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Plants as Biofactories: Physiological Role of Reactive Oxygen Species on the Accumulation of Phenolic Antioxidants in Carrot Tissue under Wounding and Hyperoxia Stress

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ABSTRACT: Plants subjected to postharvest abiotic stresses synthesize secondary metabolites with health-promoting properties. Here, we report the potential use of carrots (*Daucus carota*) as biofactories of caffeoylquinic acids when subjected to wounding and hyperoxia stresses. Wounding stress induced an increase of ~287% in total phenolic content (PC) in carrots stored for 48 h at 20 °C. This increase was higher (~349%) in the wounded tissue treated with hyperoxia stress. To further understand the physiological role of reactive oxygen species (ROS) as a signaling molecule for the stress-induced accumulation of phenolics in carrots, the respiration rate as well as the enzymatic activities of NADPH oxidase, superoxide dismutase, ascorbate peroxidase, and catalase were evaluated. Likewise, shredded carrots were treated with diphenyleneiodonium chloride solution to block NADPH oxidase ROS productions, and the phenylalanine ammonia lyase activity and total PC were evaluated. Results demonstrated that ROS play a key role as a signaling molecule for the stress-induced accumulation of PC in carrots.

KEYWORDS: Carrot (*Daucus carota*), hyperoxia, phenolic antioxidants, postharvest abiotic stresses, reactive oxygen species, wounding

INTRODUCTION

The application of postharvest abiotic stresses in fresh fruits and vegetables induces the accumulation of antioxidants.[1] Carrots (*Daucus carota*) respond to wounding stress, accumulating phenolic compounds such as caffeoylquinic acids (CQAs) and ferulic acid (FA).[2] These antioxidant compounds are highly absorbed and metabolized in humans [3−5] and have potential uses in the treatment and/or prevention of chronic-diseases such as obesity,[6] diabetes,[7] hepatitis B,[8] cardiovascular diseases,[9] neurodegenerative diseases,[10,11] and HIV.[12,13]

Little is known about the physiological basis for the accumulation of antioxidants as a postharvest stress response. Increasing the scientific knowledge in this area is critical to envisage strategies that permit the effective use of crops as biofactories of nutraceuticals. Reactive oxygen species (ROS) are signaling molecules associated with the wound-induced activation of phenylalanine-ammonia lyase (PAL) gene expression [14] and with the accumulation of phenolic compounds.[15,16] Different sources of ROS are activated upon the application of wounding stress. These ROS sources include the reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase localized at the plasma membrane and the respiration occurring at the mitochondria.[17] NADPH oxidase and respiration produce a superoxide radical ($O_2^{\cdot-}$) which is transformed into O_2 and H_2O_2 by superoxide dismutase (SOD). High levels of ROS ($O_2^{\cdot-}$ and H_2O_2) are toxic for plants, and thus the activity of detoxifying enzymes such as SOD, ascorbate peroxidase (APX) and catalase (CAT) is needed for a fine modulation of low ROS levels.[17]

The wound-induced accumulation of phenolic compounds in carrots is affected by the application of additional stresses in the wounded tissue. For instance, the application of UV-light and phytohormones (i.e., ethylene and methyl jasmonate) in wounded-carrots induces modifications in the total amount and type of phenolics accumulated.[2,15] Hyperoxia stress (high-atmospheric oxygen storage conditions) is reported to induce accumulation of phenolics in whole blueberries,[18] strawberries [19] and Chinese Bayberries.[20] However, there are no previous reports regarding the effect of hyperoxia on the accumulation of phenolics in wounded-carrot tissue. In addition, the physiological basis for the hyperoxia-induced accumulation of phenolics remains uknown.

Previous investigations on the application of abiotic stresses on carrots to increase their nutraceutical content used mild conditions of stress to preserve quality attributes such as flavor

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and color.[2,15,16] However, the genetic potential of carrots as biofactories of phenolics can be further exploited if extreme stress conditions are applied in the tissue. The resultant tissue can be subsequently subjected to downstream processing in order to recover and purify antioxidant phenolic compounds with application in the pharmaceutical and dietary supplement industries.

The objectives of this research were: (i) to evaluate the use of extreme conditions of wounding and hyperoxia stresses to produce high amounts of antioxidant phenolic compounds in carrots and (ii) to determine the physiological role of ROS on the accumulation of phenolics induced by wounding and hyperoxia stresses. This paper is based on presentations given at the 2009 Annual Meeting of the Institute of Food Technologist and at the sixth International Postharvest Symposium.[21,22]

MATERIALS AND METHODS

Chemicals. FA, Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3), 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox), 2,2′-Azobis (2-amidinopropane)-dihydrochloride (AAPH), fluorescein sodium salt, polyvinylpyrrolidine (PVPP), boric acid, ss-mercaptoethanol, L-phenylalanine, *t*-cinnamic acid, diphenyleneiodonium chloride (DPI), Na_2EDTA, ascorbic acid, diethyl dithiocarbamate, NADPH, Tris-HCL, sucrose, riboflavin, methionine, methanol (HPLC grade), and acetonitrile (HPLC grade), were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Chlorogenic acid, nitrotetrazolium blue chloride (NBT), and Triton X-100, were purchased from Fisher Scientific (Houston, TX, USA).

Plant Material and Processing. Carrots (*D. carota*) were obtained from a local market (HEB, Bolthouse Farms, Bakersfield, CA), sorted, washed, and disinfected with chlorinated water (250 ppm, pH 6.5). To prepare the carrot cuts, whole carrots were wounded with a food processor to produce slices (3−4 mm thickness), pie cuts (1/4 sections from a slice of 3−4 mm thickness), and shreds (2 mm × 3 mm × 40−60 mm). Shredded carrots treated with DPI were prepared by submerging the wounded tissue for 3 min in a DPI solution (315 μM). The DPI concentration was selected based on previous dose−response experiments.[16] In addition to the DPI treatments, shredded carrots were submerged in nanopure water and used as control samples.

Storage Studies. Two independent experiments were conducted. On the first experiment, carrots were subjected to different wounding intensities and stored for 48 h at 20 °C under two different atmospheric conditions: air and hyperoxia (80% O_2, balance N_2). The total phenolic content (PC) and antioxidant activity (AOX) of these samples were determined before and after storage. Likewise, the phenolic compounds accumulated after storage were identified and quantified. To generate the hyperoxia conditions, carrot tissue was placed in glass jars and connected to an air-flow-through system supplied with humidified flows of either air or a gas mixture containing 80% O_2 + 20% N_2. The concentrations of CO_2 were kept <0.15% to avoid any physiological effect exerted by CO_2 such as anaerobic metabolism.[23]

In the second experiment, the role of ROS as a signaling molecule for the accumulation of phenolics as a response to wounding and hyperoxia stresses was studied. For this experiment, whole and shredded carrots were prepared and stored as mentioned above. The activities of enzymes involved in ROS generation and modulation/detoxification (SOD, APX, and CAT) were evaluated. In addition, just after wounding and prior to storage, a portion of the shredded carrots was treated with either a DPI solution (315 μM) or water. Variables such as PAL activity, NADPH oxidase activity, PC, and AOX were determined before and during storage of the different treatments.

