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Recovery of ripening capacity in 'Rocha' pears treated with 1-MCP through the application of 1-NAA: Physiological and molecular analysis insights

Cindy Dias [a], Teresa R.S. Brandao [a], Ana S. Salsinha [a], Ana L. Amaro [a], Marta W. Vasconcelos [a], António Ferrante [b], Manuela Pintado [a,*]

[a] Universidade Católica Portuguesa, CBQF- Centro de Biotecnologia e Química Fina- Laboratório Associado, Escola Superior de Biotecnologia, Rua Diogo Botelho 1327, Porto, 4169-005, Portugal

[b] Institute of Crop Science, Sant'Anna School of Advanced Studies of Pisa, Piazza Martiri della Libertà 33, 56127, Pisa, Italy

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ABSTRACT

Storing 'Rocha' pear treated with 1-methylcyclopropene (1-MCP) in controlled atmosphere is a common commercial strategy to extend pear storage time and prevent postharvest disorders. However, this strategy represents a challenge to the fruit industry because 1-MCP treatment obstructs the normal fruit ripening, potentially affecting the quality to consumers. To explore possible mechanisms to reactivate ripening, 'Rocha' pears treated with 1-MCP were exposed to 2 and 4 mM 1-naphthaleneacetic acid (1-NAA) and stored at 20 ± 2 °C for 15 days. Typical ripening indicators, such as firmness, skin color, ethylene and aroma volatiles production, sugar content, and the genetic expression of ethylene-related enzymes (ACS and ACO) and receptors (*PcETR1*, *PcETR2*, and *PcETR5*) were determined over the 15 days of storage. A PCA analysis incorporating both physiological and biochemical data showed that 1-NAA promoted the recovery of ripening capacity in 1-MCP treated pears. Treating pears with 1-NAA led to increased activity of genes like *PcACS1*, *PcACS4*, and *PcETR2*, which are involved in ethylene signalling and production. This resulted in higher levels of ethylene and compounds associated with ripening, as well as softer texture, more yellow color, and higher sucrose content. The boost in ethylene-related gene activity likely heightened ethylene sensitivity and production in the treated pears. Consequently, these fruits showed accelerated softening, color change, and aroma development. This suggests that 1-NAA treatment can reverse the ripening inhibition caused by 1-MCP, possibly by enhancing ethylene sensitivity and production. This mechanism could enable consistent ripening of 'Rocha' pears after they are taken out of cold storage, and it may have similar effects on other fruits.

1. Introduction

The 'Rocha' pear (*Pyrus communis* L. cv Rocha) is a Portuguese variety, owning a Protected Designation of Origin (PDO), and being one of the most significant pear cultivars in Europe. This cultivar is renowned for its ability to withstand long cold-storage. However, it is also highly susceptible to postharvest injuries, as for example superficial scald and internal browning (Deuchande et al., 2016).

Since the banning of diphenylamine (DPA) due to legislative changes, the postharvest sector, particularly in the realm of pome fruits, has relied heavily on the ethylene inhibitor 1-methylcyclopropene (1-MCP) (Blankenship and Dole, 2003; Dias et al., 2021). This derivative of a cyclopropene, is instrumental in controlling postharvest disorders and extending the shelf life of climacteric fruits (Dias et al., 2021; Hu et al., 2019). However, in 'Rocha' pears, the concentration of 1-MCP required to effectively manage postharvest disorders also hampers the natural ripening process of the fruit (Rizzolo et al., 2014). In climacteric fruits, characterized by a surge in ethylene production known as system 2, and a simultaneous increase in respiration, 1-MCP binds irreversibly to the fruit's ethylene receptors (ETRs). This binding renders the fruit insensitive to ethylene, thereby halting downstream ripening processes (Watkins, 2006; Hewitt et al., 2020). This situation has posed a significant challenge for the Portuguese pear industry. 1-MCP prevents pears from achieving the desired organoleptic quality profile, making them easily rejected by consumers and leading to substantial postharvest losses (Gago et al., 2015). Amid growing consumer concerns over the declining flavor and texture of 'Rocha' pears, the industry is actively seeking effective methods to counteract the ethylene-blocking effects of

* Corresponding author.
E-mail address: mpintado@ucp.pt (M. Pintado).

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1-MCP (Zhou et al., 2015). Various approaches have been explored to circumvent or reverse the ripening inhibition in pears treated with 1-MCP, primarily involving physical treatments such as applying exogenous ethylene (Pongprasert et al., 2020; Minas et al., 2018), raising temperatures, or a combination of both (Rizzolo et al., 2018; Zhou et al., 2015). Nevertheless, these approaches frequently require substantial energy consumption and frequently fail to trigger ripening initiation.

It is well known that phytohormones serve as key regulators of fruit development and ripening (Cui et al., 2018; Siebeneichler et al., 2020). Recent studies have demonstrated that auxin, a phytohormone predominantly associated with plant development processes (Kende and Zeevaart, 1997), has an impact on ripening by accelerating fruit development and ethylene production (El-Sharkawy et al., 2014; Yue et al., 2020). Some authors have shown that the exogenous application of auxin accelerated ethylene production in apples (Yue et al., 2020), plums (El-Sharkawy et al., 2014), and peach cultivars (Tatsuki et al., 2013). In all studies, the authors observed that auxin triggered the expression of key ripening genes, such as 1-aminocyclopropane-1-carboxyl acid synthase (*ACS*) and ethylene response factors (*ERF*), coding ethylene biosynthesis enzymes and ethylene responses components, respectively (Bleecker et al., 1988; Yue et al., 2020; El-Sharkawy et al., 2014; Tatsuki et al., 2013).

Ethylene production involves two sequential steps: first the ethylene precursor S-adenosyl-methionine (SAM) transforms into 1-aminocyclopropane-1-carboxyl acid (ACC) through the action of ACC synthase (ACS). Subsequently, ACC oxidase (ACO) oxidizes ACC into ethylene (Bleecker et al., 1988; Zhang et al., 2017). Once synthesized, ethylene binds to ethylene receptors (ETRs), activating ethylene response factors (ERFs) that orchestrate biological changes, particularly ripening regulation (Chen et al., 2002; Fabi and do Prado, 2019). These changes include softening, starch-to-sugar conversion, impacting fruit texture, flavour, and aroma (Iqbal et al., 2017; Tucker et al., 2017).

Numerous studies have shown that 1-MCP inhibits the expression of genes responsible for initiating ripening and encoding ethylene biosynthesis enzymes by binding to ethylene receptors (ETRs) (Hao et al., 2018; Li et al., 2017, 2020).

Despite this, there have been no studies examining the impact of this auxin on 'Rocha' pears. Consequently, this study seeks to test the novel hypothesis that applying the synthetic auxin 1-NAA can counteract the ripening inhibition induced by 1-MCP. The research specifically aimed to assess if auxin could trigger the expression of ETR genes in 'Rocha' pear. This could lead to an increase in ethylene receptor sites, thus enhancing the ripening processes. Furthermore, key ripening indicators, including fruit firmness, soluble solids content, volatile organic compounds (VOCs), and ethylene production, were monitored throughout the ripening phase after treatmentwith 1-NAA.

2. Materials and methods

2.1. Fruit material and storage conditions

Pears (*Pyrus communis* L., variety Rocha) were handpicked from an orchard situated in Cadaval, in the West Region of Portugal (latitude N 39° 25'; longitude W 8° 54'; elevation 120 m) from August 1st to August 20th' 2020. The harvesting was conducted at the optimal stage as recommended by local growers, primarily considering parameters such as firmness (between 54 and 64 N), starch content (starch index between 5 and 7), sugar content (measured as soluble solids content, SSC, between 11 and 13 %), and titratable acidity (TA, between 2 and 3 g L^{-1} malic acid).

