Postharvest Biology and Technology 117 (2016) 9–20

Contents lists available at ScienceDirect

Postharvest Biology and Technology

journal homepage: www.elsevier.com/locate/postharvbio

Dynamics of metabolic adaptation during initiation of controlled atmosphere storage of 'Jonagold' apple: Effects of storage gas concentrations and conditioning

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ARTICLE INFO

Article history:
Received 30 October 2015
Received in revised form 25 January 2016
Accepted 1 February 2016
Available online xxx

Keywords:
Metabolic profiling
Metabolomics
Controlled atmosphere
Primary metabolism
'Jonagold'

ABSTRACT

Controlled atmosphere (CA) storage reduces respiration, ethylene production and related biochemical and physiological changes. The dynamics of the metabolic changes accompanying initial periods of CA storage of 'Jonagold' apples have been studied. The apples were exposed to 1 kPa O_2, 3 kPa CO_2; 3 kPa O_2, 3 kPa CO_2; 1 kPa O_2, 10 kPa CO_2 and air (20.8 kPa O_2, 0.03 kPa CO_2) was used as a control. The effect of air storage preceding CA storage was also investigated. In response to CA, metabolic changes were observed in glycolysis, tricarboxylic acid cycle, amino acids and other metabolites linked with these pathways. In general, stress response patterns of immediate and delayed CA stored apples were similar. Aspartate and 1-aminocyclopropane-1-carboxylic acid were positively correlated with O_2 concentration during the first two days and after one week of storage, respectively, while glucose-6-phosphate and some amino acids such as proline, alanine and threonine were negatively correlated with O_2 concentration. Glutamate and succinate were correlated with CO_2 concentration. Galactinol substantially increased with storage time. The observed metabolic changes are discussed in the context of adaptation mechanisms of the fruit to CA storage.

1. Introduction

Controlled atmosphere (CA) storage is predominantly used to prolong the storage life of apples by reducing respiration, ethylene production and the associated biochemical and physiological changes. The tolerance of the fruit to CA depends on several factors including cultivar, maturity, concentrations of O_2 and CO_2, length of storage time (Bai et al., 2005; Bulens et al., 2012; Watkins, 2000) and timing of CA application (Elgar et al., 1998). As suboptimal harvest timing and storage conditions adversely affect the fruit quality, these parameters need to be optimised to maintain as much as possible the initial quality attributes of the fruit while inducing minimal stress. To this end, fundamental understanding of the biochemical processes involved in adaptation to CA is of vital importance.

Temperature and gas concentrations (O_2 and CO_2) affect respiratory metabolism in several ways: by increasing or decreasing the activity of pre-existing enzymes, by inducing or suppressing the biosynthesis of new enzymes, or directly participating as a substrate and as product of the reaction (Geigenberger, 2003; Plaxton and Podestá, 2006). Low temperature reduces the activity of biochemical reactions including respiration and ethylene production (Duque et al., 1999; Kruse et al., 2011; Saltveit, 1999). However, depending on the length of the storage period, low temperature is known to stimulate ethylene production in some apple cultivars such as Braeburn, although the increase in ethylene production is still considerably lower than ethylene production at higher temperature (Tian et al., 2002). In addition to temperature, the metabolism of the fruit could be reduced by lowering the O_2 concentration in the storage atmosphere. O_2 serves as a final electron acceptor in the mitochondrial electron transport chain (mETC), and it is linked to glycolysis and the tricarboxylic acid (TCA) cycle through regeneration of nicotinamide adenine dinucleotide (NAD). O_2 is also required for the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene. On the other hand, CO_2 induces cytoplasmic acidification which impacts the activity of several enzymes. High concentrations of CO_2 have been shown to inhibit the activities of cytochrome c oxidase (COX) (Gonzàlez-Meler et al., 1996), TCA cycle dehydrogenases (Mathooko, 1996), and glycolytic

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http://dx.doi.org/10.1016/j.postharvbio.2016.02.003
0925-5214/

enzymes (Kerbel et al., 1988), while increasing the activity of enzymes involved in the fermentative pathway (Saquet and Streif, 2008). In addition, CO_2 is known to regulate the activity of ACC synthase (ACS) and ACC oxidase (ACO) in pears (De Wild et al., 2003; Gorny and Kader, 1997). CA has also been shown to affect the activities of enzymes involved in antioxidant metabolism (Larrigaudiere et al., 2001).

In addition to the CA conditions employed in storage, the timing of CA induction also influences the response of fruit to CA. In apples, immediate initiation of CA has been shown to result in a better quality of apples, even though, delayed application of CA has been shown to be beneficial under some circumstances. Delayed CA application decreased incidence and severity of flesh browning (De Castro et al., 2007; Saquet et al., 2003), core breakdown in 'Conference' pears (Verlinden et al., 2002), and external CO_2 injury in 'Empire' apples (Watkins et al., 1997). These studies highlight that the first few weeks of storage might have a large impact on the stress tolerance of apples.

In postharvest application, metabolic profiling has been widely applied to study the response of plants to diverse environmental perturbations including temperature (Kaplan et al., 2004), low O_2 concentration (Ampofo-Asiama et al., 2014; Pedreschi et al., 2009), oxidative stress (Baxter et al., 2007) and storage disorders (Lee et al., 2012; Leisso et al., 2015; Rudell et al., 2009; Vandendriessche et al., 2013). Recently, we applied a similar approach to reveal ethylene mediated metabolic changes during ripening and low O_2 stress (Bekele et al., 2015). The study showed reconfiguration of central metabolism towards maximising energy production as the apples were introduced to CA (1 kPa O_2, 3 kPa CO_2). In the current study, the separate role of O_2 and CO_2 on central metabolism was investigated by including additional CA conditions.

The objectives of this study were (i) to gain insight into the biochemical basis for the effect of various CA conditions on the primary metabolism of 'Jonagold' apple, and (ii) to study the effect of air storage preceding CA initiation on the apple's metabolic response.

2. Materials and methods

2.1. Plant material and experimental design

Apple fruit (*Malus* × *domestica* Borkh., cv. Jonagold) were harvested from a commercial orchard in Rotselaar, Belgium on September 9, 2011, during the optimal harvest window determined by Flanders Centre of Postharvest Technology (VCBT, Heverlee, Belgium).

To obtain a homogeneous sample, the fruit were picked from the same side of the trees and were sorted for uniform size. For gas measurement and metabolite analysis eight and five individual fruit were used as a biological replicates, respectively.

The experiment consisted of two parts: (i) at the same day of harvest, the apples were immediately transferred into one of the following three CA conditions (1 kPa O_2, 3 kPa CO_2; 3 kPa O_2, 3 kPa CO_2 and 1 kPa O_2, 10 kPa CO_2) at 1 °C. Fruit stored in air (20.8 kPa O_2, 0.03 kPa CO_2) at 1 °C were used as a control. Samples were collected immediately after harvest and after 1, 2, 8, 15, 19, 26 and 36 days of CA storage. (ii) the apples were transferred to the same three CA conditions as above after two weeks of air storage at 1 °C. Fruit stored in air (20.8 kPa O_2, 0.03 kPa CO_2) at 1 °C were used as a control. Samples were collected after 1, 2, 7, 15 and 23 days of CA storage. The control apples were sampled on day 1, 4, 7, 14, 15, 16, 19, 21, 26, 29, 36 and 37 of storage.

The CA conditions were established by maintaining a continuous flow of humidified gas mixture which was prepared by mixing pure gases using automatic gas mixing panels (Ho et al., 2007). The apples were placed inside six litres airtight plastic jars and continuously flushed with the targeted composition. To avoid interruption of the CA atmosphere while sampling, the jars were connected in series and samples were always taken from the last jar leaving the others intact.

2.2. Gas measurements

Respiration and ethylene production rates were measured by analysing the headspace of individual intact apples kept inside 1.7 L air tight jars fitted with inlet and outlet connection using a compact gas chromatograph (Interscience, Louvain La Neuve, Belgium) as previously described by Bulens et al. (2011). Briefly, after an initial flushing period of three hours at 1 °C with the target gas composition, the gas flow was stopped and the gas composition inside the jars was measured. After an additional 14–16 h incubation period at 1 °C, the changed gas composition was measured again. The respiration and ethylene production rates were calculated by taking the difference of the gas measurements between the two time points and normalized by the weight of the individual apples and incubation time.

2.3. Metabolite analysis

The fruit were cut longitudinally, from stem end to calyx-end, at two opposite sides of the apple, and samples were collected from the outer cortex of the middle slice using a four mm diameter cork borer. The samples were frozen by immediately immersing in liquid nitrogen and were stored at −80 °C until further analysed. The frozen tissue was crushed to a fine powder using a mixer mill (MM200, Retsch, Haan, Germany). The extraction and derivatization steps were conducted using a recently optimized gas chromatography–mass spectrometry (GC–MS) protocol for the analysis of polar metabolites from apple tissue (Bekele et al., 2014).

