# 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.

**Keywords:** Antioxidants, ethylene, *Malus domestica* Borkh., oxidative stress and respiration

## 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-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 et al., 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 aim 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 (20ºC) 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 ºC 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 0.5ºC (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 20ºC 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 20ºC 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 acclimatized chamber at 20 ºC. After each sampling point, apples were placed within sealed flasks equipped with a silicon septum for sampling the gas of the headspace after 2h 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 x 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 ºC, 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 (2g) was added

to 5 mL of HPLC-grade water. Samples were kept at room temperature (25 ºC) for 10 min and then centrifuged at 24,000 x g for 7 min at 20 ºC. Glucose and fructose were extracted from fresh-frozen material as described elsewhere (Terry et al., 2007). Briefly, 2g of sample were dissolved in 5 mL of 62.5% (v/v) aqueous methanol solvent and placed in a thermostatic bath at 55 ºC 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 (0.5g) was homogenized in 4 mL of 0.1% trichloroacetic acid (TCA) solution. The homogenate was then centrifuged at 18,800 x 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 x g for 10 min at 4 ºC, 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 x g for 15 min at 4 ºC. Quantification of $H_2O_2$ was 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 ºC with constant shaking for 2h 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 x 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 20ºC. 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 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 20ºC, a very high incidence of superficial scald was observed in 'GS' fruit (98%) if compared to 'ERO' (12% incidence; Figure 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 20ºC and slightly declining thereafter. In contrast, no ethylene peak and basal levels of this hormone were observed in 'GS' fruit stored at 20ºC 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 et al., 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 20ºC 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 expoliniar (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 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.

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 et al., 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 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

400 negative correlations were observed between the fruit respiration pattern and the content
401 of monosaccharides (glucose + fructose) and sucrose (Fig. S3) highlighting the role of
402 these compounds as important respiratory substrates in apple.
403 Malic acid is by far the main organic acid present in ripe apple fruit (Suni et al., 2000;
404 Jing et al., 2016) and its concentration is known to drastically vary among different apple
405 cultivars (Suni et al., 2000). Malic acid, as well as most organic acids present in the apple
406 flesh, are not imported but rather synthesised from imported sugars (Famiani et al., 2012).
407 Accordingly, in 'GS' but not in 'ERO', negative correlations were found between malic
408 acid and sugar content throughout fruit development (Fig. S3). The concentration (mg $g^{-1}$)
409 of this compound remained relatively unchanged during the growth of 'ERO' fruit (24-
410 36 mg $g^{-1}$) whereas a peak at 50 DAFB was observed in 'GS' apples (*ca*. 145 mg $g^{-1}$)
411 declining to constant levels (*ca*. 70 mg $g^{-1}$) thereafter and until the time of harvest. These
412 results are in agreement with those reported for other apple varieties (i.e. 'Golden
413 Delicious'; Jing et al., 2016) in which malic acid remained relatively unchanged from 90
414 DAFB to fully ripe fruit. On a fruit basis (mg $apple^{-1}$), however, the content of malate
415 steadily increased during growth and ripening of both varieties being always 2 to 3-fold
416 higher in 'GS' than in 'ERO' fruit (Fig. 5). In grapes, malic acid is thought to be an
417 important respiratory substrate (Famiani et al., 2014) and postharvest studies on apples
418 also pointed out the importance of this compound in fruit respiration (Liu et al., 2016). In
419 accordance to that recently reported in peaches (Famiani et al., 2016) and cherries (Giné-
420 Bordonaba et al., 2017), our results indicate that the amount of malate accumulated during
421 fruit growth, may contribute little or nothing to the net substrate requirements of apple
422 metabolism since this compound was constantly synthesized rather than degraded
423 throughout fruit development and on-tree ripening.

424 ***3.5 Changes in oxidative stress markers and antioxidants during fruit ripening***

425 It is generally recognized that $H_2O_2$ at low concentrations may act as a messenger
426 molecule involved in adaptive responses whereas higher concentrations of this compound
427 may lead to programmed cell death. In this work, $H_2O_2$ concentrations differently
428 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
430 two clear peaks from 40 to 80 DAFB and prior to commercial harvest were observed in
431 'GS' fruit. Theoretically, the higher $H_2O_2$ levels observed in 'GS' together with higher
432 amounts of malate might point out a higher mitochondrial function for this apple variety.
433 Thus said, fruit respiration in 'GS' was not substantially different to that of 'ERO' and
434 hence it is unlikely that the burst of $H_2O_2$ is related to an overfunctioning of the
435 mitochondrial machinery but rather to the inability of the fruit to scavenge this compound.
436 Indeed, when analysed on a fruit basis, $H_2O_2$ content (nmol $apple^{-1}$) constantly increased
437 during fruit development and ripening especially in 'GS' fruit, a result that was consistent
438 with the significant inhibition of POX activity observed in this cultivar during all the
439 growing phase (Fig. 6).

