Adv. Hort. Sci., 2023 37(1): 89-99 DOI: 10.36253/ahsc-14180

AHS
Advances in Horticultural Science

Ethanol fermentation- and ethylene physiology-related gene expression profiles in Red Delicious apples stored under variable hypoxic conditions and protocols

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Citation:
SALAMÉ E., BRIZZOLARA S., RODRIGUES M., IOB M., TONUTTI P., RUPERTI B., 2023 - *Ethanol fermentation- and ethylene physiology-related gene expression profiles in Red Delicious apples stored under variable hypoxic conditions and protocols.* - Adv. Hort. Sci., 37(1): 89-99.

Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

Competing Interests:
The authors declare no competing interests.

Received for publication 12 January 2023
Accepted for publication 18 February 2023

Key words: Dynamic Controlled Atmosphere, ERF, low oxygen, *Malus domestica*, postharvest.

Abstract: Dynamic Controlled Atmosphere (DCA) is beneficial in maintaining specific quality parameters but, due to the extreme oxygen levels applied, can cause adverse effects on the fruit by inducing excessive anaerobic metabolism and the production of off-flavors. The metabolic adaptation and responses of apples (*Malus domestica* Borkh.) cv. Red Delicious to static or dynamic oxygen concentrations (0.3 and 0.8%, with sequential shifts) during cold storage for 7 months were studied by monitoring quality parameters and the expression of genes involved in sugar, fermentative metabolism, and ethylene physiology. Ethanol content reached the highest levels (around 400 mg/kg FW) under 0.3% oxygen concentration and fruit firmness appeared to be reduced in samples accumulating the highest levels of ethanol. The oxygen switch was effective in reducing the ethanol concentrations with timing-dependent variable effects. The expression of fermentative (*alcohol dehydrogenase, lactate dehydrogenase, pyruvate decarboxylase)* and sugar metabolism *(β-amylase; phosphofructokinase*; *sucrose synthase)* genes resulted to be differently affected by the hypoxic conditions imposed, in particular during the early stages of storage. *Sucrose synthase* expression appeared to be highly sensitive to changes in low oxygen concentration. Ethylene biosynthesis (*ACC synthase* and *oxidase*) genes showed marked differences in their expression in relation to the static and dynamic protocols and the hypoxic conditions, as well as six Ethylene Responsive Factors (ERF) genes, some of them possibly involved in the oxygen sensing mechanism operating in fruit tissues.

1. Introduction

Dynamic Controlled Atmosphere (DCA) represents one of the latest technical innovations for the long storage of apples (and few other fruit crops) (Tonutti, 2015). With this technology, fruits are kept under extremely low oxygen concentrations (0.4 kPa or lower) that are beneficial for maintaining specific quality parameters (e.g., flesh firmness, acidity). However, by activating anaerobic metabolism, an accumulation of ethanol takes place. Low concentrations of ethanol are desirable in terms of improving organoleptic traits, reducing the incidence of chilling injuries (e.g., superficial scald) and limiting ethylene biosynthesis (Dixon and Hewett, 2000; Scott *et al*., 2000; Weber *et al*., 2020). Yet, the accumulation of excessive ethanol results in the appearance of off-flavors and physiological disorders (Pedreschi *et al*., 2009). Thus, based on different stress indicators (chlorophyll fluorescence -CF-, respiratory quotient -RQ-, and ethanol concentration), oxygen must be promptly adjusted (increased) to reach "safe" concentrations. The imposed extreme hypoxic conditions induce selective responses of apple tissues starting from the modulation of gene expression involved in particular in primary metabolism and hormone (mainly ethylene) physiology (Cukrov *et al*., 2019). In Granny Smith, one of the apple cultivar most frequently stored in DCA, differential expression of *sucrose synthase (SuSy), alcohol dehydrogenase (ADH)* and pyruvate-related metabolism (*lactate dehydrogenase, LDH, pyruvate decarboxylase, PDC,* and *alanine aminotransferase, AlaAT*) genes was detected when comparing 0.4 with 0.8 kPa oxygen concentration (Cukrov *et al*., 2016). When, according to the DCA protocol, oxygen level is increased from the lowest applied concentrations, molecular and metabolic rearrangements rapidly occur, with changes in both primary and secondary metabolism (Brizzolara *et al.*, 2019), indicating that highly reactive mechanisms and oxygen sensors are present in apple cortex. The expression of genes involved in fermentative metabolisms (e.g., *ADH*), in secondary metabolism (e.g., phenylpropanoid pathway), hormonal responses and regulatory mechanisms (ethylene biosynthesis, ERFs) resulted to be affected by the oxygen switch.

The duration of the storage and the oxygen concentrations applied obviously play a key role in determining the fruit metabolic responses and the dynamics of fermentative metabolite accumulation. In addition, apple varieties react differently to extremely low oxygen conditions during storage, in particular in terms of fermentation, ethanol production and accumulation (Thewes *et al.*, 2019; Brizzolara *et al*., 2020; Thewes *et al.*, 2021 a; Park *et al*., 2022). Zanella and Stürz (2015) showed that, differently from eight other varieties, 'Red Delicious' apples react significantly and accumulate higher ethanol levels under hypoxia, and in a specific comparison between Granny Smith and Red delicious (Brizzolara *et al*., 2017), it was reported that the latter considerably accumulated ethanol under both ULO (0.9 kPa oxygen) and DCA (0.2-0.55 kPa oxygen) conditions, as also observed by Lumpkin *et al*. (2014).

Among important commercial apple varieties, the responses of Red Delicious to controlled atmosphere (CA) and DCA still need to be compared and clarified, which makes this cultivar a genotype of interest in terms of both applied aspects and physiological studies related to DCA conditions and different oxygen regimes and concentration adjustment protocols.

