5b	5.2a
	40	2.2d	0.8b	3.0b	2.5f	13.5b	3.8d	21.1b	3.6a,b
	80	2.2d	0.9a,b	3.1b	2.4f	38.7a	7.2a,b	53.1a	5.3a
7 d	0	1.8e	0.6c	2.4c	3.0d,e	14.8b	4.9c,d	24.6b	3.0b
	40	1.9d,e	0.6c	2.5c	3.2c	11.2b	4.9c,d	21.0b	2.2b,c,d
	80	3.3b	0.8b	4.1a	4.1a	6.6c	7.9c,d	22.4b	0.9d
14 d	0	2.5c	0.9a,b	3.4b	2.8e	15.5b	4.9b,c,d	25.3b	3.1b,c
	40	2.8b,c	0.9a,b	3.7a	3.1c,d	3.7c	4.9c,d	13.5c	0.8c,d
	80	3.8a	1.0a	4.8a	3.8b	3.5c	6.9a,b,c	17.3c	0.5d

Values represent the means \pm SD of five different samples. Means within a column without a common letter are significantly different by Tukey's test ($P < 0.05$).

Moreover, Na^+ influx depolarizes the plasma membrane and induces K^+ efflux through plasma membrane K^+ -permeable channels (Shabala *et al.*, 2006).

Long-term salt-treated broccoli roots showed a significant reduction of calcium. These results are in agreement with those published by Ashraf *et al.* (2001) in *B. oleracea* roots salinized at 100 mM NaCl during 28 d. At the cellular level, Halperin *et al.* (2003) have observed in hair root cells that the calcium concentration was reduced, which was correlated with the reduction of cell elongation. However, other authors have observed an increment of calcium concentration under salt treatments (Yang *et al.*, 2007). These authors have correlated this increment with a higher NADPH oxidase activity of the plasma membrane and the accumulation of H_2O_2 . It must be taken into consideration that total Ca^{2+} does not reflect cytosolic or apoplastic Ca^{2+} levels. Modifications in total Ca^{2+} may show changes in apoplastic Ca^{2+} binding capacity, Ca^{2+} binding systems in cytosol, or vacuolar calcium.

Salt stress induces accumulation of H_2O_2 and oxidative damage

H_2O_2 was accumulated by salt treatments in both root zones analysed in broccoli. If these results are compared with the literature, it is found that different effects have been observed. Tsai *et al.* (2004) have observed a progressive H_2O_2 accumulation in salt-treated (150 mM NaCl) roots of

rice. Similarly, Panda and Upadhyay (2003) observed a higher content of H_2O_2 in *Lemna minor* roots treated with a progressively increased concentration of NaCl. However, other authors observe no changes (Lee *et al.*, 2001) or a significant reduction of the H_2O_2 concentration (Kim *et al.*, 2005). In the results presented here, H_2O_2 was rapidly accumulated in both root zones during the first 24 h of both salt treatments but was only maintained during long-term salt treatments at 80 mM NaCl. It is possible that the accumulation of H_2O_2 at 24 h observed in salinized broccoli roots is mainly due to the osmotic stress induced by the external NaCl concentration. This H_2O_2 can act as signal, so setting off the defence system in different parts of the plants.

In general, MDA accumulation is considered to be a marker of oxidative damage. In broccoli roots, lipid peroxidation was significantly increased under salt stress treatments and it was well correlated with H_2O_2 accumulation at 80 mM NaCl. Interestingly, 40 mM NaCl induced lipid peroxidation in zone I in long-term salt treatment but H_2O_2 was not accumulated. However, lipid peroxidation can also be induced via an enzymatic pathway by the activity of lipoxigenases, which have been observed to be induced by salt stress (Mittova *et al.*, 2002; Molina *et al.*, 2002). It is possible that zone I is more sensitive to the oxidative damage, so affecting cell integrity and elongation (Panda and Upadhyay, 2003; Li *et al.*, 2007). Similarly, Rubio *et al.* (2009) have observed greater oxidative damage

