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The effect of ethylene on transgenic melon ripening and fruit quality

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Melons are good models used for explaining the physiological and biochemical changes in postharvest ripening. In this study, transgenic melons expressing apple ACC oxidase gene when treated with ethylene (AS3) were evaluated. Cell wall expression (MPG1; MPG2), ethylene synthesis ACC oxidase (ACCO1), flavour synthesis AAT (alcohol acyltransferase) and physiochemical parameters such as pulp firmness, titratable acidity (TA), soluble solid content (SSC), volatile esters, ethylene productions, antioxidant assay capacity and ascorbic acid content were evaluated. In cell wall expression analysis, MPG1 increased when fruits of transgenic melons were exposed to ethylene; showing they are ethylene-dependent. MPG2 decreased gradually when fruits were subjected to ethylene application. Fruit firmness was modified in transgenic fruits when ethylene was applied. There was a great reduction similar to that of non- transgenic fruits. However, TA in transgenic fruits remained lower than in non-transgenic fruit. The ethylene applied in transgenic fruit made the titratable acid to increase during 48 h and after it, a reduction was observed. In relation to soluble solid contents, transgenic fruits treated with or without ethylene did not reduce gradually compared to the wild type melons in all the periods. Ethylene productions in transgenic fruits were reestablished when ethylene was applied, exhibiting the same behavior as transgenic fruits. Antioxidant assay levels were more active in transgenic fruits when ethylene was applied than in control fruits, and it was only in transgenic fruits without ethylene. Ascorbic acid was kept in transgenic fruits with or without the application of ethylene. Results obtained show that the application of ethylene in transgenic ACC oxidase melons is able to change the metabolism of the cell wall, flavors and antioxidant capacity levels in fruit during the ripening process.

Key words: Esters, antioxidant, solid soluble content, ascorbic acid.

INTRODUCTION

Melon (*Cucumis melo* L.) var. *cantalupensis* Naudin is a climacteric fruit characterized by its peak of respiration and an autocatalytic ethylene production during ripening (Périn et al., 2002; Kays and Paul, 2004). The genetic

transformation of melons with antisense ACC oxidase gene reduces ethylene production and increases marketable postharvest preservation (Ayub et al., 1996; Silva et al., 2004; Nuñez-Palenius et al., 2007). In similar studies, Ayub et al. (1995) used antisense ACC oxidase gene from melons, that were isolated and characterized by Balagué et al. (1993); other authors (Silva et al., 2004) used a clone antisense ACC oxidase pAP4 from 'Royal Gala' apple constitutively expressed in climacteric ripening index.

The ethylene-suppressed ACC oxidase gene in melons allows the studying of ethylene-dependent and independent ripening pathways. Skin coloration and sugar accumulation are ethylene-independent, whereas yellowing of the rind, flesh softening, peduncle development abscission zone, volatile flavour compounds and climacteric respiration are totally or partially ethylene-dependent (Guis et al., 1997; Bauchot et al., 1998; Bower et al., 2002). Climacteric and non-climacteric regulation coexists during climacteric fruit ripening (Pech et al., 2008). Similar observations were made in *Charentais cantaloupensis* melons transformed with an antisense ACCO from apple (Silva et al., 2004). These authors showed prolonged fruit ripening cycle in an average of 10 days later, which supports the highest accumulation of sugars, in an average of 2.5°Brix higher than untransformed melons. Moreover, important phetotipics changes were observed; for example, vegetative cycle prolongation, increased fruits size, increased extensive root growth and minor leaves senescence. These characteristics were not described in earlier studies (Ayub et al., 1996; Bauchot et al., 1998).

Climacteric melons such as *cantaloupensis* are aromatic, but the ethylene suppressed to extend shelf-life can affect sensory qualities, especially aroma responsible for sensitive flavor (Pech et al., 2008). The synthesis of volatile compounds was significantly reduced in transgenic melons of Ayub et al. (1996), Bauchot et al. (1998) and Silva et al. (2004).

Bauchot et al. (1998), studying the behavior of transgenic melons by applying ethylene, verified that flavor intensity was restored by increase in the production of volatile compounds and induction of the peduncle abscission zone. In comple-mentary studies, Flores et al. (2002) and Yahyaoui et al. (2002) verified that the reestablishment of the overall production of volatile compounds and esters, in particular, was the consequence of alcohol acyltrans-ferases synthesis (AAT) induction and enzyme-key in the biosynthesis pathway of these compounds.

As a result, four clones of AAT (Cm-AAT1, Cm-AAT2, Cm-AAT3 and Cm-AAT4) were isolated and partially characterized in melons. Cm-AAT1 and cm-AAT4 are stronger and they are expressed during the ripening and under ethylene action (Yahyaoui et al., 2002; El-Sharkawy et al., 2005; Lucchetta et al., 2007).

However, in preliminary assays with transgenic melons (AS3 clone) (Silva et al., 2004), the responses to ethylene treatment were different from those observed by Bauchot et al. (1998), Flores et al. (2002) and Yahyaoui et al. (2002). The ripening was not completely re-established, where aroma intensity restoration was partially complete; although the treatment conditions with ethylene were similar to the ones described by other authors.

Some authors like Buttery and Ling (1993) and Goff and Klee (2006) state that, the improvement of plants to obtain a cultivar that is more productive, resistant to diseases and/or with extended shelf life can generate physiological changes and make the product to lose some important qualitative attributes. Silva et al. (2004) found that, *cantaloupensis* melons transformed (AS3) showed extended shelf life in postharvest. However, there was a significant reduction of its aroma intensity and low succulence of the fruits compared to control fruits. Goff and Klee (2006) co-related the volatile compounds production with the nutritional and functional quality of fruits. Also, they cited that the emission of volatile compounds results in the functional quality potential of fruits. Buttery and Ling (1993) observed that, the tomatoes selected for prolongation of shelf life have lesser nutritional quality and volatile compounds production than wild type tomatoes. In addition, it was verified that aroma, besides being the determinant of consumers' preference, can be associated with the best nutritional quality, essentials fatty acids, vitamins, carotenoids, licopens, folates, and other molecules with antioxidants properties (Goff and Klee, 2006).

