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Function of antioxidant enzymes and metabolites during maturation of pea fruits

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Abstract

In plant cells, antioxidants keep reactive oxygen species at low concentrations, avoiding oxidative damage while allowing them to play crucial functions in signal transduction. However, little is known about the role of antioxidants during fruit maturation, especially in legumes. Snap pea (*Pisum sativum*) plants, which have edible fruits, were grown under nodulating and non-nodulating conditions. Fruits were classified in three maturity stages and antioxidants were determined in the seeds and seedless pods. Maturation or prolonged storage of fruits at 25 °C led to a decline in antioxidant activities and metabolites and in γ-glutamylcysteine synthetase protein. Notable exceptions were superoxide dismutase activity and glutathione peroxidase protein, which increased in one or both of these processes. During maturation, cytosolic peroxiredoxin decreased in seeds but increased in pods, and ascorbate oxidase activity was largely reduced in seeds. In stored fruits, ascorbate oxidase activity was nearly abolished in seeds but doubled in pods. It is concluded that symbiotic nitrogen fixation is as effective as nitrogen fertilization in maintaining the antioxidant capacity of pea fruits and that, contrary to climacteric fruits, a general decrease in antioxidants during maturation does not involve oxidative stress. Results underscore the importance of the antioxidant system in reproductive organs and point to ascorbate–glutathione metabolism and cytosolic peroxiredoxin as key players in pea fruit development.

Key words: Antioxidants, ascorbate, fruit maturation, fruit storage, glutathione, nitrogen fixation, pea, peroxiredoxins.

Introduction

Leguminous plants such as pea (*Pisum sativum*), bean (*Phaseolus vulgaris*), or alfalfa (*Medicago sativa*) are crops of major economical value as a protein source for human and animal consumption (Graham and Vance, 2003). They are also essential for sustainable agricultural systems because of their ability to establish nitrogen-fixing symbioses with soil bacteria, thus providing a biological alternative to chemical fertilization. The site of nitrogen fixation by the rhizobia–legume symbiosis is the root nodule, where bacterial nitrogenase produces ammonia which is assimilated in the host cells and exported to the shoot in the form of amides or ureides. Thus, rhizobia provide the plant with fixed nitrogen, whereas the plant supplies the nodule with carbon substrates, mainly sucrose, derived from photosynthesis.

These two processes, nitrogen fixation and photosynthesis, along with respiration and peroxisomal metabolism, involve electron transfer reactions that give rise to reactive oxygen species (ROS). In general, ROS are potentially toxic and their uncontrolled production can result in oxidative damage of cellular components (Halliwell and Gutteridge, 2007). However, some ROS such as the superoxide radical

and hydrogen peroxide (H_2O_2), at low concentrations, fulfil important roles in stress perception, photosynthesis regulation, pathogen recognition, programmed cell death, and plant development (Mittler *et al.*, 2004). Antioxidants modulate the steady-state concentrations of ROS, avoiding their potential cytotoxicity while allowing them to function as signal molecules (Mittler *et al.*, 2004). Antioxidant enzymes include the superoxide dismutases (SODs), catalases, peroxiredoxins (PRXs), glutathione peroxidases (GPXs), and the four enzymes of the ascorbate–glutathione pathway (Dietz, 2003; Matamoros *et al.*, 2003; Mittler *et al.*, 2004; Navrot *et al.*, 2006). In this pathway, ascorbate peroxidase (APX) catalyses the reduction of H_2O_2 to water by ascorbate producing monodehydroascorbate and dehydroascorbate. Ascorbate is regenerated by monodehydroascorbate reductase (MR) and dehydroascorbate reductase (DR) using NADH and reduced glutathione, respectively. The oxidized form of glutathione generated by DR activity is reduced by glutathione reductase (GR) using NADPH (Dalton *et al.*, 1986; Jiménez *et al.*, 2002a).

Two key metabolites of this pathway, ascorbate and glutathione, are major water-soluble antioxidants and redox buffers in plant cells but also have crucial functions in stress responses and organ development (Arrigoni and De Tullio, 2002; Noctor *et al.*, 2002). Ascorbate can be oxidized to monodehydroascorbate by ascorbate oxidase (AO) in the apoplast. This enzyme can therefore modulate the redox state of the apoplastic ascorbate pool, which is important for controlling cell elongation and triggering signal transduction cascades in response to external stimuli (Kato and Esaka, 2000; Pignocchi *et al.*, 2006). Glutathione participates in the induction of defence genes, sulphur transport and storage, and heavy metal detoxification, and its concentration needs to be tightly controlled at several levels. Major mechanisms are the transcriptional, translational, and post-translational regulation of the enzyme γ-glutamylcysteine synthetase (γECS), which catalyses the first step of glutathione biosynthesis in all organisms (May *et al.*, 1998; Xiang and Oliver, 1998; Noctor *et al.*, 2002; Hicks *et al.*, 2007).

The antioxidants of legume leaves and nodules have been examined in considerable detail (see, for example, Matamoros *et al.*, 2003; Palma *et al.*, 2006), but similar information on legume fruits is lacking. In fact, most extensive studies on the role of ROS and antioxidants in fruit development and maturation (ripening) have been conducted on climacteric fruits such as pear (*Pyrus communis*; Brennan and Frenkel, 1977), saskatoon fruit (*Amelanchier alnifolia*; Rogiers *et al.*, 1998), and tomato (*Lycopersicon esculentum*; Jiménez *et al.*, 2002a). Ripening of these fruits is characterized by a burst of ethylene production and respiratory activity. The involvement of ROS and antioxidants during fruit ripening has been also investigated in the chloroplasts (Bouvier *et al.*, 1998) and mitochondria (Jiménez *et al.*, 2002b) of pepper (*Capsicum annuum*) fruit, which exhibits non-climacteric physiology. In this case, ripening involves an intense chloroplast-to-chromoplast (green-to-red fruit) transition (Bouvier *et al.*,

1998). Many non-climacteric fruits like those of pea (*Pisum sativum*) do not exhibit those major changes during maturation, and hence the conclusion drawn from the above studies that there is an oxidative process needs to be assessed.

