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Superficial Scald Symptoms in 'Granny Smith' Apples Associated with Reactive Oxygen Species (ROS) Accumulation

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For many crops, low-temperature storage results in oxidative stress and chilling injury, caused by increased production of superoxide anions, which in turn leads to the generation of other dangerous reactive oxygen species (ROS). The apple cultivar Granny Smith is the most sensitive cultivar to chilling injury, which is expressed as superficial scald symptoms during cold storage and shelf life. It is already known that using antioxidant chemicals or an ethylene inhibitor (1-methylcyclopropene) prevent superficial scald symptoms in apple. In this work we showed that application of friendly organic treatment, 0.5% low-oxygen (LO2) atmosphere for 10 d at 20 °C prior to cold storage at 0 °C, was effective in preventing superficial scald symptoms. LO2-pretreated fruit maintained a healthy appearance, which was exhibited by nice green peel color without superficial scald symptoms for more than 6 months at 0 °C plus 1 week at 20 °C. Using confocal laser-scanning microscopy, fluorometer and H_2O_2 measurements of apple peel, we succeeded in determining ROS accumulation in control fruit, while none were found in LO2 treated fruit. We assume that LO2-pretreated fruit remained healthier due to reduced production of ethylene and reduced formation of α-farnesene oxidation products, which appear as ROS during cold storage and enhance peel damages in 'Granny Smith' apples.

'Granny Smith' apples are very susceptible to superficial scald, a chilling injury symptom, when stored in regular air at 0 °C for several months. It has been hypothesized that ethylene-induced α-farnesene is oxidized in the fruit peel to several reactive oxygen species (ROS) of conjugated trienes (CT), leading to superficial scald development (Anet, 1972; Ghahramani and Scott, 1998; Pechous and Whitaker, 2004; Rao et al., 1998; Rowan et al., 2001; Whitaker, 2004). Also an end-product volatile of α-farnesene oxidation, 6-methyl-5-hepten-2-one (MHO), was shown to be associated with scald symptoms (Mir et al., 1999; Whitaker, 2004), and its production decreased following low oxygen (LO2) or 1-methylcyclopropene (1-MCP) pretreatments (Pesis et al., 2010; Sabban-Amin et al., 2011; Watkins and Nock, 2005). In addition to the oxidative products of α-farnesene, oxidative stress from prolonged cold storage is also considered to play a role in scald development (Rao et al., 1998; Watkins and Nock, 2005). Chilling injury in fruit has been associated for many years with membrane disruption and production of ROS (Lyons, 1973; Prasad et al., 1994; Purvis and Shewfelt, 1993). The ROS comprise various compounds, including $OH\cdot$, $O_2\cdot$, H_2O_2, and various CTs (Rowan et al., 2001). Zubini et al. (2007) showed a correlation between scald appearance and high H_2O_2 levels, despite the high levels of anti oxidative enzymes in these fruit.

It was shown in the past that LO2 pretreatment for relatively short term reduced superficial scald in 'Granny Smith' apples, probably by reducing ethylene accumulation, which leads to less α-farnesene formation and its oxidation products (Ghahramani and Scott, 1998, 2000; Pesis et al., 2010; Sabban Amin et al., 2011; Scott et al., 1995; Wang and Dilley, 2000). In this work, we further studied the role of ROS accumulation in the development of superficial scald in 'Granny Smith' apple, and ways how to determine it.

Materials and Methods

Plant material. 'Granny Smith' apples (*Malus ×domestica*) from northern Israel were brought to the laboratory and treated on the day of harvest. Control fruits were immediately stored at 0 °C. The LO2 treatment was applied by flushing two 250-L chambers (40 kg of fruit per chamber in four plastic crates) with N_2 gas from a cylinder until the O_2 concentration reached 2%, then sealing the chambers and holding the temperature at 20 °C for 10 d, during which O_2 concentration decreased to 0.5% (Pesis et al., 2010). After the LO2 treatment, the chambers were opened and the fruits were transferred to cardboard boxes and stored in air at 0 °C.

