Antioxidative enzymes from chloroplasts, mitochondria, and peroxisomes during leaf senescence of nodulated pea plants

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Abstract

In this work the influence of the nodulation of pea (Pisum sativum L.) plants on the oxidative metabolism of different leaf organelles from young and senescent plants was studied. Chloroplasts, mitochondria, and peroxisomes were purified from leaves of nitrate-fed and Rhizobium leguminosarum-nodulated pea plants at two developmental stages (young and senescent plants). In these cell organelles, the activity of the ascorbate–glutathione cycle enzymes ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR), and the ascorbate and glutathione contents were determined. In addition, the total superoxide dismutase (SOD) activity, the pattern of mitochondrial and peroxisomal NADPH-generating dehydrogenases, some of the peroxisomal photorespiratory enzymes, the glyoxylate cycle and oxidative metabolism enzymes were also analysed in these organelles. Results obtained on the metabolism of cell organelles indicate that nodulation with Rhizobium accelerates senescence in pea leaves. A considerable decrease of the ascorbate content of chloroplasts, mitochondria, and peroxisomes was found, and in these conditions a metabolic conversion of leaf peroxisomes into glyoxysomes, characteristic of leaf senescence, took place.

Key words: Ascorbate–glutathione cycle, chloroplasts, mitochondria, peroxisomes, root nodules, senescence.

Introduction

Leaf senescence is a genetically regulated process characterized by oxidative stress and chlorophyll and protein breakdown. During senescence, an enhancement of reactive oxygen species (ROS) is produced along with an increase in proteolytic activity (del Río et al., 1998; Palma et al., 2002). ROS are maintained under certain levels by a battery of molecules with antioxidant capacity. The most important antioxidative enzymes are catalase, peroxidases, superoxide dismutase (SOD), and those of the ascorbate–glutathione cycle, a series of coupled redox reactions involving four enzymes: ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR). The main non-enzymatic antioxidant molecules are ascorbate and glutathione, which are also integrated in the cycle mentioned above, and α-tocopherol, β-carotene, and flavonoids (Noctor and Foyer, 1998; Halliwell and Gutteridge, 2000).

The ascorbate–glutathione cycle is an important antioxidant protection system against H2O2 generated in different cell compartments. Its occurrence has been reported in chloroplasts, mitochondria, peroxisomes, and cytoplasm (Dalton et al., 1993; Jiménez et al., 1997; Noctor and...
Foyer, 1998; Asada, 2000). SOD is also present in these four compartments. Chloroplasts contain basically the CuZn-SOD and Fe-SOD isoforms, whereas in mitochondria only the Mn-SOD isozyme has been clearly localized (Alscher et al., 2002; del Río et al., 2003a). In peroxisomes, both CuZn-SOD and Mn-SOD have been found either as soluble proteins in the organelle matrix, or bound to membranes in the case of Mn-SOD (del Río et al., 2003a, b). Catalase is localized in peroxisomes and is the most characteristic marker enzyme of these organelles (Baker and Graham, 2002).

In chloroplasts, leaf senescence produces the disorientation of the grana, dilation of thylakoids, and a considerable loss of photosynthetic pigments with a concomitant formation of plastoglobuli (Smart, 1994; del Río et al., 1998). However, mitochondria remain relatively intact until the last stages of senescence (Quirino et al., 2000). In peroxisomes, the photorespiratory pathway is diminished during leaf senescence whereas the glyoxylate cycle enzymes are induced (Buchanan-Wollaston, 1997), thus allowing the energy provision by a combined action of the β-oxidation and glyoxylate cycle (Pistelli et al., 1996; del Río et al., 1998). In this process, leaf peroxisomes are metabolically converted into glyoxysome-type peroxisomes (Nishimura et al., 1996), and this is accompanied by a proliferation of the peroxisomal population (Droillard and Paulin, 1990; Pastori et al., 1997).

In leguminous plants, nodules are able to fix atmospheric nitrogen by the action of nitrogenase, but in the absence of oxygen which is a strong ROS-mediated inhibitor of this enzyme (Gage and Margolin, 2000; Santos et al., 2000). The nodule bacteria and plant metabolism interact in a complex process where a number of genes and signal molecules belonging to both counterparts are involved (Schultze and Kondorosi, 1996; Hirsch, 1999; Gage and Margolin, 2000). Finally, nitrogen fixed in root nodules is exported to the aerial part of the plant and used for plant growth and development (Gage and Margolin, 2000; Schultze and Kondorosi, 1996; Puppo et al., 2005).

