Cold storage demand for 'Rocha' pear ripening: a comparison between a shorter and longer cold period

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Abstract

'Rocha' pear is a cultivar that when harvested at the recommended maturity stage (physiological condition that allows resisting to prolong cold storage), requires chilling exposure after harvest, to induce an autonomously ripening appreciated by the consumer. Fruit were stored immediately after harvest, for 6 d (batch 1) or 26 d (batch 2), in normal cold atmosphere storage, to further our understanding of 'Rocha' pear ripening under different short cold storage durations. The ripening events were then monitored at 0, 3, 7, and 10 d of shelf-life at room temperature (± 20 °C) through physicochemical and biochemical changes, including firmness, soluble sugars, malic acid, esters profile, and ethylene metabolism (1-aminocyclopropane-1-carboxylic acid (ACC) and 1-aminocyclopropane-1-carboxylic acid oxidase (ACO)). We found that ripening behaviour upon rewarming from both cold storage periods was similar, with fruit ripening with a typical pattern of ethylene production and respiration rates concomitant with a higher ACO activity. Soluble sugar and esters emission were not influenced by cold storage duration, but interestingly butyl acetate levels were significantly higher on pear, which was cold stored for 26 d.

Our data provide more novel information about Rocha' pear's ripening physiology, indicating for the first time that approximately one week of chilling temperature is

enough to promote ripening rate processes. Such knowledge could be an asset to the efficient management of 'Rocha' pear storage.

Keywords: Chilling requirements; Fruit quality; Fruit ripening; *Pyrus communis*

1. Introduction

European pear (*Pyrus communis* L.) cultivars are climacteric fruit with a peak of ethylene and respiration during ripening. Several ripening-related events are regulated by autocatalytic ethylene biosynthesis and action. It is worldwide accepted that ethylene production and action leads to several downstream metabolic processes essential for the ripening phenomena, including skin color changes, flesh softening, aroma volatiles emission, and flavour (Fan et al., 2018; Lelièvre et al., 1997). In pear, ripening promotes a burst in ethylene production, respiration rate, and aroma volatiles production in particular esters, alcohols, particularly ethanol (Villalobos-Acuña & Mitcham, 2008).

European pear (both Winter and Summer cultivars) needs low-temperature conditions to produce ethylene at a sufficient rate to stimulate ripening (Chiriboga et al., 2013). However, the requirements for a satisfactorily ripening varies among cultivars, maturity at harvest, length of cold storage, and temperature at storage (Villalobos-Acuña & Mitcham, 2008). Indeed, based on their ripening capacity after cold storage, pear is classified as Winter pear (such as 'Comice' and 'Beurré d'Anjou'), which demand prolonged exposure (minimum one moth depending on the cultivar) to cold temperatures to induce the autocatalytic production of ethylene and Summer pear (such as 'Blanquilla' and 'Conference'), which require short periods (few days or weeks depending on the cultivar) under cold temperatures (Dias et al., 2021). 'Rocha' is a Portuguese pear that has a tremendous economic impact (Almeida et al., 2016b; Saquet

& Almeida, 2017a) and, it is a cultivar with potential for long-cold storage but only under appropriate cold storage conditions (Almeida et al., 2016b; Saquet & Almeida, 2017a) and if harvested at a recommended maturity (pre-climacteric stage): firmness between 54 and 64 N, soluble solids content (SSC) between 11 and 13 %, titratable acidity (TA) between 2-3 g L^{-1} malic acid and starch index between 5 and 7 (ANP, 1997; Avelar and Rodrigues, 1999; Fonseca et al.,2005), generally designated as physiological maturity (Blanckenberg et al., 2016). Otherwise, the fruit would not be resistant to prolonged cold storage (Saquet, 2019). According to Agar et al. (1999), the consequences of cold storage at an advanced maturity stage may limit to reach the required organoleptic attributes for marketing (Silva et al., 2010; Villalobos-Acuña & Mitcham, 2008). When 'Rocha' pear is harvested at recommended maturity stage, a chilling postharvest exposure is needed to acquire the capability of ripening with quality, likewise other pear varieties (Dias et al., 2021; Avelar et al.,1994; Almeida et al., 2016a). And, according to producers it requires an exposure of approximately one month to chilling temperatures to ripen with quality. But reports can sometimes be contradictory on this matter. Saquet and Almeida (2017b), e.g., stated that 'Rocha' pear does not need chilling postharvest exposure to ripen. However, this is an exception, because in this study pear were late harvested (for example, starch index > 8) not meeting the parameters above mentioned for an optimal harvest. And, as referred above, pear harvested out of the optimal parameters are not suitable for long-cold storage. It has been demonstrated that pear at this late harvest maturity are more susceptible to pathological decay and physiological disorders (Avelar et al., 1994; Alpalhão et al., 2008) and are immediately sent to the retail market after harvest (ANP,1997).

77 According to the literature, cold storage is crucial because it activates the
78 autocatalytic ethylene production through the induction of the accumulation of the
79 enzymes involved in its biosynthesis: (i) ACC synthase (ACS), responsible for the
80 production of the substrate aminocyclopropane-1-carboxylic acid (ACC) and (ii) ACC
81 oxidase (ACO), responsible for the oxidation of ACC into ethylene (Blankenship &
82 Richardson, 1985; Chen, Mellenthin, & Borgic, 1983; Knee, 1987). Some authors claim
83 that ripening requirement differences between cultivars are connected to their ACC
84 content and, therefore, their sensitivity to ethylene (Biale & Young, 1981; McGlasson,
85 1985; Kevany et al., 2007). Also, low-temperature conditioning has proven to induce
86 other transcription factors related to fruit softening and sugar accumulation (Mitalo et
87 al., 2019).
88 Hence in this study we have focused on documenting the duration of cold exposure
89 necessary for ‘Rocha’ pear proper ripening when harvested at the optimal harvest. To
90 the best of our knowledge, there is no study supporting the capacity of 'Rocha' pear to
91 ripen under cold storage shorter than one month, which would be useful for the
92 industry. Accordingly, here we hypothesized that, much like Summer pear, 'Rocha' pear
93 harvested at the optimal harvest time can ripen under a short chilling storage period of
94 approximately one week compared to a more traditional extended period. Emphasis
95 was given to monitor global quality traits and physiological and biochemical changes,
96 like aroma volatiles production, sugars, organic acids profile, ACC content, and ACO
97 activity.