440 Whether the higher levels of $H_2O_2$ detected in 'GS' may be associated to
441 superficial scald susceptibility (Fig. 1) is still debatable but it is widely accepted that
442 increased $H_2O_2$ levels create oxidative stress leading to a diversity of physiological
443 damages (Wang and Jiao, 2001). Indeed, higher activity of $H_2O_2$-scavenging enzymes
444 (POX), as those reported herein (Fig. 6), together with lower $H_2O_2$ values were reported
445 by Rao et al. (1998) in superficial scald resistant seedlings. Moreover, application of
446 exogenous $H_2O_2$ to harvested 'GS' fruit leads to a fast development of superficial scald
447 symptoms (Giné-Bordonaba and Larrigaudière, unpublished). Collectively these results
448 indicate that the continuous higher $H_2O_2$ levels observed during growth in 'GS' may
449 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 (Fig. S3). 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

474 such ethylene inhibition at the time of harvest in this apple variety are still unknown and
475 warrant further investigation.
476 In addition, the higher susceptibility of 'GS' if compared to 'ERO' to superficial scald,
477 was not associated to peroxidative damage (malondialdehyde accumulation) or higher
478 levels of α-farnesene during growth, but rather to a fruit antioxidant imbalance resulting
479 from higher $H_2O_2$ levels and lower peroxidase activity. A greater knowledge on the major
480 physiological and biochemical events occurring during the growth and on-tree ripening
481 of apple fruit may ultimately lead to better postharvest management strategies for each
482 variety.
483

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**AUTHOR'S CONTRIBUTION**

JGB and CL conceived and designed the experiment. JGB and GE analyzed 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.

***LIST OF FIGURES:***

**Figure 1:** Fruit firmness (A) and DA-value (B) at harvest (optimal harvest date), 15 days earlier and during postharvest ripening of ‘Granny Smith; GS’ and ‘Early Red One;

691 ERO' fruit after 4 months of cold storage. (C) Superficial scald incidence and severity in
692 'GS' and 'ERO' fruit after 4 months of cold storage.

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

697 **Figure 3**: Morphological (fruit weight; A) and quality changes (B: Fruit firmness; C:
698 Titratable acidity; D: Total soluble solids) during growth and ripening of two different
699 apple (Granny Smith (GS: ○) and Early Red One (ERO: ●)) varieties. Values represent
700 the mean ± stdev (n =6). LSD values ($P<0.05$) for the interaction cultivar*sampling point
701 in figures A, B, C and D were 11.50, 3.18, 0.14 and 1.39, respectively. (E) Image of the
702 different phenological stages corresponding to each sampling point (Days after full bloom
703 (DAFB) are given for 'Early Red One; ERO' and 'Granny Smith; GS', respectively).

