

Biochemical analysis of reactive oxygen species production and antioxidative responses in unripe avocado (*Persea americana* Mill var Hass) fruits in response to wounding

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Abstract We analyzed the production of reactive oxygen species (ROS) and of detoxifying enzymes and enzymes of the ascorbate (ASC) acid cycle in avocado fruit (*Persea Americana* Mill cv Hass) in response to wounding. The levels of superoxide anion (O_2^-), hydroxyl radicals (OH \cdot) and hydrogen peroxide (H_2O_2) increased at 15 min and 2 and 15 h post-wounding. Peroxidase (POD) activity had increased to high levels 24 h after wounding; in contrast, catalase and superoxide dismutase (SOD) levels had decreased significantly at 24 h post-treatment. Basic POD was the major POD form induced, and the levels of at least three apoplastic POD isozymes –increased following wounding. Using specific inhibitors, we characterized one MnSOD and two CuZnSOD isozymes. CuZnSOD activities decreased notably 12 h after treatment. The activities of dehydroascorbate reductase and glutathione reductase increased dramatically following the wounding treatment, possibly as a means to compensate for the redox changes due to ROS production.

Keywords Antioxidative enzymes · Avocado · Reactive oxygen species · Wounding

Introduction

Post-harvest losses in avocado resulting from mechanical injuries, such as bruising and cutting, are enormous, and the consumer is frequently unaware of these injuries at the time

the food is purchased since bruised tissue is not visible until the fruit is peeled. Enzymatic darkening, which occurs following bruising, markedly reduces the acceptability of fresh avocados since it not only results in the formation of undesirable dark tissues but may also reduce nutritive values (Hofman et al. 2002). Most internal disorders involve flesh browning that is, at least partly, catalyzed by polyphenol oxidase (Bower and Cutting 1988).

Wounding produced as a result of abiotic stress factors, such as wind, rain, and hail, and biotic factors, especially insect feeding, is also a potential infection site for pathogens. Consequently, the responses activated at the wound site are a barrier against opportunistic microorganisms (Cheong et al. 2002).

Responses to wounding have been extensively studied in plants, and such responses may elicit pathways that interact with pathogen resistance and possibly other signaling pathways. A long list of molecules, such as peptides, hormones, and reactive oxygen species (ROS), have been implicated in wound responses (Ryan 2000; Leon et al. 2001). Plants respond to pathogen infection and mechanical or herbivore-induced wounding (Yahraus et al. 1995; Orozco-Cárdenas et al. 2001) by the localized production of ROS, a phenomenon often referred to as the “oxidative burst”. The ROS formed in these reactions are involved in defense responses (e.g., direct effects on the pathogen, increased oxidative cross-linking of cell-wall polymers, induction of pathogen response expression (Rustérucci et al. 2001; Shah 2003).

The major enzyme families of the defense system, which are capable of removing ROS directly, are the superoxide dismutases (SOD; Kliebenstein et al. 1998), catalases (CAT; Loprasert et al. 1996) and ascorbate peroxidases (Kvaratskhelia et al. 1999), whereas antioxidants such as ascorbic acid and glutathione (GSH) are

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involved in the neutralization of secondary products of ROS reactions (Noctor and Foyer 1998; Conklin et al. 2000). During prolonged periods of oxidative stress, however, these detoxification systems become overwhelmed and tissue damage results.

The aim of the study reported here was to better understand how avocado responds to mechanical wounding and how the antioxidative mechanisms are regulated by this stress.

Materials and methods

Plant material

Fresh unripe avocado fruits (*Persea americana* Mill. cv Hass) were purchased at a local market and used immediately.

Wounding treatment

Avocado fruits were longitudinally wounded with a sterile blade and incubated for 24 h at 25°C. We then removed 0.5 g of tissue at distances of 1, 2, and 2 cm, respectively, from the wound and used these tissue samples in various assays.

Avocado fruits also were halved longitudinally, and each half cut along its length into 3-mm-thick slices. These slices were then punched, yielding discs 14 mm in diameter. A total of 50 discs were obtained per fruit, depending on its size. Discs from all fruit were incubated at 25°C for various lengths of time. After incubation, the slices were immediately frozen in liquid nitrogen and stored at -80°C until the assay were to be carried out.

In vivo detection of ROS

The production of O_2^- was analyzed by the method described by Doke (1983). Five green fruit discs were immersed in 10 ml 0.01 M potassium phosphate buffer (pH 7.8) containing 0.05% nitroblue tetrazolium (NBT) (Sigma, St. Louis, MO) for 1 h. Stained discs were cleared by boiling in acetic:glycerol:ethanol (1:1:3, v/v/v) solution before photographs were taken.

