

# Ascorbic acid biosynthesis: a precursor study on plants

Anderson D. Barata-Soares<sup>1</sup>, Maria Luiza P. A. Gomez<sup>1\*</sup>, Carlos Henrique de Mesquita<sup>2</sup> and Franco M. Lajolo<sup>1</sup>

<sup>1</sup>Departamento de Alimentos e Nutrição Experimental, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, Av. Lineu Prestes 580, Bloco 14, CEP 05508-970, São Paulo, SP, Brasil; <sup>2</sup>Instituto de Pesquisas Energéticas e Nucleares, Av. Professor Lineu Prestes 2242, Cidade Universitária, CEP 05508-000, São Paulo, SP, Brasil. \* Corresponding author: malusp@usp.br

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Since the first isolation of ascorbic acid (AsA) in 1928, few papers have been published regarding the biosynthesis of AsA in plants, especially in fruits. It took as long as 1998, before Wheeler, Jones and Smirnoff, based on a study with *Arabidopsis* leaves, proposed what can be considered the main pathway of biosynthesis of AsA, in which L-galactose (L-GAL) is a key precursor. This paper reports the effectiveness of some precursors (cold or radiolabeled) in the biosynthesis of AsA in different plants: green sweet pepper, white-pulp guava, red-pulp guava, papaya and strawberry at two ripening stages (mature green and ripe for papaya and mature green and half red for strawberry) and broccoli. The ‘Smirnoff-Wheeler’ pathway was functioning and active in all sources studied, as demonstrated by the increase in AsA contents and incorporation of labeled precursors into AsA. In papaya, the AsA content in the ripe fruit was higher than in the mature green, indicating the synthesis of AsA during ripening. On the other hand, the AsA content in the mature green strawberry was similar to that of the half red fruits. Our data demonstrate that L-GAL and L-Galactono-1,4-lactone (L-GL) are effective precursors for the biosynthesis of AsA in fruits and also provided additional evidence for the participation of D-mannose (D-MAN) and D-glucose-1P in the biosynthesis of AsA in plants.

**Key words:** ascorbate, ascorbate biosynthesis pathway, antioxidant, precursor infiltration.

**Biossíntese de ácido ascórbico - um estudo com precursores em plantas:** Apesar da importância do ácido ascórbico (AA) para os organismos animais e vegetal, sua biossíntese somente foi elucidada em 1998, quando Wheeler, Jones e Smirnoff demonstraram, em folhas de *Arabidopsis*, que L-galactose (L-GAL) é um precursor chave. Neste trabalho, investigou-se a atuação de supostos precursores na síntese do AA em diferentes fontes vegetais: pimentão-verde, goiabas de polpa branca e vermelha, mamão e morango em dois estádios do amadurecimento: verde e maduro para o mamão e verde e rosa para o morango e em brócolis, verificando-se a atuação da via “Smirnoff-Wheeler” mediante a constatação do aumento dos conteúdos de AA e incorporação de precursores radiativamente marcados. O conteúdo de AA no mamão maduro apresentou-se maior do que no verde, indicando que há predomínio da síntese durante o amadurecimento desse fruto. O mesmo não ocorreu com o morango, onde não houve diferenças significativas entre os conteúdos de AA nos frutos verde e intermediário. Os resultados confirmaram que a L-GAL e a L-galactono-1,4-lactona (L-GL) são precursores bastante eficientes do AA, e também que há síntese de AA a partir de D-manose (D-MAN), L-GAL e D-glicose-1P nos vegetais estudados.

**Palavras-chave:** antioxidantes, ascorbato, biossíntese do ascorbato, infiltração de precursores.

## INTRODUCTION

Ascorbic acid (AsA) plays important roles in the human organism, such as conjunctive tissue formation, ion transportation, and cell protection against free radicals. In

plants, it also plays a protective role against reactive oxygen species that are formed from photosynthetic and respiratory processes. AsA is linked to cell growth, being involved in the cell cycle and other mechanisms of plant cell growth and division, as well as acting as a co-factor for many enzymes (Smirnoff, 1996; Lee and Kader, 2000).