2. Materials and Methods

Experimental design and sampling

Organic apple (*Malus domestica* Borkh., cv. Red delicious) fruit were harvested in correspondence of an average TSS value of 10.8°Brix. Fruit were selected for their uniformity and absence of physical defects/decay and then kept for 3 days of acclimation at low temperature (0°C).

Control atmosphere storage was applied by dividing the fruit into two groups in two different cold chambers. The first group (about 300 fruit) was initially stored under oxygen concentration of 0.3% (0.3ox) while the second group (60 fruit) was stored under the safer oxygen concentration of 0.8% (0.8ox) for a total period of 218 DIA (days in atmosphere). Samples were collected at harvest and T0 sampling was carried out after 24h in atmosphere (1 DIA). To simulate the dynamic changes in oxygen concentrations applied during a DCA storage, at T1 (10 DIA) 60 fruit originally stored under 0.3ox were shifted to 0.8% oxygen level and kept under these conditions for the whole period of storage (218 DIA). These samples were called Shift 1 and were sampled successively at the following time points. At T2 (20 DIA), another 60 fruit were moved from 0.3% to 0.8% oxygen and were called Shift 2. Shift 3 was performed at T3 (31 DIA) and Shift 4 took place at T4 (110 DIA). A

schematic diagram of the experimental design is reported in figure 1. At each sampling point, three biological replicates taken from three different fruit for each treatment were considered. Samples of cortex tissue were collected, immediately frozen in liquid nitrogen, and stored at -80°C.

Flesh firmness, total soluble sugars content and ethanol quantification

Flesh firmness was measured at harvest (T0) and at the end of the storage at T5 (218 DIA). Measurements were taken at the two opposite sides of the equatorial part using a fruit penetrometer (mod. FT 327, 3-27 Lbs) with a large plunger tip (11 mm-diameter) after removing 1 mm of the peel.

Total soluble solid content (TSS, °Brix) was determined in the flesh juice of apples using a portable refractometer (Sinergica Soluzioni, Pescara, Italy); measurements were performed at harvest and the end of the storage at T5 (218 DIA) on pulp juice samples taken from the opposite sides of the fruit.
Ethanol content was measured by using a TectroniK (Tectronik, Padova, Italy) Senzytec analyser, following the instructions of the manufacturer and using 100µL of juice obtained by collectively pressing portions (approximately 1/3) of the cortex of three apples representing the biological replicate.

RNA extraction and cDNA synthesis

Total RNA was isolated from cortex tissue using 'SIGMA -Aldrich' RNA extraction kit following the manufacturer instructions. Total RNA was quantified (ng/µL) using UV spectrophotometry calibrated with RNase-free water. RNA purity was assessed by evaluating the absorbance ratio at 260/280 and 260/230 nm. Ribosomal RNA bands integrity was verified using GelRed™-stained 1% agarose gel (Aranda *et al.*, 2012).

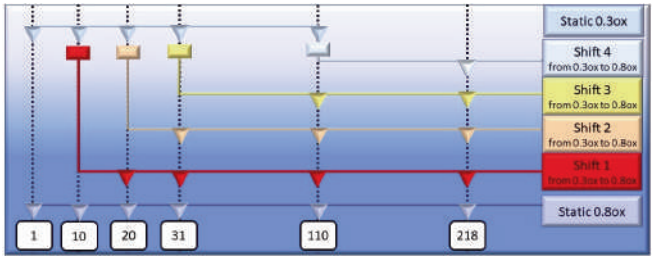

Fig. 1 - Red delicious DCA storage experimental design. The numbers indicate the days in atmosphere (DIA), ▽ Indicates the sampling with color coded for different samples, 0.3ox (Blue), 0.8ox (Purple), Sh1 (Red), Sh2 (Orange), Sh3 (Yellow) and Sh4 (Light blue).

RNA was reverse-transcribed into first-strand cDNA using 4 µL ReadyScript™ (Sigma, RDRT-500RxN), starting from 400 ng of total RNA in a final volume of 20 µL using DEPC treated water. The reaction was incubated at 25°C for 5 minutes, then at 46°C for 20 min and then heated at 95°C for 1 min and finally at 4°C for 15 min. The synthetized cDNA was diluted 1:5 by adding sterile water.

Gene expression analysis by real-time PCR

Quantitative Real-time PCR was performed using three biological replicates and two technical replicates for each sample. Based on the paper by Cukrov *et al.* (2016), primer pairs of genes related to sucrose/starch metabolism (*β-amylase, MdBAM; phosphofructokinase, MdPFK*; *sucrose synthase, MdSuSy*), the fermentative/pyruvic acid metabolism (*alcohol dehydrogenase, MdADH; lactate dehydrogenase, MdLDH; pyruvate decarboxylase, MdPDC; alanine aminotransferase, MdAlaAT*), ethylene biosynthesis (*ACC synthase, MdACS; ACC oxidase, MdACO*), and 6 *Ethylene Responsive Factors* (ERFs) were used (Table 1). *Actin* was used as a housekeeping gene.

Reaction mixtures were prepared, under sterile conditions, for the target and reference genes, containing each 5 µL of 2XSYBR® Green qPCR ReadyMix™ (SIGMA), 1 µL of each primer (Forward and Reverse) (10 µM), 2 µL RNase-free water and 1 µL of cDNA. The automated thermal cycler was programmed according to the following conditions: initial denaturation of 95°C for 30 sec followed by 40 cycles of: denaturation at 95°C for 10 sec, primer annealing (according to primer Tm) for 30 sec and extension at 72°C for 30 sec. Finally, melt-curve stage at 65°C for 0.5 sec followed by 95°C for 0.5 sec.

The Ct-values generated were used to evaluate the results of the gene expression levels comparing the expression of each target gene to the housekeeping gene (*Actin*). For samples under static 0.8ox, data were expressed with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and normalized to the corresponding sample at T1 (1 DIA). Data of samples under 0.3ox and the shifts were expressed as fold change of the expression level using the formula: $FC=Log_2(2^{-\Delta\Delta Ct})$ normalized to the corresponding sample of 0.8ox at each time point starting from 10 DIA.

