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Role of Ascorbate on the Fruit Physiology of Pepper (*Capsicum annuum* L.)

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ABSTRACT

Ascorbate (vitamin C) is essential for cell metabolism, and its supply to the human diet derives basically from the consumption of fruits and vegetables. Several biosynthetic pathways of ascorbate have been reported in plants, the major physiological one being the so-called Smirnoff-Wheeler pathway. Ascorbate is a powerful antioxidant in plants, although it also plays an important role in hormone synthesis, cell signalling, gene expression, cell division and growth, and programmed cell death. Based on this multiplicity of functions, nowadays it is considered that ascorbate works as a redox buffer in the plant cell. Bell pepper (*Capsicum annuum* L.) is the second most consumed vegetable worldwide, and its fruit is one of the richest sources of vitamin C. In our laboratory, we have analysed a number of enzymatic and non-enzymatic antioxidants in fruits from different pepper cultivars under distinct developmental and environmental conditions. Unlike the majority of parameters determined, total ascorbate levels underwent few changes during the fruit ripening process. Likewise, under certain stress situations, provoked by environmental variations, total ascorbate remained practically invariable, and only the ascorbate/dehydroascorbate ratio was modified. Therefore, ascorbate seems to play a relevant role in the physiology of pepper fruit, possibly functioning as a regulator of processes which imply important metabolic alterations, thus contributing to the stability of fruits, a typical feature of peppers which commonly display a long life-span after harvest.

Keywords: antioxidants, ascorbate, *Capsicum annuum* L., pepper fruits, redox state

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ASCORBATE: METABOLISM AND FUNCTION

Ascorbate (ascorbic acid; vitamin C) is a non-enzymatic ubiquitous antioxidant, present in many cell compartments and essential for the cell metabolism. In plant cells, ascorbate can be mainly found as two redox forms either as reduced or oxidised ascorbate (dehydroascorbate) (**Fig. 1**) (Halliwell and Gutteridge 2007; Tripathi *et al*. 2009). In human beings, the deficiency of vitamin C is associated to certain pathologies such as scurvy, and epidemiological studies also suggest that vitamin C plays an important role in the prevention of cardiovascular diseases (Berrougui *et al.* 2009; Shannahan *et al*. 2010), especially those which involve an endothelial dysfunction (Kris-Etherton *et al*. 2004; Cash *et al.* 2010).

Several biosynthetic pathways of ascorbate have been reported thus far in plants and a number of animal species, excepting humans and other primates, guinea pigs, bats and some birds (Nishikimi *et al*. 1976; Kiuchi *et al*. 1982; Davey *et al*. 2000; Valpuesta and Botella 2004; Mateos 2006; Tripathi *et al*. 2009; Li *et al*. 2010). In animal cells, the synthesis of ascorbate involves the conversion of D-glucose to ascorbic acid through different hydrocarbon intermediate molecules such as D-glucuronate, L-gulonate and L-gulono-1,4-lactone, among others (Burns *et al*. 1960; Mahan *et al*. 2004; Valpuesta and Botella 2004; Chan *et al*. 2005; Tripathi *et al*. 2009). This last precursor of the pathway is converted to ascorbic acid by the L-gulono-1,4-lactone oxidase (GulLO; EC 1.1.3.8). All primates, including humans, have lost the capacity to synthesize vitamin C since they lack the presence of GulLO (Tripathi *et al*. 2009). Thus, in the human diet, the supply of vitamin C derives basically from the consumption of fruits and vegetables what makes us strictly dependent on the plant kingdom. In fungi and algae, where ascorbate is also absent, the presence of the analogous erythroascorbate has been reported with an alternative synthesis pathway whose last enzyme is D-arabino-1,4-lactone oxidase, different from the GulLO (Smirnoff *et al*. 2001).

