Role of peroxisomes in the oxidative injury induced by 2,4-dichlorophenoxyacetic acid in leaves of pea plants

I. McCARTHY-SUÁREZ*, M. GÓMEZ, L.A. Del RÍO and J.M. PALMA

Departamento de Bioquímica, Biología Celular y Molecular de Plantas, Estación Experimental del Zaidín, CSIC, Apartado 419, E-18080 Granada, Spain

Abstract

The role of peroxisomes in the oxidative injury induced by the auxin herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) in leaves of pea (*Pisum sativum* L.) plants was studied. Applications of (2,4-D) on leaves or to root substrate increased the superoxide radical production in leaf peroxisomes. Foliar application also increased H_2O_2 contents in leaf peroxisomes. Reactive oxygen species (ROS) overproduction was accompanied by oxidative stress, as shown by the changes in lipid peroxidation, protein carbonyls, total and protein thiols, and by the up-regulation of the activities of superoxide dismutase, ascorbate peroxidase, glutathione reductase, catalase, glucose 6-phosphate dehydrogenase and $NADP^+$-dependent isocitrate dehydrogenase. Foliar or root 2,4-D applications also induced senescence symptoms in pea leaf peroxisomes, as shown by the decrease of protein content and glycolate oxidase and hydroxypyruvate reductase activities, and by the increase of endopeptidase, xanthine oxidase, isocitrate lyase and acyl-CoA oxidase activities as well as of 3-ketoacyl-CoA thiolase and thiol-protease protein contents. 2,4-D did not induce proliferation of pea leaf peroxisomes but induced senescence-like morphological changes in these organelles. Results suggest that peroxisomes might contribute to 2,4-D toxicity in pea leaves by overproducing cell-damaging ROS and by participating actively in 2,4-D-induced leaf senescence.

Additional key words: antioxidative enzymes, herbicides, hydrogen peroxide, reactive oxygen species, senescence.

Introduction

Oxidative stress has been implicated in the phytotoxicity of auxin herbicides (Segura-Aguilar *et al.* 1995, Grossmann *et al*. 2001, McCarthy-Suárez 2004, Romero-Puertas *et al.* 2004, Simonovičová *et al.* 2004, Sunohara and Matsumoto 2004, 2008). The high contents of H_2O_2, lipid peroxidation products and protein carbonyls in the leaves of sensitive plants treated with auxin herbicides have been linked to the accelerated senescence symptoms and to the death triggered by these compounds. However, neither the mechanisms for reactive oxygen species (ROS) overproduction nor their subcellular localization in the leaves of auxin herbicide-treated plants have been completely elucidated. Only two ROS-generating enzymes ($O_2^{\bullet-}$-producing xanthine oxidase, XOD, and H_2O_2-producing acyl-CoA oxidase, ACOX) have been identified as involved in the induction of oxidative stress in leaves and cotyledons of auxin herbicide-treated plants (Segura-Aguilar *et al.* 1995, McCarthy-Suárez 2004,

Received 23 May 2010, *accepted* 26 August 2010.

Abbreviations: ACOX - acyl-CoA oxidase; APX - ascorbate peroxidase; CAT - catalase; C=O - protein carbonyls; DAB - 3,3'-diaminobenzidine; 2,4-D - 2,4-dichlorophenoxyacetic acid; EP - endoproteolytic activity; F4d - 4-d foliar treatment; GOX - glycolate oxidase; G6PDH - glucose 6-phosphate dehydrogenase; GR - glutathione reductase; HPR - hydroxypyruvate reductase; ICDH - $NADP^+$-dependent isocitrate dehydrogenase; ICL - isocitrate lyase; JA - jasmonic acid; KAT - 3 keto-acylCoA thiolase; Leu-AP - leucine aminopeptidase; LP - lipid peroxidation; $O_2^{\bullet-}$ - superoxide radical; R4d - 4-d root treatment; R7d - 7-d root treatment; ROS - reactive oxygen species; SOD - superoxide dismutase; TP - thiol-protease; XOD - xanthine oxidase.

Acknowledgements: This work was supported by ERDF-cofinanced grants AGL2002-00988 and BIO-192 from the Ministry of Education and Science and Junta de Andalucía, respectively. I. McCarthy-Suárez wishes to acknowledge the Fundación Ramón Areces for a PhD fellowship. The valuable comments of Dr. J. Carbonell and Dr. M.A. Blázquez, Instituto de Biología Molecular y Celular de Plantas, CSIC-UPV, Valencia, Spain, are also appreciated.

* Corresponding author present address: Departamento de Biología del Desarrollo, Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia, E-46022 Valencia, Spain; fax: (+34) 96 3877859, e-mail: ivmcsua@upvnet.upv.es

Romero-Puertas *et al.* 2004). Also, neither the role of the non-enzymatic ROS-producing mechanisms nor that of the different cell organelles in the oxidative stress promotion in leaves of auxin herbicide-treated plants has been studied. Given that auxin herbicide-induced XOD is localized in peroxisomes apart from chloroplasts (Corpas *et al.* 2008), that ACOX is a peroxisomal enzyme (Gerhardt 1983, Palma *et al.* 1991), that oxidative stress triggers peroxisome biogenesis (López-Huertas *et al.* 2000), and that auxin herbicides induce peroxisome proliferation in animals (Espandiari *et al.* 1995), we decided to study the oxidative metabolism in leaf peroxisomes of pea plants treated by the auxin herbicide 2,4-D, which is also a positive growth regulator in tissue cultures (Sivakumar *et al.* 2010). To our knowledge, there are no studies on the effect of 2,4-D on the oxidative metabolism and the structure of leaf peroxisomes. Also, as peroxisomes have a role in leaf senescence (Del Río *et al.* 1998) we decided to study some senescence markers in leaf peroxisomes of 2,4-D-treated pea plants. During leaf senescence, peroxisomes help to convert lipids into sugars through the co-induced fatty acid β-oxidation pathway and glyoxylate cycle (Del Río *et al.* 1998) and have a role in the production of jasmonic acid (JA) by fatty acid β-oxidation (Delker *et al.* 2007). They also collaborate in the dismantlement of the cell by overproducing ROS and degrading proteins and nucleic acids by peroxisomal endoprotease (EP) and XOD activities (Palma *et al*. 2002). Thus, high contents of H_2O_2, membrane $O_2^{\cdot-}$ production, proteolytic and XOD activities, as well as low protein content, have been reported in peroxisomes during pea leaf senescence (Del Río *et al.* 1998, Distefano *et al.* 1999). Moreover, inhibition of H_2O_2-scavenging enzyme catalase (CAT) and stimulation of H_2O_2-producing superoxide dismutase (SOD) have also been described (Del Río *et al.* 1998). The changes leading to transformation of leaf peroxisomes into glyoxysomes have also been documented in pea during senescence (Del Río *et al.* 1998). Typical peroxisomal enzymes, such as CAT and photorespiratory glycolate oxidase (GOX) and hydroxypyruvate reductase (HPR), disappeared, whereas typical glyoxysomal enzymes, such as isocitrate lyase (ICL), rose up. Peroxisomes also proliferated and distributed in two populations, one of normal size but low matrix electron density and another of smaller size but high matrix electron density (Pastori and Del Río 1997). Thus, assessing the ROS-producing mechanisms and their subcellular localization would be of interest not only to clarify the mode of action of these compounds but also to design auxin herbicide-resistant plants.