Different studies have focused on the alterations of nodules during senescence, which are associated to oxidative stress and proteolytic processes and have some similarities to the process of leaf senescence (Pladys and Vance, 1993; Kardailsky and Brewin, 1996; Matamoros et al., 2003; Sheokand and Brewin, 2003; Puppo et al., 2005). Nevertheless, the influence of nodulation on leaf senescence and its associated oxidative metabolism has not been studied in nodulated plants at the subcellular level.

In this work, the influence of nodulation on leaf senescence was investigated in different cell compartments of pea plants. For this purpose, a battery of antioxidative enzymes and the non-enzymatic antioxidants ascorbate and glutathione, distributed in chloroplasts, mitochondria, and peroxisomes, were analysed in young and senescent leaves from both nitrate-fed and Rhizobium-nodulated pea plants.

**Materials and methods**

**Plant material and growth conditions**

Pea seeds (*Pisum sativum* L., cv. Phoenix) were kindly supplied by Dr Peter Römer from Südwesterdeutsche Saatzeucht (Rastatt, Germany). Seeds were surface-sterilized with ethanol for 3 min and were germinated in sterilized vermiculite for 14 d in controlled-environment chambers. Healthy and vigorous seedlings were then selected and planted in two sets of vermiculite pots. One set was grown with nitrogen-free Hoagland’s nutrient solution (Olivares et al., 1980) and these seedlings were inoculated with 60 ml of a suspension of *Rhizobium leguminosarum* biovar. *viciae* (c. 10^6 cells per plant) obtained from Microbio Rhizogen Corporation, UK. The second set of plants was grown with the same solution, supplemented with 5 mM KNO₃, and was not inoculated (controls). Plants were grown in a controlled environmental chamber with a day/night temperature cycle of 25/19 °C, 60/85% relative humidity, 600 μmol m⁻² s⁻¹ (incandescent 40 W Philips; fluorescent: Sylvania 24-P96T12VHO/CW) and 14 h photoperiod. Plants were grown for 12 weeks until an advanced fructification stage when both nodules and leaves had fully senesced. Nine-week-old plants, which were initiating the flowering stage, were considered as young plants.

**Purification of cell organelles**

All operations were performed at 0–4 °C. Chloroplasts, mitochondria, and peroxisomes were purified by differential and density-gradient centrifugations. For each organelle, the whole purification procedure was carried out both in the absence and the presence of ascorbate. Samples for the determination of ascorbate peroxidase activity were prepared in the presence of ascorbate. Thus, extraction media of leaves contained either 0 or 20 mM ascorbate, whereas all the other media used in each purification procedure, including the gradient layers, contained either 0 or 2 mM ascorbate.

**Chloroplasts**

For the purification of chloroplast from pea leaves, the method based on the centrifugation in Percoll density-gradients (21–60%, p/v) was used (Palma et al., 1986). This method results in two chloroplast bands in the Percoll gradients with different densities but sharing similar intactness (78–85%) (Palma et al., 1986). To remove Percoll from the gradient samples, the chloroplast bands were diluted five times with a medium containing 20 mM MOPS-KOH, pH 7.2, 0.3 M mannitol, 1 mM EDTA, and either 0 or 2 mM ascorbate, and they then were centrifuged at 12 000 g for 20 min. The pellets were resuspended in the same medium and used for further assays.

**Mitochondria**

Mitochondria were purified from pea leaves by a method which involves a self-generated Percoll gradient, as described by Jiménez et al. (1997). This method supplies mitochondrial fractions with less than 10% cross-contamination by peroxisomes and intactness percentages between 70–90%.

**Peroxisomes**

For the purification of peroxisomes from pea leaves two different methods were used. To determine the activity of different characteristic peroxisomal enzymes (catalase, hydroxyproryvate reductase, glycolate oxidase, and malate synthase), organelles were purified by sucrose density-gradient centrifugation (López-Huertas et al., 1995). Peroxisomes purified by this method showed less than 2% contamination by other organelles and intactness percentages of about 90%. However, for the analysis of superoxide dismutase activity, H₂O₂ content, and levels of ascorbate and glutathione, a method based on Percoll density-gradient centrifugation was used which
supplies 70% intact peroxisomes (Sandalio et al., 1987). Percoll was removed from gradient samples as indicated above for chloroplasts.

**Enzyme assays**

Ascorbate peroxidase (APX; EC 1.11.1.11) was determined by monitoring the initial ascorbate oxidation by H2O2 at 290 nm (Hossain and Asada, 1984). To test the feasibility of the assay, 20 mM p-chloromercuriphenylsulphonic acid (pCMS), a specific inhibitor of APX, was used (Mittler and Zilinskas, 1991). Monodehydroascorbate reductase (MDHAR; 1.6.5.4) was assayed by measuring the monodehydroascorbate-dependent NADH oxidation, and monodehydroascorbate was generated by the ascorbate/ascorbate oxidase system (Arrigoni et al., 1981). The rate of monodehydroascorbate-independent NADH oxidation (without ascorbate and ascorbate oxidase) was subtracted from the monodehydroascorbate-dependent reaction.