This study explains the hypothesis which states that, the transformation with antisense ACC oxidase gene promotes other physiological modifications in melons. The practical non-existence of similar studies on fruits suggests that studies on the possible inter-relations between changes in ethylene production reduced (greater than 99%) as well as the postharvest behavior of melons.

In this work, the authors studied the effect of ethylene reduction and the exogenous treatment of this hormone on physiochemical characteristics, volatile compounds and expression of some genes with ethylene characteristics regulated during fruit ripening such as ACC oxidase (ACO), alcohol aciltransferase (AAT) expression and polygalacturonase genes (MPG1 and MPG2). Exogenous ethylene treatment was performed in AS3 fruits in order to verify if it was possible to restore the condition of ripening similar to that of WT.

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Table 1. Specific primers used for RT-PCR analysis of target genes.

Gen	Primers (5' - 3')	Author
CM-ACOO1	(F) AAG GAT CCG CAC AAA CCA AAT CTT GTA C (R) AAG GAT CCT AAG CTG AAA GTG AAT TTA AAT TA	Lassère et al., 1996
CM-AAT	(F) GTGATGGTGTGAGTCACACTGTTC (R) CGACCAGCAAGGTCCAAAC	John 1997
MPG1	(F) CTCTCATGCGCTGCAGTCTG (R) GCTTGGGCAATTTGATCCTT	Hadfield et al., 1998
MPG2	(F) CCGCATGGAAGCAGGCTTGT (R) CCATGTCAACAGTAGAGCCT	
ACTIN	(F) GAT GAC GCA GAT AAT GTT TGA GAC (R) AAG GTC ACG ACC AGC AAG GTC C	Bouquin et al., 1997

MATERIALS AND METHODS

Plant material

Non- transformed fruit (WT) and ACC oxidase antisense (AS3) Cantaloupe melons (*Cucumis melo* var. Cantalupensis, Naud cv.Vedrantais) were used (Silva et al., 2004). They were grown in a greenhouse under standard cultural practices for fertilization and pesticide treatments. Hermadrofite flowers were tagged on the day and self-hand pollinated. After this step, non- transgenic fruit plants were monitored during the period just to get to the actual 32 days after day pollination (DAP). During delayed ripening, AS3 fruits were harvested, 42 DAP and immediately exposed to 100 $\mu L.L^{-1}$ ethylene for 24, 48 and 120 h in vessels of 7.2L. Vegetative root tissues were picked up from control and AS3 plants immediately after the harvesting of 2nd fruit per plant. After treating the fruits with ethylene, pulp firmness, total soluble solids, titratable acid and samples were frozen in liquid N and stored at - 80°C prior to analysis.

Soluble solid content (SSC), titratable acidity (TA) and pulp firmness

Fresh pulp firmness was determined using an 11-mm Effegi tester penetrometer and the results were expressed in Newton (N). Soluble solid content was measured by a digital refractometer (ATAGO PR-101, Tokyo, Japan), using filtered juice; the results were expressed by percentage (m/m). Titratable acidity was perfomed by titulometric method, using NaOH (0, 1 N) with pH 8.1. The results were expressed in mg citric acid g FW^{-1}.

Measurements of ethylene production

The ethylene content was determined by gas chromatography (Varian® 3300). The treatments were replicated three times, and values represented the mean ± SE. The results were expressed in nL of ethylene.$g^{-1}.h^{-1}$.

RT-PCR of ACC oxidase and CmAAT e polygalacturonase (MPG1 e MPG2)

Total RNA was extracted from 50 mg of frozen melon pulp with TRIZOL® Reagent (I*nvitrogen*) buffer according to the manufacturer's instruction. First strand cDNA was synthesized from 1 µg of total RNA (DNAse treated) using a poly (T) 15 as a primer and Kit SuperScript™ First-Strand System for RT-PCR (Invitrogen). The reaction was stopped by heating at 70°C for 10 min, and treated with RNAse H. Forward (F) and reverse (R) primers (50 nM) used for RT-PCR amplification of the target genes in each RNA sample are described in Table 1.

The RT-PCR conditions were: 35 cycles at 95°C for 30 s (2 min for the first cycle), 47°C for Cm-AAT at 1 min and 53°C for β-Actin and CM-ACO1, and 72°C for 1 min (5 min for the last cycle). MPG1 and MPG2 cycle performance was: 35 cycles at 95°C for 1min (2 min for the first cycle), 47°C for 1:30 min and 72°C for 2 min (5 min for the last cycle). Actin gene was used as constitutive promoter.

Volatile compounds (esters)

All analyses were performed as described by Bauchot et al. (1998), with minor changes. SPME carboxen-PDMS (0.75 m × 1 cm, Supelco, USA) was used as the adsorbent matrix. All analyses were performed on a Varian 3800 gas chromatograph interfaced with a Shimadzu QP-50000 mass spectrometer. Volatiles were identified by comparing each mass spectrum with spectra from authentic compounds analyzed with spectra in reference collections (NIST/ EPA/NIH Mass Spectral database).

Antioxidant assay activity

Antioxidant activities were determined as a free radical according to Brand-Willams et al. (1995), by using 2, 2- Diphenyl-1-picrilhidrazil (DPPH) D-9132, Sigma-Aldrich, Dorset, UK. The samples analyzed were obtained from 100 g of fruits pulp dissolved in 250 ml of ultrapure water and centrifuged at 14000 × *g* for 15 min. The measurement of reduction absorbance was processed with 3.9 ml of free radical DPPH (100 µM) dissolved in 80% methanol. Then 0.1 ml of sample or standard was added to homogenize the mixture carefully. It was left in the dark for 30 min at a wavelength of 517 nm. The DPPH concentration in reaction was calculated by a linear regression obtained from calibrated curve. The results were expressed in TEAC activity equivalent to Trolox (acid 6-hidroxi-2, 5, 7, 8-tetramethylcrome-2-acid carboxilic, 97%; µM g of fresh $weight^{-1}$). The antioxidant synthetic Trolox was used based on calibrated curve.