In plants, the main ascorbate synthesis pathways reported thus far are depicted in **Fig. 2** (Valpuesta and Botella 2004; Smirnoff 2005, Mateos 2006; Giovannoni 2007; Li *et al*. 2010), although the major physiological one is the so-called Smirnoff-Wheeler pathway (**Fig. 2**, route 1) that involves a series of monosaccharides such as GDP-mannose, GDP-L-galactose, L-galactose-1-phosphate, L-galactose, and L-galactono-1,4-lactone, which is finally oxidised to ascorbic acid through the galactono-lactone dehydrogenase en-

Received: 19 October, 2010. Accepted: 29 June, 2011.

Invited Mini-Review

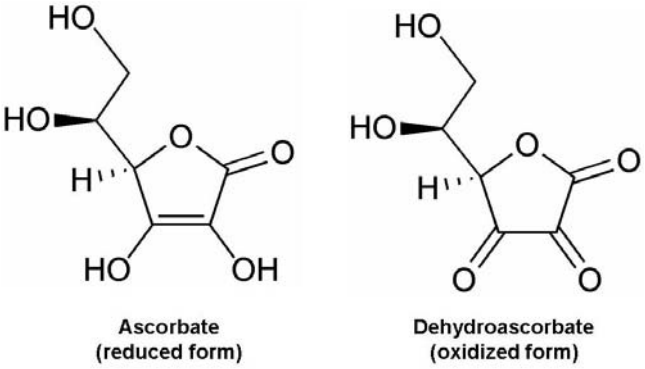

Fig. 1 Scheme of the molecular structure of reduced and oxidized forms of ascorbate.

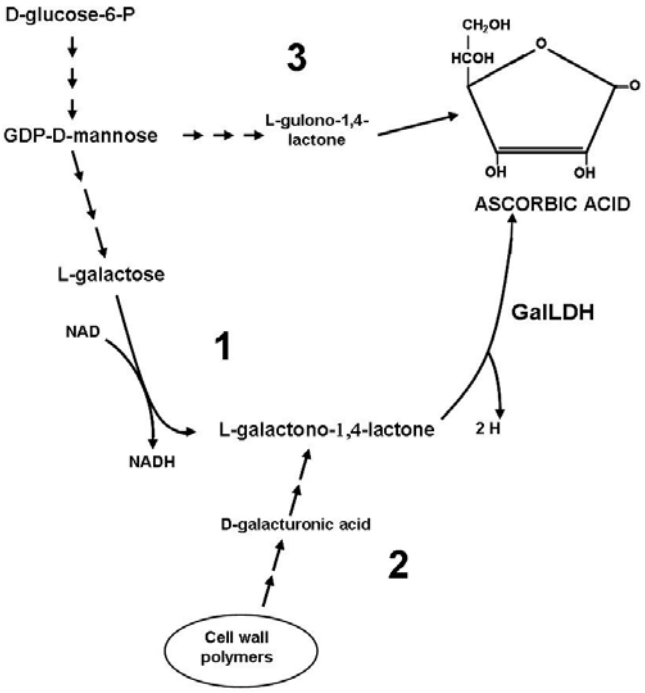

Fig. 2 Biosynthetic pathways of ascorbate in plant cells. GalLDH, L-galactono-1,4-lactone dehydrogenase. 1, Smirnoff-Wheeler pathway that involves a series of monosaccharides with the final oxidation reaction to ascorbic acid through the GalLDH. 2, Alternative pathway derived from the cell wall pectins which implies the final conversion of D-galacturonic acid to L-galactono-1,4-lactone. 3, Additional pathway of ascorbate which takes place in a very restricted number of plant species.

zyme (GalLDH; EC 1.3.2.3) (Wheeler *et al*. 1998; Conklin *et al*. 1999; Ishikawa *et al*. 2006; Tripathi *et al*. 2009; Li *et al*. 2010). An alternative pathway which derives from the cell wall pectins with successive conversions to methyl-D-galacturonate, D-galacturonic acid, L-galactonic acid and L-galactono-1,4-lactone (**Fig. 2**, route 2) has also been reported in some species (Agius *et al*. 2003; Valpuesta and Botella 2004). Additionally, other pathways seem to occur in plants, but are restricted to some species (**Fig. 2**, route 3; Loewus 1988; Saito *et al*. 1990; Smirnoff 2001).

