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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}.