The study of antioxidants in fruits is important for several reasons. First, antioxidants may protect fruit tissues from potentially toxic ROS and thereby contribute to the stress tolerance of crops (Mittler *et al.*, 2004; Van Breusegem *et al.*, 2008). Second, fruits may have nutritional value for animal and/or human consumption. Third, in many cases fruits have a relatively short shelf-life following harvest, during which they undergo changes in texture, colour, and flavour, which may be accompanied by a decline in antioxidants (Davey and Keulemans, 2004; Malacrida *et al.*, 2006; Stevens *et al.*, 2008). However, the role of antioxidants in the development, maturation, and post-harvest storage of legume fruits is poorly defined. The implication of antioxidants and oxidative stress in these processes has been investigated here using a commercial variety of pea with edible seeds and pods (Basterrechea and Hicks, 1991). In addition, the effect of sustainable agricultural practices (nitrogen fixation versus combined nitrogen) on the antioxidant composition, nutritional value, and post-harvest shelf life of pea fruits has been assessed.

Materials and methods

Plant growth and metabolic parameters

Sugar snap pea (*Pisum sativum* L. cv. Sugar Lace) seed was provided by Bonduelle (Milagro, Navarra, Spain). Nodulated plants (inoculated with *Rhizobium leguminosarum* bv. *viciae* strain NLV8) were grown in pots containing a 1:1 (v:v) mixture of Perlite and Vermiculite under controlled environment conditions (photon flux density of 350 µmol m^{-2} s^{-1}, day length of 16 h, day/night regime of 25/20 °C temperature, and 70/85% relative humidity). Non-nodulated plants were grown under the same conditions, except that the nutrient solution (Gogorcena *et al.*, 1997) was supplemented with 2.5 mM NH_4NO_3. After 45–60 d, fruits were harvested and classified into three maturity stages based on the L/N ratio (Fig. 1). In the immature stage (I) the seeds do not fill the hull, in the mature stage (M) the seeds fill the hull without causing deformation, and in the overmature stage (O) the seeds deform the hull (Basterrechea and Hicks, 1991).
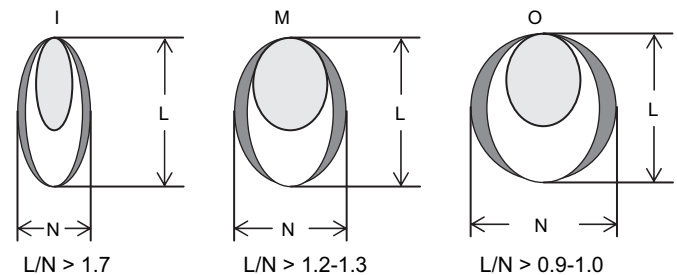
Fig. 1. Developmental stages of pea fruits. These were harvested from nodulated plants being 45–60-d-old and were classified as immature (I), mature (M), or overmature (O) according to their L/N ratios. The scheme shows transverse sections of pods.

To characterize these maturity stages further, fruits were separated into seed and seedless pods, and the fresh weights and water and protein contents were determined. Total protein was quantified by the Bradford microassay (Bio-Rad) using bovine serum albumin as the standard. The content of H_2O_2 in whole fruits was measured, just before the beginning of the photoperiod, as an indicator of metabolic activity. Extraction of H_2O_2 from pea fruits was performed immediately after harvest with trichloroacetic acid and activated charcoal. Extracts were adjusted to pH 8.4 with ammonia, and H_2O_2 was quantified following its reaction with 4-aminoantipyrine and phenol to form a stable red product in the presence of peroxidase (Zhou *et al.*, 2006). Blanks containing catalase were run for each sample, as well as for the calibration with H_2O_2 standards which were added to the extraction medium in parallel to the samples.

For biochemical and molecular analyses, samples of seeds and seedless pods of representative fruits of the three maturity stages were flash-frozen in liquid nitrogen immediately after harvest. To investigate the effects of storage, fruits were kept at 25 °C for 4 d prior to freezing in liquid nitrogen. All plant material was stored at –80 °C.

Antioxidant enzymes

For enzyme extraction, 100 mg of seeds or seedless pods were ground in liquid nitrogen; this was left to boil dry and the powder was homogenized in 500 μl of the following optimized media.

SOD: 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 1% (w/v) soluble polyvinylpyrrolidone (PVP-10), and 0.1% (v/v) Triton X-100.

APX and catalase: 50 mM potassium phosphate buffer (pH 7.0) and 0.5% PVP-10 (for the APX assay, this buffer was supplemented with 5 mM ascorbate to avoid the inactivation of the plastidic isoform).

MR, DR, and GR: 50 mM potassium phosphate buffer (pH 7.8), 1% PVP-10, 0.2 mM EDTA, and 10 mM β-mercaptoethanol.

AO: 10 mM sodium phosphate buffer (pH 6.5).

The extracts were centrifuged (13 000 *g* for 10 min at 4 °C) and the enzyme activities were assayed in the supernatants. All activities were assayed at 25 °C within linear range.

Total SOD activity was assayed by a method based on the inhibition of cytochrome *c* reduction by superoxide at 550 nm and catalase activity by following the decomposition of H_2O_2 at 240 nm (Rubio *et al.*, 2002). One SOD unit (U) was defined as the amount of SOD required to inhibit the reduction of ferric cytochrome *c* by 50% (McCord and Fridovich, 1969). APX and DR activities were determined by monitoring ascorbate oxidation at 290 nm (Asada, 1984) and ascorbate formation at 265 nm (Nakano and Asada, 1981), respectively. MR and GR activities were assayed by following the oxidation of NADH (Dalton *et al.*, 1993) and NADPH (Dalton *et al.*, 1986) at 340 nm, respectively. To assay AO activity, extracts were centrifuged (13 000 *g* for 15 min) and the pellets were resuspended by vigorous shaking for 10 min in 10 mM sodium phosphate buffer (pH 6.5) containing 1 M NaCl. The extracts were centrifuged again and AO activity was determined in the supernatant by following ascorbate oxidation at 265 nm (Pignocchi *et al.*, 2003).

