Relationship of Superficial Scald in 'Fuji' Apples to Changes of Reactive Oxygen Species Metabolism in Response to Ventilation Treatment

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'Fuji' apples stored at 0 °C under non-ventilated or ventilated air after harvest were used to investigate the responses of superficial scald susceptibility to reactive oxygen species (ROS) metabolism. Ventilation decreased the concentrations of α -farnesene and conjugated trienol in apples in comparison with static storage and conjugated trienols were correlated with scald development. In both treatments, the levels of O_2^- and H_2O_2 increased during storage, whereas, ventilated apples kept higher O_2^- but lower H_2O_2 contents than non-ventilated ones, which resulted in a positive relationship between O_2^- and H_2O_2 . The activities of antioxidant enzymes all peaked before/when scald appeared and decreased while scald sustainably developed. Superoxide dismutase and catalase activities were higher in non-ventilated than ventilated apples early in storage, but no significant difference was observed between two treatments during scald symptom expression. Difference of guaiacol peroxidase activities was only observed after 15 weeks. These results indicated that conjugated trienols and reactive oxygen species produced during α -farnesene oxidation were related with scald development. It can be concluded that scald development is a long-term event induced by reactive oxygen species metabolism and could probably be aggravated by increased generation of reactive oxygen species, which attributes to the break of the balance between α -farnesene production and oxidation and antioxidative defense system during cold storage.

Key Words: Superficial scald, α -Farnesene, Conjugated trienol, Reactive oxygen species, Antioxidant enzymes.

INTRODUCTION

Superficial scald is a commercially important physiological disorder that occurs in apples and pears during storage. Symptoms manifest as brown or black patches on the skin and typically worsen after removal from storage, rendering the fruit unmarketable¹. Largely on the basis of correlative data, superficial scald is widely believed to be induced by the sesquiterpene α -farnesene and more directly its conjugated trienol (CTol) oxidation products^{2,3}. However, Rao *et al.*⁴ suggested that abundant synthesis of α -farnesene and accumulation of its conjugated trienol oxidation products in the epidermis and cuticle are not closely correlated with scald susceptibility. Whitaker *et al.*⁵ have also reported that oxidation products of α -farnesene are not essential for scald development in fruits with severely compromised antioxidative defenses, but free radicals and/or toxic volatiles generated by α -farnesene oxidation can cause an increase in the expression and intensity of the symptoms of scald.

Production of reactive oxygen species (ROS) in all respiring cells is a continuous process and accumulations of reactive oxygen species occur during senescence or under

physiological stress. Reactive oxygen species such as hydroxyl ($\cdot OH$), hydrogen peroxide (H_2O_2) and superoxide (O_2^-) can attack and destroy lipid and protein macromolecular structures essential for cellular function⁶ and subsequently lead to scald symptom development. Plant cells are protected against the effects of reactive oxygen species by complex antioxidant systems. These systems involved both water and lipid-soluble antioxidants such as ascorbate or tocopherols and carotenoids and antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (POX), ascorbate peroxidase (APX) and glutathione reductase (GR)⁷. Superoxide dismutase is the primary defense against free radicals and specifically and efficiently catalyzes conversion of O_2^- into H_2O_2 ⁸, which is then reduced to water by a wide range of enzymes such as catalase and guaiacol peroxidase⁹. Reactive oxygen species metabolism as production and scavenging in plant cells generally keeps a dynamic equilibrium and when the production exceeds the scavenging, the balance is broken down, leading to a rapid accumulation of reactive oxygen species and causing a serious oxidative stress, such as superficial scald. Though several studies have pointed the potential role of antioxidant enzymes such as superoxide dismutase, catalase,

ascorbate peroxidase and guaiacol peroxidase^{4,10} and antioxidants¹¹ for the fruit susceptibility to scald. However, to date, there have been no reports on the entire picture of the balance between reactive oxygen species production and the antioxidant defense system involved in scald in 'Fuji' apples.

Due to the potential causal of α -farnesene metabolism in scald development, as well as its correlation with reactive oxygen species production, the major objective of the study was to investigate the response of reactive oxygen species metabolism to α -farnesene and conjugated trienol accumulations affected by ventilation and their roles in scald development of 'Fuji' apples during long-term cold storage.

