Metabolism of the Flavonoid Epicatechin by Laccase of *Colletotrichum gloeosporioides* and Its Effect on Pathogenicity on Avocado Fruits

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ABSTRACT

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During avocado fruit ripening, decreasing levels of the flavonoid epicatechin have been reported to modulate the metabolism of preformed antifungal compounds and the activation of quiescent *Colletotrichum gloeosporioides* infections. Epicatechin levels decreased as well when *C. gloeosporioides* was grown in the presence of epicatechin in culture. Extracts of laccase enzyme obtained from decayed tissue and culture media fully metabolized the epicatechin substrate within 4 and 20 h, respectively. Purified laccase protein from *C. gloeosporioides* showed an apparent MW of 60,000, an isoelectric point at pH 3.9, and maximal epicatechin degradation at pH 5.6. Inhibitors of fungal laccase such as

EDTA and thioglycolic acid reduced *C. gloeosporioides* symptom development when applied to ripening susceptible fruits. Isolates of *C. gloeosporioides* with reduced laccase activity and no capability to metabolize epicatechin showed reduced pathogenicity on ripening fruits. On the contrary, Mexican isolates with increasing capabilities to metabolize epicatechin showed early symptoms of disease in unripe fruits. Transcript levels of *cglac1*, encoding *C. gloeosporioides* laccase, were enhanced during fungal development in the presence of epicatechin at pH 6.0, where avocado fruits are susceptible to fungal attack. But transcript increase was not detected at pH 5.0, where the fruit is resistant to fungal attack. The present results suggest that biotransformation of epicatechin by *C. gloeosporioides* in ripening fruits is followed by the decline of the preformed antifungal diene compound, resulting in the activation of quiescent infections.

The phytopathogenic fungus *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. (teleomorph: *Glomerella cingulata*) is an important postharvest pathogen that attacks a wide variety of tropical and subtropical fruits (27,30,35). *C. gloeosporioides* germinates on avocado (*Persea americana* Miller var. *drymifolia* (Schltdl. & Cham.) S.F. Blake) fruit peel and forms appressoria. The infection hyphae then penetrate into the epidermal cells of the avocado exocarp but remain quiescent until the fruit ripens (5). The resistance of unripe avocado fruit to fungal attack during quiescence has been reported to depend on the presence of preformed antifungal compounds (32) and the consequent lack of secretion of fungal pathogenicity factors occurring during quiescent infections (34,47).

The level of the preformed antifungal 1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene (antifungal diene, AFD) is affected by the relative rates of synthesis and metabolism of the compound (29,31). In unripe-resistant fruits enhanced resistance to the pathogen is characterized by the activation of synthesis of the AFD in parallel to enhanced expression of key genes of its biosynthetic pathway including Δ^9 desaturation (17), Δ^{12} desaturation (43), and elongation (45). In ripe-susceptible fruits, however, the decline in concentration of AFD has been attributed to its metabolism through the oxidative activity of lipoxygenase (15). The

activity of lipoxygenase is modulated by the level of its inhibitor, the flavan-3-ol-epicatechin (epicatechin) (27), a natural antioxidant that is generated in the phenylpropanoid pathway, and that declines in concentration during fruit ripening. Decline in epicatechin levels leads to increase in lipoxygenase activity, along with metabolism of the AFD (4,32,33). Thus, epicatechin is an important factor that modulates avocado fruit resistance to postharvest attack.

Fungal pathogenicity may, however, not only depend on induced metabolism of natural compounds by the plant, as reported in avocado fruits (4), but pathogens might have strategies for circumventing the effects of preformed or inducible compounds (23). Some fungal pathogens can avoid exposure to antifungal compounds, and others may tolerate and even combat the toxic effects of these compounds by detoxification, and so enable fungal attack. Induced antifungal compounds may be avoided or their induction may be delayed by (i) evasion by the pathogen of the host's recognition capability (22); (ii) production of suppressors of the plant defense responses (46); (iii) detoxification of antifungal compounds (e.g., tomatinase, avenacinase, laccases, etc.) (20,21,25,38,42); or (iv) use of an efflux mechanism that involves active efflux by ATP-binding cassette (ABC) transporters (14).