Sample Preparation for Phytochemical Analyses. Carrot tissue (5 g) was homogenized with methanol (20 mL) using an Ultra-Turrax homogenizer (IKA Works, Inc., Wilmington, NC) and centrifuged at 29000*g* for 15 min at 4 °C. The clear supernatant (methanolic extract) was used for the analyses of total soluble phenolics (PC) and antioxidant activity (ORAC value). For the identification and quantification of the individual phenolic compounds, the methanolic extracts were passed through nylon membranes (0.2 μm) prior to injection to the chromatographic systems.

Analysis of Total Soluble Phenolics (PC) and Determination of Antioxidant Activity [Oxygen Radical Absorbance Capacity (ORAC) Value]. Total soluble phenolics were determined using the method described by Swain and Hillis.[24] Methanolic extracts (15 μL) were diluted with nanopure water (240 μL) in a 96-well microplate well, followed by the addition of 0.25 N Folin−Ciocalteu reagent (15 μL). The mixture was incubated for 3 min, and then, 1 N Na_2CO_3 (30 μL) was added. The final mixture was incubated for 2 h at room temperature in the dark. Spectrophotometric readings at 725 nm were collected using a plate reader (Synergy HT, Bio-Tek Instruments, Inc., Winooski, VT). Total phenolics were expressed as mg chlorogenic acid equivalents/kg of fresh tissue. The antioxidant activity was determined with the ORAC assay. The ORAC value was obtained using the procedure of Wu et al.[25] for hydrophilic ORAC with a slight modification as described by Villarreal-Lozoya et al.[26]

Identification and Quantification of Phenolic Compounds by High-Performance Liquid Chromatography−Diode Array Detection (HPLC-DAD) and HPLC−Electrospray Ionization (ESI)−MS^n. The HPLC system was composed of two 515 binary pumps, a 717-plus autosampler, and a 996-photodiode array detector (Waters Corp., Mildford, MA). Phenolic compounds were separated on a 4.6 mm × 150 mm, 5 μm, C-18 reverse-phase Atlantis column (Waters Corp.) that was maintained at 40 °C by a SpectraPhysics SP8792 column heater. The mobile phases consisted of water adjusted to pH 2.4 with HCl (phase A) and acetonitrile (phase B) at a flow rate of 1 mL/min. The gradient solvent system was 0/85, 5/85, 30/0, and 35/0 (min/% phase A). Data were processed with the Millennium software v3.1. The identification of individual phenolics was based on their PDA spectra and ESI-MS^- fragmentation patterns as compared with authentic standards. Mass spectrometric analyses were performed on a Thermo Finnigan LCQ Deca XP Max Ms^n ion trap mass spectrometer equipped with an ESI ion source (Thermo Finnigan, San Jose, CA). Separations were conducted using the Phenomenex (Torrance, CA) Synergi 4 μ Hydro-RP 80A (2 mm × 150 mm) with a C18 ward column. Mobile phases consisted of water/formic acid 1% (99:1, v/v, phase A) and acetonitrile (phase B) at a flow rate of 200 μL/min. The gradient solvent system was 0/85, 5/85, 30/0, and 35/0 (min/% phase A). ESI was performed in the negative ion mode using the following conditions: sheath gas (N_2), 50 arbitrary units; auxiliary gas (N_2), 0 arbitrary units; spray voltage, 4 kV; capillary temperature, 250 °C; capillary voltage, −21 V; and tube lens offset, −60 V.

PAL Activity Determination. PAL was extracted and assayed as previously described.[2,27] Briefly, carrot tissue samples (4 g) were added with polyvinylpolypyrrolidone (0.4 g) and homogenized in ice-cold 50 mM borate buffer, pH 8.5 (16 mL), containing 400 μL/L of β-mercaptoethanol. Homogenizations were preformed under low-light conditions. Homogenates were filtered through four layers of cheesecloth and then centrifuged at 32000*g* for 15 min at 4 °C. Supernatants were collected as PAL assay solutions, maintained under refrigeration (4 °C), and promptly assayed. A fresh 100 mM L-phenylalanine substrate solution was prepared in nanopure water before each assay. The PAL assay was performed by pipetting 50 mM borate buffer, pH 8.5 (235 μL), and 100 mM L-phenylalanine substrate solution (35 μL) into a well of a 96-well microplate (Greiner bio-one #65580, Monroe, NC). The reaction was started by the addition of PAL assay solution (80 μL). Spectrophotometric readings at 290 nm were registered before and after 1 h of incubation at 40 °C in a plate reader with temperature control

(Synergy HT, Bio-Tek Instruments, Inc.). The PAL activity was calculated as μmol of *t*-cinnamic acid synthesized per hour using a *t*-cinnamic acid standard curve (0−0.15 μmol/mL)

Reduced Nicotinamide Adenine Dinucleotide Phosphate (NADPH) Oxidase Activity Determination. NADPH oxidase was extracted and assayed as previously described.[28] Crude extracts containing membrane, microsomal, and cytosolic fractions were prepared by homogenizing carrot tissue samples (2 g) with ice-cold extraction buffer (50 mM Tris-HCl, pH 7.5, 250 mM sucrose, 3 mM Na_2EDTA, 10 mM ascorbic acid, and 5 mM diethyl dithiocarbamate). Extracts were filtered through four layers of cheesecloth and centrifuged at 10000*g* for 10 min at 4 °C. Supernatants were collected and further centrifuged at 65000*g* for 10 min. Supernatants were discarded, and the pellets (enriched with the crude microsomal and plasma membrane fractions) were resuspended in a phosphate buffer solution (5 mM K_2HPO_4, pH 7.8, 250 mM sucrose, and 3 mM KCl) and used as NADPH oxidase assay solutions. The NADPH-dependent $O_2^{\bullet-}$-generating activity of the crude microsomal and plasma membrane extracts was determined by reduction of NBT using NADPH as electron donor.[28] The NADPH oxidase activity was determined by mixing the assay buffer (240 μL; 50 mM Tris-HCl, pH 8.6, and 364.6 mM sucrose), 0.58 mM NBT (60 μL), NADPH oxidase assay solutions (30 μL), and either nanopure water (10 μL) or 0.35 mM DPI (10 μL) into a well of a 96-well microplate. The reaction mixtures were incubated for 1 min at room temperature. Reactions were started by the addition of 3.5 mM NADPH (10 μL) substrate solution. Spectrophotometric readings at 530 nm were collected every 30 s during 5 min in a plate reader (Synergy HT, Bio-Tek Instruments, Inc.). The enzyme activity (ΔA/min) was estimated by the initial velocities method from the linear portion of the curves. One unit of activity was defined as an increase in absorbance of 0.001 min^{-1}. Spectrophotometric readings obtained in the presence of DPI were subtracted from those obtained without DPI. A well containing the reaction mixture and nanopure water (10 μL) instead of the substrate solution was used as the blank.

SOD, APX, and CAT Activity Determination. Antioxidant defense system enzymes were extracted as described by Kang and Saltveit.[29] All enzyme activity assays were carried out on 96-well microplates, and absorbance readings were conducted using a microplate reader (Synergy HT, Bio-Tek Instruments, Inc.).

