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Changes in Growth Pattern, Enzymatic Activities Related to Ascorbate Metabolism, and Hydrogen Peroxide in Onion Roots Growing Under Experimentally Increased Ascorbate Content

M. del Carmen Córdoba-Pedregosa · José M. Villalba · Francisco Córdoba · José A. González-Reyes

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Abstract Onion (*Allium cepa* L.) roots treated with external ascorbate or with the immediate precursor of its synthesis, L-galactono-γ-lactone, increased root development measured as an increase in fresh and dry weights after 48-h treatments compared to controls. Also, treatments induced changes in extracellular (apoplastic) and cytosolic (symplastic) enzyme activities related to ascorbate metabolism and antioxidant protection, such as ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase, glutathione reductase, and catalase. Finally, we have found that both chemicals induced increased content of hydrogen peroxide in well-differentiated zones of the root, and local increases in meristematic and elongation zones were detected by cytochemistry as well. The results are discussed on the basis of changes in the root growth rate and other physiologic processes mediated by ascorbate in higher plants.

Keywords Ascorbate · Hydrogen peroxide · L-galactono-γ-lactone · Onion root growth · Plant development

M. del C. Córdoba-Pedregosa · J. M. Villalba · J. A. González-Reyes (✉)
Departamento de Biología Celular, Fisiología e Inmunología, Universidad de Córdoba, Campus de Rabanales, Edificio Severo Ochoa, Planta 3, 14014 Córdoba, Spain
e-mail: bc1gorej@uco.es

F. Córdoba
Departamento de Biología Ambiental y Salud Pública, Universidad de Huelva, Huelva, Spain

Introduction

Ascorbic acid or ascorbate is an essential molecule in plant metabolism. Besides its defensive role against biotic and abiotic oxidative damage and its involvement in processes such as cell proliferation and elongation or vegetative growth, which have been extensively documented (see Córdoba and González-Reyes 1994; De Gara 2004; Noctor and Foyer 1998; Potters and others 2002; Smirnoff 2000), ascorbate has been proposed to be directly or indirectly involved in other processes such as cell signaling, differentiation, or programmed cell death (De Pinto and De Gara 2004; Foyer and Noctor 2005; Pastori and others 2003).

In higher plants, ascorbic acid is synthesized in the mitochondria (Siendones and others 1999) and then sorted to different cell compartments, including the cell wall (Horemans and others 2000). Thus, the different redox forms of ascorbic acid (the reduced form ASC, and the fully oxidized form, dehydroascorbate or DHA) can be found in symplastic and apoplastic compartments of each organ. However, the symplastic concentration exceeds that found in the apoplast (Vanacker and others 1998; Córdoba-Pedregosa and others 2003a). In each compartment, ASC seems to play different roles according to the type of cell or tissue (Córdoba-Pedregosa and others 2003a; De Pinto and De Gara 2004).

In onion roots, apoplastic ascorbate (ASC+DHA) accounts for 2–8% of total ascorbate measured in homogenates (see Córdoba-Pedregosa and others 2003a, b). However, the distribution of both forms along the root axis is not uniform. For example, symplastic ASC and DHA concentrations are higher in the root apex and then gradually decrease toward the onion crown, whereas apoplastic concentrations follow an opposite pattern (Córdoba-Pedregosa and others 2003a). A similar result has been obtained after

measuring enzymatic activities related to ascorbic acid metabolism or to antioxidative defense such as ascorbate peroxidase (APX), DHA reductase (DHAR), monodehydroascorbate reductase (MDHAR), glutathione reductase (GR), and catalase (CAT) or different peroxidases against natural or artificial substrates (guaiacol, ferulic acid, and coniferyl alcohol). Also, by means of a cytochemical technique, hydrogen peroxide has been detected in roots and a specific distribution pattern has also been reported (Córdoba-Pedregosa and others 2003a, b). These findings suggest the existence of an accurate equilibrium of ascorbate redox forms, redox enzymes, and hydrogen peroxide along the root axis.

Experimentally changing ASC and DHA contents constitutes an important tool for studying the relationship of the ASC/DHA redox pair and their related enzymes in plant metabolism. Thus, the ASC-deficient *Arabidopsis* mutants *vtc1* and *vtc2*, which possess between 10% and 25% of wild-type ascorbic acid in leaves, show a markedly decreased growth and changes in the distribution and activities of enzymes related to the antioxidant system (Veljovic-Jovanovic and others 2001; Pavet and others 2005). Also, using these mutants, a close relationship between the ASC level and disease resistance has been reported (Pavet and others 2005; see also Foyer and Noctor 2005).

On the other hand, the experimental increase in ASC content also induced relevant changes in plant metabolism. Thus, onion roots growing in a culture medium containing ASC or L-galactono-γ-lactone (GalL, the immediate precursor of ASC synthesis in higher plants) showed a significant increase in ASC and DHA contents in both symplastic and apoplastic compartments as well as in root sprouting at the onion bulb base, and exhibited changes in the distribution pattern of peroxidase activity along the root axis (Córdoba-Pedregosa and others 2005). Some of these changes were also observed in detached tomato leaves under treatments with ASC and/or GalL (Kuzniak 2004). However, in onion roots the effect of increased ASC on the activity of related enzymes, its distribution along the root axis, and the possible relationship between high ASC level and hydrogen peroxide production at the different zones of the root have not been investigated so far.