2. Material and methods

2.1. Fruit material and experimental design

'Rocha' pear (*Pyrus communis* L.) were harvested on August 2020 from three commercial orchards located in the Portuguese "West Region" of Cadaval (N 39° 25'; W 8° 54'; 120 m), at the optimal harvest period (flesh firmness of 59 N; SSC of 11.7 %; starch index of 7.05 ± 0.8 (1-10 scale); TA of 0.16 % expressed in malic acid equivalents. After harvest, fruit were divided into two batches of 80 pear each and placed under normal cold atmosphere storage (0 °C and 90-95 % relative humidity) for 6 d to mimic approximately one week (batch 1) and 26 d to mimic approximately one month (batch 2). After each batch, 10 fruit per time-point were removed from storage and analysed for ripening capacity after 0, 3, 7, and 10 d at room temperature (RT, ± 20 °C).

2.2. Assessment of quality attributes

Fruit quality was evaluated for firmness, skin colour, SSC, and TA. Firmness (expressed as N) was measured using a penetrometer (T.A. XT plus Texture Analyser, Stable Micro Systems, Cardiff, UK) fitted with an 8 mm diameter probe. Firmness was measured twice, on opposite equatorial sides of each fruit, in 10 fruit per time-point. Fruit skin surface colour was measured with a CR-400 colourimeter (Konica Minolta, Osaka, Japan) using the D65 illuminant and the CIE (Commission Internationale de l'Eclairage) parameters (L*, a*, b*). Results were expressed as hue angle (*h* °= arctan(b*/a*)), a colour coordinate suitable to monitor colour changes from the green hue of unripe fruit to the yellow hue of ripe fruit (McGuire, 1992). Two measurements were performed on opposite sides of the widest part of 10 replicates fruit per time-point.

SSC was measured in the fruit juice (3 replicates of 3 fruit each) using a digital refractometer PR1ATAGO CoLTD (Japan). TA was determined by homogenizing 10 g of pear with 90 mL of distilled water (blend of 3 fruit per replicate and 9 replicates per time-point) and titration of an aliquot with 0.1 M NaOH until pH 8.1. Results are expressed as g of malic acid per L of pear juice (g malic acid L^{-1}).

2.3. Ethylene and respiration rate measurements

Ethylene and respiration rate were measured according to Saquet et al. (2017a), with some modifications. Ethylene production (µg kg^{-1} h^{-1}) was measured in six replicates of 2 fruit each, at each time-point. Fruit were sealed inside 1.5 L glass jars at 23 °C for 2 h. A headspace of 1 mL was removed with an air-tight syringe via a rubber septum and injected into a Varian CP-3380 gas chromatograph (Walnut Creek, CA, USA) fitted with a capillary column TG bond alumina (Na2SO4) 50 m length and 0.53 mm i.d. (Thermo Fisher Scientific Inc., Marietta, USA). Hydrogen was used as carrier gas at a flow rate of 15 mL min^{-1}. The injector temperature was set at 160 °C, a flame ionization detector (FID) at 180 °C, and the following oven temperature program: hold time of 1 min at 40 °C, followed by 20 °C min^{-1} to reach 100 °C, and a holding time of 2 min at 100 °C.

Respiration rate (mg CO_2 kg^{-1} h^{-1}), represented by the release of CO_2, was measured in the same fruit samples used for ethylene. An infrared sensor (Dansensor CheckMate 3, METEK, USA) in a close circulation circuit was used to measure the headspace CO_2 concentration.

2.4. Determination of 1-aminocyclopropane-1-carboxylic acid (ACC) and ACC oxidase activity (ACO)

143 Fruit flesh tissue from 3 individual fruit per replicate and 9 replicates per time-point
144 was frozen in liquid nitrogen after each time-point and kept at –80 °C to conduct further
145 biochemical assessments.
146 The direct precursor of the plant hormone ethylene 1-aminocyclopropane-1-
147 carboxylic acid (ACC) was extracted according to Bulens et al. (2011) with some
148 alterations. The sample (6 g of frozen tissue) was homogenized in 8 mL of 80 % ethanol.
149 The homogenate was gently shaken for 30 min in melted ice and then centrifuged for
150 10 min at 12000 *g* and 4 °C. ACC extract was then mixed with 10 mmol L^{-1} of $HgCl_2$ and
151 NaOCl (5 % v/v)-NaOH (6 mol L^{-1}) (2:1) in a vial with an air-tight septum containing cap
152 and incubated in melted ice for 4 min. One mL headspace gas sample was removed from
153 the vial and injected into a gas chromatograph fitted with a FID detector. ACC
154 concentration (nmol ACC kg^{-1} of fresh weight) was determined by the ethylene
155 formation.
156 ACC oxidase activity was determined using 5 g of frozen flesh tissue homogenized
157 with 10 mL of a buffer containing 400 mmol L^{-1} at pH 7.2, 10 % glycerol, 30 mmol L^{-1}
158 ascorbic acid sodium salt, and 2 % PVPP. The homogenate was gently shaken for 10 min
159 in melted ice and then centrifuged for 30 min at 28000 *g* and 4 °C. Subsequently, the
160 supernatant was stored at – 80 °C until analysis. Enzyme activity was analysed as Bulens
161 et al. (2011). The mixture was incubated for 60 min at 30 °C, after which 1 mL of
162 headspace gas sample was injected into a gas chromatograph for ACC determination.
163 The results were expressed as nmol C_2H_4 per kg^{-1} L^{-1} on a fresh weight basis.
164 ***2.5. Esters determination by SPME-GC-MS***

