Molecular analysis of alternative oxidase and ascorbic acid biosynthesis in two acerola clones (*Malpighia emarginata* DC)

Análise molecular da biossíntese alternativa de oxidase e ácido ascórbico em dois clones de acerola (Malpighia emarginata DC)

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ABSTRACT

The acerola, *Malpiguia emarginata* DC is a fruit small endowed with enviable nutritional qualities. The main feature that stands out from other species is its enormous capacity to synthesize ascorbic acid. For this study, we analyzed the gene expression of enzymes of the way Wheeler/Smirnoff well as the Alternative Oxidase (AOX). The last step this pathway ends in the inner mitochondrial membrane, due the presence of transmembrane enzyme L-Galactono -1,4 - Lactone dehydrogenase (L-GalLdh), located between the complex III and IV. Since the alternative oxidase is an enzyme uncoupling, no phosphorylating and insensitive to cyanide, present between the complexes II and III. This study aimed to identify the main gene expression responsible to biosynthesis of ascorbic acid and two isoforms of AOX (1 and 2), on flowers and fruits at different development stages, on two clones the acerola called Cereja and Roxinha. The

determination of ascorbic acid levels was done by titration of Tillman, on two clone's fruits. Total RNA from different tissues for two clones was isolated, and primers were used for semi-quantitative RT-PCR assays. To determine the multigene family for AOX, DNA genomic was isolated and degenerate primers were used on semi quantitative PCR. Specific and degenerate primers were used for gene expression the Wheeler/Smirnoff pathway. The ascorbic acid levels on both clones showed that the Roxinha clone had lower levels on all stages. The gene expression of three enzymes stood out from others, these showed a synergism with the expression levels of vitamin C contained in each tissue. In addition, gene expression in two clones showed differences in tissues that favor of Cereja clone. They are Mannose pyrophsphorylase, GDP-Mannose 3'5' epimerase and GDP-Galactose phosphorylase. With greater emphasis on GDP-Mannose 3'5' epimerase and GDP-Galactose phosphorylase. AOX1 gene expression increased on both clones during the fruit ripening, but revealed some differences. In green fruit of Cereja (C) clone the expression was lower than Roxinha (R), increasing the expression on unripe and ripe fruits. The AOX2 expression appeared constant during fruit ripening on Roxinha clone, increasing only in ripe fruit while on the Cereja clone the AOX2 gene expression gradually decreased with maturation.

Keywords: Acerola, Ascorbic acid, Alternative Oxidase.

RESUMO

A acerola, Malpiguia emarginata DC é uma fruta pequena dotada de qualidades nutricionais invejáveis. A principal característica que se destaca de outras espécies é a sua enorme capacidade de sintetizar o ácido ascórbico. Para este estudo, analisámos a expressão genética das enzimas da forma Wheeler/Smirnoff bem como a Oxidase Alternativa (AOX). O último passo desta via termina na membrana mitocondrial interna, devido à presença da enzima transmembrana L-Galactono -1,4 - Lactone desidrogenase (L-GalLdh), localizada entre o complexo III e IV. Uma vez que a oxidase alternativa é um desacoplamento enzimático, sem fosforilação e insensível ao cianeto, presente entre os complexos II e III. Este estudo visou identificar a principal expressão genética responsável pela biossíntese do ácido ascórbico e duas isoformas de AOX (1 e 2), em flores e frutos em diferentes fases de desenvolvimento, em dois clones a acerola chamada Cereja e Roxinha. A determinação dos níveis de ácido ascórbico foi feita por titulação de Tillman, em dois clones de frutos. O RNA total de diferentes tecidos para dois clones foi isolado, e foram utilizados iniciadores para ensaios semi-quantitativos de RT-PCR. Para determinar a família multigene para AOX, foi isolada a genómica do ADN e foram utilizados iniciadores degenerados em PCR semi-quantitativos. Primários específicos e degenerados foram utilizados para a expressão genética a via Wheeler/Smirnoff. Os níveis de ácido ascórbico em ambos os clones mostraram que o clone Roxinha tinha níveis mais baixos em todas as fases. A expressão genética de três enzimas destacou-se das outras, estas mostraram uma sinergia com os níveis de expressão de vitamina C contidos em cada tecido. Além disso, a expressão génica em dois clones mostrou diferenças nos tecidos que favorecem o clone de Cereja. São elas a Manose pirofosforilase, a GDP-Mannose 3'5' epimerase e a GDP-Galactose fosforilase. Com maior ênfase no GDP-Mannose 3'5' epimerase e GDP-Galactose fosforilase. A expressão do gene AOX1 aumentou em ambos os clones durante o amadurecimento do fruto, mas revelou algumas diferenças. No fruto verde de Cereja (C) clone a expressão era inferior à de Roxinha (R), aumentando a expressão em frutos não maduros e maduros. A expressão AOX2 apareceu constante durante a maturação do fruto no clone da Roxinha, aumentando apenas em frutos maduros enquanto que no clone da Cereja a expressão do gene AOX2 diminuiu gradualmente com o amadurecimento.

