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Research article

# A comprehensive study on the main physiological and biochemical changes occurring during growth and on-tree ripening of two apple varieties with different postharvest behaviour

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ABSTRACT

Apple quality and the storage potential likely depend on a range of physiological and biochemical events occurring throughout fruit development and ripening. In this study, we investigated the major physiological (ethylene production and respiration) and biochemical changes (related to sugar and malic acid content as well as antioxidant metabolism) occurring during growth and on-tree ripening of two apple varieties ('Granny Smith' (GS) and 'Early Red One' (ERO)) with known differences in their postharvest behaviour, mainly firmness loss and susceptibility to superficial scald. Our results demonstrate that the higher storability and the limited loss of firmness of 'GS' fruit was associated to a higher acid content, mainly malic acid, that seemed to be regulated already at fruit set (20 DAFB). The reduced loss of firmness during storage in 'GS' was also associated to the fruit inability to produce ethylene upon harvest resulting from very low 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) activity. Sugar accumulation, on the other hand, was similar among both varieties as was also observed for the rate of fruit growth or the fruit respiration pattern. In addition, the higher susceptibility of 'GS' if compared to 'ERO' to superficial scald was not associated to peroxidative damage (malondialdehyde accumulation) nor to higher levels of the sesquiterpene α-farnesene but rather mediated by a fruit antioxidant imbalance resulting from higher $H_2O_2$ levels and lower antioxidant (peroxidase) enzymatic capacity. The interplay between ethylene, respiration and antioxidants or sugars and organic acids during apple growth and development is further discussed.

## 1. Introduction

Apple development takes over 150 days from pollination to fully ripe fruit owning a typical and well characterised simple sigmoidal growth curve common for most, if not all, apple varieties (Pratt, 1988). During on-tree ripening numerous physiological and biochemical changes occur leading to the final fruit quality at harvest as well as to the fruit postharvest behaviour. Apple cultivars largely vary in their physicochemical characteristics, in their texture as well as in their storage performance (Johnston et al., 2009; Singh et al., 2017). For instance, spring or summer cultivars (i.e. 'Gala' or 'McIntosh') are characterised by poor postharvest performance, showing fast ripening and softening, if compared to mid-late season varieties (i.e 'Golden Delicious', 'Red Delicious' or 'Granny Smith'). Thus said, differences also exist when comparing mid-late season varieties, such as the ones used in this study, since some cultivars will need cold storage to initiate ripening or initiate its autocatalytic ethylene production (i.e. 'Granny Smith'; Larrigaudière and Vendrell, 1993; Lara and Vendrell, 2003) while most other cultivars ('Red Delicious', 'Golden Delicious'; Tong et al., 2016) will immediately do so following harvest. Like 'Granny Smith' apples, most cultivated European pear varieties own varying degrees of resistance to ripening even when harvested at the appropriate maturity and a postharvest chilling period is often required to induce ripening (Villalobos-Acuña and Mitcham, 2008).

Whether such differences are strictly regulated by ethylene itself (Singh et al., 2017) or related to specific changes occurring during fruit development, is somehow unclear. The role that ethylene plays in fruit development and its relationship with the fruit postharvest behaviour remains to be elucidated. It is generally recognized that the climacteric process takes place through the consecutive induction of two ethylene-

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producing systems referred as System 1 and System 2 (El-Sharkawy et al., 2004). System 1 is non-autocatalytic and operates in immature fruit whereas System 2 operates during ripening to induce autocatalytic ethylene production and the climacteric burst observed in climacteric fruit (reviewed in Pech et al., 2012). In apples, as typical climacteric fruit, the increase in ethylene production at the latest stages of ripening is accompanied by an increase in the fruit respiratory activity (Busatto et al., 2017). In turn, numerous metabolic processes are altered by fruit respiration, including chloroplastic, mitochondrial and plasma membrane-linked electron transport chains leading to the production of reactive oxygen species (ROS) such as $H_2O_2$ and $O_2^-$ (Apel and Hirt, 2004; Foyer and LelandaisKunert, 1994). Under normal physiological conditions, oxidative damage may be curtailed by antioxidant defences that scavenge or prevent the generation of ROS, as well as repair or degrade the oxidized molecules (Jamieson, 1998). Thus said, an inappropriate antioxidant system within the fruit during on-tree ripening may contribute to the development of oxidative-mediated postharvest physiological disorders (i.e. superficial scald). In accordance, previous research have shown that exogenous applications of antioxidants reduce the incidence of oxidative-stress mediated disorders in apples and pears (Jung and Watkins, 2008; Mattheis and Rudell, 2008) and other fruit (i.e. green pepper; Purvis, 2002), hence corroborating the importance of the fruit antioxidant defences to sustain a good storage potential.

To date, no other studies have investigated and compared the growing and ripening-related events between different apple varieties and its relationship with the final fruit quality and the postharvest behaviour (softening or susceptibility to superficial scald). Accordingly, this study aimed to determine if the existing differences in quality and superficial scald incidence between 'Granny Smith' and 'Early Red One' apples upon storage are triggered by specific biochemical and metabolic changes occurring during on-tree growth and ripening.

## 2. Materials and methods

### 2.1. Plant material, storage protocol and standard quality evaluations

'Early Red One' (ERO) and 'Granny Smith' (GS) apples (30 fruit per replicate and 6 replicates from at least 3 trees per variety) were picked at different developmental stages from commercial orchards in Torregrossa (Lleida, NE Spain). The stages of fruit development (S1 to S6; Fig. 4) were based on days after full bloom (DAFB), being full bloom defined as the time when over 50% of the flowers were fully open. After each harvest, apples were immediately transported to the laboratory, under acclimatised conditions (20 °C) and reaching the laboratory in less than 30 min. Upon arrival at the laboratory, 20 fruit per replicate were used for $CO_2$ and ethylene measurements whereas the remaining 10 fruit were immediately snap-frozen with liquid nitrogen and kept at −80 °C or immediately used for biochemical measurements. Fruit weight, firmness (Effegi penetrometer FT 327), diameter, colour (portable spectrophotometer CM-2600d; Konica Minolta Sensing, Japan) and DA-value (DA-meter; Turoni, Italy) were measured on 20 individual fruit per replicate. Standard quality parameters, including total soluble solids content (TSS; %) and fruit acidity (g malic 100 g fruit), were measured in the juice obtained from 5 individual fruit (n = 4 per replicate) as described elsewhere (Giné-Bordonaba et al., 2016).

At commercial harvest, an additional 180 fruit per each variety (30 fruit per replicate) was harvested and stored at 0.5 °C (95% RH) during four months. After this period, superficial scald incidence and severity was determined on 120 fruit after 0 and 7days of storage at 20 °C as described elsewhere (Giné-Bordonaba et al., 2013). Quality changes upon removal from cold storage (same parameters as described above) were also determined after 0, 7 and 10 days of storage at 20 °C on 30 individual fruit for each variety.