The SOD activity was determined by measuring its capability to inhibit the photochemical reduction of NBT.[29,30] The assay mixture (350 μL) consisted of 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT, 2 μM riboflavin, 0.1 mM EDTA, and SOD assay solution (6 μL). Riboflavin was added at the end, and the reaction mixture was shaken and immediately placed 30 cm below a light bank composed of two 15 W fluorescent lamps for 10 min. The absorbance at 560 nm was determined. In addition, a reaction mixture was placed in the dark and used as a control. One unit of activity (U) was defined as the Δabsorbance between the reaction mixtures exposed to the light and the nonirradiated controls.

The CAT activity was determined by monitoring the decrease in absorbance due to the H_2O_2 disappearance.[29,31] CAT was assayed by mixing 50 mM phosphate buffer, pH 7.4 (330 μL), CAT assay solution (6 μL), and 1% H_2O_2 (13 μL). Spectrophotometric readings were taken at 240 nm in a plate reader every second during 1 min. Samples were read against a blank containing water instead of the CAT assay solution. The enzyme activity (ΔA/s) was estimated by the initial velocities method from the linear portion of the curves. One unit of activity (U) was defined as a decrease in absorbance of 1 s^{-1}.

The APX activity was determined by its capability to oxidize ascorbate in the presence of H_2O_2 as previously described.[29,32] The reaction mixture consisted of 50 mM phosphate buffer (285 μL; pH 7.0, 0.1 mM EDTA, 0.5 mM ascorbate, and 1.54 mM H_2O_2) and APX assay solution (15 μL). The decrease in absorbance was monitored at 240 nm every 5 min for 60 min. Samples were read against a blank containing water instead of the APX assay solution. The enzyme activity (ΔA/min) was estimated by the initial velocities method from the linear portion of the curves. One unit of activity (U) was defined as a decrease in absorbance of 1/min.

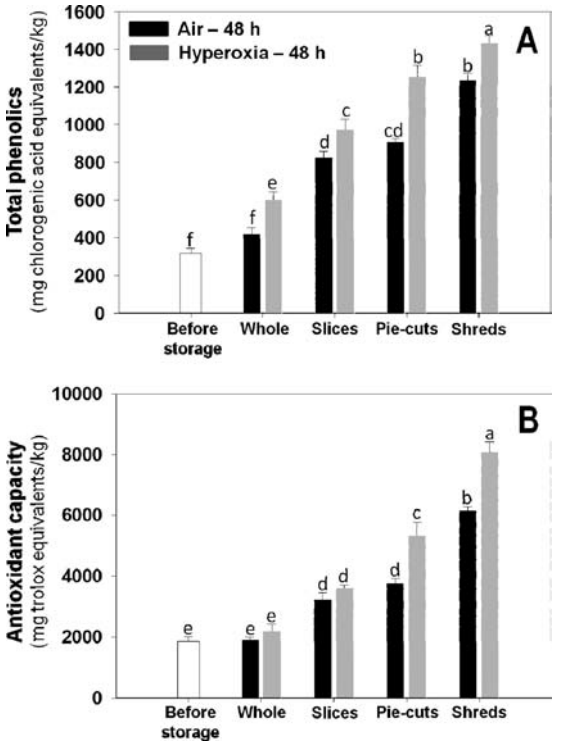

Figure 1. Effect of wounding and hyperoxia stresses on the total PC (A) and AOX (B) of carrots stored at 20 °C for 48 h. Whole, slices, pie cuts, and shredded carrots were stored under two different atmospheres: air and hyperoxia (80% O_2). Samples were evaluated at 0 h and after 48 h of storage. Data represent the means of five replicates and their standard errors. Bars with different letters indicate statistical difference by the LSD test ($p < 0.05$).

Respiration Rate. The respiration rates in the shredded and whole carrots were obtained from carrot tissue (~50 g) placed in glass jars (500 mL) connected to an air-flow-through system supplied with humidified flows of either air or a gas mixture containing 80% O_2 + 20% N_2. The concentrations of CO_2 were kept <0.15%. Respiration rates were obtained every 6 h during 48 h by disconnecting the jars from the air-flow-through system. Jars were closed, and samples were allowed to accumulate CO_2 for 30 min to concentrations <1%. Gas samples were taken using syringes through a septum port placed on the lid. Collected samples were analyzed with a Horiba CO_2 infrared gas analyzer (model PIR-2000, Horiba Instruments Inc., Irvine, CA).

Statistical Analysis. Analyses were performed using five replicates, unless otherwise indicated. Data represent the mean values of samples, and bars indicate their standard error. Analyses of variance (ANOVA) was conducted using JMP software version 5.0 (SAS Institute Inc. Cary, NC), and mean separation was performed using the LSD test ($p < 0.05$).

RESULTS

Effect of Wounding and Hyperoxia Stresses on the Accumulation of Total PC and on the Antioxidant Capacity (AOX) of Carrot Tissue. The total PC and AOX of the samples was determined before and after storage (Figure 1). The application of wounding produced a significant increase ($p < 0.001$) on the total PC and AOX values in carrots stored at 20 °C for 48 h. In

particular, the accumulation of total PC was intensified with increasing wounding intensity (Figure 1A). As compared with whole carrots before storage, total PC in the air-treated wounded tissue was increased by ~158, 185, and 287% for slices, pie cuts, and shreds, respectively. Likewise, the application of hyperoxia stress on wounded tissue increased the total PC by ~204, 293, and 349% in the same order as above (Figure 1A). The whole carrots stored under air conditions did not show a significant increase in the total PC ($p > 0.05$). However, when the whole carrots were subjected to hyperoxia stress, the total PC was increased by ~100%. The increased PC amounts in carrots generated by wounding and hyperoxia stresses resulted in an enhancement of their AOX values (Figure 1B). Among the samples stored under air, the shredded carrots showed the highest increase in AOX value (~240% higher) as compared with wholes before storage, followed by pie cuts and slices. Likewise, the pie cuts and shreds treated with hyperoxia showed significant higher AOX values increases than the air-treated samples.