Hydrogen peroxide (H_2O_2) was visually detected in green fruit discs using 3,3-diaminobenzidine (DAB) (Sigma) as substrate by the method described by Orozco-Cárdenas and Ryan (1999). Five green fruit discs were placed in 10-cm-diameter petri dishes containing DAB solution (1 mg/ml, pH 3.8) for 2 h under light at 25°C. The assay was based on the instant polymerization of DAB (to form a reddish-brown complex that is stable in most solvents) when it comes into contact with H_2O_2 in the presence of peroxidases. To enable H_2O_2 accumulation or peroxidase activity to be recognized,

we also stained the samples in the presence of different concentration of *n*-propyl gallate, an guaiacol-dependent peroxidase activity inhibitor (Mika and Luthje 2003), or catalase (Sigma–Aldrich, St Louis, MO). The experiment was terminated by immersing the discs in boiling ethanol (96%) for 20 min. After cooling, the discs were photographed and preserved at room temperature in ethanol.

The level of lipid peroxidation was measured in terms of malondialdehyde (MDA) according to the protocol proposed by Rustérucchi et al. (1996). Fruit tissue samples were ground in liquid N_2 and homogenized in 1 ml of 20% (w/v) trichloroacetic acid (TCA). The mixture was then centrifuged for 20 min at 10,000 g, and the pellet was discarded. A 200- μ L aliquot of the extract was mixed with 300 μ L of TCA (20%) and 500 μ L of thiobarbituric acid (TBA) (0.67%) and heated in boiling water bath for 15 min, cooled and centrifuged at 3000 g for 10 min. Absorbances were read at 532 and 600 nm. The concentration of lipid peroxides was expressed as the amount of MDA formed.

Enzyme activity assays

Green avocado tissue was homogenized on ice in 0.1 M K-phosphate buffer, pH 7.0, containing 1% polyvinyl pyrrolidone in a ratio of 1:4 (w/v). Homogenates were centrifuged, and the cleared supernatant was used immediately as the enzyme source.

Spectrophotometric determination of ascorbate (ASC) redox enzymes was assayed by using a Beckman (Fullerton, CA, USA) DU 680 spectrophotometer.

Dehydroascorbate reductase (DHAR) (EC 1.8.5.1) activity was assayed following the increase in absorbance at 265 nm owing to the GSH-dependent production of ASC (Arrigoni et al. 1997). The reaction mixture contained 0.1 M phosphate buffer, pH 6.2, mM GSH, and 50–100 μ g protein. The reaction was started upon the addition of 1 mM DHA, and the rate of non-enzymatic DHA reduction was subtracted (extinction coefficient 14 $mM^{-1} cm^{-1}$).

Glutathione reductase (GR) (EC 1.6.4.2) activity was determined as described previously (Foyer and Halliwell 1976). The oxidized glutathione (GSSG)-dependent oxidation of NADPH was followed at 340 nm in a 1-ml reaction mixture containing 100 mM sodium phosphate buffer, pH 7.8, 0.5 mM GSSG, 50 μ l extract, and 0.1 mM NADPH.

Ascorbate oxidase (AO) (EC 1.10.3.3) activity was assayed as described by García-Pineda et al. (2004). The oxidation of ascorbate to dehydroascorbate was followed at 265 nm. The reaction mixture (1 ml) consisted of 935 μ l of buffer (0.025 M citrate, 0.05 M phosphate, pH 5.6), 30 μ l of substrate solution (0.15 mM L-ascorbic acid, 0.5 mM neutralized disodium EDTA), and 15 μ l 1% bovine serum albumin (BSA) solution.

The CAT (EC 1.11.1.6) activity assay was performed according to De Gara et al. (2000) by following the H_2O_2 dismutation at 240 nm in a reaction mixture composed of 0.1 M phosphate buffer, pH 7.0, 50–100 μg protein, and 18 mM H_2O_2 (extinction coefficient $23.5 \text{ mM}^{-1} \text{ cm}^{-1}$).

Soluble peroxidase (POD) (EC 1.11.1.7) activity was analyzed by following the formation of tetraguaiacol in a Beckman (Fullerton, CA, USA) DU 680 spectrophotometer (Hammerschmidt et al. 1982). Each reaction mixture (1 ml) consisted of 10 μL enzyme extract and 990 μL guaiacol solution containing 0.25% guaiacol (v/v) in 10 mmol/L sodium phosphate buffer, pH 6.0, and 0.125% H_2O_2 (v/v). The POD activity in the extracts was measured as an increase in absorbance at 470 nm. For POD electrofocusing, proteins (50 μg) were focused (4°C, 60 min, 0.125 W cm^{-2}) in wide range, non-denaturing isoelectric-focusing polyacrylamide gels (PAGs) containing ampholines in the pH range 3.5–9.5 (Amersham, Piscataway, NJ). After focusing, the gels were soaked for 30 min in 10 mM Na-phosphate, pH 6.0, to remove the ampholines and equalize the pH throughout the gel. A protein standard mixture (Isoelectric focusing calibration kit; Pharmacia, GE Healthcare, Uppsala, Sweden) was included in each electrofocusing experiment for determination of the isoelectric points.