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

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

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

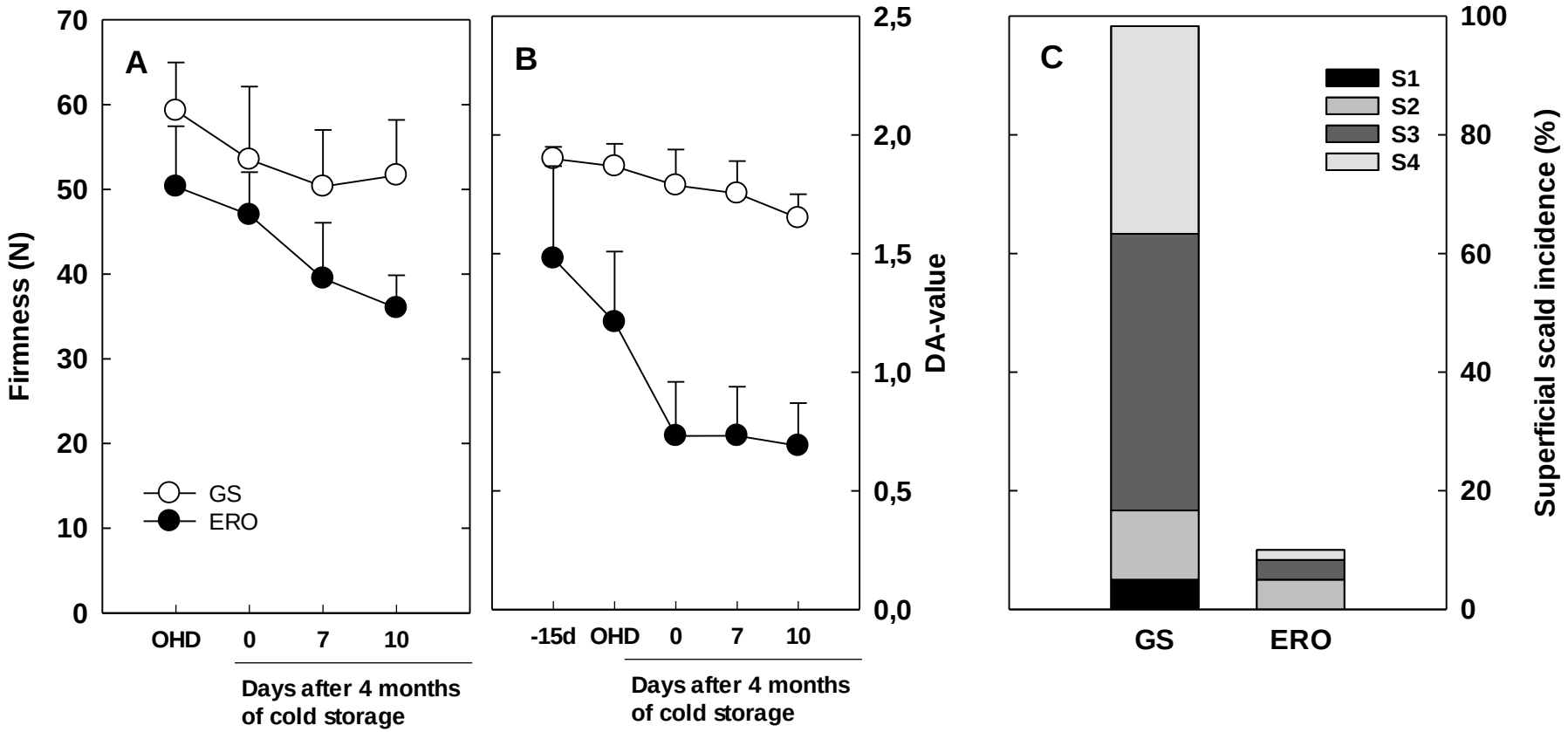
A
B
C
Firmness (N)
DA-value
Superficial scald incidence (%)
GS
ERO
S1
S2
S3
S4
OHD
0
7
10
-15d
Days after 4 months
of cold storage

**Figure 1:**

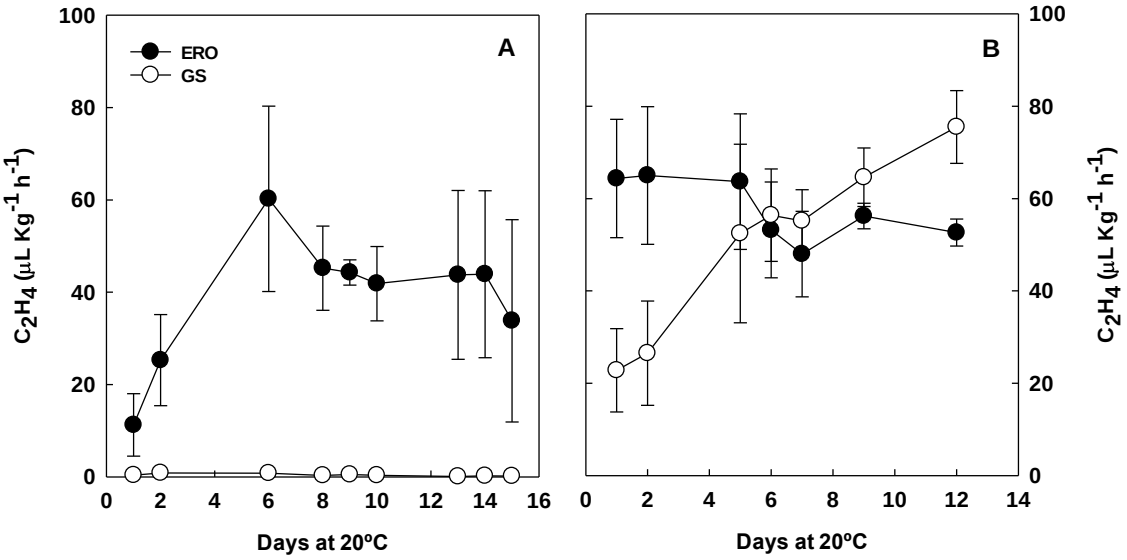
ERO
GS
A
B
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
Days at 20ºC
C2H4 (µL Kg-1 h-1)

