

Early Events During Quiescent Infection Development by *Colletotrichum gloeosporioides* in Unripe Avocado Fruits

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ABSTRACT

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Inoculation of avocado pericarp tissue with *Colletotrichum gloeosporioides* and treatment of avocado cell cultures with the cell wall elicitor of *C. gloeosporioides* both increased the production of reactive oxygen species (ROS). However, whereas the production of ROS could be detected within minutes in avocado cell suspensions, it was detected only after 2 h following inoculation of pericarp tissue. Protein kinase inhibitors such as K-252a and staurosporine and the phosphatase inhibitor microcystin-LR inhibited the release of H₂O₂ from avocado cell suspensions. When 1 mM H₂O₂ was exogenously applied to pericarp tissue, it enhanced ROS, phenyl-

alanine ammonia lyase (PAL) activity, and epicatechin levels. But, when H₂O₂ treatment was applied following staurosporine treatment, PAL activity was no longer induced. The uninduced ROS production in pericarp tissue of freshly harvested, unripe, resistant fruit was twice as high as in ripe, susceptible fruit. Challenge inoculation of resistant fruit further increased the ROS level; however, this increase did not occur in susceptible fruits. The current findings are consistent with the hypothesis that production of ROS is induced by fungal infection of unripe fruits and, consequently, may modulate resistance, resulting in the inhibition of fungal development and quiescence.

Additional keywords: fruit resistance, preformed resistance.

Colletotrichum gloeosporioides Penz. (teleomorph: *Glomerella cingulata* (Stoneman) Spauld. et H. Schrenk) attacks unripe avocado fruits in the orchard. Germinated spores produce appressoria that germinate and breach the cuticle, but the resultant subcuticular hyphae become quiescent and do not develop further until fruit is harvested and ripens (38). Significant physiological changes occur in the host fruit that enable activation of the quiescent pathogen. Four mechanisms have been hypothesized to explain the resistance of unripe fruit to fungal attack: (i) lack of nutritional requirements for the pathogen; (ii) presence of preformed antifungal compounds; (iii) presence of inducible antifungal compounds; and (iv) lack of activation of fungal pathogenicity factors.

Resistance of unripe avocado to attack by *C. gloeosporioides* is correlated with the presence of fungitoxic concentrations of the preformed antifungal compound 1-acetoxy-2-hydroxy-4-oxoheneicos-12,15-diene (diene) in the pericarp of unripe fruits (44). Decrease of diene concentration and resultant increased susceptibility has been attributed to the activity of the host-derived enzyme lipoxygenase, which oxidizes the compound (43). The activity of lipoxygenase is regulated by the levels of the flavan-3-ol epicatechin (22), a natural antioxidant present in the pericarp that declines during fruit ripening (43). Increased diene concentration was accompanied by an increase in the transcriptional activation of genes involved in the biosynthesis of the flavonoid epicatechin (2). However, no increase in epicatechin could be induced in ripe fruit (22). Differences in host gene expression during fruit ripening seem to be the rule in the host-pathogen interactions of harvested produce.

The transient and rapid production of reactive oxygen species (ROS) including superoxide, H₂O₂, and hydroxyl radical by various elicitors is well documented (1,3,7,32,34,50,51). It is not clear

whether ROS produced in plants in response to a pathogen or elicitor have direct antimicrobial effects (5). But the ROS are considered to be involved in various defensive reactions such as lipid peroxidation (23,53), oxidative cross-linking of cell wall proteins (9), peroxidative polymerization of cinnamoyl alcohol, and reinforcement of plant cell walls by the formation of lignin polymers (19,24), phytoalexin production (1,13,53), and programmed cell death (28). No reports have been published on the importance of ROS in the initial stages of quiescence development in fruits by pathogens during postharvest.

Several reports indicate that ROS synthesis occurs at the extracellular surface of the plasma membrane via a one-electron reduction of molecular oxygen (3). It has been previously reported that the transfer of electrons from a cytosolic electron donor (NADPH) to electron acceptors at the extracellular surface of the plasma membrane may lead to (i) acidification of the cytosol, (ii) alkalization of the extracellular medium, and (iii) decrease in the plasma membrane potential (17,18,31,48). These changes induced by pathogens and biotic elicitor compounds mediate a variety of early cellular events, including membrane depolarization, and changes in the permeability of the plasma membrane to calcium ions, protons, potassium, and chloride ions (35). All these effects could contribute to transduction and amplification of signals (27).

