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Role of the Ascorbate-Glutathione Cycle of Mitochondria and Peroxisomes in the Senescence of Pea Leaves[1]

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We investigated the relationship between H_2O_2 metabolism and the senescence process using soluble fractions, mitochondria, and peroxisomes from senescent pea (*Pisum sativum* L.) leaves. After 11 d of senescence the activities of Mn-superoxide dismutase, dehydroascorbate reductase (DHAR), and glutathione reductase (GR) present in the matrix, and ascorbate peroxidase (APX) and monodehydroascorbate reductase (MDHAR) activities localized in the mitochondrial membrane, were all substantially decreased in mitochondria. The mitochondrial ascorbate and dehydroascorbate pools were reduced, whereas the oxidized glutathione levels were maintained. In senescent leaves the H_2O_2 content in isolated mitochondria and the NADH- and succinate-dependent production of superoxide ($O_2^{\cdot-}$) radicals by submitochondrial particles increased significantly. However, in peroxisomes from senescent leaves both membrane-bound APX and MDHAR activities were reduced. In the matrix the DHAR activity was enhanced and the GR activity remained unchanged. As a result of senescence, the reduced and the oxidized glutathione pools were considerably increased in peroxisomes. A large increase in the glutathione pool and DHAR activity were also found in soluble fractions of senescent pea leaves, together with a decrease in GR, APX, and MDHAR activities. The differential response to senescence of the mitochondrial and peroxisomal ascorbate-glutathione cycle suggests that mitochondria could be affected by oxidative damage earlier than peroxisomes, which may participate in the cellular oxidative mechanism of leaf senescence longer than mitochondria.

In plant cells, chloroplasts, mitochondria, and peroxisomes are intracellular generators of activated oxygen species such as H_2O_2, superoxide ($O_2^{\cdot-}$) radicals, hydroxyl radicals (·OH), and singlet oxygen ($^1O_2$) (Boveris, 1984; Fridovich, 1986; Elstner, 1991; del Río et al., 1992; del Río and Donaldson, 1995; López-Huertas et al., 1996, 1997).

Some of these activated oxygen species are highly reactive and, in the absence of protective mechanisms, can produce damage to cell structure and function (Halliwell and Gutteridge, 1989; Elstner, 1991). Under nonstressing conditions the antioxidative defense system provides adequate protection against activated oxygen species (Foyer and Halliwell, 1976; Fridovich, 1986; Asada and Takahashi, 1987). SOD catalyzes the dismutation of $O_2^{\cdot-}$ radicals to molecular oxygen and H_2O_2, thus playing a key role in this defense mechanism (Fridovich, 1986). H_2O_2 scavenging is accomplished by catalase, various peroxidases, and the ascorbate-glutathione cycle, a series of coupled redox reactions involving four enzymes, APX, MDHAR, DHAR, and GR (Foyer and Halliwell, 1976; Nakano and Asada, 1981).

Susceptibility to oxidative stress depends on the overall balance between factors that increase oxidant generation and those cellular components that exhibit an antioxidant capability (Foyer et al., 1994). Oxidative damage in plant tissues is especially important during senescence and is characterized by a notable increase in the metabolism of activated oxygen species (Kar and Feierabend, 1984; Thompson et al., 1987; Halliwell and Gutteridge, 1989). In plants senescence symptoms include chlorophyll and protein loss and increases in lipid peroxidation and membrane permeability, all of which lead to a progressive decrease in the photosynthetic capacity (Thompson et al., 1987).

In a previous study of dark-induced senescence of pea (*Pisum sativum* L.) leaves, it was proposed that peroxisomes play an activated-oxygen-mediated role in the oxidative mechanism of this type of senescence (Pastori and del Río, 1994a). More recently it was reported that natural senescence of pea leaves causes essentially the same changes in peroxisome-activated oxygen metabolism as dark-induced senescence (Pastori and del Río, 1997).

Little is known about the mechanisms involved in the deterioration of mitochondrial electron transport during senescence (Thompson et al., 1987) and the activated-oxygen-related function of mitochondria in leaf senescence. This contrasts with animal systems, in which a role for the generation of mitochondria-activated oxygen species in the aging process has been suggested (Nohl, 1986; Muscari et

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Abbreviations: APX, ascorbate peroxidase; ASC, ascorbate, reduced form; DHA, ascorbate, oxidized form (dehydroascorbate); DHAR, dehydroascorbate reductase; GR, glutathione reductase; HPR, hydroxypyruvate reductase; MDA, malondialdehyde; MDHAR, monodehydroascorbate reductase; SOD, superoxide dismutase.

al., 1990; Yen et al., 1994; Herrero and Barja, 1997). The importance of mitochondria, specifically mitochondrial proteins and DNA, as targets of age-associated free radical attack has been postulated (Yen et al., 1994) and is receiving much attention. A striking relationship between mtDNA damage and glutathione oxidation during aging has also been reported (García de la Asunción et al., 1996).