Effect of Wounding and Hyperoxia Stresses on the Accumulation of Individual Phenolic Compounds in Carrot Tissue. To identify and quantify the phenolic compounds produced and accumulated by wounding and hyperoxia stresses in carrots, the methanolic extracts from stressed carrot tissue were analyzed by HPLC-DAD and HPLC-ESI-MS$^-$ (Figure 2 and Table 1). The phenolic compounds in the stressed tissue were identified as 3-*O*-caffeoylquinic acid (3-CQA), FA, 3,5-dicaffeoylquinic acid (3,5-diCQA), and 4,5-dicaffeoylquinic acid (4,5-diCQA). The 3-CQA and FA were identified based on their spectra characteristics and ESI-MS$^-$ fragmentation patterns as compared with authentic standards. The tentative identification of the CQAs derivatives 3,5- and 4,5-diCQA was based on similarities with previously reported spectra characteristics and ESI MS$^-$ fragmentation patterns.[33]

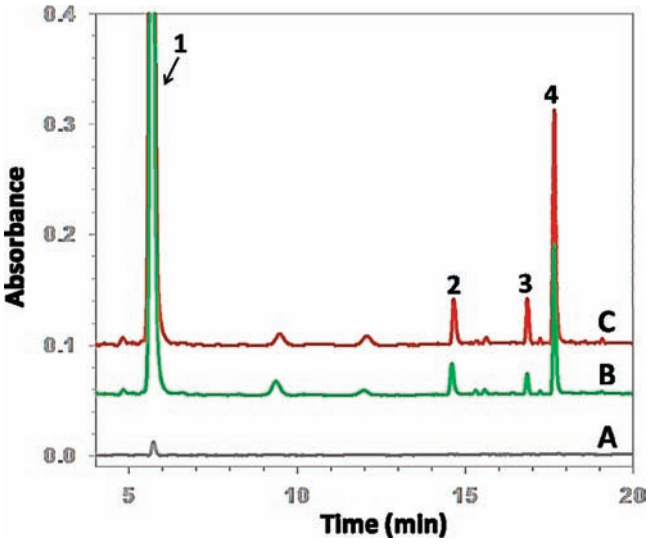

Figure 2. Typical HPLC-DAD chromatogram (shown at 320 nm) of methanolic extracts from shredded carrots before (A) and after 48 h of storage under air (B) and hyperoxia (80% O_2) conditions (C) for 48 h at 20 °C. Peak assignments: (1) 3-CQA, (2) FA, (3) 3,5-diCQA, and (4) 4,5-diCQA.

The concentration of individual phenolic compounds produced and accumulated by wounding and hyperoxia stresses in carrots is shown in Figure 3. The accumulation of 3-CQA, 3,5-diCQA, and 4,5-diCQA was enhanced as the wounding intensity increased (Figure 3A,C,D). Among the samples stored under air, the shredded carrots induced the highest production and accumulation of 3-CQA with ~2000% of increase as compared with wholes before storage (Figure 3A). The 3,5-diCQA was not detected in the whole carrots, and the application of wounding stress produced ~34 mg/kg of this compound as shown for shredded carrots stored under air (Figure 3C). The concentration of 4,5-diCQA was increased by ~1680% with wounding stress on the shredded carrots (Figure 3D). In the particular case of 4,5-diCQA, no significant difference was detected between the shreds and the pie cuts ($p > 0.05$), indicating that both wounding intensities are equally efficient to produce and accumulate this compound. On the other hand, the application of wounding stress in samples stored under air only enhanced the concentration of FA in the sliced and pie cuts carrots by ~156 and ~72%, respectively, as compared with wholes before storage (Figure 3B). The concentration of this compound among the other samples treated with air remained unchanged (Figure 3B).

The application of hyperoxia stress in the wounded tissue increased the concentration of 3-CQA in the pie cuts and shreds by ~1775 and ~2966% as compared to wholes before storage (Figure 3A). In addition, hyperoxia positively affected the accumulation of 3,5-diCQA and 4,5-diCQA in the pie cuts (Figure 3C,D). The concentration of 3,5-diCQA in the hyperoxia-treated pie cuts samples were similar to the concentration obtained in the shredded carrots stored under air (Figure 3C), while for 4,5-diCQA, this increase was even higher (Figure 3D). However, hyperoxia did not significantly affect ($p > 0.05$) the accumulation of 3,5-diCQA and 4,5-diCQA on the shredded and sliced tissue (Figure 3C,D). Interestingly, the application of hyperoxia in the whole carrots increased the concentration of FA and 4,5-diCQA by ~158 and 1293%, respectively, as compared with wholes before storage (Figure 3B,D). Therefore, the increased concentration of total PC induced by hyperoxia in the whole tissue (Figure 1A) may be mainly attributed to the stress-induced accumulation of FA and 4,5-diCQA.

Effect of Wounding and Hyperoxia Stresses on the Respiration Rate of Carrot Tissue. To further understand some of the physiological events involved in the stress-induced accumulation of phenolics in carrots subjected to wounding and hyperoxia, the respiration rates during the storage of shredded

Table 1. HPLC-DAD and HPLC-ESI-MS$^-$ Analyses of Phenolic Compounds from Methanolic Extracts of Carrots

peak no. (retention time, min)	λ_{max} (nm)	compound	$[M - H]^-$ (m/z)	MS^2 (m/z)[a]	MS^3 (m/z)
1 (5.7)	244, 247, 326	3-CQA[b]	353	179.11, (191.11)	
2 (14.6)	239, 261, 302	FA[b]	193		
3 (16.8)	327, 297, 247, 224	3,5-diCQA[c]	515	353	179, (191)
4 (17.6)	327, 297, 248, 224	4,5-diCQA[c]	515	353	173, (179), 191

[a] Ions in parentheses represent the most intense product ions. [b] Identified based on their spectra characteristics and ESI-MS$^-$ fragmentation patterns as compared with authentic standards. [c] Tentative identification based on similarities of spectra characteristics and ESI MS$^-$ fragmentation patterns.[33].

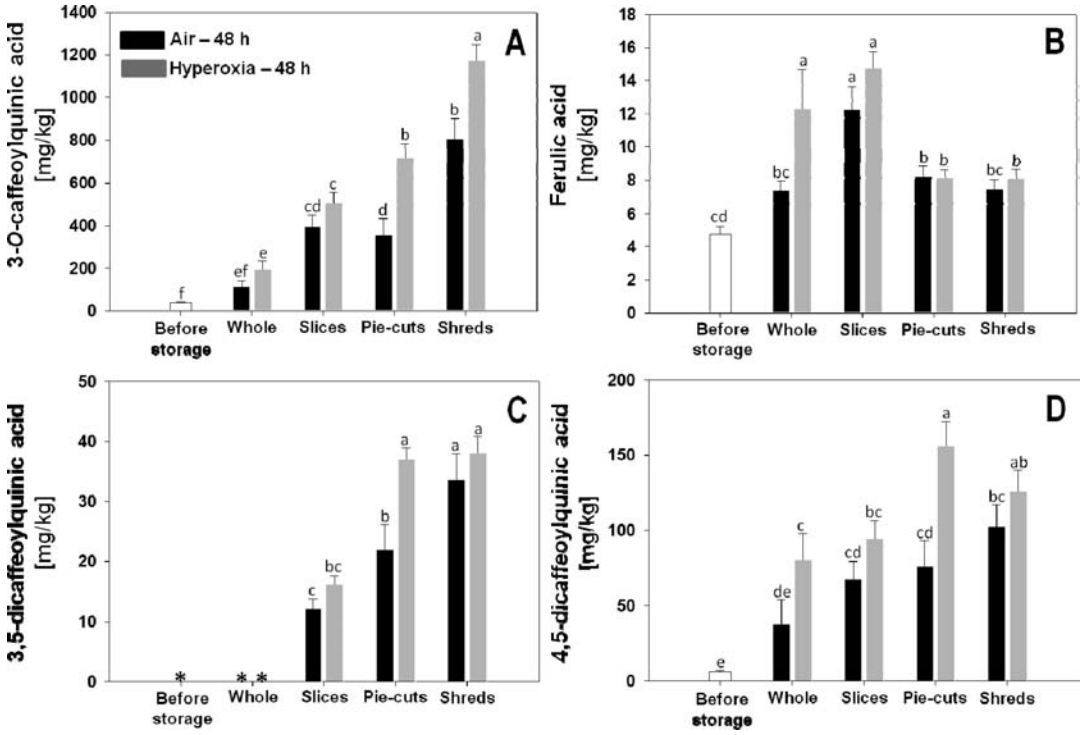

Figure 3. Effect of wounding and hyperoxia stresses on the production and accumulation of 3-CQA (A), FA (B), 3,5-diCQA, and 4,5-diCQA of carrots stored at 20 °C for 48 h. Data represent the means of five replicates and their standard errors. Bars with different letters indicate statistical difference by the LSD test ($p < 0.05$). *Not detected.

and whole carrots treated with air and hyperoxia were studied (Figure 4). The respiration rate of carrots was increased by the application of wounding stress, and after 18 h of storage, the whole and shredded carrots treated with hyperoxia showed higher respiration rate values as compared with samples treated with air ($p < 0.05$, Figure 4).

