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Ozone-induced kiwifruit ripening delay is mediated by ethylene biosynthesis inhibition and cell wall dismantling regulation

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ABSTRACT

Ozone treatments are used to preserve quality during cold storage of commercially important fruits due to its ethylene oxidizing capacity and its antimicrobial attributes. To address whether or not ozone also modulates ripening by directly affecting fruit physiology, kiwifruit (*Actinidia deliciosa* cv. 'Hayward') were stored in very low ethylene atmosphere at 0 °C (95% RH) in air (control) or in the presence of ozone (0.3 μL L^{-1}) for 2 or 4 months and subsequently ripened at 20 °C (90% RH) for up to 8 d. Ozone-treated kiwifruit showed a significant delay of ripening during maintenance at 20 °C, accompanied by a marked decrease in ethylene biosynthesis due to inhibited *AdACS1* and *AdACO1* expression and reduced ACC synthase (ACS) and ACC oxidase (ACO) enzyme activity. Furthermore, ozone-treated fruit exhibited a marked reduction in flesh softening and cell wall disassembly. This effect was associated with reduced cell wall swelling and pectin and neutral sugar solubilization and was correlated with the inhibition of cell wall degrading enzymes activity, such as polygalacturonase (PG) and endo-1,4-β-glucanase/1,4-β-glucosidase (EGase/glu). Conclusively, the present study indicated that ozone may exert major residual effects in fruit ripening physiology and suggested that ethylene biosynthesis and cell walls turnover are specifically targeted by ozone.

1. Introduction

Fruit development and ripening are highly regulated processes, including a number of physical and chemical changes that would transform a fertilized ovary into a pleasant, tasty, nutritious and appealing fruit [1]. However, over-ripening is one of the major causes of the burden of postharvest losses occurring worldwide [2,3]. Hence, the major area of fruit ripening research involves the application of effective postharvest management, since fruit industry is facing huge economic losses every year as a result of ethylene-induced senescence [2].

Ozone (O_3), or triatomic oxygen, is naturally produced by electrical discharges or UV light [4]. It has several applications in the food industry and have been cited as a promising alternative to traditional sanitizers for postharvest treatments on a plead of fresh fruit and vegetables [5]. Ozone is also used in a number of commercially important fruit species to delay ripening. It is well known

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; ACO, ACC oxidase; ACS, ACC synthase; AIR, alcohol-insoluble residue; d, day(s); EGase/glu, endo-1,4-β-glucanase/1,4-β-glucosidase; KSF, 4M KOH-soluble fraction; NS, neutral sugars; NSF, Na_2CO_3-soluble fraction; PG, polygalacturonase; PVP, polyvinylpyrrolidone; PVPP, polyvinylpolypyrrolidone; RCL, red cell lysis; RH, relative humidity; SSC, soluble solids content; TA, titratable acidity; UA, uronic acids; WSF, water-soluble fraction; α-ara, α-arabinofuranosidase; β-gal, β-galactosidase.

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that ozone can directly oxidize ethylene and in fact, this is considered the major mechanism by which it prevents ripening [2,6]. Ozone has been used extensively during kiwifruit storage due to its high ethylene-sensitivity [7], whereas it has been also shown that it effectively complements low-temperature storage in reducing spoilage caused by *Botrytis cinerea* [8].

Studies with whole plants and model species, mainly in relation to environmental pollution, suggested that ozone can also have profound effects on plant cell metabolism [9]. In leaves, ozone induced an oxidative burst, which resulted in the accumulation of superoxide (O_2^-) and hydrogen peroxide (H_2O_2) and mimicking some oxidative events occurring in response to other stressors [10]. However, in detached fruits in which ozone exposure is widely used for postharvest management little research has been done. Indeed, most of the studies conducted to date have focused in the optimization of ozone treatment rather than in the evaluation of ozone impact in fruit physiology. Recent work documented that ozone exposure during cold storage could modulate a number of ripening-associated changes in kiwifruit, even after kiwifruit removal from the ozone-enriched atmosphere [11], thereby suggests that ozone may directly modulate kiwifruit ripening. Thus, it is apparent that postharvest ozone application provides an excellent scientific challenge for ripening studies which, among others, could significantly improve our understanding of how fruit cells effectively respond to environmental ozone.

The biochemical changes accompanying softening vary considerably among fruit species and even between cultivars of the same species [12,13]. For instance, in some climacteric fruit, such as tomato and avocado, most of the softening changes are coincident with the increase in ethylene production, whereas in kiwifruit extensive softening occurs prior to these ethylene-regulated events [14]. This ripening behavior also renders kiwifruit an interesting experimental system in terms of softening phenomena [15]. In addition, kiwifruit is characterized by a massive dismantling of the cell wall composite. Fast solubilization of both pectic and cross-linking glycans was observed together with a large increase in the viscosity of the cell wall material and its ability to swell in water [14]. Extensive levels of galactose likely removed from RG-I-type pectins are lost. The enzyme β-galactosidase (β-gal) was suggested to be involved in cell wall swelling and softening in kiwifruit [16,17], however this function was not always fully supported [18]. Only a few other cell wall degrading proteins have been characterized in kiwifruit and their involvement in kiwifruit softening is far from being understood [19–21]. Although ozone treatments have been shown to affect polyuronide disassembly in tomato [22], no research has been conducted to analyze its impact on cell wall turnover in kiwifruit, one of the commodities in which ozone is widely used.

In the current study, we evaluated the influence of ozone, applied during cold storage, on kiwifruit post-storage ripening and on several key components of ethylene biosynthetic pathway in order to determine whether besides its known direct ethylene oxidizing properties it also directly modulates fruit physiology. The effect of ozone exposure on cell wall polysaccharides and cell wall degrading enzymes during kiwifruit ripening was also investigated.

2. Materials and methods

2.1. Fruit material and experimental design

Kiwifruit (cv. 'Hayward'), grown under standard cultural practices, were harvested from a commercial orchard (Fresno County, CA, USA) at physiological mature stage [mean weight: 90 ± 5 g, tissue firmness: 58.1 ± 1.5 N, soluble solids content (SSC): 6.7 ± 0.3%, titratable acidity (TA): 2.0 ± 0.1%, dry weight: 15.9 ± 0.4%]. Subsequently, fruit were divided into 25 lots of 30 fruits each. One lot was analyzed at the time of harvest and the other lots (12 + 12) were subjected to cold storage (0 °C, 95% RH) in a room were ethylene was oxidized through $KMnO_4$ filters (Purafil, CA, USA, control) or in a room were fruit exposed to continuous supply of gaseous ozone (0.3 μL L^{-1}, ozone treatment). Ozone supply and monitoring was performed using a Purfresh Cold Storage Generator incorporated to an online Intellipur Command Center installed from Purfresh, Inc. (Fremont, CA, USA) at the Postharvest Lab located at UC Davis (Davis, CA, USA). Ethylene levels within the cold storage rooms were monitored and were below the generally accepted levels for long-term kiwifruit storage (10 nL L^{-1}), without differences among the storage conditions applied (Supplementary Fig. S1, Supplementary data associated with this article can be found, in the online version). Fruit were removed from cold storage after 2 or 4 months and subsequently were transferred at room temperature (20 °C, 90% RH) and analyzed after 0, 2, 4, 6 or 8 d, respectively. At each ripening day (0, 2, 4, 6, 8), ethylene production, respiration rate, tissue firmness, SSC, TA, were monitored. Outer pericarp flesh samples were collected from each replication per sample (3 batches of tissue from 10 fruits) and subsequently were frozen with liquid nitrogen and stored at −80 °C for further analysis. The experimental set up is presented in Fig. S2.