Statistical analysis

For gene expression, data analysis was performed on six replicates (3 biological x 2 technical replicates). RStudio was used with external package "pcr" to calculate the relative expression and fold change. For

Table 1 - Primers pairs used in the RT-qPCR analysis

Genes	Primer sequence 5'-3'	Length (bp)	GC content (%)	Tm (°C)
ACC synthase MD15G1302200	F AAGTGGCGAACTGGAGTCGA	20	55	67.2
	R GGTTTGATGGGTTCGTGACC	20	55	66.4
ACC oxidase MD10G1328100	F CAGTCGGATGGGACCAGAA	19	57.8	66.3
	R GCTTGGAATTTCAGGCCAGA	20	50	66.3
Pyruvate decarboxylase MD04G1160100	F CAAGGCAGTAAAGCCGGTTA	20	50	63.9
	R AAATCGGTCCAGCAAACAAG	20	45	63.9
Alcohol dehydrogenase MD10G1014200	F GGAAGCACTGAAGCCATGAT	20	50	64.2
	R CTCCACGACAGAGGGAATGT	20	55	64.2
Lactate dehydrogenase MDP0000143956	F CATAAAACTCCTTCAGGCTCCA	22	45.4	64.1
	R GTGSGGTCTTGGGTGAGGAT	22	55	63.9
Beta-amylase 6 MD09G1103800	F CTATGTGCCGATCTTCGTGA	20	50	63.8
	R ACTGCTTGAAACACGCTCCT	20	50	63.9
Sucrose synthase MD15G1223500	F TCCGTGTTCACTGCTACGAG	20	55	64.1
	R GCCTCAAGAAGGTCCAACAG	20	55	63.8
Phosphofructokinase MD04G1042400	F AGTCGTGGAGTGGTGGAATC	20	55	64.1
	R TAGAGGGTGAGGGCTTCAGA	20	55	63.9
Alanine aminotransferease MD09G1173000	F TGCTGTCCGAGGTGAAATCGTC	22	54.5	70.6
	R AGCCCGGATTCGCCTTTAACTC	22	54.5	69.9
Ethylene response factor MD11G1306500 variant	F CGGTGGTGCTATAATCTCCG	20	55	64.2
	R GGAATTGAGTCGGTGTGAGTAGTT	24	45.8	64.3
Ethylene response factor MD11G1306500	F CTCCCTTCGCCAAGTTCG	18	61.1	65.9
	R TTGAGTCGGTGCGATTAACC	20	50	64.9
Ethylene response factor MD16G1162900	F CCAGAAGCCCAAACCATCAG	20	55	66.7
	R TTCCTCGGCGGTGTTGTA	18	55.5	65.4
Ethylene response factor MD13G1163300	F GGTGGGGAAATGTATGCTAAGA	22	45.4	63.7
	R GTCATCCAGCATCCACAGG	19	57.8	64.4
Ethylene response factor MD17G1152400	F CTTCTGCAAAGCGTTCTGTG	20	50	63.7
	R GGCAGGATCGGATGGAG	17	64.7	64.5
Ethylene response factor MD09G1174400	F TTCTGCAAAGCGTTCCATC	19	47.3	64
	R TTCATTGGCAGGGAAGGTG	19	52.6	66
Actin MD04G1127400	F TGACCGAATGAGCAAGGAAATTA	25	40	67.4
	R TACTCAGCTTTGGCAATCCACATC	24	45.8	68

The table reports the genes nomenclature according to https://iris.angers.inra.fr/gddh13/, the primers sequence, length, GC content and melting temperature (Tm)

fruit firmness, TSS and ethanol content 3 biological replicates were used. Internal statistical functions and external package "agricolae" were used to analyze the data (Kronthaler and Zoellner, 2021). All data were analyzed using *t-test* for samples at 1 DIA to compare 0.3 to 0.8ox samples. One-way ANOVA and mean comparison with Least significant difference (LSD) post-hoc test ($p \leq 0.05$) was used to compare different samples of shifts to the corresponding 0.8ox at each time point starting from 10 DIA. A Kruskal-Wallis test was applied to non-parametric data ($p \leq 0.05$).

3. Results

Flesh firmness, TSS and ethanol production

At the end of the storage period and after five days of shelf life at room temperature no physiological disorders or external/internal defects were observed in all analysed samples collected from the different protocols.

Concerning technological parameters, Table 2 reports apple firmness and TSS values for samples taken at harvest (T0) and at the end of the trial at 218 DIA. Results showed that samples stored for 30

Table 2 - Firmness and total soluble solids (TSS). Mean values (±SE) of apple samples at harvest (T0) and after 218 DIA of static (0.8ox) and dynamic atmosphere storage (Shift 1-4) are reported in the table

Samples	DIA	Firmness (N)	TSS (°Brix)
At harvest	0	75.10 ± 0.96 a	10.85 ± 0.45
Static 0.8ox	218	67.67 ± 2.85 ab	11.40 ± 0.06
Shift 1	218	62.52 ± 3.51 ab	11.76 ± 0.32
Shift 2	218	67.91 ± 0.73 ab	11.53 ± 0.24
Shift 3	218	58.10 ± 6.76 b	10.86 ± 0.13
Shift 4	218	61.30 ± 5.83 b	11.40 ± 0.06

Different letters indicate significant differences among samples (ANOVA, LSD post-hoc test (p≤0.05).

(Sh3) and 110 (Sh4) days under 0.3% oxygen before being shifted to 0.8% oxygen showed the lowest values of firmness at the end of the trial. While 0.8ox, Sh1 and Sh2 samples maintained firmness values not significantly different from those detected in T0 samples.

No significant difference (*p=0.414, alpha level ≤ 0.05)* was recorded for TSS levels over time or between the different applied protocols.