In this report, we found that infection of avocado fruit by *C. gloeosporioides* or treatment of avocado cell suspensions with cell wall elicitor induced ROS production. Ripe and unripe fruit tissue differ with regard to ROS production in response to pathogen attack. The unripe, resistant fruits are physiologically able to react and produce high levels of ROS that can enhance the phenylpropanoid pathway, regulate the levels of the antifungal compound diene, and inhibit fungal development, resulting in its quiescence (42).

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MATERIALS AND METHODS

Plant material and experimental treatments. Experiments were carried out with freshly harvested avocado fruits (*Persea ameri-*

cana Miller var. *drymifolia* (Schldl. and Cham.) S.F. Blake) cv. Fuerte from an orchard at Kibbutz Givat Brenner, Rehovot, Israel. Experiments were carried out during three consecutive harvesting seasons and repeated at least three times per season. The average value of three to five replications of one single experiment is usually presented. In some cases, the average of all the replications from repeated experiments are presented with their pooled standard error. Fruits of fairly uniform size and weight (250 to 270 g) were harvested, washed in water, and used 1 to 2 h after harvest for experiments. Pericarp tissue strips (about 1 mm thick, almost free of mesocarp tissue) were cut, washed several times with water, and aerated for 20 h in 0.2 mM CaSO₄. The pericarp strips were then placed with the interior side in contact with the filter paper saturated with 0.2 mM CaSO₄, and the upper cuticular side was used for fungal inoculation.

Suspension cultures were generated from pericarp callus cultures by transferring 1 cm³ of callus tissue into 40 ml of the medium described by Prusky et al. (44), with the growth regulator picloram at a concentration of 50 µg/liter of medium, without agar, in a 250-ml Erlenmeyer flask. Cells for experiments were grown at room temperature with continuous agitation at 96 rpm on a rotary shaker.

Pericarp inoculation and H₂O₂ treatment. A single-spore isolate of *C. gloeosporioides* isolate Cg-14 (39) was obtained previously from decayed avocado fruits. The fungus was normally maintained on potato dextrose agar at 20°C. A spore suspension (2 × 10⁶ spores per ml) was brushed over pericarp strips (41). In some cases, pericarp strips were also brushed with a freshly prepared solution of 1 mM or 5 mM H₂O₂ (E. Merck AG, Darmstadt, Germany). As a control, pericarp strips were brushed with distilled water only. Following inoculation or H₂O₂ treatment, plasma membranes were isolated.

Extraction of epicatechin. Epicatechin was extracted from 1-mm-thick avocado pericarp, as previously described (39). Three grams (fresh weight) of pericarp was ground in 95% ethanol (1:10, wt/vol). The ethanol extract was dried in a rotary evaporator, and the residue was redissolved in 10 ml of 1 mM NaCl and extracted twice with 90 ml of ethyl acetate. Epicatechin was quantitated by reverse phase-high-performance liquid chromatography (HPLC) (RP-18) in a column eluted with 55% methanol and 1% acetic acid in double-distilled water. Calculation of the epicatechin level was based on comparison of the HPLC peak areas quantitated at 275 nm to those of the standard.

Extraction and activity of phenylalanine ammonia lyase (PAL). Samples for PAL activity were extracted by blending 5 g of avocado

pericarp in 50 ml of cold acetone (−20°C) and the homogenate filtered out with Whatman No. 1 (Whatman International, Ltd., Maidstone, England). This process was carried out four times. The pericarp powder was dried at room temperature overnight and stored at −20°C until use. PAL was extracted from 100 mg of pericarp powder by stirring for 2 h at 4°C with 5 ml of 0.1 M sodium borate buffer, pH 8.8. The extract was centrifuged at 10,000 × g for 20 min at 4°C, and the supernatant was used as an enzyme source. The reaction mixture for PAL consisted of 10 mM L-phenylalanine (Aldrich Chemical Co., Milwaukee, WI), 0.1 M borate buffer (pH 8.8), and 1.5 ml of enzyme extract in a final volume of 5 ml. The reaction was incubated at 37°C for 60 min and terminated by adding 0.1 ml of 5 N HCl. The product cinnamic acid was extracted into 7 ml of diethyl ether and quantitated by measuring the amount of *trans*-cinnamic acid formed at 269 nm (30).