After harvest, fruits were immediately stored at 0 °C and 90–95 % relative humidity. After 7 days of cold storage, to ensure consistent temperature among fruits, the pears were treated with 312 ppb of 1-MCP (Smartfresh™, AgroFresh Inc.) for 24 h, following the manufacturer's guidelines. After the treatment, the containers were aerated, and pears were stored under normal atmosphere conditions (0 °C and 90–95 % relative humidity) for two months. After this period, the pears were moved to controlled atmosphere (CA) conditions maintained at −0.5 °C with 4 % oxygen and 0.5 % carbon dioxide levels for around 2 months until the initiation of treatments.

2.2. Pear treatments

Two experiments were conducted using pears previously treated with 1-MCP. Each experiment involved duplicate treatments with the auxin, carried out on two separate days. In each experiment, the pears were divided into two groups: the control group (CTRL) and the treated group (1-NAA), each consisting of 54 pears. In the 1st experiment, pears were immersed in a solution containing 4 mM 1-NAA for 2 h, based on previous studies using 1-NAA treatment in apples (Yue et al., 2020). In the 2nd experiment, pears were submerged in a solution containing 2 mM 1-NAA for 2 h to explore the possibility of reducing the concentration of 1-NAA. After treatments, all fruits were subjected to current industry practice conditions for ready to eat or ripen (20 ± 2 °C) for 15 days and sampled on days 0, 7 and 15. On each sampling day, eighteen fruits from each group were used for color, ethylene, respiration, and aroma measurements. Another set of eighteen fruits was used for firmness, soluble solids content (SSC), and titratable acidity (TA) measurements. Additionally, pear tissue was immediately frozen in liquid nitrogen and kept at −80 °C for biochemical and genetic analysis.

2.3. Evaluation of firmness, skin colour, soluble solids, and titratable acidity

Firmness (expressed as Newtons, N) was determined using the penetration test on a texturometer (T.A. XT plus Texture Analyser, Stable Micro Systems, Cardiff, UK), equipped with an 8 mm diameter probe. Measurements were taken at two opposite equatorial sides of each fruit, and the data were averaged from eighteen fruits per time-point and per treatment.

The pear peel color was assessed on opposite sides of the widest section of eighteen fruits from each condition using a CR-400 colorimeter (Konica Minolta, Osaka, Japan) with the D65 illuminant and the CIE (Commission Internationale de l'Eclairage) parameters (L*, a*, b*). Results were presented as hue angle ($h^\circ = \arctan(b^*/a^*)$), a parameter that indicates colour changes from the green hue of unripe fruit to the yellow hue of ripe fruit (McGuire, 1992).

SSC of the fruit was assessed using a digital refractometer (PR1A-TAGO CoLTD, Japan)), with the juice from three fruits (six replicates of three fruits each).

TA was measured by titration with 0.1 M NaOH until pH 8.1, after homogenization of 10 g of pear tissue with 90 mL of distilled water (six replicates of three fruits each). Results were expressed as g of malic acid equivalents per kg of pear on a fresh weight basis (g kg^{-1}).

2.4. Ethylene production and respiration measurements

Ethylene produced at each time-point was measured in six replicates of three fruits each. Three fruits were incubated in 1.5 L glass jars at 23 °C for 2 h. Following this, 1 mL of headspace was collected and injected into a gas chromatograph (Varian CP-3380 gas chromatograph Walnut Creek, CA, USA)) equipped with an activated alumina column (50 m length and 0.53 mm i. d. Thermo Fisher Scientific Inc., Marietta, USA) and a flame ionization detector (FID) set at 180 °C Saquet and Almeida (2017). Results were expressed as μg per kg of pear per h. Respiration rate (mg CO_2 kg^{-1} h^{-1}) was assessed using the same fruit samples used for ethylene measurement, employing an infrared sensor (Dansensor CheckMate 3, METEK, USA) in a closed circulation circuit.

2.5. Esters determination by SPME-GC-MS

2.5.1. Extraction and concentration of volatile compounds

Extraction and concentration of the volatile compounds were performed using headspace-solid phase microextraction (HS-SPME). 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, following previous works (Dias et al., 2022), and activated according to the manufacturer's instructions. For each extraction, 5 g of pear pulp tissue were placed in 20 mL screw-cap vials containing 1.8 g NaCl, as per Qin et al. (2012), along with 20 μL of 50 mg L^{-1} 3-octanol as an internal standard. Each vial was then placed at 40 °C for 2 min, followed by exposure of the SPME fibre to the headspace for 40 min to adsorb the volatiles. Finally, the fibre was introduced to the heated injector port of the chromatograph for desorption at 220 °C for 10 min.

2.5.2. GC-MS conditions

Volatiles were identified and quantified using a Bruker EVOQ Triple Quadropole mass selective detector, coupled with a Bruker 456-GC gas chromatograph equipped with a 50 m × 0.25 mm x 0.2 μm CP WAX-58 (FFAP) column. Helium, flowing at a rate of 1.0 mL min $^{-1}$, served as the carrier gas. The injector temperature was set at 250 °C. The oven temperature program was as follows: 40 °C for 1 min, increased at a rate of 2 °C min^{-1} to 220 °C, and held at 220 °C for 5 min. Mass spectra were scanned in the *m/z* range 40–350 amu. The ion source temperature and the transfer line temperature were maintained at 230 °C and 250 °C, respectively. Mass spectra were scanned in the *m/z* range of 33–350. Identification and quantification of ester compounds was achieved using authentic standards curves, and the results were expressed in nmol of volatile per kg of pear on a fresh weight basis (nmol kg^{-1}). The reported values represent the mean of six biological replicates, each consisting of three pears.

2.6. Determination of malic acid and sugars

Malic acid and free sugars contents were obtained following the methodology described by Lindo-García et al. (2019) and Giné-Bordonaba et al. (2017). Approximately 2 g of frozen tissue was homogenised in 5 mL of 62.5 % (v/v) aqueous methanol and heated to 55 °C for 15 min, with the solution mixed every 5 min to prevent layering. The samples were centrifuged at 20000 *g* for 7 min at 20 °C and the supernatant was evaporated using a speed vacuum and subsequently dissolved in water. The HPLC analysis was performed using a Beckman Coulter System Gold HPLC (Knauer, Berlin, Germany), coupled to RI and UV detector. Malic acid quantification was conducted using an Aminex 37-H column (Bio-Rad, Berkeley, USA) at 40 °C, and 5 mM H_2SO_4 as the mobile phase (flow rate: 0.6 mL min^{-1}). Free sugars profiling was determined using an Aminex HPX-87P column (Bio-rad, Berkeley, USA) at 85 °C, with ultra-pure water as the mobile phase, at a flow rate of 0.6 mL min^{-1} flow rate. The quantification of malic acid and free sugars was performed with standard calibration curves (0.1–40 g L^{-1}). The results represent the mean of six replicates of three fruit each and expressed as grams of sugar or malic acid per kg of pear on a fresh weight basis (g kg^{-1}).

2.7. RNA extraction and qRT-PCR

Around 100 mg of frozen ground tissue was submitted for the extraction of total RNA using the Spectrum Plant Total RNA Kit (Sigma, Italy), according to the manufacturer's instructions, except that the final elution volume was 30 μL. RNA concentration and quality were determined using a NanoDrop N-100 spectrophotometer (NanoDrop technologies). For reverse transcription, 100 ng of RNA was used with the SuperScript® IV cDNA Synthesis Kit, following the manufacturer's methods (Invitrogen) and oligo(dT) primers. qRT-PCR analyses were carried out using iTaq™ Universal SYBR® Green Supermix (BIO-RAD) in 20 μL on a plate for the ABI7300 (Applied Biosystems) equipment, consisting of 2 μL of c-DNA (1/3 dilution), 10 μL of Green Supermix, 0.2 μM of forward and reverse primer, and 7.2 μL of sterile water. Primers were obtained from conserved regions of the multigene family of *PcACS*, *PcACO,* and *PcETR,* which were available in gene data banks or the literature (Xie et al., 2017). *PcActin* was used as the housekeeping gene to normalize the expression of the target genes. The specificity of the primers was checked by the melting curve analysis. The PCR reaction was carried out with an initial step at 50 °C for 2 min, followed by denaturation at 95 °C for 10 min, and by 40 cycles of denaturation (95 °C for 15 s) and annealing/extension for 1 min at 60 °C. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method using samples at 0 days after treatment calibrated as 1. The relative expression for each gene was calculated in relation to the values at 0 days of shelf-life. The values are average of three independent runs performed for each biological replicate. Each treated group consisted of three biological replicates, each comprising three fruits.