Apoplasmic fluid was obtained to assay POD activity in the avocado mesocarp following wounding (Córdoba-Pedregosa et al. 2005). Briefly, apoplasmic soluble components were obtained from vacuum-infiltration (4°C, 5 min, 60 kPa) in medium composed of 0.01 M phosphate buffer and 1 M NaCl, followed by centrifugation at 1500 *g* for 5 min. Using this procedure, we obtained 70–100 μl of apoplasmic fluids for 1 g of tissue. As there was no detectable glucose 6-phosphate (Creissen et al. 1999) in the apoplasmic fluid, we conclude that this procedure did not result in any contamination with intracellular material (data not shown).

Superoxide dismutase activity was assayed using the method described by Beauchamp and Fridovich (1971). In brief, samples of the supernatant (100 μg per lane) were separated by polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions. Following electrophoresis in 12.5% (w/v) native PAGs at 100 V and 4°C, the gel was immersed in 2.45 mM nitroblue tetrazolium for 20 min, followed by a 15-min soak in a solution containing 28 mM tetramethylethylenediamine, 28 μM riboflavin, and 36 mM potassium phosphate, pH 7.8. Superoxide dismutase activity was detected by illuminating the gel with bright light, which caused the gel to turn uniformly blue except at positions exhibiting SOD activity. When a maximum contrast was achieved, the reaction was stopped by rinsing the gel with water. CuZn, Fe, or MnSOD activities were distinguished from each other based on their sensitivity to

4 mM KCN or 5 mM H_2O_2 (Scandalios 1993). In brief, following electrophoresis, the gels were incubated with the inhibitors for 30 min at room temperature and then examined for SOD activity.

The protein content of the extracts was determined according to Bradford (1976) using the Bio-Rad dye reagent (Bio-Rad, Hercules, CA) with BSA as the standard.

Results

Specificity of responses to wounding

The specificity of the responses to the wounding treatment was determined by measuring POD enzyme activity and H_2O_2 production at different distances from the wounding (Fig. 1a). Increased POD enzyme activity was observed 1 cm from the wounding site, but decreased with increasing distance from the wound (2 and 3 cm; Fig. 1b). There was a large increase in H_2O_2 production within the first 0.5 cm analyzed; thereafter, the H_2O_2 levels were low up to a distance of 2 cm, which as the total distance assayed (Fig. 1c). Based on these results we can deduce that these processes are locally regulated by the wound in unripe avocado fruit.

Induction of ROS production

The kinetics of O_2^- production was measured at several time points following mechanical stimulation (Fig. 1). Figure 1a shows that the level of O_2^- increased significantly 15 min after stimulation and decreased thereafter. The level of H_2O_2 also increased 15 h after stimulation, but there was no return to the baseline level after this time.

Because the DAB assay is also used to measure peroxidase enzyme activity, discs were incubated with the addition of catalase or *n*-propyl gallate in order to eliminate H_2O_2 production or peroxidase activity, respectively (Fig. 1b). Catalase inhibited H_2O_2 accumulation, indicating that the DAB reaction is due to H_2O_2 production in response to wounding and not to peroxidase activity, as supported by the observation that the phenolic compound *n*-propyl gallate had no effect in decrease the DAB reaction.

The induction of lipid peroxidation was assessed by determining the accumulation of thiobarbituric acid reactive species (TBARS) at various times following the wounding (Fig. 1c). Increasing levels of TBARS were detected 2 h after wounding, decreasing to control levels at 24 h.

Catalase, POD and SOD activities

Specific activities of the antioxidant enzymes CAT, POD and SOD were measured in response to wounding (Table 1).