2.2. Physicochemical characterization of kiwifruit ripening

Tissue firmness was measured at the two opposite sides of each fruit after removal of peel (1 mm thick), using a fruit texture analyzer (FTA, model GS, Güss Manufacturing Ltd., Strand, South Africa) with a 8.0-mm probe. Data were recorded as Newton (N) and tissue firmness was expressed as the mean of 30 fruits.

SSC and TA were assessed in juice obtained from three groups of 10 fruits as previously described [23]. Briefly, the upper and lower parts of each fruit (stem-end and calyx-end) were removed from each fruit group and pooled to form a composite sample and pressed through cheesecloth using a juicer. The SSC of the juice was measured with a temperature compensated digital refractometer (PR 32α, Atago, Tokyo, Japan) and expressed as percentage (%, w/v). TA was measured using an automatic titrator (model TitraLab 850, Radiometer Analytical SAS, Lyon, France) connected to a sample changer (model SAC 80, Radiometer Analytical SAS, Lyon, France) with 0.1 M NaOH to pH 8.2 and expressed as percentage of citric acid (%, w/v).

2.3. Ethylene production and respiration rate

For each treatment, ten replications of three fruit were weighed and placed into separate 1.8 L volume airtight jars connected in an open flow through system with a flow of humidified air at 30 mL min^{-1}. Ethylene production was measured using two-dimensional gas chromatography (GC x GC) by withdrawing a 1 mL headspace gas sample from each jar and injecting it into a gas chromatograph (model Carle AGC-211, EG&G Chandler Engineering, Tulsa, OK, USA) equipped with two stainless steel columns (1.22 m and 0.305 m) packed with 8% NaCl on Alumina F-1 80/100 DV (EG&G Chandler Engineering, Tulsa, OK, USA) and a flame ionization detector. Nitrogen was used as the carrier gas at a flow rate of 30 mL min^{-1} while O_2 and H_2 were used to create the flame of the detector at flow rates of 300 and 30 mL min^{-1}, respectively. Injector, oven and detector temperatures were 80 °C. Respiration rate (RR) was calculated by carbon dioxide concentration in the gas phase of the jars, determined by withdrawing a 1 mL headspace gas sample from each jar and injecting it into an infrared gas analyzer (Horiba PIR-2000R, Horiba Instruments Inc., Irvine, CA, USA). Nitrogen was used as the carrier gas at a flow rate 20 mL min^{-1}.

Ethylene production was expressed as C_2H_4 μL kg^{-1} h^{-1} and RR as CO_2 mL kg^{-1} h^{-1}.

2.4. ACC and 1-malonyl-ACC (MACC) content determination

ACC and MACC were quantified according to Bullens et al. [24] with slight modifications, using 2 g of ground frozen tissue and 4 mL of 5% sulfosalicylic acid. The homogenized mixture was incubated for 30 min at 4 °C while gently shacked, centrifuged 10 min at 3100 × *g* (4 °C) and the supernatant was stored at −80 °C. Acidic hydrolysis to liberate the MACC and estimate the ACC + MACC content was performed using safe lock microcentrifuge tubes containing 0.5 mL of ACC extract by adding 0.2 mL of 6 M HCl in a water bath at 99 °C for 3 h. After boiling, samples were cooled and neutralized with 0.2 mL of 6 M NaOH, centrifuged for 5 min at 22,000 × *g* and the supernatant was collected and stored at −20 °C. The reaction to convert ACC into ethylene was performed twice per sample (either spiked or not with 20 μL of 50 μM ACC solution in order to calculate the reaction efficiency) using 1.4 mL of ACC extract and 0.4 mL 10 mM $HgCl_2$ in a 12 mL vial (BD Vacutainer®, Becton, Dickinson and Company, Plymouth, UK), which was immediately sealed with a septum-containing cap. Right after, 0.2 mL of 6 M NaOH – 5% v/v NaOCl (1:2 v/v) was injected through the septum of the cap using a needle and syringe, allowed to incubate for 4 min on melting ice and a 1 mL headspace gas sample was withdrawn from the vial through the septum and injected to the gas chromatograph for ethylene analysis, as previously described. For the determination of ACC + MACC 0.1 mL of hydrolyzed ACC extract plus 0.6 mL distilled water, 0.2 mL 10 mM $HgCl_2$, 0.1 mL 6 M NaOH – 5% v/v NaOCl (1:2 v/v) were used. The free ACC content was determined by the ACC extract, while MACC by subtracting the free ACC content from the total hydrolyzed ACC and expressed as nmol g^{-1}.

2.5. ACS and ACO enzyme activity

ACS and ACO enzyme *in vitro* activities were analyzed according to Bullens et al. [24] with slight modifications. For ACS enzyme activity 3 g of frozen tissue was ground and 3 mL of extraction buffer [200 mM Tricine extraction buffer pH 8.5 containing 2 mM pyrodoxal-L-phosphate (PLP) and 10 mM dithiothreitol (DTT)] and 15 mg polyvinylpyrrolidone (PVP). The sample was vortexed and centrifuged for 20 min at 18,500 × *g* at 4 °C and 2.5 mL of the supernatant cleaned up with a Sephadex G-25 desalting column at 4 °C. The sample was eluted from the column with 3.5 mL of column solution (Tricine buffer pH 8.0 containing 2 mM PLP and 1 mM DTT in 5 mM), collected and stored at −80 °C. The reaction mixtures contained 1.5 mL of enzymatic extract, 150 μL of Tricine reaction buffer (200 mM, pH 8.0) and 150 μL S-(5-adenosyl)-L-methionine chloride at 25 °C for 2 h. The reaction was stopped by adding 200 μL of 100 mM $HgCl_2$ and samples were kept on ice. To release ethylene from the reaction mixture, 850 μL of distilled water and 950 μL of reaction mixture was transferred into capped vial containing a septum. Two hundred microliters of 6 M NaOH – 5% v/v NaOCl (1:2 v/v) were injected through the septum and samples were incubated for 4 min on melting ice. Ethylene concentration analyzed as described above and readings were performed in duplicate on each reaction mixture. Three biological replications were analyzed for each treatment and ripening stage and the *in vitro* activity of ACS expressed as nmol ACC kg^{-1} s^{-1}.

