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Physiological implications of controlled atmosphere storage of 'Conference' pears (*Pyrus communis* L.): A proteomic approach

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ABSTRACT

Two-dimensional electrophoresis (2-DE) coupled to a robust statistical approach comprising univariate and multivariate statistics and LC–ESI-MS/MS protein identification was used to improve the understanding of the physiology of pears submitted to four different controlled atmosphere (CA) conditions. Optimal commercial CA conditions (2.5% O_2, 0.7% CO_2, with pre-cooling and fruit from the optimal harvest date), browning-inducing CA conditions (1% O_2, 10% CO_2, using no pre-cooling and fruit from a late harvest) and two intermediate CA conditions (2.5% O_2, 10% CO_2 and 15% O_2, 0.6% CO_2, both including pre-cooling and fruit from the optimal harvest date) were evaluated. The combination of oxygen and carbon dioxide concentrations, pre-cooling period and harvest time plays a key role in core breakdown presumably in the protein levels during controlled atmosphere storage of pears. Our results show that impaired respiration is highly related to protein synthesis alterations, and activation of defense mechanisms. Triosephosphate isomerase, a key enzyme of the energy metabolism was up-regulated under browning-inducing conditions in an attempt to use alternative more efficient anaplerotic pathways to cope with the applied stresses. The changes in the accumulation of proteins related to ethylene biosynthesis (ACC oxidase) and allergens (major allergen Pyrc 1) were highly dependent on the oxygen and carbon dioxide concentrations. ACC oxidase and the major allergen Pyrc 1 were clearly down-regulated under low oxygen or high carbon dioxide concentrations. Their involvement in metabolic disturbances cannot be discarded.

1. Introduction

Fruit are often stored under controlled atmosphere (CA) conditions to extend their storage and shelf-life. For any given commodity, optimal oxygen and carbon dioxide concentrations must be determined in order to reduce respiration, ethylene production rates and action, delay ripening and senescence, as well as to reduce the growth of pathogens (Kader, 2002). However, even when the applied external gas concentrations are relatively high, the oxygen concentration across plant tissues may fall because of the large diffusion gradients that are required to direct oxygen across the tissue at a rate fast enough to maintain the rate of oxygen consumption (Geigenberger, 2003; Ho et al., 2006, 2008). Thus, if the CA conditions applied are not appropriate, hypoxic zones can develop and induce fermentation-related disorders and accumulation of off-flavors. The hypothesis of the induction of anoxic zones when 'Conference' pears are stored under reduced oxygen concentrations has been extensively explained in a diffusion context (Lammertyn et al., 2003a,b; Ho et al., 2006), but the physiological interpretation and consequences have so far been largely ignored.

The use of proteomic approaches in the area of fruit and vegetable physiology has been increasing over recent years (Hjernø et al., 2006; Rocco et al., 2006). However, in the area of postharvest physiology, proteomics is a fairly new approach. Research conducted on other commodities and physiological disorders has been limited to isolated assays trying to find explanations for the disorders under study (Burmeister and Dilley, 1995; Alférez et al., 2005; Lurie and Crisoto, 2005; Sala et al., 2005). Only a limited number of comprehensive studies aimed at understanding the physiology behind postharvest disorders have been reported so far (Franck, 2004; Pedreschi et al., 2007). Proteomics is a promising tool to identify marker proteins for specific physiological disorders of a wide range of commodities and to identify high risk batches at an early stage to reduce economic losses. In a previous proteomics study conducted by our research group, focus was given to the characterization of browning-related proteins (Pedreschi et al., 2007) in stored pears. However, a specific focus on the controlled atmosphere conditions applied at an early stage, before browning is evident, has not been conducted so far.

The objective of this research was to study the effects of four different controlled atmosphere conditions on protein levels to better

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understand the physiological effects of controlled atmosphere storage on 'Conference' pears.

2. Materials and methods

2.1. Fruit material

Pears (*Pyrus communis* L cv. Conference) were harvested in the orchard of the Centre for Fruit Culture in Rillaar (Belgium). The trees in this orchard are planted along the north–south axis and hence, with exception of the corner trees, have a west and east side. Pears were picked randomly from the east side at a height of 2 m. One batch of pears was picked at the commercial harvest date (8 September 2004) as determined by the Flanders Centre of Postharvest Technology (Belgium). These fruit were submitted to pre-cooling in air at −1 °C for 3 weeks before applying controlled atmosphere conditions of either 2.5% O_2 and 0.7% CO_2 (commercial condition), 15% O_2 and 0.6% CO_2 (high O_2 condition) and 2.5% O_2 and 10% CO_2 (high CO_2 condition). Pears from these CA conditions did not present any visual symptoms of disorders. A second batch of pears was picked 2 weeks after the commercial harvest date (22 September 2004) from the same trees and immediately stored under 1.0% O_2 and 10% CO_2 at −1 °C with no pre-cooling period, to induce the browning disorder. After 6 months of CA storage, pears from the different conditions were sampled. Pears were cut perpendicularly to the stem-calyx axis at 5 cm from the bottom of a pear. Tissue samples were taken from the equatorial region excluding the skin and core. From amongst all the pears, only sound (non-brown) tissue was sampled. Samples were immediately frozen in liquid nitrogen and kept at −80 °C until further analysis. Six independent biological replicates of tissue samples were prepared based on pooled tissue from six individual pears.