2.8. Statistical analysis

The average values of each variable across shelf-life time were compared by analysis of variance (one-way ANOVA), with post-hoc Tuckey's test for significant means distinction. Additionally, the independent samples *t*-test was used to detect significant differences between the two conditions for each experiment. The significance level was set at 1 % in all cases.

A Principal Component Analysis (PCA) with varimax rotation was conducted to reduce the number of all studied variables by considering their correlations and generating principal components, which represent clusters. The criteria used to determine the number of relevant principal components included identifying eigenvalues higher than 1 and assessing the proportion of variance explained by each component. A threshold of 64 % cumulative variance of the components was applied to guide this decision. Variables were included in the principal components if their loadings exceeded 0.6 in the component's matrix.

Data analyses were executed using IBM SPSS Statistics 27 for Windows® (SPSS Inc., Armonk, NY, USA).

3. Results

To provide an initial comprehensive assessment of the impact of the auxin 1-NAA on restoring 'Rocha' pear ripening following 1-MCP treatment, a PCA was conducted. This analysis encompassed the two experiments with 1-NAA (2 and 4 mM) along with their corresponding control groups (CTRL2 and CTRL4). To address the natural biological variability in data from the two experiments, namely the different physiological statuses of pears at the beginning of each experiment, and to allow a more logical interpretation of the data, the data was normalized by the values observed at day 0 of shelf-life in each experiment. In this data-set, 32 variables were used for the PCA: four physicochemical parameters (firmness, hue angle, SSC and TA), respiration rate, ethylene production, fourteen VOC's, seven genes, three free sugars, sorbitol and malic acid concentrations. Data was reduced to two components capturing 64.34 % of total variability (PC1 = 40.55 % and PC2 = 23.80 %; Fig. 1A). The scores biplot (Fig. 1B) showed mainly three groups along the first component, differentiating samples across shelf-life time.

On the positive side of PC1, we find the samples treated with 1-NAA at 15 days (for both concentrations), exhibiting lower firmness values, higher ethylene and VOC production, and increased respiration rates. Furthermore, the application of auxin to 1-MCP-treated fruits led to heightened genetic expression of key genes involved in ethylene biosynthesis and signalling, including *PcACS's*, *PcACO*, and *PcETR2*. Conversely, on the negative side of the PC1 axis, we observe samples at 0 days of shelf-life, characterized by higher firmness and a greener color,

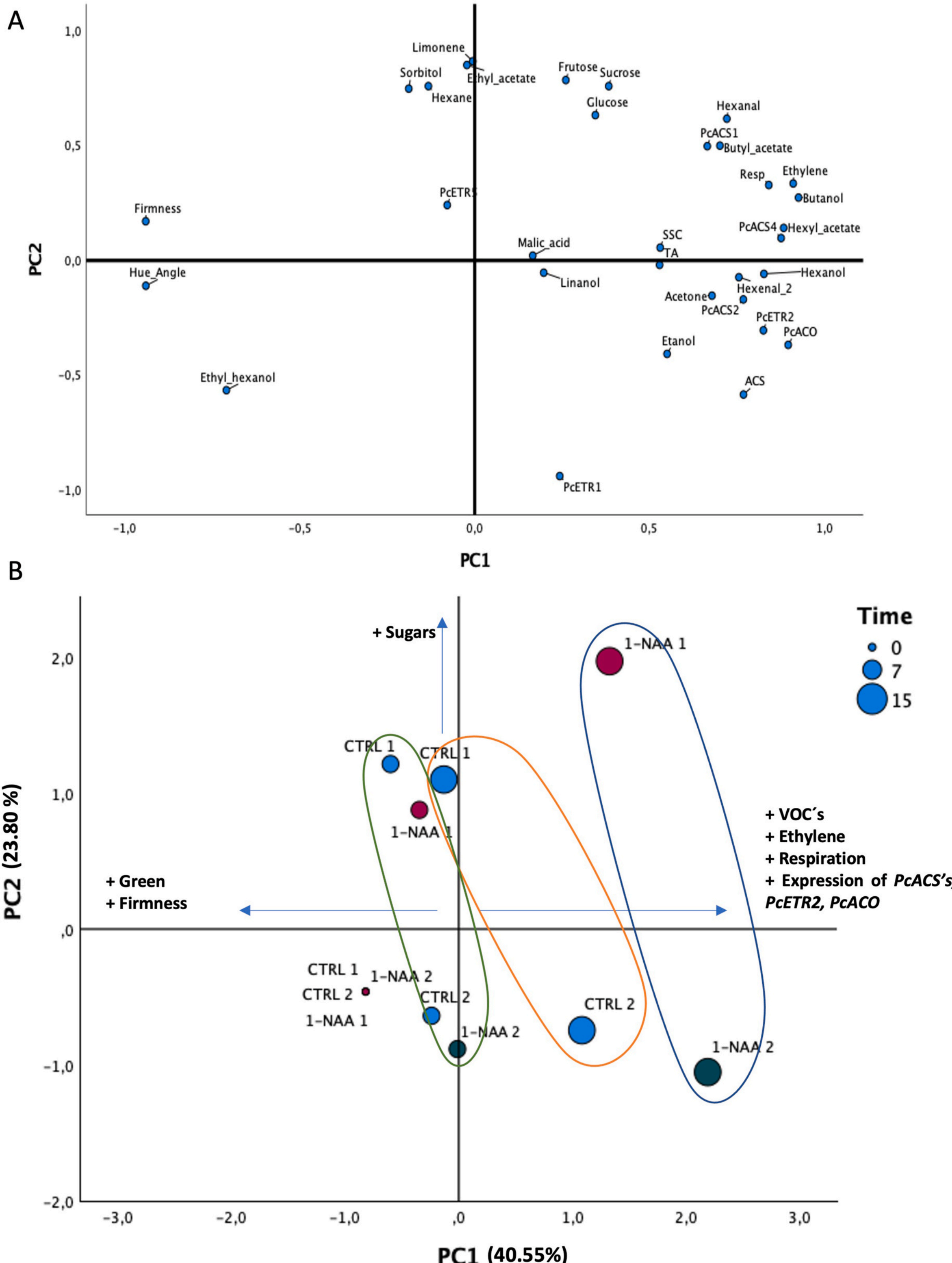

Fig. 1. Principal component analysis biplot: **A** – distribution of variables loadings along PC1 and PC2 and **B** - data scores along PC1 and PC2. CTRL 1 (blue circles) and 1-NAA 1 (red circles) represent, respectively, control and treatment with 1-NAA at 4 mM in the 1st experiment. CTRL 2 (blue circles) and 1-NAA 2 (green circles) represent, respectively, control and treatment with 1-NAA at 2 mM in the 2nd experiment. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

as expected. Those closer to the origin represent samples at 7 days of shelf-life and the CTRL groups at 15 days. At 7 days of shelf-life, samples treated with 1-NAA are not significantly separated from the CTRL group compared to what is observed at 15 days of shelf-life, indicating that the effect of 1-NAA was more pronounced between 7 and 15 days of shelf-life. Additionally, the CTRL groups at 15 days closely located to their respective groups at 7 days of shelf-life, suggesting that between 7 and 15 days, the CTRL groups (i.e., non-treated samples) did not undergo significant changes in their ripening status. PC2 distinguishes between different groups based on sugar and some VOC levels. Samples from experiment 1 (1-NAA at 4 mM) are positioned on the positive axis, displaying higher levels of sorbitol, free sugars, and the volatiles limonene, ethyl acetate, and hexane, in contrast to samples from experiment 2 (1-NAA at 2 mM).