ACO activity was analyzed using 0.5 g of ground frozen tissue, 50 mg polyvinylpolypyrrolidone (PVPP) and 1 mL of 3-morpholinopropane-1-sulfonic acid (MOPS) extraction buffer (400 mM MOPS, pH 7.2), containing 10% v/v glycerol and 30 mM sodium ascorbate. The homogenized sample was incubated while gently shacked at 4 °C for 10 min, centrifuged for 30 min at 22,000 × *g* (4 °C) and the supernatant was collected and stored at −80 °C until analysis. The enzyme reaction mixtures contained 3.6 mL of 50 mM MOPS, pH 7.2; 10% v/v glycerol; 5 mM sodium ascorbate; 20 mM sodium bicarbonate; 0.02 mM iron sulfate; 1 mM ACC and 1 mM DTT and 400 μL fruit enzymatic extract. The reaction was conducted in capped vials containing a septum for sampling. The reaction mixture was incubated for 1 h at 30 °C. After incubation, gas samples were taken form the headspace and analyzed on a GC as previously described. Ethylene readings were performed on three biological replications for each treatment and ripening stage and the *in vitro* activity of ACO was expressed as nmol C_2H_4 kg^{-1} s^{-1}.

2.6. RNA isolation, cDNA synthesis and real time quantitative RT-PCR analysis of ethylene biosynthesis genes

RNA extraction was done with Omega Bio-tek E.Z.N.A SQ Total RNA extraction kit with slight modifications. The red cell lysis (RCL) buffer was prepared with addition of 2% PVP-40 (w/v). Fifty to one hundred mg of finely ground samples were transferred to 1.5 mL microfuge tubes containing 600 μL of RCL buffer. The tubes were inverted to mix and placed on ice for 1–3 min until 200 μL protein/DNA precipitation solution was added and placed on ice for 10 min. The tubes were centrifuged at 14,000 × *g* (4 °C) for 10 min and supernatant was transferred to a clean 1.5 mL tube. Then, 750 μL of cold (−20 °C) 100% (v/v) isopropanol was added and tube was inverted several times to mix before incubating at −20 °C for 30 min. After, incubation tubes were centrifuged at 14,000 × *g* (4 °C) for 10 min. The supernatant was discarded and 0.5 mL 70% (v/v) ethanol was added to wash the pellet and centrifuged at 14,000 × *g* (4 °C) for 5 min. The supernatant was discarded and the clean white pellet was dried out and suspended in 50 μL DEPC H_2O. RNA concentration and quality integrity were checked as elsewhere described [25]. RNA was purified with RNA clean and concentrate kit™ (Zymo Research, Irvine, CA, USA) and DNASE I treated (Promega, Madison, WI, USA), as per manufacturer's directions before cDNA synthesis. The cDNA synthesis was done with QuantiTect Reverse Transcription Kit (Qiagen) with integrated removal of genomic DNA contamination for use in real-time two-step RT-qPCR reaction according to manufacturer's directions.

The *Actinidia deliciosa* ACC synthase (*AdACS1*), ACC oxidase (*AdACO1*) and *ACT1* mRNA assay was designed by the Real-time PCR Research and Diagnostic Core Facility (Genome Center, University of California, Davis, CA, USA) utilizing Primer Express 3 from the sequence of M97961 and AB005722 (Table S1 and S2). The *q*PCR systems were validated using 2-fold dilutions of a known positive control. The dilutions were analyzed in triplicate and a standard curve plotted against the dilutions. The slope of the standard curve was used to calculate amplification efficiencies using the formula $E = 10^{1/-s} - 1$. Positive and negative (water) controls was included for the assay. The efficiency for *AdACS1*, *AdACO1* and *ACT1* mRNA, complete cds assays were 94.7, 91.1 and 93.5% respectively with a standard deviation of less than 0.4 for all triplicates.

The two-step Real-time PCR analysis was done using the regular 12 μL format as stated [26]. The samples were placed in a 384 well plate and amplified in an automated fluorometer (7900HT FAST Real-Time PCR System, AB). ABI's standard amplification conditions were used: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Fluorescent signals were collected during the annealing step and Cq values were exported with a threshold of 0.1 and a baseline of 3–15. The amplification plot and multi-component of each reaction were checked to confirm all Cq values and all questionable wells were repeated in the regular 12 μL TaqMan® format for the reference gene (*ACT1*) and the gene of interest. In addition, positive and negative controls were run for the gene of interest and

reference gene. Cq values of the repeats were imported using the same range and baseline listed above.

The relative amount of PCR product generated from each primer set was determined on the basis of the cycle threshold (Ct) value and was reported as relative transcription or the *n*-fold difference relative to a calibrator cDNA. In brief, the housekeeping gene, *AdACT1*, was used to normalize the Cq values of the target genes (ΔCq). The ΔCq was calibrated against the average of the controls at 0 d for each gene. The relative linear amount of target molecules relative to the calibrator was calculated by $2^{-\Delta\Delta Cq}$ [27]. The qPCR experiments were repeated three times and all gene transcription is expressed as an *n*-fold difference relative to the calibrator. Linearized values >1.0 indicates increased expression and linearized values <1.0 indicates decreased expression.

2.7. Cell wall isolation, swelling, fractionation and metabolite analysis

For cell wall isolation, approximately 15 g of kiwifruit pulp was placed in absolute ethanol to limit the action of cell wall modifying enzymes and was then homogenized in an Ultra-Turrax Polytron (model T 25 D, IKA, Werke GmbH & Co. KG, Staufen, Germany) with 100 mL of ethanol and boiled for 30 min to ensure the inactivation of enzymes, to extract low molecular weight solutes and to prevent autolysis. The insoluble material was vacuum filtered and sequentially was washed with 40 mL of ethanol, 40 mL of chloroform:methanol (1:1, v/v), and 40 mL of acetone and dried at 37 °C, yielding the alcohol insoluble residue (AIR). The dried residue was weighed. Three independent extractions were made for each treatment and storage time considered. Results were estimated as g of AIR per 100 g of fresh fruit and expressed as % AIR.

Cell wall (CW) swelling *in vitro* analyzed as previously described with slight modifications [28]. Briefly, 20 mg of AIR added to 15 mL falcon tubes with 10 mL of distilled water and shacked horizontally for 24 h (4 °C) in order to allow AIR hydration. After incubation tubes placed vertically and CW swelling assessed based on the volume occupied by the swollen AIR (mL) and the electric conductivity of the supernatant recorded ($\mu S\,m^{-1}$).

Fractions of different cell wall components were obtained by sequential chemical extraction of the cell wall material (AIR) according to Vicente et al. [29]. Approximately 40 mg of AIR residue from each sample was suspended in 10 mL of water and stirred at room temperature for 3 h under continuous shaking, then centrifuged at 6000 × *g* and vacuum filtered. The filtrate was taken to 14 mL with distilled water and designated as WSF. The residue was then extracted with 10 mL of 50 mM Na_2CO_3 for 1 h under continuous shaking. The slurry was centrifuged and the supernatant was taken to 14 mL with distilled water and designed as NSF. The Na_2CO_3 insoluble pellet was then extracted with 10 mL of 4 M KOH for 1 h, under continuous shaking, and the extracted solution was designated as the KSF. The 4 M KOH insoluble residue was then washed with 10 mL of 50% (v/v) ethanol and centrifuged at 6000 × *g*. This procedure was repeated three times. The pellet was dried at 60 °C, weighed and designated as α-cellulose. Samples of the different cell wall fractions obtained were assayed for uronic acids (UA) and neutral sugars (NS) content.

UA were measured according to Blumenkrantz, and Asboe-Hansen [30]. Absorbance was measured at 520 nm and the readings were calculated on the basis of a galacturonic acid standard curve. Three independent samples were analyzed for each treatment and storage condition and measurements were done in duplicate. Results were expressed as mg of galacturonic acid per g of fruit fresh weigh (FW). NS were measured by the anthrone method [31]. Results were expressed as mg of glucose per g FW.

