

Irradiation reduces superficial scald by downregulating ethylene and α -farnesene biosynthetic
enzymes in 'Granny Smith' apples

Anderson Adriano Martins Melo^{a1}, Paul Nifemi Olabode^a, Hagop S. Atamian^b, Brian Nyakundi^a,
Criselda Toto Pacioles^c, and Anuradha Prakash^{a2}

^aFood Science Program

^bBiological Sciences Program

^cMathematics Program

Schmid College of Science and Technology, Chapman University, One University Drive,
Orange, CA, 92866, USA

¹Present address: Embrapa Western Amazon | Rod. AM 010 km 29, Manaus, AM, 69010-970,
Brazil

Correspondence to: Anuradha Prakash

E-mail: prakash@chapman.edu

²Permanent Address: One University Drive, Orange, CA 92866

ABSTRACT

Superficial scald is a postharvest disorder of apples related to increased ethylene production in stored fruit which leads to α -farnesene accumulation and oxidation. Ionizing irradiation inhibits ethylene production and has been shown to reduce superficial scald, but this phenomenon has not been explored at the molecular level. The goal of this study was to determine the effect of irradiation on gene expression of enzymes related to ethylene and α -farnesene in ‘Granny Smith’ apples. Irradiation at 310 Gy controlled scald severity and incidence and inhibited the rise in *AFS1* expression up to 90 d of cold storage followed by 7 d at room temperature, while 1000 Gy suppressed scald symptoms for either 90 and 180 d of cold storage, but showed internal browning.

Irradiation at both doses reduced the formation of α -farnesene by more than 50 % with concomitant decline of ACO enzyme activity and ethylene production as compared to the control, and suppressed the increase in *ACSI* gene expression, but without the same effect on *ACO1* gene. Irradiation treatment at 310 Gy and 1000 Gy may reduce superficial scald in ‘Granny Smith’ apples through inhibition of gene expression of enzymes related to ethylene and α -farnesene biosynthesis.

Keywords: α -farnesene; ACC synthase; ACC oxidase; *MdAFS1*; *MdACSI*.

1. INTRODUCTION

Superficial or storage scald is a major physiological disorder that affects quality of ‘Granny Smith’ apples (*Malus domestica* Borkh) after prolonged cold storage (Lurie and Watkins, 2012).

Scald symptoms develop after 2-4 months of cold storage and severity intensifies after warming (Rudell et al., 2005). The disorder is related to chilling injury (Watkins et al., 1995), and involves both the accumulation and oxidation of the sesquiterpene α -farnesene ([3E,6E]-3,7,11-trimethyl-1,3,6,10-dodecatetraene). α -farnesene, a volatile compound naturally formed in the hypodermal cells, accumulates in the epicuticular layer of the apple peel during storage (Rupasinghe et al., 1998; Huelin and Coggiola, 1968). Synthesis and accumulation of α -farnesene after harvest in apple peels have been linked to an increase in ethylene concentration (Du and Bramlage, 1994; Moggia et al., 2010). Oxidation of α -farnesene results in the production of conjugated trienes (CTs) and trienols (CTols) (Whitaker, 2013). These compounds participate in further redox reactions, producing free radicals which lead to lipid peroxidation and loss of membrane integrity of the hypodermal cell layers of susceptible apple cultivars such as 'Granny Smith' (Lurie and Watkins, 2012; Rowan et al., 2001). Loss of membrane integrity allows mixing of PPO with phenolic substrates and subsequent browning (Lurie and Watkins, 2012), leading to the characteristic necrotic lesions, the intensity of which depends on the duration of cold storage followed by exposure at room temperature, as illustrated in Fig.1:



Fig.1. Superficial scald ranging from none to severe in Granny Smith apples.

Oxygen deprivation as well as the use of antioxidants can inhibit apple scald by reducing ethylene and α -farnesene production and oxidative stress. Controlled atmosphere storage is used commercially in the Pacific Northwest to limit scald incidence. Reduced scald was observed at standard controlled atmosphere storage with ventilation or low pO_2 (Anet, 1972; Whitaker, 2000), ultra-low oxygen storage alone (Zanella, 2003) or combined with pre-storage low temperature conditioning (Zoffoli et al., 2018). Diphenyl-amine (DPA) is also used commercially to reduce scald incidence. As a lipid soluble antioxidant, DPA prevents oxidative processes related to scald, reducing the formation of CTs and CTols (Lurie et al., 1990; Whitaker, 2000). However, DPA is considered a pollutant and a potential carcinogen and banned by the European Commission (EC) and in 28 EU countries (Calvo and Kupferman, 2012).

Control of ethylene perception using 1-methylcyclopropene (1-MCP) has proven to decrease the incidence of superficial scald in 'Granny Smith' apples (Fan et al., 1999) by downregulating

genes responsible for α -farnesene synthesis (*MdAFS*) (Pechous et al., 2005; Tsantili et al., 2007). However, the efficacy of 1-MCP diminishes during cold storage. For example, in ‘Law Rome’ and ‘Cortland’ apples, 1-MCP showed transient effect with recovery of ethylene evolution and expression of scald-related genes in green sections of peel during long term storage (Tsansili et al., 2007).

Irradiation is increasingly used as a phytosanitary treatment to prevent the introduction and spread of quarantine pests so that horticultural products that are at risk of carrying the pests may be traded. Irradiation accomplishes this by inhibiting development and reproduction of these pests (FAO, 2003). At the low doses utilized for quarantine purposes, irradiation has been reported to lower ethylene levels ‘Gala’ and ‘Fuji’ apples, irradiation (Fan et al., 2001; Kheshti et al., 2019), and reduce the activity of 1-aminocyclopropane-1-carboxylic acid oxidase (ACO, a key enzyme in ethylene synthesis) in papaya fruit during storage (D’Innocenzo and Lajolo, 2001). Ionizing irradiation has been observed to reduce scald incidence in ‘McIntosh’, ‘Cortland’, and ‘Rome Beauty’ apples at doses of 0.5-1 kGy (Massey, Parsons and Smock, 1964). However, there are no reports of the mechanism by which superficial scald is inhibited at the molecular level. Specifically, there are no studies that evaluate the effect of irradiation on the gene expression of enzymes related to ethylene and α -farnesene synthesis. Thus, the goal of this study is to explore the molecular mechanisms by which irradiation inhibits ethylene production and mitigates superficial scald in ‘Granny Smith’ apples.

