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RESEARCH IN CONTEXT: PART OF A SPECIAL ISSUE ON REACTIVE OXYGEN AND NITROGEN SPECIES

Physiology of pepper fruit and the metabolism of antioxidants: chloroplasts, mitochondria and peroxisomes

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• **Background and Aims** Pepper (*Capsicum annuum*) contains high levels of antioxidants, such as vitamins A and C and flavonoids. However, information on the role of these beneficial compounds in the physiology of pepper fruit remains scarce. Recent studies have shown that antioxidants in ripe pepper fruit play a key role in responses to temperature changes, and the redox state at the time of harvest affects the nutritional value for human consumption. In this paper, the role of antioxidant metabolism of pepper fruit during ripening and in the response to low temperature is addressed, paying particular attention to ascorbate, NADPH and the superoxide dismutase enzymatic system. The participation of chloroplasts, mitochondria and peroxisomes in the ripening process is also investigated.
• **Scope and Results** Important changes occur at a subcellular level during ripening of pepper fruit. Chloroplasts turn into chromoplasts, with drastic conversion of their metabolism, and the role of the ascorbate–glutathione cycle is essential. In mitochondria from red fruits, higher ascorbate peroxidase (APX) and Mn-SOD activities are involved in avoiding the accumulation of reactive oxygen species in these organelles during ripening. Peroxisomes, whose antioxidant capacity at fruit ripening is substantially affected, display an atypical metabolic pattern during this physiological stage. In spite of these differences observed in the antioxidative metabolism of mitochondria and peroxisomes, proteomic analysis of these organelles, carried out by 2-D electrophoresis and MALDI-TOF/TOF and provided here for the first time, reveals no changes between the antioxidant metabolism from immature (green) and ripe (red) fruits.
• **Conclusions** Taken together, the results show that investigation of molecular and enzymatic antioxidants from cell compartments, especially chloroplasts, mitochondria and peroxisomes, is a useful tool to study the physiology of pepper fruit, particularly in the context of expanding their shelf-life after harvest and in maintaining their nutritional value.

Key words: Antioxidants, ascorbate, *Capsicum annuum*, chloroplasts, low temperature, mitochondria, NADPH, pepper fruit, peroxisomes, proteomics, reactive oxygen and nitrogen species, ripening, superoxide dismutase.

PEPPER FRUIT: MAIN FEATURES AND RIPENING

Pepper (*Capsicum annuum* L.) is one of the most widely consumed vegetables worldwide, mainly due to the diversity of culinary purposes and its handling plasticity. Thus, besides being used raw in many diets, pepper fruits are subjected to several industrial transformations to convert them to conserves, condiments, spices, etc. Much of the nutritional value of pepper fruits resides in their low calorie content and high antioxidant levels, especially ascorbic acid (vitamin C) and β-carotene (provitamin A). In fact, pepper fruits are one of the agricultural products, including fruits and vegetables, with the highest ascorbate content (Palma *et al*., 2009, 2011*a*; Martí *et al*., 2011*a*) (Table 1). One hundred grams of pepper fruit provides approx. 25 % of the recommended daily amount (RDA) of vitamin A, but 50 g of fresh fruit is enough to overpass the RDA for vitamin C (Howard *et al*., 2000; Proteggente *et al*., 2002; Hassimotto *et al*., 2009; Mateos *et al*., 2013).

Pepper fruits also display high antioxidant activity, as determined by gallic acid equivalents. In pepper fruit, numerous compounds are potential contributors to total antioxidant capacity (TAC), including ascorbate, flavonoids, carotenoids, phenolics and capsaicinoids. Fraga *et al*. (2014) have shown that *in vitro* TAC assays usually have limitations as they exclude some compounds such as antioxidant enzymes, metal-binding proteins and other antioxidants. It is clear that pepper fruits are one of the main sources of vitamin C and A in the human diet.

Note also that in many cases, in pepper, tomato, mango, orange, lemon and other fruits and vegetables, ascorbate values depend on the cultivar/variety, developmental stage, environmental conditions, crop season, production practice, and maturation and storage conditions (Jiménez *et al*., 2002, 2003; Deepa *et al*., 2006; Ribeiro *et al*., 2007; Ghasemnezhad *et al*.,

TABLE 1. *Total antioxidant capacity and total ascorbate in some fruits and vegetables*

	Total antioxidant activity [mg GAE (100 g f. wt)$^{-1}$]	Total antioxidant activity [FRAP: μmol (kg f. wt)$^{-1}$]	Total ascorbate [mg (100 g f. wt)$^{-1}$]
Fruit			
Apple	48	4200–6300	6–60
Banana	38	4200	10–11
Grape	80	4160–4780	2–3
Grapefruit	ND	8080	36
Kiwi	ND	8200	59
Lemon	ND	10400	58
Mandarin	ND	5400	20
Mango	ND	5060	37
Orange	126	9420	46–54
Peach	38	ND	6
Pear	60	4080	3–6
Pineapple	ND	3480	12
Plum	320	9280	4–5
Raspberry	228	ND	26
Strawberry	330	15940	61–77
Vegetable*			
Aubergine	45	ND	22
Broccoli	128	2940–7480	45–87
Cauliflower	30	2840–5580	15–43
Cabbage	ND	3500–5000	49
Carrot	ND	1660–2400	6
Celery	ND	1340–1560	8
Garlic	ND	2400–2680	17
Leek	22	ND	16
Lettuce	14	580–880	<2–3
Onion	88	2880–4320	5–6
Pea	32	ND	22
Pepper (green)	119	ND	92
Pepper (red)	131	ND	105
Potato	ND	1440–2320	7
Spinach	72	ND	7
Tomato	30	2360–3120	17–18

Data were collected from Szeto *et al*. (2002), Proteggente *et al*. (2002) and Hassimotto *et al*. (2009). Total antioxidant activity is expressed as gallic acid equivalent (GAE) and as FRAP (ferric reducing–antioxidant power) per fresh weight. ND, not determined.