We recently demonstrated the presence of the ascorbate-glutathione cycle in mitochondria and peroxisomes of pea leaves, and the participation of this cycle in the control of H_2O_2 concentration in both cell organelles has been proposed (Jiménez et al., 1996, 1997). There is considerable evidence supporting the importance of the intracellular distribution of antioxidative enzymes in the mechanisms of protection and cell response to oxidative stress conditions (del Río et al., 1991; Bowler et al., 1992; Hérouart et al., 1993; Edwards et al., 1994; Foyer et al., 1994; Mullineaux and Creissen, 1997).

We studied the response of the ASC-GSH cycle components in mitochondria and peroxisomes during dark-induced senescence of pea leaves with the aim of establishing a relationship between H_2O_2 metabolism and the senescence process. Using soluble fractions, mitochondria, and peroxisomes purified from senescent pea leaves, we analyzed the level of antioxidants and antioxidative enzymes, $O_2^{\cdot -}$ radical production, H_2O_2 concentration, and other oxidative damage parameters.

MATERIALS AND METHODS

Plant Material

Pea (*Pisum sativum* L. cv Lincoln) seeds were obtained from Royal Sluis (Enkhuizen, The Netherlands). Plants were grown in pots containing aerated nutrient solution in a growth chamber under optimal conditions for 20 d, as described by Hernández et al. (1995).

Induction of Senescence

Excised leaves (about 50–60 g fresh weight) from 15- to 17-d-old pea plants (control) were placed in trays floating in air-saturated distilled water and incubated in permanent darkness at 28°C for 11 d (Pastori and del Río, 1994a). Leaves were then washed and used for the different assays.

Purification of Cell Organelles

All operations were performed at 0°C to 4°C. Mitochondria and peroxisomes were isolated from pea leaves after 0 and 11 d in darkness by differential centrifugation, and the washed 12,000*g* particulate pellet, enriched in mitochondria and peroxisomes, was centrifuged in self-generated Percoll gradients (28%, v/v) as previously described (Jiménez et al., 1997). In this isolation procedure the 12,000*g* supernatant was considered to be the soluble fraction. After the sample was centrifuged, fractions of 1.5 mL were collected dropwise by puncturing the bottom of the tubes using a fractionator (model 185, Isco, Lincoln, NE) equipped with an optical unit and an absorbance detector. The integrity of the outer mitochondrial membrane was estimated from the succinate to Cyt *c* oxidoreductase activity, as described by Hernández et al. (1993). The purified mitochondria had intactness between 70% and 90%, and on the basis of the specific activity of HPR, there was a peroxisome contamination of about 13% (Jiménez et al., 1997). Similarly, the mitochondrial contamination of peroxisomes was less than 10%, and the intactness of the purified peroxisomes was between 65% and 70%.

For studies of APX activity, an independent organelle-isolation procedure was used: 20 mM sodium ascorbate was added to the extraction medium, and all other solutions contained 2 mM ascorbate to prevent possible inactivation of APX.

Preparation of Submitochondrial Particles

To obtain submitochondrial particles the method described by Hernández et al. (1993) was followed with some modifications. Particles were prepared by sonication of mitochondria in a disruption medium with a high salt content (20 mM $MgCl_2$), which produced a high percentage of inside-out submitochondrial particles (Petit et al., 1991).

Enzyme Assays

Unless otherwise indicated, the activities of all enzymes were assayed in organelle samples diluted 2- to 5-fold with 50 mM phosphate buffer, pH 7.8, containing 0.1% (v/v) Triton X-100. APX (EC 1.11.1.11), MDHAR (EC 1.6.5.4), DHAR (EC 1.8.5.1), and GR (EC 1.6.4.2) were assayed as described previously (Jiménez et al., 1997).

SOD (EC 1.15.1.1) was analyzed by the ferricytochrome *c* method using xanthine/xanthine oxidase as the source of superoxide radicals (McCord and Fridovich, 1969). SOD isozymes (Cu,Zn-SOD and Mn-SOD) were separated by PAGE on 10% gels, as described by Hernández et al. (1994).

The activities of the organelle-marker enzymes catalase (EC 1.11.1.6), Cyt *c* oxidase (EC 1.9.3.1), fumarase (EC 4.2.1.2), and HPR (EC 1.1.1.29) were assayed as described previously (Hernández et al., 1993; Pastori and del Río, 1994a).