Briefly, the metabolites were analysed using a GC 7890A coupled with 5975C MS mass selective detector (Agilent Technologies, Palo Alto, CA, USA) equipped with an auto-sampler. The GC separation was performed on a 30 m HP-5MS column (Supelco, Bellefonte, CA, USA) with 0.25 mm internal diameter and 0.25 μm film thickness. The samples were volatilized at the injection temperature of 220 °C inside a deactivated glass liner (SGE Analytical Science, Australia). The mass selective detector (MSD) was operated in electron ionization mode with quadrupole mass analyser and source temperature maintained at 150 and 230 °C respectively. The mass spectra ranging from m/z 30 to m/z 600 were recorded. The identification of compounds was performed by comparing retention indexes and the deconvoluted mass spectra (AMDIS, National Institute of Standards, Gaithersburg, MD, USA) against an in-house built mass spectra library and Fiehn (Kind et al., 2009) and NIST (National Institute of Standards and Technology, Gaithersburg, MD, USA) commercial libraries. The metabolites were quantified based on the peak area of selected characteristic ions for the respective compounds using the MSD ChemStation software (Agilent Technologies, Palo Alto, CA, USA). To normalise for small variations due to derivatization and GC–MS analysis, phenyl-β-D-glucopyranoside and 3-(4-hydroxyphenyl) propionic acid were used as internal standards for high and less abundant metabolites, respectively. In addition to the internal standards, peak areas were also normalized by the fresh weight of the sample. Quality control samples were included with each injection sequence containing around 20 samples. These include a solvent blank containing hexane, a method blank which contains all the solvents used for analysis and quality control (QC) sample representative of all the samples. The QC samples were run after every five samples and were used to correct for small gradual decrease in instrument response. This was done by dividing the daily average peak area of the particular metabolite in the QC

samples by the average peak area of that same metabolite across all QC samples. The inlet liner and septum were monitored daily for contamination and changed roughly every 40 injections.

2.4. Statistical analysis

In order to visualize the structure of the data set, principal component analysis (PCA) and partial least square discriminant analysis (PLS-DA), a supervised regression method, were employed using Unscrambler® X software (version 10.3, CAMO A/S, Trondheim, Norway). The variables were mean centred and weighted by their standard deviations. Metabolites were used as predictor variables while the concentrations of O_2 and CO_2, and storage time were used as response variables. Important variables to discriminate between the CA conditions and storage time were selected based on jack-knife based resampling (Martens and Martens, 2000). Subsequently, metabolites with stable ($p < 0.05$) regression coefficient were selected to display structured variations in the PLS-DA biplot. Moreover, a one way ANOVA was conducted to investigate which metabolites were significantly different between the immediate and delayed CA stored apples.

3. Results

The physiological status of the apples was assessed by measuring the respiration and ethylene production rates (Fig. 1). Low ethylene production rate of fruit at harvest remained low in immediate CA fruit (Fig. 1A), except for the increase observed in apples stored at 3 kPa O_2 and 3 kPa CO_2. In contrast, ethylene production increased in air stored apples. While delayed application of CA gradually arrested ethylene production of apples stored at 3 kPa CO_2, the effect was stronger for apples stored at 10 kPa CO_2 (Fig. 1B). As compared to immediate CA stored apples, delayed CA stored apples had higher ethylene production, particularly in apples stored at 1 kPa O_2, 3 kPa CO_2 and 3 kPa O_2, 3 kPa CO_2 during the first two weeks of CA storage.

Apple stored under air had higher respiration rate than apples stored in CA in both immediate and delayed applications (Fig. 1C and D). In both groups, the respiration rates of the apples stored under the three CA conditions were similar.

3.1. Metabolic response to immediate CA storage

Polar metabolites from diverse metabolic classes including organic acids, amino acids, sugars, sugar phosphate, sugar alcohols

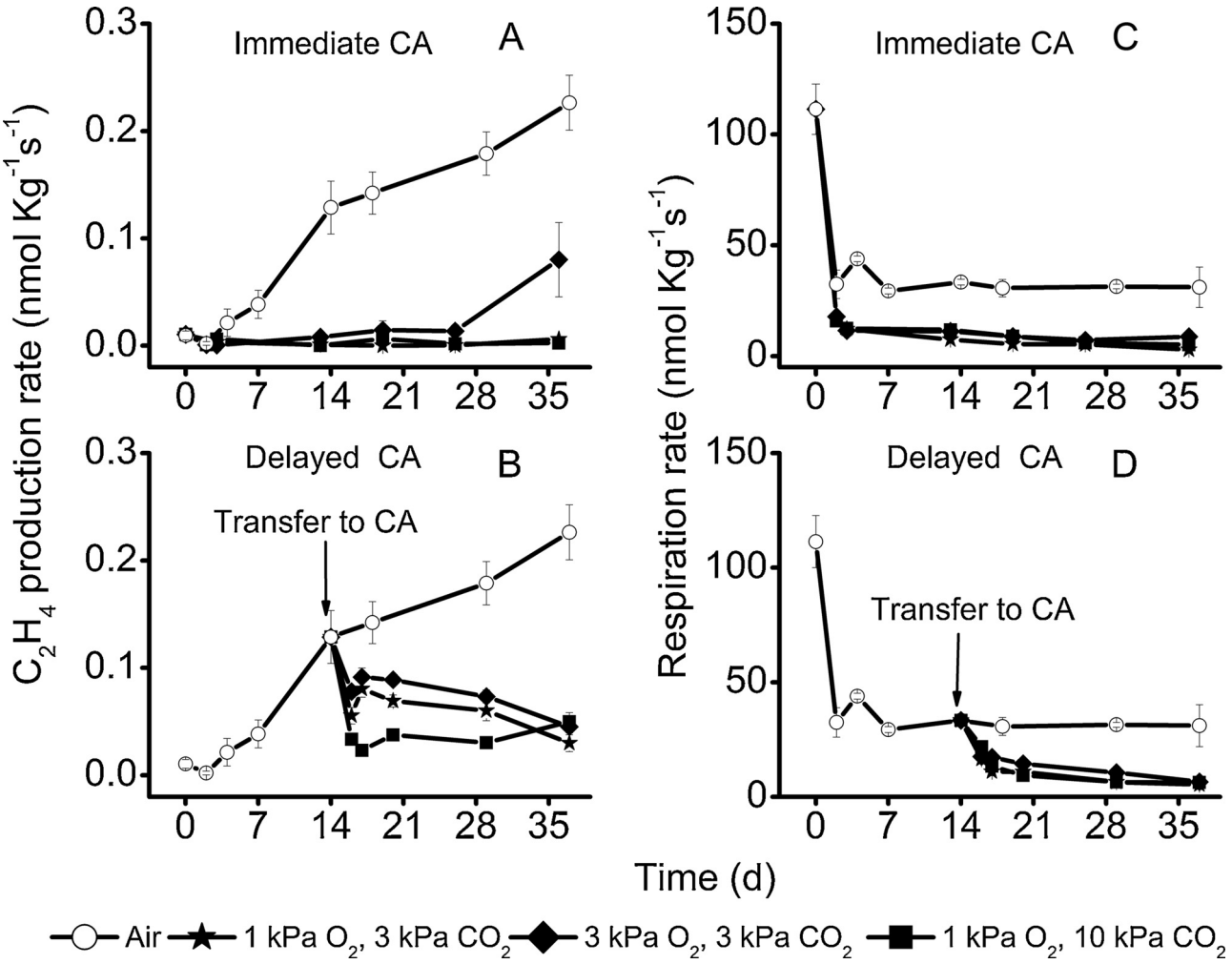

Fig. 1. The respiration (O_2 consumption) and ethylene production rates of 'Jonagold' apples stored in 1 kPa O_2, 3 kPa CO_2; 3 kPa O_2, 3 kPa CO_2; 1 kPa O_2, 10 kPa CO_2 and air (20.8 kPa O_2, 0.03 kPa CO_2) at 1 °C. The top (A and C) and the bottom (B and D) figures correspond to apples that were transferred to CA storage immediately after harvest and after two weeks of air storage, respectively. The error bars represent standard errors of the mean of 8 individual apples.