Effect of Wounding and Hyperoxia Stresses on the Enzymatic Activities of SOD, APX, and CAT. In addition to the ROS potentially generated by the increased respiration rate in the wounded carrots (Figure 4), other sources of ROS, such as the plasma membrane NADPH oxidase, may be activated by wounding and hyperoxia stresses. NADPH oxidase as well as respiration produce superoxide radical ($O_2^{\bullet -}$), which is converted into hydrogen peroxide (H_2O_2) by SOD. These ROS ($O_2^{\bullet -}$ and H_2O_2) may be acting as signaling molecules activating the phenylpropanoid metabolism.[34] Because high levels of H_2O_2 are toxic for plants, the activity of detoxifying enzymes such as APX and CAT is needed for a fine modulation of H_2O_2 levels below toxic concentrations.[17]

To understand the effect of wounding and hyperoxia stresses on SOD, APX, and CAT, the activity of the enzymes was evaluated on the whole and the shredded carrots before and during storage under air and hyperoxia conditions (Figure 5). Wounding stress induced an immediate activation of SOD (Figure 5). The shredded carrots showed ~113% higher SOD activity ($p < 0.05$) than the whole tissue at 0 h. However, no immediate effect of wounding was observed on the activities of APX and CAT (Figure 5B,C).

An increase in SOD activity was observed in whole and shredded carrots during the evaluated period (Figure 5A). A ~50% increase in SOD activity was observed in the whole carrots after 24 h of storage, and a decrease in SOD activity to the initial levels was observed at 36 h. Hyperoxia stress did not affect significantly ($p > 0.05$) SOD activity. The SOD activity in shredded carrots decreased during the first 12 h of storage. After 12 h, the activity increased, reaching its maximum values at 36 h of storage with a subsequent decrease in activity to the initial levels at 48 h.

A significant increase in APX activity was observed through storage time (Figures 5B, $p < 0.05$). In general, whole and shredded carrots stored under air showed higher APX activity as compared with hyperoxia-treated samples ($p < 0.05$). An increase in CAT activity (~78%) was observed in the shredded carrots stored under air at 12 h of storage (Figure 5C). However, the activity returned to its original value at 24 h, and a significant increase ($p < 0.05$) in the activity of the enzyme was observed at 48 h in all samples with the exception of wholes stored under hyperoxia conditions. This activation was higher in shreds (~189% of increase) as compared with whole carrots stored under air (~55% of increase). The later activation of CAT as compared with APX can be related with the concentration of H_2O_2 present in the plant cell. APX has affinity for H_2O_2 at micromolar ranges, while the affinity of CAT for H_2O_2 is at the millimolar range. Mittler [17] suggested that this difference in substrate affinity might be related with the function of the enzyme. APX is responsible to finely modulate ROS to serve as signaling molecule. On the other hand, CAT is responsible for removal of ROS when toxic levels are reached. Those toxic levels may be reached at 48 h of storage where CAT activation is observed.

Effect of Wounding, Hyperoxia, and DPI Treatments on NADPH Oxidase Activity. The ROS generated by the plasma membrane NADPH oxidase may be acting as signaling molecules that are triggering the stress-induced accumulation of phenolic

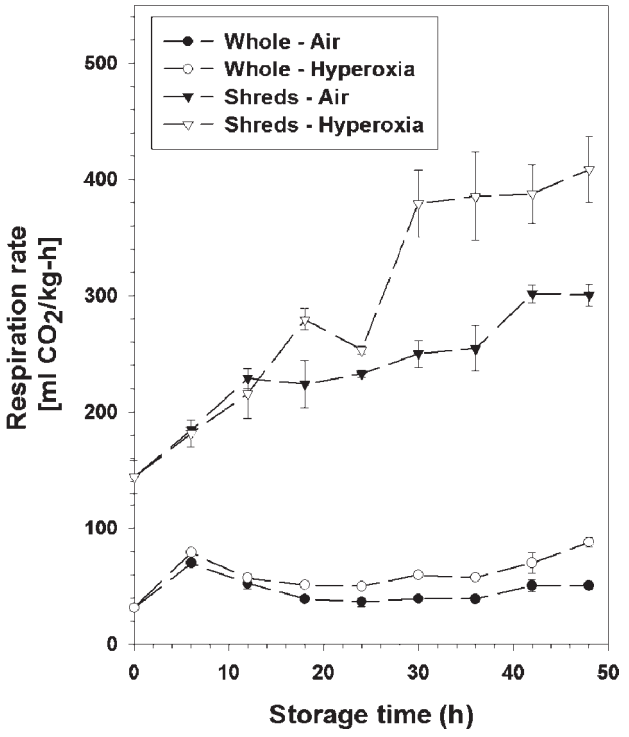

Figure 4. Effect of wounding and hyperoxia on the respiration rate of carrots stored at 20 °C for 48 h. Whole and shredded carrots were stored under two different atmospheres: air and hyperoxia (80% O_2). Data represent the means of three replicates and their standard errors.

compounds in carrots (Figure 1). Therefore, the effect of wounding and hyperoxia stresses on NADPH oxidase activity was studied (Figure 6). The activity of the enzyme was evaluated before and immediately after wounding in the shredded carrots. In addition, the activity of NADPH oxidase was determined during the storage of the shredded and whole carrots treated with air and hyperoxia. Likewise, shredded carrots were dipped in either a DPI solution or water immediately after wounding, and the effect of both treatments on NADPH oxidase was evaluated (Figure 6).

A ~72% increase in NADPH oxidase activity was observed immediately after wounding, suggesting an instant $O_2^{\bullet -}$ production (Figure 6). Dipping the shredded carrots in water or DPI inhibited the wound-induced activation of NADPH oxidase. Likewise, water and DPI dips reduced the activity of the enzyme in the shredded carrots to ~27 and ~4%, respectively, as compared with the initial activity in the whole carrots.