Fruit-quality criteria. Fruit were stored in cardboard boxes in air at 0 °C for up to 8 months. Three trays (containing 10 fruit each) from each treatment were transferred to 7 d of shelf life at 20 °C after 2, 4, and 6 months of cold storage for superficial scald measurements after cold and room temperature shelf life. The superficial scald index was assessed according to percentage of visible damage to the peel on three trays, using a 10-point scale (0 = no scald; 1= <25%; 5 = 25% to 50%; and 10 = > 50%) (Pesis et al., 2010). Additional fruit were removed from storage at the

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same time for all other measurements. Peel color was recorded on two diametrically opposite sides of each fruit (20 measurements per treatment) by a colorimeter (CR-310; Minolta, Osaka, Japan). Results were expressed as hue angle, H°, where 90° = full yellow and 180° = full green. The data were analyzed with JPM 8.0 software (SAS, Cary, NC) and separated according to Tukey's highest significant difference test at $P < 0.05$.

Confocal microscopic analyses of ROS production. ROS were detected as described by Sabban-Amin et al. (2011), using the fluorescent probe 2,7-dichlorodihydrofluorescein diacetate (H_2DCF-DA), in which the measurement of DCF fluorescence is used to quantify general oxidative stress (Halliwell and Whiteman, 2004). The relative intensity of the fluorescence signal was estimated by calculating average pixel intensity from each successive focal plane of the apple peel slice, in 5-µm steps, using MICA software (Multi-Image Analysis, CytoView, Israel). The final value of each measurement represents the average result and standard error (SE) from measurements of four to six different slices, with the total number of focal planes ranging from 60 to 130.

Fluorometry analyses of ROS production. H_2DCF-DA was loaded as described for confocal microscopy, except that disk incubation with the dye was extended to 30 min to ensure H_2DCF-DA penetration into all cells. Apple peel disks were incubated in a 24-well plate. Intracellular ROS was determined by measuring fluorescence with an FL600-Microplate Fluorescence Reader (BioTek Instruments, Winooski, VT) equipped with a 485-nm excitation and a 530-nm reading filter.

Assay of hydrogen peroxide concentration. The H_2O_2 concentration was measured with 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent) (Invitrogene), which is used in combination with horseradish peroxidase (HRP) to detect H_2O_2 released from biological samples, including cells. Discs of apple peel were incubated in reaction buffer containing 0.1 M sodium phosphate buffer, pH 7.4, in 24-well plates shaken in the dark for 15 min. After incubation, 50 µL of the extract was transferred to a 96-well plate containing 10 mM Amplex Red, HRP stock at 10 U·mL^{-1} and reaction buffer, and were incubated for 30 min in darkness. In the presence of peroxidase, the Amplex Red reagent reacts with H_2O_2 in a 1:1 stoichiometry to produce the red-fluorescent oxidation product. Fluorescence was detected with an EbSpire 2300 Multilabel Reader (Perkin-Elmer, Billerica, MA). Excitation was in the range of 530–560 nm and emission detection at 590 nm.

Results

Superficial scald analysis. Scald development in 'Granny Smith' apples was described in terms of scald-severity index and peel color (Fig. 1). Unlike the control fruits in which scald symptoms appeared at an early stage, i.e., after 2 months in cold storage at 0 °C plus 1 week at 20 °C, apples pretreated with LO2 showed very minor scald symptoms only after 6 months of storage (Fig. 1A, 1B). The appearance of scald symptoms was correlated with a reduction in the green color of the apple peel as expressed by lower Hue angles (Fig. 1C).

ROS detection and quantification. Using confocal microscopy we detected elevated ROS levels already after 2 months in cold storage as green fluorescence in the control fruit (Sabban-Amin et al., 2011). We are showing here the amount of fluorescence in the control and LO2-treated fruits, after 4 months of cold storage plus one week at shelf life (Fig. 2). It can be noticed that the cell of the control and LO2-treated fruit are quite different even without the addition of the fluorescence dye (Fig. 2, black and white photo). However, applying the dye revealed that the control peel cells were fully fluorescent (Fig. 2), while in the LO2-treated fruit, there were only small dots of fluorescence, which were due to the chlorophyll fluorescence (Sabban-Amin et al., 2011).