Elicitor preparation from cell walls of *C. gloeosporioides*. Conidia of *C. gloeosporioides* from single-spore cultures were used to inoculate in Erlenmeyer flasks (500 ml) containing 200 ml of potato dextrose broth. The flasks were incubated at 25°C with continuous agitation at 100 rpm for 7 days. Harvesting and disruption of the hyphae were carried out as described by Prusky et al. (39) with glass beads (0.45 to 0.5 mm) agitated at maximum speed in a Braun MSK homogenizer (Braun, Melsungen, Germany) for four periods of 45 s each, with the tube being cooled in ice (6). The broken cell walls were sediments in centrifuge tubes centrifuged at 1,000 × g for 5 min and washed six times with 50 ml of distilled water. The residual walls were lyophilized, resuspended in distilled water (10 mg/ml), and autoclaved for 30 min. The autoclaved suspension was centrifuged at 5,000 × g for 10 min at 4°C. The supernatant was filtered through a Millipore filter (0.2 µm; Gelman Sciences, Ann Arbor, MI), and the collected fraction was evaporated to 2% of its initial volume. The sample was loaded on a DEAE-Diethylaminoethyl Cellulose column (Sigma Chemical Co., St. Louis) washed with 100 ml of 10 mM phosphate buffer at pH 8.0. The fraction was eluted first with 50 ml of 25 mM phosphate buffer, pH 8.0, and secondly by 50 ml of 500 mM NaCl (4). Both fractions were pooled, concentrated partially by lyophilization, and finally dialyzed twice against 2 liters of double-distilled water and used as the elicitor source. Soluble sugars were quantified by the anthrone method as a parameter for elicitor concentrations.

H₂O₂ determination by spectrofluorimeter and diaminobenzidine. H₂O₂ production in cultured avocado cells was measured by monitoring the scopoletin conversion to nonfluorescent product (47). The cell suspensions were washed twice with a medium containing 2% sucrose, 1 mM NaH₂PO₄, 1 mM (NH₄)₂SO₄, 1 mM MgSO₄, 1 mM CaCl₂, 25 mM KNO₃, and 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) adjusted to pH 6.6. The cells were concentrated to 10⁶ cells per ml with washing medium. The cells were allowed to stand for 0.5 h at room temperature, 200 µl of cell suspensions were placed in enzyme-linked immunosorbent assay plates, and 0.1 mM scopoletin (10 µl) and 30 µM horseradish peroxidase (10 µl) were

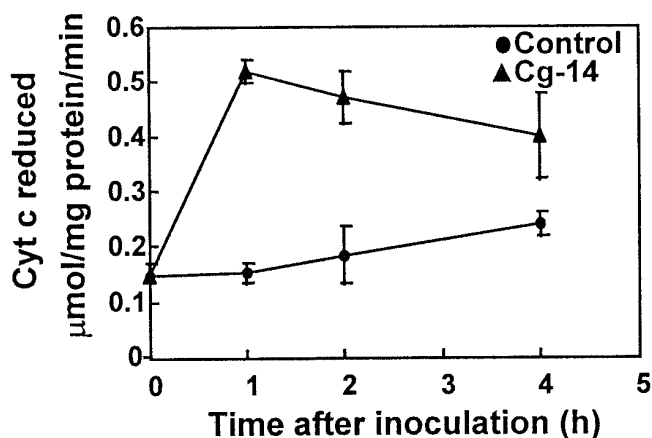


Fig. 1. Time course of reactive oxygen species (ROS) production in plasma membrane isolated from unripe pericarp tissue inoculated with *Colletotrichum gloeosporioides* strain Cg-14. Bars represent standard error from one representative experiment run four times with five replications. ROS was determined as cytochrome *c* reduction in the absence and presence of superoxide dismutase according to the method of Owen and Hancock (36). Each molecule of ROS reduces one molecule of cytochrome *c* (33).

TABLE 1. Effect of cell wall extract from *Colletotrichum gloeosporioides* on reactive oxygen species production in avocado cell suspensions

	Cytochrome <i>c</i> reduction (µmol) ^a		
	30 min	60 min	120 min
Elicitor (15 µg/equivalent Glu)	0.00	0.34 ± 0.06	0.91 ± 0.21
Elicitor (30 µg/equivalent Glu)	0.00	0.84 ± 0.25	2.11 ± 0.48
Ionophore A-23187 ^b (1 µM)	1.14 ± 0.019	NT	NT

^a The difference in cytochrome *c* reduction Δ_{OD(530-570)} in the absence and presence of superoxide dismutase is used as a measurement of O₂⁻ production, since each O₂⁻ reduces ferric cytochrome *c* to its ferrous form (33). Cytochrome *c* reduction was measured in 10⁶ avocado cells. Means and standard errors are based on the data from one experiment of four that gave similar results. Each treatment was replicated five times. NT = not tested.