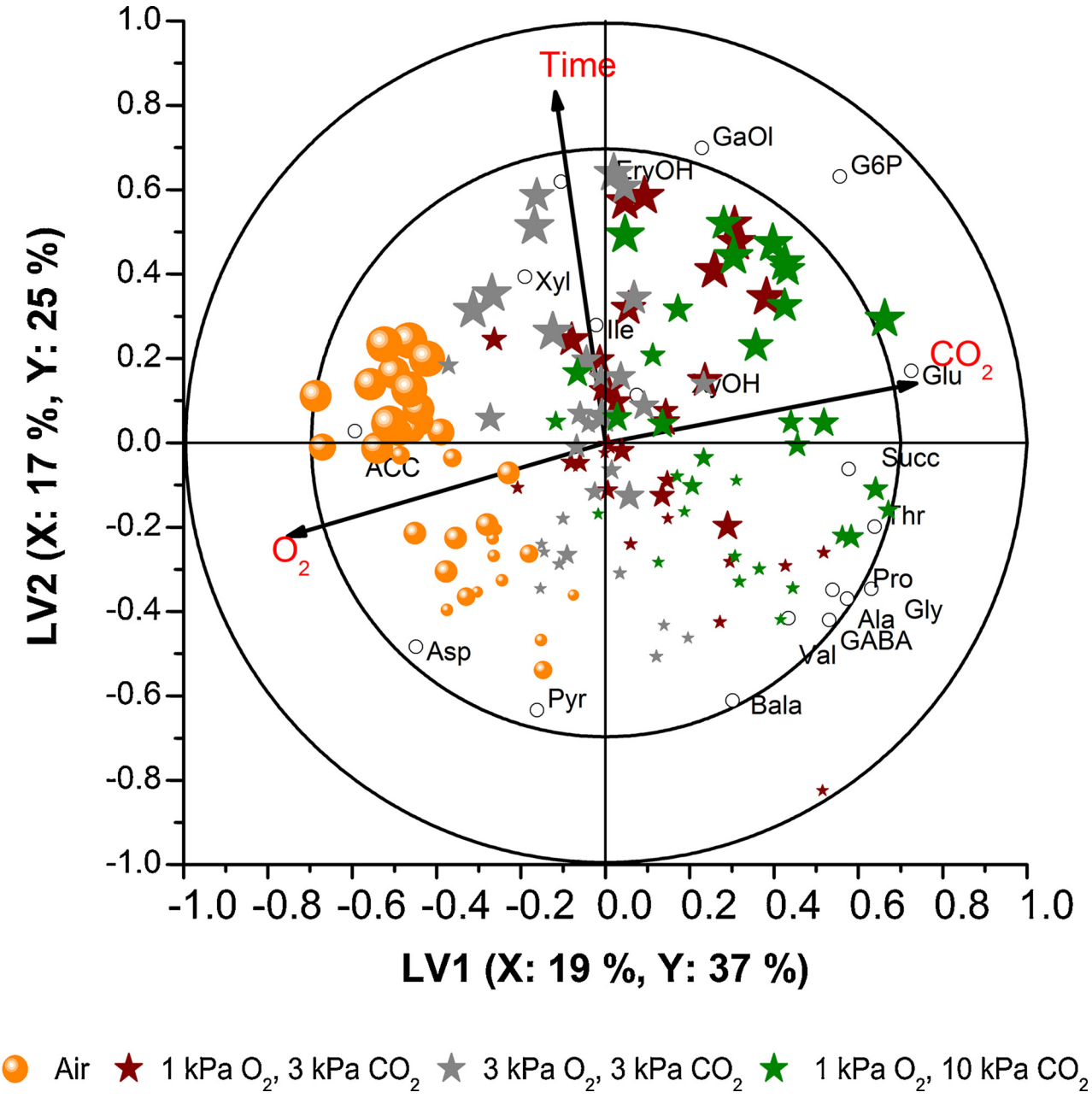

Fig. 2. The PLS-DA biplot of the metabolic profiles of 'Jonagold' apples immediately stored in 1 kPa O_2, 3 kPa CO_2; 3 kPa O_2, 3 kPa CO_2; 1 kPa O_2, 10 kPa CO_2 and air (20.8 kPa O_2, 0.03 kPa CO_2) at 1 °C for a period of five weeks. The metabolites were selected based on the stability ($p < 0.05$) of the PLS-DA model regression coefficients (Martens and Martens, 2000). The spheres and stars represent apples stored under air and CA respectively. The scores (closed symbols) and loadings (open symbols) are superimposed. The size of the symbols denotes the length of the storage period.
Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; Ala, alanine; Asp, aspartate; Bala, β-alanine; G6P, glucose-6-phosphate; GABA, γ-aminobutryic acid; GaOl, galactinol; Glu, glutamate; Gly, glycine; Ile, isoleucine; LV, latent variable; Pro, proline; Pyr, pyruvate; Succ, succinate; Thr, threonine; EryOH, erythritol; Val, valine; Xyl, xylose; XyOH, xylitol.

and polyphenols were detected and quantified. The PCA score plots of immediate and delayed CA stored apples are provided in Supplementary Fig. S1 and S2. The plots show the separation of air stored apples from CA stored apples. In order to further investigate the relationship between the metabolites and the response variables, PLS-DA was conducted. The PLS-DA (Fig. 2) on metabolic profiles of the fruit revealed the diverse patterns of metabolic responses to the imposed CA conditions and storage time. The scores showed the clear separation between apples stored in air (spheres) and CA (stars) based on their metabolic profile. Generally, closely clustered scores suggest a similarity in the metabolic profile of the apples involved. The correlation loadings revealed the correlation of the metabolites with the main factors (level of O_2, level of CO_2, storage time). However, as the y-loadings for low O_2 concentration (opposite direction to the displayed line arrow for O_2) and y-loadings for high CO_2 concentration pointed almost exactly towards the same direction, the effect of O_2 and CO_2 might not be completely separable from the PLS-DA biplot alone.

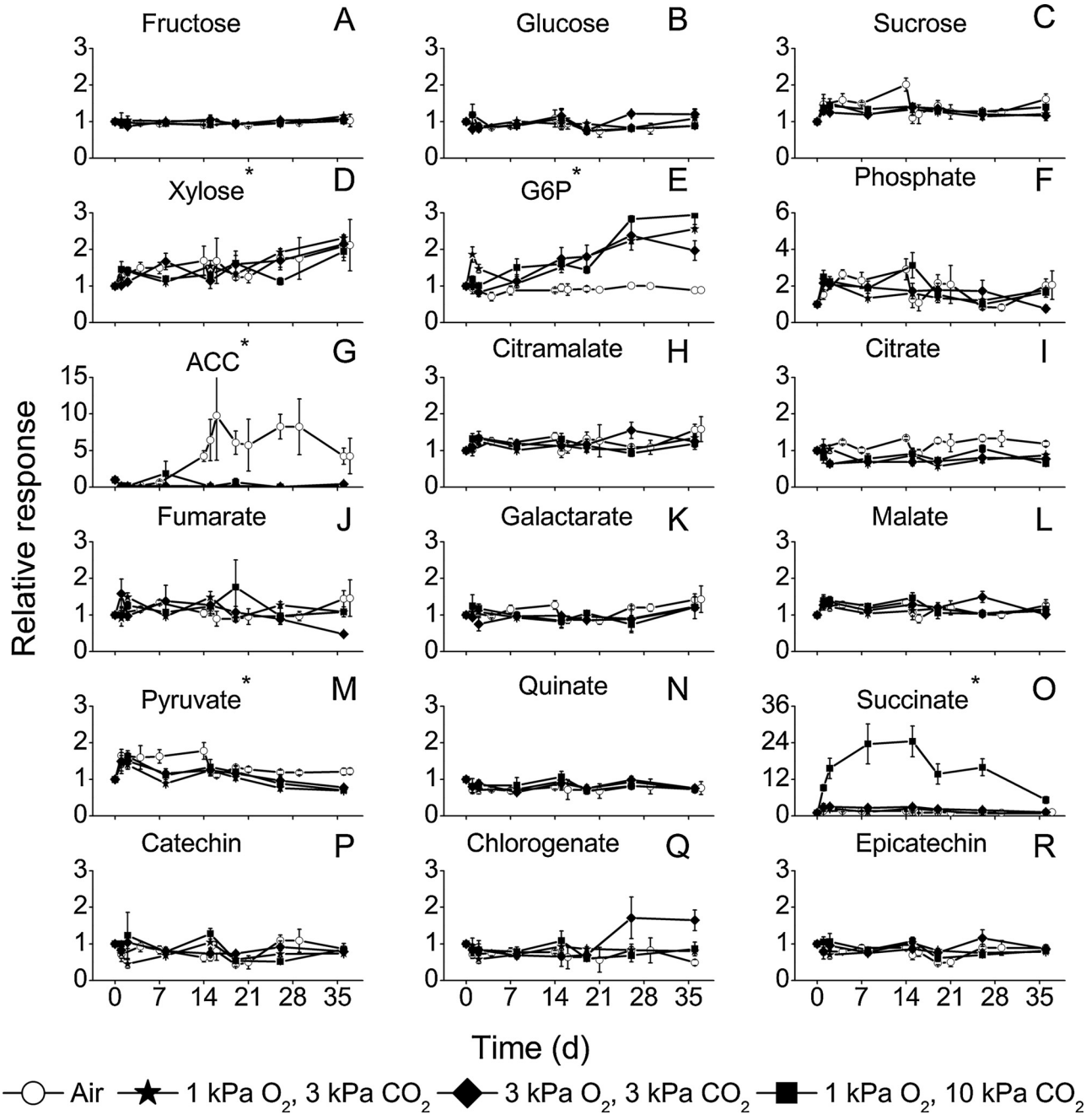

Fig. 3. Dynamic metabolic changes of 'Jonagold' apples immediately stored in 1 kPa O_2, 3 kPa CO_2; 3 kPa O_2, 3 kPa CO_2; 1 kPa O_2, 10 kPa CO_2 and air (20.8 kPa O_2, 0.03 kPa CO_2) at 1 °C for a period of five weeks. Metabolites are ordered alphabetically in the order of their metabolic classes (sugars, G6P, organic acids and polyphenols). Metabolites with stable ($p < 0.05$) PLS-DA model regression coefficients (Martens and Martens, 2000) are marked with asterisk. The relative responses denote the metabolites concentration normalized by their concentration at harvest. The error bars represent the standard error of the mean of 5 individual apples.
Abbreviation: ACC, 1-aminocyclopropane-1-carboxylic acid; G6P, glucose-6-phosphate.

Additional information was required to dissect the two effects, and is provided in the detailed time profiles of the metabolites (Figs. 3 and 4).

While some metabolites were directly associated with the concentrations of O_2, concentrations of CO_2 and storage time (Fig. 2), the interaction between the gas concentrations and storage time also affected most metabolites.

Aspartate and ACC were positively correlated with O_2 concentration at beginning of storage and after one week of storage, respectively. In contrast, glucose-6-phosphate (G6P) was negatively correlated with O_2 concentration. Similarly, threonine, proline, glycine, alanine, γ-aminobutyrate (GABA) and valine were negatively associated with O_2 particularly at the onset of CA. Glutamate and succinate were positively associated with CO_2 concentration. On the other hand, galactinol, erythritol, G6P and xylose were positively correlated with storage time in low O_2 concentration stored apples, while pyruvate and β-alanine showed a negative correlation.