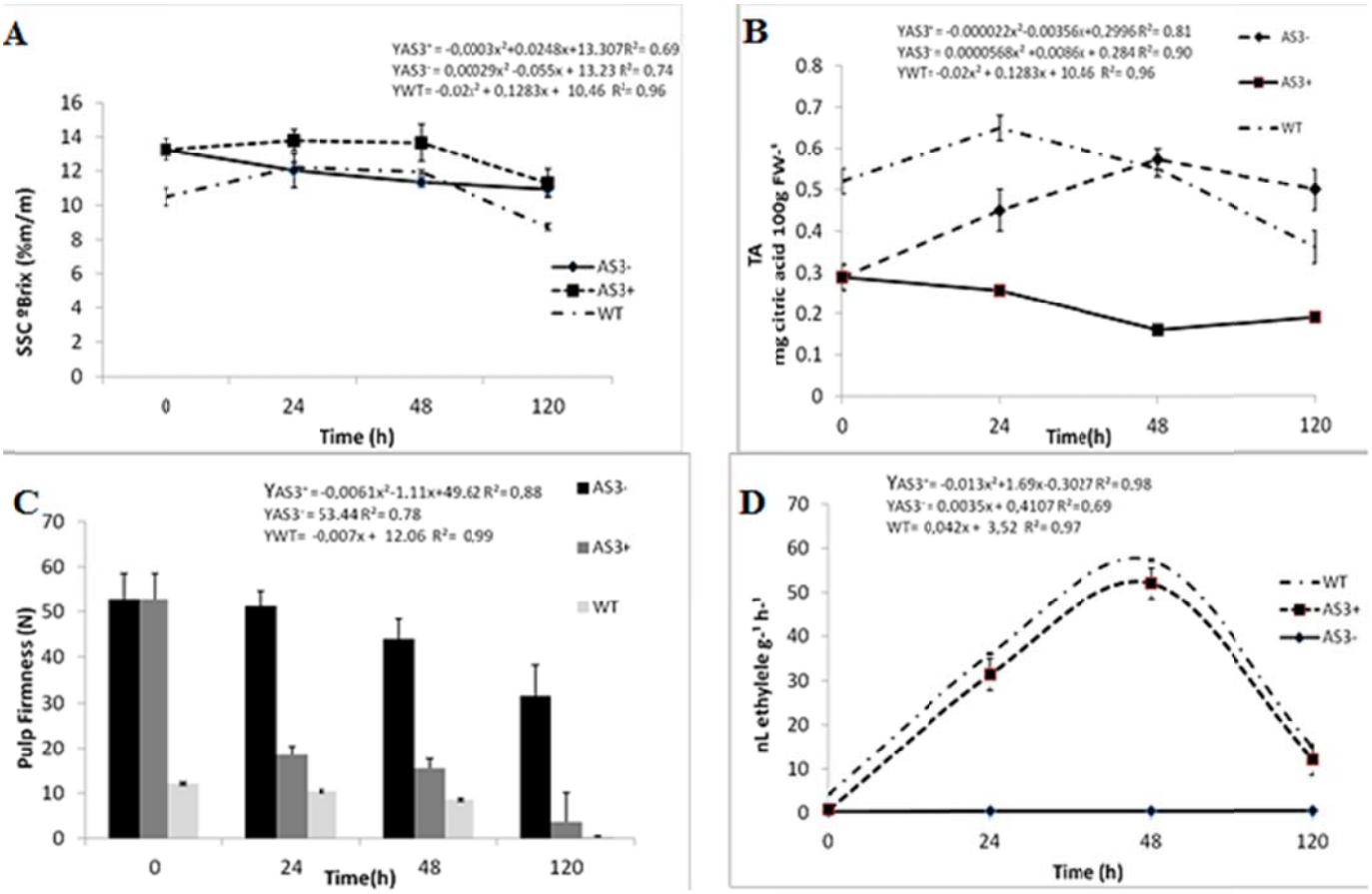

Figure 1. Characterization for Soluble solids content (A), titratable acidity (B), Pulp firmness (C) and Ethylene production (D) in melons in wild-type (WT) (C. melo, L. var. *cantalupensis* Naudin cv. Vedrantais) and transgenic melons (AS3) treated with ethylene for 0, 24, 48 and 120 h.

Ascorbic acid content

Ascorbic acid or vitamin C content in the melon pulp was measured using a high-performance liquid chromatography (HPLC) system. A Shimadzu liquid chromatography equipped with an auto sampler and a detector of 254 nm was used. A reversed-phase RP-18 column (5 mm particle size, 4.6 mm diameter, 150 mm length) with octadecyl stationary phase, operating at 25°C with a flow of 0.8 ml^{-1} min^{-1} was used.

Along with RP-18 guard column, it was used to separate the vitamin C using methanol (100%) and acidified water as a mobile phase. The ultra-pure water was acidified with acetic acid (0.1%, v/v). The mobile phase was filtered using a 0.45 *ím* membrane filter and degassed using helium gas before passing through the column at 25°C with a flow of 0.8 ml^{-1} min^{-1}. A standard calibrated curve was obtained using L-ascorbic acid (Sigma Chemical, 99.97% of purity) in the following concentrations: 10, 25, 50, 75 and 100 mg 100 ml^{-1}. The method was adapted from Vinci et al. (1995) and Ayhan et al. (2001).

A portion of 10 g of melon pulp was cut into small pieces and diluted to 30 ml of phosphoric acid solution (4.5%). This sample with phosphoric acid solution was filtered and the volume was completed to 50 ml with ultrapure water. An aliquot of 1.5 ml of this mixture was centrifuged at 10 000 rpm for 10 min (T = 20°C). The volume of the supernatant after centrifugation was always accurately measured. A fraction of 10 µL of the supernatant was injected into the HPLC chromatograph.

Statistical analysis

Experimental setup was performed in a completely randomized blocks with 3 mode of treatments [Control fruit, transformed without ethylene ($AS3^-$) and transformed with ethylene ($AS3^+$)]; it was evaluated four times when ethylene was applied. Each treatment is made up of three replicates. Four fruits were used for the evaluations. The data were subjected to variance analysis by Test-F p< (0.05). For each treatment, regression analyses were performed to represent time index analysis.