^b The ionophore A-23187 was used to test the capability of the avocado cell suspensions to release reactive oxygen species.

added. When fluorescence values had stabilized (5 to 10 min), the stimulus was added. The effect of specific inhibitors was tested by adding the inhibitors 5 min before the addition of the stimulus. Staurosporine, K-252a, and microcystin-LR were added from stock solutions prepared in dimethyl sulfoxide (DMSO). A similar amount of DMSO was added to the control treatment. The optimal inhibitor's concentration was determined by testing a series of concentrations. Higher concentration of the inhibitor did not further decrease the H_2O_2 production by avocado cell suspensions. Oxidation of scopoletin by H_2O_2 , catalyzed by peroxidase, was measured as the decrease in fluorescence recorded by an automatic reading Microplate-Fluorometer-FLUOstar (BMG Labtechnologies, Offenburg, Germany). The sample was excited at 350 nm and the emission measured at 460 nm. The fluorometric units were calculated according to the standard curve of H_2O_2 concentrations.

For in vivo ROS production, disks of pericarp tissue (1 cm in diameter) were placed in 3,3'-diaminobenzidine (DAB; Sigma Chemical Co.) solution containing 0.2 mM $CaSO_4$ according to Thordal-Christensen et al. (52). ROS generated in the tissue reacted with DAB, resulting in a dark brown pericarp. At various times after inoculation, transferring the disks to 20 ml of 96% ethanol stopped the reaction. The tissue was boiled and the metric lightness units were determined with a Minolta Chroma Meters (CR-200; Minolta Camera, Ltd., Osaka, Japan). Maximal value of metric lightness (45 units) was obtained in the untreated control tissue. Disks of pericarp placed in DAB solution treated with increasing concentrations of H_2O_2 (0 to 50 mM) resulted in darker tissue and a reduction of lightness units (the value with 50 mM was 31 units). The regression coefficient between H_2O_2 concentrations (0 to 50 mM) and the units of lightness was $r = 0.934$. Similar regression coefficients were obtained in three independent experiments.

Preparation of microsomes and isolation of plasma membranes. Plasma membranes were isolated from 40 g of pericarp strips after treatment with 0.2 mM $CaSO_4$ for 24 h, as described above. Plasma membrane was isolated at several periods after inoculation with *C. gloeosporioides*. Tissue was homogenized in a Waring blender (Waring Products, New Hartford, CT) at 4°C, and the microsomes were prepared according to Giannini et al. (16). The microsomes were suspended in suspension medium (330 mM sucrose, 5 mM KCl, and 5 mM potassium phosphate buffer, pH 7.8), and the plasma membranes were separated by two-phase partitioning (26). The final composition of the phase system was 6.4% (wt/wt) of each of dextran T500 and polyethylene glycol (PEG 3350) in the suspension medium. The final upper phase was diluted

1:10 with resuspension medium/330 mM sucrose, 1 mM dithiothreitol (DTT), 5 mM NaF, and 5 mM MES-bis-tris propane (MES-BTP), pH 7.8, and the purified plasma membranes were pelleted at $55,000 \times g$. The pellet was redissolved and brought to a concentration of 1 to 3 mg of protein per ml and adjusted to pH 7.0 with 330 mM sucrose, 5 mM MES-BTP, and 1 mM DTT. Protein was assayed according to Bradford (8). ATPase activity in these plasma membranes was about 87% vanadate sensitive.

ROS production measured by cytochrome c-reducing activity. ROS production was determined by cytochrome c reduction dependent on NADPH oxidation according to Owen and Hancock (36). By this method, for each molecule of ROS production, one molecule of cytochrome c reduction is detected (33). The assay consisted of 20 μ M cytochrome c (Sigma type VI; Sigma Chemical Co.), 100 μ M NADPH, 25 mM potassium phosphate buffer (pH 7.5), and 10 μ g of plasma membrane protein in a total volume of 1 ml. The reaction was started with the addition of NADPH, and the rate of cytochrome c reduction was measured on a dual-wavelength spectrophotometer at 550 nm by the difference in the absence and presence of 100 μ g of superoxide dismutase (14,36).

RESULTS

ROS production in avocado fruit tissue triggered by fungal infection and fungal cell wall elicitor. Plasma membranes of isolated pericarp tissue that had been preinoculated with *C. gloeosporioides* showed a rapid increase in superoxide production, measured by cytochrome c reduction. ROS production by the isolated plasma membrane of inoculated pericarp from unripe avocado fruits increased by threefold within 1 h and remained higher up to 4 h after inoculation compared with that of the uninoculated pericarp tissue (Fig. 1). Addition of fungal cell wall elicitor to avocado cell suspensions enhanced the cytochrome c reduction within 1 h after treatment (Table 1).