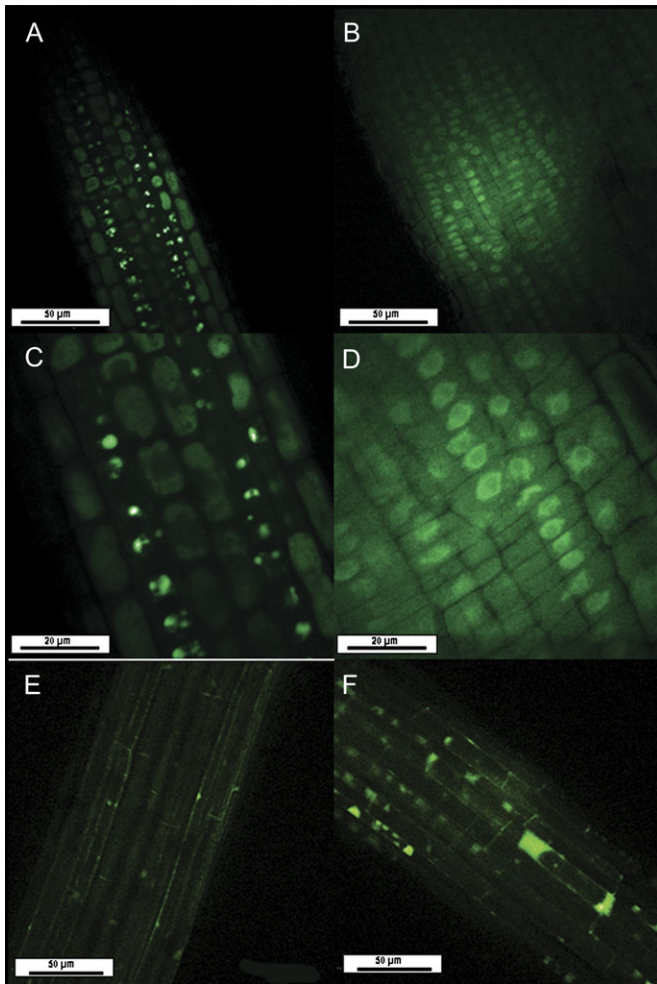


Fig. 8. *In situ* location of glutathione in control (A, C, and D) and salt-treated roots (80 mM NaCl, E and F) of *Brassica oleracea* during 24 h. Root sections were treated with dye solution (monochlorobimane) and images were taken by confocal laser scanning microscopy after an incubation period of 1 h. The fluorescent GSB conjugate was visualized in a single optical section of root. Zone I (A and B; C and D are magnifications of the same image in C and D) and zone II (E and F).

in *Lotus japonicus* exposed to a high saline concentration, despite the maintenance of antioxidant levels. They suggest two possible explanations: (i) MDA was accumulated due to the fact that cellular membranes are particularly sensitive to ROS attack; and (ii) the oxidative damage is the result of an excess of ROS production rather than insufficient antioxidant protection.

H_2O_2 accumulation was confirmed with different histochemical techniques. The analysis of H_2O_2 distribution using the DAB technique demonstrated a higher accumulation of H_2O_2 in the root tip (zone I) of salt-treated roots. Moreover, H_2O_2 was also highly accumulated in zone II in the stele. This accumulation can be also observed in the newly formed vasculature in zone I (see Fig. 2B, arrows). Fernandez-Garcia *et al.* (2009) demonstrated that salt-treated roots (80 mM NaCl) showed a higher lignification

of the xylem and phi thickenings and, therefore, it is possible that this accumulation of H_2O_2 is participating in the lignification of these structures. Lignification is produced by the action of class III peroxidases and H_2O_2 , and under salt stress seems to be induced in many species (Cachorro *et al.*, 1993; Neumann *et al.*, 1994; Jbir *et al.*, 2001; Sanchez-Aguayo *et al.*, 2004). In broccoli roots, lignification of phi thickening seems to affect the movement of cations from the cortex to the endodermis (Fernandez-Garcia *et al.*, 2009). Moreover, the biochemical data demonstrate a higher POX activity at 7 d and 14 d than was correlated with the H_2O_2 accumulation in the stele observed with the DAB technique. The analysis of the isozyme pattern demonstrates a higher increment of a basic POX in salt-treated roots. Similarly, Quiroga *et al.* (2001) have observed that a basic isozyme (pI 9.1) of tomato root is induced by salt treatments.