RESULTS

Pulp firmness, soluble solid content (SSC), titratable acidity (TA) and ethylene production

The AS melon, Cantaloupe cv. Vedrantais had low production of ethylene, approximately 0.5 $nL.h.g^{-1}$ (Figure 1A). This result is in accordance with that described by Silva et al. (2004) in previous studies, where it was verified that AS melon had 99.5% lower ethylene produc-

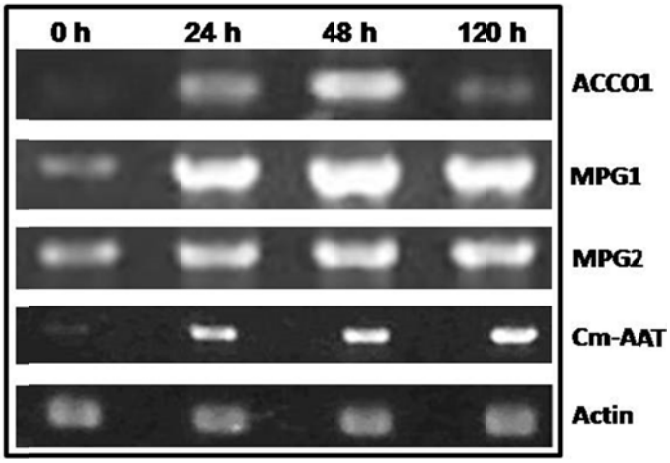

Figure 2. Expression of mRNA transcripts of ACC oxidase (ACCO1), poligalacturonase MPG1 and MPG2, alcohol acyl transferase (CmAAT) and Actin (Cm-Actin) in transgenic cantaloupe Vedrantais melon fruit (AS3) treated by ethylene exogenous for 0, 24, 48 and 120 h.

tion than control fruits. In other studies, Ayub et al. (1996) showed that transgenic melons expressing ACO anti-sense gene reduced ethylene production by 95%. AS melon treated with ethylene for 24 and 48 h and non-transformed fruits had similar ethylene production, but it declined in both materials at 120 h.

The wild-type melon quickly lost pulp firmness after harvest (Figure 1C). The main important characteristic of AS cantaloupes melon is that it maintains high pulp firmness in postharvest (Figure 1C). In AS fruits (AS3-), reduced firmness was lower than in control fruits during all the time of the analysis. The dates showed reduced pulp firmness in AS fruits of 52, 51, 48 and 32 N (harvest), respectively in 24, 48 and 120 h after harvest. This rise in the values obtained can be considered because 10 N is the minimum limit for the commercialization of this fruit. Otherwise, the presence of exogenous ethylene contributed to the reduction of pulp firmness significantly (Figure 1C). After 24 h of exogenous ethylene treatment, there was reduction in pulp firmness of 51 to 14 N. At the end of 120 h of exogenous ethylene treatment, pulp firmness was lower than 10 N reaching a value of 4 N. These results show that the strong correlation between pulp firmness and ethylene treatment was characterized by an ethylene-dependent event (Figure 1B).

In relation to SSC contents, $AS3^-$ increased during the first 24 h and kept on until the 48th hour; thereafter; there was a slight reduction of sugar contents. Slight reduction in AS3+ was observed until 120 h and was equal at the end. This is similar to AS3-. Also, in WT fruits, there was a slight increase in SS contents during 24 and 48 h and a strong reduction at the end of 120 h with value lower than 10°Brix (%m/%m).

Titratable acid (TA) in $AS3^-$ increased until the 48^{th} hour. It decreased after this period slightly until the 120th hour. On the contrary, when these fruits were exposed to exogenous ethylene treatment, there was a reduction in TA at the 48th hour and its establishment at the 120th hour. These results suggest that this reduction in TA content can be explained by higher respiration rate (data not shown), which must be in accordance with ethylene peak as shown in Figure 1D. Organic acids made part of the respiratory pathways. Already in non-transgenic fruit, there was a slight increase in acid within the first 24 h and a considerably decline at 120 h. Perhaps, in these fruits, sugars were transformed in organic acids and were used in the respiratory pathway.

ACC oxidase (ACCO1), polygalaturonase MPG1 and MPG2, and alcohol acyl transferase (CmAAT) gene expression

When evaluating the expressions of MPG1 and MPG2 genes in cantaloupensis melon under the action of ethylene, it was verified that both increased in the transcription genes; however, high levels of MPG1 gene were observed (Figure 2). In addition, the biggest difference in expression occurred within the first 24 h of ethylene treatment. These results suggest that MPG1 gene can be strongly involved with cell wall hydrolysis that leads to reduced pulp firmness (Figure 1C).

Considering that MPG1 and MPG2 correspond to an endo and exo-PG, respectively (Hadfield et al. 1998), it was expected that the increased expression of these genes would lead to reduction in pulp firmness. In fact, it happened so fast (Figure 2 and 1C). The ACC oxidase gene transcription in AS3 melons was lower than that in untransformed fruits (Figure 2). The ethylene applied in AS3 melons induced the transcription of ACC oxidase gene for 24 and 48 h (Figure 1A). The long exposure to ethylene (120 h) resulted in low amount of transcripts. Ethylene treatment induces the production of phytohormone like ACCO1 gene transcription (Figure 2). ACCO1 is a key enzyme in ethylene biosynthesis that shows the effect of induction treatment and recovery on the maturation of melons WT.

The mRNAs transcription of Cm-AAT1 was strongly expressed in control melons but weakly expressed in AS3 melons. This is because Cm-AAT1 gene is induced during ripening and under ethylene action. The ethylene applied in AS3 melons induced the transcription of Cm-AAT1, led to high expression of mRNAs and the attaining of similar levels with WT melons at 24 h. These elevated levels were maintained until 120 h (Figure 2).