Antioxidant metabolites

Ascorbate was extracted from 100 mg of plant tissue with 500 μl of $HClO_4$. The extract was centrifuged (13 000 *g* for 10 min at 4 °C) and the supernatant neutralized with 1 M K_2CO_3. For determination of total ascorbate (reduced ascorbate+dehydroascorbate), samples were incubated for 15 min at room temperature in the dark with 0.4 mM dithioerythritol; then 100 μl of the mixture was added to 900 μl of 100 mM HEPES (pH 5.6) and the decrease of ascorbate concentration was monitored at 265 nm after the addition of 0.05 units of AO (Sigma-Aldrich). For the determination of reduced ascorbate, the same procedure was used omitting the incubation with dithioerythritol, and dehydroascorbate was calculated as the difference between the concentrations of total and reduced ascorbate.

Glutathione was quantified after derivatization with monobromobimane using an HPLC with fluorescence detection (Matamoros *et al.*, 1999). The concentrations of reduced and oxidized forms of glutathione were determined spectrophotometrically using an enzymatic cycling assay (Griffith, 1980). The redox states of ascorbate and glutathione were calculated as 100× [ascorbate/ (ascorbate+dehydroascorbate)] and 100×[reduced glutathione/ (reduced+oxidized glutathione)], respectively.

Markers of oxidative stress

The oxidative damage of lipids was estimated as the content of malondialdehyde after reaction with thiobarbituric acid (TBA) by HPLC (Iturbe-Ormaetxe *et al.*, 1998). Briefly, lipid peroxides were extracted with 5% (w/v) metaphosphoric acid and 0.04% (w/v) butylated hydroxytoluene. After centrifugation, the chromogen was formed by mixing 100 μl of supernatant, 10 μl of 2% butylated hydroxytoluene, 50 μl of 1% (w/v) TBA, and 50 μl of 25% (v/v) HCl, and by incubating the reaction mixtures at 95 °C for 30 min. The chromogen was extracted with 1-butanol and the organic phase was evaporated under N_2 and kept at –80 °C until analysis. The samples were resuspended in 60 μl of HPLC solvent and the $(TBA)_2$-malondialdehyde adduct was resolved on an Ultrasphere C_{18} column (5 μm, 25 cm×4.6 mm; Beckman) and was eluted with 5 mM potassium phosphate buffer (pH 7.0) containing 15% acetonitrile and 0.6% tetrahydrofuran. The flow rate was 1 ml min^{-1} and detection was at 532 nm. Calibration curves were made using 1,1,3,3-tetraethoxypropane. The oxidative damage of proteins was estimated as the content of total carbonyl groups. Proteins were separated on 12.5% sodium dodecyl sulphate (SDS) gels, and carbonyls were quantified by derivatization with 2,4-dinitrophenylhydrazine using the OxyBlot Protein Oxidation Detection kit following the manufacturer's instructions (Chemicon, Temecula, CA, USA).

Immunoblot analyses

Proteins were extracted, separated on 15% SDS gels, and transferred onto polyvinylidene fluoride membranes as described by Loscos *et al.* (2008). Equal loading of lanes and transfer quality were verified by Ponceau staining of membranes. Immunoblot analyses were performed using rabbit polyclonal antibodies against γECS of common bean (*Phaseolus vulgaris*; MR Clemente, unpublished results), GPX3.2 of poplar (*Populus trichocarpa*; Navrot *et al.*, 2006), or 2-CysPrx (Laxa *et al.*, 2007), PrxQ (Lamkemeyer *et al.*, 2006), PrxIIC (Horling *et al.*, 2003), PrxIIF (Finkemeier *et al.*, 2005), and plastidial DR (DRp) of *Arabidopsis* (*Arabidopsis thaliana*). For immunoblots of cytosolic DR (DRc), a guinea pig polyclonal antibody generated against *Arabidopsis* DRc (Eltayeb *et al.*, 2006) was used. The antibody against PrxIIC also recognizes two other PRX isoforms of *Arabidopsis*, PrxIIB and PrxIID, that are localized to the cytosol (Horling *et al.*, 2003). Consequently, cytosolic PrxII isoforms will collectively be designated as PrxIIc in this work. Primary antibodies were used at dilutions of 1:1000 (γECS and GPX), 1:500 (2-CysPrx), 1:3000 (PrxQ, PrxIIc, PrxIIF, and DRp), or 1:2500 (DRc). Secondary antibodies were goat anti-guinea pig (DRc) or anti-rabbit (other enzymes) IgG horseradish peroxidase conjugated antibodies and were used at dilutions of 1:5000 or 1:20000, respectively. Incubations with the primary and secondary antibodies were performed in TTBS [20 mM TRIS-HCl (pH 7.8), 0.5 M NaCl, 0.05% (v/v) Tween-20] supplemented with 5% (w/v) skimmed milk to

reduce background signal. Immunoreactive proteins were visualized using the SuperSignal West Pico (Pierce) chemiluminescent reagent for peroxidase detection.

Gene expression analyses

Total RNA was isolated from seeds using the RNAqueous kit (Ambion, Cambridgeshire, UK) and processed as described by Loscos *et al.* (2008). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis was carried out with the iCycler iQ System using the iQ SYBR-Green Supermix (Bio-Rad) and gene-specific primers for γECS (5′-CCCTCTTGAAACCCTGCATC-3′; 5′-CTCCCACTTTGGCTGGAAAC-3′) and *actin* (5′-GTGTCT-GGATTGGAGGATCAATC-3′, 5′-GGCCACGCTCATCATAT-TCA-3′). The PCR programme was as previously reported (Loscos *et al.*, 2008) and the amplification efficiency of the primers, calculated by serial dilutions of seed cDNA, was >90%. The γECS mRNA levels were normalized with *actin* as the reference gene and were expressed using the method of Livak and Shmittgen (2001). All the reactions were set up in duplicate (two technical replicates) using three RNA preparations (biological replicates) from different fruits.