EXPERIMENTAL

'Fuji' apples were harvested at commercial maturity from a commercial orchard in Shaanxi, China. Apples were transported immediately after harvest to laboratory and equally stored in two 25 m³ chambers, one under static (non-ventilated) and another with ventilated (air quantity, 1831 m³ h⁻¹, wind velocity, 2 m s⁻¹, six times per day, three minutes for each time) air at 0 °C with a relative humidity of *ca.* 90 % for up to 30 weeks. There were three replicates for each experimental treatment and each replicate contained 275 fruit.

For each treatment, there were 10 fruit per replicate for measurement of α -farnesene and conjugated trienol concentrations at 5 week intervals of storage, plus 100 fruit for evaluation of scald after 30 weeks storage plus 7 days at 20 °C. Then fruit peel, respectively removed from the equatorial region on opposite sides of extra 15 fruits per replicate, was frozen in liquid nitrogen at each interval of storage and stored at -85 °C until used for extraction and measurement of O₂⁻, H₂O₂ and malondialdehyde contents and determination of superoxide dismutase, catalase and guaiacol peroxidase activities.

Extraction and analysis of α -farnesene and conjugated trienes: Extraction and analysis of α -farnesene and conjugated trienols were performed as described by Isidoro and Almeida¹². Peel was removed from the equatorial region on opposite sides of 10 fruit. After carefully scraping off all cortical tissue, a disk 10 mm in diameter was excised from the peel. Two disks excised from each fruit were immersed in 10 mL of hexane and incubated for 10 min at 22 °C with agitation. After incubation the solvent was filtered through a cellulose paper filter and the final volume adjusted to 10 mL. Immediately after filtration, absorbance of the extract was measured at 232 nm and in the range 281-290 nm with a UV-visible spectrophotometer (UV-1700 Pharmaspec, Shimadzu, Tokyo, Japan). α -Farnesene and conjugated trienols concentrations were calculated using molar extinction coefficients $\epsilon_{232\text{ nm}} = 27,740$ for α -farnesene and $\epsilon_{281-290\text{ nm}} = 25,000$ for the conjugated trienes¹³ and expressed as $\mu\text{g g}^{-1}$ on a fresh weight basis.

Determination of H₂O₂ and O₂⁻: The H₂O₂ content was determined according to a method of Loreto and Velikova¹⁴. 2 g FW of frozen peel were homogenized at 4 °C in 0.1 % (w/v) trichloroacetic acid. The homogenate was centrifuged at 12,000 $\times$ g for 15 min and 1 mL of the supernatant was mixed with 1 mL of 10 mM potassium phosphate buffer pH 7 and 2 mL of 1 M KI. The H₂O₂ content of the supernatant was evaluated by comparing its absorbance at 390 nm with a standard calibration curve and expressed as $\mu\text{mol g}^{-1}$ FW.

For O₂⁻ determination, 1 g FW of frozen peel was homogenized at 4 °C with 10 mL of cold 50 mM potassium phosphate buffer, pH 7.8. The homogenate was centrifuged at 10,000 $\times$ g for 20 min at 4 °C and the supernatant used to determine superoxide anion content by hydroxyl amide oxidation method¹⁵. Superoxide was expressed as nmol g⁻¹ FW.

Superoxide dismutase, guaiacol peroxidase and catalase assays: Superoxide dismutase was extracted from 1 g FW of frozen peel tissue with 10 mL of cold 50 mM potassium phosphate buffer, pH 7.8 in a mortar and pestle at 4 °C. The homogenate was twice centrifuged at 20,000 $\times$ g for 15 min at this temperature. Superoxide dismutase activity was assayed by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium (NBT) according to the method of Oberley and Spitz¹⁶. One unit of superoxide dismutase activity was defined as the amount of enzyme required to cause 50 % inhibition of the reduction of the nitro blue tetrazolium monitored at 560 nm.

Guaiacol peroxidase and catalase were extracted from 1 g FW of frozen peel tissue using 10 mL of ice-cold 50 mM potassium phosphate buffer, pH 7, containing 1 mM EDTA and 5 % (w/v) PVPP at 4 °C. The homogenate was twice centrifuged at 20,000 $\times$ g for 15 min at this temperature. Guaiacol peroxidase activity was determined using the method described by Rao⁹. One unit of guaiacol peroxidase was defined as the amount of enzyme responsible for the changes of optical density (OD) min⁻¹ at 470 nm. Catalase activity was determined by the method of Klapheck¹⁷. One unit of catalase was defined as the amount of enzyme which disintegrates 1 mmol H₂O₂ min⁻¹ at 25 °C.