Ethanol levels have been monitored along the entire experimental period to assess the activation of fermentative metabolism under static and dynamic CA storage (Fig. 2). 0.3ox samples already showed higher levels than those of the 0.8ox sample at 10 DIA, and a further increase from 10 to 20 DIA with values around 400 mg/kg FW up to 110 DIA (last sampling time for this specific treatment). On the other hand, 0.8ox samples showed a similar trend in

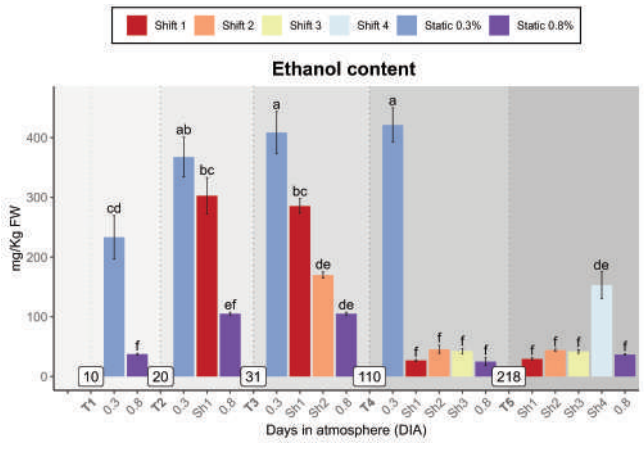

Fig. 2 - Ethanol content (mg/Kg FW). Samples stored in 0.3ox (Blue), 0.8ox (Purple), Sh1 (Red), Sh2 (Orange), Sh3 (Yellow) and Sh4 (Light blue) analysed from 10 to 218 days in atmosphere (DIA). Different letters indicate significant differences among samples (ANOVA, LSD post-hoc test (p ≤ 0.05).

terms of ethanol accumulation but with significantly lower amounts compared to 0.3ox samples and a decreasing trend after the highest concentrations detected at 20 and 31 DIA. Samples subjected to partial re-oxygenation at different time points (Sh1, Sh2, Sh3 and Sh4) showed a different behaviour: Sh1 did not show significant difference from 0.3ox at 20 DIA , but displayed a reduced amount at 31 and at 110 DIA as also observed for Sh2.

Interestingly, at 110 DIA ethanol levels were similar in all shifted and in 0.8ox samples. This was also observed at 218 DIA, except for Sh4 apples that displayed still significant higher levels (Fig. 2).

Effect of different protocols on sugar metabolism- and fermentation-related gene expression

For gene expression data analysis, 0.8ox samples, kept under a static concentration throughout the experiment (from 1 to 218 DIA), were considered as a reference for the other storage protocols (0.3ox, Sh1, Sh2, Sh3 and Sh4). Consequently, the gene expression levels of these latter samples were expressed as fold change in relation to 0.8ox.

Considering sugar metabolism, the expression levels of three genes were monitored throughout storage (Fig. 3). In 0.8ox samples, *MdBAM* gene revealed a significant up regulation, compared to T0, at 31 and 110 DIA. A significantly lower expression level was recorded in 0.3ox samples at 10 and 110 DIA. Sh1, Sh2, Sh3 and Sh4 samples had significantly lower levels of expression at 110 DIA. At the last sampling time, 218 DIA, all shifted samples, except Sh3, revealed significantly higher expression values compared to 0.8ox. *MdPFK* gene expression showed a steady state in samples stored at 0.8ox. A lower expression level of this gene was detected at 10 DIA in 0.3ox samples. Sh1 samples showed higher expression at 31 DIA, Sh2 had lower expression level at 31 DIA and higher at 110 and 218 DIA, while Sh3 samples showed higher expression levels at 218 DIA. Considering *MdSuSY, in* 0.8ox apples the expression showed a significant induction at 10 and 20 DIA, followed by a basal expression level at all the other sampling points. Samples stored under 0.3ox revealed two marked and significant peaks of induction, at 10 and 110 DIA. Interestingly, Sh1 showed significantly reduced levels of expression at 20 DIA, while Sh2 and Sh4 revealed significantly higher expression at 218 DIA.

Concerning the gene related to the fermentative/pyruvic acid metabolism (Fig. 4), *MdLDH* expression showed, in 0.8ox samples, a significant increase