The main role of ascorbate in plants is functioning as a powerful antioxidant. Thus, besides its capacity to directly scavenge several reactive oxygen species (ROS) such as superoxide radicals, hydroxyl radicals, hydrogen peroxide and singlet oxygen, ascorbate is able to regenerate other antioxidants previously oxidised such as vitamin E, and this confers to vitamin C an additional power (Padh 1990; Halliwell and Gutteridge 2007; Ahmad *et al*. 2010; Gill and Tuteja 2010). In addition, ascorbate participates in the xanthophyll cycle, a process which usually takes place at the thylakoidal lumen of chloroplasts and implies the regeneration of violaxantine at acidic pH through the violaxantine

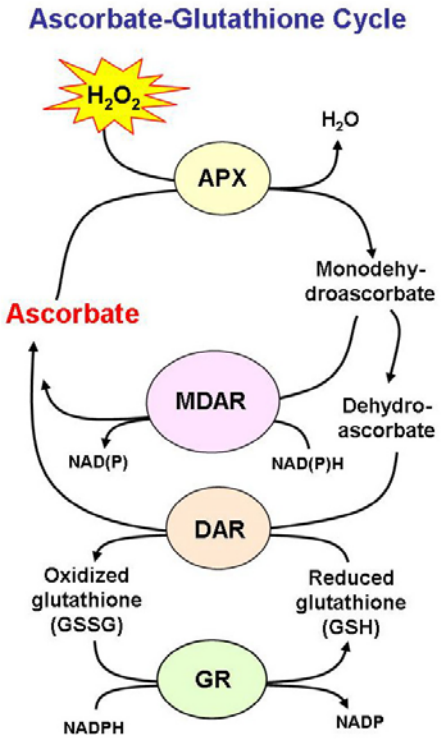

Fig. 3 Plant ascorbate-glutathione cycle. Hydrogen peroxide is removed in the presence of ascorbate by ascorbate peroxidase (APX) with the final consumption of reducing power as NADPH. MDAR, monodehydroascorbate reductase; DAR, dehydroascorbate reductase; GR, glutathione reductase.

de-epoxigenase activity (Smirnoff 2000).

However, the most important antioxidant role of ascorbate is its involvement in the ascorbate-glutathione cycle (AGC) also called the Asada-Halliwell-Foyer pathway (**Fig. 3**). This pathway is exclusive for plants and involves four antioxidative enzymes and two low molecular weight non-enzymatic antioxidants which synergically remove hydrogen peroxide. The enzymes of this pathway are ascorbate peroxidase (APX; EC 1.11.1.11), monodehydroascorbate reductase (MDAR; EC 1.6.5.4), dehydroascorbate reductase (DAR; EC 1.8.5.1), and glutathione reductase (GR; EC 1.6.4.2). The two small molecules are ascorbate and glutathione, the latter being a tripeptide composed by glutamic acid, cysteine and glycine. The different components of this cycle are interconnected in such a way that hydrogen peroxide is reduced to water and the electrons necessary for this reaction are provided by NADPH (Noctor and Foyer 1998; Mittler 2002; Asada 2006; Halliwell and Gutteridge 2007; van Breusegem *et al*. 2008; Kavitha *et al*. 2010).

This role as an antioxidant in plants confers to ascorbate a central role in the defense against oxidative stress situations, especially in photosynthetic tissues (Foyer *et al*. 1983; Smirnoff 2000). Thus, although ascorbate is synthesized in mitochondria, it has been reported that its concentration in chloroplasts reaches values ranging from 20-300 mM in some alpine species (Foyer *et al*. 1983; Rautenkranz *et al*. 1994; Streb *et al*. 1997; Smirnoff 2000).

In plants, it has been reported that ascorbate, besides being a powerful antioxidant, also plays an important role in other important processes inside and outside the cell. Actually, although most ascorbate is localized within distinct cell compartments, mainly chloroplasts, mitochondria, peroxisomes and cytosol, a proportion is exported to the apoplast, where it can be found at millimolar concentrations (Horemans *et al*. 2000a, 2000b). This makes indispensable a transport system through the plasma membrane, where some Na-dependent transporters (NAT)-like have been reported (Argyoru *et al*. 2001).

