Oligogalacturonides stimulate antioxidant system in alfalfa roots

D. CAMEJO[1], M.C. MARTÍ[1], E. OLMOS[1], W. TORRES[2], F. SEVILLA[1] and A. JIMÉNEZ[1]*

Department of Stress Biology and Plant Pathology, CEBAS-CSIC,
P.O. Box 164, E-30100 Murcia, Spain[1]
Department of Plant Physiology and Biochemistry, INCA,
P.O. Box 1, 23700 S. José de las Lajas, La Habana, Cuba[2]

Abstract

Alfalfa (*Medicago sativa* L.) roots were treated with 50 and 100 μg cm^{-3} of oligogalacturonide (OGA) solutions with a degree of polymerization between 7 and 15. Changes in the activities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) as well as ascorbate (ASC) content were determined in crude extract of alfalfa roots after 30, 60 and 120 min of treatment. An increase in the SOD activity was observed in roots treated with 50 and 100 μg cm^{-3} OGA, which could be related to its $O_2^{\bullet-}$ scavenging function. As concern H_2O_2 scavenging, CAT activity was increased in the first 30 min by both OGA concentrations, while POX was a key enzyme at higher OGA concentration and treatment duration. ASC content firstly increased upon exposure to high OGA concentration, and then decreased after longer treatment while low OGA concentration had no effect on ASC content.

Additional key words: ascorbate, ascorbate peroxidase, catalase, dehydroascorbate reductase, monodehydroascorbate reductase, reactive oxygen species, peroxidase, superoxide dismutase.

Introduction

Oligogalacturonic acids (OGAs) are released upon fragmentation of homogalacturonan from the plant primary cell wall. The OGA released are used as a carbon source but are also detected by plants as signals to initiate defence responses (Côté *et al*. 1994). Different biological responses to OGA have been reported, and the particular response observed depends on the plant species, the bioassay, and the chemical structure of the OGA used. The first response observed after the addition of OGA is the production of reactive oxygen species (ROS), including superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (Low and Merida 1996), which are known to be involved in the plant defence, as well as in plant growth and developmental processes (Camejo *et al*. 2007, Dunand *et al*. 2007, Díaz-Vivancos *et al*. 2010). This response, termed the oxidative burst, is an immediate and localized reaction and occurs within a few min of the addition of OGA to suspension-cultured soybean (Legendre *et al*. 1993), tobacco (Binet *et al*. 1998) and tomato (Stennis *et al*. 1998) or alfalfa seedling (Camejo *et al*. 2011). Several enzymes are involved in apoplastic ROS production following successful pathogen recognition. Recent reports have demonstrated that members of the *Rboh* family mediate the production of apoplastic ROS during the defence response (Torres and Dangl 2005) and also in response to abiotic stresses (Hernández *et al*. 2001). Generally the NADPH oxidases are considered the highest enzymatic source of ROS in the oxidative burst of plant cell in response to pathogen or elicitors (Torres *et al*. 2006). Other enzymes appear to be important in the oxidative burst induced by elicitors, including apoplastic peroxidases (Bolwell *et al*. 1995, Ros Barceló 2000), apoplastic oxidases (Dumas *et al*. 1993), and amine oxidase (Allan and Fluhr 1997), which generate either $O_2^{\bullet-}$ and H_2O_2. We recently showed that OGA in a concentration range (25 - 100 μg cm^{-3}) induce the $O_2^{\bullet-}$

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Abbreviations: ASC - ascorbate; APX - ascorbate peroxidase; CAT - catalase; DHAR - dehydroascorbate reductase; MDHAR - monodehydroascorbate reductase; OGA - oligogalacturonide; ROS - reactive oxygen species; POX - peroxidase; SOD - superoxide dismutase.
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* Corresponding author; fax: (+34) 968 396213, e-mail: ajimenez@cebas.csic.es

accumulation in alfalfa root and proposed that this oxidative burst could be a signal to drive the promoting effect of OGA on root growth (Camejo *et al*. 2011).