### 2.2. Fruit ethylene production, respiration and ACO activity

Fruit respiration (mg $CO_2$ $kg^{-1}$ $h^{-1}$ or mg $CO_2$ $apple^{-1}$ $h^{-1}$) and ethylene production (μL $g^{-1}$ $h^{-1}$ or μL $apple^{-1}$ $h^{-1}$) were measured on a standard weight basis ($kg^{-1}$) or on a fruit basis ($apple^{-1}$) on fruit kept in an acclimatised chamber at 20 °C. After each sampling point, apples were placed within sealed flasks equipped with a silicon septum for sampling the gas of the headspace after 2 h incubation. Gas samples (1 mL) were taken daily from the headspace and injected into a gas chromatograph fitted with a FID detector (Agilent Technologies 6890, Wilmington, Germany) and an alumina column 80/100 (2 m × 3 mm) (Teknokroma, Barcelona, Spain) as previously described (Giné Bordonaba et al., 2014). Fruit respiration was determined by quantifying the $CO_2$ concentration within the flask with an $O_2/CO_2$ gas analyser (CheckPoint $O_2/CO_2$, PBI Dansensor, Ringsted, Denmark).

1-aminocyclopropane-1-carboxylic acid oxidase (ACO) activity was extracted as described by Chiriboga et al. (2013) and the enzyme activity analysed mixing 400 μl aliquot of the enzyme extract with 50 mM MOPS reaction buffer pH 7.2, 10% glycerol, 5 mM ascorbic acid sodium salt, 20 mM sodium bicarbonate, 0.02 mM iron sulphate, 1 mM ACC and 1 mM DTT. The mixture was aired and incubated for 60 min at 30 °C, after which a 1 mL headspace gas sample was injected into a gas chromatograph and the results were expressed as nmol $C_2H_4$ $g^{-1}$ $h^{-1}$ or nmol $C_2H_4$ $apple^{-1}$ $h^{-1}$.

### 2.3. Determination of fruit malate and sugar content

Extracts for malate determination were prepared as described in Giné-Bordonaba and Terry (2010) with some modifications. Briefly, fresh frozen fruit tissue (2 g) was added to 5 mL of HPLC-grade water. Samples were kept at room temperature (25 °C) for 10 min and then centrifuged at 24,000 × *g* for 7 min at 20 °C. Glucose and fructose were extracted from fresh-frozen material as described elsewhere (Terry et al., 2007). Briefly, 2 g of sample were dissolved in 5 mL of 62.5% (v/v) aqueous methanol solvent and placed in a thermostatic bath at 55 °C for 15 min, mixing the solution with a vortex every 5 min to prevent layering. Then, samples were centrifuged as described above. The supernatant from each extraction was recovered and used for enzyme-coupled spectrophotometric determination of malate (L-malate dehydrogenase) and glucose and fructose (hexokinase/phosphoglucose isomerase) as described by Giné-Bordonaba et al. (2017) using commercial kits (BioSystems S.A., Barcelona, Spain) and following the manufacturer instructions.

### 2.4. Determination of malondialdehyde and $H_2O_2$ content

Malondialdehyde (MDA) was quantified in fruit as an index of lipid peroxidation using the thiobarbituric acid reactive substrates (TBARS) assay as described elsewhere (Martínez-Solano et al., 2005). Briefly, frozen fruit tissue (0.5 g) was homogenized in 4 mL of 0.1% trichloroacetic acid (TCA) solution. The homogenate was then centrifuged at 18,800 × *g* for 20 min and 0.5 mL of the supernatant was added to 1.5 mL 0.5% thiobarbituric acid (TBA) in 20% TCA. A second aliquot (0.5 mL) of the supernatant was added to a mixture containing only 20% TCA as a control. The mixture was incubated at 90 °C for 30 min until stopped by placing the reaction tubes in an ice-water bath. Samples were then centrifuged at 18,800 × *g* for 10 min at 4 °C, and the absorbance of the supernatant was read at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The amount of MDA-TBA complex (red pigment) was calculated using the extinction coefficient 155 $mM^{-1}$ $cm^{-1}$ and the results expressed as nmol $g^{-1}$ $h^{-1}$ or nmol $apple^{-1}$ $h^{-1}$.

To determine $H_2O_2$ levels, 2.5 g of fresh frozen fruit tissue were homogenized in 10 mL of 5% trichloroacetic acid, filtered through two layers of Miracloth (Textil Planas Oliverassa, Manresa, Spain) and centrifuged at 20,000 × *g* for 15 min at 4 °C. Quantification of $H_2O_2$ was

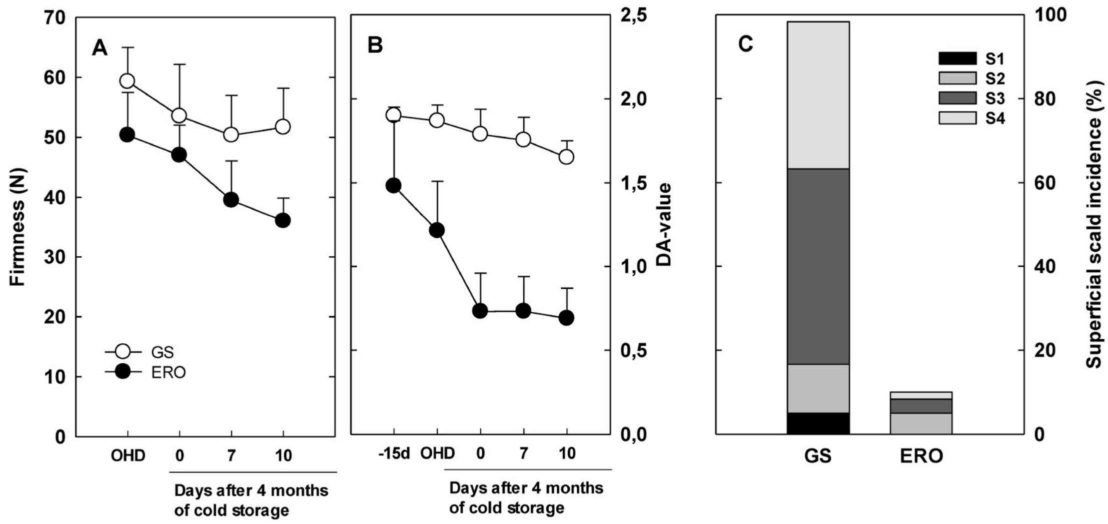


**Fig. 1.** Fruit firmness (A) and DA-value (B) at harvest (OHD: optimal harvest date), 15 days earlier and during postharvest ripening of 'Granny Smith; GS' and 'Early Red One; ERO' fruit after 4 months of cold storage. (C) Superficial scald incidence and severity in 'GS' and 'ERO' fruit after 4 months of cold storage.

determined using the Bioxytech $H_2O_2$-560 (OXIS International Inc., Portland, OR USA) colorimetric assay following the manufacturer's instructions as described elsewhere (Giné-Bordonaba et al., 2017).