2. MATERIALS AND METHODS

2.1. Fruit source, transport and treatments

Over 1000 early season ‘Granny Smith’ apples were harvested on September 14th, 2017 in a commercial orchard located in Linden, CA. Titratable acidity of the fruit was 0.71 g 100 g⁻¹ and TSS content was 8.3 g 100 g⁻¹. The fruit were loose filled, approximately 100 to a box, in each of ten boxes (51 cm x 33 cm x 30.5 cm) without cleaning or additional processing. Log Tags[®] (Auckland, New Zealand) were placed in each case to monitor the temperature during subsequent transport, treatment, and storage. The apple boxes were transported a distance of 130 km to Steri-Tek (Fremont, CA) for x-ray treatment. Two boxes of fruit were placed sideways for each dose to receive two-sided x-ray radiation from a dual beam 10 MeV, 20 kW MEVEX linear accelerator (Stittsville, Canada). Dose mapping was conducted on a box of apples using Gafchromic[™] HD-V2 film dosimeters (Ashland Specialty Ingredients, Bridgewater, New Jersey) placed on either side of 12 apples within the box and a dose uniformity ratio of 1.33 was obtained. The film dosimeters were calibrated against alanine pellet dosimeters (Kodak, Rochester, NY). Verification of dose during the treatments was done by placing a Gafchromic dosimeter in a pocket on top of the conveyor rack. The racks were conveyed at different speeds in order to achieve the minimum target doses of 250 and 1000 Gy. The dose of 250 Gy was selected based on the approval by USDA APHIS for for phytosanitary treatment of apples by the California apple industry (USDA, 2021) and 1000 Gy to exaggerate the effect of irradiation. When applied commercially, a target dose of 250 Gy will result in some apples absorbing twice or even more than that, depending on the physical arrangement of the load. One thousand gray is the current maximum absorbed dose allowed by the US FDA and similar agencies in other countries for fruit irradiation. Apples that were subjected to a target dose of 250 Gy received a

dose of 310 Gy (as absorbed by the reference dosimeter) and a range of 300-390 Gy. Apples irradiated at a target dose of 1000 Gy absorbed a range of 1000-1310 Gy.

After irradiation treatment, the apples were transported to Chapman University and stored at 0-1 °C and 95 % humidity for 180 d. On the day of arrival (day 0) and after 90 and 180 d, two sets of 18 apples per treatment were randomly removed from each of the two boxes in cold storage and placed at room temperature (22 °C) for 7 d prior to the analyses. One set of 18 apples was divided into three sets of six apples each and used for measurement of carbon dioxide and ethylene production. These apples were then halved for observation of internal browning. The peels were frozen at -80 °C for subsequent enzymatic and gene expression analyses. Another set of eighteen apples, divided into three sets of six apples each, were used for superficial scald evaluation, followed by α -farnesene and conjugated trienol analyses.

2.2. Respiration and ethylene production rate

Carbon dioxide and ethylene production rates were determined using a static system as described by Sea et al. (2015). On day 0 and after, 90, and 180 d, 18 apples per treatment were selected at random from two boxes and stored at room temperature. After 7 d, six apples each were placed in three 3.7 L glass jars per treatment and the jars sealed with a rubber stopper. After one hour, 1 mL of headspace air was withdrawn from each jar and injected into a SRI 8610C gas chromatograph (SRI Instruments Inc., Torrance, CA, USA) equipped with a 1.83 m \times 3.18 mm HayeSep-D 80/100 mesh column (Restek CO., Bellefonte, PA, USA) maintained at 80 °C for separation, and an FID/TCD for ethylene/carbon dioxide detection, respectively, both at 150 °C. Hydrogen was used as carrier and make up gas at a flow rate of 15 mL min⁻¹. Ethylene and

carbon dioxide were quantified by measuring peak area in relation to the area of a standard gas (20,000 $\mu\text{L L}^{-1}$ of CO_2 and 100 $\mu\text{L L}^{-1}$ of C_2H_4) used to create calibration curves.

2.3. Superficial scald and internal browning

Superficial scald incidence was observed on apples stored for 90, and 180 d at 0-1 °C plus room temperature for 7 d and recorded as % of 18 apples per treatment at each time point. To measure the severity of superficial scald, a four-point scale was adopted, where 0 = no injury; 1 = slight injury (1 to 25 % of surface affected); 2 = moderate injury (26 to 50 % of surface affected) and 3 = severe (> 50 % of surface affected). Scald severity was expressed as Scald Index = [(% fruit grade 1) + (2 x % fruit grade 2) + (4 x % fruit grade 3)]/4 (Lurie et al., 1990).

To evaluate internal browning incidence, the same apples were halved at the equator and the number of fruits exhibiting internal browning were counted and reported as % of fruit exhibiting internal browning.

2.4. α -farnesene and Conjugated Trienol (CTol) concentration

Concentrations of α -farnesene and CTol were measured in 18 apples per treatment (3 sets of 6 apples) stored for 90 and 180 d at 0-1 °C plus room temperature for 7 d. Hexane extracts were obtained by dipping fruit individually in 100 mL of HPLC grade hexane and constantly turning them for 3 min to maximize extraction from the entire fruit surface. The absorbance spectrum of hexane extracts was measured from 195 to 300 nm. The concentration of α -farnesene was calculated from the absorbance peak at 232 nm using the molar extinction coefficient $\epsilon_{232} =$

29,000, as proposed by Anet (1972). Concentrations of all CTols in hexane extracts were assumed to have an extinction coefficient of 25,000 and were calculated from the absorbance maxima at 281 subtracted from 290 (non-specific absorbance) (Anet, 1972). The surface area of the fruits was calculated from the volume using the equation $A = 5.3 x^e$, where A is the area (cm^2), x is the volume (cm^3) and e is the non-linear coefficient for 'Granny Smith' apples proposed by Clayton et al. (1995). Results were expressed as nanomoles per square centimeter of fruit.

2.5. ACO activity

ACO enzyme activity was determined as described by Bulens et al. (2011) with slight modifications. Frozen peel was crushed in a mortar and pestle using liquid nitrogen. The ground peel, 0.1 g, and 0.3 g of polyvinylpyrrolidone (PVPP) were weighed out in a falcon tube while keeping cold on ice. Six mL of extraction buffer was added to the falcon tube and the slurry was placed in a 1.5 mL micro-centrifuge tube and centrifuged at 21,000 g for 30 min at 4 °C. 800 μL of supernatant was collected and transferred into a 15 mL glass vial where 3.2 mL of MOPS reaction buffer was added. The vial was immediately capped and vortexed for 5 s, then incubated in a shaking water bath (30 °C) at 55 rpm for 40 min. After incubation, the vial was vortexed to release all the gas into the headspace and 1 mL of gas was withdrawn with a syringe and injected into the gas chromatograph to measure ethylene production as described above. The amount of ethylene released (mol) was used to calculate the in vitro activity of ACO using the equation proposed by Bulens et al. (2011). ACO activity was expressed as $\text{U min}^{-1} \text{mL}^{-1}$. One

unit was defined as the amount of enzyme that catalyzes the conversion of 1 nmol of ACC per min under the assay conditions.

2.6. RNA extraction and DNase treatment

Total RNA was isolated from frozen peel tissue using a modified CTAB protocol (Bekesiova et al., 1999). Briefly, 700 μL of RNA extraction buffer (2 g 100 mL^{-1} CTAB, 2 g 100 mL^{-1} PVP, 100 mmol L^{-1} Tris-HCl (pH 8.0), 25 mmol L^{-1} EDTA (pH 8.0), 2 mol L^{-1} NaCl, 2 mL 100 mL^{-1} β -mercaptoethanol) was added to frozen ground peel tissue and heated at $65\text{ }^{\circ}\text{C}$ for 10 min. The samples were extracted twice with chloroform (Sigma–Aldrich, St. Louis, MO) and precipitated with $\frac{1}{4}$ volume of 8 mol L^{-1} LiCl overnight at $-20\text{ }^{\circ}\text{C}$. Followed by washing the RNA pellet with $700\text{ }\mu\text{L}$ of $70\text{ mL } 100\text{ mL}^{-1}$ ethanol, air drying at room temperature for 15 min and resuspending the RNA pellet in $20\text{ }\mu\text{L}$ of nuclease free water. The quality and quantity of the extracted RNA was measured using a Nanodrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). High quality RNA extractions were subjected to on-column DNase treatment using the Spectrum Plant Total RNA Kit (Sigma–Aldrich, St. Louis, MO) following the manufacturer protocol.