Dehydroascorbate reductase (DHAR; EC 1.8.5.1) was determined according to the method of Dalton et al. (1993) by following the increase of ascorbate formation at 265 nm using N2-saturated buffer. The reaction rate was corrected by the non-enzymatic reduction of dehydroascorbate by glutathione (GSH). A factor of 0.98, to account for the small contribution to the absorbance by GSSG, was also considered. Glutathione reductase (GR; EC 1.6.4.2) was assayed by monitoring the NADPH oxidation coupled to the reduction of GSH (Edwards et al., 1990). The reaction rate was corrected for the small, non-enzymatic oxidation of NADPH by GSSG. Superoxide dismutase (SOD; EC 1.15.1.1) was determined by the ferricytochrome c reduction method using xanthine/xanthine oxidase as the source of superoxide radicals (McCord and Fridovich, 1969).

NADP-dehydrogenases were determined by monitoring the NADPH formation at 340 nm, but using their specific substrates, and following basically the methods previously described for these enzymes (Corpas et al., 1998, 1999). The substrates used were: oxaloacetate for NADP-malate dehydrogenase (NADP-MDH; EC 1.1.1.82), malate for the malic enzyme (ME; EC 1.1.1.40), glucose-6-phosphate for glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49), 6-phosphogluconate for 6-phosphogluconate dehydrogenase (6PGDH; EC 1.1.1.44), and isocitrate for the NADP-isocitrate dehydrogenase (NADP-ICDH; EC 1.1.1.42). The total NADPH rate formation in purified organelles was calculated by the sum of their respective dehydrogenase activities, NADP-MDH, ME, and NADP-ICDH in mitochondria and G6PDH, 6PGDH and NADP-ICDH in peroxisomes.

Hydroxyproylurate reductase (HPR; EC 1.1.1.29) was assayed by following the oxidation of NADH (Schwitzguebel and Segenthaler, 1984), and glycolate oxidase (GOX; EC 1.1.3.1) was determined by monitoring the formation of the complex glyoxylate–phenylhydrazone at 324 nm (Kerr and Groves, 1975). Malate synthase (MS; EC 4.1.3.2) activity was analysed by measuring the absorbance at 412 nm of the mercaptide group formed by 5,5’-dithio-bis-(2-nitrobenzoic) acid and the free acetyl-CoA generated by the enzyme (Hock and Beevers, 1966). The determination of catalase activity was performed by the method of Aebi (1984). Protein concentration was determined according to Bradford (1976) using BSA as standard.

**Determination of total ascorbate and glutathione in organelles**

The antioxidants ascorbate and glutathione were determined in chloroplasts, mitochondria, and peroxisomes immediately after the purification of these organelles by Percoll density-gradient centrifugation. All media used for these determinations were free of ascorbate and cysteine. Basically, the methods described by Jiménez et al. (1997) were followed.

Total ascorbate was extracted with 5% (v/v) phosphoric acid, and the levels of reduced (ASC) and oxidized (DHA) ascorbate in organelles were determined by HPLC (Castillo and Greppin, 1988). GSH and GSSG were extracted with 12% perchloric acid containing 2 mM bathophenanthroline disulphonic acid. Then samples were frozen, centrifuged and derivatized (Farris and Reed, 1987), and an internal γ-Glu-Glu-Glu standard was used. HPLC analysis was performed following the protocol described by Asensi et al. (1994).

**Results**

In recent studies carried out in our laboratory in pea plants, the effect of nodulation with *Rhizobium leguminosarum* on the relationship between leaf senescence and oxidative stress was studied (Vanacker et al., 2006). In nitrate-fed (controls) and nodulated pea plants different physiological parameters were investigated during plant growth, and in leaf crude extracts the level of H2O2, and the extent of lipid peroxidation and protein oxidation were also determined. The results obtained established that plants grown for 9 weeks were at the initial flowering stage, and after 12 weeks they were at advanced fructification and were clearly senescent. Accordingly, in this work for comparative purposes 9-week-old plants were considered as young plants, whereas 12-week-old plants were regarded as senescent plants.

**Chloroplasts**

The activity of the ascorbate–glutathione cycle and other organelle-specific enzymes was determined in chloroplasts, mitochondria, and peroxisomes isolated from pea leaves of nitrate-fed and nodulated young and senescent plants. In chloroplasts from nitrate-fed plants, APX, MDHAR and DHAR activities notably decreased in senescent plants compared with young plants (Fig. 1). However, in nodulated plants only DHAR was clearly affected by senescence, and, to a lower extent, APX, GR activity did not change with time in both plant treatments. In general, all enzyme activities were higher in nitrate-fed than in nodulated young plants, especially APX and MDHAR.