Ascorbate has been proved to participate also in the mechanisms of cell signalling. Thus, it plays a role in the

resistance against pathogens attack modulating the content of some signal molecules such as salicylic acid, abscisic acid, ethylene and gibberellins in a series of steps which activate the MAP kinases (Conklin and Bath 2004). Besides, ascorbic acid has been reported to be involved in hormone synthesis, gene expression, cell division and growth, programmed cell death or apoptosis, and stomatal movement of guard cells, among others (Chen and Gallie 2004; Foyer and Noctor 2005a). Based on this multiplicity of functions, nowadays it is considered that ascorbate acts as a redox buffer in the plant cell (Foyer 2004; Foyer and Noctor 2005a, 2005b; Smirnoff 2005; de Gara *et al*. 2010).

ASCORBATE IN FRUITS AND VEGETABLES

The main source of antioxidants in the human diet has a plant origin, basically fruits and vegetables. These antioxidants contribute to maintaining the health status and complement the cell defence mechanisms. One of the most important and abundant antioxidants present in plant products is vitamin C what considerably influences their total antioxidant activity. Nevertheless, a positive correlation among total antioxidant capacity (TAC) and ascorbate content does not always occur. As shown in **Table 1**, in natural products, the highest contents of vitamin C are detected in pepper, either green or red, strawberry, broccoli, citrus and others (Proteggente *et al.* 2002; Mariko *et al*. 2005; Mateos 2006). Thus, most data indicate that 0.1-0.2% of the pepper fresh weight corresponds to ascorbic acid. The amount of ascorbic acid in this vegetable is such that about 80 g (1/4 of a California-type pepper fruits) would be enough to satisfy our vitamin C diary needs (Mateos 2006).

These data suggest an important role for ascorbic acid in the biochemistry and physiology of pepper fruits where huge amounts of this vitamin are found.

ASCORBATE IN PEPPER FRUITS

In our laboratory, the metabolism of ascorbate has been studied in pepper fruits under distinct conditions: 1) in fruits harvested at two environmental conditions (both green and fully mature red fruits); 2) in mature (red and yellow) fruits which underwent different environmental conditions; and 3) in green and red fruits stored at 20°C. The involvement of this antioxidant in the metabolism of chloroplasts, mitochondria and peroxisomes from pepper fruits was also investigated. In most cases, where applicable, besides the content of both ascorbate (ASC) and dehydroascorbate (DHA), the activity of APX, MDAR, DAR and GalLDH was also studied.

Our results showed that, similarly to previous findings (Howard *et al*. 2000), green and red peppers showed comparable contents of ascorbate, determined as total ascorbate (ASC + DHA) (**Table 2**) (Mateos *et al*. 2004; Martí *et al*. 2009). It means that during ripening of pepper, where green fruits shift to red or yellow colour (depending on the cultivar) no important changes occur in the ascorbate pool. Only differences were found when the ratio ASC/DHA was calculated, with a tendency to decrease this ratio during ripening, and this indicates that oxidation processes undergo. Nevertheless, in this process, important metabolic changes take place, the conversion of chloroplasts to chromoplasts being one of the major events. In most *Capsicum* species, ripening is also characterized by a very intense metabolism, emitting volatile organic compounds associated to respiration, with destruction of chlorophyll, synthesis of new pigments (red/yellow carotenoids plus related xanthophylls, anthocyans), formation of pectins, protein synthesis, taste alteration as a consequence of modification in acidity, pH and astringency, and changes in total soluble reducing equivalents (Camara *et al*. 1995; Markus *et al*. 1999; Howard *et al*. 2000; Manirakiza *et al*. 2003; Mateos *et al*. 2003, 2009). Therefore, the absence of changes in the ascorbate concentration in fruits during ripening of pepper fruits implies that vitamin C might have an important role in this physiological process, and this gains relevance at the chloroplast/chromoplast sites where ascorbate displays the highest subcellular concentration within plant cells.