In this article we study the effect of ASC and GalL pretreatments on root development and on the distribution pattern of ascorbic acid-related enzymes and hydrogen peroxide along the root axis. The results show that both molecules stimulate the production of root biomass and induce changes in the activity and/or distribution patterns of the analyzed enzymes and hydrogen peroxide. The results are discussed in relation to the regulatory role of ascorbate on plant development.

Materials and Methods

Growth Conditions and Treatments

Onion (*Allium cepa* L.) roots were grown hydroponically in containers of about 1.5-L capacity (6–8 bulbs per container) in the dark at 25°C and aerated by continuous bubbling at the rate of 30–40 ml air/min. Once roots had reached about 3 cm in length, some bulbs were transferred to a culture medium containing 1 mM ascorbate (ASC) or 2 mM L-galactono-γ-lactone (GalL; both from Sigma-Aldrich, St. Louis, MO, USA) for 48 h. Solutions were renewed at 24 h. Roots were then detached from the bulbs and cut into three zones of 2-cm length each starting from the root apex. The remaining root was discarded. The zone size was the smallest possible to still be able to handle without appreciable damage to the root and yielding apoplastic fluids with low amounts of cytosolic contamination (Córdoba-Pedregosa and other 2003a).

Biomass Calculation

In another set of experiments, onions were cultivated and treated as above. After 48-h treatments the whole set of roots sprouted from each bulb were blotted onto filter paper and weighed (fresh weight). Afterward, roots were placed in a desiccation chamber at 65°C until a constant dry weight was achieved (about 48 h were required). Data obtained with this procedure were expressed in grams of roots per grams of bulb. Fresh weight of each bulb was also determined before and after the treatments.

Isolation of Apoplastic Fluid and Soluble Symplastic Fractions

About 2 g of each type of root segment was quickly washed in distilled water, placed in Petri dishes in 10 mM sodium phosphate (pH 6) containing 1.5% (w/v) polyvinylpolypyrrolidone, 1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride, and submitted to vacuum (60 kPa) for 5 min at 4°C. Afterward, root segments were carefully blotted onto filter paper and placed in syringes, which were placed in centrifugation tubes. Roots were centrifuged at 150*g* for 5 min and the apoplastic fluid (AF) recovered at the bottom of the tubes. With this procedure we obtained 70-110 µl of AF for 1 g fresh weight of each zone. The remaining roots were used to obtain the soluble symplastic fraction (SSF) after homogenization in the same medium with an Ultraturrax T-25 (IKA Labortecnik, Staufen, Germany) and centrifugation at 15,000*g* for 30 min. Cytosolic contamination of AF was monitored by

assaying glucose-6-phosphate dehydrogenase activity as a marker.

Enzymatic Activities

Enzymatic activities were spectrophotometrically assayed for AF and SSF obtained from each root zone. Reactions were developed at 25°C for 5 min, with stirring, in a final volume of 1 ml containing 25-35 μg of protein.

The glucose-6-phosphate dehydrogenase (G6PDH) assay was carried out in 100 mM Tris-HCl (pH 8) containing 1 mM $MgCl_2$, 0.2 mM $NADP^+$, and 1 mM glucose-6-phosphate. Generation of NADPH was measured at 340 nm, and the extinction coefficient was 6.2 mM^{-1} cm^{-1} (Weimar and Rothe 1986).

For ascorbate peroxidase (APX) determination, root pieces were vacuum-infiltrated in phosphate buffer containing 5 mM ASC. For SSF, homogenization buffer also contained 5 mM ASC. The activity was measured by the method of Nakano and Asada (1981). The reaction mixture contained 50 mM phosphate buffer (pH 7), 1 mM sodium ASC, and 2.5 mM H_2O_2. After the addition of ASC to the mixture, the reaction was followed at 290 nm (extinction coefficient of ASC = 2.8 mM^{-1} cm^{-1}).

Monodehydroascorbate reductase (MDHAR) was assayed following the method of Hossain and others (1984). The reaction mixture contained 50 mM Tris-HCl (pH 7.5), 0.2 mM NADH, 2.5 mM ASC, and 0.15 unit of ASC oxidase. The reaction was initiated by adding ASC oxidase to the mixture, thus generating the substrate MDHA. Activity was measured as the ASC oxidase-induced oxidation of NADH. The reaction was monitored at 340 nm (extinction coefficient for NADH = 6.2 mM^{-1} cm^{-1}).

Dehydroascorbate reductase (DHAR) was assayed according to Hossain and Asada (1984) by measuring the reduction of DHA to ASC in a reaction mixture containing 50 mM potassium phosphate (pH 7), 0.5 mM DHA, and 2.5 mM GSH. The reaction was followed at 265 nm (extinction coefficient of ASC = 14 mM^{-1} cm^{-1}).