*Values given on FRAP were obtained in vegetables extracted in water and acetate buffer (pH 3.6).

2011; Martí *et al*., 2011*a*). Thus, in tomato fruits, ascorbate content is linked to a combination of genetic factors (with cultivars displaying large variability), environmental factors (environmental conditions plus cultural practices) and post-harvest storage conditions (García *et al*., 2009). In mango fruits, ascorbate content varies depending on environmental factors, i.e. temperature and solar radiation, but also on fruit position within the canopy, while it was independent of the stage of maturity (Léchaudel *et al*., 2013).

One of the most important features of pepper fruits is the presence of capsaicin. This is an alkaloid of phenylpropanoid nature responsible for the pungency associated with hot peppers. Capsaicin is a member of the family of capsaicinoids consisting of 22 substances that derive from a structure comprising vanillylamine linked to a 9–11-carbon branched fatty acid (Ishikawa, 2003). The major (about 90 %) capsaicinoids found in pepper are capsaicin and dehydrocapsaicine (Reyes-Escogido *et al*., 2011), and they are mainly localized in the vacuoles of the epidermal cells of the placenta and the septum (Ishikawa, 2003; Garcés-Claver *et al*., 2007). The concentration of these compounds increases throughout fruit development, reaching a maximum at the highest fruit size. They then become degraded by the action of peroxidases (Estrada *et al*., 2002; Ishikawa, 2003). Regardless, capsaicinoid levels depend on genotype (DeWitt and Bosland, 1993), developmental stage (Estrada *et al*., 2002), and environmental and nutritional conditions, among other factors (Estrada *et al*., 1999; De, 2003; Sung *et al*., 2005; Garcés-Claver *et al*., 2007).

Fruit ripening is a much orchestrated complex process involving many genes and proteins (Klie *et al*., 2014). During ripening important visual and metabolic changes occur in most *Capsicum* species: intense metabolism, emission of volatile organic compounds associated with respiration, destruction of chlorophylls and synthesis of new pigments (red/yellow carotenoids plus xanthophylls and anthocyans) responsible for the colour shift, protein degradation/synthesis, taste alteration and changes in total soluble reducing equivalents (Camara *et al*., 1995; Markus *et al*., 1999; Howard *et al*., 2000; Manirakiza *et al*., 2003; Palma *et al*., 2011*b*; Mateos *et al*., 2013).

Pepper fruits have been investigated mainly due to their culinary and gastronomic value. Thus, attention has been paid to the complexity of the mechanisms which take part in the process of biosynthesis of capsanthin, a typical pepper colorant (De, 2003). Given the importance of pepper in agriculture and nutrition, a better understanding of the molecular changes associated with fruit ripening will provide useful information regarding varieties and harvesting times to improve fruit quality and to set target features such as levels of aroma, pungency, sweetness and colour (De, 2003; Martí *et al*., 2011*a*). With the aim of investigating the redox processes that help pepper fruits to cope with oxidative stress triggered by changes in the environment and during ripening – thus rendering fruits of good quality – our group has been working at biochemical and cell and molecular levels on the synergistic role of antioxidant metabolites and enzymes in this crop species.

ASCORBATE AND OXIDATIVE METABOLISM OF PEPPER FRUITS DURING RIPENING AND IN THE RESPONSE TO LOW TEMPERATURE

Non-enzymatic antioxidants such as ascorbic acid, glutathione, phenolic compounds and carotenoids are important in the metabolism of fruits and vegetables, but also for their beneficial effects in certain human diseases and pathologies (Namiki, 1990; Byers and Perry, 1992; Rimm *et al*., 1996; Gil *et al*., 2002; Palma *et al*., 2009; Martí *et al*., 2011*a*). The presence of carotenoids is a typical feature of pepper fruit, and for many years they have been the subject of extensive research aimed at determining their physiological role and characterizing their biosynthetic pathways (Bouvier *et al*., 1998; Paran and van der Knaap, 2007; Gómez-García and Ochoa-Alejo, 2013). However, less attention has been paid to other molecules involved in the redox and oxidative metabolism. This paper thus investigates the roles of ascorbate and NADPH/NADP metabolism in ripening of pepper fruits and in the response to low temperature.

Ascorbate metabolism and fruit ripening

In pepper, although it is commonly assumed that the highest total ascorbate content is associated with ripe fruits (Jiménez *et al*., 2002; Zhang and Hamauzu, 2003; Navarro *et al*., 2006), other reports, including our data, have found no changes between immature green and mature peppers (Simonne *et al*., 1997; Howard *et al*., 2000; Martí *et al*., 2009, 2011*a*). Similarly, contradictory results have been reported for cultivars which shift to different colours at ripening. Thus, whereas it has been observed in ripe fruits that ascorbic acid content in red pepper cultivars was higher than in yellow cultivars (Matsufuji *et al*., 2007), other recent research did not find such differences (Martí *et al*., 2011*a*). Likewise, in a study that reviewed five pepper cultivars, with ripe phenotypes being orange, purple, dark violet, red and yellow, a variable ascorbate pattern between immature and mature fruit was reported (Ghasemnezhad *et al*., 2011).