Palavras-chave: Acerola, ácido ascórbico, oxidase alternativa.

1 INTRODUCTION

Two important systems present on plant mitochondria are important to avoid the synthesis of reactive oxygen species (ROS). The first is function by Alternative oxidase (AOX) that dissipates the electrochemical gradient generated by respiration (Considine *et al*., 2002) and be the last step the Wheeler/Smirnoff pathway which synthesize ascorbic acid, by presence the L-Galactono 1,4 Lactono dehydrogenase (Wheeler & Smirnoff, 1998). Climacteric fruits such as acerola exhibit biochemical changes on ripening, as the expression of genes for enzymes that prevent the formation this reactive oxygen species (ROS). The expression of genes belonging to the AOX multigene family and the enzymes responsible by ascorbic acid biosynthesis is regulated during fruit development, depending on the gradual production of ROS and others factors. For this work, we analyzed the gene expression the AOX genes and Wheeler/Smirnoff pathway on two clones of acerola with different levels of ascorbic acid on flowers and fruits on three stages of developmental (Moura, 2007), (Bartoli *et al*., 2000), (Bartoli *et al*., 2005).

2 OBJECTIVES

This study aimed to identify the main gene expression the Wheeler/Smirnoff pathway who can generate differences on biosynthesis of ascorbic acid, and gene expression of two isoforms to AOX (1 and 2), on flowers and fruits at different development stages and on two clones to acerola called Cereja (C) and Roxinha (R).

3 MATERIALS AND METHODS

3.1 TISSUES OF ACEROLA (*MALPIGHIA EMARGINATA* DC).

Flowers fully developed, with all the whorls formed and fruits on three developmental stages (unripe, semi mature fruit and ripe fruits) collected at Embrapa Pacajús of the state of Ceará. For the analysis were chosen clones Cereja or BRS 236 and Roxinha or BRS 237 that differ by up to 50% on the amount of ascorbic acid accumulated. The cereja is the clone that accumulates more ascorbic acid. The tissues collected immediately were cut and frozen in liquid nitrogen.

3.2 DETERMINATION THE ASCORBIC ACID ON FRUITS

After harvested the ascorbic acid levels on unripe, semi mature and mature fruits of the two clones are determinates by titration of Tillman.

3.3 ISOLATION OF GENOMIC DNA TO DETERMINATE MULTIGENE FAMILY

To evaluate the presence the multigene family of AOX on acerola the genomic DNA isolated by CTAB method.

3.4 ISOLATION OF TOTAL RNA

Total RNA was isolated from tissues using RNeasy plant mini kit from Quiagen, following the manufacturer's specifications. The integrity of the total RNA isolated from the tissues observed on a 1.5% agarose gel diluted in MOPS buffer, observing the ribosomal subunits.

Figure 1. Sequence of total RNA extraction using the RNeasy plant Mini kit (Quiagen®).