Results and discussion

Antioxidant protection is similar in pea fruits from nodulated and non-nodulated plants

The first objective of this study was to evaluate the effect of symbiotic nitrogen fixation on the antioxidant composition of pea fruits. To this end, the antioxidant enzymatic activities and metabolite contents of fruits were determined at the mature stage from nodulated plants and from plants fed combined nitrogen (Fig. 2). For all measurements, fruits were separated into seeds and seedless pods immediately after harvest. In general, the antioxidant content, when expressed on a fresh weight basis, was greater in the seeds, but there were also significant amounts in the pods. For example, DR activity was 5-fold greater in the seeds than in the pods, whereas catalase activity and glutathione content were only *c.* 1.7-fold greater in the seeds (Fig. 2). However, in an average mature fruit, the pod accounts for *c.* 70% of the fresh weight (Table 1). These data should be taken into consideration when calculating the absolute values of antioxidants present in the seeds and pod of an individual fruit. Also, it can be concluded that the fruits from plants that were dependent on nitrogen fixation had similar antioxidant levels to those that were supplied with ammonium nitrate. In fact, the DR activity and glutathione content of the seeds were *c.* 22% and 17% higher, respectively, in the nodulated plants. In seedless pods, the antioxidant content was roughly similar in plants grown on any of the two nitrogen nutrition sources, with a slight increase of GR activity (14%) in nodulated plants (Fig. 2). It is also worth noting that nodulation led to an increase in the specific activities of the antioxidant enzymes in the seeds (data not shown). Consequently, the loss of 30% of the total protein content in the mature seeds of nodulated plants (Fig. 2) did not compromise their overall antioxidant capacity. Recently, Palma *et al.* (2006) compared the oxidative metabolism of leaves of nodulated and non-nodulated pea, and concluded
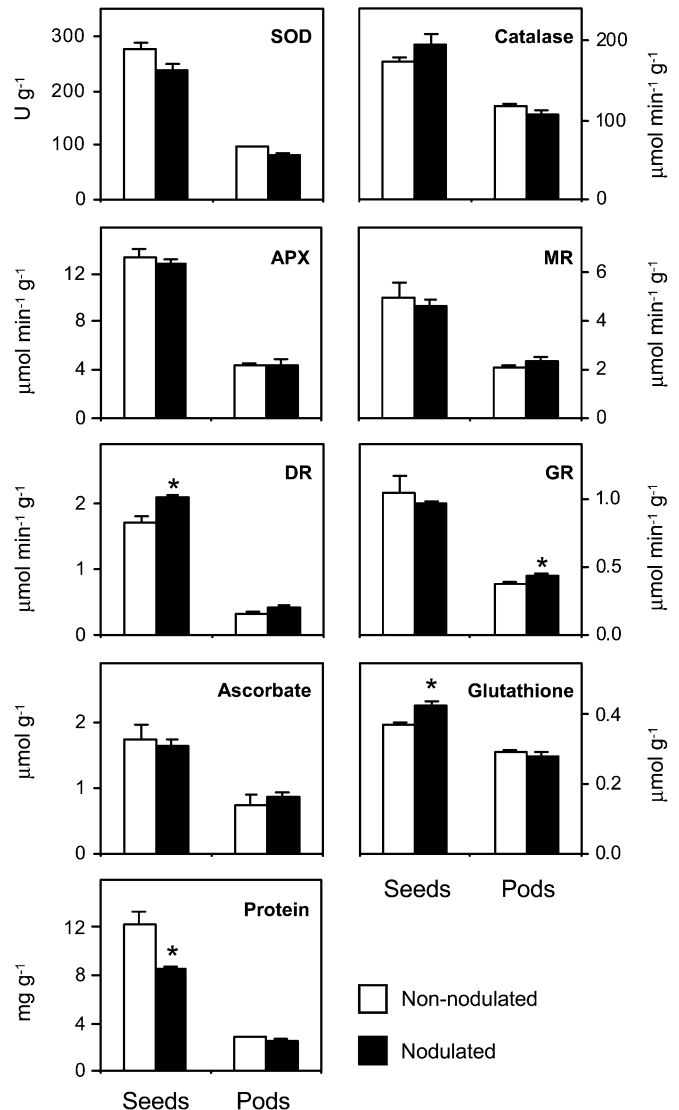
Fig. 2. Antioxidants in seeds and seedless pods of non-nodulated (nitrogen-fed) and nodulated (nitrogen-fixing) pea plants. All parameters are expressed per gram of fresh weight. The contents of ascorbate and glutathione are the sum of the corresponding reduced and oxidized forms. Values are means ±SE of 4–6 replicates from at least three series of plants grown independently. For each tissue, means marked with an asterisk are significantly different at *P* <0.05 based on Student's *t* test. Abbreviations: SOD, superoxide dismutase; APX, ascorbate peroxidase; MR, mono-dehydroascorbate reductase; DR, dehydroascorbate reductase; GR, glutathione reductase.

that nodulation accelerates the decline in the antioxidant capacity of leaves during senescence. Also, Vanacker *et al.* (2006) observed higher lipid and protein oxidation levels in senescing leaves of nodulated plants compared with nitrate-fed plants, and suggested that systemic signals produced by the breakdown of the symbiosis were the responsible factors. Instead it was found here that the pea fruits of nodulated plants retained a similar antioxidant capacity to those of non-nodulated plants. This discrepancy between the responses of the antioxidant capacity of leaves and

Table 1. Some growth and metabolic parameters of seeds and seedless pods of pea fruits

| Parameter	Units[a]	Developmental stage		
		Immature	Mature	Overmature
Fresh weight (seeds)	g FW per seed	0.04±0.01 a	0.24±0.01 b	0.56±0.02 c
Fresh weight (pods)	g FW per pod	1.81±0.20 a	2.81±0.19 b	2.66±0.24 b
Water content (seeds)	%	82.48±0.49 a	82.60±0.13 a	75.74±0.77 b
Water content (pods)	%	87.69±0.50 a	84.91±0.79 b	85.56±0.64 b
Protein (seeds)	mg g^{-1} FW	11.00±0.53 a	8.59±0.13 b	9.28±0.73 b
Protein (pods)	mg g^{-1} FW	3.24±0.13 a	2.51±0.23 b	2.07±0.08 b
H_2O_2 (whole fruits)	µmol g^{-1} FW	1.30±0.34 a	1.21±0.25 a	1.10±0.43 a

[a] FW, fresh weight.

fruits during senescence and maturation, respectively, may be attributed to metabolic features inherent in these two plant organs and underscores the importance of ROS protection in reproductive tissues.