Ascorbate redox state, including both reduced ascorbate (ASC) and the oxidized form (dehydroascorbate; DHA), has been reported to change during the process of maturation in a sequence similar to what occurs in plant senescence where oxidation processes take place (Tan *et al*., 2012; Gapper *et al*., 2013; Gómez *et al*., 2014). The decrease in ASC and/or the increase of DHA contents usually coincide with maturation, with an increment of either ascorbate peroxidase (APX) activity (Camejo *et al*., 2010; Tan *et al*., 2012), which oxidizes ascorbate concomitantly with the removal of hydrogen peroxide, or ascorbate oxidase, which oxidizes ASC to monodehydroascorbate (Alós *et al*., 2013). In both cases ascorbate is regenerated for new use through the ascorbate–glutathione cycle (AGC), also called the Foyer–Halliwell–Asada pathway, which, besides APX, involves the enzymes mondehydroascorbate reductase (MDAR), dehydroascorbate reductase (DAR) and glutathione reductase (GR) which consumes NADPH (Smirnoff, 2005; Asada, 2006; del Río, 2011; Foyer and Noctor, 2011; Corpas and Barroso, 2014). The multiple data provided so far indicate that there is no clear pattern of the effect of ripening on ascorbate redox state in pepper fruits. Different factors seem to operate during maturation affecting ascorbate oxidation, such as the cultivar and environmental (temperature and sun radiation) and culture conditions (Lee and Kader, 2000; Martí *et al*., 2011*a*).

Regarding the synthesis of ascorbate, an inverse correlation between expression of the biosynthetic genes and ASC concentrations was found during ripening of pepper fruits. Thus, ascorbate content increased at ripening, whereas expression levels of the biosynthetic pathway genes *GDP-mannose pyrophosphorylases 1* and *2*, *GDP-mannose-3′-5′-epimerase, GDP-L-galactose transferase* and *L-galactono-1,4-lactone dehydrogenase* (*GalLDH*) decreased (Alós *et al*., 2013). The authors postulated that a feedback mechanism by which ASC content may control its own biosynthesis could be taking place. Furthermore, they found that ascorbate oxidase seemed to play a critical role in the regulation of the ASC pool during fruit ripening (Alós *et al*., 2013).

Therefore, a universal description of how ascorbate evolves at ripening and development of pepper fruits cannot be given as many factors influence this profile. Rather, a potential question that need to be addressed is the role that ascorbate might have in fruit physiology. In our view, ascorbate seems to act as a redox buffer which cushions the important metabolic changes occurring during ripening. There, ascorbate participates as a preservative which contributes to expand the shelf life of fruits. In fact, pepper fruits are one of the fresh plant products with a long shelf life.

The use of proteomics as a powerful high-throughput tool to gain deeper understanding of the redox metabolism during ripening and development of fruits, including pepper, has been proposed (Palma *et al*., 2011*b*). More recently, the transcriptomic analysis of genes involved in the biosynthesis, recycling and degradation of L-ascorbic acid in pepper fruits has been accomplished, paying particular relevance to the profile of expression of APX (Alós *et al*., 2013). Likewise, in tomato fruits, the genes involved in the biosynthesis of ascorbic acid and redox reactions under cold storing conditions were investigated, and a post-transcriptional up-regulation of those genes was reported (Tsaniklidis *et al*., 2014). Accordingly, the study of ascorbate metabolism, from biosynthesis to degradation, confers a body of knowledge which is gaining attention in crop species.

NADPH/NADP metabolism and fruit ripening

NADPH is necessary for the regeneration of ascorbate through the AGC (del Río, 2011; Foyer and Noctor, 2011; Corpas and Barroso, 2014). Besides this role in plant cells, NADPH is an important molecule which participates in other cell detoxification processes as a co-factor of NADPH-dependent thioredoxin reductases, NADPH-cytochrome P450 reductases, NADPH oxidases and the L-arginine-dependent nitric oxide synthase. Furthermore, this coenzyme is involved in specific events associated with cell growth and development such as fatty acid biosynthesis, sugar biosynthesis in the Calvin–Benson cycle, carotenoid biosynthesis, conversion of ribonucleotides to deoxyribonucleotides and chloroplast protein import through the Tic complex (Corpas and Barroso, 2014). In pepper plants, NADPH has been shown to be involved in the response to stress by high Cd concentrations (León *et al*., 2002), and to treatment with the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) (unpublished). In mature pepper fruits, NADPH and NADP also displayed a noteworthy increase with respect to green immature fruits. Analysis of the expression, enzyme activity and protein content of glucose 6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH), NADP-dependent isocitrate dehydrogenase (ICDH) and NADP-dependent malic enzyme (ME) suggested that, besides being key elements in the mechanism of response to nitro-oxidative stress situations (Corpas and Barroso, 2014), these NADPH-generating enzymes could be involved in the maturation of pepper fruits (Mateos *et al*., 2009). These enzymes seem also to have a function in the maturation of peach fruits (Etienne *et al*., 2002; Kong *et al*., 2007), during development of sour lemon (Sadka *et al*., 2000), in the physiology of banana (Carpentier *et al*., 2010), in tomato fruit under ripening (Gallardo *et al*., 1995), and in olive fruits where they may participate in the regulation mechanisms that modulate the antioxidant composition of olive oil (López-Huertas and del Río, 2014), among others.