The plant cell is equipped with various ROS-scavenging systems, including superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR), which regulate the overproduction of ROS and oxidative damage in different compartments of the plant cell (Jiménez *et al*. 1997, Mittler *et al*. 2004). Differential regulation of these enzymes could restrict the ROS-dependent damage and control signal transduction, and may contribute to the activation of defence mechanisms following infection (Mittler *et al*. 1999, Klessig *et al*. 2000). As an example, the reduction of APX and catalase activities resulted in a tobacco hypersensitive response to pathogens (Mittler *et al*. 1999).

It is well known that OGAs initiate signalling cascades that induce defence genes and proteins in plants: the exogenous application of OGA elicit the expression of proteinase inhibitors in tomato plants (Bishop *et al*. 1984), and also the activity of the chalcone synthase in bean cotyledons (Rose *et al*. 1999), the casbene synthetase in castor bean seedlings (Walker-Simmons *et al*. 1984) and the phenylalanine ammonia lyase (PAL) in suspension culture of carrot (Messiaen and Van Cutsem 1994). As regards changes in the antioxidant enzymes related with ROS scavenging, OGA have been described as inducing a series of cell wall peroxidase isoenzymes in suspension-cultured castor bean cell (Bruce and West 1989). Other woks demonstrated the interaction between calcium-pectate-binding anionic isoperoxidase (APRX) and partially or totally unesterified polygalacturonates (Penel and Greppin 1994) and it has been suggested that pectins can influence the distribution of proteins such as apoplast APRX through different factors such as their conformation depending on the Ca^{2+} concentration, their degree of polymerization and esterification (Penel *et al*. 1999). However, the changes induced by exogenous OGAs application on the antioxidant system related to the superoxide metabolism in intact plants are aspects that nowadays are not well established. In this work we examined the changes in the activities of SOD, CAT, POX, APX, MDHAR and DHAR as well as ascorbate (ASC) content in crude extract of alfalfa roots after incubation with OGA for 30, 60 and 120 min.

Materials and methods

OGAs were prepared by degradation of citric pectin with an endo-polygalacturonase using a slight modification of the method described by Spiro *et al*. (1993), as in Camejo *et al*. (2011). OGAs with a degree of polymerization of 7 to 15 (OGA pool) were selected.

Alfalfa (*Medicago sativa* L.) seeds were sterilized with 10 % (v/v) bleach for 10 min and placed on Petri dishes with filter paper in the dark at 25 °C. After 48 h, the seedlings with straight roots and cotyledon leaves were used for the bioassays, and for the OGA treatments they were submerged in 5 cm^3 of OGA solutions at 50 and 100 μg cm^{-3} (immediately prepared and dissolved in distilled water) in a *Pyrex* vessel for 120 min. Incubations were carried out at room temperature with slow shaking. A group of untreated seedlings was incubated in distilled water in the same conditions and was identified as a control. The biochemical determinations were realized after 30, 60 and 120 min of incubation with OGA or water.

For the root enzyme extraction, all operations were performed at 4 °C. The root tissue was homogenised in a mortar using 50 mM potassium phosphate buffer (pH 7.8), containing 0.1 mM EDTA, 5 mM cysteine, 0.2 % (v/v) *Triton X-100*, 1 % (m/v) soluble polyvinylpyrrolidone (PVP) and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was filtrated through two layer of nylon. After centrifugation for 10 min at 15 000 *g*, the supernatant was recovered and filtrated through a *Sephadex G50* (*PD10*) column (*Pharmacia Biotech AB*, Piscataway, NJ), equilibrated with the same buffer used for homogenisation. The supernatant collected constituted the protein extract that was immediately used for the enzyme activity measurement. Total soluble proteins were measured according to Bradford (1976), using bovine serum albumin as standard.

SOD (EC 1.15.1.1) activity was assayed according to Jimenez *et al*. (1997), following the ferricytochrome *c* reduction using xanthine/xanthine oxidase as the source of superoxide radicals (McCord and Fridovich 1969). One SOD unit (U) was defined as the quantity of enzyme that produces a 50 % inhibition of the reduction of cytochrome *c*.