Determination of Total GSH and Total ASC

Total GSH and total ASC were extracted at 0°C from soluble fractions of pea leaves and from mitochondria and peroxisomes isolated from pea leaves at 0 and 11 d of senescence using a medium without ASC and Cys. For each antioxidant, mitochondria and peroxisomes obtained from two different gradients were combined to obtain a higher yield of purified organelles. GSH and GSSH were extracted from samples by mixing with an equal volume of 12% (v/v) perchloric acid containing 2 mM bathophenanthroline disulfonic acid. The resulting acid extract was frozen, thawed, and centrifuged at 12,000*g* for 5 to 10 min. Derivation of the supernatant was carried out as described previously (Farris and Reed, 1987). HPLC analysis was conducted following the protocol described by Asensi et al. (1994). HPLC profiles of these samples were compared

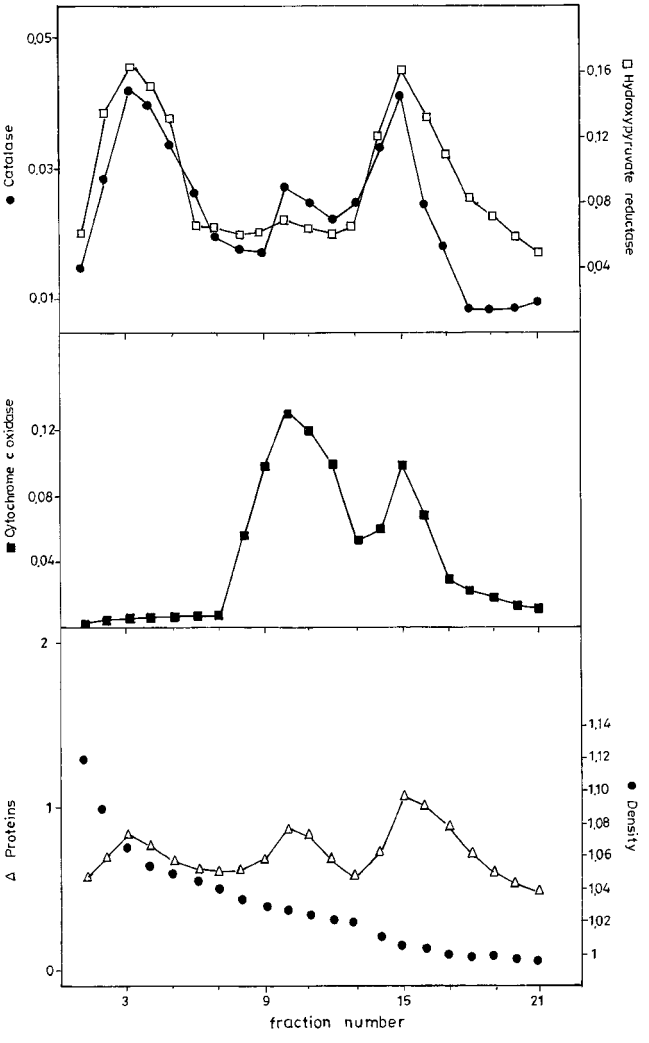

Figure 1. Purification of mitochondria and peroxisomes from dark-induced senescent leaves (11 d of dark incubation). Fractions of 1.5 mL were eluted with a gradient fractionator and the activity of different marker enzymes was assayed. Enzyme activities are expressed in micromoles per minute per milliliter, protein content as milligrams per milliliter, and density as grams per cubic centimeter.

with the profiles of samples treated with the acid solution containing 40 mM *N*-ethylmaleimide before its derivation (Asensi et al., 1994).

Ascorbate was extracted from the soluble fractions and purified mitochondria and peroxisomes by mixing the samples with an equal volume of 10% *m*-phosphoric acid and incubating for 30 min. The mixture was diluted with distilled water to give a final concentration of 2% *m*-phosphoric acid and centrifuged at 12,000*g* for 10 min. ASC and DHA levels in the supernatant were determined by HPLC as described by Castillo and Greppin (1988).

Other Analytical Methods

The H_2O_2 concentration was determined in soluble fractions, mitochondria, and peroxisomes purified from pea leaves at 0 and 11 d of senescence by a peroxidase-coupled assay using 4-aminoantipyrine and phenol as donor substrates (Frew et al., 1983). Soluble fractions (50–100 μL), intact mitochondria (50–200 μL), and peroxisomes (50–200 μL) were added to a reaction mixture containing 25 mM phenol, 5 mM 4-aminoantipyrine, 0.1 M potassium phosphate buffer (pH 6.9), 0.02 μM peroxidase, and 2.5 μM H_2O_2. Quinone-imine formation was measured at 505 nm.