Regarding to the couple NADH/NAD and its influence on the metabolism of fruits from crop species, it has been reported

that the plastidial NADH dehydrogenase that supports non-photochemical electron fluxes could be essential for ripening (Nashilevitz *et al*., 2010). However, most references on this subject pay attention to the enzymes which use NADH/NAD as co-factors rather than to the relevance of each nucleotide in ripening stages (Manriquez *et al*., 2006; Singh *et al*., 2010; Yamaya and Kusano, 2014).

Oxidative metabolism under low temperature

Pepper has a tropical origin but it grows and develops under mild temperature conditions (16–28 °C). In pepper plants, low temperature (LT) was found to promote both oxidative and nitrosative stress shortly after treatment, although these effects reverted after a period of acclimation. Overall, plants subjected to LT increased their contents of ascorbate and glutathione in leaves as well as the activity of several NADP-dehydrogenases (Airaki *et al*., 2012). In pepper fruits, LT provokes many physiological disorders (Airaki *et al*., 2012), thus producing negative impact in crops and economical losses for farmers. Chilling injury under post-harvest conditions showed an increase in ethylene production and lipid peroxidation and lower ascorbate content in pepper fruits (Sánchez-Bel *et al*., 2012).

The oxidative metabolism of fruits from pepper plants subjected to LT was investigated. Thus, fruits that were set, developed and ripened *in planta* at an average temperature 4–6 °C below that of fruits grown under control conditions (average temperature, 16 °C) were studied, and it was found that their antioxidative systems were involved in the response to lower temperature, thus avoiding injuries and oxidative stress. In this case, principal components analysis of up to 15 reactive oxygen species (ROS)-related parameters – including enzymatic and non-enzymatic antioxidants and oxidative stress indexes – showed that only the ascorbate pool remained unaffected after fruits from the four cultivars underwent LT (Mateos *et al*., 2013). The NADP-dehydrogenases referred to above were involved in the response to LT of pepper fruits (Mateos *et al*., 2013), tomato fruits (Knee *et al*., 1996) and Valencia orange fruits (Falcone-Ferreyra *et al*., 2006). In this last species it was reported that the NADP-dehydrogenases also participated after heat treatment of fruits during post-harvest cold storage (Perotti *et al*., 2015).

Proteomic studies have been carried out to advance understanding of chilling injury in pepper fruits, and alteration of the redox homeostasis and carbohydrate metabolism has been described (Sánchez-Bel *et al*., 2012), but high-throughput approaches are still lacking on this issue for this plant material.

Overall, the data reported above, which are representative of the many references that can be found in the literature, indicate the relevance of studying the metabolism of ascorbate and NADPH in fruits, particularly those that are important contributors to the human diet. One aspect that has to be taken into account on this subject is the cell compartments where the metabolism of these molecules is distributed, which commonly involves chloroplasts/chromoplasts, mitochondria, peroxisomes, cytosol and the apoplast. Therefore, investigation at the subcellular level using cell biology and molecular approaches such as the purification of cell organelles combined with proteomic/metabolomic tools, the use of specific genes or microscopy techniques (confocal, fluorescence and electron) will provide greater knowledge of the metabolic and physiological processes that take place in fruits under normal and anomalous situations.

RIPENING OF PEPPER FRUITS AND ANTIOXIDATIVE METABOLISM AT SUBCELLULAR LEVEL

An overall picture of the ROS and antioxidative metabolism in different cell organelles, mainly from leaves, has been issued based on results obtained from different plant sources (Jiménez *et al*., 1997; Foyer and Noctor, 2003, 2013; Palma *et al*., 2006; Locato *et al*., 2009). Pepper is perhaps the only plant species where the antioxidative metabolism during ripening and development of fruits has been thoroughly investigated at the subcellular level. Thus, besides the specific metabolic pathways that take place in plastids, mitochondria and peroxisomes from immature and ripe fruits, the reactions in which distinct antioxidative enzymes are involved in these organelles have been analysed in recent years (Jiménez *et al*., 2002; Mateos *et al*., 2003; Martí *et al*., 2009). The partial distribution of ascorbate in these organelles has been evaluated, and it was concluded that chromoplasts from red pepper fruits were the cell compartments where ascorbate accumulates most, two-fold the levels obtained in chloroplasts from immature green fruits (Palma *et al*., 2011*a*). Interestingly, these authors also found that peroxisomes contained higher amounts of ascorbate than mitochondria, organelles where the synthesis of this antioxidant occurs (Horemans *et al*., 2000; Smirnoff *et al*., 2001; Millar *et al*., 2003; Bartoli *et al*., 2006; Smirnoff, 2011). These data on the content of ascorbate (and other compounds) in isolated cell organelles are not definite as loss of metabolites throughout the respective purification procedures takes place, but are consistent enough to compare the same type of organelles in fruits at different ripening and developmental stages.