Laccases (p-diphenol:dioxygen oxidoreductases) are part of a larger group of multicopper enzymes, detected in several fungal species, that act on a variety of polyphenol substrates that have been implicated in a wide range of biological processes that affect fungal virulence (3,11,20,39,43,44). We hypothesize that metabolism of epicatechin by laccase secreted by *Colletotrichum* spp.

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during the initial stages of pathogenicity leads to enhanced lipoxygenase activity, decreased level of the preformed AFD, and increased susceptibility of the fruit. Our objectives are to determine the contribution of laccase in decay development of *Colletotrichum* spp. in avocado fruits. In the present paper, we describe the decline in concentration of the flavonoid epicatechin by *C. gloeosporioides* laccase and the importance of this process for the pathogenicity of *C. gloeosporioides* in ripening avocado fruits.

MATERIALS AND METHODS

Avocado fruit, fungal isolates, growth media, fruit inoculation, and epicatechin extraction. Freshly harvested avocado fruits (cv. Fuerte) from an orchard at Kibbutz Givat Brenner, Israel were used in the experiments. A single-spore isolate of *C. gloeosporioides*, isolate Cg-14, obtained from decayed avocado fruits was used in the experiments (32). Three-week-old harvested conidia from Mathur's (MS) medium plates (41) were used for conidial germination and appressorial formation assays and fruit inoculation. Conidial germination and appressorial formation were tested on glass slides and incubated at 22°C, 95% relative humidity (RH) for 20 h. Fruits were inoculated by placing 10 µl of a conidial suspension (10^6 conidia per ml) at six longitudinally spaced inoculation spots, three on each side of the fruit. Following inoculation, fruits were incubated at 22°C, 95% RH for 24 h, in covered plastic containers containing wet paper towels; they were then transferred to ambient RH (70 to 80% RH) at 22°C until fruit ripening and symptom development were observed (28). The average diameter of decay of 10 fruits was reported. Inoculation experiments were repeated three times.

Epicatechin was extracted from avocado pericarp (2 g of tissue, 1- to 2-mm-thick slices). Pericarp tissue was homogenized in 95% ethanol in an Omni-Mixer (Sorvall Instruments Division, Dupont Co., Newton, CT) at full speed for 3 min. The extract was filtered through filter paper, and the ethanol sample was dried under vacuum in a Rota evaporator at 40°C, dissolved in 10 ml of 1 mM NaCl, and fractionated twice with ethyl acetate (4). The pooled organic phases were dried with anhydrous $MgSO_4$ and evaporated to dryness. The samples were dissolved in ethyl acetate/dichloromethane (1:3) and subjected to flush chromatography, as described previously (4). The collected samples were dried, dissolved in 1 ml of the running solvent, and analyzed by high-performance liquid chromatography (HPLC); the column was eluted with 55% methanol and 1% acetic acid in double-distilled water. Each experiment was run in four replicates. The epicatechin levels were calculated by comparison with a commercial epicatechin standard ([2R,3S]-2-[3,4-dihydroxyphenyl]-3,4-dihydro-1[2H]-benzopyran-3,5,7-triol) (Sigma, Jerusalem, Israel). Concentrations were expressed as micrograms per gram fresh weight of fruit peel. The experiments were carried out during two consecutive harvesting seasons and repeated twice in each season. The results corresponding to one representative experiment are presented.

***C. gloeosporioides* laccase: purification, characterization, activity, and inhibition.** Purification of laccase secreted to the culture media by *C. gloeosporioides* was carried out following the inoculation of spores (1×10^6 spores per flask) into 40 ml of MS medium (40) at pH 5.0 according to Yakoby et al. (47) (primary growth culture). Cultures were incubated at 25°C in a shaking incubator at 150 rpm for 5 days. The culture was harvested by filtration in a sterile Büchner funnel fitted with a filter paper, and the hyphae remaining on the filter were washed twice with 40 ml of sterile distilled water. The washed mycelia were resuspended in 50 ml of fresh medium (secondary growth culture or laccase-inducing medium) containing, per liter, 10 g of KH_2PO_4 , 5 g of $MgSO_4 \cdot 7H_2O$, 12.5 g of KNO_3 , 0.75 g of $CaCl_2 \cdot 2H_2O$, 0.025 g of $FeCl_3$, 25 g of glucose, and 5.5 mg of epicatechin. Twenty-nine