Superoxide radical generation by purified submitochondrial particles was determined by the method of the SOD-inhibitable oxidation of epinephrine using NADH and succinate as respiratory substrates, according to the method of Boveris (1984). The amount of $O_2^{\cdot -}$ radicals produced was calculated using an ϵ_{480} of 4.0 mM^{-1} cm^{-1} for epinephrine (Boveris, 1984). The extent of lipid peroxidation in the different cell compartments was estimated by determining the concentration of substances reacting to thiobarbituric acid (Buege and Aust, 1972). Chlorophyll and proteins were quantified as described by Hernández et al. (1995).

RESULTS

In a previous study two stages in the dark-induced senescence of detached pea leaves were established by measuring various senescence parameters (Pastori and del Río, 1994a). Detached pea leaves were kept in permanent darkness for 14 d, and two clear peaks of ethylene production and O_2 consumption were found at d 3 and 11. The typical symptoms of leaf senescence developed as the dark treatment progressed. Chlorophyll loss, lipid peroxidation, and proteolysis increased throughout the process of dark-induced senescence, especially at d 11, when more than one-half of the chlorophyll had been lost and the formation of MDA and soluble amino acids had increased about 2- to 3-fold (Pastori and del Río, 1994a). In the present study pea leaf organelles were isolated from dark-induced senescent leaves at the second well-defined stage of senescence (11 d of dark treatment), the stage at which severe changes take

Table I. *Specific activities of SOD and ascorbate-glutathione cycle enzymes in mitochondria isolated from young and dark-induced senescent pea leaves*

Values are expressed as the means ± SE of at least four independent experiments.

Senescence	Mn-SOD	APX	MDHAR	DHAR	GR
d	*units* mg^{-1} *protein*		*nmol* min^{-1} mg^{-1} *protein*		
0	189.0 ± 12.0	161 ± 11	98.6 ± 10.5	210 ± 40	25.5 ± 3.2
11	40.6 ± 6.3[a]	78 ± 7[a]	19.5 ± 3.1[a]	30 ± 5[b]	8.7 ± 1.8[b]

[a] Significantly different from control at $P < 0.001$. [b] Significantly different from control at $P < 0.01$.

Table II. *Superoxide production in submitochondrial particles of young and dark-induced senescent leaves*

NADH (50 μM) and succinate (7 mM) were used as substrates, and rotenone (1 μM) and antimycin (1 μM) were used as inhibitors for the superoxide generation assays. Values are expressed as the means ± SE of four different experiments.

Senescence	NADH	NADH + Rotenone	Succinate	Succinate + Antimycin
d	*nmol $O_2^{\cdot-}$ · min^{-1} mg^{-1} protein*			
0	50.4 ± 6.3	56.1 ± 7.3	41.81 ± 8.0	52.3 ± 8.45
11	99.9 ± 12.0[a]	119.7 ± 7.6[b]	85.9 ± 9.7[c]	123.7 ± 10.7[c]

[a] Significantly different from control at $P < 0.05$. [b] Significantly different from control at $P < 0.001$. [c] Significantly different from control at $P < 0.01$.

place, probably leading to cell death (Pastori and del Río, 1994a).

A comparison of Percoll-density gradients of young (control) and dark-induced senescent leaves (11 d of dark treatment) was conducted using marker enzymes of peroxisomes (HPR and catalase) and mitochondria (Cyt *c* oxidase). In the Percoll-density gradients of young leaves, the main peak of peroxisomes was found in fractions 4 to 8, with a maximum equilibrium density of 1.070 g cm^{-3} (Jiménez et al., 1997), a value similar to that previously determined for pea leaf peroxisomes using a different Percoll gradient (Sandalio et al., 1987). These organelles were well separated from intact mitochondria, which banded in fractions 10 to 13 and had an equilibrium density of 1.025 g cm^{-3} (Jiménez et al., 1997). In dark-induced senescent leaves the pattern of the Percoll-density gradients showed some minor changes, with the presence of a broad peak of peroxisomes in fractions 2 to 6, with an equilibrium density of 1.062 g cm^{-3} (Fig. 1). In these leaves, intact mitochondria were also detected as a broad peak that banded in fractions 8 to 13, with a maximum equilibrium density of 1.026 g cm^{-3} (Fig. 1). The specific activity of Cyt *c* oxidase was lower than 10% in the peroxisomal peak, a value similar to that found for control leaves, whereas the peroxisomal contamination of mitochondria was about 15% to 18%, based on the HPR-specific activity. This mitochondrial contamination by peroxisomes was slightly higher than that in mitochondria from young leaves.

