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Escola Superior de Biotecnologia

INTERNAL BROWNING DISORDERS OF 'ROCHA' PEAR DURING LONG-TERM STORAGE

Thesis submitted to *Universidade Católica Portuguesa* to attain the degree of PhD in Biotechnology, with specialization in Food Science and Engineering

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RESUMO

A pera 'Rocha' (*Pyrus communis* L.) é uma cultivar Portuguesa que pode ser armazenada em atmosfera controlada (AC) durante 10 meses. No entanto, sabe-se que é suscetível ao acastanhamento interno (AI), uma das principais causas de perdas económicas durante o armazenamento prolongado. Embora muitos estudos tenham sido realizados para melhor compreender o AI em peras, os mecanismos subjacentes não permanecem por elucidar. O objetivo desta tese é investigar os mecanismos fisiológicos, bioquímicos e moleculares envolvidos no desenvolvimento do AI em pera 'Rocha', estabelecendo a base científica para o desenvolvimento de modelos preditivos para o AI e recomendações de manuseio pós-colheita, visando reduzir a incidência de AI em um ambiente regulatório em que as ferramentas químicas convencionais já não se encontram disponíveis.

O AI em pera pode ter vários sintomas e foi demostrado que a suscetibilidade da pera para desenvolver este acidente fisiológico é afetada pela maturação dos frutos à colheita. Neste trabalho, os AIs em pera 'Rocha' foram classificados em duas categorias: decomposição necrótica húmida e cavidades secas, que podem coexistir no mesmo fruto. A ocorrência de cavidades foi associada a períodos de armazenamento mais prolongados e à exposição dos frutos a alto CO_2. Os resultados mostraram claramente que as peras colhidas tardiamente eram mais suscetíveis ao AI do que as colhidas num estado de maturação precoce ou ótimo.

Para o estudo da base bioquímica do AI foram conduzidos três ensaios em três anos consecutivos. Na nossa primeira avaliação, realizada numa base de longo prazo, a fermentação desempenhou um papel importante no AI, mas o sistema antioxidante não. No entanto, no segundo ensaio, realizado numa base de curto prazo, os resultados sugeriram que o AI em pera 'Rocha' era desencadeado por stress oxidativo. Finalmente, no terceiro ensaio, a base bioquímica do AI relacionado com o CO_2 em pera 'Rocha' foi estabelecida. Os resultados mostraram que o mecanismo subjacente ao AI em pera 'Rocha' envolve a conjugação de ambos os metabolismos, antioxidante e fermentativo. A partir dos resultados obtidos um mecanismo o para o desenvolvimento do AI relacionada com o CO_2 foi proposto.

Neste estudo também se analisou pela primeira vez a regulação molecular de genes que codificam enzimas antioxidantes e fermentativas. As diferenças encontradas na regulação da transcrição destes genes apoiaram os dados bioquímicos. Os resultados

também mostraram que o armazenamento dos frutos em alto CO_2 leva a uma disfunção do sistema antioxidante e que, quando o alto CO_2 é combinado com níveis muito baixos de O_2 a fermentação é altamente induzida, com ambos os fatores a atuar sinergicamente no desenvolvimento de IBD.

Também trabalhamos para identificar marcadores de predisposição dos frutos para desenvolver AI, o que poderá ser útil para uma avaliação precoce do risco de desenvolvimento de AI. Entre os marcadores bioquímicos, os resultados evidenciaram o acetaldeído, o etanol e o ácido ascórbico como os mais promissores, ao passo que, entre os minerais, o cobre (Cu) era o melhor candidato. Em geral, modelos de previsão do AI em pera 'Rocha' foram desenvolvidos e validados neste trabalho, representando um importante passo na previsão do AI em pera 'Rocha'.

De forma a encontrar estratégias de controlo eficazes para prevenir o AI em pera 'Rocha' foram realizados três ensaios em dois anos consecutivos. Dois dos ensaios foram realizados no primeiro ano e o outro no ano seguinte. No primeiro ensaio, atmosferas controladas dinâmicas monitorizadas por fluorescência da clorofila (ACD-FC) e etanol (ACD-EtOH) foram avaliadas para a prevenção do AI. Pela primeira vez mostramos que o armazenamento dos frutos em ACD-FC conduz a uma incidência reduzida de AI, sendo esta uma metodologia promissora para o armazenamento prolongado de pera 'Rocha'. Pelo contrário, mostramos também que o armazenamento em ACD-EtOH poderá não ser adequado para prevenir o AI. No segundo e terceiro ensaios, a eficácia do tratamento com 1-metilciclopropeno (1-MCP) e do armazenamento em atmosfera controlada diferida na prevenção do AI foi avaliada. Pela primeira vez, mostrámos que a eficácia do 1-MCP depende da maturidade dos frutos à colheita e que, quando aplicado em frutos tardios, induz uma maior incidência de AI.

Os resultados também mostraram que o armazenamento de frutas atmosfera controlada diferida, em contraste com o observado para outras cultivares de pera, não foi eficaz na prevenção do AI em pera 'Rocha', podendo mesmo induzir a sua incidência.

Em geral, os resultados obtidos no decorrer desta tese proporcionaram novo conhecimento e uma melhor compreensão do AI em pera 'Rocha'. Os mecanismos bioquímicos subjacentes foram estabelecidos e pistas moleculares foram desvendadas; novas perspetivas para a modelação preditiva do AI foram dadas; uma nova e promissora metodologia para a prevenção do AI foi revelada e as limitações das estratégias de controlo atualmente usadas, quando aplicadas à pera 'Rocha', foram evidenciadas.

ABSTRACT

'Rocha' pear (*Pyrus communis* L.) is a Portuguese native cultivar which can be stored under controlled atmosphere (CA) for up to ten months. However, it is known to be susceptible to internal browning disorders (IBD), one of the major causes of economic losses during long-term storage. Although many studies have been conducted to better understand IBDs in pears, the underlying mechanisms of this disorder remains to be elucidated. The aim of this thesis is to investigate the physiological, biochemical and molecular mechanisms involved on IBD development in 'Rocha' pear, establishing the scientific basis for the development of IBD predictive models and postharvest handling recommendations aiming at reducing the incidence of IBD in a regulatory environment where conventional chemical tools are no longer available.

IBDs in pear can have various symptoms and pear susceptibility to develop this disorder has been shown to be affected by fruit maturity at harvest. In the current work IBDs in 'Rocha' pear were classified into two categories: wet necrotic breakdown and dry cavities, which may coexist in the same fruit. The occurrence of cavities was associated to longer storage durations and fruit exposure to high CO_2. The results clearly showed that late harvested pears were more susceptible to IBD than early and optimally harvested.

For the study of the biochemical basis of IBD three trials were conducted in three consecutive years. In our first assessment, conducted on a long-term basis, fermentation played a major role on IBD whereas the antioxidant system did not. However, in the second trial, conducted on a short-term basis, the results suggested that IBD in 'Rocha' pear was triggered by oxidative stress. Finally, in the third trial, the biochemical basis of CO_2-related IBD in 'Rocha' pear was established. The results showed that the underlying mechanism of IBD in 'Rocha' pear involves the conjugation of both, the antioxidant and fermentative metabolisms. From the results obtained a mechanism of CO_2-related IBD development was proposed.

In this study we also looked for the first time to the molecular regulation of genes codifying antioxidant and fermentative enzymes. The differences found in the transcriptional regulation of these genes supported the biochemical findings. The results also showed that fruit storage under high CO_2 leads to an impairment of the antioxidant system and that, when high CO_2 is combined with very low O_2 levels, there is a highly induction of fermentation with both factors acting synergistically on IBD development.

We also worked to identify markers of fruit predisposition to develop IBD, which could be useful for an early assessment of the risk of IBD development. Among the biochemical markers, the results evidenced acetaldehyde (AcDH), ethanol (EtOH) and ascorbic acid (AA) as the most promising ones, whereas, among the minerals, copper (Cu) was the best candidate. Overall, IBD prediction models for 'Rocha' pear have been developed and validated in this work, representing a major step forward in the prediction of IBD in 'Rocha' pear.

In order to find effective control strategies to prevent IBD in 'Rocha' pear three trials were conducted in two consecutive years. Two trials were conducted in the first year and the other in the following year. In the first trial, dynamic controlled atmospheres monitored by chlorophyll fluorescence (DCA-CF) and ethanol (DCA-EtOH) were evaluated for IBD prevention. For the first time, we showed that fruit storage under DCA-CF leads to a reduced IBD incidence, making it a promising methodology for 'Rocha' pear long-term storage. On the contrary, we also showed that storage under DCA-EtOH may not be suitable to prevent IBD. In the second and third trials, the efficacy of 1-MCP treatment and storage under delayed CA, in preventing IBD was assessed. For the first time, we showed that 1-MCP efficacy is dependent on fruit maturity at harvest and that, when applied to late harvested fruits, induces higher IBD incidence. The results also showed that fruit storage under delayed CA, in contrast to the observed for other pear cultivars, was not effective in preventing IBD in 'Rocha' pear, and may even induce its incidence.

Overall the results obtained in the course of this thesis provided novel knowledge and a better understanding of IBDs in 'Rocha' pear. The underlying biochemical mechanisms were established and molecular clues were unraveled; new perspectives for IBD predictive modelling were provided; a new promising methodology for IBD prevention was revealed and the limitations of currently used control strategies, when applied to 'Rocha' pears, were evidenced.

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LIST OF ABBREVIATIONS

1-MCP	1-methylcyclopropene
AcDH	Acetaldehyde
ACP	Anaerobic compensation point
ADH	Alcohol dehydrogenase
APX	Ascorbate peroxidase
AsA	Ascorbic acid
CA	Controlled atmosphere
CAT	Catalase
DCA	Dynamic controlled atmosphere
DCA-CF	Dynamic controlled atmosphere monitored by chlorophyll fluorescence
DCA-EtOH	Dynamic controlled atmosphere monitored by ethanol
DCR	Dynamic control respiration
DHA	Dehydroascorbate
DHAR	Dehydroascorbate reductase
EtOH	Ethanol
GR	Glutathione reductase
H_2O_2	Hydrogen peroxide
$HO_2^{\cdot -}$	Perhydroxyl radicals
IBD	Internal browning disorder
ILO	Initial low oxygen stress
MDHA	Monodehydroascorbate
MDHAR	Monodehydroascorbate reductase
O_2^-	Superoxide anion radical
OH·	Hydroxyl radical
PDC	Pyruvate decarboxylase
POX	Peroxidase
PPO	Polyphenoloxidase
RMSEP	Root mean square error of prediction
ROS	Reactive oxygen species
TAA	Total ascorbate
ULO	Ultra-low oxygen
CO_2	Carbon dioxide

O_2	Oxygen
PDO	Protected designation of origin
Cu	Copper
Fe	Iron
Zn	Zinc
P	Phosphorous
K	Potassium
Ca	Calcium
Mg	Magnesium
Mn	Manganese
B	Boron
Na	Sodium
PLS	Partial least square
PCA	Principal component analysis
VIP	Variable importance in the projection

CHAPTER 1.

General Introduction

Parts of this chapter were taken from:

Deuchande, T., Carvalho, S.M.P., Larrigaudière, C. and Vasconcelos, M. W. 2015. Advances in refrigerated and controlled atmosphere storage of fruits and vegetables. In: Gaspar, P.D. and Dinho, P. (Eds.), Handbook of Research on Advances and Applications in Refrigeration Systems and Technologies (pp 457-489). Hershey, PA: IGI-Global. doi:10.4018/978-1-4666-8398-3.ch013

1.1. GENERAL INTRODUCTION

In a global market a high postharvest quality and increased postharvest life of fresh fruits is of utmost importance for meeting consumers' demand for the availability of many types of high quality fresh fruits throughout the year (Thompson 2010). Most fruits have a relatively narrow harvest period where the supply largely exceeds the demand. Fruit ripening is a process that occurs from the latter stages of fruit growth and development until the early stages of senescence. This process involves many changes on the produce, including colour and texture changes, tissue softening, volatiles' production, and changes on sugar content, respiratory metabolism and ethylene production, among others (DeEll et al. 2003). In climacteric fruits, such as pears, which are capable to ripen to good quality after detached from the plant, it is essential to avoid the ripening process in order to maximize the retention of quality attributes.

'Rocha' pear is a member of the Rosacea family and belongs to the species *Pyrus communis* L. It is a Portuguese native cultivar mostly produced in Portugal (99% of world 'Rocha' pear production) (ANP, 2013) and most of its production is located in the West Region of the country, in a narrow coastal strip near the Atlantic sea, extending from Sintra to Alcobaça. This region has singular conditions for 'Rocha' pear production and therefore this pear variety has been classified as a PDO (Protected Designation of Origin) product.

'Rocha' pear is predominantly round, oval, presenting an average size between 60 and 65 mm and a weight of around 130 g, its skin is smooth and may have a yellow or green light. Its white pulp may be crunchy or soft depending on the ripening state, it is sweet, not acidic and very juicy (ANP, 1997). The combination of these features with its good resistance to handling, transport and storage, makes this pear cultivar a very interesting variety for both the domestic and international market.

'Rocha' pear is usually harvested in mid-August and can be stored for up to ten months under recommended storage conditions, being available almost the whole year. To ensure that fruits are harvested at the optimal harvest date for fruit long-term conservation a set of parameters are measured, including pulp firmness (which may be between 55 and 65 N), total soluble solids (ideally between 11 and 13%) and titratable acidity (between 2-3 g L^{-1} malic acid) (ANP, 1997) and an average starch index of 6.5 (Avelar & Rodrigues 1999). In general, these values are reached about 135 to 140 days after flowering (ANP, 2013).

1.2. 'ROCHA' PEAR ECONOMIC IMPORTANCE

In Portugal, 93% of the total fruit pear production comes from 'Rocha' pear produced in this PDO region (INE, 2012). Currently, there are approximately 11,000 hectares of crop area, capable of producing between 100-230 thousand tons of pear fruit per year (INE, 2015) with a market value at the farm gate estimated at 140 million euro and exporting over 70 million euros (INE, 2015). 'Rocha' pear is therefore one of the few export-oriented products of the Portuguese Agriculture and the most important fruit produced in the country.

Among the countries of the European Union (EU), Portugal is ranked in fifth place as one of the major pear producers also occupying the fourth place as one of the most important pear exporters (Fig. 1).

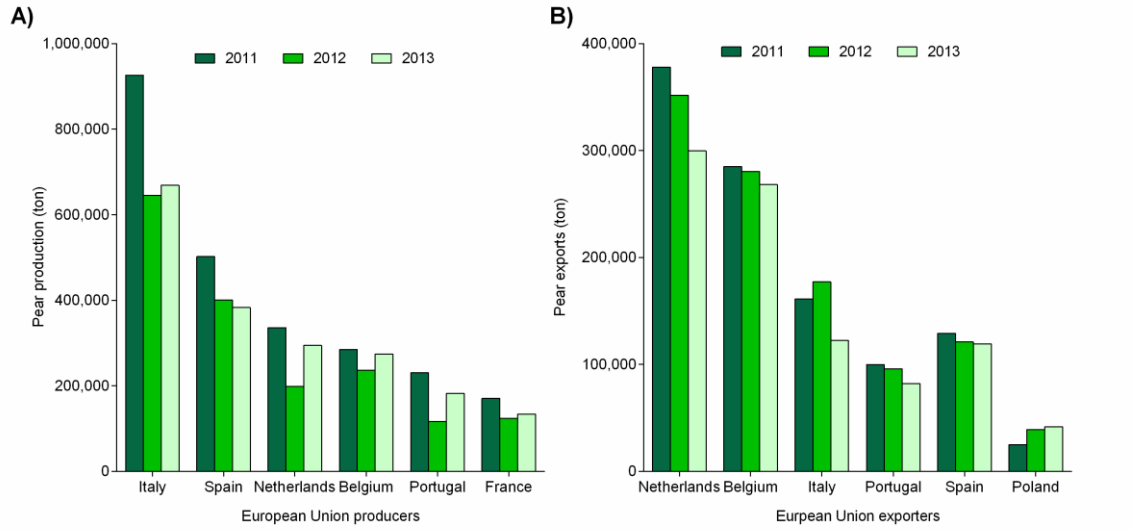

Figure 1: Pear production (A) and pear exportations (B) in the countries of the European Union from 2011 to 2013 (WAPA, 2016).

The production of 'Rocha' pear in Portugal has a considerable weight in the national economy, as it represents 95% of total pear produced across the country. In the campaign of 2013/2014 the production exceeded the 195 000 tonnes (ANP, 2013) and nearly 95,000 tonnes were directed to exportation mainly to Brazil (35%), UK (21%) and France (17%) (INE, 2015). With the domestic market absorbing the remaining 100 000 tonnes, the trade of 'Rocha' pear totalized a global revenue of around 140 million euros (INE, 2015).

In Portugal, this sector is well organized by cooperatives with packing houses equipped with cold rooms of 100 to 400 ton of capacity. High storage capacity is essential for provision of fresh fruits out of the harvest season. Long term storage of 'Rocha' pear is critical to face the seasonality associated to pear production. Its use allows the producers to respond to the market demand for 'Rocha' pear regarding the national and international markets, also avoiding the need for importation from the southern hemisphere.

1.3. COLD STORAGE

Cold storage is an important aspect when considering long term storage of fruits, particularly 'Rocha' pear. Fresh fruits need low temperature and high relative humidity to reduce respiration, slowing down the fruit's metabolism and simultaneously preserve quality and physiological characteristics such as taste, aroma, acidity, soluble solids content and nutritional properties. However, pears are very sensitive to temperature and are normally stored for long time periods at temperatures between -1 and 0 ºC with current recommendation for 'Rocha' pear being -0.5 ºC . Since each fruit variety has its optimal storage temperature considering the susceptibility to develop physiological disorders associated to cold storage, care has to be taken on maintaining temperature stability. Even a decline of 0.5 °C on storage temperature could result on physiological damage. Accordingly, it is crucial to uphold the refrigeration parameters under strict control to ensure that during and after storage, as well as during the shelf life period at room temperature the fruits maintain their quality. When setting up the refrigeration system there are three other very important parameters to have into consideration: relative humidity, ventilation and air circulation. High relative humidity inside the chamber is required to prevent loss of moisture from the fruit during storage and this is a challenging point since values of relative humidity near 100% are hard to achieve. Ventilation is also essential to restore the atmosphere inside the chamber. Although fruit metabolism is reduced to a minimum, during cold storage there is still consumption of oxygen (O_2) and production of carbon dioxide (CO_2) and ethylene (responsible for fruit ripening), which need to be replaced and removed, respectively. Air circulation ensures the uniform cooling of the stored fruits but it should be kept slow in order to avoid transpiration phenomena that may cause water loss with consequent fruit dehydration.

1.4. CONTROLLED ATMOSPHERE STORAGE

Cold storage is oftentimes combined with controlled atmosphere (CA) in an attempt to extend fruits storability. Although there is no formal definition of CA storage, this technology involves a constant monitoring and adjustment of the CO_2 and O_2 levels within gas tight chambers (Thompson 2010). In CA there is a need for precise control of the levels of O_2, CO_2 and ethylene since these gases have a high impact on fruit physiology during CA storage, particularly, during long term storage. There are also ethylene analyzers that continuously measure ethylene concentration in the storage chamber. In storage rooms where low ethylene is essential, checks can be made to verify if ventilation and ethylene removal systems are operating correctly.

1.4.1. Historical context

The first scientific studies on the use of modified atmospheres to preserve fruit were conducted by Berard (1821), when demonstrated that fruits consume O_2 and produce CO_2 and that the reduction of O_2 during storage would prevent ripening. Later, Kidd & West (1927) demonstrated for the first time that reduced O_2 and high CO_2 during storage would prolong the storage life of apple fruit. They also considered that CA was a complement to refrigeration and that it would not be successful if applied on its own (Kidd & West 1927). As a result of this study, after two years, the first CA storage was constructed for storage of apples in England (Beaudry 1999). Afterwards, Burg & Burg (1967) established that reduced O_2 and high CO_2 partial pressures during storage resulted in an inhibition of ethylene responses, concluding that, in the case of climacteric fruits, O_2 and CO_2 likely delay ripening, not only due to their effect on respiration, but also through their inhibitory effect on ethylene action.

1.4.2. Effect of CA storage on fruit physiology and quality

The main advantages of CA during long-term storage of fruits are: i) considerable decrease in respiration rate, with a reduction in climacteric maximum, accompanied by an expansion of both pre-climacteric and post-climacteric periods; ii) reduction of ethylene production (low O_2) and ethylene action (high CO_2, low temperature); iii) extension in storage life, which can be doubled; iv) preservation of flesh firmness, due to the effect of CO_2 on the enzymes acting on cellular membranes; v) high turgidity, such that fruits are more juicy and crisp; vi) preservation of quality attributes: smaller loss of acidity, sugars and vitamin C; vii) higher nutritional and sensory quality as well as limited degradation of

chlorophyll, with a consequent higher color retention; viii) fewer physiological alterations (e.g. chilling injuries, spot, decay, browning, water core and scald) ; ix) less decay by fungal contaminations.

Some of the main physiological disorders which are alleviated by CA include: chilling injury, ethylene injury, storage scald in pome fruits, core browning, also called brown core, brown heart or core flush, bitter pit, flesh breakdown, overstorage (a physiological disorder of pear characterized by the loss of capacity of pears to ripen with good eating quality when held at ripening temperatures (15–20°C) after prolonged storage at –1 to 0°C), pitting, spotted necrosis and discolouration (Prange & DeLong 2006). However, in certain situations, CA can induce some disorders such as internal browning.

1.4.2.1. Effect of high CO_2

Plant tissues can respond to elevated CO_2 levels in many ways. One of them is induction of fermentation, with decreased production of ATP, accumulation of glycolytic and Krebs cycle intermediates and acidifying intracellular compartments (Kader 1995). CO_2 was believed to reduce the ethylene production via an effect on respiration. Some studies of CO_2 effects on apples and pears showed that it inhibits several respiratory enzymes of the Krebs cycle (Kerbel et al. 1988; Knee 1973; Lange & Kader 1997; Monning 1983). However, De Wild, Otma, & Peppelenbos (2003) demonstrated, based on respiration measurements, that the effect of CO_2 on ethylene production did not operate via an effect on respiration.

1.4.2.2. Effect of low O_2

Low O_2 concentrations during storage allow a further reduction on respiration; however the levels of O_2 should be kept above the anaerobic compensation point (ACP). The ACP corresponds to the O_2 concentration at which the evolution of CO_2 levels is minimal (Boersig et al. 1988). As the oxygen level surrounding the fruit is reduced, the respiration decreases (Chervin et al. 1996). During long term storage the levels of O_2 should be sufficient to avoid fermentation. Therefore, levels of O_2 commonly used in CA storage are between the 2 and 3kPa. Despite these levels are much higher than the tolerated by the fruits, which are generally in the range of the 0.2 and 0.7 kPa for climacteric fruits, it is important to ensure that O_2 is uniformly distributed within the fruit for the long term storage. Levels of O_2 below 1 kPa around the produce may not be sufficient to achieve this requirement during long term CA storage. Conditions of hypoxia within the fruit may lead

to changes in the respiratory metabolism, causing the switch from aerobic to anaerobic metabolism (Ke et al. 1990). It may lead to the accumulation of potentially harmful fermentative metabolites (ethanol and acetaldehyde) and products of glycolysis, such as lactate or acetaldehyde.

Ethanol is usually the major product of the pathway in low O_2-stressed fruits (Ke & Kader 1992), but acetaldehyde is considered more toxic (Chervin et al. 1996). Furthermore, the excessive production of fermentative metabolites may lead to the development of off-flavours. To avoid fermentation, it is important to set up the gas concentration in the storage chamber according to the ACP of the specific product to be stored.

There are interactive effects of the two gases in extending the storage life fruits. It has been shown that the effects of reduced O_2 and elevated CO_2 on metabolic changes and respiratory activity are additive. Burg & Burg (1967) demonstrated that storage under 10 kPa CO_2 influences respiratory metabolism to about the same extent as 2 kPa O_2 and that a combination of 2 kPa O_2 + 10 kPa CO_2 has approximately twice the effect of either component.

1.4.2.3. The influence of pre- and post-harvest factors

The effects of CA storage on fruit preservation are also dependent on factors other than temperature and gas composition, including: 1) fruit species and cultivar; 3) climatic conditions; 4) growth conditions (orchard management and soil); 5) stage of fruit maturity at harvest; 6) temperature of the produce before storage under CA; 7) temperature and RH inside the storage chamber; 8) postharvest treatments. Among the pre-harvest factors, maturity at harvest is one of the most important, oftentimes determining the storage life and final quality of fruits. Fruits harvested before and after the optimal harvest date are commonly more prone to develop physiological disorders and generally have shorter storage life and inferior quality (Kader 1999). Considering the postharvest factors, pre-cooling and postharvest treatments such as application of fungicides and ethylene inhibitors, wax emulsions, ethylene absorbents, anti-transpirants, senescence retardants are also very important to extend the storability and marketability of fresh fruit.

1.5. LONG TERM STORAGE OF 'ROCHA' PEAR UNDER CA

Pears are very sensitive to temperature and are normally stored for long time periods at temperatures between -1 and 0 ºC and 90 to 95% RH with current recommendation for 'Rocha' pear being -0.5 ºC and 95% RH (Silva et al. 2010). Cold storage of 'Rocha' pear is combined with CA in an attempt to extend its storability preserving its quality attributes. During CA storage, fruit ripening is delayed and hence the ethylene production is lowered, ensuring a better retention of the quality attributes during long-term storage. Current recommended atmosphere for CA storage of 'Rocha' pear is 2.5-3 kPa O_2 + 0.5-0.7 kPa CO_2, but these values may range from 1 - 3 kPa O_2 and 0 - 5 kPa CO_2, depending on pear cultivar (Silva et al. 2010).

Although 'Rocha' pear can be stored for prolonged time periods, it is known to be susceptible to IBD and superficial scald, the two major causes of postharvest losses during long-term storage under CA (Silva et al. 2010; Isidoro & Almeida 2006).

The diphenylamine (DPA), a synthetic antioxidant, was previously applied to pome fruits to prevent these physiological disorders. It was the only method available to effectively prevent superficial scald in pome fruits and despite not indicated in the registered use, DPA was also beneficial in the prevention of IBD (Silva et al. 2008; Silva et al. 2010). However, in 2009, by decision of the European Commission, the application of this product was prohibited (EC 2009) and after the campaign of 2013 its application was effectively banned. In the absence of DPA treatment, fruits may develop IBD just after three to four months of storage under CA, depending on fruits specific susceptibility, reducing the potential of 'Rocha' pear for exportation and availability during the whole year (Almeida 2010). After the effective prohibition on the use of DPA, the European Union allowed the use of ethoxyquin during the campaign of 2014 (EC 2014). Ethoxyquin is a synthetic antioxidant considered as effective as DPA in preventing superficial scald albeit less effective in controlling IBD in pears (Kupferman & Gutzwiler, 2003). However, this product is also considered potentially harmful and hence its application was also prohibited by the European Union (EC 2014; EC 2016). The impact of storage disorders in 'Rocha' pear in the absence of DPA treatment was estimated in 39 million euros or 28% of the total crop value (Almeida 2010).

In the absence of DPA or ethoxyquin treatment, 'Rocha' pears may develop IBD just after three to four months of storage under CA, depending on fruits specific

susceptibility, reducing the potential of 'Rocha' pear for exportation and availability throughout the whole year.

Considering that ripening and postharvest pear behaviour is variety-specific, the current restriction on the application of synthetic antioxidants generated a need for understanding the physiological and biochemical bases of IBD in 'Rocha' pear in order to develop postharvest handling recommendations aiming at reducing postharvest losses. Given the geography of 'Rocha' pear production, this cultivar was rarely used as model system to study postharvest physiological disorders. Therefore, there is scarce information on the physiological, biochemical and molecular mechanisms involved on the occurrence and development of IBD in this pear cultivar. To date, there is no effective strategy to prevent IBD in 'Rocha' pear during long-term storage under CA and hence there is an urgent need for establishing the scientific basis for the development of control strategies to prevent IBD in this pear cultivar also ensuring the maximum retention of quality.

1.6. INTERNAL BROWNING DISORDERS

Internal browning is a physiological disorder occurring mainly during long term storage under CA but fruit stored in air may also develop symptoms (Franck et al. 2007). This disorder has no external expression even when it is widely spread from the core to the cortex; hence the specific symptoms and rate of development of this disorder have not been clearly established. To date, although at laboratory scale non-destructive methods for IBD detection exist, using for example X-ray technologies, magnetic resonance and infrared spectroscopy reflection, there is no non-destructive technology available for its detection at a commercial scale (Franck et al. 2007). Furthermore, mathematical models were not yet developed for this cultivar, which would allow an early assessment of the fruits risk for IBD development in different fruit batches, allowing the selection of the ideal storage conditions.

The symptomatology of IBDs include radial or asymmetrical browning, dry brown spots or randomly dry cavities in the pulp tissue (Franck et al. 2007).

Evidence suggests that, in 'Conference' pear, CO_2-related disorders, which appear associated to the appearance of dry cavities in the carpelar region which can be extended to the mesocarp, differ from senescence related disorders, often associated to wet necrotic breakdown of the flesh (Larrigaudière et al. 2004). However, both symptoms, wet necrotic breakdown and dry cavities, may coexist in the same fruit and limited gas diffusion and

hypoxia at the centre of the fruit is likely involved in both instances (Lammertyn et al. 2003). Therefore, although internal browning can have various symptoms, whether these symptoms are related to the same or to distinct physiological disorders is a matter of debate (Franck et al. 2007; Larrigaudière et al. 2004). Arguably, brown heart, which is often associated to high CO_2, and core browning, which is related to senescent processes, are two physiological disorders with different etiology (Larrigaudière et al. 2004). Decreased levels of ascorbate and subsequent lipid peroxidation are likely early events leading to brown heart (Veltman, Sanders, et al. 1999; Larrigaudière et al. 2004) whereas evidence suggests that core browning is more related to fermentative metabolism (Larrigaudière et al. 2001; Pintó et al. 2001; Larrigaudière et al. 2004). However, the same data are susceptible to other interpretations. For instance, core browning and brown heart may be different developmental stages of the same physiological process.

Symptoms of IBDs in 'Rocha' pear are not yet precisely described and a detailed description of symptomatology would be useful to better understand the development of this disorder in this pear cultivar.

Figure 2: Rocha pear exhibiting internal browning disorder.

1.6.1. Biochemistry of internal browning

IBDs are firstly related to membranes' damage which allows the polyphenols previously retained into the vacuoles and the polyphenol oxidases (PPOs) mainly located in the plastids to be released into the cytosol where they become into contact, leading to the enzymatic browning (Nicolas et al. 1994). The PPOs comprise two distinct enzymes, the laccase (EC 1.10.3.2) and tyrosinase (EC 1.14.18.1), which differ on their specificity for substrates (Vámos-Vigyázó 1995) (Fig. 3). The laccase has its action restricted to the oxidation of ortho and para-diphenols to form the respective o-quinones (catecholase

activity). The tyrosinase is responsible for the oxidative browning of the tissues by hydroxylation of monophenols to *o*-diphenols (cresolase activity) and subsequent oxidation of the *o*-diphenols to *o*-quinonas (catecholase activity) which by subsequent oxidation and polymerization form the browning compounds called melanins (Veltman, Larrigaudière, et al. 1999).

It is known that *o*-quinones could be reduced back to *o*-diphenols through the action of ascorbic acid (AsA) and that PPO, as a copper-containing enzyme, may have its activity inhibited by AsA since it may act as a copper chelating agent (Macheix et al. 1990; Di Guardo et al. 2013). Under oxidative stress the cells may use their AsA pool in these processes in order to avoid IBD. In this sense, a continuous exposure of the fruits to oxidative stress may lead to a depletion of AsA and afterwards to an increased susceptibility to develop IBD since the increase of DHA/AsA ratio may impair the functioning of the antioxidant system. Despite crucial to IBD development, PPO has been shown not to be a limiting factor on the development of this disorder (Veltman, Larrigaudière, et al. 1999). Therefore, in the origin of IBD there are the factors affecting the integrity of the intracellular membranes.

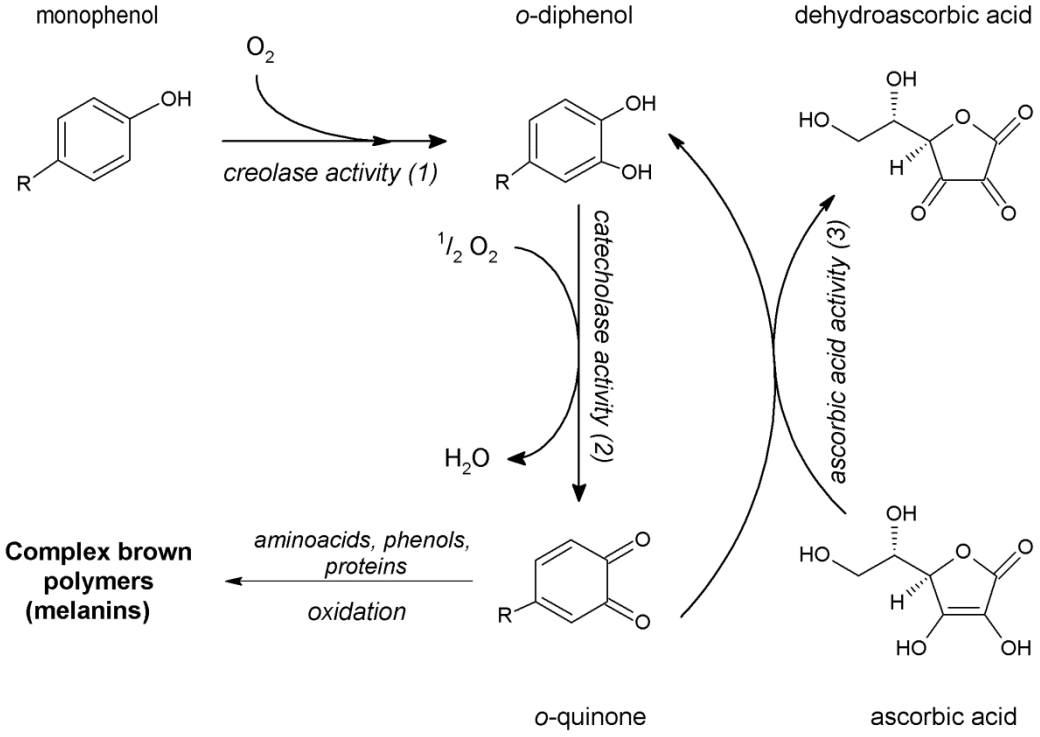

Figure 3: Reactions catalysed by PPO and ascorbic acid. Monophenols are hydroxylated to *o*-diphenols by PPO (creolase activity) (1). *o*-diphenols are oxidized to *o*-quinones through PPO catecholase activity (2). Ascorbic acid reduces the *o*-quinones back to *o*-diphenols (3) forming dehydroascorbic acid.

Peroxidase (POX) is also alleged to be related to IBD development in pears by causing the oxidation of phenolic compounds in the presence of H_2O_2 (Tomás-Barberán & Espín 2001). The POX appears to act synergistically with the PPO in the formation of browning, but it is dependent on the initial activity of PPO generating H_2O_2 via oxidation of phenolic compounds (Tomás-Barberán & Espín 2001). These enzymes are found in two distinct forms in plants, in its soluble form at the level of the cytoplasm of the cells and insoluble form bound to the cell walls (Vámos-Vigyázó 1981). To the best of our knowledge the study of the involvement of PPO and POX on IBD development in 'Rocha' pear during long term storage under CA was not addressed.

1.6.2. The primary cause of IBD: investigating membrane damage causes

Many studies have been carried out to explain membrane damage during pear storage under CA and some hypotheses have been drawn. During storage under CA, i.e. high CO_2 and low O_2 levels fruits are exposed to highly hypoxic conditions particularly at the fruit core. Under hypoxia or anoxia, fruits' respiration may change from aerobic to predominantly anaerobic. As a result, cells may be unable to generate the necessary energy for membrane regeneration as well as maintenance of the antioxidant system, accumulating reactive oxygen species (ROS) (Peppelenbos & Oosterhaven 1998; Larrigaudière et al. 2001). ROS are highly reactive molecules that can indiscriminately react with almost all the cellular components, causing lipid peroxidation and protein denaturation (Halliwell & Gutteridge 1989; Apel & Hirt 2004), thus compromising membrane integrity (Nicolas et al. 1994; Franck et al. 2007). If on the one hand the respiratory change towards fermentation may lead to a cell incapacity to sustain an efficient functioning of the antioxidant system, on the other hand it induces an excessive accumulation of fermentative metabolites, that beyond altering membranes fluidity and gas diffusivities within the fruit may also be toxic to the cells, contributing to membrane damage (Chervin et al. 1999; Ke & Kader 1989; Chervin et al. 1996).

There are some studies establishing the biochemical basis of IBD in some pear cultivars but there is scarce information on the biochemical underlying mechanisms involved on IBD development in 'Rocha' pear.

1.6.2.1. Oxidative stress

ROS, being partially reduced forms of molecular oxygen (O_2) formed due to electron transfer, are the by-product of the normal metabolic process (Foyer & Noctor

2005). As mentioned above, a highly hypoxic environment within the fruit tissue, particularly in the inner part of the fruit, reached during long term storage under CA, may lead to an excessive production of these reactive oxygen molecules resulting in oxidative stress (Møller 2001). These oxygen species include the superoxide anion radical (O_2^-), perhydroxyl radicals (HO_2^-), the highly toxic hydroxyl radical (OH·) and hydrogen peroxide (H_2O_2), among others (Apel & Hirt 2004). In order to protect the cell against the excessive production of ROS, the antioxidant defence system increases several of its enzymatic and non-enzymatic components (Gill & Tuteja 2010). The enzymatic components include antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and peroxidase (POX). These enzymes prevent the initiation of oxidation and tissue deterioration by scavenging ROS before they start reacting with cellular components. Among the non-enzymatic antioxidants, ascorbic acid is definitely the most important being capable of quenching ROS directly by reacting and removing them and/or indirectly by donating electrons to APX (Gill & Tuteja 2010).

In the following sections further details on the functioning of the antioxidant system are given.

1.6.2.1.1. Reactive oxygen species and the role of superoxide dismutase

The superoxide anion radical (O_2^-) is formed when there is a transfer of an electron to O_2 and it is very unstable having a very short life (2-4 μs) (Bhattacharjee 2010). A major sources of O_2^- in fruits is the electron transport chain of mitochondria but it can also be produced in the peroxisome and cytosol. O_2^- is not able to cross the phospholipid bilayer of membranes and thus its action is restricted to the place of formation (Bhattacharjee 2010). This radical may act as either a univalent oxidant or reductant (Fig. 4). O_2^- can be converted to H_2O_2 by electron transfer or SOD action, or be protonated to form the HO_2• radical (the conjugate acid of superoxide) which under acidic conditions is the dominant form of superoxide (Gill & Tuteja 2010). HO_2• radical is more reactive than superoxide itself (Elstner 1987) and may initiate lipid peroxidation (Aikens & Dix 1991) contributing to membrane damage.

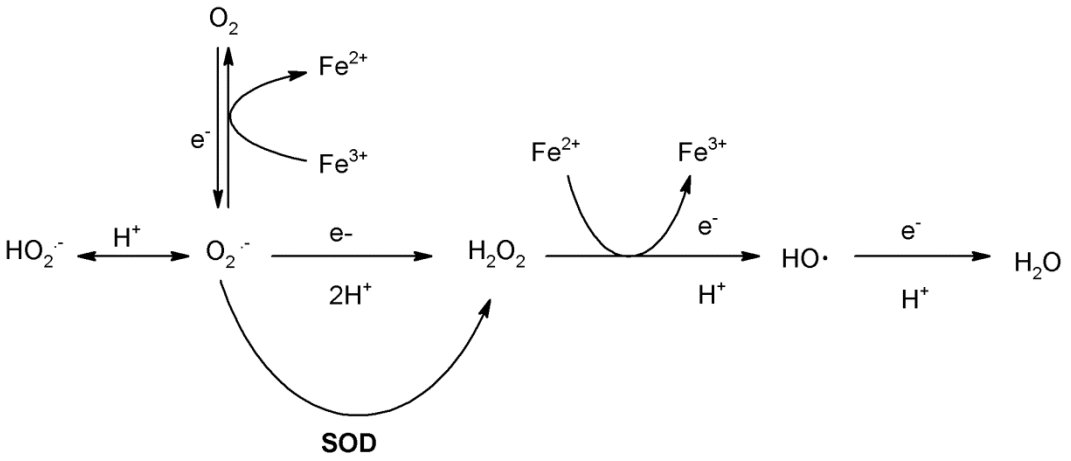

Figure 4: Generation of Reactive oxygen species through energy transfer and SOD action.

The enzyme SOD (EC 1.15.1.1) can be regarded as the first line of defense of the antioxidant system, and can be found in the chloroplasts, mitochondria, peroxisomes or apoplasts (Bhattacharjee 2010). These enzymes are responsible for the conversion of $O_2^{\cdot -}$ to H_2O_2 (Fig. 4). SODs comprise three groups of isoenzymes, Cu/Zn-SOD, Mn-SOD, Fe-SOD, which act differently depending on the metal molecule to which they are associated (co-factor). Cu/Zn-SOD is mostly located in the cytosol, mitochondria and plastids; Mn-SOD is located in the mitochondria and peroxisomes and Fe-SOD can be found in the chloroplasts, cytosol, mitochondria and peroxisomes (Mittler 2002).

Later on, the formed H_2O_2, which is also potentially toxic, is further metabolized to water by the action of a number of enzymes including CAT, APX and POX.

1.6.2.1.2. H_2O_2 and H_2O_2-scavenging enzymes

H_2O_2 is generated in the chloroplasts, mitochondria, peroxisomes, cytosol, cell walls and apoplastic space as a response to several abiotic stresses (Bhattacharjee 2010). OH· is generated by H_2O_2 and $O_2^{\cdot -}$ in the presence of iron and copper via the Haber-Weiss or Fenton reaction (Bhattacharjee 2010). This ROS is highly reactive and can therefore oxidize all biological molecules, because the plant cells antioxidant machinery is not able to scavenge it (Gill & Tuteja 2010). Its formation is prevented through the concerted action of SOD, CAT, APX and POX as well as ferritin, which sequesters Fe preventing to some extent the Fenton reaction (Mylona & Polidoros 2010).

Unlike the oxygen radicals, H_2O_2 can diffuse across biological membranes possibly causing oxidative stress far from the site of formation or may act as a signal molecule (Mhamdi et al. 2010). It has been shown to act as a key regulator in a broad range of

physiological processes, such as senescence (Peng et al. 2005), cell cycle (Mittler et al. 2004) and growth and development (Foreman et al. 2003). It has been established that excess of H_2O_2 in the plant cells leads to the occurrence of oxidative stress (Gill & Tuteja 2010) and may induce the programmed cell death) as a defence response. However, ROS are not only destructive, but at sublethal levels, they have been shown to act as signal molecules activating defense responses during oxidative stress (Ahmad et al. 2009). Prasad et al. (1994) reported that enhanced levels of H_2O_2 induce metabolic events associated to increased chilling tolerance in maize seedlings. This means that at certain levels, H_2O_2 might be an active signal molecule, involved on the regulation of antioxidant enzyme activity and physiological or molecular processes rather than an adverse ROS.

CAT detoxifies H_2O_2 converting it into water and oxygen (Fig. 5). This enzyme is found mainly in the peroxisomes, but it can also be located in the cytoplasm and mitochondria (Scandalios et al. 1997). This enzyme is unique among H_2O_2-scavenging enzymes since it is able to degrade H_2O_2 without consuming cellular reducing equivalents (Mittler 2002). Hence, when there is a lack of available energy and H_2O_2 is rapidly being generated through catabolic processes, H_2O_2 may be degraded by CAT in an energy efficient manner. CAT has a very fast turnover rate, but a much lower affinity for H_2O_2 compared to APX (Mittler 2002). However, while CAT is able to rapidly and directly reduce H_2O_2 into water and O_2, reduction by APX requires ascorbate as a reducing equivalent (Fig. 5).

APX is an enzyme which includes the Class I heme peroxidases, and uses ascorbic acid as the electron donor to convert H_2O_2 into water, oxidizing ascorbate and forming two molecules of monodehydroascorbate (MDHA) (Fig. 5). Given its high specificity to ascorbate, its activity is highly dependent on the substrate concentration. This enzyme can be found in several locations in the cell including the cytosol, mitochondria, chloroplast stroma and in the case of its soluble isoenzymes, peroxisomes and chloroplast thylakoid (Shigeoka et al. 2002).

POX belongs to the group of the oxidoreductases. This enzyme comprises a group of non-donor peroxidases for which guaiacol is a common donor. It catalyses breakdown of H_2O_2 into H_2O and O_2 and can be found mainly in cytosol and cell wall (Mylona & Polidoros 2010).

1.6.2.2. Ascorbic acid and ascorbate recycling enzymes

Ascorbic acid (AA) is a molecule that can be synthesized by most animals and plants, and is the most important reducing substrate in plant cells to eliminate H_2O_2 (Noctor & Foyer 1998). AA can be found in its reduced form (AsA) and in its two oxidized forms (monodehydroascorbate (MDHA) and dehydroascorbate (DHA). This molecule can be found in many cell compartments, such as chloroplasts, mitochondria and peroxisomes, at intermediate concentrations, and also in vacuoles and cell walls at lower concentrations (Ahmad et al. 2010).

In addition to the conversion of H_2O_2 into water, ascorbic acid also acts on ROS detoxification, either directly or through the ascorbate-glutathione cycle, being also involved in other plant processes such as photosynthesis and cell growth and development (Ahmad et al. 2010). Ascorbate is capable of destroying ROS directly by reacting and removing them and/or indirectly by donating electrons to APX. Importantly, ascorbate has the ability to reduce the formed *o*-quinones back to *o*-diphenols preventing the development of browning (Fig. 3). It has been shown that during storage, the levels of ascorbate decrease (Veltman, Sanders, et al. 1999; Zerbini, Rizzolo, et al. 2002) which may compromise the antioxidant potential of the fruit.