### 2.5. *Fruit antioxidant capacity, total phenolic content and enzymatic antioxidants*

Total phenolic concentrations and antioxidant capacity of the apples through development were quantified from freeze-dried material as described earlier (Giné Bordonaba and Terry, 2008) by mixing 50 mg of freeze-dried fruit sample with 1.5 mL of 79.5% (v/v) methanol and 0.5% (v/v) HCl in HPLC-grade water. Sample extraction was held at 25 °C with constant shaking for 2 h and mixing the samples every 15 min (Giné Bordonaba and Terry, 2016). From the same extract, total phenolic compounds (mg gallic acid equivalents (GAE) $g^{-1}$ FW or mg GAE $apple^{-1}$) were measured by means of the Folin-Ciocalteu method and total antioxidant capacity (mg $Fe^{2+}$ per $g^{-1}$ FW or $apple^{-1}$) measured by the Ferric Reducing Antioxidant Power (FRAP) assay as described in recent works (Giné Bordonaba and Terry, 2016).

Total peroxidase (POX, EC 1.11.1.7) extractions were carried out as described in Giné-Bordonaba et al. (2017) based on the protocols previously reported by Lurie et al. (1997) and Vilaplana et al. (2006) and using fresh-frozen fruit.

### 2.6. *Determination of cell wall-modifying enzyme activities*

Pectin methyl esterase (PME; EC 3.1.1.11) enzyme was extracted using the method described by Plaza et al. (2003). PME was extracted by homogenisation of 2 g of frozen ground sample with 6 mL of an extraction solution (1 M NaCl in 0.2 M sodium phosphate buffer pH 7.5). The resulting mixture was shaken for 10 min at 4 °C, centrifuged at 16,000 × *g* for 20 min at 4 °C and then the supernatant filtered through six cheesecloth layers. Finally, PME activity from the resulting extract was quantified by titration as described elsewhere (Yeom et al., 2000). The PME activity unit (AU) was expressed as the amount of enzyme necessary to release 1 μmol galacturonic acid $min^{-1}$ $g^{-1}$ fresh weight.

Polygalacturonase (exo-PG; EC 3.2.1.67 and endo-PG; EC 3.2.1.15) extraction and determination was conducted by following the methods described by Van Linden et al. (2008) with some modifications. PG activity unit (AU) was calculated as the release of reducing groups per unit of time and per fresh weight (μmol $min^{-1}$ $g^{-1}$ FW) based on the two reaction periods as described in Giné-Bordonaba et al. (2017).

### 2.7. *Data analysis*

All data, except that referring to antioxidant enzymes activity, is presented both in terms of standard concentrations (i.e. mg of the analyte $g^{-1}$) and per fruit basis (i.e. mg of the analyte $apple^{-1}$) aiming to understand the net assimilation of the target compounds without considering the increase in fruit volume occurring during fruit growth. In all cases, data were subjected to analysis of variance (ANOVA) tests using JMP 8.0.1 SAS Institute Inc. Least significant difference values (LSD; $P \leq 0.05$) were calculated for mean separation using critical values of t for two-tailed tests. Correlations between experimental variables were made using Spearman's Rank Correlations and, if required, presented as Spearman's Correlation Coefficient (r) and P value based on a two-tailed test. Unless otherwise stated, significant differences were $P \leq 0.05$.

## 3. Results and discussion

### 3.1. *Quality characteristics at harvest and postharvest behaviour*

Fruit firmness along with total soluble solid and the starch content are among the main quality parameters used by apple growers to determine the optimum harvest date. In this work, fruit firmness at the time of commercial harvest was similar for both apple varieties being slightly higher in 'GS' (59.8 N) than in 'ERO' (51.2 N) fruit (Fig. 1). The higher firmness observed in 'GS' at the time of harvest was accompanied by lower TSS (1.14-fold) and higher acidity (2.2-fold) if compared to 'ERO'. The starch content, determined by the iodine staining method, and which may be a good indicator for the fruit physiological maturity stage, was also similar for both varieties (6.1 ± 1.12 in 'GS' and 7.45 ± 1.9 in 'ERO'; data not shown). Overall, quality parameters for both varieties at the time of harvest were within the standards used by growers in the region of Lleida (Lara and Vendrell, 2003; Villatoro et al., 2008) and show that both cultivars were harvested at similar physiological maturity stages.

Both apple varieties experienced little or no firmness loss during cold storage (up to 4 months) yet differences between varieties appeared upon removal from cold storage and ripening at 20 °C. In this case, firmness loss gradually decreased in 'ERO' fruit (−1.3 N/day) while it remained fairly constant in 'GS'. In this later variety the lack of firmness loss during cold storage or shelf-life was mimicked by no significant changes in the $I_{AD}$ values for the same period (changing from 1.78 to 1.65) and thereby indicating a better storage potential of this

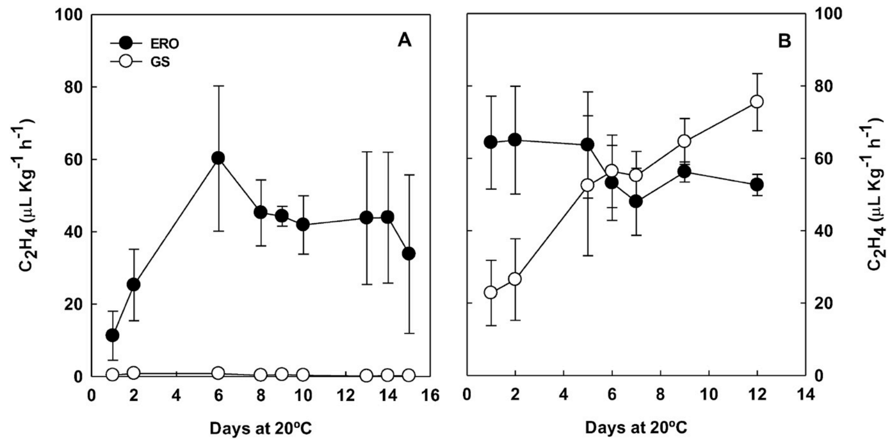


**Fig. 2.** Ethylene production (μL Kg$^{-1}$ h$^{-1}$) of two different apple (Granny Smith (GS: ○) and Early Red One (ERO: ●)) varieties during storage at 20 °C immediately after harvest (A) or after 4 months of cold storage (0.5 °C; 95% RH; B). Values represent the mean ± stdev (n = 3).

variety in terms of limited softening and ripening. In contrast, the decrease in $I_{AD}$ values in 'ERO' indicated that this variety, albeit not losing firmness, ripens to some extent during cold storage (1.7-fold lower values upon removal from cold storage than at harvest) as was also observed during the last stages of on-tree development (Fig. 1).