The activities of the enzymes involved in the metabolism of activated oxygen were measured in soluble fractions, mitochondria, and peroxisomes from dark-induced senescent pea leaves. After 11 d of senescence the activities of Mn-SOD, APX, MDHAR, DHAR, and GR had substantially decreased by about 78%, 50%, 80%, 85%, and 65%, respectively, in mitochondria (Table I). These changes were parallel to a significant increase in Cyt *c* oxidase-specific activity, which was 3 times higher in senescent mitochondria than in control mitochondria (from 0.18 ± 0.012–0.50 ± 0.04 μmol min^{-1} mg^{-1} protein).

In submitochondrial particles from young leaves $O_2^{\cdot-}$ radicals were generated at slightly higher rates with NADH than with succinate as the substrate, but differences were not statistically significant. In the presence of rotenone the $O_2^{\cdot-}$ production with NADH was slightly increased (up to 11%), whereas antimycin enhanced the $O_2^{\cdot-}$ generation with succinate by about 25%. After 11 d of senescence both the NADH- and the succinate-dependent production of $O_2^{\cdot-}$ radicals showed a significant increase of up to 2-fold in submitochondrial particles. In these senescent conditions, rotenone and antimycin also increased the $O_2^{\cdot-}$ generation rates by NADH and succinate, respectively, although the increase produced by antimycin was higher (about 44%) than that by rotenone (Table II).

Parallel to all of these changes, the H_2O_2 concentration in isolated mitochondria almost doubled during senescence, although the peroxidation of lipids in the membranes expressed as MDA production was apparently unaffected (Table III). In addition to all of these effects induced by leaf senescence, the intactness of the external mitochondrial membrane was reduced, from 70% to 90% in mitochondria from young leaves to 55% to 60% in mitochondria from senescent leaves.

The content of the antioxidants ascorbate and glutathione was also measured in mitochondria after 11 d of leaf senescence, and a large decrease in total ascorbate was found. The mitochondrial ratio of ASC to DHA was reduced, mainly due to the loss of ASC, which was diminished by 70% at 11 d. The GSH pool also decreased after 11 d of dark incubation, to about 60% of the level found in mitochondria from fresh leaves, whereas the GSSH levels were maintained and the ratio of GSH to GSSH decreased (Table IV).

In peroxisomes from senescent pea leaves, the activities of APX and MDHAR were decreased up to 70% and 83%, respectively, compared with those from young leaves; GR activity was similar in peroxisomes of senescent and young leaves; and DHAR activity increased in peroxisomes of senescent leaves, although not significantly (Table V). The peroxisomal content of H_2O_2 increased significantly after

Table III. *H_2O_2 concentration and lipid peroxidation in mitochondria isolated from young and dark-induced senescent leaves*

Values are expressed as the means ± SE of at least six different experiments.

Senescence	MDA	H_2O_2
d	*nmol mL^{-1}*	
0	1.06 ± 0.21	2.83 ± 0.30
11	1.26 ± 0.18[a]	4.33 ± 0.47[b]

[a] Not significantly different from control. [b] Significantly different from control at $P < 0.05$.

Table IV. *Ascorbate and glutathione content in mitochondria isolated from control and dark-induced senescent pea leaves*

Antioxidants were extracted and determined as described in "Materials and Methods." For each antioxidant, mitochondria obtained from two density gradients were combined and processed. Values are expressed as the means ± SE of at least four different experiments.

Senescence	ASC	DHA	ASC/DHA	GSH	GSSG	GSH/GSSG
d	*nmol mg^{-1} protein*			*nmol mg^{-1} protein*		
0	21.63 ± 2.95	2.34 ± 0.57	9.2	5.92 ± 0.12	0.21 ± 0.05	28.2
11	0.11 ± 0.005[a]	0.46 ± 0.17[b]	0.24	3.52 ± 0.13[a]	0.22 ± 0.04[c]	16

[a] Significantly different from control at $P < 0.001$. [b] Significantly different from control at $P < 0.01$. [c] Not significantly different from control.

11 d of senescence. The rate of lipid peroxidation, expressed as MDA production, an indicator of oxidative damage, also significantly increased (about 4-fold) in peroxisomes of dark-induced senescent leaves (Table VI). In contrast to mitochondria, in peroxisomes from leaves at d 11 of senescence, no decreases in the total ascorbate content was found and no changes in the ratio of ASC to DHA took place. Moreover, in senescent peroxisomes a large increase in the total glutathione content, which was mainly GSSH, was observed. This fact was reflected in a significant decrease in the ratio of GSH to GSSH in senescent peroxisomes, although the GSH content was also enhanced.

