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A metabolomics approach to elucidate apple fruit responses to static and dynamic controlled atmosphere storage

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ABSTRACT

The response of apple fruit to storage conditions based on low oxygen protocols depends on their genetic background. In order to elucidate common and divergent processes characterizing the metabolic changes under hypoxia, fruit of two apple (*Malus domestica*) varieties ('Granny Smith', GS, and 'Red Delicious', RD) were stored under two different low oxygen protocols (Ultra Low Oxygen, ULO, at 0.9 kPa oxygen, and Dynamic Controlled Atmosphere based on chlorophyll fluorescence, DCA-CF, between 0.2 and 0.55 kPa oxygen) for up to 200 and 214 days of storage for GS and RD samples, respectively. Through an integrated metabolomics approach (^{1}H NMR, GC–MS, HS-SPME-GC–MS analyses) a total of 130 metabolites (volatiles and non-volatiles) were identified. Most of them (117) were common to both cultivars; 95 were significantly different between both cultivars when comparing the whole set of data (ULO + DCA-CF), whereas 13 volatile compounds, identified via HS-SPME-GC–MS, were specific for either GS or RD. Multivariate analyses (PCA and PLS) of the whole dataset allowed to clearly discriminate between GS and RD samples. When storage condition was used as a categorical response variable, a lower percentage explained variance was obtained as this effect was overshadowed by the large effect of storage time. After 4 months of storage, RD underwent more pronounced metabolic compositional changes of the cortex, possibly associated with the evolution of ripening. Based on the accumulation pattern of pyruvate-derived metabolites (ethanol, acetaldehyde, lactate, alanine) it can be hypothesized that there are two main metabolic reconfiguration strategies in GS and RD to regenerate NAD^+ and cope with energy crisis under hypoxia. GS showed more pronounced responses through changes in the nitrogen metabolism and limited induction of the ethanol fermentation while the latter was highly induced in RD under both ULO and DCA-CF. Marked differences were detected between the VOC profiles of the two cultivars regardless storage conditions. Ethyl esters and 2-methylbutyl derivatives appeared finely modulated by the oxygen level in GS and RD apples, respectively.

1. Introduction

A decreased oxygen level, coupled with refrigeration and increased carbon dioxide concentration, is commonly applied in order to prolong the market life of fruits such as apples, kiwifruit, and winter pears in so-called controlled atmosphere (CA) systems. Since the earliest commercial applications, the benefits of CA technology in apple, as compared to regular atmosphere storage, are the delay of ripening and senescence and a better maintenance of quality due to the synergistic effects of low temperature, increased carbon dioxide concentration and reduced levels of oxygen (Yahia, 2009). The discovery that oxygen concentrations of around 1 kPa improve storability has led to the worldwide application of Ultra Low Oxygen (ULO, 0.8–1.2 kPa) protocols (Dilley, 2006). In general, these hypoxic conditions are maintained from the beginning until the end of storage (static CA). This static approach does not always provide optimal post-storage results

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(Prange et al., 2013). A further improvement of CA technology is realised through the development of dynamic CA (DCA) in which storage conditions change over time, to adapt to the changing fruit physiology. With this technology, fruit are kept at oxygen concentrations of 0.4–0.8 kPa (depending on the lowest threshold tolerated by the different apple cultivars) that correspond or are close to the anaerobic compensation point (ACP) where CO_2 production reaches its minimum (Gasser et al., 2008; Hoehn et al., 2009). The ACP can also be taken as the Lower Oxygen Limit (LOL) that represents the concentration below which the fermentation level becomes unacceptable, causing tissue damage and loss of quality. While O_2 concentrations corresponding to or slightly above the ACP results in reduced respiration and maximal storability of apple fruit (Prange et al., 2003), the risk of severe quality losses due to the anaerobic metabolism (e.g., fermentation) and stress conditions increases; in this context, the evaluation of the fruit's metabolic activity becomes crucial. The typical parameters used to monitor the fruit's metabolic responses are ethanol production, chlorophyll fluorescence (DCA-CF) and the Respiratory Quotient (RQ-DCA) (Maxwell, 2000; Prange et al., 2002; Veltman et al., 2003; Gasser et al., 2008; Bessemans et al., 2016). Based on these physiological responses the oxygen concentration is set in order to reach levels deemed "safe".

As compared to ULO, the extremely low oxygen concentrations used in DCA protocols have been effective in retaining firmness and acidity in specific varieties such as 'Holsteiner Cox', 'Boskoop' (Hennecke et al., 2008), 'Ariane' (Gasser and Von Arx, 2015) and 'Braeburn' (Zanella and Rossi, 2015), and reducing the incidence of storage disorders such as superficial scald as demonstrated in cv 'Granny Smith' (Zanella et al., 2005). However, DCA has not always been as successful showing limited or no significant added effect of DCA-CF over ULO in cvs such as 'Topaz', 'Otava' (Gasser and Von Arx, 2015), 'Fuji' and 'Gala' (Zanella and Rossi, 2015). This indicates that, within the same species, a pronounced variability is present in terms of the capacity to reduce firmness loss in response to postharvest low oxygen applications. This is probably due to different hypoxia-related regulatory mechanisms present in apple varieties. Slight changes in low oxygen concentrations may induce different physiological and metabolic responses depending on the genotypes. Zanella and Stürz (2015) showed that, differently from eight other varieties, 'Red Delicious' apple reacts significantly and accumulates higher ethanol levels when stored in DCA-CF as compared to ULO. Besides the main quality parameters, limited information is available concerning the effects of different hypoxic conditions on metabolic processes and composition profiling of apples. Cukrov et al. (2016) recently reported that 'Granny Smith' apples stored at 0.4 or 0.8 kPa oxygen markedly differ in terms of metabolic and transcriptome profiling. More than 1000 genes were differentially expressed after 24 d of storage under these different hypoxic conditions and the involvement of ethylene responsive factors (ERFs) in modulating hypoxia-dependent gene expression has been hypothesized. In addition to the observed difference in terms of primary metabolism (e.g., pyruvate, ethanol) in 'Granny Smith' (Cukrov et al., 2016), oxygen levels ranging from 1.0 to 0.5 kPa differentially affected the volatile profile of 'Royal Gala' with some negative aspects on ester production induced by the lowest oxygen concentration (Both et al., 2014).