We further investigated the potential impact of 1-NAA on the ripening recovery of 1-MCP-treated 'Rocha' pears by examining selected key parameters identified from the PCA analysis.

In both experiments (1 and 2), 1-NAA treatment significantly boosted ethylene production, reaching levels approximately twice as high as those of the CTRL pears after 15 days of shelf-life conditions (Fig. 2A and Table S1). As expected, the exclusive effect of 1-MCP on the CTRL pears suppressed ethylene production, particularly during the initial 7 days of shelf-life, where no significant increase was observed. However, the application of auxin to 1-MCP-treated pears restored ethylene biosynthesis during the shelf-life period (Fig. 2A). Similarly, 1-NAA treatment of 'Rocha' pears led to an increase in the respiration rate compared to control fruit, with increments of 38.4 % and 36.6 % observed after 15 days, respectively (Fig. 2B–Table S1). This indicates that the respiration rate exhibited a similar pattern to ethylene production as ripening progressed. Regardless of concentration, treatment with 1-NAA notably accelerated fruit softening, particularly evident after 15 days of storage, coinciding with increased ethylene production. Meanwhile, the rate of firmness loss in pears solely under the influence of 1-MCP (i.e., CTRL condition) was comparatively lower (Fig. 3A). In experiment 1, fruit treated with 1-MCP retained firmness, whereas in experiment 2, there was a slight decrease (Fig. 3A). Another important ripening indicator is the hue angle, a color parameter that adequately explains the surface color changes from green to yellow (Saquet and Almeida, 2017). Upon observing Fig. 3B, distinct differences are evident in the first experiment between the CTRL and the 4 mM 1-NAA treatment groups. After 15 days, following 1-NAA treatment, skin color hue values decreased rapidly to 80°, indicating a ripe yellow color (Table S1), while 1-MCP significantly inhibited the fruit's yellowing process. In the second experiment, despite the higher ethylene production and firmness decrease observed in pears treated with 1-NAA 2 mM (Fig. 1A and 2A), no significant differences between the CTRL and the 1-NAA treated pears hue angle were found.

As ethylene production and respiration rates increased, the sucrose content also rose steadily throughout storage in fruits treated with 4 mM 1-NAA, surpassing levels observed in the control group, which remained relatively stable (Fig. 4A). However, when treated with 2 mM 1-NAA,

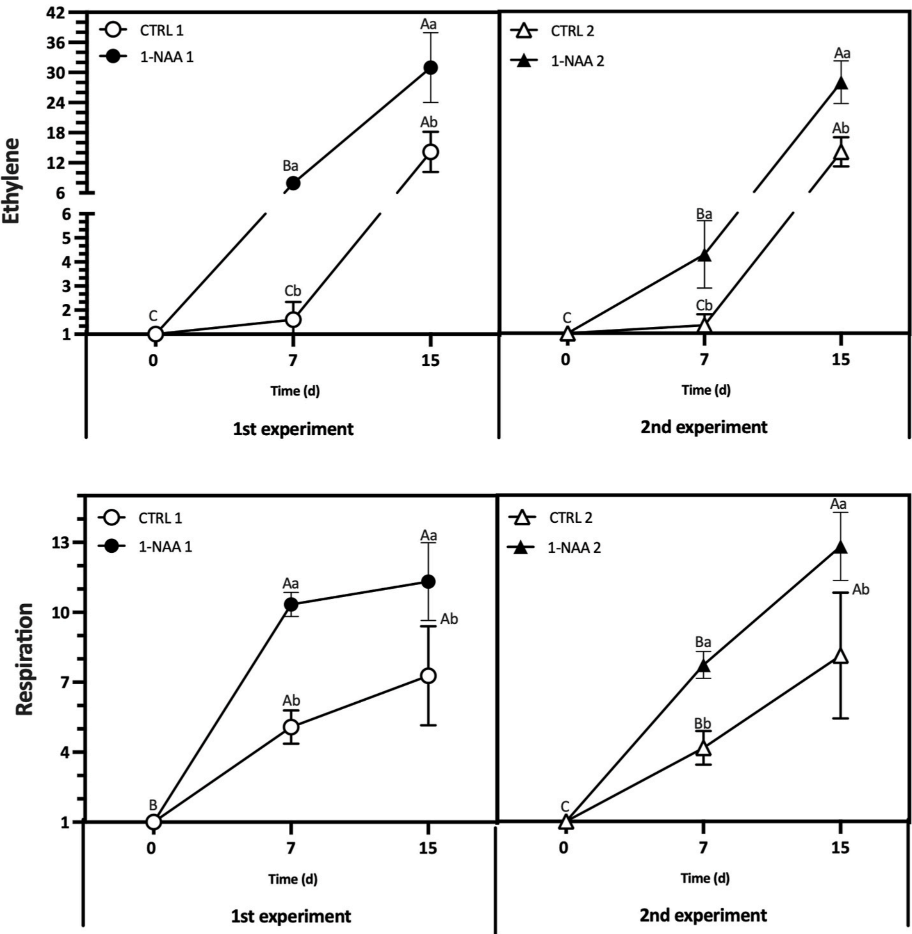

Fig. 2. **A-** Ethylene production and **B-** Respiration rate of 'Rocha' pear during shelf-life ripening at 20 °C after treatments with 1-NAA. The left side of the graph represents the 1st experiment, with pears treated with 1-NAA 4 mM (1-NAA 1) and the respective control (CTRL 1). On the right side of the graph, it is represented the 2nd experiment, with pears treated with 1-NAA two mM (1-NAA 2) and the respective control (CTRL 2). Pears were kept at CA storage before treatments at shelf-life conditions. Values are means ± SD of normalized data from six replicates of three fruits each. Different capital letters indicate significant differences (Tuckey, $p < 0.01$) across time for each condition. Different small letters indicate significant differences (*t*-test, $p < 0.01$) between conditions at each time-point.