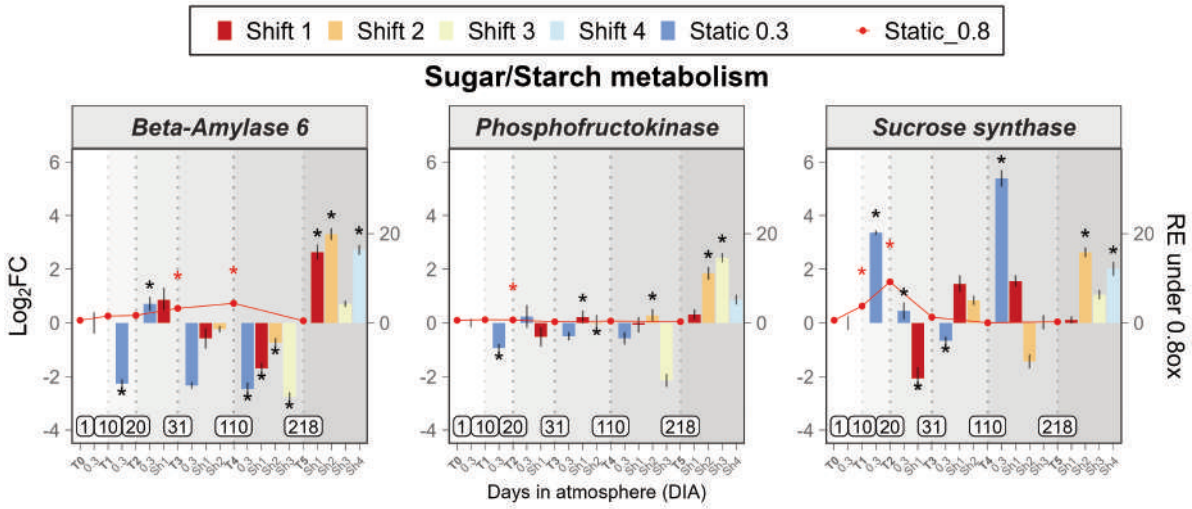

Fig. 3 - Relative expression of genes related to sugar metabolism and energy production β-amylase, phosphofructokinase, and sucrose synthase. For samples 0.3ox (Blue), Sh1 (Red), Sh2 (Orange), Sh3 (Yellow) and Sh4 (Light blue) the expression level is reported from 1 to 218 days in atmosphere (DIA) as *log$_2$ FC* normalized on 0.8ox expression level at each time point. The red line represents gene relative expression in 0.8ox samples. Black asterisks indicate significant differences (ANOVA, LSD post-hoc test (p≤0.05) comparing each sample to 0.8ox level at the same sampling time. Red asterisks indicate significant differences (ANOVA, LSD post-hoc test (p≤0.05) between 0.8ox samples at the specific time points and 0.8ox apples at 1 DIA.

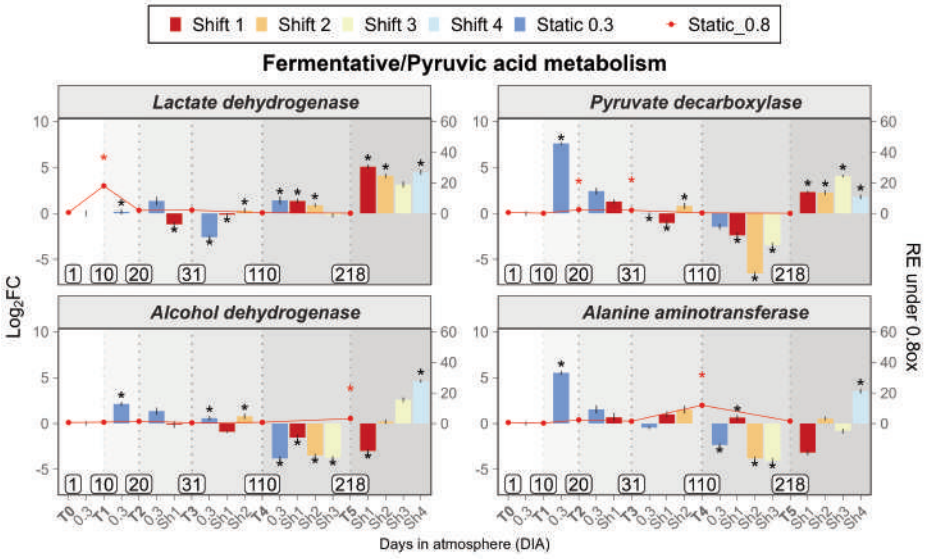

Fig. 4 - Relative expression of genes related to fermentative/pyruvic acid metabolism, lactate dehydrogenase, pyruvate decarboxylase, alcohol dehydrogenase and alanine aminotransferase. For samples 0.3ox (Blue), Sh1 (Red), Sh2 (Orange), Sh3 (Yellow) and Sh4 (Light blue) the expression level is reported from 1 to 218 days in atmosphere (DIA) as *log$_2$ FC* normalized on 0.8ox expression level at each time point. The red line represents gene relative expression in 0.8ox samples. Black asterisks indicate significant differences (ANOVA, LSD post-hoc test (p≤0.05) comparing each sample to 0.8ox level at the same sampling time. Red asterisks indicate significant differences (ANOVA, LSD post-hoc test (p ≤ 0.05) between 0.8ox samples at the specific time points and 0.8ox apples at 1 DIA.

of expression only at 10 DIA. Compared to 0.8ox samples, 0.3ox treatment induced higher expression at 10 and 110 DIA, and a general up-regulation in shifted samples at 218 DIA. *MdPDC*, involved in the production of acetaldehyde, increased its expression at 20 and 31 DIA in 0.8ox samples. 0.3ox apples revealed an earlier increase in the expression of *MdPDC* gene, significant at 10 DIA. Among samples that underwent partial re-oxygenation, a general lower expression, always compared to 0.8ox, was observed at 110 DIA, followed by increased levels at 218 DIA. Acetaldehyde can be further converted to

ethanol under low oxygen levels by the enzyme coded by *MdADH* genes, and this is a crucial reaction in apple tissue under hypoxia. Compared to T0 samples, the selected *MdADH* gene showed a significant increase only at 218 DIA in 0.8ox sample. The application of 0.3% oxygen resulted in general higher levels of expression until 31 DIA, significant at 10 and 31 DIA. At 110 DIA, a significant decrease in *MdADH* expression was recorded for 0.3ox apples as well as for all shifted samples.

MdAlaAT is involved in the reversible transfer of an amino group from glutamate to pyruvate, which in turn forms 2-oxoglutarate and alanine. In 0.8ox samples, this gene showed a peak of expression at 110 DIA. The expression in 0.3ox samples is highly induced at 10 DIA, while it strongly decreased at 110 DIA, similarly to Sh2 and Sh3. Higher expression of this gene was detected at 218 in Sh4 sample (Fig. 4).

Ethylene biosynthesis and ERFs gene expression

The expression of two genes involved in ethylene biosynthesis, namely *MdACS* and *MdACO*, has been analysed (Fig. 5). These two genes appeared to be highly affected during storage under 0.8% oxygen concentration. The expression of both genes increased with time, constantly for *MdACO,* which also reached the highest recorded levels of expression, while, in the case of *MdACS,* a peak at 110 DIA was detected. Regarding *MdACS* gene expression, 0.3ox samples showed increased values (compared to 0.8ox) at 10 DIA, but lower levels at 20, 31 and 110 DIA, when also Sh2 and 3 showed low expression levels.