2.5.1 Extraction and concentration of volatile compounds

HS-SPME was used for the extraction and concentration of the volatile compounds. SPME fibres with a 50/30 µm thickness of divinylbenzene/ carboxen/polydimethylsiloxane (DVB/CAR/PDMS; Supelco Co., Bellefonte, PA, USA) were used in this study. Fibres were activated before sampling according to the manufacturer's instruction. Esters concentration was determined in six replicates of 1 fruit each at each time-point according to Fonseca et al. (2020). Each analysed pear was randomly selected and sealed in a 1.5 L air-tight glass jar fitted with a rubber septum in the lid. Jars were closed and kept overnight at room temperature to reach equilibrium. Before sealing the jars, 20 µL of 500 mg L^{-1} 3-octanol was added as internal standard. The jar was then immersed in a water bath adjusted to 25.0 ± 0.1 °C, and the DVB/CAR/PDMS SPME fibre was inserted in the headspace through the septum for 1h. The volatiles adsorbed and absorbed on the SPME fibre coating were determined. After the extraction/concentration step, the SPME fibre was manually introduced into the port at 220 °C for 5 min for analytes desorption in a splitless mode.

2.5.2 GC-MS conditions

The volatiles were analysed with a Varian 240-IT-MS mass selective detector coupled with a Varian 450-GC gas chromatograph (Walnut Creek, CA, USA) equipped with a 50 m x 0.32 mm x 0.25 µm BP-21 column. A constant column flow of 1.0 mL min^{-1} helium was used as carrier gas. The oven temperature program was 40 °C for 1 min, increased at 2 °C min^{-1} to 220 °C, 220 °C for 30 min. Mass spectra were recorded at 70 eV in electron impact (EI) ionization mode. The ion source temperature was 210 °C, and the transfer line temperature was 160 °C. Mass spectra were scanned in the *m/z* range of 33-350. Tentative identification of the esters compounds was made by comparing mass

189 spectra of the samples with the data system library (NIST 98). Quantification was done
190 by the internal standard method, where the concentration of each volatile aromatic
191 compound was normalized to that of 3-octanol. The results are presented in nmol of
192 ester per kg of pear (nmol kg^{-1}).

193 ***2.6. Extraction and quantification of malic acid and sugars***

194 The free sugars and organic acid composition were obtained by extraction following the
195 methodology described by Lindo-García et al. (2019) and Giné-Bordonaba et al. (2017).
196 Briefly, freeze-dried flesh tissue (300 mg) was added to 2 mL of 62.5 % (v/v) aqueous
197 methanol solvent and placed in a thermostatic bath at 55 °C for 15 min, mixing the
198 solution with a vortex every 5 min to prevent layering. Then, samples were centrifuged
199 at 20000 g for 7 min at 20 °C. The supernatant was recovered, followed by solvent
200 evaporation using a speed vacuum and subsequent dissolution in water. The HPLC
201 analysis was performed using a Beckman Coulter System Gold HPLC (Knauer, Berlin,
202 Germany) coupled to RI and UV detector. The malic acid quantification was obtained
203 using an Aminex 37-H column (Bio-Rad, Berkeley, USA) at 40 °C and 5 mM H_2SO_4 as
204 mobile phase (flow rate: 0.6 mL min^{-1}). The free sugar profile was determined using an
205 Aminex HPX-87P column (Bio-rad, Berkeley, USA) at 85 °C and ultra-pure water as
206 mobile phase at a flow rate of 0.6 mL min^{-1}. The quantification of malic acid and free
207 sugars was achieved using standard calibration curves (0.1 – 40 mg mL^{-1}). The results are
208 represented as g of the sugar per kg of pear on a fresh weight basis (g kg^{-1}).

209 ***2.7. Statistical analysis***

210 The experiment was set up using a completely randomized design. Data were
211 subjected to analysis of variance (one-way ANOVA) for the effect of shelf-life time at
212 20 °C in the ripening of pear from each batch. The t-student independent samples test

was used to analyse the differences between batches at each time using the SPSS software. Significant differences for the effect of the storage atmosphere were determined by calculating Tukey's post-test significant difference at $p < 0.01$.

3. Results and discussion

According to the industry recommendations, 'Rocha' pear was harvested at the optimal harvest stage for this pear cultivar: flesh firmness of 59 N; SSC of 11.7%; starch index of 7.05 ± 0.8 (1-10 scale) and TA of 0.16%. According to 'Rocha' pear growers, pear harvested at this early stage needs a chilling exposure to ripen uniformly with the development of a pleasing aroma and texture after rewarming. In this way, 'Rocha' pear were stored for 6 d (batch 1) and 26 d (batch 2) at 0 °C and after this time removed from cold storage and evaluated for their ripening capacity upon rewarming.

3.1. Changes in the quality attributes

Since pear are climacteric and, thus, very sensitive to ethylene, ethylene dependent ripening phenomena's like softening, SSC increase and colour change are significant fruit attributes (Villalobos-Acuña & Mitcham, 2008; Itai et al., 2015; Lindo-García et al., 2019).