APX (EC 1.11.1.11) activity was assayed according to Jiménez *et al*. (1997). For APX activity, the oxidation rate of ASC was estimated between 1 and 60 s after starting the reaction by the addition of H_2O_2. Coefficient of absorbance (ε) was 2.8 mM^{-1} cm^{-1}.

MDHAR (EC 1.1.5.4) activity was assayed by the decrease in absorbance (A_{340}) due to the NADH oxidation (ε = 6.22 mM^{-1} cm^{-1}; Jiménez *et al*. 1997). Monodehydroascorbate (MDH) was generated by the ASC/ascorbate oxidase system. To determine the MDHAR activity, the rate of MDH-independent NADH oxidation (without ASC and ascorbate oxidase) was subtracted from the initial MDH-dependent NADH oxidation rate (with ASC and ascorbate oxidase).

DHAR (EC 1.8.5.1) activity was determined according to the method of Dalton *et al*. (1993) by following the increase in A_{265} due to ASC formation using N_2-bubbled buffer (ε = 14 $mM^{-1}cm^{-1}$). The reaction rate was corrected for the non-enzymatic reduction of dehydroascorbate (DHA) by reduced gluthatione (GSH). A factor of 0.98,

to account for the small contribution to the absorbance by oxidized glutathione (GSSG), was also considered.

POX (EC 1.11.1.7) activity was determined in an assay medium containing 50 mM Tris-acetate buffer, (pH 5.0) and 10 mM H_2O_2, using 100 mM 4-methoxy-α-naphtol as electron donor ($\varepsilon = 21.6\ mM^{-1}\ cm^{-1}$; Ferrer and Ros Barceló 1999). The reaction was followed for 1 to 5 min. A control reaction was carried out in absence of H_2O_2.

CAT (EC 1.11.1.6) activity was determined according to Aebi (1984) by measuring the decrease in A_{240} due to the disappearance of H_2O_2 ($\varepsilon = 39.58\ M^{-1}\ cm^{-1}$).

The determination of ASC was performed from 0.1 g of root tissue which were extracted with 1 cm^3 of 5 % *m*-phosphoric acid (m/v) and incubated on ice in the dark for 30 min. The homogenate was centrifuged at 15 000 *g* for 10 min and the supernatant obtained was filtrated through 0.22 µm *Millex* filters (*Millipore*, Bedford, USA). ASC and DHA in the supernatant were determined immediately by HPLC and the DHA was quantified from ASC data by incubating the samples for 24 h at room temperature with 1 mM dithiothreitol (DTT). The DHA concentration was measured as ASC after rechromatography (Jiménez *et al*. 1997).

The H_2O_2 accumulation in OGA-treated and control roots was tested in 0.1 g of fresh tissue which was immediately submerged in 1 cm^3 acetone acidified with 25 mM H_2SO_4 and frozen in liquid nitrogen. Samples were thawed at room temperature for 45 min. Thereafter, 0.05 cm^3 of extract was mixed with 0.5 cm^3 of eFOX medium according Cheeseman (2006) containing 1 % ethanol to increase the sensibility of the assay. The method is based on the peroxide-mediated oxidation of Fe^{2+}, followed by the reaction of Fe^{3+} with xylenol orange (*o*-cresolsulfonephthalein-3′,3′′-bis[methylimino] diacetic acid tetrasodium salt, *Sigma-Aldrich*, Canada), which was detected after 45 min. H_2O_2 was determined based on the difference in absorption at 550 and 850 nm using a standard curve that covered the range of 0 - 200 µM. The specificity for H_2O_2 was tested by adding bovine catalase (*Sigma*) at the same time as OGA at a final concentration of 600 U cm^{-3}.

Each experiment was repeated at least three times with three replicates per treatment and a mean of three seedlings were analyzed in each replication. The significance of any differences between mean values was determined by the one-way analysis of variance; Duncan's multiple range tests was used to compare the means when necessary.