hours after transfer to the inducing medium, the hyphae were filtered through a 0.45-µm-pore-size membrane filter (Supor-450; Gelman Sciences, Ann Arbor, MI). The filtrate was further concentrated four- to fivefold by ultrafiltration at 4°C through a cellulose ester membrane (Filtron Technology Corp., Northborough, MA) with a 10-kDa cutoff (Amicon Rotterdam, The Netherlands). In some experiments the fungus was grown in 50 ml of the minimal medium, and the medium was concentrated 10-fold in a rotary evaporator as described above.

Extraction of laccase from *C. gloeosporioides* mycelia was carried out after growing the fungus in inducing liquid media containing epicatechin. Mycelia (2.0 g fresh weight) were grounded in liquid N_2 and the enzyme was extracted by mixing thoroughly with 3 ml of 0.2 M phosphate buffer, at pH 6.0, containing 1% insoluble polyvinyl pyrrolidone (PVP) (Sigma, St. Louis). The slurry was centrifuged at $10,000 \times g$ for 10 min at 4°C and the supernatant was used as an enzyme source.

For extraction of laccase from avocado tissue, 5 g of decayed pericarp was ground with a mortar and pestle in the presence of liquid nitrogen. The enzymes were extracted by adding 5 ml of phosphate buffer (0.2 M, pH 6.0) containing 1% insoluble PVP. The slurry was centrifuged at $10,000 \times g$ for 10 min at 4°C, and 0.1 g of activated charcoal (Sigma, Jerusalem, Israel) was added to the supernatant. The supernatant was then mixed gently, centrifuged at $10,000 \times g$ at 4°C, and loaded on a Sephadex G-50 Fine column (5 cm length, 1 cm diameter) (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The protein was eluted with 0.1 M phosphate buffer, pH 6.0, and was collected in 5-ml fractions to be used as a laccase source. The concentrated products were dialyzed against 3 liters of 10 mM sodium acetate (pH 6.0) and the extracts were loaded on a MonoQ anion-exchange column (Pharmacia) in a fast protein liquid chromatography (FPLC) unit (Akta, Pharmacia). Protein fractions (1.5 ml each) were eluted with a linear gradient of sodium acetate (pH 6.0) containing 1 M NaCl and used for determinations of laccase activity. Protein concentrations in the samples were determined according to Bradford (6). In some experiments, partial purification of laccase was carried out with a Rotaphor purification system (Bio-Rad Laboratories, Hercules, CA). Eighteen milliliters of concentrated protein extracts (approximately 1,000 µg/ml protein) from decayed avocado fruit extracts (as described below) or from liquid *C. gloeosporioides* media was separated by isoelectric focusing (IEF, Bio-Rad) at 15 W using ampholytes (Bio-Rad) in the pH range of 4 to 10, according to the manufacturer's instructions.

Activity assays for laccase consisted of a reaction mixture of 100 µl of 26-mg/ml 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) (Sigma), 800 µl of 0.1 M phosphate buffer, pH 6.0, and 100 µl of enzyme extract in a final volume of 1 ml. The reaction was incubated at room temperature (22°C), up to 60 min, where the reaction is linear and quantified by measuring the optical density at 436 nm (24). Activity of laccase was also tested by analyzing by HPLC chromatography the remaining amount of phenol substrates. For that purpose, *C. gloeosporioides* spores were grown in primary growth culture for 5 days and then transferred for 24 h to a secondary growth culture containing, per liter, 10 g of KH_2PO_4 , 5 g of $MgSO_4 \cdot 7H_2O$, 12.5 g of KNO_3 , 0.75 g of $CaCl_2 \cdot 2H_2O$, 0.025 g of $FeCl_3$, 25 g of glucose, and 30 mg of either epicatechin, DL-catechin, p-coumaric acid, or caffeic acid. In some experiments, the amount of epicatechin added was 25 mg/liter of culture.