2.7. Complementary DNA (cDNA) synthesis and gene expression analysis

cDNA was synthesized from 100 ng DNase treated RNA using Superscript III reverse transcriptase (Invitrogen™, San Diego, CA) according to the manufacturer's instructions. Quantitative PCR (qRT-PCR) was performed using iQ SYBR Green Supermix (Bio-Rad). The

amplification conditions were 3 min at 95 °C, 39 cycles of 15 s at 95 °C and 1 min at 60 °C. The relative fold difference of mRNA levels and was calculated using a modified 2–DDCT formula (Livak and Smittgen, 2001) with *Protein disulphide isomerase (MdPDI)* and *Ubiquitin (MdUBI2)* used as reference to normalize gene expression. Samples from day 0+7 d at room temperature were assigned an arbitrary quantity of “1” and used as a calibrator to calculate relative gene expression. Three biological replicates (each one a composite sample of 6 different apples) and two technical replicates were used. The sequences of the primers used are listed in Table 1.

Table 1. Gene specific primers used for this study

Genes	Primers	Sequences (5'-3')
<i>MdACS1</i>	ACS1-F ACS1-R	CTCCTCCTTTCCTTCGTTGA ACCATGTCGTCGTTGGAGTAG
<i>MdACO1</i>	ACO1-F ACO1-R	GTTCTACAACCCAGGCAACG TCTCAGAGCTCAGGCAGTTG
<i>MdAFS</i>	AFS-F AFS-R	GCTTCCATCACACCGCAGAG CCCAGATTTGCCACCACTTG
<i>MdUBI2</i>	UBI2-F UBI2-R	TTGATCTTTGCTGGGAAACAG CACCACCATCATTC AACACC
<i>MdPDI1</i>	PDI1-F PDI1R	TGCAAATCCCTTGCTCCTAC CCCTCCTTGTTGTTCTTTGG

2.8. Statistical analysis

The experiment was set up in a completely randomized design. Three replicates of 6 fruit per treatment were used for all the analyses. Analysis of variance (ANOVA) was performed to determine the effect of irradiation and storage as well as differences in gene expression using R statistical Software (program R), version 3.42. (RStudio, 2017). Differences among means were compared by Tukey's least significant difference (LSD) test at $\alpha = 0.05$. The data from visual assessment of superficial scald and internal browning were submitted to the Shapiro-Wilk normality test. Given the non-normal distribution of data, these were submitted to the Kruskal-Wallis non-parametric test, and the means compared by the Bonferroni test at 5 % significance.

3. RESULTS

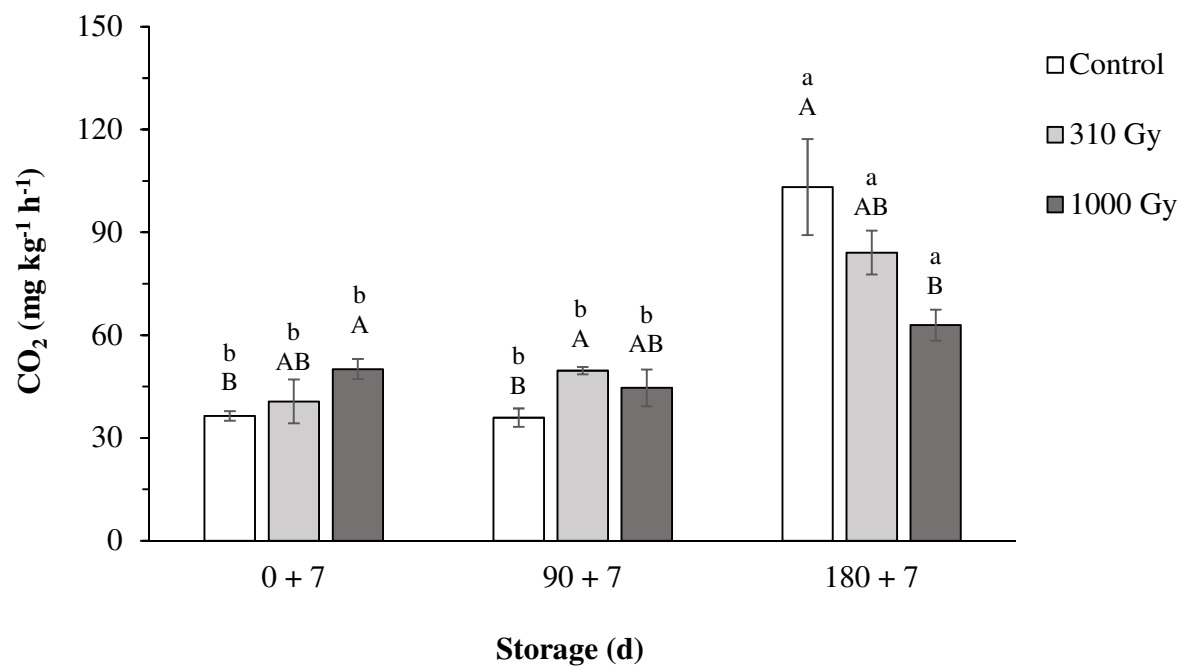
3.1. Respiration rate, and ethylene production rate

No differences were observed in respiration rates between the irradiation doses on day 0 plus 7 d at room temperature ($p > 0.05$), but the 1000 Gy showed higher rates when compared to control. After storage for 90 d at 0-1 °C plus room temperature for 7 d, apples irradiated at 310 Gy showed higher respiration than non-irradiated apples. However, after 180 d at 0-1 °C plus room temperature for 7 d, respiration rates increased for all treatments, especially the control, and fruit

irradiated with 1000 Gy had significantly lower respiration rate compared to control ($p < 0.05$) (Fig. 2 A).

Cold storage induced a large increase in ethylene synthesis, and this effect was attenuated by irradiation (Fig. 2 B). The levels of ethylene production increased 32-fold for control and 310 Gy irradiated apples, while the 1000 Gy irradiated apples increased 16-fold during the three first months of storage. After 180 d at 0-1 °C plus room temperature for 7 d, a decrease in ethylene production was observed in non-irradiated apples, while the amount of the hormone produced by irradiated fruit remained unchanged.