In chloroplasts, the content of reduced ascorbate (ASC) did not vary in nitrate-fed and nodulated plants of the same age (Table 1). However, in both treatments a considerable decline of ASC was observed in senescent plants. The oxidized form of ascorbate (dehydroascorbate, DHA) was only detected in senescent plants, and the ratio ASC/DHA was similar in both sets of plants (Table 1). In senescent plants the content of reduced glutathione (GSH) was slightly higher in nodulated than in nitrate-fed plants. The content of oxidized glutathione (GSSG) considerably decreased with time in nitrate-fed plants, but in nodulated plants this parameter was not modified. Comparison among treatments revealed that the GSSG content was negatively affected by nodulation (Table 1). In all types of plants, reduced glutathione (GSH) was the predominant form of this non-enzymatic antioxidant. The GSH/GSSG ratio was considerably higher in nodulated than in nitrate-fed
plants, and this ratio was strongly enhanced by senescence in nitrate-fed plants.

**Mitochondria**

In mitochondria isolated from pea leaves, most of the ascorbate–glutathione cycle enzyme activities increased with time in both sets of plants, the most apparent response being observed in nodulated plants (Fig. 2). Nevertheless, APX activity diminished with senescence in the nitrate-fed treatment. Comparisons between both plant treatments showed that in senescent nodulated plants MDHAR, DHAR, and GR activities were much higher than in nitrate-fed ones. In mitochondria from young plants the ascorbate content increased with nodulation, but this parameter declined in senescent nodulated plants (Table 2). No DHA could be detected in mitochondria of both nitrate-fed and nodulated plants. GSH was the predominant form in mitochondria from the two treatments and decreased with time in nitrate-fed plants. The GSH/GSSG ratio increased with senescence in nitrate-fed plants but under the same conditions diminished in nodulated plants.

In purified mitochondria, the activities of NADP-dependent malate dehydrogenase (NADP-MDH) and NADP-dependent malic enzyme (NADP-ME) notably decreased in senescent leaves of both nitrate-fed and nodulated plants, while an opposite pattern was observed for the NADP-isocitrate dehydrogenase (NADP-ICDH) which increased in senescent plants (Fig. 3). The comparison between both plant treatments showed that only NADP-MDH activity was negatively affected by nodulation, especially in young plants. As indicated in Fig. 3, the theoretical total amount of NADPH generated in leaf mitochondria by these three dehydrogenases did not vary significantly as a consequence of either the plant nitrogen regime or age.

**Peroxisomes**

The ascorbate–glutathione cycle was also studied in peroxisomes isolated from leaves of nitrate-fed and nodulated plants. APX and MDHAR activities were reduced in

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**Table 1. Ascorbate and glutathione contents in chloroplasts from nitrate-fed and nodulated pea plants at two different growth times**

<table>
<thead>
<tr>
<th></th>
<th>ASC (µg 100⁻¹ g leaves)</th>
<th>DHA (µg 100⁻¹ g leaves)</th>
<th>ASC/DHA</th>
<th>GSH (ng 100⁻¹ g leaves)</th>
<th>GSSG (ng 100⁻¹ g leaves)</th>
<th>GSH+GSSG (ng 100⁻¹ g leaves)</th>
<th>GSH/GSSG</th>
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<tr>
<td><strong>Nitrate-fed</strong></td>
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<tr>
<td>9 weeks</td>
<td>48.38±2.25</td>
<td>ND</td>
<td></td>
<td>1.52±0.40</td>
<td>0.075±0.002</td>
<td>1.595</td>
<td>20.3</td>
</tr>
<tr>
<td>12 weeks</td>
<td>7.13±0.38</td>
<td>1.71±0.43</td>
<td>4.17</td>
<td>1.42±0.09</td>
<td>0.013±0.001</td>
<td>1.433</td>
<td>109.2</td>
</tr>
<tr>
<td><strong>Nodulated</strong></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>9 weeks</td>
<td>48.00±2.25</td>
<td>ND</td>
<td></td>
<td>1.49±0.07</td>
<td>0.004±0.001</td>
<td>1.526</td>
<td>413.9</td>
</tr>
<tr>
<td>12 weeks</td>
<td>6.75±0.38</td>
<td>1.71±0.43</td>
<td>3.95</td>
<td>1.88±0.11</td>
<td>0.004±0.001</td>
<td>1.923</td>
<td>437.2</td>
</tr>
</tbody>
</table>