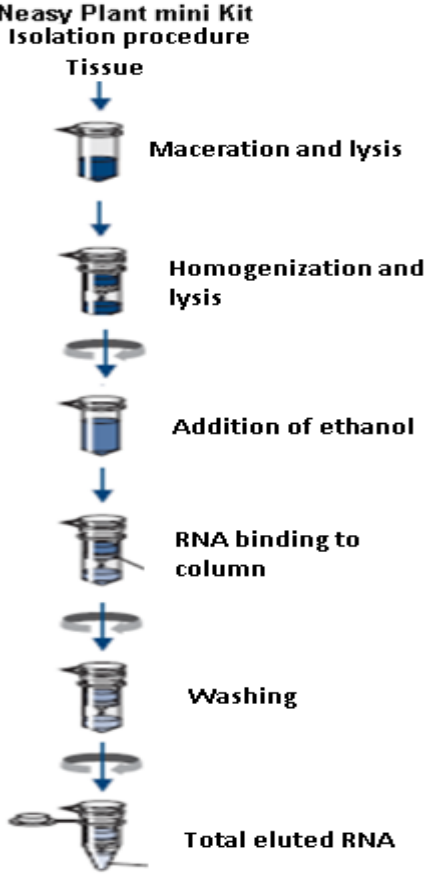
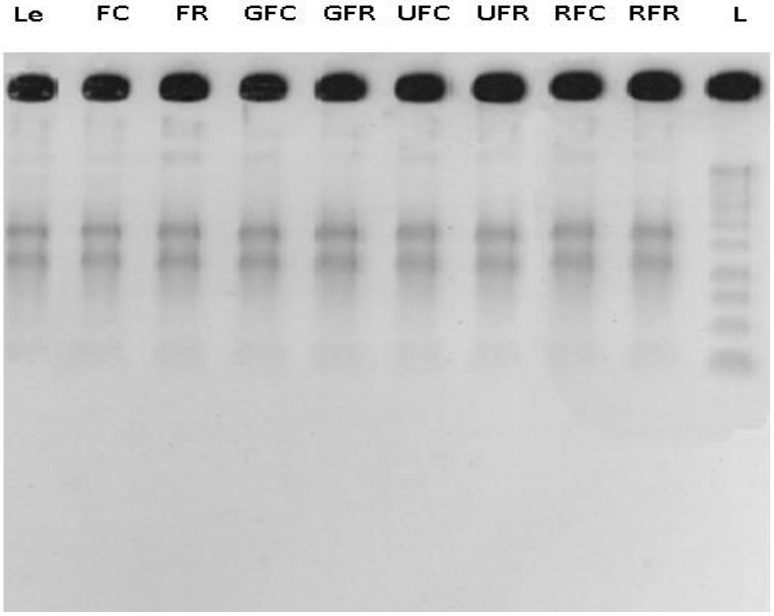


Figure 1. Electrophoresis in 1.5% agarose gel diluted in MOPS buffer of total RNA extracted from common acerola leaves (Le), flowers (Cereja and Roxinha) FC and FR, green fruits (GFC and GFR), unripe fruits (UFC and UFC) and ripe fruits (RFC and RFR), showing the ribosomal subunits. The bands visualized in ethidium bromide 0.5 µg / ml, under ultraviolet light. M- 1 Kb Ladder (L) molecular weight marker (Invitrogen®).



3.5 SYNTHESIS OF THE FIRST TAPE BY REVERSE TRANSCRIPTION

The synthesis of the cDNA tape performed using 0.5µg of total RNA from tissues evaluated, added reagents to kit from Promega for reverse transcription.

3.6 PCR REACTIONS

The PCR reactions developed with thirty cycles, where denaturation was 94ºC for 1 minute, annealing at 55ºC for 1m15s, and extension at 72ºC with 1m10s. For these reactions were used specific and degenerate primers.

4 RESULTS

Table 1. Pulp weight and DFI volumes used to calculate the title and the amount of ascorbic acid (mg) in 100g of pulp.

	BRS 236 Cereja			**BRS 237 Roxinha**		
Development stage	**Green fruit**	**Unripe fruit**	**Ripe fruit**	**Green fruit**	**Unripe fruit**	**Ripe fruit**
Pulp weigth (g)	**0,54**	**0,58**	**0,56**	**0,57**	**0,56**	**0,5**
DFI volume used for holder Repetition (ml)	4,7	3,0	2,3	2,2	1,8	1,4
	4,6	3,0	2,3	2,3	1,7	1,5
	4,5	3,1	2,3	2,3	1,7	1,5
Average	**4,6**	**3,03**	**2,3**	**2,26**	**1,73**	**1,46**