MdACO gene revealed marked higher levels in 0.3 ox samples at 10 DIA and in Sh1 samples at 20, 31, and 110 DIA. Lower expression levels were detected in 0.3ox apples at 20, 31 and 110 DIA. Interestingly, Sh1 samples showed higher levels of expression, compared to 0.8ox, at 20, 31, and 110 DIA. All shifted samples had lower expression level than 0.8ox apples at 218 DIA.

The ethylene signalling and response pathway includes *Ethylene Response Factors* (ERFs), which belong to the transcription factor family APETALA2/ERF that plays important roles in stress-related responses. The effect of the different applied storage protocols on the expression level of six ERF genes has been investigated throughout the experiment (Fig. 6). In general, samples showed relatively similar responses in terms of ERF expression. Overall, considering 0.8ox samples a general increase of ERF genes expression was observed up to 110 DIA, which was significant at different time points for the different analysed ERFs (*MD09G1174400* gene at 110 DIA; *MD13G1163300* gene at 10, 20 and 31 DIA; *MD11G1306500* gene at 10, 20 and 31 DIA; *MD16G1162900* gene at 10, 31 and 110 DIA; *MD11G1306500 VARIANT* gene at 10, 20 and 31 DIA;

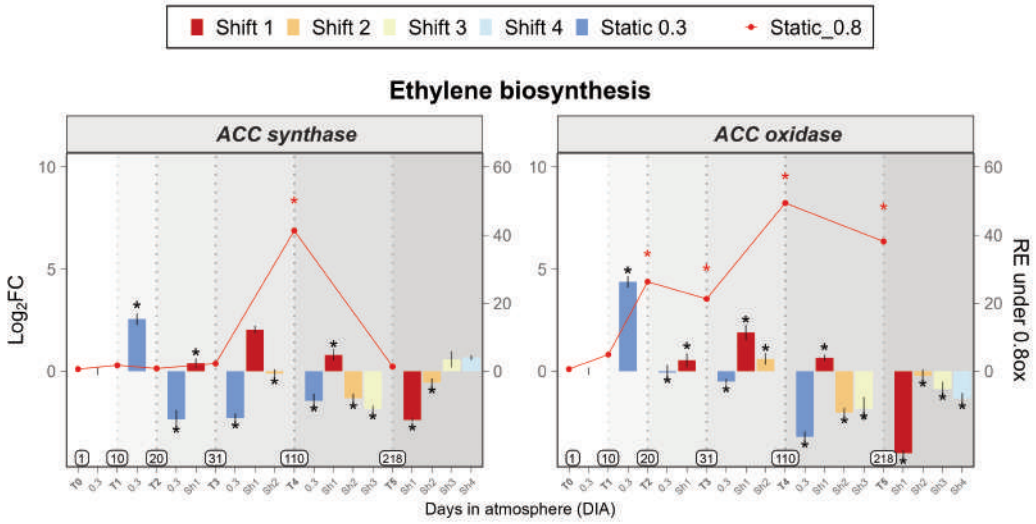

Fig. 5 - Relative expression of ethylene biosynthesis genes ACC-synthase and ACC-oxidase. For samples 0.3ox (Blue), Sh1 (Red), Sh2 (Orange), Sh3 (Yellow) and Sh4 (Light blue) the expression level is reported from 1 to 218 days in atmosphere (DIA) as *log2 FC* normalized on 0.8ox expression level at each time point. The red line represents gene relative expression in 0.8ox samples. Black asterisks indicate significant differences (ANOVA, LSD post-hoc test ($p \leq 0.05$) comparing each sample to 0.8ox level at the same sampling time. Red asterisks indicate significant differences (ANOVA, LSD post-hoc test ($p \leq 0.05$) between 0.8ox samples at the specific time points and 0.8ox apples at 1 DIA.