While fruit quality (firmness and acid content) was better maintained in 'GS' than in 'ERO' during cold storage and further shelf-life at 20 °C, a very high incidence of superficial scald was observed in 'GS' fruit (98%) if compared to 'ERO' (12% incidence; Fig. 1). This result is not surprising since 'GS' fruit are generally referred as very susceptible to this physiological disorder (Giné-Bordonaba et al., 2013). Thus said, α-farnesene content at or prior to harvest, a compound intimately related to superficial scald development (Giné-Bordonaba et al., 2013), was greater in 'ERO' than in 'GS' fruit (Supplementary Figure 1).

After harvest, a typical climacteric ethylene production pattern was observed in 'ERO' fruit, with a peak in ethylene production (62 μL kg$^{-1}$ h$^{-1}$) occurring after 6 days of storage at 20 °C and slightly declining thereafter. In contrast, no ethylene peak and basal levels of this hormone were observed in 'GS' fruit stored at 20 °C following harvest (Fig. 2). In agreement with these results, it is well documented that 'GS' fruit, if compared to other apple varieties (Tong et al., 2016), and similarly to many European pear varieties (Villalobos-Acuña Mitcham, 2008), requires cold storage to initiate its autocatalytic ethylene production (Larrigaudière and Vendrell, 1993; Lara and Vendrell, 2003). This specific behaviour of 'GS' apples is not strictly related to the fruit maturity stage at the time of harvest since fruit harvested at starch indexes close to 8 also fail to produce ethylene when placed at 20 °C immediately following harvest (Giné Bordonaba and Larrigaudière, unpublished).

Upon removal, differences in the ethylene production pattern between both varieties remained noticeable, highlighting a typical climacteric behaviour (increase in ethylene production) in 'GS' and a post-climacteric behaviour (no increase and even slight decrease) in 'ERO' fruit. This result further confirm the data from the $I_{AD}$ values and clearly reflect that 'ERO' apples ripen during cold storage.

Overall, our results demonstrate that quality traits at harvest but mainly during postharvest storage and further shelf-life were clearly distinct among the studied varieties. Whether such differences are related to the specific growth pattern or some physiological events occurring during the fruit development of each variety, is analysed in the following sections.

### *3.2. Morphological and quality changes during fruit growth*

Notwithstanding the observed differences in fruit quality at the time of harvest or the different postharvest behaviour, both apple varieties showed a similar growth pattern (typical sigmoidal growth curve) and hence in agreement with that reported in earlier studies (Pratt, 1988; Whale and Singh, 2007). In both apple varieties the period of maximal growth rate was from 90 to 120 DAFB (2.3 g day$^{-1}$; Fig. 3). Other authors have described apple growth either as curvilinear in the initial stages (up to 35 DAFB) followed by a steady linear increase until the time of harvest (Assaf et al., 1982) or expolinear (Lakso et al., 1995), depending on the cultivar or the agro-climatic conditions being tested. In any case, our data confirm for both apple varieties three clearly differentiated growth phases being: (I) a period of limited growth (up to 40–50 DAFB) likely attributed to a period of rapid cell division, (II) a period of fast growth rate generally referred to the period of cell elongation and enlargement (Austin et al., 1999; from 50 to 150 DAFB), and (III) a short period of fruit maturation where fruit growth does no longer occur (from 150 to 175 DAFB). In other apple cultivars (i.e. the summer cultivar 'Gala'), fruit growth was arrested much earlier, at 90–100 DAFB, hence up to 40 days prior to commercial harvest (Goulao et al., 2007).

For both varieties, fruit firmness was maximal at 90 DAFB, when the fruit had reached only 25–30% of its final fruit size (Fig. 3) and declined thereafter thereby in contrast to other fruit such as plums, peaches, dates or loquats where the loss of fruit firmness is initiated at later developmental stages and generally when the fruit is no longer growing (Serrano et al., 2001; Amorós et al., 2003; Zuzunaga et al., 2001). The loss of firmness during the last stages of apple development and ripening have been associated with the solubilisation of pectins through a complex and coordinated action of several cell wall modifying enzymes (Goulao et al., 2007). Our data on PG or PME activities, either in absolute concentrations or on a fruit basis (Supplementary Figure 2), did not support this idea since the loss of firmness from 90 DAFB onwards was not consistently paralleled by higher enzyme activities in any of the varieties investigated.

The fruit acid content (g malic g$^{-1}$) steadily declined for both varieties from 60 DAFB to harvest, whereas total soluble solids (TSS) content remained fairly unchanged until 120 DAFB and increased later on (Fig. 3). The increase in TSS was especially noticeable in 'ERO' fruit (1.35-fold higher at 160 DAFB than at 120 DAFB) if compared to 'GS' (1.19-fold) and agrees with that reported for several apple varieties (Villatoro et al., 2008; Molina-Delgado et al., 2009; Ortiz et al., 2011). It is important to remark the sound differences in the acid content between both varieties being consistently 2.5-fold higher in 'GS' than in 'ERO' throughout development. The involvement of the fruit acidity on the storage potential of different apples was already discussed in an old paper (Plagge and Gerhardt, 1930), yet it was never since addressed in detail. Data from earlier studies suggest a strong negative correlation between the rate of firmness loss observed during cold storage and the