**Figure 2**:

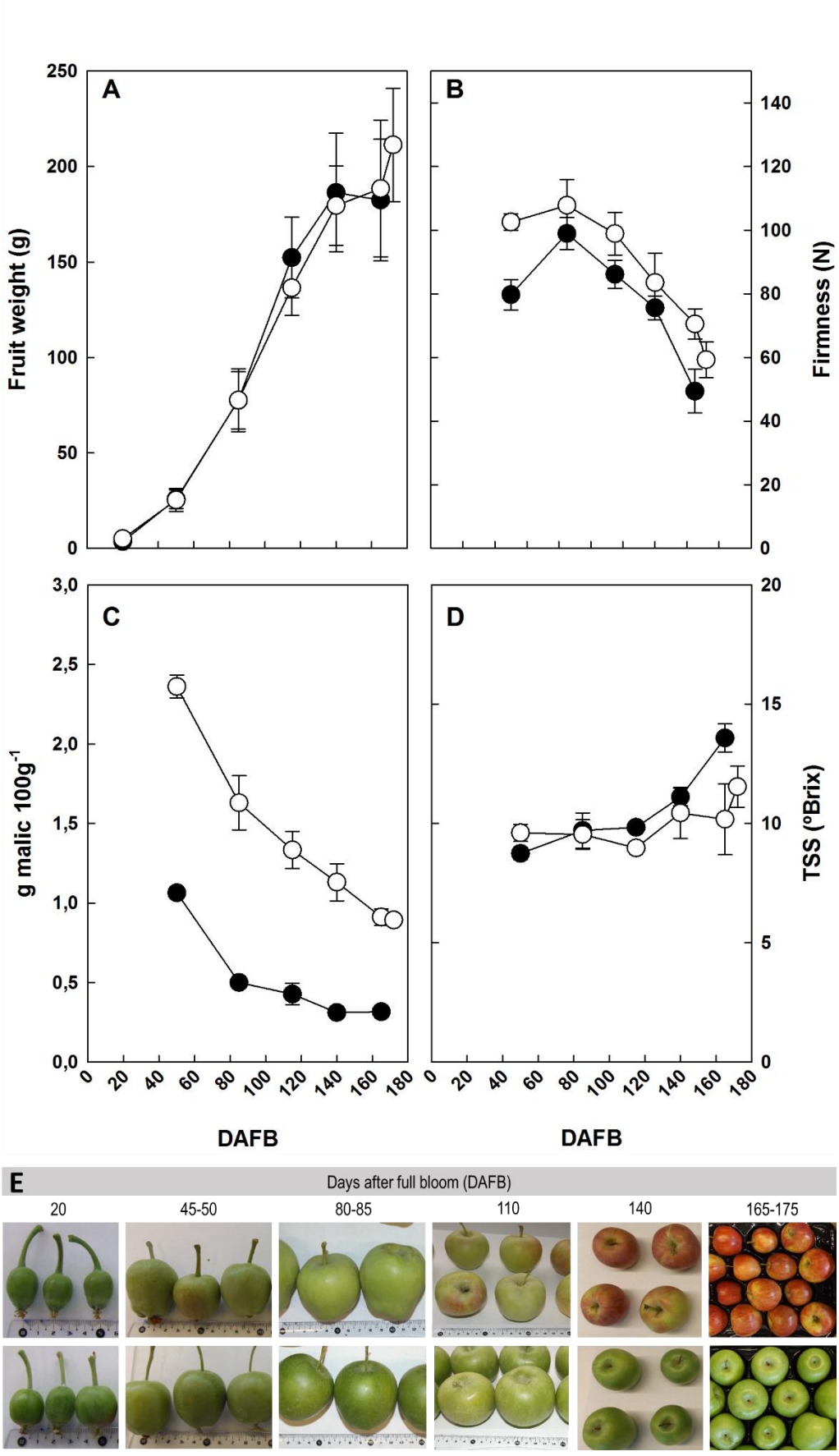
A
B
C
D
E
Fruit weight (g)
Firmness (N)
g malic 100g-1
TSS (ºBrix)
DAFB
DAFB
Days after full bloom (DAFB)
20
45-50
80-85
110
140
165-175
ERO
GS