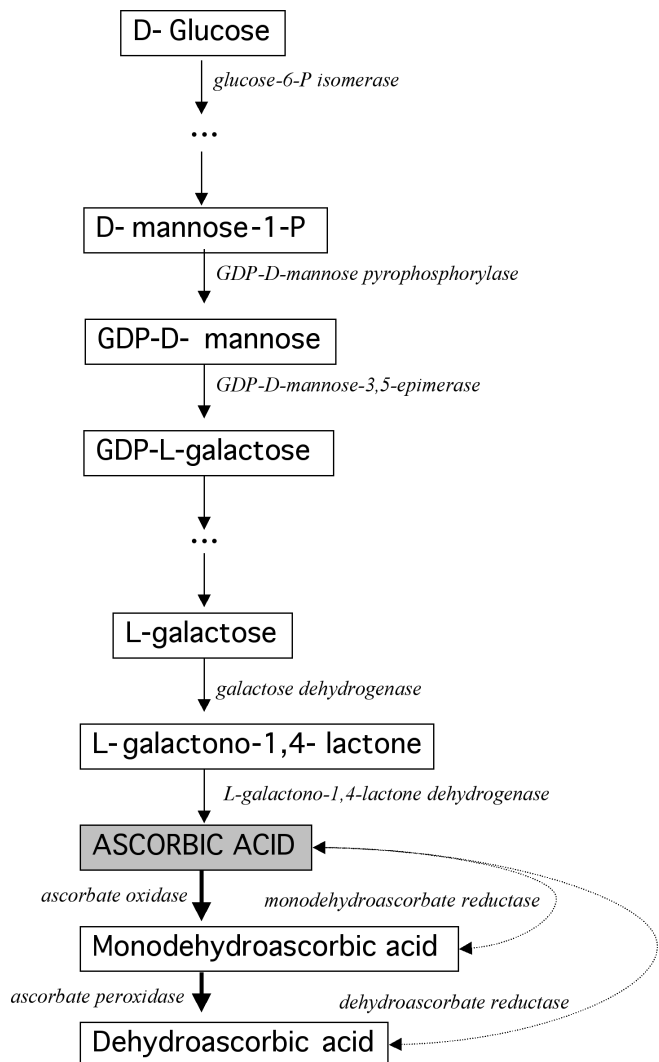
Despite the importance of AsA, its biosynthetic pathway in different plant parts is not completely understood. In 1998, Wheeler and colleagues proposed the first pathway to gain acceptance. Before this, many other pathways and mechanisms were studied, but no consensus was reached (Loewus et al., 1956; Isherwood and Mapson, 1962).

The so-called “Smirnoff-Wheeler” pathway for AsA biosynthesis has as its immediate precursor L-galactono-1,4-lactone (L-GL), and the intermediates involved are phosphorylated sugars and nucleotide-linked sugars (figure 1). Several studies have confirmed this mechanism, and some of the enzymes involved have been detected and described (Oba et al., 1995, Gatzek et al., 2002). This pathway would appear to be the main one for the biosynthesis of AsA, but other pathways cannot be discarded. One example is the conversion of D-galacturonic acid (D-GalUA) into L-GL, first shown by Loewus and Kelly (1961) and confirmed by Davey et al. (1999). D-GalUA and, to a lesser extent, L-Gal are constituents of the cell wall, which may be the source of this secondary pathway for the biosynthesis of AsA in plants (Smirnoff et al., 2004). Recently, a specific D-GalUA reductase was cloned and overexpressed in strawberry, leading to higher contents of AsA (Agius et al., 2003). Other pathways, in which AsA is derived from gulonic acid, gluconic acid and araboascorbate, and glucosone and sorbosone (Loewus et al., 1990, Saito et al., 1990, Saito, 1996) may occur, but they appear to be of lesser importance (Smirnoff et al., 2004).