H_2O_2 production in unripe avocado fruit tissue elicited by direct fungal infection and fungal cell wall elicitor. ROS was initially detected by DAB staining 2 h after unripe avocado pericarp was inoculated with *C. gloeosporioides*. Avocado pericarp inoculated with *C. gloeosporioides* at 0 time showed 42.7 ± 2.3 metric lightness units, and 2 h later, it decreased to 40.1 ± 1.3 units. The lightness further decreased to 37.8 ± 2.8 and 35.6 ± 1.3 units, 10 and 20 h after inoculation, respectively. When cultured avocado cell suspensions were treated with cell wall elicitor (6 μ g of glucose equivalents), release of H_2O_2 was detected within a few minutes, as determined by the loss of scopoletin fluorescence (Fig. 2). Increasing amounts of elicitor (15 and 30 μ g of glucose equivalents) further increased the generation of H_2O_2 by the avocado cell cultures.

Effect of protein kinase and phosphatase inhibitors on H_2O_2 release by avocado cell suspension system treated with fungal cell wall elicitor. Incubation of cultured avocado cells in the presence of protein kinase inhibitor, such as K-252a (1 μ M) and staurosporine (10 μ M), inhibited H_2O_2 release by 70 to 94% (Table 2).

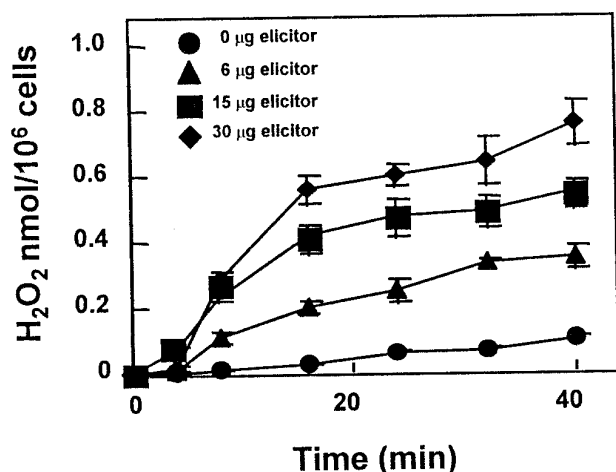


Fig. 2. Effect of *Colletotrichum gloeosporioides* elicitor on the release of H_2O_2 from avocado cell suspensions. Different concentrations of elicitor were added to the avocado cell suspensions (10^6 cells) and their effects were compared with that of the untreated control. Bars represent standard errors. The data from four experiments with three replications per treatment were pooled.

TABLE 2. Effect of cell wall extract from *Colletotrichum gloeosporioides* on the release of H_2O_2 from avocado cell suspensions^a

	H_2O_2 (nmol) \pm SE
Cells only	0.14 \pm 0.022
Elicitor (60 μ g/equivalent Glu)	1.01 \pm 0.26
Staurosporine (10 μ M) + elicitor	0.306 \pm 0.092
K-252a (1 μ M) + elicitor	0.15 \pm 0.068
Microcystin-LR (1 μ M) + elicitor	0.254 \pm 0.076
Ionophore A23187 (5 μ M)	2.87 \pm 0.402

^a Cell wall elicitor from *C. gloeosporioides* prepared as described in text. Release of H_2O_2 from suspensions of 10^6 avocado cells within 10 min after adding elicitor and various inhibitors. The release of H_2O_2 was measured as oxidation of scopoletin 350/460 nm in the presence of peroxidase. Means and standard errors (SE) are based on the results of one experiment of four that gave similar results. Each treatment was replicated three times.

The phosphatase inhibitor microcystin-LR (1 μ M) also inhibited H_2O_2 release by 75%. While the Ca^{2+} ionophore A-23187 alone stimulated the release of H_2O_2 from cultured avocado cells almost threefold.

Effects of challenge inoculation of *C. gloeosporioides* and H_2O_2 on enhancement of epicatechin, PAL, and ROS production. Early reports showed that challenge inoculation of whole avocado fruits enhanced the level of epicatechin and the activity of PAL in the fruit pericarp within 24 h (2,42). Inoculation of pericarp tissue from unripe avocado fruits with *C. gloeosporioides* increased the epicatechin level by twofold within 6 h (Fig. 3). During the same period, PAL activity increased as measured by the increment of cinnamic acid from 3.5 to 6 μ mol per mg of protein per h (data not shown).