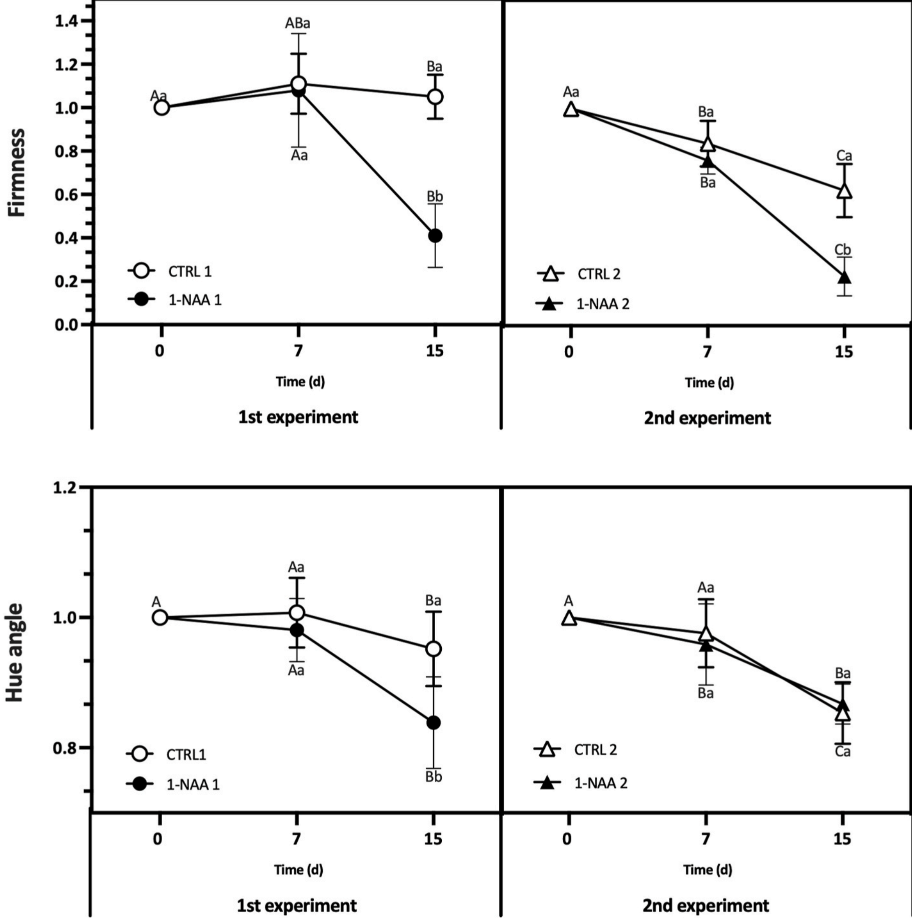

Fig. 3. **A-** Firmness and **B-** Hue angle variation of 'Rocha' pear during shelf-life ripening at 20 °C after treatments with 1-NAA. The left side of the graph represents the 1st experiment, with pears treated with 1-NAA 4 mM (1-NAA 1) and the respective control (CTRL 1). On the right side of the graph, it is represented the 2nd experiment, with pears treated with 1-NAA 2 mM (1-NAA 2) and the respective control (CTRL 2). Pears were kept at CA storage before treatments at shelf-life conditions. Values are means ± SD of normalized data from eighteen replicates. Different capital letters indicate significant differences (Tuckey, $p < 0.01$) across time for each condition. Different small letters indicate significant differences (*t*-test, $p < 0.01$) between conditions at each time-point.

the sucrose content in 'Rocha' pears remained similar to that of pears treated only with 1-MCP (CTRL group), although an increase in sucrose content was noted in both conditions after 15 days of storage. Sorbitol levels were not influenced by 1-NAA treatment, as no significant differences were detected when compared to the control. During storage, glucose concentrations exhibited significant increases, while fructose concentrations followed a consistent pattern in both experiments. However, the auxin treatment did not result in significant differences compared to the control in these sugars (Fig. 4B).

To examine the contribution of auxin to the fruit aroma, the VOC's emission was analyzed, and those quantitatively more important and following some trend along shelf-life period, are shown in Table 1. The main aldehydes detected in our study were hexanal and (E)-2-hexenal. Among them, hexanal registered a significant increase along the shelf-life in 1-NAA treated pears, while in the CTRL group, 1-MCP blocked a higher production of this aldehyde beyond 7 days of shelf-life.

This study also quantified the production of alcohols, including hexanol, 2-ethyl-hexanol, butanol, and ethanol. Among these, butanol and ethanol exhibited the highest variability in response to 1-NAA treatment. In the first experiment, butanol production was significantly influenced by 1-NAA treatment, particularly evident after 15 days of shelf-life, with noticeably higher production compared to the CTRL group. Conversely, in the second experiment, the impact of 1-NAA treatment on butanol production was more prominent at 7 days of shelf-life, continuing to increase as the storage period progressed. Although variations in ethanol were less pronounced compared to butanol, higher ethanol production was observed in pears treated with 2 mM of 1-NAA after 7 days of storage. Additionally, butyl, ethyl, hexyl, and pentyl acetates were identified in the VOC analysis. Over the course of shelf-life, a significant increase in the concentration of butyl, hexyl, and pentyl acetate was observed in pears treated with 1-NAA, while CTRL pears did not exhibit similar changes (Table 1 and Table S1). In the terpenes group, limonene and linanol were identified, but no significant variation was observed across shelf-life or between the 1-NAA and CTRL groups.

Auxin exogenous application stimulated an increase in ethylene production. Thus, the genetic expression of key ethylene genes was studied to further explore the increased ethylene production promoted by 1-NAA treatment.

1-NAA at 4 mM significantly stimulated the expression of *PcACO*, mainly after 15 days (Fig. 5A), which coincides with the higher ethylene production (Fig. 2) observed for this condition.

However, 1-NAA at 2 mM was insufficient to affect the expression of *PcACO*.

During storage at shelf-life temperature, all fruits treated with 1-NAA exhibited significant increase in *PcACS1* and *PcACS4* expression (Fig. 5B,D), showing similar trends to those for ethylene production (Fig. 2). In contrast, 1-MCP suppressed their expression. Regarding *PcACS2*, another gene within this multigene family, its expression was not significantly affected by 1-NAA treatment like the other *PcACS* genes analyzed. However, a slight increase was observed after 15 days of shelf-life, particularly in the 1-NAA 2 mM treatment. It can be observed that

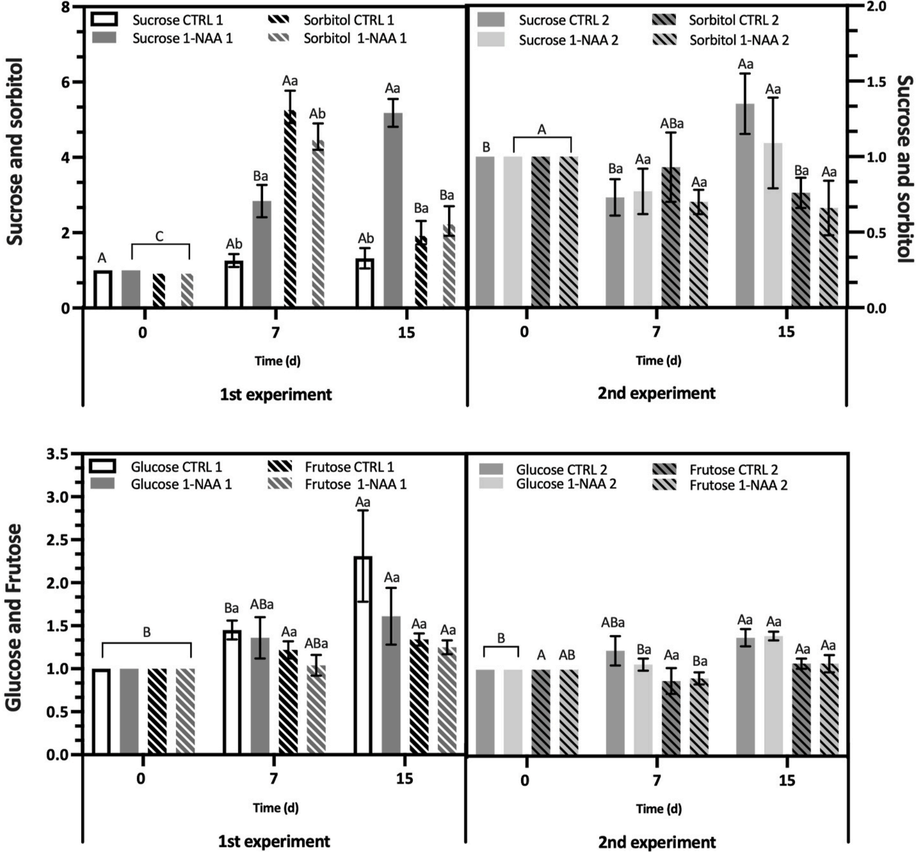

Fig. 4. **A**-Sucrose and Sorbitol, and **B**- Fructose, and Glucose content variation 'Rocha' pear during shelf-life ripening at 20 °C after treatments with 1-NAA. The left side of the graph represents the 1st experiment, with pears treated with 1-NAA 4 mM (1-NAA 1) and the respective control (CTRL 1). On the right side of the graph, it is represented the 2nd experiment, with pears treated with 1-NAA 2 mM (1-NAA 2) and the respective control (CTRL 2). Pears were kept at CA storage before treatments at shelf-life conditions. Values are means ± SD of normalized data from six replicates of three fruits each. Different capital letters indicate significant differences (Tuckey, $p < 0.01$) across time for each condition. Different small letters indicate significant differences (*t*-test, $p < 0.01$) between conditions at each time-point.

once the expression of *PcACS1* and *PcACS4* was initiated (Fig. 5B,D) immediately after 1-NAA treatment, ethylene production was significantly induced (Fig. 2). In 1-NAA treated pears, the higher ethylene production was accompanied by a significant increase in ACS transcript levels. Regarding the expression of *PcACO*, it was mostly induced from 7 days of shelf-life onwards (Fig. 5A).