Materials and methods

Pea (*Pisum sativum* L. cv Lincoln) seeds (*Royal Sluis*, Enkhuizen, The Netherlands) were surface-sterilized with tetramethylthiuram disulfide (4 g kg^{-1} seeds), treated 1 h with 0.2 mM $CaSO_4$ to favour imbibition, and germinated in *Vermiculite* (25 ºC, darkness) for 14 d. The healthiest seedlings were transferred to 2.5 dm^3 PVC pots containing aerated nutrient solution (4 plants per pot) and grown in greenhouse (day-night temperatures 26/18 ºC, relative humidity 70 % and photosynthetic photon flux density 420 μmol m^{-2} s^{-1}). 42-d- old plants were treated with 2,4-D either by foliar spray (10 cm^3 of 22.62 mM solution per pot, followed by 4-d plant growth - treatment F4d) or by adding to nutrient solution (45.2 μM final concentration per pot, followed by 4-d or 7- plant growth - treatments R4d and R7d, respectively). These 2,4-D concentrations and times of exposure were sufficient to cause oxidative stress on pea leaves, as shown earlier (McCarthy-Suárez 2004, Romero-Puertas *et al.* 2004). Untreated plants served as the controls. F7d treatment was not included in the experimental design as sprayed plants died. Herbicide solution was prepared by dissolving 2,4-D first in ethanol (50 %; v/v) and then in water, with 1 % (v/v) of surfactant (*Agridex®*, *Bayer*, Germany) in the case of foliar application. After 2,4-D treatments, leaves were detached, rinsed with distilled water and used for peroxisome isolation.

The whole procedure was conducted at 4 °C to preserve organelle integrity. Isolation and purification of pea leaf peroxisomes by differential and sucrose density gradient centrifugation were performed according to López-Huertas *et al.* (1995). This method provides less than 2 % contamination by other organelles and intactness percentages of about 90 %. In those cases where APX activity had to be determined, ascorbate was added to the extraction media (20 mM) and to all sucrose gradient layers (2 mM).

Separation of membranes and matrices of peroxisomes by osmotic shock of isolated organelles and ultracentrifugation was carried out at 4 °C as described by López-Huertas *et al.* (1995). Peroxisomal membranes, recovered as a pellet and resuspended according to López-Huertas *et al.* (1995), were used to determine APX activity, NADH-dependent $O_2^{\cdot-}$ radical production and protein carbonyls. Peroxisomal matrices, recovered as a supernatant, were 20-fold concentrated by sequential centrifugation in *Amicon® Centriplus YM-10* and *Centricon 10* (*Millipore*, Billerica, MA, USA) centrifugal filter devices (2.700 *g* and 5.000 *g* respectively; cut-off molecular mass 10 kDa) and used in protein carbonyl determination.

NADH-dependent $O_2^{\cdot-}$ radical production by peroxisomal membranes was measured by monitoring

ferricytochrome *c* reduction at 550 nm (López-Huertas *et al.* 1999). Contents of H_2O_2 within intact pea leaf peroxisomes were determined by the peroxidase-catalyzed 4-aminoantipyrine oxidation method (Frew *et al.* 1983).

Membrane lipid peroxidation was measured in intact pea leaf peroxisomes by the thiobarbithuric acid-reacting substances method (Buege and Aust 1978). Protein carbonyls were determined in matrices and membranes of peroxisomes by measuring the complex formed with 2,4-dinitrophenylhydrazine (Levine *et al.* 1991). Total-, protein-, and non-protein-thiols were measured as the complex formed with 5,5'-dithiobis(2-nitrobenzoic) acid (Patra *et al.* 1994).

All activities of antioxidative enzymes were calculated per mg of peroxisomal protein. Catalase (CAT; EC 1.11.1.6) was measured by monitoring H_2O_2 decomposition at 240 nm (Aebi 1984). Superoxide dismutase (SOD; EC 1.15.1.1) was assayed by the McCord and Fridovich method (1969) but with final concentrations of cytochrome (Cyt) *c* and xanthine in the reaction mixture of 13.5 and 96.7 μM, respectively. The assay measured the rate of inhibition of Cyt *c* reduction by xanthine oxidase (XOD)-derived $O_2^{\bullet -}$ radicals at 550 nm. One unit of SOD activity was defined as the amount of enzyme needed to inhibit by 50 % the maximum rate of Cyt *c* reduction resulting from XOD control reaction plus sample blank reaction. Ascorbate peroxidase (APX; EC 1.11.1.11) was assessed by monitoring ascorbate oxidation at 290 nm in the presence of H_2O_2 (Jiménez *et al.* 1997). Glutathione reductase (GR; EC 1.6.4.2) was assayed by following NADPH oxidation coupled to oxidized glutathione reduction at 340 nm (Edwards *et al.* 1990). Glucose 6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) was analyzed by following $NADP^+$ reduction coupled to glucose-6P oxidation to 6-phosphogluconate (Corpas *et al.* 1998). Isocitrate dehydrogenase ($NADP^+$-ICDH; EC 1.1.1.42) was assayed by monitoring $NADP^+$ reduction coupled to the decarboxylative oxidation of isocitrate to 2-oxoglutarate (Goldberg and Ellis 1983).