Genetic evidence for AsA biosynthesis has been obtained with the help of an *Arabidopsis thaliana* mutant, which shows AsA levels about 30 % lower than the wild type. This deficiency is due to a lower activity of GDP-D-mannose-3,5-epimerase, which catalyzes the conversion of D-mannose-1-phosphate to GDP-D-mannose (Conklin et al., 1999). This enzyme also participates in other processes, such as cell wall carbohydrate biosynthesis and protein glycosylation in eukaryotic cells and the product GDP-Man is a source of mannose for the cell wall (Smirnoff and Wheeler, 2000; Smirnoff et al., 2004).

The infiltration of AsA precursors has been used to study and establish pathways of biosynthesis and degradation in plants. Infiltration of D-[6-<sup>14</sup>C]-glucose and D-[6-<sup>14</sup>C]-glucosone in bean seedlings led to a 0.4 % conversion into AsA (Loewus et al., 1987). Furthermore, D-[1-<sup>14</sup>C]-glucose infiltration in watercress, parsley and geranium leaves has confirmed the conversion of D-glucose into AsA (Loewus and Jang, 1957; Williams and Loewus, 1978; Helsper et al., 1982). In geranium leaves, Loewus et al. (1975) reported that

D-[1-<sup>14</sup>C]-glucose infiltration formed about 9-fold more radiolabeled oxalic acid (AOx, an AsA degradation product) than D-[6-<sup>14</sup>C]-glucose infiltration, which formed 82% of the AsA marked with the radioactive carbon in the position 6. The synthesis of AOx from AsA has been an object of several studies (Keates et al., 2000; Kostman et al., 2001; DeBolt et al., 2004) where it was demonstrated by radiolabel infiltration and detection of labeled AOx and other AsA cleavage products, such as tartaric acid (TA). In *Vitis vinifera*, DeBolt et al. (2004) demonstrated that AsA cleavage may occur in different plant organelles, originating both AOx and TA. This seems to be a directed process, which may be important for the formation of calcium oxalate and calcium tartrate crystals for the control of calcium concentration in the cells, as well as remobilization of calcium at specific stages of fruit development. In fact, the formation of AOx, TA and other



**Figure 1.** Pathway of ascorbic acid biosynthesis and biodegradation.

organic acids is a result of AsA metabolism in grape berries and other plants (Wagner and Loewus, 1974; Saito and Loewus, 1989; DeBolt et al., 2004), and may contribute to the balance of AsA content in plants.

The conversion of L-GL into AsA is consensual, and has been demonstrated in several studies with different plant sources (Baig et al., 1970; Arrigoni, 1994; Smirnov et al., 2004). The reaction is catalyzed by L-galactone-1,4-lactone dehydrogenase (GLDHase), an enzyme found in the inner membrane of mitochondria (Oba et al., 1995; Smirnov et al., 2001). In sweet pepper, the activity of GLDHase accompanied ripening and was associated with the increase in the AsA levels (Imahori et al., 1998).

Besides strawberries (Agius et al., 2003), to date there are no available studies concerning possible pathways of the biosynthesis of AsA in fruits. The purpose of this investigation was to study the biosynthesis of AsA in different fruits by precursor infiltration, with or without radioactive labeling, in order to corroborate the "Smirnov-Wheeler" pathway for AsA synthesis in fruits. In addition, we also included broccoli in our study. To our knowledge there is not such information for edible flowers.

## MATERIAL AND METHODS

*Plant material:* Green sweet pepper, guavas, papaya and broccoli were obtained from a local market. Strawberries, var. Dover, were obtained from a plantation in Atibaia (São Paulo State, Brazil). The ripening stages were defined according to Paull et al. (1996).

*Chemicals:* L-Galactose (L-GAL), D-mannose (D-MAN), D-glucose (D-GLU), L-galactono-1,4-lactone (L-GL) and metaphosphoric acid were purchased from Sigma Chemical Co. The L-ascorbic acid used as standard was purchased from Merck. Other reagents used were of analytical grade. D-[U-<sup>14</sup>C]-Mannose (7.4 MBq.mL<sup>-1</sup>) was purchased from Amersham Biosciences and L-[1-<sup>14</sup>C]-galactose (3.7 MBq.mL<sup>-1</sup>) from Amersham Radiolabeled Chemicals.