Regarding the soluble fractions (12,000*g* supernatants) at 11 d of senescence, a significant decrease was found in APX (90%) and GR (70%) activities (Table VII), similar to that occurring in mitochondria. MDHAR activity was slightly decreased (15%), whereas DHAR activity showed a significant 4-fold increase in relation to the controls. Nearly a 2-fold increase of the MDA content in the soluble fraction was observed, and the concentration of H_2O_2 did not change very much after 11 d of senescence (Table VIII). In the soluble fraction the ascorbate content was diminished, with a greater reduction in DHA than in ASC, which was reflected in an increased ratio of ASC to DHA in senescent leaves. Unlike ascorbate, the GSSH content was considerably enhanced by senescence, which was reflected by a decrease in the ratio of GSH to GSSH. This pattern was very similar to that found in peroxisomes of senescent leaves (Table VI).

DISCUSSION

We recently identified the ascorbate-glutathione cycle in mitochondria and peroxisomes of pea leaves and proposed that this cycle could represent an important antioxidant protection system against H_2O_2 generated in both plant cell organelles (Jiménez et al., 1997). During senescence strong oxidative damage takes place, and in this situation the mitochondrial and peroxisomal ascorbate-glutathione cycle could help to inhibit any enhancement of activated oxygen species production.

Analysis of self-generated Percoll-density gradients of dark-induced senescent leaves showed a unique and broad area corresponding to the mitochondrial fraction, which had an equilibrium density very similar to that of the mitochondria of young leaves. This agrees with the results reported by Pastori and del Río (1994a, 1997) in dark-induced and naturally senescent pea leaves.

Dark-induced senescence produced a significant reduction of Mn-SOD, DHAR, and GR activities present in the mitochondrial matrix, as well as APX and MDHAR activities localized in the mitochondrial membrane (Jiménez et al., 1997). The decrease of these enzyme activities seems to be a specific response induced by senescence in the mitochondrial antioxidative enzymes, since Cyt *c* oxidase activity was significantly increased in senescent leaves.

Both ASC and DHA decreased significantly in mitochondria of dark-induced senescent leaves, probably because of the H_2O_2-mediated oxidation of ASC and the degradation of DHA. As suggested by Foyer et al. (1994) and Smirnoff and Palanca (1996), the ASC pool could be reduced by oxidative stress when the capacity of regenerative systems is exceeded. The content of GSH was also significantly decreased in pea leaf mitochondria during senescence, which was probably due to the decrease in GR activity that took place under the same conditions.

In dark-induced senescent leaves a significant increase in the mitochondrial H_2O_2 concentration was found. Similar results have been described by Boveris et al. (1978) in aged potato tuber submitochondrial particles, in which $O_2^{\cdot-}$ generation was also increased. In the present study rotenone increased the NADH-dependent $O_2^{\cdot-}$ generation in submitochondrial particles from young and senescent pea leaves. At least four NADH dehydrogenases have been

Table V. *Specific activities of ascorbate-glutathione cycle enzymes in peroxisomes isolated from young and dark-induced senescent pea leaves*

Values are expressed as the means ± SE of at least four different experiments.

Senescence	APX	GR	MDHAR	DHAR
d	*nmol min^{-1} mg^{-1} protein*			
0	281 ± 43	12.7 ± 2.3	32.8 ± 3.6	25 ± 5
11	83 ± 13[a]	14.6 ± 2.6[b]	5.6 ± 0.9[c]	40 ± 7[b]

[a] Significantly different from control at $P < 0.01$. [b] Not significantly different from control. [c] Significantly different from control at $P < 0.001$.

Table VI. *Lipid peroxidation, H_2O_2, ascorbate, and glutathione content in peroxisomes isolated from young and dark-induced senescent pea leaves*

Antioxidants were extracted and determined as described in "Materials and Methods." For each antioxidant, peroxisomes obtained from two density gradients were combined and processed. Values are expressed as the means ± SE of at least six (MDA and H_2O_2) and four (ascorbate and glutathione) different experiments.

Senescence	MDA	H_2O_2	ASC	DHA	ASC/DHA	GSH	GSSG	GSH/GSSG
d	*nmol mL^{-1}*		*nmol mg^{-1} protein*			*nmol mg^{-1} protein*		
0	3.6 ± 0.3	2.9 ± 0.3	3.7 ± 0.7	5.4 ± 0.9	0.68	4.1 ± 0.8	0.15 ± 0.03	27.3
11	17.6 ± 1.9[a]	17.3 ± 1.4[b]	4.9 ± 0.9[c]	7.2 ± 1.1[c]	0.68	33.6 ± 9.6[a]	51.8 ± 10.2[b]	0.64

[a] Significantly different from control at $P < 0.01$. [b] Significantly different from control at $P < 0.001$. [c] Not significantly different from control.

reported in plant mitochondria (Douce, 1985; Møller, 1997). In the present study the sites where the electrons from NADH entered the transport chain of the submitochondrial particles were not determined. Exogenous NADH may have entered at more than one site (Møller, 1997). Rotenone inhibits strongly but not completely the NADH oxidation by inside-out submitochondrial particles (Petit et al., 1991) at the complex I segment of the respiratory chain (Douce, 1985; Siedow and Umbach, 1995).

