Transcriptome and metabolic survey disclose the mode of action of static and dynamic low oxygen postharvest storage strategies to prevent the onset of superficial scald disorder in fruit of 'Granny Smith' apple cultivar

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Keywords:

Apple, hypoxia, postharvest, superficial scald, transcriptomic signature, metabolomics

ABSTRACT

To preserve quality features and ensuring availability of fresh fruit on the market, apples need to be stored after harvest. The low temperature applied during storage, beside avoiding important fruit loss, can also promote the development of serious chilling injury disorders, such as superficial scald. One of the strategies largely employed to prevent the development of this phenomenon is the control of the storage atmosphere by lowering down the oxygen concentration. In this work, a multifaceted survey was carried out to investigate transcriptome variation together with three categories of metabolites (phenolics, lipids and volatile organic compounds, VOCs) in fruit of 'Granny Smith' apple cultivar stored in both static and dynamic controlled hypoxia atmosphere for five and seven months, respectively. The global transcriptome survey identified a core set of differentially expressed genes in three main functional groups, revealing as the duration of storage had an important effect in the coordination of gene expression. The effect of the length of storage was furthermore highlighted by the DEG-network analysis that identified a distinct number and type of transcriptomic hubs. Samples characterized by the development of superficial scald were distinguished by a higher concentration of chlorogenic acid and a higher expression of *phenylalanine ammonia lyase* (*PAL*) and *polyphenol oxidase* (*PPO*) in a time-dependent fashion. The prevention of this disorder was instead related to distinctive re-programming events, involving the accumulation of specific antioxidant types of metabolites, very long chain fatty acids (VLCFAs) and the expression of genes coordinating a hypoxia acclimation process, such as *RAP2-like* and *plant cysteine oxidase* (*PCO*).

1. INTRODUCTION

Apple is one of the most grown and cultivated fruit crops world-wide, especially in the temperate areas. Besides quality attributes, the economic success of this species is ensured by its long-lasting storability, which guarantees an almost year-round availability of fresh fruit on the market (El-Ramady et al., 2015; Tadiello et al., 2016). The apple marketability is mainly governed by both, the genetic control of each specific cultivar and the variability of the metabolic processes that regulate, for instance, the dismantling of the cell-wall polysaccharide structure, leading, in the end, to mealy or crispy types of fruit texture (Costa et al., 2011, 2012). The maintenance of these quality features is today secured by several postharvest strategies, commonly characterized by the lowering down of the storage temperature. The control of temperature and atmosphere are hereby two main factors promoting an extension of the postharvest life while delaying fruit ripening. However, storage at low temperature can also induce the onset of important physical and biochemical modifications, due to non-freezing chilling injury phenomenon (Sevillano et al., 2009; Wills, R. B. H., Golding, 2016; Cainelli & Ruperti, 2019). Among the postharvest chilling types of disorders, superficial scald is one of the most serious in pome fruits (Busatto et al., 2018; Giné-Bordonaba et al., 2020), which is manifested with the development of dark-discolored area on the peel of the fruit, compromising, in the end, its entire marketability (Brooks et al., 1919; Little, C.R. and Holmes, 2000; Tsantili et al., 2007; Busatto et al., 2014). The most accredited physiology behind the onset of superficial scald was focused on the role of the acyclic sesquiterpene α-farnesene, synthesized in the cytosolic mevalonate pathway (MVA) from the 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) (Rupasinghe et al., 1998 and 2001; Lange et al., 2000). Previous studies indicated a positive correlation between the accumulation of α-farnesene and the production of the hormone ethylene (Barden & Bramlage, 1994), since the expression of *AFS1* (*α-farnesene synthase 1*) in apple, the gene encoding the key enzyme in the α-farnesene

biosynthesis, was increased after harvest at the time of the climacteric ripening (Du Zhanyuan & Bramlage, 1994; Watkins et al., 1995; Ju & Curry, 2000) and repressed by the exogenous application of ethylene inhibitor (AVG: aminoethoxyvinylglycine) or competitor (1-MCP: 1-methylcyclopropene) (Ju & Curry, 2000; Lurie et al., 2005; Gapper et al., 2006). α-farnesene can undergoes into oxidation process generating conjugated trienols (CTols), which can be converted into the volatile ketone 6-methyl-5-hepten-2-one (MHO) (Mir et al., 1999; Wang & Dilley, 2000) by a non-enzymatic oxidation. This mechanism has been however recently reviewed, since the accumulation of both, α-farnesene and MHO during the onset of superficial scald was not fully elucidated or exhaustively established (Lurie & Watkins, 2012).

Alternative mechanisms have been therefore proposed, shedding light on a scenario involving a more complex transcriptional and metabolic re-programming (Gapper et al., 2017), especially focused on the role of phenolic compounds, which can be oxidized by the action of the polyphenol oxidase (PPO), contributing to the development of symptoms proper of superficial scald (Busatto et al., 2014 and 2018). In the work of Busatto et al. (2018) it was observed that scalded peel tissue of 'Granny Smith' apples showed a higher concentration of phenolic compounds. In this regard, it was also supposed that the antioxidant role of these class of metabolites exerted a protective mechanism towards the accumulation of ROS (reactive oxygen species) by scavenging the free radicals produced during the oxidative process (Di Meo et al., 2013). Among the several phenolics, chlorogenic acid is one of the most accumulated in apple. This hydroxycinnamic acid can further reacts with the polyphenol oxidase enzyme in apple (Taranto et al., 2017), encoded by *PPO* gene highly expressed during the shelf-life period following cold storage (Golding et al., 2001; Rocha and Morais, 2001; Busatto et al., 2014). Moreover, the expression pattern of the *PPO* gene in apple skin of 'Granny Smith' cultivar turned out to be ethylene-dependent, since application of 1-MCP down regulated its transcript accumulation (Busatto et al., 2014, 2018; Vittani et al., 2023).

Despite most of the scientific works oriented to prevent superficial scald have been mainly focused on the application of exogenous molecules competing with the ethylene perception (1-MCP), or playing an antioxidant role (DPA), also the storage in controlled atmosphere resulted to be an efficient strategy towards the control of this disorder. Modern postharvest technologies enabling a decrease of pO_2 below 1kPa, such as ultra-low oxygen (ULO) or dynamic controlled atmosphere (DCA; bringing the oxygen level almost to the Anaerobic Compensation Point-ACP), prevented, in fact, the development of superficial scald as much as the application of the aforementioned molecules (Zanella, 2003; Whitaker, 2004; Lurie & Watkins, 2012).