Values are expressed as the mean±SEM of at least three independent experiments. ND, not detected.
senescent plants of both treatments (Fig. 4). On the contrary, DHAR and GR increased with time in both treatments. The effect of nodulation on these enzymes was very clear since both DHAR and, to a lower extent, GR were reduced in comparison with nitrate-fed plants. In peroxisomes only the reduced ascorbate form (ASC) was detected by HPLC (Table 3). The ASC content was higher in nitrate-fed plants that in nodulated ones. As reported for mitochondria, no DHA could be detected in leaf peroxisomes from both sets of plants. No changes were observed in the reduced form of glutathione (GSH) as a result of nodulation or age. The GSH/GSSG ratio was enhanced at the senescence stage in both nodulated and nitrate-fed plants.

Some of the enzymes involved in the main metabolic pathways of peroxisomes were analysed. The photorespiratory enzymes glycolate oxidase (GOX) and hydroxypropyruvate reductase (HPR) diminished in senescent plants of both treatments (Fig. 5). Comparison between nitrate-fed and nodulated plants showed that the activity of these two photorespiratory enzymes was negatively affected by nodulation. The activity of the glyoxylate cycle enzyme malate synthase (MS) behaved in an opposite way to that of the photorespiratory enzymes. Considerable increases were observed in senescent plants, and this effect was more dramatic in nodulated plants. With respect to catalase, a characteristic enzyme of the oxidative metabolism of peroxisomes, its activity pattern was similar to that of GOX and HPR, with notable decreases as a result of plant senescence and nodulation.

The activity of the NADPH-generating dehydrogenase from leaf peroxisomes, glucose-6-phosphate dehydrogenase (G6PDH) decreased with time but not with plant nodulation (Fig. 6). 6-Phosphogluconate dehydrogenase (6PGDH) was not modified by senescence in both treatments, although this enzyme activity was much lower in nodulated plants compared with the nitrate-fed ones. The NADP-dependent isocitrate dehydrogenase activity (NADP-ICDH) was higher in nitrate-fed plants and was

Table 2. Ascorbate and glutathione content in mitochondria from nitrate-fed and nodulated pea plants at two different growth times
Values are expressed as the mean ± SEM of, at least, three independent experiments. ND, not detected.

<table>
<thead>
<tr>
<th></th>
<th>ASC (ng 100⁻¹ g leaves)</th>
<th>DHA (ng 100⁻¹ g leaves)</th>
<th>GSH (ng 100⁻¹ g leaves)</th>
<th>GSSG (ng 100⁻¹ g leaves)</th>
<th>GSH+GSSG (ng 100⁻¹ g leaves)</th>
<th>GSH/GSSG</th>
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<tr>
<td>Nitrate-fed</td>
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<tr>
<td>9 weeks</td>
<td>92.5±12.3</td>
<td>ND</td>
<td>0.83±0.13</td>
<td>0.018±0.006</td>
<td>0.848</td>
<td>46.1</td>
</tr>
<tr>
<td>12 weeks</td>
<td>97.9±21.4</td>
<td>ND</td>
<td>0.45±0.05</td>
<td>0.002±0.001</td>
<td>0.452</td>
<td>225</td>
</tr>
<tr>
<td>Nodulated</td>
<td></td>
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<tr>
<td>9 weeks</td>
<td>235.5±29.6</td>
<td>ND</td>
<td>0.75±0.03</td>
<td>0.019±0.004</td>
<td>0.769</td>
<td>39.5</td>
</tr>
<tr>
<td>12 weeks</td>
<td>109.5±25.1</td>
<td>ND</td>
<td>0.80±0.08</td>
<td>0.073±0.019</td>
<td>0.873</td>
<td>11.0</td>
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Antioxidative enzymes in nodulated peas

Fig. 2. Activities of the ascorbate–glutathione cycle enzymes in mitochondria from nitrate-fed and nodulated pea plants at two different growth times. Values are expressed as the mean ±SEM of at least three independent experiments. APX, ascorbate peroxidase; MDHAR, monodehydroascorbate reductase; DHAR, dehydroascorbate reductase; GR, glutathione reductase.
slightly lowered by senescence in both plant treatments. As a consequence of all these changes, in peroxisomes, the theoretical amount of NADPH generated by the endogenous NADP-dehydrogenases slightly decreased with senescence in both treatments, and was negatively affected by nodulation (Fig. 6).

Finally, considering the localization of superoxide dismutase isozymes in different cell compartments, the effect of nodulation on superoxide dismutase (SOD) activity was analysed in chloroplasts, mitochondria, and peroxisomes purified from the two sets of pea plants. In chloroplasts, SOD decreased both with time and with nodulation, but the opposite pattern was observed in peroxisomes (Table 4). In these organelles, SOD showed a considerable increase in senescent plants from both treatments, and this activity was also enhanced by nodulation. By contrast, no significant differences were observed in the mitochondrial SOD activity of nitrate-fed and nodulated plants.