Plastids

Due to the molecular architecture and composition of their membranes, chloroplasts (plastids) are the organelles most prone to be affected by ROS under certain conditions, such as stress promoted by diverse agents, and also during senescence and development. These organelles contain powerful antioxidative tools to cope with those situations, including low-molecular-weight compounds (ascorbate, glutathione, carotenoids, α-tocopherol, etc.) and enzymes (Asada, 2006; Locato *et al*., 2009; Foyer and Noctor, 2013; Corpas *et al*., 2015). In pepper fruits, chloroplasts are converted to chomoplasts during ripening and this event is associated with the destruction of chlorophyll and the synthesis of carotenoids (Camara *et al*., 1995; Bouvier *et al*., 1998; Markus *et al*., 1999; Manirakiza *et al*., 2003; Mateos *et al*., 2003, 2013). In studies performed in chloroplasts and chromoplasts isolated from green (immature), red and yellow fruits, respectively, it was found that all enzymes of the AGC underwent up-regulation (Table 2) at ripening, while decreases of about 20 % of superoxide dismutase (SOD) activity were observed in chromoplasts from mature fruits, either red or yellow (Table 3). It was concluded that

TABLE 2. *Activity of the ascorbate–glutathione cycle enzymes in plastids, mitochondria and peroxisomes from pepper fruits at different ripening stages*

Fruit ripening stage	Organelle	APX	MDAR	DAR	GR
		nmol 10^{-6} org min^{-1}			
Green (immature)	Chloroplasts	8·5 ± 0·7	1·4 ± 0·3	2·4 ± 0·2	2·3 ± 0·1
Red (ripe)	Chromoplasts	49·8 ± 3·0***	5·1 ± 0·5***	17·7 ± 1·0***	7·1 ± 1·3**
Yellow (ripe)	Chromoplasts	57·0 ± 1·5***	9·0 ± 0·5***	18·7 ± 0·6**	23·1 ± 0·4***
		nmol mg^{-1} min^{-1}			
Green	Mitochondria	7·9 ± 0·4	4·37 ± 0·4	1·8 ± 0·2	2·9 ± 0·9
Red	Mitochondria	23·2 ± 2·4**	2·9 ± 0·5*	0·9 ± 0·3*	0·9 ± 0·2*
Green	Peroxisomes	927 ± 84	ND	ND	113·1 ± 18·6
Red	Peroxisomes	232 ± 18***	ND	ND	20·0 ± 7·3***

Data are the means ± s.e. of four different experiments and asterisks indicate statistically significant differences between organelles from green, red and yellow pepper fruits at $*P < 0·05$, $**P < 0·01$ and $***P < 0·001$. Plastids (chloroplasts and chromoplasts), mitochondria and peroxisomes were statistically analysed among each other. APX, ascorbate peroxidase; MDAR, monodehydroascorbate reductase; DAR, dehydroascorbate reductase; GR, glutathione reductase. Data reported on chloroplasts and mitochondria were obtained from results given in Jiménez *et al*. (2002) and Martí *et al*. (2009), respectively. Results of GR from peroxisomes were taken from Mateos *et al*. (2003), and those of peroxisomal APX are reported here for the first time. ND, not determined.

TABLE 3. *Superoxide dismutase (SOD) in organelles from pepper fruits*

Organelle	Fruit ripening stage	Phenotype of fruits at ripe stage	SOD activity (units mg^{-1} protein)	SOD isozyme
Chloroplasts	Green (immature)	Red	38·9 ± 6·1	Fe-SOD, CuZn-SOD I
Chromoplasts	Red (ripe)	Red	30·4 ± 3·9	Fe-SOD, CuZn-SOD I
Chloroplasts	Green (immature)	Yellow	23·8 ± 2·4	Fe-SOD, CuZn-SOD I
Chromoplasts	Yellow (ripe)	Yellow	19·0 ± 0·5	Mn–SOD, Fe-SOD, CuZn-SOD I
Mitochondria	Green	Red	102·7 ± 8·9	Mn-SOD
	Red	Red	337·0 ± 22·3	Mn-SOD
Peroxisomes	Green	Red	370·0 ± 28·5	Mn-SOD
	Red	Red	227·2 ± 19·6	Mn-SOD

Data given here were summarized from results obtained by Jiménez *et al*. (2002), Mateos *et al*. (2003) and Martí *et al*. (2009). Data on chloroplasts and chromoplasts correspond to two different cultivars: in one of them fruits ripen as a red phenotype, and the other one as a yellow phenotype. Results from mitochondria and peroxisomes were obtained from the cultivar whose ripe fruits are red.

these enzymatic systems could function as modulators of signal molecules such as superoxide radicals and hydrogen peroxide during fruit maturation (Martí *et al*., 2009). Besides the typical presence of CuZn-SOD and Fe-SOD activities in plastids, these authors also reported, by using biochemical and immunocytochemical approaches, the unequivocal localization of an Mn-SOD in chromoplasts from a cultivar whose fruits ripe as a yellow phenotype (Martí *et al*., 2009). In Table 3, the identity of the SODs in organelles from pepper fruits described thus far is also given. This is an interesting peculiarity of plastids in pepper fruits as the localization of Mn-SODs has been commonly associated with mitochondria and peroxisomes (Palma *et al*., 1998; del Río *et al*., 2003; Rodríguez-Serrano *et al*., 2007). As the Mn-SOD is a nuclear-encoded protein, it implies that the potential chromoplastic Mn-SOD from yellow pepper fruits should harbour the specific targeting signal at the N terminus to address the protein to the organelle. A possible dual-targeting event may occur as it was postulated for the peroxisomal and mitochondrial Mn-SODs from pea leaves, where a process of alternative splicing seemed to be involved (Palma *et al*., 1998). Import assays carried in isolated organelles from *Arabidopsis* revealed a dual targeting of APX, MDAR and GR gene products to mitochondria and chloroplasts, whereas a putative DAR protein was only imported into mitochondria (Chew *et al*., 2003). Nevertheless, this subject needs further research as it indicates new genetic, physiological and evolutionary perspectives in the biology of superoxide dismutases.