Optimal pH activity for epicatechin degradation was determined by assaying epicatechin residue following incubation of enzyme extract in the presence of 30 mg of epicatechin per liter in 0.1 M phosphate buffer at pH ranging from 4.8 to 6.2. Assay reactions at each pH with boiled enzyme extract were used as controls. The laccase activity enhancer Cu^{++} was tested either by adding $CuSO_4$ to a final concentration of 2 mM to the reaction mixture or by growing the fungus in the inducing media amended

with 2 mM CuSO₄. The laccase inhibitors EDTA and thioglycolic acid were included at increasing concentrations from 1 to 25 mM in the reaction mixture.

Inhibitors of laccase were tested for their effect in vivo on decay development. EDTA at concentrations of 20 or 50 mM and 5 mM thioglycolic acid solutions in 0.05 M phosphate buffer, pH 5.0, were infiltrated in freshly harvested avocado fruits 1 day before inoculation with 10 µl of a *C. gloeosporioides* conidial suspensions containing 10⁶ spores per ml. The infiltration was done by dipping one longitudinal half of the fruit in the solution, reducing the atmospheric pressure to 20 mm Hg within 20 s, and then releasing the vacuum. The other half of the fruit was then similarly infiltrated with water as a control (31).

Generation of a laccase probe for northern analysis. Degenerate primers corresponding to the conserved sites of several fungal laccases (2) were used to generate a probe for northern analysis. The sequence of the degenerate primers was 5'-GGAAT-TCTGGTAYCACAGCCACTT-3' (forward primer) and 5'-GGA-ATTCTCRTGGCCRTGAAGRTG-3' (reverse primer). The polymerase chain reaction (PCR) contained 100 ng of genomic DNA of *C. gloeosporioides* and was prepared according to Yakoby et al. (47). The cycling conditions were as follows: 95°C for 1 min in 1 cycle; 94°C for 1 min, 58°C for 40 s, and 72°C for 1 min in 35 cycles; and finally, 72°C for 8 min. The PCR product was cloned into pGEM-T-Easy (Promega, Madison, WI) and purified plasmid DNA was sequenced using the universal primers for this vector. The sequence data were analyzed with the BLAST package at the National Center for Biotechnology Information (NCBI) and the nucleotide sequence was deposited as GenBank accession no. AY853696. Deduced amino acid sequence of the PCR fragment from the laccase gene of *C. gloeosporioides* was aligned to the corresponding region in the laccase of *Neurospora crassa* using the BioEdit program (13).

Southern blot analysis was performed according to the protocol of Amersham Pharmacia Biotech (Buckinghamshire, UK), for Hybond-N⁺. Genomic DNA of *C. gloeosporioides* (20 µg) was digested with *Xho*I, *Hind*III, and *Eco*RI; hybridization was performed at 65°C with the 908-bp probe of *cglac1*; and the membrane was washed at high stringency. The probed membrane was exposed to Kodak BioMax MR Film (Eastman Kodak Co., Rochester, NY).

Northern blot analysis was performed following the isolation of total RNA of *C. gloeosporioides* with the RNeasy Plant Mini Kit (QIAGEN Sciences, Hilden, Germany). Total RNA was run in standard formaldehyde denaturing gels (36). After separation, the RNA was transferred to a Hybond-N⁺ membrane, hybridized with a ³²P-dCTP-labeled-*cglac1* probe, and exposed to Kodak BioMax MR Film (36). Hybridization signals were quantified and the percentage intensity value of the signal was derived by subtracting background intensity and comparing to the hybridization signal from the rDNA probe, as determined with MacBAS software version 2.3 (Fujifilm, Koshing Gafic Systems, Inc., Tokyo, Japan).