Protein content was determined using bovine serum albumin as standard (Bradford 1976). Glycolate oxidase (GOX; EC 1.1.3.1) was measured by following the oxidation of glycolate to glyoxylate and formation of a complex of it with phenylhydrazine chlorhydrate (Archer and Ting 1996). Hydroxypyruvate reductase (HPR; EC 1.1.1.29) was assayed by monitoring at 340 nm the oxidation of NADH coupled to hydroxypyruvate reduction to glycerate (Schwitzguebel and Siegenthaler 1984). Endopeptidase (EP; EC 3.4.24.11) was assayed at 324 nm by the azocasein degradation method (Carrasco and Carbonell 1990). One unit of EP activity was defined as the amount of enzyme required to generate an increase of absorbance of 0.01 every 2 h. Leucine aminopeptidase (Leu-AP; EC 3.4.11.1) was estimated by using leu-*p*-nitroanilide as substrate (Corpas *et al.* 1993). Xanthine oxidase (XOD; EC 1.1.3.22) was quantified by following the formation of uric acid from hypoxanthine at 293 nm (Rajagopalan 1985). Acyl-CoA oxidase (ACOX; EC 1.3.3.6) was assayed by following at 500 nm the palmytoyl-CoA-dependent production of H_2O_2 through peroxidase-catalysed 4-aminoantipyrine oxidation (Gerhardt 1983). Isocitrate lyase (ICL; EC 4.1.3.1) was determined by measuring the glyoxylate generated from isocitrate breakdown through complexation with phenylhydrazine chlorhydrate (Archer and Ting 1996).

Contents of peroxisomal specific proteins (catalase, thiol-protease, 3-keto-acyl-CoA thiolase, isocitrate lyase) were estimated as described by Corpas *et al.* (1998). Proteins were separated by SDS-electrophoresis (*Mini-Protean II* slab gel, *BioRad,* Hercules, USA) in 12 % polyacryl-amide gels and transferred to a polyvinylidene fluoride membrane (*Inmobilon* P transfer membrane, *Millipore*) with a *BioRad* semi-dry transfer cell. After incubating the membrane with the primary and secondary antibodies, immunodetection was accomplished by using a chemiluminescent or chromogenic reaction (Corpas *et al.* 1998, Bunkelman and Trelease 1996). Primary antibodies used were those against pumpkin catalase (1:2000 dilution; Nishimura *et al.* 1993), senescent pea ovaries thiol-protease (1:500 dilution; Carrasco and Carbonell 1990), pumpkin 3 keto-acylCoA thiolase (1:500 dilution; Kato *et al.* 1996) and *Ricinus* isocitrate lyase (1:500 dilution; Baker and Graham 2002). Secondary antibodies used were those linked to horseradish peroxidase (chemiluminescence) or alkaline phosphatase (chromogenic detection).

Cytochemistry of pea leaf peroxisomes was carried out in leaves from control, F4d and R4d plants. Leaf peroxisomes were identified by transmission electron microscopy (TEM) by using the cytochemical method of Palma *et al.* (1991). This method evidences the peroxidase activity of catalase at basic pH by using 3,3'-diaminobenzidine (DAB) and H_2O_2 as substrates. Nonspecificity was checked by control reactions without H_2O_2 or including the catalase inhibitor aminotriazole. Polymers of enzymically-formed oxidized DAB were precipitated with OsO_4 to form TEM-visible amorphus precipitates of "osmium black". Leaf cuts were then dehydrated with a series of ethanol and included in Epon resine. Ultrathin leaf cuts, obtained by using a *Reichert-Jung Ultracut E* (Viena, Austria), were contrasted with uranyl acetate and lead citrate and observed under a *Zeiss EM 10C* (Oberkochen, Germany) transmission electron microscope. Counting of peroxisomes in leaf cells of control and 2,4-D-treated plants F4d and R4d was performed by TEM visualization of ultrathin leaf cuts stained for catalase cytochemistry. Average size of leaf peroxisomes was assessed by using 20 000× TEM micrographs. Paper copies of micrographed peroxisomes were weighed in a 0.1 mg-sensitive electronic balance and their area was calculated by using as reference the average mass of 10 paper square pieces of 1 cm^2.

Results and discussion

Pea leaves overproduce ROS in response to 2,4-D (McCarthy-Suárez, 2004; Romero-Puertas *et al.* 2004). To test whether peroxisomes are involved in the overproduction of ROS, we analyzed the ROS production in leaf peroxisomes isolated from 2,4-D-treated pea plants. Results showed that the cytosol-oriented NADH-dependent $O_2^{\bullet-}$ production by peroxisomal membranes increased under the 4-d foliar (F4d) and 7-d root (R7d) treatments, whereas intra-peroxisomal contents of H_2O_2 increased under F4d treatment (Table 1). As $O_2^{\bullet-}$ quickly dismutate to membrane-diffusible H_2O_2, either spontaneously or by the action of SOD, H_2O_2 overproduced in the membrane or the matrix of peroxisomes could migrate to the cytosol and/or other organelles and contribute to the oxidative stress (McCarthy-Suárez 2004, Romero-Puertas *et al*. 2004). In fact, several plant stresses have been reported to enhance the peroxisomal membrane $O_2^{\bullet-}$ production and the peroxisomal matrix H_2O_2 generation (Palma *et al.* 1991, Del Río *et al.* 1996). Moreover, membrane $O_2^{\bullet-}$ could react in the cytosol with the H_2O_2, through Fe^{2+}/Cu^{2+}-catalysed Haber-Weiss reactions, to generate deleterious hydroxyl radicals ($OH^{\bullet}$) which are able to damage DNA, lipids and proteins (Halliwell and Gutteridge 2007). In that sense, several works have demonstrated the implication of ROS overproduction by organelle membranes in the damaging mechanisms of different types of stress. Plasma membrane $O_2^{\bullet-}$ have been generated after wound and copper and nickel stress (Minibayeba *et al.* 1998, Quartacci *et al.* 2001, Hao *et al.* 2006) whereas chloroplast and mitochondrial membrane-generated $O_2^{\bullet-}$ overproduction has been related to chilling injury in sensitive plants (Hodgson and Raison 1991, Purvis *et al.* 1995).