The ratio of DHA/AsA has been reported to be an important factor in plant resistance to oxidative stress (Waskiewicz et al. 2014). Therefore, the enzymes responsible for the regeneration of AsA, monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) have a significant impact on the fruit's capacity to respond to oxidative stress.

MDHAR is the major constituent of the glutathione ascorbate cycle, using monodehydroascorbate (MDHA) as an electron acceptor and NAD(P)H as an electron donor to regenerate ascorbic acid. This enzyme is localized in different cell organelles such as chloroplasts, cytosol, mitochondria and peroxisomes (Bhattacharjee 2010). Similarly, DHAR is localized in different cell organelles (Bhattacharjee 2010) and it uses reduced glutathione (GSH) as a co-factor to convert DHA to AsA. GSH as a co-factor in this reaction may limit the cell capacity to regenerate AsA. In order to maintain the GSH pool, glutathione reductase (GR) catalyzes the reduction of glutathione disulphide (GSSG) to glutathione (GSH), using NADPH as the reducing agent in the reaction (Fig. 5). GR is a flavo-protein oxidoreductase and can be found mainly in the chloroplast, but it can also be found in lower quantities in the mitochondria, cytosol and peroxisome (Kocsy et al. 2001).

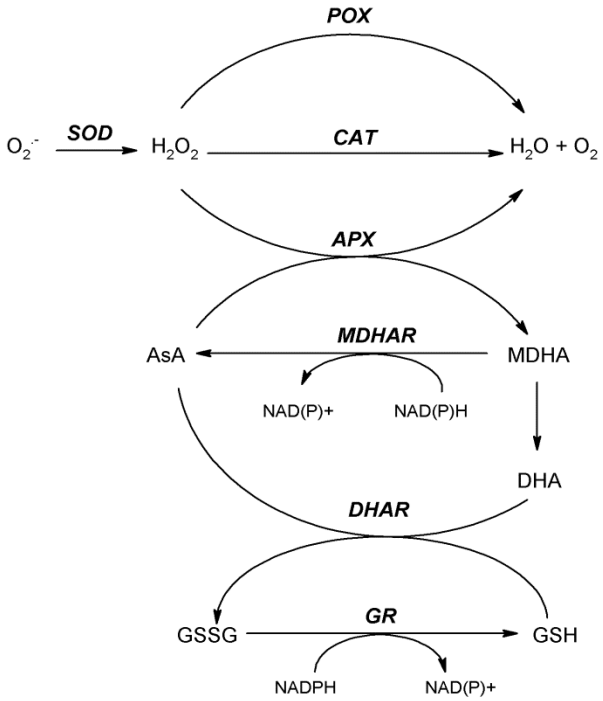

Figure 5: Scheme of the the functioning of the antioxidant defence system

1.6.2.3. Changes in respiration towards fermentation

The storage of the fruits under high CO_2 and low O_2 at low temperature may lead to a shift from an aerobic to an anaerobic metabolism with accumulation of fermentative metabolites, such as ethanol and acetaldehyde. These, when present above tolerated levels, become toxic, causing changes on membrane fluidity and gas diffusivity in the fruit tissues (Chervin et al. 1999; Ho et al. 2008; Ho et al. 2009; Ke et al. 1994). It may for instance cause membrane damage with subsequent loss of integrity of intracellular structures.

During long term storage under CA, respiration may switch from aerobic to anaerobic as a result of hypoxia within the fruit. This switch may involve an induction of a transient lactic fermentation with accumulation of lactic acid and consequent reduction of cytosolic pH to an optimal level for pyruvate decarboxylase (PDC), directing pyruvate towards ethanolic fermentation (Davies 1980). PDC converts pyruvate to acetaldehyde which is then further metabolized by alcohol dehydrogenase (ADH) to ethanol, the major and ultimate end-product product of fermentation (Fig. 6). The fermentative metabolites formed through ethanolic fermentation are important to several processes in the fruit tissue, particularly ripening. However, when there is an excessive accumulation, these metabolites

become toxic to the cells (Chervin et al. 1996; Kimmerer & MacDonald 1987; Perata & Alpi 1991).

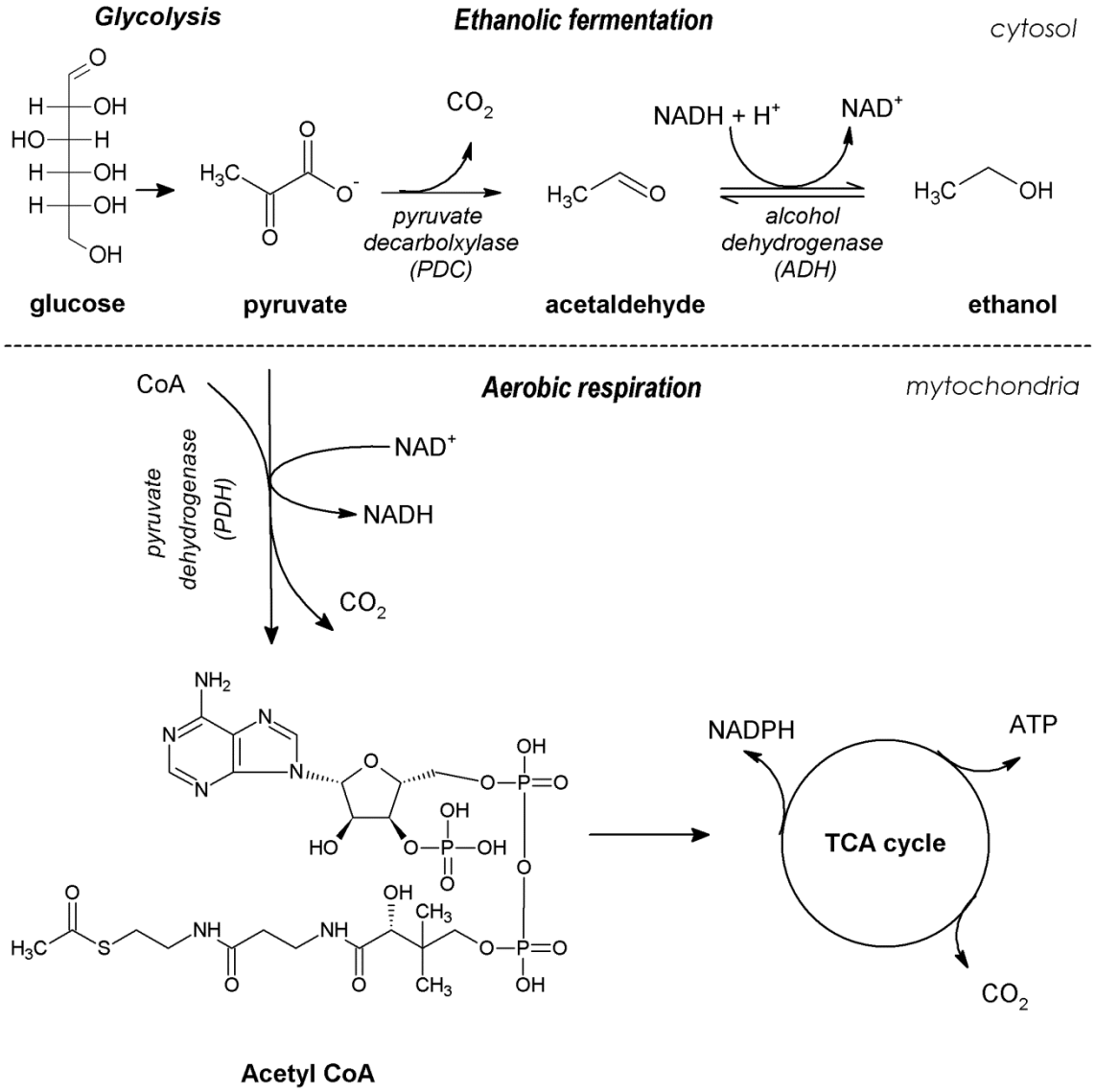

Figure 6: Schematic representation of aerobic respiration and ethanolic fermentation pathways.

Under hypoxia, glycolysis is highly up-regulated in order to produce maximum energy when ATP production via oxidative phosphorylation in the mitochondria is at risk (Pedreschi et al. 2007). Furthermore, the fermentative reactions make use of NADH regenerating NAD^+ which is needed to sustain glycolysis. However, as the fermentation proceeds, there is an accumulation of acetaldehyde which in turn may inhibit the PDC activity (Juni 1961).

1.6.2.4. Fruit mineral composition

Fruit mineral composition have also previously been shown to influence the incidence of IBD in apples and pears (Xuan et al. 2003; Xuan et al. 2001; Streif et al. 2001; Fallahi et al. 2010), but their role on IBD development is less well described. For instance boron, (B) has been widely reported to reduce the incidence of IBD in pears and apples stored under CA (Xuan et al. 2001; Xuan et al. 2003; Streif et al. 2001; Neuwald et al. 2014; Fallahi et al. 2010), but excessive amounts of B have also been reported to increase the incidence of watercore and flesh breakdown in apples and pears (Marlow & Loescher 1984; Kupferman 2002; Watkins 2009). The tolerance to boron was also very dependent on the cultivar.

Also, considering that iron (Fe) mediates the formation of ROS, under stressful conditions it high concentrations of Fe may favour membrane damage with consequent increased IBD incidence. Phosphorous (P) may also influence IBD since it is involved in the energy metabolism processes (R. H. Veltman et al. 2003) and it is known to affect enzyme phosphorylation status as well as modulate the action of Ca as a secondary messenger. All these factors have been previously suggested to influence IBD incidence (Larrigaudiere et al. 1996). Sodium (Na) is an important electrolyte with osmoregulatory function also contributing to the maintenance of an adequate cellular pH (Hawkesford et al. 2012). Adequate Na levels may prevent pH changes in fruit tissue as a consequence of fruits exposure to high CO_2 during CA storage (Lange & Kader 1997). Changes in the pH of fruits tissues may impair the electron transport chain in the mitochondria leading to deregulation of aerobic respiration. Calcium (Ca) is another mineral reported to influence fruit susceptibility to IBD. It is an important constituent of membranes as it stabilizes the membranes' phospholipids. Low Ca concentration has been previously linked to increased incidence of disorders in apples and pears (Ferguson et al. 1999; Streif et al. 2001; Broadley et al. 2012). However, these effects seem to be dependent on the cultivar since contradictory results were reported (James & Jobling 2009). In sum, the role of mineral concentrations on the prevention or induction of IBD is still a greatly unexplored are of research.

1.6.3. Molecular mechanisms involved on IBD development

Little is known about the molecular regulation of antioxidant and fermentative enzymes of pears during storage under CA and on the effect of such regulation on IBD development. It has previously been shown that the regulation of the enzymes involved in

the ascorbate recycling pathway may play a relevant role in the development of IBD in pears. For instance, APX is up-regulated at the transcriptional level under high CO_2 conditions in three pear cultivars (Cascia et al. 2013). Cytosolic APX has been shown to be the most stress responsive isozyme of the APX family (Cascia et al. 2013; Davletova et al. 2005; Wang & Dilley 2000) being up-regulated in sound tissue of 'Conference' pears when compared to brown tissue (Pedreschi et al. 2007). MDHAR and SOD have also been shown to be up-regulated in brown tissue but for SOD its transcription appeared to be up-regulated in sound tissues. In browning affected apples APX expression has been shown to be up-regulated in the inner cortex and SOD and CAT in the outer cortex (Mellidou et al. 2014).

Regarding fermentation there is no information concerning the regulation of this pathway in pears stored under CA. For instance, increased transcript levels of alcohol dehydrogenase (ADH), the enzyme responsible for the conversion of acetaldehyde to ethanol, were reported in the inner cortex of affected apples, during storage under ultra-low O_2 (Mellidou et al. 2014).

To the best of our knowledge no study was previously conducted in 'Rocha' pear addressing how the transcriptional regulation of the enzymes of the antioxidant and fermentative metabolisms may contribute for IBD development during CA storage. Therefore, any study conducted on this topic is a major step forward when trying to better understand the underlying mechanisms involved on IBD development in 'Rocha' pear.

1.7. CURRENT STRATEGIES UNDER INVESTIGATION TO PREVENT IBD

To date, no effective strategy exists to prevent IBD in 'Rocha' pear during long-term storage under CA. Currently, most promising strategies are the fruit treatment with 1-methylcyclopropene (1-MCP), and fruit storage under delayed CA or dynamic controlled atmosphere (Larrigaudière et al. 2004; Prange et al. 2011).

1.7.1. Dynamic controlled atmospheres

Storage under dynamic controlled atmosphere (DCA) storage is another alternative under investigation for long term storage of 'Rocha' pear. During DCA, the O_2 is gradually reduced to below 1 kPa, far below the values used in static CA (generally above the 2 kPa), in order to maximize the maintenance of the fruit quality during storage. Once the critical level of O_2 is reached, which is based on monitoring of the fruit physiological responses to low-O_2 stress, the levels of the gases inside the storage room are adjusted. In order to use

DCA, some specialized sensors have been developed. For instance, these sensors are capable of detecting the peak of the respiratory quotient (ratio of CO_2 produced/ O_2 consumed) (Gasser et al. 2010), the chlorophyll fluorescence signal (CF) (Prange et al. 2003; DeEll et al. 2003) or the ethanol production (Schouten et al. 1997; R.H. Veltman et al. 2003). The increases in the respiratory quotient, in the chlorophyll fluorescence and in the production of ethanol correspond to the transition from aerobic to anaerobic metabolism. Thus, according to the information given by the specific sensor used, the O_2 levels are adjusted in order to restore the optimal conditions of storage. These sensors must be accurate enough to ensure that the atmosphere is efficiently adjusted before damage occurs in fruits resulting from the use of low O_2. The setting of storage conditions can be automatic, i.e., the sensors can be connected to a monitoring system able to automatically adjust the levels of O_2 in the chamber or it can be manually operated.

There is scarce information available for the effect of dynamic controlled atmospheres on browning development in pome fruits, but some studies report contradictory effects on the prevention of IBD (Lafer 2011; DeEll et al. 2000; Mattheis et al. 2013). Considering the potential of this technology to prevent IBD and the lack of any study on the effect of these atmospheres in 'Rocha' pear browning it is important to evaluate its potential for the long term-storage.

1.7.2. 1-MCP treatment

The 1-MCP has been suggested as an alternative to DPA, to ensure the maintenance of fruit quality parameters and to prevent the incidence of physiological disorders during long-term storage. 1-MCP acts as an inhibitor of ethylene, and being structurally similar to this hormone it has the ability to bind irreversibly to ethylene receptors inhibiting its action and thus the ripening process. The inhibition of ethylene production is dependent on the 1-MCP dose applied, duration and storage conditions (Chiriboga, Schotsmans, et al. 2013). The application of 1-MCP has been shown to decrease the rate of respiration and ethylene production, maintain firmness and acidity, as well as reduce the incidence of superficial scald in 'Empire' apple (DeEll et al. 2005; Kochhar et al. 2003; Elgar et al. 1998). Its application has also been shown to contribute for a better quality retention and reduced incidence of physiological disorders in some pear varieties (Isidoro & Almeida 2006; Ke et al. 1991; Chiriboga, Saladié, et al. 2013).

However, in 'Rocha', pear, as observed in other pear varieties such as 'Conference' (Chiriboga et al. 2011), oftentimes the "evergreen" phenomenon occurs which is a major

drawback. This event is characterized by a permanent inhibition of the ability of the fruit to ripen. Thus, after storage, fruits remain green and hard during the shelf life never reaching the desired ripening stage (Chiriboga et al. 2011). This factor is an obstacle to the application of this product. For instance, it has been reported that storage of 1-MCP treated 'Rocha' pear under CA at 2.5 ºC provides an adequate post-storage ripening behaviour but also induced IBD incidence (Gago et al. 2013). Thus, further studies are needed to define a protocol for 1-MCP application in 'Rocha' pear, adjusting the concentration and duration of the treatment having into account pre and post-harvest factors capable of affecting the effectiveness of the treatment or prompt the impairment of ripening. For instance, the effect of 1-MCP treatment has not yet been studied in 'Rocha' pears harvested at different maturity stages, and it is likely that the fruit maturity at harvest may influence the efficacy of 1-MCP treatment.

1.7.3. Delayed CA

Delayed CA is another alternative to control IBD during long-term storage that is being investigated. Delayed CA, usually implies a three to four weeks storage under normal cold before imposing the CA regime, and it has been shown to reduce the incidence of brown heart in 'Conference' pears. Various authors consider that delayed CA is very important in preventing browning without significant quality losses during storage (Larrigaudière et al., 2004; Verlinden, Jager, Lammertyn, Schotsmans, & Nicolai, 2002; Zerbini, Rizzolo, Brambilla, Cambiaghi, & Grassi, 2002). However, the use of this method is still under investigation.

Saquet et al. (2003) suggested that the prior period of storage under cold would allow the maintenance of a higher energy charge facilitating the fruit adaptation to the subsequent CA conditions. In 'Rocha' pear harvested at optimal maturity delayed CA conditions did not confer enhanced resistance to IBD development compared to immediate CA (Morais et al. 2001; Silva et al. 2010; Almeida et al. 2015). Considering the beneficial effects of delayed CA observed in other pear cultivars and having into account that in all the studies optimally harvested 'Rocha' pears were used, it is highly recommended to conduct more studies evaluating this control strategy using 'Rocha' pears harvested at early and late maturity stages.

1.8. SCOPE AND OUTLINE OF THE THESIS

This study aimed at elucidating the mechanism of internal browning disorders of 'Rocha' pear and provides the scientific basis for the development of recommendations to mitigate them in a regulatory environment where conventional chemical tools are no longer available. The high variability among pear cultivars regarding ripening and postharvest behaviours warrants the study of 'Rocha' pear since due to the geography of 'Rocha' pear production this pear cultivar was not previously used as a system to study postharvest physiological disorders. Specific objectives were: 1) to characterize the internal browning disorders in 'Rocha' pear and determine their etiology; 2) to elucidate the physiological, biochemical and transcriptional bases of internal browning disorders in 'Rocha' pear, with emphasis on the antioxidant defense system and fermentative metabolism; 3) to develop models for predicting the incidence of internal browning disorders during storage; 4) to test postharvest handling recommendations aiming at reducing the incidence of internal browning disorders.

In Chapter 1 there is a brief overview about the importance of 'Rocha' pear in Portugal and the current technologies used for long term storage of 'Rocha' pear. A literature review of the state of the art regarding the underlying mechanisms believed to be involved in the development of internal browning disorders in pears is also given.

In Chapter 2, there is a first assessment on the effects of harvest date and storage conditions, particularly storage under high CO_2, on fruits susceptibility to IBD during long term storage, and a preliminary identification of the main metabolic pathways involved on IBD development in 'Rocha' pear.

From the first assessment conducted in a long-term storage basis (Chapter 2), it was concluded that fermentation was probably the main cause of IBD in 'Rocha' pear during storage under CA, and that the antioxidant system would be barely involved. However, considering previous findings on the involvement of the antioxidant system on IBD development in other pear cultivars, another trial was designed in a short-time basis. Hence, in Chapter 3, the short term effects of fruit storage under high CO_2 on the antioxidant system were studied considering the previous observations on the possible no involvement of the antioxidant system in the development of IBD in 'Rocha' pear.

In Chapter 4, the biochemical basis of CO_2-related internal browning disorders were established, based on the study of the antioxidant and fermentative metabolisms, as well as on the involvement of PPO on browning development.

In Chapter 5, the transcriptional regulation of the enzymes of the antioxidant defence system and ethanolic fermentation in fruits stored under different storage regimes was assessed to better understand how transcriptional regulation is affected by the storage atmosphere and how the regulation of the genes codifying these enzymes may influence the development of internal browning disorders.

In Chapter 6, models for predicting internal browning disorders in 'Rocha' pear during long term storage were developed based on changes in the levels of fermentative metabolites and ascorbic acid during storage and mineral concentrations at harvest.

In Chapter 7 the effect of storage under dynamic controlled atmospheres monitored by chlorophyll fluorescence (DCA-CF) and ethanol levels (DCA-EtOH) in preventing IBD were evaluated

The Chapter 8 was dedicated to test the efficacy of two control strategies to prevent IBD during long term storage, fruit treatment with 1-MCP and fruit storage under delayed CA.

A general discussion, conclusions and future prospects are given in Chapter 9.

Figure 7 gives a schematic overview of the thesis structure.

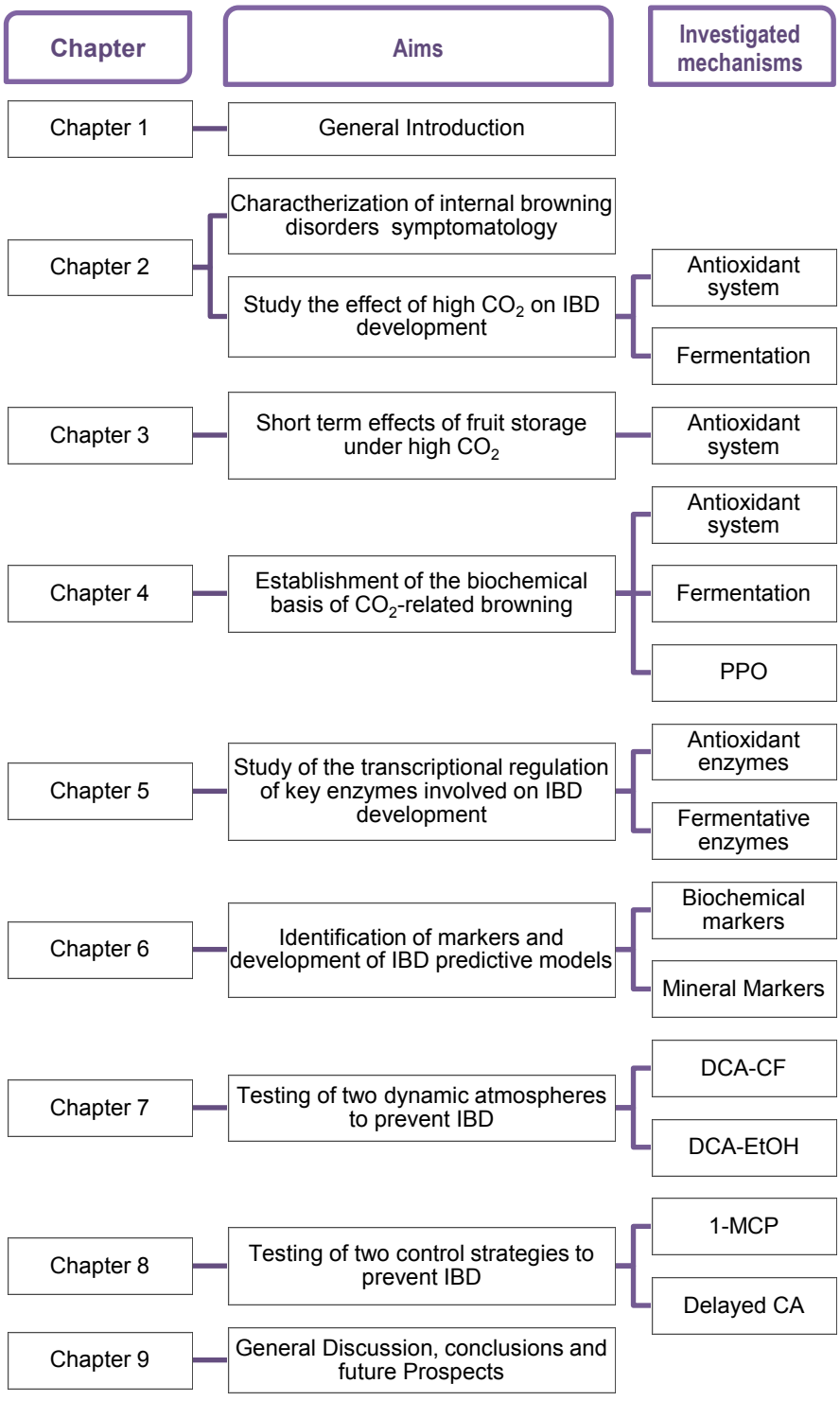

Figure 7: General outline of the thesis structure, according to the main aims of each chapter and listing the main investigated mechanisms.

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CHAPTER 2.

Internal Browning Disorders During Storage of 'Rocha' Pear: Effects of Harvest Maturity and CO_2 Partial Pressure

Oral Communication:

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2.1. ABSTRACT

The aim of this work was to study the development and biochemical basis of internal browning in 'Rocha' pear (*Pyrus communis* L. cv. Rocha) in relation to storage atmosphere and harvest maturity. Pears were harvested at an early, optimal, and late maturity stages and stored for 6 months at $0^{o}C$ + 3% O_2 + 0.5% CO_2 (normal CA storage) and up to 2 months at $0^{o}C$ + 1% O_2 + 10% CO_2 (browning inducing CA storage). Internal browning symptoms were classified into two categories: wet necrotic breakdown and dry cavities. After 4 months of storage under normal CA, fruit from the early and optimal harvests did not develop browning, while 25% of the late harvested fruits were affected with an index of ca. 0.1. After 2 months under high CO_2, internal browning disorders affected, on average, 90% of the fruit. Late harvested fruits had the highest browning incidence (98%) and index (0.72), while early-harvested fruit and fruit picked at optimal maturity had 90 and 92% incidence and indices of 0.46 and 0.67, respectively. Disorder incidence was positively correlated with increased levels of acetaldehyde and ethanol in late harvested fruits both at harvest and after 2 months of storage, but not with changes in H_2O_2-scavenging potential (CAT, APX and POX activities). Hence, we proposed to use the fermentative markers to predict the sensitivity of 'Rocha' pear to browning disorders.

Keywords: *Pyrus communis,* CA-storage, carbon dioxide, oxygen, fermentative metabolism, physiological disorders.

2.2. INTRODUCTION

'Rocha' pear is susceptible to internal browning disorders (IBD), a major cause of postharvest losses during long-term storage under controlled atmosphere (CA) (Veltman et al. 2000). The main factors affecting the incidence and severity of IBD during long term storage are: fruit maturity at harvest, imbalance between oxidative and reductive processes, fermentative metabolism, partial pressures of the gases used in CA storage and temperature (Veltman et al. 1999; Veltman et al. 2000; Larrigaudière et al. 2001). Combining high CO_2 and low O_2 with low temperatures reduces the metabolic activity of the fruit extending its storability. However, under hypoxia cells may be unable to generate the necessary energy for membrane regeneration as well as maintenance of the antioxidant system, accumulating reactive oxygen species (Larrigaudière et al. 2001), which may cause lipid peroxidation and protein denaturation (Halliwell & Gutteridge 1989). The shift to anaerobic metabolism

may also lead to the accumulation of toxic fermentative metabolites and alteration on gas diffusivities in the fruit tissue. Together, all these factors may cause membrane damage with subsequent decompartmentation of intracellular structures and consequent development of physiological disorders. Although IBD can have various symptoms (Franck et al. 2007), it is still a matter of debate whether these symptoms are related to the same or to two distinct physiological disorders (Larrigaudière et al. 2004; Franck et al. 2007). Evidence suggests that, in 'Conference' pear, CO_2-related disorders differ from senescence related disorders (Larrigaudière et al. 2004), but also that limited gas diffusion is likely involved in both instances (Lammertyn et al. 2000). The aim of this study was to describe the symptoms of IBD and their evolution during CA storage of 'Rocha' pear with special emphasis on the effects of storage atmosphere, harvest maturity and relation with fermentative metabolism.

2.3. MATERIALS AND METHODS

2.3.1. Plant material, postharvest treatment and storage conditions

Pears (*Pyrus communis* L. 'Rocha') were harvested from one orchard located in Cadaval (39º 16' N, 9º 8' W), Portugal. Pears were harvested at early, optimal, and late maturity stages, with corresponding firmness averages of 80, 70, and 55 N respectively. Fruit were then immersed in an aqueous solution of imazalil (375 $\mu g\ l^{-1}$) and stored at 0 ^{o}C, under two different atmospheres: 3 kPa O_2 + 0.5 kPa CO_2 (normal CA storage) and 1kPa O_2 + 10 kPa CO_2 (browning inducing CA storage).

2.3.2. Estimation of internal browning disorders

To evaluate the incidence of IBD, 60 fruits from each stage of maturity and storage atmosphere, randomly selected, were observed after removal and after 7 days of shelf life at room temperature. The incidence of IBD was reported as the percentage of individual fruits affected by the total of fruits observed. Severity was classified considering the extent of the damage according to the scale: (0) no internal disorders; (I) slightly damaged fruits: <25% of the fruit affected; (II) moderate: 25-50% of the fruit affected; (III) severe: >50% of the fruit affected. The browning index was calculated as described by (Veltman et al. 1999).

2.3.3. Fermentative metabolism

Ethanol and acetaldehyde were measured as described by (Ke et al. 1994) with slight modifications. Juice samples (10 ml) (4 replicates) were stored at -25 °C until analysis. Samples were placed in a 20 ml test tube with a screw cap and incubated in a water bath at 65 °C for 1 hour, after which time 1 ml of headspace sample was taken with a 2.5 ml glass syringe for chromatographic determination of ethanol and acetaldehyde. A gas chromatograph (HP 5890 II, Hewlett Packard) equipped with a CP-WAX 57 CB chromepack column (0.25 mm x 50 m x 0.2 μm, Agilent Technologies Inc., USA) and a flame ionization detector was used. Nitrogen was used as the gas carrier, and the operating conditions were as follows: oven temperature 90 ºC, injector temperature 250 ºC, detector temperature 220 °C. The concentrations were calculated using a standard curve generated by injecting standard solutions of known concentrations.

2.4. RESULTS AND DISCUSSION

IBD in 'Rocha' pear was classified into two categories: wet necrotic breakdown and dry cavities, which may coexist in the same fruit. Both symptoms can have their occurrence restricted to the carpelar region or occur also in the mesocarp (Fig. 1). After 1 month storage there was a high incidence of wet necrotic breakdown and low incidence of cavities. The incidence of cavities increased at the end of the storage period.

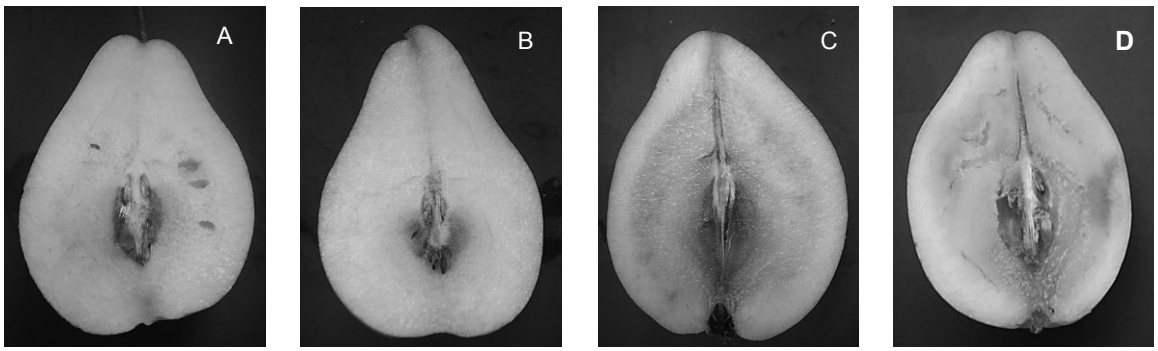

Figure 1: Browning disorders in 'Rocha' pears during storage under CA: (A) slight browning in the core and dry cavities in the flesh; (B) slight browning and dry cavities in the core; (C) wet necrotic breakdown in the core, and browning extended to the flesh; (D) wet necrotic breakdown in the core and flesh with cavities.

Under normal CA, IBD started appearing after six months storage in early-harvested fruits and two months storage in fruits picked at optimal maturity with

incidences of ca. 3%, while in late-harvest fruits IBD appeared after 1 month storage with an incidence of 25%. High CO_2 atmosphere enhanced the incidence and severity of IBD which occurred much earlier under these conditions (90% of incidence after 2 months of storage) than under normal CA (13% of incidence after 6 months of storage) (Fig. 2). Late harvested fruits had the highest browning incidence and index in both atmospheres (Fig. 2).

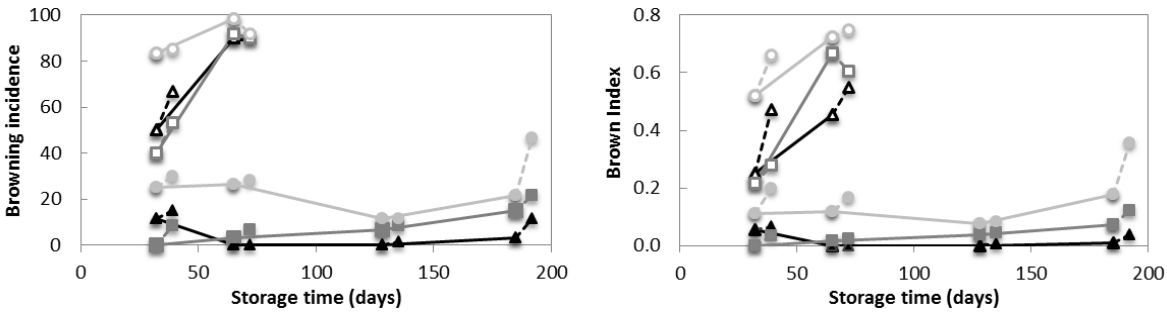

Figure 2: Internal browning incidence, expressed as percentage, (A) and index (B) in 'Rocha' pears harvested at three maturity stages [(Δ) early; (□) optimal; (○) late harvests] during storage period (solid line) and after 7 days of shelf life (dashed line), under normal CA (solid symbols) and high CO_2 atmosphere (open symbols).

Similar results have been also reported for 'Fuji' apples (Grant et al. 1996), 'Yali' and 'Seuri' Chinese pears (Crisosto et al. 1994) and 'Conference' pears (Lammertyn et al. 2000). Under high CO_2 atmosphere the percentage of damaged fruits from the late harvest with dry cavities at the end of storage was much higher (40%) than that of the fruits under normal CA (ca. 15%).

Induction of fermentative metabolism under high CO_2 concentrations may be the underlying mechanism to initiate IBD (Larrigaudière et al. 2004). At harvest, the concentration of ethanol in late-harvested fruits was about 30 μl l^{-1} and in early and optimally harvested were approximately 4 μl l^{-1}, whereas acetaldehyde concentrations were 3.09 μl l^{-1} and ca. 1 μl l^{-1}, respectively (Fig. 3). During storage, fruits exhibited a general increase in the levels of fermentative metabolites. The levels of ethanol were tenfold higher than acetaldehyde and this difference was maintained during storage.

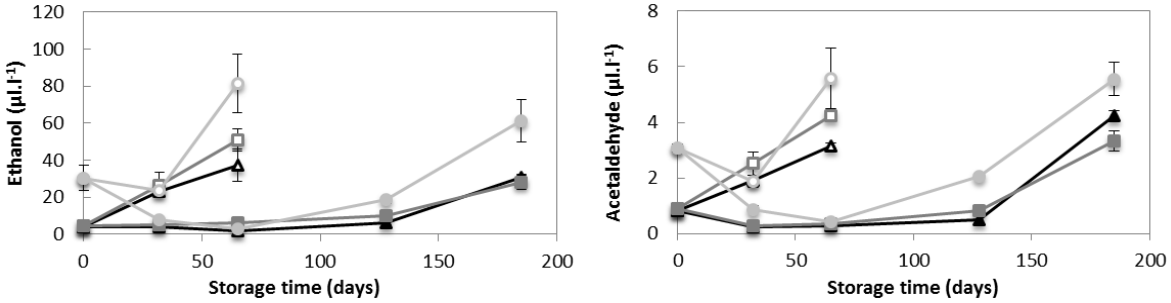

Figure 3: Concentrations of ethanol (A) and acetaldehyde (B) in 'Rocha' pears harvested at three maturity stages [(Δ) early; (□) optimal; (○) late harvests] during storage period under normal CA (solid symbols) and high CO_2 atmosphere (open symbols). Values are means ±SD (n=10).

Ethanol is usually the major product of the pathway in low O_2-stressed fruit (Ke & Kader 1992), but acetaldehyde is considered more toxic (Chervin et al. 1996). Late-harvested fruits had significantly higher levels of fermentative metabolites both at harvest and during storage. The high concentration at harvest suggests that fermentative metabolites accumulate during initial ripening on the tree (Pesis et al. 2002), possibly causing premature damage on membranes and increasing the susceptibility of pears to develop IBD during long term storage. Disorder incidence was positively correlated with increased levels of acetaldehyde and ethanol in late harvested fruits (Fig. 2 and Fig. 3). Accordingly, in fruits stored under high CO_2 atmosphere levels of ethanol and acetaldehyde increased very rapidly compared to the fruits under normal CA (Fig. 3). It has been shown that exposure of the fruits to low O_2 and high CO_2 atmospheres reduces respiration and increases ethanol and acetaldehyde concentrations on Bartlett' pears (Ke et al. 1990).

It is not still clear if the accumulation of fermentative metabolites is directly related with the incidence of IBD. Pintó et al. (2001) suggested that ethanol and acetaldehyde may be involved in the development of IBD but considered these metabolites insufficient to explain the disorder considering their findings on oxidative damage. In our study there were no significant changes in H_2O_2-scavenging potential (CAT, APX and POX activities) (results not shown). Despite the current uncertainties, the positive correlation found in this study concerning disorder incidence with increased levels of acetaldehyde and ethanol suggest that these fermentative metabolites may be used as markers to predict the sensitivity of 'Rocha' pear to IBD.

2.5. ACKNOWLEDGEMENTS

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CHAPTER 3.

Internal Browning Disorders in 'Rocha' Pear Stored Under High CO_2 Atmospheres are Triggered by Oxidative Stress

Poster presentation:

Deuchande, T., Fidalgo, F., Vasconcelos, M., Costa, D.V.T.A. and Larrigaudière, C. 2013. Internal Browning Disorders in 'Rocha' Pear Stored under High CO_2 Atmospheres are triggered by Oxidative Stress. Poster presented at International Controlled & Modified Atmosphere Research Conference, June 3-7, Trani, Italy.

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3.1. ABSTRACT

The aim of this work was to study the physiological basis, with special emphasis on antioxidant metabolism, of CO_2-related internal browning disorders in 'Rocha' pear (*Pyrus communis* L. cv. Rocha) during storage under different controlled atmosphere (CA) conditions. Pears were stored for 5 months at 0 ºC and 3 kPa O_2 + 0.5 kPa CO_2 (normal CA storage) or up to 4 months at 0°C and 1kPa O_2 + 10 kPa CO_2 (browning inducing CA storage) and the internal browning disorders were evaluated after 30, 60 and 120 days of storage. Changes in antioxidant enzyme activities (SOD, CAT, APX and POX), ascorbic (AsA) and dehydroascorbic acid (DHA) were also evaluated after 7, 15, 30 and 60 days of storage aiming at assessing the relationship between oxidative stress and internal disorders. During the entire storage period under normal CA up to 5 months, fruits did not develop browning disorders. In contrast, fruit stored under high CO_2 atmospheres exhibited high levels of internal browning (70% of damaged fruit) after 4 months of storage. High disorder incidence at high CO_2 levels was related to a decrease of total ascorbate levels during storage. Internal disorder incidence was also related to greater SOD, and to some extent APX activities as well as higher DHA/AsA ratios. Collectively these results suggest that internal disorders in Rocha pear under high CO_2 atmosphere are the consequence of an oxidative stress within the fruit.

Keywords: Antioxidant metabolism, ascorbic acid, CA-storage, carbon dioxide, physiological disorders, *Pyrus communis*

3.2. INTRODUCTION

'Rocha' pear (*Pyrus communis* L. cv. Rocha) is the most widespread variety of pear grown in Portugal. It is known that 'Rocha' pear is susceptible to internal browning disorders (IBD), a major cause of postharvest losses during long-term storage under controlled atmosphere (CA) (Veltman et al. 2000). Many factors including maturity at harvest, postharvest storage conditions and certain treatments affect internal browning development (Franck et al. 2007). CA is applied to fresh pears aiming to extend its storability. The storage of the fruits under high CO_2 and low O_2 at low temperature reduces the fruit´s metabolic activity and aerobic respiration, and may cause the reduction on the available energy for metabolism and cell regeneration as well as for the maintenance of the antioxidant system (Peppelenbos & Oosterhaven 1998; Larrigaudière et al. 2001). In such a

condition, the production of reactive oxygen species (ROS), including singlet oxygen ($^1O_2$), superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and highly toxic hydroxyl radicals (OH·) is increased. These compounds are very reactive and can indiscriminately react with almost all the cellular components causing lipid peroxidation and protein denaturation (Halliwell & Gutteridge 1989), thus compromising membrane integrity and leading to development of physiological disorders (Franck et al. 2007). IBD are associated with enzymatic oxidation of phenolic compounds by polyphenoloxidase to o-quinones, which polymerize to produce brown pigments. Under oxidative stress the phenolic compounds initially stored in vacuoles may be released into the cytosol causing enzymatic browning reactions (Nicolas et al. 1994).

ROS are the byproduct of the normal metabolic process and the cell has an efficient antioxidant system to regulate their excessive production (Foyer & Noctor 2005). Nonetheless, environmental changes such as low temperatures or exposure to CA, cause stress and are known to induce their accumulation (Møller 2001; Yong et al. 2008). In order to protect the cell against ROS, the antioxidant defense system increases several of its enzymatic and non-enzymatic components. The enzymatic components include antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (POX). Antioxidant enzymes prevent the initiation of oxidation and tissue deterioration by scavenging ROS before they start reacting with cellular components. SOD constitutes the first line of defense against O_2^-, spontaneously dismutating it to H_2O_2. H_2O_2, which is also potentially toxic, is further metabolized to water by the action of a number of enzymes such as CAT, APX and POX. CAT has a key role on the degradation of H_2O_2 and APX is also involved in the regeneration of ascorbate. Ascorbate is one of the non-enzymatic components of the antioxidant system and plays a key role in the destruction of ROS, directly by reacting and removing them and/or indirectly by donating electrons to APX, an H_2O_2-scavenging enzyme.

Although CO_2-induced browning during CA storage has been observed in many varieties of apples (Elgar et al. 1998; Lau 1998; Volz et al. 1998; Castro et al. 2007) and pears (Drake 1994; Veltman et al. 1999), the underlying mechanism remains to be clearly established in 'Rocha' pear. It is believed to be related with pre- and postharvest factors that affect the physiological state of the fruit at harvest and resistance to postharvest stresses. The aim of this work was to study the physiological basis, with special emphasis on the antioxidant metabolism, of CO_2-related internal browning disorders in 'Rocha' pear

(*Pyrus communis* L. cv. Rocha) during storage under different controlled atmosphere (CA) conditions.

3.3. MATERIALS AND METHODS

3.3.1. Plant material

Pears (*Pyrus communis* L. cv Rocha) were harvested from one orchard located in Cadaval (39º 16' N, 9º 8' W), Portugal, at an optimal maturity stage (62 N). After harvest, the fruits were sorted by hand to select the undamaged fruits, washed, and stored at 0^{o}C + 3% O_2 + 0.5% CO_2 (normal CA storage) and at 0^{o}C + 1% O_2 + 10% CO_2 (browning inducing CA storage), during 5 and 4 months, respectively. Samples of pulp tissue from the inner part of 4 individual fruits were pooled and used for biochemical analyses after 7, 15, 30 and 60 days storage.

3.3.2. Estimation of internal browning disorders

To evaluate the incidence of IBD, 60 fruits from each storage atmosphere, randomly selected, were observed after 30, 60 and 120 days of storage immediately after removal from chambers. The incidence of IBD was established as the percentage of individual fruits affected by the total number of observed fruits.

3.3.3. Extraction of Enzymes

Pulp tissue (10 g fresh weight) previously frozen in liquid nitrogen and crushed in a kitchen mixer was ground at 0 °C with a mortar and pestle and homogenized with quartz sand in 15 ml of 100 mM potassium phosphate buffer (pH 7.3), 1 mM EDTA, 8% glycerol 87% (v/v), 1 mM phenylmethanesulfonyl fluoride (PMSF), 5 mM ascorbic acid, 2% (w/v) polyvinylpolypyrolidone (PVPP). The homogenate was centrifuged at 8000 rpm (Thermo Scientific Heraeus Centrifuge Multifuge X3FR, United Kingdom) for 30 min at 4 ºC and the resulting supernatant was used as enzyme extract for the determination of CAT, APX, POX and SOD activities.

3.3.4. Protein Determination

Total protein in the enzyme extract was determined according to the method described by Bradford (1976) with modifications (Pierce Coomassie Plus Protein Assay kit) according to manufacturer's indications, using bovine serum albumin (BSA) as a standard.

3.3.5. Enzymatic assays

The activity of SOD was determined by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm after exposure to light for 15 min using 4 fluorescent lamps of 15 W (Giannopolitis & Ries 1977). The reaction mixture consisted in 100 mM phosphate buffer (pH 7.8), 1.3 μM riboflavin, 13 mM methionine and 122 μM NBT. The mixtures with and without enzyme were illuminated and non-illuminated and identical solutions served as blanks. One unit of SOD was considered to be the amount of enzyme required to inhibit 50% of NBT reduction. CAT activity was measured by the method of Clairbone (1985), following the disappearance of H_2O_2 at 240 nm in a reaction mixture containing 100 mM potassium phosphate buffer with pH 7.0 and 40 mM H_2O_2. To determine APX activity the reaction mixture consisted of 100 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid and 1 mM H_2O_2. APX activity was measured by following the decline in absorbance at 290 nm as ascorbate was oxidized, using the method of Nakano & Asada (1981). POX activity was determined by the method of Rao et al. (1996) with some modifications. The reaction mixture consisted in 100 mM phosphate buffer (pH 6.8), 10 mM guaiacol and 10 mM H_2O_2. Peroxidase activity was measured by monitoring the increase in absorbance resulting from guaiacol oxidation in the presence of H_2O_2 for 10 min, and expressed as change in optical density at 470 $nm.mg^{-1}$ protein.

