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, 2011a; Martí et al., 2011a) (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.,...

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

<table>
<thead>
<tr>
<th>Fruit</th>
<th>Total antioxidant activity</th>
<th>Total antioxidant activity</th>
<th>Total ascorbate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[mg GAE (100 g f. wt)⁻¹]</td>
<td>[FRAP: µmol (kg f. wt)⁻¹]</td>
<td>[mg (100 g f. wt)⁻¹]</td>
</tr>
<tr>
<td>Apple</td>
<td>48</td>
<td>4200–6300</td>
<td>6–60</td>
</tr>
<tr>
<td>Banana</td>
<td>38</td>
<td>4200</td>
<td>10–11</td>
</tr>
<tr>
<td>Grape</td>
<td>80</td>
<td>4160–4780</td>
<td>2–3</td>
</tr>
<tr>
<td>Grapefruit</td>
<td>ND</td>
<td>8080</td>
<td>36</td>
</tr>
<tr>
<td>Kiwi</td>
<td>ND</td>
<td>8200</td>
<td>59</td>
</tr>
<tr>
<td>Lemon</td>
<td>ND</td>
<td>10400</td>
<td>58</td>
</tr>
<tr>
<td>Mandarin</td>
<td>ND</td>
<td>5400</td>
<td>20</td>
</tr>
<tr>
<td>Mango</td>
<td>ND</td>
<td>5060</td>
<td>20</td>
</tr>
<tr>
<td>Orange</td>
<td>126</td>
<td>9420</td>
<td>46–54</td>
</tr>
<tr>
<td>Peach</td>
<td>38</td>
<td>ND</td>
<td>6</td>
</tr>
<tr>
<td>Pear</td>
<td>60</td>
<td>4480</td>
<td>3–6</td>
</tr>
<tr>
<td>Pineapple</td>
<td>ND</td>
<td>3480</td>
<td>12</td>
</tr>
<tr>
<td>Plum</td>
<td>320</td>
<td>9280</td>
<td>4–5</td>
</tr>
<tr>
<td>Raspberry</td>
<td>228</td>
<td>ND</td>
<td>26</td>
</tr>
<tr>
<td>Strawberry</td>
<td>330</td>
<td>15940</td>
<td>61–77</td>
</tr>
<tr>
<td>Vegetable*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aubergine</td>
<td>45</td>
<td>ND</td>
<td>22</td>
</tr>
<tr>
<td>Broccoli</td>
<td>128</td>
<td>2940–7480</td>
<td>45–87</td>
</tr>
<tr>
<td>Cauliflower</td>
<td>30</td>
<td>2840–5580</td>
<td>15–43</td>
</tr>
<tr>
<td>Cabbage</td>
<td>ND</td>
<td>3500–5000</td>
<td>49</td>
</tr>
<tr>
<td>Carrot</td>
<td>ND</td>
<td>1660–2400</td>
<td>6</td>
</tr>
<tr>
<td>Celery</td>
<td>ND</td>
<td>1340–1560</td>
<td>8</td>
</tr>
<tr>
<td>Garlic</td>
<td>ND</td>
<td>2400–2680</td>
<td>17</td>
</tr>
<tr>
<td>Leek</td>
<td>22</td>
<td>ND</td>
<td>16</td>
</tr>
<tr>
<td>Lettuce</td>
<td>14</td>
<td>580–880</td>
<td>&lt;2–3</td>
</tr>
<tr>
<td>Onion</td>
<td>88</td>
<td>2880–4120</td>
<td>5–6</td>
</tr>
<tr>
<td>Pea</td>
<td>32</td>
<td>ND</td>
<td>22</td>
</tr>
<tr>
<td>Pepper (green)</td>
<td>119</td>
<td>ND</td>
<td>92</td>
</tr>
<tr>
<td>Pepper (red)</td>
<td>131</td>
<td>ND</td>
<td>105</td>
</tr>
<tr>
<td>Potato</td>
<td>ND</td>
<td>1440–2320</td>
<td>7</td>
</tr>
<tr>
<td>Spinach</td>
<td>72</td>
<td>ND</td>
<td>7</td>
</tr>
<tr>
<td>Tomato</td>
<td>30</td>
<td>2360–3120</td>
<td>17–18</td>
</tr>
</tbody>
</table>

Data were collected from Szeto et al. (2002), Proteggente et al. (2002) and Hassimoto 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).

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., 2011a). 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., 2011a). 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; Parán 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, 2011a). 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., 2011a). 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 monodehydroascorbate 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., 2011a).

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-β-galactose transferase and α-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., 2011b). 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 chromatoplasts 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., 2011a).

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 chromoplasts 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
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-SOD 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 galactono-γ-lactone dehydrogenase have been characterized in immature and ripe fruits (Jiménez et al., 2002).