During the first hours of storage, the activity of NADPH oxidase rapidly decreased in the air and hyperoxia-treated shreds to levels even lower than the whole carrots before storage (Figure 6). After 4 h of storage, the activity of NADPH oxidase started to increase. This reactivation of NADPH oxidase occurred more rapidly in the hyperoxia-treated shredded carrots (Figure 6B) as compared with the air-treated samples (Figure 6A). The maximum NADPH oxidase activity during storage was observed at 12 and 36 h for the hyperoxia- and air-treated samples, respectively. A decrease in the activity of the enzyme was observed in the air-treated samples at 48 h of storage (Figure 6A).

Shredded carrots treated with water showed a decrease in NADPH oxidase activity over time (Figure 6). The inactivation

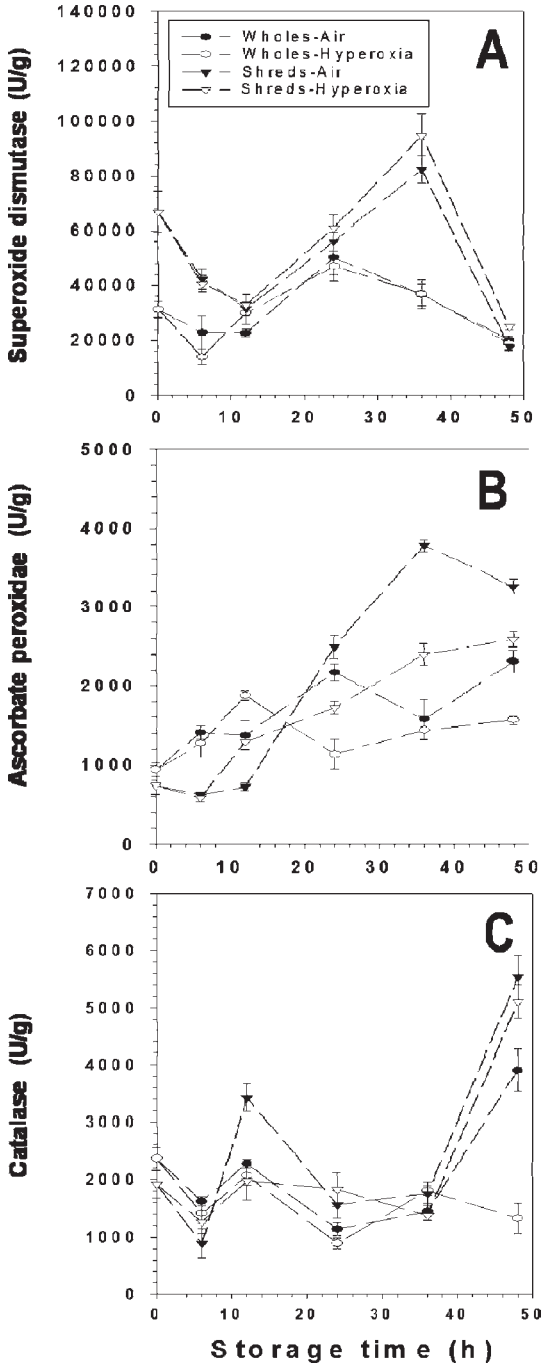

Figure 5. Effect of wounding and hyperoxia on the activity of carrot SOD (A), APX (B), and CAT (C). Whole and shredded carrots were stored at 20 °C for 48 h under two different atmospheres: air and hyperoxia (80% O_2). Data represent the means of five replicates and their standard errors.

of NADPH oxidase by the application of DPI was irreversible during the evaluated period. The DPI and water-treated shreds showed lower NADPH oxidase activity as compared with wholes before and during storage (data not shown).

Effects of Wounding, Hyperoxia, and DPI Treatments on Phenylalanine Ammonia Lyase (PAL) Activity. To understand the potential role of ROS produced by NADPH oxidase on the stress-induced synthesis of phenolics in carrots, the activity of

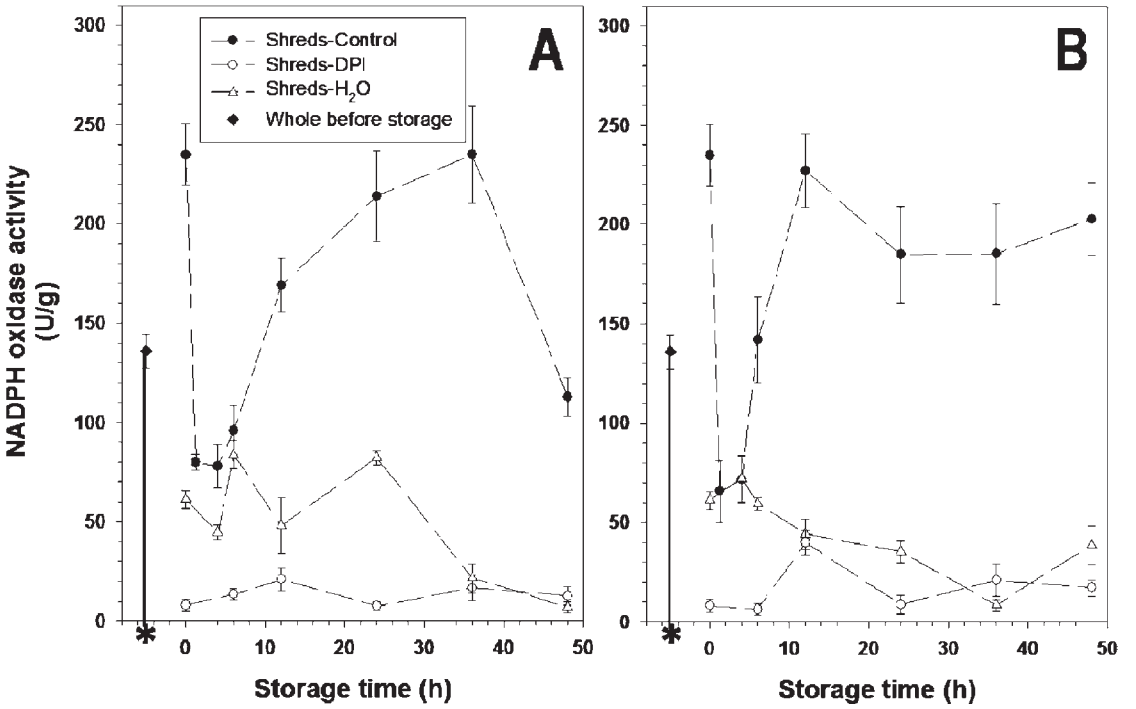

Figure 6. Effect of dipping shredded carrots in H_2O or in a solution with DPI (317 μM) on nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity. Control and treated samples were stored at 20 °C for 48 h in two different atmospheres: air (A) and hyperoxia (80% O_2) (B). Data represent the means of five replicates and their standard errors. *Whole carrots before storage.

PAL was determined during the storage of shredded tissue dipped on either a DPI solution or water and stored under air and hyperoxia conditions. In addition, the PAL activity was determined on the wholes and nondipped shreds (controls) stored under both atmospheric conditions.

Wounding did not induce an immediate activation of PAL activity (Figure 7). Similarly, an immediate effect of DPI or water dips on PAL activity was not observed. An increase in PAL activity was observed in all samples during storage (Figure 7). The maximum activity of PAL in the control carrot shreds was reached at 24 h of storage.