3.3.6. Ascorbic and dehydroascorbic acids

Ascorbic acid (AsA) and dehydroascorbic acid were determined as described by Zapata & Dufour (1992). Pulp tissue (15 g fresh weigh) previously frozen in liquid nitrogen and crushed in a kitchen mixer was homogenised with 15 ml of methanol/water (5:95 v/v) with 10 g l^{-1} of HCl, using an Ultra-Turrax (IKA Labortechnik model T 25, Staufen, Germany). The homogenate was centrifuged at 5.000 rpm (Universal 320 R cooled, Hettich, Zentrifugen) for 15 min at 4 ºC and the supernatant was filtered through a fluted filter. Three ml extracts were incubated with 1 ml of a solution of 1,2-phenylenediamine dihydrochloride (OPDA, from Sigma-Aldrich), for 30–40 min and then filtered through a 0.45 μm filter. All the procedures were performed in darkness at 4 ºC. The samples were analyzed with an HPLC system equipped with a UV-Vis detector (UV-1575), an auto sampler (AS-1555) and a pump (PU-1580), all controlled by appropriate software (Borwin v. 1.5, from Jasco Corporation, Tokyo, Japan). Separation of AsA and DHA was achieved in a reverse-phase C18 column (250 × 4.6 mm Nucleosil 100-10 from

Macherey-Nagel, GmbH & Co. KG, Germany). The mobile phase was methanol/water (5:95, v/v) containing 5 mM cetrimide and 50 mM potassium dihydrogen phosphate. The flow rate was 1.8 ml.min^{-1} and the injection volume was 20 µl. The detector was set at 348 nm for DHA and 261 nm for AsA detection. Four individual fruits were pooled and three analytical replicates of each storage condition were analysed. AsA and DHA contents were expressed in mg.100 g^{-1} fresh weight.

3.4. RESULTS AND DISCUSSION

During the entire storage period under normal CA, up to five months, fruit did not develop IBD. In contrast, fruit stored under high CO_2 atmospheres exhibited high levels of internal browning (ca. 70% of damaged fruit) after four months of storage (Fig. 1). Similar results have been described in many pear varieties including Conference (Veltman et al. 1999) and 'Anjou' pears (Drake 1994).

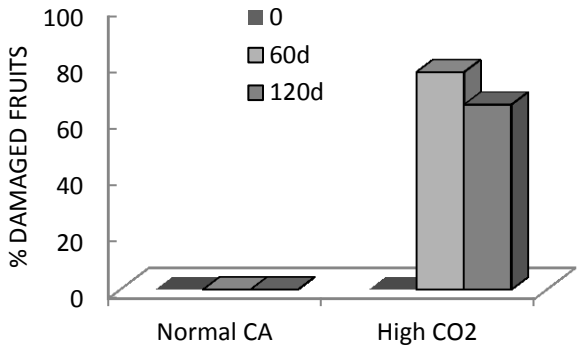

Figure 1: Incidence of internal browning disorders during storage (0, 60 and 120 days of storage) under normal CA and high CO_2 atmosphere. Values are in percentage of individual fruits affected by the total number of observed fruits (60 fruits from each storage condition in each date).

The activities of the antioxidant enzymes showed variations during the storage period. After a decrease in the first 14 days of storage, SOD activity slightly increased after 30 days of storage in both atmospheres with a two-fold higher activity observed in fruits stored under high CO_2 atmosphere. However, after 60 days of storage the activity of SOD in fruits from both storage conditions reached similar values (ca. 60 Ua mg^{-1}) (Fig. 2). The higher SOD activity and lower CAT and APX activities detected in fruits stored under high CO_2 after 7 days of storage and the severe decline of CAT activity at this point, may have led to the accumulation of the H_2O_2. However, while CAT activity stabilized during storage, APX activity seemed to act concomitantly with SOD to compensate oxidative

stress caused by accumulation of H_2O_2 generated by SOD (Fig. 2). Larrigaudière et al. (2004) found a negative correlation between APX and CAT activities and the incidence of IBD in 'Blanquilla' pears. POX activity, after a decrease during the first 14 days of storage, remained relatively stable after 30 and 60 days of storage in both atmospheres (Fig.25). A decrease in the activity of POX and SOD has been related to the development of IBD in pears (Larrigaudière et al. 2004).

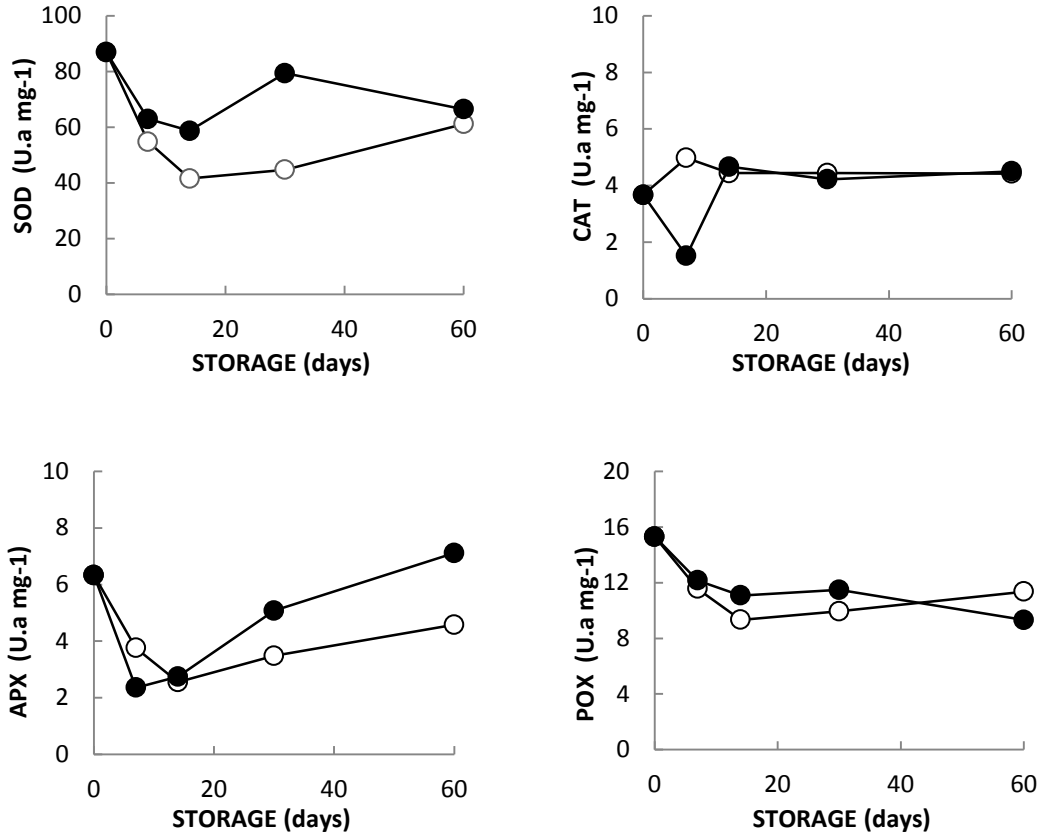

Figure 2: Activities of antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (POX) in fruits stored under normal CA (○) and high CO_2 atmosphere (●) and at different storage times. Values represent an average of three analytical replicates of 4 pooled fruits.

According to our results, in general, the greater differences concerning the activity of the enzymatic antioxidant system under high CO_2 atmosphere occur at the beginning of storage, during the first 7 days. After 60 days of storage the antioxidant enzymes activities tend to assume similar values in both storage conditions, with exception of APX activity which remained significantly higher in fruits stored under higher CO_2 after 30 and 60 days of storage.

The levels of total ascorbate decreased during storage in both atmospheres, with a more severe decline in fruits stored under high CO_2 (1.45 mg 100 g^{-1}) compared to fruits

stored under normal CA (2.13 mg 100 g^{-1}) after 60 days of storage (Fig. 3). It has been shown that storage of 'Conference' pears under cold temperatures and high CO_2 concentrations also lead to a decrease in the ascorbate levels (Larrigaudière et al. 2001) and was associated to a higher susceptibility to develop browning symptoms (Veltman et al. 1999; Zerbini et al. 2002). According to these later authors, IBD appears when the ascorbate concentration drops below a certain threshold value and therefore, loss of AsA has been considered to be directly related to the development of IBD (Franck et al. 2003; Zerbini et al. 2002). A similar pattern has been observed in 'Rocha' pear during long term storage under CA (Veltman et al. 2000). The results observed in our study are in accordance with those found on 'Conference' pears suggesting that susceptibility of 'Rocha' pear to develop IBD is well correlated with decreasing ascorbate levels.

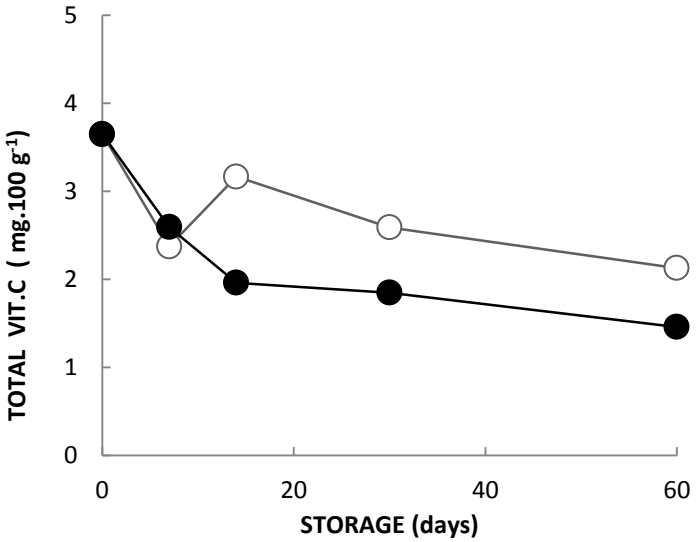

Figure 3: Total ascorbate levels in fruits stored under high CO_2 (●) and normal CA (○). Values represent an average of three analytical replicates of 4 pooled fruits.

In both atmospheres, the ratio between the oxidized and reduced form of ascorbate (DHA/AsA ratio) increased after 7 days of storage, from 0.84 at harvest to 2.32 in fruits stored under high CO_2 and 1.7 in fruits stored under normal CA. The DHA/AsA ratios decreased thereafter with higher ratios observed in fruits stored under high CO_2, when compared to fruits stored under normal CA (Fig. 4). These results indicate a change in the redox status directed towards an accumulation of the oxidized form and a clear reduction of the antioxidant potential.

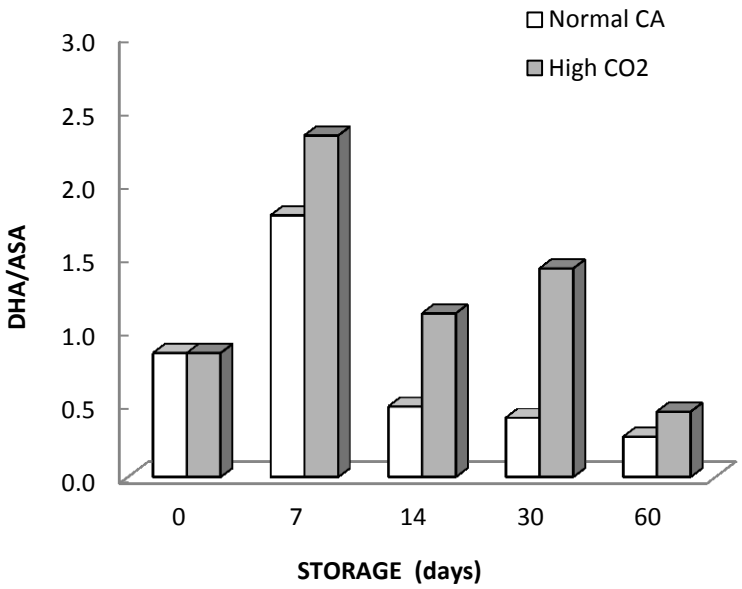

Figure 4: Ratio of DHA/AsA in fruits stored under high CO2 (grey squares) and normal CA (white squares).

Altogether these results show that high disorder incidence at high CO_2 levels were related to a significant decrease of total ascorbate levels during storage. Internal disorder incidence was also related to greater SOD and to some extent APX activities as well as higher DHA/AsA ratios. They also suggest that internal disorders in Rocha pear under high CO_2 atmosphere are the consequence of an oxidative stress within the fruit.

3.5. ACKNOWLEDGEMENTS

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CHAPTER 4.

Biochemical Basis of CO_2-related Internal Browning in Pears (*Pyrus communis* L. cv. Rocha) During Long-Term Storage

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4.1. ABSTRACT

This study aimed at understanding the biochemical basis of internal browning disorders (IBDs) in 'Rocha' pear. For this purpose, the effects of storage under normal controlled atmosphere (CA) (3 kPa of O_2 + 0.5 kPa of CO_2) and IBD- inducing CA (1 kPa of O_2 + 10 kPa of CO_2) on the antioxidant and fermentative metabolisms and polyphenol oxidase (PPO) activity and phenolics concentration were studied. The higher IBD incidence in high CO_2-stored fruits was positively correlated with fermentative metabolites and negatively with ascorbate and H_2O_2 concentrations, and it was linked to PPO activation. These results indicate that both the antioxidant and fermentative metabolisms are involved in the occurrence of IBD in 'Rocha' pear.From the integration of the biochemical and enzymatic data, a schematic model illustrating the effects of high CO_2 and low O_2 in 'Rocha' pears during long-term storage was constructed.

Keywords: antioxidant system, controlled-atmosphere storage, fermentative metabolism, hydrogen peroxide, polyphenol oxidase, *Pyrus communis*.

4.2. INTRODUCTION

'Rocha' pear (*Pyrus communis* L.) is a Portuguese native pear cultivar with Protected Designation of Origin (PDO) and one of the most important pear cultivars produced in Europe. With a production exceeding the 200 thousand ton and exporting over 67 million euros in 2013, 'Rocha' pear is one of the few export-oriented Portuguese agricultural products (INE - Instituto Nacional de Estatística 2015). Although 'Rocha' pear can be stored for up to ten months under controlled atmosphere (CA) (2.5-3.0 kPa O_2 + 0.5-0.7 kPa CO_2), it is known to be susceptible to internal browning disorders (IBD) and superficial scald, the major causes of postharvest losses during long-term storage under CA (Silva et al. 2010; Isidoro & Almeida 2006). Diphenylamine (DPA) and ethoxyquin, two synthetic antioxidants, have been previously applied to pome fruits to prevent the incidence of superficial scald during long term storage under CA and, despite specifically indicated for prevention of superficial scald, DPA seemed to be also effective in reducing IBD in 'Rocha' pear (Silva et al. 2010). However, the current restrictions imposed by the European Commission on their use generated a need for understanding the physiological and biochemical and molecular bases of IBD in 'Rocha' pear in order to find effective

strategies to prevent the incidence of physiological disorders and better preserve the fruit quality during long term storage.

In 'Rocha' pear, IBDs are characterized by tissue browning in the carpelar region which can be extended to the mesocarp. Oftentimes, internal browning is accompanied by wet necrotic breakdown or dry cavities (Deuchande et al. 2012). CA storage has been shown to limit gas diffusion in the pulp tissue prompting the incidence of IBD due to the hypoxic conditions reached within the fruit, particularly at the inner cortex (Ho et al. 2006; Ho et al. 2008).

The incidence of IBDs are related to membranes' damage which allows the release of the polyphenols from the vacuoles and polyphenol oxidases (PPOs) mainly located in the plastids, to the cytosol, where they react causing the oxidation of phenols with formation of *o*-quinones which, by subsequent polymerization, form the browning compounds, melanins (Vámos-Vigyázó 1981). Despite involved in IBD development, PPO is not considered a limiting factor for IBD development in other pear cultivars, including 'Conference' and 'Blanquilla' (Larrigaudière et al. 1998; Veltman, Larrigaudière, et al. 1999).

The cold storage of fruits under high CO_2 and low O_2 may lead to a shift from an aerobic to a predominantly anaerobic metabolism with a consequent reduction of the energy charge and accumulation of fermentative metabolites. On the one hand, excessive concentrations of ethanol and particularly acetaldehyde become toxic to the cells causing changes on membrane fluidity and gas diffusivities in the fruit tissues which may lead to membrane damage (Ke et al. 1994; Ho et al. 2008; Ho et al. 2009; Chervin et al. 1999). On the other hand, the reduced energy charge makes the cells unable to maintain membranes through activation of the antioxidant system, also leading to the accumulation of reactive oxygen species (ROS). The accumulation of ROS such as superoxide anion radical ($O_2^{\cdot-}$), perhydroxyl radicals ($HO_2^{\cdot-}$), the highly toxic hydroxyl radical (OH·) and hydrogen peroxide (H_2O_2) may cause lipid peroxidation and protein denaturation with consequent cell damage (Halliwell & Gutteridge 1989). Unlike the oxygen radicals, H_2O_2 can diffuse across biological membranes, oftentimes causing oxidative stress far from the site of formation, acting as a signal molecule (Mhamdi et al. 2010). In order to protect the cell against the excessive ROS production, the antioxidant defence system increases several of its enzymatic and non-enzymatic components. The enzymatic components include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase

(DHAR) and guaiacol peroxidase (POX). These enzymes prevent tissue oxidation and peroxidation by scavenging ROS before they start reacting with cellular components. A decrease in the activity of these antioxidant enzymes has previously been linked to the occurrence of IBD in pears (Larrigaudière et al. 2004). Among the non-enzymatic antioxidants, ascorbic acid plays an important role since it is capable of destroying ROS directly by reacting and removing them or indirectly by donating electrons to APX. Besides, ascorbic acid has the ability to reduce the *o*-quinones, formed by oxidation of the phenols through the action of PPO, back to *o*-diphenols preventing the development of browning. Accordingly, the loss of ascorbic acid has been directly related to the initiation and development of IBD in 'Conference' pears (Veltman, Sanders, et al. 1999; Zerbini et al. 2002; Franck et al. 2003). Therefore, the regulation of the enzymes involved in the ascorbate recycling pathway may also play an important role in the development of IBD.

The storage behavior of pears is cultivar-specific and in contrast to other cultivars such as 'Conference' (Veltman, Sanders, et al. 1999; Veltman et al. 2003; Larrigaudière et al. 2004) and 'Blanquilla' (Pintó et al. 2001; Larrigaudière et al. 1998) for which the biochemical basis of IBD are described, scarce information is available for 'Rocha' pear, one of the European pear cultivars with the longest storage life. This characteristic of Rocha' pear makes this cultivar an interesting model to study IBD development during long term storage under CA. Therefore, our study aimed at investigating the underlying biochemical mechanisms involved in IBD occurrence and development in this pear cultivar.

4.3. MATERIALS AND METHODS

4.3.1. Plant material and storage conditions

'Rocha' pears were harvested from an orchard located in Cadaval (39º 16' N, 9º 8' W), Portugal, at a slightly late maturity stage with an average firmness value of 54 N. After harvest, fruits were stored at -0.5 ºC under normal CA (3 kPa O_2 + 0.5 kPa CO_2) and under browning inducing CA (1 kPa O_2 + 10 kPa CO_2; hereafter referred as high CO_2 atmosphere) for 145 days. Two experimental chambers of two ton capacity fully loaded with 'Rocha' pears were used in this experiment. To reach the 10% CO_2 in the high CO_2 storage condition, CO_2 was added from an external source until the defined level was achieved. The gas composition in the CA chambers was carefully controlled and checked with centralized analyzers, supervised by special Fruit Control Equipment software.

4.3.2. Samples preparation for biochemical analyses

Fruit samples were prepared at harvest and after 45, 95, 125 and 145 days of storage. During storage, after fruits removal, the chambers were resealed and the atmosphere was immediately re-established, reaching the settled conditions in about two hours. At each removal time the picked fruits were allowed to warm up for up to six hours at room temperature before sampling. Samples of pulp tissue (three replicates of three fruits) prepared by selecting the inner part of the fruit (Fig. 1), were immediately frozen under liquid nitrogen and kept at -80 ºC until analysis. Pear juice samples (three replicates of five fruits) were also prepared using a commercial blender, filtered through cellulose paper filter and stored at -25 °C for the analysis of fermentative metabolites (ethanol and acetaldehyde).

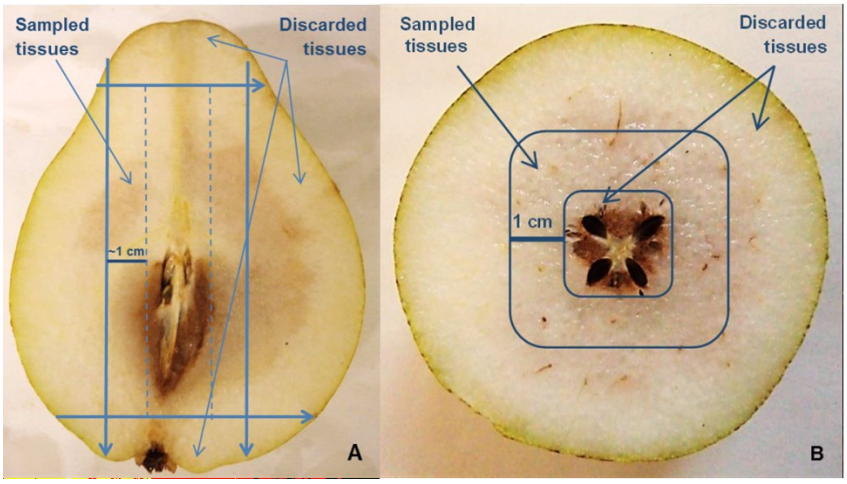

Figure 1: Schemes of cut and tissue collected for the biochemical analysis. (A) Longitudinal cuts made to collect the tissue from the inner region of the pear excluding the external pulp tissue and the top and bottom of the fruit and (B) transversal perspective of the sampled tissues. The sample included healthy and affected tissues depending on the degree of browning severity.

4.3.3. Assessment of initial maturity

The assessment of initial maturity was carried out on 30 fruits immediately after harvest. Firmness was analyzed on two opposite sides of the fruit, after peel removal and using a penetrometer (Turoni, Italy), fitted with an 8 mm Magness Taylor probe. For the assessment of sugars and acidity, juice samples were prepared from wedges of cut fruit (three replicates of nine fruits each), homogenized and filtered through cellulose paper filter. Total soluble solids content (SSC) was measured in each juice sample with an Atago PR-100 palette refractometer (Tokyo, Japan), and titratable acidity was determined by titration with 0.1N NaOH to pH 8.1. The starch index was determined by iodine staining

(Avelar & Rodrigues 1999) using a reference chart scored from 1 (maximum starch concentration) to 10 (no starch).

4.3.4. Estimation of internal browning disorders

To evaluate the incidence and severity of IBDs, 60 randomly selected fruits from each storage condition were observed after 45, 95, 125 and 145 days of storage. For the visual evaluation pears were cut longitudinally and transversely. IBD incidence was reported as the percentage of individual fruits affected. The severity of the symptoms was classified considering the extent of the damage according to a scale: (0) no internal disorders; (I) slightly damaged fruits: < 25% of the fruit affected; (II) moderate: 25-50% of the fruit affected; (III) severe: > 50% of the fruit affected. The browning index was calculated as previously described (Veltman, Sanders, et al. 1999), where 0, number of healthy pears; I, number of class I pears; II, number of class II pears; III, number of class III pears. The index value 0 means 'no browning' and brown index 1 means 'maximal browning'.

$$\text{Browning Index} = \frac{\text{I} + 2\text{II} + 3\text{III}}{3(0 + \text{I} + \text{II} + \text{III})}$$

4.3.5. Determination of H_2O_2

For the extraction of H_2O_2, fresh pulp tissue (15 g) was ground (Ultra-Turrax, IKA Labortechnik T 25, Staufen, Germany) in 20 mL of 5% trichloroacetic acid. The homogenate was centrifuged at 4,000 *g* for 30 min at 4 ºC and the resulting supernatant was used for the determination of H_2O_2 using the PeroxiDetect™ kit from Sigma-Aldrich, Inc. (Missouri, USA), following the manufacturer instructions. All the extraction procedures were conducted at 4 ºC in the absence of light.

4.3.6. Extraction and analysis of ascorbic and dehydroascorbic acids

Ascorbic acid (AsA) and dehydroascorbic acid (DHA) were determined as described in a previous work (Zapata & Dufour 1992) with slight modifications. Pulp tissue (15 g) was homogenized with 15 mL of methanol/water (5:95 v/v) plus 10 g L^{-1} of HCl. The homogenate was centrifuged at 4,000 *g* for 15 min, filtered and 3 ml of the resulting extract plus 1 mL of 1,2-phenylenediamine dihydrochloride solution were incubated for 30 to 40 min. The samples were analyzed with an HPLC system equipped with a UV-Vis detector (UV-1575), an auto sampler (AS-1555) and a pump (PU-1580), all controlled by appropriate software (Borwin v. 1.5, from Jasco Corporation, Tokyo, Japan). Separation of AsA and DHA was achieved in a Spherisorb ODS2 5 μm (250 x 4.6 mm da

Waters Corporation, USA). The detector was set at 348 nm for DHA and 261 nm for AsA detection. All the procedures were conducted in the absence of light at 4 ºC.

4.3.7. Extraction of enzymes

The extraction of the enzymes of the antioxidant system, superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (POX), was carried out as previously described (Larrigaudière et al. 2001) with slight modifications. Samples of pulp tissue (10 g fresh weight) were ground using a mortar and pestle with 15 mL of extraction buffer containing 100 mM potassium phosphate pH 7.3, 1 mM EDTA, 8% glycerol 87% (v/v), 1 mM phenylmethanesulfonyl fluoride (PMSF), 5 mM ascorbic acid and 2% (w/v) polyvinylpolypyrolidone (PVPP). The homogenate was centrifuged at 10 000 *g* for 30 min at 4 ºC and the resulting supernatant was collected. Polyphenoloxidase (PPO) extraction was carried out as previously shown (Galeazzi & Sgarbieri 1981). Total protein in the enzyme extract was determined using the Pierce Coomassie Plus Protein Assay kit, based on the Bradford method (Bradford 1976), and following the manufacturer's instructions. Bovine serum albumin was used as a standard.

4.3.8. Enzymatic assays

APX activity was determined following the decline in absorbance as ascorbate was oxidized (Nakano & Asada 1981). CAT activity was measured by following the reduction of H_2O_2 (Clairbone 1985). POX activity was determined by monitoring the increase in absorbance resulting from guaiacol oxidation in the presence of H_2O_2 (Rao et al. 1996). SOD activity was determined by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) after exposure to light for 15 min using four fluorescent lamps(Giannopolitis & Ries 1977). One unit of SOD was considered to be the amount of enzyme required to inhibit 50% of NBT reduction(Giannopolitis & Ries 1977). The PPO activity was measured following the increase in absorbance resulting from phenolics oxidation (Galeazzi & Sgarbieri 1981).

Except for SOD, all the enzyme activities were expressed as the change in optical density min^{-1} mg^{-1} protein.

4.3.9. Fermentative metabolites

The determination of ethanol and acetaldehyde was performed as described in previous work (Ke et al. 1994) with slight modifications. From each sample, 10 mL of juice were placed in a 20 mL test tube with a screw cap and a septum. After sample

incubation in a water bath at 65 °C for 1 hour, 1 mL of headspace sample was taken to determine the acetaldehyde and ethanol concentrations. For this purpose a gas chromatograph (HP 5890 II, Hewlett Packard) equipped with a column CP-WAX 57 CB chromepack (0.25 mm x 50 m x 0.2 μm; Agilent Technologies Inc., USA) and a flame ionization detector was used.

4.3.10. Phenolics content

Pulp tissue (3 g) was homogenized with 15 mL of a solution of 79.5:0.5:20 (v/v/v) of methanol: HCl: H_2O by mixing for 2 hours at 250 rpm. The homogenate was centrifuged at 4,000 *g* for 30 minutes and the supernatant was collected for phenolics determinations. The amount of total phenolics was determined using the Folin–Ciocalteu method (Obanda et al. 1997).

4.3.11. Statistical analyses

The data analysis and the analysis of variance (ANOVA) were performed using the IBM SPSS Statistics, version 21.0. (Armonk, NY: IBM Corp.). The analysis of variance and the evaluation of the effect of the atmosphere during storage were performed using General Linear Model. Mean comparisons were done by calculating the Tukey's honestly significant difference (HSD) at P=0.05. The correlation values presented correspond to two-tailed Pearson correlation coefficients calculated using the same software.

4.4. RESULTS

4.4.1. Fruit maturity

Maturity indices at harvest were 53.8N, 12.9 ºBrix for SSC, 1.3 g malic acid L^{-1} and an averaged starch index of 8.0.

4.4.2. Incidence and severity of IBD

Fruits stored under normal CA had no browning or less than 5% incidence during the entire storage period, while fruits stored under high CO_2 had an increasing incidence and severity of the disorder which began just after 45 days with ca. 15% of fruits affected with an index of 0.1, reaching at the end of the storage period, ca. 70% incidence and a severity index of 0.5 (Fig. 2).

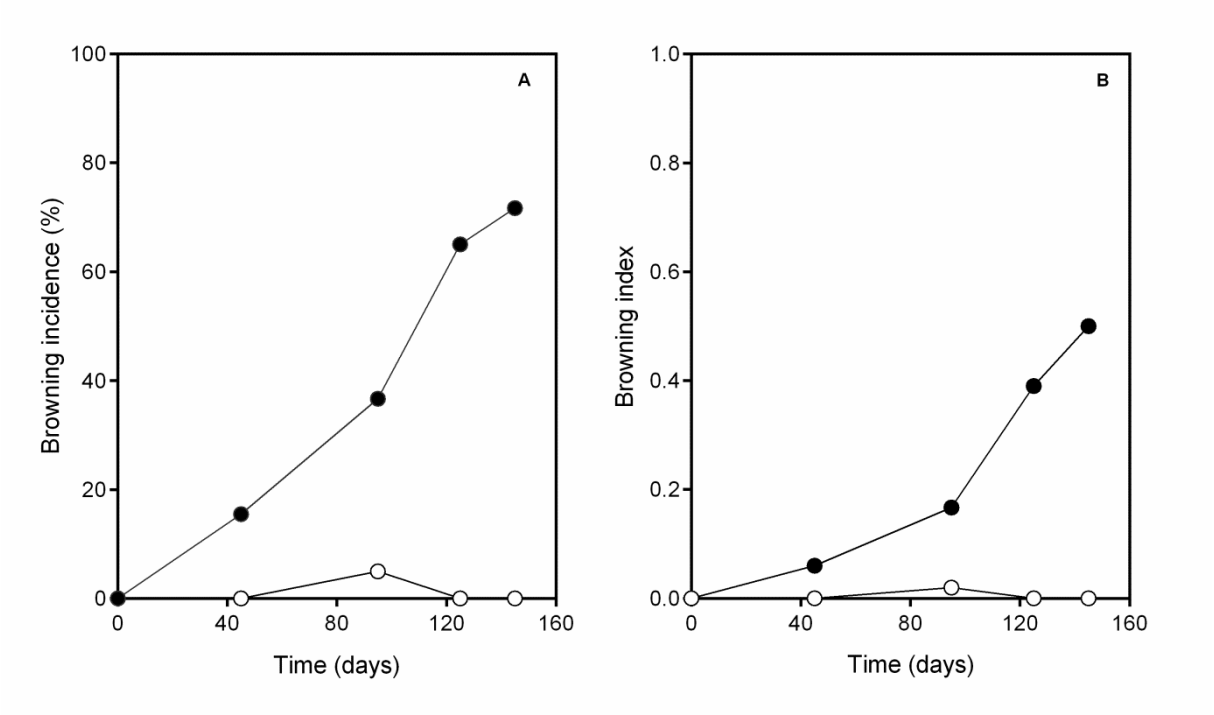

Figure 2: Internal browning incidence (A) and severity (B) during storage under normal CA (open symbols) and high CO_2 (solid symbols) storage coditions. Browning incidence was expressed as percentage of damaged fruits and browning severity classified as an index (0 = no browning; 1 = maximum browning).

4.4.3. Changes in H_2O_2 levels and antioxidant compounds

4.4.3.1. Hydrogen peroxide

The concentration of H_2O_2 remained stable during the first 45 days of storage and sharply decreased thereafter in both storage atmospheres. However, the decline rate between the 45 and 95 days of storage was much higher in the fruits stored under high CO_2 (0.42 µmol $g^{-1}day^{-1}$) than in normal CA stored fruits (0.25 µmol g^{-1} day^{-1}) (Fig. 3). From 95 days of storage until the end of the experiment, despite the opposite tendency, the H_2O_2 levels remained fairly constant under both conditions but significantly lower in fruits stored under high CO_2 (Fig. 3).

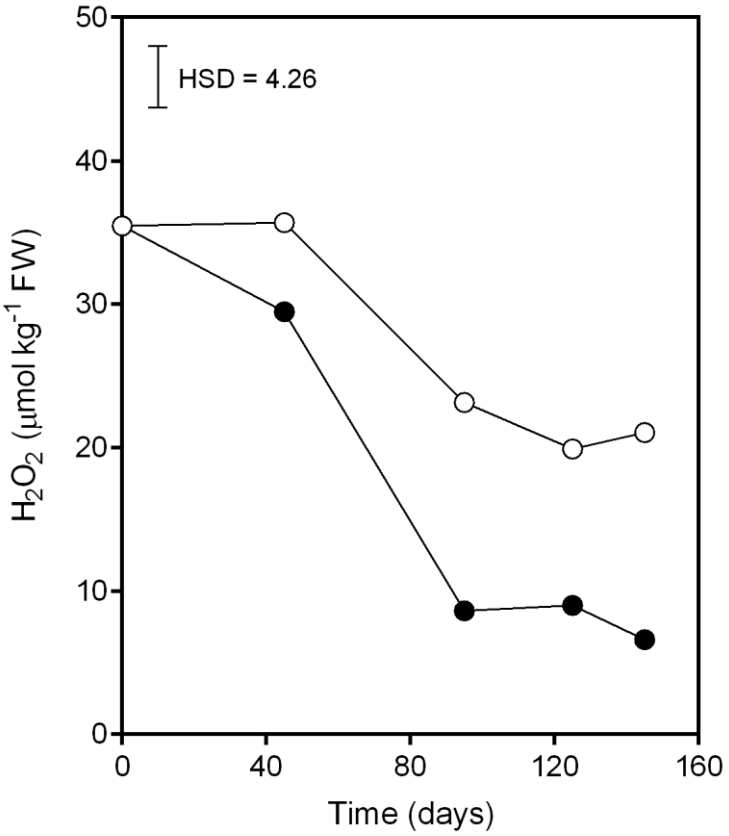

Figure 3: Levels of H_2O_2 during storage under normal CA (open symbols) and high CO_2 (solid symbols) storage coditions. Values represent the means of 3 replicates of 5 fruits. Vertical bar represents the HSD (p=0.05).

4.4.3.2. Ascorbic and dehydroascorbic acids

Although of total ascorbic acid (TAA) levels progressively decreased during the entire storage period in both storage atmospheres, the levels of TAA in the fruits stored under high CO_2 were always significantly lower than in the fruits stored under normal CA (Fig. 4A). The ratio between the oxidized and reduced forms of ascorbate (DHA/AsA ratio) in the fruits of normal CA sharply decreased between 45 and 95 days of storage (from 3.75 to 0.36) remaining stable until 125 days of storage and increasing thereafter (Fig. 4B). In contrast, in the fruits stored under high CO_2 this ratio strongly increased between 45 and 95 days of storage from 2.8 to 5.5, decreasing to 2.0 thereafter until the 125 days of storage (Fig. 4B). At the end of the storage period this ratio increased again and was similar to the ratio registered in the fruits of normal CA (Fig. 3).

AsA levels, after an initial similar decrease in both storage conditions, increased in the normal CA-stored fruits between the 45 and 95 days, decreasing thereafter, while in the high CO_2-stored fruits the AsA levels were fairly constant until day 125 decreasing at the

end of storage (Fig. 4C). DHA concentration decreased in the fruits of both conditions (Fig. 4D).

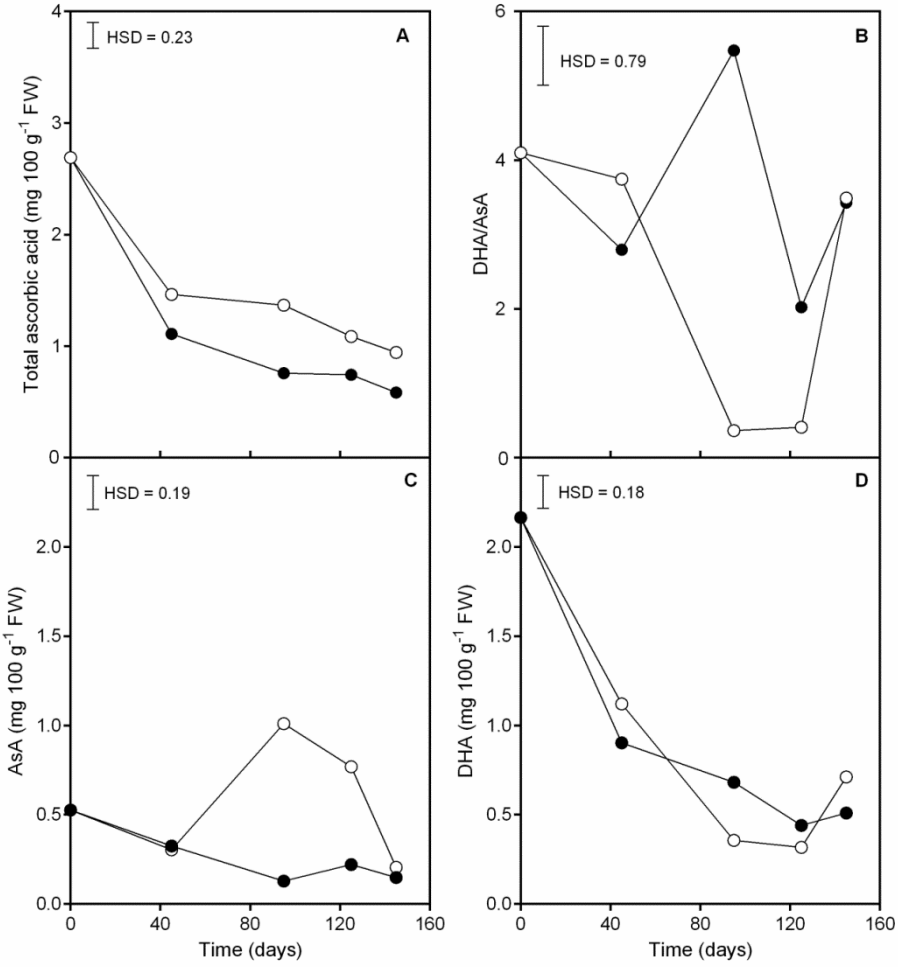

Figure 4: Total ascorbic acid concentration (A), ratio of oxidized and reduced form of ascorbate (DHA/AsA) (B), concentration of ascorbic acid (AsA) (C) and dehydroascorbic acid (DHA) (D) during storage under normal CA (open symbols) and high CO_2 (solid symbols) storage coditions. Values represent the means of 3 replicates of 3 fruits. Vertical bars represent the HSD (p=0.05).

4.4.3.3. Activity of antioxidant enzymes

SOD activity in the fruits stored under high CO_2 decreased during the first 45 days of storage and then remained stable until the end of the experiment. In contrast, under normal CA, SOD activity steadily increased up to day 95 and decreased thereafter (Fig. 5A) remaining higher than in the fruits stored under high CO_2 during the entire storage period (Fig. 5A). A completely different behavior was observed for CAT. Except on day 125, CAT activity was higher in high than low CO_2 treated fruits (Fig. 5B). APX activity slightly increased in the fruits stored under high CO_2 during the entire storage period

whereas it peaked at day 45 in fruits stored under normal CA, decreasing thereafter until the end of the storage period (Fig. 5C). POX activity decreased during storage under both storage atmospheres. Similarly to SOD, POX activity in the fruits stored under high CO_2 was lower compared to normal CA- stored fruits, except at the end of the storage period, when the activity of POX in the fruits of both conditions reached similar values (Fig. 5D).

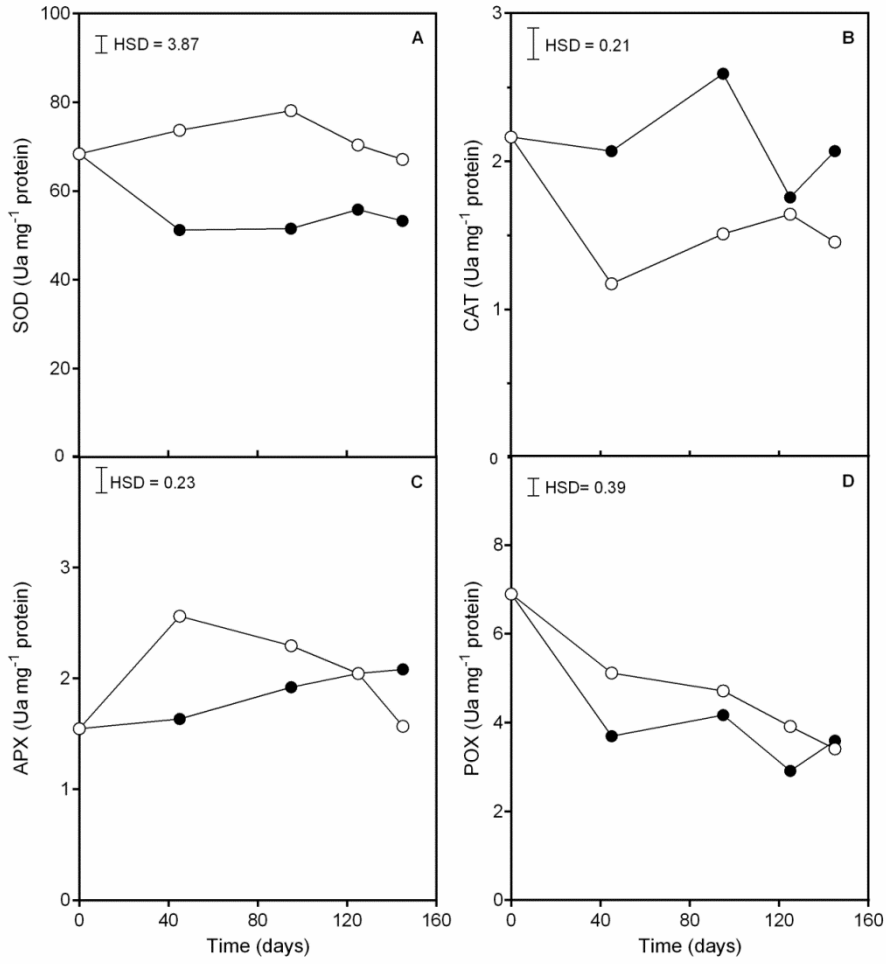

Figure 5: Activity of superoxide dismutase (SOD) (A), catalase (CAT) (B), ascorbate peroxidase (APX) (C) and peroxidase (POX) (D) during storage under normal CA (open symbols) and high CO_2 (solid symbols) storage coditions. Values represent the means of 3 replicates of 3 fruits. Vertical bars represent the HSD (p=0.05).

4.4.4. Changes in fermentative metabolites

The concentrations of ethanol and acetaldehyde in normal CA-stored fruits remained low (< 2 µL ethanol L^{-1} and < 0.4 µL acetaldehyde L^{-1}) and stable until the 95 days of storage, slightly increasing thereafter to a maximum level of 11.46 µl L^{-1} for ethanol and 3.97 µL L^{-1} for acetaldehyde (Fig. 6). In contrast, in fruits stored under high

CO_2 both fermentative metabolites increased during the first 95 days in a rate of 4.6 µL ethanol L^{-1} day^{-1} and 0.28 µL acetaldehyde L^{-1} day^{-1}(increasing from 2.1 to 90µL ethanol L^{-1} and 0.69 to 8.1 µL acetaldehyde L^{-1}) (Fig. 6). From 95 days until the end of the storage period, the accumulation rates of ethanol increased by 4.5 fold and acetaldehyde by 3.5 fold with the levels of these metabolites reaching the 298 µL ethanol L^{-1} and 22.2 µL acetaldehyde L^{-1} at the end of storage.

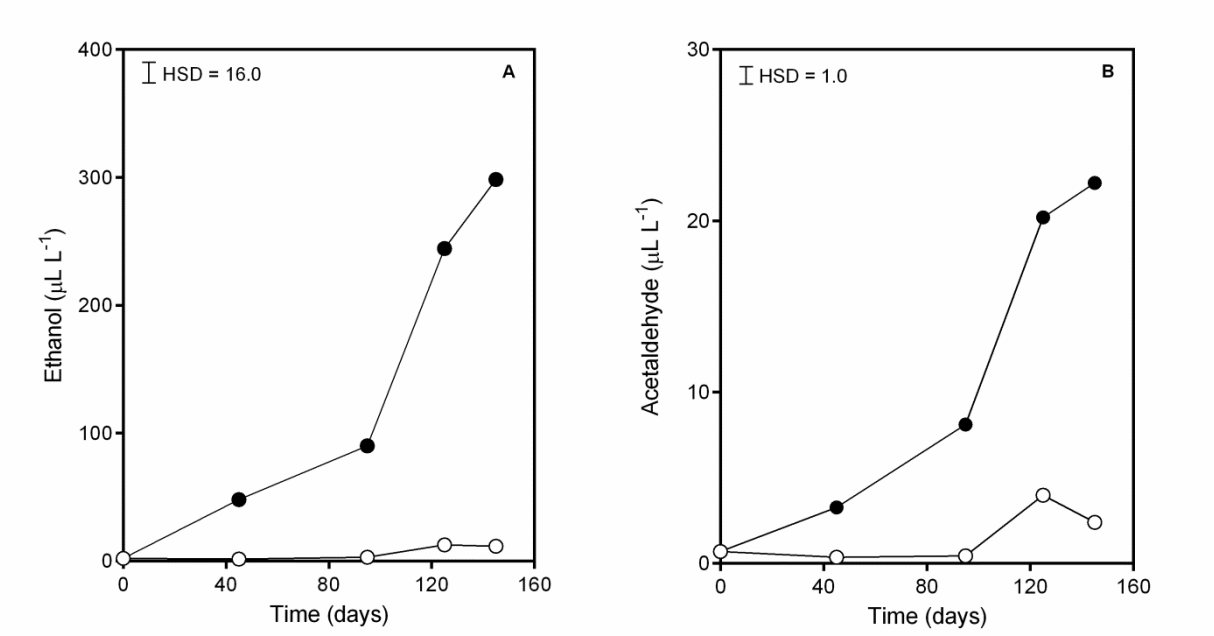

Figure 6: Levels of ethanol (A) and acetaldehyde (B) during storage under normal CA (open symbols) and high CO_2 (solid symbols) storage conditions. Values represent the means of 3 replicates of 5 fruits. Vertical bars represent the HSD (p= 0.05).