Fig. 3. Mitochondrial NADPH-generating enzymes from nitrate-fed and nodulated pea plants at two different growth times. Values are expressed as the mean ± SEM of at least three independent experiments. NADP-MDH, NADP-dependent malate dehydrogenase; NADP-EM, NADP-dependent malic enzyme; NADP-ICDH, NADP-dependent isocitrate dehydrogenase.

Fig. 4. Activities of the ascorbate–glutathione cycle enzymes in peroxisomes from nitrate-fed and nodulated pea plants at two different growth times. Values are expressed as the mean ± SEM of at least three independent experiments. APX, ascorbate peroxidase; MDHAR, monodehydroascorbate reductase; DHAR, dehydroascorbate reductase; GR, glutathione reductase.
The effect of soil nitrogen limitation on some foliar antioxidants distributed in chloroplasts and mitochondria from spinach leaves was studied by Logan et al. (1999), and there are some reports on antioxidants of legume nodule organelles and their role in nitrogen fixation (Dalton et al., 1993; Iturbe-Ormaetxe et al., 2001). However, as far as is known, this is the first report on the effect of nodulation on the relationship between leaf antioxidants and senescence in different cell compartments.

Results obtained on the metabolism of leaf chloroplasts, mitochondria, and peroxisomes indicate that nodulation of pea plants with *Rhizobium* accelerates its progression towards senescence. Chloroplasts of mesophyll cells are dismantled in the early phases of senescence, when degradation pathways for chlorophyll and proteins are interconnected (Inada et al., 1998; Quirino et al., 2000; Hortensteiner and Feller, 2002). The rate of senescence and the remobilization of leaf nitrogen are related to the nitrogen nutrition status of the plant and the source/sink relationships (Crafts-Brandner et al., 1998; Masclaux et al., 2000; Hortensteiner and Feller, 2002). Therefore, in nodulated plants, chloroplasts gain special relevance since in mesophyll cells of C₃ plants these organelles accumulate as much as 75% of the total cell nitrogen as part of the Rubisco and other stromal proteins (Hortensteiner and Feller, 2002).

Chloroplasts are one of the target organelles of age-associated oxidative stress, and some of their antioxidant barriers have been studied (Munne-Bosch and Alegre, 2002). Our results indicate that the loss of ascorbate in chloroplasts from both nitrate-fed and nodulated young plants might contribute to the age-promoted oxidative stress response. Ascorbate is one of the main antioxidants present in chloroplasts, which can regenerate the thylakoidal α-tocopherol and directly scavenge the excess superoxide ($O_2^-$) and hydroxyl (‘OH) radicals and singlet oxygen ($^1O_2$).
during senescence (Smirnoff, 2000). Our results suggest that, in senescent plants, ascorbate cannot perhaps be used to remove \( \text{H}_2\text{O}_2 \) by the chloroplastic ascorbate–glutathione cycle since all enzyme activities involved in this pathway are diminished. Moreover, in these organelles nodulation seems to produce a similar effect to senescence in all components of the ascorbate–glutathione cycle. In addition, the decrease in SOD activity suggests that chloroplasts from senescent nodulated and nitrogen-fed plants are less efficient than those from young plants to remove the \( \text{O}_2^- \) radicals generated in the photosynthetic electron transport chain. The decrease in SOD activity, together with that observed in the enzymes of the ascorbate–glutathione cycle, suggests that an overproduction of \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) probably takes place in chloroplasts. Recently, an increase in the \( \text{H}_2\text{O}_2 \) content, associated with a reduction in the ascorbate level was reported in chloroplasts from pea plants subjected to salt stress (Gómez et al., 2004). However, the pattern of both SOD and APX activities described by these authors was different from that described in this work.

Glutathione distribution among the organelles was more homogeneous than ascorbate, although in all types of plants chloroplastic GSH+GSSG represents about 50% of the total cell content. Mitochondria and peroxisomes contributed in a similar proportion to the overall glutathione bulk. Contrary to what happened with ASC, total glutathione increased with senescence in chloroplasts from nitrogen-fed and nodulated plants (Table 5). In mitochondria, glutathione percentages only varied with time in nitrate-fed plants, showing a similar pattern to that reported for glutathione in mitochondria from senescent Cucurbita pepo leaves (Zechmann et al., 2005). The level of peroxisomal glutathione was differently affected by senescence in the two treatments (Table 5). Thus, the contribution of peroxisomes to the cellular glutathione pool increased in nitrate-fed plants, whereas in nodulated plants these organelles contained less glutathione.