*Precursor infiltration:* Labeled precursors were fed to strawberries and broccoli florets by immersing the petiole in a solution containing 0.5 % of each precursor, as described by Baig et al. (1970). The green sweet pepper, papaya and guavas were sliced and immersed in the precursor solutions. The zero-time samples were frozen just before infiltration. Controls were immersed in water. After a 24 h period at room temperature and under artificial light (fluorescent illumination

of 40W, at a distance of 40 cm), samples were frozen in liquid nitrogen and kept in freezer until analysis.

*Labeled precursor infiltration:* Infiltration of labeled precursors was carried out according to Loewus (1963), with some modifications. For the strawberry samples at the mature green stage, sixteen fruits were separately immersed by the petiole in 1 mL of distilled and deionized water containing 74 kBq of D-[U-<sup>14</sup>C]-mannose or L-[1-<sup>14</sup>C]-galactose, until it was totally taken up. Then, infiltration continued with pure water (no isotope). Samples were taken every 24 h, using 8 fruits. Broccoli samples were treated the same way, but the radioactivity was 37 kBq. For the red-pulp guavas, at the mature green stage, and papaya, at the mature green and ripe stages, circular slices from the pulp were taken, in which 3.7 kBq of D-[U-<sup>14</sup>C]-mannose, D-[1-<sup>14</sup>C]-glucose-P or L-[1-<sup>14</sup>C]-galactose were infiltrated. The conversion rate was determined after a 24 h period.

*Ascorbic and dehydroascorbic acids determination:* The AsA and DHA were determined as described by Rizzolo et al. (1984). Samples were ground under liquid nitrogen, and homogenized with a 0.1 % metaphosphoric acid solution, in appropriated proportions. Then, the homogenate was centrifuged (12.000 g<sub>n</sub>, 10min) and the supernatant filtered through a Millipore membrane (0.45 μm), and diluted with more metaphosphoric acid for AsA determination and with dithiothreitol for the total AsA analysis. The DHA content was determined by the difference between AsA and total AsA contents. The extracts were analyzed by HPLC, using a μBondapak C<sub>18</sub> column, and the mobile phase was 0.2 mol.L<sup>-1</sup> acetate buffer pH4.5, at a flow rate of 1.5 mL.min<sup>-1</sup>. The compounds eluting from the column were detected at 254 nm. A standard curve was obtained from 10 to 100 mmol.L<sup>-1</sup> AsA.

*Labeled AsA analysis:* The labeled AsA determination was carried out as described by Keates et al. (2000), with some modifications. The AsA peaks were collected after HPLC separation (injection volume 100 μL). The AsA fractions were mixed with 4 mL of scintillation fluid (ASC<sup>®</sup>NASC104, Amersham Biosciences) and counted in a liquid scintillation counter (LSC – TriCarb 1900, Canberra Packard).

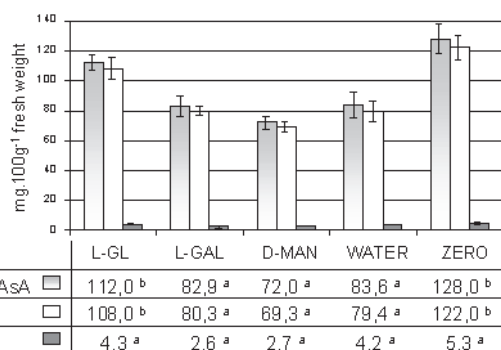
*Statistical Analysis.* All experimental data were assumed to follow a normal distribution and were subjected to an analysis of variance using a fully randomized design. The Tukey test was applied (p≤0.5) to compare means for significant differences.