Harvest time and pre-cooling regime were not used as real independent treatment factors but were just part of the standard protocol to induce the browning disorder. The aim was to use pears from the browning-inducing conditions (that would definitely develop browning) as a reference for those fruit stored under the three other CA conditions where no visible browning was induced, but where protein changes would be triggered to various degrees.

2.2. Protein extraction

Proteins were extracted with a modified phenol extraction/methanol-ammonium acetate precipitation method optimized for pear tissue by Pedreschi et al. (2007). Two hundred milligrams of powdered frozen pear tissue were homogenized in 500 μL of cold protein extraction buffer (PEB composed of 0.7 M sucrose, 100 mM KCl, 1 mM PMSF, 500 mM EDTA, 50 mM Tris–HCl pH 8.5, 1% DTT) for 30 min at 4 °C. Five hundred microliters of ice-cold Tris-buffered phenol (pH 8.0) was added and the sample was vortexed (MS2, IKA Works, Inc., Wilington, NC, USA) thoroughly for 5 min at 4 °C. After centrifugation (Sanyo Hawk 15/05, UK) of the sample at 7000 × *g* for 10 min at 4 °C, the phenol phase was collected and re-extracted with an equal volume of protein extraction buffer and an extra 50 μL of ice-cold Tris-buffered phenol. The water phase containing the cellular debris was re-extracted with an equal volume of ice-cold Tris-buffered phenol. Both phases were thoroughly vortexed and shaken for 5 min at 4 °C. After centrifugation at 7000 × *g* for 10 min at 4 °C, both phenol phases were combined and 900 μL of PEB and an extra 100 μL of ice-cold Tris-buffered phenol were added. The mixture was vortexed for 5 min at 4 °C, and after centrifugation at 7000 × *g* for 10 min at 4 °C the phenol phase was recovered and precipitated overnight with five volumes of 100 mM ammonium acetate in methanol at −20 °C. After centrifugation at 21,900 × *g* for 30 min, the supernatant was removed and the protein pellet washed twice with methanol and twice with acetone containing 0.1% DTT. After washing, the pellet was allowed to dry in air until the remaining acetone was evaporated, and then stored at −80 °C until used.

2.3. Two-dimensional electrophoresis (2-DE)

Protein pellets were redissolved in rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.2% IPG buffer, 75 mM DTT and 0.002% bromophenol blue) for 1 h and quantified by using a modified Bradford dye-binding procedure (Bradford, 1976). Thirty-five micrograms protein samples for silver staining at pH 5–8 were rehydrated in 150 μL and applied via anodic cup loading. Strips of 24 cm of pH 5–8 (Bio-Rad, Nazareth, Belgium) were rehydrated for at least 8 h in 460 μL rehydration buffer. Proteins were isoelectrically (IEF) focused in an IPGphorII System (Amersham Biosciences, Uppsala, Sweden) at 20 °C and with a 50 μA per strip limit. IEF was carried out in four steps: 3 h at 300 V, 6 h at 1000 V, 3 h at 8000 V and 24,000 V h at 8000 V. After IEF completion, strips were equilibrated individually for 15 min in 8 mL equilibration buffer (6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, 50 mM Tris–HCl pH 8.8) containing 2% (w/v) DTT, and subsequently for 15 min in 8 mL equilibration buffer containing 2.5% iodoacetamide. Second dimension separation was performed in an Ettan DALT System (Amersham Biosciences) with lab cast 1.5 mm SDS polyacrylamide gels (12.5%) for 45 min at 12 W and subsequently for 5 h at 100 W. Six replicate gels were run for each condition.

2.4. Protein visualization and image analysis

Proteins were visualized by silver staining (Blum et al., 1987) or by colloidal Coomassie Brilliant Blue (CBB) G-250 (Neuhoff et al., 1988). Image analysis was performed with the Image Master 2-D platinum software 6.0 (Amersham Biosciences). Spots were detected without spot editing and quantified as percentage volume. Silver stained gels were used as analytical gels and Coomassie stained gels as preparative gels for further LC–ESI-MS/MS protein identification.

2.5. Statistical data analysis

The 2-DE data calculated as spot percentage volume were pre-processed before multivariate statistical analysis. Gels were matched to a reference gel composed of equal amount of samples from all the different treatments. Missing values were imputed with the BPCA (Bayesian Principal Component Analysis) method described by Oba et al. (2003) as detailed in Pedreschi et al. (2008). Data were log transformed to stabilize variance and outlying gels were removed with the 95% Hotelling's T^2 limit (Johnson and Wichem, 1998). Data were also mean-centered and variables weighed to give them equal (unity) variance. Partial least square discriminant analysis (PLS-DA) was performed to cluster the individual gels according to similar protein expression profiles. The correlation loading plots were used to identify the proteins which significantly contributed to class discrimination. The variable important plot (VIP) was used as a formal tool (Karp et al., 2005) based on the correlation loadings to identify the most important proteins describing the differences in protein expression profiles among the treatments. After selection of 100 proteins with PLS-DA and VIP procedures, these proteins were checked for downstream processing. Only successfully identified proteins were