In apples stored under different CA conditions, small differences were observed in sugars, some organic acids and polyphenols (Fig. 3). However, clear divergence of stress responses was observed in other metabolites. In contrast to a sharp increase of

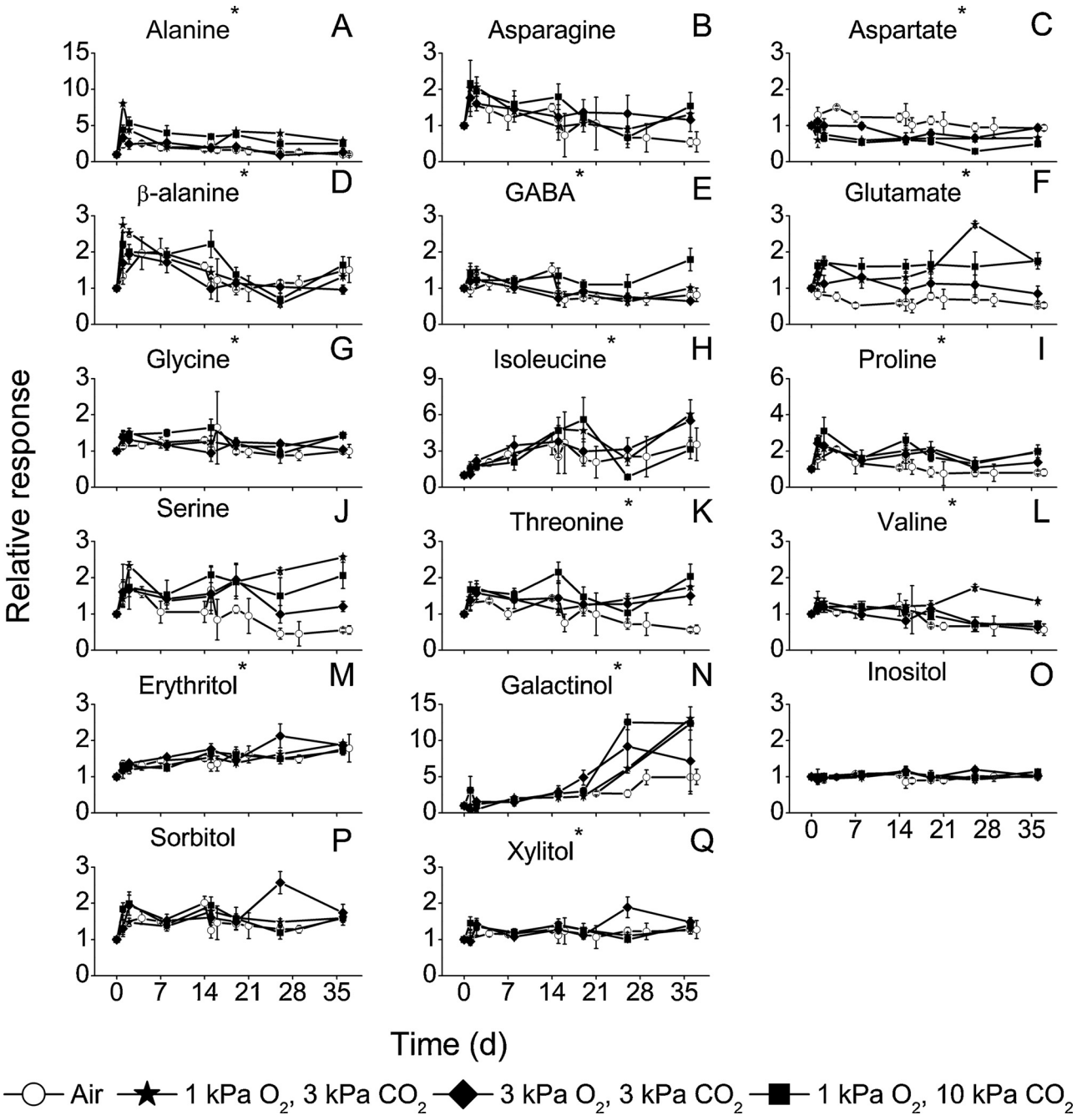

Fig. 4. Dynamic metabolic changes of 'Jonagold' apples immediately stored in 1 kPa O_2, 3 kPa CO_2; 3 kPa O_2, 3 kPa CO_2; 1 kPa O_2, 10 kPa CO_2 and air (20.8 kPa O_2, 0.03 kPa CO_2) at 1 °C for a period of five weeks. Metabolites are ordered alphabetically in the order of their metabolic classes (amino acids and sugar alcohols). Metabolites with stable ($p < 0.05$) PLS-DA model regression coefficients (Martens and Martens, 2000) are marked with asterisk. The relative responses denote the metabolites concentration normalized by their concentration at harvest. The error bars represent the standard error of the mean of 5 individual apples.
Abbreviation: GABA, γ-aminobutryic acid.

ACC (Fig. 3G) in air stored apples, apples stored in CA maintained lower levels of ACC throughout the storage period. Also, apples stored under air had higher level of pyruvate (Fig. 3M) as compared to CA stored apples. One of the most notable changes in the metabolic profile of apples stored under high CO_2 (1 kPa O_2, 10 kPa CO_2) environment was the accumulation of succinate (Fig. 3O). As compared to decreasing the O_2 concentration from 21 kPa to 1 kPa, increasing the CO_2 concentration from 3 kPa to 10 kPa had a remarkable effect on the accumulation of succinate. Succinate increased drastically during the first week of storage, levelled off during the second week and declined afterwards. In addition to the effect of the gas concentrations, some metabolites exhibited strong association with storage time. While xylose (Fig. 3D) increased with storage time irrespective of storage conditions, G6P increased steadily in all three CA conditions but not under air storage (Fig. 3E).

Apples stored under 10 kPa CO_2 had higher levels of alanine (Fig. 4A) and GABA (Fig. 4E) as compared to apples stored in air. While aspartate (Fig. 4C) rapidly declined and stabilized in apples stored at 1 kPa O_2 at both 3 kPa and 10 kPa CO_2, it decreased gradually in apples stored at 3 kPa O_2 and air. β-alanine (Fig. 4D) increased at the onset of CA but declined gradually with storage

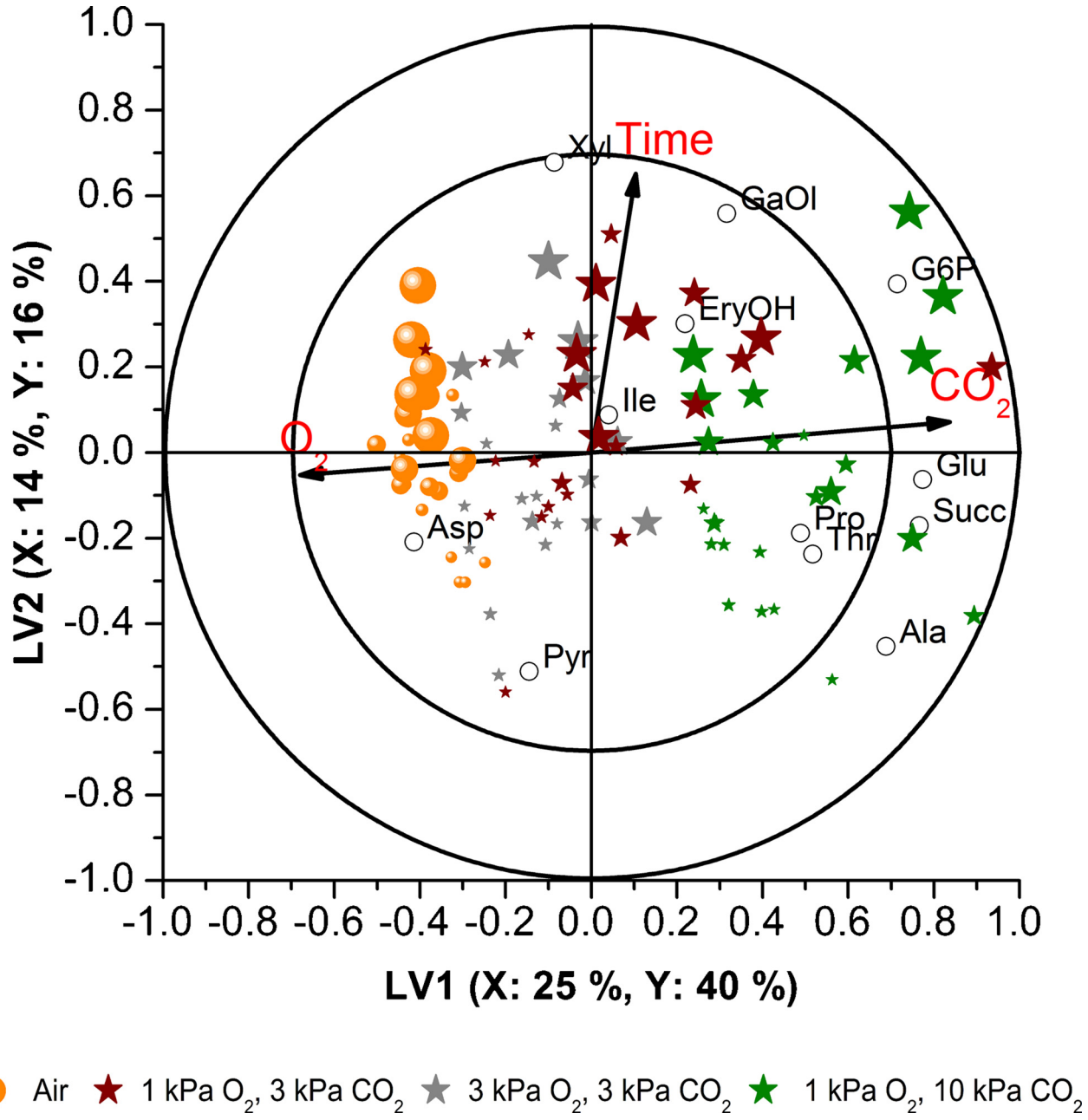

Fig. 5. The PLS-DA biplot of the metabolic profiles of 'Jonagold' apples stored in 1 kPa O_2, 3 kPa CO_2; 3 kPa O_2, 3 kPa CO_2; 1 kPa O_2, 10 kPa CO_2 and air (20.8 kPa O_2, 0.03 kPa CO_2) at 1 °C for three weeks after two weeks of air storage at 1 °C. The metabolites were selected based on the stability ($p < 0.05$) of the PLS-DA model regression coefficients (Martens and Martens, 2000). The scores (closed symbols) and loadings (open symbols) are superimposed. The spheres and stars represent apples stored under air and CA respectively. The size of the symbols denotes the length of the storage period.
Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; G6P, glucose-6-phosphate.

time. Unlike aspartate, the level of glutamate (Fig. 4F) in air stored apples was lower than in CA stored apples. As compared to apples stored at 1 kPa O_2, apples stored at 3 kPa O_2 had lower glutamate level, particularly after two weeks of storage. Similarly, apples stored under air had lower level proline (Fig. 4I) and threonine (Fig. 4K) particularly during later storage periods. On the other hand, while erythritol (Fig. 4M) increased with storage time irrespective of storage conditions, galactinol (Fig. 4N) steeply increased in apples stored under CA. Isoleucine (Fig. 4H) tends to increase with storage time without showing a clear pattern.