In addition, the expression profiles of ethylene receptors genes *PcETR1* and *PcETR2* were examined (Fig. 5E,F). The expression level of *PcETR1* remained low throughout the fruit shelf-life and did not increase after 1-NAA treatment. In contrast, the expression of *PcETR2* increased after auxin application as the shelf-life progressed, along with ethylene production (Fig. 2A).

Following the visual evolution of the pears after treatment in both experiments, it was observed that 1-NAA at 4 mM caused some phytotoxicity in fruit skin after 15 days, while 1-NAA 2 mM did not have a negative impact (Fig. 6).

4. Discussion

The disruptive effect of 1-MCP on the normal ripening of pear fruit is well-documented (Ekman et al., 2004; Gago et al., 2015; Rizzolo et al., 2014). During normal ripening, a typical climacteric fruit undergoes significant ethylene-dependent physiological changes in fruit respiration, color, aroma, flavor, and texture, resulting in the typical ripening sensorial quality. Consistent with previous findings in other cultivars, treatment of 'Rocha' pears with exogenous auxin increased ethylene production (El-Sharkawy et al., 2014; Tatsuki et al., 2013; Shi and Zhang, 2014). This increase induced by 1-NAA treatment was accompanied by elevated respiration rates, fruit pulp softening, epicarp yellowing, changes in sugar composition, and the production of ripening aroma compounds.

In a previous study conducted by our research group, the normal ripening phenotype of 'Rocha' pear was characterized (i.e., without 1-MCP), yielding similar physiological transformations to those observed in this study for 1-NAA treatment (Dias et al., 2022). These findings suggest that 1-NAA may have restored the typical ripening process of 'Rocha' pear. For example, following treatment with 1-NAA, pears treated with 1-MCP softened to firmness levels characteristic of the eating ripe stage (firmness <20 N; Isidoro and Almeida, 2006)), after being held at 20 °C for 15 days.

In our previous study, 'Rocha' pears without 1-MCP required the same time to achieve a similar ripe stage (Dias et al., 2022). As expected, 1-MCP treatment delayed the fruit ripening (Yue et al., 2020; Zhang et al., 2020), evidenced by lower ethylene production, as well as reduced softening and yellowing levels in the fruit, as observed in other pear cultivars (Xie et al., 2017; Dong et al., 2018).

The sugar content and production of VOC's are important molecular indicators of fruit flavor quality. Concerning the sugar concentration fructose, glucose, and sucrose are the main simple sugars in pear fruit, increasing markedly with the progression of shelf-life as a consequence of starch degradation (Pasquariello et al., 2013; Tucker et al., 2017; Zhu et al., 2020). Recent studies have demonstrated that ethylene positively regulates sucrose biosynthesis (Farcuh et al., 2018; Choudhury et al., 2008), stimulating the activities of starch-degrading enzymes

Table 1
Major VOC emissions in 'Rocha' pear during shelf-life ripening at 20 °C after treatments with 1-NAA. On the left side of the table, it is represented the 1st experiment, with pears treated with 1-NAA 4 mM and the respective control. On the right side of the table, it is represented the 2nd experiment, with pears treated with 1-NAA 2 mM and the respective control. Pears were kept at CA storage before treatments at shelf-life conditions. Values are means ± SD of normalized data from six replicates of three fruits each. Different capital letters indicate significant differences (Tuckey, $p < 0.01$) across time for each condition. Different small letters indicate significant differences (t-test, $p < 0.01$) between conditions at each time-point.

		1st experiment			2nd experiment		
Aroma Volatile		0 d	7 d	15 d	0 d	7 d	15 d
Hexanal	CTRL	1^{B}	11.91 ± 4.01^{Aa}	9.97 ± 3.48^{Aa}	1^{B}	8.93 ± 2.31^{Aa}	9.48 ± 3.04^{Aa}
	1-NAA	1^{C}	10.64 ± 3.19^{Ba}	31.96 ± 11.01^{Ab}	1^{C}	8.94 ± 2.50^{Ba}	15.56 ± 4.21^{Ab}
Hexanol	CTRL	1^{A}	1.48 ± 0.49^{Aa}	1.29 ± 0.40^{Aa}	1^{B}	0.70 ± 0.25^{Ba}	2.82 ± 0.62^{Aa}
	1-NAA	1^{B}	1.41 ± 0.16^{ABa}	1.67 ± 0.35^{Aa}	1^{B}	0.62 ± 0.22^{Ba}	3.20 ± 0.62^{Aa}
Butyl acetate	CTRL	1^{B}	1.16 ± 0.20^{Ba}	2.86 ± 0.93^{Ab}	1^{B}	0.49 ± 0.11^{Bb}	4.61 ± 1.48^{Ab}
	1-NAA	1^{B}	0.94 ± 0.29^{Ba}	21.32 ± 7.20^{Aa}	1^{B}	1.02 ± 0.23^{Ba}	7.97 ± 3.03^{Aa}
Hexyl acetate	CTRL	1^{A}	1.26 ± 0.45^{ABa}	1.83 ± 0.32^{Bb}	1^{B}	0.88 ± 0.34^{Ba}	1.92 ± 0.67^{Ab}
	1-NAA	1^{B}	1.41 ± 0.12^{Ba}	7.20 ± 3.30^{Aa}	1^{B}	1.15 ± 0.44^{Ba}	9.39 ± 3.11^{Aa}
2-Ethyl-hexanol	CTRL	1^{A}	0.32 ± 0.20^{ABa}	0.18 ± 0.08^{B}	1^{A}	0.84 ± 0.32^{Aa}	0.52 ± 0.21^{Aa}
	1-NAA	1^{A}	0.34 ± 0.12^{Aa}	nd	1^{A}	0.58 ± 0.16^{Bb}	0.10 ± 0.02^{Cb}
Butanol	CTRL	1^{B}	2.00 ± 0.52^{Ba}	3.73 ± 0.84^{Ab}	1^{B}	0.95 ± 0.20^{Bb}	6.29 ± 1.59^{Aa}
	1-NAA	1^{B}	1.60 ± 0.47^{Ba}	8.45 ± 2.79^{Aa}	1^{C}	2.45 ± 0.64^{Ba}	6.99 ± 1.42^{Aa}
Ethanol	CTRL	1^{A}	1.43 ± 0.23^{Aa}	1.09 ± 0.30^{Aa}	1^{B}	1.49 ± 0.46^{Bb}	3.02 ± 0.49^{Aa}
	1-NAA	1^{A}	1.43 ± 0.18^{Aa}	1.33 ± 0.34^{Aa}	1^{C}	3.49 ± 0.25^{Aa}	2.43 ± 0.48^{Ba}
Acetone	CTRL	1^{B}	nd	3.89 ± 1.15^{Aa}	1^{B}	1.14 ± 0.18^{Bb}	6.55 ± 1.17^{Aa}
	1-NAA	1^{B}	2.34 ± 0.91^{A}	2.13 ± 0.44^{Ab}	1^{C}	2.80 ± 0.73^{Ba}	3.90 ± 1.08^{Ab}
(E)-2-Hexenal	CTRL	1^{B}	4.38 ± 1.78^{Aa}	2.93 ± 0.61^{Ab}	1^{B}	8.24 ± 2.46^{Aa}	12.46 ± 4.49^{Aa}
	1-NAA	1^{B}	3.92 ± 0.86^{Ba}	7.81 ± 3.06^{Aa}	1^{B}	6.85 ± 0.82^{Aa}	7.73 ± 1.99^{Aa}
Limonene	CTRL	1^{A}	1.08 ± 0.05^{Aa}	1.04 ± 0.05^{Aa}	1^{A}	1.05 ± 0.04^{Aa}	1.02 ± 0.05^{Aa}
	1-NAA	1^{A}	1.04 ± 0.03^{Aa}	1.11 ± 0.09^{Aa}	1^{A}	0.96 ± 0.08^{Aa}	0.96 ± 0.04^{Aa}
Hexane	CTRL	1^{B}	2.09 ± 0.63^{Aa}	1.71 ± 0.59^{ABa}	1^{A}	1.22 ± 0.17^{Aa}	1.38 ± 0.42^{Aa}
	1-NAA	1^{B}	1.97 ± 0.34^{Aa}	1.55 ± 0.31^{ABa}	1^{B}	1.69 ± 0.36^{Aa}	0.72 ± 0.16^{Bb}
Linanol	CTRL	1^{B}	0.80 ± 0.09^{Ba}	1.77 ± 0.34^{Aa}	1^{A}	0.73 ± 0.04^{Bb}	0.99 ± 0.16^{Aa}
	1-NAA	1^{A}	0.57 ± 0.11^{Bb}	0.89 ± 0.12^{Ab}	1^{A}	1.04 ± 0.21^{Aa}	1.10 ± 0.12^{Aa}
Ethyl acetate	CTRL	1^{B}	10.79 ± 2.84^{Aa}	9.85 ± 2.90^{Aa}	1^{B}	0.90 ± 0.20^{Ba}	3.25 ± 0.99^{A}
	1-NAA	1^{B}	5.77 ± 1.60^{Ab}	6.09 ± 1.60^{Aa}	1^{A}	1.37 ± 0.44^{Aa}	nd
Pentyl acetate	CTRL	1^{A}	nd	7.92 ± 2.49^{Ab}	1^{A}	nd	12.28 ± 2.82^{Ab}
	1-NAA	1^{A}	nd	55.51 ± 24.01^{Aa}	1^{A}	2.01 ± 0.72^{B}	37.08 ± 12.83^{Aa}