In vivo labelling of H_2O_2 and the use of laser confocal location confirm a higher production of H_2O_2 in salt-treated roots in both zones, showing an accumulation in the apoplast and cytoplasm. To verify its subcellular location, H_2O_2 was also located by the precipitation technique using cerium chloride. In the cytoplasm of salt-treated roots, the most interesting finding was that mitochondria accumulated H_2O_2 in the cristae and external membranes. These results are in agreement with those observed in purified mitochondria in a tomato NaCl-sensitive cultivar and in cucumber, which accumulate H_2O_2 under salt stress (Mittova *et al.*, 2004; Shi *et al.*, 2007). Similarly, Leshem *et al.* (2007), using *in vivo* techniques, observed that the mitochondria of *Arabidopsis* roots accumulated H_2O_2 under salt stress.

On the other hand, H_2O_2 can also act as a secondary messenger under stress conditions (Quan *et al.*, 2008). Some authors consider that H_2O_2 accumulation under high saline concentrations may be a signal for an adaptive response to stress (Foyer *et al.*, 1997). It has been demonstrated that H_2O_2 accumulation is involved in stomata closure induced by abscisic acid (ABA) signalling (Zhang *et al.*, 2001). It has been observed that stomatal conductance was reduced in salt-treated broccoli plants (Fernandez-Garcia *et al.*, 2009) and this parameter was directly correlated with stomatal closure. Moreover, it was observed that the ABA concentration is highly increased in the xylem of salinized broccoli plants under short- and long-term salt treatments (data not shown).

Salt stress effect on the enzymatic antioxidative system

Recently, Jiang *et al.* (2007) analysed the proteome of *Arabidopsis* roots under NaCl stress and showed that detoxifying enzymes such as APXs, glutathione peroxidases and SODs are up-regulated by salt stress.

The present results demonstrate a differential effect of salt stress according to the duration of salt treatments and the root zone. Short salt treatments reduced the capacity to eliminate H_2O_2 by inhibition of the activity of CAT, POX, and APX. However, long-term treatments result in recovery of the activities of CAT and POX, but not of APX, which is

drastically reduced. In this study, the pattern of changes of total SOD activity and that of its isoforms indicates that the activity was unaltered during short salt treatments. Two isoforms of Cu,Zn-SOD, one Mn-SOD, and Fe-SOD were detected in the native gel where Fe-SOD was the main isoform in both root zones. Therefore, the accumulation of H₂O₂ observed could be due to reduced capacity to eliminate H₂O₂ by the deactivation of APX, POX, and CAT and the maintained activity of SOD in both root zones.

However, the total SOD activity was different after 7 d of salt treatments; zone I showed a high increment of total SOD activity that was mainly due to a higher activity of Fe-SOD and Cu,Zn-SOD I, while Mn-SOD was slightly induced and Cu,Zn-SOD II was unaltered. However, in zone II the total SOD activity was greatly reduced by salt treatments, showing a high level of inhibition of Fe-SOD. It is possible that a higher amount of the superoxide radical is induced in the root tip to maintain root growth during salt stress, so SOD was induced to dismutate superoxide radicals to H₂O₂. After 14 d of salt treatments, SOD activity showed a similar trend in zone I but, surprisingly, was unaltered in zone II. It can be argued that in zone I the SOD activity is increased to maintain the active root growth, in contrast to zone II, where tissues are at a mature stage.