In this work, we aimed to investigate the transcriptional and metabolic changes occurring during the onset and prevention of superficial scald following both static and dynamic postharvest storage strategies.

2. MATERIAL and METHODS

2.1 Fruit storage and experimental design

Fruit from 'Granny Smith' apple cultivar were collected at the optimal harvest date for long-term CA storage from trees planted at the experimental orchard of the Laimburg Research Centre (Ora-BZ, North of Italy). Apples were harvested according to the following maturity index: starch level of 1.7-2.5 (based on a 1-5 scale), flesh firmness of 8.5 $kgcm^{-2}$, acidity of 10 gl^{-1} of malic acid, and a brix value of 9. After harvest, fruit were immediately transported to the postharvest facility, randomized and divided in two batches, stored for five (T1) and seven (T2) months, respectively. The storage followed three main procedures: cold storage at 1.3°C in i) regular atmosphere (RA); ii) static ultra-low oxygen atmosphere (1 kPa O_2 and 1.3 kPa CO_2; ULO) and iii) dynamic controlled atmosphere based on chlorophyll fluorescence (0.4 kPa O_2 and 0.9 kPa CO_2; DCA). At the end of each storage period, fruit were moreover divided in

two additional sub-batches. Fruit of the first sub-batch were assessed immediately after cold storage (CS), while the apples included in the second sub-batch were maintained for an additional period of seven days of post-storage shelf-life (SL) at the controlled temperature of 20°C. In total, the experimental design was represented by twelve samples: three storage strategies (RA, ULO and DCA) × two storage periods (T1 and T2) × two post-storage conditions (CS and SL) (Supp. Tab. 1). The incidence of the superficial scald symptoms was assessed on 130 apples/time points (T1, T1-SL, T2, T2-SL)/storage strategy (RA, ULO and DCA). Skin tissue collected from each sample were immediately frozen in liquid nitrogen and stored at -80°C until processing for metabolic and transcriptomic analysis. For each analysis, three biological replicates made by a batch of ten homogeneous apples/sample were used.

2.2 Metabolic assessments

In this work, three classes of metabolites were assessed, such as phenolic compounds, lipids and volatile organic compounds (VOCs).

From each frozen sample, 100 milligrams of powdered tissue were initially used to isolate and characterize the array of phenolic compounds, extracted with a methanol/chloroform (2:1) solution followed by a second extraction with methanol/water (2:1) mixture, as previously illustrated by Tadiello et al., (2016) and Busatto et al., (2021). After extraction and purification, each phenolic compound was separated and characterized using an ultra-high-performance liquid chromatograph (UHPLC) Water Acquity coupled with a Xevo TQMS-ESI system and characterized according to the protocol described by Vrhovsek et al. (2021).

The second category of metabolites was represented by lipids, extracted using a second aliquot (100 mg) of the frozen powder collected and stored from each sample as previously described. The extraction and analysis of lipids followed the procedure reported by Della Corte

et al. (2015) and Busatto et al. (2021). After an initial chloroform extraction, performed with the additions of butylhydroxytoluene (BHT, 500 mg L^{-1}) as antioxidant, a second extraction was performed with a chloroform (with BHT)/methanol/water (86:14:1) mixture. The extract from each sample was analyzed with a UHPLC Dionex 300 (Thermo Fisher Scientific, MA, USA) coupled with a Mass Spectrometer API5500. Both phenolic compounds and lipids were expressed in terms of μg g^{-1} of fresh weight.

The third aliquot of tissue dedicated for the metabolic profiling was employed to target the entire apple volatilome. The comprehensive VOC fingerprinting was profiled and characterized through a PTR-ToF-MS 8000 device (Ionicon Analytik GmbH, Austria) coupled with an autosampler enabling the automated high-throughput fingerprinting of the sample headspace (Li et al. 2020). The direct injection mode of this instrument enabled a comprehensive inspection of the mass spectrum up to m/z=400 with a sampling rate of 1 Hz (one spectrum per second). The analytical procedure adopted to characterize the whole volatilome was detailed in Di Pierro et al. (2022) and Vittani et al. 2023, while the data extraction, analysis and correlation were performed following the protocol reported by Cappellin et al. (2011a and 2011b).

2.3 Transcriptome analysis through RNA-Seq profiling

From each sample, the total RNA was isolated using the Spectrum™ Plant Total RNA Kit (Sigma Aldrich, MO, USA) according to the manufacturing instruction. The concentration of total RNA was evaluated with the Nanodrop 8000 Spectrophotometer (Thermo Fisher Scientific, MA, USA), while the quality assessment was verified with a TapeStation 2200 (from Agilent Technologies, CA; USA). For RNA-Seq analysis, only samples showing a RIN value higher than 7 were used. The NEB Next Ultra II kit® (BioLabs inc., New England) was employed for libraries preparation according to the instructions reported by the manufacturer.

Each library was performed by using 800 ng of total RNA. Libraries were further quantified through qPCR and Nanodrop 8000 Spectrophotometer (Thermo Fischer Scientific, MA; USA). RNA libraries were processed at the Functional Genomics Lab of the University of Verona (Italy) with a NovaSeq 6000 (Illumina).

2.4 Data analysis

The reads obtained from the RNA-Seq sequencing were further analyzed with the New Tuxedo protocol freely available in the cloud pipeline made accessible at the CyVerse on-line infrastructure (Merchant et al., 2016). Through this pipeline, sequences were assessed employing FastQC, Scythe, Sickle, HiSAT and StringTie software to assess the quality and subsequentially performing sequence trimming, alignment and read count, respectively. Sequences were aligned over the reference apple genome (assembly ASM211411v1) as available at the NCBI database. Transcripts quantification was finally obtained with the DESeq2 R package with default parameters (Love et al., 2014). The final list of genes differentially expressed (DEGs) were implemented in a functional characterization of genes by interrogating the Gene Ontology and KEGG pathway databases, with the aim to perform a gene enrichment analysis and to identify the most representative pathways involved in this process (False Discovery Rate - FDR p-adjusted$\leq$0.05). The query of the databases and the gene enrichment analysis were carried out with iDEP, an on-line suite of applications dedicated to the analysis of transcripts and database (Ge et al., 2018). The global gene expression was illustrated through a heat-map organized into several clusters of transcription profiles established according to the K-means clustering approach. The analysis of the dis-similarity matrix performed through three R packages (tidyverse, cluster and factorextra) allowed, through the generation of the Average Silhouette method, the inspection of the Silhouette

profile for the definition of the most significant number of subgroups, with a prior number of K-means cluster set from a minimum of 2 to a maximum of 10.