Catalase (CAT) activity was estimated using the method of Aebi (1983). The reaction mixture contained 50 mM potassium phosphate (pH 7) and 10 mM H_2O_2. After enzyme addition, the reaction was monitored by following decomposition of H_2O_2 at 240 nm (extinction coefficient of H_2O_2 = 43.6 mM^{-1} cm^{-1}).

Glutathione reductase (GR) was measured according to Foyer and Halliwell (1976). The reaction was developed in 50 mM Tris-HCl (pH 7.5) containing 2.5 mM $MgCl_2$, 0.5 mM GSSG, and 0.2 mM NADPH. Oxidation of NADPH was followed at 340 nm (extinction coefficient = 6.2 mM^{-1} cm^{-1}).

Hydrogen Peroxide Determination

Root hydrogen peroxide content was determined by spectrophotometry using the method of Jana and Choudhuri (1981) with some modifications. After the treatments, hydrogen peroxide was extracted by homogenizing 500 mg of tissue from each root zone in 5 ml phosphate buffer (50 mM, pH 6.5). The homogenate was filtered through four layers of sterile gauze and then through a 0.20-μm filter (Minisart; Sartorius AG, Göttingen, Germany). Then, 750 μl of the resulting fraction were mixed with 250 μl of 0.1% titanium sulfate in 20% H_2SO_4 (v/v) and the mixture was centrifuged at 6000*g* for 5 min. The entire process was carried out at 4°C. The intensity of the yellow color of the supernatant was measured at 410 nm. H_2O_2 levels were calculated using the extinction coefficient 0.28 μmol^{-1} cm^{-1}.

Electron Microscopy

Hydrogen peroxide was also detected by cytochemistry. Roots were detached from the bulbs and five to six pieces (0.5 mm long) were processed. The detection of H_2O_2 is based on the formation of cerium perhydroxide from exogenous cerium chloride and endogenous H_2O_2, as described by Bestwick and others (1997). In brief, pieces from different zones of the root were obtained and preincubated in 50 mM MOPS buffer (pH 7) containing 5 mM $CeCl_3$ for 1 h. Afterward, samples were quickly washed in the buffer and fixed in 2.5% (w/v) glutaraldehyde-2% (w/v) paraformaldehyde mixture in 0.1 M sodium cacodylate buffer (pH 6.8) for 4 h at 4°C. Afterward, samples were washed in buffer and postfixed in 1% (w/v) osmium tetroxide, dehydrated in an ethanol series, treated with propylene oxide, and embedded in Epon 812. After curing, transverse sections of about 60-nm thicknesses were obtained in an ultramicrotome, mounted on nickel grids, observed unstained, and photographed in an electron microscope (Philips EM 300, Eindhoven, The Netherlands). For control, some pieces were preincubated for 15 min in 10 mM sodium pyruvate (Sigma-Aldrich) because this molecule has been reported to be a strong hydrogen peroxide scavenger (Li and others 1998).

Protein Determination

Protein was determined by the dye-binding method of Bradford (1976) using γ-globulin as a standard.

Statistical Analysis

In all experiments, mean values were compared using Student's *t* test. Significance levels of 95% ($p < 0.05$) or

99% ($p < 0.01$) are indicated in figure legends or in the text.

Results

Effect of ASC and GalL on Root Biomass

Treatments with ASC and GalL increased root development measured as root fresh and dry weights. Forty-eight hours of treatment with 1 mM ASC increased biomass by 22% compared to untreated controls, whereas GaIL treatment enhanced biomass by 32%. Both effects were statistically significant ($p < 0.01$). For dry weight the increases were 18% and 24% after ASC and GalL, respectively, compared to untreated roots. Hence, both chemicals induced increases in root biomass, but this effect was higher after GalL treatment.

After weighing the roots, we also weighed the remaining plant (mainly bulb and in some cases short shoot sprouts) but no differences were found in ASC or GalL pretreated roots compared to control conditions (data not shown).

Effect of ASC and GalL on Enzyme Activities Related to ASC Metabolism and Antioxidant Protection at the Different Root Zones

Roots were treated with ASC and GalL as described in Materials and Methods and then separated into three 2-cm-long segments. AFs and SSFs were obtained from these zones. In both fractions we measured enzyme activities related to ascorbate metabolism and antioxidant function. In addition, G6PDH was measured as a marker for cytosolic contamination of the apoplasts.

Glucose-6-Phosphate Dehydrogenase (G6PDH)

In SSF from control roots, G6PDH activity was higher in zone I and decreased toward the bulb base, with values ranging from 400 ± 30 to 200 ± 15 nmol min^{-1} g^{-1} FW. Treatments with ASC or GalL did not change that trend but increased the activities about 50% on average. On the other hand, AF showed an opposite trend with sequentially increasing values from zone I to zone III. However, the activities were significantly less compared to SSF and ranged from 0.8% to 1.5% less than the corresponding SSF. These percentages were similar to those obtained by other authors in other materials (see, for example, Hernández and others 2001; Vanacker and others 1998, 1999; Veljovic-Jovanovic and others 2001) and were used to correct data on other activities for cytosolic contamination.