The values of ascorbate and glutathione contents determined in this work in chloroplasts, mitochondria, and

<table>
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<tr>
<th>Table 4. Superoxide dismutase (SOD) activity in chloroplasts, mitochondria and peroxisomes from nitrate-fed and nodulated pea plants at two different growth times</th>
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<tbody>
<tr>
<td>Values are expressed as the mean ± SEM of, at least, three independent experiments.</td>
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<tr>
<td>SOD (U mg(^{-1}))</td>
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<tr>
<td><strong>Chloroplasts</strong></td>
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<tr>
<td>Nitrate-fed</td>
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<tr>
<td>9 weeks</td>
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<tr>
<td>12 weeks</td>
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<tr>
<td>Nodulated</td>
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<td>9 weeks</td>
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<td>12 weeks</td>
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<tr>
<td><strong>Mitochondria</strong></td>
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<td>Nitrate-fed</td>
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<td><strong>Peroxisomes</strong></td>
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<tr>
<td>Nodulated</td>
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<td>9 weeks</td>
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Fig. 6. Enzyme activities of NADPH-generating dehydrogenases in peroxisomes from nitrate-fed and nodulated pea plants at two different growth times. Values are expressed as the mean ± SEM of at least three independent experiments. G6PDH, glucose-6-phosphate dehydrogenase; 6PGDH, 6-phosphogluconate dehydrogenase; NADP-ICDH, NADP-dependent isocitrate dehydrogenase.
peroxisomes differ substantially from the data obtained for these antioxidants in crude extracts of leaves from nitrate-fed and nodulated plants (Vanacker et al., 2006) which are about three orders of magnitude higher. However, there are several reason to explain these differences: (i) the relatively low purification yield of cell organelles, as a result of their lability during the isolation procedure, particularly in the case of peroxisomes (Palma et al., 1986; López-Huertas et al., 1995; Jiménez et al., 1997); (ii) the permeability of chloroplastic, mitochondrial, and peroxisomal membranes to both ascorbate and glutathione can produce losses to the medium of these metabolites during their purification procedures; and (iii) the data obtained in crude extracts, besides ascorbate and glutathione from the three cell organelles analysed in this work, also include the contents of other cell compartments such as the nucleus, cytoplasm, and apoplast. Therefore, the ascorbate and glutathione data reported in this work, although do not strictly represent real values, for the reasons mentioned above they can be useful from a comparative viewpoint to obtain information about change tendencies in the antioxidant levels of cell organelles from the different plant treatments.

In root nodules, the mitochondrial ascorbate–glutathione cycle functions as a detoxifying mechanism involved in the protection against oxidative processes which interfere with nitrogen fixation (Iturbe-Ormaetxe et al., 2001). It has been also found that this pathway, together with other antioxidative systems from root and foliar mitochondria are up-regulated by salinity in salt-tolerant Lycopersicon plants (Mittova et al., 2003, 2004). In the experimental conditions in this study, the capability of scavenging hydrogen peroxide by mitochondrial APX was limited in nodulated and senescent plants. Under these conditions, a possible increase in H$_2$O$_2$ concentration might occur in mitochondria.

In plant systems, mitochondrial MDH, ME, and ICDH have been reported to regenerate NADPH for use in organelle metabolism (Møller and Rasmusson, 1998). Thus, the NADPH necessary for GR activity is provided by the NADPH-generating enzymes malate dehydrogenase, malic enzyme, and isocitrate dehydrogenase. In pea plants, the activity of these enzymes varied greatly with nodulation and time. However, in the overall balance the total NADPH generation rate did not show variations with treatments.

In animal mitochondria, the role of senescence-associated oxidative stress has been thoroughly reviewed (Sastre et al., 2003; Huang and Manton, 2004). In plants, mitochondria remain intact until the last stages of senescence (Inada et al., 1998; Quirino et al., 2000), and in pea leaves a role for the mitochondrial ascorbate–glutathione cycle and SOD during this physiological process was reported (Jiménez et al., 1998). These authors found that dark-induced leaf senescence was characterized by a decrease of SOD, APX, MDHAR, DHAR, and GR mitochondrial activities as well as a reduction in the ascorbate and glutathione pools of these organelles. However, in the experimental conditions used here, natural senescence was studied, which might explain the slight but not dramatic changes that were observed in the antioxidant systems.