The multivariate statistical analysis of Principal Component Analysis (PCA) for the visualization of the distribution of the mean value for each sample according to the variability present within each dataset was performed with 'prcomp' function of tidyverse R package (Wickham et al., 2019).

The set of DEGs detected across the several samples were finally employed to construct two gene networks, for T1 and T2, respectively, based on a partial correlation applied to identify relevant links (Schäfer & Strimmer, 2005; Opgen-Rhein & Strimmer, 2007). The two networks were designed employing the functions 'ggm.estimate.pcor' and 'network.test.edges', implemented in the R package GeneNet.

The normality of the datasets was explored with the Shapiro test. For parametric and not parametric datasets, the homogeneity of variances was assessed with Bartlett and Brown-Forsythe tests respectively. For the normally distributed data, one-way ANOVA and the post hoc HSD Tukey's test were employed for multiple comparison. The heteroscedastic datasets were instead analyzed by Welch one-way ANOVA and the post hoc Games-Howell's test. For all tests, the statistical significance was defined by a p-value ≤ 0.01.

3. RESULTS

3.1 Assessment of superficial scald incidence and transcriptional variations in skin tissue of 'Granny Smith' apples stored in controlled atmosphere with different levels of oxygen

Fruit from each one of the twelve samples defined in this study were visually inspected to evaluate the incidence of superficial scald. After both T1 and T2 storage duration, RA apple samples (both CS or SL) showed severe superficial scald symptoms, while storage at low

oxygen concentration (ULO and DCA) efficiently prevented the development of this disorder (Fig. 1).

The RNA-seq analysis carried out on the samples defined in the experimental scheme, generated an average of 22,131,227 reads sequenced/sample. The alignment of the reads over the assembled genome and the counting of the fragments enabled the identification of 57,424 genes, out of which 8100 genes resulted statistically significant (DEGs-differentially expressed genes) in at least one of the pair-wise comparisons. The number of DEGs (Supp. Fig. 1A) highlighted that besides the modification of the transcriptome related to the oxygen concentration and the post-storage shelf-life, also the length of storage had a significant effect. For instance, the comparison between the two samples stored in controlled atmosphere with different oxygen level (ULO and DCA) showed a much higher number of DEGs in T2 than in T1 (T1: 55 DEGs; T2: 366 DEGs), while after one week of shelf-life at room temperature and atmosphere conditions the higher number of genes differentially expressed was observed in T1 (T1: 349 DEGs; T2: 14 DEGs). In the analysis of the transcriptome modification, in response to the different length of storage, the ULO samples showed the highest number of DEGs, with 4548 and 1020 DEGs identified between T1 and T2 and T1-SL and T2-SL, respectively (Supp. Fig. 1B). In general, samples after cold storage showed a higher number of DEGs between the two storage time points (T1 and T2) when compared to the samples assessed after shelf-life, with a fold change of 6.9, 4.5 and 1.5 for RA, ULO and DCA, respectively.

The effect of the duration of storage on the global transcriptome was also illustrated in the gene network analysis distinctively carried out on the two sub-cores of DEGs for T1 and T2, respectively. The two networks revealed, in fact, a distinct node-organization that identified unique hub, defined as genes showing a higher level of correlation connectivity, and common nodes (Fig. 2 A and B). As unique hub, a *nuclear pore complex protein NUP93A-like* (LOC103430281) was identified in the T1 network, while a *probable protein phosphatase 2c*

(LOC103430685) and a *sugar transporter 14-like* (LOC103400631) were identified in the T2 network. The common nodes between T1 and T2 networks were instead represented by 4 genes, such as *ubiquitin conjugate E2* (LOC103437801), *mRNA export factor SUS-1* (LOC103411295), *histone deacetylase HDT1-like* (LOC103427240) and an *abscisic acid ripening protein 1-like* (LOC103444659).

3.2 The different grades of hypoxia during storage and post-storage shelf-life grouped the gene expression profiles in three main clusters

The overall transcriptional variability was initially assessed through a multivariate Principal Component Analysis (PCA) (Fig. 3A). The PCA plot efficiently discriminated the type of atmosphere employed during the three different storage strategies. In the negative area of the PC1 (accounting for 37% of the global transcriptome variability) were in fact positioned the samples stored at regular atmosphere (RA), with a clear distinction between cold stored and shelf-life samples. In the positive area of the PC1, were instead grouped the samples stored in hypoxia conditions, regardless the type of strategy (static or dynamic). The PC2 (accounting for 26%) was instead mainly associated to the level of temperature and re-oxygenation, since cold stored samples were plotted on the PC2 positive area of the 2D-PCA plot, while shelf-life samples were allocated in the PC2 negative part. The distribution of the samples over the 2D-PCA plot also revealed as a prolonged storage reduced the transcriptional differences between cold stored and post-storage shelf-life samples. Indeed, cold stored samples for five months (T1) were more distantly plotted from their corresponding shelf-life samples with regards to the samples stored for seven months (T2).

The analysis of the expression pattern between the samples was performed through a hierarchical clustering and visualized as a heatmap, which enabled the distinction of the several DEGs into three main clusters (Fig. 3B), organized according to the K-means clustering

Silhouette method (Supp. Fig. 2). The heatmap visualization of the genes expression profile for each cluster unraveled a distinct behavior in response to storage condition (RA-ULO-DCA) and storage duration (T1-T2).