Treatment of pericarp tissue (unripe avocado fruits) with exogenous H_2O_2 (1 mM) doubled ROS production by the plasma membrane within 30 min (Fig. 4). H_2O_2 treatment induced a 1.5-fold increase in PAL activity within 60 min and a 2.5-fold increase 6 h after treatment (Fig. 5B). A similar pattern of increase was observed in the epicatechin level, which increased from an initial value of 400 to 1,200 μ g/g fresh weight 6 h after treatment (Fig. 5A). Treat-

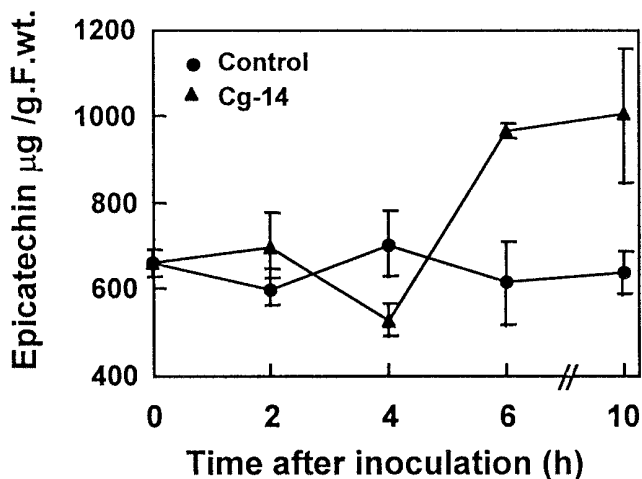


Fig. 3. Epicatechin levels in avocado fruit pericarp (unripe) in response to inoculation with *Colletotrichum gloeosporioides* strain Cg-14. Pericarp was sampled from freshly harvested fruits, incubated for 24 h in an aerated solution of 0.2 mM $CaSO_4$, and then inoculated as described in text. Bars represent standard errors. The data from three experiments with three replications per treatment were pooled.

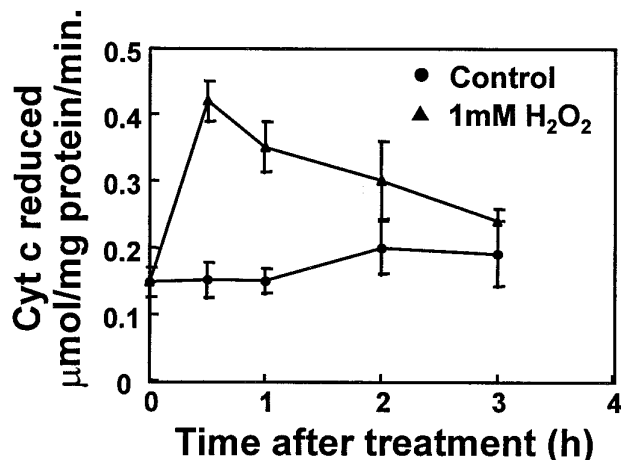


Fig. 4. Time course of reactive oxygen species production in plasma membrane isolated from unripe pericarp tissue treated with 1 mM H_2O_2 . Bars represent standard errors from one representative experiment run four times with three replications.

ment of pericarp tissue with 5 mM H_2O_2 induced faster increases in PAL activity and epicatechin levels than did 1 mM H_2O_2 1 h after treatment. However, PAL activity and epicatechin levels in the 5 mM H_2O_2 exogenously treated pericarp started to decline 1 h after treatment, while at 1 mM H_2O_2 , the activity increased continuously during the 5 h following treatment. When avocado pericarp was treated with 1 μ M staurosporine by brushing the pericarp 20 min before its exposure to 1 mM H_2O_2 , PAL activity was inhibited and was similar to that found in untreated pericarp tissue (Fig. 6).

ROS production in pericarp of unripe, resistant and of ripe, susceptible avocado fruits. ROS levels in uninoculated plasma membranes from pericarp of unripe, resistant fruits was 2.3-fold higher than ROS production in ripe, susceptible fruits (Fig. 7). Inoculation of pericarp in resistant fruits showed that ROS levels in the resistant tissue was 4.3-fold higher than in ripe fruits (Fig. 7).

DISCUSSION

ROS produced in response to pathogen attack or elicitor treatment have been hypothesized to have direct antimicrobial effects and play a role in defense mechanisms by triggering lignin accumulation, lipid peroxidation, phytoalexin production, and the hypersensitive response (5). One of the probable sources of ROS induced by early interactions with fungal infection is NADPH oxidase located in the plasma membranes (45). In our experiments, *C. gloeosporioides* infection activated the production of ROS threefold in plasma membranes of avocado pericarp within 1 h after inoculation. Production of ROS was also detected in cultured avocado cells 8 min after treatment with cell wall elicitor prepared from *C. gloeosporioides*. Production of ROS is, thus, one of the most rapid biochemical responses detected in avocado pericarp tissue (1,14).