Results and discussion

OGA are pectic fragments of the plant cell wall that are perceived as signalling molecules, and their biological effects in plants are diverse. They can alter plant growth and development, including the inhibition of auxin-induced cell elongation and ethylene accumulation in pea stem (Branca *et al*. 1988), regulate organogenesis in tobacco explants (Bellincampi *et al*. 1993) and stimulate flower formation (Marfà *et al*. 1991). The responses induced by OGA are also related to an increase in protein phosphorylation in tomato and tobacco membranes (Farmer *et al*. 1991), transmembrane ion flux in tobacco suspension cells (Mathieu *et al*. 1991) and hydrogen peroxide accumulation in suspension-cultured soybean cells (Spiro *et al*. 2002). In a recent work, an $O_2^{\bullet-}$ burst in alfalfa roots at 30 min after the addition of 50 and 100 µg cm^{-3} OGA has been demonstrated (Camejo *et al*. 2011), and from these initial results, we were interested in the possible involvement of some components of the antioxidant system related to the oxidative metabolism in alfalfa roots treated with 50 and 100 µg cm^{-3} OGA.

SOD activity is known to be induced by $O_2^{\bullet-}$ under stress situations (Hassan and Fridovich 1977, Mittova *et al*. 2003, Gómez *et al*. 2004, Maia *et al*. 2010) and in the apoplast, appears to function also in the biosynthesis of lignin by causing rapid disproportionation of the $O_2^{\bullet-}$ prior to its interaction with cellular components (Vuletić *et al*. 2003). In this sense, apoplastic Cu,Zn-SOD has been shown to provide the H_2O_2 required for lignification in spinach leaves and hypocotyls tissues (Ogawa *et al*. 1996). In the present work, the analysis of SOD activity revealed an increase in alfalfa roots treated with both 50 and 100 µg cm^{-3} OGA concentrations at 30 and 60 min (Fig. 1*A*), while it did not change at 120 min. As we commented before, an induction of $O_2^{\bullet-}$ burst in alfalfa roots was dependent on OGA concentration (Camejo *et al*. 2011). We proposed then that the $O_2^{\bullet-}$ accumulation in alfalfa roots treated with 50 µg cm^{-3} OGA could be related to the observed promoting effect on the root growth (Camejo *et al*. 2011). The differential activation of SOD activity found in the present work in roots treated with 50 and 100 µg cm^{-3} OGA could be related to the induced $O_2^{\bullet-}$ accumulation and the biological function of $O_2^{\bullet-}$ and SOD in the root. Thus, in roots treated with 50 µg cm^{-3} OGA the slower SOD activation and a higher $O_2^{\bullet-}$ accumulation could drive processes related with development and growth while the higher activation of the SOD activity at 100 µg cm^{-3} OGA, could be activating cellular events related to a defence response. It has been demonstrated that the concentrations at which OGA regulate developmental processes are usually lower than those required to induce defence responses, suggesting the existence of different classes of receptors involved in these processes (Messiaen and Van Cutsem 1993). The activation of SOD activity in the presence of OGA would avoid a toxic $O_2^{\bullet-}$ accumulation in the root, preventing a high oxidative damage.

In the plant cell, there are different enzymatic systems scavenging H_2O_2 generated by SOD action or other cell processes and among them, CAT and POX play an important role preventing destructive oxidation of

important metabolites. CAT and POX are antioxidant enzymes that have often been used as indicators of metabolic activities related to growth, senescence and stress response (Pastori *et al*. 1994, Mittova *et al*. 2003, Vanacker *et al*. 2006). An increase in the CAT activity was observed in roots treated with both OGA concentrations at 30 min and this increase was observed when the SOD activity was increased. However, the protective effect of CAT enzyme on the root was only maintained in 50 µg cm^{-3} OGA-treated roots at 60 min while it was similar to that in control and 100 µg cm^{-3} OGA-treated roots (Fig. 1*B*). No-significant changes in CAT activity were observed at 120 min in any of the treatments. Peroxidases are a group of plant proteins with a large number of isoenzymes, some of them located in the apoplast (Córdoba-Pedregosa *et al*. 2003, Hernández *et al*. 2001). The analysis of this activity in OGA-treated alfalfa roots revealed a significant increase only in roots treated with 100 µg cm^{-3} OGA at 30 min, while 50 µg cm^{-3} OGA did not modify this activity (Fig. 1*C*).