Using another method for ROS detection, incubating peel disks with the same dye as in the confocal microscopy (H_2DCF) and measuring fluorescence by fluorometer revealed the same result (Fig. 3). It was shown that after 4 months at 0 °C the levels of ROS in both control and LO2-treated fruit were the same. However, during shelf life at 20 °C there was gradual increase in fluorescence in control peel, which was the highest after 7 d at 20 °C (Fig. 3). In contrast, ROS level was decreasing in the LO2-treated fruit, probably because of some breakdown of chlorophyll during shelf life. It is to be noted that the relative fluorescence units are 10 times higher with the fluorometer compared to the units

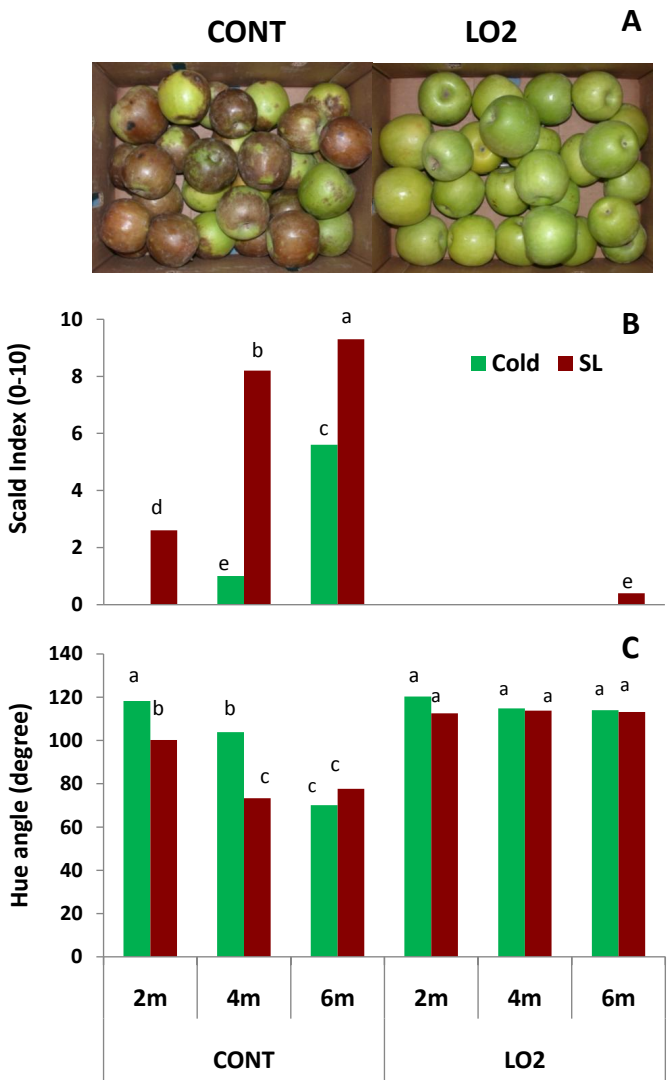

Fig. 1. Effects of LO2 pretreatments on superficial scald appearance (**A**) and on superficial scald index (**B**) and Hue angle (**C**) of 'Granny Smith' apples after 2, 4, and 6 months in cold storage at 0 °C plus 7 d shelf life (SL) at 20 °C. Picture (**1A**) was taken after 6 months at 0 °C + 1 week at 20 °C. Superficial scald index is an average of 3 trays containing 10 fruit per tray. Hue angle data are average of 20 fruit per treatment. Values followed by the same letter within a column do not differ significantly according to Tukey's test ($P < 0.05$).