Figure 2. Ascorbic acid levels on two clones and three stages of development

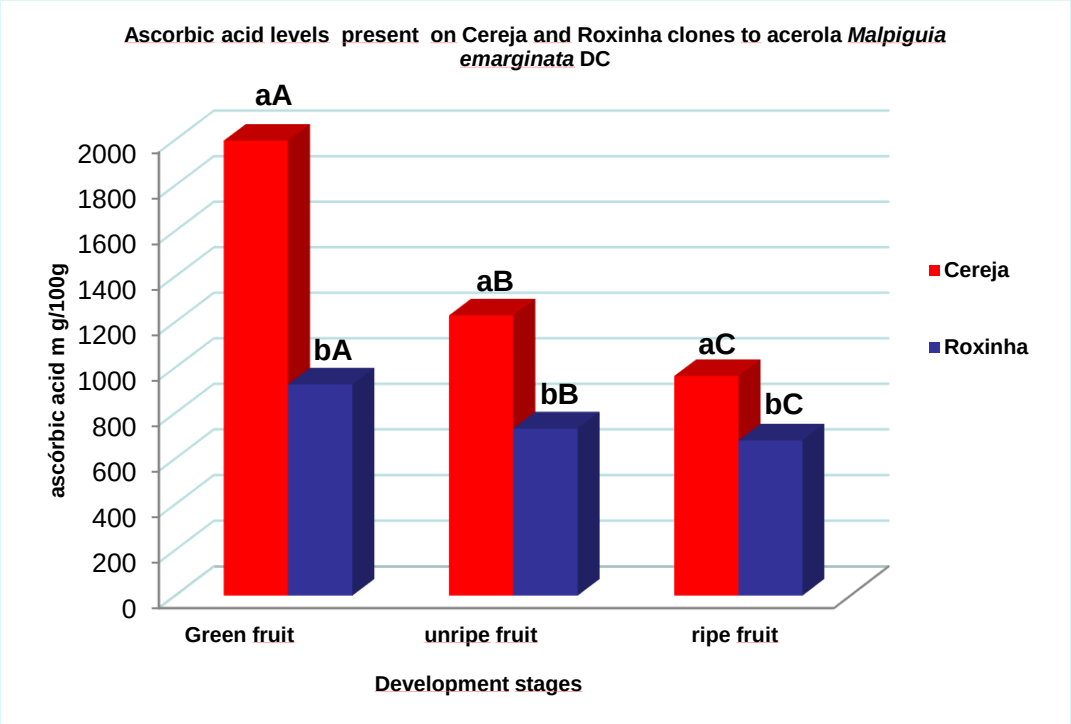
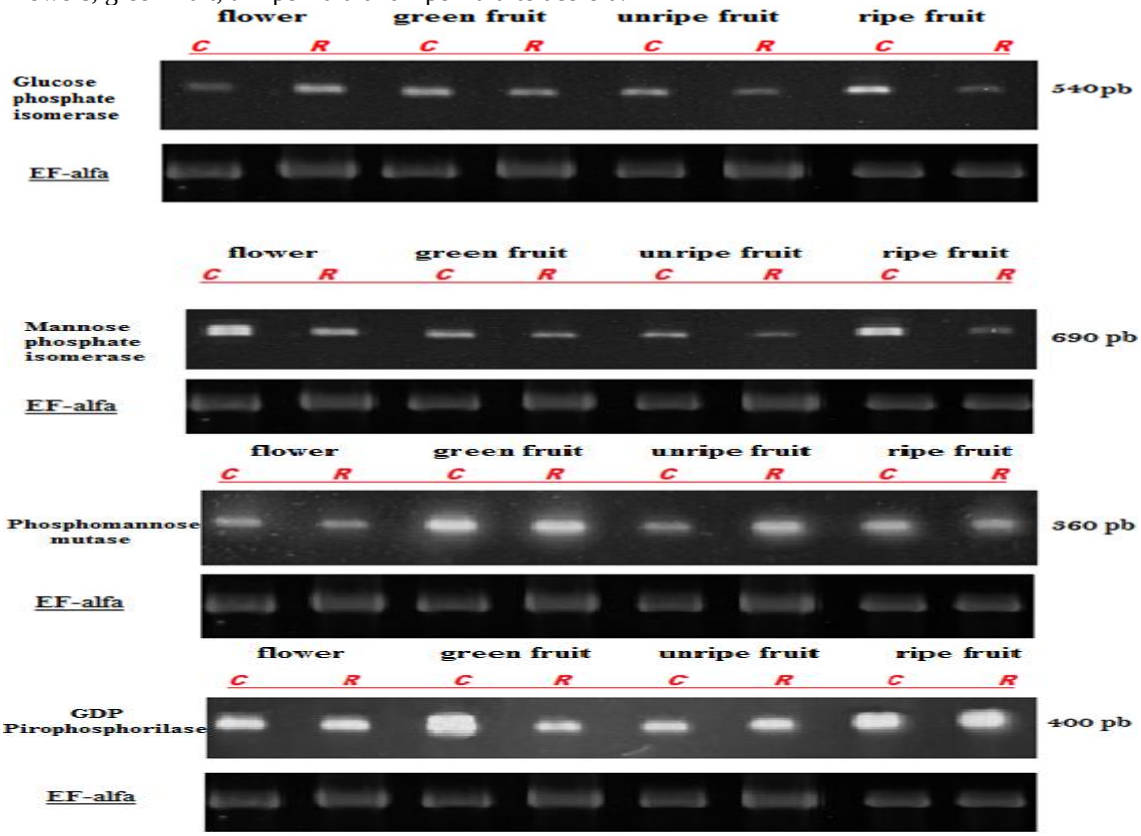