Since there was a high proportion of inside-out submitochondrial particles in the pea leaves in our study, rotenone should have inhibited a considerable part of the electron transport from the internal dehydrogenase to oxygen. Therefore, the main $O_2^{\cdot-}$ production we found in submitochondrial particles with NADH and rotenone could have been derived from the dehydrogenase region. Antimycin A, by blocking the electron flow to Cyt *c* oxidase, should enhance $O_2^{\cdot-}$ production by ubisemiquinone (Rich and Bonner, 1978). Our results for $O_2^{\cdot-}$ radical production by submitochondrial particles with succinate plus antimycin showed that in senescent mitochondria the antimycin-inhibited region, i.e. ubiquinone-Cyt *b*, is also involved in the increase of $O_2^{\cdot-}$ generation.

Although in submitochondrial particles from senescent leaves the $O_2^{\cdot-}$ generation with succinate and antimycin was higher than with NADH and rotenone, differences were not statistically significant. Similarly, no significant differences were found for $O_2^{\cdot-}$ generation with NADH and succinate. Although some NADH-dependent $O_2^{\cdot-}$ production could have taken place after complex I, our results suggest that both the flavoprotein dehydrogenase and the antimycin-inhibited regions are involved in the increased $O_2^{\cdot-}$ generation during senescence. These results disagree with those found in submitochondrial particles from potato tubers (Boveris et al., 1978). Taking into account that $O_2^{\cdot-}$ radicals are considered the main precursors of mitochondrial H_2O_2 (Boveris and Cadenas, 1982; Nohl, 1986), we are tempted to propose that the senescence-induced increase in pea mitochondrial H_2O_2 might be due to higher rates of $O_2^{\cdot-}$ production in mitochondria from senescent pea leaves.

However, in mitochondria from senescent leaves, the MDA content, an indicator of lipid peroxidation, showed no significant increase. This could be explained by the fact that MDA can be readily metabolized by mitochondria, as was reported by Muscari et al. (1990). Conversely, in mitochondria from senescent leaves we found a loss in the integrity of the outer mitochondrial membrane, which has usually been considered to be an intrinsic feature of oxidative damage in senescent and/or stressed tissues (Thompson et al., 1987; Buchanan-Wollaston, 1997). The loss of membrane integrity agrees with previous results from electron microscopy studies showing that senescence of pea leaves induced a deterioration in the mitochondrial membrane structure and a slight disorganization in the matrix and cristae (Pastori and del Río, 1994a). A decrease in mitochondrial membrane integrity could allow the leakage of H_2O_2 from the mitochondria into the cytosol during senescence. This extrusion of H_2O_2 could be favored by the decrease of APX and MDHAR activities in mitochondrial membranes.

In the Percoll-density gradients of dark-induced senescent leaves, a unique and broad band of peroxisomal fraction was found, which had an equilibrium density similar to peroxisomes of young and naturally senescent pea leaves (Pastori and del Río, 1997). In contrast to mitochondria, ultrastructural membrane alterations in senescent peroxisomes were not particularly intense.

In peroxisomal matrices of senescent leaves DHAR activity was slightly enhanced, whereas the GR activity re-

Table VII. *Specific activities of the SOD isozymes and ascorbate-glutathione cycle enzymes in the soluble fraction of young and dark-induced senescent pea leaves*

Values are expressed as the means ± SE of at least three different experiments.

Senescence	Mn-SOD	Cu,Zn-SOD I	Cu,Zn-SOD II	APX	MDHAR	DHAR	GR
d	*units mg^{-1} protein*			*nmol min^{-1} mg^{-1} protein*			
0	1.9 ± 0.2	10.6 ± 0.4	10.3 ± 0.1	258 ± 40	69.0 ± 4.0	5.6 ± 1.3	34.6 ± 9.2
11	nd[a]	9.7 ± 0.3[b]	10.4 ± 0.2[b]	24 ± 4[c]	57.6 ± 6.1[b]	20.7 ± 1.2[d]	9.7 ± 0.9[e]

[a] nd, Not detected. [b] Not significantly different from control. [c] Significantly different from control at $P < 0.01$. [d] Significantly different from control at $P < 0.001$. [e] Significantly different from control at $P < 0.05$.