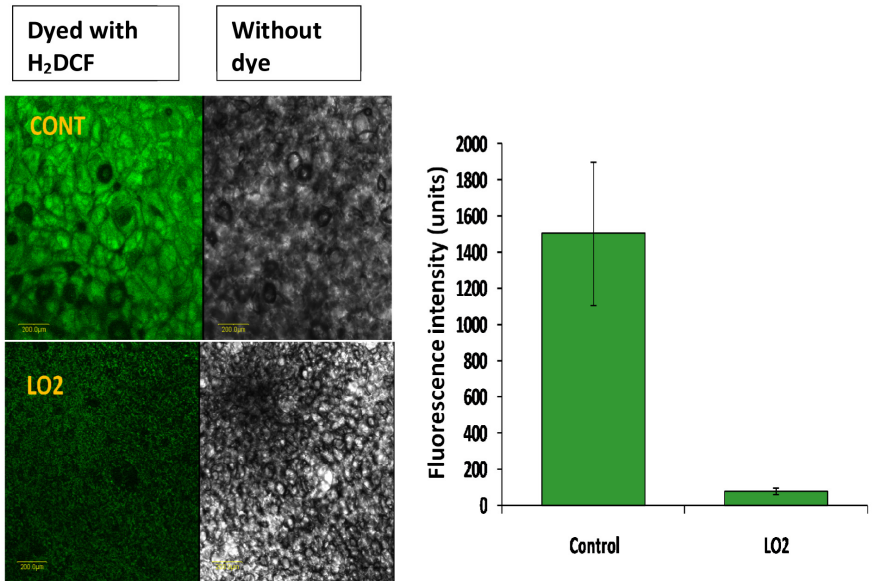

Fig. 2. Laser-scanning confocal fluorescence images and fluorescence intensity of 'Granny Smith' peel of control and LO2-treated fruit after 4 months at 0 °C +7 d at 20 °C. Pictures on the left side colored with specific dye H_2DCF and on the right side the picture is without a dye. Data are average of 4 measurements ± SE.

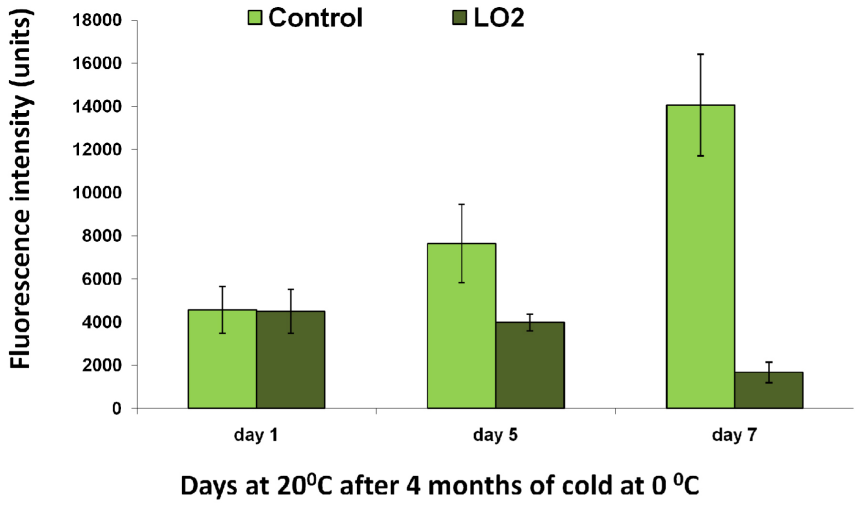

Fig. 3. ROS accumulation detected by fluorometer in 'Granny Smith' peel of control and LO2 treated fruit after 4 months at 0 °C +7 d at 20 °C. (ROS detected by dye H_2DCF). Data are average of 4 measurements ± SE.

obtained from the confocal microscopy, but the ratio between control and LO2-treated fruit are the same in both instruments (Fig. 2 vs. Fig. 3).

Hydrogen peroxide accumulation. The H_2O_2 levels in 'Granny Smith' apple peel, as measured 1 d after harvest (time zero) by fluorescence of Amplex Red reagent oxidation products, were relatively low (Fig. 4). During cold storage at 0 °C the level of H_2O_2 in the control fruits increased to a peak after 2 months at 0 °C, and then decreased after 6 and 8 months (Fig. 4). The H_2O_2 levels measured in the peel of LO2-treated fruits behaved differently: the level rose during 8 months of cold storage to a level similar to that observed in control fruits after only 2 months in cold storage (Fig. 4).

Discussion

In this study we highlighted the effectiveness of application of LO2 pretreatment prior to cold storage at 0 °C in maintaining the quality of 'Granny Smith' apples during cold storage (Fig. 1). Our present results confirm those obtained earlier by an Australian group and by ourselves on 'Granny Smith' apples (Ghahramani and Scott, 1998, 2000; Pesis et al., 2010; Sabban-Amin, 2011; Scott et al., 1995).