ROS overproduction was accompanied by oxidative stress. F4d and R7d treatments increased the lipid peroxidation (LP) and the matrix and membrane protein carbonyls (C=O), and decreased the total and protein thiols (Table 1). Moreover, the protein thiol decrease under these treatments confirmed the ROS overproduction in pea leaf peroxisomes, as oxidation of cysteine residues of proteins is an immediate sensor of

Table 1. Effect of the herbicide 2,4-D on the $O_2^{\bullet-}$ production [nmol($O_2^{\bullet-}$) mg^{-1} min^{-1}], H_2O_2 content [µmol dm^{-3}], lipid peroxidation (LP) [nmol(MDA) mg^{-1}(protein)], protein carbonyls (C=O) [nmol mg^{-1}(protein)] and total-, protein-, and non-protein thiols (-SH) [µg(Cys) g^{-1}], the activities of CAT and APX [µmol mg^{-1}(protein) min^{-1}], SOD [U mg^{-1}(protein)], GR, G6PDH and ICDH [nmol mg^{-1}(protein) min^{-1}]), protein content [mg cm^{-3}] and GOX, HPR, ACOX [µmol mg^{-1}(protein) min^{-1}], EP [U mg^{-1} (protein)], Leu-AP, XOD and ICL [nmol mg^{-1}(protein) min^{-1}] and number [$cell^{-1}$] and size [$µm^2$] of peroxisomes. Values are means ± SE of four independent experiments.

	Control	F4d	R4d	R7d
$O_2^{\bullet-}$	58.1 ± 12.6	351 ± 70.9	63.9 ± 11.0	318 ± 21.6
H_2O_2	0.83 ± 0.07	1.08 ± 0.03	0.91 ± 0.08	0.84 ± 0.02
LP	1.18 ± 0.11	1.87 ± 0.05	1.24 ± 0.07	5.73 ± 0.27
$C{=}O_{matrix}$	13.7 ± 1.49	47.7 ± 5.22	13.9 ± 1.53	31.6 ± 3.46
$C{=}O_{membrane}$	19.5 ± 1.21	33.9 ± 2.10	19.6 ± 1.22	41.6 ± 2.58
$-SH_{total}$	3.72 ± 0.24	3.25 ± 0.45	3.60 ± 0.19	2.64 ± 0.05
$-SH_{protein}$	2.54 ± 0.29	2.09 ± 0.02	2.55 ± 0.23	1.34 ± 0.12
$-SH_{non\ protein}$	1.19 ± 0.07	1.16 ± 0.07	1.11 ± 0.02	1.34 ± 0.09
CAT	1010 ± 73	791 ± 128	1325 ± 41	1396 ± 137
SOD	27.1 ± 2.79	38.2 ± 5.37	29.0 ± 4.36	43.3 ± 7.30
APX	1.46 ± 0.14	3.20 ± 0.55	1.49 ± 0.02	4.47 ± 0.02
GR	55.2 ± 3.19	87.7 ± 10.6	79.6 ± 11.6	100.8 ± 24.8
G6PDH	4.46 ± 0.58	6.01 ± 0.76	4.36 ± 0.43	6.07 ± 0.86
ICDH	35.1 ± 3.10	43.6 ± 2.19	35.7 ± 3.49	45.3 ± 5.84
Protein content	0.18 ± 0.01	0.14 ± 0.01	0.16 ± 0.02	0.10 ± 0.01
GOX	1.03 ± 0.07	0.85 ± 0.02	1.29 ± 0.10	0.89 ± 0.04
HPR	5.23 ± 0.30	3.92 ± 0.29	6.41 ± 0.15	4.11 ± 0.35
EP	11.2 ± 0.05	28.5 ± 7.77	13.0 ± 1.99	20.8 ± 1.57
Leu-AP	22.8 ± 0.04	8.00 ± 0.63	23.1 ± 2.14	10.5 ± 1.18
XOD	2.23 ± 0.23	3.24 ± 0.57	2.48 ± 0.35	3.46 ± 0.52
ACOX	1407 ± 142	2911 ± 151	2981 ± 418	3284 ± 487
ICL	12.8 ± 1.30	26.7 ± 3.19	11.7 ± 1.66	35.7 ± 7.19
Peroxisome number	1.81 ± 0.18	1.68 ± 0.23	1.55 ± 0.15	-
Peroxisome size	0.50 ± 0.16	0.55 ± 0.18	0.45 ± 0.17	-

H_2O_2 stress (Hung *et al.* 2005). On the other hand, the higher membrane $O_2^{\cdot-}$ production under these treatments might have explained their higher lipid peroxidation. In fact, lipid peroxidation did not increase if membrane $O_2^{\cdot-}$ were not overproduced, as seen in R4d treatment. Also, under R7d treatment, the higher membrane $O_2^{\cdot-}$ production might have accounted for the higher matrix protein oxidation. Besides promoting lipid peroxidation, $O_2^{\bullet-}$ radicals can diffuse through membranes and cause soluble protein oxidation (Dat *et al.* 2000). In turn, under F4d treatment, the increased matrix protein oxidation might have resulted from both a higher membrane $O_2^{\bullet-}$ production and a higher matrix H_2O_2 generation.

To determine which of the peroxisomal ROS-producing enzymes might be contributing to promote oxidative stress, the H_2O_2-producing activities of GOX, SOD and ACOX, and the $O_2^{\bullet-}$-producing activity of XOD, were analyzed in leaf peroxisomes of 2,4-D-treated pea plants (Table 1). Results showed that SOD, ACOX and XOD activities increased under the F4d and R7d treatments, whereas GOX increased slightly under the R4d treatment. Thus, although R7d treatment did not increase H_2O_2 contents in leaf peroxisomes, ROS overproduction in the peroxisomal matrix under this treatment was inferred not only from the increase of matrix C=O, but also from the stimulation of ROS-producing activities of SOD, ACOX and XOD. Moreover, considering the stimulation of H_2O_2-producing ACOX and SOD, and of $O_2^{\bullet-}$ -producing XOD, $OH^{\bullet}$ radicals could also appear in the peroxisomal matrix under F4d and R7d treatments, thereby enhancing the oxidative stress within the organelle. Thus, results suggest that peroxisomes might contribute to 2,4-D-induced oxidative stress in pea leaves by overproducing membrane and matrix ROS able to reach the cytosol giving rise to $OH^{\bullet}$ radicals.