Ascorbate Peroxidase (APX)

The pattern of APX activity varied depending on the root zone and treatment. In control conditions APX activity from SSF significantly decreased from zone I to zone III. Treatments with ASC or GalL resulted in significantly enhanced activity in all the three zones but showed a similar decreasing spatial pattern as for controls (Figure 1A). Although ASC and GalL treatments also augmented apoplastic APX activity, the lowest values were found in zone I and then gradually increased to zone III (Figure 1B), showing an opposite pattern to that described in symplastic fractions.

Monodehydroascorbate Reductase (MDHAR) and Dehydroascorbate Reductase (DHAR) Activities

A similar pattern was found for MDHAR activity measured in SSF from all three experimental conditions, that is, significantly higher values in zone I and gradual decreases in zones II and III. However, the activities measured after ASC and GalL treatments in zones II and III were significantly higher than those obtained in controls (Figure 2A). An opposite trend was found in AF in which the activity

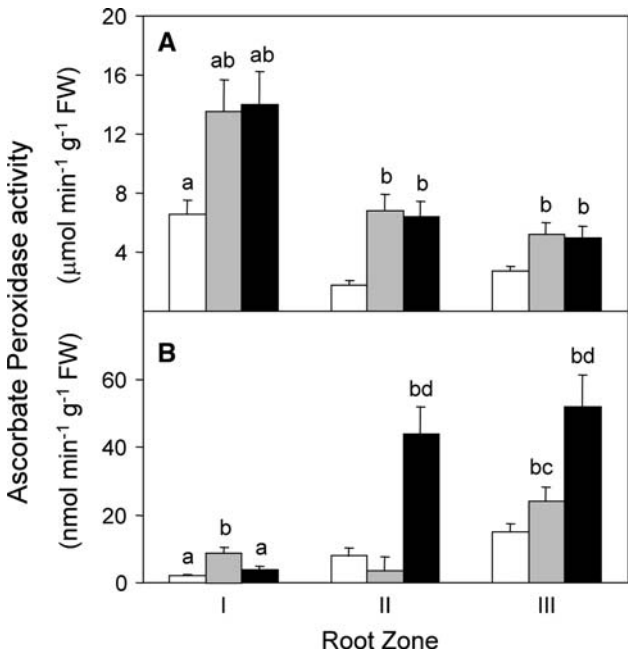

Fig. 1 Ascorbate peroxidase (APX) activity measured at the different fractions and root zones in control (white bars) and after 48-h treatments with ASC (gray bars) or GalL (solid bars). **A** Data for APX from SSF. **B** The results for AF. Values are mean ± SE of four different experiments. [a] $p < 0.01$ versus zones II and III in the same treatment. [b] $p < 0.01$ versus the same zone of control. [c] $p < 0.01$ versus zones I and II in the same treatment. [d] $p < 0.01$ versus the same zone of ASC treated roots

gradually increased from zone I to zone III, being comparatively and significantly higher in those roots treated with ascorbate in comparison to control and GalL-treated roots (Figure 2B).

DHAR activity showed a similar pattern as described for MDHAR. In SSF the activity decreased gradually from zone I to zone III in all three experimental conditions, although the differences between zones were not so pronounced as in MDHAR (Figure 2C). Significant increases from zone I to zone III were found in AF for control and ASC treatments, whereas GalL showed an increase from zone I to zone II and a drop in zone III to reach similar values to those found in zone I (Figure 2D).

Glutathione Reductase (GR) and Catalase (CAT) Activities

In SSF from all three experimental conditions, GR activity was higher in zone I and decreased toward the root base, this phenomenon being more pronounced in control and GalL-treated roots. However, values obtained for ASC and GalL in zones II and III remained higher than those calculated for controls (Figure 3A). On the other hand, in AF the activity followed an opposite pattern as described above. Thus, for control and ASC-treated roots, lower values were measured in zone I and increased toward the root base (zones II and III). However, ASC induced a significant activity increase in all three zones (Figure 3B). Finally, GalL treatments induced increase in zone I compared to controls, but the activity remained constant along the root axis (Figure 3B).

CAT activity detected in SSF did not show striking changes along the root axis after the treatments. Thus, in control conditions the activity was nearly constant along the root and remained roughly constant after the treatments with ASC or GalL (Figure 3C). However, the activity measured in AF changed at the different zones of the root depending on the treatment. Thus, for controls the highest activities were found at zone I and markedly decreased toward the root base. ASC treatment enhanced the activity in all three zones, which was more pronounced in zone II (Figure 3D). On the contrary, GalL incubation resulted in a significantly decreased activity in zones I and III and an increase in zone II compared to controls (Figure 3D).

Determination of Hydrogen Peroxide in Root Homogenates

Previous determinations failed to detect any hydrogen peroxide in AF or SSF fractions. This was probably due to the time that elapsed while handling the root fragments for vacuum infiltration, loading into the syringes, centrifugation, and so on. Thus, we measured this molecule in filtered total homogenates from the different zones of the root and after the treatments. These homogenates were quickly obtained, avoiding prolonged handling of the plant material. The results are displayed in Figure 4. In control conditions hydrogen peroxide content increased gradually from zone I to zone III, in which differences were significantly higher (Figure 4). Treatments with ASC or GalL resulted in a similar pattern, but peroxide content was significantly higher at every root zone compared to untreated roots. No significant changes were found when comparing ASC and GalL treatments among them (Figure 4).