4.4.5. Polyphenoloxidase activity and total phenolics content

In fruits stored under normal CA, PPO activity gradually decreased until day 125, sharply increasing thereafter, while in high CO_2 stored fruits, after a slight increase until day 95, PPO activity slightly decreased until day 125, highly increasing thereafter (Fig. 7A). Fruits stored under high CO_2 had higher PPO activity during the entire storage period (Fig. 7A).

Total phenolics content in the fruits stored under high CO_2 decreased between 45 and 95 days of storage increasing after 125 days and decreasing thereafter (Fig. 7B). In fruits stored under normal CA a sharp decrease was observed during the first 45 days followed by an increase (1.8-fold) until the end of the storage period (Fig. 7B).

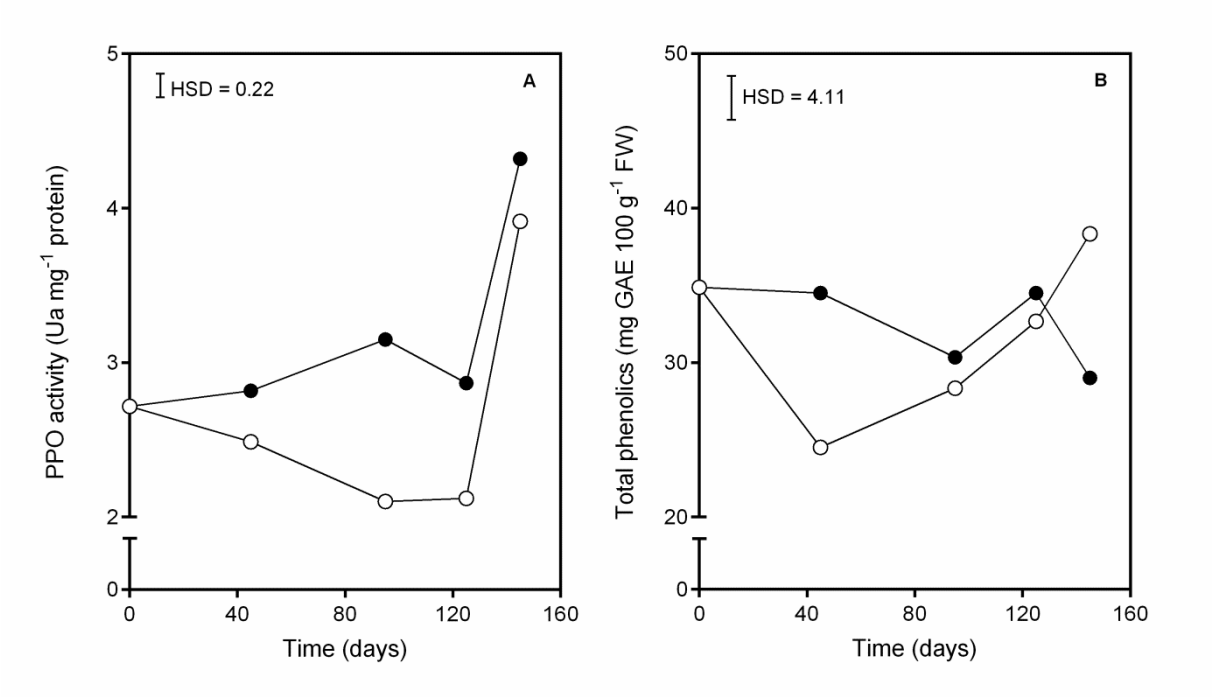

Figure 7: Polyphenoloxidase (PPO) activity (A) and total phenolics content (B) during storage under normal CA (open symbols) and high CO_2 (solid symbols) storage conditions. Values represent the means of 3 replicates of 3 fruits each. Vertical bars represent the HSD (p= 0.05).

4.5. DISCUSSION

'Rocha' pears stored under high CO_2 had higher IBD incidence compared with normal CA-stored fruits. Higher incidence of IBD during storage under high CO_2 atmospheres has been previously described for many cultivars of apples (Argenta et al. 2000; L. Argenta et al. 2002; L. C. Argenta et al. 2002; Castro et al. 2007; Mellidou et al. 2014) and pears including 'Rocha' (Veltman et al. 2000; Deuchande et al. 2012), 'Conference' (Veltman, Sanders, et al. 1999; Verlinden et al. 2002), 'Blanquilla' (Pintó et al. 2001) and d'Anjou' pears (Drake 1994).

4.5.1. Influence of H_2O_2 on IBD development in 'Rocha' pear

It is widely recognized that ROS and especially H_2O_2 tend to accumulate as a result of biotic and abiotic stresses (Ahmad et al. 2009; Gill & Tuteja 2010). It has been shown that the accumulation of H_2O_2 in 'Conference' pears was higher during the first 15 days of storage under CA (2 kPa O_2 + 5 kPa CO_2) than under cold storage (Larrigaudière et al. 2001). Higher levels of H_2O_2 were also reported in 'Pink Lady' apples stored for two months under CA (1.5 kPa O_2 + 5 kPa CO_2) with higher IBD incidence compared to cold stored apples (de Castro et al. 2008). These studies indicated that fruits stored under high

CO_2 were under oxidative stress earlier during storage, suggesting that enhanced levels of H_2O_2 had a significant impact on IBD development. In our study H_2O_2 levels were significantly lower in the fruits stored under high CO_2 compared to normal CA-stored fruits, with an earlier and sharper decline registered in the high CO_2-stored fruits (Fig. 3). Also, here we found for the first time that H_2O_2 levels were negatively correlated to IBD incidence in pears (Table 1). A downward trend on H_2O_2 concentration accompanying the development of IBD in peach fruit during cold storage was also recently reported (Ding et al. 2009). The reasons that explain this correlation are not totally known, and some hypotheses can be drawn. It has been established that excessive levels of H_2O_2 in plant cells lead to the occurrence of oxidative stress (Gill & Tuteja 2010) and may induce the programmed cell death as a defence response. However, at sub-lethal levels, ROS and particularly H_2O_2 have been shown to act as signal molecules activating defense responses during oxidative stress (Foyer & Noctor 2005; Ahmad et al. 2009). One hypothesis to explain the negative correlation between the H_2O_2 and IBD incidence, is that when the H_2O_2 depletion rate during storage under CA increases above a certain threshold value, H_2O_2 may be unable to act as a signaling molecule, effectively triggering a defense response against oxidative stress. Another hypothesis, considering the aforementioned results found for 'Conference' pears, regarding the existence of an H_2O_2 peak during the first days of storage under high CO_2, is that the H_2O_2 peak in high CO_2 stored 'Rocha' pear occurred much earlier during storage (before day 45). In this case the earlier and sharper reduction of the H_2O_2 levels in the high CO_2-stored fruits would be following the typical pattern of a stress curve, peaking very rapidly and decreasing later.

The lower levels of H_2O_2 in fruits stored under high CO_2 may also be the result of several factors such as respiration, oxidation of ascorbic acid and/or the activity of the H_2O_2-scavenging enzymes. Regarding respiration, it is known that under CA fruit tissues of the inner cortex are exposed to severe hypoxia or anoxia leading to respiratory chain inactivation in the mitochondria (Ho et al. 2010). Since the main radical formation is expected to occur in the mitochondria, its inactivation implies that there are almost no oxygen radicals forming (Veltman et al. 2000). This may partly explain why H_2O_2 did not accumulate under such high CO_2 (10 kPa) and low O_2 (1 kPa) levels. The ascorbate oxidation and further degradation may also contribute to reduced H_2O_2 levels. The DHA/AsA ratio was higher in the fruits stored under high CO_2 indicating a further oxidization of AsA (Fig. 5A). DHA is known to be less stable than AsA in the presence of H_2O_2 and could be further hydrolyzed to form degradative products which cannot be

reduced back to AsA (Deutsch 1998). The lower SOD and higher CAT activity found in the fruits stored under high CO_2 (Fig. 6) may also contribute to the sharper lowering of H_2O_2 levels. In contrast, APX and POX may not be directly involved (Fig. 6).

4.5.2. Influence of ascorbic acid metabolism and oxidative damage on IBD in 'Rocha' pear

The levels of TAA strongly decreased during the first 45 days of storage in both storage conditions but more sharply in the fruits stored under high CO_2. The faster depletion of ascorbate in the fruits stored under high CO_2 correlated to the higher incidence of IBD (Table 1). A decrease in the TAA levels during storage has been associated to a higher susceptibility of fruits to develop internal browning (Veltman, Sanders, et al. 1999; Zerbini et al. 2002; Deuchande et al. 2015). IBD was reported to occur in 'Conference' pears when the TAA concentration drops below a certain threshold value, determined as 1.3 mg 100 g^{-1} FW (Veltman, Sanders, et al. 1999).

Regarding the ratio of oxidized and reduced form of ascorbate (DHA/AsA), higher ratios in the fruits stored under high CO_2 (mainly after 95 and 125 days of storage) indicated that there was a further oxidization and subsequent degradation of ascorbate in these fruits (Fig. 4 and Fig. 5). It may be a consequence of fruit inability to recycle ascorbic acid through the ascorbate-glutathione cycle which in turn may have contributed to an acceleration of the browning process (Fig. 10).

4.5.3. Fermentation as an underlying mechanism involved in IBD development in 'Rocha' pear

During long term storage under CA, respiration may switch from aerobic to predominantly anaerobic as a result of hypoxia within the fruit tissue. This switch induces the accumulation of acetaldehyde and ethanol and may cause membrane damage (Chervin et al. 1996; Kimmerer & MacDonald 1987; Perata & Alpi 1991).

The levels of ethanol and acetaldehyde were higher in fruits stored under high CO_2 and a strong positive correlation was found between these levels and IBD incidence ($r > 0.9$; $P<0.001$) (Table 1). Accordingly, earlier studies have found that fermentation may be the underlying factor involved in IBD development both in 'Rocha' (Deuchande et al. 2012) and 'Blanquilla' [17] pear. The fermentative metabolites formed through ethanolic fermentation are important to several processes in the fruit tissue but their excessive accumulation is toxic to the cells and may affect membranes' fluidity and gas diffusivities

in the fruit tissues, causing membrane damage (Chervin et al. 1996; Kimmerer & MacDonald 1987; Perata & Alpi 1991).

4.5.4. New insights on the role of PPO

PPO activity was higher in the high CO_2-stored fruits during the entire storage period compared to the values registered in the fruits stored under normal CA (Fig. 7) and PPO activity was positively correlated to IBD incidence (Table 1). These findings are in agreement with previous studies conducted in other pear cultivars stored under cold conditions. For instance, increased PPO activity was correlated to increased IBD incidence in 'Shahmiveh' pears (Yazdani et al. 2014) and to an higher severity of IBD in Asian pears (Arzani et al. 2009). In contrast, for 'Conference' and 'Blanquilla' pears stored under CA, PPO activity has been shown not to be directly correlated with IBD incidence (Veltman, Larrigaudière, et al. 1999; Larrigaudière et al. 1998). To the best of our knowledge, this is the first study showing a direct relationship between PPO activity and the incidence of IBD in pears stored under CA (Fig. 7, Table 1). However, at the end of storage, PPO activity increased in the fruits of both conditions, suggesting that its activation at the end of storage may be independent of storage condition. Furthermore, under high CO_2 fruits were affected by IBD before PPO activation occurs whereas PPO activation in the normal CA stored fruits was not related to an increased browning incidence. This same trend was reported in apples and the authors hypothesized that PPO activation at the end of storage would be related to a genetically determined accumulation of the PPO enzyme, which could differ greatly depending on apple cultivars (Di Guardo et al. 2013).

Up-regulation of PPO expression has been previously reported in apples affected by flesh browning (Mellidou et al. 2014) and has been linked to the activation of the polyphenol signalling pathway, an important pathway involved on defence response against biotic and abiotic stress (Di Guardo et al. 2013).

Overall, the results of our study reinforce the hypothesis that the polyphenol signaling pathway may have an important role on defense response against abiotic stress such as high CO_2.

Table 1: Two tailed Pearson correlation coefficients and respective p-values for the biochemical parameters analyzed against the browning incidence during storage under both conditions, normal CA and high CO_2. The *, **, *** represent *P* values < 0.05, <0.01 and <0.001, respectively.

Biochemical parameter	Pearson correlation coefficient	*P*-value
H_2O_2	- 0.748*	0.020
Total ascorbic acid	- 0.682*	0.043
SOD	- 0.873**	0.002
CAT	0.683*	0.043
APX	- 0.029	0.945
POX	- 0.693	0.057
Ethanol	0.982***	< 0.001
Acetaldehyde	0.960***	< 0.001
PPO	0.694*	0.038
Phenolics	0.119	0.779

4.5.5. Proposed mechanism of IBD occurrence and development in 'Rocha' pear

Based on our results, a proposed mechanism of CO_2-related IBD development is presented in Fig. 8. In brief, during storage under high CO_2, a deregulation of mitochondrial function with consequent induction of fermentation occurs. As a result, there is an increased production of fermentative metabolites and a reduced energy charge. Due to the reduced energy production cells may not be able to maintain membrane integrity and antioxidant system. Therefore, TAA may be irreversibly oxidized and further degraded in the presence of H_2O_2, leading to decreased levels of both ascorbate and H_2O_2. H_2O_2 levels may be further reduced through the action of the antioxidant enzymes (SOD, CAT, APX, POX). It could be observed that the activity of the H_2O_2-scavenging enzymes (CAT, APX and POX) was up-regulated whereas SOD was down-regulated (Fig. 5), contributing to decreased H_2O_2 levels (Fig. 3 and Fig. 8).

The reduction of TAA and H_2O_2 as a result of the aforementioned factors may in turn lead to a fruits' inability to develop an adequate defense response against ROS which

in last instance causes oxidative damage. Ultimately, the excessive accumulation of fermentative metabolites, and the decreased concentrations of ascorbate and H_2O_2, may lead to loss of intracellular structure allowing PPO and phenolics to react, forming the browning compounds.

The results of this study indicate that the underlying mechanism involved in the occurrence and development of IBD in 'Rocha' pear involves the conjugation of both, the antioxidant and fermentative metabolisms and also indicate that PPO may play a role in IBD development.

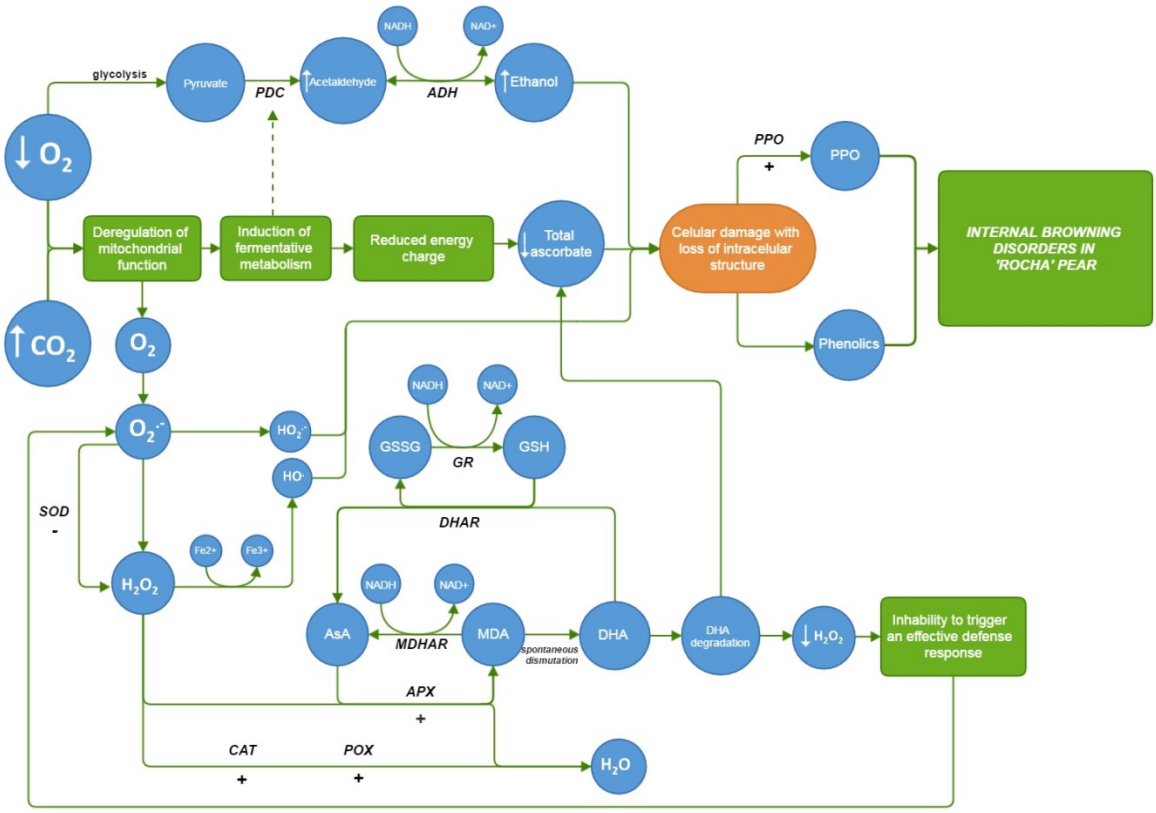

Figure 8: Schematic representation of the effects of high CO_2 and low O_2 in 'Rocha' pears during long term storage. The key enzymes believed to be involved in browning occurrence and development (SOD, superoxide dismutase; CAT, catalase; APX, ascorbate peroxidase; POX, peroxidase; GR, glutathione reductase; MDHAR, monodehydrooascorbate reductase; DHAR, dehydroascorbate reductase; PDC, pyruvate decarboxylase; ADH, alcohol dehydrogenase and PPO, polyphenoloxidase) are represented in the scheme. The results obtained for the antioxidant enzymes (SOD, CAT, APX and POX), PPO and total ascorbic acid, H_2O_2, ethanol and acetaldehyde, at the end of storage are also shown.

4.6. ABBREVIATIONS

TAA, total ascorbic acid; ADH, alcohol dehydrogenase; APX, ascorbate peroxidase; AsA, L-ascorbic acid; CAT, catalase; DHA, dehydroascorbic acid; DHAR, dehydroascorbate reductase, GR, glutathione reductase; IBD, internal browning disorder; MDHAR, monodehydroascorbate reductase, PDC, pyruvate decarboxylase; POX, guaiacol peroxidase; PPO, polyphenoloxidase; ROS, reactive oxygen species; SOD, superoxide dismutase.

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4.9. FUNDING

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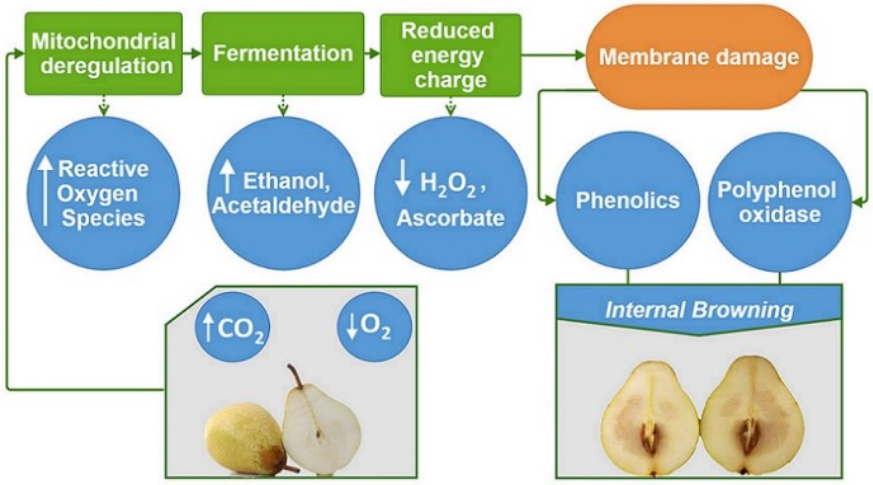
4.10. TABLE OF CONTENTS GRAPHICS



CHAPTER 5.

The Role of Transcriptional and Biochemical Regulation of the Antioxidant and Fermentative Metabolisms on Internal Browning Development in 'Rocha' Pear

To be submitted as:

Deuchande, T., Carvalho, S.M.P., Giné-Bordonaba, Jordi, Vasconcelos, M.W. and Larrigaudière, C. The role of transcriptional and biochemical regulation of the antioxidant and fermentative metabolisms on internal browning development in 'Rocha' pear.

5.1. ABSTRACT

The aims of this study were 1) to investigate the underlying mechanisms involved in IBD occurrence and development, focusing on the transcriptional regulation of the antioxidant and fermentative enzymes and 2) better understand the effect of high CO_2 and low O_2 on IBD development. For this purpose, pears were harvested from one orchard and stored under four different storage conditions: 1) cold air (-0.5 ºC), 2) normal CA (2 kPa O_2 + 0.5 kPa CO_2), 3) high CO_2 (2 kPa O_2 + 10 kPa CO_2) and 4) high CO_2 (O_2-switch) [60 days of storage under high CO_2 + 80 days of storage under high CO_2 plus ultra-low O_2 (1 kPa O_2 + 10 kPa CO_2)]. Fruit stored under both high CO_2 conditions were affected by internal browning disorders (IBDs) and the incidence was two-fold higher in the switched fruits. The highest IBD incidence was related to the lower levels of H_2O_2 and ascorbic acid and higher levels of ethanol and acetaldehyde. In the high CO_2 stored fruits the fast depletion of ascorbate was associated to the down-regulation of *Pc*MDHAR whereas in the switched fruits it was related to the down-regulation of *Pc*DHAR and *Pc*GR. A strong negative correlation between the expression of *Pc*MDHAR and *Pc*GR was also found suggesting that transcription of these genes may be regulated based on a competition for NAD(P)H. Increased SOD expression and reduced expression of the H_2O_2-scavenging enzymes (APX, CAT and POX), indicating fruits inability to detoxify H_2O_2 through the ascorbate-glutathine cycle, may have also contributed for the higher IBD incidence in the fruits stored under both high CO_2 conditions. Altogether, these results suggest that oxidative stress and reduced energy charge are the main factors involved in the occurrence and development of IBD and increased levels of fermentative metabolites significantly accelerate the browning process. A synergistic effect between high CO_2 levels and low O_2 levels leading to IBD was hence evidenced.

Keywords: ascorbic acid, controlled atmosphere storage, ethanol, internal browning, oxidative stress, *Pyrus communis*.

5.2. INTRODUCTION

Rocha pear is a Portuguese native pear recognized for its excellent organoleptic properties and also its high resistance to handling and long storage life. ‘Rocha’ pear maintains an high quality standard even upon storage for up to ten months under the recommended controlled atmosphere (CA) (2-3 kPa O_2 + 0.5-0.7 kPa CO_2 at -0.5 º C)

(Silva et al. 2010). Nevertheless, the prohibition on the application of synthetic antioxidants, such as diphenylamine and ethoxyquin, made these fruits more susceptible to develop superficial scald but also internal browning disorder (IBD) during long term storage under CA, causing considerable economic losses (Almeida 2010).

IBD in 'Rocha' pear is characterized by two types of symptoms: wet necrotic breakdown and dry cavities, which may coexist in the same fruit. Both symptoms can have their occurrence restricted to the carpelar region or be extended to the mesocarp.

It is known that IBD results from the action of polyphenoloxidase (PPO) which oxidizes the phenols forming the *o*-quinones that spontaneously polymerize to form the brown colored compounds, melanins. Although crucial on IBD development, PPO has been shown not to be a limiting factor in IBD development in 'Conference' and 'Blanquilla' pears (Veltman, Larrigaudière, et al. 1999; Larrigaudière et al. 1998). In fact, PPO and phenolics cannot react before membrane damage occurs since they are located in different cell compartments (Tomás-Barberán & Espín 2001).

During CA storage fruits are exposed to hypoxia, particularly in the fruit core which may lead to a shift in respiration towards fermentation. As a result, there is 1) a reduction of energy charge, which may lead to a cell incapacity to maintain the functionality of the antioxidant system leading to the accumulation of reactive oxygen species (ROS) which may cause lipid peroxidation and protein denaturation (Halliwell & Gutteridge 1989) and consequently membrane damage; 2) an accumulation of fermentative metabolites which above tolerated levels are known to be phytotoxic to the cells, particularly acetaldehyde (Chervin et al. 1996), also contributing to membrane destabilization. The high CO_2 levels, on the other hand, also cause changes in gas diffusion within the fruit and cytoplasmic acidification, contributing to a higher susceptibility to membrane damage (Ho et al. 2006; Pintó et al. 2001).

Anaerobic respiration and the dysfunction of the antioxidant system are, hence, believed to be main causes of IBD development. For instance, it has been shown that ascorbate depletion and initial accumulation of H_2O_2 during CA storage were related to increased IBD incidence and appearance (Veltman, Sanders, et al. 1999; Franck et al. 2003; Pedreschi et al. 2009; Zerbini et al. 2002; Larrigaudière et al. 2004). Also, increases on ethanol and acetaldehyde levels have been shown to be highly correlated to IBD in 'Blanquilla' and 'Rocha' pear (Pintó et al. 2001; Deuchande et al. 2016).

Despite of this general understanding, the exact etiology of IBD in pears remains unclear and little is known on the molecular regulation of these pathways in pears (Cascia

et al. 2013; Pedreschi et al. 2007). For instance, in 'Williams Bon Chretien', 'Doyenne du Comice' and 'Beurre Bosc, it has been shown that ascorbate peroxidase (APX) responsible for scavenging H_2O_2 using ascorbate as co-factor is up-regulated at the transcriptional level under high CO_2 conditions (Cascia et al. 2013). Cytosolic APX has been shown to be the most stress responsive isozyme of the APX family (Cascia et al. 2013; Davletova et al. 2005). Also, the expression of some monodehydroascorbate reductase (MDHAR) and superoxide dismutase (SOD) isoforms have been shown to be differently regulated in healthy and IBD affected tissues of 'Conference' pears (Pedreschi et al. 2007). Regarding fermentation, it was found that expression of alcohol dehydrogenase (ADH), the enzyme responsible for converting acetaldehyde to ethanol, was repressed in the outer and induced in the inner cortex of apples affected by browning (Mellidou et al. 2014).

In contrast to other cultivars, for which some studies on molecular regulation were conducted (Cascia et al. 2013; Pedreschi et al. 2009), scarce or no information is available for 'Rocha' pear. To the best of our knowledge, this is first study on transcriptional regulation of genes encoding antioxidant and fermentative enzymes in 'Rocha' pear during storage. Therefore, the aim of our study was to gather more information on these regulatory mechanisms in order to better understand and later control IBD in 'Rocha' pear during long term storage.

5.3. MATERIALS AND METHODS

5.3.1. Plant material and storage conditions

Pears (*Pyrus communis* L. cv. 'Rocha') were harvested from one orchard located in Cadaval, Portugal, at the beginning of the harvest season and stored under four different conditions for 140 days: 1) cold air (-0.5 ºC and 95% relative humidity), 2) normal CA (2 kPa O_2 + 0.5 kPa CO_2, 95% RH), 3) browning inducing CA_1 (2 kPa O_2 + 10 kPa CO_2, 95% RH hereafter referred to as high CO_2 atmosphere; and 4) browning inducing CA_2 (2 kPa O_2 + 10 kPa CO_2, 95% RH for 60 days + 80 days under 1 kPa O_2 + 10 kPa CO_2) hereafter referred to high CO_2 (O_2-switch).

5.3.2. Samples preparation for molecular and biochemical and analyses

Samples of pulp tissue were prepared at harvest and along storage for the molecular and biochemical analyses. The samples (3 replicates of 3 fruits each) were frozen under liquid nitrogen and ground using an electric mill. The frozen pulp tissue was used for

measuring enzyme activities. For the molecular analyses and some of the biochemical determinations (ascorbate, antioxidant scavenging capacity and phenols), ground lyophilized pulp tissue was used. Samples of pear juice (4 replicates of 3 fruits each) were also prepared for the determination of ethanol and acetaldehyde concentrations.

5.3.3. Estimation of incidence of internal browning disorders

To evaluate the incidence of IBD, 50 fruits from each storage condition were observed after 30, 60, 100 and 140 days of storage, immediately after removal from the storage chambers. The incidence of IBD was reported as the percentage of individual fruits affected by the total of fruits observed.

5.3.4. Hydrogen peroxide

H_2O_2 was extracted as previously described (Deuchande et al. 2016) and its concentration was determined using the Bioxytech H_2O_2-560 from OXIS International Inc. (Portland, OR USA) and following the manufacturer instructions. The assay is based on the oxidation of ferrous ions (Fe^{2+}) to ferric ions (Fe^{3+}) by hydrogen peroxide under acidic conditions. The ferric ions bind with the indicator dye xylenol orange to form a stable, coloured complex which can be measured at 560nm. The levels of H_2O_2 were expressed as μmol g^{-1} of fresh weight (FW). All the procedures were performed at 4 ºC in the absence of light.

5.3.5. Ascorbic and dehydroascorbic acids

Ascorbic acid (AsA) and dehydroascorbic acid (DHA) were determined as described by Zapata and Dufour (1992) with slight modifications. Freeze dried pulp tissue (150 mg) was homogenized with 1.5 ml of methanol:water (5:95 v/v) plus HCl (10 g l^{-1}) using an Ultra-Turrax (IKA Labortechnik, T 25, Germany). The homogenate was centrifuged at 4000 ×*g* for 15 min at 4ºC. To 600 μl of supernatant, 200 μl of a solution of 1,2-phenylenediamine dihydrochloride (OPDA, from Sigma-Aldrich) were added being the mixture incubated for 30–40 min and then filtered through a 0.22 μm filter. The samples were analyzed with an HPLC system equipped with a UV-Vis detector (UV-1575), an auto sampler (AS-1555) and a pump (PU-1580), all controlled by appropriate software (Borwin v. 1.5, from Jasco Corporation, Tokyo, Japan). Separation of AsA and DHA was achieved in a reverse-phase column Spherisorb ODS2 5 μm (250 x 4.6 mm da Waters Corporation, USA). The detector was set at 348 nm for DHA and 261 nm for AsA detection. AsA and

DHA contents were expressed in mg 100 g^{-1} dry weight (DW). All the procedures were conducted in the darkness at 4 ºC.

5.3.6. RNA extraction and cDNA synthesis

Total RNA was extracted as described by (Chang et al. 1993) with modifications. In brief, samples of freeze dried pulp tissue (300 mg) were washed twice with ethanol 80% and the float cell debris discarded. After the washing step, 3 ml of preheated extraction buffer (100 mM Tris–HCl, pH 8.0, 2 M NaCl, 25 mM EDTA, pH 8.0, 2% (w/v) cetyltrimethylammonium, 2% (w/v) polyvinylpyrrolidone 40, 500 mg L^{-1} spermidine, 2% (v/v) β-mercaptoethanol) were added and further volumes were proportionally adjusted. RNA quality and quantity were checked by UV-spectrophotometry, using a nanophotometer (Implen, Isaza, Portugal) and the RNA integrity was verified by agarose gel electrophoresis staining with red gel. Single strand cDNA was synthetized from 1 μg of total RNA, using First Strand cDNA Synthesis Kit (Fermentas UAB, Cat. Nr. #K1612) in a thermal cycler (VWR, Doppio, Belgium), following the manufacturer instructions.

5.3.7. Primer Design and qRT-PCR

The transcript levels of six antioxidant enzymes [(SOD, catalase (CAT), APX, peroxidase (POX), MDHAR, glutathione reductase (GR) and dehydroascorbate reductase (DHAR)] and two fermentative enzymes (pyruvate decarboxylase (PDC) and ADH were analysed, using PP2A and SAND as reference genes (Cascia et al. 2013). Sequences for CAT, APX, PDC and GR were obtained from the Genome database for Rosaceae (GDR) using the Pyrus Unigenes V5 (Jung et al. 2014). Sequences with an *e* value < 10^{-38} were considered homologs. For SOD (Wang et al. 2012), POX (Ou & Jiang 2012) and ADH (Chervin et al. 1999), sequences were retrieved from a search on NCBI and the Basic Local Alignment Search Tool (BLAST) was used to find regions of local similarity with organisms belonging to the Rosaceae family, selecting the most preserved part of the sequence to design the primers. In all cases, primers were designed using the Primer-Blast tool from NCBI. Primers for the MDHAR, DHAR and housekeeping genes (PP2A and SAND) were retrieved from available published literature (Cascia et al. 2013). The primer pairs used for the qPCR analysis are listed in Table 1. The RT-qPCR analyses were performed in a CFX96 Touch™ Deep Well Real-Time PCR Detection System (Bio-Rad Laboratories Inc., CA, USA) using iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, CA, USA). Primer efficiency was determined for all primers by qPCR analysis of a standard curve, constructed by serial dilutions of the synthetized cDNA from one test

sample. The amplification protocol was set to cycle as follows: 95 °C denaturation for 10 min; 45 cycles of 95 °C for 15 s followed by 58–60 °C (depending on primers used) for 30 s; followed by melt curve stages to check that only single products were amplified. The CFX Manager™ software, version 3.1 (Bio-Rad Laboratories Inc., CA, USA) was used to verify the stability of the reference genes.

Table 1: Primers used for quantitative qPCR analysis.

Gene name	Primer pairs	Pear contig and/or closest organism, homolog gene, GenBank Acc. No.	Fragment size (bp)	References
Antioxidant system				
PcSOD	GCCATCAAGTTCAATGGCGG; CAAGCCACACCCATCCAGAA	*Pyrus calleryana,* MnSOD, JQ014284.1	202	-
PcAPX	GTCCCATTCCACCCAGGAAG; CCACCGGAGAGAGCAACAAT	Contig 134, *Pisum sativum,* cytosolic APX1, X62077.1	149	-
PcCAT	CTAGGGGAGCCAGTGCAAAA; CATGGATCACGTCCGGGAAT	Contig 239, *Gossypium hirsutum,* CAT1, X52135	273	-
PcGR	CATCCATGTGTGGGCCAGAT; GTACCTGCAGCAATACGCCT	*Pisum sativum,* cytosolic GR, X98274	166	-
PcMDHAR	TGGTGTCAAAGGAGCTGATG; ACCAAGCTCGAGACCAATGT	*Arabidopsis,* MDAR4, At3g52880	142	Cascia et al. (2013)
PcDHAR	TCTCGAGGTCGCTGTCAAG; GGGGTTTGTCGCTGAGATT	*Arabidopsis,DHAR3,* At1g75270	140	Cascia et al. (2013)
PcPOX	CCCGGAGTAGTCTCATGTGC; AGGAATGGAGGTGTTGGCAG	*Pyrus communis,* POX2, KC153030.1	144	-
Fermentative metabolism				
PcPDC	GAAGCGATTGAGACCGCAAC; AACAGACCGCAGGGAAATGT	Contig 95, *Pisum sativum,* PDC2, Z66544	247	-
PcADH	TTGCCAAGACCAATCCCCTG; AATCCGTGATGCCCCACAAA	Contig161, *Pyrus communis* (Adh3) AF031899.1	188	-
Housekeeping genes				
PP2A	GAATCCAGAACTTGCCATTCA; TGCTCTATAGTTGCATCCTTTCC	At1g13320	141	Cascia et al. (2013)
SAND	TCCAGATATGGAGATGAACACAAG; GGTCCCTTGACAAGAAAAATCA	At2g28390	140	Cascia et al. (2013)

All expression data were normalized against the geometric mean of the expression of the two stable reference genes, using the delta CT method incorporating individual amplification efficiencies. Three biological replicates were analyzed and two technical repetitions of each biological replicate were performed. Non-template controls (NTC) were

included in each plate to discard the presence of primer dimers and/or primer contamination.

5.3.8. Fermentative metabolites

Ethanol and acetaldehyde were measured as described by Ke et al. (1994) with slight modifications. Juice samples (5 ml) (4 replicates of 5 fruits each) were prepared and stored at -25 °C until analysis. Samples were placed in a 10 ml test tube with a screw cap and incubated in a water bath at 65 °C for 1 hour, after which, 1 ml of headspace sample was taken with a 1 ml glass syringe for chromatographic determination of ethanol and acetaldehyde. The concentrations were calculated using a standard curve generated by injecting standard solutions of known concentrations.

5.3.9. Statistical analysis

The data was subjected to analysis of variance (two-way ANOVA) for the effect of the storage atmosphere and storage time using the GraphPad Prism version 6.0 (San Diego, CA, USA). Significant differences for the effect of the storage atmosphere were determined by calculating the Tukey's honestly significant difference (HSD) at P=0.05.

Partial least square (PLS) regression was performed using Tanagra software, version 1.4.50 (Ricco Rakotomalala, Lyon, 2003; http://chi-rouble.univ-lyon2.fr/~ricco/tanagra), using 16 *X*-variables (H_2O_2, AsA, DHA, total ascorbic acid (AA) and DHA/AsA ratio, ethanol and acetaldehyde levels, and relative gene expression of CAT, APX, DHAR, MDHAR, GR, ADH and PDC)] and five *Y*-variables, (percentage of browning incidence and four categories of storage conditions [cold air, normal CA, high CO_2 and high CO_2 (O_2-switch)]. For each storage condition was attributed a discrete value of 1 when the sample was included in the category and 0 when it was not. All the X-variables and the percentage of browning were continuous whereas storage conditions corresponded to categorical variables.

When not related to the PLS model the correlations mentioned correspond to pairwise two-tailed Pearson correlations including the data during the entire storage period.

5.4. RESULTS AND DISCUSSION

5.4.1. Initial fruit maturity and IBD incidence

For 'Rocha' pear, the recommended optimal values for harvest range from 55 to 65 N for firmness, a SSC between 11 to 13%, acidity of 2-3 g malic acid l^{-1} (ANP, 1997) and

a starch index averaged at about 6.5 (Avelar & Rodrigues 1999). According to our results and considering the reference values, the fruits used in this experiment were harvested at an early maturity stage (Table 2). Early and optimally harvested fruits have been shown to be less susceptible to develop IBD during CA storage than late harvested fruits (Deuchande et al. 2012).

Table 2: Initial maturity indexes. Values of firmness, weight and colour (lightness (L*), chroma (C*) and hue angle (hº) represent the mean of 30 fruits ±SE and the values of titratable acidity (TA) and soluble solids content (SSC) represent the mean of 3 replicates of 5 fruits each ± SE.

Firmness (N)	Weight (g)	Starch	TA (g malic acid l^{-1})	SSC (%)	Colour		
					L*	C*	hº
66.2 ±0.7	118±3.4	5±0.19	1.94±0.02	13.4±0.1	66.6±0.31	45.92±0.22	108.3±0.3

Table 3: Internal browning incidence in pears during storage under cold air, normal CA, high CO_2 (2 kPa O2 + 10 kPa CO_2) and high CO_2 (O_2-switch) (60 days under 2 kPa O_2 + 10 kPa CO_2 plus 80 days under 1 kPa O_2 + 10 kPa CO_2).

Storage condition	**Storage period (days)**	**Browning incidence (%)**
Cold air	140	0
Normal CA	140	0
High CO_2	60	5.4
	100	0
	140	14.8
High CO_2 (O_2-switch)	100	3.8
	140	26.6

No browning disorder was observed after 140 days of storage under air and normal CA (Table 3). In contrast significant incidence was observed in fruit stored under high CO_2 and high CO_2 (O_2-switch) that exhibited 15% and 27% of incidence respectively (Table 3). These results are in accordance to our previous study, confirming the role that high CO_2 levels play on IBD induction (Deuchande et al. 2016), but also shows for the first time the synergistic effect of O_2 levels when combined with high CO_2 on further induction of IBD.

5.4.2. Regulation of the antioxidant defence system

5.4.2.1. The role of H_2O_2 on IBD

It is widely recognized that H_2O_2 may accumulate as a result of oxidative stress (de Castro et al. 2008; Larrigaudière et al. 2001) when the rate of accumulation of reactive oxygen species (ROS) is higher than the fruit capacity to scavenge these harmful molecules.

In this work, two peaks of H_2O_2 were observed in the fruits of all the different storage conditions, the first after 7 days and the second after 30 days of storage (Fig. 2). At day 7 no significant differences were observed between treatments but at 30 days significant higher H_2O_2 levels were observed in fruit stored under normal CA and cold air (ca. 30 μmol kg^{-1} FW), compared to the levels reached in the high CO_2 stored fruits (21 μmol kg^{-1} FW). Despite of these differences the rate of H_2O_2 increase was similar in the fruits of all storage conditions between day 14 and day 30 (ca. 1.06 ±0.19 μmol kg^{-1} day^{-1} FW) (Fig. 2). From day 60 up to the end of the storage period the H_2O_2 levels slightly decreased in the fruits stored under cold air whereas in the fruits stored under three CA conditions the levels sharply decreased (Fig. 2).

An increase in the H_2O_2 levels at the beginning of storage have already been reported for 'Conference' pears (Larrigaudière et al. 2001). However, in contrast to our results, in this previous study H_2O_2 levels were higher in the fruits stored under CA (2 kPa O_2 + 5 kPa CO_2) than in the fruits stored under cold air. This interesting result suggests that in 'Rocha' pear, contrarily to the observed in 'Conference' pear, CA may not be the main responsible for the stress that leads to H_2O_2 accumulation. Other processes, mainly related to the maintenance of energy charge and/or induction of fermentation are involved and likely play a predominant role in the induction of IBD in 'Rocha' pear.

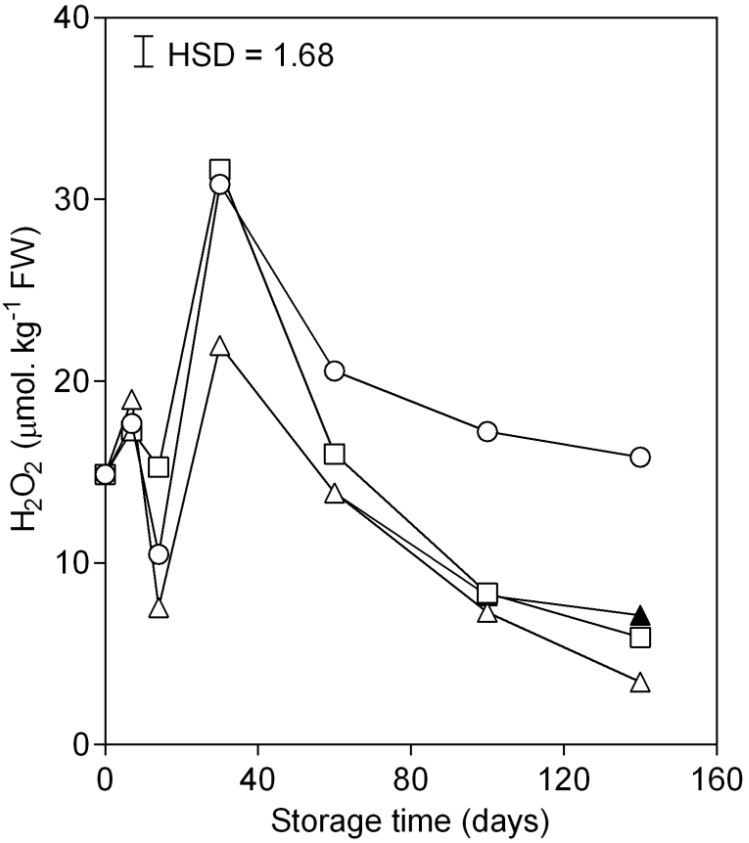


Figure 1: Hydrogen peroxide (H_2O_2) levels in pears during storage under cold air (○), normal CA (□), high CO_2 (△) and high CO_2 (O_2-switch) (▲). Symbols represent the means of 4 replicates of 5 fruits each. Vertical bars represent the HSD (P=0.05). For details concerning storage conditions see Table 3.

During storage under cold air the higher H_2O_2 levels may be due to fruits' exposure to higher O_2 levels and hence higher rate of ROS formation but since these fruits may be well energetically charged ensuring an efficient functioning of the antioxidant system, the redox status of the fruits may be maintained as well as appropriate levels of H_2O_2. Proper levels of H_2O_2 may contribute to IBD prevention, since, by acting as a signaling molecule, H_2O_2 mediates fruit response to oxidative stress. Regarding the fruits stored under CA, the lower levels of H_2O_2 may be the result of fruit exposure to hypoxia. Under these conditions, if from one hand ROS are barely formed, on the other hand the energy status of the cells may be insufficient to maintain the antioxidant system and hence an unbalanced redox status may contribute for membrane damage and ultimately to IBD development.

5.4.3. Influence of transcriptional regulation of SOD and H_2O_2 scavenging enzymes on IBD

SOD is the enzyme responsible for converting the superoxide anion radical to H_2O_2 comprising the first line of defence against oxidative stress (Gill & Tuteja 2010). In the fruits stored under both high CO_2 conditions the expression of SOD was higher compared to the expression measured in the fruits stored under cold air and normal CA (Fig. 4A). These results showed that fruits stored under high CO_2 were under higher oxidative stress and trying to respond to the stressful conditions through SOD activation. The differences in *Pc*SOD regulation between the fruits of each high CO_2 condition, particularly between day 100 and day 140 at the end of storage [(up-regulation of *Pc*SOD by 2.5 fold in the fruits stored under high CO_2 (O_2-switch) and down-regulation by 1.6 fold in the fruits stored under high CO_2] (Fig. 4A) also suggest that fruit storage under different low O_2 levels combined with high CO_2 have an important influence in the regulation of SOD at the transcriptional level which may influence the fruits susceptibility to IBD. Although SOD expression was mainly up-regulated in the fruits stored under both high CO_2 conditions an induction of gene expression of the H_2O_2-scavenging enzymes (*Pc*CAT, *Pc*APX and *Pc*POX) did not occur (Fig. 4A, B, C, D) probably due to the already very low H_2O_2 levels (Fig. 2). Regarding *Pc*APX, the lower levels of AsA may have also determined the down-regulation of this gene (Fig. 3C and Fig. 4C). The up-regulation of *Pc*APX in the fruits stored under normal CA support this hypothesis. Although H_2O_2 levels in these fruits were as low as the detected in the fruits stored under high CO_2 the levels of ascorbate were, in general, significantly higher and this factor may have contributed to the general up-regulation of *Pc*APX in these fruits (Fig. 2, 3A, 4C). Regarding *Pc*POX its expression was highly down-regulated in the fruits stored under the three CA conditions whereas it was up-regulated in the fruits stored under cold air (Fig. 4D). These results suggest that regulation of *Pc*POX expression was mainly affected by the low O_2 levels rather than high CO_2 levels. Also, the very low levels of H_2O_2 in the CA stored fruits may have also determined the lowest *Pc*POX expression.