After removal from storage, firmness from both pear batches rapidly decreased from 3 to 7 d (ca. 6 and 7 N per day, respectively) and then more slowly until reaching a firmness below 10 N at 10 d (Fig. 1A). Albeit there were some significant differences between the two batches at times 0, 7, and 10 d, a similar pattern could be observed, demonstrating a significant firmness loss, characteristic of pear ripening upon removal from cold. Both stored pear batches achieved equal eating firmness (< 20 N) after 7 d at room temperature (Fig. 1A) (Cavaco et al., 2009; Nham et al., 2017). The softening behaviour reported herein is consistent with observations of previous studies in 'Rocha' pear harvested at physiological maturity, removed from cold storage after 96 d (Almeida et al., 2016b). Also, similar results were obtained by Chiriboga et al. (2013) on 'Conference' pear (Summer pear cultivar), cold-stored for 90 d. This information supports the evidence for the first time that a shorter (6 d) or more extended cold storage can

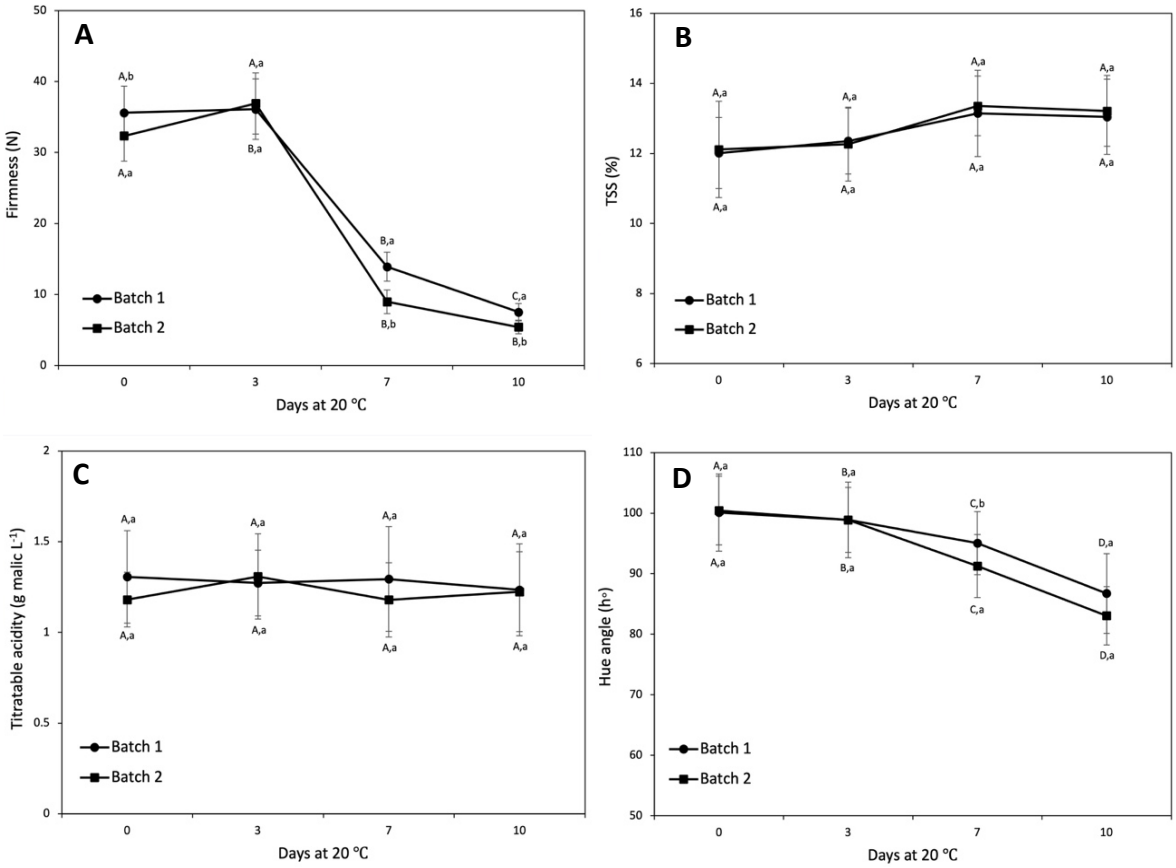

Fig. 1. Changes in flesh firmness (A), total soluble solids (B), titratable acidity (C) and hue angle (D) of 'Rocha pear at 20 °C after removal from storage of batch 1 (6 d cold) and batch 2 (26 d cold). Values are means ± standard deviation. Different capital letters indicate significant differences ($p<0.01$) across time for each batch. Different small letters indicate significant differences ($p<0.01$) between batches at each time-point.

promote a similar softening rate on 'Rocha' pear, which is a unique feature of this cultivar. For example, softening rate of ‘Bartlett’ pear (Summer cultivar) was much slower after 2 than 4 weeks under cold storage (Agar et al., 2000). It is interesting that softening started during cold storage since a decrease of ca. 20 % firmness was observed compared with the pear immediately after harvest.

In fruit with high sensitivity to ethylene, like pear, softening during low temperature is stimulated by basal levels of system 1 ethylene (system responsible for the basal levels of ethylene activated by low temperature and essential in fruit ripening upon warming) (Dias et al., 2021; Mitalo et al., 2019).

SSC in flesh fruit gives an estimation of dissolved sugar level (sucrose, glucose, and fructose) (Pasquariello et al., 2013). In this study, TSS hardly changed in both batches across the 10 d of ripening (Fig. 1B), which, according to the producer, is expected due to the low starch content (Rodrigues, 2005). Despite not having significant differences, a slight increase (ca. 10 %) on TSS was observed after day 3 and remained relatively constant after that (Fig. 1B). This increase in sugar content is another ripening indicator of 'Rocha' pear, and it is a consequence of the breakdown of complex carbohydrates, namely starch hydrolysis, into simple sugars. It is also an essential quality parameter since it reflects fruit sweetness (Taiti et al., 2017). These results were also confirmed in other studies on 'Rocha' pear (Saquet & Almeida, 2017b) and other pear cultivars, for example ‘Carmen’, ‘Coscia’ and ‘Anjou’ (Pasquariello et al., 2013; Xie, Zhao, & Wang, 2016). Moreover, according to Brackmann et al. (2005), significant changes in TSS usually occur when respiration starts since these sugars are used as substrates. But this only happens after a marked consumption of organic acids (the primary substrates for respiration).