Cytochemical Localization of Hydrogen Peroxide After ASC and GalL Treatments

Hydrogen peroxide was also detected at the electron microscope by cytochemistry as described in Materials and

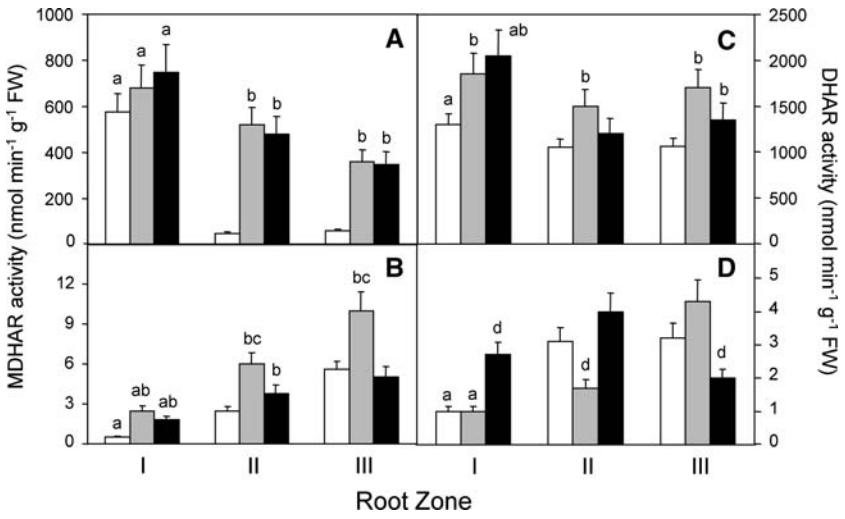

Fig. 2 Monodehydroascorbate reductase (MDHAR, left axis) and dehydroascorbate reductase (DHAR, right axis) activities in SSF (**A, C**) and AF (**B, D**) from the different root zones in control (white bars), ASC (gray bars), and GalL (solid bars) treated roots. Values are mean ± SE of four different experiments. [a] $p < 0.01$ versus zones II and III in the same treatment. [b] $p < 0.01$ versus the same zone of control. [c] $p < 0.01$ versus the same zone of GalL treated roots. [d] $p < 0.01$ versus the other treatments at the same zone

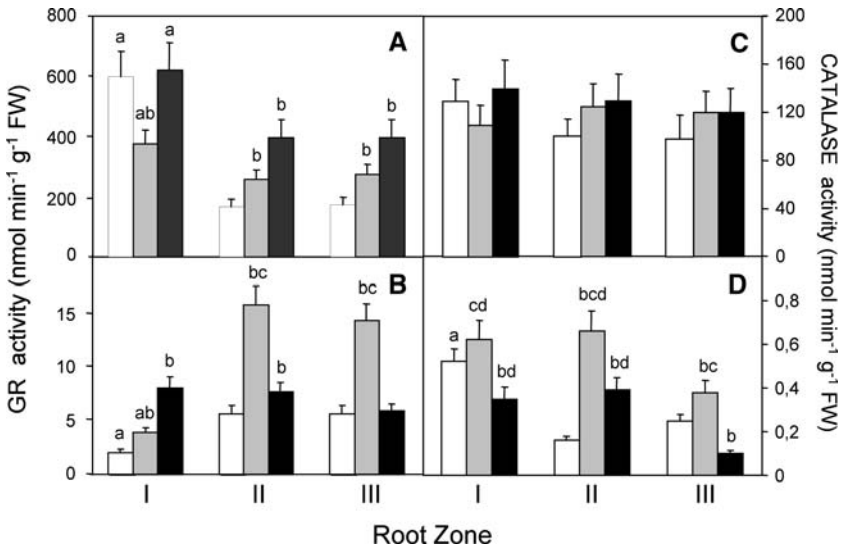

Fig. 3 Glutathione reductase (GR, left axis) and catalase (right axis) activities measured in SSF (**A, C**) and AF (**B, D**) from the different zones of control (white bars), ASC-treated (gray bars), and GalL-treated (solid bars) roots. Values are mean ± SE of four different experiments. [a] $p < 0.01$ versus zones II and III in the same treatment. [b] $p < 0.01$ versus the same zone compared to controls. [c] $p < 0.01$ versus the same zone of GalL-treated roots. [d] $p < 0.01$ versus zone III in the same experimental condition

Methods. In our specimens H_2O_2 was revealed as electron-dense spots located mainly at the cell wall and/or attached to the plasma membrane. However, the spot localization pattern changed depending on the root zone, the treatment, and the relative position of the cell in the root (epidermal, cortical, or central localization). In control conditions the meristematic and elongation zones showed reaction mainly at the radial walls of epidermal (or rhizodermal) cells (Figure 5A) as well as in walls and intercellular spaces from cortical and central cells (Figure 5B, C). Although this pattern was constant along zone I, the staining density and the number of cells showing spots decreased toward the root base.