In spite of its steady levels during fruit ripening, the analysis of the internal ascorbate concentration in chloroplast/chromoplasts, mitochondria and peroxisomes from green and red peppers showed that organelles in red fruits contained higher amounts of this antioxidant, excepting

Table 1 Total antioxidant activity and ascorbate content in extracts from several fruits and vegetables.

Fruit/Vegetable	Total antioxidant activity (mg GA/100 g FW)	Total ascorbate (mg/100 g FW)
Strawberry	330 ± 4	61
Red plum	320 ± 12	5
Raspberry	228 ± 6	26
Red pepper	131 ± 12	105
Orange	126 ± 6	46
Broccoli	128 ± 44	45
Green pepper	119 ± 410	92
Onion	88 ± 1	6
Grape	80 ± 4	2
Spinach	71 ± 1	7
Pear	60 ± 3	3
Apple	48 ± 1	6
Aubergine	45 ± 2	22
Peach	38 ± 1	6
Banana	38 ± 4	10
Pea	32 ± 1	22
Tomato	30 ± 1	18
Cauliflower	30 ± 1	15
Leek	22 ± 1	16
Lettuce	14 ± 1	<2

Total antioxidant activity is expressed as phenolic content in gallic acid (GA) equivalents (mean ± SEM). Data collected from Proteggente *et al.* 2002, Mariko *et al.* 2005, and Mateos 2006. FW, fresh weight.

Table 2 Total ascorbate content in several cultivars of pepper fruits (*Capsicum annuum* L.).

Cultivar	Total ascorbate (ASC + DHA) mg/100 g FW	Reference
Herminio (red fruits)	148 ± 4.80	Martí *et al.* 2009a
Herminio (green fruits)	126 ± 26.6	Martí *et al.* 2009a
Biela (yellow fruits)	81 ± 3.1	Martí *et al.* 2009a
Biela (yellow fruits)	126.7 ± 11.20	Mateos *et al.* 2004
Biela (green fruits)	89 ± 1.96	Martí *et al.* 2009a
Biela (green fruits)	143.7 ± 10.7	This work
Vergasa (red fruits)	111.8 ± 5.5	This work
Vergasa (green fruits)	149.8 ± 6.5	Mateos *et al.* 2004
Galileo (red fruits)	108.4 ± 6.8	Mateos *et al.* 2004
Dulce italiano (green fruits)	105.9 ± 17.8	Mateos *et al.* 2004

Cultivars Herminio, Biela and Vergasa correspond to California type fruits, Galileo to Lamuyo and Dulce italiano to type with same name. a, Data modified from Martí *et al.* (2009) *Plant Biology* **11**, 613-624.

Table 3 Total ascorbate content from plastids (chloroplasts and chromoplasts), mitochondria and peroxisomes of pepper fruits (*Capsicum annuum* L.).

Cell organelle	Total ascorbate (ASC + DHA) nmol/ml	Reference
Chloroplasts	7.9 ± 0.6	Martí *et al.* 2009
Chromoplasts	16.4 ± 2.9	Martí *et al.* 2009
Mitochondria (green peppers)	1.6 ± 0.2	Jiménez *et al.* 2002
Mitochondria (red peppers)	1.9 ± 0.3	Jiménez *et al.* 2002
Peroxisomes (green peppers)	2.9 ± 0.2	Mateos *et al.* 2003
Peroxisomes (red peppers)	8.8 ± 0.2	Mateos *et al.* 2003

Data from chloroplasts, chromoplasts and mitochondria are modified from Martí *et al.* (2009) *Plant Biology* **11**, 613-624 and Jiménez *et al.* (2002) *Plant Physiology and Biochemistry* **40**, 515-520, respectively. Data from peroxisomes were obtained from Mateos *et al.* (2003) *Journal of Plant Physiology* **160**, 1507-1516.