Volatile compounds quantitation in AS melons

WT fruits had high production of esters. Ethylene production was reduced by transformed plants. It was observed as a significant reduction of volatile compounds synthesis

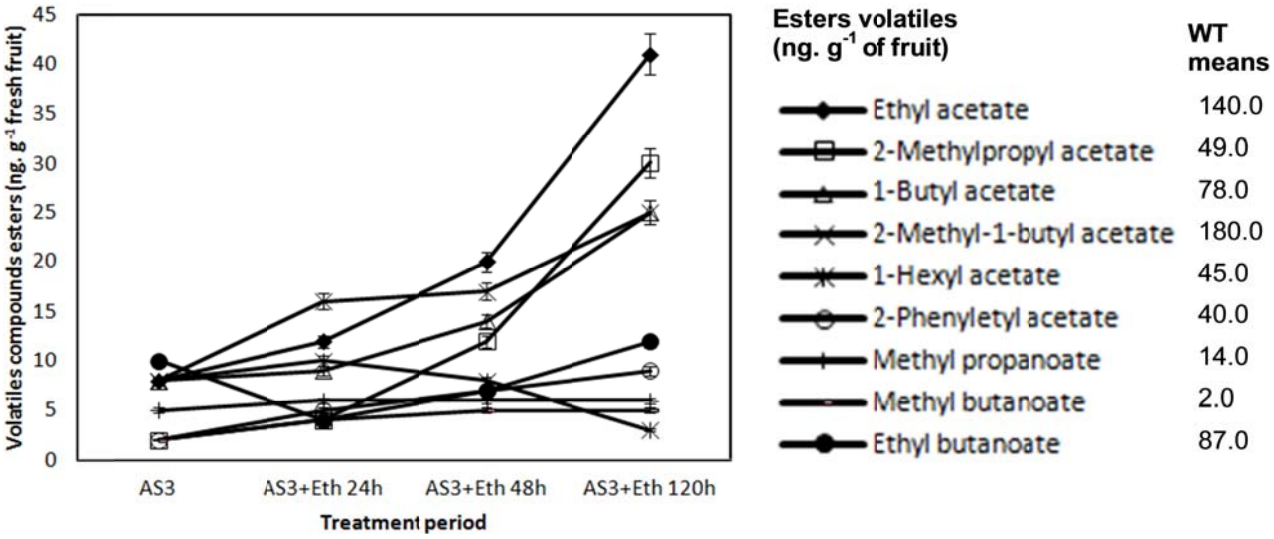

Figure 3. Contents of esters volatiles quantified (means) in melons wild-type (WT - right column) (C. melo, L. var. *cantalupensis* Naudin cv. Vedrantais) and transgenic melons (AS3) treated with ethylene for 0, 24, 48 and 120 h. Vertical bars represent standard error of the mean.

(Figure 3). Ethylene treatment restored the production of volatile compounds completely. This also happened in the study of Flores et al. (2002). It had partial effect, having recovered about 30% of volatile compounds; the fruits were maintained under ethylene action for 120 h (Figure 3).

Exogenous ethylene induced Cm-AAT1 transcription (Figure 2), but there was not a total effect on volatile compounds production (Figure 3). Within the compounds analyzed, 2-methylpropyl acetate, 1-butyl acetate, 2-Methyl-1-butyl acetate, 1-hexyl acetate, Methyl propanoate, ethyl propanoate, methyl butanoate and ethyl butanoate were more expressive than those treated with ethylene. By studying the possible cause of this behavior, ethylene was applied to enhance ACC oxidase expression in the proper phytohormone biosynthesis pathway, Cm-AAT1 and esters. The mRNAs expression of these genes was stimulated (Figure 2), which is in line with that of Yahyaoui et al. (2002).

Antioxidant total activity (TEAC) and ascorbic acid content

Also, in this study, some components of melon fruits that have nutritional and/or functional importance were evaluated. AS3 fruits that were harvested 42 DAA showed potential antioxidants levels higher than WT fruits (Figure 4A). The exposure of the fruits to exogenous ethylene for 24 h to 120 h enhanced antioxidants activity of AS3 melons. Moreover, to extend exposure of fruits to ethylene treatment, AS3 fruit exhibits values significantly higher than antioxidants activity compared to WT fruits.

Another component with antioxidant activity is ascorbic acid. In transgenic plants, ascorbic acid is kept almost constant for 120 h. These results make one to believe that ethylene is able to regulate other genes related to secondary metabolism such as phenolic compounds. The ascorbic acid content (Vitamin C) was strongly influenced by genetic transformation (AS), resulting in drastic reduction in the fruits transformed (Figure 4B). The AS fruits with low ethylene production had significantly lower vitamin C than WT fruits during the harvest and after treating with exogenous ethylene.

DISCUSSION

The reduction of ethylene production in transgenic plants reduced ACC oxidase gene expression (Hamilton et al., 1991; Ayub et al., 1996; Silva et al., 2004) and led to improved long shelf life in postharvest. But with these changes in ethylene metabolism, the changes that occur by ethylene action were also modified. For example, majority of these studies verified lower chlorophyll degradation but not induced abscission zone, delayed leaves senescence, less pulp softening and reduced volatile compounds synthesis (Ayub et al., 1996; Bauchot et al., Silva et al., 2004).

Flores et al. (2002) demonstrated that when the transformed plants with antisense ACC oxidase gene from melon were exposed, ethylene was seen; this reestablished the ripening of the process. This also occurred in AS3 melons obtained by Silva et al. (2004).

The fruits treated with ethylene for 120 h had low production of ethylene. This is probably due to the induction of the ACCO1 gene expression (Figure 2); and production of ethylene was strong during the first 48 h.