In zone I from ASC- or GalL-treated roots, the localization pattern was similar, but some relevant differences were found when compared to the controls. Thus, at the meristematic and elongation zones the number of epidermal cells showing staining and their intensity were markedly higher than in control conditions (Figure 5D, E). In the middle area of zone I the number of spots in cortical and central cell walls decreased, but the staining density was higher compared to control roots (Figure 5F, G).

In zones II and III from control and pretreated roots, very few cells showed walls with a significant number of spots. Thus, the reaction was practically absent in epidermal and cortical cell walls from all three experimental conditions. However, after ASC or GalL treatments, a relatively high number of intercellular spaces showed cerium chloride deposits (Figure 5H). Finally, in the central area of these zones, most of the cells showed no perceptible reaction (Figure 5K). However, some central cells showed cerium chloride spots attached to the plasma membrane or located at the intercellular spaces (Figure 5I, J). A quantitative analysis revealed that in zones II and III, although very low in all three cases, the number of cells showing cerium chloride deposits was higher in ASC- or GalL-treated cells (results not shown).

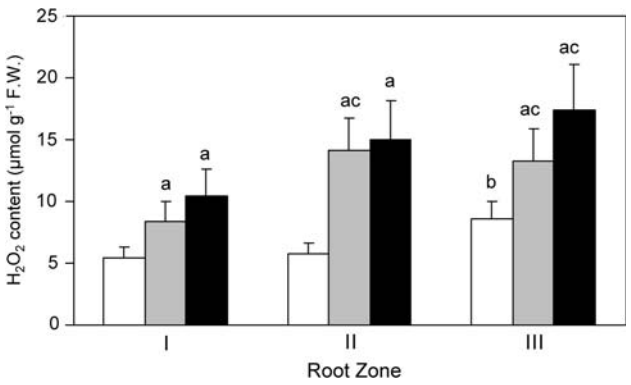

Fig. 4 Hydrogen peroxide content along the root axis measured in total homogenates from control (white bars), ASC-treated (gray bars), and GalL-treated (solid bars) roots. Values are mean ± SE of five different experiments. [a] $p < 0.01$ versus the same zone of control. [b] $p < 0.05$ versus zones I and II in the same treatment. [c] $p < 0.05$ versus zone I in the same experimental condition

Discussion

In this article we show a correlation between ASC and GalL treatments, the increase in root biomass production, changes in the production of hydrogen peroxide, and changes in ASC-related enzymatic activities along the root axis in onion roots growing hydroponically. Although we discuss these points separately, all these aspects are closely related because ASC plays a pivotal role in plant development and constitutes the major low-molecular-weight antioxidant present in symplastic and apoplastic compartments of higher plants.