Peroxiredoxins are differentially expressed in pea tissues

Thiol peroxidases, which include the closely related GPX and PRX families, catalyse the reduction of peroxides and play important roles in antioxidant defence and stress signalling. Several GPX and PRX isoforms can be distinguished based on their catalytic mechanism, substrate specificity, and subcellular localization (Dietz, 2003; Navrot *et al.*, 2006). The information on the distribution of thiol peroxidases in legumes is scarce and the relative abundance of these proteins has therefore been examined in seeds and seedless pods of mature pea fruits using immunoblots. This was a necessary prerequisite to study the effects of fruit development and storage on these proteins. For comparison, leaves, roots, and nodules harvested at the same time as the fruits were included in the screening of thiol peroxidases. It was found that GPXs are expressed in the five plant organs examined (data not shown), but the presence of specific isoforms could not be ascertained because the antibody against poplar GPX3.2 may recognize several of them (Navrot *et al.*, 2006). Immunolocalization studies showed that, in legumes, this antibody detects at least a GPX isoform in leaf chloroplasts and in root and nodule plastids (Ramos *et al.*, 2009), and hence it was concluded that pea fruits contain GPX in the plastids of seeds and seedless pods.

By contrast, the high specificities of the four antibodies available against *Arabidopsis* PRXs allowed the localization of the proteins to be investigated in different organs of pea plants (Fig. 3). Our results indicate that the PrxQ and 2-CysPrx isoforms are abundantly expressed in leaves, in
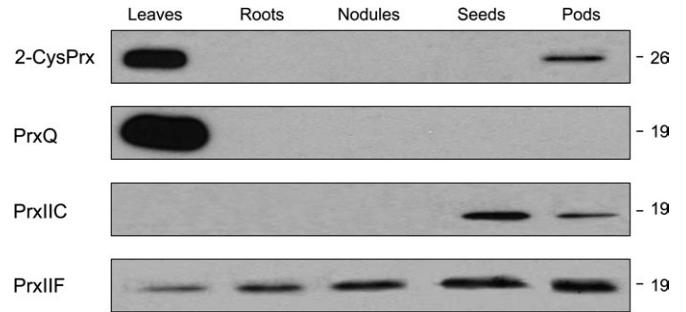
Fig. 3. Relative abundance of peroxiredoxin proteins in leaves, roots, nodules, seeds, and seedless pods of pea plants. Gels were loaded with 15 µg of protein per lane. The apparent molecular mass of the proteins (kDa) is indicated on the right. Blots are representative of three to five gels loaded with extracts from different plants.

agreement with their proposed role in the protection of photosynthesis against ROS (Dietz, 2003). Notably, the PrxQ protein was not detected in roots, nodules, seeds, or seedless pods, whereas the 2-CysPrx protein was detectable in pods, albeit at low levels. The PrxIIc proteins immunologically related to *Arabidopsis* PrxIIC were clearly present in seeds and pods, but were barely detectable in roots, nodules, or leaves (Fig. 3). It should be noted, however, that these organs were harvested from the same plants, and at the same time, as fruits and hence PrxIIc might well be detectable in younger tissues. Contrary to the pattern observed for the other PRXs, it was found that the mitochondrial isoform PrxIIF was expressed in all five pea organs examined (Fig. 3). Groten *et al.* (2006) have reported that the PrxIIF isoform of pea nodules is not regulated by ascorbate and its level does not change appreciably with senescence. Together with our results, it is concluded that PrxIIF, along with manganese–SOD and the ascorbate–glutathione pathway enzymes (Jiménez *et al.*, 2002b), represents a general defence mechanism of fruit mitochondria against ROS generated during respiration.

### Pea fruit maturation involves a general decrease in antioxidant capacity but not oxidative stress

Maturation (ripening) has been described as an oxidative process in climacteric fruits (Brennan and Frenkel, 1977; Rogiers *et al.*, 1998). Conceivably then, the antioxidant system may be involved in the control of fruit maturation. To gain an insight into the mechanisms underlying the maturation of pea fruits, antioxidant activities and metabolites were quantified in seeds and seedless pods at the three maturity stages described in Fig. 1. Fruit maturation caused a decrease in the water and protein contents of seeds and pods but had no significant effect on the H_2O_2 content of whole fruits (Table 1).

Although all determinations of antioxidants were performed both in nodulated and non-nodulated plants, the results were very similar and only those with nodulated plants are presented here for simplicity. Antioxidant enzyme activities and metabolites decreased during the maturation

of pea seeds, except APX activity, which was not affected, and SOD and DR activities, which increased by 30–50% from the immature to the overmature stages (Fig. 4A). Thus, catalase and MR activities and ascorbate and glutathione contents were *c*. 30–50% lower in the mature and/or overmature seeds than in immature seeds, whereas GR activity declined by *c*. 10–25% (Fig. 4A). The availability of antibodies against the DRc and DRp of *Arabidopsis* enabled us to investigate whether the high DR activity observed in overmature seeds was due to an increase in the protein level. Surprisingly, during seed development, the content of DRp protein did not change and that of DRc protein was even moderately reduced (data not shown). This suggests that DR activity is post-translationally regulated during fruit development or that the fruit tissue contains additional proteins with DR activity. For example, thioredoxins, glutaredoxins, disulphide isomerases, and glutathione peroxidases display DR activity in plants and animals (De Tullio *et al.*, 2002). The high DR activity in overmature seeds could be explained as a response to the low levels of ascorbate at this developmental stage. If dehydroascorbate is not rapidly reduced back to ascorbate, it may be catabolized to oxalate and tartrate; therefore, an improved efficiency of dehydroascorbate recycling could influence the ascorbate pool size and delay its degradation.