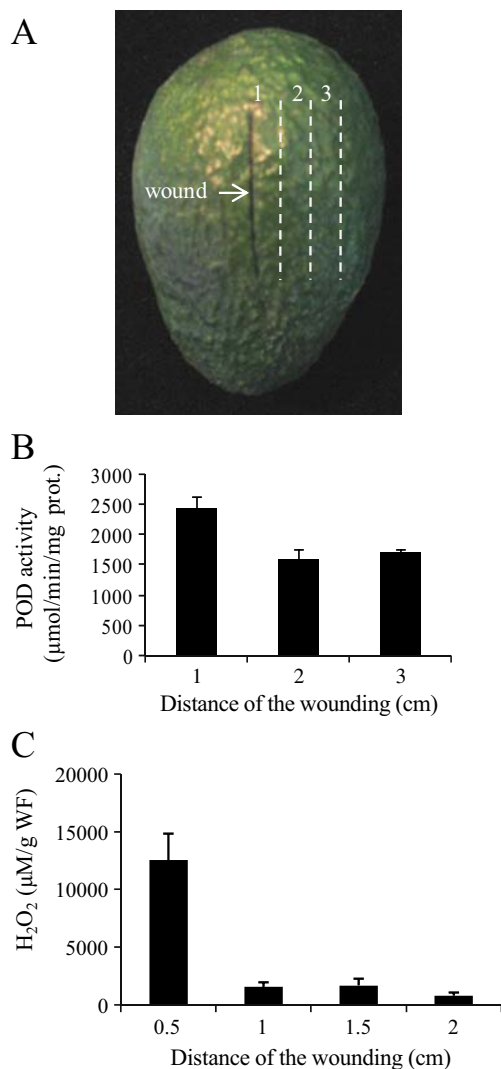


Fig. 1 Responses to wounding treatment in unripe avocado fruit. **a** Unripe avocado fruit was longitudinally wounded with a sterile blade, and samples of tissue were collected at 1-cm intervals from the wound 24 h after wounding. Soluble peroxidase (POD) activity (**b**) and hydrogen peroxide (H₂O₂) production (**c**) were analyzed different distances from the wound. Data are means \pm standard deviation (SD) of at least four independent experiments

Catalase activity had decreased by 38% at 1 h post-treatment, remaining lower than the controls at 24 h post-treatment. A significant increase in POD activity (150%) was observed 24 h after wounding; in contrast, SOD activity had increased only slightly (8.5%) 1 h after treatment, and had decreased by 37% relative to the control 24 h after wounding.

Enzymes that metabolize ASC

The wounding caused increases in the activity of dehydroascorbate (DHR), GR, and AO 2 h after treatment (Table 2), and the activities of these enzymes remained high until 24 h post-treatment (extent of study period). Glutathi-

Table 1 Effect of wounding treatment on activities of some protective enzymes in avocado fruits

Time after wounding ^a (h)	Enzyme		
	CAT (U mg ⁻¹ protein)	POD (μmol mg ⁻¹ protein min ⁻¹)	SOD (U mg ⁻¹ protein)
0	317.09 \pm 19.53	203.50 \pm 7.19	2817 \pm 49.34
0.25	221.53 \pm 9.65	176.19 \pm 9.71	3017 \pm 71.27
0.5	231.57 \pm 20.53	182.70 \pm 3.70	3180 \pm 50.12
1	198.03 \pm 35.18	157.43 \pm 5.84	3057 \pm 160.94
2	219.00 \pm 25.71	169.00 \pm 9.57	2747 \pm 108.28
4	225.53 \pm 15.54	172.53 \pm 13.08	2512 \pm 151.43
8	218.87 \pm 23.21	236.34 \pm 16.41	2130 \pm 80.12
12	231.73 \pm 19.55	308.26 \pm 12.46	1061 \pm 157.48
24	224.33 \pm 25.00	453.94 \pm 25.16	1458 \pm 120.30

Data are given as the average of three independent experiments (means \pm standard deviation)

^aMesocarp discs were incubated at different times post-treatment and CAT (catalase), POD (peroxidase) and SOD (superoxide dismutase) assayed

one reductase showed the highest, followed by AO and DHR.

Effect of wounding on POD, SOD and CAT expression

Figure 2 shows typical patterns of the most active total isoperoxidases obtained at different times after avocado wounding. Native PAGE revealed no evident changes in isozyme composition after wounding (Fig. 2a). In the apoplast (Fig. 2b), two isozymes showed the greatest increase in activity as a consequence of the treatment, and a new isozyme was observed 24 h after wounding.

Table 2 Wounding effect on enzymes that metabolize ascorbic acid in avocado fruits