The cluster A was represented by 3344 DEGs (Supp. Tab. 2) more expressed during the post-storage shelf-life period but showing a specific regulation between T1 and T2. While in T1 all the genes included in this cluster were coordinately stimulated by the restore of the room temperature and regular atmosphere (shelf-life), in T2 these genes started to be actively expressed already during the postharvest cold storage at low oxygen concentration, and further enhanced during shelf-life. The most enriched KEGG pathways identified in this cluster were related to photosynthesis processes (mdm00195, mdm00710 and mdm00196), carbon metabolism (mdm01200), biosynthesis of secondary metabolites (mdm01110), fatty acid metabolism (mdm01212), and plant hormone signal transduction (mdm04075) (Supp. Tab. 3). These pathways were furthermore validated by the Gene Ontology enrichment analysis that identified important biological processes, related to photosynthesis (GO:0015979), oxidation-reduction process (GO:0055114), carboxylic acid metabolic process (GO:0019752), fatty acid metabolic and biosynthetic process (GO:0006631 and GO:0006633, respectively), pyruvate metabolic process (GO:0006090), cell redox homeostasis (GO:0045454), response to reactive oxygen species (GO:0000302) and response to cold (GO:0009409) (Supp. Tab. 4). Related to these processes, we identified genes annotated as *cold shock protein* (LOC103407010), *linoleate 13S-lipoxygenase* (LOC103427024, LOC103418601), *11-beta-hydroxysteroid dehydrogenase 1B-like* (LOC103431105, LOC103407818), *phosphoenolpyruvate carboxylase kinase* (LOC103451062) and *acetyltransferase* (LOC103445094). In addition to this group, a set of transcription factors, such as *MADS box-AGL11* (LOC103441806), *LOB domain* (LOC103401493), *AP2* (LOC103405594) and *cytochrome P450* (LOC103448666, LOC103419418) were also identified, together with two genes involved in chloroplast

299 metabolism, such as *photosystem I chlorophyll a/b-binding protein* (LOC103451376) and
300 *STAYGREEN* (LOC103444765) (Supp. Fig. 3).

301 Cluster B, composed by 2501 DEGs (Supp. Tab. 2), was distinguished by genes only
302 expressed during cold storage in hypoxia conditions (ULO and DCA), in both T1 and T2. This
303 cluster was mainly represented by KEGG pathways involved in biosynthesis of secondary
304 metabolites (mdm01110), glycolysis/gluconeogenesis (mdm00010), pyruvate metabolism
305 (mdm00620), citrate cycle-TCA cycle (mdm00020), fatty acid degradation (mdm00071) and
306 biosynthesis of unsaturated fatty acids (mdm01040) (Supp. Tab. 5). The GO enrichment
307 analysis (Supp. Tab. 6) consistently identified elements involved in biological processes related
308 to response to abiotic stimulus (GO:0009628), generation of precursor metabolites and energy
309 (GO:0006091), glycolytic process (GO:0006096), response to hypoxia (GO:0001666), organic
310 acid metabolic process (GO:0006082), response to temperature stimulus (GO:0009266) and
311 response to cold (GO:0009409). The genes identified within the most enriched categories
312 belong to three mains pathways (Supp. Fig. 4). The first one was represented by elements
313 involved in the glycolysis and the shift from the aerobic to anaerobic respiratory metabolism,
314 such as *glucose-phosphate isomerase* (LOC103440507), *alcohol dehydrogenase*
315 (LOC103432769, LOC103439766, LOC103450330) and *pyruvate decarboxylase*
316 (LOC103425929, LOC103414051, LOC103419936). The second group was represented by
317 regulatory elements assigned to the ethylene responsive factor *ERF* family, such as *ERF003*
318 (LOC103440753), *RAP2-like* (LOC103438804) and *ERF073-like* (LOC103432615). *RAP2* is
319 a transcription factor belonging to the *ERF-VII* family involved in an oxygen-sensing post-
320 translational modification process triggering a molecular acclimation to low oxygen condition.
321 In this regard it is also worth noting the identification of another element playing an important
322 role in this mechanism, the *Plant Cysteine Oxidase (PCO)* gene (LOC103449336) identified
323 within a highly enriched Gene Ontology category (GO:0055114) related to oxidative-reduction

process (Fig. 4). The last group of elements, instead, was represented by genes involved in response to cold, such as *cold-regulated plasma membrane (*LOC103449792), *temperature-induced lipocalin-1-like* (LOC103400012, LOC103431387) and *early response to dehydration* (LOC103432879) (Supp. Fig. 4).

Cluster C, with 2255 genes (Supp. Tab. 2), showed instead an opposite transcriptomic behavior with regards to cluster B. The DEGs of this cluster were in fact more specifically expressed in cold stored RA samples. The difference between samples stored in normoxic and hypoxic condition showed a higher magnitude in T2 rather than in T1. The KEGG analysis (Supp. Tab. 7) identified as the most enriched pathways were involved in the biosynthesis of secondary metabolites (mdm01110), such as phenylpropanoid biosynthesis (mdm00940) and flavonoid biosynthesis (mdm00941), plant-pathogen interaction (mdm04626), glycolysis/gluconeogenesis (mdm00010), amino acids metabolism (*i.e.* tyrosine metabolism-mdm00350, phenylalanine metabolism-mdm00360, phenylalanine, tyrosine and tryptophan biosynthesis-mdm00400, valine, leucine and isoleucine degradation-mdm00280) and fatty acid metabolism (alpha-Linolenic acid metabolism-mdm00592 and fatty acid degradation-mdm00071). These pathways were also represented by the GO enrichment analysis carried out on these elements (Supp. Tab. 8), which identified biological processes related to defense response (GO:0006952), response to biotic stimulus (GO:0009607), response to alcohol (GO:0097305), response to lipid (GO:0033993), lipid metabolic process (GO:0006629), response to hypoxia (GO:0001666), regulation of secondary metabolic process (GO:0043455), flavonoid biosynthetic process (GO:0009813) and response to hormone (GO:0009725). About hormones, the most enriched and represented genes were related to the ethylene biosynthetic, perception and response pathways, such as *ACS*, *ACO* and *ERF* transcription factor. For the regulation of the secondary metabolism, a high number of elements were also identified. Among them it is important to underline the activation in RA samples of *phenylalanine*

ammonia-lyase (*PAL*), *polyphenol oxidase* (*PPO*), *farnesyl pyrophosphate synthase* and *glutathione transferase*. In addition to these elements, also the *cytochrome P450* family was largely represented. This cluster was also distinguished by the expression of a large number of genes encoding for the pathogenesis-related protein (PR-10) known to be the major allergen in apple, such as *Mal d1* and *Pru ar/av 1-like,* and *DMR6-like* elements (Supp. Fig. 5).

3.3 Metabolite profiling

The samples defined in the experimental scheme, were also employed to assess the concentration of three specific categories of metabolites, such as phenols, lipids and volatile organic compounds (VOCs).