Mitochondria

Very recently, it has been reported that the number of energized mitochondria strongly decreased in tomato fruit during ripening and that there was an important contribution of chromoplasts to total fruit respiration in late ripening stages (Renato *et al*., 2014). However, the important role of mitochondria in fruit ripening has been also reviewed, where the alternative oxidase (AOX) and the plant uncoupling mitochondrial protein (PUMP) are involved (Perotti *et al*., 2014; see also Holtzapffel *et al*., 2003). Also, it has been indicated that one of the major factors associated with senescence in fruits is the ROS-mediated impairment of mitochondrial function (Tian *et al*., 2013).

Mitochondria undergo serious alteration in fruits exposed to different types of stress, both biotic and abiotic, with ROS and the antioxidant organelle battery being key pieces (Kan *et al*., 2010; Perotti *et al*., 2014). In pepper, most antioxidant enzymatic systems, including the AGC, SODs and the ascorbate synthesizing galactone-γ-lactone dehydrogenase have been characterized in immature and ripe fruits (Jiménez *et al*., 2002).

Activity of MDAR, GR and DAR was higher in mitochondria isolated from green immature than from red fruits, while APX and Mn-SOD were much higher in red fruits (Tables 2 and 3). It has been also shown that the mitochondrial Mn-SOD is one of the enzymes affected by oxidative damage during senescence of apple fruits besides other mitochondrial enzymes such as malate dehydrogenase and aconitase (Qin *et al*., 2009). A role for ROS and some antioxidative enzymes in fruit physiology has therefore been proposed. Mn-SOD, the only universally accepted SOD enzyme to be located in mitochondria as it occurs in pepper fruits (Table 3), seems to be a key point for regulation of ROS metabolism during ripening along with APX, but this possibly depends on the plant species and developmental stage. Besides, other proteins involved in the antioxidative machinery of mitochondria such as thioredoxins, peroxiredoxins, glutaredoxins and sulfiredoxins might also have important functions in the fruit. In fact, these proteins from mitochondria of higher plants have been reported to participate in the response to stress conditions (Martí *et al*., 2011*b*; Lázaro *et al*., 2013).

Peroxisomes

Peroxisomes are single membrane-bound organelles which contain essential enzymes for plant metabolism involved in photorespiration, β-oxidation, glyoxylate cycle, ureide metabolism and oxidative metabolism, among others (del Río, 2011, 2013). These organelles are characterized by their high plasticity, so their composition is prone to change depending on the tissue/organ, developmental stage and growth conditions (Palma *et al*., 2009; del Río, 2013; Corpas and Barroso, 2014). Most reports concerning peroxisomal metabolism in plants have been obtained from vegetative tissues, mainly leaves, while little is known on how these cell compartments contribute to fruit physiology.

The full characterization of peroxisomes from fruits of a higher plant was first reported in pepper (Mateos *et al*., 2003). The potential involvement of olive peroxisomes in the beneficial qualities of olive oil has since been reported (López-Huertas *et al*., 2014). In both cases, the contribution of their respective antioxidative machinery in the ripening of fruits has been postulated, and the participation of peroxisomal metabolism in fruit physiology under several conditions has been reported elsewhere (Di Matteo *et al*., 2012; Sánchez-Bel *et al*., 2012; Gest *et al*., 2013). In pepper fruits, the peroxisomal matrix is mostly occupied by a crystalline core that takes variable shapes, although no specific content is attributable to this structure (Mateos *et al*., 2003). Comparison between the peroxisomal metabolism from immature green fruits and ripe red fruits showed no qualitative differences, so the same pathways are operative in the two ripening stages (Fig. 1).

Peroxisomes from both green immature and red ripe fruits contain enzymes of the β-oxidation of fatty acids, but also those of the glyoxylate cycle and photorespiration. In this model, the hydrogen peroxide (H_2O_2) generated by the glycollate oxidase (GOX; photorespiration) and the acyl-CoA oxidase (ACOX; β-oxidation) is removed by catalase (CAT) and APX. This APX, integrated in a peroxisomal AGC, functions by the continuous provision of NADPH to be used for GR. The NADPH is produced by the battery of NADP-dehydrogenases (G6PDH, 6PGDH and ICDH) located in this organelle. As indicated in Tables 2 (APX and GR) and 3 (SOD), and reported elsewhere for GOX, ACOX, G6PDH, 6PGDH and ICDH (Mateos *et al*., 2003), all these enzymes displayed higher activities in green immature fruits than in red fruits, as depicted in Fig. 1. On the other hand, the detected xanthine oxidase (XOD) activity would generate superoxide radicals ($O_2^{\cdot-}$), which would be the substrate of the peroxisomal Mn-SOD, thus closing the oxygen cycle in the organelle (Fig. 1). The identical specific activity of XOD obtained in peroxisomes from green immature and red fruits suggests that this enzyme may have an important role in the ripening process in this plant species. This is under study in our laboratory.