According to our results the up-regulation of SOD expression indicates increased exposure to oxidative stress and in combination with a simultaneous down-regulation of the expression of the H_2O_2-scavenging enzymes it may indicate an unbalance of the antioxidant system which may determine an increased susceptibility to IBD.

5.4.4. The role of ascorbic and dehydroascorbic acid on IBD

The levels of total ascorbate (TAA) in the fruits stored under cold air were the highest and remained almost stable during the entire storage period (Fig. 3A). A similar trend was observed for the fruits stored under normal CA, albeit with more oscillations (Fig. 3A). In contrast, the TAA levels in the high CO_2 stored fruits after 60 days up to the end of the storage period were lower when compared to the levels registered in the fruits of the other storage conditions (Fig. 3A). These results indicate that the fruits stored under cold air and normal CA were able to regenerate ascorbate, while the fruits stored under high CO_2 were not and, hence, in these last, TAA was oxidized and further hydrolyzed to form degradative products which could not be reduced back to ascorbic acid (AsA) (Deutsch 1998). Our results are in accordance with previous studies reporting a faster TAA loss in 'Conference' pears stored under CA than under cold air (Larrigaudière et al. 2001) and, a more pronounced TAA decrease in 'Rocha' pear stored under high CO_2 than under normal CA (Deuchande et al. 2015; Deuchande et al. 2016). The two DHA/AsA peaks observed in the normal CA and cold air stored fruits after 30 and 100 days of storage support this conclusion, clearly showing that at the end of storage these fruits were still able to regenerate ascorbate through the ascorbate-glutathione cycle (Fig. 3B). A stress peak in the fruits stored under high CO_2, after 30 days of storage, was also observed (Fig. 3B). However, from day 60 until the end of storage the ratio of DHA/AsA increased in these fruits and even further in the fruits stored under high CO_2 (O_2-switch) (Fig. 3B). Considering that AsA levels decreased and DHA did not accumulate in the fruits stored under high CO_2 these results indicate that the increased DHA/AsA ratios were related to AsA degradation due to an impairment of AsA regeneration. Between day 60 and day 100, the up-regulation of *Pc*DHAR expression by 3.8 fold in the high CO_2 stored fruits and the up-regulation of *Pc*MDHAR by 2.2 fold in the fruits stored under high CO_2 (O_2-switch) combined with the low *Pc*APX expression in the fruits of both conditions, followed by decreasing levels of AsA at the end of storage support this observation (Fig. 4 C, E, F and Fig. 3C).

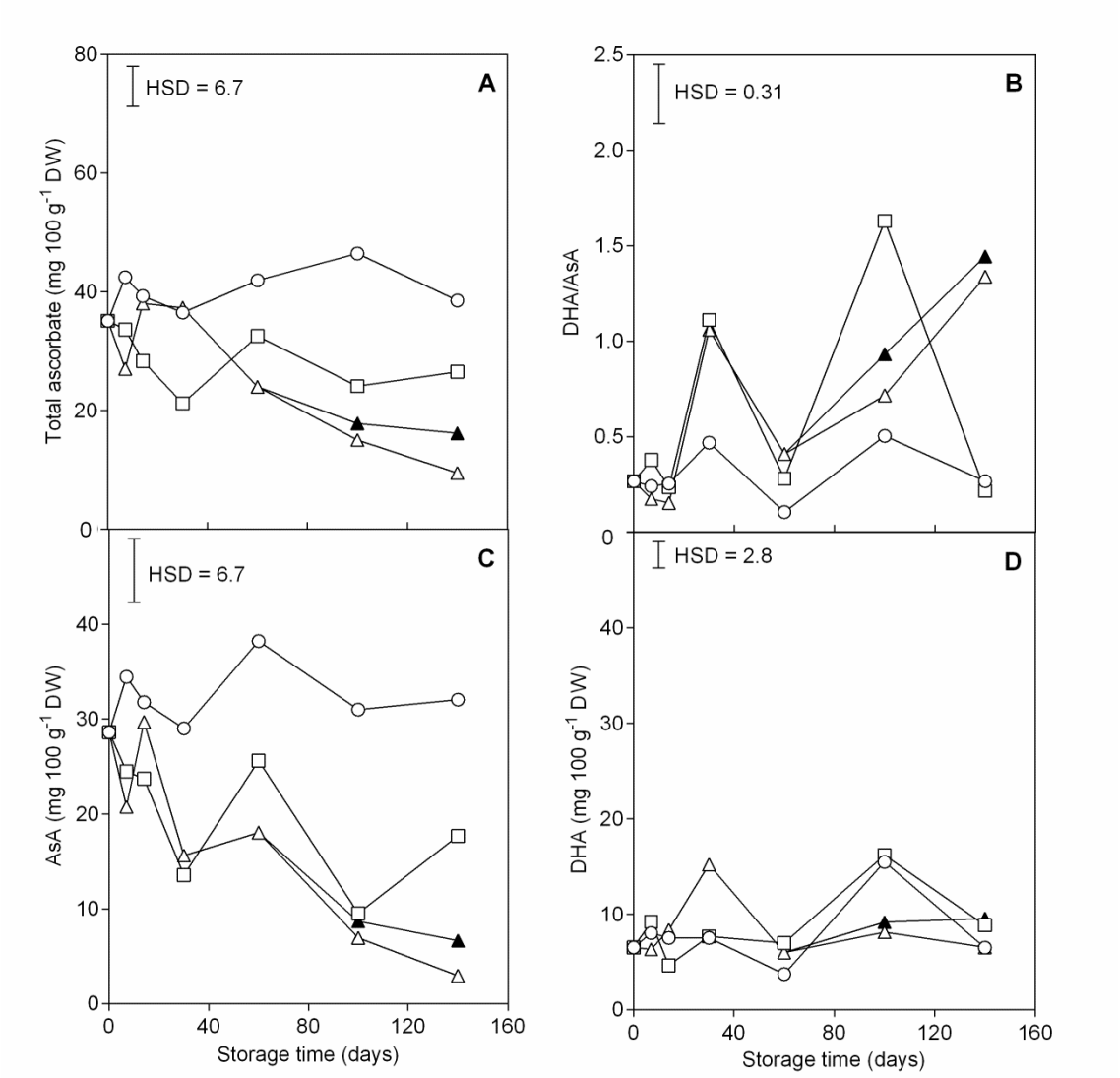


Figure 2: Concentration of total ascorbate (A), ratio between oxidized and reduced form of ascorbate (DHA/AsA) (B), level of ascorbic acid (AsA) (C) and dehydroascorbic acid (DHA) (D) in pears during storage under cold air (○), normal CA (□), high CO_2 (△) and high CO_2 (O_2-switch) (▲). Symbols represent the means of 3 replicates of 3 fruits each. Vertical bars represent the HSD (P=0.05). For details concerning storage conditions see Table 3.

5.4.5. Influence of transcriptional regulation of the ascorbate recycling pathway on IBD

According to our results, the fast reduction in the levels of AsA in the fruits stored under high CO_2 (O_2-switch) did not result in higher DHA levels probably due to the up-regulation of *Pc*MDHAR in these fruits between day 60 and day 140 (Fig. 4E). However, the levels of AsA remained low (Fig. 3C) indicating that MDHAR activation was not sufficient to regenerate all MDHA and the proportion of MDHA oxidized to DHA was

then rapidly degraded. In the fruits stored under high CO_2 the decrease in the levels of AsA did not also result in increased levels of DHA (Fig. 3C,D). Although there was an increased expression of *Pc*DHAR at day 100, the levels of AsA further decreased up to the end of storage (Fig. 4F). These results showed that these fruits were not able to regenerate ascorbate and that once formed, DHA was rapidly degraded, possibly due to the inability of DHAR to regenerate AsA. For its activity DHAR requires reduced glutathione as a co-factor and therefore, low GSH levels may possibly have limited DHAR action. It may also explain the up-regulation of GR by 2.5 fold between day 100 and day 140 as an attempt to supress the requirement for GSH (Fig. 7A).

Considering the involvement of the ascorbate recycling pathway on the development of IBD, when comparing the results for fruits stored under cold air and normal CA to those obtained for fruits stored under the two high CO_2 conditions, the higher IBD incidence in the fruits stored under high CO_2 (O_2-switch) was well correlated to the lower expression levels of *Pc*GR and *Pc*DHAR, whereas in the fruits stored under high CO_2 it was well correlated to the significantly lower expression levels of *Pc*MDHAR. In both cases the lowering of AsA levels due to the down-regulation of genes codifying the enzymes involved in AsA regeneration may have led to the lower expression of *Pc*APX which also appeared correlated to the higher IBD incidence (Table 3, Fig. 4C, E, F, G and Fig. 6).

An interesting result was the highly negative correlation between *Pc*GR and *Pc*MDHAR in the fruits stored under cold air ($r = 0.806$; $p = 0.009$) and normal CA ($r = 0.890$; $p= 0.003$) during the entire storage period (Fig. 4E, G). The inverse regulation of these genes suggests that their expression is interdependent. Considering that MDHAR and GR require NAD(P)H to reduce ascorbate and glutathione, respectively, we can hypothesize that differences found on transcript levels of these enzymes may result from a competition for NAD(P)H. There are evidences in the literature that NAD(P)H affects gene expression in plants but the mechanisms by which NAD influences plant gene regulation, metabolism and physiology still remain unclear (Pétriacq et al. 2012). However, if we compare the expression of the same genes in the fruits stored under the two high CO_2 conditions this relation is not that clear, indicating that other factors are involved in the regulation of these genes probably related to changes in respiration and energy charge caused by fruits' exposure to high CO_2 and low O_2 levels.

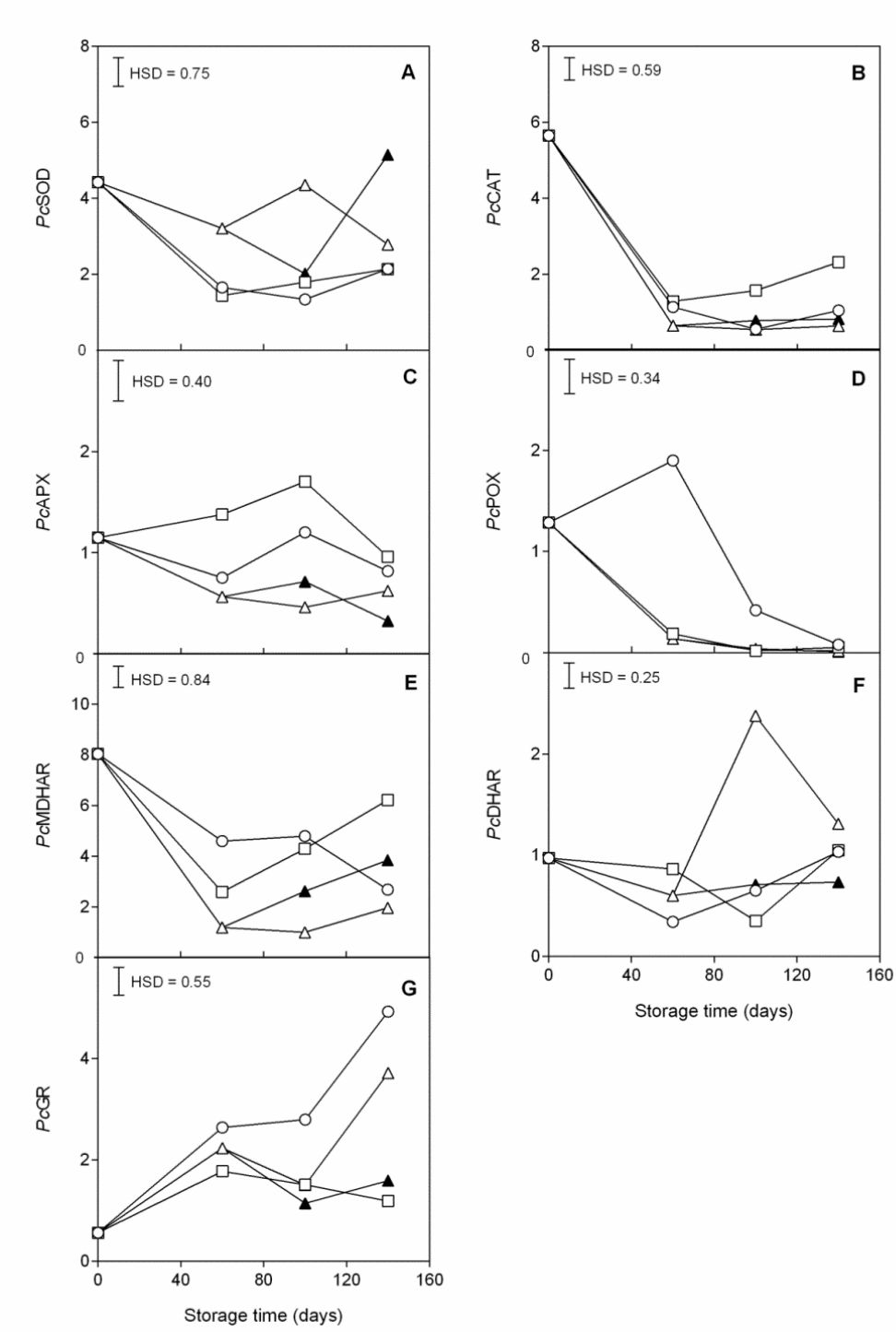


Figure 3: Relative gene expression of superoxide dismutase (*Pc*SOD) (A), catalase (*Pc*CAT) (B), ascorbate peroxidase (*Pc*APX) (C), peroxidase (*Pc*POX) (D), monodehydroascorbate reductase (*Pc*MDHAR) (E), dehydroascorbate reductase (*Pc*DHAR) (F); glutathione reductase (*Pc*GR) (G), in pears during storage under cold air (○), normal CA (□), high CO_2 (△) and high CO_2 (O_2-switch) (▲). Symbols represent the means of 3 replicates of 3 fruits each. Vertical bars represent the HSD (*P*=0.05). For details concerning storage conditions see Table 3.

5.4.6. Regulation of fermentation

5.4.6.1. The role of fermentative metabolites on IBD

The levels of ethanol and acetaldehyde in the fruits stored under cold air and normal CA were fairly low until day 60 slightly increasing thereafter, with higher levels registered in the fruits stored under cold air at the end of storage (Fig. 5). These results were expectable since, despite there is a reduction on metabolic activity under cold air, the ripening process still occurs. It is also known that CA storage further reduces fruit's metabolisms and hence, ripening and senescence occur earlier during storage under air than under CA with an higher natural production of fermentative metabolites (Ke et al. 1991; Pesis et al. 2002).

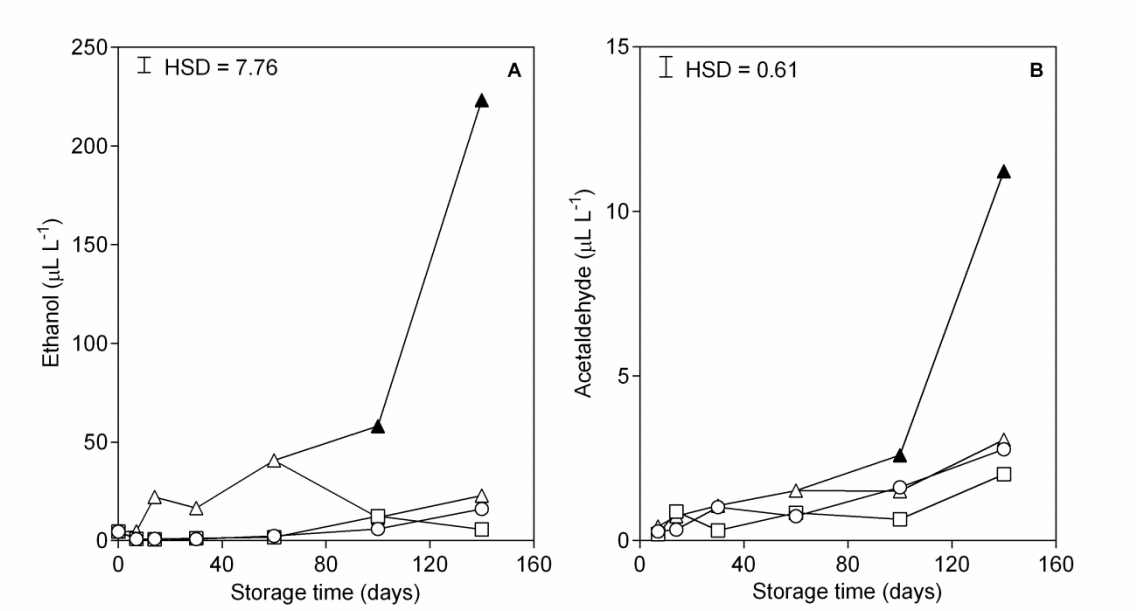


Figure 4: Concentrations of ethanol (A) and acetaldehyde (B) in pears during storage under cold air (○), normal CA (□), high CO_2 (△) and high CO_2 (O_2-switch) (▲). Symbols represent the means of 3 replicates of 3 fruits each. Vertical bars represent the HSD (P=0.05). For details concerning storage conditions see Table 3.

Initially, in the high CO_2 stored fruits the ethanol levels were higher than in the fruits of the other storage conditions, increasing until day 60. However, at day 100 up to the end of the storage period the levels of ethanol dropped to values similar to the registered in the fruits stored under normal CA and cold air, slightly increasing thereafter (Fig. 5A). On the contrary, at day 100 the levels of ethanol increased in the fruits stored under high CO_2 (O_2-switch) and by about four-fold thereafter (Fig. 5A). Similarly, the

acetaldehyde levels in these fruits increased in the same rate (Fig. 5B), being far higher than the registered in the fruits of the other storage conditions. This sharp increase in the levels of fermentative metabolites indicates a shift in the respiratory metabolism of these fruits towards fermentation also evidencing that fruits' exposure to very low O_2 levels more than to high CO_2 levels is critical for fruits respiration and hence, in keeping the energy status of the cells.

5.4.7. The involvement of transcriptional regulation of fermentative enzymes on IBD

A further induction of fermentation in the fruits stored under high CO_2 would be expected to lead to an increased biosynthesis of fermentative enzymes. Accordingly, in these fruits *Pc*ADH expression was higher than in the fruits of the other storage conditions (Fig. 6B). However, the expression *Pc*PDC in the high CO_2 stored fruits was the lowest and was kept almost constant during the entire storage period (Fig. 6A). It has previously been shown that high CO_2 levels affect the functioning of enzymes involved in the glycolytic pathway leading to the repression of this pathway (Kerbel et al. 1988; Ke et al. 1993) and hence reduced formation of pyruvate. Hence, PDC, as an allosteric enzyme may have its activity and expression inhibited by the reduced levels of substrate under high CO_2.

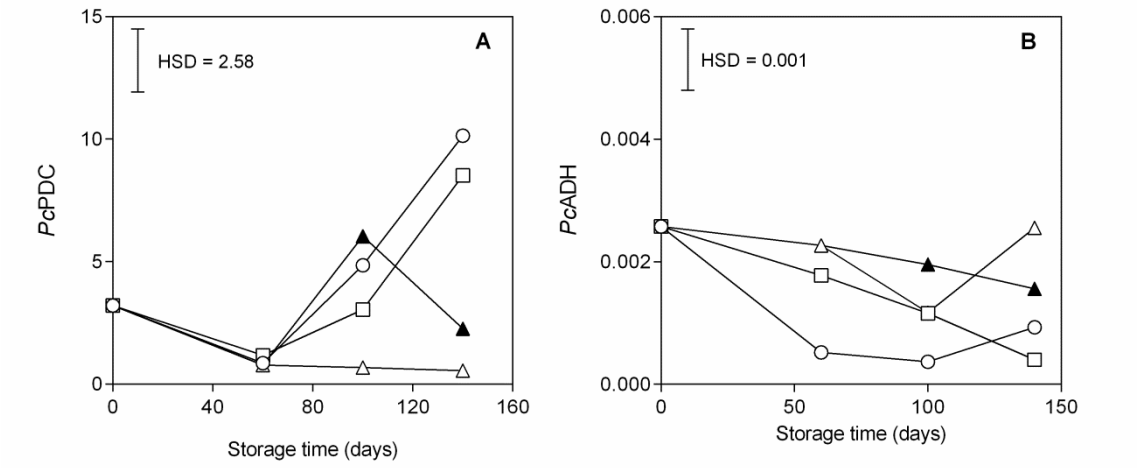


Figure 5: Relative gene expression of enzymes of fermentative metabolism, pyruvate decarboxilase (*Pc*PDC) (A) and alcohol dehydrogenase (*Pc*ADH) (B), in pears during storage under cold air (○), normal CA (□), high CO_2 (△) and high CO_2 (O_2-switch) (▲). Symbols represent the means of 3 replicates of 3 fruits each. Vertical bars represent the HSD (P=0.05). For details concerning storage conditions see Table 3.

Contrarily to the observed for the fruits stored high CO_2, in the fruits stored under high CO_2 (O_2-switch) the expression of *Pc*PDC was up-regulated by 9 fold between day 60 and day 100 (Fig. 6A). Under hypoxia glycolysis has been shown to be highly up-regulated in order to produce maximum energy (Pedreschi et al. 2007). Therefore, the increased build-up of pyruvate due to glycolysis up-regulation may have activated *Pc*PDC expression in these fruits. However, as the fermentation proceeded, there was an accumulation of acetaldehyde which in turn may have then inhibited PDC activity. The retro-inhibition of acetaldehyde on enzyme transcription may explain the down-regulation of *Pc*PDC in the fruits stored under high CO_2 (O_2-switch) between day 100 and day 140.

5.4.8. PLS regression

In order to correlate the analysed variables with the incidence of IBD and atmosphere storage conditions a PLS regression model using the percentage of browning incidence and the four storage conditions as targets was conducted. The PLS loading plot (Fig. 6) clearly shows that the main factors related to IBD incidence are the concentrations of ascorbic and dehydroascorbic acid and the levels of ethanol and acetaldehyde. Increased expression levels of *Pc*SOD also appeared strongly related to increased IBD incidence. In contrast, reduced expression of *Pc*APX, *Pc*CAT and *Pc*POX were significantly correlated to increased IBD incidence. The expression of the genes involved in ascorbate regeneration (*Pc*MDHAR, *Pc*GR, *Pc*DHAR) did not appeared directly correlated to IBD since the negative correlation found was not significant. Regarding the fermentative enzymes the expression *Pc*PDC appeared negatively correlated to IBD whereas the expression of *Pc*ADH, appeared positively correlated. Considering the influence of the storage conditions, the results showed that fruit storage under both high CO_2 conditions were separated mostly in relation to the LV2. However, differences in fruits behaviour can be observed in the fruits of these conditions. According to the PLS results the highest incidence in the fruits stored under high CO_2 (O_2-switch) was positively correlated with *Pc*SOD expression and levels of H_2O_2, DHA, ethanol and acetaldehyde and negatively with *Pc*POX, *Pc*APX, *Pc*GR and *Pc*DHAR expression. In contrast, fruit storage under high CO_2, was positively correlated with *Pc*GR, *Pc*DHAR *Pc*ADH expression and DHA/AsA ratio and negatively with the levels of H_2O_2, ascorbic acid and total ascorbate as well as with the expression of *Pc*MDHAR, *Pc*CAT and *Pc*PDC.

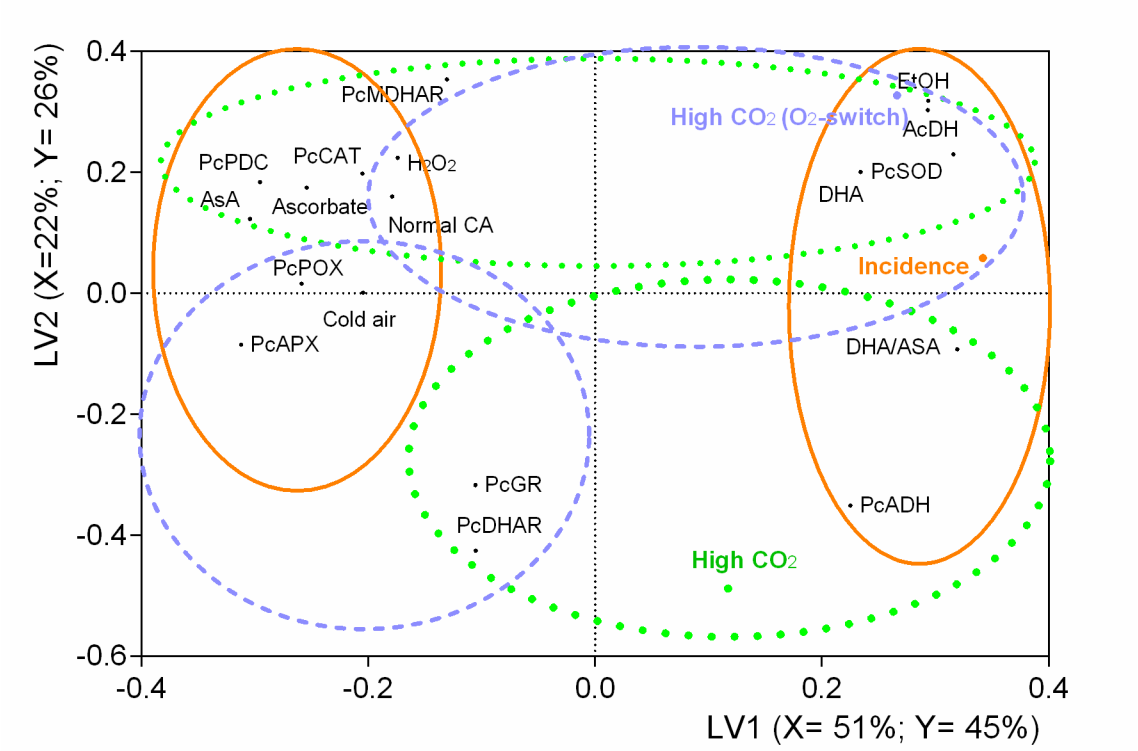


Figure 6: Loading plot of PLS model containing *X*-variables [concentrations of hydrogen peroxide (H_2O_2), ascorbic acid (AsA), dehydroascorbic acid (DHA), total ascorbic acid (AA) and DHA/AsA ratio, ethanol (EtOH) and acetaldehyde (AcDH) levels, and relative gene expression of catalase (*Pc*CAT), ascorbate peroxidase (*Pc*APX), dehydroascorbate reductase (*Pc*DHAR), monodehydroascorbate reductase (*Pc*MDHAR), glutathione reductase (*Pc*GR), alcohol dehydrogenase (*Pc*ADH) and pyruvate decarboxylase (*Pc*PDC)] and *Y*-variables (percentage of browning incidence and the different storage conditions [cold air, normal CA, high CO_2 and high CO_2 (O_2-switch)] after 140 days of storage.

5.4.9. Proposed mechanisms of IBD development under high CO_2 and very low O_2 levels combined with high CO_2

'Rocha' pear storage under high CO_2 (10 kPa) determines crucial changes on fruit biochemistry and transcription but in this study we clearly showed that, when high CO_2 levels are combined with very low O_2 levels (1 kPa) additive effects contribute for increased IBD incidence. Figure 7 shows the effects of these two types of storage atmospheres on the antioxidant system and fermentation and how the regulation of these pathways influences the incidence of IBD.

During storage under high CO_2 (Fig. 7A) the expression of SOD is activated due to fruit exposure to oxidative stress. However, the expression of the H_2O_2-scavenging enzymes is repressed probably due to an insufficient energy charge resulting from the

down-regulation of glycolysis or maybe because the H_2O_2 levels were already too low. The levels of AsA are reduced in these fruits but the levels of DHA did not accumulate indicating that once formed DHA was rapidly degraded in the presence of H_2O_2 leading to decreased levels of H_2O_2 and ascorbate. Despite PcDHAR was up-regulated at day 100, *Pc*MDHAR and *Pc*GR were down-regulated, determining an impairment of the antioxidant system leading to oxidative damage and ultimately to IBD. Under these conditions fermentation was slightly activated and low ethanol and acetaldehyde slightly accumulated which may also have contributed to increased fruit susceptibility to IBD due to possible changes in gas diffusivities within the fruit and changes in membranes fluidity.

During storage under low O_2 levels combined with high CO_2 (Fig. 7B), the regulation of SOD and H_2O_2-scavenging enzymes was similar to the observed in the fruits stored under high CO_2 indicating that those fruits were also under oxidative stress and not able to detoxify H_2O_2. The ascorbate levels also rapidly decreased in these fruits, despite differences in the regulation of enzymes involved on ascorbate regeneration. In these *Pc*DHAR and *Pc*GR were down-regulated and the up-regulation of *Pc*MDHAR from day 60 up to the end of storage was not sufficient for ascorbate regeneration. The most significant difference between the fruits stored under the high CO_2 conditions is related to fermentation. In the fruits stored under very low O_2 levels there is an induction of fermentation showing that fruits switched from a predominantly aerobic respiration to an anaerobic respiration. This change on respiration may have prompted glycolysis and subsequently the expression of *Pc*PDC and *Pc*ADH with high accumulation of acetaldehyde and ethanol which are known to affect gas diffusivities within the fruit and membranes fluidity, hence also contributing for the highest IBD incidence observed in these fruits.

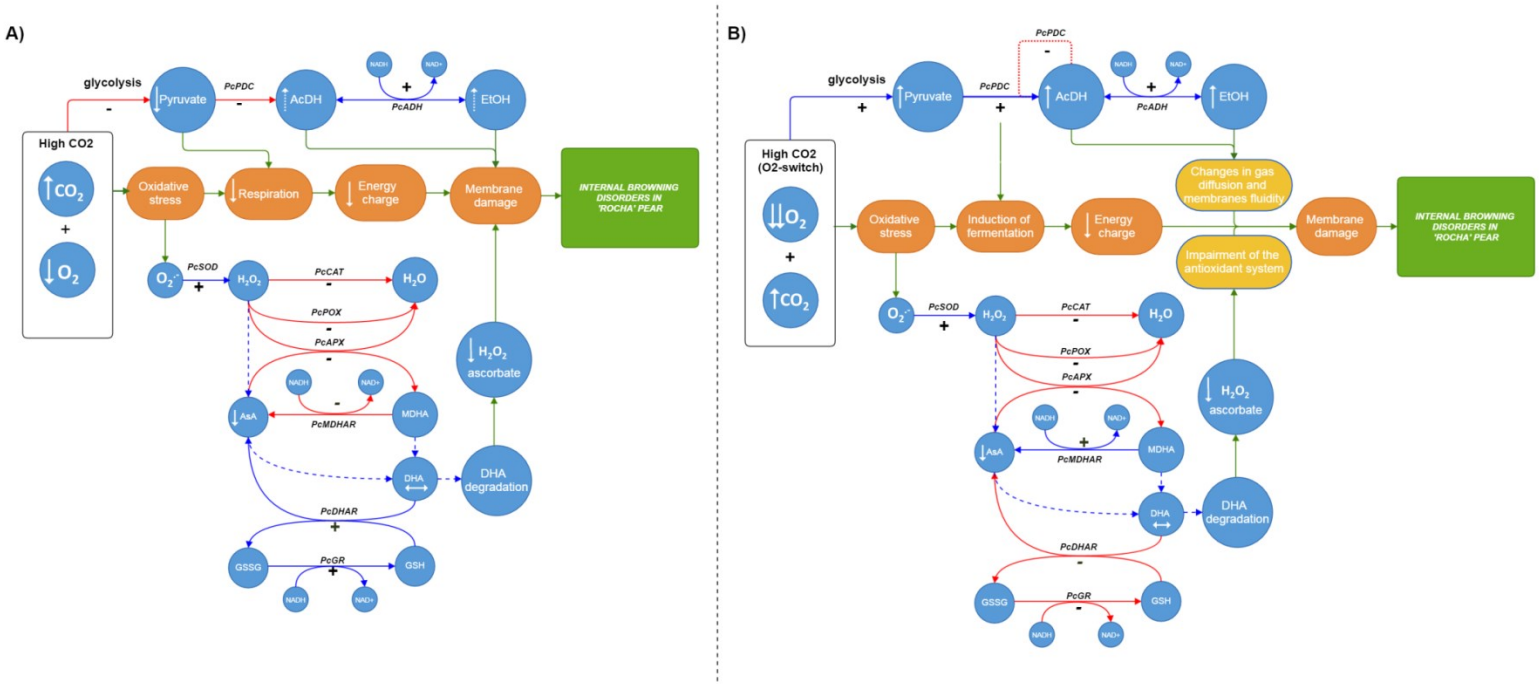


Figure 7: Schematic representation of the effects of high CO_2 (2 kPa O_2 + 10 kPa CO_2) (A) and high CO_2 (O_2-switch) (1 kPa O_2 + 10 kPa CO_2) (B) in 'Rocha' pears after 60 days until the end of the storage period. The transcriptional regulation of key enzymes believed to be involved in browning occurrence and development including the antioxidant enzymes [superoxide dismutase (SOD); catalase (CAT); ascorbate peroxidase (APX); peroxidase (POX); monodehydrooascorbate reductase (MDHAR); dehydroascorbate reductase (DHAR) and glutathione reductase (GR)] and fermentative enzymes [pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) are represented in the scheme. The results obtained for the total ascorbic acid, hydrogen peroxide (H2O2), ethanol (EtOH) and acetaldehyde (AcDH) during storage are also shown. The discontinuous lines represent spontaneous reactions. +, up-regulated; -, down-regulated; = unregulated; ↑, high accumulation; ↟slight accumulation; ↓ depletion; ↔ , maintenance. The blue arrows represents up-regulation and red arrows down-regulation.

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CHAPTER 6.

Biochemical and Mineral Markers to Predict Internal Browning Disorders in 'Rocha' Pear During Storage Under High CO_2

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6.1. ABSTRACT

This study aimed at identifying biochemical and mineral markers of internal browning disorders (IBD) in 'Rocha' pear in order to develop a model for IBD incidence. Fruits from five orchards harvested at two different maturity stages were stored for 45 days in cold air (-0.5 ºC) followed by 100 days under controlled atmosphere (CA) (1 kPa O_2 + 10 kPa CO_2 at -0.5 ºC). For predictive modelling of IBD incidence, the concentrations of ethanol (EtOH), acetaldehyde (AcDH), ascorbic acid (AA) and the activities of peroxidase (POX) and polyphenoloxidase (PPO) were analyzed at different time points. Mineral concentrations were also measured at harvest to identify additional markers of IBD incidence. The results evidenced AcDH, EtOH and AA as the most promising IBD markers, being thus used for modelling. The PLS model using EtOH and AcDH as predictors explained 89% of the variance in IBD incidence, whereas the univariate models based on EtOH and AcDH concentrations and accumulation rates explained between 89 to 94%. In contrast, the models based on AA levels and AA depletion rate showed lower predictive value, explaining 57% and 82% of the variance in IBD incidence, respectively. Models' validation confirmed the robustness of the model based on EtOH levels (R^2 = 0.91; RMSEP = 11.1) and allowed to propose a threshold level of 25 µL EtOH L^{-1} above which IBD may occur. Copper (Cu), among the ten analyzed minerals, was the most promising IBD marker. This work represents a major step forward in the prediction of IBD in 'Rocha' pear.

Keywords: acetaldehyde, ascorbic acid, ethanol, internal browning disorders, mineral concentrations, modelling.

6.2. INTRODUCTION

'Rocha' pear can be stored for up to ten months under controlled atmosphere (CA) (ANP, 1997). However, during long-term storage under CA, 'Rocha' pear, similarly to other pear cultivars, develops two major physiological disorders: internal browning and superficial scald (Silva et al., 2010). Internal browning disorders (IBD) affect the inner part of the fruit and in the most severe cases this damage could be extended up to the peel (Deuchande et al., 2012).

IBD is the result of the reaction of polyphenoloxidase (PPO) with its phenolic substrates, leading to the formation of *o*-quinones which spontaneously polymerize to form

melanins, the browning compounds (Vámos-Vigyázó, 1981). Since PPO and its substrates are naturally separated in different cellular compartments, enzymatic browning does not take place until membrane damage occurs, allowing these components to react (Yamaki, 1984). Still, despite this crucial role, PPO seems not to be a limiting factor for IBD development in some pear varieties such as 'Conference' (Veltman et al., 1999a).

Several studies have been carried out to determine the exact biochemical causes of IBD in pears and some hypothesis have been drawn, such as: (i) reduced energy charge, with consequent cellular incapacity to sustain the antioxidant system (Veltman et al., 2003); (ii) cellular inability to repair membrane damage caused by higher oxidation and degradation of ascorbate levels (Veltman et al., 1999b); (iii) accumulation of fermentative metabolites above tolerated levels (Deuchande et al., 2012); (iv) changes in membrane fluidity and gas diffusivity in the pulp tissue, with heterogeneous gas distribution within the fruit (Lammertyn et al., 2003). Associated with these hypotheses, several biochemical parameters have been correlated to IBD development. Amongst these, ascorbic acid, whose oxidation has been previously linked to increased IBD incidence (Veltman et al., 1999b; Zerbini et al., 2002), fermentative metabolites (EtOH and AcDH), reported to be highly correlated to IBD incidence in 'Rocha' pear (Deuchande et al., 2012) and the activities of PPO and peroxidase (POX) (Larrigaudière et al., 2004), are the most well established factors. Additionally, fruit mineral composition has also been shown to influence the incidence of IBD in apples and pears (Fallahi et al., 2010; Xuan et al., 2003, 2001), but their role on IBD development is less well described.

Despite this general knowledge, there are still very few studies aiming at developing predictive models for IBD incidence in pears. Generating such predictive models would allow an early selection of the most adequate storage/treatment strategies to prevent IBD during long term storage. For instance, Lammertyn et al. (2000) developed models for IBD incidence in 'Conference' pears based on intrinsic and external factors influencing disorder incidence, including sugar content, fruit firmness, size and weight, CO_2 and O_2 concentrations, harvest date and storage temperature, but the authors concluded that more parameters were required to improve their predictive performance. Veltman et al. (2000) and Zerbini et al. (2002) also developed models for IBD in 'Conference' pears as a function of AA levels and the authors proposed AA-threshold levels below which IBD occurrence was expected.

To date, the underlying factors determining the sensitivity of 'Rocha' pears to IBD remains to be clarified, and efforts should be done to identify markers and to develop

models that may allow predicting the fruit sensitivity to IBD during long term storage. To this end, the aims of this study were: (i) to develop, by means of multivariate and univariate analyses, different predictive models capable of estimating IBD incidence in 'Rocha' pear during long term storage under high CO_2; (ii) to validate the most promising predictive models using independent data-sets; (iii) to understand if mineral concentrations in pear pulp tissue at harvest can be used as novel targets for the development of IBD predictive models.

6.3. MATERIALS AND METHODS

6.3.1. Plant material and storage conditions

Two experiments were conducted in this study with 'Rocha' pears (*Pyrus communis* L. cv. 'Rocha'). Experiment 1 aimed to identify biochemical and mineral and markers of IBD and to define predictive models for IBD. The second experiment aimed at generating independent data-sets to validate the predictive models.

6.3.1.1. Experiment 1: Model development

Pears were harvested from five orchards located in Cadaval, Portugal. Fruits from two of these orchards (1 and 2) were harvested at the beginning of the commercial stage while the fruits from the other three orchards (3-5) were harvested at the end of this stage. After harvest, fruits were stored for 45 days in cold air (-0.5 ºC) followed by 100 days under CA (1 kPa O_2 + 10 kPa CO_2 at -0.5 ºC). Delayed CA has previously been shown to delay the occurrence and slightly retard the development of IBD in pears (Saquet et al., 2003; Verlinden et al., 2002). Therefore, this storage condition was selected to allow the capture of an adequate gradient of values for IBD incidence required to develop the predictive models.

6.3.1.2. Experiment 2: Model validation

Pears were harvested from two orchards (6 and 7), located in the same area as the ones from Exp. 1. Fruits from orchard 6, used to validate the predictive model for IBD based on fruit mineral composition, were harvested at the beginning of commercial stage and stored under the same conditions of Exp. 1. Fruits from orchard 7, used to validate the predictive models for IBD based on biochemical parameters were harvested at the end of the commercial stage and stored under two different conditions: (i) immediate CA (1 kPa

O_2 + 10 kPa CO_2 at -0.5 ºC) for 145 days; and (ii) 21 days in cold air (-0.5 ºC) followed by 104 days in CA.

6.3.2. Controlled atmosphere set up

Fruits were stored in experimental chambers of two tones capacity, fully loaded with 'Rocha' pears. To reach the 10 kPa CO_2, it was added from an external source until the defined level was achieved. The gas composition in the CA chambers was carefully controlled and checked with centralized analyzers, supervised by special Fruit Control Equipment software.

6.3.3. Sampling

Samples of pulp tissue (three replicates of three fruits each) and pear juices (three replicates of five fruits each) were prepared at harvest and during storage. For fruits from the Exp.1 samples were prepared after 0, 45, 60, 75, 95, 125 and 145 days of storage (orchards 1-5). For fruits from orchard 6 (Exp. 2) samples of pulp tissue were prepared only at harvest. For fruits from orchard 7 (Exp. 2), stored under immediate CA, samples were prepared after 0, 45, 75, 95, 125 and 145 days of storage whereas for the stored under 21 days delayed CA, samples were prepared after 0, 45, 95 and 125 days of storage. At each removal time for the fruits of both experiments, the picked fruits were allowed to warm up for up to six hours at room temperature before sampling. The incidence and severity of IBDs were evaluated in 60 fruits at each removal time-point for the fruits of both experiments.

Pulp tissue samples were frozen in liquid nitrogen and kept at -80 ºC for the analysis of ascorbic acid, POX and PPO activities and mineral concentrations. Pear juice samples were prepared using a commercial blender, filtered through cellulose paper filter and stored at -25 °C for the analysis EtOH and AcDH.

6.3.4. Assessment of initial maturity

The assessment of initial maturity was carried out on 30 fruits immediately after harvest. Firmness was analysed on two opposite sides of the fruit, after peel removal and using a penetrometer (T.R.Turoni srl., Italy), fitted with an 8 mm Magness Taylor probe. Surface colour was measured on opposite sides of each fruit with a Minolta CR-400 colorimeter equipped with a D_{65} illuminant using the L* a* b* colour space. The hue angle was calculated with the formula arctg b*/a*. To determine the soluble solids content (SSC) and titratable acidity (TA), juice samples were homogenized and filtered through a

cellulose filter paper (three replicates of five fruits each) measurements were conducted in triplicate. SSC was measured with an Atago PR-100 palette refractometer (Atago, Japan). TA was measured by titration with 0.1 N NaOH to pH 8.1. The starch index was assessed by iodine staining according to Avelar and Rodrigues (1999) using a reference chart scored from 1 (maximum starch concentration) to 10 (no starch).

6.3.5. Estimation of internal browning disorders

For the visual evaluation of internal browning, fruits were cut longitudinally. The incidence was reported as the percentage of damaged fruits in the total number of observed fruits. The severity of the symptoms was classified considering the extent of the damage according to Veltman et al. (1999b).

6.3.6. Mineral determination

Mineral concentrations were assessed in pear pulp tissue. Dried pulp powder (200 mg) was mixed with 5 mL of 65% HNO_3 in a Teflon reaction vessel and digested in a microwave system (Speedwave MWS-3+, Berghof, Eningen, Germany). Digestion was conducted in five steps: 130 °C for 10 min; 160 °C for 15 min; 170 °C for 12 min; 100 °C for 7 min and 100 °C for 3 min. The resulting solutions were filtered and brought up to 20 mL with ultrapure water for analysis. Mineral concentration was analysed by inductively coupled plasma argon spectrometry (ICP; ICP-OES Optima 7000 DV, PerkinElmer, Waltham, Massachusetts, USA). Three biological replicates of three fruits each were analysed in triplicate. Mineral concentrations were expressed in mg kg^{-1} dry weight.

6.3.7. Analysis of ascorbic and dehydroascorbic acid

Ascorbic acid (AsA) and dehydroascorbic acid (DHA) were determined as described by Zapata and Dufour (1992) with modifications. Pulp tissue (15 g) was homogenised in 15 mL of 50 mL L^{-1} methanol plus HCl (10 g L^{-1}), using an Ultra-Turrax (IKA Labortechnik model T 25, Staufen, Germany). The homogenate was centrifuged at 4000 *g* (Universal 320R, Hettich, Germany) for 15 min at 4 ºC and the supernatant was filtered through a fluted cellulose paper filter. To 3 mL of supernatant, 1 mL of 0.6 g L^{-1} 1,2-phenylenediamine dihydrochloride (OPDA, from Sigma-Aldrich) solution was added and the resulting mixture was incubated for 30–40 min at 4 ºC in the darkness. After the incubation period the mixture was filtered through a 0.22 μm filter and analysed with an HPLC system equipped with a UV-Vis detector (UV-1575), an auto sampler (AS-1555) and a pump (PU-1580), all controlled by appropriate software (Borwin v. 1.5, from Jasco

Corporation, Tokyo, Japan). The separation of AsA and DHA was achieved in a Spherisorb ODS2 5 μm (250 x 4.6 mm da Waters Corporation, USA) column. The mobile phase was 50 mL L^{-1} methanol containing 5 mmol L^{-1}cetrimide and 50 mmol L^{-1} potassium dihydrogen phosphate. The flow rate was 1.8 mL min^{-1} and the injection volume was 20 μL. The detector was set at 348 nm for DHA and 261 nm for AsA detection. AsA and DHA contents were expressed in mg kg^{-1} fresh weight (FW). Three biological replicates of three fruits each were analysed in triplicate.