The phenols were represented by 18 compounds (Supp. Tab. 9) and their quantification enabled the projection of the different samples over a 2D-PCA plot defined by 2PCs (PC1: 40% and PC2: 19%) (Fig. 5A). The first principal component (PC1) clearly distinguished the set of RA samples from the others stored in controlled atmosphere. This distribution was mainly assigned to the higher concentration of chlorogenic, neochlorogenic acids and vanillin in RA samples (Fig. 6A, B and E). Chlorogenic acid, moreover, showed a higher accumulation in shelf-life samples compared to cold stored apples, for both storage time points, with a fold change of 2.17 and 4.05 for T1 and T2, respectively. On the contrary, ULO and DCA samples were distinguished by a higher concentration of epicatechin and catechin (Fig. 6C and D), with a fold change of 1.56 and 1.4 for T1 and T1 shelf-life, and 2.6 and 2.81 for T2 and T2-SL, respectively.

The second category of metabolite was represented by the targeted characterization of 15 lipid compounds (Supp. Tab. 10). The samples distribution on the lipids-based PCA, defined by the implementation of the first two PCs (PC1: 53%; PC2: 32%; Fig. 5B), was consistent with the plot obtained for the phenolic compounds, with the PC1 distinguishing the normoxic

374 RA samples from those stored under hypoxia conditions (ULO and DCA), and the PC2
375 characterizing cold stored from shelf-life samples, in particular for the samples stored in
376 hypoxia conditions. To this end it is also worth noting a progression of the shelf-life samples
377 according to the storage duration. The T2-SL samples were more distantly plotted from their
378 respective cold-stored samples with regards to T1, which were instead more closely grouped.
379 The distinguished position of the RA samples over the 2D-PCA plot was mainly attributed to
380 the accumulation of specific categories of lipids, such as palmitic, margaric, stearic, arachidic
381 and linolenic acids that were more accumulated in this group of samples stored at regular
382 atmosphere compared to the others, with an averaged fold change spanning from 2.1 to 7.9
383 (Fig. 7). On the contrary, behenic, lignoceric and oleanolic acids were generally more
384 accumulated in the hypoxia set of samples. It is also interesting to note the accumulation of
385 oleic and linoleic acids. Although they were already accumulated during cold storage at T1 and
386 T2 in RA samples, a specific increase in their concentration was observed in the shelf-life
387 period, especially in samples stored at low oxygen (ULO and DCA).
388 The samples comprehended in the experimental design were also employed to
389 characterize the overall volatilome (Supp. Tab. 11). The PCA analysis (carried out employing
390 PC1: 47% and PC2: 25%, Fig. 5C) reveal as also in this class of secondary metabolite, the PC1
391 clearly distinguished the RA samples together with the T2 shelf-life sample, for DCA and
392 ULO, from the rest. Within the 98 VOCs masses profiled, also the hormone ethylene was
393 assessed, which revealed a time dependent regulation (Supp. Fig.6). While at T1 all the samples
394 showed a similar and basal level of ethylene, in T2 a higher concentration was instead observed.
395 For RA and ULO, the re-establishment to normal atmosphere (shelf-life) stimulated an increase
396 and comparable concentration of ethylene, while in DCA the difference in ethylene production
397 between cold stored and post-storage shelf-life samples was lower. Within the volatilome
398 profiling, the α-farnesene and the 6-methyl-5-hepten-2-one (MHO) were specifically assessed

399 (Supp. Fig. 7 and Supp. Tab. 11). Between the two VOCs, MHO resulted to be more correlated
400 with the onset of superficial scald, being more accumulated in the symptomatic RA samples
401 with regards to the other samples, where the development of this disorder was controlled by
402 the low level of oxygen in all the time points considered (T1, T1-SL, T2 and T2-SL). On the
403 contrary, the accumulation of α-farnesene was consistent with the incidence of the superficial
404 scald only at T1, where the accumulation in the RA sample was higher than ULO and DCA,
405 while at T1-SL and T2 it was more accumulated in the ULO samples, whereas at T2-SL it was
406 not observed any significant differences.
407
408 **4. DISCUSSION**
409 ***4.1 The transcriptome is influenced by the oxygen level, length of postharvest storage and***
410 ***post-storage restore of shelf-life room atmospheric conditions***
411 The genome-wide transcriptome was affected by each of the variables implemented in
412 this study: the oxygen concentration in the storage chamber atmosphere and the length of
413 postharvest cold storage together with the post-storage shelf-life. Within each of the two-
414 storage time points (T1 and T2), the greater transcriptional variation was associated with the
415 diminished concentration of oxygen. In both time points, the highest number of DEGs were in
416 fact identified in the comparison between RA and DCA, which is characterized by the lowest
417 level of oxygen, rather than between RA and ULO. The lower number of DEGs was instead
418 detected in the comparison between the two hypoxia postharvest strategies (ULO and DCA),
419 meaning that a reduced level of oxygen from 1% to 0,4% modified only marginally the global
420 transcriptome. Beside the reduction of the oxygen concentration during storage, it is important
421 to underline as the duration of storage and shelf-life had also an important role in the definition
422 of the transcriptomic signature of each sample. This can also be the reason of the different
423 observation proposed by Cukrov et al., (2016), which in a similar comparison between ULO

and DCA storage (on the same cultivar, 'Granny Smith'), a much higher number of genes was reported. Apart from the different tissues considered in the analysis (cortex, while in this work only skin tissues were processed), Cukrov and colleagues profiled the transcriptome after 24 d of storage, while in our study the samples were assessed after five and seven months, respectively, simulating a commercial storage situation. The length of storage has thus an important impact in the postharvest fruit physiology, affecting considerably the global transcriptome variation. To this end, it is also important to underline that in the transcriptome comparison carried out between the two storage time ponts (T1/T2), ULO samples showed a much higher number of DEGs (4548) than DCA (829), indicating as a small reduction in oxygen concentration can in fact determine important variation in the transcriptome, but in a dynamic scenario, thus considering different storage periods. The number of DEGs resulted instead lower in the post-storage shelf-life condition, as effect of the restoration of the normal temperature and atmospheric condition (re-oxygenation).

The actual impact of the length of storage in regulating the genome-wide transcriptome was also depicted through the gene network analysis. The two storage time points (T1 and T2) were in fact characterized by a distinctive gene network-architecture, as well as by a different number and type of unique gene-hub. In addition to this, it is also worth noting that among the set of common nodes, a *ubiquitin conjugate E2* and an *abscisic acid ripening protein 1-like* were identified. The *ubiquitin E2 factor* has been already proposed as a member involved in the conjugation of ubiquitin and SUMO, which has been shown to play important role in the oxygen sensing and response in both plants and mammals (Park et al., 2011; Crozet et al., 2016; Tomanov et al., 2018; Filippopoulou et al., 2020). The other node, represented by the *abscisic acid ripening protein 1-like,* might also have a crucial role in hypoxia adaptation, since oxygen depletion can lead to an enhanced ABA sensitivity (Giuntoli and Perata, 2018).