**Figure 3**:

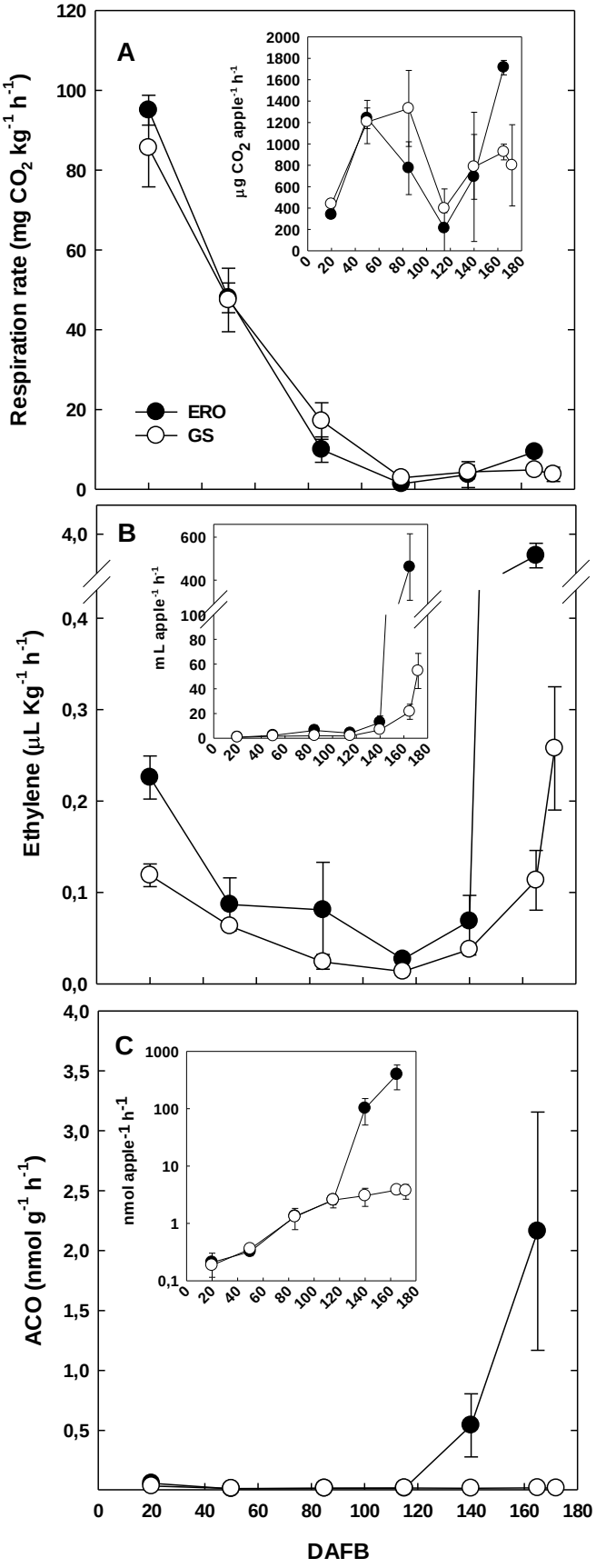
A
B
C
Respiration rate (mg CO2 kg-1 h-1)
Ethylene (µL Kg-1 h-1)
ACO (nmol g-1 h-1)
µg CO2 apple-1 h-1
mL apple-1 h-1
nmol apple-1 h-1
ERO
GS
DAFB

**Figure 4**:

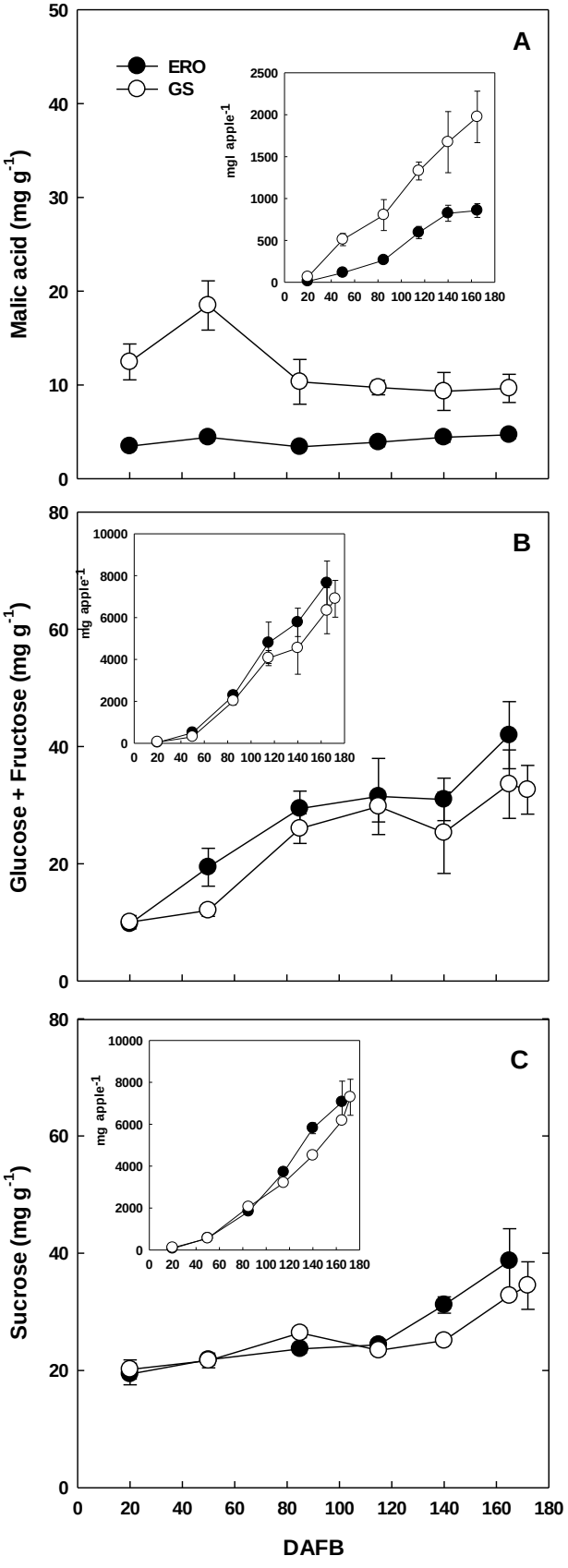
A
ERO
GS
Malic acid (mg g-1)
mgl apple-1
B
Glucose + Fructose (mg g-1)
mg apple-1
C
Sucrose (mg g-1)
mg apple-1
DAFB