(A)



(B)

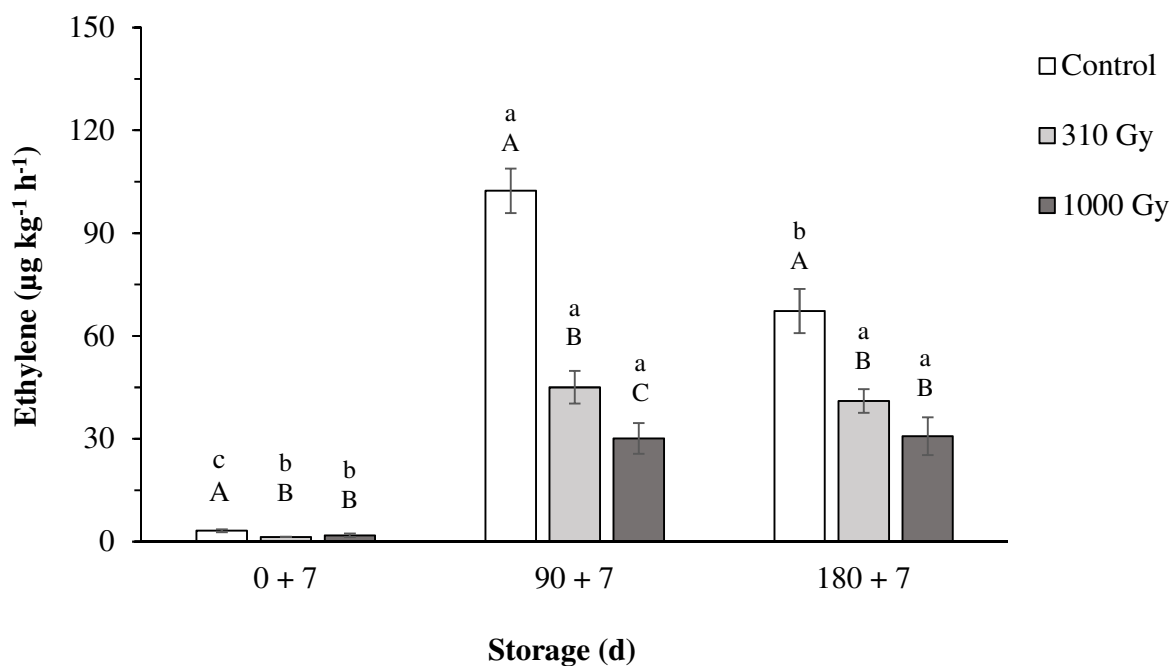


Fig. 2. Effect of irradiation (310 and 1000 Gy) on the respiration rate (A) and ethylene production rate (B) of 'Granny Smith' apples stored for 90 or 180 d at 0-1 °C followed by seven

days at room temperature. Results are represented as mean values of three replicates of six fruit each and the error bars show standard deviations. The letters A, B, C show differences due to treatment on the given day and the letters a, b, c show differences during storage for any given treatment ($P < 0.05$).

3.2. Accumulation of α -farnesene and conjugated trienols (CTols)

Initial concentrations of α -farnesene were low but increased significantly after three months of storage at 0-1 °C plus room temperature for 7 d (Fig. 3 A). Irradiation at both doses significantly suppressed the rise in α -farnesene concentration by more than 50 % as compared to the control. After 180 d at 0-1 °C plus room temperature for 7 d, the levels of α -farnesene decreased in all fruit, but irradiated fruit still maintained significantly lower α -farnesene concentration. The differences in the concentration of CTols showed a similar trend to that of α -farnesene (Fig. 3 B). The highest concentrations of CTols were observed at 90 d at 0-1 °C plus room temperature for 7 d for all treatments. Fruit irradiated at 1000 Gy had significantly lower concentration of CTols as compared to control fruit, independent of storage time.

(A)

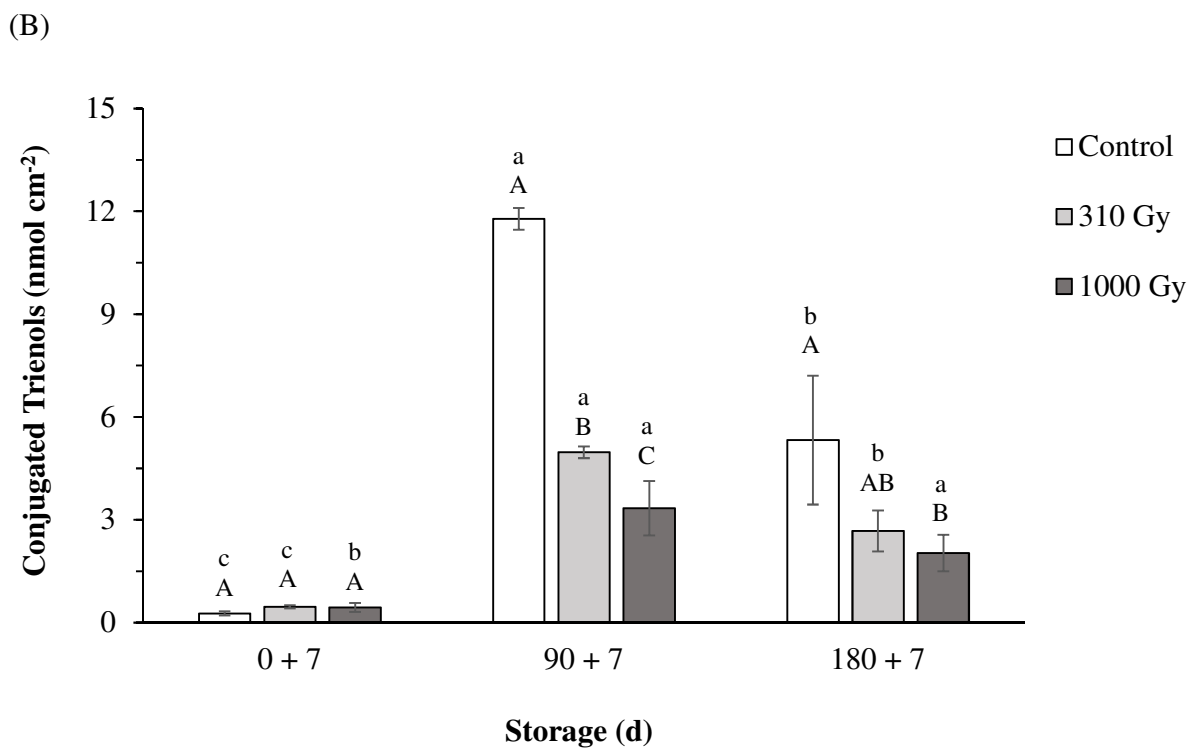
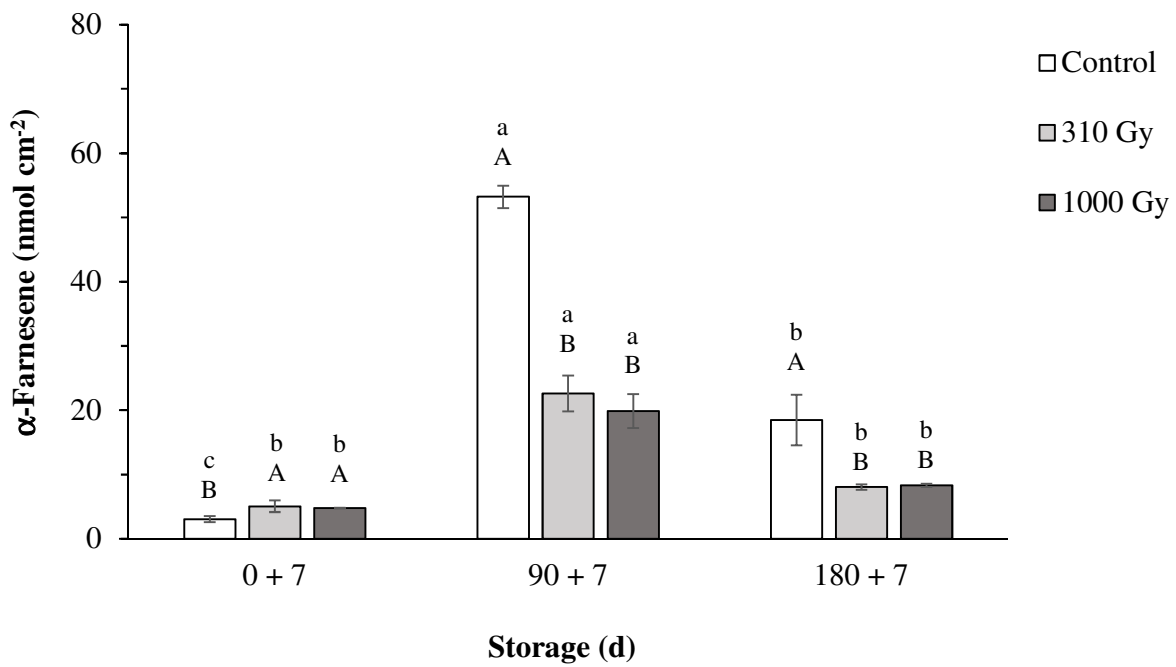