4.2 Postharvest cold storage induces a transcriptional re-programming oriented to activate an acclimation process to cold stress

The total number of DEGs was organized based on their transcriptional pattern into three main clusters. The first group, cluster A, was represented by 3344 DEGs specifically influenced by the length of storage and shelf-life, regardless the type of atmosphere (normoxia or hypoxia). The replacement of room temperature condition (shelf-life at 20°C) stimulated the transcription of these genes, with a higher magnitude in T2 than in T1. Beside shelf-life, the expression level of these DEGs was also affected by the duration of storage, since at T2 these genes were more expressed than in T1. Within this group, a *cold shock protein C120-like* was found. Cold shock domains are highly conserved among taxa and play important role in preventing the molecular folding of RNA molecules into stable secondary structure, which can in turn impede gene transcription and translation (Sasaki & Imai, 2012). Due to their function, cold shock proteins expressed at the initial response of a cold shock have a primary role in cold adaptation, stimulating the downstream expression of cold inducible genes (Bae et al., 2003). The increased expression of this gene after the application of cold temperature was already observed by Lee et al., (2021), which investigating the role of cold stress in fruit dropping, reported an increased expression of *MdCS120-like* over 24h at room temperature following an initial application of cold. The other genes with a similar expression pattern and involved in lipid metabolism were annotated as *linoleate 13S-lipoxygenase* and *hydroxysteroid dehydrogenase*. While the first element is involved in the oxygenation of fatty acids to produce jasmonates, a class of hormone known to be involved in abiotic stress including cold (González-Aguilar et al., 2000; Ding et al., 2002; Turner et al., 2002), the second is a $NAD^+/NADP^+$ oxidoreductase involved in the metabolism of lipids, especially in the production of very long chain fatty acids (VLCFAs) constituents of cuticular wax (Zhang et al., 2016) and lipid homeostasis. This expression pattern was also consistent with the

metabolite assessment of two specific VLCFAs (behenic acid C22:0 and lignoceric acid C24:0) that resulted to be specifically accumulated in the shelf-life samples, in particular at T2 stage for DCA and ULO samples. Another element supporting a reinforcement of the cuticle in the low oxygen samples is represented by the accumulation of one of its fundamental components, oleanolic acid (Tsubaki et al., 2013; Zhang et al., 2020), in particular at T1 stage. Moreover, this pentacyclic terpenoid is capable of modulating the fluidity and stability of liposomal membranes, thereby enhancing their performance during temperature variations (Han et al., 1997). In the context of plant response and adaptation to environmental stress, it is also worth noting the identification of an *acetyltransferase* gene. This gene is needed to catalyze the acetylation of specific lysine residues on the histone H3 (H3K9 and H3K19) enhancing the transcriptional activity at specific loci related to the activation of abiotic stress related genes, towards the improvement of the thermotolerance mechanism (Abdullah et al., 2022). The re-establishment of normal atmosphere also showed to have an important influence in the plastid metabolism, as supported by the expression of a *photosystem I chlorophyll a/b binding protein* and *STAYGREEN* genes, involved in both chlorophyll antenna complex activity and degradation (Liu et al., 2013; Jiao et al., 2020; Shin et al., 2020). The simultaneous expression of this group of genes indicate that the harvested fruit subjected to low temperature, activate a transcriptional machinery addressed to modulate a series of acclimation processes, possibly coordinated by regulatory genes involved in the response to abiotic stresses.

4.3 Hypoxia conditions during controlled atmosphere storage regulates the change in the central carbon metabolism and triggers the acclimation process to low oxygen

Cluster B was represented by 2501 DEGs strongly influenced by a low concentration of oxygen during storage (ULO and DCA), especially in T1. Among these genes, a group of six elements particularly stimulated by hypoxia condition and belonging to *pyruvate*

499 *decarboxylase* and *alcohol dehydrogenase* gene families, were identified. These two classes of
500 genes are major drivers guiding the shift in the central carbon metabolism, coordinating the
501 transition from the aerobic respiration towards an anaerobic metabolism (fermentation). The
502 catabolism of pyruvate into acetaldehyde (operated by pyruvate decarboxylase) and
503 subsequently into ethanol (through the action of the alcohol dehydrogenase; Supp. Fig. 8) is
504 established by the plant in order to produce energy in case of oxygen deprivation, ensuring
505 basal physiological activity (Perata and Alpi, 1993). In agreement with this mechanism, the
506 volatilome assessment we performed revealed as both acetaldehyde and ethanol were
507 accumulated in ULO and DCA shelf-life samples, but only in T2 (Supp. Fig. 8A and B),
508 additionally validating the role of the length of storage in the control of the general metabolism.
509 It is also important to note that the entire volatilome was however dependent to the oxygen
510 concentration, since all the samples stored in regular atmosphere (RA) and the ULO and DCA
511 samples at T2-SL were clearly characterized by a higher production of VOCs with regards to
512 other samples (Supp. Tab. 12). The highest accumulation of fermentative metabolites in RA
513 samples could be however attributed to a fermentation process initiated by a prolonged storage
514 at room atmosphere (postharvest over-ripening). The highest production of fermentative
515 metabolites, in particular acetaldehyde and ethanol, in ULO-SL and DCA-SL samples was
516 related to an enhanced vaporization of ethanol at higher temperature. Already in the work of
517 Thewes et al., (2023) it was observed that in fruit of 'Maxi Gala' and 'Cripps Pink' apple
518 cultivars, the emission of ethanol was higher at 20°C (the same temperature applied here in the
519 week of room temperature post-storage shelf-life) than at 2°C. In our work, however, the
520 highest detection of these two metabolites was observed at T2 for the RA_SL sample. Although
521 these two VOCs are stimulated by low oxygen as result of the transition from the aerobic
522 respiration to the fermentation, the concentration of ethanol can also be highly induced in over-
523 ripe fruits. Due to a general fruit decay and loss of internal cellular compartmentation, the