Table VIII. *Lipid peroxidation, H_2O_2, ascorbate, and glutathione content in the soluble fraction of young and dark-induced senescent pea leaves*

Values are expressed as the means ± SE of at least four different experiments.

Senescence	MDA	H_2O_2	ASC	DHA	ASC/DHA	GSH	GSSG	GSH/GSSG
d	*nmol mL^{-1}*		*nmol mg^{-1} protein*			*nmol mg^{-1} protein*		
0	21.5 ± 2.1	0.34 ± 0.02	320.4 ± 38.8	9.7 ± 1.1	33	11.8 ± 1.7	0.32 ± 0.05	36.8
11	38.5 ± 4.8[a]	0.20 ± 0.03[b]	45.4 ± 11.3[c]	0.5 ± 0.2[c]	90	30.4 ± 5.2[b]	51.4 ± 9.1[c]	0.6

[a] Significantly different from control at $P < 0.05$. [b] Significantly different from control at $P < 0.01$. [c] Significantly different from control at $P < 0.001$.

mained unchanged. A slight increase of the total ASC pool was found in senescent leaf peroxisomes, without any significant change in the ratio of ASC to DHA. An important increase in the total GSH was detected, although the ratio of GSH to GSSH was strongly decreased. These results are consistent with GR and DHAR acting against ASC oxidation probably produced by H_2O_2 and/or $O_2^{\cdot-}$ radicals in senescent leaf peroxisomes. The increase of the H_2O_2 concentration and MDA content, an indicator of lipid peroxidation, found in peroxisomes of senescent pea leaves is in accordance with previous results showing increases in $O_2^{\cdot-}$ radical production, H_2O_2 concentration, and lipid peroxidation in peroxisomes of dark-induced and naturally senescent pea leaves (Pastori and del Río, 1994a, 1997). As a result, a rapid, activated-oxygen-mediated oxidation of GSH could take place in leaf peroxisomes during senescence. The possibility of a higher rate of GSH synthesis and/or a more efficient import of GSH into peroxisomes from chloroplasts or cytosol should also be considered, since the total GSH pool was considerably increased in peroxisomes from senescent leaves. The transport of GSH and GSSH through different cellular membranes has previously been described in plant systems (Schneider et al., 1992; Tommasini et al., 1993; Jamai et al., 1996).

In previous reports it was proposed that during pea leaf senescence an enhancement of the extrusion of peroxisomal membrane-generated $O_2^{\cdot-}$ radicals into the cytosol could occur, along with the leakage of overproduced H_2O_2 out of peroxisomes (Pastori and del Río, 1994a, 1997). In our study the decrease of APX and MDHAR activities, both localized in the peroxisomal membranes, could have enhanced this potentially serious situation for all of the cellular compartments because of the possible formation of the strongly oxidizing ·OH radicals produced by the reaction of H_2O_2 with $O_2^{\cdot-}$ radicals (Elstner, 1987; Halliwell and Gutteridge, 1989). Moreover, it is very likely that the peroxisomal NADH-dependent production of $O_2^{\cdot-}$ radicals is intensified by the reverse transition of leaf peroxisomes to glyoxysomes that occurs during senescence (Landolt and Matile, 1990; Pistelli et al., 1996; Pastori and del Río, 1997), since more NADH would be available as a result of the induction of fatty acid β-oxidation and the glyoxylate cycle.

$O_2^{\cdot-}$, H_2O_2, and GSH can act as messenger molecules in cellular signal transduction pathways and also as factors in plant defense responses (Saran and Bors, 1989; Hérouart et al., 1993; Levine et al., 1994; Prasad et al., 1994; del Río et al., 1996; Karpinski et al., 1997). Mitochondria and peroxisomes could play a role in the oxidative mechanism of senescence in pea leaves (Pastori and del Río, 1994a, 1994b, 1997) by favoring the leakage into the cytosol of $O_2^{\cdot-}$ and H_2O_2, two activated-oxygen transduction signals that could lead to the expression of specific genes involved in leaf senescence.

Our results suggest that during senescence oxidative injuries could be accelerated in mitochondria in relation to peroxisomes because of the depression of the antioxidant system of mitochondria, resulting in an enhanced H_2O_2 production and membrane damage. Peroxisomes, however, may function longer than mitochondria in the oxidative mechanism of senescence, because they are able to respond to increased activated-oxygen production with increased synthesis of antioxidant systems that could partly counteract the accumulation of activated oxygen species.

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