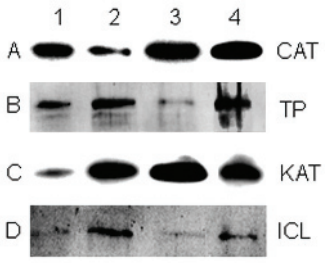

Fig. 1. Effect of the herbicide 2,4-D on the proteins of CAT (*A*), TP (*B*), KAT (*C*) and ICL (*D*) in leaf peroxisomes of pea plants. *Lanes*: Control (1), F4d treatment (2), R4d treatment (3), R7d treatment (4). Protein loaded per lane: 4.5 μg (CAT), 3 μg (TP), 4.7 μg (KAT) and 3 μg (ICL). Bands were revealed by chemiluminiscence (CAT and KAT) or chromogenic detection (TP and ICL).

As H_2O_2 and stress up-regulate antioxidant activities (Fedina *et al.* 2009, Wang *et al*. 2009, Maia *et al*. 2010), some of them peroxisomal (Del Río *et al.* 2006), oxidative stress in leaf peroxisomes of 2,4-D-treated pea was also confirmed by the analysis of the antioxidant activities of CAT, SOD, APX, GR, G6PDH and ICDH (Table 1). SOD, APX, G6PDH and ICDH activities increased under F4d and R7d treatments, especially the membrane-localized APX activity, whereas GR increased with all 2,4-D-treatments. However, CAT activity increased under the root treatments (R4d and R7d) but decreased under the foliar treatment (Table 1), in parallel to CAT protein levels (Fig. 1*A*). As CAT has the main H_2O_2-scavenging role in peroxisomes, inhibition of CAT under the foliar treatment might also promote oxidative stress in these organelles. The results mentioned above clearly show that the oxidative stress developed in leaf peroxisomes differed between leaf and root 2,4-D treatments.

Table 2. The size distribution of leaf peroxisomes of pea plants. Peroxisomes were counted on 58 control cells, 41 F4d cells and 68 R4d cells. To assess peroxisome size distribution in leaves, size of peroxisomes was expressed arbitrarily (ranging 1 to 7 in increasing order). Percentages were calculated by referring the number of peroxisomes of a particular size to the total number of peroxisomes analyzed in each treatment.

Size class	1	2	3	4	5	6	7
Control	0	13.5	37.8	21.6	18.9	8.1	0
F4d	2.6	10.5	18.4	36.8	18.4	10.5	2.6
R4d	8.2	20.4	24.4	26.5	18.4	2.0	0

Pea leaf peroxisomes displayed structural changes in response to 2,4-D. In spite of the peroxisome-proliferating effect of 2,4-D in animals (Abdellatif *et al.* 1990), neither F4d nor R4d treatments induced peroxisome proliferation in pea leaves (Table 1). Also 2,4,5-trichloro-phenoxyacetic acid did not induce peroxisomal proliferation in cotyledons of *Picea abies* seedlings (Segura-Aguilar *et al.* 1995). In animals, xenobiotic-induced peroxisome proliferation has been associated with a 30-fold induction of the H_2O_2-producing ACOX activity (Lock *et al.* 1989). However, in F4d pea plants, leaf ACOX activity increased only 2-fold and treatment of *Picea abies* seedlings with 2,4,5-trichlorophenoxy-acetic acid stimulated cotyledonal ACOX only 7-fold (Segura-Aguilar *et al.* 1995). Thus, although H_2O_2 overproduction is able to induce peroxisomal proliferation in plants (López-Huertas *et al.* 2000), it is possible that H_2O_2 contents and H_2O_2-producing ACOX activity in leaves of 2,4-D-treated pea plants were insufficient to stimulate the biogenesis of peroxisomes. As H_2O_2 in pea leaves accumulated preferentially in leaf veins (Romero-Puertas *et al.* 2004) and leaf cells used in our study for peroxisomal counting were only parenchymatic, it is possible that extra-vascular levels of H_2O_2 in pea leaves were insufficient to promote peroxisomal proliferation in parenchymatic

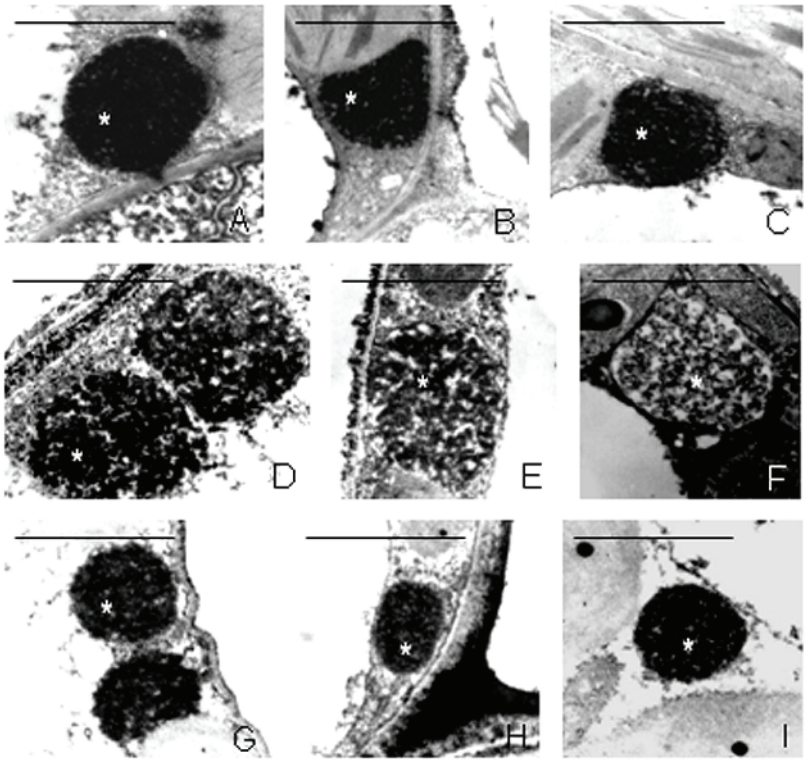

Fig. 2. Effect of the herbicide 2,4-D on the morphology of leaf peroxisomes of pea plants. *A* - *C*: control peroxisomes; *D* - *F*: F4d peroxisomes; *G* - *I*: R4d peroxisomes (*bar* = 0.5 μm, *asterisk* indicates the peroxisomal matrix).