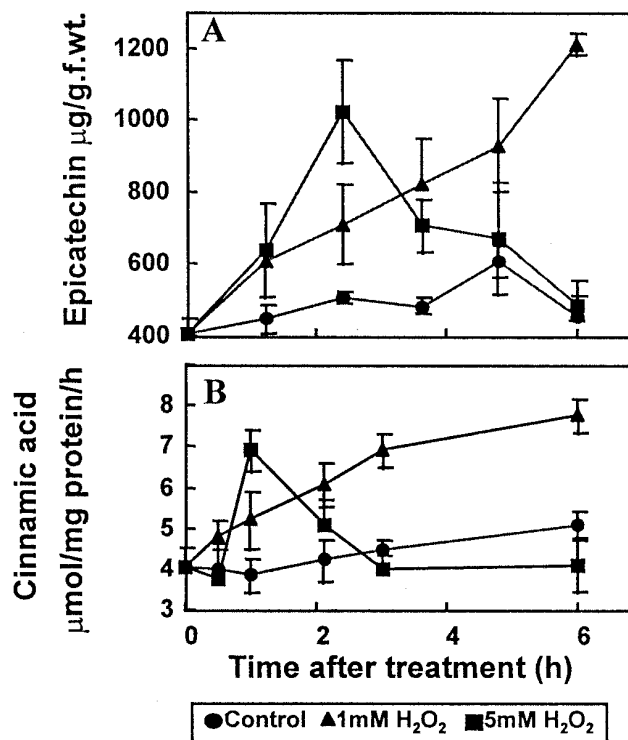


Fig. 5. Epicatechin levels and phenylalanine ammonia lyase (PAL) activity in the pericarp of unripe avocado fruit cv. Fuerte treated with H_2O_2 . Pericarp was sampled from freshly harvested fruits, incubated in an aerated solution of 0.2 mM $CaSO_4$ for 24 h, and then treated with H_2O_2 as described in text. A, Epicatechin levels, and B, PAL activity. Bars represent standard errors. The data from three experiments with five replications per treatment were pooled.

Programmed cell death induced by H_2O_2 has been reported in several plant systems (20,28). However, in the current study, no obvious cell death was observed in the avocado pericarp tissue or in cultured avocado cells where ROS production was detected. H_2O_2 was easily detected in cultured avocado cells, as reported for other cell suspension systems (29,49). Eight minutes after cultured avocado suspension cells were exposed to the cell wall elicitor of *C. gloeosporioides*, a 10-fold increase in H_2O_2 production was observed. In inoculated pericarp, however, release of ROS was detected by DAB staining only after several hours. It might be possible that the delay in H_2O_2 detection in tissue is a result of partial destruction of the generated ROS, since significant activities of catalase and peroxidase are found in the avocado pericarp (D. Beno-Moualem and D. Prusky, unpublished data).

The ability of the cell wall elicitor to enhance H_2O_2 release in a cultured avocado cell suspension system is promoted by the rapid binding to, or interaction with, the plasma membrane. Binding of several oligo- or polyglucans from fungal origin to soybean microsomal membranes has been reported (10,11). Avocado fruit pericarp, similar to other plant organs (34), might recognize the pathogen or the cell wall elicitors at very early stages in the infection process and react with rapid generation of ROS. These findings imply that the plasma membranes of avocado fruit pericarp acts as a site for the initial binding of elicitor molecules that triggers the defense response (42). In previous work, it was found that influx of Ca^{2+} , exchange of H^+/K^+ across the cell membrane, and activation of ion channels occurred within 10 min after elicitor treatment, before ROS were released (35). We demonstrated here that the avocado cell suspensions could be activated to generate H_2O_2 by the addition of the Ca^{2+} ionophore A-23187, which allows the influx of the Ca^{2+} ions. In other systems in which specific elicitors were used, increased endogenous Ca^{2+} also was found to stimulate the release of H_2O_2 (21,35,49). Additionally, protein kinase inhibitors such as staurosporine and K-252a significantly reduced ROS production in cultured avocado cell suspensions induced by the *Colletotrichum* cell wall. The phosphatase inhibitor microcystin-LR also inhibited ROS production. From the action of kinase and phosphatase inhibitors, one can deduce that different processes (phosphorylation and dephosphorylation) might regulate the induction of ROS in avocado fruit tissue.

Prusky et al. (39) reported that challenge inoculation of intact fruits with pathogenic or nonpathogenic *Colletotrichum* strains enhanced PAL activity and other phenylpropanoid biosynthetic en-

zymes within 24 h. Following the early reports of Doke (14), the connection between ROS production and phytoalexin synthesis has been explored in several interactions (1,12,25,46). Apostol et al. (1) reported that exogenous H_2O_2 (0.5 to 1 mM) added to soybean suspension cells was sufficient to induce glyceollin. In this work, we found that avocado fruit, similar to other plant organs, increased ROS production, PAL activity, and epicatechin levels after fungal inoculation. We found that application of exogenous H_2O_2 to pericarp tissue also induced an increase of the epicatechin level, which indicates that the reactive oxygen level produced during the *Colletotrichum*-avocado interaction leads to the activation of phenylpropanoid pathway. However, inhibition of this process by pretreatment of pericarp with staurosporine before H_2O_2 application inhibited PAL activity, suggesting that protein kinase activity is required for the activation of phenylpropanoid biosynthesis in the avocado system. It is possible that the application of H_2O_2 may trigger a second oxidative burst as shown by Park et al. (37) in potato slices. In their system also, staurosporine inhibited the induction of subsystemic oxidative burst by H_2O_2 . This might indicate that H_2O_2 stimulates a novel secondary signaling process in avocado tissue.