TA estimates the content of organic acids in pear flesh and is also a crucial ripening trait. Generally, malic acid is the main organic acid in pear, so TA is commonly expressed in g malic acid L^{-1} (Eccher Zerbini, 2002). No significant differences were found for TA between batch 1 and batch 2 (Fig 1 C). TA at harvest was 1.6 g malic L^{-1} and decreased during cold storage until reaching values of ca. 1.25 g malic L^{-1}. After removal, there was no significant variation throughout time. TA found no marked differences in another study with 'Rocha' pear (Almeida et al., 2016a). However, due to ripening, a reduction of acidity resulting from organic acids metabolism conveyed by the respiration or their conversion into sugars by gluconeogenesis was expected (Halinska & Frenkel, 1991; Díaz-Mula et al., 2009). For example, in 'Blanquilla' pear (summer cultivar), a decrease of TA during ripening was reported (Lindo-García et al., 2019). Still, the lack of TSS increase is coherent with the lack of TA reduction.

Colour change from green to yellow is another fruit ripening marker of European pear due to chlorophyll degradation by the enzyme chlorophyllases (Martin et al., 2017). This enzyme is the first enzyme belonging to the chlorophyll catabolic pathway and is induced by ethylene and accompanied by the biosynthesis of carotenoids in fruit (Xie et al., 2017; Gago et al., 2015). Chlorophyll degradation measured by the hue angle reflects the ripening-related yellowing of 'Rocha' pear in both batches, highlighting the adequate ripening of pear stored only for 6 d (Fig. 1D). The initial hue angle was around 100°, corresponding to the greenish colour of the fruit. As the pear ripened at room temperature, the hue angle significantly decreased in both batches until the end of the ripening period evaluated herein. After 10 d at room temperature, pear from both batches registered a hue angle around 85°, corresponding to a fully visually ripe yellow pear (Fig. 1D) (Almeida et al., 2016a; Saquet & Almeida, 2017b).

3.2. Changes in respiration rate and ethylene production

Ripening of 'Rocha' pear is mainly associated with respiration and ethylene production. Both factors are influenced by cold storage and are responsible for triggering and maintaining many aspects of ripening reported herein (Agar et al., 1999). Respiration rate, which is measured by the production of CO_2, is very ripening-dependent (Fig. 2A). Both batches exhibited the same pattern during shelf-life, a continuous and significant increase upon rewarming of the fruit (Fig. 2A). Similar tendencies and values were observed in another work with 'Rocha' pear during ripening at shelf-life (Saquet & Almeida, 2017b). Despite respiration being a biological phenomenon that uses organic acids as primary substrates, the observed rise in respiration did not trigger a significant increase in TA.

Ethylene production followed a similar respiration pattern, a well-known behaviour in climacteric fruit (Fig. 2B). As expected, the autocatalytic rise in ethylene seems to follow the respiration spike after the third day of rewarming. The ethylene reached the highest rate at 10 d coincident with the maximum respiration rate, lower firmness and hue angle, and higher TSS. Albeit exhibiting a similar trend, differences in

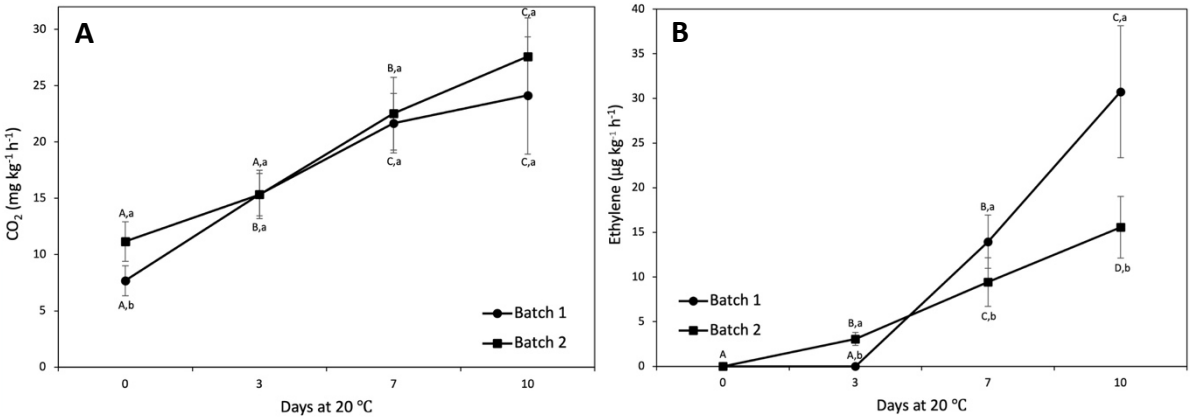

Fig. 2. Respiration rate (A) and ethylene production (B) of 'Rocha pear at 20 °C after removal from storage of batch 1 and batch 2, respectively. Values are means ± standard deviation. Different capital letters indicate significant differences ($p<0.01$) across time for each batch. Different small letters indicate significant differences ($p<0.01$) between batches at each time-point.

305 the kinetics of ethylene production were found between the two batches (Fig. 2B). Pear
306 from batch 1 showed a typical pre-climacteric behaviour with a delay (3 d) in the
307 initiation of ethylene production, in opposition to pear from batch 2. But then a steady
308 increase with time up to 30.74 μg kg^{-1} h^{-1} at 10 d was observed for pear with 6 d of cold
309 storage (batch 1) compared to pear with more time under cold storage (2-fold lower).
310 Lower ethylene production should trigger a lower ripening rate (Lelièvre et al., 1997),
311 but this was not verified for batch 2, because at 7 and 10 d at room temperature, a
312 significant loss of firmness (Fig. 1A) and colour fading (Fig. 1D) was observed for batch 2
313 compared to batch 1. Probably, since pear from batch 2 were stored for longer under
314 normal cold storage, some ripening could have started during cold storage, although at
315 a much slower rate than at room temperature, explaining the higher respiration rate,
316 lower hue value, and firmness, and lower ethylene production observed for batch 2. The
317 softening and chlorophyll degradation are coincident with the ethylene production
318 increases after 3 d at room temperature on batch 2, corroborating that this could be a
319 consequence of the significant increase of ethylene synthesis observed at this time
320 point.