The changes in the antioxidants of pods were also studied during fruit development (Fig. 4A). Except for SOD, MR, and DR activities, which remained constant or slightly decreased in pods, the antioxidant activities and metabolites followed similar trends in seeds and pods. However, the content of PrxIIc protein showed a contrasting pattern during fruit development (Fig. 4B). The protein decreased in seeds but consistently accumulated in pods from overmature fruits (Fig. 4B), which also contained less ascorbate than the immature or mature pods (Fig. 4A). Because oxidative damage was not detected in seeds or pods at any maturity stage, PrxIIc may be differently regulated in pea fruits and in *Arabidopsis* leaves, and this regulation could involve roles during seed and pod growth. This hypothesis is consistent with the proposal of Bréhélin *et al.* (2003) that the expression of three cytosolic PrxII isoforms have developmental functions in *Arabidopsis*. The results suggest that ascorbate or oxidative stress, described as regulatory cues in leaves (Horling *et al.*, 2003) and nodules (Groten *et al.*, 2006), do not regulate expression of PrxIIc in pea fruits.
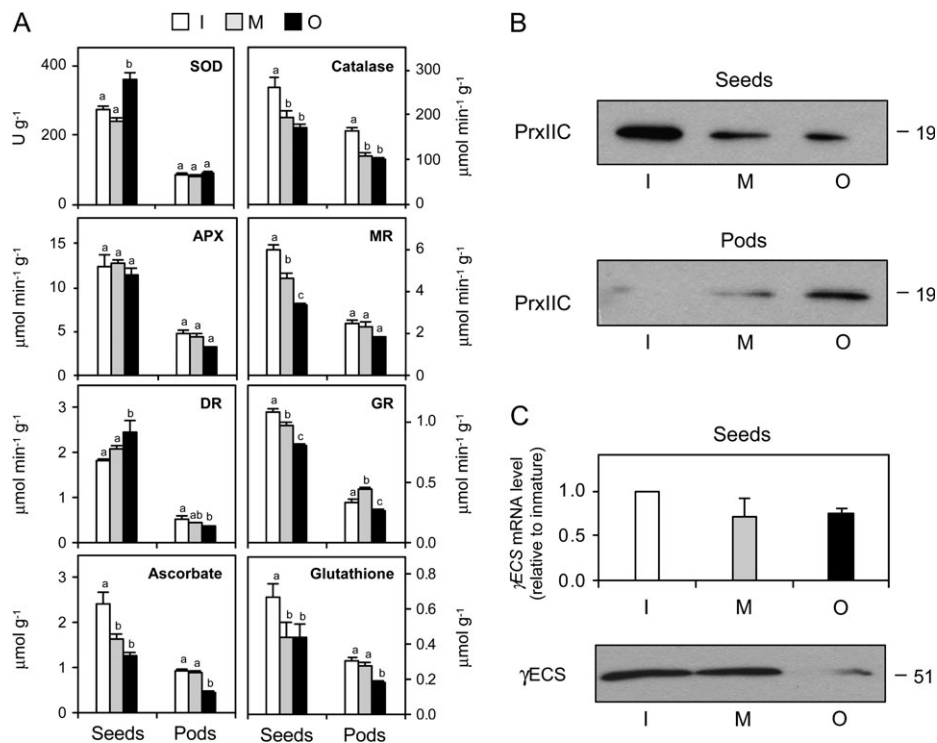
Fig. 4. Changes in antioxidant levels during the maturation of pea fruits. These were classified as immature (I), mature (M), or overmature (O) as described in Fig. 1. (A) Enzyme activities and metabolite contents of seeds and seedless pods. Abbreviations and other details are as described in Fig. 2. Values are means ±SE of 4–6 replicates from at least three series of plants grown independently. For each tissue, means denoted by the same letter do not significantly differ at $P<0.05$ based on Duncan's multiple range test. (B) Immunoblot analysis of cytosolic peroxiredoxin II (PrxIIc) in seeds and pods. Gels were loaded with 15 µg of protein per lane, and blots are representative of three to five gels loaded with extracts from different plants. (C) Expression (mRNA level and protein) of γ-glutamylcysteine synthetase (γECS) in seeds. The γECS mRNA levels were normalized with *actin* as the internal reference gene and were expressed relative to those of immature seeds, which were arbitrarily given a value of 1. Values represent means ±SE of 3–4 RNA extractions from seeds of at least two series of plants grown independently. For immunoblots, gels were loaded with 50 µg of protein per lane. The blot shown in (C) is representative of three independent blots. For (B) and (C), the apparent molecular mass (kDa) of the proteins is indicated on the right.

Despite our finding that the maturation of pea fruits is accompanied by a decrease in antioxidant defences, progressive oxidative damage was not detected using lipid and protein oxidation as markers (data not shown). The lack of accumulation of oxidatively damaged products is consistent with the observation that H_2O_2 levels remained rather constant during maturation (Table 1). In addition, although the ascorbate and glutathione contents declined during fruit development, their redox states remained largely unchanged. Thus, >90% of the ascorbate and glutathione pool were in a reduced form in immature, mature, and overmature fruits. However, it should be kept in mind that the glutathione redox potential is sensitive to concentration since two molecules of reduced glutathione are oxidized to one molecule of glutathione disulphide; thus, with decreasing glutathione concentration the redox midpoint potential increases to more positive values even if the ratio of reduced to oxidized glutathione remains unchanged (Schafer and Buettner, 2001). Because the drop in concentration was less than 2-fold, this effect is <20 mV. Despite this, and contrary to the situation described for climacteric fruits, a decrease in the antioxidant capacity does not trigger oxidative stress in developing fruits of nodulated pea plants. Also, it is surmised that the low levels of protein and lipid oxidation during the reproductive phase may be part of a strategy to limit the transfer of oxidatively damaged components to the offspring.

To investigate the mechanism behind the decrease in glutathione in seeds and seedless pods during pea fruit maturation further, the expression of γECS was analysed at the mRNA and protein levels (Fig. 4C). It was found that the γECS mRNA level remained fairly constant during seed maturation, whereas the corresponding protein level sharply decreased at the overmature stage, indicating that the γECS activity of pea fruits is regulated, at least in part, at the translational level. These results, together with our previous work with bean nodules (Loscos *et al.*, 2008), are consistent with the proposal that a translational repression of γECS is part of a multilevel regulation of glutathione homeostasis in plant cells (Xiang and Oliver, 1998; Noctor *et al.*, 2002). Our results also suggest that the significant decline of glutathione in overmature seeds was caused by a decrease in the content of γECS protein and that additional factors, such as glutathione degradation, utilization or transport, may be involved in the decrease in glutathione in mature seeds.