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

<table>
<thead>
<tr>
<th>Fruit ripening stage</th>
<th>Organelle</th>
<th>APX</th>
<th>MDAR</th>
<th>DAR</th>
<th>GR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>nmol 10⁻⁶ org min⁻¹</td>
<td>nmol mg⁻¹ min⁻¹</td>
<td>nmol min⁻¹</td>
<td>nmol mg⁻¹ min⁻¹</td>
</tr>
<tr>
<td>Green (immature)</td>
<td>Chloroplasts</td>
<td>8.5 ± 0.7</td>
<td>1.4 ± 0.3</td>
<td>2.4 ± 0.2</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td>Red (ripe)</td>
<td>Chromoplasts</td>
<td>49.8 ± 3.0***</td>
<td>5.1 ± 0.5***</td>
<td>17.7 ± 1.0***</td>
<td>7.1 ± 1.3**</td>
</tr>
<tr>
<td>Yellow (ripe)</td>
<td>Chromoplasts</td>
<td>57.0 ± 1.5***</td>
<td>9.0 ± 0.5***</td>
<td>18.7 ± 0.6**</td>
<td>23.1 ± 0.4***</td>
</tr>
</tbody>
</table>

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.

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

<table>
<thead>
<tr>
<th>Organelle</th>
<th>Fruit ripening stage</th>
<th>Phenotype of fruits at ripe stage</th>
<th>SOD activity (units mg⁻¹ protein)</th>
<th>SOD isozyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroplasts</td>
<td>Green (immature)</td>
<td>Red</td>
<td>38.9 ± 6.1</td>
<td>Fe-SOD, CuZn-SOD I</td>
</tr>
<tr>
<td>Chloroplasts</td>
<td>Red (ripe)</td>
<td>Red</td>
<td>30.4 ± 3.9</td>
<td>Fe-SOD, CuZn-SOD I</td>
</tr>
<tr>
<td>Chloroplasts</td>
<td>Green (immature)</td>
<td>Yellow</td>
<td>23.8 ± 2.4</td>
<td>Fe-SOD, CuZn-SOD I</td>
</tr>
<tr>
<td>Chloroplasts</td>
<td>Yellow (ripe)</td>
<td>Yellow</td>
<td>19.0 ± 0.5</td>
<td>Mn-SOD, Fe-SOD, CuZn-SOD I</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>Green</td>
<td>Red</td>
<td>102.7 ± 8.9</td>
<td>Mn-SOD</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>Red</td>
<td>Red</td>
<td>337.0 ± 22.3</td>
<td>Mn-SOD</td>
</tr>
<tr>
<td>Peroxisomes</td>
<td>Green</td>
<td>Red</td>
<td>370.0 ± 28.5</td>
<td>Mn-SOD</td>
</tr>
<tr>
<td>Peroxisomes</td>
<td>Red</td>
<td>Red</td>
<td>227.2 ± 19.6</td>
<td>Mn-SOD</td>
</tr>
</tbody>
</table>

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.
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., 2011b; 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 olives 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$_2$O$_2$) 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$_2^-$), which would be the substrate of the peroxisomal Mn-SOD, whose product, the H$_2$O$_2$, closes the oxygen cycle in the organelle.

![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$_2$O$_2$) 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$_2^-$), which would be the substrate of the peroxisomal Mn-SOD, thus closing the oxygen cycle in the organelle.](https://academic.oup.com/aob/article-abstract/116/4/627/94361)
Table 4. Identification of oxidative metabolism-related proteins from mitochondria and peroxisomes of pepper fruits through proteomic analysis combining 2-D and MALDI-TOF.

<table>
<thead>
<tr>
<th>Organelle</th>
<th>Identified protein</th>
<th>Accession no./UniProt</th>
<th>Plant species</th>
<th>Protein score CI %/pept. count</th>
<th>Mw/pl</th>
<th>Other subcellular localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondria</td>
<td>Mn-superoxide dismutase</td>
<td>O49066, T08045</td>
<td>Capsicum annuum</td>
<td>100/12</td>
<td>25610-2/8-39</td>
<td>Peroxisomes</td>
</tr>
<tr>
<td>Peroxisomes</td>
<td>Catalase</td>
<td>P49319, P49316</td>
<td>Nicotiana tabacum</td>
<td>100/25</td>
<td>56967-2/6-72</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Q9M5L6</td>
<td>Nicotiana plumbaginifolia</td>
<td>100/14</td>
<td>57317-6-6-75</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fe-superoxide dismutase</td>
<td>Q6XIDO</td>
<td>Capsicum annuum</td>
<td>100/25</td>
<td>56957-4/7-31</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mn-superoxide dismutase</td>
<td>O49066, T08045</td>
<td>Capsicum annuum</td>
<td>100/8</td>
<td>25610-2/8-39</td>
<td>Mitochondria</td>
</tr>
</tbody>
</table>

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 gel-cine 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 (Genomics 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. pl, 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., 2011b). 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., 2011b). 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., 2011b). 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., 2011b), 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., 2011b; 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 (Alvarez 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 isoform 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 isoform 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., 2011).
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 Rio et al., 2003). These data, together with references which report the presence of the isozyme Cu/Zn-SOD in plant peroxisomes (Bueno et al., 1995; Corpas et al., 1998; del Rio et al., 2003; Sandalio et al., 2013), reinforce the metabolic plasticity of peroxisomal metabolism. Catalase, as the marker enzyme of peroxisomes (del Rio, 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.

ACKNOWLEDGEMENTS

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