In this paper the results of a comparative metabolomics approach on two apple varieties ('Granny Smith' and 'Red Delicious') kept under two different oxygen protocols (static ULO, at 0.9 kPa, and DCA-CF, between 0.2 and 0.55 kPa) throughout 6 months of storage are reported. The goal of this work was to better clarify the responses of the selected cultivars, differently sensitive to hypoxic conditions, to slight changes in terms of oxygen level and to the different atmosphere management, highlighting the differences that characterise both varieties and storage protocols.

2. Materials and methods

2.1. Plant material and treatments

Apple fruit (*Malus domestica* Borkh., cv 'Granny Smith', GS, and 'Red Delicious', RD) were harvested in the province of Bolzano (Italy) at the optimum harvest date for long-term storage, according to the recommended maturity parameters for long term CA storage by the Laimburg Research Centre for Agriculture and Forestry [GS: Starch pattern index (1–5) 2.1–2.5, Firmness 67–76 N, Total Soluble Solids: 10.0–11.0%; RD: Starch pattern index (1–5) 2.0–2.5, Firmness 66–74 N, Total Soluble Solids: 11.0–12.0%]. In case of GS, apple fruit were sampled in the commercial packing house 'OG Kaiser Alexander' (Latitude: 46.4226°; Longitude: 11.3269°), whereby apples were harvested in a representative orchard for the Adige valley (≈200 m a.s.l.). RD were sampled in the commercial packing house 'GEOS' (Latitude: 46.6262°; Longitude: 10.7646°), whereby apples were harvested in a representative orchard for the higher altitude in the Venosta valley (≈660 m a.s.l.). After harvest, homogeneous fruit in terms of size and color were selected and immediately pre-refrigerated at 2.5 °C for seven days under normoxia (21 kPa, regular air), in order to simulate the room filling time of commercial packing-houses. Afterwards fruit were stored at 1.0–1.3 °C and about 98% relative humidity. After one week of acclimation to low temperature the first sampling (0 DIA, days in atmosphere) was performed. Starting from this point the level of oxygen was slowly lowered down from 21 kPa to about 0.9 kPa in four days (4 DIA). Then a static 0.9 kPa oxygen atmosphere with 1.3 kPa (GS-ULO) or 1.5 kPa (RD-ULO) carbon dioxide concentration was applied (ULO treatments), whereas, for the DCA-CF treatments, apples were kept under a dynamic oxygen concentration, ranging between 0.2 and 0.55 kPa (GS-DCA-CF and RD-DCA-CF). The experimental chambers for DCA-CF storage were equipped with the HarvestWatch system (Satlantic Inc., Halifax, N. S., Canada). It consists of the hourly assessment of chlorophyll fluorescence (F-α) by means of FIRM (fluorescence interactive response monitor, Satlantic Inc.) sensors during the whole storage period on samples of six apples (DeLong et al., 2004). Supplementary Fig. S1A shows the oxygen levels applied throughout the experimental period; the oxygen concentration was set dynamically based on the chlorophyll fluorescence signal. Starting from 5 DIA the two varieties have been treated independently according to the onset of the chlorophyll fluorescence peak.

In the GS-DCA-CF chamber, the chlorophyll fluorescence peak appeared at 5 DIA at a level of 0.3 kPa that was maintained for one day, after which the concentration was brought to 0.45 kPa and kept until 119 DIA. A rise of fluorescence was later detected again and the oxygen level was raised up to 0.55 kPa (119 DIA), which was maintained until the end of the experiment (200 DIA).

Similarly, for RD-DCA-CF samples, after one day at 0.9 kPa the oxygen concentration was lowered to 0.4 kPa (5 DIA), thereafter (7 DIA) it was reduced to 0.2 kPa. The chlorophyll fluorescence peak appeared one day later (8 DIA) when the oxygen pressure was set at 0.55 kPa and maintained until the end of the experiment (214 DIA).

Since no fluorescence peak was detected afterwards, that oxygen concentration was maintained until the end of the trial. The temperature of the storage chambers was maintained at 1–1.3 °C throughout the experimental period. For metabolomics analyses, fruit samples were collected after 0, 4, 5, 7, 14, 21 and 26 (for GS and RD samples, respectively), 35, 119, 174 DIA. Supplementary Fig. S1B shows the experimental design and the sampling collection times.