Selection of *C. gloeosporioides* isolates with reduced and increased pathogenicity. Isolates of *C. gloeosporioides* showing reduced pathogenicity were isolated from a healthy part of the exocarp tissue (i.e., where no symptoms were expressed) of ripe, decayed cv. Fuerte avocado fruits, obtained from an orchard in the Tzrifim farm, Israel. Tissue samples (1 × 1 mm²) were exposed to 1% Na hypochlorite, washed twice in sterile water, and transferred to MS media. Single-spore cultures of *Colletotrichum* spp. isolated from developing colonies were confirmed as *C. gloeosporioides* by means of PCR with taxon-specific amplification primers that included the internal transcribed spacer 4 primer (5'-TCCTCCGCTTATTGATATGC-3') coupled with the specific primer for *C. gloeosporioides* (CgInt) (5'-GGCCTCCGCGCTC-CGGGCGG-3') (10). Conidial germination, germ tube elongation, and appressorium formation of the reduced pathogenicity

C. gloeosporioides strains were compared with the wild-type Cg-14 isolate as described previously.

Single spores of *C. gloeosporioides* isolates obtained from avocado orchards in Michoacan, Mexico, showing symptoms of anthracnose in unripe or in ripening fruit were compared. Tissue samples (1 × 1 mm²) were initially exposed to 1% Na hypochlorite, washed twice in sterile water, and transferred to MS medium for isolation.

Data analysis. Statistical analyses of the data were performed using the JMP-in software, version 5 for Windows (SAS Institute Cary, NC). Standard errors of the means or least significant difference tests were reported.

RESULTS

Effect of *C. gloeosporioides* on epicatechin levels. When *C. gloeosporioides* hyphae were transferred from primary growth cultures to secondary growth cultures containing epicatechin, the concentration of epicatechin level declined by 50% during the first 5 h of incubation, and a 50% reduction of the remaining level was observed 17 h later (Fig. 1A). Five hours after transfer of the fungus to the secondary growth culture containing epicatechin, activity of laccase was detectable, but the highest activity (25,000 µmol ABTS per min) was observed 45 h later (Fig. 1B). No remnants of laccase activity was detectable in mycelial extracts of *C. gloeosporioides* obtained from secondary growth cultures containing epicatechin.

Epicatechin levels in the pericarp of healthy cv. Fuerte avocado fruits decreased from 998 to 153 µg g⁻¹ fresh weight during fruit ripening (Fig. 2). Epicatechin levels in the pericarp of ripe fruit decayed by *C. gloeosporioides* were only 1.5 µg g⁻¹ fresh weight (Fig. 2) and showed a laccase activity of 22,321 µmol ABTS per

Fig. 1. Epicatechin concentrations and cumulative laccase activity in culture medium containing epicatechin at 30 mg/liter. **A**, Epicatechin concentrations were measured by high-performance liquid chromatography. **B**, Laccase activity was measured by using 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) as a substrate. Bars represent standard error. The data from one out of three experiments with five replications per treatment are presented.

min per μg of protein. This extract of laccase from decayed tissue reduced epicatechin concentrations in the medium by a factor of 7.5 during the first 30 min of incubation at 25°C. However, extracts of healthy ripe pericarp did not contain any laccase activity and did not affect the decline in epicatechin concentration in vitro.

Characterization of laccase from *C. gloeosporioides*. Purified laccase from decayed pericarp extracts obtained by IEF or FPLC showed a single active band at 60 kDa when the protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) and compared to native PAGE with ABTS used as a developer (Fig. 3A and B). The isoelectric point of the purified protein was pH 3.9 and the optimal pH for enzyme activity (from purified culture filtrates or decayed fruit extracts) was 5.4. The maximal epicatechin degradation by *Colletotrichum* sp. in culture was 93% of the original epicatechin content and occurred at pH 5.6 (Fig. 4).

Secretion of laccase by *C. gloeosporioides* 21 h after culture inoculation was highest if epicatechin and DL-catechin were included in the medium as a substrate, whereas activities of lower intensity were obtained when p-coumaric acid or caffeic acid were used as substrates (Table 1). The residual concentrations of the nonmetabolized phenols were 12.5% for epicatechin, 1.1% for DL-catechin, and 63 and 96% for p-coumaric and caffeic acid, respectively (Table 1).