Enzyme activities were all based on total protein. Protein content was determined according to the method of Bradford¹⁸ using bovine serum albumin as a standard.

Malondialdehyde content assay: Malondialdehyde content was determined by the thiobarbituric acid (TBA) reaction according to Imahori *et al.*¹⁹. Frozen peel tissues (2 g) were homogenized in a cooled mortar and pestle with 10 mL of ice-cold 0.1 % trichloroacetic acid (TCA). The homogenate was centrifuged at 20,000 $\times$ g for 10 min at 4 °C. A 1 mL aliquot of the supernatant was thoroughly mixed with 4 mL of 20 % trichloroacetic acid containing 0.5 % thiobarbituric acid. The mixture was incubated at 95 °C for 0.5 h and then quickly cooled in an ice-bath. After centrifugation at 20,000 $\times$ g for 10 min at 4 °C, the absorbance of the supernatant at 532 nm was recorded and corrected for non-specific absorption at 600 nm using a spectrophotometer (UV-1700 Pharmaspec, Shimadzu, Tokyo, Japan). The malondialdehyde concentration was calculated using the extinction coefficient 155 mM⁻¹ cm⁻¹.

Assessment of superficial scald: Scald was defined by incidence and severity. Incidence was evaluated as all fruit with symptoms. Scald severity was evaluated visually using the percentage of the fruit surface area affected, where none = 0, 1-10 % = 1, 11-33 % = 2, 34-66 % = 3 and 67-100 % = 4 and divided by the number of affected fruit.

Statistical analysis: The experimental design was a completely randomized one with three replications. Data was subjected to analysis of variance (ANOVA) with treatment and storage time. Mean comparisons were performed using the least significant difference (LSD) method $p = 0.05$. Pearson

correlations were used to quantify the relationships among factors assessed.

RESULTS AND DISCUSSION

Scald incidence and severity both increased greatly during cold storage. Though no significant difference was found at week 15th, ventilated apples showed nearly two-fold lower levels in scald incidence and severity than non-ventilated ones after 20 weeks or more storage.

Ventilation could reduce the concentrations of α -farnesene and conjugated trienol²⁰, which might be the reason why ventilation was effective in scald reduction. Though α -farnesene levels of two treatments both increased and progressed to a maximum level after *ca.* 15 weeks, the effect of ventilation on its accumulations had become apparent since the first five weeks and non-ventilated apples subsequently kept *ca.* 1.5-fold more than ventilated ones (Fig. 1A). The concentrations of conjugated trienol increased with prolonged storage time and showed a closely correlation with scald incidence ($r = 0.745$, $p < 0.05$) and severity ($r = 0.711$, $p < 0.05$). Throughout the storage, non-ventilated apples kept more conjugated trienol accumulations than ventilated ones (Fig. 1B). Conjugated trienols are not considered to be volatile²¹ and so any suppression of their accumulation by ventilation might well result from depletion of more α -farnesene²⁰.

Fig. 1. Changes in the concentrations of α -farnesene (panel A) and conjugated trienol (panel B) in peel tissue of non-ventilated (■) and ventilated (▲) apples during storage at 0 °C. Vertical bars represent LSD at 5 % level

Though the accumulations of conjugated trienol resulting from oxidation of α -farnesene correlative with scald are fact-based, there are reports to the contrary^{4,5}. In fact, conjugated trienols are barely digitally relative with superficial scald and

never confirmed to be the infectious agent. In contrast, it is widely believed that scald development may be also related to the production of free radicals through α -farnesene catabolism⁴. Anet¹³ suggested that free radicals generated from the decomposition of peroxides and hydroperoxides produced by the autoxidation of α -farnesene are contributory cause of the scald. Du and Bramlage²² have also hypothesized that low-temperature-induced reactive oxygen species is responsible for the development of physiological disorders such as superficial scald. Therefore, the generation of reactive oxygen species could serve as a potential primary cause for the development of superficial scald.