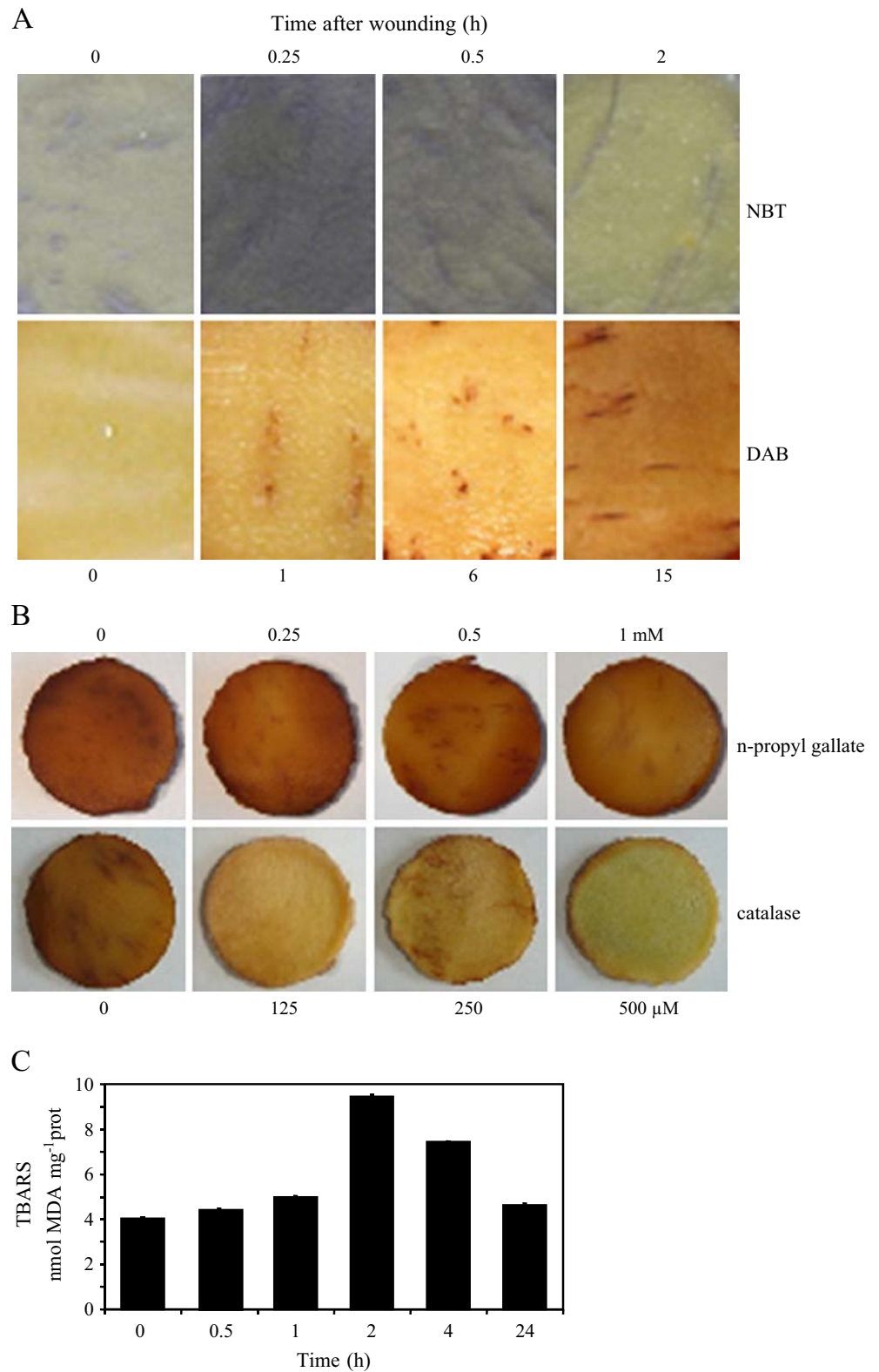
Time after wounding (h)	Enzyme		
	DHR (μmol min ⁻¹ mg ⁻¹ protein)	GR (nmol min ⁻¹ mg ⁻¹ protein)	AO (nmol min ⁻¹ mg ⁻¹ protein)
0	97.70 \pm 32.84	0.44 \pm 3.20	52.02 \pm 16.67
2	134.65 \pm 13.06	7.16 \pm 5.30	276.29 \pm 16.49
8	108.11 \pm 1.65	7.37 \pm 8.12	389.59 \pm 40.52
12	nm	3.56 \pm 3.95	247.61 \pm 68.89
24	125.26 \pm 17.50	4.55 \pm 6.63	111.39 \pm 43.03

nm, Not measured

Results are means \pm SD of four determinations

DHR, Dehydroascorbate reductase; GR, glutathione reductase; AO, ascorbate oxidase

Fig. 2 Reactive oxygen species production in avocado fruit tissue after wounding. **a** O_2^- and H_2O_2 were detected with nitroblue tetrazolium (*NBT*) and 3,3-diaminobenzidine (*DAB*) staining, respectively. **b** Different concentrations of inhibitors of POD activity or H_2O_2 accumulation were added to avocado discs, and DAB staining was realized 24 h after treatment. **c** Changes in lipid peroxidation expressed as equivalents of thiobarbituric acid reactive species (*TBARS*). Data are means \pm SD of at least four independent experiments



To determine which peroxidase isozymes were affected by wounding, tissue samples were analyzed by isoelectric focusing (Fig. 2c, Fig. 3). At least eight isozymes were observed at all of the time points

assayed, with the expression changing with the incubation time. Wounding induced an increase in the activity of at least three isozymes, pI 8.5, pI 8.8, and pI 9.4, at 24 h post-treatment. The activity of some isozymes,