cells. Moreover, as auxin herbicides increase JA contents in leaves of sensitive plants (Grossmann *et al.* 2004), JA might have inhibited peroxisome proliferation in 2,4-D-treated pea plants as it has been reported in other plants (León 2008). Treatments F4d and R4d changed the size and matrix electron density of pea leaf peroxisomes (Table 1 and Fig. 2). Under F4d, peroxisomes were almost 10 % bigger, whereas under R4d they were 10 % smaller (Table 1). Moreover, peroxisome size distribution in pea leaf cells (Table 2) showed that while the majority of control peroxisomes had an arbitrary size 3, most F4d peroxisomes had size 4. Also, only F4d treatment produced peroxisomes of size 7. R4d treatment, in turn, gave rise to a high proportion (20 %) of peroxisomes of size 2 and almost 10 % of size 1. However, control peroxisomes ranged between size 2 and 6. In that sense oxidative stress and JA have been shown to increase the size of plant peroxisomes (Schrader and Fahimi 2006, Castillo *et al.* 2008). F4d and R4d treatments also altered the electron density of the matrix of pea leaf peroxisomes (Fig. 2). While F4d gave rise to peroxisomes of a less electron-dense matrix as compared to control peroxisomes, R4d produced peroxisomes of a highly electron-dense matrix, similar to the control.

Pea leaf peroxisomes showed symptoms of senescence in response to 2,4-D as the morphological changes induced by 2,4-D treatment resembled those occurring in peroxisomes during leaf senescence (Del Río *et al.* 1998). Analogous to senescence, also protein content and photorespiratory activities GOX and HPR decreased under F4d and R7d treatments. These treatments also increased EP activity, thiol-protease (TP) protein level (Fig. 1*B*) and $O_2^{\bullet-}$-producing XOD activity, involved in the catabolism of nucleic acids. However, they decreased the activity of Leu-AP, which could be involved in protein import to peroxisomes (Corpas *et al.* 1993). As aminopeptidase activity and protein levels decrease during senescence, whereas thiol-protease activity increases (Fischer *et al.* 1998), it is possible that lower peroxisomal protein content under F4d and R7d treatments might have resulted from the lower Leu-AP activity and the higher EP activity. Moreover, oxidative vulnerability of Leu-AP, due to its essential thiol groups (Corpas *et al.* 1993), might have prompted its degradation, thereby affecting protein import and protein contents of peroxisomes. In fact, in leaf peroxisomes of cadmium-treated pea plants, higher Leu-AP activity was accompanied by increased protein contents (McCarthy *et al.* 2001). 2,4-D treatments also stimulated the fatty acids β-oxidation in pea leaf peroxisomes. All treatments increased the H_2O_2-producing ACOX activity, first enzyme of the route (Table 1), and the protein levels of 3-keto-acylCoA thiolase (KAT), last enzyme of the pathway (Fig. 1*C*). F4d and R7d treatments also

increased the activity (Table 1) and the protein levels (Fig. 1*D*) of glyoxylate cycle ICL activity. Thus, a co-stimulation of fatty acid β-oxidation and glyoxylate cycle took place in leaf peroxisomes under F4d and R7d treatments, as shown during senescence. As auxin herbicides increase the content of senescence/stress hormone JA in leaves of sensitive plants (Grossmann *et al.* 2004), and JA is synthesized in peroxisomes through fatty acid β-oxidation (Delker *et al.* 2007), up-regulation of the fatty acid β-oxidation pathway suggests that peroxisomes might be essential to the onset of senescence in the leaves of auxin-herbicide treated plants. Moreover, as auxin herbicides induce the senescence-related activities DNAse (Grossmann *et al.* 2001) and XOD (McCarthy-Suárez 2004, Romero-Puertas *et al.* 2004) in leaves of sensitive plants, stimulation of peroxisomal XOD suggests that these organelles might also be important to the senescence-associated degradation of nucleic acids induced by auxin herbicides. Thus, results of this study suggest that peroxisomes might have a role in the oxidative stress and senescence induced by auxin herbicides in the leaves of sensitive plants.

References

Abdellatif, A.G., Préat, V., Vamecq, J., Nilsson, R., Roberfroid, M.: Peroxisome proliferation and modulation of rat liver carcinogenesis by 2,4-dichlorophenoxyacetic acid, 2,4,5-trichlorophenoxyacetic acid, perfluorooctanoic acid and nafenopin. - Carcinogenesis **11**: 1899-1902, 1990.

Aebi, H.: Catalase *in vitro*. - Methods Enzymol. **105**: 121-126, 1984.

Archer, E.K., Ting, B.L.: A virescent plastid mutation in tobacco decreases peroxisome enzyme activities in seedlings. - J. Plant Physiol. **149**: 520-526, 1996.

Baker, A., Graham, I.A. (ed.): Plant Peroxisomes, Biochemistry, Cell Biology and Biotechnological Applications. - Kluwer Academic Publishers, Dordrecht 2002.

Bradford, M.M.: A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. - Anal. Biochem. **72**: 248-254, 1976.

Buege, J.A., Aust, S.D.: Microsomal lipid peroxidation. - Methods Enzymol. **52**: 302-310, 1978.

Bunkelman, R.J., Trelease, R.N.: Ascorbate peroxidase: a prominent membrane protein in oilseed glyoxysomes. - Plant Physiol. **110**: 589-598, 1996.

Carrasco, P., Carbonell, J.: Changes in the level of peptidase activities in pea ovaries during senescence and fruit set induced by gibberellic acid. - Plant Physiol. **92**: 1070-1074, 1990.

Castillo, M.C., Sandalio, L.M., Del Río, L.A., León, J.: Peroxisome proliferation, wound activated responses and expression of peroxisome-associated genes are cross-regulated but uncoupled in *Arabidopsis thaliana*. - Plant Cell Environ. **31**: 492-505, 2008.