Superoxide (O_2^-) contents increased in both treatments throughout the storage and were significantly higher ventilated than non-ventilated apples from 15 to 30 weeks (Fig. 2A). Superoxide dismutase specifically and efficiently catalyzes conversion of O_2^- and thus prevents the toxic effect of active species such as those generated by oxidation of α -farnesene¹⁰. In the non-ventilated apples, superoxide dismutase activities increased faster and kept higher levels in comparison with those in ventilated ones for the first 10 weeks, however, no significant difference was observed between two treatments afterwards (Fig. 3A). The levels of reactive oxygen species *in vivo* depend upon the balance between their generation and the capacity to remove them²⁰. The higher levels of O_2^- content and superoxide dismutase activity in non-ventilated apples than ventilated ones, as well as their increase with time in the first 10 weeks storage indicated that the balance had been destroyed after harvest and the reactive oxygen species generation exceeded scavenging, to some degree, promoting the oxidation of α -farnesene.

Fig. 2. Changes in O_2^- (panel A) and H_2O_2 (panel B) levels in peel tissue of non-ventilated (■) and ventilated (▲) apples during storage at 0 °C. Vertical bars represent LSD at 5 % level

O_2^- can be dismutated to H_2O_2 , a potentially toxic compound, by superoxide dismutase. The maintaining of H_2O_2 levels depends on the balance between H_2O_2 -generating superoxide dismutase and H_2O_2 -degrading catalase and guaiacol peroxidase enzyme activities. Catalase is an enzyme that catalyzes the decomposition of H_2O_2 to oxygen and water, whereas guaiacol peroxidase decomposes H_2O_2 by a hydrogen donor molecule, such as ascorbate, phenolic and/or several aromatic compounds²³. The increased H_2O_2 content in fruit of two treatments after 5 weeks storage (Fig. 2B), as well as the advanced catalase and guaiacol peroxidase activities (Fig. 3B-C) indicated that the H_2O_2 metabolic balance *in vivo* had been destroyed yet. Though no significant differences were statistically found in the contents of H_2O_2 between two treatments for the first 10 weeks storage (Fig. 2B), the higher catalase activities (Fig. 3B) also made it clear that the reactive oxygen species metabolic in non-ventilated apples exhibited a more notable departure from balance than those in ventilated ones. Guaiacol peroxidase activities did not contribute to the H_2O_2 variances because of their insignificant difference during this process (Fig. 3C).

As the results above, oxidative stress had occurred as the generation of reactive oxygen species exceeded the capacity

of the fruit to maintain cellular redox homeostasis, or, more simply, the production of reactive oxygen species exceeded the capacity of the apple to scavenge them. Reactive oxygen species are able to initiate lipid peroxidation reactions and the increase in peroxidation products may have been associated with changes in cellular conditions that result in symptom development²⁴. Lipid peroxidation can be indicated by measuring the content of malondialdehyde, because malondialdehyde is a secondary end product of polyunsaturated fatty acid oxidation¹⁹. Malondialdehyde contents gradually increased for the first 10 weeks storage, which indicated that increased reactive oxygen species levels (Fig. 2) aggravated the lipid peroxidation, but there was no significance difference between two treatments (Fig. 4) because of the indifference reactive oxygen species levels at this time of storage (Fig. 2). Though the deterioration of the integrity of cell membrane had been initiated once after harvest, no scald symptom was observed until the 15th week (Fig. 5).

Fig. 3. Changes in superoxide dismutase (SOD) (panel A), catalase (CAT) (panel B), guaiacol peroxidase (POX) (panel C) activities in peel tissue of non-ventilated (■) and ventilated (▲) apples during storage at 0°C. Vertical bar represents LSD at 5 % level

Fig. 4. Changes in malondialdehyde (MDA) content in peel tissue of non-ventilated (■) and ventilated (▲) apples during storage at 0 °C. Vertical bar represents LSD at 5 % level

Fig. 5. Scald incidence (solid lines) and severity (dotted lines) in non-ventilated (■) and ventilated (▲) 'Fuji' apples after 30 weeks storage at 0 °C plus 7 days at 20 °C in air. Vertical bars represent LSD at 5 % level

Malondialdehyde contents significantly increased from 10 to 20 weeks, after which decreased in both treatments, but kept more in non-ventilated fruit than ventilated ones (Fig. 4). During this stage, scald incidence and severity gradually increased and was higher in non-ventilated apples than ventilated ones (Fig. 5). The changes of malondialdehyde content suggested that the deterioration of cell membrane due to lipid peroxidation was a gradual process, rather than a transient reaction, which coincided with the phenomenon that superficial scald always occurs after a certain storage period.