The production of ROS in avocado fruit is reported, for the first time, to depend on the physiological stage of fruit ripening. In pericarp of freshly harvested unripe, resistant fruits, ROS production was 0.168 ± 0.026 compared with only 0.072 ± 0.014 μmol of cytochrome *c* per mg of protein per min in the ripe, susceptible tissue. It is possible that one of the factors affecting the induction of resistance in unripe fruit is the capability to react and the presence of naturally high ROS levels, compared with the reduced capability to react in ripe fruits (40,41). Induction of H_2O_2 production has been described in parsley cell suspensions and cucumber, in which the response can be further enhanced by 2,6-dichloroisonicotinic acid, salicylic acid, or methyl jasmonate, and also by cuticle abrasion (15). In avocado fruit, *C. gloeosporioides* spores that land on the fruit cuticle germinate within a few hours and produce appressoria from which penetration hyphae breach the fruit cuticle. We hypothesize that the infection point will subsequently become the local source for ROS production that will activate the phenylpropanoid pathway, preserve or increase the levels of the antifungal compound diene, and ultimately inhibit fungal development, resulting in its quiescence (2).

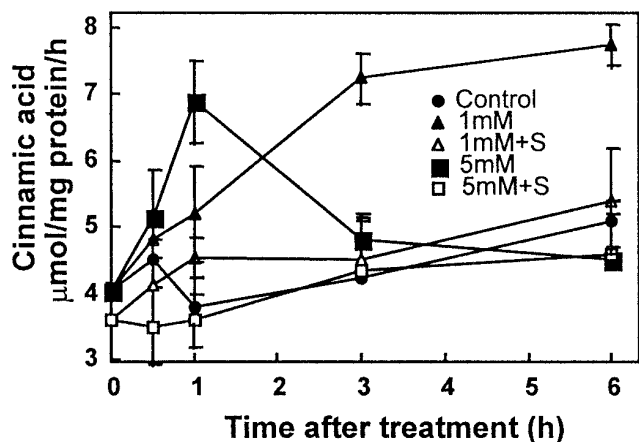


Fig. 6. Effect of staurosporine on the activity of PAL, induced by H_2O_2 , in isolated pericarp tissue of unripe avocado fruits. Pericarp tissue was sampled from freshly harvested fruits and incubated in an aerated solution of 0.2 mM $CaSO_4$ for 24 h. The pericarp was brushed four times during 20 min with 1 μM staurosporine (S), followed by treatment with 1 mM and 5 mM H_2O_2 . Bars represent standard errors. The data from three experiments with three replications per treatment were pooled.

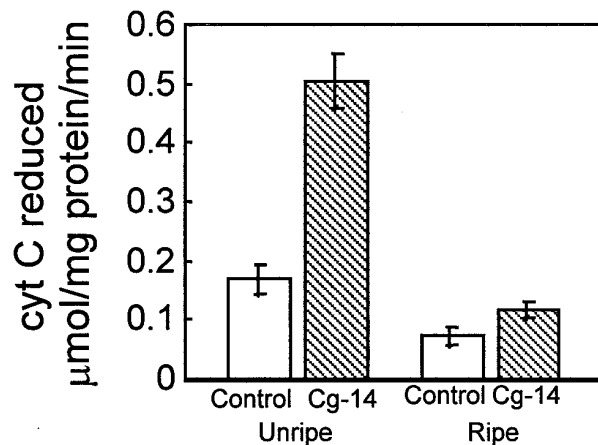


Fig. 7. Reactive oxygen species production in plasma membrane isolated from pericarp of unripe, resistant fruits and of ripe, susceptible fruits. The resistant fruits and the susceptible fruits were harvested on the same day. The susceptible fruit were stored for 12 days to ripen completely and become soft. Both pericarp tissues were inoculated with *Colletotrichum gloeosporioides* strain Cg-14. Activity was tested 4 h after inoculation, at the same time as was non-inoculated fruit. Bars represent standard errors. The data from four experiments with five replications per treatment were pooled.

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