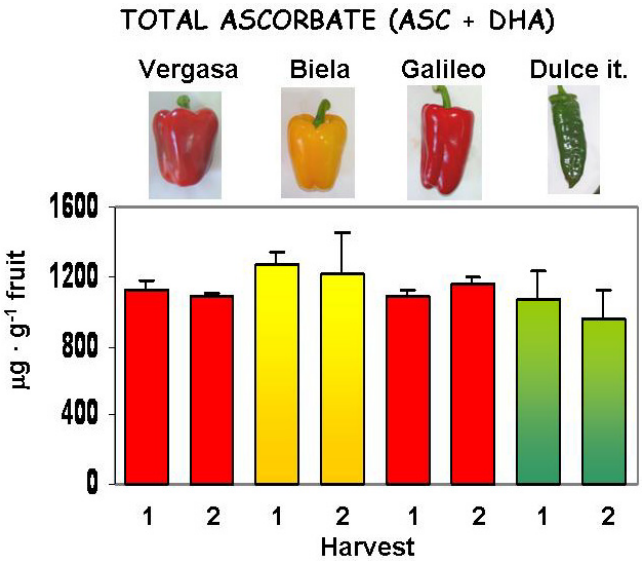

Fig. 4 Total ascorbate content (reduced ascorbate plus dehydroascorbate) in mature fruits from four pepper cultivars harvested at two different environmental conditions. Cultivars studied were from California type (Vergasa and Biela), Lamuyo type (Galileo) and Dulce italiano type (Dulce italiano). As indicated in the pictures, peppers from Vergasa and Galileo cultivars were red, those from Biela were yellow, and Dulce italiano were green. Data were obtained from Mateos *et al*. 2004.

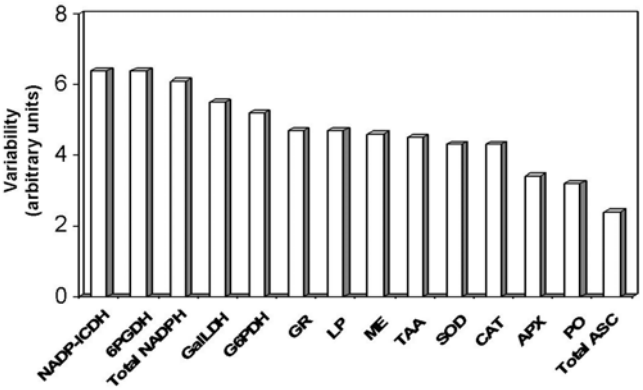

Fig. 5 Variability of ROS-related parameters studied in mature fruits from four pepper cultivars harvested at two different environmental conditions. Cultivars studied were fom California type (Vergasa and Biela), lamuyo type (Galileo) and dulce italiano type (Dulce italiano). The variability values, in arbitrary units, were obtained by analyzing all data obtained from the parameters in the four cultivars and the two harvests by the mathematic test Principal Components Analysis. NADP-ICDH, NADP-dependent isocitrate dehydrogenase; 6PGDH, 6-phosphogluconate dehydrogenase; GalLDH, L-galactono-1,4-lactone dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; GR, glutathione reductase; LP, lipid peroxidation; ME, malic enzyme; TAA, total antioxidant activity; SOD, superoxide dismutase; CAT, catalase; APX, ascorbate peroxidase; PO, protein oxidation.

mitochondria from both types of fruits which displayed no changes (**Table 3**; Jiménez *et al*. 2002; Mateos *et al*. 2003; Martí *et al*. 2009). An overall comparison among the results obtained in ascorbate content in fresh fruits and the studied cell organelles provides a big discrepancy, since data from purified organelles are much lower than those from crude extracts. Several reasons can justify these differences: the own stability of ascorbate that allows obtaining the best results in recently extracted materials than in a series of samples (purified organelles) obtained after several centrifugations; the low purification yield of cell organelles resulting from their lability during the isolation procedure; and the permeability of organelles to ascorbate which leads to leakage of vitamin C from the organelles and considerable losses during the respective purification procedures. Nevertheless, our approach is valid to carry out comparative studies among organelles from both green and red fruits, since it can be expected that similar losses could occur in the purification from both types of fruits.