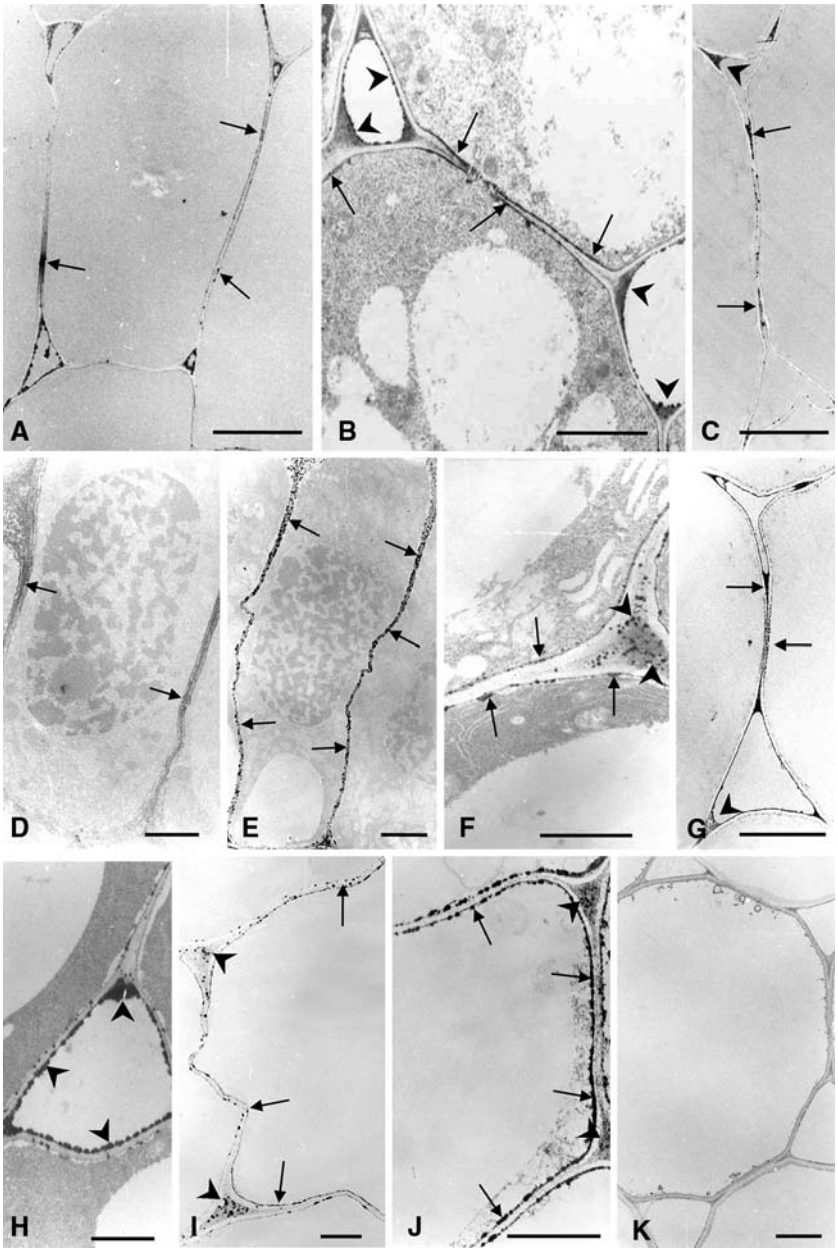

Fig. 5 Ultrastructural localization of hydrogen peroxide in transverse sections along the root axis of *Allium cepa.* L. Arrows indicate electron-dense spots in plasma membrane and/or cell walls; arrowheads denote the presence of deposits in intercellular spaces. **A**, **B, C** Epidermal, cortical, and central cells, respectively, from the meristematic area of control roots. **D, E** Epidermal cells from the meristematic area of ASC-pretreated (**D**) and GalL-pretreated (**E**) roots. **F, G** Portions of cortical and central cells located at the middle region of zone I from GalL-treated (**F**) and ASC-treated (**G**) roots. **H** An intercellular space from an ASC-treated root obtained from the cortical area of zone II showing profuse electron-dense spots. **I, J** Portions of central cells obtained from zones II (**I**) and III (**J**) from GalL-treated roots and control, respectively, with abundant cerium spots in cell walls and intercellular spaces. **K** A typical central cell from zone III without remarkable cerium spots. In **A**, **D**, **E**, **I**, **J,** and **K**, scale bar = 2 μm; in **B**, **F**, and **H**, scale bar = 0.25 μm; and in **C** and **G**, scale bar = 1 μm

The Effect of Ascorbate and GalL on Root Biomass Production

Recently, evidence has accumulated for a role for ASC in plant growth control. For example, the reduced growth displayed by the ASC-deficient *Arabidopsis* mutants *vtc1* and *vtc2* has made it possible to establish an unequivocal correlation between ASC content and plant growth and has also revealed the close relationship between ASC levels and oxidative stress resistance in plants (Veljovic-Jovanovic and others 2001; Barth and others 2004; Pavet and others 2005).

In onion ASC and GalL increase root sprouting and elongation (González-Reyes and others 1994; Córdoba-Pedregosa and others 1996, 2005), but little is known about the effects of these two chemicals in biosynthetic processes leading to the stimulation of root development. In this article we show that a 48-h treatment with both chemicals results in an increased root biomass. In higher plants a significant part of growth is due to water uptake and accumulation. However, we show here that in addition to the expected increase in fresh weight, ASC and GalL also stimulated root dry weight, suggesting stimulated biosynthesis metabolism during the treatments.

The Effect of Ascorbate and GalL on Antioxidant Enzyme Activities

In previous papers we have shown that ASC content is not uniform along the root axis, being more concentrated at the apical zone and gradually decreasing toward the bulb base (Córdoba-Pedregosa and others 2003a, b). More recently we reported that ASC and GalL treatments increase ASC contents in the roots, while maintaining the concentration gradient described above (Córdoba-Pedregosa and others 2005). In this article we show that most of the studied enzyme activities change along the root axis following distribution patterns related to the ASC content at every root zone.

In SSF treatments with ASC and GalL induced an additional increase in APX, MDHAR, DHAR, and GR activities, but the differential zonal activities remained nearly identical as in controls, that is, decreasing toward the root base. These results were partially in accordance with those reported by De Pinto and De Gara (2004) in pea shoot cytosolic fractions. These authors found higher amounts of ASC and enhanced APX and MDHAR activities in nondifferentiated tissues such as meristems and a gradual decline toward elongation and differentiation zones, as described here for onion roots. However, in pea shoots DHAR activity increased in the same direction (De Pinto and De Gara 2004).

The presence of most of the enzymes studied in this article (APX, DHAR, MDHAR, and GR) in the symplastic compartment has been correlated with ASC regeneration in the so-called "ascorbate-glutathione cycle," constituting one of the major antioxidant mechanisms in plants cells (Noctor and Foyer 1998). Thus, experimental situations leading to oxidative stress (such as pathogen infection or saline treatments) induce changes in symplastic ASC content as well as in the activity of these enzymes (Vanacker and other 1998, 1999; Hernández and others 2001; Veljovic-Jovanovic and others 2001). However, in most of these cases the response of the plant to oxidative injury was not uniform because different effects were found in different cultivars from the same species submitted to the same experimental conditions (Vanacker and other 1998, 1999; Hernández and others 2001). Nevertheless, these results reveal a strong correlation between antioxidative enzyme activities and ASC concentration in the different plant tissues.