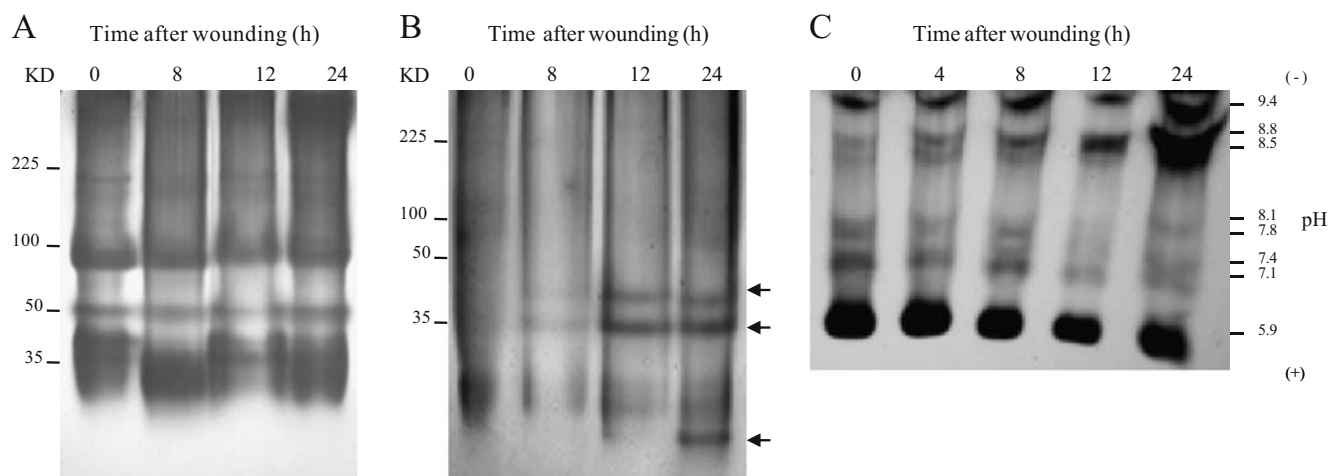


Fig. 3 Effect of wounding on avocado POD isoforms. Total POD isozymes were visualized with DAB and H_2O_2 from the tissue (**a**) or apoplastic fluid (**b**) at different times after wounding. Size of the Mr markers is indicated on the *left*. Arrows indicate the POD isozymes

differentially expressed in the apoplast. **c.** Isoelectric focusing of POD isozymes from the tissue after wounding. The isoelectric points are marked on the *right side* of the figure. The experiments were repeated at least three times with the same results

such as pI 5.9–8.1, appeared to decrease with increasing incubation time.

Changes in the activities of SOD isozymes were followed following wounding (Fig. 4). Three major SOD activities were observed, denoted SOD1, SOD2, and SOD3.

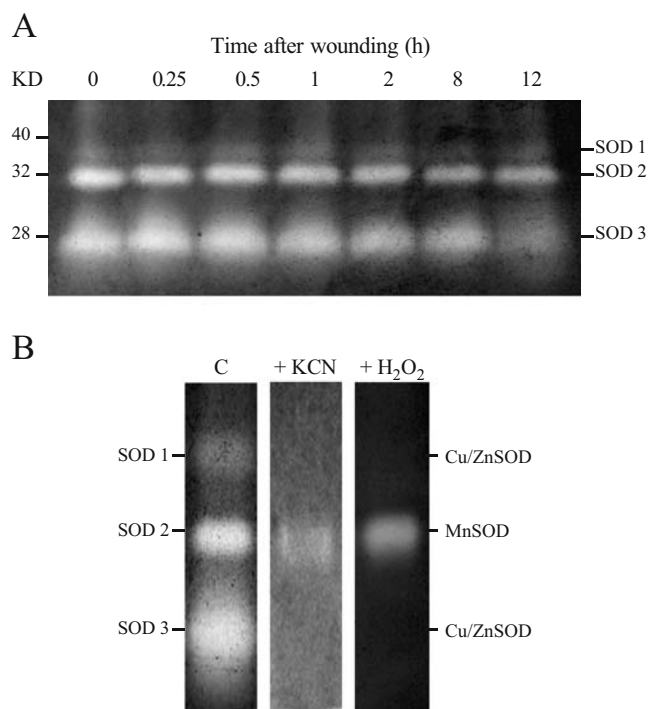


Fig. 4 Effect of wounding on avocado superoxide dismutase (SOD) activity. **a.** Total activity. Size of Mr markers is indicated on the *left*. **b.** The SOD isozymes are identified on the basis of native polyacrylamide gel electrophoresis analysis. The positions of Cu/ZnSOD and MnSOD on the activity gel are indicated. The experiments were repeated at least three times showing the same results

The activities of all SOD isozymes decreased progressively with increasing incubation time (Fig. 3a). The SOD isoforms were classified according to their sensitivity to cyanide and H_2O_2 . When the SOD were incubated in KCN or H_2O_2 , SOD1 and activities SOD3 were abolished, identifying this activity as a Cu/ZnSOD. SOD2 was resistant to both inhibitors, a characteristic of MnSOD (Fig. 4b).