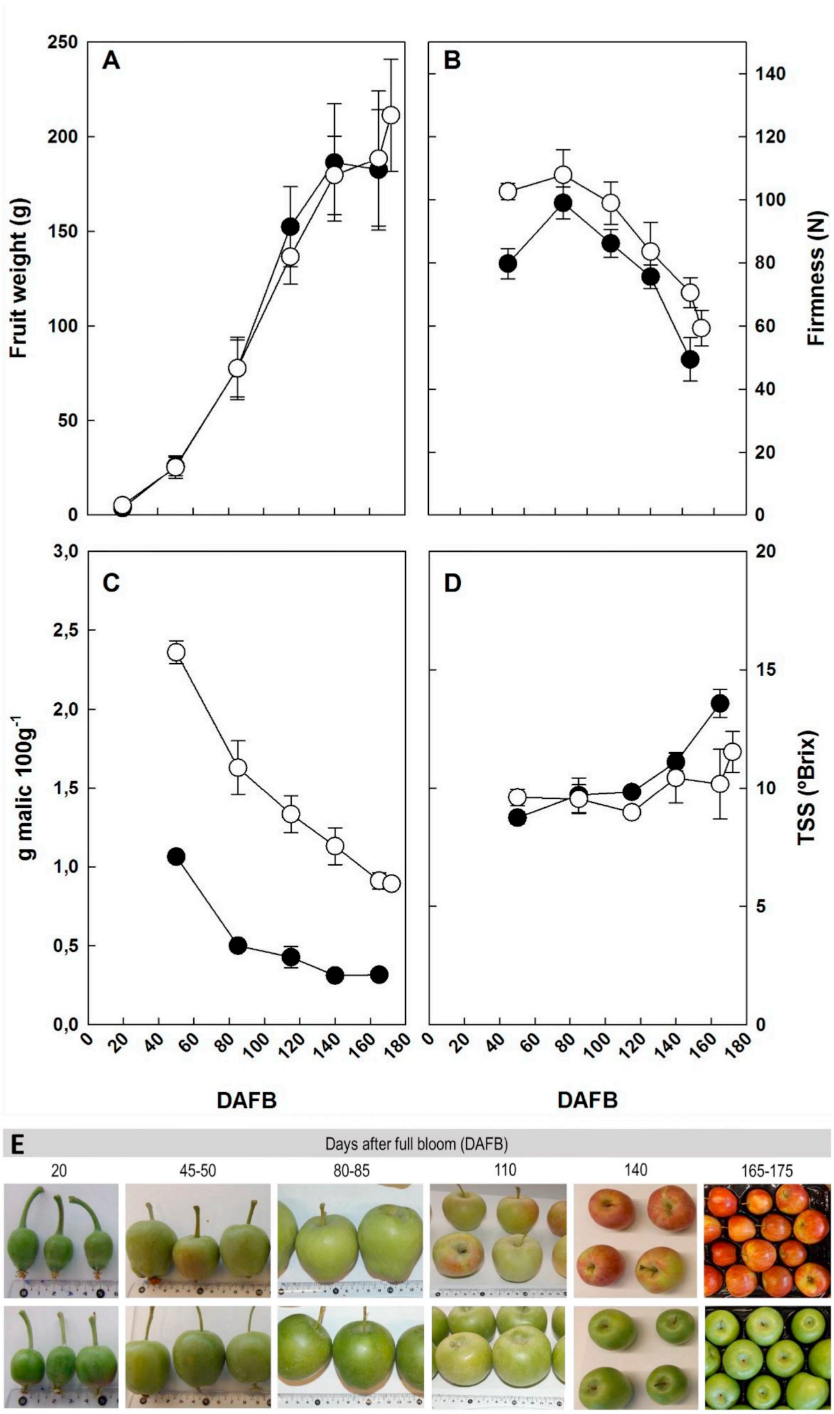


**Fig. 3.** Morphological (fruit weight; A) and quality changes (B: Fruit firmness; C: Titratable acidity; D: Total soluble solids) during growth and ripening of two different apple (Granny Smith (GS: ○) and Early Red One (ERO: ●)) varieties. Values represent the mean ± stdev (n = 6). LSD values ($P < 0.05$) for the interaction cultivar*sampling point in figures A, B, C and D were 11.50, 3.18, 0.14 and 1.39, respectively. (E) Image of the different phenological stages corresponding to each sampling point (Days after full bloom (DAFB) are given for 'Early Red One; ERO' and 'Granny Smith; GS', respectively). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

acid content of 'GS' apples from seven different orchards ($R^2 = 0.67$; $P < 0.05$; Giné Bordonaba and Larrigaudière, unpublished). Therefore, it is likely that the higher acid content observed in 'GS' may account, in part, for the good storage performance, in terms of firmness maintenance, of this apple variety (aside the high superficial scald susceptibility). However, future studies are encouraged to confirm this relationship in other apple cultivars.

### 3.3. Respiration pattern and ethylene production during fruit growth

Fruit respiration, as determined by the amount of $CO_2$ released per $Kg^{-1}\ h^{-1}$, of both varieties was maximal at 20 DAFB and decreased up to 110 DAFB with little changes thereafter (Fig. 4). As observed in plums (Famiani et al., 2012) or peaches (Famiani et al., 2016), it is probable that this decrease results from the higher ratio of the vacuole to the cytoplasm of the pericarp cells during growth since the vacuole is the actual site of $CO_2$ release during respiration (Famiani et al., 2016). The changes in the amount of $CO_2$ released on a fruit basis (μL per $fruit^{-1}\ h^{-1}$), were, however, completely different, showing an initial peak of $CO_2$ released per fruit around 40 and 80 DAFB for 'ERO' and 'GS', respectively, followed by a sudden decline and then a second peak occurring close to the time of commercial fruit harvest. The pattern of $CO_2$ released on a fruit basis was similar between the two varieties but different to that shown in other fruit such as peaches (Famiani et al., 2016), cherries (Giné-Bordonaba et al., 2017) or grapes (Famiani et al., 2014) where $CO_2$ production tend to constantly increase during fruit ripening.