2.8. Cell wall modifying enzymes in vitro activity

Cell wall modifying enzymes extraction procedure and *in vitro* activity analysis performed as described elsewhere [32]. Approximately 8 g of fruit tissue were homogenized in an Ultra-Turrax Polytron with 20 mL of 50 mM HAc–NaAc pH 5.0 containing $10\,g\,L^{-1}$ PVPP, 1 M NaCl and 1 mM of phenylmethanesulfonyl fluoride. The homogenates were then stirred at 4 °C for 1 h and centrifuged (5100 × *g*, 20 min, 4 °C). The supernatant was filtrated and dialysed against 10 mM NaAc pH 5.0 and subsequently was used for assays of enzymatic activities. Three independent extracts were prepared for each treatment and storage time considered.

For β-gal, reaction mixtures containing 100 μL of enzyme extract, 1000 μL of 50 mM HAc-NaAc buffer pH 5.0 and 200 μL of 3 mM *p*-nitrophenylgalactopyranoside were incubated at 37 °C. For α-ara, reaction mixtures containing 700 μL of enzyme extract, 800 μL of 50 mM HAc-NaAc buffer pH 5.0 and 200 μL of 3 mM *p*-nitrophenylarabinofuranoside were incubated at 37 °C. Two hundred μL aliquots were taken at different time intervals, 1000 μL of 0.4 M Na_2CO_3 were added and absorbance at 410 nm was measured to determine the linear reaction rate of each enzyme-substrate combination.

PG activity was measured in reaction mixtures containing 500 μL of 50 mM NaAc–HAc buffer pH 5.0, 200 μL of 0.15% (w/v) polygalacturonic acid and 500 μL of enzymatic extract. The mixtures were incubated at 37 °C. At different time intervals, 200 μL aliquots were taken and 1 mL of 0.1 M sodium borate was added. Reducing sugars liberated were measured with the 2-cyanoacetamide method [33].

Finally, for EGase/glu activity the reaction mixtures contained 400 μL of 50 mM NaAc–HAc buffer pH 5.5, 400 μL of 0.2% (w/v) carboxymethyl-cellulose and 200 μL of enzymatic extract. The mixtures were incubated at 37 °C. At different time intervals, 200 μL aliquots were taken and assayed for reducing sugars as described for PG activity. In all cases three protein extracts were prepared for each ripening stage and treatment analyzed. Results were expressed as ΔOD per $kg^{-1}\,s^{-1}$.

2.9. Statistical analysis

Statistical analysis performed using SPSS 19.0 for Mac OS X (SPSS, Chicago, IL, USA). Data (means consisted by 3 biological replications) were subjected to analysis of variance and least significant differences (LSD) at 5% level were used for means comparison. Graphs were created using Prism v5.0 for Mac OS X (Graph Pad Inc., San Diego, USA).

3. Results and discussion

3.1. Ozone delays ripening and inhibits ethylene production and respiration rate

An accepted mode of ozone's beneficial action in postharvest life of fruit involves its ability to reduce postharvest decay and to oxidize ethylene in the atmosphere of storage rooms, thus delaying ripening occurring during cold storage [5,34,35]. To characterize the ripening performance of kiwifruit previously stored (0 °C, 95% RH, 2 or 4 months) in an ozone-enriched atmosphere ($0.3\,\mu L\,L^{-1}$) or in air with $KMnO_4$ oxidizers (control), we ensured that both of the storage systems maintained very low ethylene levels in the atmosphere (<$5.0\,nL\,L^{-1}$, Supplementary Fig. S1). Upon transfer to 20 °C after 2 months, control fruit exhibited a typical climacteric rise in ethylene production, as opposed to ozone-treated fruit, which consistently showed basal levels of ethylene production (Fig. 1A). A more pronounced inhibition

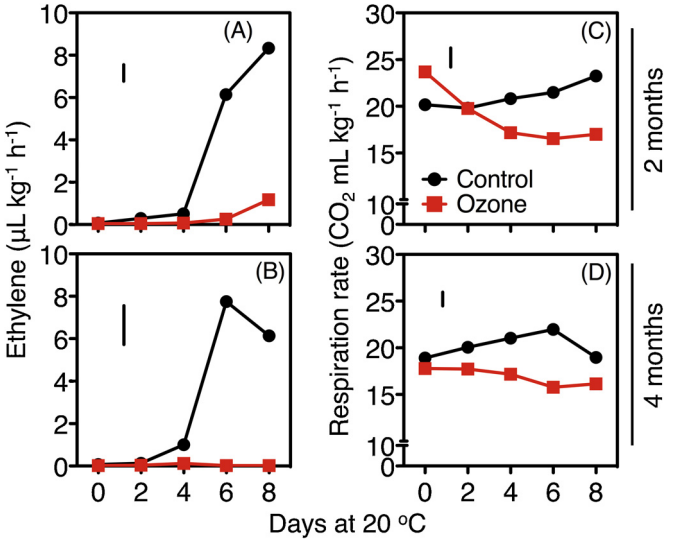

Fig. 1. Changes in ethylene production (A, B) and respiration rate (C, D) of kiwifruit (cv. 'Hayward') during ripening (20 °C, 90% RH) for up to 8 d following 2 (A, C) or 4 (B, D) months of cold storage (0 °C, 95% RH) in the absence (control) or in the presence of ozone (0.3 μL L^{-1}). Each value represents the mean of 10 replications of 3 fruits analyzed at each ripening stage after cold storage. The vertical bars in each particular figure represent the least significant difference (LSD, $P = 0.05$).

($P = 0.05$) of ethylene production was observed during ripening at 20 °C and following 4 months of cold storage (Fig. 1B), suggesting that the outcome of ozone treatments is dependent on the treatment duration. Higher respiration rate (RR) was recorded in ozone-treated fruit subjected to 2 months of cold storage than in the respective controls immediately upon transfer to 20 °C. This higher RR in fruit exposed to ozone may represent an initial response of kiwifruit to oxidative stress conditions caused by ozone treatment. However, during ripening at 20 °C the RR increased in the control fruit, maintaining lower values in ozone-treated kiwifruit (Fig. 1C). The latter, showed lower RR throughout the ripening period after 4 months at 0 °C (Fig. 1D), possibly due to the absence of climacteric ethylene production (Fig. 1B).