3.2. Metabolic response to delayed application of CA

Similar to metabolic responses of the fruit to immediate CA storage, the PLS-DA (Fig. 5) on the metabolic profiles of delayed CA stored apples revealed diverse effects of O_2, CO_2 and storage time. Also, additional information required to separate the effect of low O_2 from that of high CO_2 is provided in the detailed dynamic time profiles of the metabolites (Figs. 6 and 7).

Aspartate was positively associated with O_2 particularly at the initiation of CA storage (Fig. 5). Proline, threonine, alanine and G6P

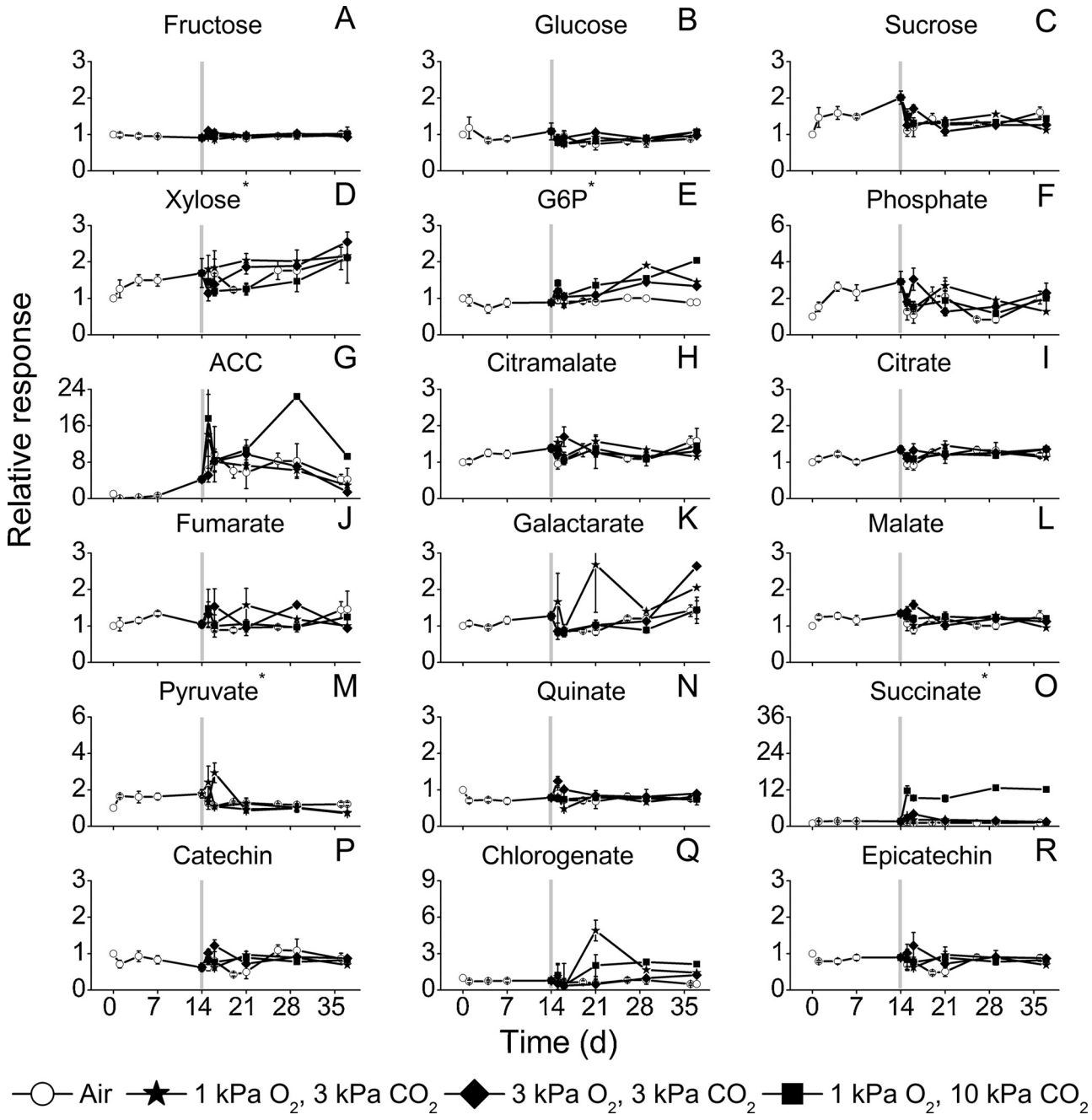

Fig. 6. Dynamic metabolic changes of 'Jonagold' apples stored for three weeks in 1 kPa O_2, 3 kPa CO_2; 3 kPa O_2, 3 kPa CO_2; 1 kPa O_2, 10 kPa CO_2 and air (20.8 kPa O_2, 0.03 kPa CO_2) at 1 °C after two weeks of air storage at 1 °C. Metabolites are ordered alphabetically in the order of their metabolic classes (sugars, G6P, organic acids and polyphenols). Metabolites with stable ($p < 0.05$) PLS-DA model regression coefficients (Martens and Martens, 2000) are marked with asterisk. The relative responses denote the metabolites concentration normalized by their concentration at harvest. The vertical lines indicate the time point at which the apples were transferred to CA. The error bars represent the standard error of the mean of 5 individual apples.
Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; G6P, glucose-6-phosphate.

were negatively associated with O_2. However, unlike G6P, all the three amino acids were negatively correlated with storage time. On the other hand, succinate and glutamate were positively correlated to CO_2. While erythritol was positively correlated with storage time, pyruvate exhibited an opposite trend.

Similar to immediate CA stored apples, apples stored in delayed CA showed no appreciable differences in sugars, some organic acids and polyphenols. However, metabolic changes that can be attributed to the concentrations of O_2, CO_2 and storage time were observed (Figs. 6 and 7). Apples stored in CA had higher G6P (Fig. 6E) than those stored in air, but there was a subtle difference among the three CA conditions. Although pyruvate (Fig. 6M) tended to increase as CA was initiated, it immediately declined and stabilized. On the other hand, succinate (Fig. 6O) sharply increased in fruit stored at high CO_2 concentration on the first day of CA storage. Xylose (Fig. 6D) increased with storage time irrespective of the storage conditions.

Alanine (Fig. 7A) accumulated in all CA stored apples during the first week of storage, being relatively higher in apples stored in high CO_2 storage (1 kPa O_2, 10 kPa CO_2), which was followed by a