321 Contrasting results regarding ethylene rate were demonstrated by Saquet et al.
322 (2017b), which have tested the ripening of 'Rocha' pear immediately after harvest, i.e.,
323 without chilling exposure. These authors verified that 'Rocha' pear achieved the peak of
324 ethylene production, 14.7 μg kg^{-1} h^{-1}, after 19 d at 20 °C, which is relatively lower and
325 later than our results. However, pear from their study were late-harvested, which could
326 explain their slower ripening capacity compared to chilling exposed pear from the
327 present study and also evidence the real need for chilling exposure for a more rapid
328 ripening. Besides that, according to literature and manufacturers, late harvest stage

329 pear is not appropriate for long cold storage (Saquet, 2019). Short exposure to chilling
330 temperatures was needed to induce a quicker ripening (Almeida et al., 2016b). Similar
331 results to ours were obtained by other authors working with different Summer cultivars
332 cold-stored for several weeks. In those, the ethylene production was significantly
333 increased after rewarming the cold-exposed pear compared to non-cold stored (Agar et
334 al., 2000; Lindo-García et al., 2021; Chiriboga et al., 2013). The fact that 'Rocha' pear
335 with only 6 d of cold storage was capable of producing large amounts of ethylene could
336 be due to the chilling stress promoted followed by the rapid exposure to room
337 temperature and also due to a higher sensitivity to ethylene of this cultivar which is
338 regulated at the receptor level (Kevany et al., 2007). Hence to better understand the
339 distinct behaviour of batch 1 and 2 regarding ethylene production, we have analysed
340 changes in ACC metabolism (Fig. 3).

341 ***3.3. Changes in ethylene synthesis***

342 ACC is the critical substrate for ethylene biosynthesis through the action of the
343 ACO enzyme. In this way, as expected, an increase in ACC content was observed
344 throughout storage accompanied by a greater ACO activity, especially after 3 d, parallel
345 to ethylene production increase in both batches (Fig. 3), which is in agreement with
346 previous reports on European pear (Villalobos-Acuña et al., 2011; Xie et al., 2016). This

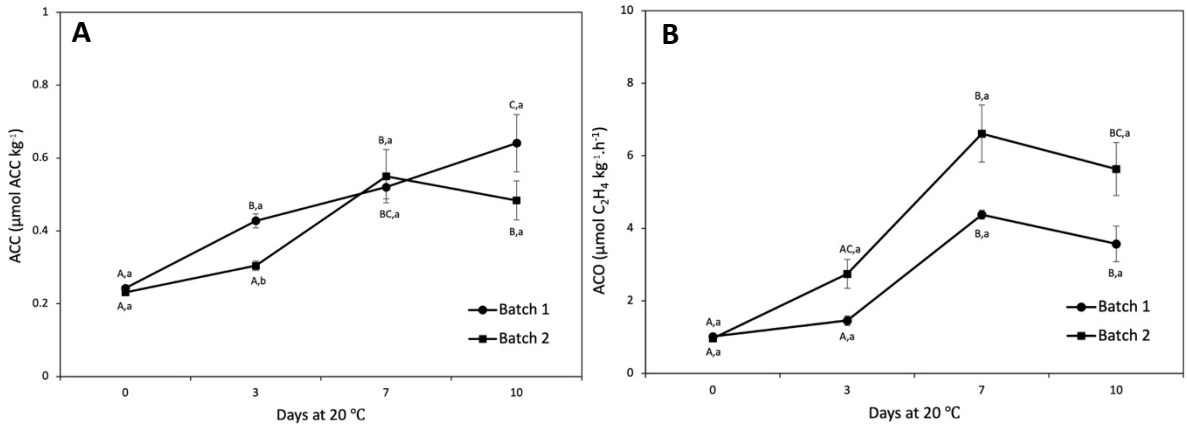

Fig. 3. Changes in ACC levels (A) and ACO enzyme activity (B) of 'Rocha pear at 20 °C after removal from storage of batch 1 and batch 2. Values are means ± standard error. Different capital letters indicate significant differences ($p<0.01$) across time for each batch. Different small letters indicate significant differences at $p<0.05$ for ACC and <0.01 for ACO between batches at each time-point.

ACC accumulation can be due to the different enzyme activities concerning temperature exposure. In batch 1 samples, a higher content of ACC after 10 d compared to batch 2.

Despite the difference in ACC content not being statistically different, it agrees with the higher values of ethylene observed at this time-point for batch 1. Moreover, ACO activity remained low in the first 3 d and sharply increased after that until day 7 (Fig. 3B) in agreement with the ethylene production registered in the first 3 d at 20 °C. It is important to highlight that, although not statistically different from batch 1, ACO activity in samples from batch 2 was better. This result could be an effect of the longer cold storage since, according to the literature, ACO enzymes production is strongly stimulated by cold treatment (Lelièvre et al., 1997) and corroborates the previous statement in section 3.2. In 'Barlett' pear, a similar cold storage effect on promoting ACO activity was reported for 12 weeks cold-stored pear upon removal from cold storage than those stored for shorter periods (Agar et al., 1999; Villalobos-Acuña et al., 2011). However, in our study, this higher activity has not been reflected in a higher ripening rate compared to batch 1. Also, *in vitro* enzyme activity can exceed that *in vivo*. The differences in ethylene production between batches are not exclusively explained by

differences in ACO activity, suggesting that differences observed between fruit from both batches could be due to intra-species variability.

Fonseca et al. (2005) verified that pre-climacteric 'Rocha' pear under chilling conditions for 2 months had an increase of ACO activity, hence consistent with our findings with the important difference that we have demonstrated the production of ACC and activation of ACO enzyme with only 6 d of chilling exposure, for the first time, advising a proper ripening. With this in mind, we analysed the changes in fruit assimilates (sugars and organic acids) and changes in fruit volatiles.

3.4. Changes in major sugars and malic acid

Since the content of soluble sugars was unchanged across time, we analysed the major sugars. Among the total soluble sugars in pear fruit, fructose, sucrose, glucose, and sorbitol are the predominant (Pasquariello et al., 2013) (Fig. 4 A, B), resulting from the hydrolysis of starch during ripening.