6.3.8. Analysis of and fermentative metabolites

Determination of fermentative metabolites, ethanol and acetaldehyde, was performed according to Ke et al. (1994) with slight modifications. From each juice sample, 10 mL of juice was placed in a 20 mL test tube with a screw cap with a septum and incubated in a water bath at 65 °C for 1 hour, after which 1 mL of headspace sample was taken with a 1 mL glass syringe for determining acetaldehyde and ethanol concentrations. For this purpose a gas chromatograph (HP 5890 II, Hewlett Packard) equipped with a column CP-WAX 57 CB chromepack (0.25 mm x 50 m x 0.2 μm; Agilent Technologies Inc., USA) and a flame ionization detector were used. The operating conditions were as follows: oven temperature 90 ºC, injector temperature 250 ºC, detector temperature 220 °C. The concentrations of ethanol and acetaldehyde were calculated using a standard curve generated by injecting standard solutions of known concentrations. Ethanol and acetaldehyde concentrations were expressed in μl L^{-1}. Three biological replicates of five fruits each were analysed in triplicate.

6.3.9. Enzymes extraction

For the extraction of POX, samples of pulp tissue (10 g) previously stored at -80 ºC were ground using a pre-chilled mortar and pestle with 15 mL of an ice-cold medium containing 100 mM potassium phosphate buffer pH 7.3, 1 mM EDTA, 8% (v/v) glycerol 87% (v/v), 1 mM phenylmethanesulfonyl fluoride (PMSF), 5 mM AA and 2% (w/v) polyvinylpolypyrolidone (PVPP). The homogenate was centrifuged at 10,000 x g for 30 min at 4 ºC and the resulting supernatant was used to measure enzymatic activity.

PPO was extracted as described by Galeazzi and Sgarbieri (1981). Samples of pulp tissue (10 g) were homogenised in 10 mL of 200 mM sodium phosphate buffer pH 6.5, 0.25% (v/v) Triton X-100 and 2% (w/v) of PVPP, using an Ultra-Turrax for 2 min in an ice bath. The homogenate was centrifuged at 4,000 ×*g* for 20 min at 4 °C. The supernatant was filtered through two layers of miracloth and used as enzyme extract.

6.3.10. Enzymatic activities

POX activity was determined by the method of Rao et al. (1996) with some modifications. The reaction mixture consisted of 100 mM phosphate buffer pH 6.8, 10 mM guaiacol and 10 mM H_2O_2. Peroxidase activity was measured by monitoring the increase in absorbance resulting from guaiacol oxidation in the presence of H_2O_2 for 10 min, at 470 nm. PPO activity was measured according to Galeazzi and Sgarbieri (1981). The reaction buffer consisted of 200 mM catechol and 200 mM of sodium phosphate buffer pH 6.5. The PPO activity was measured at 420 nm for 3 minutes.

Enzymatic activity was expressed as the change in optical density mg-1 protein min-1. Total protein in the enzyme extracts was determined using the Pierce Coomassie Plus Protein Assay kit, based on the method described by Bradford (1976), and following the manufacturer instructions. Bovine serum albumin (BSA) was used as a standard.

Three biological replicates of three fruits were analysed in triplicate.

6.3.11. Statistical analysis

Principal component analysis (PCA) was performed to establish the relationships among the different variables. The data set included nine variables of which eight were continuous (concentrations of EtOH, AcDH and AA, PPO and POX activities, IBD incidence and severity and storage time) and one was categorical (initial fruit maturity). The categorical variable was transformed in a continuous variable by attributing a numeric value to each maturity stage previously identified based on the quality parameters [early harvested fruits (orchards 1 and 2) were attributed the value 1 and late harvested fruits (orchards 3, 4 and 5) were attributed the value 2 (see section 3.1)]. Each maturity stage was separated by one unit. The resulting matrix contained 35 averaged values corresponding to 105 samples (three biological replicates of three fruits each per orchard and time-point). The averaged data was mean centred and weighted with the inverse of the standard deviation before applying the PCA to allow all variables to have the same chance to influence the model. This analysis was performed using the IBM SPSS Statistics, version 21.0. (Armonk, NY).

Partial least square regression analysis (PLSR) was used to identify biochemical markers of IBD and to quantify the correlation found in the PCA analysis using Tanagra software, version 1.4.50 (Ricco Rakotomalala, Lyon, 2003; http://chi-rouble.univ-lyon2.fr/~ricco/tanagra). The PLSR was first performed including five continuous *X*-variables (concentrations of EtOH, AcDH and AA and PPO and POX activities delayed

CA storage) and then re-run repeatedly using the stepwise method to exclude non-significant *X*-variables, until the best explaining significant predictors remain, making the model more robust. The Variable Importance in the Projection (VIP) was used to evaluate the influence of the variables in the model and when this value was below 0.8 the variable was excluded from the model. The PLSR containing *X*-variables (mineral concentrations) and *Y*-variables (IBD incidence after 95, 125 and 145 days of storage) was performed to identify mineral markers of IBD incidence, using the same software. For this model, a different approach was used: mineral data included the average of the analytical replicates measured in each biological replicate (three biological replicates per orchard). Therefore, the resulting matrix contained 15 averaged measurements corresponding to 15 samples (three samples per orchard).

The curve fitting of the sigmoidal models was performed using the GraphPad Prism version 6.0 (San Diego, CA, USA). The determination coefficient (adjusted R^2) was used to determine the accuracy of the fitted model. In order to have a reliable validation an external data-set obtained from experiment 2 was used to calculate the root mean square error of prediction (RMSEP) and evaluate the robustness of the models. The lower the RMSEP value, the higher the accuracy of the model, as given by the following equation:

$$RMSEP = \sqrt{\frac{1}{N}\left(\sum_{i=1}^{n}(y_m - y_p)^2\right)} \quad (Eq.\,1)$$

where y_m is the observed value, y_p is the corresponding predicted value and N is the number of observed data.

The analysis of variance (ANOVA) and the evaluation of orchards effect on fruit mineral composition was performed using General Linear Model of IBM SPSS Statistics, version 21.0. (Armonk, NY). Mean comparisons were done by calculating the Fisher Least Significant Difference (LSD) at P=0.05.

6.4. RESULTS AND DISCUSSION

6.4.1. Postharvest characterization of the orchards

'Rocha' pear is usually harvested when the fruit firmness is between 55 and 65 N, SSC is between 11 and 13%, TA reaches 2-3 g L^{-1} malic acid and the average starch index is 6.5 (Avelar and Rodrigues, 1999). According to these values, fruits from orchards 1, 2 and 6 were harvested at an early maturity stage with an average firmness of 66 N; whereas

fruits from orchards 3, 4, 5 and 7 were harvested at a late maturity stage with an average firmness of 55 N (Table 1). However, when considering the other quality parameters, particularly the ratio between the SSC and TA, the fruits from orchard 7 were harvested in a more mature stage if compared to fruits from orchards 3, 4 and 5 (Table 1). In various pear cultivars initial fruit maturity has been shown to influence fruit susceptibility to IBD. For instance, late harvested 'Rocha' pear was reported to be more susceptible to develop IBD during storage under CA, compared to early and optimally harvested (Deuchande et al., 2012). Therefore, the variability in the fruit maturity at harvest may be important for the development of robust IBD models and ensure a broader applicability.

Table 1: Initial maturity indexes of fruits from different orchards. Values of firmness, weight and colour (lightness, chroma and hue angle) represent the mean of 30 fruits ±SE and the values of titratable acidity (TA) and soluble solids content (SSC) represent the mean of 3 replicates of 5 fruits each ± SE.

Orchard	Firmness (N)	Weight (g)	Starch index	TA (g malic acid L^{-1})	SSC (%)	$\frac{SSC}{TA}$	Lightness (L*)	Chroma (C*)	Hue angle (h^o)
1	66.2±0.7	118±3	5.5±0.2	1.94±0.02	13.4±0.1	6.9	66.6±0.4	45.9±0.3	108.3±0.4
2	66.1±0.7	138±5	5.8±0.1	2.26±0.02	13.9±0.1	6.2	67.8±0.4	46.4±0.4	109.7±0.3
3	53.7±0.7	156±5	6.5±0.2	1.57±0.01	13.1±0.1	8.3	71.2±0.5	45.4±0.3	104.2±0.6
4	55.7±0.6	157±8	8.1±0.2	1.62±0.02	12.3±0.1	7.6	69.3±0.5	46.1±0.3	108.3±0.3
5	56.8±0.7	142±5	7.1±0.3	1.66±0.00	13.6±0.1	8.2	65.9±0.5	46.7±0.3	107.0±0.5
6	67.2±0.9	130±3	5.1±0.3	2.36±0.03	15.1±0.1	6.4	69.6±0.5	47.5±0.4	107.0±0.5
7	53.8±0.6	165±5	8.0±0.3	1.31±0.02	12.9±0.1	9.8	68.7±0.5	46.5±0.3	107.2±0.5

6.4.2. PCA model including all biochemical data

An initial PCA model was performed to extract the most important information from the data-set, reducing the number of variables to PC1 and PC2. The resulting PCs explained 86% of the total variance in the data with PC1 explaining 55% and PC2 31% of the variance (Fig. 1).

The loading plot of PC1 vs PC2 showed a high correlation between the levels of fermentative metabolites and IBD incidence (Fig. 1) indicating that fermentation may be the main process involved on IBD development. In contrast, the levels of AA correlated negatively with browning incidence and severity and were clearly associated to healthy

fruits. This negative correlation has been extensively reported in the literature (Deuchande et al., 2016a; Franck et al., 2003; Larrigaudière et al., 2001; Veltman et al., 2000), but thus far it was not possible to establish a direct link between the levels of AA and the development of IBD. Veltman et al. (2000) suggested that other factors, mainly related to fruit maturity at harvest may be involved in IBD development. Although there are some studies reporting an influence of fruit maturity at harvest with IBD susceptibility (Deuchande et al., 2012), in this study, initial fruit maturity was not directly correlated to fruit sensitivity to IBD. Nevertheless, the model (Fig. 1) shows a significant negative correlation of AA concentration, with PPO activity and initial fruit maturity. This suggests that fruits harvested at an earlier maturity stage, due to their higher AA concentration, may have reduced PPO activity and thus delayed browning initiation. It is known that *o*-quinones could be reduced back to *o*-diphenols through the action of AA and that PPO, being a Cu-containing enzyme, may have its activity inhibited by AA, a Cu chelating agent (Macheix et al., 1990).

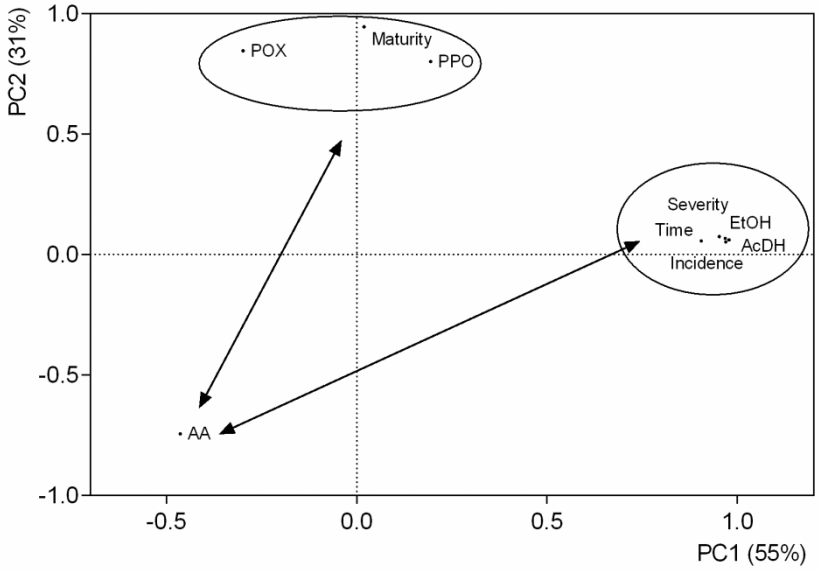

Figure 1: Loading plot of PC1 vs PC2 from PCA model containing all variables (storage time; fruit maturity at harvest; concentrations of ethanol (EtOH), acetaldehyde (AcDH) and total ascorbic acid (AA); polyphenoloxidase (PPO) and peroxidase (POX) activities; internal browning incidence and severity, during storage under 45 days delayed CA. The model explains 86 % of the total variance in the data.

6.4.3. PLS model with biochemical variables to predict IBD incidence

6.4.3.1. Model development

The PLS regression model developed for predicting IBD incidence, containing five *X*-variables (concentrations of EtOH, AcDH, AA and PPO and POX activities) and one *Y*-variable (IBD incidence) during delayed CA storage (Fig. 2A), was in accordance with the results of the PCA (Fig. 1). This model also showed that the principal factors influencing IBD incidence were the levels of EtOH, AcDH and AA whereas the activity of PPO and POX only partially accounted for the disorder incidence (Fig. 2A).

The PLS model (Fig. 2A) including all variables explained 87% of the total variance in disorder incidence and showed that only three of the five variables were significant (AA, EtOH and AcDH concentrations) (Table 1S). Furthermore, only two of them, EtOH and AcDH concentrations, had a VIP value higher than 0.8 (Table 1S). Therefore, in order to simplify the model for predicting IBD incidence during storage, another model was developed using the variables which proved to have a closer relationship with IBD incidence (concentration of both fermentative metabolites) (Fig. 2B). The resulting model explained 90% of the total variance in disorder incidence (Fig. 2B; Table 1S) and was defined by the following equation:

$$y = -3.894 + 2.480 * [AcDH] + 0.786 * [EtOH] \quad (Eq. 2)$$

6.4.3.2. Validation of the model

Fig. 4 shows the relationship between predicted and measured IBD incidence using the validation samples of Exp. 2. This validation plot allows evaluating the performance of the model when applied to an independent data-set (i.e. fruits grown and stored in different conditions as compared to the ones used for model development). The high determination coefficient (R^2 = 0.8914), the slope close to 1, the low intercept value and the reasonable root mean square error of the prediction (RMSEP = 14.28) show the high predictive value of this IBD model (Fig. 2C). The PLS model clearly showed that fermentative metabolites are the most important factors involved on IBD incidence in 'Rocha' pear and are the most adequate parameters to develop a robust predictive model.

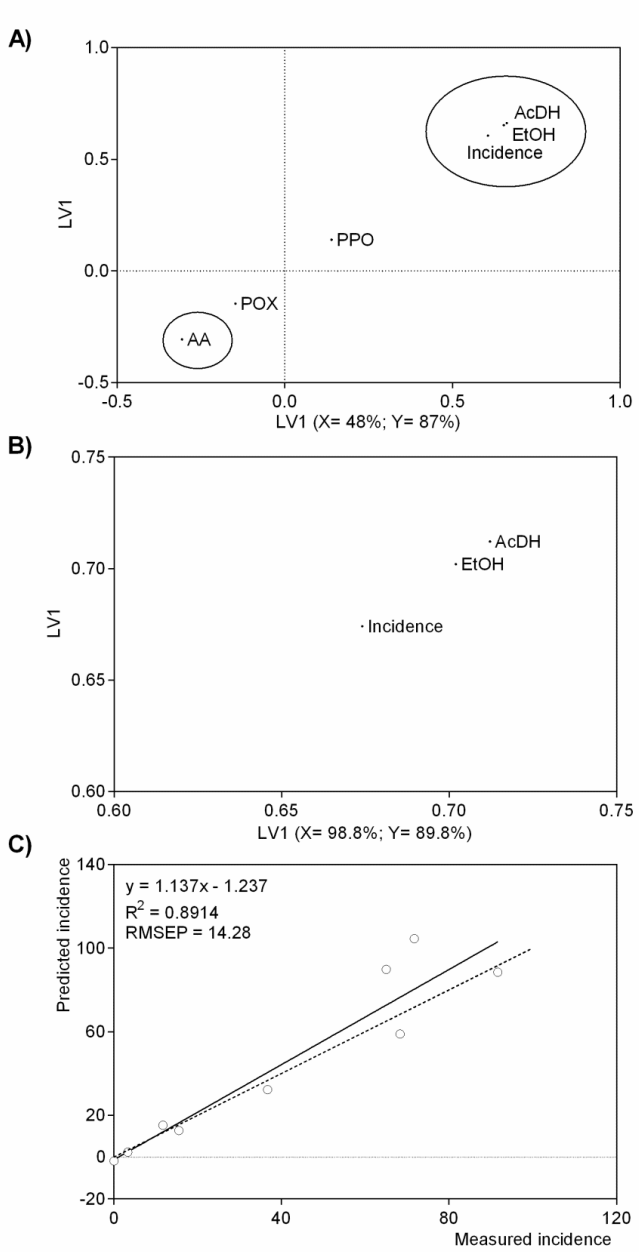

Figure 2: (A) PLS loading plot from model containing all *X*-variables (concentrations of ethanol (EtOH), acetaldehyde (AcDH) and total ascorbic acid (AA); polyphenoloxidase (PPO) and peroxidase (POX) activities) and *Y*-variable (internal browning incidence) obtained from fruits of five orchards (orchard 1-5) during storage under 45 days delayed CA; (B) PLS loading plot from model containing the selected *X*-variables; and (C) validation plot of the model containing EtOH and AcDH as predictors fitted using the validation samples obtained from fruits of orchard 7 during storage under immediate CA and 21 days delayed CA. Each data point in Fig. C corresponds to the average of three replicates of three fruits for each orchard at each time-point. The solid line represents the linear regression and the dotted line represents the 1:1 relationship. The linear regression equation, determination coefficient and root mean square error of prediction (RMSEP) are given.

6.4.4. Ethanol, acetaldehyde and ascorbic acid as independent IBD predictors

Considering the proved role of the fermentative metabolites and AA on IBD we developed models for predicting IBD incidence as a function of the concentration of those metabolites during fruit storage and a as function of the accumulation rates of fermentative metabolites and depletion rate of AA. The model for predicting IBD incidence as a function of the metabolites' concentration (Fig. 3A, C, E), would allow to assess the risk of IBD development at any time point during storage, thus helping in the decision support system concerning the selling priority of the different fruit batches and the transferability of the fruits to appropriate storage conditions. The models based on the kinetics of accumulation of EtOH and AcDH and depletion of AA during storage (δ[EtOH]/δtime or δ[AcDH]/δtime or δ[AA]/δtime) would have the same function but would require the measurement of the parameters at least at two time-points during storage (Figs. 3B, D, F). For the construction of these models the data of each of the five orchards was fitted using a standard four parameter sigmoidal curve (Fig. 3) described by the following equation:

$$Browning\ incidence = \frac{\alpha_i}{1 + \left(\frac{\gamma_i}{[EtOH\ or\ AcDH\ or\ AA] or \frac{\delta[EtOH\ or\ AcDH\ or\ AA]}{\delta time}} \right)^{\beta_i}} \quad (Eq. 3)$$

where α_i is an estimated maximum value of browning incidence depending on orchard, β_i is the slope and γ_i corresponds to the inflection point of the curve. For both models the data including the combined results of all orchards was also fitted using the Eq. 2 (Fig. 3, bold solid line). The estimated parameters and respective standard errors, as well as the root mean square error of prediction (RMSEP) of each model, calculated using the validation samples are given in Table 2S.

6.4.4.1. Ethanol models

The model based on the EtOH levels (Fig. 3A) had a percentage variance accounted for (adjusted R^2) of 91% whereas the model based on EtOH accumulation rate (Fig. 3B) had a percentage of 89% (Table 2S). An IBD percentage below 4% has been considered acceptable when proposing metabolite thresholds for IBD prevention (Giné Bordonaba et al., 2013; Veltman et al., 1999b). Here, for both models, it was possible to propose threshold values above which IBD occurs, assuming a more conservative incidence value of 2% (Fig. 3A, B*)*. The proposed thresholds would allow the assessment of the risk of IBD development to an extent capable of compromising the marketability of the stored fruit batches. Regarding the model based on EtOH concentration, IBD occurred only when

the levels of EtOH rose above 25 μL L^{-1} (Fig. 3A). For instance, IBD incidence occurred to an extent of up to 27% when there was a two-fold increase on the ethanol levels above the established threshold (Fig. 3A). For the model based on the EtOH accumulation rate (Fig. 3B), fruits with accumulation rates higher than 0.34 μL L^{-1} day^{-1} showed symptoms of IBD towards the end of storage whereas less than 2% incidence was detected in fruits with values below this threshold. For this model a 60% EtOH increase above the proposed threshold level would be needed to observe an increase of up to 28% in IBD incidence (Fig. 3B).

The threshold values proposed for both models were consistent and could be used to define corrective measures to avoid IBD during storage under the conditions of this experiment.

6.4.4.2. Acetaldehyde models

The model based on AcDH levels, had a higher determination coefficient (R^2 = 0.9279) compared to the one based on EtOH concentration (R^2 = 0.9110; Fig. 3A, C and Table 2S) but it was not sensitive enough to predict IBD in its first stages of development (Fig. 3C). As observed in Fig. 3C, fruits with similar levels of AcDH were either healthy or moderately affected by IBD (up to 30% damaged fruits). Therefore, this model did not allow establishing a secure threshold value for AcDH concentration (Fig. 3C). Instead, a proposed threshold of 3.7 μL L^{-1} above which IBD occurs could be suggested (Fig. 3C).

Even though the measured levels of AcDH in the fruit tissues were very low compared to the EtOH levels, small increases in the levels of AcDH seemed to be sufficient to cause increased browning incidence (Fig. 3C). This may be explained by the reported higher toxicity of AcDH compared to EtOH (Chervin et al., 1996).

The model based on AcDH accumulation rate (Fig. 3D), despite its high determination coefficient (Adjusted R^2= 0.9489), did not allow establishing a threshold value above which IBD would occur. IBD incidence up to 28% occurred when AcDH accumulation rate ranged between 0.04-0.09 μL L^{-1} day^{-1} and the fitted model was only able to estimate IBD incidence when this value was above 0.08 μL L^{-1} day^{-1} (Fig. 3D).

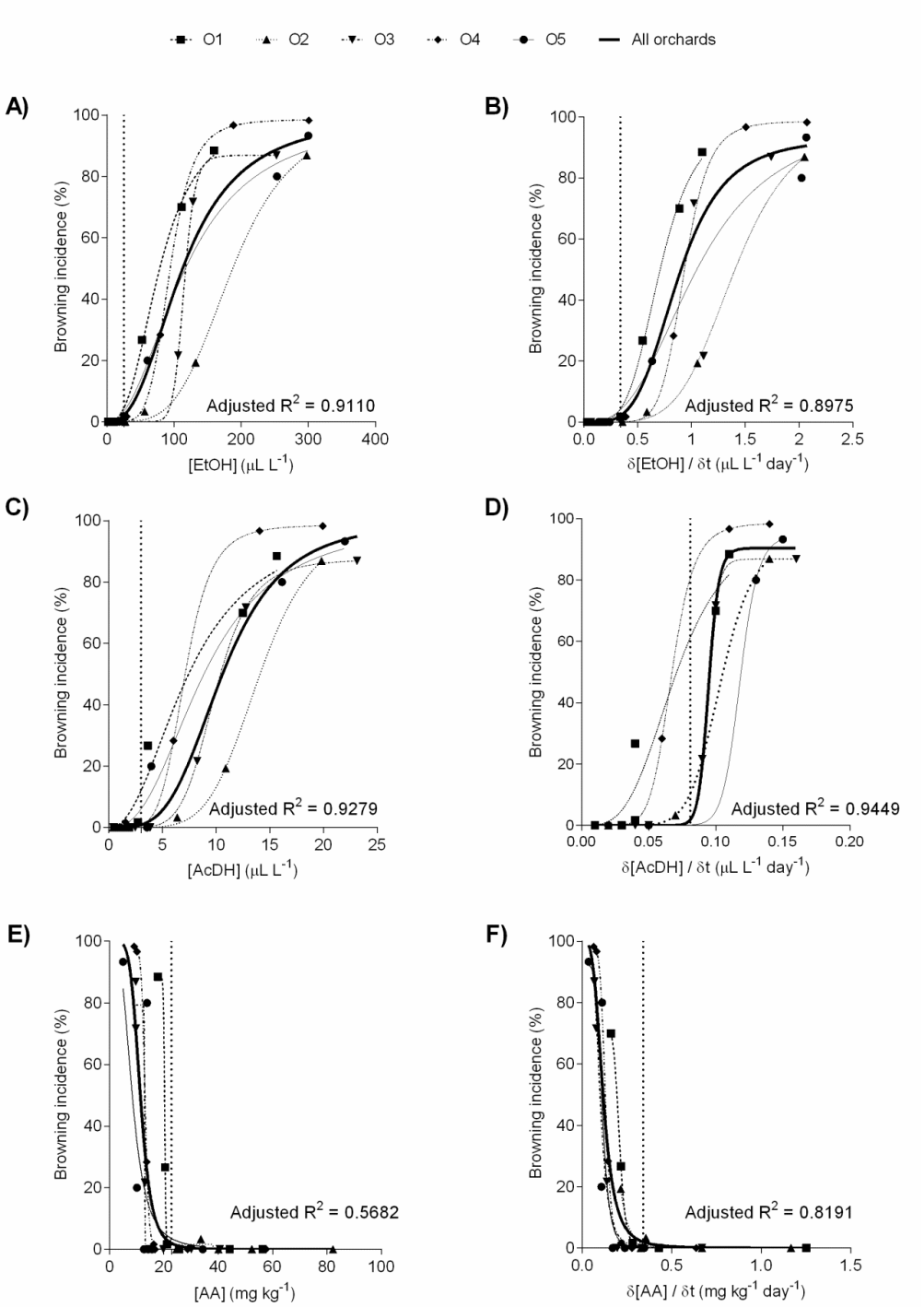

Figure 3: Models for predicting browning incidence during storage as a function of ethanol concentration ([EtOH]) (A), ethanol accumulation rate (δ[EtOH]/δt) (B), acetaldehyde concentration ([AcDH]) (C) and acetaldehyde accumulation rate (δ[AcDH]/δt) (D), ascorbic acid concentration ([AA]) (E) and ascorbic acid depletion rate (δ[AA]/δt) (F), in fruits from five orchards (orchard 1-5) during storage under delayed CA. Each data-point represents the average of three replicates of three fruits for each orchard at each time-point. The data was fitted for each orchard and for the combined data (all orchards) using a four parameter sigmoidal curve. The dotted vertical lines represent the metabolites level corresponding to 2% IBD incidence and for

some models (A, B, C, E), proposed threshold values above or below which internal browning is expected to occur.

6.4.4.3. Ascorbic acid models

The correlation between AA concentration and the incidence of IBD during storage under CA has been extensively described (Franck et al., 2003; Pedreschi et al., 2009; Veltman et al., 2000, 1999b; Zerbini et al., 2002). In our first assessment through the PCA and PLS analysis, we found a significant negative correlation between the concentration of AA and the incidence and severity of IBD. However, these correlations were not as high as the ones found for the fermentative metabolites.

Contrarily to fermentative metabolites the timepoint at which the levels of AA drop below a certain level appeared to be the most important parameter for IBD prediction (Fig. 3E, F). This is in accordance with previous studies in 'Conference' pears (Zerbini et al., 2002).

The model based on AA levels (Fig. 3E) did not allow establishing a secure threshold level below which IBD occurs. The model fit was not adequate, indicating that this biomarker was not suitable for IBD prediction (Fig. 3E). In contrast, the model based on the AA depletion rate allowed establishing a threshold level of 0.34 mg kg^{-1} day $^{-1}$, below which IBD may develop during storage under high CO_2 (Fig. 3F).

6.4.4.4. Validation of ethanol and acetaldehyde based models

The validation of these models (Fig. 4) showed that the model based on EtOH levels was the most accurate to predict IBD incidence with an RMSEP far lower than the observed for the other models. Therefore, the proposed threshold level defined by the model was effectively validated (Fig. 4A). In contrast, the validation plot of the model based on EtOH accumulation rate (Fig.4B) showed the lowest R^2 and the highest intercept and RMSEP values, being the less accurate and overestimating the disorder incidence.

The model developed based on AcDH levels also allowed to establish a threshold level but this model was less accurate compared to the model based on EtOH concentration (Fig. 4A, C).

The model developed using the AcDH accumulation rate (Fig. 3D) had the highest variance accounted for (adjusted R^2 = 94%, Fig. 3D, Table 2S) but after validation, this model revealed low accuracy (RMSEP = 18.6) and a pronounced tendency to highly underestimate IBD incidence (intercept = -15.45) at the beginning of storage (Fig. 4D).

These results indicated that this model was not sensitive enough to predict IBD incidence during storage.

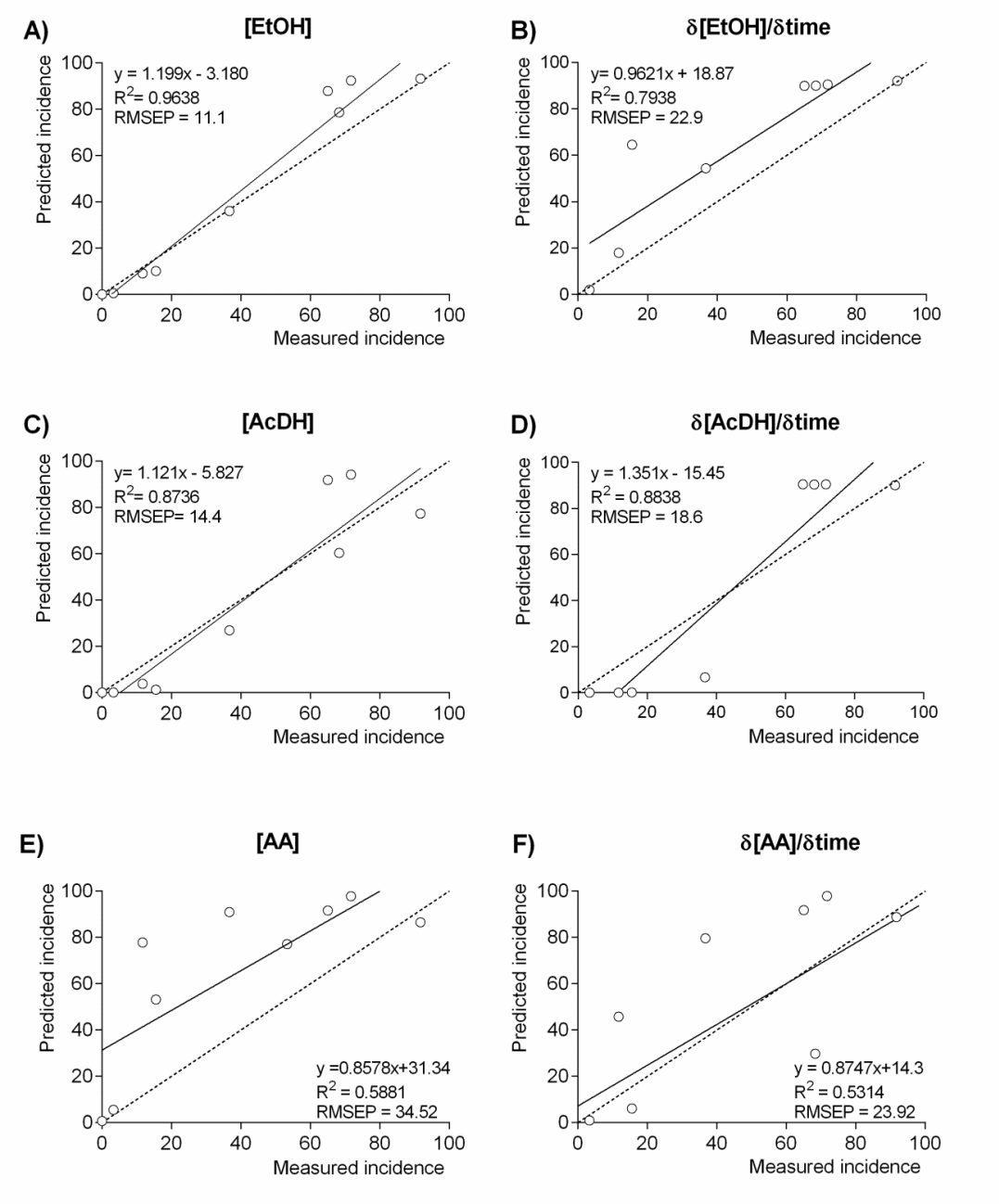

Figure 4: Plots of predicted vs measured incidence of internal browning disorders based on ethanol concentration ([EtOH]) (A), ethanol accumulation rate (δ[EtOH]/δt) (B), acetaldehyde concentration ([AcDH]) (C) and acetaldehyde accumulation rate (δ[AcDH]/δt) (D), ascorbic acid concentration ([AA]) (E) and ascorbic acid depletion rate (δ[AA]/δt) (F) during storage under 45 days delayed CA. The validation plots were fitted using the validation samples obtained from orchard 7 during storage under immediate CA and 21 days delayed CA. Each data-point represents the average of three replicates of three fruits. The solid lines represent the linear regression and the

dotted lines represent the 1:1 relationship. The linear regression equation, determination coefficient and root mean square error of prediction (RMSEP) are given.

6.4.4.5. Validation of AA based models

The validation plot for the model based on AA levels confirmed that this model is not accurate in predicting IBD (RMSEP = 34.52) and overestimates the levels of IBD incidence (intercept = 31.34) (Fig. 4E).

The validation plot for the model based on AA depletion rate showed that this model was more accurate than the one based on AA levels but also tended to overestimate IBD, albeit to a lesser extent (Fig. 4E, F). Since the measured IBD incidence for the validation samples only occurred when AA depletion rate increased above the proposed threshold value, this model should be considered valid for the fruits harvested within the maturity ranges used in this study.

6.4.5. Minerals as novel markers for IBD prediction and future model development

6.4.5.1. PLS model based on fruit mineral concentrations

The mineral composition differed significantly among orchards in relation to copper (Cu), manganese (Mn), iron (Fe), boron (B), sodium (Na) and potassium (K) (Table 3S). Since IBD incidence also varied among orchards (Fig. 1S), these minerals were thus considered as promising IBD predictors.

For the development of this PLS model the variables were considered independently of significance and VIP values because mineral concentrations may be interdependent and even if a certain mineral has not a direct effect on IBD incidence it may affect the concentration of other minerals and indirectly the disorder incidence (Marschner, 2012).

The PLS loading plot of fruit mineral concentrations at harvest and IBD incidence during storage (Fig. 5A) showed significant relationships between the concentration of some minerals and fruits sensitivity to IBD (Table 4S). For instance, this is the case for Cu concentration at harvest that was significantly correlated to increased IBD incidence during the entire storage period (Fig. 5A, Table 4S). This is not surprising given coppers' role on: i) the lignification of xylem vessels (Broadley et al., 2012); ii) as a constituent of ethylene receptors and co-factor of several antioxidant and oxidant enzymes such as superoxide dismutase and PPO (Macheix et al., 1990); and iii) in the oxidation of AA which is Cu-chelating agent. The strong correlation between Cu at harvest and IBD incidence during

storage suggests that this micronutrient may be a potential marker of IBD in 'Rocha' pear, whose potential should be further studied.

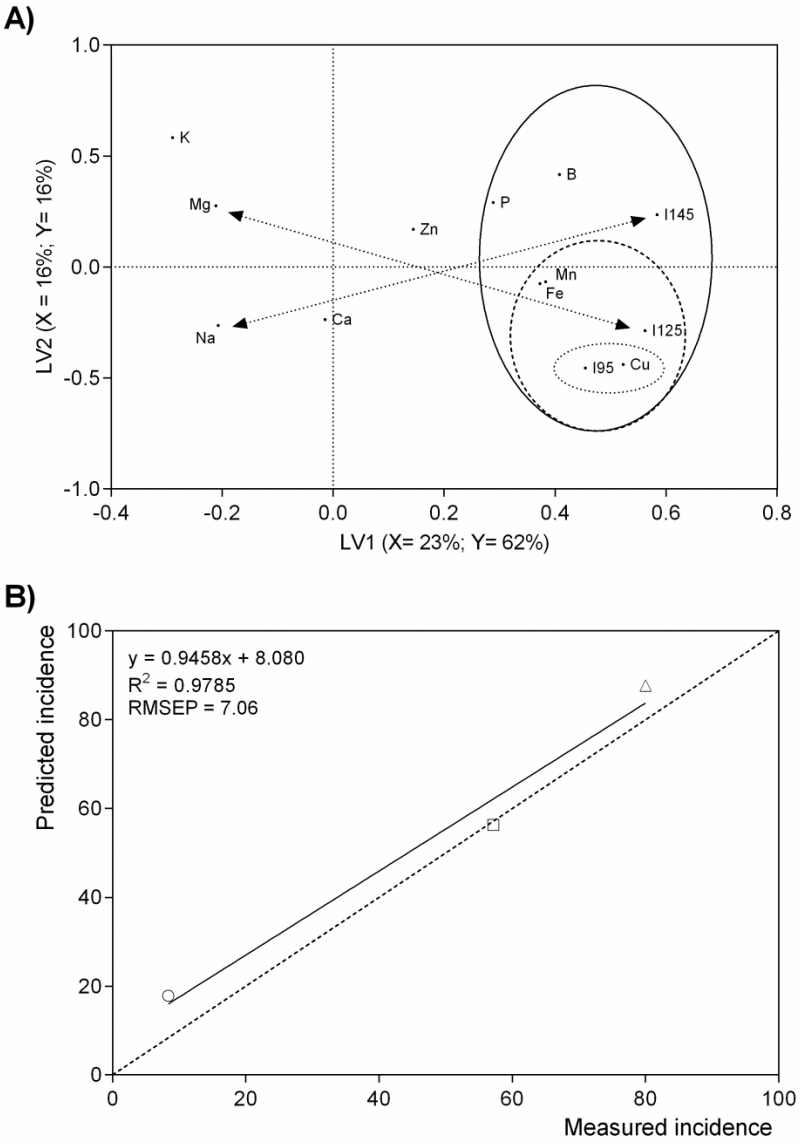

Figure 5: PLS loading plot from model containing *X*-variables (mineral concentrations) and *Y*-variables (internal browning incidence after 95 (I95), 125 (I125) and 145 (I145) days of storage) (A); and plot of predicted vs measured incidence of internal browning disorders after 95 days (○), 125 days (□) and 145 days (△) of storage, using samples from orchard 6 (B). Each data-point represents the average of three predicted values for incidence obtained from the measurement of mineral concentration in each of the three biological replicates. The solid line represents the linear regression and the dotted line represents the 1:1 relationship.

Mn, Fe, B and P were also positively correlated to IBD (Fig. 5A). However, B and P were significantly correlated to IBD only at the end of storage whereas Fe and Mn were also significantly correlated after 125 days of storage (Table 4S).

The way by which these minerals could be involved in IBD development is very complex. However, it can be highlighted that high Mn concentrations are, in general associated with high PPO activity (Fecht-Christoffers et al., 2007) and thus likely with higher IBD incidence. Fe, on the other hand, can mediate the formation of reactive oxygen species such as the highly reactive hydroxyl radical which can cause protein denaturation and lipid peroxidation (Halliwell and Gutteridge, 1989) and in general oxidative damage associated to IBD. Therefore, under stress conditions, such as storage under high CO_2, the presence of high concentrations of Fe may favor membrane damage with consequent increased IBD incidence. B has been widely reported to reduce the incidence of IBD in pears and apples (Fallahi et al., 2010; Neuwald et al., 2014; Streif et al., 2001; Xuan et al., 2003, 2001). However, excessive amounts of B have also been reported to increase the incidence of flesh breakdown in apples and pears (Kupferman, 2002; Marlow and Loescher, 1984), which may explain the positive correlation observed in this work.

P is involved in the energy metabolism linked to the appearance of IBD (Veltman et al., 2003) and it is known to affect enzyme phosphorylation status as well as modulate the action of Ca as a secondary messenger (Hawkesford et al., 2012).

In this work, magnesium (Mg) and Na were also negatively correlated to disorder incidence after 125 and 145 days of storage, respectively (Fig. 5A). Na is an important electrolyte for osmotic regulation also contributing to the maintenance of cellular pH (Hawkesford et al., 2012). Adequate Na levels may prevent pH changes in fruit tissue and thus IBD incidence. Mg for its part interacts with Ca in the cells and at high concentration induces Ca deficiency. Low Ca concentration has been previously linked to increased incidence of disorders in apples and pears (Broadley et al., 2012; Xuan et al., 2001).

The correlation between Cu concentration at harvest and IBD incidence during storage is new and of interest but needs to be further studied for the establishment of a useful IBD prediction model for 'Rocha' pear.

3.5.2. Validation of PLS model based on fruit mineral concentrations

The high R^2 and low RMSEP of the validation plot (Fig. 5B) suggests that a multivariate model based on the initial concentration of minerals in fruit pulp tissue at harvest may be a useful tool to predict the sensitivity of the fruits to IBD during storage. Nonetheless, this type of modelling is not adapted to all storage conditions and further

studies performed in different years and using different storage conditions would be required to optimize the model.

6.5. CONCLUSION

The results of this study showed that fermentative metabolites and AA concentrations during storage clearly influenced IBD incidence but also that the concentrations of some minerals, especially Cu at harvest, may have a significant impact on IBD development in 'Rocha' pear. Overall, IBD prediction models for 'Rocha' pear based on the concentration or accumulation/depletion rates of fermentative metabolites, ascorbic acid and mineral concentrations have been developed and validated in this work. Among the biochemical parameters used for the modelling the results evidenced EtOH and AcDH as the most promising IBD markers. The PLS model using EtOH and AcDH as predictors explained 89% of the variance in IBD incidence, whereas the univariate models based on EtOH and AcDH concentrations and accumulation rates explained between 89 to 94%. In contrast, the models based on AA levels presented a low predictive value. Among the univariate models, the one based on EtOH levels showed the highest potential to predict IBD in 'Rocha' pear during storage explaining 91% of the variance in disorder incidence and presenting the highest accuracy after validation (RMSEP= 11.1). This model also allowed proposing a threshold level of 25 μL EtOH L^{-1} above which IBD may occur in 'Rocha' pear. Regarding minerals, Cu showed the highest potential to be used as an IBD marker. Nonetheless, further studies are required to assess the suitability of these models in fruits stored under standard CA conditions.

6.6. ACKNOWLEDGMENTS

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6.8. SUPPLEMENTARY MATERIAL

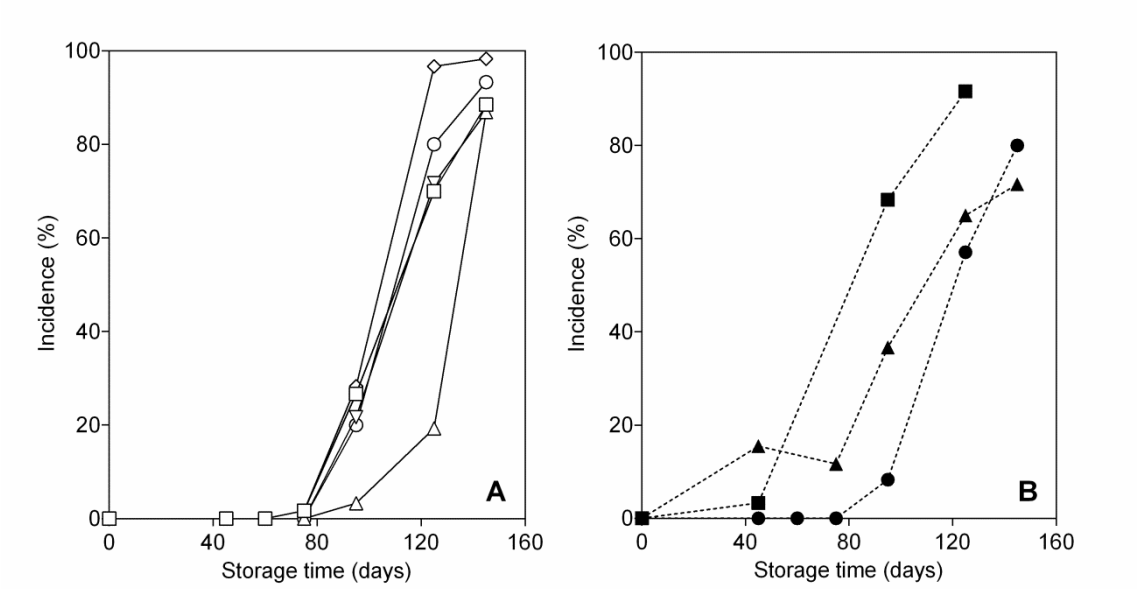

Figure 1S: Incidence of internal browning disorders in fruits of Exp. 1 (A) stored under 45 delayed CA including the data-sets for orchard 1 (□), 2 (△), 3 (▽), 4 (◇) and 5 (○); and Exp. 2 (B) including the data-sets for orchard 6 stored under 45 days delayed CA (●) and orchard 7 stored under immediate CA (▲) and 21 days delayed CA (■) The storage time presented in the figures includes the storage period under cold air prior to CA storage.

Table 1S: PLS model including *X*-variables [ascorbate, ethanol and acetaldehyde concentrations, and polyphenoloxidase (PPO) and peroxidase (POX) activities] and *Y*-variable (incidence of internal browning). The variable importance in the projection (VIP) and regression coefficients are presented for both models, the PLS model including all variables and the final model including the selected variables.

PLS model	***X*- variables**	**VIP**	**Standardized Regression coefficients**	***p-value***
All variables included [z]	Acetaldehyde	1.4810	0.4014	<0.001
	Ethanol	1.4599	0.3956	< 0.001
	Ascorbic acid	0.6851	- 0.1857	< 0.001
	POX	0.3294	-0.0893	> 0.05
	PPO	0.3121	-0.0846	> 0.05
Selected variables [w]	Ethanol	0.9928	0.4732	< 0.001
	Acetaldehyde	1.0072	0.4801	< 0.001

[z] X = 48.3%, Y= 86.8%; Latente variables (LVs) extracted: 1

[w] X = 98.8%, Y= 89.8%, Latente variables (LVs) extracted: 1

Table 2S: Estimates of the fitted parameters of Eq. (2) ± SE and adjusted R^2 resulting from the combined analysis of the data obtained from fruits of five different orchards at harvest and during storage. The root mean square error of prediction (RMSEP) calculated using the validation samples is given.