Fig. 2. Epicatechin concentrations in healthy ripe and unripe, and decayed ripe avocado fruits cv. Fuerte infected by *Colletotrichum gloeosporioides*. Bars represent the least significant differences (at $P \leq 0.05$) of epicatechin concentrations between treatments.

Fig. 3. Purified laccase from *Colletotrichum gloeosporioides* decayed pericarp extracts run in activity gel and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). **A**, Activity gel detected by 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) (as a green color); and **B**, SDS-PAGE gel silver stained.

Copper was also shown to activate laccase activity, as reported previously (38) for other laccases. Addition of 2 mM CuSO_4 to the reaction mixture increased activity fourfold and a similar concentration added to the minimal growth medium increased the activity fivefold. On the other hand, laccase activity extracts from culture filtrate or from decayed fruits were inhibited by 50% in the presence of 20 mM EDTA or 1 mM thioglycolic acid. Infiltration of 50 mM EDTA or 5 mM thioglycolic acid into freshly harvested fruits reduced decay development from 7.07 ± 0.4 mm in control fruits to 1.3 ± 0.4 and 3.5 ± 0.6 mm, respectively. Similar concentrations of EDTA or thioglycolic acids did not affect fungal germination or growth of *C. gloeosporioides* germ tubes.

Generation of a *C. gloeosporioides* laccase probe and northern analysis. Degenerate primers were designed in order to obtain a DNA fragment that could be used to study the expression of *C. gloeosporioides* laccase. The cloned PCR products were similar among themselves except that one of the clones contained a shorter fragment (data not shown). A clone of 908 bp (*cglac1*)

Fig. 4. Optimal pH for epicatechin degradation by laccase extracted from decayed avocado pericarp. Pericarp extracts were incubated at different pH ranges in the presence of epicatechin at 30 mg/liter, and the residual epicatechin concentrations were determined after 24 h. Bars represent standard errors. The data from one out of three experiments with five replications per treatment are presented.

TABLE 1. Laccase activity and metabolism of different phenol substrates by *Colletotrichum gloeosporioides*

Phenols substrates	Laccase activity ^a	Remaining phenol	
	$\mu\text{mole ABTS}/\text{min}/\mu\text{g of protein}$	$\mu\text{g/ml}^b$	% Initial concentration
Epicatechin	$2,082.3 \pm 229.5$	3.70 ± 0.38	12.5
DL-Catechin	971.5 ± 162.2	0.33 ± 0.05	1.1
Caffeic	176.8 ± 32.7	28.67 ± 8.12	95.8
Coumaric	301.5 ± 29.8	18.86 ± 1.85	62.9

^a Mycelia were transferred from primary growth cultures to secondary growth cultures amended with 30 mg of epicatechin, p-coumaric acid, DL-catechin, or caffeic acid per liter. Laccase activity was determined as described in the text following 24 h of growth of *C. gloeosporioides* in secondary growth culture. ABTS = 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid). The average mean of three replications $\pm$ SE is presented.

^b Remaining substrate concentrations after 24 h of growth of *C. gloeosporioides* in the presence of different phenol substrates. The amount of remaining substrate was determined by high-performance liquid chromatography analysis. The mean of three replications $\pm$ SE is presented.

was selected for further sequencing and it showed considerable homology to fungal laccases, including the conserved Cu-oxidase domain of the multicopper oxidase enzymes (2). The deduced amino acid sequence of *cglac1* was aligned to the *N. crassa* laccase (accession no. M18333) (Fig. 5). Amino acid identity to other fungal laccases was 48% for *Gaeumannomyces graminis* (accession no. AJ437320) and *Botryotinia fuckeliana* (accession no. AF243855). Southern analysis of *C. gloeosporioides* genomic DNA with the 908-bp *cglac1* probe under high-stringency hybridization revealed one band for the *XhoI* digest, one main and a second minor band for the *HindIII* digest, and two bands of relatively low intensity for the *EcoRI* digest. The sequence of *cglac1* did not contain sites for the aforementioned restriction enzymes. Northern blot analysis yielded a single band and a relatively high basal expression of *cglac1* transcripts (Fig. 6). Exposure to epicatechin at pH 4.0 did not enhance *cglac1* expression. However, *cglac1* transcripts accumulated in the presence of epicatechin as the pH increased from 4.0 to 5.6 (Fig. 6).