responsible for the transformation of complex carbohydrates into simple sugars (Asiche et al., 2018; Cordenunsi-Lysenko et al., 2019). Thus, the above-mentioned studies support the higher sucrose contents observed in pears treated with 1-NAA 4 mM compared to the CTRL ones. However, regarding sorbitol, fructose, and glucose, no significant increase was observed across shelf-life compared to the CTRL pears (Fig. 4). In fact, our results are consistent with previous studies suggesting that starch metabolism during fruit ripening has a component independent of ethylene (Saquet and Almeida, 2017; Defilippi et al., 2004; Gao et al., 2007). The enzymes involved in starch metabolism have a highly complex and coordinated function and regulation, thus requiring further exploration.

During ripening, significant shifts in fruit volatile profiles occur, which play a crucial role in determining fruit aroma. Aroma volatiles consist of a mixture of esters, aldehydes, and alcohol compounds, among others (Zhu et al., 2020). Variations in aroma, especially esters, result from metabolic pathways strongly regulated by the ethylene signalling pathway (Mitalo et al., 2019; Pott et al., 2020). Esters are recognized as the main contributors to ripe pear aroma (Wang et al., 2019). Particularly, butyl, pentyl and hexyl acetate are among the predominant and characteristic esters of ripe 'Rocha' pear (Avelar et al., 1994; Barbosa, 2020; Dias et al., 2022). These previous findings corroborate the observation that 1-NAA recovered the ripening aroma of 'Rocha' pear. Besides their higher concentration compared to pears under only the effect of 1-MCP, these esters steadily increased with the progression of shelf-life. In combination, these compounds contribute to the "pear", "floral", and "fruity" aroma attributes (Rizzolo et al., 2005). In contrast, ethyl acetate and acetone are considered off-flavour volatiles (Pott et al., 2020), and their accumulation was higher in the CTRL pears. Aldehydes produce a green and an herbaceous aroma (Hendges et al., 2018). Hexanal reached higher emission after 1-NAA treatment, but the differences only became significant after 15 days. Indeed, high hexanal production is also associated with increased stress experienced by the fruit (Torregrosa et al., 2020). These results suggest that auxin treatment might have induced a stress in 1-MCP treated pears, likely attributable to ripening, as ripening is a stress leading to senescence. During normal ripening, alcohols increase with storage time, while 1-MCP generally lowers their production (Rizzolo et al., 2005). While significant changes between the CTRL group and 1-NAA group were not observed, overall, the auxin treatment was found to slightly enhance alcohol production.

Specifically, butanol, a compound contributing to the "fruity" aroma of pears, was higher in the 1-NAA treated group at the end of storage. This finding aligns with observations in other pear cultivars during natural ripening processes. (Rizzolo et al., 2005).

Key genetic changes underlying ethylene biosynthesis and perception during shelf-life were studied to explore the mechanism by which 1-NAA induces ethylene biosynthesis in 'Rocha' pear. As shown, 1-NAA treatment increased the expression of related ethylene biosynthesis genes *PcACO*, *PcACS1*, *PcACS2,* and *PcACS4* in a concentration-dependent manner compared to the CTRL. These genes have been demonstrated to play crucial roles, becoming increasingly upregulated as ripening progresses, thus highlighting their central involvement in climacteric ethylene production (Liu et al., 2015; Nakatsuka et al., 1998; Zhu et al., 2020). The results from this study are supported by other investigations reporting that 1-NAA induced the expression of *ACS* and *ACO* genes families, while 1-MCP decreased their expression (Tatsuki et al., 2013; Yue et al., 2020).

Considering that 1-NAA significantly increased ethylene production when *PcACS1* and *PcACS4* were overexpressed compared to the CTRL, while 1-MCP inhibited their expression, and also taking into account that *PcACO* expression was only up-regulated by 1-NAA 4 mM treatment from the 7th day onward, it is possible to suggest that *PcACS1* and *PcACS4* may play a significant role in system II of 'Rocha' pear. This suggest that these genes could be useful ripening markers in this pear cultivar (Li et al., 2015).

At the receptor level, 1-NAA also induced significant changes in gene expression. 1-NAA treatment was associated with an up-regulation of *PcETR2*, while 1-MCP reduced *PcETR2* expression, as observed in other findings (Yang et al., 2013; Zhou et al., 2017). In fruits, climacteric ethylene production during shelf-life is mainly associated with the