We previously reported that H_2O_2 accumulation was not detected in alfalfa root using the sulphate/xylenol orange (FOX) method (Camejo *et al*. 2011). In the present work we used this method modified according to Cheeseman (2006) in which 1 % ethanol was included in the medium reaction (eFOX) to increase the sensibility of the method minimizing the interference with ASC and other root constituents. H_2O_2 accumulation was found similar in control and OGA-treated alfalfa roots, except in roots treated with 100 µg cm^{-3} OGA at 30 min (Table 1). Low H_2O_2 accumulation could be explained by the activation of CAT and POX enzymes observed in 100 µg cm^{-3} OGA-treated roots at 30 min, while in 50 µg cm^{-3} OGA-treated roots, the CAT seems to be the key enzyme to scavenge H_2O_2 generated by SOD action. H_2O_2 could be also scavenged by non-enzymatic antioxidants as ASC or transported out of the root to be used in other cellular functions. Previous evidence has demonstrated that $O_2^{\bullet-}$ was accumulated in the elongation and meristematic zones in *Arabidopsis* roots, while H_2O_2 predominated in the differentiation zone, where cell elongation ceases (Dunand *et al*. 2007). H_2O_2 can be used by apoplastic peroxidases to reinforce the cell wall catalyzing the cross-linking between the structural polymers and the oxidative polymerization of cinnamyl alcohol to lignin (Ros Barceló

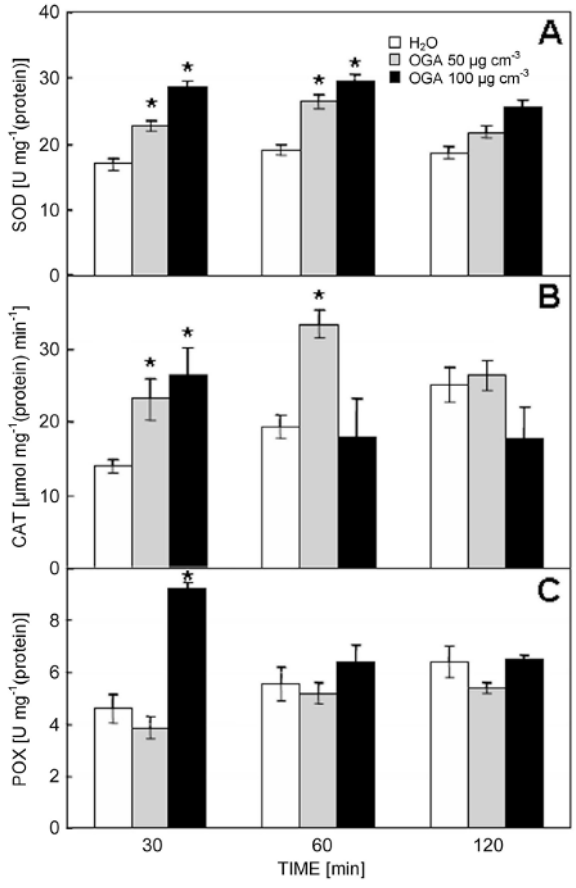

Fig. 1. Changes in the superoxide dismutase (SOD) (*A*), catalase (CAT) (*B*) and peroxidase (POX) (*C*) activities in alfalfa roots incubated in water (control), 50 and 100 µg cm^{-3} OGA for 30, 60 and 120 min. Means ± SE, *n* = 9, * - differences from control values significant at $P < 0.05$.