## RESULTS

### *The influence of precursor infiltration on AsA and DHA levels:*

The results obtained from infiltration experiments with green sweet pepper, mature green and ripe papaya, white-pulp and red-pulp guavas, mature green and half red strawberries and broccoli florets are shown in figures 2 to 6. In figure 2, it may be seen that for the green sweet peppers the pool size of AsA diminished during the 24 h infiltration period, although this decline was not statistically significant when L-GL was used as precursor. This indicates that L-GL was more effective in maintaining the original AsA levels, while L-GAL and D-MAN appeared to be totally inefficient, producing AsA levels similar to the control infiltrated with water. Perhaps the L-GL to AsA step is more active in the green sweet pepper, since the other precursors were unable to sustain the initial AsA concentration. The decline in total AsA may be due to a higher degradation involving both ascorbate oxidase (AO) and ascorbate peroxidase (APX), possibly activated by the stress of slicing and the infiltration technique used for the experiment. Evidently, over the infiltration period, the biosynthetic activity was not capable of counter-balancing the catabolic activity, except perhaps for L-GL as precursor.

For papaya, it is interesting to observe that there was substantial synthesis of AsA during ripening, as shown by the 4-fold higher AsA level in the ripe fruit compared with the mature green one (cf. zero-time data of figures 3A and 3B). There was a significant increase in total AsA levels only with the L-GL infiltrated sample, both for the mature green and the ripe fruits. Despite the higher level of AsA in the ripe fruits, there was no significant difference in the biosynthetic capacity of mature green and ripe fruits, that were about 12.5

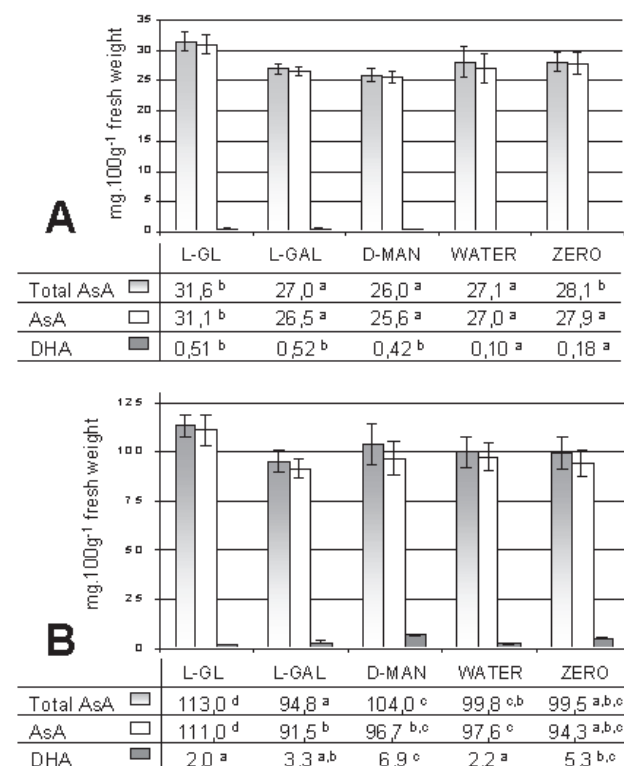


**Figure 2.** Total ascorbic acid, ascorbic acid and dehydroascorbic acid contents in green sweet pepper infiltrated with different ascorbic acid precursors. The results are the mean of three repetitions; means with different letters within rows are statistically different.

% and 13.6 %, respectively. The infiltration with the other precursors seemed to be less effective, showing no significant AsA increase in relation to the controls.

Figures 4A and 4B show that there were differences between the white-pulp and the red-pulp guava fruits at the mature green ripening stage, respectively. In the case of white-pulp guava, there was no detectable increase associated with precursor infiltration. On the other hand, in red-pulp guavas there was a significant increase of the total AsA contents when the fruit were infiltrated with either L-GAL or L-GL. Lee and Kader (2000) showed that the AsA content in fruits may vary according to the plant cultivar, this being possibly associated with modifications in composition, tissue structure and other intrinsic factors.