Ethylene production is associated with a stronger starch enzyme-degradation activity, responsible for cell wall degradation and transformation of complex carbohydrates into simple sugars (Asiche et al., 2018). The cell wall degradation was verified throughout the higher firmness loss between 3 and 7 d, but the increase in soluble sugars did not occur. These observations suggest that variations in these sugars might involve other regulatory mechanisms that are not regulated by ethylene and low temperature per se (Mitalo et al., 2019). In fact, previous studies in other fruit crops revealed that starch metabolism and sugar accumulation during fruit ripening have a component independent of ethylene (Defilippi, Dandekar, & Kader, 2004; Gao et al., 2007). Notwithstanding, a slight increase until 7 d in sucrose and fructose

concentrations was detectable, especially on batch 1, in line with the observed TSS values (Fig. 1B) and simultaneously with the burst in ethylene production (Fig. 2B).

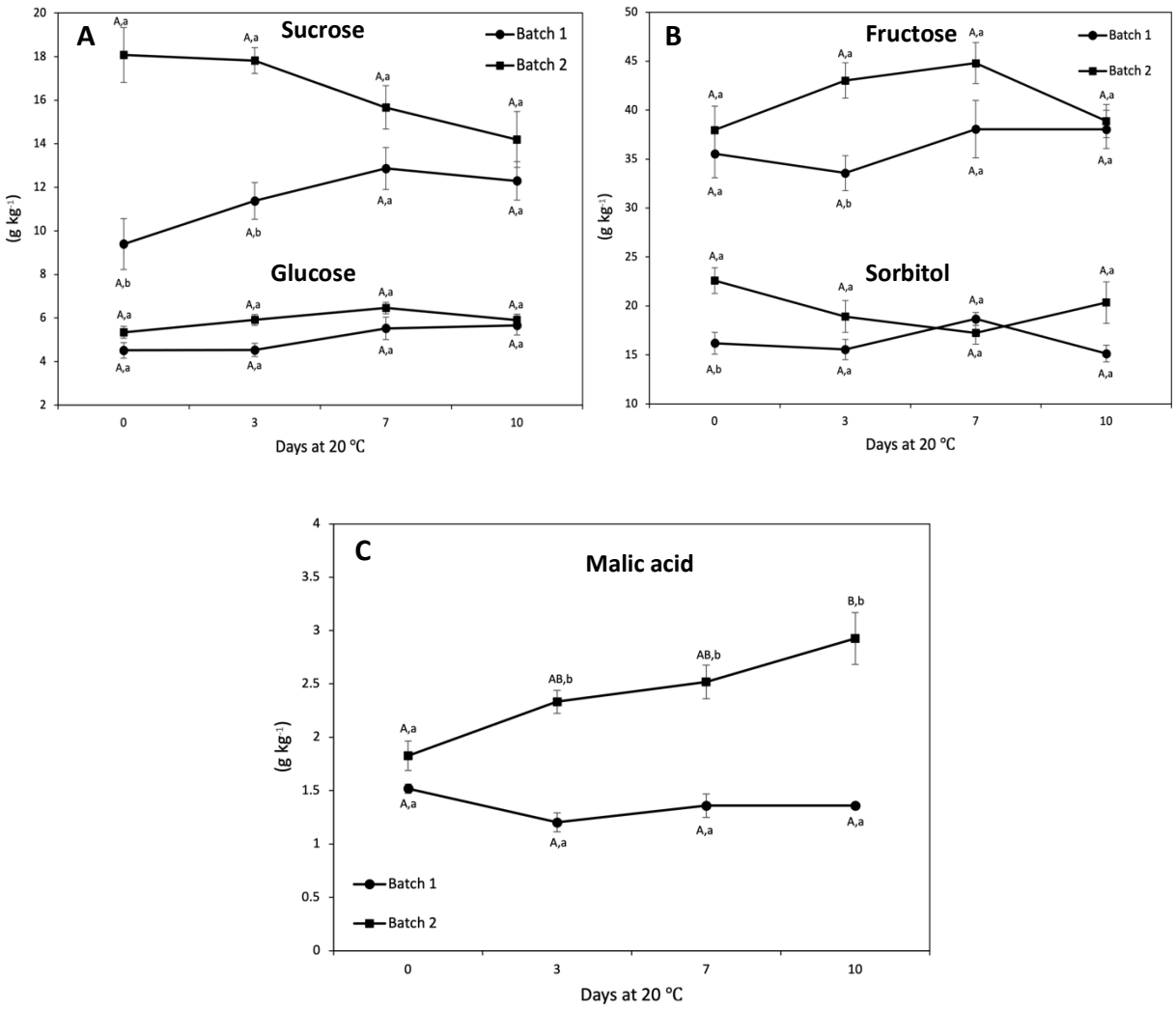

Fig 4. Changes in soluble sugars and malic acid of 'Rocha pear at 20 °C after removal from storage of batch 1 and batch 2. Values are means ± standard error. Different capital letters indicate significant differences ($p<0.01$) across time for each batch. Different small letters indicate significant differences at ($p<0.01$) between batches at each time-point.