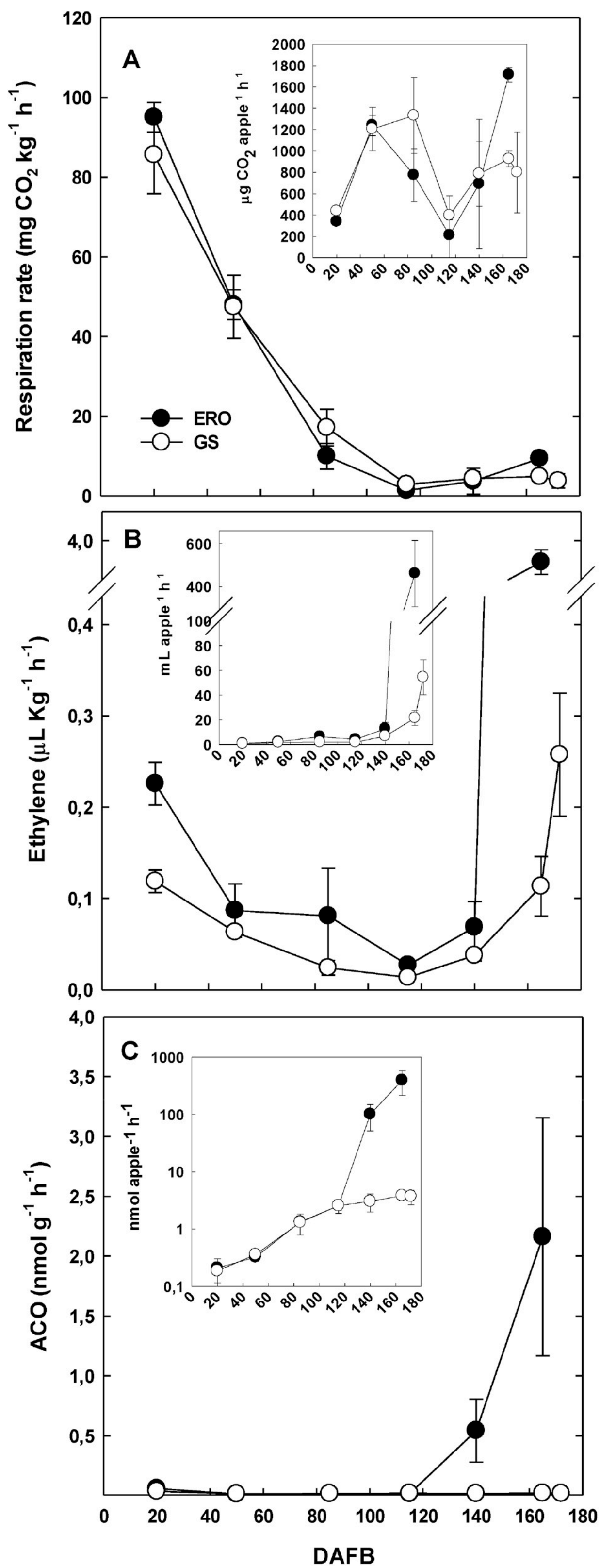


**Fig. 4.** Dynamicchanges in fruit respiration (A), ethylene production (B) and 1-Aminocyclopropane-1-carboxylic acid oxidase (ACO; C) during growth and ripening of two different apple (Granny Smith (GS: ○) and Early Red One (ERO: ●)) varieties. Values represent the mean ± stdev (n = 6). Inserts in each graph show the results on a fruit basis. LSD values ($P < 0.05$) for the interaction cultivar*sampling point in figures A, B and C were 7.451, 0.218 and 0.512, respectively.

Ethylene production, on a concentration basis (μL kg$^{-1}$ h$^{-1}$), was also higher at earlier fruit developmental stages showing a steady decrease in both varieties until 90 or 110 DAFB (basal ethylene levels values) followed by a sharp increase thereafter. The peak in ethylene production at later developmental stages for both species occurred later than the observed changes in firmness loss (Fig. 4); thereby indicating that non-ethylene dependent fruit softening occurs already during on-tree apple ripening. As pointed out earlier, the observed on-tree fruit softening was neither mediated by the activity of PG or PME (Supplementary Figure 2). A similar ethylene production pattern during development of different apple and pear varieties has already been reported (Walsh and Solomos, 1987; Dal Cin et al., 2007; Whale and Singh, 2007) demonstrating that ethylene production is greater at the development stages of cell division (up to 50 DAFB) and later on prior to commercial harvest. Such increase in the ethylene production towards the time of commercial harvest was especially noticeable in 'ERO' fruit being 11-fold greater than in 'GS' apples. The higher ethylene production in 'ERO' was, in turn, coupled to a drastic activation of 1-aminocyclopropane-1-carboxylic acid oxidase (ACO), the enzyme responsible for the synthesis of ethylene from ACC. In contrast, in 'GS' apples ACO activity remained unchanged from 50 DAFB to the time of harvest.

On a fruit basis (μL fruit$^{-1}$ h$^{-1}$), ethylene production remained very low for both varieties during the whole growing period and peaked just prior to the time of harvest. The burst in ethylene production in 'ERO' fruit prior to harvest agrees with earlier studies in that anthocyanin accumulation during apple ripening is triggered by ethylene (Faragher and Brohier, 1984). Indeed, anthocyanin accumulation during apple growth owns two well-differentiated peaks; the first occurring in young fruitlets during cell expansion and the second one during ripening prior to commercial harvest (Saure, 1990), both peaks coinciding with periods of high ethylene production (Fig. 4).

Generally, our results demonstrate that ethylene and respiratory metabolism was similar during the growth and development of both varieties except at the time of fruit ripening. The burst in ethylene production and the higher activation of ACO observed in 'ERO' fruit if compared to 'GS' clearly explain the different ethylene production capacity and ripening behaviour (changes in $I_{AD}$ and softening) upon harvest (Fig. 2). Our results also point out that other hormones (i.e. ABA, giberillins) or its crosstalk are likely responsible for the ethylene inhibition via inactivation of ACO observed in 'GS' fruit prior to harvest. In addition, the lack of ethylene at the time of harvest in 'GS' also explains the lower α-farnesene content (Supplementary Figure 1) in this variety since ethylene promotes the enzymatic synthesis of this compound via AFS1 (α-farnesene synthase 1; Tsantili et al., 2007). Indeed, in the later study the authors found that changes in the expression patterns of the α-farnesene synthase gene *MdAFS1*, the ethylene receptor gene *MdERS1*, and the ethylene biosynthetic genes *MdACS1* and *MdACO1* were highly related to the observed patterns of α-farnesene accumulation and ethylene production.

### *3.4. Changes in sugar and organic acid content during fruit growth*

In most cultivated apple varieties, sucrose and fructose are the predominant sugars followed closely by glucose and other minor sugars such as sorbitol (Doerflinger et al., 2015; Ma et al., 2015; Jing et al., 2016). Sugar accumulation during the growth and ripening of both cultivars (Fig. 5) followed similar kinetics both on a concentration (mg g$^{-1}$) or fruit basis and with values slightly higher in 'ERO' than in 'GS' fruit. The concentration of monosaccharides (glucose + fructose; Fig. 5) increased mainly during the periods of slower growth rate from 20 to 90 DAFB, and then from 140 DAFB to harvest, whereas sucrose concentration (Fig. 5) remained relatively unchanged until 120 DAFB and sharply increased thereafter showing a positive and strong correlation ($r^2 = 0.83$; $P < 0.01$) with the changes observed in the fruit TSS content (Fig. 3). On a fruit basis, changes in monosaccharides and