Figure 3. Gene expressions to enzymes by Wheeler/Smirnoff pathway ,on Cereja (C) and Roxinha (R) clones in flowers, green fruit, unripe fruit and ripe fruit to acerola.



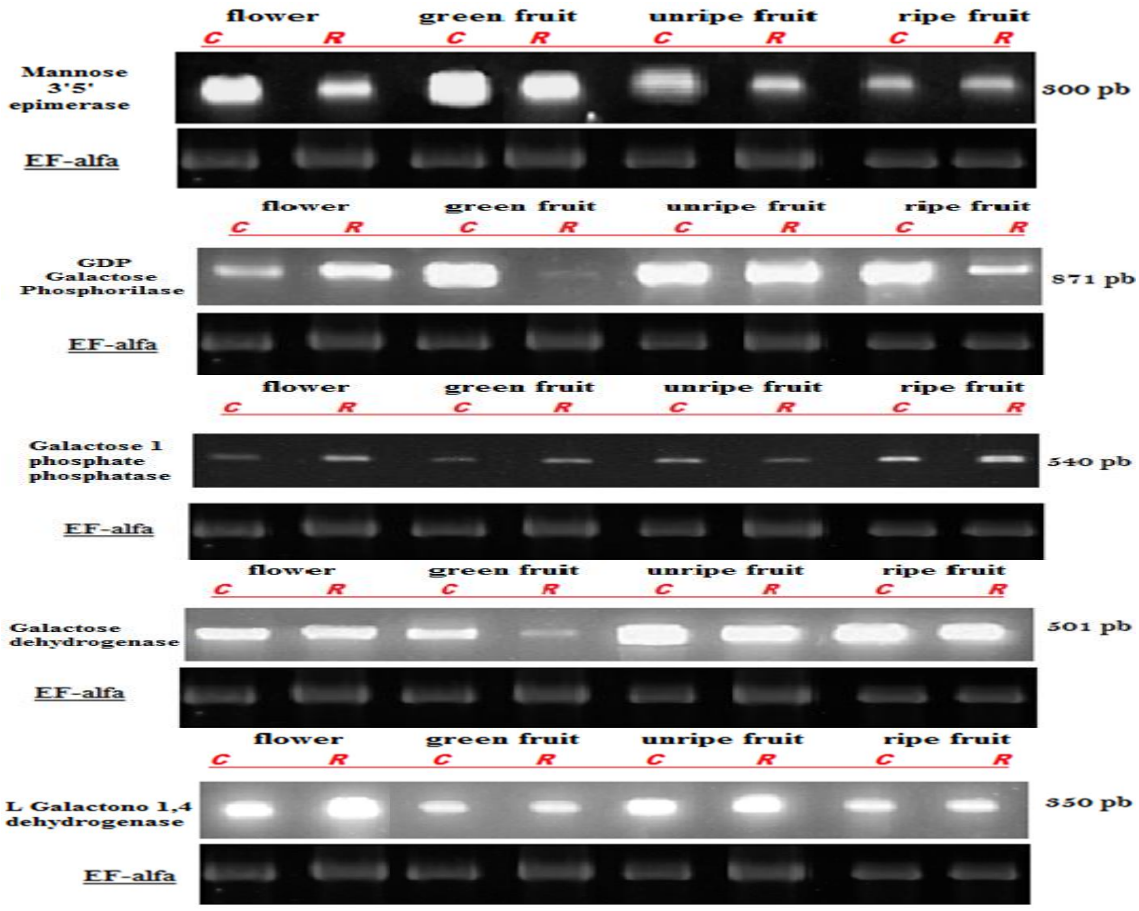
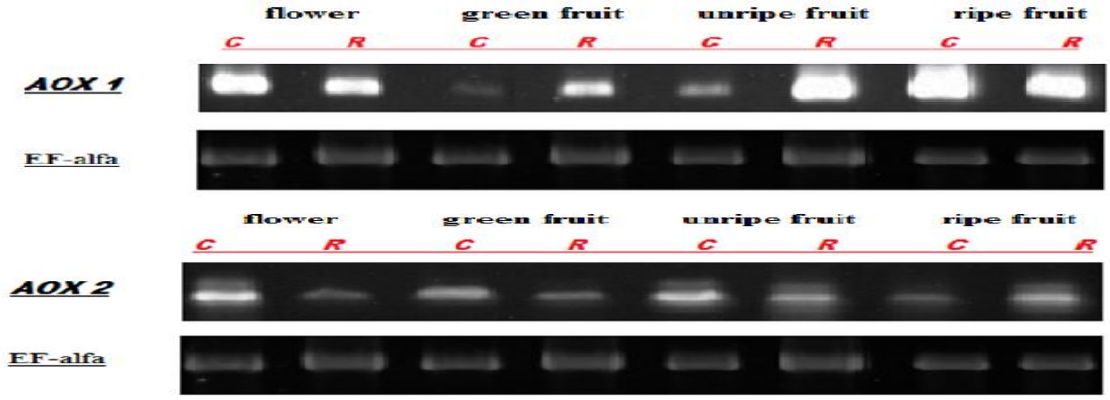