Tissue firmness decreased during cold storage to the same extent in control and treated fruit (*ca.* 29 and 75% after 2 and 4 months of cold storage, respectively relative to the values recorded at harvest, Fig. 2A). Interestingly, upon transfer to 20 °C the fruit refrigerated in ozone-enriched atmosphere maintained higher tissue firmness than control, in both ripening periods studied (after 2 or 4 months of cold storage). Untreated fruit entered the 'eating-ripe window' tissue firmness stage (the fruit are acceptably soft for consumers, produce ethylene and the characteristic ripe fruit aroma volatiles, phase 3; [15]) after 4 d and 2 d of ripening, following 2 and 4 months of cold storage, respectively. However, continued to soften rapidly, leading to an over-ripe flesh (unacceptably soft for consumers, phase 4; [15]) after 8 and 6 d following 2 and 4 months of cold storage, respectively. In contrast, ozone-treated fruit remained in the 'eating-ripe window' of tissue firmness throughout the 8-day ripening period following storage at 0 °C for 2 and 4 months. The delayed softening of ozone-treated fruit was correlated with the inhibition of ethylene production (Fig. 1A and B). Some softening events in kiwifruit have been shown to be ethylene independent [15]. However, the fact that in ACO knockdown kiwifruit lines (*Actinidia chinensis*, cv. 'Hort16A') and 1-methylcyclopropene (1-MCP, an inhibitor of ethylene action) treated fruit yield improved firmness retention suggests that at least part of kiwifruit textural changes occur during ripening are ethylene-regulated [15,19].

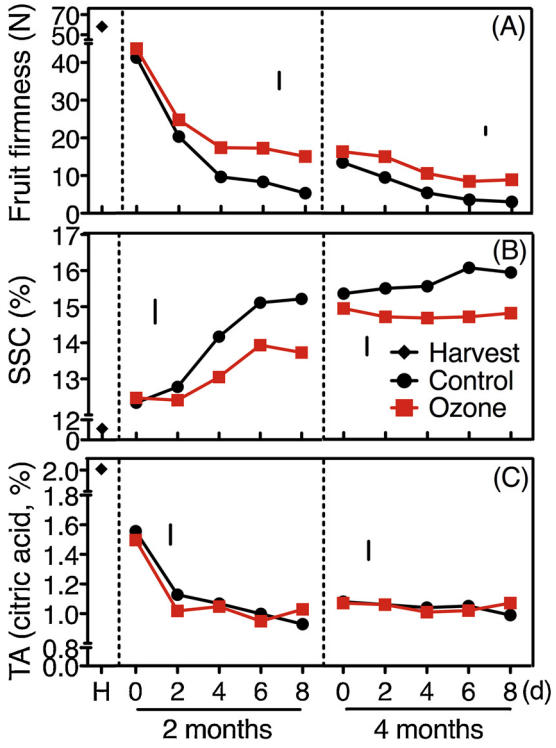

Fig. 2. Changes of tissue firmness (A) soluble solids content (SSC, B) and titratable acidity (TA, C) in kiwifruit (cv. 'Hayward') at harvest (H) and during ripening (20 °C, 90% RH) for up to 8 d following 2 or 4 months of cold storage (0 °C, 95% RH) in the absence (control) or in the presence of ozone (0.3 μL L^{-1}). Each value represents the mean of 3 biological replications of 10 fruits analyzed at each ripening stage after cold storage. The vertical bars in each particular figure represent the least significant difference (LSD, $P = 0.05$).

Soluble solids content increased from 6.7% at harvest to approximately 12.5 and 15% after 2 and 4 months of cold storage, respectively. Storage in ozone-enriched atmosphere led to lower SCC in kiwifruit compared to control during ripening at 20 °C, but in

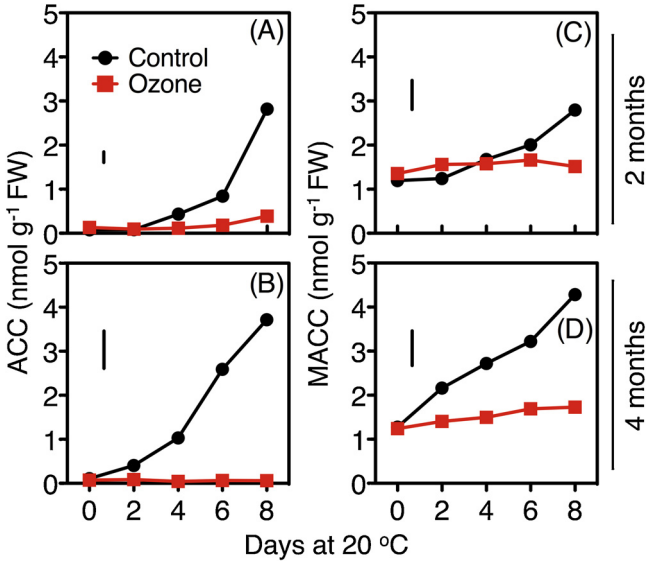

Fig. 3. Changes of ACC (A, B) and MACC (C, D) content in kiwifruit (cv. 'Hayward') during ripening (20 °C, 90% RH) for up to 8 d following 2 (A, C) or 4 (B, D) months of cold storage (0 °C, 95% RH) in the absence (control) or in the presence of ozone (0.3 μL L^{-1}). Each value represents the mean of 3 biological replications analyzed at each ripening stage after cold storage. The vertical bars in each particular figure represent the least significant difference (LSD, $P = 0.05$).

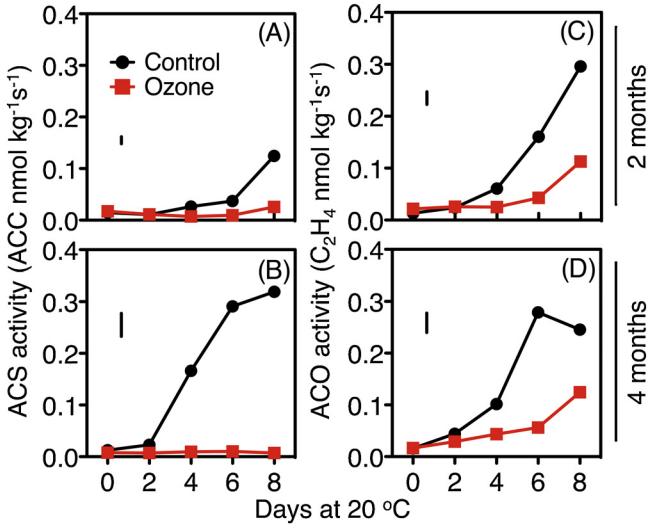

Fig. 4. Changes of ACC synthase (A, B) and ACC oxidase (C, D) *in vitro* enzyme activities in kiwifruit (cv. 'Hayward') during ripening (20 °C, 90% RH) for up to 8 d following 2 (A, C) or 4 (B, D) months of cold storage (0 °C, 95% RH) in the absence (control) or in the presence of ozone (0.3 μL L^{-1}). Each value represents the mean of 3 biological replications analyzed at each ripening stage after cold storage. The vertical bars in each particular figure represent the least significant difference (LSD, $P = 0.05$).

acceptable for consumption levels (>12.5%) (Fig. 2B) [36]. Titratable acidity decreased by 23 and 45% after 2 and 4 months of cold storage, respectively compared to harvest. However, no difference was observed between control and ozone treatment during storage and the subsequent ripening period (Fig. 2C). The fact that the kiwifruit ripening delay, expressed as reduced fruit softening and SSC accumulation, was observed during the ripening periods which occurs under ozone-free conditions, indicates that ozone exerts residual effects in the fruits.