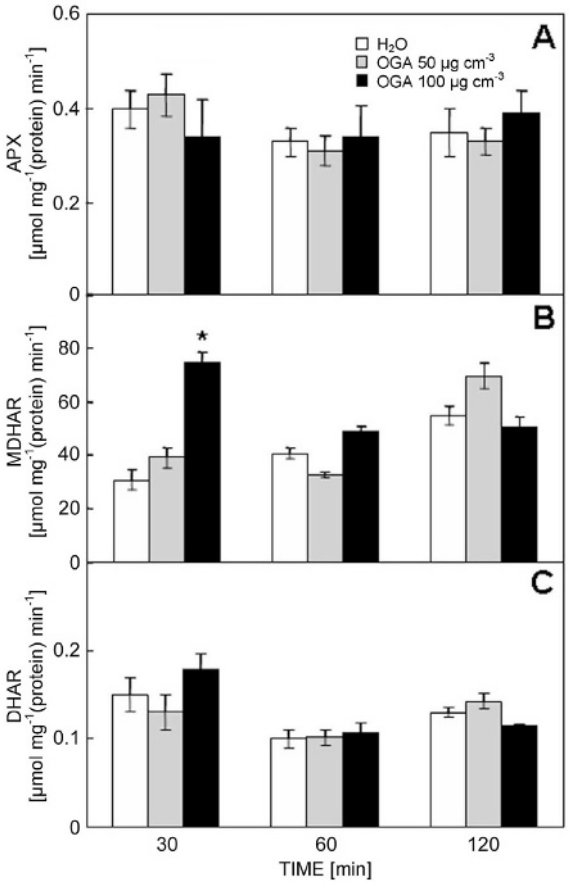

Fig. 2. Changes in the ascorbate peroxidase (APX) (*A*), monodehydroascorbate reductase (MDHAR) (*B*) and dehydro-ascorbate reductase (DHAR) (*C*) activities in alfalfa roots incubated in water (control), 50 and 100 µg cm^{-3} OGA for 30, 60 and 120 min. Means ± SE, n = 9, *- differences from control values significant at $P < 0.05$.

Table 1. Changes in contents of H_2O_2 [nmol g^{-1} (f.m.)], reduced ascorbate (ASC) and oxidised ascorbate (DHA) [μg g^{-1} (f.m.)] in alfalfa roots incubated with water (control), 50 and 100 μg cm^{-3} OGA for 30, 60 and 120 min. Means ± SE, $n = 9$, * - significant differences from control values at $P < 0.05$.

Time	OGA conc.	H_2O_2	ASC	DHA
30	0	4.52 ± 0.01	3.0 ± 0.7	165.0 ± 21.0
	50	4.19 ± 0.07	2.9 ± 0.3	158.3 ± 11.0
	100	3.95 ± 0.04*	3.8 ± 0.2*	172.9 ± 19.0
60	0	5.23 ± 0.84	2.5 ± 0.2	162.0 ± 11.3
	50	4.93 ± 0.37	3.0 ± 0.3	136.3 ± 17.7*
	100	4.68 ± 0.27	2.2 ± 0.1	185.8 ± 11.1
120	0	4.88 ± 0.12	2.6 ± 0.3	180.0 ± 19.8
	50	5.17 ± 0.25	3.0 ± 0.2	106.9 ± 8.5*
	100	4.87 ± 0.14	1.6 ± 0.1*	161.8 ± 6.4

1997), provoking a cessation of cellular elongation.

The different functions attributed to ASC have led to the suggestion that ASC may, in addition to being a powerful antioxidant and redox buffer, be a signalling molecule involved in the regulation of complex processes such as the senescence of plants, abiotic stress response, or pathogen attack (Smirnoff 2000, Hernández *et al*. 2001, Pastori *et al*. 2003, Puppo *et al*. 2005, Locato *et al*. 2006) and low contents of ASC in *vitc* mutants has been shown to lead to microlessions, enhanced basal resistance against pathogens and programmed cell death (Pavet *et al*. 2005). Changes in the ASC content were also analysed in control and OGA-treated roots considering ASC as being involved also in growth and development. The presence of OGA in the incubation medium provoked a significant increase in the reduced ASC content in 100 μg cm^{-3} OGA-treated roots in the first 30 min of the treatment (Table 1). This increase in ASC could contribute to the observed decrease in H_2O_2 accumulation. However, this difference disappeared at 60 min. A significant decrease in reduced ASC content was observed in the 100 μg cm^{-3} OGA-treated root at 120 min. Low OGA concentration, such as 50 μg cm^{-3}, had no effect on the reduced ASC at any time (Table 1). However, on analysing DHA, it was found that the presence of 100 μg cm^{-3} OGA in the incubation medium had no effect on its content, while a significant reduction was only observed in 50 μg cm^{-3} OGA-treated roots at 60 and 120 min, which was not correlated with significant changes in ASC (Table 1). These results suggested that 50 μg cm^{-3} OGA stimulated a rapid use of DHA in the growing roots and could be related to the observed physiological effects of 50 μg cm^{-3} OGA promoting root growth (Camejo *et al*. 2011). It is noted, that in our experimental conditions, the ASC/DHA ratio is quite low due to a high DHA content. It has been previously demonstrated that ASC should be oxidized prior its uptake by the cells and that ASC in the uncharged acid form which exists at pH values below 5, can move according to a concentration and pH gradient, as do plant auxins (Horemans *et al*. 2000). It is well known that apoplastic ASC may function as a redox buffer and that many processes occurring in the apoplast depend to great extent on ASC oxidation (Pignocchi and Foyer 2003). Regulated changes in the apoplastic ASC to DHA seem to be of particular importance in the transition from cell division to cell elongation (Córdoba and González-Reyes 1994, Kato and Esaka 1999).