Fig. 3. Concentration of α -farnesene (A) and conjugated trienols (B) in irradiated (310 Gy, 1000 Gy) 'Granny Smith' apples stored for 90 or 180 d at 0-1 °C followed by seven days at room

temperature. Results are represented as mean values of three replicates of six fruit each and the error bars show standard deviations. The letters A, B, C show differences due to treatment on the given day and the letters a, b, c show differences during storage for any given treatment ($P < 0.05$).

3.3. Superficial scald and internal browning

No visual scald symptom was observed in apples of any treatment stored for 7 d at room temperature immediately after treatment (Table 2). After cold storage for 90 d followed by a week at room temperature, 67 % of the control apples showed scald symptoms, with a scald severity index of 29.2, while no scald was observed in irradiated apples. After 180 d at 0-1 °C plus room temperature for 7 d, all control apples and 72 % of the apples irradiated at 310 Gy showed scald, with a severity index of 94.4 and 23.6, respectively, while apples irradiated at 1000 Gy showed no scald incidence.

Table 2. Effect of post-harvest irradiation on Scald Incidence (%), Scald Severity Index and Internal Browning (IB) Incidence (%) in ‘Granny Smith’ apples stored for 0, 90 and 180 d at 1 °C followed by 7 d at room temperature.

Scald Incidence			
<i>Treatments</i>	<i>Days</i>		
	0 + 7	90 + 7	180 + 7
Control	0 aB	66.67 aAB	100 aA
310 Gy	0 aB	0 aB	72.22 abA
1000 Gy	0 aA	0 aA	0 bA

Scald Severity^a			
<i>Treatments</i>	<i>Days</i>		
	0 + 7	90 + 7	180 + 7
Control	0 aB	29.17 aAB	94.45 aA
310 Gy	0 aB	0 aB	23.61 abA
1000 Gy	0 aA	0 aA	0 bA

IB Incidence			
<i>Treatments</i>	<i>Days</i>		
	0 + 7	90 + 7	180 + 7
Control	0 aB	0 bB	100 aA
310 Gy	0 aB	0 bB	66.67 aA
1000 Gy	0 aB	88.89 aA	94.44 aA

^aScald severity index was calculated as $[(1 * \% \text{ fruit grade 1} + (2 * \% \text{ fruit grade 2}) + (4 * \% \text{ fruit grade 3})]/4$. Means followed by the same letters, lowercase vertically and uppercase horizontally, are not significantly different as per the Bonferroni test ($p < 0.05$).

Unlike superficial scald, treatment at 1000 Gy induced internal browning in 88.9 % after 90 d at 0-1 °C plus room temperature for 7 d (Table 2). Interestingly, no internal browning was observed in control and in fruit treated with 310 Gy. After 180 d at 0-1 °C plus room temperature for 7 d, internal browning incidence increased in all the treatments and fruit subjected to 310 Gy

irradiation showed internal browning in 66.7 % of the fruit, but not significantly different ($p>0.05$) from the control (100 %) fruit and 1000 Gy treated fruit (94.4 %), by the Bonferroni test.

3.4. Effect of irradiation on ACO enzyme activity

ACO activity and ethylene levels were low on day 0 at 0-1 °C plus room temperature for 7 d (Fig. 2 B, Fig. 4) indicating that the apples were in a pre-climacteric stage. After 90 d of cold storage, when the apples were warmed at room temperature for seven days, ACO activity increased by seven-fold in the control apples suggesting that the apples entered a climacteric stage. The irradiated samples also exhibited increased enzyme activity, however irradiation seemed to dampen the increase. Upon 90 d of cold storage followed by seven days of room temperature, the 310 Gy samples showed a five-fold increase and the 1000 Gy exhibited a three-fold increase as compared to the first time point. At 180 d at 0-1 °C plus room temperature for 7 d, ACO activity did not show significant difference between treatments and the control.