**Figure 5**:

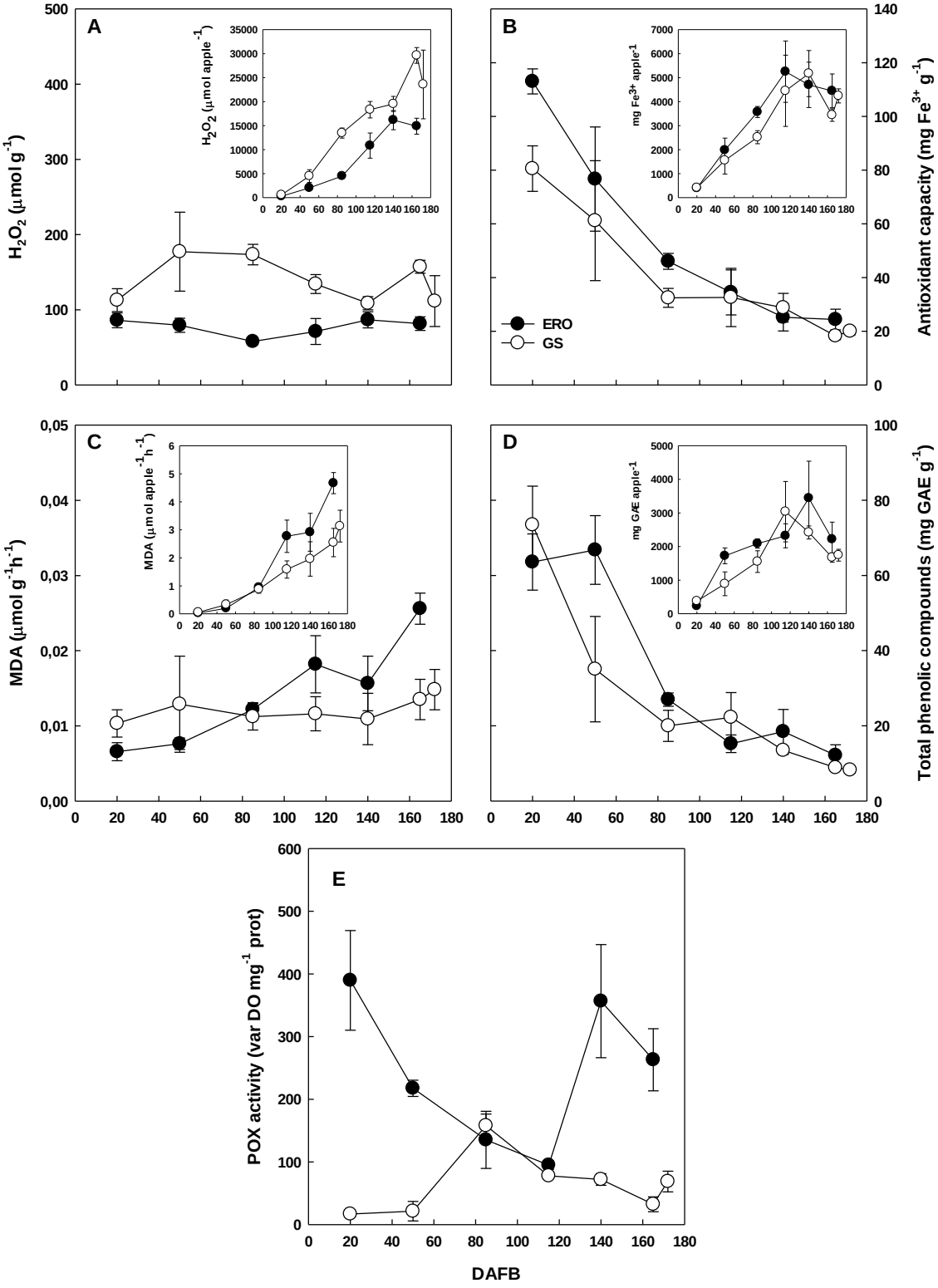
A
B
C
D
E
ERO
GS
H2O2 (µmol g-1)
Antioxidant capacity (mg Fe3+ g-1)
MDA (µmol g-1h-1)
Total phenolic compounds (mg GAE g-1)
POX activity (var DO mg-1 prot)
DAFB