deterioration of the mitochondrial membranes can lead to a decreased energy production for a compromised aerobic respiration. In consequence of this phenomenon, a cytoplasm-located anaerobic respiration (not requiring membrane integrity) is further stimulated towards the maintenance of minimal quote of energy production (Pesis et a., 2005). In this cluster, three *ERF* transcription factors, especially *ERF073* and *RAP2-13-like* were also identified. These elements belong to the VII subgroups of *ERFs* that during normoxia are degraded through a protein degradation process guided by the N-end rule mechanism (Licausi et al., 2011; Gibbs et al., 2014). In hypoxia condition, instead, these *ERF* elements trigger the response of hypoxia/anaerobic related genes towards a possible acclimation process to low oxygen availability (Loreti et al., 2018). The existence of a fruit-dependent oxygen-sensing mechanism guiding the acclimation response was also supported by the expression pattern of *PCO,* a *Plant Cysteine Oxidase* gene belonging to a family of plant specific metalloproteins involved in the oxygen sensing machinery coordinating the oxygen-dependent generation of N-degron leading to the proteolysis of ERF-VII protein (Weits et al., 2014). As it has been discovered in *Arabidopsis, PCOs* are induced by hypoxia and *RAP2* and control the lifetime of the ERF-VII protein, by guiding a feedback loop mechanism towards the homeostatic control of the anaerobic response. The *PCO* element identified in this work (LOC1034493), showed a protein-based sequence similarity with hypoxia-inducible *PCO1* (At5G15120.4, $4e^{-71}$) and *PCO2* (At5G33890.1, $7e^{-79}$) in *Arabidopsis* (Weits et al., 2014). This suggests that *PCO* and *ERF-VII* play a fundamental role in the hypoxia response also in apple fruit stored in controlled atmosphere at low oxygen concentration (ULO and DCA).

4.4 The onset of superficial scald is related to the accumulation of chlorogenic acid and its oxidation catalyzed by polyphenol oxidase in an oxygen-dependent manner

548 The cluster C, comprehending 2255 DEGs, showed an opposite behavior with regards
549 to cluster B. This set of DEGs, in fact, resulted specifically expressed in all the RA samples,
550 underlining as this category of genes was highly influenced by the level of oxygen present in
551 the storage atmosphere. Within this set, the most enriched elements were represented by
552 ethylene related genes, involved in both ethylene production (*ACS* and *ACO*) and signal
553 transduction (*ERF*). The ethylene biosynthetic genes were highly expressed in the RA cold
554 stored stages at both T1 and T2, and almost entirely represented by *1-aminocyclopropane-1-*
555 *carboxylate oxidase (ACO)* genes. This is consistent with the mechanism that ACO require
556 oxygen as activator to catalyze ACC into ethylene (Houben & Van de Poel, 2019; Riaño et al.,
557 2022). Similar to these genes, also the *ERF* elements were repressed in the stage characterized
558 by an oxygen limitation. The expression pattern of the ethylene related genes was consistent
559 with the ethylene profile across the several samples. Especially in T2, the RA-SL sample
560 showed in fact the highest production of ethylene, while in T1 it was not observed any
561 significant difference among the samples. The oxygen concentration effectively controlled the
562 amount of ethylene, with ULO being more effective with regards to DCA, but only in T1. It
563 has been already documented that ethylene can control the onset of the superficial scald (Lurie
564 & Watkins, 2012), but the lack of a direct relationship between these two phenomena in T1
565 suggest the possible existence of other additional mechanism. To support the hypothesis that
566 the onset of superficial scald is primarily associated to the role of chlorogenic acid and its
567 oxidation by the polyphenol oxidase, it is important to underline that in this cluster several
568 genes annotated as *polyphenol oxidase* were identified, with a higher expression in RA cold
569 stored samples as well as in shelf-life, and more in T2 than in T1. The effect of the duration of
570 storage on the expression of the *PPOs* also finds consistency with the accumulation pattern of
571 its substrate chlorogenic acid, and the incidence of this disorder. Although it is known that
572 superficial scald is a chilling injury phenomenon, it is also true that it is highly affected by the

time of exposure to low temperature, since prolonged cold storage enhanced the severity of the symptoms. This dependency can thus be attributed to the progressive increase and accumulation of this metabolite during storage and its subsequent oxidation by PPO, although the typical symptom of superficial scald generated by the action of this enzyme (dark coloration) appears only after the establishment of the chilling injury at the early stage of the cold storage. The exposure at low temperature for a long period, beside affecting the stability of the internal membranes, can thus stimulate the accumulation of this polyphenolic compound, which in turn represent the main substrate for the development of this oxidation phenomenon. The accumulation of this hydroxycinnamic acid, together with other compounds such as vanillin, was moreover consistent with other previous works (Busatto et al. 2018 and Vittani et al., 2023) and with the expression profile shown by the cluster of the *phenylalanine ammonia-lyase 1* gene, the key gene upstream the entire pathway of the phenylpropanoids. The expression profile of *PAL* was also consistent with the observation of Cukrov et al., (2016) that reported, for this gene, a higher expression in DCA samples compared to ULO. Our results confirm these observations, additionally supporting the effect of the length of storage on the transcriptome variation. The accumulation of polyphenolic compounds can also represent a response mechanism operated by the fruit to prevent oxidative damage. To this end, it is also worth noting the accumulation in the asymptomatic samples of polyphenolic compounds with known antioxidant activity, such as catechin and epicatechin. The wide response mechanism activated by the fruit to prevent damage from abiotic stress can also be largely coordinated by the *cytochrome P450*, a class of oxidoreductase involved in many responsive pathways (Pandian et al., 2020). The active coordination of this mechanism also in stored apples, is supported by the finding that *cytochrome P450* represents one of the largest categories of elements identified in this cluster. Another class of genes highly expressed in this group was represented by the *glutathione S-transferase*. In plants, glutathione, within the ascorbate-