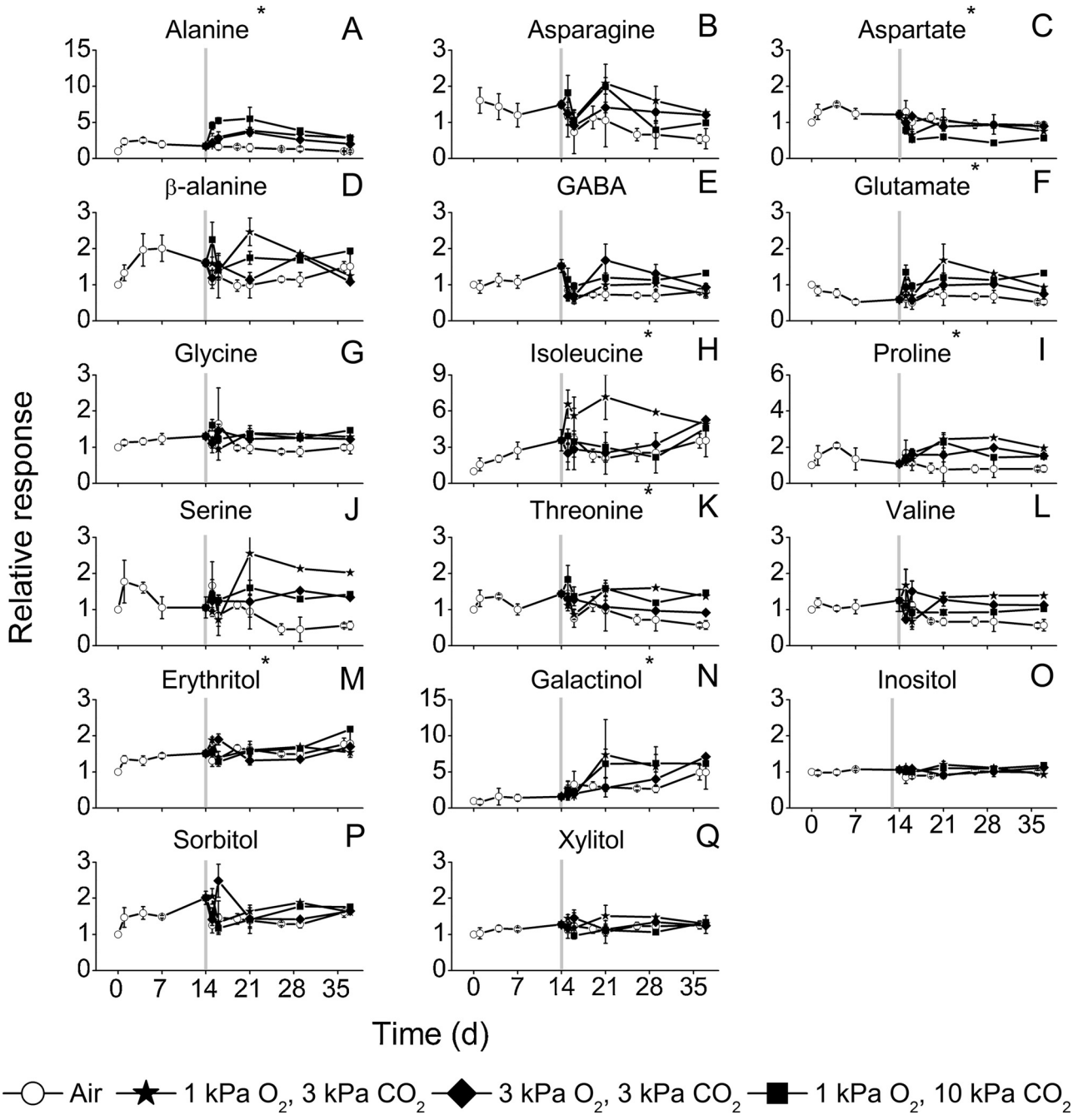

Fig. 7. Dynamic metabolic changes of 'Jonagold' apples stored for three weeks in 1 kPa O_2, 3 kPa CO_2; 3 kPa O_2, 3 kPa CO_2; 1 kPa O_2, 10 kPa CO_2 and air (20.8 kPa O_2, 0.03 kPa CO_2) at 1 °C after two weeks of air storage at 1 °C. Metabolites are ordered alphabetically in the order of their metabolic classes (amino acids and sugar alcohols). Metabolites with stable ($p < 0.05$) PLS-DA model regression coefficients (Martens and Martens, 2000) are marked with asterisk. The relative responses denote the metabolites concentration normalized by their concentration at harvest. The vertical lines indicate the time point at which the apples were transferred to CA. The error bars represent the standard error of the mean of 5 individual apples.
Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; G6P, glucose-6-phosphate.

decline. In contrast, at the onset of CA, aspartate (Fig. 7C) declined rapidly particularly in apples stored at high CO_2 condition (1 kPa O_2, 10 kPa CO_2). Apples stored at this condition maintained lower aspartate level than at the other two CA conditions (1 kPa O_2, 3 kPa CO_2; 3 kPa O_2, 3 kPa CO_2). As compared to apples stored at 1 kPa O_2, glutamate (Fig. 7F) was lower in apples stored under air and 3 kPa O_2. While erythritol (Fig. 7M) increased with storage time irrespective of the storage conditions, galactinol (Fig. 7N) was higher in apples stored at 1 kPa O_2 as compared to apples stored at 3 kPa O_2. Proline (Fig. 7I) and threonine (Fig. 7K) were lower in air storage than CA storage. Apples stored at 1 kPa O_2, 3 kPa CO_2 fruit had higher isoleucine (Fig. 7H) levels than those in other storage conditions.

3.3. The effect of the timing of CA application on metabolic response

In contrast to immediate CA stored apples, which were exposed to a simultaneous stress from low temperature, low O_2 and high

CO_2, delayed CA stored apples were exposed to these stresses in two steps. Nonetheless, the metabolic responses between the two groups were in general similar (Figs. 3, 4, 6 and 7), except for a few peculiar differences on some metabolite levels. Also, no visual sign of storage disorder such as flesh browning was observed. In apple stored in 1 kPa O_2, 3 kPa CO_2, delayed CA stored apples had significantly higher level of ACC, isoleucine, citramalate, erythritol, aspartate, xylose, citrate and galactarate but lower level of G6P as compared to immediate CA stored apples. The ACC level of delayed CA stored apples were also higher in apples stored in 3 kPa O_2, 3 kPa CO_2 and 1 kPa O_2, 10 kPa CO_2. However, in the latter storage condition, delayed CA stored apples exhibited lower succinate level.

4. Discussion

In this study, a detailed analysis of the effect of O_2, CO_2 and storage time on the metabolic profiles of 'Jonagold' apple during the initial periods of CA storage was conducted. In response to the imposed CA conditions, metabolic reconfigurations were observed in glycolytic pathway, the TCA cycle, and the biosynthesis of ethylene and amino acids branching from these pathways.

4.1. The effect of storage gas concentrations

The suppression of ethylene production in CA stored apples as compared to air stored apples is in agreement with previous reports (Bekele et al., 2015; Bulens et al., 2012; Gorny and Kader, 1997). The differences in ethylene production between air and CA stored apples paralleled the difference observed in their ACC level. In contrast to the steady increase of ACC observed in air stored apples, CA storage maintained the ACC level of the apples close to their harvest level. The accumulation of ACC during air storage is in agreement with previous reports (Bekele et al., 2015; Tian et al., 2002).

As compared to air stored apples, G6P was higher in CA stored apples, suggesting the consumption of G6P in CA is lower than its production. Similar accumulation of G6P and fructose-6-phosphate (F6P) with a decline in the level of fructose-1,6-bisphosphate was observed in pears stored at 10 kPa CO_2 (Kerbel et al., 1988). In the glycolytic pathway, the step converting F6P to fructose-1,6-bisphosphate is the key metabolic regulator (Plaxton and Podestá, 2006). In plants, this reaction could be catalysed by either or both ATP-dependent phosphofructokinase (ATP-PFK) and PPi-dependent phosphofructokinase (PFK) (PPi-PFK). Low O_2 and high CO_2 atmospheres may lower cytoplasmic pH (Lange and Kader, 1997; Nanos and Kader, 1993) resulting in the inhibition of these enzymes. In apples, the activity of ATP-PFK was shown to be strongly dependent on pH, the optimum being at pH 7.8 (Gyulakhmedov et al., 2006). In line with this, the activity of both enzymes were inhibited in pears exposed to 10 kPa CO_2 (Kerbel et al., 1988).

In contrast to G6P, pyruvate was higher in air stored apples as compared to CA stored apples. Pyruvate plays a central role in the regulation of respiratory metabolism as it links glycolytic pathway to the TCA cycle, fermentative pathway and mETC. The low level of pyruvate in CA stored apples suggested reduced production of pyruvate as compared to its consumption. In addition to reduced carbon flux through the glycolytic pathway, the metabolism of pyruvate to alanine accounts for the low level of pyruvate observed in CA stored apples. It is known that higher pyruvate level stimulates the less efficient alternative oxidase pathway (Oliver et al., 2008) and, therefore, plants exposed to hypoxic stress reduce pyruvate by metabolizing it to alanine. The higher level of alanine in apples stored in 10 kPa CO_2 suggested a highly positive correlation between accumulation of alanine and the CO_2 concentration.

Among the TCA cycle intermediates, succinate was highly positively correlated to CO_2. Succinate ubiquitously accumulates in plants exposed to high CO_2 (Fernández-Trujillo et al., 1999; Ke et al., 1993; Shipway and Bramlage, 1973; Vandendriessche et al., 2013) and low O_2 storage (Rocha et al., 2010). High CO_2 and low O_2 concentrations reduce the regeneration of nucleotides in mETC (Saquet et al., 2000) initiating wider effects in the glycolysis and TCA cycle. High CO_2 exposure is known to reduce the activity of succinate dehydrogenase (Zeylemaker et al., 1970), COX (Gonzàlez-Meler et al., 1996), and to lower the capacity of the mitochondria to oxidize pyruvate, NADH and TCA cycle intermediates (Bruhn et al., 2007; Shipway and Bramlage, 1973). In addition, during hypoxia, NAD^+ regeneration by partial reversal of the TCA could keep the metabolic flux from 2-oxoglutarate to succinate (Rocha et al., 2010). The steep increase of succinate followed by a saturation and decline could suggest the time dependent inhibition kinetics of succinate producing or consuming enzymes.

Amino acids metabolism linked to the TCA cycle is an integral component of respiratory metabolism. In this study, clear differences in aspartate, glutamate and GABA were found between apples stored in air and CA storage. Aspartate was highly positively correlated with O_2 concentration in the storage atmosphere. The depletion of aspartate was previously observed at the onset of anaerobiosis of barley root tissue (Good and Muench, 1993), under low O_2 treatment of *Arabidopsis* seeds (Gibon et al., 2002), in high CO_2 storage of broccoli florets (Hansen et al., 2001), and CA storage (1 kPa O_2, 3 kPa CO_2) of apples (Bekele et al., 2015). Aspartate could transfer its amino group to alanine in transamination reactions involving aspartate and alanine aminotransferases.

Apples stored at high CO_2 concentration (1 kPa O_2, 10 kPa CO_2) exhibited higher GABA level, particularly after the second week of storage. GABA is dominantly produced from glutamate, and to a lesser extent from polyamines (Deyman et al., 2014). It is further metabolized to succinic semialdehyde (SSA) and succinate through the GABA shunt. Activation of glutamate decarboxylase (GAD) due to cytoplasmic acidification (Carroll et al., 1994; Trobacher et al., 2013) and reduced activity of SSA dehydrogenase and GABA transaminase to metabolize GABA (Busch and Fromm, 1999; Streeter and Thompson, 1972) are the most probable reasons for the accumulation of GABA observed, in this study, in high CO_2 stored apples. Previous research also showed the accumulation of GABA in four high CO_2 treated strawberry cultivars (Deewatthanawong et al., 2010a), and tomatoes (Deewatthanawong et al., 2010b).