As compared with wholes before storage, the control shreds stored under air for 24 h showed ~7020% higher activity. The induction of PAL was even higher when the shredded carrots were stored for 24 h under hyperoxia conditions (Figure 7). After 24 h of storage, the activity of PAL on the control shreds remained constant.

The shredded carrots dipped on water showed lower PAL activity as compared with the controls (Figure 7). Their maximum PAL activity was reached at 36 h of storage where ~6900% higher activity was observed as compared with whole carrots before storage. Interestingly, no significant effect of hyperoxia ($p > 0.05$) on PAL activity was observed at 36 h of storage of the shredded carrots dipped on water. At 48 h of storage, the activity of PAL decreased on these samples, showing a higher decrease in the samples stored under air as compared with those under hyperoxia. A slight increase in the activity of PAL was observed during storage of the shreds dipped on DPI solution (Figure 7).

Effect of DPI and Water Treatments on the Stress-Induced Accumulation of Total PC in Carrots. The total PC values were determined after 48 h of storage of the shredded carrots dipped on DPI and water and treated with air and hyperoxia (Figure 8). The water dips reduced the accumulation of total PC by ~36% in both the hyperoxia- and the air-treated samples. The accumulation of phenolic compounds was almost completely blocked on

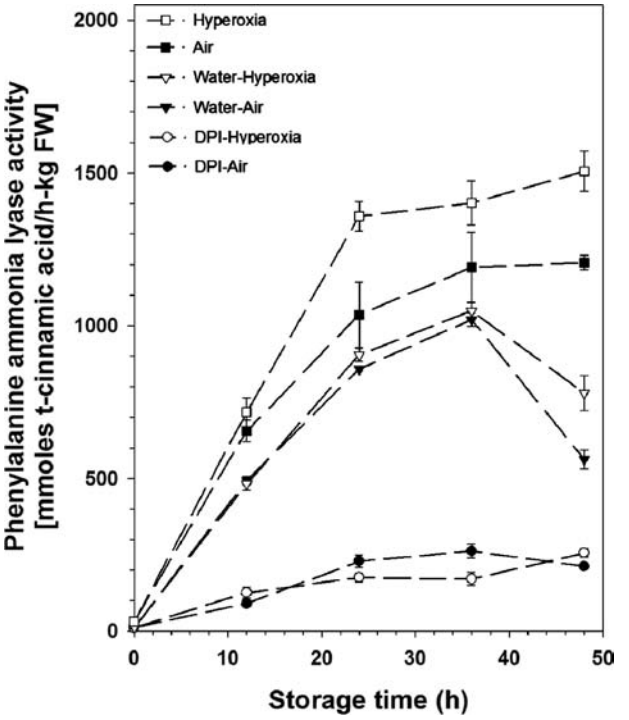

Figure 7. Effect of dipping shredded carrots in H_2O or in a solution with DPI (317 μM) on PAL activity. Control and treated samples were stored at 20 °C for 48 h in two different atmospheres: air and hyperoxia (80% O_2). Samples were evaluated at 0 h and after 48 h of storage. Data represent the means of five replicates and their standard errors.

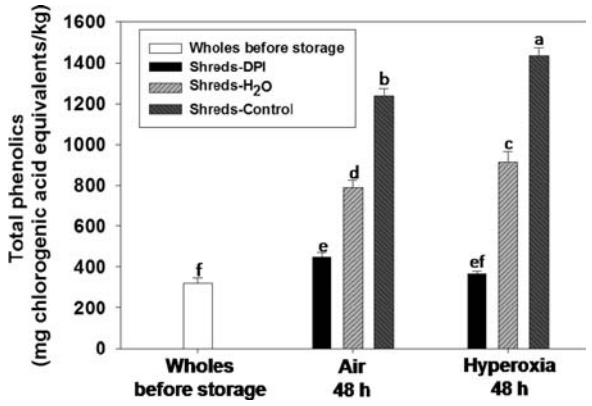

Figure 8. Effect of dipping shredded carrots in H_2O or in a solution with DPI (317 μM) on the accumulation of total phenolic compounds. Treated samples were stored at 20 °C for 48 h in two different atmospheres: air and hyperoxia (80% O_2). Samples were evaluated at 0 h and after 48 h of storage. Data represent the means of five replicates and their standard errors.

the DPI-treated shreds where only a slight ~40% of increase was observed on their total PC value as compared with wholes before storage. No significant difference on the total PC values between the DPI-treated shreds stored under air and hyperoxia conditions was observed, suggesting that the initial oxidative burst produced by NADPH oxidase is needed to trigger the higher accumulation of total PC produced by hyperoxia on wounded tissue (Figure 8). The reduced accumulation of total PC observed on the DPI-treated samples (Figure 8) matches with their reduced activation of PAL and NADPH oxidase as compared with the controls (Figures 6 and 7).

DISCUSSION

Wounding stress induced the accumulation of phenolic compounds in carrots. Whole and wounded carrots treated with hyperoxia stress showed higher accumulation of phenolics as compared with air-treated samples. Previous reports on hyperoxia-treated blueberries,[18] strawberries,[19] and Chinese Bayberries[20] also showed higher accumulation of phenolic compounds, suggesting that both stresses, wounding and hyperoxia, may induce the activation of the phenylpropanoid metabolism through a common signal. Phenolics are identified as the compounds with the highest AOX value in fruit and vegetables and thus are regarded as the main contributors to their AOX.[35] Therefore, the stress-induced production of phenolics resulted in an enhancement of the AOX value on the tissue (Figure 1B). Interestingly, although whole and sliced carrots treated with hyperoxia showed higher accumulation of total PC, the AOX value of the tissue was not affected during the storage period. These observations suggest that although hyperoxia increased the total PC of wholes and sliced carrots (Figure 1A), the type of phenolic compounds produced and accumulated by hyperoxia resulted in a phenolic mixture with similar AOX value as compared with air-treated samples (Figure 1B). Because each phenolic compound has a particular AOX based on its chemical structure, the AOX value of fruits and vegetables not only depends on their total PC, it is also affected by their phenolic profiles (type of phenolics present and their relative amounts or proportions). [36−39] When the AOX value against the total PC of the samples under investigation was correlated (Figure 1), a R^2 value of 0.8 was obtained, suggesting differences in their phenolic profiles induced by wounding and hyperoxia. The application of wounding and hyperoxia stresses induced the accumulation of 3-CQA, FA, 3,5-diCQA, and 4,5-diCQA. With the exception of FA, the highest accumulation of the phenolic compounds was observed on the shredded carrots. The lack of accumulation of FA in shredded carrots may be explained in terms of rate of production and utilization. It is well-known that wounding stress induces the production of lignin and suberin during wound healing in carrots. [40] Lignin is composed of monolignols residues that are synthesized from hydroxycinnamic acid precursors such as FA. [41] Therefore, FA in the shredded carrot may be synthesized at the same rate as it is utilized for lignin and suberin synthesis. The concentrations of CQAs reached in the stressed carrot tissue are similar to those found on the main natural sources of hydroxycinnamates. [46,47] Furthermore, the concentration of 3-CQA produced in the stressed carrots is higher as compared to that reported for coffee beans and artichoke. [46,47]

ROS activate plant defense genes and are also related with the accumulation of antioxidants such as phenolics in plants subjected to environmental stresses.[34,42] Respiration is known as one of the sources of ROS.[17,43] Results demonstrated that hyperoxia induced a higher respiration rate in whole and shredded carrots. Likewise, hyperoxia inhibited the wound-induced APX activation through time and the activity of SOD in air, and hyperoxia-treated shreds were similar (Figure 5B). These results suggest that hyperoxia-treated samples had higher accumulation of H_2O_2. Given that H_2O_2 may be activating the phenylpropanoid metabolism, the higher respiration rate and lower H_2O_2 detoxification ability of hyperoxia-treated samples may be inducing the higher total PC observed in the hyperoxia-treated wholes and shreds.