In the case of strawberry at mature green and half red stages increases were again found for the AsA and total AsA levels in fruits infiltrated with L-GAL and L-GL (figures 5A and 5B), but not for D-MAN. The increase caused by the L-GL infiltration was higher in the half red fruits than in the mature green (78 % and 58 %, respectively), yet there was no



**Figure 3.** Total ascorbic acid, ascorbic acid and dehydroascorbic acid contents in mature green (A) and ripe papaya fruit (B) infiltrated with different ascorbic acid precursors. The results are the mean of three repetitions, and means with different letters within rows are statistically different.

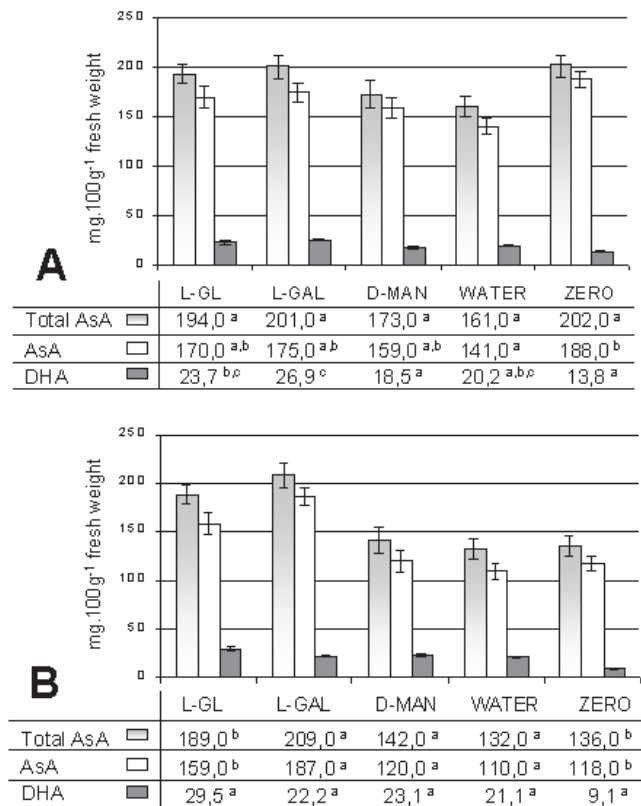
difference between the initial AsA content of the mature green and half red fruits. This result might suggest more active degradation in the half red fruits but also that a stronger biosynthetic or regeneration system was present that maintained the AsA content unchanged. Kim and Chung et al. (1998) reported higher APX activity during strawberry ripening.

Since broccoli shows high contents of AsA, it was also included in our studies. There was a great increase in the total AsA in the L-GL and L-GAL infiltrated samples, as compared to the water-infiltrated sample (figure 6). On the other hand, there was no statistical difference in the D-MAN infiltrated sample compared to the water-infiltrated sample. The initial AsA content was higher than in some of the fruits studied, and the increase in rate of synthesis was around 86%.

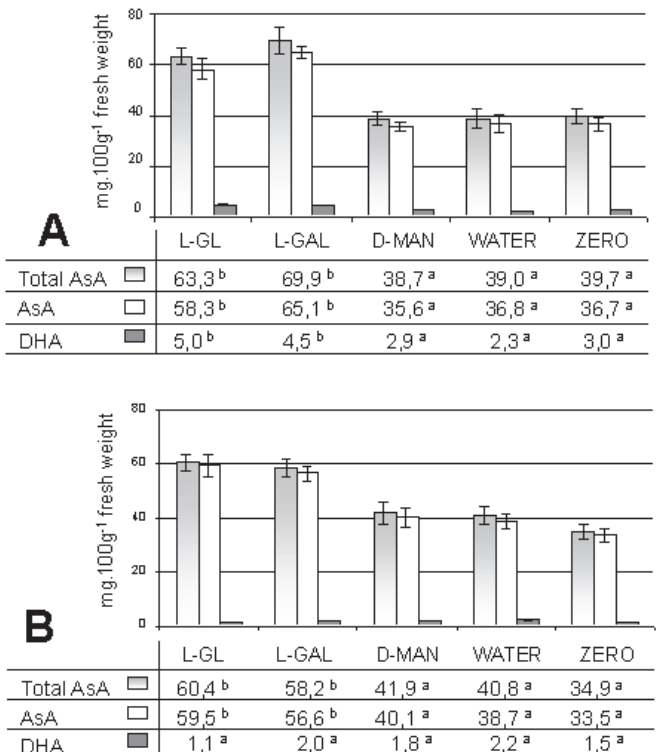
*Conversion of labeled precursor into AsA:* The results of the conversion of labeled precursors into AsA are shown in table 1. In all cases, it may be seen that L-GAL infiltration resulted in highly labeled AsA. For ripe papavas, there was a smaller