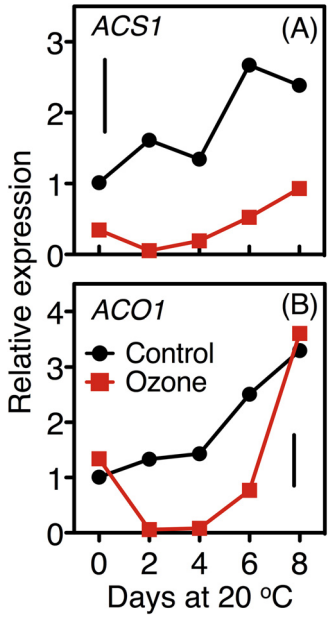

Fig. 5. Relative transcript levels of ACC synthase (A) and ACC oxidase (B) in kiwifruit (cv. 'Hayward') during ripening (20 °C, 90% RH) for up to 8 d following 2 months of cold storage (0 °C, 95% RH) in the absence (control) or in the presence of ozone (0.3 μL L^{-1}). The experiment was carried out in triplicate. The vertical bars in each particular figure represent the least significant difference (LSD, $P = 0.05$).

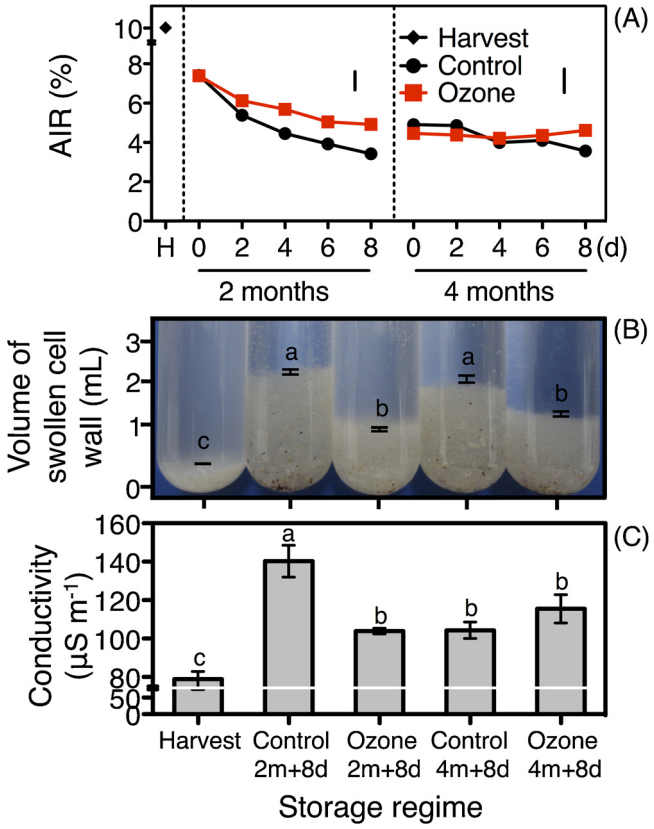

Fig. 6. (A) Changes of alcohol insoluble residue (AIR) content in kiwifruit (cv. 'Hayward') at harvest (H) and during ripening (20 °C, 90% RH) for up to 8 d following 2 or 4 months of cold storage (0 °C, 95% RH) in the absence (control) or in the presence of ozone (0.3 μL L^{-1}). Each value represents the mean of 3 biological replications analyzed at each ripening stage after cold storage. The vertical bars in each particular figure represent the least significant difference (LSD, $P = 0.05$). (B) Cell wall swelling of kiwifruit measured as volume of swollen cell wall and (C) electric conductivity of the water suspension at harvest and after 8 d ripening upon removal from of cold storage after 2 or 4 months (m) in control or in ozone conditions. Each value (±SE) represents the mean of 3 biological replications analyzed at each ripening stage. Values followed by the same letter do not differ significantly according to the least significant difference test (LSD, $P = 0.05$).

3.2. *Ozone blocks ethylene biosynthesis by preventing ACC accumulation through the inhibition of ACS expression and activity*

The rates of ethylene production in a plant cell are controlled by its immediate precursor ACC levels [37]. Whereas MACC have not be considered an important storage form of ACC, malonylation of ACC can regulate ACC levels [37,38], while recently has been reported that can be transported between different tomato tissues, providing a potential mechanism to control ethylene biosynthesis [39]. To characterize the effects of ozone exposure on ethylene biosynthesis, we determined the changes in ACC and MACC contents. Kiwifruit presented very low ACC content immediately after removal from cold storage (Fig. 3A and B). The content of ACC in control kiwifruit was increased after 2 months of cold storage plus 4 d of ripening, as well as following 4 months of cold storage plus 2 d of ripening. In contrast, in fruit cold stored with ozone and subsequently ripened at room temperature in air, ACC accumulation was greatly suppressed. Malonyl-ACC content increased during both ripening periods studied in control fruit, however extended cold storage (4 months) led to faster and to higher MACC accumulation over the 8-day ripening period compared to the 2-month cold storage (Fig. 3C and D). The levels of MACC remained

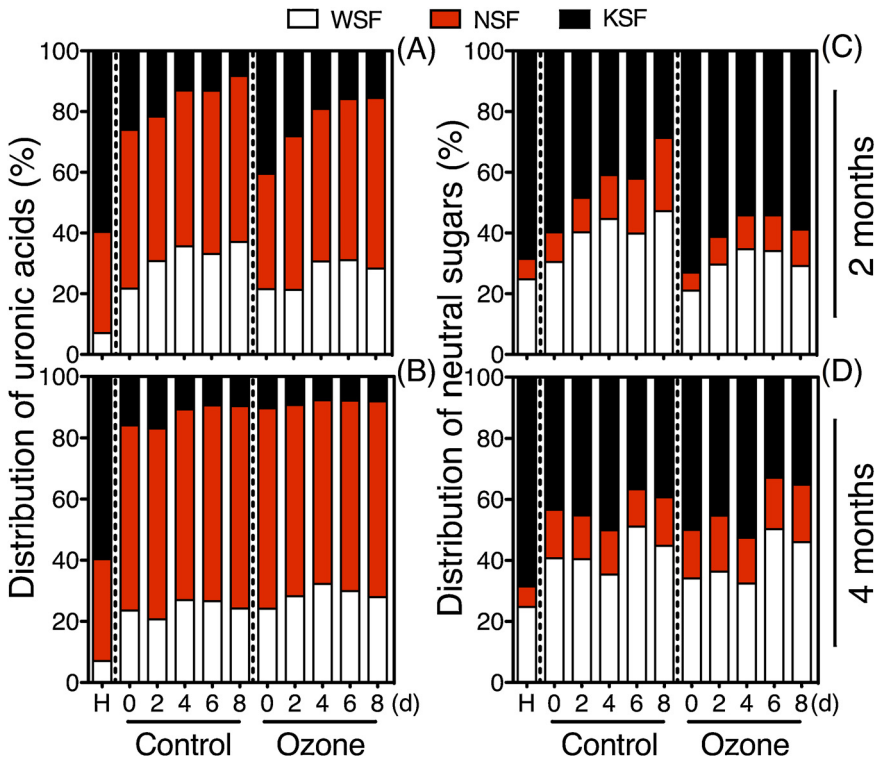

Fig. 7. Uronic acids (A, B) and neutral sugars (C, D) in water- (WSF), Na_2CO_3- (NSF), 4KOH-soluble fractions and the corresponding residue of the insoluble cell wall of at harvest (H) and during ripening (20 °C, 90% RH) for up to 8 d following 2 (A, C) or 4 (B, D) months of cold storage (0 °C, 95% RH) in the absence (control) or in the presence of ozone (0.3 μL L^{-1}). Each value represents the mean of 3 biological replications analyzed at each ripening stage after cold storage.

unchanged in ozone-treated kiwifruit throughout both ripening periods at 20 °C.