Concerning the apoplastic compartment, it should be noted that the presence of only a few enzymes has been demonstrated. Thus, in relation to the ascorbate-glutathione cycle enzymes and CAT, most of them have been shown to be present in barley and oat leaves (Vanacker and other 1998, 1999), whereas in other cases such as pea leaves, only DHAR has been detected (Hernández and others 2001). In shoots from the same plants, only APX was reported to be present (De Pinto and De Gara 2004).

As mentioned for the symplastic compartment, the apoplastic enzyme activity pattern changed after oxidative injury (Vanacker and other 1998, 1999; Hernández and other 2001) or at the different zones of roots and shoots depending on the tissue differentiation degree (Córdoba-Pedregosa and other 2003a, 2005; De Pinto and De Gara 2004). Our results on apoplasts from roots after ASC and GalL treatments show that the activities increase at every zone and toward the root base. Thus, for most of the cases, these activities were higher in more differentiated tissues (as occurs for APX, MDHAR, DHAR, or GR) in an opposite way, as mentioned for the symplastic fraction. This effect was also found for APX activity in the pea shoot apoplastic fraction (De Pinto and De Gara 2004).

We have to take into account that some of the activities found in onion root apoplast are within the range of the AF purity tested using G6PDH as marker. Among them CAT, DHAR, and in some zones APX seem to be the more scarcely represented, and although we made the appropriate correction to the data, the possibility exists that these activities could be due to symplastic contamination. On the other hand, the presence of NADH or NADPH (which are substrates for MDHAR and GR) has never been demonstrated in the cell walls, making the existence of both activities in the apoplast intriguing.

Hydrogen Peroxide in Roots

Onion roots contain similar amounts of hydrogen peroxide as roots of other species such as rice (Lin and Kao 2001) and barley (Kim and others 2005). However, we have found that the concentration of this metabolite increases toward the bulb base. On the other hand, treatments with ASC and GalL increased peroxide content in roots, but its distribution pattern along the root axis remained as in controls. To our knowledge this is the first time that zonal changes in hydrogen peroxide concentration and its stimulated production after ASC and GalL treatments have been described in roots.

Traditionally, the production of hydrogen peroxide and other reactive oxygen species (ROS) has been considered to be part of a response (oxidative burst) to harmful situations (Foyer and Noctor 2005). However, evidence has accumulated recently that points out specific roles for ROS in plant growth regulation and in cell signaling in general (Foreman and others 2003; Foyer and Noctor 2005). Therefore, the distribution of hydrogen peroxide along the root axis probably corresponds to the roles played by this metabolite in physiologic phenomena taking place at the different zones of the root.

Besides the determination of hydrogen peroxide along the root axis by spectrophotometry, we have studied its subcellular localization using electron microscopy. In this regard, we confirm the increased accumulation of this metabolite in cell walls and intercellular spaces at the meristematic and elongation zones of the root, and report additional increases in these zones after ASC and GalL treatments. Because both chemicals induce an increase in the cell elongation rate and in ASC and DHA concentrations at the root apex (Córdoba-Pedregosa and others 1996, 2005), the possibility exists that enhanced ASC/DHA and hydrogen peroxide concentration and stimulated cell elongation are correlated.

Fry (1998) and Miller and Fry (2001) proposed that apoplastic ascorbate and hydrogen peroxide can react to form hydroxyl radicals which can be responsible for polysaccharide cleavage resulting in cell wall relaxation. Furthermore, Liszkay and others (2004) reported that experimentally increased ROS induced wall loosening, whereas its scavenging resulted in cessation of maize root growth. Thus, a direct relationship seems to exist between enhanced ascorbate, hydrogen peroxide at the cell wall, and cell elongation in roots. Interestingly, a recent paper (Kärkönen and Fry 2006) demonstrates that DHA, the predominant form in the apoplast (see, for example, Vanacker and other 1998, 1999; Veljovic-Jovanovic and others 2001; De Pinto and De Gara 2004), and its product of degradation may also be involved in the modulation of hydrogen peroxide concentration at the apoplast.

We have detected peroxide also in some cells from zones II and III and then not submitted to elongation. However, the number of cells showing cerium spots was higher after ASC or GalL pretreatments, which is compatible with the increased content of hydrogen peroxide detected in these zones. Here, the presence of peroxide could be related to some other processes such as cell differentiation or lignification in which hydrogen peroxide can also play a relevant role (Ros-Barceló 2005). However, this hypothesis should be investigated more deeply.

Conclusions

The data presented here demonstrate a close relationship between ASC and GalL treatments, increased root biomass and hydrogen peroxide content, and changes in ASC-related enzymes in symplastic and apoplastic compartments. Because each compartment and root zone is characterized by a distinct degree of metabolic activity (cell proliferation and elongation in root apex, or lignification and differentiation in other zones toward the onion crown, for example), an accurate regulation of the presence and activities of metabolites involved in these functions is expected to occur along the root axis. Our results showing differential distribution of enzyme activities and hydrogen peroxide along the root axis are compatible with this hypothesis and point out the pivotal role of ASC in processes related to plant growth.

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