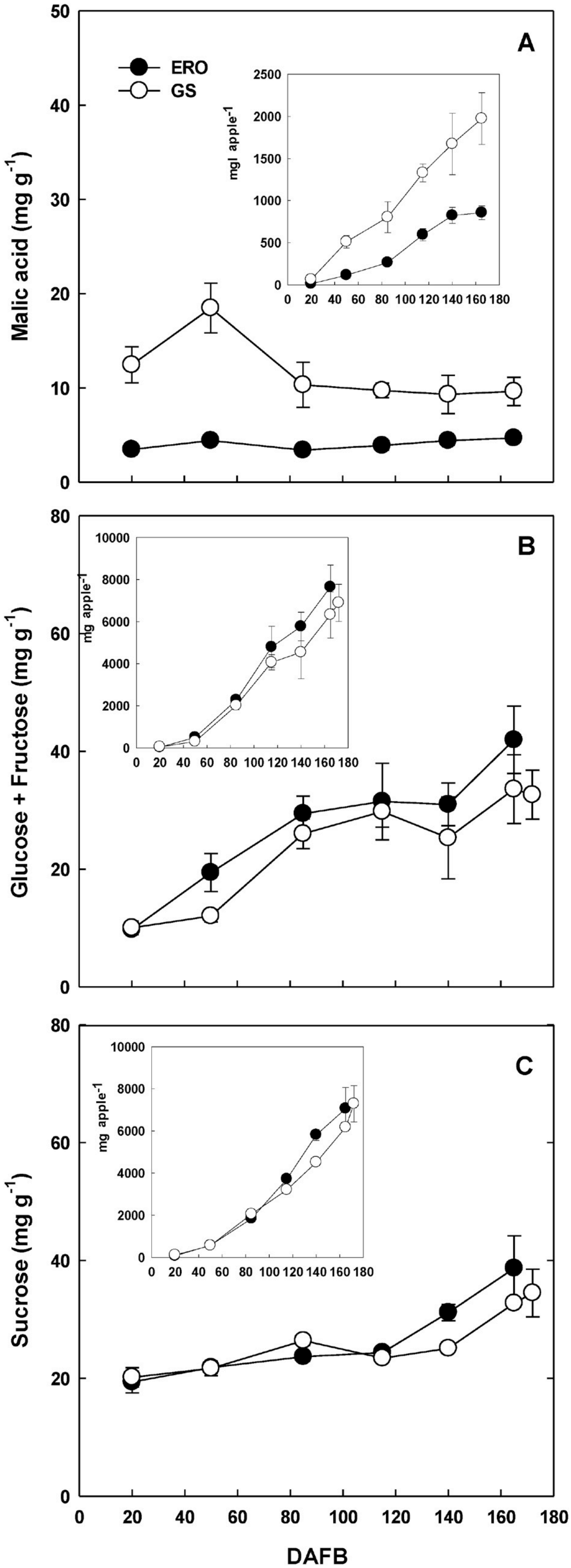


**Fig. 5.** Changes in the concentration (mg $g^{-1}$ FW) of malate (A), glucose + fructose (B) and sucrose (C) during growth and ripening of two different apple (Granny Smith (GS: ○) and Early Red One (ERO: ●)) varieties. Values represent the mean ± stdev (n = 6). Inserts in each graph depict the temporal changes of each parameter on a fruit basis. LSD values ($P < 0.05$) for the interaction cultivar*sampling point in figures A, B and C were 2.506, 6.704 and 3.284, respectively.

sucrose were well correlated ($r^2 = 0.98$; $P < 0.01$) and maximal sugar accumulation within the fruit occurred during the period of maximum fruit growth, hence suggesting that the faster fruit growth was accompanied by a faster mobilization of assimilates from source to sink tissues. For both apple varieties strong negative correlations were observed between the fruit respiration pattern and the content of monosaccharides (glucose + fructose) and sucrose (Supplementary Figure 3) highlighting the role of these compounds as important respiratory substrates in apple.

Malic acid is by far the main organic acid present in ripe apple fruit (Suni et al., 2000; Jing et al., 2016) and its concentration is known to drastically vary among different apple cultivars (Suni et al., 2000). Malic acid, as well as most organic acids present in the apple flesh, are not imported but rather synthesised from imported sugars (Famiani et al., 2012). Accordingly, in 'GS' but not in 'ERO', negative correlations were found between malic acid and sugar content throughout fruit development (Supplementary Figure 3). The concentration (mg $g^{-1}$) of this compound remained relatively unchanged during the growth of 'ERO' fruit (24–36 mg $g^{-1}$) whereas a peak at 50 DAFB was observed in 'GS' apples (*ca.* 145 mg $g^{-1}$) declining to constant levels (*ca.* 70 mg $g^{-1}$) thereafter and until the time of harvest. These results are in agreement with those reported for other apple varieties (i.e. 'Golden Delicious'; Jing et al., 2016) in which malic acid remained relatively unchanged from 90 DAFB to fully ripe fruit. On a fruit basis (mg $apple^{-1}$), however, the content of malate steadily increased during growth and ripening of both varieties being always 2 to 3-fold higher in 'GS' than in 'ERO' fruit (Fig. 5). In grapes, malic acid is thought to be an important respiratory substrate (Famiani et al., 2014) and postharvest studies on apples also pointed out the importance of this compound in fruit respiration (Liu et al., 2016). In accordance to that recently reported in peaches (Famiani et al., 2016) and cherries (Giné-Bordonaba et al., 2017), our results indicate that the amount of malate accumulated during fruit growth, may contribute little or nothing to the net substrate requirements of apple metabolism since this compound was constantly synthesised rather than degraded throughout fruit development and on-tree ripening.

### *3.5. Changes in oxidative stress markers and antioxidants during fruit ripening*

It is generally recognized that $H_2O_2$ at low concentrations may act as a messenger molecule involved in adaptive responses whereas higher concentrations of this compound may lead to programmed cell death. In this work, $H_2O_2$ concentrations differently changed during the fruit growth of both cultivars. In 'ERO', $H_2O_2$ concentration (nmol $g^{-1}$ FW) remained fairly constant throughout development (ca. 80 nmol $g^{-1}$ FW) whereas two clear peaks from 40 to 80 DAFB and prior to commercial harvest were observed in 'GS' fruit. Theoretically, the higher $H_2O_2$ levels observed in 'GS' together with higher amounts of malate might point out a higher mitochondrial function for this apple variety. Thus said, fruit respiration in 'GS' was not substantially different to that of 'ERO' and hence it is unlikely that the burst of $H_2O_2$ is related to an overfunctioning of the mitochondrial machinery but rather to the inability of the fruit to scavenge this compound. Indeed, when analysed on a fruit basis, $H_2O_2$ content (nmol $apple^{-1}$) constantly increased during fruit development and ripening especially in 'GS' fruit, a result that was consistent with the significant inhibition of POX activity observed in this cultivar during all the growing phase (Fig. 6).