Our results proved that the concentration of ascorbate in plastids, either chloroplasts or chromoplasts, was much higher than in mitochondria and peroxisomes, and this correlates with previous data reported in organelles isolated from pea leaves (Palma *et al*. 2006). Our data also indicate that ascorbate plays an important role at subcellular level during fruit ripening both in plastids and peroxisomes. Interestingly, the ascorbate levels in mitochondria from both green and red peppers are similar. This might indicate that, although mitochondria synthesize and export ascorbate continuously, due to the enzymatic activity of the GalLDH (Bartoli *et al.* 2000), ascorbate is perhaps exerting its function in other cell compartments. But this absence of differences could also be a simple consequence of the continuous synthesis of ascorbate within the organelle, what keeps its mitochondrial concentration at constant values in spite of the drastic changes occurring during ripening. In fact, when the activity of the GalLDH was investigated in mitochondria isolated from green and red pepper fruits, no significant changes were observed (Jiménez *et al*. 2002).

Regarding to results obtained in fully mature peppers from four different cultivars harvested at two environmental conditions, it was found that the total ascorbate content did not vary in any of the cultivars studied, although slight differences were observed among them (**Fig. 4**, comparisons between Harvest 1 and 2; Mateos *et al.* 2004). In this work, we analyzed two pepper cultivars from the California type (Vergasa and Biela; red and yellow fruits, respectively), one from the Lamuyo (Galileo; red fruits), and another one from the Dulce italiano (Dulce italiano; green fruits) types. In all cultivars, a plethora of ROS-related parameters were studied including catalase, superoxide dismutase, APX, MDAR, DAR, GR, GalLDH, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, NADP-isocitrate dehydrogenase, malic enzyme, total ascorbate content, total NADPH content, lipid peroxidation, total antioxidant activity, and protein oxidation. The Principal Components Analysis (PCA) mathematic pack was used to investigate the correlation among all the parameters, the cultivars selected, and the two harvesting conditions (Mateos *et al.* 2011). The analysis showed that ascorbate content was the most stable parameter in our experimental design (**Fig. 5**), thus indicating the important role of vitamin C and its contribution to the fruit stability throughout ripening and the prevailing seasonal conditions.

Table 4 Effect of storage at 20ºC on total ascorbate content of pepper fruits (*Capsicum annuum* L.).

		Treatment at 20ºC		
		0 days	7 days	19 days
Ascorbate (mg/100 g FW)	green fruits	136 ± 0.1	215 ± 0.5	221 ± 0.2
	red fruits	204.4 ± 0.2	204 ± 0.2	318 ± 0.1

Data modified from Jiménez *et al*. (2003) *Journal of Agricultural Food and Chemistry* **51**, 6293-6299. FW, fresh weight.

Finally, the effect of storing green and red California peppers at 20°C for 7 and 19 days was also investigated. It was found that storage of fruits notably increased ascorbate content, although the pattern varied depending on the fruit. Thus, storage was influential in green peppers after 7 days treatment, whereas in red fruits the increases occurred after 19 days with higher values than in green fruits (**Table 4**; Jiménez *et al*. 2003). Nevertheless, the ascorbate redox state did not change with storage (Jiménez *et al*. 2003). This also provides evidence on the importance of ascorbate in post-harvest fruit metabolism under storage conditions.

CONCLUSIONS

An overall overview of the metabolism profile of ascorbate in pepper leads to the conclusion that this antioxidant is essential for the physiology of fruits, and not only due to its high content. This parameter is quite constant and even increases under certain conditions where important oxidative events take place. Among the potential roles that ascorbate could play in pepper physiology, it is very relevant its possible function as a redox buffer (Palma *et al*. 2009), as it has postulated earlier for this vitamin in plant cells (Foyer and Noctor 2005a, 2005b). This role might also contribute to the stability of fruits, a typical feature of peppers which commonly display long life-span after crops.

ACKNOWLEDGEMENTS

This work was supported by ERDF-cofinanced grants AGL2008-00834 and AGL2005-00101 from the Ministry of Science and Innovation, Spain. Pepper fruits were provided by Syngenta Seeds Ltd. (El Ejido, Almería, Spain)

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