Figure 4. AOX1 and AOX2 gene expressions on flowers, green fruits, unripe fruits and ripe fruits for Cereja (C) and Roxinha (R) clones. The expression standardized using the elongation alpha factor as a constituent element.



5 CONCLUSIONS AND DISCURSION

The gene expression of three enzymes stood out from others, these showed a synergism with the expression levels of vitamin C contained on each tissue. On addition, gene expression in two clones showed differences in tissues that favor of Cereja clone. They are Mannose pyrophosphorylase, GDP-Mannose 3'5' epimerase and GDP-Galactose phosphorylase. With greater emphasis on GDP-Mannose 3'5' epimerase and GDP-Galactose phosphorylase. Another important conclusion was that Alternative

oxidase gene expressions showed that AOX1 and AOX2 had behaviors that suggest some relation with the biosynthesis of vitamin C. The MeAOX1 gene expression increased on both clones during the fruit ripening, but revealed some differences. On green fruit of Cereja (C) clone the expression was much lower than Roxinha (R), increasing the expression in unripe and ripe fruits. However, on unripe fruits was lower on Cereja (C) clone. Only on ripe fruit, the gene expression was higher than Cereja (C) clone. The MeAOX2 expression appeared constant during fruit ripening in Roxinha clone, increasing only in ripe fruit while in the Cereja clone the MeAOX2 mRNA gradually decreased with maturation. The differential MeAOX expression between contrasting acerola clones suggest a possible AOX interaction with the ascorbate biosynthesis.

Apparently, the regulation provided by AOX seems to lead to an increase in the synthesis of vitamin C, or at least prevents a decrease in the synthesis. This fact can to be confirm when comparing the differences between the contents of ascorbic acid and the gene expression in the stages of semi-ripe and ripe fruit. In this evaluation, it can to be verify that the differences in the levels of ascorbic acid between the semi-mature and ripe fruits of the Roxinha clone are statistically significant, but these contents show a difference that when compared with the gene expression in these two stages there is a much greater proximity of expression than in the Cherry clone. By observing the difference in the expression of AOX1 in the clone Cereja and the levels of ascorbic acid in these two stages, it can be seen that the greatest difference in expression is directly proportional to the greatest difference in the levels of ascorbic acid, that is, the greatest expression of AOX1 in the Roxinha clone it does not provide an elevation of the ascorbic acid synthesis sufficient to overcome the levels of the Cereja clone, but it positively favors by avoiding the same level of decline in the synthesis that occurs in the Cereja clone. The behavior shown by the increase in AOX1 and AOX2 gene expression in *Malpiguia emarginata* DC seems to be a proportional response to the amount of L-Galactone-1,4 lactone produced, the result of an integrated set of enzyme activities of its gene expression, or both present on the Wheeler / Smirnoff route.

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