Prolonged storage of pea fruits decreases antioxidant capacity but does not cause oxidative damage

Post-harvest storage of fruits usually results in physiological disorders that affect both commercial and nutritional qualities. On the other hand, the antioxidant capacity has been positively correlated with better storage properties (Davey and Keulemans, 2004; Stevens *et al.*, 2008). Thus, it was decided to assess the involvement of ROS and oxidative damage in the deterioration of fruits from nodulated pea plants during storage at room temperature. For this purpose, the antioxidant content of mature fruits that ha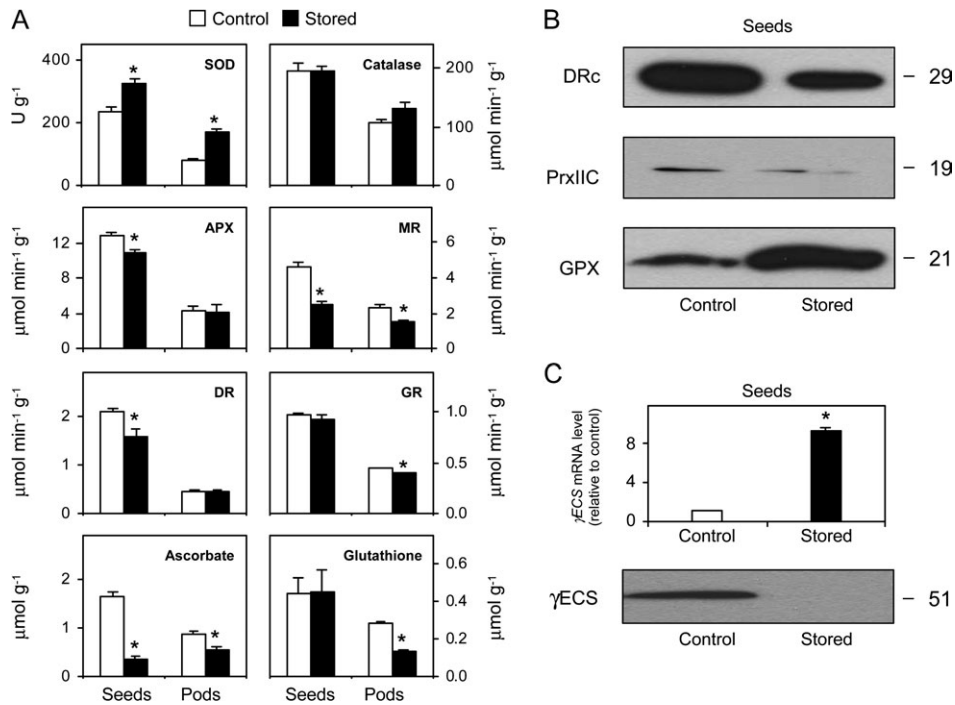d been flash-frozen in liquid nitrogen immediately after harvest was compared with that of fruits that had been kept at 25 °C for 4 d. In addition, oxidative damage levels were determined in both sets of fruits. These results showed that the storage of pea fruits at room temperature caused a general decrease in their antioxidant content. In seeds from stored fruits, APX, MR, and DR activities were 16%, 25%, and 47% lower, respectively, than in seeds from frozen fruits (Fig. 5A). Similarly, the ascorbate content of seeds decreased by 78% with storage. By contrast, glutathione did not vary and SOD activity was enhanced by 37% (Fig. 5A). In pods, prolonged storage of fruits decreased the ascorbate and glutathione contents by 35–55% and MR and GR activities by 10–35%, whereas it doubled the SOD activity and had no effect on the activities of catalase or the other enzymes of the ascorbate–glutathione pathway (Fig. 5A).

Further studies on the effects of pea fruit storage on selected antioxidant enzymes were performed using immunoblots (Fig. 5B). These revealed that the decrease of DR activity in the seeds during storage was caused, at least in part, by a consistent decrease in DRc protein. Similarly, fruit storage decreased the content of PrxIIc protein in the seeds but had the opposite effect on GPX protein (Fig. 5B). The levels of γECS protein were also determined in the seeds during fruit storage, along with those of the corresponding transcript (Fig. 5C). Most notably, the mRNA and protein levels showed contrasting patterns to the point that the γECS protein was hardly detectable in extracts of stored fruits, further confirming a translational regulation of the enzyme. As concluded above from the results of fruit maturation, the lack of correlation among γECS mRNA, γECS protein, and glutathione in stored fruits reflects a high degree of complexity in the regulation of glutathione homeostasis in plant cells.

To determine whether the decreases in ascorbate, glutathione, and some enzyme activities of the ascorbate–glutathione pathway caused oxidative stress in the stored fruits, lipid peroxidation and protein oxidation were measured as markers. Instead of the accumulation of oxidative products, a decrease of 68% was found in malondialdehyde (from 25.6 to 8.2 nmol g^{-1} fresh weight) and of 61% in protein carbonyl groups (as estimated by densitometric analysis of Western blots), and the pools of ascorbate and glutathione remained >90% in the reduced form. The more likely explanation for the absence of measurable oxidative damage or altered redox poise in cells is a general slow-down of metabolism during storage. These fruits would exhibit lower ROS production, yet they maintain sufficient antioxidant protection to cope with them. In fact, there were marked increases in SOD activity and GPX protein content in the stored fruits (Fig. 5B), which are enzymes involved in the detoxification of superoxide radicals and lipid peroxides, respectively.

Previous results about the effects of storage on the antioxidants of fruits are difficult to interpret because of their considerable variability. This may be due to differences in the plant species, type of fruits, experimental designs, or plant growth conditions (for example, see Jiménez *et al.*, 2003; Malacrida *et al.*, 2006). Interestingly,

Fig. 5. Changes in antioxidants during the storage of pea fruits. Control fruits were frozen in liquid nitrogen immediately after harvest, whereas stored fruits were kept at 25 °C for 4 d. (A) Enzyme activities and metabolite contents in seeds and seedless pods. Abbreviations and other details are as described in Fig. 2. Values are means ±SE of 4–6 replicates from at least three series of plants grown independently. For each tissue, means marked with an asterisk are significantly different at P <0.05 based on Student's t test. (B) Immunoblot analysis of cytosolic dehydroascorbate reductase (DRc), cytosolic peroxiredoxin II (PrxIIc), and glutathione peroxidase (GPX) in seeds. Gels were loaded with 50 μg (DRc, GPX) or 15 μg (PrxIIc) of protein per lane. (C) Expression (mRNA level and protein) of γ-glutamylcysteine synthetase (γECS) in seeds. The γECS mRNA levels were normalized with *actin* as the internal reference gene and were expressed relative to those of control seeds, which were arbitrarily given a value of 1. Values are means ±SE of 3–4 RNA extractions from seeds of at least two series of plants grown independently, and asterisks denote up-regulation of the gene (R >2). For immunoblots, gels were loaded with 50 μg of protein per lane. For (B) and (C), blots are representative of four gels loaded with seed extracts from different plants, and the apparent molecular mass (kDa) of the proteins is indicated on the right.