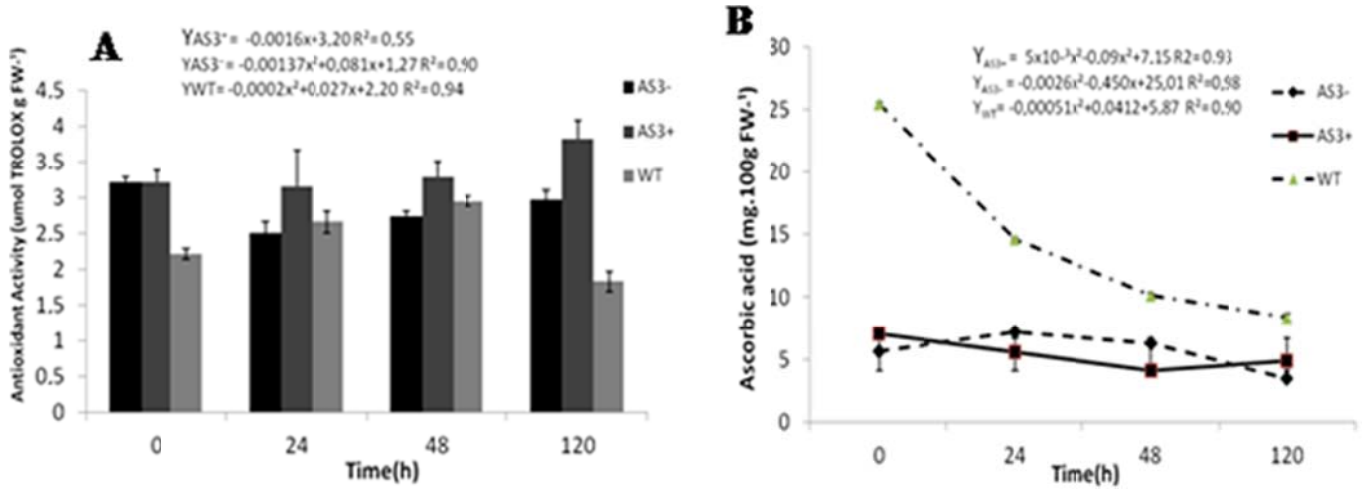

Figure 4. Characterization of antioxidant activity (A) and ascorbic acid (B) in melons in wild-type (WT) (*C. melo*, L. var. *cantalupensis* Naudin cv. Vedrantais) and transgenic melons (AS3) treated with ethylene for 0, 24, 48 and 120 h. Vertical bars represent standard error of the mean.

For 120 h, there were mature stadium and deterioration symptoms in this phase. They were characterized by low pulp firmness (Figure 1C). The effect of ethylene on the transcription induction of this gene was demonstrated by Lassere et al. (1996). However, these authors had studied the expression of this and other genes in vegetal models without genetic modification.

Ayub et al. (1996) and Bauchot et al. (1998), studying ACC oxidase melons antisense, related that the flesh softening in transformed fruits were practically inhibited. After 10 days of storage at 25°C, transgenic fruit remained fully firm, with a green rind and unaltered shape; whereas, wild-type fruit displayed senescence profile with a shriveled yellow rind, fungal infection, soft flesh and squashed shape. The continuous treatment of detached transgenic fruit with 100 ppm ethylene reversed the antisense phenotype result, which led to the activation of abscission zone melon, declined firmness and increased yellowing, like in control fruits.

The perception of autocatalytic ethylene is given by a set of receptor which transduces signal through a cascade of factors such as ctr, ein2, ein3/EIL and finally ethylene response factors (ERFs) (Bapat et al., 2010). In general, reducing the production and/or the action of ethylene, such as cold storage (CS), controlled atmosphere (CA) and the use of control systems (Zhou et al., 2000b; Dong et al., 2001) result in more maintenance of pulp firmness. Chaves et al. (1997) had verified, however, that the benefits of genetic modification in pulp firmness manifest just in fruits harvested in the stadium "breaker". When the fruits were matured, this did not present differences between fruits with low and high ethylene production. In AS3 melons, this did not take place. The reduction of ethylene production led to the maintenance of pulp firmness and exogenous phytohormone treatment softens the fruits (Figure 1C). Nishiyama et al. (2007) also demonstrated that in transgenic melon with ACO suppressed expression gene, there was a complete inhibition of softening of pulp, but was restored by exogenous ethylene treatments. In control fruits, when 1-MCP was applied, there was a significant reduction in the loss of firmness, which suggests an ethylene-dependent event.

In the harvesting of AS3 melons (42 DAA), the fruits did not show the peak of climateric maturity, which occurs in the maximum accumulation of reserves. Melons transformed with ACO1 antisense demonstrated greater accumulation of SS, which was also observed in previous experiments (Ayub et al., 1996; Silva et al., 2004; Grumet et al., 2007). The AS3 fruit was treated with exogenous ethylene, and rates of SS and TA were changed because there was an acceleration of maturation process. Probably, the solubilization of pectin that occurs in this period can explain the increase of SS and TA (Figure 1A and B). The solubilized carbohydrates, although less in melons, are in the order of 0.5 to 1.2%. Temporarily, during the post-harvest, this can result in the accumulation of SS content and increased acidity. In the reduction, when the fruit was treated with ethylene, between 48 and 120 h, there was consumption of sugars through the maintenance of aerobic respiration and senescence process.

The variations of pulp firmness are related with hydrolytic enzymes, where transcription can be ethylene-dependent (α-L-arabinosidase and β-D-galactosidase, endo-poligalactuonase) (Guis et al. 1997, 1999; Pech et al., 2008), ethylene-independent (pectil-metil-esterase) (Guis et al., 1997, 1999 and exo-poligalacturonase (Lelièvre et al., 1997). In melons, the main hydrolytic enzymes of cell wall are pectilmehyl esterase, endo and

exo-poligalacturonase, β-galactosidases/β-galactanases, expansins, endo-1, 4-b-glucanases, and xyloglucan endotransglycosylases (Rose et al., 1998; Hadfield et al., 1998).

Earlier, Gonçalves et al. (2013) showed that PG1 responds to ethylene treatment in pMEL1AS and pAP4AS cloned fruits. The regulation of gene expression during maturation and senescence related as wall cellular enzymes has generated a lot of discussion. Sitrit and Bennett (1998), studying polygalacturonase behavior gene in tomatoes expressing an ACC synthase gene with low ethylene production, verified that polygalacturonase mRNA gene was suppressed but when ethylene was applied, there was increase in mRNA transcripts levels of PG. However, in both cases, there was fruit softening. Regarding the gene MPG2, cited by Hadfield et al. (1998) as a possible exo-PG, the accumulation of mRNAs was gradual under the action of ethylene.