Corpas, F.J., Barroso, J.B., Sandalio, L.M., Distefano, S., Palma, J.M., Lupiáñez, J.A., Del Río, L.A.: A dehydrogenase-mediated recycling system of NADPH in plant peroxisomes. - Biochem. J. **330**: 777-784, 1998.

Corpas, F.J., Palma, J.M., Del Río, L.A.: Evidence for the presence of proteolytic activity in peroxisomes. - Eur. J. Cell Biol. **61**: 81-85, 1993.

Corpas, F.J., Palma J.M., Sandalio, L.M., Valderrama, R., Barroso, J.B., Del Río, L.A.: Peroxisomal xanthine oxidoreductase: characterization of the enzyme from pea (*Pisum sativum* L.) leaves. - J. Plant Physiol. **168**: 1319-1330, 2008.

Dat, J.F., Vandenabeele, S., Vranová, E., Van Montagu, M., Inzé, D., Van Breusegem, F.: Dual action of the active oxygen species during plant stress responses. - Cell. Mol. Life Sci. **57**: 779-795, 2000.

Delker, C., Zolman, B.K., Miersch, O., Wasternak, C.: Jasmonate biosynthesis in *Arabidopsis thaliana* requires peroxisomal β-oxidation enzymes – additional proof by properties of pex6 and aim1. - Phytochemistry **68**: 1642-1650, 2007.

Del Río, L.A., Palma, J.M., Sandalio, L.M., Corpas, F.J., Pastori, G.M., Bueno, P., López-Huertas, E.: Peroxisomes as a source of superoxide and hydrogen peroxide in stressed plants. - Biochem. Soc. Trans. **24**: 434-438, 1996.

Del Río, L.A., Pastori, G.M., Palma, J.M., Sandalio, L.M., Sevilla, F., Corpas, F.J., Jiménez, A., López-Huertas, E., Hernández, J.A.: The activated oxygen role of peroxisomes in senescence. - Plant Physiol. **116**: 1195-1200, 1998.

Del Río, L.A., Sandalio, L.M., Corpas, F.J., Palma, J.M., Barroso, J.B.: Reactive oxygen species and reactive nitrogen species in peroxisomes. Production, scavenging, and role in cell signaling. - Plant Physiol. **141**: 330-335, 2006.

Distefano, S., Palma, J.M., McCarthy, I., Del Río, L.A.: Proteolytic cleavage of plant proteins by peroxisomal endo-proteases from senescent pea leaves. - Planta **209**: 308-313, 1999.

Edwards, E.A., Rawsthorne, S.R., Mullineaux, P.M.: Subcellular distribution of multiple forms of glutathione reductase in leaves of pea (*Pisum sativum* L.). - Planta **180**: 278-284, 1990.

Espandiari, P., Thomas, V.A., Glauert, H.P., O´Brien, M., Noonan, D., Robertson, L.W.: The herbicide dicamba (2-methoxy-3,6-dichlorobenzoic acid) is a peroxisome proliferator in rats. - Fund. appl. Toxicol. **26**: 85-90, 1995.

Fedina, I.S., Nedeva, D., Çiçek, N.: Pre-treatment with H_2O_2 induces salt tolerance in barley seedlings. - Biol. Plant. **53**: 321-324, 2009.

Fischer, A., Brouquisse, R., Raymond, P.: Influence of senescence and of carbohydrate levels on the pattern of leaf proteases in purple nutsedge (*Cyperus rotundus*). - Physiol. Plant. **102**: 385-395, 1998.

Frew, J.E., Jones, P., Scholes, G.: Spectrophotometric determination of hydrogen peroxide and organic hydroperoxides at low concentrations in aqueous solutions. - Anal. chim. Acta **155**: 139-150, 1983.

Gerhardt, B.: Localization of β-oxidation enzymes in peroxisomes isolated from nonfatty plant tissues. - Planta **159**: 238-246, 1983.

Goldberg, D.M., Ellis, G.: Isocitrate - In: Bergmeyer H.U. (ed.): Methods in Enzymatic Analysis. Pp. 183-190. Academic Press, New York 1983.

Grossmann, K., Kwiatkowski, J., Tresch, S.: Auxin herbicides

induce H_2O_2 overproduction and tissue damage in cleavers (*Galium aparine* L.). - J. exp. Bot. **52**: 1811-1836, 2001.

Grossmann, K., Rosenthal, C., Kwiatowski, J.: Increases in jasmonic acid caused by indole-3-acetic acid and auxin herbicides in cleavers (*Gallium aparine*). - J. Plant Physiol. **161**: 809-814, 2004.

Hao, F., Wang, X., Chen, J.: Involvement of plasma-membrane NADPH-oxidase in nickel-induced oxidative stress in roots of wheat seedlings. - Plant Sci. **170**: 151-158, 2006.

Halliwell, B., Gutteridge, J.M.C. (ed.): Free Radicals in Biology and Medicine. - Oxford University Press, Oxford 2007.

Hodgson, R.A.J., Raison, J.K.: Superoxide production by thylakoids during chilling and its implication in the susceptibility of plants to chilling-induced photoinhibition. - Planta **183**: 222-228, 1991.

Hung, S.H., Yu, C.W., Lin, C.H.: Hydrogen peroxide functions as a stress signal in plants. - Bot. Bull. Acad. sin. **46**: 1-10, 2005.

Jiménez, A., Hernández, J.A., Del Río, L.A., Sevilla, F.: Evidence for the presence of the ascorbate-glutathione cycle in mitochondria and peroxisomes of pea leaves. - Plant Physiol. **114**: 275-284, 1997.

Kato, Y., Hayashi, M., Takeuchi, Y., Nishimura, M.: cDNA cloning and expression of a gene for 3-ketoacyl-CoA thiolase in pumpkin cotyledons. - Plant mol. Biol. **31**: 843-852, 1996.

León, J.: Peroxisome proliferation in *Arabidopsis*. The challenging identification of ligand perception and downstream signalling is closer. - Plant Signal. Behaviour **3**: 671-673, 2008.

Levine, R.L., William, J.A., Stadtman, E.R., Shacter, E.: Carbonyl assays for determination of oxidatively modified proteins. - Methods Enzymol. **233**: 346-363, 1991.

Lock, E.A., Mitchell, A.M., Elcombe, C.R.: Biochemical mechanisms of induction of hepatic peroxisome proliferation. - Annu. Rev. Pharmacol. Toxicol. **29**: 145-163, 1989.