Exposure of plants to low temperatures can cause increases in ROS production in various plant tissues (Prasad et al., 1994; Purvis and Shewfelt, 1993). It was suggested earlier that superficial scald symptoms in apples stored at low temperature were

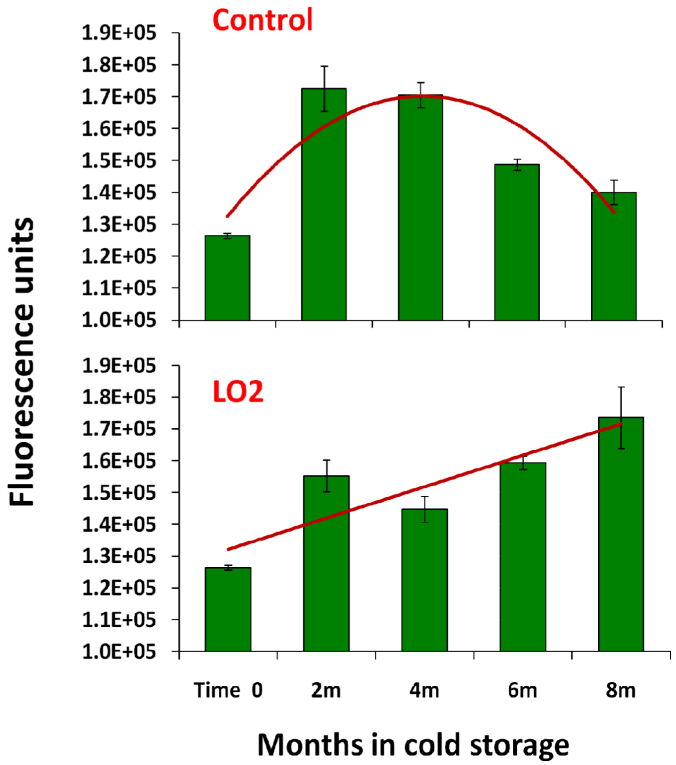

Fig. 4. Hydrogen peroxide fluorescence measured by Amplex Red reagent during 8 months at 0 °C in 'Granny Smith' peels. Excitation in the range of 530–560 nm and emission detection at 590 nm. Data are average of 4 measurements ± SE.

due to chilling injury caused by oxidative stress products (Rao et al., 1998; Watkins et al., 1995). In our present study, we detected accumulation of ROS during cold storage by three different detection methods: confocal microscopy, fluorometer and H_2O_2 assay (Figs. 2–4). The accumulation of ROS was much lower in LO2-treated fruit than in control fruit after 4 months of cold storage and with additional 7 d at room temperature, and was associated with superficial scald development (Fig. 1 vs. Figs. 2 and 3). This result is in agreement with previous findings of ROS accumulation in apple cv. White Angel × Rome Beauty during cold storage (Rao et al., 1998) and with Rowan et al. (2001), who showed that farnesyl hydroperoxide and other CTs are the immediate causal agents of superficial scald in 'Granny Smith' apple. Moreover, it was shown that superficial scald was correlated with the accumulation of MHO, the end product of α-farnesene oxidation process (Mir et al., 1999; Pesis et al., 2010; Whitaker, 2004). Recently, Sabban-Amin et al. (2011) showed low levels of ROS and MHO in both 1-MCP and LO2-treated fruit, which was correlated to the very low level of scald symptoms in these treated apples.

The increased amount of H_2O_2 in control fruit (Fig. 4) during cold storage is in agreement with previous work of Rao et al. (1998) and Zubini et al. (2007), who showed association between H_2O_2 production and superficial scald development. However, in certain cases, H_2O_2 was shown to protect the plant cell from damages (Quan et al., 2008), which can serve as a possible explanation why in LO2 treated fruit after 8 months of cold storage, there were high levels of H_2O_2 that were not correlated to scald appearance.

In conclusion, application of low oxygen (<1%) atmosphere for 10 d at 20 °C to 'Granny Smith' apple prior to cold storage maintained healthier fruit with no superficial scald symptoms, probably due to reduction in ROS accumulation during cold storage.

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