4.2. Dynamics of metabolic changes with storage time

Galactinol increased with storage time in all conditions, but to a higher extent in fruit stored under CA. Galactinol is produced from UDP-galactose and myo-inositol by galactinol synthase, and is used as a precursor for the biosynthesis of raffinose family oligosaccharides (Saravitz et al., 1987). Galactinol has a capacity to scavenge hydroxyl radicals, and has been suggested to protect plants from oxidative damage (Nishizawa et al., 2008). The accumulation of galactinol in fruit stored under CA may be beneficial in reducing oxidative damage of the fruit. In transgenic *Arabidopsis thaliana*, the accumulation of galactinol and raffinose was correlated with cold and drought tolerance (Taji et al., 2002). Erythritol increased with storage time under all storage conditions. The metabolism of erythritol is not well elucidated in plants but erythritol levels are known to decrease in the brown tissues of pears due to low O_2 stress (Pedreschi et al., 2009). The accumulation of G6P in CA stored apples could be explained by its reduced consumption for respiration and for biosynthesis of

other metabolites such as aromatic amino acid through the pentose phosphate pathway.

4.3. The effect of conditioning

Generally, the initial responses of the apples to CA were preserved between immediate and delayed application of CA. Previous reports showed that ethylene could be involved in enhancing cold tolerance (Zhang and Huang, 2010) by expressing cold related genes through ERF protein TERF2/LeERF2, and acclimatisation to hypoxia by expressing ERF transcription factor RAP2.2 (Hinz et al., 2010). Also, a previous report showed higher level of ATP in delayed CA stored 'Braeburn' apples as compared to immediate CA stored apples (Saquet et al., 2003).

The level of some metabolites including sucrose, xylose, phosphate, β-alanine, isoleucine, serine and sorbitol changed during the first two weeks of air storage. However, the change in the above mentioned metabolites generally had minor effect on metabolic levels after CA induction. The lower accumulation of succinate in delayed application of CA (1 kPa O_2, 10 kPa CO_2) might be explained by less energy demand of delayed CA stored apples, as they were already acclimatized to the cold temperature.

5. Conclusion

Metabolic profiling was applied to understand the biochemical basis for CA storage of 'Jonagold' apples. Clear metabolic differences were observed in glycolysis, TCA cycle and amino acids metabolic pathways in air and CA stored apples. By interpreting the dynamics of the metabolic changes in the metabolic pathways useful insights into the potential regulatory points and underlying control mechanism of apples stored under CA was deduced. The induction of CA resulted in the accumulation of some metabolites involve in the glycolysis such as G6P and some amino acids such as alanine while amino acids such as aspartate decreased. CA storage of the apples also induced a considerable accumulation of galactinol which serves as a radical scavenger, suggesting that the galactinol metabolism might play an important role in the response of apples to CA.

Similar stress response patterns were observed between immediate and delayed CA storage. However, as this study focused on the first five weeks of storage, some slow metabolic changes might not be detected. In this study, the metabolite levels were measured at the tissue level, and hence subcellular metabolic levels were lumped together. As the control of plant respiratory metabolism involves subcellular compartmentalization, additional insights about the control of metabolic responses could be obtained by extending the metabolic analysis to subcellular levels. Furthermore, metabolic flux analysis and kinetic models could provide quantitative information about the metabolic fluxes and help to get full understanding of CA stress response of the apples.

Acknowledgements

We would like to thank the European Union (Grant agreement FP7/2007-2013—Frisbee), the Research Council of the KU Leuven (OT 12/055) and the Research Fund Flanders (Project G.0645.13) for the financial support. This research was carried out in the context of the European COST Action FA1106 ('QualityFruit'). Flanders Centre of Postharvest Technology (VCBT) is gratefully acknowledged for providing the CA storage facility. The opinions expressed in this document do by no means reflect the official opinion of the European Union or its representatives.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.postharvbio.2016.02.003.

References

Ampofo-Asiama, J., Baiye, V.M.M., Hertog, M.L.A.T.M., Waelkens, E., Geeraerd, A.H., Nicolai, B.M., 2014. The metabolic response of cultured tomato cells to low oxygen stress. Plant Biol. 16, 594–606.

Bai, J., Baldwin, E.A., Goodner, K.L., Mattheis, J.P., Brecht, J.K., 2005. Response of four apple cultivars to 1-methylcyclopropene treatment and controlled atmosphere storage. HortScience 40, 1534–1538.

Baxter, C.J., Redestig, H., Schauer, N., Repsilber, D., Patil, K.R., Nielsen, J., Selbig, J., Liu, J., Fernie, A.R., Sweetlove, L.J., 2007. The metabolic response of heterotrophic *Arabidopsis* cells to oxidative stress. Plant Physiol. 143, 312–325.

Bekele, E.A., Annaratone, C.E.P., Hertog, M.L.A.T.M., Nicolai, B.M., Geeraerd, A.H., 2014. Multi-response optimization of the extraction and derivatization protocol of selected polar metabolites from apple fruit tissue for GC–MS analysis. Anal. Chim. Acta 824, 42–56.

Bekele, E.A., Beshir, W.F., Hertog, M.L.A.T.M., Nicolai, B.M., Geeraerd, A.H., 2015. Metabolic profiling reveals ethylene mediated metabolic changes and a coordinated adaptive mechanism of 'Jonagold' apple to low oxygen stress. Physiol. Plant 155, 232–247.

Bruhn, D., Wiskich, J.T., Atkin, O.K., 2007. Contrasting responses by respiration to elevated CO_2 in intact tissue and isolated mitochondria. Funct. Plant Biol. 34, 112–117.

Bulens, I., Van de Poel, B., Hertog, M.L.A.T.M., De Proft, M.P., Geeraerd, A.H., Nicolai, B. M., 2012. Influence of harvest time and 1-MCP application on postharvest ripening and ethylene biosynthesis of 'Jonagold' apple. Postharvest Biol. Technol. 72, 11–19.

Bulens, I., Van de Poel, B., Hertog, M.L.A.T.M., De Proft, M.P., Geeraerd, A.H., Nicolaï, B. M., 2011. An updated integrated methodology for analysis of metabolites and enzyme activities of ethylene biosynthesis. Plant Methods 7, 17.

Busch, K.B., Fromm, H., 1999. Plant succinic semialdehyde dehydrogenase. Cloning, purification, localization in mitochondria, and regulation by adenine nucleotides. Plant Physiol. 121, 589–598.

Carroll, A.D., Fox, G.G., Laurie, S., Phillips, R., Ratcliffe, R.G., Stewart, G.R., 1994. Ammonium assimilation and the role of γ-aminobutyric acid in pH homeostasis in carrot cell suspensions. Plant Physiol. 106, 513–520.

De Castro, E., Biasi, B., Mitcham, E., Tustin, S., Tanner, D., Jobling, J., 2007. Carbon dioxide-induced flesh browning in Pink Lady apples. J. Am. Soc. Hortic. Sci. 132, 713–719.

De Wild, H.P.J., Otma, E.C., Peppelenbos, H.W., 2003. Carbon dioxide action on ethylene biosynthesis of preclimacteric and climacteric pear fruit. J. Exp. Bot. 54, 1537–1544.

Deewatthanawong, R., Nock, J.F., Watkins, C.B., 2010a. γ-Aminobutyric acid (GABA) accumulation in four strawberry cultivars in response to elevated CO_2 storage. Postharvest Biol. Technol. 57, 92–96.

Deewatthanawong, R., Rowell, P., Watkins, C.B., 2010b. γ-Aminobutyric acid (GABA) metabolism in CO_2 treated tomatoes. Postharvest Biol. Technol. 57, 97–105.

Deyman, K.L., Brikis, C.J., Bozzo, G.G., Shelp, B.J., 2014. Impact of 1-methylcyclopropene and controlled atmosphere storage on polyamine and 4-aminobutyrate levels in 'Empire' apple fruit. Front. Plant Sci. 5, 1–9.

Duque, P., Barreiro, M.G., Arrabac, D., 1999. Respiratory metabolism during cold storage of apple fruit: I. Sucrose metabolism and glycolysis. Physiol. Plant. 107, 14–23.

Elgar, H.J., Burmeister, D., Watkins, C.B., 1998. Storage and handling effects on a CO_2-related internal browning disorder of 'Braeburn' apples. HortScience 33, 719–722.

Fernández-Trujillo, J.P., Nock, J.F., Watkins, C.B., 1999. Fermentative metabolism and organic acid concentrations in fruit of selected strawberry cultivars with different tolerances to carbon dioxide. J. Am. Soc. Hortic. Sci. 124, 696–701.

Geigenberger, P., 2003. Response of plant metabolism to too little oxygen. Curr. Opin. Plant Biol. 6, 247–256.

Gibon, Y., Vigeolas, H., Tiessen, A., Geigenberger, P., Stitt, M., 2002. Sensitive and high throughput metabolite assays for inorganic pyrophosphate ADPGlc, nucleotide phosphates, and glycolytic intermediates based on a novel enzymic cycling system. Plant J. 30, 221–235.

Gonzàlez-Meler, M.A., Ribas-carbó, M., Siedow, J.N., Drake, B.G., 1996. Direct inhibition of plant mitochondrial respiration by elevated CO_2. Plant Physiol. 112, 1349–1355.