Variable used to develop the model	α_i	β_i	γ_i	Adjusted R^2	RMSEP
[Ethanol]	≈100	2.56±0.74	112.6±15.3	0.9110	11.07
δ[ethanol]/ δtime	93.4±7.8	4.03±1.15	0.87±0.07	0.8975	22.90
[Acetaldehyde]	≈100	3.74±1.04	10.6±0.9	0.9279	14.39
δ[acetaldehyde]/ δtime	90.5±3.7	24.4±7.0	0.095±0.002	0.9449	18.61
[total ascorbic acid]	≈100	5.67±2.30	1.136±0.145	0.5682	34.52
δ[total ascorbic acid]/ δtime	≈100	3.61±1.05	0.012±0.002	0.8191	23.92

Table 3S: Mineral composition of 'Rocha' pears from five orchards, measured at harvest in the fruit pulp tissue. Means followed by different letters indicate significant differences according to Tukey's test ($p < 0.05$). The *, **, *** represent p values < 0.05, <0.01 and <0.001, respectively.

	Fruit mineral concentration (mg kg^{-1})									
Orchard	**Zn**	**P**	**B**	**Mn**	**Fe**	**Mg**	**Ca**	**Cu**	**Na**	**K**
1	32	7,316	69^{a}	39^{a}	95^{abc}	3,895	4,437	84^{a}	3,663^{a}	98,843^{ac}
2	29	7,638	100^{ab}	17^{bc}	34^{ac}	3,933	4,528	46^{b}	1,772^{b}	110,708^{a}
3	20	7,395	100^{ab}	05^{b}	80^{ac}	3,433	5,548	50^{b}	1,550^{b}	72,460^{b}
4	35	8,408	154^{b}	36^{a}	99^{abc}	3,588	4,750	76^{ac}	438^{b}	93,235^{c}
5	43	8,180	157^{b}	32^{ac}	173^{b}	3,758	4,167	66^{bc}	2,068^{b}	88,045^{cd}
6	19	7,542	105^{b}	04^{b}	51^{c}	3,309	3,782	50^{b}	1,644^{b}	76,854^{bd}
$LSD_{0.05}$	3.1	2,131	33	1.5	7.9	541	1,334	17	1,342	14,977
Significance	n.s	n.s	**	***	*	n.s	n.s	**	**	**

Table 4S: PLS model including x-variables (Zn, P, B, Mn, Fe, Mg, Ca, Cu and Na concentrations at harvest) and y-variables (incidence of internal browning after 95 (I95), 125 (I125) and 145 days (I145) of storage). The variable importance in the projection (VIP) and the standardized regression coefficients and respective significance level are presented. The *, **, *** represent p values < 0.05, <0.01 and <0.001, respectively.

***X*-variables**	**VIP**		**Standardized Regression coefficients and significance**		
	LV1	LV2	I95d	I125d	I145d
Cu	1.6524	1.5549	0.4376***	0.4195**	0.2013**
B	1.2891	1.345	0.0047	0.1097	0.3358**
Mn	1.2107	1.0785	0.2044	0.2343*	0.2076**
Fe	1.1776	1.0491	0.2034	0.2308*	0.1995*
K	0.9145	1.1169	0.396531	0.3291	0.0317
P	0.9122	0.9485	0.0013	0.0789	0.2366*
Mg	0.6689	0.6875	0.2216	0.1977*	0.0586
Na	0.6549	0.7252	0.0262	0.0408	0.1829
Zn	0.4564	0.4935	0.0117	0.0325	0.1241
Ca	0.0463	0.3466	0.1013	0.0596	0.0643

CHAPTER 7.

Dynamic Controlled Atmosphere for Prevention of Internal Browning Disorders in 'Rocha' Pear

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7.1. ABSTRACT

This study aimed to evaluate the potential of two dynamic controlled atmospheres, DCA-CF (chlorophyll fluorescence sensor) and DCA-EtOH (ethanol sensor) when compared to controlled atmosphere (CA), in the prevention of internal browning disorders (IBD) in 'Rocha' pear stored under commercial conditions. Pears harvested at optimal maturity were stored for 145 days at -0.5 ºC and 95% relative humidity, under three atmospheres: (1) CA (3 kPa O_2 + 0.5 kPa CO_2), (2) DCA-CF and (3) DCA-EtOH. At the end of storage, fruits in DCA-CF did not develop IBD while fruits in DCA-EtOH had an IBD incidence of 15 and 20% after 125 and 145 days of storage, respectively. The higher incidence of IBD under DCA-EtOH may be related to the higher levels of fermentative metabolites and to the lower ascorbate content. In contrast, the higher levels of ascorbate in DCA-CF showed that this technology contributes to maintaining the fruit´s antioxidant potential. Collectively our results suggest that DCA-CF is an effective strategy to prevent IBD in 'Rocha' pear. On the contrary, the DCA-EtOH is not suitable to prevent the induction of fermentation and IBD development. The results also suggest that the IBD development in 'Rocha' pear is related to fermentative metabolism.

Keywords: ascorbic acid, fermentative metabolites, low O_2, physiological disorders, *Pyrus communis*

7.2. INTRODUCTION

'Rocha' pear (*Pyrus communis* L. cv. Rocha) is a Portuguese native variety with a Protected Designation of Origin (PDO) and one of the few export-oriented products of Portuguese agriculture. 'Rocha' pear can be stored for up to 10 months under CA (2-3 kPa O_2 + 0.5-0.7 kPa CO_2 at -0.5 ºC and 95% relative humidity), but it is known to besusceptible to internal browning disorders (IBD) and superficial scald, the major causes of postharvest losses during long-term storage under CA (Silva, Gomes, Fidalgo, Rodrigues, & Almeida, 2010). The diphenylamine (DPA) and ethoxiquin as synthetic antioxidants were applied to pome fruits to prevent these physiological disorders during long term storage. However, the current restrictions on their use may have a significant impact on 'Rocha' pear exportation potential. Hence, there is a need to find novel strategies for long term storage of 'Rocha' pear.

The incidence of IBD is mainly related to the gas concentrations in the CA chamber. The reduced O_2 levels can lead to hypoxia within the fruits causing oxidative stress and inducing fermentation. Also, the high levels of CO_2 have been shown to further induce the development of IBD (Deuchande, Fidalgo, Larrigaudière, & Almeida, 2012; Deuchande, Fidalgo, Vasconcelos, Costa, & Larrigaudière, 2015). IBDs are firstly related to membranes damage which may occur as a result of the shift from a predominantly aerobic to an anaerobic metabolism. This metabolic shift may lead to: 1) a decreased energy assumption making it insufficient for cell regeneration and maintenance of the antioxidant system (Franck et al., 2007; Veltman, Lenthéric, Van Der Plas, & Peppelenbos, 2003); and 2) accumulation of fermentative metabolites (ethanol and acetaldehyde), which at high concentration may be toxic to the cells (Chervin, Brady, Patterson, & Faragher, 1996; Ho, Verlinden, Verboven, & Nicolaï, 2006; Ke & Kader, 1992; Ke, Van Gorsel, & Kader, 1990). These factors together may contribute to loss of cell compartmentalization and consequent development of IBD.

Dynamic controlled atmosphere (DCA) aims to provide optimal storage conditions improving the quality and preventing the development of physiological disorders. During storage under DCA, the concentration of O_2 is gradually reduced to the lower limit tolerated by the fruit (less than 1 kPa), the anaerobic compensation point (ACP), below which the respiratory metabolism switches from aerobic to anaerobic. When the critical level of O_2 is reached, the O_2 concentration is automatically or manually adjusted in order to restore optimal storage conditions. The critical level of O_2 is determined by monitoring the physiological fruit responses to stressfullow O_2 levels in the storage atmosphere, through the use of specialized sensors. These sensors must be sufficiently precise and reliable to ensure that the atmosphere is adjusted before irreversible fruit damage occurs. Currently, there are three methods capable of detecting significant changes in the following parameters: (1) respiratory quotient (ratio of CO_2 produced/O_2 consumed) (Gasser, Eppler, Naunheim, Gabioud, & Bozzi Nising, 2010); (2) chlorophyll fluorescence (Prange, Delong, Leyte, & Harrison, 2002; Prange, DeLong, Leyte, & Harrison, 2003) and (3) ethanol production (Schouten, Prange, Verschoor, Lammers, & Oosterhaven, 1997; Veltman, Verschoor, & Van Dugteren, 2003). Prange et al. (2002, 2003) and DeLong, Prange, Leyte, and Harrison (2004) suggested the use of chlorophyll fluorescence sensors as a quick and non-destructive method to determine the minimum acceptable level of O_2 for storing fruits and vegetables, leading to the development of the HarvestWatch™ system for use in CA rooms. DeLong, Prange, and Harrison (2007) demonstrated for two varieties

of apples, 'Cortland' and 'Delicious', that storage under DCA using the HarvestWatch ™ system maintained the fruit quality better than storage under CA and reduced the incidence of superficial scald. The same effect was reported for ‘Abbé Fétel’ and ‘Conference’ pears (Folchi, Bertolini, & Mazzoni, 2015; Rizzolo, Buccheri, Bianchi, Grassi, & Vanoli, 2015; Vanoli, Rizzolo, & Grassi, 2015). Nevertheless, the storage of ‘Abbé Fétel’ pears under DCA-CF was reported to induce the incidence of soft scald compared to CA and air storage (Folchi et al., 2015; Vanoli et al., 2015).

Schouten et al. (1997) reported that the storage of 'Elstar' apples in DCA-EtOH led to a better maintenance of the quality attributes than storage under ULO (1.2% O_2 + 2.5% CO_2). Veltman, Verschoor, et al. (2003) have also demonstrated for the same apple variety, that storage under DCA-EtOH contributes to a greater firmness and colour retention after 7 months of storage plus 10 days at room temperature when compared to the CA storage, also leading to a decreased incidence of 'skin spots' a specific physiological disorder of 'Elstar' apple. However, even during storage under air conditions or under levels of O_2 above the lower limit tolerated by the fruits, sometimes there are production of fermentative metabolites, which means that ethanol may not be a reliable indicator to detect changes on the respiratory metabolism (Peppelenbos & Oosterhaven, 1998). Furthermore, in order to ensure reliable ethanol measurements, allowing an adequate adjustment of the O_2 levels in the CA room as function of fruit responses, destructive sampling of the fruit is required

Regarding ‘Rocha’ pear, to the best of our knowledge, the use of DCA for long term storage has not been studied yet. Given the beneficial effects of storage under DCA reported for other fruits, this study aimed at evaluating the potential of this technology monitored by two types of sensors (ethanol and chlorophyll fluorescence) in the prevention of IBD in ‘Rocha’ pear. Emphasis was given to assess the effect of DCA on IBD development and the relationships with the levels of fermentative metabolites and ascorbate during storage. To the best of our knowledge, this is the first time that such an assessment is reported in commercial scale units.

7.3. MATERIALS AND METHODS

7.3.1. Plant material and experimental design

Pears (*Pyrus communis* L. cv Rocha) were collected from one orchard located in Cadaval (39º 16’ N, 9º 8’ W). As this experiment was conducted at commercial scale and it

was intended to store the fruits for an extended time period, optimally harvested fruits, which have been shown to be less susceptible to IBD (Deuchande et al., 2012), were selected to ensure fruits' marketability upon storage. The fruits were stored in three commercial CA chambers under the following conditions: (1) CA (3 kPa O_2 + 0.5 kPa of CO_2); (2) DCA monitored by a chlorophyll fluorescence sensor (DCA-CF) using an HarvestWatch^{TM} system and (3) DCA monitored by an ethanol biosensor (Tectronick, Senzytech, Italy) (DCA-EtOH). The storage conditions were equally settled concerning temperature (-0.5 °C), relative humidity (95%) and CO_2 concentration (0.5 kPa). The gas composition in the CA chambers was carefully controlled and checked with centralized analysers, supervised by special Fruit Control Equipment software for storage under DCA-EtOH and by an Isolcell software for storage under CA and DCA-CF, both systems with set points and alarms.

The capacity of the chambers used for CA and DCA-CF storage was 150 ton while that used for DCA-EtOH was 400 ton. All the chambers were fully loaded with 'Rocha' pear and were gas-tight allowing levels of O_2 as low as 0.2 kPa to be reached inside the storage rooms. In the two DCA chambers the O_2 levels were adjusted manually according to the physiological fruit responses monitored by the respective sensors.. In the DCA-CF chamber the levels of O_2 were reduced to 1 kPa and subsequently 0.2 kPa every two days until the chlorophyll fluorescence peak was detected. Once detected a buffer of 0.2 kPa O_2 was added to the atmosphere until the fluorescence signal was reduced to its initial level. During the remaining storage period the levels of O_2 were adjusted following chlorophyll fluorescence signal (Fig. 1A).

In the DCA-EtOH chamber the pull-down strategy was the same as for DCA-CF and the atmosphere was adjusted to maintain the ethanol level below 20 μL L^{-1}, which was defined as the critical level. At the beginning of storage fruit ethanol was measured every two days and then every ten days. After set point adjustments the levels were measured every two days until a reduction below the established threshold was observed. Since the fruits were stored in commercial chambers, the main doors of all chambers were opened between the 75 days and 125 days of storage to verify the condition of the fruit and therefore there was an O_2 peak between those time points (Fig. 1).

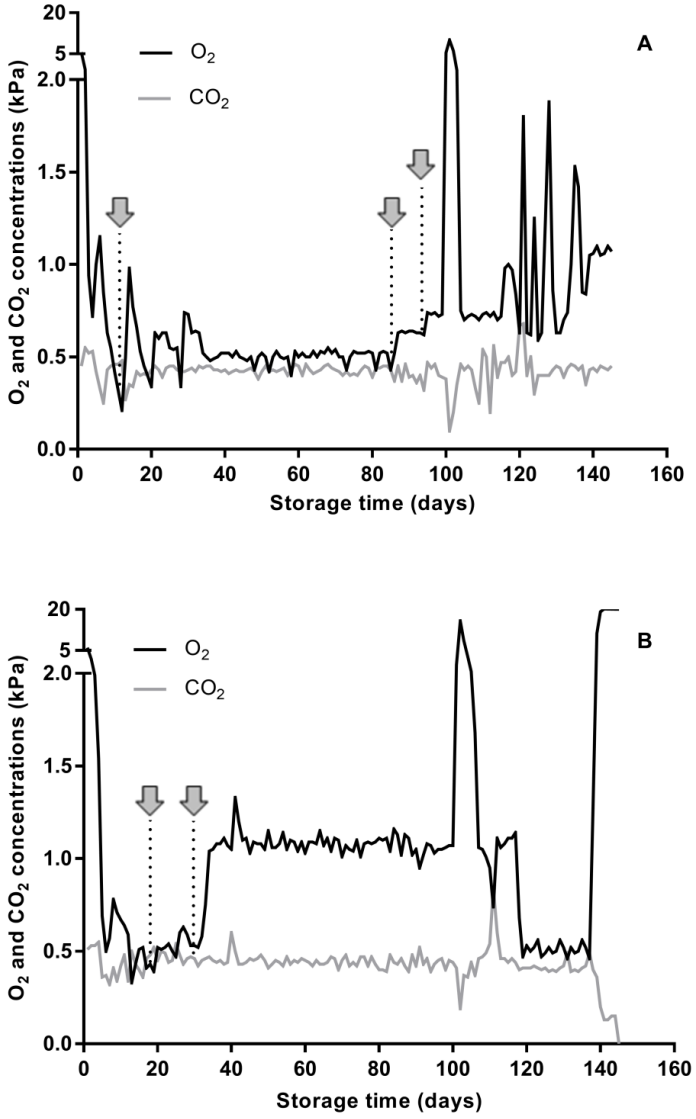

Figure 1: Changes in atmospheric concentrations of O_2 (black line) and CO_2 (grey line) during storage of 'Rocha' pear under dynamic controlled atmospheres determined by monitoring the chlorophyll fluorescence (DCA-CF) (A) and the fruit ethanol (DCA-EtOH) (B).The arrows represent the time points at which stress peaks were detected by the respective methods and after which the O_2 levels were adjusted.

In each chamber, batches of 70 fruits were placed in front of the inspection port-holes in order to easily remove themat the sampling time points avoiding changes in the storage atmosphere. Periodically, during the storage the incidence and severity of IBD were evaluated and samples of pear pulp for the analyses of ascorbate (3 replicates of 3 fruits each) and pear juice (3 replicates of 10 fruits each) for the analysis of fermentative metabolites (ethanol and acetaldehyde) were prepared. The pulp samples were frozen in

liquid nitrogen and ground in a commercial grinder and then stored at -80 °C until analysis. The pear juices were prepared using a commercial blender and filtered through cellulose paper filter and stored at -25 °C until analysis.

7.3.2. Determination of fruit quality

Fruit quality determinations at harvest were done on 30 fruit.. Firmness was assessed on opposite sides of the fruit, after peel removal and using a penetrometer (Turoni, Italy) fitted with an 8 mm Magness Taylor probe. Surface colour was measured on opposite sides of each fruit with a Minolta CR-400 colorimeter equipped with a D_{65} illuminant using the L* a* b* colour space. The hue angle was calculated with the formula arctg b*/a*. Juice samples were And total soluble solids content was measured in each juice sample (3 replicates of 10 fruits each) with an Atago PR-100 palette refractometer (Tokyo, Japan), and titratable acidity was determined by titration with 0.1 mol/L NaOH to pH 8.1. The starch index was determined by iodine staining according to Avelar & Rodrigues (1999) using a reference chart scored from 1 (maximum starch concentration) to 10 (no starch).

7.3.3. Estimation of physiological disorders

To evaluate the incidence of IBDs, 60 fruits from each storage condition, were checked after 45, 75, 125 and 145 days of storage. For the visual evaluation of IBD, fruits were cut longitudinally and transversally. The incidence was reported as the percentage of individual fruits affected by the total of fruits observed. The severity of the symptoms was classified considering the extent of the fruit affected according to the scale: (0) no internal disorders; (I) slightly damaged fruits: <25%; (II) moderate: 25-50%; (III) severe: >50%. The severity index was calculated as described by Veltman, Sanders, Persijn, Pemppelenbos, and Oosterhaven (1999).

$$Brown\ Index = \frac{\mathrm{I} + 2\mathrm{II} + 3\mathrm{III}}{3(0 + \mathrm{I} + \mathrm{II} + \mathrm{III})}$$

0, number of pears without browning; I, number of class I pears; II, number of class II pears; III, number of class III pears. The index value 0 means ‘no browning’ and brown index 1 means ‘maximal browning’.

7.3.4. Fermentative metabolites

Determination of ethanol and acetaldehyde was performed according to Ke, Yahia, Mateos, and Kader (1994) with slight modifications. For the analysis 10 mL of juice placed in a 20 mL test tube with a screw cap with a septum were incubated in a water bath at 65

°C for 1 hour, after which 1 mL of headspace sample was taken with a 1 mL glass syringe for determining acetaldehyde and ethanol concentrations. For this purpose a gas chromatograph (HP 5890 II, Hewlett Packard) equipped with a column CP-WAX 57 CB chromepack (0.25 mm x 50 m x 0.2 μm; Agilent Technologies Inc., USA) and a flame ionization detector were used.

7.3.5. Analysis of ascorbate

Ascorbic acid (AsA) and dehydroascorbic acid (DHA) were determined as described by Zapata and Dufour (1992) with modifications. Pulp tissue (15 g) was homogenised in 15 mL of 50 mL L^{-1} methanol plus HCl (10 g L^{-1}), using an Ultra-Turrax (IKA Labortechnik model T 25, Staufen, Germany). The homogenate was centrifuged at 3857 x g (Universal 320R, Hettich, Germany) for 15 min and the supernatant was filtered through a fluted cellulose paper filter. To 3 mL of supernatant, 1 mL of 1,2-phenylenediamine dihydrochloride (OPDA, from Sigma-Aldrich) solution was added and the resulting mixture was incubated for 30–40 minThe mixture was then filtered through a 0.22 μm filter and analysed with an HPLC system equipped with a UV-Vis detector (UV-1575), an auto sampler (AS-1555) and a pump (PU-1580), all controlled by appropriate software (Borwin v. 1.5, from Jasco Corporation, Tokyo, Japan). The separation of AsA and DHA was achieved in a Spherisorb ODS2 5 μm column (250 x 4.6 mm da Waters Corporation, USA). The mobile phase was 50 mL L^{-1} methanol containing 5 mmol L^{-1} cetrimide and 50 mmol L^{-1} potassium dihydrogen phosphate. The flow rate was 1.8 mL min^{-1} and the injection volume was 20 μL. The detector was set at 348 nm for DHA and 261 nm for AsA detection. All the procedures were conducted in darkness at 4 ºC.

7.3.6. Statistical analysis

The data was analysed for significant differences by analysis of variance (ANOVA) using the GraphPad Prism version 6.0 (San Diego, CA, USA). The data presented in the figures was subjected to separation of means calculating the Tukey HSD value (honestly significant difference) ($P = 0.05$).

7.4. RESULTS AND DISCUSSION

7.4.1. Postharvest fruit characterization

The analysis of initial fruit maturity indicate that fruit were harvested at an optimal maturity stage (Table 1). ‘Rocha’ pear harvested at optimal harvest dates may have a

firmness between 54 N and 64 N, a soluble solids content between 11 and 13 ºBrix, acidity of 2-3 g L^{-1} malic acid (ANP, 1997) and a starch index averaged at about 6.5 (Avelar & Rodrigues, 1999). Although the firmness value is in the range of optimally harvested fruits (Table 1), the other parameters analysed reflect a slightly advanced maturity stage. These slight differences determined are probably a consequence of the cold storage period before CA implementation.

Table 1: Initial maturity indices of 'Rocha' pear. Values of firmness, weight and colour (lightness, chroma and hue angle) represent the mean of 30 fruits ±SE and the values of titratable acidity and soluble solids content represent the mean of 3 replicates of 10 fruits each ± SE.

Firmness (N)	Weight (g)	Colour			Titratable acidity (g/L malic acid)	Soluble solids content (º Brix)	Starch index
		Lightness (L*)	Chroma (C*)	Hue (hº)			
54.5±0.7	151.8±4.5	69.1±0.5	45.8±0.3	105.3±0.5	1.45±0.01	13.6±0.0	7.7±0.1

7.4.2. Storage conditions

At the beginning of storage under DCA-EtOH and DCA-CF the levels of O_2 were reduced until the lowest O_2 limit tolerated by the fruits (Fig. 1). During storage under DCA-CF after the first sharp peak and subsequent adjustment of the O_2 set point there was a fluctuation in the O_2 levels inside the chamber (Fig.1A). This was probably due to the dimension of the commercial chamber, in which the time period required to stabilize the O_2 levels after set point adjustments may be longer. Throughout storage there were some slight increases in chlorophyll fluorescence signal represented in Fig 1A by the arrows, implying adjustments to the O_2 set point during storage to reduce the fluorescence signal to its initial level.

Regarding storage under DCA-EtOH, after the first stress peak, represented by the first arrow in Fig. 1B, the O_2 levels were slightly increased in order to reduce the ethanol levels below the established threshold value (20 μL L^{-1}). However, the ethanol levels did not stabilize increasing above it latter, occurring the second stress peak. Therefore, the O_2 levels were increased again to 1 kPa O_2 until the ethanol levels were reduced below the established threshold value being maintained thereafter.

Comparing the two DCA storage conditions (Fig. 1) it could be observed that the fruit response to the stress caused by low oxygen levels in the storage atmosphere was

detected earlier in the fruits stored under DCA-CF than under DCA-EtOH and that the lowest O_2 concentration reached in the chamber under DCA-CF was 0.2 kPa compared to the 0.3 kPa reached in the chamber under DCA-EtOH. These results indicate that the ethanol and the chlorophyll fluorescence methods represented differently the stress conditions to which the fruit were exposed, providing different values of critical oxygen concentration or ACP (Fig. 1). This difference may result of different levels of accuracy between the two methods used to monitor the fruit stress responses or may be related to the use of different CA chambers.

7.4.3. Incidence and severity of internal browning disorders

During storage under CA and DCA-CF fruit did not develop IBD. In contrast, the fruit stored under DCA-EtOH exhibited 15% of IBD with a severity index of 0.1 after 125 days of storage and 20% of incidence and a severity index of 0.12 at the end of the experiment. DeEll, Prange, and Murr (1995) have previously demonstrated that the chlorophyll fluorescence methodology is capable of detecting stress situations in apples 'Marshall McIntosh' caused by low O_2 and high CO_2 levels, preventing the incidence of skin discoloration or cavities in the cortex during storage. Lafer (2011) also reported a reduction in the incidence of IBD in 'Uta' pear stored during six months under DCA-CF compared to CA. On the contrary, Mattheis, Felicetti, and Rudell (2013) reported a higher incidence of IBD in pears 'd'Anjou' stored under DCA-CF than under CA. Moreover, they observed that the development of IBD did not produce any change in the chlorophyll fluorescence signal, suggesting that this sensor cannot detect the stress condition that leads to the development of IBD in this pear cultivar. Accordingly, Vanoli et al. (2015) also reported an higher incidence of internal disorders in ‘Abbé Fétel’ pears stored under DCA-CF than under air .

7.4.4. Changes in fermentative metabolites

There was a significant effect of storage condition on the ethanol and acetaldehyde concentrations after 125 and 145 days of storage (Fig. 2). After 125 days of storage higher levels of fermentative metabolites were found in fruit stored under DCA-EtOH (50±3.6 µl ethanol L^{-1} and 7.6±0.58 µl acetaldehyde L^{-1}) compared to DCA-CF (24.0±0.73 µl ethanol L^{-1} and 6.03±0.09 µl acetaldehyde L^{-1}) and CA (3.1±0.41 µl ethanol L^{-1} and 4.4±0.58 µl acetaldehyde L^{-1}). Storage under DCA-EtOH may have induced a switch in the respiratory metabolism between the 75 and 125 days of storage (Fig. 2).

Although the low O_2 levels (ca. 0.5 kPa) at the beginning of storage did not immediately induced fermentation, the very low O_2 levels (0.3-0.5 kPa) maintained up to 30 days may have induced the biosynthesis of fermentative enzymes. Afterwards, the sharp increase up to 1 kPa allowed aerobic respiration to be restored but when the O_2 levels were reduced, after ca. 120 days of storage, fermentation may have been induced with the pooled fermentative enzymes previously synthetized triggering an intensive production of fermentative metabolites (Fig. 2).

Contrary to DCA-EtOH, under DCA-CF the levels of O_2 remained constant up to 75 days and were slightly increased later until the end of the experiment. This difference in the regulation of O_2 levels in DCA-CF may have favoured the fruit physiology, considering the lower levels of ethanol (*P<0.001*) and acetaldehyde (*P=0.0069*) (Fig. 2) and significantly higher ascorbate levels (*P= 0.0086*) (Fig. 3) after 125 days of storage. These differences were related to the differences in IBD incidence observed up to the end of the storage.

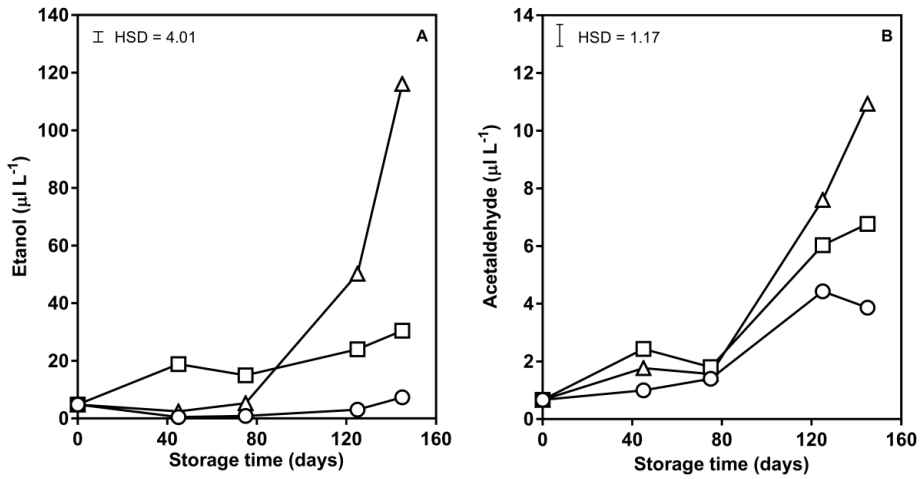

Figure 2: Effects of storage under controlled atmosphere (CA) (○) and dynamic controlled atmospheres implemented by monitoring chlorophyll fluorescence (DCA-CF) (□) and fruit ethanol (DCA-EtOH) (△), on the concentrations of ethanol (A) and acetaldehyde (B) in 'Rocha' pear. The symbols represent the mean of 3 replicates of 3 fruits each and the vertical bars represent the Tukey HSD values (P=0.05).

The increased levels of fermentative metabolites found in the fruits stored under DCA-EtOH were well correlated with the higher incidence of IBD. Similar results were previously found by Deuchande et al. (2012) in 'Rocha' pear. Mattheis et al. (2013) also found a significant relationship between fruit ethanol content and pithy brown core in

'd'Anjou' pear. Pintó, Lentheric, Vendrell, and Larrigaudière (2001) suggested that fermentative metabolites might be partially involved in the development of IBD in 'Blanquilla' pear. However, in the later study, the increased levels of ethanol and acetaldehyde were not sufficient to explain the incidence of IBD and the authors suggested the involvement of oxidative processes to explain this disorder in 'Blanquilla' pear.

7.4.5. Changes in total ascorbic acid and redox form

During storage total ascorbic acid decreased in the fruit regardless of the storage conditions (Fig. 3). This reduction was sharper in the fruits stored under CA with a decrease of about the 60% at the end of the storage while this decrease was about 40% in the fruits stored under DCA (Fig. 3).

The lowering of ascorbate levels in fruit has been associated with an increased susceptibility to the development of IBD (Veltman, Kho, Van Schaik, Sanders, & Oosterhaven, 2000). According to Veltman et al. (1999), the IBD appears when the ascorbic acid concentration during storage is reduced below a certain limit determined as 1.3 mg 100 g^{-1} FW for 'Conference' pears.

After 125 days of storage there were significant differences in the concentrations found in the fruits stored at the three storage atmospheres with higher concentrations of total ascorbate in the fruits of DCA-CF (2.3 mg 100 g^{-1} FW) followed by CA (1.8 mg 100 g^{-1} fresh weight) and finally DCA-EtOH (1.3 mg 100 g^{-1} FW) (Fig. 3). Although the levels found in the fruits stored under DCA-EtOH were the lowest after 125 days of storage reaching the threshold limit determined for 'Conference' pear (Veltman et al., 1999), at the end of the experiment the ascorbate levels in these fruits increased and were higher than in CA (Fig. 3). This rise may be related to the transient increase of O_2 levels between the 140 and 145 days of storage (Fig. 1B). This last probably allowed a higher energy assumption through aerobic respiration permitting the recovery of at least part of the antioxidant potential.

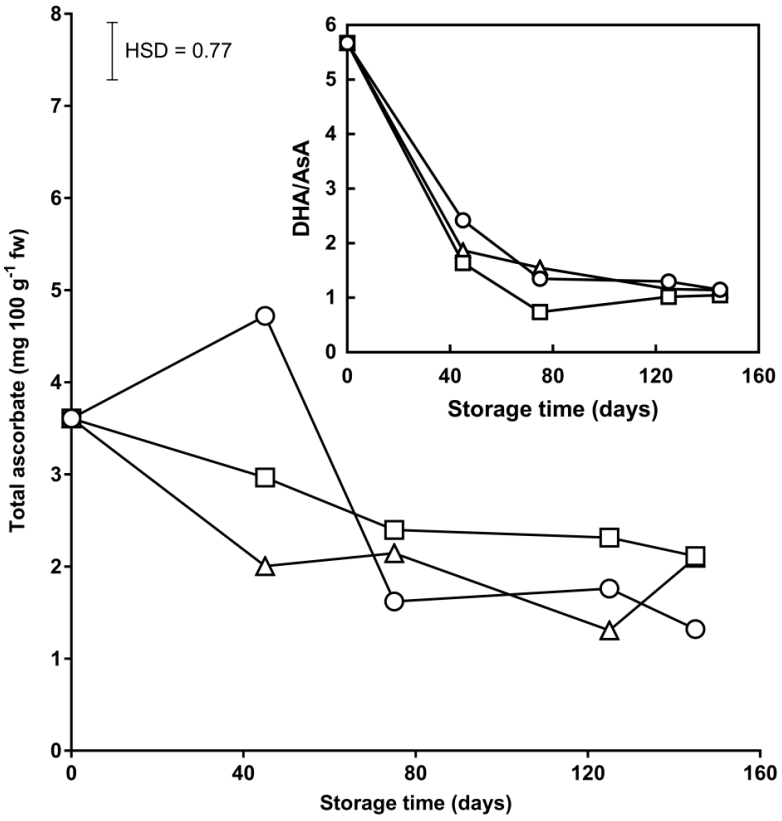

Figure 3: Effects of storage under controlled atmosphere (CA) (○) and dynamic controlled atmospheres implemented by monitoring chlorophyll fluorescence (DCA-CF) (□) and fruit ethanol (DCA-EtOH) (△), on the concentrations of total ascorbic acid in 'Rocha' pear. The insert represents the ratio between the oxidized and reduced forms of ascorbate (DHA/AsA) during storage. The symbols represent the mean of 3 replicates of 3 fruits each and the vertical bar represents the Tukey HSD value (*P=0.05*).

The significantly higher levels of ascorbate in the fruits of DCA-CF compared to DCA-EtOH after 125 days and to the fruits of CA at the end of the experiment, suggest that DCA-CF may contribute for a better quality maintenance compared to the others storage conditions. Furthermore, the ratios of ascorbic acid in the oxidized and reduced form (DHA/AsA) were, in general, lower in the fruits stored under DCA-CF compared to the others storage conditions (Fig. 3). These results indicate that storage under DCA-CF may contribute to reduce the exposure of fruit to oxidative stress, allowing the maintenance of the antioxidative potential (Larrigaudière, Lentheric, Pintó, & Vendrell, 2001).

7.5. CONCLUSION

In this work the absence of IBD in the fruit stored under DCA-CF and CA has been related to lower levels of fermentative metabolites at the end of storage and in the case of fruit stored under DCA-CF also to the higher levels of total ascorbate and lower oxidation of this compound. The use of DCA-CF appears an effective strategy in preventing IBD in 'Rocha' pear. In contrast, DCA-EtOH, which was associated with production of high levels of fermentative metabolites and high IBD incidence, was not of interest. The results finally suggest that the development of IBD in 'Rocha' pear is mainly determined by the changes in fermentative metabolism and that storage under DCA-CF may promote the maintenance of 'Rocha' pear quality.

7.6. ACKNOWLEDGEMENTS

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CHAPTER 8.

The efficacy of 1-methylcyclopropene (1-MCP) Treatment in Preventing Internal Browning Disorders in 'Rocha' Pear During Storage Depends on Fruit Maturity at Harvest

Submitted as:

Deuchande, T., Larrigaudière, C., Carvalho, S.M.P.and Vasconcelos, M.W. The efficacy of 1-methylcyclopropene (1-MCP) treatment in preventing internal browning disorders in 'Rocha' pear depends on fruit maturity stage at harvest.

8.1. ABSTRACT

This study aimed to define the potential of 1-methylcyclopropene (1-MCP) treatment and delayed controlled atmosphere (CA) storage in preventing internal browning disorders (IBDs) in 'Rocha' pear, focusing on the effects of these treatments in fruit quality and levels of ascorbic acid and fermentative metabolites. For this purpose, two experiments were conducted including fruit from three orchards picked at three different maturity stage and subjected to three different treatments: i) high CO_2 CA storage; ii) delayed high CO_2 CA storage; and iii) treatment with 300 nL L^{-1} 1-MCP and subsequent storage under high CO_2 CA. For early harvested fruit, the 1-MCP treatment prevented IBD incidence when compared to control and delayed CA stored fruit. In contrast, both strategies tested herein did not control IBD in late and very late harvested fruit. The 1-MCP treatment in late and very late harvested fruit resulted in more than three-fold higher IBD incidence when compared to untreated fruit after 75 days of storage. Delayed CA, contrarily to the observed for other pear varieties, did not prevent IBD in 'Rocha' pear, and an even higher incidence was found comparatively to control and 1-MCP treated fruit.

Keywords: 1-MCP, ascorbate, delayed CA, fermentative metabolites, internal browning, quality

8.2. INTRODUCTION

'Rocha' pear (*Pyrus communis* L. cv. Rocha) can be stored for up to 10 months under the recommended controlled atmosphere (CA) of 2.5-3.0 kPa O_2 and 0.5-0.7 kPa CO_2).[1] However, this cultivar is susceptible to internal browning disorders (IBD) and superficial scald, leading to important postharvest losses during long-term storage under CA.[2]

Many factors, including fruit maturity at harvest, postharvest treatments and storage conditions affect the internal browning development.[1,3–6] It has been shown that pear storage under CA at low temperature leads to a shift from an aerobic to an anaerobic metabolism with accumulation of fermentative metabolites and changes in membranes fluidity and gas diffusivities in fruit tissues.[7–9] Furthermore, CA may cause membrane damage with subsequent loss of the potential to generate sufficient energy for membrane regeneration and maintenance of the antioxidant system.[10,11] Despite this current

knowledge, the underlying mechanisms of IBD in pears remains unclear. Recently, it has been found that in 'Rocha' pear the underlying mechanisms involved in the occurrence and development of CO_2-related IBD are associated with the antioxidant and fermentative metabolisms.[12]

Until recently, diphenylamine (DPA) and ethoxyquin were applied to apples and pears to prevent physiological disorders. However, due to the current restrictions on their use imposed by the European Commission, the application of these synthetic antioxidants to pome fruits is no longer allowed. This prohibition generated an urgent need for finding new strategies to control superficial scald and IBD. The application of 1-methylcyclopropene (1-MCP), an inhibitor of ethylene action, emerged as one of those strategies. It has been shown that the application of 1-MCP in 'Rocha' pear reduced IBD and superficial scald in optimally harvested fruit stored in air.[13] However, some concerns in terms of inadequate fruit ripening after long-term storage remain. 1-MCP treated 'Rocha' pear has been shown to be affected by this phenomenon, the so called 'evergreen'.[14] In order to avoid this critical problem, it has been suggested to store the 1-MCP treated fruit at 2.5 ºC instead of -0.5 ºC but this protocol despite promoting an adequate ripening also led to higher IBD incidence.[15] A possible alternative to promote an adequate ripening after long-term storage may be the application of 1-MCP in fruits at advanced maturity stages followed by storage under CA.[16]

The use of delayed CA storage (i.e. storing fruit for a certain period in cold air before being transferred to CA storage), was proposed as another possible alternative for preventing IBD and superficial scald. The 21 days delayed CA storage reduced IBD incidence by more than 60% in 'Conference' pears, even in late harvested fruit, which were previously identified has being more susceptible to IBD.[17,18] In contrast, for optimally harvested 'Rocha' pear, no differences in IBD incidence were found after 15-20 days in fruits stored under delayed CA compared to immediate CA stored fruits.[1,13,19]

To the best of our knowledge, there are no previous studies describing the relationship between the effectiveness of these strategies on IBD prevention and initial fruit maturity. Moreover, the few available studies on the use of 1-MCP or delayed CA for preventing IBD in 'Rocha' pear are not conclusive;[1,19,20] and were conducted using only optimally harvested 'Rocha' pears. It has previously been shown that late harvested 'Rocha' pears are much more susceptible to develop IBD during long term storage than early and optimally harvested fruit.[21] Therefore, it is important to analyse the possible interaction existing between fruit maturity stage and the effectiveness of the control

strategies. Considering that the occurrence of IBD in 'Rocha' pear is correlated with increased levels of fermentative metabolites and reduced levels of ascorbate, [12] it is also important to clarify how these treatments affect the antioxidant and fermentative metabolisms.

The aims of this study were to: i) analyse the effect of 1-MCP treatment and delayed CA storage on IBD prevention and maintenance of fruit quality during storage in fruits of different maturity stages; ii) evaluate the effect that both treatments may have on ascorbate, dehydroascorbate, ethanol and acetaldehyde levels, correlating the results with IBD susceptibility.

8.3. MATERIALS AND METHODS

For this study, two experiments were conducted with pears (*Pyrus communis* L. cv. 'Rocha') harvested in different years at different maturity stages.

8.3.1. Plant material, treatments and storage conditions

In Experiment 1, pears were harvested in 2013 from two orchards located in Cadaval (Portugal) at the end of the harvest season (orchards 1 and 2). Fruit were subjected to three treatments: 1) immediate storage under browning inducing conditions (CA) (1 kPa O_2 + 10 kPa CO_2) which corresponded to the control; 2) treatment with 300 nL L^{-1} 1-MCP for 24 h and then storage under CA and 3) delayed CA storage (21 days storage in cold air prior to CA storage). For the 1-MCP treatment, fruits were placed in two containers of 30 L of capacity (60 fruit in each container) and stored at 0 ºC. After two days of storage, fruits were treated with 1-MCP using Smartfresh™ (AgroFresh Inc.) and according to the manufacturer's recommendations. Briefly, inside each container, a flat flask with a septum containing the reaction mixture [1-MCP as a powder (0.5 g) and water at 30 ºC added with a syringe] was opened and the containers were immediately sealed. After 24 h, the containers were well aerated and the fruits were stored under the defined CA condition.

In Experiment 2, pears were harvested in 2014 from one orchard at the same location as in Exp.1 but at the middle of the harvest season in order to obtain less mature fruit (orchard 3). Similarly to Exp. 1, fruits were subjected to the same treatments and atmospheres, but the oxygen concentration was 2 kPa instead of 1 kPa.

8.3.2. Samples preparation for biochemical analysis

In Exp. 1, pulp tissue samples for the analysis of ascorbate, and pear juices for the analysis of ethanol and acetaldehyde, were prepared at harvest and after 45, 75, 95 and 125 days of storage, whereas in Exp. 2 the samples were prepared at harvest and at the end of storage (after 140 days of storage). In both cases, the pulp tissue samples (3 replicates of 3 fruit each) were frozen in liquid nitrogen and ground using an electric mill and then stored at -80 ºC until analysis. Pear juice samples (3 replicates of 9 fruits each) were stored at -25 ºC until analysis.

8.3.3. Assessment of initial maturity and fruit quality during storage

The assessment of initial maturity was carried out on 30 fruit immediately after harvest for both experiments. Firmness was analysed on two opposite sides of the fruit, after peel removal and using a penetrometer (Turoni, Italy), fitted with an 8 mm Magness Taylor probe. Surface colour was measured on opposite sides of each fruit (Minolta CR-400 colorimeter equipped with a D_{65} illuminant), using the L* a* b* colour space. The hue angle was calculated with the formula arctg b*/a*. For the assessment of sugars and acidity, juice samples were prepared from wedges of cut fruit (3 replicates of 9 fruits each), homogenized and filtered through cellulose filter paper. Soluble solids content (SSC) was measured in each juice sample with an Atago PR-100 palette refractometer (Tokyo, Japan), and titratable acidity (TA) was determined by titration with 0.1N NaOH to pH 8.1. The starch index was determined by iodine staining using a reference chart scored from 1 (maximum starch concentration) to 10 (no starch).[22]

8.3.4. Estimation of incidence and severity of IBD

Incidence and severity of IBD were evaluated on 50 fruits immediately after removal from the storage chambers. The incidence of IBD was reported as the percentage of individual fruit affected by the total of fruits observed. The severity of the disorders was classified considering the extent of the damage as previously described.[23]

8.3.5. Extraction and analysis of ascorbic and dehydroascorbic acids

Ascorbic acid (AsA) and dehydroascorbic acid (DHA) were determined as previously described [24] with slight modifications. Pulp tissue (15 g) was homogenized with 15 mL of methanol:water (5:95 v/v) plus 10 g L^{-1} of HCl. The homogenate was centrifuged at 4,000 *g* for 15 min, filtered and 1 mL of 1,2-phenylenediamine dihydrochloride solution was added to 3 mL of the resulting extract and further incubated for 30 to 40 min. The

samples were analysed with an HPLC system equipped with a UV-Vis detector (UV-1575), an auto sampler (AS-1555) and a pump (PU-1580), all controlled by appropriate software (Borwin v. 1.5, from Jasco Corporation, Tokyo, Japan). Separation of AsA and DHA was achieved in a Spherisorb ODS2 5 μm (250 x 4.6 mm da Waters Corporation, USA). The detector was set at 348 nm for DHA and 261 nm for AsA detection. All the extraction procedures were conducted in the absence of light at 4 ºC.

8.3.6. Fermentative metabolites

The determination of ethanol and acetaldehyde was performed as previously described [7] with slight modifications. From each sample, 10 mL of juice were placed in a 20 mL test tube with a screw cap and a septum. After sample incubation in a water bath at 65 °C for 1 hour, 1 mL of headspace sample was taken to determine the acetaldehyde and ethanol concentrations. For this purpose a gas chromatograph (HP 5890 II, Hewlett Packard) equipped with a column CP-WAX 57 CB chromepack (0.25 mm x 50 m x 0.2 μm; Agilent Technologies Inc., USA) and a flame ionization detector was used.

8.3.7. Statistical analysis

The analyses of variance (ANOVA) for the effects of atmosphere and storage time were performed using the GraphpPad Prism version 6.0 (San Diego, CA, USA). Mean comparisons for the effect of the atmosphere were done by calculating the Tukey's least significant difference (LSD) at P=0.05.

8.4. RESULTS AND DISCUSSION

8.4.1. Initial fruit maturity

Analyses of initial fruit maturity (firmness, colour, TA, SSC and starch index) were performed just after harvest. 'Rocha' pear harvested at commercial harvest dates may have a firmness between 55 and 65 N, a soluble solids content (SSC) between 11 and 13%, acidity of 2-3 g L^{-1} malic acid [25] and an averaged starch index of about 6.5.[22] Accordingly, in this work the fruit of orchard 1 and 2 corresponded to fruits picked at late and very late maturity stages, whereas fruits of orchard 3 were harvested at an early maturity stage (Table 1).

Table 1: Initial maturity indices of the fruit from three different orchards. Values of firmness, weight and colour (lightness, chroma and hue angle) represent the mean of 30 fruits ±SE and the values of titratable acidity (TA) and soluble solids content (SSC) represent the mean of 3 replicates of 9 fruits each ± SE measure in triplicate.