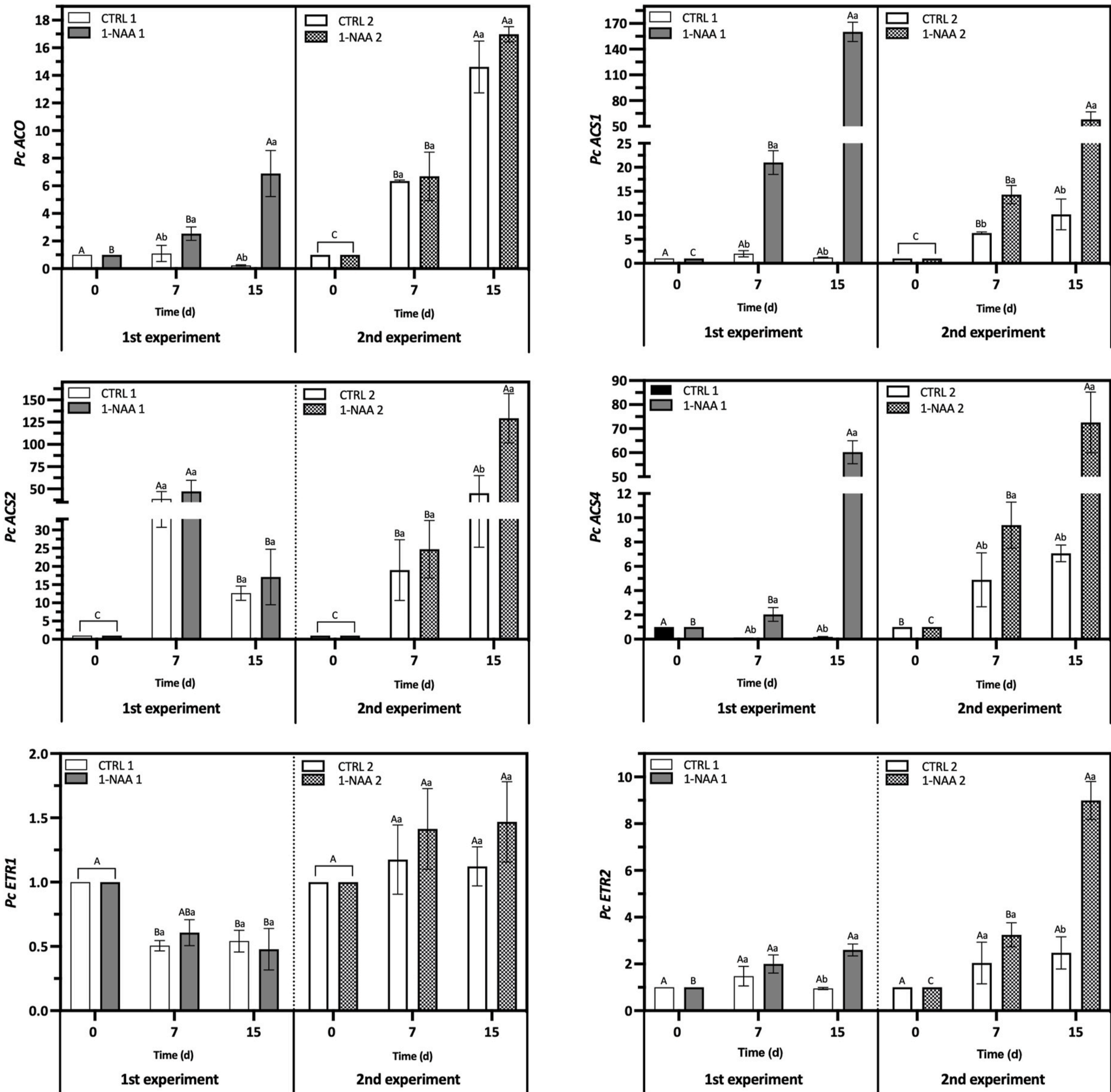

Fig. 5. Expression levels variation of **A**-*PcACO*, **B**-*PcACS1*, **C**-*PcACS2*, ***D***-*PcACS4*, **E**-*PcETR1;* **F**-*PcETR2* in 'Rocha' pear during shelf-life ripening at 20 °C after treatments with 1-NAA. On the left side of each graph, it is represented the 1st experiment, with pears treated with 1-NAA 4 mM and the respective control. On the right side of each graph, it is represented the 2nd experiment, with pears treated with 1-NAA 2 mM and the respective control. Values are means ± SD of normalized data from three replicates of three fruits each. Different capital letters indicate significant differences (Tuckey, $p < 0.01$) across time for each condition. Different small letters indicate significant differences (*t*-test, $p < 0.01$) between conditions at each time-point.

greater expression of *PcETR2* (Chiriboga et al., 2013; Tadiello et al., 2016). It is noteworthy that 1-MCP did not completely inhibit the expression of *PcETR* genes in CTRL pears, which aligns with the expression of some *PcACS* genes and the ethylene production observed in CTRL pears, though lower than 1-NAA group. It is hypothesized that the treatment with 1-MCP after harvest and prior to storage could only block the existing ethylene receptors, but new receptors could be slowly formed during cold storage (Tsantili et al., 2007). In the deficiency of ethylene, ethylene receptors act as negative regulators of signaling processes (Chen et al., 2018). This implies that when ethylene is present, it attaches to its receptors, allowing for subsequent signaling processes. However, newly formed ethylene receptors in 1-MCP treated pears are not sufficient to bring back the typical ripening process. Therefore, the treatment with 1-NAA might have promoted the generation of new ethylene receptors, which can then interact with the ethylene produced as a result of the increased expression of ethylene biosynthetic genes triggered by 1-NAA treatment. This interaction kickstarts the ethylene signalling pathway, which is essential for triggering a series of reactions leading to the formation of ethylene response factors (ERFs). ERFs, in turn, activate enzymes that contribute to physiological processes such as softening, yellowing, and aroma production (Gwanpua et al., 2017). Thus, in addition to stimulating ethylene biosynthesis, 1-NAA may have had the ability to induce the production of new receptors. In fact, this study found that significant phenotypic changes occurred only when the expression of ethylene receptors increased. Therefore, as important as stimulating internal ethylene production is the production of new receptors that enable ethylene binding and initiate the observed ripening process.

In the present study, the effect of 2 and 4 mM 1-NAA treatments was investigated. Overall, it was found that both concentrations of 1-NAA may have accelerated the onset of 'Rocha' pear ripening under all the conditions tested. However, the 4 mM 1-NAA treatment resulted in some skin injuries to the fruit surface, whereas the 2 mM treatment did not exhibit similar effects in certain parameters such as color, sucrose

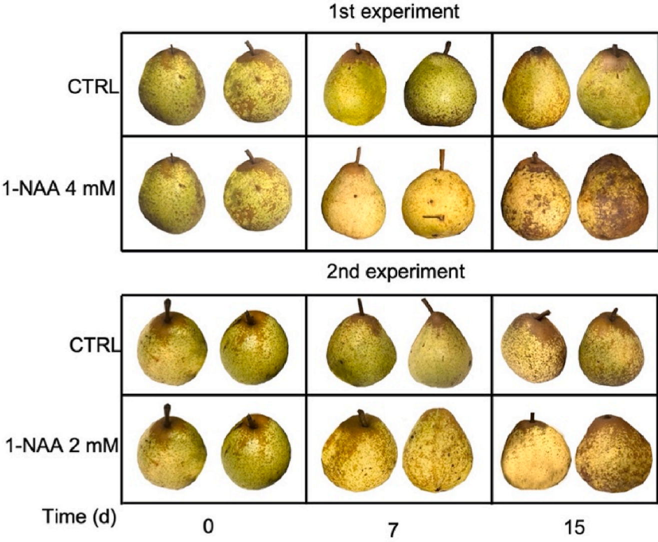

Fig. 6. Pictures of 'Rocha' pear from both experiments after treatment with 1-NAA and during shelf-life. Pears from this experiment presented a height between 55 and 65 mm.

content, and *PcACO* expression. Therefore, an intermediate concentration should be considered for further evaluation.

5. Conclusion

This study aimed to investigate whether 1-NAA treatment could counteract the inhibitory effect of 1-MCP on 'Rocha' pear ripening. The results revealed that increased ethylene production was accompanied by heightened respiration, fruit softening, skin yellowing, changes in sugar content, and the production of ripening aroma compounds. These observed changes in 1-NAA-treated pears closely resembled the natural ripening process. Furthermore, the study demonstrated that 1-NAA treatment stimulated ethylene biosynthesis and signalling genes, providing evidence of the role played by auxin in fruit ripening and shedding light on how to counteract the effects of 1-MCP. Nevertheless, further research is necessary to determine the many complex regulatory processes' precise nature.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2025.109921.

Data availability

Data will be made available on request.

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