Whether the higher levels of $H_2O_2$ detected in 'GS' may be associated to superficial scald susceptibility (Fig. 1) is still debatable but it is widely accepted that increased $H_2O_2$ levels create oxidative stress leading to a diversity of physiological damages (Wang and Jiao, 2001). Indeed, higher activity of $H_2O_2$-scavenging enzymes (POX), as those reported herein (Fig. 6), together with lower $H_2O_2$ values were reported by Rao et al. (1998) in superficial scald resistant seedlings. Moreover, application of exogenous $H_2O_2$ to harvested 'GS' fruit leads to a fast

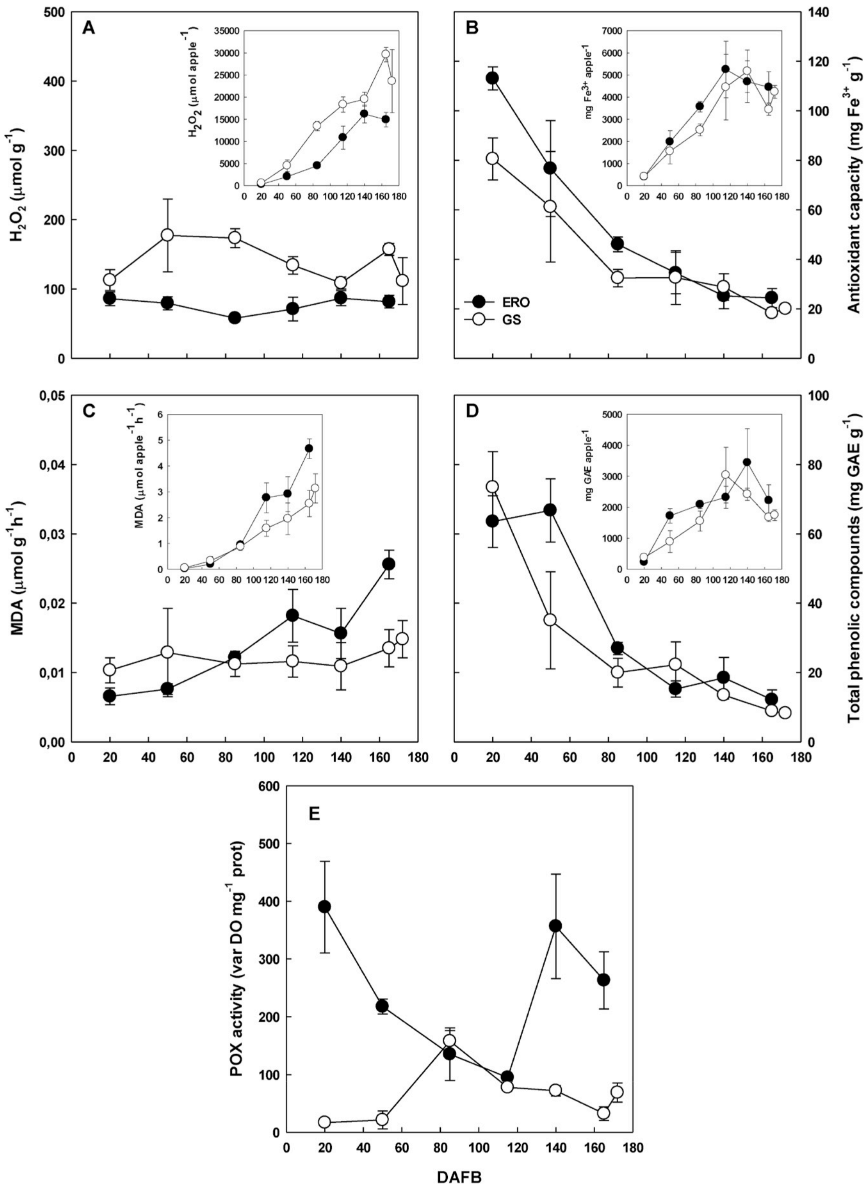


**Fig. 6.** Changes in the concentration oxidative stress markers ($H_2O_2$ (A) and MDA (C) (μmol g-1), total antioxidant capacity (B; mg $Fe^{3+}$ $g^{-1}$) and total phenolic compounds (D; mg GAE $g^{-1}$) and peroxidase (POX; E) enzyme activity during growth and ripening of two different apple (Granny Smith (GS: ○) and Early Red One (ERO: ●)) varieties. Values represent the mean ± stdev (n = 6). Inserts in each graph depict the temporal changes of each parameter on a fruit basis. LSD values ($P < 0.05$) for the interaction cultivar*sampling point in figures A, B, C, D and E were 32.002, 18.347, 0.005, 16.409, and 57.950, respectively.

development of superficial scald symptoms (Giné-Bordonaba and Larrigaudière, unpublished). Collectively these results indicate that the continuous higher $H_2O_2$ levels observed during growth in 'GS' may contribute, at least in part, to the higher sensitivity of this cultivar to superficial scald.

In contrast to that observed in other fruit species (i.e. cherries; Giné-Bordonaba et al., 2017), the increase in $H_2O_2$ levels observed in 'GS' was not paralleled with higher MDA content. In 'ERO' fruit, MDA constantly increased during growth but remained partly unchanged in 'GS'. A positive and strong correlation was found between MDA content and ethylene production in 'ERO' ($r^2 = 0.695$; $P < 0.05$) but not in 'GS' fruit (Supplementary Figure 3). Furthermore, the lower membrane lipid peroxidation in 'GS' could not be explained by a higher content in fruit antioxidants (Fig. 6). On a concentration basis, both the fruit antioxidant capacity and the total phenolic composition steadily declined during fruit growth in both varieties while the opposite trend was observed if considering the results on a fruit basis (Fig. 6).

Collectively, these results may indicate that the accumulation of $H_2O_2$ and the lower enzymatic antioxidant capacity observed in 'GS' fruit during growth and ripening may play an important role in determining the sensitivity of this cultivar to superficial scald.

## 4. Conclusions

The results from this study demonstrate that differences in quality traits or storage performance at the time of harvest, understood as the capacity of the fruit to soften or to suffer some physiological disorders, may partially be explained by a range of physiological and biochemical changes occurring during apple fruit growth and on-tree ripening. The limited firmness loss experienced by 'GS' apples, if compared to other varieties, may be related to a higher acid content, which based on malic acid accumulation seemed to be regulated already at fruit set (20 DAFB) as well as to its inability to produce ethylene at the time of harvest, which was in turn associated to reduced ACO activity. Thus said, the precise mechanisms or substances accounting for such ethylene inhibition at the time of harvest in this apple variety are still unknown and warrant further investigation.

In addition, the higher susceptibility of 'GS' if compared to 'ERO' to superficial scald, was not associated to peroxidative damage (malondialdehyde accumulation) or higher levels of α-farnesene during growth, but rather to a fruit antioxidant imbalance resulting from higher $H_2O_2$ levels and lower peroxidase activity. A greater knowledge on the major physiological and biochemical events occurring during the growth and on-tree ripening of apple fruit may ultimately lead to better postharvest management strategies for each variety.

## Author's contribution

JGB and CL conceived and designed the experiment. JGB and GE analysed all the data. ED and GB performed the biochemical and physiological measurements. JG wrote the article and all authors contributed in improving and revising the manuscript.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2018.10.035.

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