598 glutathione cycle, play a fundamental role as antioxidant molecule towards the scavenging of
599 ROS molecules (especially H_2O_2) and the maintenance of the cellular redox state (Noctor &
600 Foyer, 1998; Hasanuzzaman et al., 2019). The mode of action of this gene family observed in
601 this work is consistent with previous findings reporting an increased level of glutathione in
602 scalded apple of 'Granny Smith' apple cultivar (Zermiani et al., 2015; Karagiannis et al., 2018,
603 2020). In these studies, the level of glutathione, or genes involved in its metabolism, resulted
604 to be repressed by the application of the ethylene competitor 1-MCP. The level of glutathione
605 can therefore be related to the amount of ethylene despite the mechanism of regulation, either
606 controlled by the exogenous application of its competitor or the decreased concentration of
607 oxygen interfering with the activity of ACO. The RA samples were also distinguished by a
608 higher expression of *Mal d-1 like* and *Pru av 1-like* genes. *Mal d-1* refers to a pathogen-related
609 protein and allergen in apple, but its involvement in abiotic stress and accumulation during
610 postharvest has been also recently reported (Matthes & Schmitz-Eiberger, 2009; Ahammer et
611 al., 2017). *DMR-6 LIKE OXYGENASE* is instead a gene of susceptibility negatively affecting
612 pant defense. Although its role is more related to pathogenic process, its involvement has also
613 been recently documented during abiotic stresses (de Toledo Thomazella et al., 2021). The
614 expression of these elements, specifically in the symptomatic samples, valorize the hypothesis
615 that the development of superficial scald and its prevention involve a complex of regulatory
616 mechanism, leading to a specific transcriptome signature related to the physiological
617 predisposition of the fruit to develop this disorder or triggering specific networks towards its
618 prevention.
619

620 **5. CONCLUSION**

621 Postharvest storage is nowadays a fundamental step in the entire horticultural production chain
622 to reduce/avoid wastage while promoting fruit security. The improvement of storability is

however not exempted by important drawbacks, such as the development of superficial scald that can seriously limit the consumers' appreciation of apple. The storage in controlled atmosphere is representing a valuable alternative to other chemistry-based methods currently applied. With this work we attempted to provide additional knowledge about the transcriptomic and metabolite re-programming occurring in the fruit during storage and oriented to induce an active protecting mechanism to superficial scald and adaptation to low oxygen. The information presented here can enable a better comprehension of the physiological processes occurring in stored apples towards a promotion of the postharvest quality.

CRediT authorship contribution statement

Fabrizio Costa, Angelo Zanella, Nicola Busatto: Conceptualization, Design, Planning; **Francesca Populin, Lorenzo Vittani, Stefan Stuerz, Ilaria Folie, Iulia Komhenko, Franco Biasioli, Domenico Masuero, Urska Vrhovsek, Nicola Busatto:** Formal analysis, Investigation, Visualization; **Matthias Scholz:** Bioinformatic analysis; **Fabrizio Costa, Lorenzo Vittani, Nicola Busatto, Francesca Populin:** Writing - Original Draft; **Fabrizio Costa:** Conceptualization, Resources, Supervision, Funding acquisition; **All Authors:** Reviewing and editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data are available as supplementary files. RNA-Seq data have been deposited and made available at the GEO database (accession number: GSE225233).

Acknowledgment

This research was funded by the European Region Tyrol-South Tyrol-Trentino (EGTC) through the Euregio Science Fund, project Sald_Cold - IPN 118, 3rd call 2017. The authors also thank Assomela for supporting the PhD program of Lorenzo Vittani within the project PhD Apple.

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Figure legends

Figure 1.

Superficial scald incidence in fruit of 'Granny Smith' across the different storage conditions and time points. Fruit were stored following different strategies (RA: room atmosphere, ULO: ultra-low oxygen; DCA: dynamic controlled atmosphere) for five (T1) and seven (T2) months, respectively. The post-storage period of seven days of shelf-life (SL) at room temperature was also considered. (Color image available only in the online version).

Figure 2.

Genes expression network profiling for the core set of differentially expressed genes in T1 **(**five months of storage, **A)** and T2 **(**seven months of storage, **B)**. Positive and negative correlation are evidenced with solid and dotted lines, respectively. Red nodes were used for the hub genes, while light blue color indicate common nodes between the two networks. For the network the most relevant 150 edges have been considered. (Color image available only in the online version).

Figure 3.

Visual representation of the transcriptomic variability across different storage conditions. In panel **(A)** Principal Component Analysis (PCA) performed on all the differentially expressed genes (DEGs) is illustrated. In panel **(B)** is instead depicted the heatmap showing the expression profile of DEGs in the two time points (storage duration) in response to different storage conditions and shelf life (SL). The three clusters were defined on the base of the K-mean cluster analysis (Cluster A, B and C). RA= Regular Atmosphere; ULO= Ultra Low Oxygen; DCA= Dynamic Controlled Atmosphere condition. T1 and T2 are samples stored for five and seven months, respectively. SL = shelf-life. (Color image available only in the online version).

Figure 4.

Expression analysis of *Plant Cysteine Oxidase* (PCO) in 'Granny Smith'. The expression was reported considering five (T1) and seven (T2) months of storage and seven days of shelf-life (SL). The error bars correspond to the standard deviation. RA= Regular Atmosphere; ULO= Ultra Low Oxygen; DCA= Dynamic Controlled Atmosphere condition. FPKM= fragments per kilobase of transcript per million fragments mapped. (Color image available only in the online version).

Figure 5.

Principal Component Analysis (PCA) plot illustrating the whole variability of the different classes of metabolites. In panel **(A)**, **(B)** and **(C)** are illustrated the PCA lots for polyphenols, lipids and volatile organic compounds (VOCs), respectively. RA= Regular Atmosphere; ULO= Ultra Low Oxygen; DCA=Dynamic Controlled Atmosphere condition. T1 and T2 are samples stored for five and seven months, respectively. SL = seven days of shelf-life. Each sample

plotted is the mean value of three biological replicates. (Color image available only in the online version).

Figure 6.

Quantification of phenolic compounds. Analysis of the concentration of Chlorogenic acid **(A)**, Neochlorogenic acid **(B)**, Epicatechin **(C)**, Catechin **(D)** and Vanillin **(E)** in 'Granny Smith' samples over the storage period. The concentration was express as $\mu g\ g^{-1}$ of fresh weight. RA= Regular Atmosphere; ULO= Ultra Low Oxygen; DCA= Dynamic Controlled Atmosphere condition. T1 and T2 correspond to five and seven months of storage respectively. SL= shelf-life. The error bars correspond to the standard deviation. Different letters indicate statistically significant differences. (Color image available only in the online version).

Figure 7.

Characterization of lipids content in fruit of Granny Smith during storage. The differential lipids accumulation during the different storage condition and conservation period (T1 = five months and T2= seven months) is depicted in the heatmap. RA= Regular Atmosphere; ULO= Ultra Low Oxygen; DCA= Dynamic Controlled Atmosphere condition. SL= shelf-life. The concentration was calculated as $\mu g\ g^{-1}$ of fresh weight. (Color image available only in the online version).