Orchards	Firmness (N)	Weight (g)	Starch	TA (g malic acid L^{-1})	SSC (%)	Colour	
						L*(65)	hº
1	53.8±0.6	165± 5	8.0±0.3	1.31±0.02	12.9±0.0	68.7±0.4	107.2±0.4
2	37.7±1.1	179±6	9.5±0.9	1.40±0.03	14.1±0.0	73.4±0.5	103.0±0.5
3	66.2 ±0.7	118±3	5.0±0.2	1.94±0.02	13.4±0.1	66.6±0.3	108.3±0.3

8.4.2. Effects of postharvest treatments on IBD

8.4.2.1. 1-MCP treatment

Late harvested fruits (orchard 1) treated with 1-MCP exhibited higher IBD incidence and severity than untreated fruits (Table 2). This trend was even more pronounced in fruits harvested in a very late maturity stage after 75 days of storage (Table 2). Moreover, for both treated and untreated fruits, the very late harvested fruits were more susceptible to IBD than the late and early harvested fruits. These findings are in agreement with previous studies which showed that late harvested 'Rocha' pears are more prone to develop IBD than optimally and early harvested fruit.[21,26] Similar results were also obtained in other pear cultivars, such as Williams', 'Bosc' and 'Packhams Triumph'.[26]

Previous studies have been shown that when 1-MCP is applied to optimally harvested 'Rocha' pear IBD may be prevented.[1,13] Our results are in accordance with those findings as the early harvested fruits treated with 1-MCP (orchard 3) were less affected by IBD after 140 days of storage (Table 2).

8.4.2.2. Delayed CA storage

Some studies reported that a prior storage period in cold air before CA reduces IBD incidence in apples and pears.[17,18,27,28] In 'Conference' pear such behaviour has been associated with the maintenance of a higher energy charge, which would make the fruit better adapted to subsequent CA conditions.[28] In contrast, previous studies conducted in optimally harvested 'Rocha' pear reported that fruit storage under delayed CA (15, 20 or 60 days under cold air prior to storage under CA) did not confer enhanced resistance to

IBD.[1,13,19] Our results are in accordance with this previous observation but also indicate that for early and late harvested 'Rocha' pear, delayed CA may even increase IBD incidence and severity (Table 2).

Altogether, these results suggest that initial fruit maturity strongly influences fruit's susceptibility to IBD and the efficacy of delayed CA in preventing IBD.

Table 2: Internal browning incidence and severity during storage of late (orchard 1), very late (orchard 2) and early harvested fruit (orchard 3) stored under immediate CA (control), immediate CA after 1-MCP treatment and delayed CA.

		Early			Late			Very Late	
Parameter	**Storage time (days)**	Control	1-MCP	Delayed CA	Control	1-MCP	Delayed CA	Control	1-MCP
Internal browning incidence	45	-	-	-	15.5	24.6	3.3	23.3	79.7
	75	-	-	-	11. 7	41. 7	53.3	26. 7	85.0
	95	-	-	-	36. 7	-	68.3	-	-
	125	-	-	-	65.0	-	91.7	-	-
	140	14.8	6.7	23.1	-	-	-	-	-
Internal browning severity index	45	-	-	-	0.057	0.092	0.011	0.089	0.478
	75	-	-	-	0.061	0.172	0.233	0.178	0.517
	95	-	-	-	0.167	-	0.450	-	-
	125	-	-	-	0.390	-	0.550	-	-
	140	0.150	0.017	0.154	-	-	-	-	-

8.4.3. Effects of postharvest treatments on fruit quality

8.4.3.1. 1-MCP treatment

It is generally recognized that 1-MCP reduces firmness and acidity losses and delays yellowing during storage and shelf-life.[29,30] The efficacy of 1-MCP treatment is dependent on cultivar, initial fruit maturity[31] and number of ethylene receptors at the moment of 1-MCP application, 1-MCP concentration applied, temperature and length of storage.[15,16,32,33]

In our results, clear differences regarding quality retention were found among the fruits of the different maturity stages (Fig. 1 and Fig. 2). For late harvested fruits the rate of

firmness loss was similar in both, treated and untreated fruits (Fig. 1A). However, in the very late harvested fruits firmness decreased faster in the 1-MCP treated than in the untreated (Fig. 1A). This contradiction with the general mode of action of 1-MCP, can be explained by the fact that the fruits were picked too mature, in such a condition that 1-MCP at the dose applied in this study was ineffective to block the existing ethylene receptors. Considering that firmness loss in 1-MCP treated pears differs among cultivars, also depending on the, fruit maturity at harvest, temperature and length of storage.[15,16,32,33] Our results showed that 1-MCP efficacy in maintaining fruit firmness is highly dependent on initial fruit maturity and 1-MCP concentration applied but also indicate that initial maturity at harvest is the main determining factor of 1-MCP efficacy.

The application of 1-MCP in optimally harvested 'Rocha' pear has been reported to preserve the green colour during storage and shelf-life at 20 ºC.[15,34] In our study, the rate of yellowing of late harvested fruits treated and untreated with 1-MCP was similar (Fig 1E). Very late harvested fruits treated with 1-MCP were significantly more yellow after 75 days of storage when compared to the untreated fruits (Fig. 1E). Considering the previous observations and the results obtained in this study (Fig. 1C,E), we can conclude that fruit capacity to retain colour after 1-MCP treatment is highly dependent on initial fruit maturity and that in fruits harvested at advanced maturity stages the yellowing is faster.

In general, late and very late harvested fruits treated with 1-MCP exhibited higher TA and lower SSC during storage compared to the untreated fruits (Fig. 2 A, C). The retention of TA and SSC may result of the 1-MCP action on fruit ripening which leads to reduced respiration rates [35] and reduced conversion of acids into sugars. Although in Williams', 'Bosc' 'Packham's Triumph', 'Beurré d'Anjou' and 'Rocha' pears TA was reported to be higher in 1-MCP treated fruit than in untreated,[36] for 'Bartlett' pears the treatment with 1-MCP did not contribute for a better maintenance of this parameter.[30,37] In our study, the lowest levels of SSC in very late harvested fruits treated with 1-MCP, detected after 45 days of storage, may be related to increased fermentation (Fig. 2C, 4).

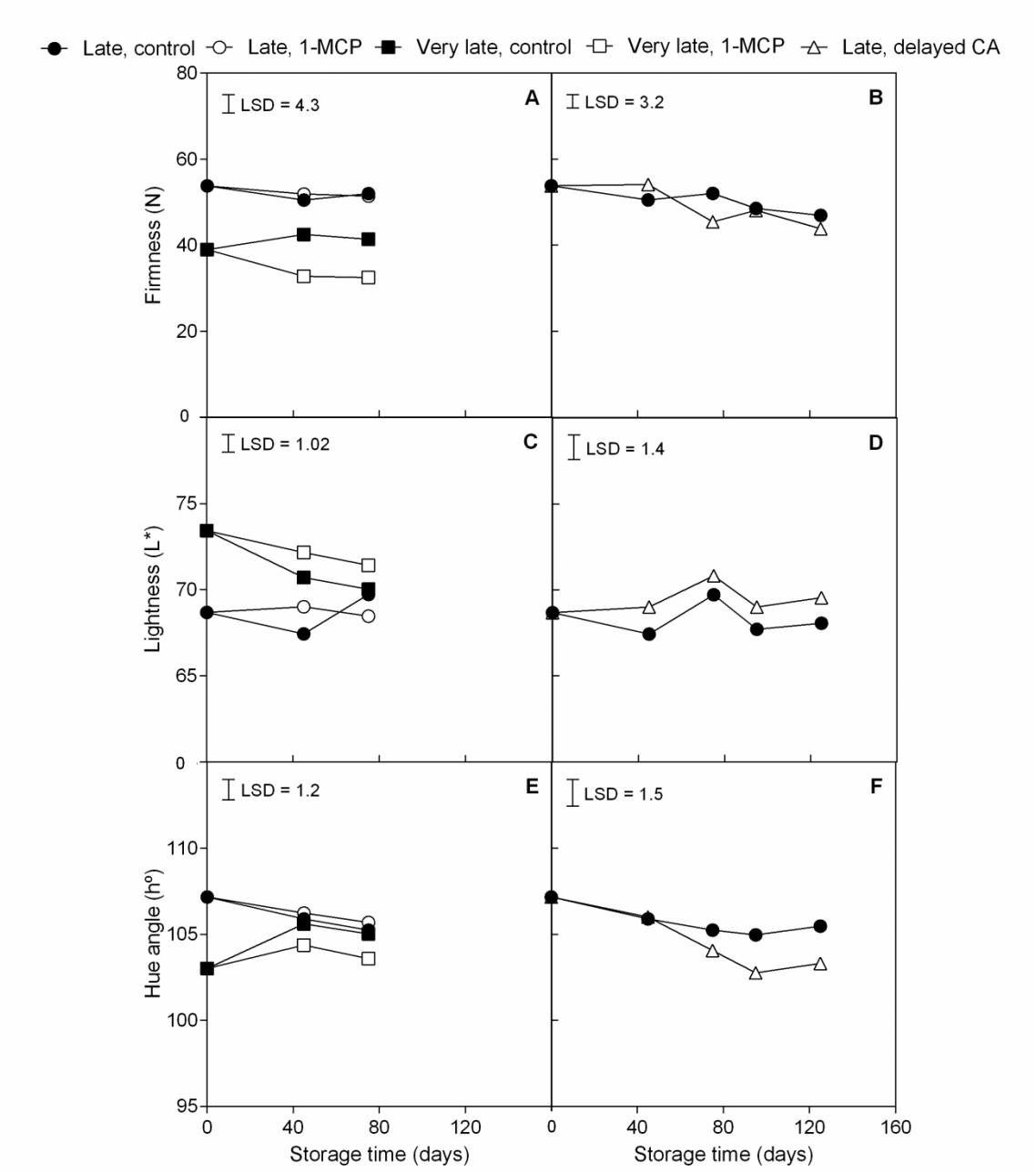

Figure 1: Effect of 1-MCP application (A, C, E) and delayed CA (B, D, F) on the firmness, lightness (L*) and hue angle (hº) (for late harvested fruit (orchard 1) and very late harvested fruit (orchard 2). Values of firmness, colour (lightness and hue angle) represent the mean of 30 fruits ±SE and vertical bars represent the Tukey LSD for the treatment effect at each time-point (p=0.05).

8.4.3.2. Delayed CA storage

The loss of firmness and lightness changes were similar in the fruits stored under delayed and immediate CA (Fig. 1B, D) but the values of lightness were higher in the fruits stored under delayed CA during the entire storage period (Fig. 1D). Only slight changes in

tonality (hue angle) were found in control fruit during all the storage period but the rate of yellowing was faster in the delayed CA stored fruits especially after 45 days of storage (Fig. 1F).

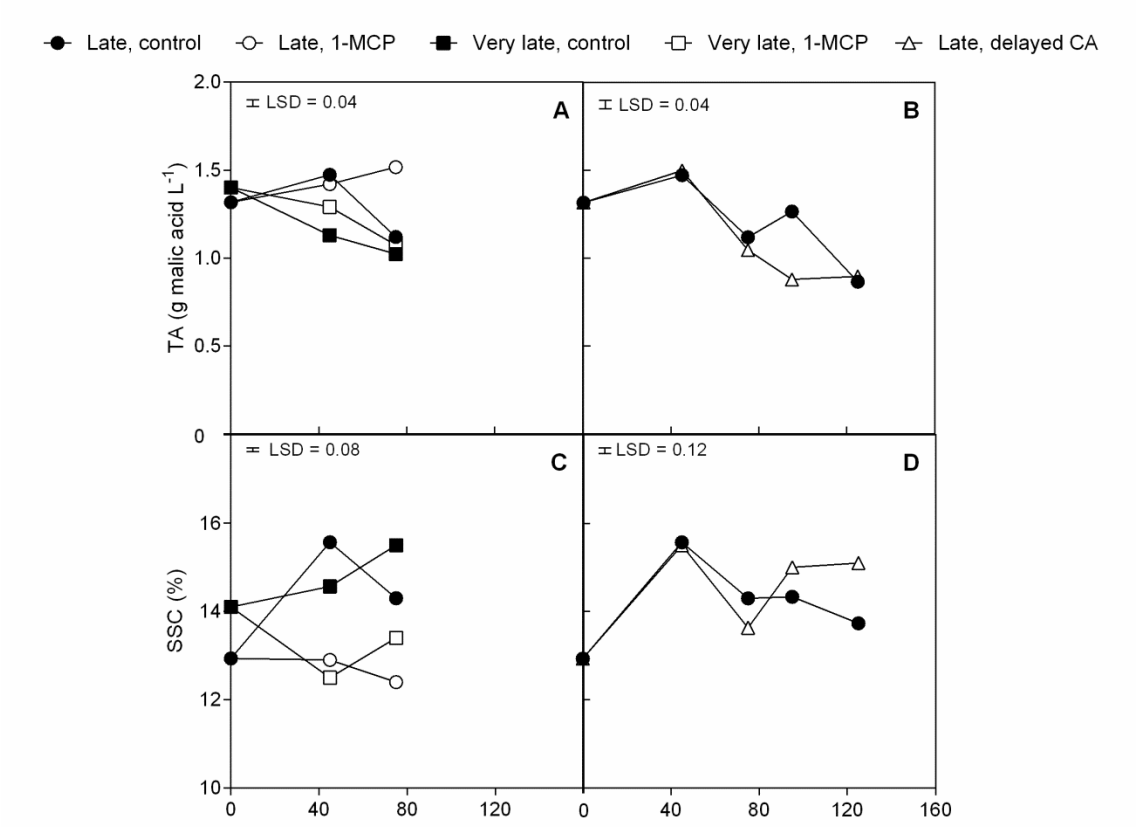

Figure 2: Effect of 1-MCP application (A and C) and delayed CA (B and D) on the titratable acidity (TA) and soluble solids content (SSC) of late harvested fruit (orchard 1) and very late harvested fruit (orchard 2). Values of TA and SSC represent the mean of 3 replicates of 9 fruits each ± SE and the vertical bars represent the Tukey LSD for the treatment effect at each time-point (p=0.05).

Under delayed CA fruit acidity decreased faster than under immediate CA, but after 125 days of storage, TA was similar in the fruit of both conditions (Fig. 2B). Concerning SSC, the levels were higher in the delayed CA stored fruits than in the control fruits from day 95 up to the end of storage (Fig. 1D). These results suggest that the ripening process occurs faster in the fruit stored under delayed CA than in control fruit.

Collectively these results showed that the 1-MCP treatment did not contribute for a better retention of quality in very late harvested fruit, whereas it was slightly beneficial in late harvested fruit. Regarding fruit storage under delayed CA, in general, this storage condition was not effective in maintaining fruit quality.

8.4.4. Effect of postharvest treatments on ascorbate, ethanol and acetaldehyde levels

8.4.4.1. Effect of 1-MCP treatment

The levels of total ascorbic acid at harvest were significantly higher in early harvested fruits than in late and very late harvested fruits which showed similar levels of ascorbate (Fig. 3A).

Regarding the early harvested fruits, although the levels of fermentative metabolites in the 1-MCP treated fruits were higher compared to the untreated and the ascorbate levels and DHA/AsA were equivalent, the treated fruits showed lower IBD incidence (Table 2). Similarly, control fruits despite presenting the lowest levels of fermentative metabolites, the lowest DHA/AsA ratio and a concentration of ascorbate similar to the registered in the other fruits (Fig. 2 and 3) had an IBD incidence of 15%, a two-fold higher incidence than the observed in the 1-MCP treated fruits (Table 2). These results indicate that other factors beyond fermentation and oxidative stress are involved in the development of IBD in 'Rocha' pear and that these factors may be related to the ethylene metabolism and length of storage under high CO_2 conditions.

In the fruits harvested at late and very late maturity stages ascorbic acid levels sharply decreased during the first 45 days of storage regardless of treatment. After 75 days of storage, late and very late harvested fruits treated with 1-MCP showed lower levels of ascorbic acid compared to control fruits but the difference was significant only for the late harvested fruits (Fig. 3A). For the fruits of both maturity stages, late and very late, no significant differences in DHA/AsA ratio were found between the control and 1-MCP treated fruits (Fig. 3B). Collectively, these results showed that 1-MCP treatment did not contribute for the maintenance of the antioxidant potential of late and very late harvested fruits (Fig. 3).

In contrast to ascorbic acid, significant differences in the levels of fermentative metabolites were observed between the fruits of the different maturity stages, especially when the fruit were treated with 1-MCP. After 45 days of storage, very late harvested fruits treated with 1-MCP had three-fold higher levels of ethanol and acetaldehyde than the other fruits (Fig. 4). High levels of these metabolites were related to higher IBD incidence (79%, Table 2). At day 75, the levels of ethanol and especially acetaldehyde decreased in the very late harvested fruit treated with 1-MCP but the ethanol levels remained significantly higher

than in control fruits (Fig. 3). The higher IBD incidence observed in the 1-MCP-treated fruits associated to the higher levels of ethanol clearly indicate that 1-MCP application in very late harvested fruits is not suitable.

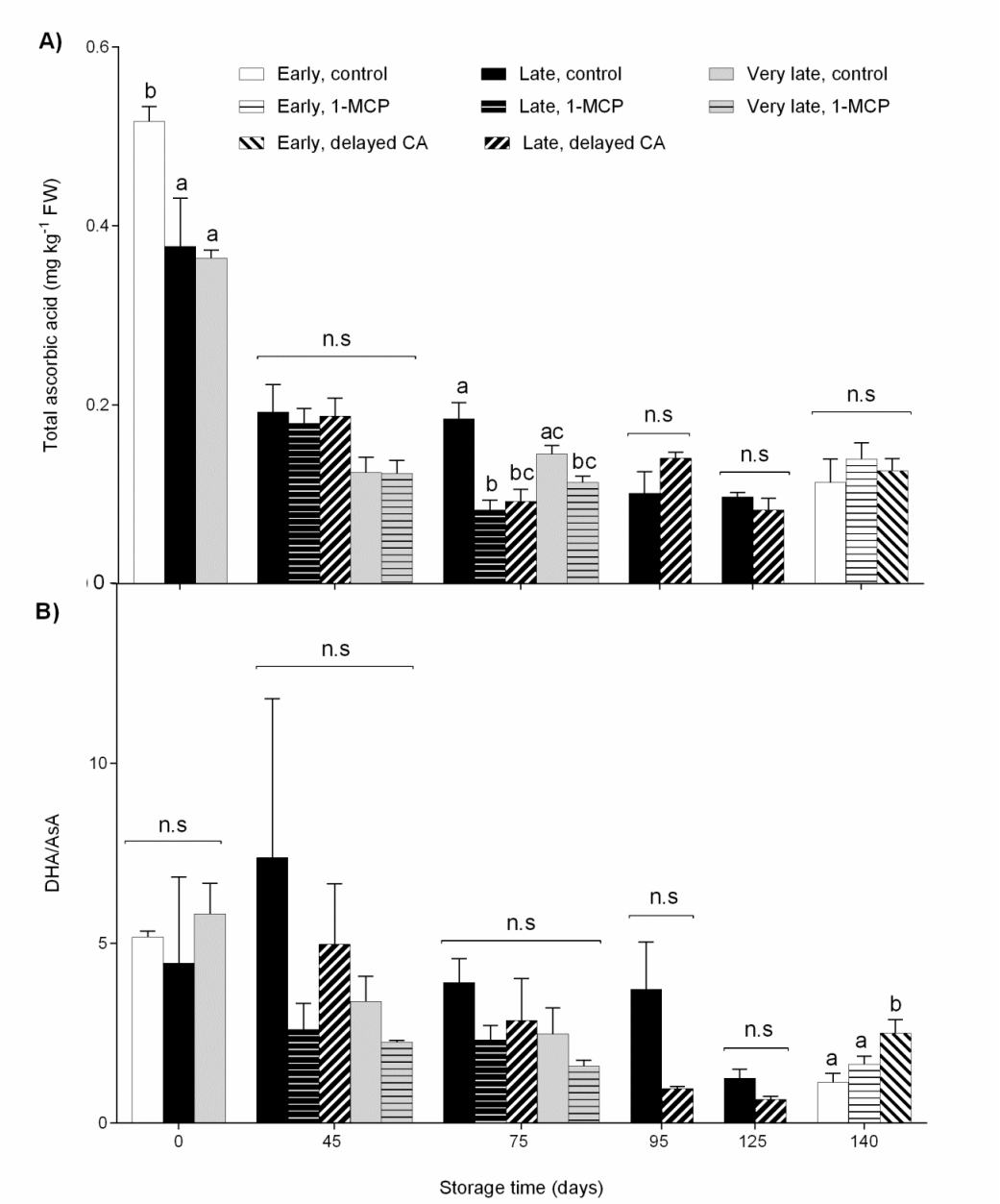

Figure 3: Changes in the levels of total ascorbic acid (A) and ratio between the oxidised and reduced form of ascorbate (DHA/AsA) (B) in late and very late harvested fruit stored under CA and treated or untreated (control) with 1-MCP. Results for late harvested fruit stored under delayed CA and early harvested fruit stored under immediate CA (control), delayed CA and CA after treatment with 1-MCP. Different letters above columns indicate significant differences among the treatments at each time point ($p \leq 0.05$).

In the late harvested fruits treated with 1-MCP, the levels of ethanol slightly increased up to day 75 and were higher than in control fruits (Fig. 4A). In contrast, the levels of acetaldehyde decreased and were lower in the 1-MCP treated fruit (Fig. 4B). The lower levels of acetaldehyde in the 1-MCP treated fruits may be related to the ethylene levels in these fruits. Indeed, it has previously been suggested that expression and activity of pyruvate decarboxylase, the enzyme responsible for the conversion of pyruvate to acetaldehyde, may be ethylene-regulated.[38] Therefore, the higher ethanol levels in conjunction with the lower levels of ascorbic acid in late harvested fruit treated with 1-MCP may have determined the higher IBD incidence (42%) compared to control fruits (12%).

8.4.4.2. Delayed CA storage

The currently accepted hypothesis for the higher IBD resistance of the fruits stored under delayed CA compared to immediate CA stored fruits is related to difference in energy charge.[28] Under delayed CA, the higher energy level maintained during the cold storage period would confer a higher fruit capacity to handle the abiotic stress resulting from the subsequent fruit exposure to CA conditions.[17,28] Under these conditions, fruits appeared to be better equipped to respond to adverse storage conditions.

The initial decrease in ascorbate levels observed in the delayed CA and control fruits after 45 days of storage may have determined the first occurrence of IBD (Fig. 3A). However, delayed CA stored fruits at this time-point had lower IBD incidence than control fruits. This may be explained by the aforementioned hypothesis that fruit would still have sufficient energy charge and be able to respond to the stress caused by CA. However, between 45 and 75 days of storage, whereas the levels of ascorbic acid in the control fruits remained constant, they sharply decreased in the delayed CA stored fruits (Fig. 3A). Such results are not in accordance with previous studies conducted in 'Conference' pears where the loss of ascorbic acid during delayed CA has been reported to occur mainly during the initial storage period before the implementation of CA.[39] It is concluded then, that the sharp decrease in the ascorbate levels combined with increased ethanol and acetaldehyde levels in the delayed CA during the first 75 days of storage may have been determinant for the high increase of IBD incidence in this work (Fig. 3, Fig. 4 and Table 2).

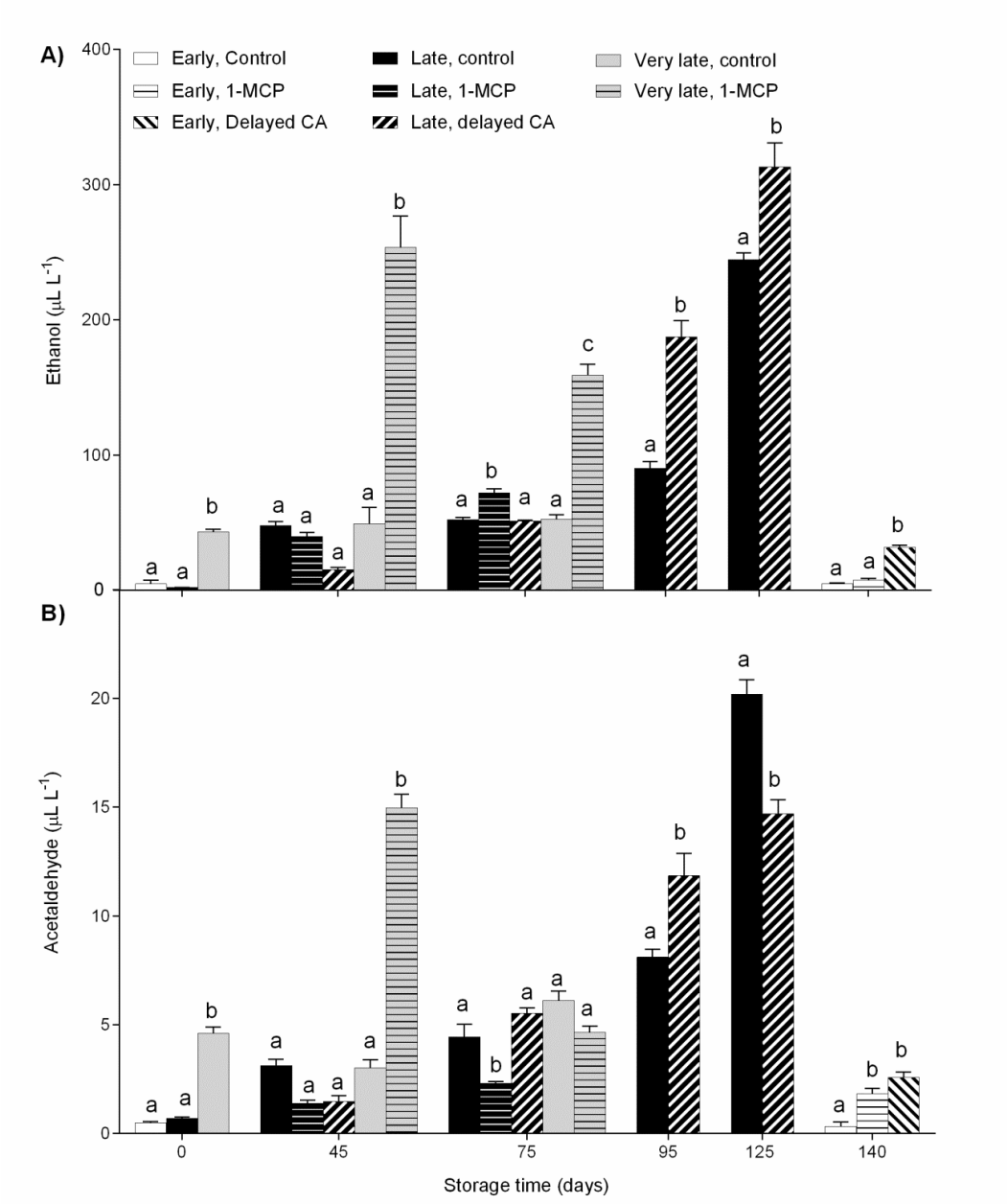

Figure 4: Changes in the levels of ethanol (A) and acetaldehyde (B) in late and very late harvested fruit stored under CA and treated and untreated (control) with 1-MCP, late harvested fruit stored under delayed CA and early harvested fruit stored under immediate CA (control), delayed CA and CA after treatment with 1-MCP. Different letters above columns indicate significant differences among the treatments at each time point ($p \leq 0.05$).

8.5. CONCLUSION

Collectively the results of this study showed that the efficacy of 1-MCP in preventing IBD in 'Rocha' pear treatment depends on initial fruit maturity, being effective on IBD prevention in early harvested fruits but not in late and very late harvested fruits.

Concerning the effect of 1-MCP treatment on maintenance of fruit it was dependent on fruit maturity at harvest with lower retention of quality attributes observed in very late harvested fruits.

'Rocha' pear storage under delayed CA, in contrast to the observed for other cultivars, was not effective in preventing IBD and may even increase its incidence in the fruits of the different maturity stages studied herein (early, late and very late). Delayed CA storage also did not contribute for a better maintenance of fruit quality in late and very late harvested fruits. According to our results the highest IBD incidence in the fruit stored in delayed CA was closely related to the elevated levels of ethanol and acetaldehyde and to a lesser extent to the reduced levels of ascorbate.

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CHAPTER 9.

General Discussion

9.1. GENERAL DISCUSSION

In the past, internal browning disorders (IBD) in pome fruits were studied in order to better understand their etiology and develop postharvest handling recommendations to reduce their occurrence during long term storage. From these previous studies it was concluded that pear behavior during long term storage is cultivar-specific. Establishing the scientific basis of internal browning disorders in 'Rocha' became even more essential when the application of diphenylamine (DPA), the only tool available to prevent IBD incidence in pears during long term storage, was prohibited by the European Commission. Due to the geography of 'Rocha' pear production few studies were conducted using this pear cultivar as a model and there is a lack of a precise description of the range of symptoms that occur in 'Rocha' pear. Also, the precise characterization of the etiology of IBD occurrence in the commercial storage settings of 'Rocha' pear was not previously done. With a proper understanding of the etiology, progress in the understanding of the mechanisms and the proposal of therapies becomes possible.

The working model used in this study to elucidate the biochemical basis and find clues on the molecular regulation of IBD in 'Rocha' pear can be summarized as follows: under CA fruits are exposed to hypoxia or even anoxia in the fruit core which may lead changes on fruit respiration and ROS accumulation (Ho et al. 2006; Pintó et al. 2001; Deuchande et al. 2016). ROS accumulation may induce the loss of membrane integrity in cells at the fruit core and fruit. Fruit inability to repair damage lead to membrane leakiness with PPO sequestered in plastids, and phenolic substrates present in vacuoles coming into contact (Nicolas et al. 1994). PPO-mediated oxidation of phenolics and subsequent polymerization of the *o*-quinones lead to the formation of the brown polymers (Vámos-Vigyázó 1981). The incapacity of the fruit to sustain the functioning of the antioxidant system may be related to the reduced energy charge derived from a change on respiration towards fermentation (R. H. Veltman et al. 2003).

9.1.1. Internal browning characterization

Internal browning disorders in pear can have various symptoms (Franck et al. 2007). Evidence suggests that, in 'Conference' pear, CO_2-related disorders differ from senescence related disorders (Larrigaudière et al. 2004), but limited gas diffusion and hypoxia at the center of the fruit is likely involved in both instances (Lammertyn et al. 2003). However, whether these symptoms are related to the same or to two distinct physiological disorders is still a matter of debate (Franck et al. 2007; Larrigaudière et al.

2004). The characterization of the symptoms of internal browning disorders occurring in in 'Rocha' pear during CA storage was established in this work (Chapter 2). We observed that IBD in 'Rocha' pear can be classified into two categories: wet necrotic breakdown and dry cavities, which may coexist in the same fruit. Both symptoms can have their occurrence restricted to the carpelar region or occur also in the mesocarp. From our study we concluded that cavities appear later during storage and that high CO_2 contributes to the development of internal browning associated to cavities. These observations are in accordance to the previously reported for 'Conference' pears (Larrigaudière et al. 2004).

9.1.2. Internal browning: the effect of fruit maturity at harvest and CO_2 partial pressure

Fruit maturity at harvest has been shown to affect fruits susceptibility to internal browning disorders during long-term storage in many pear cultivars such as 'Conference', Yali' and 'Seuri' Chinese pears (Verlinden et al. 2002; Crisosto et al. 1994; Lammertyn et al. 2000). In general, late harvested pears were more susceptible to IBD during CA storage than early and optimally harvested fruits. In this study similar results were observed for 'Rocha' pears with late harvested fruits being significantly more affected by IBD when compared to early and optimally harvested fruits (Chapter 2).

The highest incidence in the fruits stored under high CO_2 was correlated with increased levels of acetaldehyde and ethanol but not with changes in H_2O_2-scavenging potential (CAT, APX and POX activities) (results not shown). These results suggested that IBDs in 'Rocha' pear were mainly caused by changes in respiration. However, considering that this trial was conducted on a long-term basis and previous studies conducted in other pear cultivars reported an important influence of oxidative damage on IBD occurrence (Larrigaudière et al. 2001), further studies are required to address the possible involvement of oxidative stress during the first days of storage. Therefore, in Chapter 3 the short term effects of storage under high CO_2 on the antioxidant metabolism of 'Rocha' pear were studied.

9.1.3. The trigger of CO_2-related browning

Although CO_2-induced browning during CA storage has been observed in many varieties of apples (Elgar et al., 1998; Lau, 1998; Volz et al., 1998; Castro et al., 2007) and pears (Drake, 1994; Veltman et al., 1999), the underlying mechanism remains to be clearly established in 'Rocha' pear. In our first assessment on the major metabolic pathways involved on IBD (Chapter 2), IBD incidence was positively correlated to the levels of

fermentative metabolites but not with changes in the functioning of the antioxidant system. In this study the short-term effects of fruit storage under high CO_2 on the antioxidant system were evaluated and results showed that the higher disorder incidence during storage under high CO_2 was related to a significant decrease of total ascorbate levels during storage and increased DHA/AsA ratio and greater SOD and to some extent APX activities. Altogether, these results allowed concluding that IBD in 'Rocha' pear during storage under high CO_2 atmosphere is a consequence of oxidative stress within the fruit.

9.1.4. Biochemical basis of CO_2-related browning

The biochemical basis of IBD development during long-term storage under CA has been previously established for other pear varieties including 'Conference' (Larrigaudière et al. 2001; R. H. Veltman et al. 2003) and 'Blanquilla' (Pintó et al. 2001; Larrigaudière et al. 2004).

Despite of the general understanding of IBD, pear behavior is cultivar-specific and the study of the biochemical basis of IBD in 'Rocha' was not previously addressed.

In this study the biochemical basis of CO_2-related IBD in 'Rocha' pear was established (Chapter 4). The results indicated that the underlying mechanism involved in the occurrence and development of IBD in 'Rocha' pear involves the conjugation of both the antioxidant and fermentative metabolisms and also showed that PPO may play a role on IBD development. From the results, a proposed mechanism of CO_2-related IBD development was presented. In brief, during storage under high CO_2, a deregulation of mitochondrial function with consequent induction of fermentation occurs. As a result, there is an increased production of fermentative metabolites and a reduced energy charge. Due to the reduced energy production, cells may not be able to maintain membrane integrity and antioxidant system. H_2O_2 levels are reduced through the action of the antioxidant enzymes (SOD, CAT, APX, POX). Ultimately, the excessive accumulation of fermentative metabolites, and the decreased concentrations of ascorbate and H_2O_2, may lead to loss of intracellular structure allowing PPO and phenolics to react, forming the browning compounds.

9.1.5. The role of transcriptional regulation of the antioxidant and fermentative metabolisms on IBD

The exact etiology of IBD in pears remains unclear and little is known on the molecular regulation of the antioxidant and fermentative metabolisms of pears during long-term storage (Cascia et al. 2013; Pedreschi et al. 2007). Whereas for other pear cultivars,

some studies on molecular regulation of the antioxidant system were conducted (Cascia et al. 2013; Pedreschi et al. 2009), no information is available for 'Rocha' pear. In this study we investigated for the first time the underlying mechanisms involved in IBD occurrence and development, focusing on the transcriptional regulation of the antioxidant and fermentative enzymes as affected by high CO_2 and low O_2, correlating these parameters with the results of IBD incidence (Chapter 5). To the best of our knowledge, this is first study on transcriptional regulation of genes encoding antioxidant and fermentative enzymes in 'Rocha' pear during storage.

From the results obtained in this study we clearly showed that, when high CO_2 levels are combined with very low O_2 levels (1 kPa) additive effects contribute for increased IBD incidence. Based on the results two models for IBD development during storage under high CO_2 and high CO_2 combined with very low O_2 levels were proposed.

In brief, during storage under high CO_2 the expression of SOD is activated, and that of the H_2O_2-scavenging enzymes is repressed. The down-regulation of *Pc*MDHAR and *Pc*GR leads to an impairment of the antioxidant system and the levels of AsA are reduced in these fruits. Under high CO_2, fermentation is slightly activated and ethanol and acetaldehyde slightly accumulate also contributing for membrane damage.

During storage under low O_2 levels combined with high CO_2, the regulation of SOD and H_2O_2-scavenging enzymes is similar to the observed in the fruits stored under high CO_2 indicating that those fruits were also under oxidative stress and not able to detoxify H_2O_2. The ascorbate levels are also rapidly depleted in these fruits, despite differences in the transcriptional regulation of the enzymes involved on ascorbate regeneration. However, in the fruits stored under very low O_2 levels, contrarily to the observed in the high CO_2-stored fruits, there was a pronounced induction of fermentation showing that fruits switched from a predominantly aerobic to an anaerobic respiration.

9.1.6. Predictive modelling of internal browning disorders.

Despite the current knowledge regarding the factors involved on IBD development, there are still very few studies aiming at developing predictive models for IBD incidence in pears. Generating such predictive models would allow an early selection of the most adequate storage/treatment strategies to prevent IBD during long term storage. For instance, (Lammertyn et al. 2000) developed models for IBD incidence in 'Conference' pears based on intrinsic and external factors influencing disorder incidence, including sugar content, fruit firmness, size and weight, CO_2 and O_2 concentrations, harvest date and

storage temperature, but the authors concluded that more parameters were required to improve their predictive performance. Veltman et al. (2000) and Zerbini et al. (2002) also developed models for IBD in 'Conference' pears as a function of AA levels and the authors proposed AA- threshold levels below which IBD occurrence was expected.

In Chapter 6, biochemical and mineral markers of IBD were identified, and for the first time, accurate predictive models were developed and validated for IBD in 'Rocha' pear during storage. The results of this study showed that the concentrations of fermentative metabolites and AA during storage clearly influenced IBD incidence but also that the concentrations of some minerals, especially Cu at harvest, may have a significant impact on IBD development in 'Rocha' pear. Overall, IBD prediction models for 'Rocha' pear based on the concentration or accumulation/depletion rates of fermentative metabolites, ascorbic acid and mineral concentrations have been developed and validated in this work. The results evidenced AcDH, EtOH and AA as the most promising IBD markers, being thus used for modelling. The PLS model using EtOH and AcDH as predictors explained 89% of the variance in IBD incidence, whereas the univariate models based on EtOH and AcDH concentrations and accumulation rates explained between 89 to 94%. In contrast, the models based on AA levels and AA depletion rate showed lower predictive value, explaining 57% and 82% of the variance in IBD incidence, respectively. Models' validation confirmed the robustness of the model based on EtOH levels and allowed to propose a threshold level of 25 μL EtOH L^{-1} above which IBD may occur.

This work represents a major step forward in the prediction of IBD in 'Rocha' pear. Nonetheless, further studies are required to assess the suitability of these models in fruits stored under standard CA conditions.

9.1.7. Strategies to prevent internal browning during long-term storage under CA

The current restriction on the use of synthetic antioxidants such as DPA to prevent the incidence of IBD in pome fruits generated a need for developing new strategies to prevent IBD during long term storage. In this study two trials were conducted aiming at testing and understanding the effect of different control strategies on IBD development in 'Rocha' pear. Therefore, in in Chapter 7 fruit storage in two dynamic controlled atmospheres were evaluated in the prevention of IBD in 'Rocha' pear whereas in Chapter 8 the application of 1-MCP and fruit storage under delayed CA were tested, whereas as In both experiments the impact of the treatments on the antioxidant and fermentative metabolisms were analysed.

9.1.7.1. Dynamic controlled atmospheres

Dynamic controlled atmosphere (DCA) aims to provide optimal storage conditions improving the quality and preventing the development of physiological disorders. In this study, two DCAs were tested for the prevention of IBD in 'Rocha' pear: 1) DCA monitored by chlorophyll fluorescence (DCA-CF) and DCA monitored by ethanol (DCA-EtOH). To the best of our knowledge, the effect of storage under DCA in 'Rocha' pear behaviour and IBD development was not previously studied.

DeLong, Prange, and Harrison (2007) demonstrated for two varieties of apples, 'Cortland' and 'Delicious', that storage under DCA-CF resulted in better retention of fruit quality than storage under CA and reduced the incidence of superficial scald. The same effect was reported for 'Abbé Fétel' and 'Conference' pears (Folchi et al. 2015; Vanoli et al. 2015; Rizzolo et al. 2015). In our study the absence of IBD in the fruit stored under DCA-CF suggested that it may be an effective strategy to prevent IBD in 'Rocha' pear.

Regarding fruit storage under DCA-EtOH, it has been shown for 'Elstar' apples to contribute for a better maintenance of the quality attributes than storage under ultra-low oxygen, and lead to a decreased incidence of 'skin spots' than storage under CA (Schouten et al. 1997; Veltman et al. 2003). In contrast, our results showed that fruit storage under DCA-EtOH did not prevent the induction of fermentation and IBD development in 'Rocha' pear.

In this study we showed for the first time that DCA-CF, leading to reduced IBD incidence and high quality retention is a promising methodology for 'Rocha' pear long-term storage and that DCA-EtOH may not be suitable to prevent fermentation and IBD development.

9.1.7.2. 1-MCP treatment

The 1-MCP treatment has previously been shown to have beneficial effects in the prevention of IBD in optimally harvested 'Rocha' pear during long term storage (Silva et al. 2010; Almeida et al. 2015; Gago et al. 2013). However no studies were conducted in late harvested fruits which are known to be more susceptible to IBD (Deuchande et al. 2012). Our results clearly showed that the 1-MCP treatment of late and very late harvested fruits induce higher IBD and leads to a reduced retention of the quality attributes when compared to untreated fruits. In contrast, for early harvested fruits the 1-MCP treatment

resulted in reduced IBD incidence, which is in accordance to the previously observed results in optimally harvested fruits.

9.1.7.3. Delayed CA storage

Delayed CA has been previously reported to reduce IBD incidence in 'Conference' pears, even preventing its incidence in late harvested fruits which were previously identified as being more susceptible to IBD (Saquet et al. 2001; Verlinden et al. 2002). In contrast, for 'Rocha' pear no differences on IBD incidence were reported for fruits immediately stored under CA and subjected to a delay of 15-20 days before storage under CA (Morais et al. 2001; Almeida et al. 2015; Silva et al. 2010). According to our results, storage under delayed CA of late and very late harvested 'Rocha' pear, contrarily to the observed for other pear varieties such as 'Conference', is not effective in preventing IBD, and may even increase its incidence. In fact IBD incidence in these fruits was higher than the registered in the fruits immediately stored under CA and in the 1-MCP treated fruits and the highest incidence was closely related to the elevated ethanol levels combined with the reduced ascorbate levels.

Delayed CA storage also did not contribute for a better retention of the quality attributes during storage.

9.2. CONCLUSIONS

The concluding remarks resulting from this study are the following:

- IBD in 'Rocha' pear is characterized by wet necrotic breakdown or dry cavities which may coexist in the same fruit.
- The occurrence of IBD associated with dry cavities is more pronounced in fruits stored under high CO_2 conditions than under normal CA.
- Late harvested 'Rocha' pears are more prone to develop IBD than early and optimally harvested.
- High CO_2 combined with low O_2 highly accelerates IBD development acting synergistically on the induction of IBD in 'Rocha' pear but
- Oxidative stress is probably the trigger of IBD in 'Rocha' pear
- During storage under high CO_2, the switch from a predominantly aerobic respiration towards fermentation, with increased accumulation of fermentative metabolites, ethanol and acetaldehyde, highly contributes for hig IBD incidence.

- In general, IBD in 'Rocha' pear during storage under high CO_2 is also associated with reduced levels of H_2O_2 and ascorbate and increased levels of ethanol and acetaldehyde.
- PPO activity also seems to have an important involvement on IBD development
- Transcriptional regulation of genes codifying enzymes of the ascorbate-glutathione cycle and fermentation are differently regulated depending on the levels of O_2 and CO_2 used during CA storage. It suggests that the regulation of these genes is involved on the occurrence and development of IBD in 'Rocha' pear.
- High CO_2 and low O_2 have Multivariate and univariate models based on the concentrations of ethanol and acetaldehyde proved to be accurate for IBD prediction in 'Rocha' pear during storage under high CO_2.
- Multivariate models based on mineral concentrations at harvest may possibly be used to predict IBD during storage under CA.
- Cooper may be a promising mineral marker to predict at harvest, the risk of IBD development during storage under CA.
- The efficacy of 1-MCP treatment in the prevention of IBD and maintenance of quality attributes in 'Rocha' pear depends on fruit maturity at harvest. 1-MCP treatment is not effective in preventing IBD in 'Rocha' pears harvested at advanced maturity stages even inducing further browning in these fruits.
- Delayed CA induces IBD in 'Rocha' pear regardless of fruit maturity stage at harvest and does not contribute to enhanced maintenance of fruit quality
- Dynamic controlled atmosphere monitored by chlorophyll fluorescence is a promising methodology to prevent IBD in 'Rocha' pear during long term storage also contributing to a better retention of the antioxidant potential.
- Dynamic controlled atmosphere monitored by ethanol does not prevent IBD in 'Rocha' pear.

9.3. FUTURE PROSPECTS

In this work the scientific basis of IBD development in 'Rocha' pear was established representing a major step forward in regards to the understanding of the development of this disorder in this pear cultivar. However, some questions remain to be clarified and future research should focus in the following subjects:

- Development of non-destructive methods to detect IBDs at commercial scale

- PPO involvement on IBD development in 'Rocha' pear should be further studied at the biochemical and transcriptional levels.
- Develop models for IBD prediction in 'Rocha' pear using fruits from different orchards at different locations and in different years, and storing the fruits under standard CA conditions.
- The use of copper and other minerals as markers to prevent IBD during long term storage should be further studied. For instance applications of Cu to the soil can be done accompanying the uptake of this mineral by the fruit in the tree and then evaluating its influence on fruit susceptibility to IBD during storage.
- Developing protocols for 1-MCP application in 'Rocha' pear capable of promoting an adequate ripening behavior after long term storage, simultaneously preventing IBD incidence during storage and the evergreen phenomenon.
- Further studies on the use of DCA-CF for IBD prevention should be conducted with fruits from different orchards, locations, and in different years and harvested at different maturity stages to confirm the beneficial effect of this storage condition on maintaining better quality and preventing IBD across all possible scenarios.
- Another strategy that may prevent IBD in 'Rocha' pear and that has not been yet tested is fruit storage under DCA-RQ. Therefore a preliminary trial should be conducted to assess the potential of this technology for 'Rocha' pear long-term storage.

9.4. REFERENCES

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