Another key enzyme involved in the scavenging of H_2O_2 is APX, an ASC specific peroxidase whose activity was not modified in the presence of OGA in alfalfa treated roots (Fig. 2*A*). The non activation of APX enzyme and the undetectable H_2O_2 level in roots suggested that H_2O_2 generated by SOD is scavenged by other ways, as commented above, or freely diffused across membranes, possibly acting as a signal molecule to activate different response mechanisms in the root. While excess of H_2O_2 is potentially toxic to cell, the generation and control of a low steady-state concentration of H_2O_2 in plants is critical because it acts as a signal molecule which modulates gene expression in response to a pathogen attack, different stress situations and development and growth processes (Lamb and Dixon 1997, Pellinen *et al*. 2002, Hernández *et al*. 2001, Vandenabeele *et al*. 2003). The enzymatic regeneration of DHA is controlled by two enzymes of the ascorbate-glutathione cycle, MDHAR and DHAR. MDHAR activity was significantly increased in 100 μg cm^{-3} OGA-treated roots at 30 min (Fig. 2*B*), and this increase was parallel to an enhanced ASC level, suggesting that MDHAR is activated by 100 μg cm^{-3} OGA in the initial min of treatment and this possibly has an effect on the regeneration of this antioxidant. However, lower OGA concentration of 50 μg cm^{-3}, had no effect on MDHAR (Fig. 2*B*). ASC recycle pathway plays an important role in the stress response and adaptation (Badawi *et al*. 2004, Gómez *et al*. 2004, Stevens *et al*. 2008), however, little is known about the specific function of MDHAR protecting plants, although it has been shown that the over-expression of MDHAR increased tolerance to salt and osmotic stress in tobacco (Eltayeb *et al*. 2007) and chilling in tomato fruit (Stevens *et al*. 2008). The analysis of DHAR activity showed that this enzyme was not modified by any OGA treatment (Fig. 2*C*). Evidence has been reported for enhanced tolerance to low temperature, salt and ozone in tobacco (Kwon *et al*. 2003, Chen and Gallie 2005) and to salt and aluminum in *Arabidopsis* (Ushimaru *et al*. 2006, Yin *et al*. 2010) when DHAR is over-expressed. Indeed, the over-expression of DHAR in tobacco plants was reported to increase aluminum tolerance diminishing the oxidative damage with higher ASC levels and increased APX activity, but contrary, over-expression of MDHAR neither affected ASC generation nor tolerance to aluminum stress (Yin *et al*. 2010).

From this study novel aspects related with OGA effect on the antioxidant system in intact plant have been described. We may conclude that 50 and 100 μg cm^{-3} OGA application provoked changes in the antioxidant

enzymes in the first 30 min of treatment in alfalfa roots, fundamentally in the SOD and CAT activities, being these enzymes key players in changes induced by OGA in ROS metabolism at the root level. POX and CAT enzymes seem to have a role at the highest OGA concentration together with the antioxidant ASC, to maintain low content of H_2O_2. ASC recycle was differentially modified by OGA and these changes could be related to the biological effect of the OGA on alfalfa roots.

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