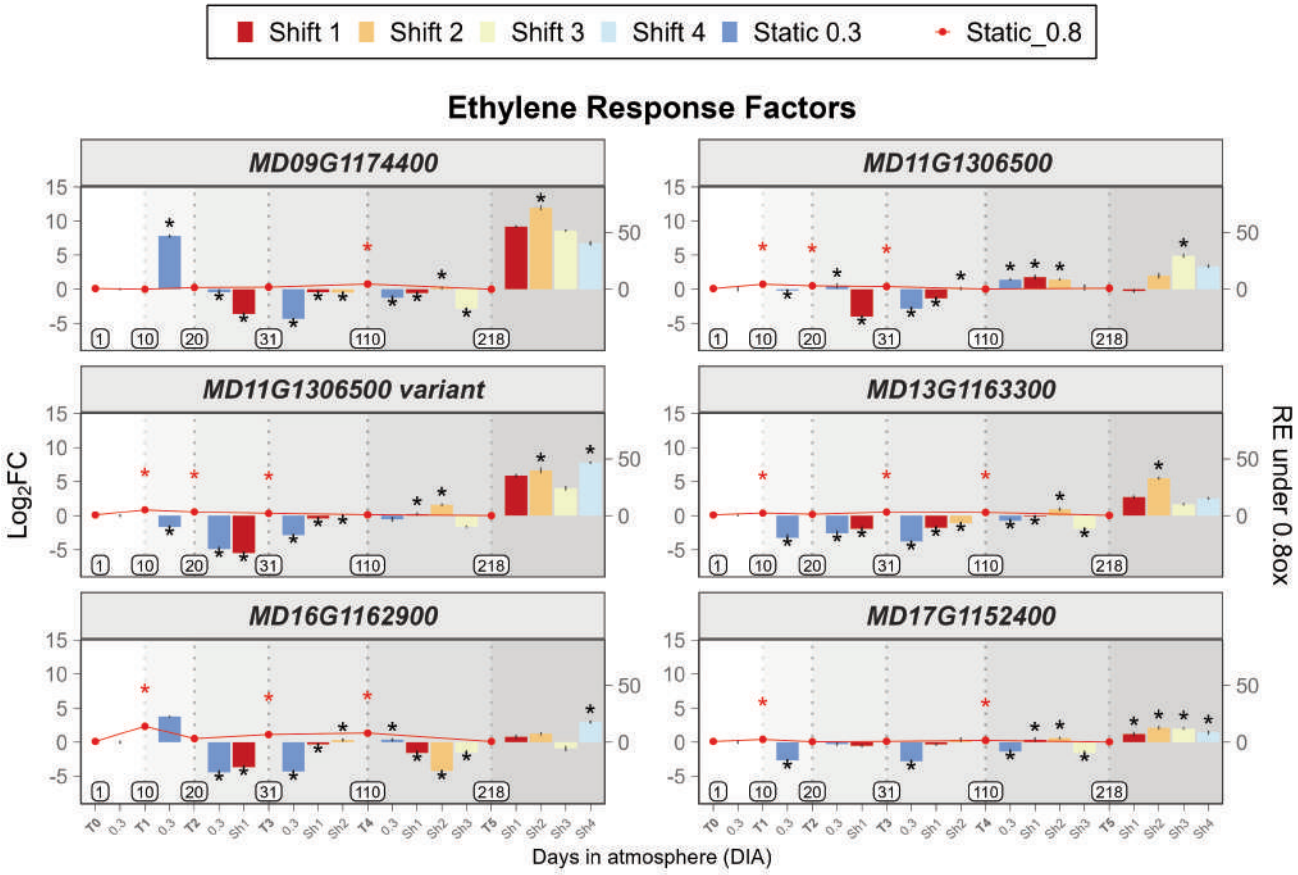

Fig. 6 - Relative expression of genes belonging to Ethylene Response Factor (ERF) gene family. For samples 0.3ox (Blue), Sh1 (Red), Sh2 (Orange), Sh3 (Yellow) and Sh4 (Light blue) the expression level is reported from 1 to 218 days in atmosphere (DIA) as *log2 FC* normalized on 0.8ox expression level at each time point. The red line represents gene relative expression in 0.8ox samples. Black asterisks indicate significant differences (ANOVA, LSD post-hoc test ($p \leq 0.05$) comparing each sample to 0.8%ox level at the same sampling time. Red asterisks indicate significant differences (ANOVA, LSD post-hoc test ($p \leq 0.05$) between 0.8ox samples at the specific time points and 0.8ox apples at 1 DIA.

MD17G1152400 gene at 10 and 110 DIA).

The expression of ERF genes in 0.3ox samples was instead characterized by significant lower levels at the different time points with only two exceptions: *MD09G1174400* gene at 10 DIA and *MD11G1306500* gene at 20 DIA, when significantly higher levels of expression than those of 0.8ox apples were recorded. On the other hand, considering apples subjected to partial re-oxygenation samplings, lower levels of expression were generally detected up to 31 DIA, with only two exceptions (MD*11G1306500* and *MD16G1162900*) concerning Sh2 samples at 31 DIA, when a significantly higher level of expression was observed.

After 110 DIA, the different samples revealed variable patterns. Concerning *MD09G1174400* and *MD13G1163300* genes, Sh1 and Sh3 showed significantly lower levels of expression, while Sh2 had significantly higher levels. For these two genes at 218 DIA only Sh2 revealed significantly higher levels of expression compared to 0.8ox samples, despite a general increasing trend in all apples subjected to partial re-oxygenation.

As far as the *MD11G1306500* gene is concerned, significant higher expression was detected for both Sh1 and Sh2 at 110 DIA, while only Sh3 had significantly higher levels at 218 DIA. *MD16G1162900* gene expression in shifted samples revealed significant lower levels at 110 DIA, while only Sh4 had significantly higher levels at 218 DIA. In the case of *MD11G1306500 VARIANT* gene Sh1 and Sh2 had higher expression levels at 110 DIA, while only Sh2 and Sh4 showed significantly higher levels at 218 DIA. Lastly, *MD17G1152400* gene had a significantly higher level of expression in samples of Sh1 and Sh2 at 110 DIA, while Sh3 apples had significantly lower

expression for this gene. On the other hand, all shifted samples showed significantly higher expression of this gene at 218 DIA compared to 0.8ox apples. A general trend was identified for all samples subjected to partial re-oxygenation: in general the expression level of the ERF gene was induced at 218 DIA.

4. Discussion and Conclusions

An in-depth understanding of low oxygen responses in fruits is of fundamental importance for the optimisation of storage approaches and for the development of protocols aimed at maintaining optimal quality while preventing the occurrence of physiological disorders associated with long term storage. Metabolic adaptation responses to hypoxic conditions have only recently started to be clarified in apple fruits and are gaining increasing interest since apples are routinely stored for very long periods of time thanks to the adoption of low oxygen (0.8 kPa oxygen, Ultra Low Oxygen, ULO) or dynamic controlled atmosphere (DCA, 0.4 or lower kPa oxygen) protocols. Primary metabolism and ethylene physiology are markedly affected by hypoxia with differences depending on the oxygen concentration and modulation, and the genotype. In this study we characterized the ethanol accumulation and the expression pattern of sugar/fermentative metabolism- and ethylene physiology-related genes of Red delicious apples in CA/DCA storage. Our goal was that of better understanding the behaviour and the responses (also in terms of specific quality parameters) of this apple variety in relation to two levels of low oxygen concentration and the variable (in terms of timing) switch from 0.3 to 0.8% oxygen levels.