Rao *et al.*⁴ have demonstrated that tissue concentrations of H₂O₂ were lower in scald-resistant than in scald-susceptible fruit at harvest and after storage and these lower concentrations were associated with less damage to cellular membranes (lipid peroxidation) and proteins. H₂O₂ contents of both treatments significantly increased after 10 weeks storage and kept at relatively high levels until the end of storage. In comparison with ventilated ones, non-ventilated apples always kept more H₂O₂ contents after 15 weeks storage (Fig. 2). And also, correlation coefficients of 0.779 and 0.838 ($p < 0.05$) were found for H₂O₂ and scald incidence, severity respectively, which suggested that H₂O₂ was potential causal agents in the development of scald. H₂O₂ could potentially diffuse throughout the cell readily, causing peroxidation of membrane lipids leading to increased membrane permeability and metabolic disturbances and eventually the visual symptoms of scald.

During the stages of scald symptom expression, superoxide dismutase activities decreased after 10 weeks storage and no significant differences were found between non-ventilated and ventilated apples (Fig. 3A). Du and Bramlage²² found no relation of total superoxide dismutase activities to scald development. As well, Rao *et al.*⁴ reported that activities of superoxide dismutase were not related to scald susceptibility or storage period. However, it was certain that decreased superoxide dismutase activities resulted in more O₂⁻ contents. Though the activities of H₂O₂-degrading enzymes, catalase and guaiacol peroxidase, were relatively high in two treatments in the 15th week (Fig. 3B-C), the increase of H₂O₂ contents (Fig. 2B) were not inhibited and scald symptoms also expressed at this time (Fig. 5). Then, the sharply decreased activities of guaiacol peroxidase and gradually decreased catalase after 15 weeks (Fig. 3B-C) indicated the continued increase of H₂O₂ contents (Fig. 2B), as well as the accelerated scald development. Higher activities of catalase and guaiacol peroxidase were related to lower H₂O₂ content and lower scald susceptibility⁴. Indeed, Rao *et al.*⁴ suggested that coordination between superoxide dismutase and H₂O₂ degrading enzymes may minimize the influence of active oxygen species on the oxidation of α -farnesene and thereby, scald. Activities of superoxide dismutase, catalase and guaiacol peroxidase all went through nearly the same pattern where they rose firstly and then dropped to relatively lower levels (Fig. 3), though not inhibiting the scald development, to some extent, their role in decreasing the degree and modifying the aspect of deviation from reactive oxygen species catabolism balance could not be ignored. And also, similar result indicated that the higher levels of H₂O₂ in 'Granny Smith' apples was positively correlated with the higher levels of mRNAs for the antioxidant enzymes catalase, glutathione reductase and MDHAR, suggesting direct regulation by the level of H₂O₂ on transcription of these antioxidant enzyme genes²⁴.

During cold storage, most of the scald appeared after α -farnesene peaked and continued to increase while α -farnesene decreased. It is probable that the production of α -farnesene is only the first stage in the development of scald and that the oxidation products of α -farnesene are the causal agents. The indifferent activities of three antioxidant enzymes (superoxide

dismutase, guaiacol peroxidase and catalase) between the non-ventilated and ventilated apples during the stages of scald symptom expression, as well as the significantly different reactive oxygen species levels suggested that α -farnesene metabolism might play a central role in reactive oxygen species production. Ventilation decreased the concentrations of α -farnesene in apples in comparison with static storage and then less α -farnesene involved in oxidative metabolism resulted in fewer conjugated trienols and reactive oxygen species. Then it can be concluded that superficial scald is closely related with reactive oxygen species metabolism in apple fruit. Scald development could probably be aggravated by increased reactive oxygen species production which attributed to the break of the balance between α -farnesene production and oxidation and antioxidative defense system after harvest.

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