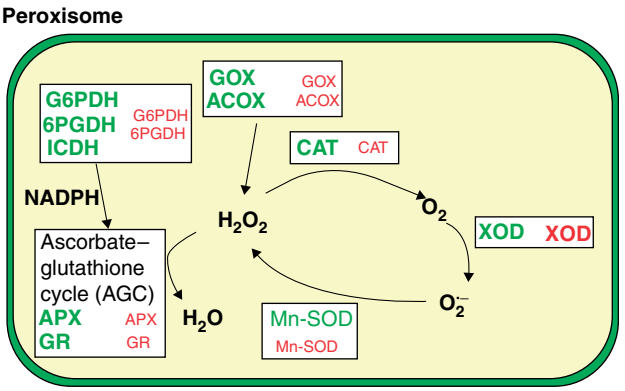

FIG. 1. Model of reactive oxygen species (ROS) metabolism in peroxisomes from pepper fruit. This model is based in the results obtained from peroxisomes isolated from green and red pepper fruits (Mateos *et al*., 2003). All the enzymes depicted here were reported in both types of fruits. Acronyms of enzymes are written in the colour corresponding to their respective fruits. All enzyme activities, except xanthine oxidase (XOD), were lower in red fruits than in green fruits and, accordingly, acronyms are written in smaller sizes that those from green fruits. The hydrogen peroxide (H_2O_2) generated by the photorespiratory enzyme glycollate oxidase (GOX) and the acyl-CoA oxidase (ACOX) from the β-oxidation of fatty acids is scavenged by the catalase (CAT) and the ascorbate peroxidase (APX) integrated in the organelle ascorbate–glutathione cycle. The glutathione reductase (GR) of this cycle uses NADPH produced by the glucose 6-phosphate dehydrogenase (G6PDH), the 6-phosphogluconate dehydrogenase (6PGDH) and the NADP-dependent isocitrate dehydrogenase (ICDH) located in this organelle. On the other hand, the peroxisomal XOD activity would generate superoxide radicals ($O_2^{\cdot-}$), which would be the substrate of the peroxisomal Mn-SOD, whose product, the H_2O_2, closes the oxygen cycle in the organelle.

Overall, comparison of the data reported on SOD activity in crude extracts from pepper fruits, where a notable increase was observed (Martí *et al*., 2009), and those from isolated organelles shown here (plastids, mitochondria and peroxisomes), attributes to mitochondrial Mn-SOD a key role in the metabolism of ROS, mainly superoxide radicals, during ripening.

The presence of photorespiratory activity in peroxisomes from red fruits is a surprising event as this pathway is commonly associated with green tissues, as a parallel pathway to the photosynthetic carbon fixation initiated at the Rubisco crossroad. This peculiarity confers to peroxisomes from pepper fruits a unique enzymatic configuration not reported in other plant species thus far. Research on the peroxisomal protein content from fruits at different developmental and ripening stages will provide more data on the metabolic plasticity of these organelles and their potential for future biotechnological applications.

TABLE 4. *Identification of oxidative metabolism-related proteins from mitochondria and peroxisomes of pepper fruits through proteomic analysis combining 2-D and MALDI-TOF.*

Organelle	Identified protein	Accession no./UniProt	Plant species	Protein score CI %/pept. count	Mw/pI	Other subcellular localization
Mitochondria	Mn-superoxide dismutase	O49066 T08045	*Capsicum annuum*	100/12	25 610·2/8·39	Peroxisomes
Peroxisomes	Catalase	P49319	*Nicotiana tabacum*	100/25	56 967·2/6·72	
		P49316	*Nicotiana plumbaginifolia*	100/14	57 317·6 6·75	
		Q9M5L6	*Capsicum annuum*	100/25	56 957·4/7·31	
	Fe-superoxide dismutase	Q6X1DO	*Solanum lycopersicum*	99·2/4	27 919/6·53	Plastids
		Q7YK44	*Solanum lycopersicum*	100/6	27 893/6·6	
	Mn-superoxide dismutase	O49066	*Capsicum annuum*	100/9		
		T08045	*Capsicum annuum*	100/8	25 610·2/8·39	Mitochondria

Concentrated mitochondria and peroxisomes from pepper fruits were subjected to 2-D electrophoresis. Isoelectric focusing was performed with precast IPG (immobilized pH gradient gels, pH 3–10), and each gel was loaded with 100 µg of organelle proteins. The second dimension separation was carried out by glycine SDS-PAGE. The gels were stained with Sypro Ruby, scanned, and analysed with the Bio-Rad PDQuest software. Identified spots in the Sypro Ruby-stained gels were automatically picked using an Investigator ProPic Protein Picking Workstation equipment (Genomic Solutions). Then, they were destained and digested with trypsin using an Investigator ProGest Protein Digestion Station (Genomics Solutions) as described (Chaki *et al*., 2009; Begara-Morales *et al*., 2013). The identified spots were analysed by MALDI-TOF/TOF mass spectrometry after trypsin digestion. The MASCOT search engine was used to parse MS data to identify proteins from primary sequence databases. The closer value of Protein Score Confidence Interval (CI) to 100 % indicates a strong likelihood that the protein is correctly matched. Pep. count., number of identified peptides. MW, molecular weight. pI, isoelectric point.