In mango, Sane et al. (2005) describe an expansion gene which correlates with the other genes of the cell wall metabolism during maturation induced by ethylene treatment. In advanced stages of ripening, endo- β-1, 4-glucanase enzymes correlate with increased activity of EGase (Chourasia et al., 2008). This study overlapping expression of cell wall enzymes shows synergistic action which explains why the change of part of the plant cell wall metabolism is directly influenced by ethylene, while another part depends on physiological factors correlated. Quesada et al. (2009) demonstrated that PG plays an important role in the ripening of strawberry and is negatively regulated by auxin. In strawberry fruits with PG transformed anti sense, the behavior is similar to that of melons, which maintain pulp firmness and increase the content of soluble solids during ripening.

MPG1 and MPG2 correspond respectively to an endo and exo-PG. Hadfield et al. (1998) state that both are involved in the reduction of pulp firmness, but MPG1 gene has stronger effect. This explains the quick and severe loss of firmness during the first 24 to 48 h of exposure to ethylene. It should be noted, however, that the interpretations of this study, which entail evaluating the mRNAs of genes and not enzymatic activity, give the assumption that the period between the transcription, translation, and post modification co-translational is long. This statement is made because in some cases, there is no relationship between the rate of transcription and enzymatic activity respectively. Like in the case of tomatoes, where the gene transcription PG is far above the máximum enzymatic activity (Sitrit and Bennett, 1998). However, Hadfield et al. (1998) and Rose et al. (1998) observed that these events are simultaneous and co-ordinated in melon.

The mRNAs transcription of Cm-AAT1 was strongly expressed in control melons than in AS3 melons. This behavior is based on the fact that, Cm-AAT1 gene is induced during the ripening and under ethylene action (Flores et al., 2002; Katzir et al., 2008); this is with pMEL clone of ACC oxidase gene (Lassere et al., 1996). The WT fruits had high aroma production. When ethylene production was reduced, volatile compounds synthesis was reduced too. This behavior has been explained earlier by other authors (Bauchot et al., 1998; Yahyaoui et al., 2002; Silva et al., 2004). The ethylene treatment did not restor the volatile compounds production completely, as seen in the study of Flores et al. (2002). The effect was partial. This behavior is not due to ethylene treatment imperfections, but because it has the same conditions described by other authors (Bauchot et al., 1998; Yahyaoui et al., 2002; Flores et al., 2002). The ethylene treatment induced Cm-AAT1 transcription (Figure 2), but there was no total response to volatile compounds production, which is contrary to the results obtained by Flores et al. (2002).

By studying the possible causes of this behavior ethylene was applied to enhance ACC oxidase expression in the proper phytohormone biosynthesis pathway, Cm-AAT1 and esters. The mRNAs expression of these genes was stimulated (Figure 2), as described by Yahyaoui et al. (2002). This behavior could have occurred by controlling the processes of post-transcriptions phases and/or other metabolism pathways that reduced levels of subtrates such as Acyl CoA, organics acids, aldehyds, alcohols from fatty acids and amino acids degradation (Song and Bangerth, 2003; Fellman et al., 2000). In general, the metabolism is lower when ethylene production is low; so it suggests that the reserved degradation that gives substrates physiological events in secondary metabolism is affected (Baldwin et al., 2000; Bauchot et al., 1998). Moreover, the effect of ethylene in the processes of CoA-SH recycled; reaction product in the esters pathway by action of AATs (Lucchetta et al., 2007) is unknown. Hypothesis is not tested in this study. In climacteric fruit, the esters volatile compounds are prevalent in strawberries (Severo et al., 2011), apple (Villatoro et al., 2008) and melon (Obando-Ulloa et al., 2008).

The volatile compound was quantified in these fruits. This has already been done by Bauchot et al. (1998). WT fruits have high esters production. When it was reduced, the ethylene produced by transformed plants was observed as a significant factor responsible for the low volatile compounds synthesis (Figure 3). This behavior has been explained earlier by other authors (Yahyaoui et al., 2002; Silva et al., 2004; Pech et al., 2008). The ethylene treatment did not restore the volatile compounds production completely, as seen in the study of Flores et al. (2002). It was partial, having recovered around 30% of volatile compounds; although this maintained the fruits under 120 h ethylene actions (Figure 3). This behavior is not due to ethylene treatments imperfections, but due to its concentrations and times of exposure (data not shown). The compounds analyzed, 2-Methylpropyl acetate, 1-butyl acetate, 2-methyl-1-butyl acetate, 1-hexyl acetate, methyl propanoate, ethyl propanoate, methyl

butanoate and Ethyl butanoate were more expressive than the one treated with ethylene.

The aroma profile of the melon decreases with maturity and senescence. In the same species, the profile of volatile compounds is different between climacteric and non-climacteric fruits (Obando-Ulloa et al., 2008). Villatoro et al. (2008) demonstrated that during the ripening of apples, there was increased esters production primarily by the accumulation of substrate for the action of the enzyme alcohol acyltransferase. This is due to the action of other enzymes such as precursor lipoxygenase (LOX), hydroperoxide lyase (HPL), pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) that give rise to the substrates.

Souleyre et al. (2005) showed that the substrate is not necessarily the profile of esters of fruit; this explains why there are no specific precursors of the esters. Severo et al. (2011) describe significant affinity between the transcription and physiological responses related to changes in sensory and nutritional strawberry, which highlights genes involved in cell wall metabolism, phenolic compounds biosynthesis, ascorbic acid and aroma (ADH and, AAT).

In earlier study (Shan et al., 2012), melons transformed with antisense AAT resulted in levels of mRNA transcripts and lower enzymatic activity than WT fruits. This caused a reduction in esters production. The reduction of esters contributed to a greater accumulation of aldehydes and alcohols that normally decrease with ripening. In previous studies, our group of collaborators (Yahayaoui et al., 2002; Ei-Sharkawy et al., 2005; Lucchetta et al., 2007) had already reported that these clones (Cm-AAT) after expression in yeast were active and showed different substrate preferences.