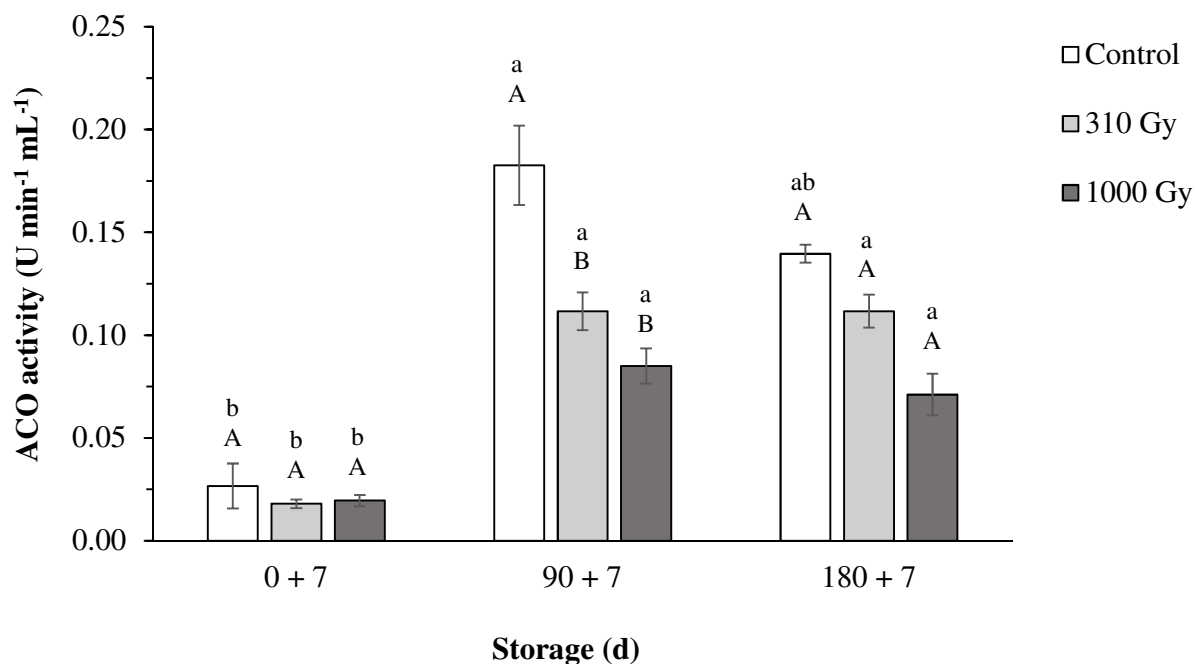


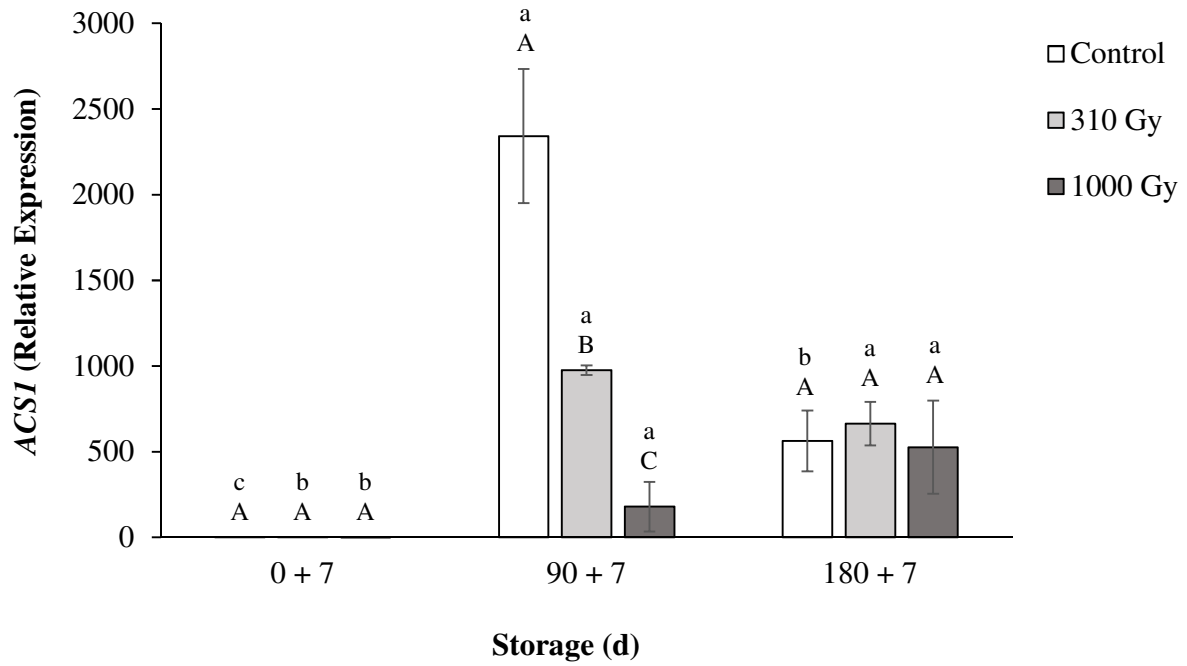
Fig. 4. Effect of irradiation (310 and 1000 Gy) on the ACC oxidase (ACO) activity of ‘Granny Smith’ apples stored for 90 or 180 d at 0-1 °C followed by seven days at room temperature. Results are represented as mean values of three biological replicates of six fruit each and the error bars show standard deviations. The letters A, B, C show differences due to treatment on the given day and the letters a, b, c show differences during storage for any given treatment ($P < 0.05$).

3.5. Expression of ethylene and α -farnesene biosynthetic genes

The expression of the *MdACS1* and *MdACO1* genes in control fruit peaked after 90 d of cold storage plus 7 d at room temperature and decreased thereafter (Fig. 5 A, B). Irradiation significantly suppressed the expression of *MdACS1* after 90 d at 0-1 °C plus room temperature

for 7 d compared to the control fruit in dose dependent manner (Fig. 5 A). Irradiation-induced reduction in *MdACO1* gene expression was not statistically significant ($p>0.05$).

(A)



(B)

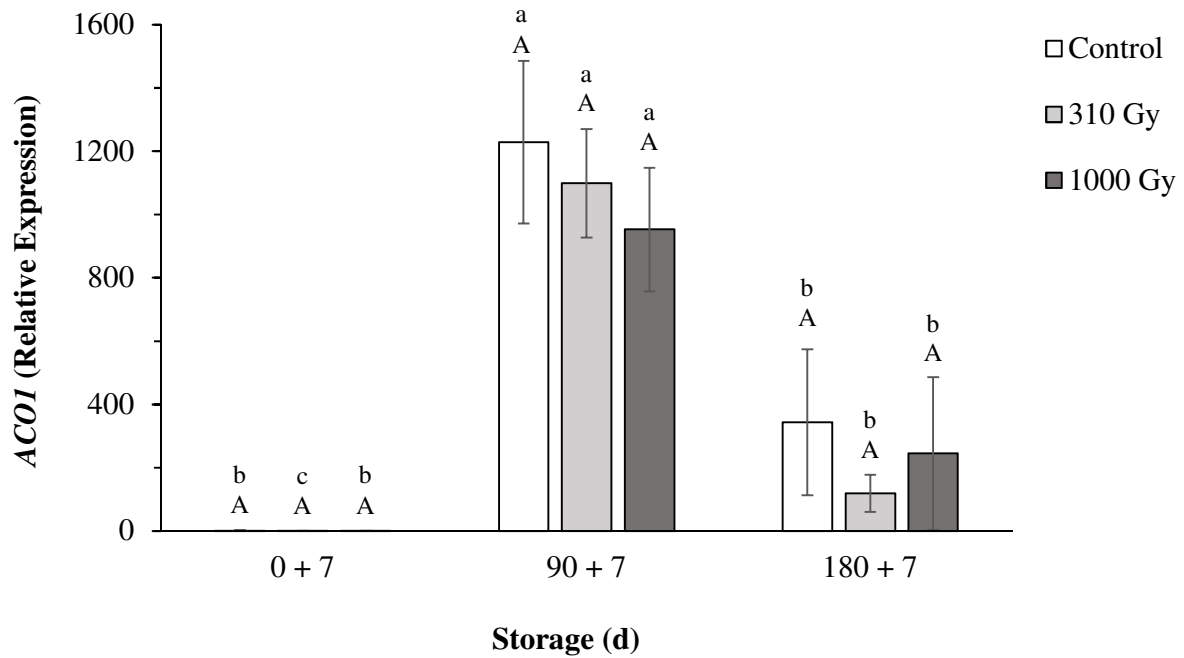


Fig. 5. Effect of irradiation on the expressions of ‘Granny Smith’ ethylene biosynthesis genes, *MdACS1* (A), and *MdACO1* (B), stored for 90 or 180 d at 0-1 °C followed by seven days at room temperature. The y-axis represents the relative fold difference of mRNA levels. Results are represented as mean values of three replicates of six fruit each and the error bars show standard deviations. The letters A, B, C show differences due to treatment on the given day and the letters a, b, c show differences during storage for any given treatment.