The activity of NADPH oxidase during storage shows a similar pattern as observed for SOD activity (Figure 5). Wounding immediately activated both enzymes, and their activity decreased during the first hour of storage. Additionally, both enzymes showed a second reactivation during storage. These data suggest that during storage SOD produces H_2O_2 from the $O_2^{\bullet-}$ generated by NADPH oxidase, and a subsequent oxidative burst may be occurring in the shredded carrots. Similar observations have been reported for potato tubers.[44] The authors found that immediately after wounding an initial burst of ROS occurs within the first hours. Furthermore, the authors reported that three other massive bursts occurred at 42, 63, and 100 h after wounding. It is suggested that the first oxidative burst may be triggering the activation of the phenylpropanoid metabolism to accumulate soluble phenolics, while the second may be associated with wound healing and is involved in the oxidative cross-linking of suberin poly(phenolics).[44] Dipping the shredded carrots on water inhibited the wound-induced activation of NADPH oxidase. These results suggest that the water dips induced removal of a chemical signal produced at the site of cutting, which is triggering the wound-induced activation of NADPH oxidase. That signal may be the extracellular ATP, since it has been reported to activate the NADPH oxidase-mediated $O_2^{\bullet-}$ production in *Arabidopsis thaliana*.[14]

Shredded carrots treated with hyperoxia showed higher PAL activity during storage. These results indicate that the wound-induced activation of the phenylpropanoid metabolism is higher when hyperoxia is applied on the wounded tissue as can be observed on the accumulation of total PC (Figure 1A). Likewise, the activity of PAL during the storage of the whole carrots

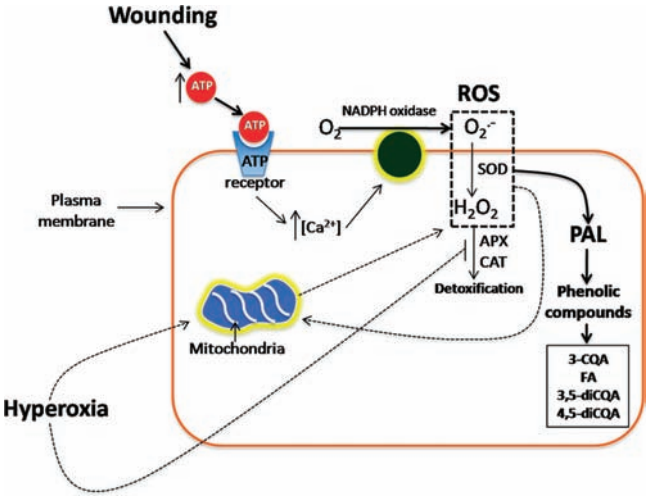

Figure 9. Hypothetical model explaining the role of ROS on the stress-induced production of phenolic compounds in carrots.

exposed to hyperoxia was slightly higher as compared with the wholes stored under air (data not shown). These increases on PAL activity and total PC in the hyperoxia-treated control shreds and whole carrots may be explained in terms of ROS concentration. Because hyperoxia increases the respiration rate of the tissue (Figure 4) and partially inhibits the wound-induced activation of APX (Figure 5), the concentration of ROS is increased, which may be acting as a signal to activate PAL. The decreased PAL activity observed during the storage of the shredded carrots dipped on water may be attributed to partial removal of the chemical signal that triggers the wound-induced activation of PAL. The exact nature of the wound signal that activates PAL remains unknown. Previous research on lettuce suggests that it is a chemical signal synthesized at the site of wounding that diffuses from the site of injury into adjacent cells.[45] This chemical signal may be partially removed from the site of injury by the water dips. As compared with the controls, the activation of PAL in the DPI-treated samples is drastically reduced, indicating that ROS produced by NADPH oxidase is playing a major role on the wound-induced activation of PAL. The activity of PAL observed on the DPI- and water-treated shreds matches with the accumulation of total PC, suggesting that ROS synthesized by NADPH oxidase are needed for the stress-induced accumulation of phenolic compounds produced by wounding and hyperoxia on carrots.

The present study generated new information that allowed the formulation of a hypothetical model explaining the role of ROS as signaling molecules for the wound-induced production of phenolics in carrots (Figure 9). Because ROS produced by NADPH oxidase play a major role on the activation of PAL and thus on the production of phenolics in wounded carrots, it is speculated that extracellular ATP accumulated at the site of injury plays a key role as primary signal triggering ROS production.[14] Upon the application of wounding stress, cell disruption occurs, inducing the liberation of cytosolic ATP into the extracellular matrix. The released ATP diffuses from the site of injury into adjacent cells, where it is recognized by its receptor at the plasma membrane. Once ATP binds to its receptor, the cytosolic Ca^{2+} concentration is increased, triggering the activation of NADPH oxidase (Figure 6) and thus $O_2^{\bullet-}$ production.[14] Superoxide radical is transformed into H_2O_2 by SOD (Figure 5A). These ROS ($O_2^{\bullet-}$ and H_2O_2) act as a signal that increase the mitochondria respiration rate in the tissue (Figure 4), inducing higher ROS levels.[17,43] Simultaneously, ROS activate the phenylpropanoid metabolism, producing phenolic compounds (Figure 1A) such as 3-CQA, FA, 3,5-diCQA, and 4,5-diCQA (Figure 3). Because high levels of ROS are toxic for plant cells, APX and CAT activities increase (Figure 5B,C, respectively) to finely tune ROS levels.[17] Hyperoxia applied in the wounded tissue induces higher ROS levels by both increasing the respiration rate and partially inhibiting the wound-induced activation of APX and CAT (Figure 5B,C, respectively). These increased ROS levels in the wounded tissue treated with hyperoxia induced a higher activation of PAL (Figure 7) and thus higher accumulation of phenolic compounds (Figure 1A).

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ABBREVIATIONS USED

APX, ascorbate peroxidase; CAT, catalase; 3,5-diCQA, 3,5-dicaffeoylquinic acid; 4,5-diCQA, 4,5-dicaffeoylquinic acid; DPI, diphenyleneiodonium chloride; FA, ferulic acid; 3-CQA, 3-*O*-caffeoylquinic acid; PC, phenolic content; PAL, phenylalanine ammonia lyase; ROS, reactive oxygen species; NADPH oxidase, reduced nicotinamide-adenine dinucleotide phosphate; SOD, superoxide dismutase

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