One of the molecules included in the antioxidant activity are phenolic compounds, although they are not evaluated in this study. These phenollic molecules can contribute to increase antioxidant activity. In studies with Kiwi fruits, Park et al. (2008) showed a strong correlation between applying ethylene with phenol compounds. The same authors showed that there are different phenolic compounds interacting in different moments at fruit ripening stage.

The evaluations also showed the functional/nutritional modifications in the melon pulp; therefore, the AS fruits had low accumulation of ascorbic acid (vitamin C), but they had a significant increased antioxidant potential. Ioannidi et al. (2009), studying the expression profile of ascorbic acid-related genes during tomato fruit development and ripening, showed that L-Galactose-1-phosphate phosphatase mRNA and transduction are dependent on ethylene. Perhaps, in our study, this gene can be changed by antisense ACC oxidase gene, showing a small loss of AA content compared to the wild type. The carotenoids contents were not altered by genetic modification and treatment with ethylene (data not shown).

The reduction of ascorbic acid contents in AS melons and genetic modification were on average five times the values found in fruits. After the harvest, in the fruits treated with exogenous ethylene, a stimulation of the maturation was verified. This led to a significant reduction of ascorbic acid contents, mainly in WT melons, that were more sensible to this phytohormone. This reduction is also observed in other fruits with mature stadium (Andrade et al., 2002) where, the oxidation of ascorbic acid (vitamin C) produces compounds with radical carbonyl that can react with amino groups and by polymerization produce dark pigments. The levels of ascorbic acid in AS fruits were practically unchanged.

An hypothesis is linked to cell wall metabolism. Di Matteo et al. (2010) verified that the up- regulation of a pectinesterase and two polygalacturonases suggests that AsA accumulation in tomato fruit is mainly achieved by increasing flux through the L-galactonic acid pathway, which is driven by pectin degradation and may be triggered by ethylene. Otherwise, in our AS3 melons, AA was kept due to low production of ethylene and consequently low expression of polygalacturonase as demonstrated in Figure 2.

The antioxidant activity of AS melons in the harvest was on average 100% more than that of WT fruits. The genetic modification resulted in reduction of ethylene production and in prolongation of maturation cycle. This led to high accumulation of compounds that result in antioxidant activity. The postharvest treatment with exogenous ethylene accelerated the process of maturation and the accumulation of compounds with antioxidant capacity. The effect of ethylene was more intense in WT fruits; however the indices of the antioxidant activity of AS continued to be significantly higher. The levels of potential antioxidant activity in cantaloupes melons have average good values in relation to some fruits commercialized; however they were lesser in red fruits (Kuskoski et al., 2005). The antioxidant capacity of fruits makes provision for some components, mainly phenols and the concentrations depend on environmental conditions, cultivar, species, etc. In this study, the genetic modification changed the composition and quantity of potential antioxidant. These differences in compounds can change the interaction for synergism or inhibitory effect (Rice-Evans et al., 1999; Robards et al., 1999).

To explain the behavior of WT melons and exogenous treatments, the possible interference of the ethylene production reduced can be related to cytokinins amounts and possible responses to the ethylene treatment (Zaicovski et al., 2008, Gonçalves et al., 2013). Zaicovski et al. (2008), evaluating different effects of depth irrigation on broccoli, showed that hydric stress was able to extend shelf life, gave high cytokinin levels and low ethylene production. Liu et al. (2013) transformed broccoli with isopentenyltransferase transformed (IPT), which encodes the key enzyme for cytokinin; and exoge-

nous treatment with N6-benzylaminopurine promoted postharvest conservation, establishing a system of protection.

The transgenic melons plants had significant phenoltypes alterations such as delaying leaves senescence, emission of more shoots and prolonging cycle of ripening. This indicates that other hormones interaction changes this phenotypes aspect, modifies ethylene sensibility, increases roots mass and more accumulation of the cytokinins levels in roots, pulp and rind of fruits (Gonçalves et al., 2013). The high accumulation of transcripts of genes involved in cytokinin synthesis shows that cytokines could be responsible for these different physiological behaviors of melon. Broccoli (Chen et al., 2001) and tomato (Martineau et al. 1995), induced to increase cytokinins, had significant effect on the ethylene responses. The irrigation management can stimulate the roots emission and increase the synthesis and translocation of cytokinins. This leads to reduction in the ethylene responses, leaves and flowers senescence (Zaicovski et al., 2008; Chang et al., 2003; Hedden and Philips, 2000; Martineau et al. 1995). In addition, cytokinins treatment in broccolis reduced the ethylene responses, which leads to the prevention of high green color degradation (Tian et al., 1995; Downs et al., 1997). In the case of melon, Gonçalves et al. (2013) applied exogenous cytokinin. But, it did not show any differences in ethylene production, firmness, soluble solids, titratable acidity, carotenoids, volatile ester compounds, or the contents of mRNA. Although the physiological mechanism has not been well described, the authors suggest the relation of cytokinins synthesis and accumulation increased with shelf life prolongation.

On the other hand, Yang et al. (2013) showed that in apple, the 1-MCP treatment induced changes in expression of genes involved in ethylene biosynthesis, perception and signal transduction. The 1-MCP blocked the system of perception and signal transduction of ethylene, resulting in decreased expression of genes involved in the ethylene response autocatalysis. In the case of AS3 melon, there were also changes in the perception and transduction system changed signal with low expression of related genes, causing a feeble response to ethylene treatment.

Conclusion

The exogenous ethylene treatments in transgenic fruits were reestablished and the metabolism was partially restored, changing fruit quality attributes. Transgenic melon expressing an antisense ACC oxidase under ethylene treatment was able to restore polygalacturonase genes (MPG1 and MPG2). Fruit firmness was greatly reduced similar to non- transgenic fruits. For CmAAT, the restoration of expression was similar to WT levels; however, there was no consistent amount of the esters productions, strengthening the hypothesis that, other factors influence the aromatic compounds production. AS3 melons showed higher total antioxidant activity than WT maintained throughout the treatment with exogenous ethylene.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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