The storage under 0.3% oxygen resulted in an early significant accumulation of ethanol already at 10 DIA and that further increased at 20 DIA. This level remained rather stable as long as the fruit were kept at 0.3% oxygen atmosphere until 110 DIA. Although to a lesser extent, ethanol content also increased in 0.8ox samples, confirming what observed by Brizzolara *et al*. (2017). In these apples, ethanol content levelled off after three months of storage. The metabolization of ethanol seemed to be more sensitive to re-oxygenation when apples had experienced a shorter period of DCA. In fact, the longest storage under 0.3% oxygen resulted in more stable ethanol levels in the cortex at the end of the storage period (218 DIA, Sh4 in Fig. 2). Considering one of the main parameters dictating the commercial life of apples, the samples Sh3 and Sh4 showed the lowest values of flesh firmness at the end of the trial. This behaviour could be associated to the highest levels of ethanol accumulated in these samples. In Braeburn apples ethanol production exceeding 472 $\mu L \cdot L^{-1}$ and the overproduction of anaerobic metabolites in Royal Gala resulted in a decrease of flesh firmness (Weber *et al*., 2020; Thewes *et al*., 2021 b). In persimmon, it has been observed that accelerated loss of flesh firmness during storage was induced by ethanol treatments applied to reduce astringency (Vilhena *et al*., 2022). These authors observed that this event is closely related to greater parenchyma degradation during storage caused by ethanol treatment. If this cellular event also occurs in apple fruit accumulating high ethanol levels following hypoxic storage conditions remains to be elucidated.

It is interesting to note that even in the samples with the highest ethanol content (0.3ox) no internal physiological disorders (e.g., flesh breakdown) were detected.

The gene expression data confirmed that the molecular regulation of hypoxic responses is overall conserved among apple varieties: the up-regulation of *MdPDC*, *MdADH* and *MdAlaAT* in response to extreme levels of hypoxia (0.3 oxygen concentration) is readily activated and peaks at 10 DIA, after which is promptly and progressively levelled down until 110 DIA, when a general low level of expression (compared to 0.8ox samples) is present in 0.3ox and shifted samples. This possibly suggests a negative feedback exerted by ethanol on its own synthesis. In agreement with these findings, one of the genes encoding group VII ethylene response factors (*MD09G1174400*), with similarity to RAP2 proteins involved in low oxygen signalling in model systems (Licausi *et al.*, 2011; Gibbs *et al*., 2011), displayed an expression pattern overlapping with that of the fermentative metabolic genes with a transient up-regulation at 10 DIA and low expression levels at 110 DIA.

It is well known that under energy shortage conditions, such as those induced by low oxygen conditions, plant tissues and organs (including fruit) instead of using invertases and hexokinases to produce hexose-phosphates to form sucrose, an ATP consuming process, can use sucrose synthase as alternative energy saving pathway (Mustroph *et al*., 2014). The activation under hypoxic conditions of sucrose synthase was already reported by Cukrov *et al.* (2016) in Granny Smith apples, and our expression

data (showing a high induction at 10 DIA in 0.3ox and a prompt reduction of expression level in Sh1 apples at 31 DIA) confirm that this gene can be considered highly sensitive to oxygen levels also in cv. Red Delicious. A cultivar-specific behaviour is, instead, observed regarding beta-amylase and phosphofructokinase. In fact, these genes in Granny Smith apples follow a similar expression pattern compared to *MdSuSY* (Cukrov *et al*., 2016), not observed in the present trials on Red Delicious.

As far as ethylene biosynthesis is concerned, the expression pattern of ACC oxidase detected in 0.8ox Red Delicious samples mirrors that observed in Granny Smith (Cukrov *et al.*, 2016), while the transient higher expression levels observed for both ACC synthase and oxidase at 10 DIA under 0.3% oxygen appear to be a specific response of cv. Red Delicious apple.

Interestingly, the transcription of both genes appeared to be re-activated exclusively in the first and second shift to 0.8ox (Sh1 and Sh2), performed after 10 and 20 DIA, respectively, and showed a peak at 31 DIA followed by lower expression levels. However, the increase of *MdACS* and *MdACO* transcript following the oxygen resupply reached a level significantly lower than that reached by apples that had been constantly kept at 0.8ox. It could be hypothesised that this may be due to the higher levels of ethanol accumulated in the pulp of DCA stored apples, which might exert a suppressive action on ethylene biosynthesis as previously shown by some authors in different apple varieties (Pesis *et al*., 2005; Thewes *et al*., 2019; Weber *et al*., 2020; Thewes *et al*., 2021 a).

Concluding, the recovery from anoxia in apple fruits is dependent on the length of exposure to the anoxic stress (Wood *et al*., 2022). Our data on ethanol accumulation and ethylene-related gene expression are in line with these findings, showing that longer periods of exposure to 0.3% oxygen result in the maintenance of higher levels of ethanol and on the prevention of transcription of ethylene biosynthetic genes. The effect of low oxygen storage of Red Delicious apples on transcript abundance of several important genes related to hypoxic stress response in apple fruit revealed both similarity with Granny Smith apples stored under the same CA protocols, and specific responses of cv. Red Delicious. In both Red Delicious and Granny Smith apples two phases can be recognized in relation to fermentative metabolism: a first phase characterised by the activation of fermentative pathways, and a second phase (from two months onward) in which a generalized de-activation of fermentative metabolism is observed.

Acknowledgements

This study was carried out by PT and SB within the Agritech National Research Center and received funding from the European Union Next-GenerationEU (PIANO NAZIONALE DI RIPRESA E RESILIENZA (PNRR) – MISSIONE 4 COMPONENTE 2, INVESTIMENTO 1.4 – D.D. 1032 17/06/2022, CN00000022). This manuscript reflects only the authors views and opinions, neither the European Union nor the European Commission can be considered responsible for them.

SB was supported by the special project of Scuola Superiore Sant'Anna ISVSTRATEGICO2019.

This work has been supported by the networking activities "Oxygen sensing a novel mean for biology and technology of fruit quality" (CA:18210) which is implemented under the COST Action "Roxy-COST," funded by the European Cooperation in Science & Technology (2019-2023).

Funding was provided by Marvil Engeneering Srl through project "Rupe Comm17_01" and by University of Padova project BIRD173975/17.

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