Peroxisomes are cell organelles bound by a single membrane and characterized by an essentially oxidative type of metabolism (del Río et al., 2003a, b). Leaf peroxisomes house many of the photorespiratory enzymes and, during leaf senescence, these organelles undergo a metabolic conversion into glyoxysome-type peroxisomes (Nishimura et al., 1996; del Río et al., 1998). Glyoxysomes are also present in cotyledons from oilseeds and contain the glyoxylate cycle enzymes. This pathway, along with the β-oxidation enzymes, converts lipids into carbohydrates, useful for germination and late stages of senescence. Thus, during leaf senescence, a decline in photorespiration and an enhancement of the glyoxylate cycle occurs. The decrease in peroxisomal GOX activity in N$_2$-fixing plants compared with nitrate-fed plants is in agreement with previous results obtained by Frechilla et al. (1999) in crude extracts from pea leaves. These results showed that pea leaf peroxisomes developed senescent symptoms in nodulated and senescent plants, as indicated by the activity pattern of the photorespiratory enzymes HPR and GOX and by the glyoxylate cycle enzyme MS. Malate synthase is an enzyme whose activity has been exclusively associated to glyoxysomes in germinating oilseeds and to peroxisomes in senescent leaves. Thus, the strongly enhanced MS activity found in nodulated plants clearly indicates that these plants are

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**Table 5. Relative ascorbate and glutathione content in chloroplasts, mitochondria and peroxisomes from nitrate-fed and nodulated pea plants**

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<th></th>
<th>ASC (%)</th>
<th>GSH+GSSG (%)</th>
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<tr>
<td></td>
<td>Chloroplasts</td>
<td>Mitochondria</td>
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<tr>
<td>Nitrate-fed</td>
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<tr>
<td>9 weeks</td>
<td>99.4</td>
<td>0.2</td>
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<tr>
<td>12 weeks</td>
<td>95.5</td>
<td>1.3</td>
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<tr>
<td>Nodulated</td>
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<tr>
<td>9 weeks</td>
<td>99.4</td>
<td>0.3</td>
</tr>
<tr>
<td>12 weeks</td>
<td>96.8</td>
<td>1.6</td>
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senescent. Catalase (CAT) and SOD results also confirm these symptoms, since a decreasing CAT activity but an enhanced SOD activity were found during both natural and induced senescence of pea plants (Pastori and del Río, 1994, 1997; Jiménez et al., 1998).

The activity pattern of peroxisomal APX, and MDHAR on one hand, and DHAR and GR on the other, correlates well with the pattern reported in pea leaves during induced senescence (Jiménez et al., 1998), and also reinforces the functional link of these enzymes: APX and MDHAR associated with the peroxisomal membrane, and DHAR and GR soluble in the organelar matrix (Jiménez et al., 1997). Overall, the ascorbate–glutathione cycle seems to be less affected by senescence than by nodulation. The presence of the ascorbate–glutathione cycle in mitochondria and chloroplasts has led to the proposal that, in Arabidopsis, an integrated ascorbate–glutathione antioxidant defence linked at the level of the genome may take place (Chew et al., 2003). The contribution of peroxisomal ascorbate and glutathione relative percentages to the total of these antioxidants in the three cell organelles, together with the high specific activity of the ascorbate–glutathione cycle enzymes, give peroxisomes a special relevance in the ascorbate–glutathione cycle of the whole cell.

Considering the relative ascorbate content in the three organelles analysed, the level of vitamin C was reduced in chloroplasts from senescent nitrate-fed and nodulated plants compared with young plants, but no specific changes due to nodulation were observed (Table 5). By contrast, the contribution of mitochondria and peroxisomes to the total ASC content was notably enhanced with time in the two treatments. In nitrate-fed plants, the ascorbate level of peroxisomes was twice that found in mitochondria, which shows the importance of peroxisomes in the ascorbate metabolism of the three cell organelles studied. Chloroplastic ascorbate represents about 95–99% of the total cell ascorbate analysed, which is consistent with chloroplasts as the main ascorbate store in leaves where this antioxidant plays an important role in photosynthesis (Noctor and Foyer, 1998; Asada, 2000; Smirnoff, 2000).

In conclusion, considering the oxidative metabolism of chloroplasts, mitochondria, and peroxisomes, it might be postulated that, in these experimental conditions, 12-week-old pea plants develop senescence symptoms in leaves at a subcellular level, which is in agreement with the physiological and biochemical analyses previously described in whole plants and leaf crude extracts, respectively (Vanacker et al., 2006). This time-dependent evolution is accelerated by nodulation with Rhizobium and is basically characterized by a decline in the leaf antioxidant capacity. The role of ROS metabolism as a source of redox signals in chloroplasts, mitochondria and peroxisomes (Corpas et al., 2001; Foyer and Noctor, 2003), has a special relevance in the symbiotic system reported in this work, and suggests that the physiological efficiency of the plant–Rhizobium association might perhaps be improved by enhancing the antioxidant capacity of the aerial parts of the plant.

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