Antioxidant proteome of mitochondria and peroxisomes from pepper fruits

The proteome is the full complement of proteins expressed by a genome at a specific point of time (Palma *et al*., 2011*b*). Accordingly, it is assumed that the proteome of each living organism is dynamic, with changes due to the metabolic state and the reception of signal stimuli (Newton *et al*., 2004; Palma *et al*., 2011*b*). At the functional level, the proteome rather than the genome provides a better picture of the metabolism as it is known that proteins undergo more than 200 post-translational modifications (Palma *et al*., 2011*b*). Applied to cell biology, analysis of organelle proteomes provides a deep knowledge of the dynamics of cell metabolism, including interactions among organelles, transit of molecules, signalling processes inside the cell, etc. In fact, this strategy, with all the approaches available for proteomic analyses, was proposed for a better understanding of the molecular physiology of fruit ripening and development (Palma *et al*., 2011*b*), and would complement the available biochemical data. Convergence of proteomics with transcriptomic and metabolomic data will provide a full picture of the interrelationship among organelles and with overall cell metabolism.

As indicated above, conversion of chloroplasts to chromoplasts is commonly associated with fruit development and ripening. It implies dismantling of the protein complement of chloroplasts and the synthesis of proteins and pigments (Egea *et al*., 2011; Palma *et al*., 2011*b*; Renato *et al*., 2014) to carry out the functions of the new organelle. Investigation of the whole plastidial proteome has been recently documented as well as its evolution through the developmental changes that occur in fruits at ripening (Barsan *et al*., 2012; Nogueira *et al*., 2012; Wang *et al*., 2013), together with the proteome of chromoplasts from pepper fruits (Ytterberg *et al*., 2006; Wang *et al*., 2013). Proteomic analysis of plastids from fruits revealed that the profile of the oxidative metabolism-related enzymes including those from the AGC (APX, MDAR, DAR, GR), lipooxygenase, CuZn-SOD, Mn-SOD and glutathione-*S*-transferase depended to a large extent on the plant species and developmental stage (Barsan *et al*., 2012; Wang *et al*., 2013). As indicated above for chromoplasts from yellow pepper fruits, the presence of an Mn-SOD in plastids is again reported. Biochemical and proteomic data converge and, accordingly, this is a subject that deserves further study.

By contrast, the mitochondrial and peroxisomal proteomes in fruits have been less well studied, and no data on pepper are available so far. Here we report for the first time the proteomes of mitochondria and peroxisomes from pepper fruits and their profile in two ripening stages. To undertake this study, mitochondria and peroxisomes from green immature and red pepper fruits were purified by differential and density-gradient centrifugations (Jiménez *et al*., 2002; Mateos *et al*., 2003, respectively). Once organelles (mitochondria and peroxisomes) were separated in their respective density gradients, they were eluted, concentrated and analysed by 2-D electrophoresis.

As indicated in Table 4, among a series of potential mitochondrial proteins (Álvarez de Morales *et al*., unpubl. res.), an Mn-SOD was detected in purified mitochondria from both green immature and red peppers as the only oxidative metabolism-related enzyme. This SOD isozyme is usually located in mitochondria and peroxisomes (Palma *et al*., 2013), and the presence in both types of pepper fruits (immature and ripe) corroborates the activity reports given a decade ago (Jiménez *et al*., 2002). This eventuality also suggests that superoxide radicals are formed throughout ripening, possibly as a result of the dysfunction of the respiratory chain. However, this aspect needs further investigation. The Mn-SOD isozyme from peach mitochondria, which undergoes oxidative damage, has been reported to be involved in fruit senescence (Qin *et al*., 2009; Tian *et al*., 2013).

An Mn-SOD protein was also detected in peroxisomes from green and red fruits by proteomic analysis (Table 4), as commonly described in plant peroxisomes (Palma *et al*., 1998, 2013; Sandalio *et al*., 2013), and in correspondence with previous data which reported the presence of Mn-SOD activity and protein content (western blotting) in pepper fruits (Mateos

et al., 2003). The latter authors did not find any Fe-SOD in peroxisomes from pepper fruits, in contrast to our recent results obtained from the proteome of these organelles (Table 4). Fe-SOD is generally located in chloroplasts although the presence of this isozyme in peroxisomes has been described in petals from carnation (Droillard and Paulin, 1990; del Río *et al*., 2003). These data, together with references which report the presence of the isozyme CuZn-SOD in plant peroxisomes (Bueno *et al*., 1995; Corpas *et al*., 1998; del Río *et al*., 2003; Sandalio *et al*., 2013), reinforce the metabolic plasticity of peroxisomal metabolism. Catalase, as the marker enzyme of peroxisomes (del Río, 2013), was also detected by matrix assisted laser desorption ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) technology in the organelles isolated from green immature and red pepper fruits, thus confirming the relevant role of this protein in cell metabolism through the development and ripening of fruits.

As inferred from the data above, the current scenario in which plant biology moves towards the combination of different approaches including proteomics, metabolomics and transcriptomic profiling through RNA-seq data from fruits at different ripening and developmental stages will contribute to a better understanding of the physiology of fruits from higher plants and to obtain better knowledge of the beneficial effects of those products destined for the human diet. It will also allow us to depict signalling networks among and within cells that could help with breeding purposes, mainly in those species of worldwide importance. Furthermore, our results show that the investigation of molecular and enzymatic antioxidants from all cell compartments, especially chloroplasts, mitochondria and peroxisomes, provides a useful tool to study the physiology of pepper fruits, particularly in the context of expanding their shelf-life after harvest and to maintain their nutritional value.

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