Davey and Keulemans (2004) reported that different apple fruit (*Malus*) cultivars differed substantially in their ability to maintain ascorbate levels during storage, and that the capacity to maintain the ascorbate and glutathione pool was related to better storage properties.

Apoplastic AO activity is important for pea fruit development

Ascorbate is not only a potent antioxidant but is also involved in the control of cell elongation as a substrate of AO (Pignocchi *et al.*, 2003). Because seed maturation, especially from the immature to the mature stage, entails a rapid growth (Fig. 1) and AO expression is high in expanding tissues (Kato and Esaka, 2000), this enzyme activity was determined during maturation (Fig. 6A) and after prolonged storage (Fig. 6B) of the fruits. Indeed, the AO activity of mature and overmature seeds was 70% and 95% lower, respectively, than that found in immature seeds; however, this activity remained constant in pods (Fig. 6A). This distinct response of AO activity in seeds and pods suggests differences in the growth pattern of both organs during maturation.

Our results are consistent with the implication of AO in cell growth and, specifically, in legume fruit development. Previous studies on the changes of AO activity in fruits showed that AO activity was high in rapidly growing young fruits of pumpkin (*Cucurbita maxima*; Esaka *et al.*, 1992) and zucchini (*Cucurbita pepo*; Lin and Varner, 1991). Likewise, AO expression was enhanced in young and mature fruits of melon (*Cucumis melo*; Sanmartin *et al.*, 2007). The major decreases in AO activity that were found in pea fruits during seed maturation or storage are consistent with an important role of the enzyme in cell growth. The observation that AO activity is very high in immature seeds, which consist of actively growing tissues (Table 1; see also Figs 1 and 2 in Dam *et al.*, 2009), supports this conclusion. The mechanism by which AO activity modulates cell growth may involve changes in the apoplastic pool of oxidized ascorbate (Pignocchi *et al.*, 2006). Despite the high AO activity in immature seeds, however, we failed to detect a corresponding increase in the dehydroascorbate levels (data not shown). This may be explained considering that the apoplastic pool of ascorbate constitutes only 5–10% of the total ascorbate cell content (Pignocchi *et al.*, 2006), and thus determination of the
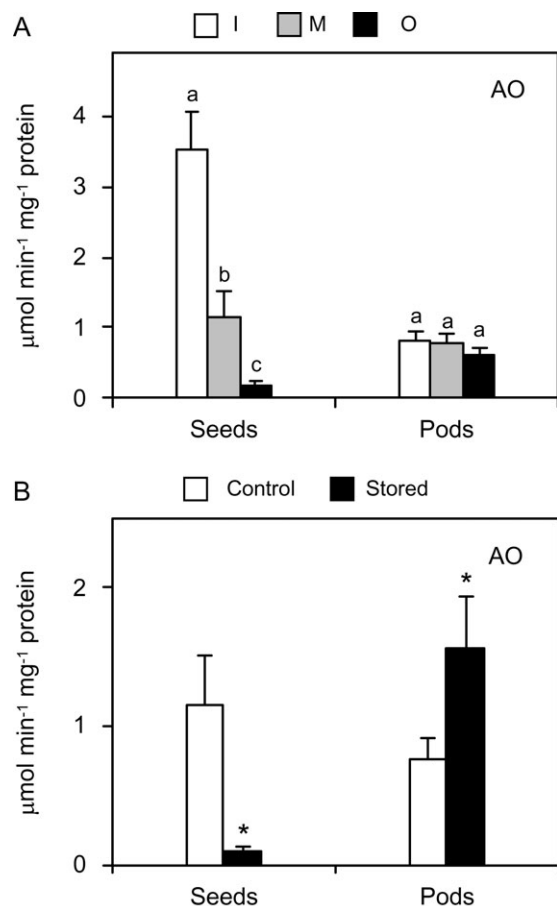
Fig. 6. Ascorbate oxidase (AO) activity in seeds and seedless pods of pea fruits. (A) Changes in AO activity during maturation. Fruits were classified as immature (I), mature (M), or overmature (O) as described in Fig. 1. Values are means ±SE of 4–6 samples from at least two series of plants grown independently. For each tissue, means denoted by the same letter do not significantly differ at $P <0.05$ based on Duncan's multiple range test. (B) AO activity in seeds and pods from control and stored fruits. Values are means ±SE of 4–6 samples from at least two series of plants grown independently, and asterisks indicate significant differences from control seeds based on Student's t test ($P <0.05$).

ascorbate redox state in whole seeds may mask detection of any increase in the oxidative state of the apoplast.

Prolonged storage of pea fruits at room temperature nearly abolished AO activity in seeds but doubled the activity in pods (Fig. 6B). The down-regulation of AO activity during storage may decrease ascorbate oxidation in the cell wall and probably contributes to improved fruit tolerance to stressful conditions imposed by detachment from the plant. Other studies have shown a relationship between AO expression and stressful conditions. Thus, AO gene expression was repressed in response to wounding (Diallinas *et al.*, 1997), and tobacco (*Nicotiana tabacum*) and *Arabidopsis* plants with suppressed expression of the apoplastic *AO* gene were more tolerant to salt stress (Yamamoto *et al.*, 2005).

In summary, in this work the impact of sustainable agricultural practices on the antioxidant content of pea fruits was assessed. This may have implications for both the nutritional value and post-harvest shelf life of the fruits. Maturation and storage of pea fruits probably entail a 'downwards' modulation of ROS and antioxidant levels, which would explain the absence of oxidative damage. These results underscore the importance of ascorbate, glutathione, PrxIIc, and AO as part of the antioxidant network in these two processes

Acknowledgements

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