**Figure 6:**

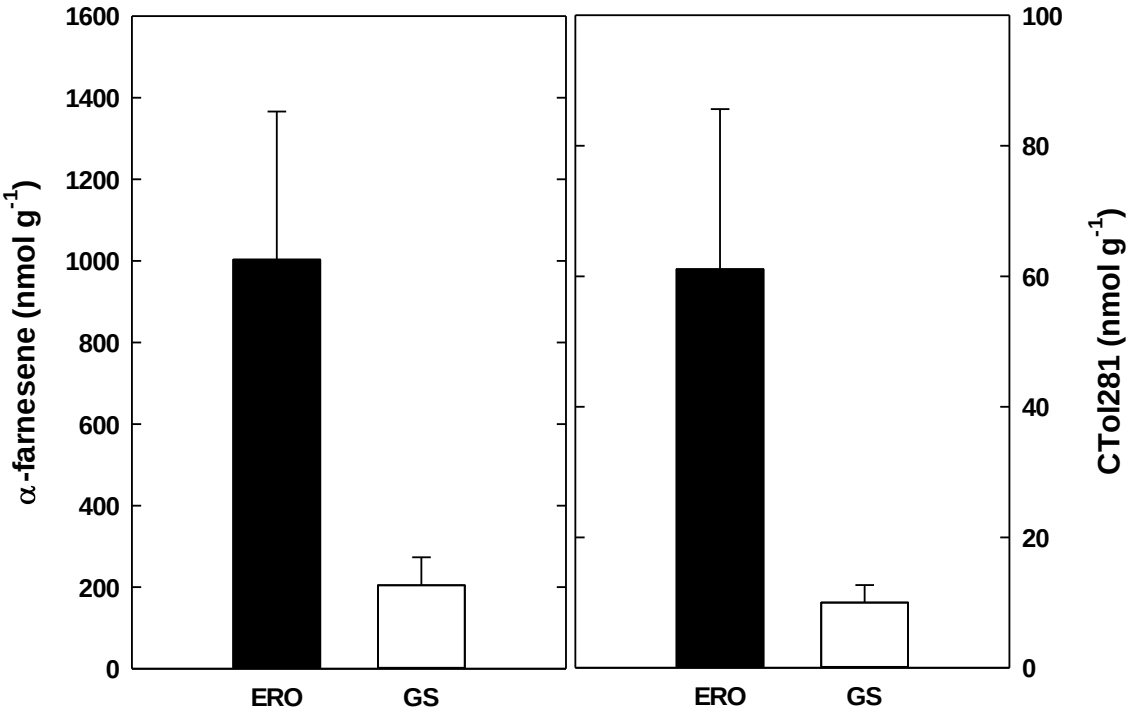


**Figure S1:** Levels of α-farnesene and CTol 281 in Granny Smith (GS) and Early Red One (ERO) apples at the time of harvest. Extraction and quantification of both analytes was done as described in Giné-Bordonaba et al. (2013). Error bars indicate the standard deviation for n=6.

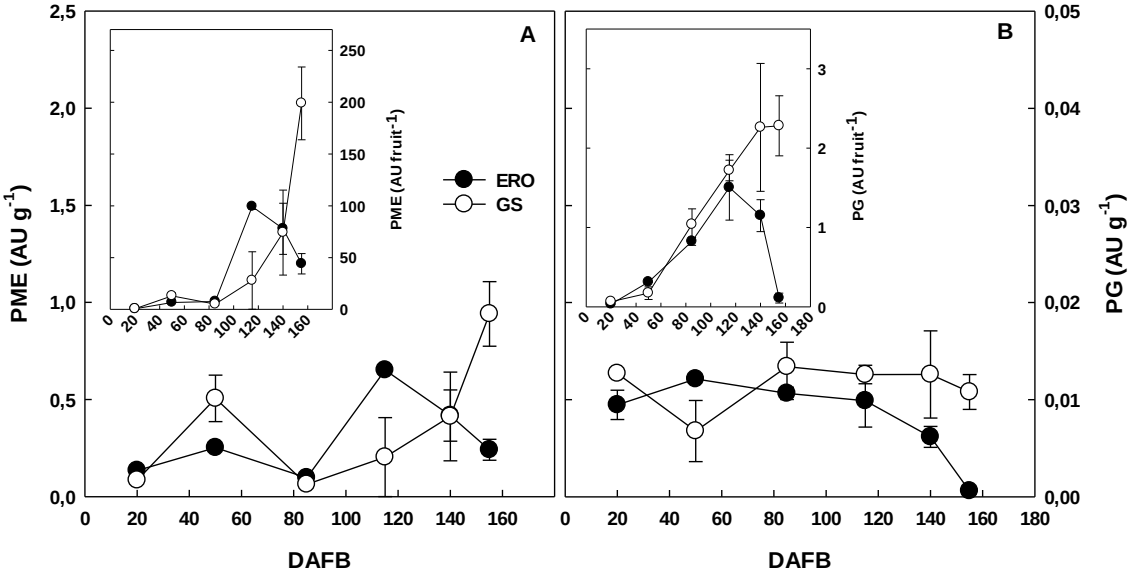


**Figure S2:** Changes in the activity of cell wall modifying enzymes (Pectin methylesterase; PME (A) and Polygalacturonase; PG (B)) during growth and ripening of two different apple (Granny Smith (GS: ○) and Early Red One (ERO: ●)) varieties. Values represent the mean ± stdev (n =6). Inserts in each graph depict the temporal changes of each parameter on a fruit basis.