In many cases, it has been proposed that salt stress tolerance is related to a higher activity of antioxidant enzymes such as APX, CAT, and SOD, and that lower activity is found in sensitive species (Shalata *et al.*, 2001). However, a direct correlation cannot always be found between salt stress tolerance and the induction of antioxidant enzymes. Transgenic plants overexpressing these enzymes did not always induce salt tolerance (Munns and Tester, 2008).

Total APX activity was dramatically affected by salt treatments in broccoli roots but this reduction cannot be attributed to a low concentration of ASC. Miller *et al.* (2007) observed that a double inhibition of the expression of a cytosolic APX and thylakoid APXs in *Arabidopsis* induces salt tolerance. These authors suggest the existence of redundant pathways of ROS protection that compensate the lack of antioxidant enzymes such as APXs.

Differential effect of short and long salt treatments in the ascorbate and glutathione pools

It has been proposed that salt-tolerant species have higher ascorbate and glutathione contents and higher redox states in comparison with salt-sensitive species (Shalata *et al.*, 2001; Chaparzadeh *et al.*, 2004; Khan and Panda, 2008). However, in *B. oleracea* roots, both antioxidants showed a different response to salt stress. Reduced ascorbate accumulation is induced by the higher saline treatment but not by the lower concentration, and oxidized ascorbate was only slightly increased by long-term salt treatments. The change in the ASC/DHA ratio, an important indicator of the redox status of the cell, suggests a greater redox capacity

under high salt concentration. The high level of inactivation of APX in salt-treated roots reduces the need for ascorbate through the ascorbate–glutathione cycle. Therefore, it is possible that ASC is directly scavenging H₂O₂ and the ASC redox state is maintained by the unaltered activities of DHAR and MDHAR during long-term salt treatments.

Brassica oleracea roots showed a rapid increment of total and reduced glutathione at high saline concentrations, although the GSH/GSSG ratio was significantly reduced. The experiments in broccoli roots showed that after 24 h of salt treatment an important amount of GSH was recruited in the nucleus of the root tip cells, altering the redox state of this organelle and probably preventing nuclear damage and/or reducing proteins that can activate the cellular defence mechanisms. However, the GSH/GSSG ratio was highly decreased by salt treatments at 7 d and 14 d in *B. oleracea* roots. In view of these results, it is possible that GSH is required during the initial phase of osmotic stress induced by salt stress, activating alternative pathways for ROS protection that compensate for the lack of APX activity.

The glutathione and ascorbate contents and their ratio are considered to have an important role in redox sensing. In ozone stress, a model of the interaction and regulation of gene expression by the interplay of glutathione and ascorbate has been proposed (Foyer and Noctor, 2005). It is considered that ascorbate modulates the intensity and outcome of oxidative signalling, affecting the glutathione content. Moreover, in *Arabidopsis thaliana* mutants (*vtc1* and *vtc2*) deficient in ascorbate a higher content of glutathione has been observed probably as a compensatory mechanism (Foyer and Noctor, 2005). It is possible that in *B. oleracea* roots under salt stress ascorbate accumulation and its redox state can modulate gene transcription or that through its role as an antioxidant ascorbate can impede processes regulated through ROS-mediated signalling (Foyer and Noctor, 2005).

In view of the results observed in this work, the enzymatic antioxidative system of broccoli roots was highly affected by salt undergoing short-term treatments (24 h) but was partially recovered with long-term salt treatments. However, the increased concentration of ASC and its redox state seem to be critical for salt tolerance in *B. oleracea* roots. Munns and Tester (2008) recently proposed that the antioxidant system is not responsible for the salt tolerance observed in many species. The reduction of Na⁺ content in the cell and prevention of K⁺ loss seem to be the most important mechanisms of plant salt tolerance. However, it is considered that the present results confirm the relevance of induction of the antioxidant system to protect the cell against the oxidative damage and the importance of maintaining the cellular redox state for root growth and development under salt stress.

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