increase, which may indicate a lower rate of AsA biosynthesis or higher degradation in this sample. In broccoli, on the other hand, the highest rate of conversion was found, suggesting an efficient formation of AsA from L-GAL. Papaya at the mature green ripening stage also showed a high conversion rate while ripe fruits showed less than half of this rate.

The conversion of labeled D-MAN into AsA was very similar for broccoli and strawberry, while for the red-pulp guava it was the lowest. For the papaya and red guava fruits at the mature green ripening stage, the conversion rate of D-glucose-1P and D-MAN were very similar. Other authors (Keates et al., 2000; Kostman et al., 2001) also found higher conversion of L-[1-<sup>14</sup>C]-GAL and lower conversion of D-[U-<sup>14</sup>C]-MAN. The experiments also showed that AsA biosynthesis took place from glucose-1-P as precursor in papaya and red-pulp guava, confirming the ‘Smirnoff-Wheeler’ scheme in these fruits. The increase in AsA contents originated by D-MAN and D-GLU-1-P were lower, possibly because these precursors are at the initial steps of the pathway.

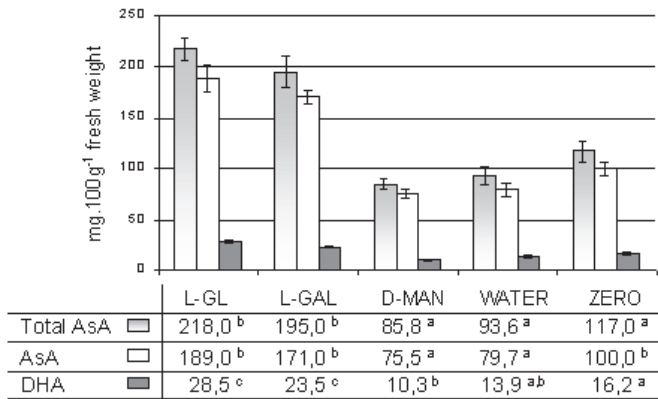


**Figure 4.** Total ascorbic acid, ascorbic acid and dehydroascorbic acid contents in white-pulp (A) and red-pulp (B) guava fruit infiltrated with different ascorbic acid precursors. The results are the mean of three repetitions, and means with different letters within rows are statistically different.



**Figure 5.** Total ascorbic acid, ascorbic acid and dehydroascorbic acid contents in mature green (A) and half red (B) strawberry fruit infiltrated with different ascorbic acid precursors. The results are the mean of three repetitions, and means with different letters within rows are statistically different.





**Figure 6.** Total ascorbic acid, ascorbic acid and dehydroascorbic acid contents in broccoli florets infiltrated with different ascorbic acid precursors. The results are the mean of three repetitions; means with different letters within rows are statistically different.

## DISCUSSION

Despite the importance of ascorbic acid, only recently has its biosynthetic pathway been elucidated. The pathway proposed by Wheeler et al. (1998) appears to be the main one for the synthesis of AsA in plants. This pathway was shown to be present in different plant sources but, until now, not in fruits. The pathways of biosynthesis and degradation of AsA have been studied with the help of tissue infiltration, sometimes with the use of radioactive labeling. Experiments involving the infiltration of D-[6-<sup>14</sup>C]-glucose and D-[6-<sup>14</sup>C]-glucosone in bean seedlings and D-[1-<sup>14</sup>C]-glucose in watercress, parsley and geranium leaves confirmed the conversion of these precursors into AsA (Loewus and Jang, 1957; Williams and Loewus, 1978; Helsper et al., 1982; Loewus et al., 1987).