The peel of the control apple showed significant increase in the expression of the α -farnesene synthase gene *MdAFS1* at 90 d at 0-1 °C plus room temperature for 7 d and returned to the initial levels by 180 d at 0-1 °C plus room temperature for 7 d (Fig. 6). Both doses of irradiation significantly suppressed this burst of *MdAFS1* gene expression at 90 d at 0-1 °C plus room temperature for 7 d.

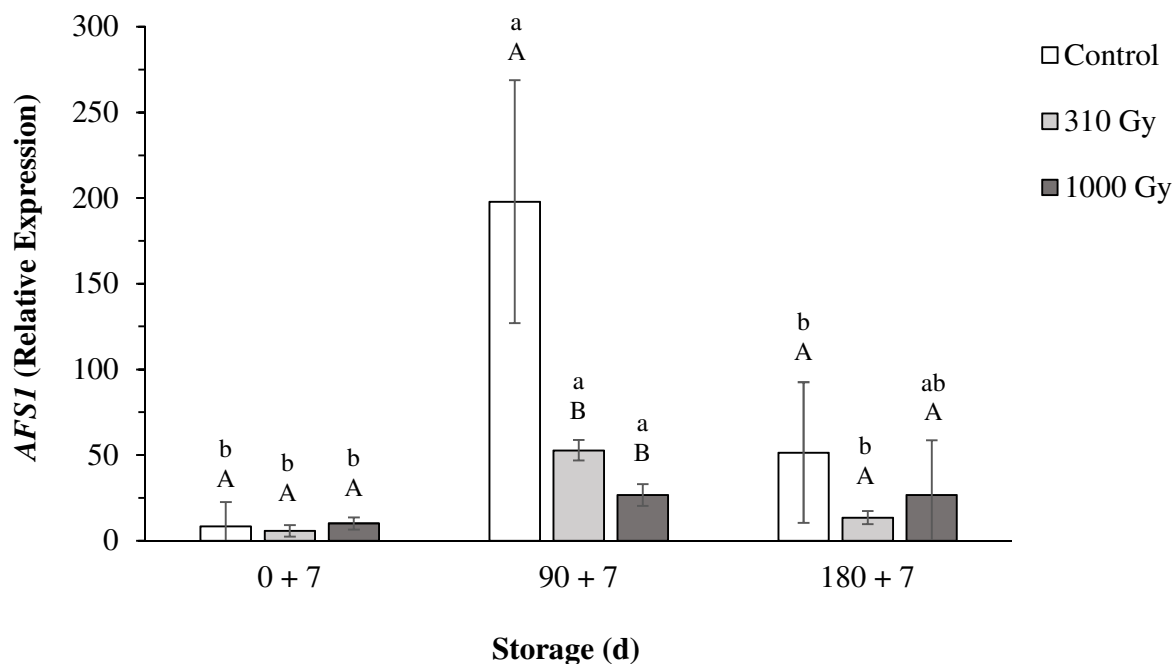


Fig. 6. Effect of irradiation on the expressions of ‘Granny Smith’ α -farnesene synthase gene, *MdAFS*, stored for 90 or 180 d at 0-1 °C followed by seven days at room temperature. The y axis represents the relative fold difference of mRNA levels. Results are represented as mean values of three replicates of six fruit each and the error bars show standard deviations. The letters A, B, C show differences due to treatment on the given day and the letters a, b, c show differences during storage for any given treatment ($P < 0.05$).

DISCUSSION

Biochemical changes associated with superficial scald

Irradiation at the doses applied in this study promoted decrease in ethylene and α -farnesene synthesis, effect that has been observed previously. Fan et al. (2001) reported a dose-dependent

decrease in ethylene produced by ‘Gala’ apples irradiated at 0.5, 1.0, and 1.5 kGy after 21 d at room temperature. Similarly, lower ethylene levels were reported in late-harvest ‘Bartlett’ pears treated at 400 Gy (Sea et al. 2015). In fruits of control stored for 90 d and 180 d at 0-1 °C followed by 7 d at 22 °C, levels of α -farnesene and CTols were lower than those found by Lurie et al. (1990) under similar storage conditions. This contrast in concentration measured in the apple skin may be due to differences in ventilation during room storage or fruit physiological maturity and ethylene synthesis and perception, both affecting the dynamics of α -farnesene biosynthesis, evaporation and oxidation.

Our results are consistent with the literature regarding the effects of irradiation on superficial scald. Al-Bachir (1999) observed that irradiation of ‘Golden Delicious’ apples at doses of 0.5, 1.0 and 1.5 kGy prevented the formation of scald. Similarly, Drake et al. (1999) reported absence of superficial scald in ‘Granny Smith’ and ‘Gala’ apples irradiated at doses up to 0.90 kGy. However, these authors did not link superficial scald incidence to ethylene production. This is the first report on the effects of ionizing radiation on the dynamics of α -farnesene and CTols metabolism in stored fruit. Our results show that the decrease of ethylene production by irradiation resulted in lower concentrations of α -farnesene and CTols in the skin of ‘Granny Smith’ apples. Ethylene regulates the synthesis of α -farnesene (Moggia et al., 2010; Ju and Curry, 2000). Ju and Curry (2000) observed that fruit producing low ethylene levels synthesized less α -farnesene and developed less scald. In our study, irradiation appeared to decrease superficial scald incidence and severity (Table 2), although the low number of apples used to monitor the incidence does not allow us to draw this conclusion definitively.

Inhibitors of ethylene perception such as 1-MCP have been shown to reduce scald incidence and α -farnesene biosynthesis in apples due to the inhibition of the expression of one isogene of the 3-

hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), a rate-limiting enzyme of the sesquiterpene biosynthesis (Rupasinghe et al., 2001). The lower respiratory rates and content of α -farnesene and oxidation products in irradiated apples after 180 d of storage, suggests that there could also be a connection between decreased respiration rate and the inhibition of α -farnesene synthesis via the mevalonate pathway. This hypothesis is supported by the findings of Rupasinghe et al. (2001), in which a 60 % inhibition in respiration by 1-MCP in apples suggests that inhibition of α -farnesene synthesis could also be mediated through respiratory regulation, with fewer C2 skeletons provided for the mevalonate pathway, and consequent lower α -farnesene biosynthesis.