Effect of differential pathogenicity isolates of *C. gloeosporioides* on epicatechin metabolism. Of 30 isolates of *C. gloeosporioides* obtained from healthy, ripe, avocado tissue, only two isolates, Cg-W7 and Cg-W10, showed no decay development when symptoms of decay of the wild-type *C. gloeosporioides* Cg-14 isolate were expressed (Fig. 7). Symptoms of decay of the reduced pathogenicity isolates occurred 6 to 7 days later and only to a minor extent. Reduced pathogenic isolates did not differ from the wild type in germination rates, germ tube elongation, or appressorium formation on fruit exocarp. However, the laccase activity in culture media 36 h after inoculation with reduced pathogenic isolates was only 20% of that produced by the wild type, Cg-14 ($9,242 \pm 21$ in the reduced pathogenic compared with $57,137 \pm 230$ μmol ABTS per min per μg of protein in the wild type) (Fig. 8). During the same period, the wild-type *C. gloeosporioides* metabolized 96% of the epicatechin substrate, whereas the reduced pathogenic isolates reduced the epicatechin concentration by only 8 to 15% of its initial value (Fig. 8).

Mexican isolates CgM-3 and CgM-6, showing early symptoms of anthracnose in unripe fruits, could metabolize epicatechin almost twofold faster than isolates CgM-4 and CgM-5, which cause anthracnose symptoms on ripe fruit only (Table 2).

DISCUSSION

Decrease in the level of the preformed AFD was suggested as the main factor modulating quiescence of *C. gloeosporioides* during ripening of avocado fruits. Factors modulating the metabolism of preformed compounds such as plant lipoxygenase and epicatechin, an inhibitor of lipoxygenase activity, modulate the quiescent period (33). Faster epicatechin metabolism during

ripening of avocado fruits increased susceptibility to decay development by *C. gloeosporioides* and shortened the quiescent period (4,33). The capability of *C. gloeosporioides* laccases to reduce epicatechin concentration in the decayed tissue suggests that laccase secretion by *C. gloeosporioides* may be a factor contributing to the activation of quiescent infections (11,43). Laccases are multicopper oxidases that are widely distributed among fungi involved in the biotransformation of polyaromatic compounds (16, 22). This biotransformation may lead *C. gloeosporioides* laccase to transform epicatechin into a product with reduced inhibition of avocado lipoxygenase.

Fig. 6. *cglac1* transcript accumulation from *Colletotrichum gloeosporioides* in response to different ambient pH conditions in the presence and absence of epicatechin. Northern analysis of total RNA isolated from *C. gloeosporioides* mycelia (20 μg loaded) after 3 days of growth in media with and without epicatechin, at the indicated pH values. The blot was hybridized with the 982-bp *cglac1* probe under high-stringency hybridization and with a ribosomal DNA (rDNA) fragment. The columns at the bottom indicate the corrected percent intensity value of *cglac1* based on the background intensity and the hybridization signal from the rDNA probe, as determined with MacBAS software version 2.3 (Fujifilm, Tokyo).