In our study, the infiltration of L-GL led to an increase of the AsA content in fruits and in broccoli florets, suggesting that the L-galactono-1,4-lactone dehydrogenase activity is responding efficiently to increases in its substrate. The

exception was the white-pulp guava, where no effect was obtained for any precursor tested. The infiltration with L-GAL also increased the AsA levels in broccoli, red-guava and strawberry (at mature green and half red stages). Although papaya (both mature green and ripe fruits) did not accumulate AsA from L-GAL infiltration, AsA did become labeled after radioactive L-GAL infiltration. This fact indicates that L-GAL is an efficient precursor, but in papaya, there may be also a higher degradation of AsA which would explain the 5-fold increase in DHA levels observed. Similar results were found for D-MAN infiltration. None of the samples accumulated AsA from D-MAN infiltration, but all samples tested using labeled D-MAN infiltration formed labeled AsA, suggesting a tighter control of the pathway at the conversion of D-mannose to AsA, together with a continuous turnover of AsA. In papaya at the mature green stage, we observed an increase in DHA levels, suggesting that D-MAN infiltration led to the biosynthesis of AsA followed by degradation. Axenic cell culture of *Pistia stratiotes* L. showed high conversion of L-GAL into AsA, the labeled carbon being also incorporated in oxalic acid. The conversion of AsA into AOx and other organic acids has already been shown for different plant sources (Keates et al., 2000; Kostman et al., 2001; DeBolt et al., 2004). The closer the precursor is to the final step of AsA biosynthesis, the higher the conversion rate into AsA. Furthermore, it is possible that the need for mannose phosphorylation is a limiting factor, since it has a high energy requirement and then mannose may be diverted to other pathways.

During fruit ripening, many reactions are still occurring, such as color transformation, sugar synthesis and cell wall degradation. All these phenomena may cause tissue stresses which would require antioxidant action, especially by ascorbate, preventing cell damage. It is conceivable that, due to these stresses, AsA levels would invariably decrease during fruit ripening. However, it would appear that, in some fruit,

**Table 1.** Percent conversion of D-[U-<sup>14</sup>C]-mannose, L-[1-<sup>14</sup>C]-galactose e D-[1-<sup>14</sup>C]-glucose-1-P for different samples, after 24 h of infiltration<sup>a</sup>.

	D-[U- <sup>14</sup> C]-mannose	L-[1- <sup>14</sup> C]-galactose	D-[1- <sup>14</sup> C]-glucose-1-P
Papaya (mature green stage)	9,0 ± 1,9 a	58,0 ± 0,6 b	7,1 ± 0,6 a
Papaya (ripe-stage)	-	25,4 ± 4,6	-
Guava (red-pulp, mature green stage)	3,0 ± 0,8 a	39,3 ± 2,8 b	3,7 ± 0,3 a
Strawberry (mature green stage)	11,2 ± 2,3 a	40,9 ± 5,4 b	-
Broccoli	11,0 ± 1,6 a	67,5 ± 10,5 b	-

<sup>a</sup> Results are the mean ± standard deviation (n=3); means followed by different letters within rows are statistically different.

AsA levels increase greatly while in others, these levels remain unchanged or decrease. Papaya presented a great increase (about 4-fold) in AsA levels from the mature green to the ripe stage of ripening. These results are in agreement with those presented by Wills and Widjanarko (1995), who also found a 4-fold increase in AsA levels during papaya ripening. On the other hand, strawberry did not show significant changes from the mature green to the intermediate stages, but Cordenunsi et al. (2002) showed an increase of about 20 % from the intermediate to the fully ripe stage in strawberry cv. Dover. The mechanisms that regulate ascorbate content in fruits are still under investigation and possibly the balance between synthesis and degradation processes, which are genetically regulated, also plays an important role.

Finally, the increase or decrease of the AsA and DHA levels from precursors also reflect both enzymatic and non-enzymatic factors. The balance between these factors assures the final content and underlies the variation of AsA levels during ripening or storage processes of different plants.

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