CAT isozymes were not distinguished, and only one smeared band was observed in all of the times assayed. The CAT activity of this band decreased with increasing time post-wounding (data not shown).

Discussion

Wounding caused by various biotic and abiotic factors is a daily stress for plants that can lead to the loss of essential organs and the easy penetration of pathogens. Plants respond to wounding by activating self-defense systems to restore damaged tissues or to defend against attacks by pathogens and herbivores.

Our data show that ROS are rapidly produced in avocado fruit following mechanical stress. We found that as early as 15 min after mechanical treatment, the levels of O_2^- in the mesocarp tissue were increased; lipid peroxidation was observed 2 h post-treatment, and H_2O_2 accumulated progressively during the time of experiment (Fig. 1). Lamb and Dixon (1997) reported that, in plants, exposure to various abiotic and biotic stresses results in the accumulation of H_2O_2 and oxidative stress.

The speed of the oxidative burst associated with the capacity of H_2O_2 to diffuse freely and rapidly across

biological membranes and to signal gene expression suggests that H_2O_2 could function as a short-lived second messenger diffusing from cell to cell. Using cDNA microarray technology Desikan et al. (2001) identified 175 non-redundant expressed sequence tags that were regulated by H_2O_2 . Of these, 113 were induced and 62 were repressed by H_2O_2 . A major proportion of these expressed sequence tags had predicted functions in cell rescue and defense processes, cell signaling, and transcription, implying that H_2O_2 does have multiple roles in plant responses to stress.

H_2O_2 affects the polyphenol oxidase (PPO) of the avocado mesocarp, which is involved in the browning response through the oxidation of phenols and their subsequent polymerization to dark-colored melanins. 3,4-Dihydroxyphenylalanine (DOPA) oxidation by avocado PPO was slightly increased by a relatively low concentration of H_2O_2 (3.3–30 mM), while higher concentrations of H_2O_2 decrease both the rate and final level of dopachrome formed (Kahn 1983). We did not study the effect of the wounding on PPO activity here, but no browning of the tissue was observed with the treatment (data not shown). The possibility that H_2O_2 production in avocado affects PPO activity or melanin accumulation, given that melanins are bleached in the presence of relatively high concentrations of H_2O_2 (50–500 mM) (Kahn 1983), could be considered.

Alternately, organisms can protect themselves against oxidative stress by the synthesis of various antioxidant enzymes. The major ROS-scavenging enzymes of plants include SOD, POD, and CAT (Mittler 2002). We observed changes in these enzyme activities in avocado fruit after wounding. The activities of POD and SOD increased and that of CAT decreased with increasing time after wounding. Cheong et al. (2002) reported on POD and CAT gene expression in *Arabidopsis* during the analysis of transcriptional profiling in response to wounding. Interestingly, no SOD gene expression was observed in *Arabidopsis* despite that analysis being carried out during the same time interval our study (30 min).

There were no changes in total POD isozymes at different times after wounding, but two apoplastic isozymes did show increased levels and one new isozyme was observed after wounding. Isoelectric focusing gels stained for activity showed a predominant expression of basic peroxidases (Fig. 2).

The POD family is a large group of proteins in plants, many of which function as isozymes, with some located in the apoplastic compartment (Andrews et al. 2000; Córdoba-Pedregosa et al. 2003). The diversity of the reactions catalyzed by plant POD provides the basis for the implication of these proteins in a broad range of physiological processes, such as auxin metabolism, lignin and suberin formation, cross-linking of cell-wall components,

defense against pathogen, or cell elongation (Hiraga et al. 2001). Peroxidase has been shown to exhibit increases in activity or mRNA levels upon mechanical wounding in various plants, including tobacco (Lagrimini and Rothstein 1987), tomato and potato (Roberts et al. 1988), cucumber (Svalheim and Robertson 1990), and sweet potato (Huh et al. 1997). Suberin is deposited in wounded tissues. Since suberin is a highly hydrophobic macromolecule composed of hydroxycinnamic acid and its derivatives contain conjugated aliphatic moieties, suberin deposition around the wounded tissue should aid and healing (Hiraga et al. 2001).