Good, A.C., Muench, D.C., 1993. Long-term anaerobic metabolism in root tissue: metabolic products of pyruvate metabolism. Plant Physiol. 101, 1163–1168.

Gorny, J.R., Kader, A.A., 1997. Low oxygen and elevated carbon dioxide atmospheres inhibit ethylene biosynthesis in preclimacteric and climacteric apple fruit. J. Am. Soc. Hortic. Sci. 122, 542–546.

Gyulakhmedov, S.G., Omarov, Y.A., Mamedov, Z.M., Kuliev, A.A., 2006. Isolation and study of active ATP-dependent phosphofructokinase from apple fruits *Pyrus domestica* Borkh. Appl. Biochem. Microbiol. 42, 468–471.

Hansen, M.E., Sørensen, H., Cantwell, M., 2001. Changes in acetaldehyde: ethanol and amino acid concentrations in broccoli florets during air and controlled atmosphere storage. Postharvest Biol. Technol. 22, 227–237.
Hinz, M., Wilson, I.W., Yang, J., Buerstenbinder, K., Llewellyn, D., Dennis, E.S., Sauter, M., Dolferus, R., 2010. *Arabidopsis* RAP2.2: an ethylene response transcription factor that is important for hypoxia survival. Plant Physiol. 153, 757–772.
Ho, Q.T., Verlinden, B.E., Verboven, P., Vandewalle, S., Nicolai, B.M., 2007. Simultaneous measurement of oxygen and carbon dioxide diffusivities in pear fruit tissue using optical sensors. J. Sci. Food. Agric. 87, 1858–1867.
Kaplan, F., Kopka, J., Haskell, D.W., Zhao, W., Schiller, K.C., Gatzke, N., Sung, D.Y., Guy, C.L., 2004. Exploring the temperature-stress metabolome of *Arabidopsis*. Plant Physiol. 136, 4159–4168.
Ke, D., Mateos, M., Siriphanich, J., Li, C., Kader, A.A., 1993. Carbon dioxide action on metabolism of organic and amino acids in crisphead lettuce. Postharvest Biol. Technol. 3, 235–247.
Kerbel, E.L., Kader, A.A., Romani, R.J., 1988. Effect of elevated CO_2 concentratons on glycolysis in intact 'Bartlett' pear fruit. Plant Physiol. 86, 1205–1209.
Kind, T., Wohlgemuth, G., Lee, D.Y., Lu, Y., Palazoglu, M., Shahbaz, S., Fiehn, O., 2009. FiehnLib: mass spectral and retention index libraries for metabolomics based on quadrupole and time-of-flight gas chromatography/mass spectrometry. Anal. Chem. 81, 10038–10048.
Kruse, J., Rennenberg, H., Adams, M.A., 2011. Steps towards a mechanistic understanding of respiratory temperature responses. New Phytol. 189, 659–677.
Lange, D.L., Kader, A.A., 1997. Elevated carbon dioxide exposure alters intracellular pH and energy charge in avocado fruit tissue. J. Am. Soc. Hortic. Sci. 122, 253–257.
Larrigaudiere, C., Lentheric, I., Pintó, E., Vendrell, M., 2001. Short-term effects of air and controlled atmosphere storage on antioxidant metabolism in conference pears. J. Plant. Physiol. 158, 1015–1022.
Lee, J., Rudell, D.R., Davies, P.J., Watkins, C.B., 2012. Metabolic changes in 1-methylcyclopropene (1-MCP)-treated 'Empire' apple fruit during storage. Metabolomics 8, 742–753.
Leisso, R.S., Buchanan, D.A., Lee, J., Mattheis, J.P., Sater, C., Hanrahan, I., Watkins, C.B., Gapper, N., Johnston, J.W., Schaffer, R.J., Hertog, M.L.A.M., Nicolaï, B.M., Rudell, D. R., 2015. Chilling-related cell damage of apple (*Malus* × *domestica* Borkh.) fruit cortical tissue impacts antioxidant, lipid and phenolic metabolism. Physiol. Plant. 153, 204–220.
Martens, H., Martens, M., 2000. Modified jack-knife estimation of parameter uncertainty in bilinear modelling by partial least squares regression (PLSR). Food Qual. Prefer. 11, 5–16.
Mathooko, F.M., 1996. Regulation of respiratory metabolism in fruits and vegetables by carbon dioxide. Postharvest Biol. Technol. 9, 247–264.
Nanos, G.D., Kader, A.A., 1993. Low O_2-induced changes in pH and energy charge in pear fruit tissue. Postharvest Biol. Technol. 3, 285–291.
Nishizawa, A., Yabuta, Y., Shigeoka, S., 2008. Galactinol and raffinose constitute a novel function to protect plants from oxidative damage. Plant Physiol. 147, 1251–1263.
Oliver, S.N., Lunn, J.E., Urbanczyk-Wochniak, E., Lytovchenko, A., van Dongen, J.T., Faix, B., Schmälzlin, E., Fernie, A.R., Geigenberger, P., 2008. Decreased expression of cytosolic pyruvate kinase in potato tubers leads to a decline in pyruvate resulting in an in vivo repression of the alternative oxidase. Plant Physiol. 148, 1640–1654.
Pedreschi, R., Franck, C., Lammertyn, J., Erban, A., Kopka, J., Hertog, M., Verlinden, B., Nicolaï, B., 2009. Metabolic profiling of 'Conference' pears under low oxygen stress. Postharvest Biol. Technol. 51, 123–130.
Plaxton, W.C., Podestá, F.E., 2006. The functional organization and control of plant respiration. Crit. Rev. Plant Sci. 25, 159–198.
Rocha, M., Licausi, F., Araújo, W.L., Nunes-Nesi, A., Sodek, L., Fernie, A.R., van Dongen, J.T., 2010. Glycolysis and the tricarboxylic acid cycle are linked by alanine aminotransferase during hypoxia induced by waterlogging of *Lotus japonicus*. Plant Physiol. 152, 1501–1513.
Rudell, D.R., Mattheis, J.P., Hertog, M.L.A.T.M., 2009. Metabolomic change precedes apple superficial scald symptoms. J. Agric. Food Chem. 57, 8459–8466.
Saltveit, M.E., 1999. Effect of ethylene on quality of fresh fruits and vegetables. Postharvest Biol. Technol. 15, 279–292.
Saquet, A.A., Streif, J., 2008. Fermentative metabolism in 'Jonagold' apples under controlled atmosphere storage. Eur. J. Hortic. Sci. 73, 43–46.
Saquet, A.A., Streif, J., Bangerth, F., 2003. Reducing internal browning disorders in 'Braeburn' apples by delayed controlled atmosphere storage and some related physiological and biochemical changes. Acta Hortic. 628, 453–458.
Saquet, A.A., Streif, J., Bangerth, F., 2000. Changes in ATP, ADP and pyridine nucleotide levels related to the incidence of physiological disorders in 'Conference' pears and 'Jonagold' apples during controlled atmosphere storage. J. Hortic. Sci. Biotechnol. 75, 243–249.
Saravitz, D.M., Pharr, D.M., Carter, T.E., 1987. Galactinol synthase activity and soluble sugars in developing seeds of four soybean genotypes. Plant Physiol. 83, 185–189.
Shipway, M.R., Bramlage, W.J., 1973. Effects of carbon dioxide on activity of apple mitochondria. Plant Physiol. 51, 1095–1098.
Streeter, J.G., Thompson, J.F., 1972. Anaerobic accumulation of γ-aminobutyric acid and alanine in radish leaves (*Raphanus sativus* L.). Plant Physiol. 49, 572–578.
Taji, T., Ohsumi, C., Iuchi, S., Seki, M., Kasuga, M., Kobayashi, M., Yamaguchi-Shinozaki, K., Shinozaki, K., 2002. Important roles of drought and cold-inducible genes for galactinol synthase in stress tolerance in *Arabidopsis thaliana*. Plant J. 29, 417–426.
Tian, M.S., Prakash, S., Zhang, N., Ross, G.S., 2002. Chilling-induced ethylene biosynthesis in Braeburn apples. Plant Growth Regul. 38, 249–257.
Trobacher, C.P., Zarei, A., Liu, J., Clark, S.M., Bozzo, G.G., Shelp, B.J., 2013. Calmodulin-dependent and calmodulin-independent glutamate decarboxylases in apple fruit. BMC Plant Biol. 13, 144.
Vandendriessche, T., Schäfer, H., Verlinden, B.E., Humpfer, E., Hertog, M.L.A.T.M., Nicolaï, B.M., 2013. High-throughput NMR based metabolic profiling of Braeburn apple in relation to internal browning. Postharvest Biol. Technol. 80, 18–24.
Verlinden, B.E., de Jager, A., Lammertyn, J., Schotsmans, W., Nicolai, B.M., 2002. Effect of harvest and delaying controlled atmosphere storage conditons on core breakdown in 'Conference' pears. Biosyst. Eng. 83, 339–347.
Watkins, C.B., 2000. Responses of horticultural commodities to high carbon dioxide as related to modified atmosphere packaging. HortTechnology 10, 501–506.
Watkins, C.B., Silsby, K.J., Goffinet, M.C., 1997. Controlled atmosphere and antioxidant effects on external CO_2 injury of 'Empire' apples. HortScience 32, 1242–1246.
Zeylemaker, W.P., Klaasse, A.D.M., Slater, E.C., Veeger, C., 1970. Studies on succinate dehydrogenase: VI: Inhibition by monocarboxylic acids. Biochim. Biophys. Acta 198, 415–422.
Zhang, Z., Huang, R., 2010. Enhanced tolerance to freezing in tobacco and tomato overexpressing transcription factor *TERF2/LeERF2* is modulated by ethylene biosynthesis. Plant Mol. Biol. 73, 241–249.