Enzyme activity and gene expression

Previous studies have associated reduced ethylene production to a decrease in ACO activity in irradiated papaya fruit (D'Innocenzo and Lajolo, 2001) and 'Fuji' apples (Kheshti et al., 2019). In apples warmed for 7 days following 90 d of cold storage, a climacteric pattern was observed, but the intensity of this burst in ethylene production was attenuated by irradiation, with corresponding inhibition of ACO activity. Our results suggest that this phenomenon can be attributed to post transcriptional events as the gene expression of *MdACO1* was not altered by irradiation. Fruit treated with ethylene inhibitor 1-MCP showed reduced ACO enzyme activity similar to our results (Vilaplana et al., 2015). Pears treated with 1-MCP before cold storage also had significantly reduced ACO activity after 105 and 180 d of storage compared to untreated pears (Villalobos-Acuña et al. 2011). These authors also observed a moderated recovery of

ethylene production at 180 d of storage paralleled with an increase in ACO activity at day 180 for 1-MCP treated pears. The recovery of ACO activity and thus increase in ethylene production at day 180 following 1-MCP treatment is believed to be due to apple fruit's ability to form new ethylene receptors (Zhou et al., 2017). This phenomenon has not been observed with irradiation, as both ACO activity and ethylene production did not show a recovery between the three and six-month testing times. This may be an indication that irradiation does not affect ethylene receptors but instead, directly affects ACO enzyme activity. The observed reduction of ACO activity in irradiated apples could be a result of irradiation-induced membrane damage, consistent with the findings of ACO associated to the external face of the plasma membrane (Ramassamy et al., 1998), although this enzyme has been also reported to be localized in the cytosol of apple fruit (Chung et al., 2002). Ethylene synthesis may not be entirely membrane dependent, but regardless, its perception, that occurs via receptors in the ER membrane may be directly or indirectly affected by irradiation. Irradiation can also affect enzyme structure which in turn will affect its function.

MdACS1 gene was expressed in control and irradiated fruit stored for three and six months (Fig. 5A) and is consistent with the fact that *ACS1* expression is only observed during the late ripening stages of fruit development after a sudden increase in ethylene production that corresponds to system 2 ethylene synthesis (Varanasi et al., 2011). The reduced *MdACS1* expression by irradiation is concomitant with the decrease in ethylene production observed in irradiated fruit. *MdACS1* expression is believed to be the rate-limiting factor for climacteric ethylene biosynthesis in apples, and silencing the *MdACS1* gene in mature apple fruit significantly decreased the ethylene production (Dandekar et al., 2004). In apples treated with 1-MCP, *MdACS1* expression was inhibited completely when fruit were tested after 20 d of storage at 24

°C (Tan et al., 2013). Our results demonstrate the long-lasting effect of irradiation in suppressing *MdACS1* expression, reducing ethylene synthesis and ultimately controlling scald formation, similar to the previously observed efficacy of 1-MCP treatment in ‘Granny Smith’ apples, stored under conditions similar to this study (Fan et al., 1999).

Finally, irradiation significantly suppressed the increase in *MdAFS1* gene expression at 90 d at 0-1 °C plus room temperature for 7 d, maintaining similar levels of *MdAFS1* up to 180 + 7 d after storage. The peak of *MdAFS1* expression in control fruit after 90 d at 0-1 °C plus room temperature for 7 d (Fig. 6) is consistent with the higher ethylene levels and higher concentration of α -farnesene and CTols at that time point. At 180 d at 0-1 °C plus room temperature for 7 d, a much lower level of *MdAFS1* expression in all treatments was accompanied by the high incidence of scald symptoms (100 %) in untreated and 72 % in 310 Gy apples confirming that α -farnesene synthesis precedes further oxidation events leading to scald development. According to Pechous et al. (2005), *AFS1* gene expression can increase prior to or at the same time as α -farnesene production.

Regulation of α -farnesene synthesis depends on ethylene perception and tissue responsiveness to this hormone (Ju and Curry, 2000). Expression of α -farnesene synthase gene is inhibited by 1-MCP in apples (Tsantili et al., 2007). Our results show that suppressed expression of *MdACS1* and *MdAFS1* genes by irradiation agrees with previous findings correlating the patterns of expression of ethylene and α -farnesene biosynthetic genes in apples (Tsantili et al., 2007).

This work is the first report on the effect of irradiation downregulating genes of ethylene and α -farnesene synthesis in harvested fruit. The inhibited expression of *MdACS1* and *MdAFS1* observed in this work is consistent with the literature regarding the prevalence of downregulation over upregulation of genes in response to ionizing radiation. Hwang et al. (2014), studied

specific responses to different ionizing radiation sources on gene expression of rice, and observed that gamma radiation at 200 Gy upregulated 385 and downregulated 806 genes. In *Arabidopsis*, a total of 496 and 1042 genes were respectively up and downregulated as a result of gamma irradiation at doses of 100 Gy and 800 Gy (Kim et al., 2014).

Sensitivity of genes to irradiation is reported to depend on their base composition. For example, A-T pairs are more sensitive than C-G pairs thus genes with more A-T base pairs are more sensitive to irradiation. Also, spatial localization of the genes in the chromosome may make them more accessible to hydroxyl radicals (Lim et al., 2006). Therefore, in the same organism, some genes may be more responsive to ionizing radiation than others. Considering the complexity of events triggered by irradiation, it would be valuable to conduct a more comprehensive data analysis including transcriptomic, proteomics and metabolomics in treated apple fruit to discover sets of genes differentially expressed and elucidate metabolic pathways up or downregulated as a result of irradiation treatment.

Internal Browning

Irradiation-induced internal browning has been observed in Gala apples (Fan and Mattheis, 2001), pineapples (Jenjob et al., 2017) and mangoes (Reyes and Cisneros-Zevallos, 2007). Franck et al. (2007) hypothesized that browning disorders are a result of imbalanced oxidative and reductive processes in response to impaired gas diffusion inside the fruit, leading to deprived energy to sustain the activity of enzymes of the ascorbate-glutathione cycle, accumulating ROS, in concentrations that overwhelms the capacity of the antioxidant systems to sufficiently remove them, causing loss of membrane integrity. A further decompartmentalization allows for

polyphenoloxidase in the cytoplasm and membrane to react with soluble phenolic compounds which undergo non-enzymatic polymerization to form brown pigments. The high incidence of internal browning (89 %) observed in the 1000 Gy fruit after 90 d at 0-1 °C plus storage at room temperature for 7 d (Table 2) can be due to similar cascade of events as described above, however with ROS accumulating as a result of irradiation as the stress trigger. The higher incidence of internal browning in apples irradiated at 1000 Gy could be linked to the downregulation of *MdACO* similar to that observed by Mellidou et al. (2014) in ‘Braeburn’ apples. These authors found reduced ACO gene expression as a result of prolonged CA storage, which they attributed to a consequence of higher H₂O₂ among other factors, and suggested a role for ACO as a browning biomarker. At the lower dose, 310 Gy, the ROS produced, including H₂O₂, could be sufficiently quenched by enzymes and antioxidant compounds, preventing an imbalance in the redox homeostasis of the cell. This study shows that the ideal dose to reduce superficial scald and prevent internal browning lies between 310 and 1000 Gy. The challenge in commercial application would be to limit the dose to a narrow range.

5. CONCLUSION

Irradiation affected the expression of ethylene biosynthesis gene *MdACS1*, however it had no effect on *MdACO1*. Suppression of *MdACS1* gene expression was concomitant with decreased activity of ACO and ethylene production. Reduced ethylene production coincided with significant decrease in *MdAFS1* gene expression, resulting in lower α -farnesene and CTol concentrations. Therefore, besides its efficacy as a phytosanitary treatment, ionizing irradiation may also decrease the incidence of superficial scald, a major postharvest disorder in ‘Granny

Smith' apples. The effects on gene expression as a result of irradiation at phytosanitary doses needs further investigation to determine the mechanism by which irradiation induces changes to individual genes in stored fruit. It would also be valuable to determine if irradiation can decrease incidence of other ethylene-mediated effects, and if similar responses are evident in other fruit.

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