To further elucidate the mechanisms governing the inhibition of ethylene production by ozone during kiwifruit ripening, we assessed the activity of the key enzymes of ethylene biosynthesis, namely ACC synthase (ACS) and ACC oxidase (ACO) (Fig. 4). ACS enzyme activity showed a ripening-dependent increase in the control fruit, whereas its activity was strongly inhibited by ozone treatment (Fig. 4A and B), thus leading to decreased ACC content in ozone-treated kiwifruit (Fig. 3B). Indeed, ACS activity remained at very low levels during ripening in kiwifruit exposed to ozone, suggesting that ACS function could be considered as a key mechanism by which ozone controls kiwifruit ripening. On the other hand, kiwifruit exposed to ozone showed lower levels of ACO activity than control fruit during the 4–8 d ripening period (Fig. 4C and D), indicating that ACO is also specifically targeted by ozone.

Previous study on kiwifruit ripening showed that the ethylene biosynthetic genes *AdACS1* and *AdACO1*, which encode ACC synthase and ACC oxidase, respectively, are closely related with climacteric ethylene production [40]. Thus, in the current study, we isolated and cloned these genes and their expression (Fig. 5) was evaluated in the samples collected during ripening after 2 months of cold storage based on ethylene production patterns (Fig. 1A and B).

Exposure of tomato leaves for some hours (2–6) to 0.08–0.2 μL L^{-1} ozone induced *ACO1* expression and increased ethylene production [41,42]. In contrast to the situation described for vegetative organs, it has been documented [43] that tomato fruit subjected to ozone-enriched atmospheres (0.05–0.1 μL L^{-1}) for up to 6 d displayed lower *LeACO1* gene expression. In the present study, gene expression analysis indicated that *AdACS1* mRNA accumulation increased during ripening at 20 °C in control kiwifruit. Of particular interest is the fact that kiwifruit stored under ozone-enriched atmosphere for 2 months exhibited a down-regulation of *AdACS1* expression (Fig. 5A). *AdACO1* was also down-regulated in ozone-treated kiwifruit after 2, 4 or 6 d ripening compared to untreated fruit. Only after 8 d at 20 °C, *AdACO1* expression in ozone-treated fruit recovered to the level of their control counterparts (Fig. 5B). The above results suggest that the inhibition of ethylene biosynthesis in kiwifruit by ozone may be attributed to a residual inhibition of ethylene biosynthetic machinery. These kind of 'priming' effects of ozone that have been reported in *Arabidopsis thaliana*, provides supporting evidence that ozone pre-treatment could modulate subsequent responses to stress events [44]. Such an ozone action could be mediated by various signaling intermediates, which were generated during the ozone-derived oxidative conditions, particularly evidenced in kiwifruit upon prolonged cold storage-originated stress situation. Indicatively, it has been suggested that the physiological action of ozone is interconnected with ethylene, salicylic acid (SA), abscisic acid (ABA) and nitric oxide (NO) during stress responses in *A. thaliana* [10,45]. Thus apart ethylene biosynthesis, ozone may regulate the kiwifruit ripening through alterations of the balance, interaction and constant recalibration of the different plant hormones [46]. Notably, the fact that the ozone-treated fruit finally ripened to an eating ripe level upon transferred to 20 °C is highly interesting from a commercial perspective and suggest that further studies are needed to address the function of ozone in kiwifruit ripening physiology.

3.3. Residual effects of ozone treatment in kiwifruit cell wall disassembly

Given the marked delay in softening pattern observed in ozone-treated fruit (Fig. 2A), we isolated the alcohol insoluble residue (AIR) representing the cell wall material and some residual starch (Fig. 6A). The AIR decreased by 25% during cold storage for 2 months, although no differences between control and

ozone-treated fruit were monitored. Upon transfer to 20 °C the AIR dropped more rapidly in the control than in fruit exposed to ozone (Fig. 6A). During 4 months storage at 0 °C, a rapid decrease in the AIR was found in both control and ozone-treated kiwifruit and no further modifications occurred within the corresponding ripening period (Fig. 6A).

The swelling ability of the cell walls of the melting flesh fruit types, such as kiwifruit and tomato, increases both *in vitro* and *in vivo* during ripening [28,47]. Swelling has been associated with the movement of water into voids left in the cellulose-hemicellulose matrix [14,47]. Though the molecular changes associated with this phenomenon have not been unequivocally determined, it has been correlated with the solubilization of pectic polysaccharides [14]. In the present project, the swelling capacity of the AIRs in the ozone-treated fruit was lower than that of the control after both ripening periods studied (Fig. 6B). The swelling capacity of native starch in cold water was extremely low and consequently the increased swelling of the control AIRs likely resulted from more extensive degradation of cell wall components compared to ozone-treated fruits. The electric conductivity of control fruit AIR dispersions was higher than that of ozone-treated kiwifruit after 2 months cold storage and 8 d ripening (Fig. 6C). Lower conductivity was measured after 4 months of cold storage, but no differences were detected between control and ozone-treated kiwifruit (Fig. 6C). The higher conductivity values have resulted from the presence of higher levels of demethoxylated GalA residues in soluble pectins raising polysaccharide net charge. Conductivity would be expected to increase during ripening, as pectins are solubilized and demethylated, however, at late stages, the conductivity could drop again as lower levels of pectins remained. This pattern is similar to that reported for fruit cell wall swelling capacity [28,47].

Although mature and ripe kiwifruit are able to synthesize cell wall polymers during ripening, the degradation rate are by far more prominent [14,47]. To further characterize the effects of ozone pre-exposure on cell wall disassembly, we sequentially solubilized the components present in the AIRs with water, Na_2CO_3 and KOH. The proportion of extractable water-soluble UA was markedly increased in control kiwifruit during the ripening period following 2 months of cold storage, possibly reflecting the drop in both KOH soluble polyuronides (Fig. 7A). Pectin solubilization in kiwifruit was reported to be an ethylene-dependent process, because 4 d after external ethylene treatment, the 60% of cell wall polyuronide was solubilized [18]. However, it is worth noting that during the ripening period following 4 months of refrigerated storage, no major differences in the relative distribution of UA were detected between control and treated fruits (Figs. 7B and S3), regardless the massive inhibition in ethylene production (Fig. 1B). After 2 months of cold storage, ozone-treated fruit maintained lower levels of both water and Na_2CO_3-soluble NS (Fig. 7C). On the other hand, in control and ozone-treated fruit following the 4-month cold storage period significant differences on NSF NS recorded at the end of the ripening period (Figs. 7D and S4B). The solubilization of NS in the WSF and NSF was less affected by the 2-month ozone exposure than that of UA (Figs. S3 and S4).