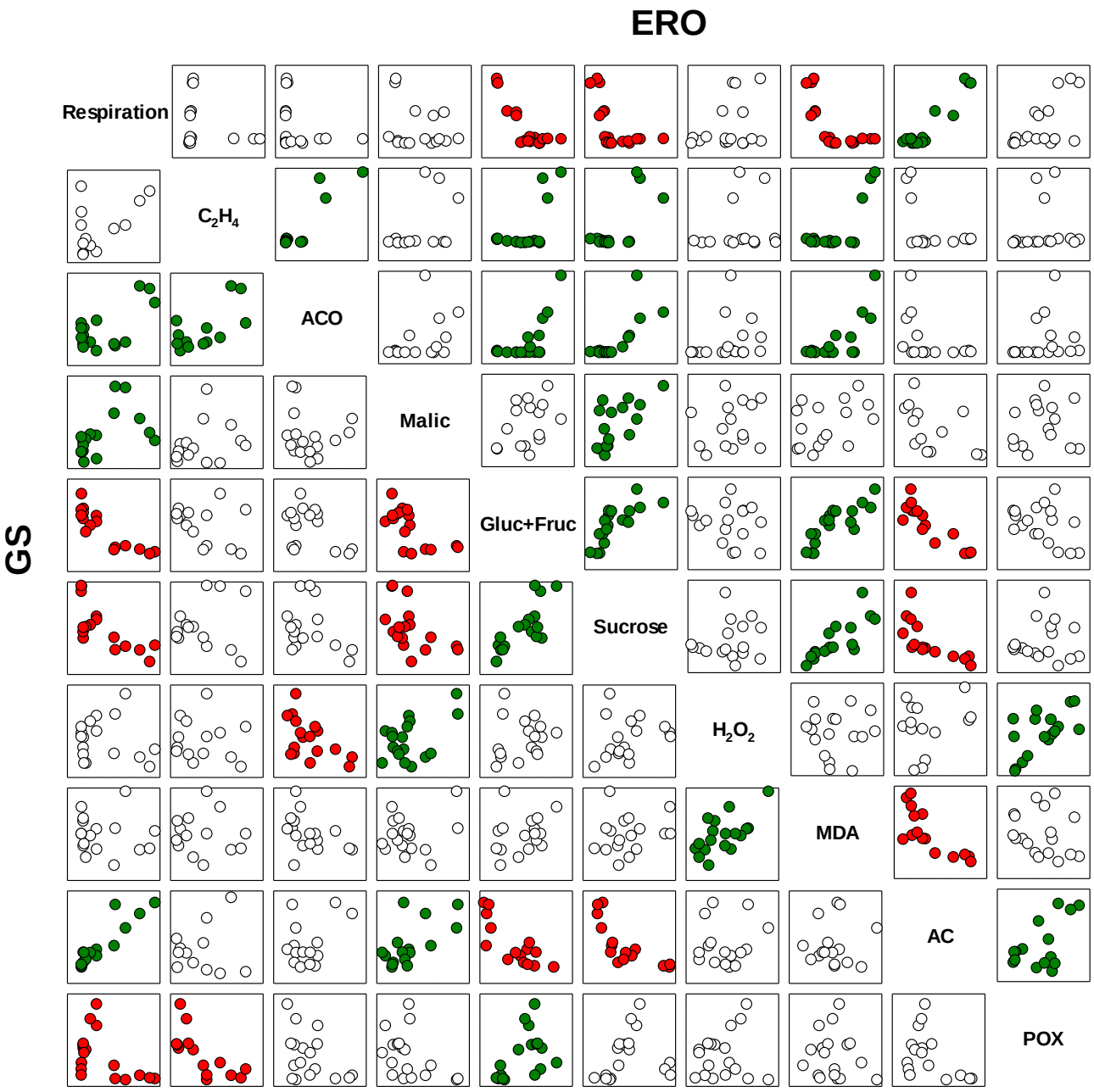


**Figure S3**: Pearson's correlation scatter matrix for ERO (scatter plots above the diagonal) and GS apples (scatter plots below the diagonal) for the different variables investigated. Coloured scatter plots depict significant correlations for P <0.05