In *Asparagus officinalis* spear discs the increase of the total peroxidase activity was found to be due to the increase in several distinct isoperoxidases. Four peroxidases with pI 8.7, 8.1, 7.4 and 6.7, respectively, showed increased activity. The histochemical stain for lignin revealed an increase in the lignified area during the time of measurement, suggesting the involvement of a set of peroxidases in the polymerization of phenolic compounds (Holm et al. 2003). Studies are in progress to determine whether the POD isozymes that increased in response to wounding in avocado are related to lignin and suberin accumulation.

In our studies SOD activity increased 30 min after wounding and then decreased to lower levels than the control thereafter (Table 2, Fig. 3). The main function of SOD is to scavenge O_2^- radicals generated in various physiological processes, thus preventing the oxidation of biological molecules, either by the radicals themselves or by their derivatives (Liochev and Fridovich 1994; Karpinska et al. 2001). We observed three SOD enzymes at all of the times assayed after wounding, SOD1, SOD2 and SOD3 (Fig. 1b), and all three activities decreased in time. Using SOD inhibitors, we characterized these activities as CuZnSOD to SOD1 and SOD3 and MnSOD to SOD2. The decrease in activity 12 h after wounding, most notably in SOD3, could be due to an increase in H_2O_2 accumulation. Strack et al. (1996) reported that, in animal cells, CuZnSOD is inactivated and degraded by the combined action of active oxygen species and proteases. H_2O_2 can reduce the enzyme-bound Cu^{+2} to Cu^+ , which in turn can form $Cu^{+2}-OH$ with an additional H_2O_2 . This OH can completely inactivate the enzyme molecule by oxidative modification of histidine residues at the active site (Hodgson and Fridovich 1975). Also, in wheat chloroplasts under light conditions, both CuZnSOD activity and enzyme content decayed with exposure time to photooxidative stress. O_2^- had no effect on either SOD activity or enzyme level. H_2O_2 and $\cdot OH$ inhibited SOD by inducing its fragmentation, as evidence on native PAGE (Cassano et al. 1997). This mechanism could explain the decrease in SOD activity after wounding because this decrease was correlated with an increase in H_2O_2 accumulation.

We observed changes in the activities of enzymes related to the metabolism of ascorbic acid (AA) such as DHR, GR, and AO, following our wounding treatment of avocado fruit. All enzyme activities increased as a result of the treatment. Ascorbic acid is the most abundant antioxidant present in all subcellular compartments, including the apoplast. It provides protection against ROS produced during normal cell metabolism or upon exposure to environmental stresses (Smirnoff 2000), and there is a growing recognition that shifts in the amount and/or redox state of AA participates in the perception of potentially stressful situations as well as in the modulation of defense responses (Pastori et al. 2003; Pignocchi and Foyer 2003; Foyer and Noctor 2005). Apoplastic AA also is believed to represent the first line of defense against ROS produced as a result of environmental perturbations (Barnes et al. 2002). In the apoplast, AO; a glycoprotein belonging to the family of blue copper oxidase enzymes) oxidizes AA to the unstable radical monodehydroascorbate (MDHA), which rapidly disproportionates to yield DHA and AA (Smirnoff 2000). The MDHA radical can be recycled back to AA by the activity of NAD(P)-dependent monodehydroascorbate reductase (MDHAR). Despite MDHA regeneration systems, rapid MDHA disproportionation results in DHA production, which can be reduced back to AA through the so-called ascorbate-glutathione (AA-GSH) cycle, which involves the co-ordinated action of DHAR and NADPH-dependent GR (Smirnoff 2000). Although the biosynthetic pathway of AA in plants has been elucidated (Wheeler et al. 1998), a detailed knowledge of the mechanisms controlling AA levels in different cell compartments is still missing (Ishikawa et al. 2006). The pool of AA in the apoplast results from the balance between inputs from newly synthesized AA transported from the cytosol and losses associated with enzymatic metabolism and oxidation by cell-wall-localized AO (Smirnoff 2000).

It has been reported that tomato MDHAR (AFR; ascorbate free radical) mRNA (Grantz et al. 1995) and *Cucumis melo* AO4 (*CmA04*) (Sanmartin et al. 2007) mRNA increased notably in response to wounding. This process may also contribute to maintaining the levels of AA for protection against wound-induced free radical-mediated damage.

Finally, Cheong et al. (2002) studied the transcriptional response of 8200 genes in *Arabidopsis* plants with aim of identifying those genes regulated by wounding. They found that approximately 8% of these genes were altered at steady-state mRNA levels. Studies of expression patterns of these genes provided new information on the interactions between wounding and other signals, such as pathogen attack, abiotic stress factors, and plant hormones. Two time points after wounding were analyzed, 30 min and 6 h. Those genes with significantly altered mRNA levels within

30 min were considered to be early response genes, whereas those responsive after 6 h were considered to be late response genes. In general the early response genes encoded to signaling or regulatory components, such as protein kinases and transcription factors, and late response genes encoded to effector proteins, such as enzymes in the metabolism.

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