Significant differences between the two batches were observed in sucrose and fructose, with batch 2 registering the higher values of soluble sugars. Glucose and sorbitol hardly changed across time. In batch 2 samples, despite no significant differences, sucrose levels slightly decrease (Fig. 4A) in parallel with a slight increase in fructose throughout the ripening time. This behaviour was also registered by Itai et al. (2015) in pear cold-stored for longer than 1 month. These authors also demonstrated

410 that more extended cold storage could result in higher sugar content, explaining the
411 higher content of sugars observed at 0 d in batch 2 samples. According to the literature,
412 a metabolic process known as cold-induced sweetening is responsible for converting
413 sucrose into glucose and fructose (Blenkinsop, Yada, & Marangoni, 2010; McKenzie et
414 al., 2013). It means that even under cold storage some ripening events starts, but at a
415 lower rate compared to ripening at room temperature. So, the higher the cold-storage
416 period the higher amount of some sugar will be found when removing pear from cold
417 storage, as observed. Despite this, at the end of 7 and 10 d, sugar content values did not
418 exhibit significant differences between the two batches.
419 Regarding malic acid content, contrasting results were obtained compared to TA
420 values (Fig. 1C), where no differences between batches were registered. The malic acid
421 content in batch 2 samples was significantly higher, and a slight increase was verified
422 between the beginning and the end of the shelf-life period. This was not demonstrated
423 in batch 1. The rise in malic acid values can be related to the increase in weight loss
424 under more extended cold storage (Sun et al., 2019) and the slower respiration that
425 probably occurred during the longer cold storage period. Malic acid content from batch
426 1 samples hardly changed during the 10 d at room temperature, but it is notable a minor
427 decrease between 0 and 10 d. Malic acid is an essential respiration substrate used as the
428 carbon source in the tricarboxylic acid cycle (Ma & Chen, 2003). Its use in the respiration
429 process can explain the observed pattern. However, a more noticeable decrease was
430 expected due to the respiration pattern observed in Fig. 2A. Further studies are needed
431 to understand other carbon sources used in the respiration process.
432 **3.5. Changes in the volatile profile**

Previous studies on fruit ripening have shown that the ripening process is characterized by the production of aroma volatiles highly regulated by ethylene production, namely esters (Mitalo et al., 2019; Wang et al., 2019). Accordingly, we analysed whether the development of 'Rocha' pear esters profile during ripening after shorter cold storage (Batch 1) impaired or enhanced ripening in comparison with the long cold storage (Batch 2) (Table 1). The predominant esters compounds in both batches were butyl acetate, hexyl acetate, and ethyl octanoate, as concluded by other studies in 'Rocha' pear (Avelar et al., 1994; Taiti et al., 2017; Barbosa, 2020). In combination, these compounds are responsible for the "pear", "floral", and "fruity" aroma. It is clear from table 1 that in both batches, in general, there was a significant increase of all esters throughout the ripening time. It is known that fully ripe pear have a higher concentration of esters (Wang et al., 2019), which corroborates the aptness of 6 d of cold storage in promoting a ripening with similar quality than a more extended cold storage. Notwithstanding the high variability observed between batches apparent differences in means, significant differences between the two batches were only observed in butyl acetate and hexyl acetate at day 0. The higher values registered on batch 2 samples suggest a role for cold storage duration in the modulation of the aroma development of 'Rocha' pear, although no higher ethylene production was observed on batch 2 samples.

Cold storage is known to markedly change volatile emissions (Gomes, Fabi, & Purgatto, 2016). In fact, our results demonstrate that butyl acetate, the ester responsible for the "pear" aroma (Wang et al., 2019), was the only compound influenced by the longer cold storage (batch 2) ($p<0.01$). Future sensorial analyses should be performed to clarify if the higher liberation of the "pear" aroma after longer cold storage was noticeable.

The high variability observed could be mainly influenced due to the biological variability plus the fact that this is SPME *in vivo* analysis, which poses some difficulties regarding the complexity and changing concentrations of target metabolites in the biological samples. However, in this study, *in vivo* analysis was carried out because it has the advantage of giving a better suggestion of what is really happing (Musteata & Pawliszyn, 2007).

Table 1. Changes in main esters of 'Rocha pear at 20 °C after removal from storage of batch 1 and batch 2. Values are means ± standard error. Different small letters indicate significant differences (at $p<0.01$) between batches at each time-point.

Days at RT	Esters (µmol kg^{-1})					
	Butyl acetate		Hexyl acetate		Ethyl octanoate	
	Batch 1	Batch 2	Batch 1	Batch 2	Batch 1	Batch 2
0	2.382 ± 0.249a	3.009 ± 0.323a	12.01 ± 0.53a	36.634 ± 0.18b	0.60 ± 0.13a	0.570 ± 0.060a
3	45.47 ± 8.14a	172.689 ± 8.111b	1437.14 ± 126.57a	6045.730 ± 189.79b	41.996 ± 3.943a	103.183 ± 14.293a
7	60.204 ± 8.381a	668.07 ± 70.50b	4495.82 ± 578.35a	3782.199 ± 189.593a	137.841 ± 29.582a	72.456 ± 4.679a
10	237.536 ± 40.995a	2295.542 ± 304.489b	28375.61 ± 6043.50a	55537.830 ± 5355.043a	73.293 ± 5.898a	173.249 ± 23.40a

4. Conclusion

European pear must be generally exposed to cold temperatures to develop the capacity to ripen with quality. Results from this study detailed information on the short cold-induced regulation of 'Rocha' pear ripening when fruit is harvested according to the local recommendations for optimum long cold storability. Ripening-related characteristics evidenced for the first time that approximately one week of cold storage can successfully promote proper fruit ripening similar to the traditional practice (approximately one month of chilling exposure). Our findings provide new information on the duration of chilling exposure after harvest of 'Rocha' pear to ripen to suitable eating quality. Data demonstrated that one week of cold storage is enough to stimulate ACO activities upon transferring the fruit to 20 °C resulting in good ripening. We

conclude that 'Rocha' pear is an early maturing pear with a more Summer than Winter ripening behaviour since this cultivar will ripen after harvest with little exposure to chilling temperatures. Further biochemical and sensorial studies are needed to understand the respiration process and the organoleptic impact of shorter and longer chilling exposure on 'Rocha pear'.

CRediT author contribution statement

Cindy Dias: Conceptualization, Methodology, Data analysis; Writing- original draft; Writing - review & editing, Investigation; **Tânia Ribeiro:** Data analysis; review; **Ana Cristina Rodrigues:** Validation; **António Ferrante:** Supervision, Methodology, Validation; **Marta W Vasconcelos:** Supervision, Methodology, Validation; **Manuela Pintado:** Conceptualization, Supervision, Methodology, and Validation.

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