Lower solubilization of KOH NS was also found in ozone treated fruit following 2 months of storage (Fig. S5A). The contents of α-cellulose displayed similar levels in control and ozone-treated fruit immediately after removal form 2-month cold storage, decreasing subsequently more rapidly in the control (Fig. S5B). Similarly to what was observed with pectic polysaccharides (Figs. 7, S3 and S4), no differences in KOH soluble NS and α-cellulose between control and ozone-treated fruit were recorded in the ripening period following 4 months at 0 °C (Fig. S5A and B). These results showed that ozone treatment impact the degradation of all major wall polysaccharides groups. A previous work focused on the analysis

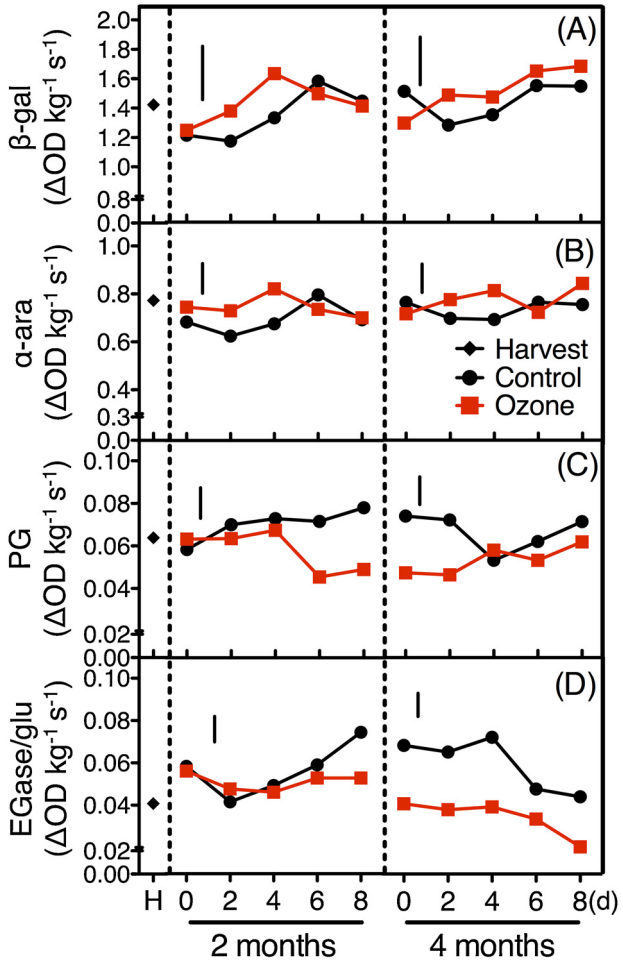

Fig. 8. Changes of *in vitro* enzymatic activities of β-galactosidase (A, β-gal), α-arabinofuranosidase (B, α-ara), polygalacturonase (C, PG) and endo-1,4-β-glucanase/1,4-β-glucosidase (D, EGase/glu) in kiwifruit (cv. 'Hayward') at harvest (H) and during ripening (20 °C, 90% RH) for up to 8 d following 2 or 4 months of cold storage (0 °C, 95% RH) in the absence (control) or in the presence of ozone (0.3 μL L^{-1}). Each value represents the mean of 3 biological replications analyzed at each ripening stage after cold storage. The vertical bars represent the least significant difference (LSD, $P = 0.05$).

of cell wall degradation process in kiwifruit treated with external ethylene to stimulate ripening immediately just after harvest [14], however scarce information is available on the effect of cold storage and postharvest treatments on kiwifruit cell wall disassembly. The lower differences in firmness (Fig. 2A), cell wall swelling (Fig. 6B and C) and polysaccharide disassembly (Fig. 7) observed during the ripening period following 4 months of cold storage confirm that ozone does not block the fruit's ripening capacity. The fact that after long-term ozone treatment (4 months) the inhibition of ethylene biosynthesis was accentuated, while the arrest of cell wall disassembly fainted has two corollaries. Ethylene biosynthetic machinery is more sensitive to ozone than cell wall metabolism. Moreover, kiwifruit may soften to the 'eating-ripe window' firmness even in the presence of extremely low levels of ethylene production (0.02–0.12 μL kg^{-1} h^{-1}) [48].

3.4. Ozone treatments reduce wall disassembly, swelling and softening by inhibiting PG and EGase/glu but not β-gal and α-ara activities

To further investigate the effect of ozone on wall disassembly, we analyzed the changes in cell wall modifying enzymes associated with degradation of pectins (α-ara, β-gal, PG) and hemicelluloses (EGase/glu) (Fig. 8). One of the most massive changes that occurs

during kiwifruit softening is the loss of galactose from type I rhamnogalacutronan side chains due to the activity of β-gal [14]. However, in this work, no differences were found either during storage or between treatments in β-gal activity (Fig. 8A). α-ara, that is associated with pectin side chain remodeling [19], showed lower activity than β-gal and was not affected by ozone exposure (Fig. 8B). In un-ripe fruit, potential substrates for β-gal and α-ara are present but are inaccessible because of their location or physical state in the wall [18]. The *in vivo* cell wall swelling and the solubilization of the pectic polymers renders them susceptible to enzyme attack [18,47] and this may be the case in our study. As shown in Fig. 8C, ozone-treated kiwifruit displayed lower PG activity than the control ripened for 6 or 8 d, following 2 months of cold storage as well as during the initial days at 20 °C following 4 months of cold storage. Similarly, to what reported herein, previous study detected very low levels of PG enzyme activity compared to other cell wall hydrolases [49], however PG activity increased in the late stages of kiwifruit softening [49,50]. Collectively, the above data suggest that PG activity was remarkably affected by ozone (Fig. 8C), highlighting a possible role for this protein in the regulation of ozone postharvest function. In addition, PG activity was correlated with the flesh breakdown at the final stage of kiwifruit softening (Fig. 2A), indicating that ozone could exert an inhibitory effect at this level. Furthermore, EGase/glu activity went descending during ripening, following 2 months at 0 °C, however, its activity was stimulated at the end of ripening period. Ozone-treated kiwifruit maintained lower EGase/glu activity during ripening at 20 °C following 4 months at 0 °C and ripening at 20 °C (Fig. 8D). Although some of the ozone-associated effects observed in cell wall degradation could result from down-regulation of ethylene-dependent events, it cannot be excluded that some of the residual ozone effects resulted from alterations in ethylene-independent processes.

4. Conclusions

Results reported herein indicate that pre-exposure to ozone at 0 °C delays kiwifruit ripening at 20 °C. Importantly, ozone-treated kiwifruit exhibited an extended 'eating-ripe window' due to the reduced softening rate, however, reached consumption maturity denoting that ozone is not blocking fruit's ripening capacity. Interestingly, it was evidenced that ozone blocked ethylene biosynthesis of kiwifruit and this was linked with the inhibition of the gene expression and activity of ACS, thus resulting in reduced ACC levels in kiwifruit cells. In addition, ozone-treated fruit exhibited a significant reduction in softening and cell wall swelling that was accompanied with delayed solubilization of pectic polysaccharides. Ozone treatment inhibited PG and EGase/glu but did not affect the activity of enzymes involved in pectin-side chain removal, such as β-gal and α-ara. Overall, this study, using kiwifruit as a model crop, provides insight into a yet unknown mechanism underlying ozone's function on ripening process that will constitute a significant contribution to our knowledge on climacteric fruit ripening.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2014.08.016.

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