López-Huertas, E., Charlton, W.L., Johnson, B., Graham, I.A., Baker, A.: Stress induces peroxisome biogenesis genes. - EMBO J. **19**: 6770-6777, 2000.

López-Huertas, E., Corpas, F.J., Sandalio, L.M., Del Río, L.A.: Characterization of membrane polypeptides from pea leaf peroxisomes involved in superoxide radical generation. - Biochem. J. **337**: 531-536, 1999.

López-Huertas, E., Sandalio, L.M., Del Río L.A.: Integral membrane polypeptides of pea leaf peroxisomes: characterization and response to plant stress. - Plant Physiol. Biochem. **33**: 295-302, 1995.

Maia, J., Costa de Macedo, C., Voigt, E., Freitas, J., Silveira, J.: Antioxidative enzymatic protection in leaves of two contrasting cowpea cultivars under salinity. - Biol. Plant. **54**: 159-163, 2010.

McCarthy, I., Romero-Puertas, M.C., Palma, J.M., Sandalio, L.M., Corpas, F.J., Gómez, M., Del Río, L.A.: Cadmium induces senescence symptoms in leaf peroxisomes of pea plants. - Plant Cell Environ. **24**: 1065-1073, 2001.

McCarthy-Suárez, I.: Estudio del Estrés Oxidativo Inducido por el 2,4-D (ácido 2,4-diclorofenoxiacético) en Plantas de Guisante (*Pisum sativum* L.) y Peroxisomas de Hojas [Study of the Oxidative Stress Induced by 2,4-D (2,4-dichlorophenoxyacetic acid) in Pea Plants and Pea Leaf Peroxisomes]. - PhD Thesis, University of Granada, Granada 2004. [In Span.]

McCord, J.M., Fridovich, I.: Superoxide dismutase: an enzymic function for erythrocuprein. - J. biol. Chem. **244**: 6049-6055, 1969.

Minibayeba, F.V., Kolesnikov, O.P., Gordon, L.K.: Contribution of a plasma membrane redox system to the superoxide production by wheat root cells. - Protoplasma **205**: 101-106, 1998.

Nishimura, M., Takeuchi, Y., De Bellis, L., Hara-Nishimura, I.: Leaf peroxisomes are directly transformed to glyoxysomes during senescence. - Protoplasma **175**: 131-137, 1993.

Palma, J.M., Garrido, M., Rodríguez-García, M.I., Del Río L.A.: Peroxisome proliferation and oxidative stress mediated by activated oxygen species in plant peroxisomes. - Arch. Biochem. Biophys. **287**: 68-74, 1991.

Palma, J.M., Sandalio, L.M., Corpas, F.J., Romero-Puertas, M.C., McCarthy, I., Del Río, L.A.: Plant proteases, protein degradation and oxidative stress: role of peroxisomes. - Plant Physiol. Biochem. **40**: 521-530, 2002.

Pastori, G.M., Del Río, L.A.: Natural senescence of pea leaves: an activated oxygen-mediated function for peroxisomes. - Plant Physiol. **113**: 411-418, 1997.

Patra, J., Lenka, M., Panda, B.B.: Tolerance and co-tolerance of the grass Chloris barbata Sw. to mercury, cadmium and zinc. - New Phytol. **128**: 165-171, 1994.

Purvis, A.C., Shewfelt, R.L., Gegogeine, J.W.: Superoxide production by mitochondria isolated from green bell pepper fruit. - Physiol. Plant. **94**: 743-749, 1995.

Quartacci, M.F., Cosi, E., Navari-Izzo, F.: Lipids and NADH-dependent superoxide production in plasma membrane vesicles from roots of wheat grown under copper deficiency or excess. - J. exp. Bot. **52**: 77-84, 2001.

Rajagopalan, K.V.: Purification of bovine milk xanthine oxidase. - In: Greenwald R.A. (ed.): Handbook of Methods for Oxygen Radical Research. Pp. 21-23. CRC Press, Boca Raton 1985.

Romero-Puertas, M.C., McCarthy, I., Gómez, M., Sandalio, L.M., Corpas, F.J., Del Río, L.A., Palma, J.M.: Reactive oxygen species-mediated enzymatic systems involved in the oxidative action of 2,4-dichlorophenoxyacetic acid. - Plant Cell Environ. **27**: 1135-1148, 2004.

Schrader, M., Fahimi, H.D.: Peroxisomes and oxidative stress. - Biochim. biophys. Acta **1763**: 1755-1766, 2006.

Schwitzguebel, J.P., Siegenthaler, P.A.: Purification of peroxisomes and mitochondria from spinach leaf by Percoll gradient centrifugation. - Plant Physiol. **75**: 670-674, 1984.

Segura-Aguilar, J., Hakman, I., Rydström, J.: Studies on the mode of action of the herbicidal effect of 2,4,5-trichlorophenoxyacetic acid on germinating Norway spruce. - Environ. exp. Bot. **35**: 309-319, 1995.

Simonovičová, M., Huttová, J., Mistrík, I., Široká, B., Tamás, L.: Peroxidase-mediated hydrogen peroxide production in barley roots grown under stress conditions. - Plant Growth Regul. **44**: 267-275, 2004.

Sivakumar, P., Gnanam, R., Ramakrishnan, K., Manickam, A.: Somatic embryogenesis and regeneration of *Vigna radiata*. - Biol. Plant. **54**: 245-251, 2010.

Sunohara, Y., Matsumoto, H.: Oxidative injury induced by the herbicide quinclorac on *Echinochloa orizycola* Vasing. and involvement of antioxidative ability in its highly selective action in grass species. - Plant Sci. **167**: 597-606, 2004.

Sunohara, Y., Matsumoto, H.: Quinclorac-induced cell death is accompanied by generation of reactive oxygen species in maize root tissue. - Phytochemistry **69**: 2312-2319, 2008.

Wang, Y., Yang, Z.M., Zhang, Q.F., Li, J.L.: Enhanced chilling tolerance in *Zoysia matrella* by pre-treatment with salicylic acid, calcium chloride, hydrogen peroxide or 6-benzyl-aminopurine. - Biol. Plant. **53**: 179-182, 2009.