<i>N. crassa</i>	169	WYHSHFSLQYA-----NGVFGGMIFNGPATANYDEDLGHFLFLSDWSHVDFVTRWFAARNGQPPAL
<i>C. gloeosporioides</i>		WYHSHFALQAWSQDPYLTDPRLNGLFGGTIIVNGPASAPYDEDLGTIMLSDFHQTSDELYPTAATNGPPTA
<i>N. crassa</i>	228	ESGLINGTNKFDCTSSTDPNCTGRGVKLTTVVEKGKKYRLRLVNAAVDGVFOFSIDKHKLKVIAADIIVP
<i>C. gloeosporioides</i>		NTGLINGTGMYN-----GGCSRFTTNFEACKSYRMRLVNGAIDTMWKFIMDNHTLEVISADEVFP
<i>N. crassa</i>	298	IKPYSTSSSLKLTGQRYDIIIFHANQAVDNYWVRAGWLNACQPNVNPDSITSVLRYNASSTLDPTSTSDVT
<i>C. gloeosporioides</i>		INPYNTSSISIGIGQRYDVIVRANQATDNYWLRAPELTCSSNENTLDIKGIVRYDSSSTADPT-TEIGT
<i>N. crassa</i>	368	VSSDCLDEPLASLVPHLKMDVTNIIKSFDTYLNIS---TTNYVHWTINTSSLLIDWNNPTLTKQVFNGESL
<i>C. gloeosporioides</i>		YMDNCLDESMSDLVPVVAIEAG--QQTYDDTLTVGLQVSSQFKWTLNNSTFLSDWGYPTVEQVIDGNDT
<i>N. crassa</i>	435	FPTDYNVLAINKTTTDDDEWIVLVIQDQSNLGLHPIHLHGH
<i>C. gloeosporioides</i>		YSTQONIVHLDQAN---VWVHFIIQN--PIGLAHPHHLHGH

Fig. 5. Comparison between the deduced amino acid sequences of the laccase of *Neurospora crassa* and the putative laccase fragment obtained from *Colletotrichum gloeosporioides*. Accession numbers are as follows: *N. crassa*, M18333; and *C. gloeosporioides*, (*cglac1*), AY85369. Black regions represent identical amino acid sequence and shaded regions represent similarity (7).

Fig. 7. Decay development by *Colletotrichum gloeosporioides* isolates with laccase reduced activity (isolate Cg-W7) in comparison with the wild-type isolate Cg-14.

Fig. 8. Effect of reduced pathogenicity *Colletotrichum gloeosporioides* isolates Cg-W7 and Cg-W10 on laccase activity and epicatechin metabolism in comparison to wild-type Cg-14 isolate. **A**, Cumulative laccase activity in culture media. Laccase activity was measured by using 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) as a substrate. **B**, Epicatechin metabolism in culture medium containing epicatechin at 25 mg/liter. Epicatechin concentrations were determined by extraction of the residual concentrations from inoculated culture media after various intervals, and detected by high-performance liquid chromatography. Bars represent the least significant differences (at $P \leq 0.05$) between epicatechin metabolism and laccase activity between the wild-type strain and the reduced pathogenicity isolates.

TABLE 2. Metabolism of epicatechin by Mexican isolates of *Colletotrichum gloeosporioides* with symptoms of disease expressed during the period of avocado fruit development or after fruit harvesting and ripening

Isolate	Cultivar	Stage of attack	Epicatechin metabolized after 24 h ^a	
			µg/ml	%
CgM-3	Fuerte	Young fruits	21.2 ± 1.7	84.9
CgM-6	Hass	Young fruits	17.4 ± 0.9	69.4
CgM-4	Criollo	Ripe fruits	7.5 ± 1.0	30.1
CgM-5	Hass	Ripe fruits	9.2 ± 0.1	36.8

^a Mycelia were transferred from primary growth cultures to secondary growth cultures amended with 25 mg of epicatechin per liter and after 24 h the remaining concentrations of epicatechin were detected by high-performance liquid chromatography. The average mean of three replications ±SE is presented.

Purified laccase from *C. gloeosporioides* showed a molecular mass of 60 kDa, a size similar to laccase isoenzymes produced by *Pleurotus eryngii*, with apparent molecular masses of 56 and 70 kDa (24), and by *Fomes lignosus*, with an apparent molecular mass of 66.5 kDa (13). *Gaeumannomyces graminis* var. *tritici*, however, produced a laccase with a higher molecular mass (190 kDa) but yielded two subunits with molecular masses of 60 kDa when denatured and deglycosylated (9).

The optimum pH for laccase production by *C. gloeosporioides*, in culture or extracted from decayed fruits, was found to be 5.4, which is similar to that for laccases of the wood-degrading fungus *Pleurotus pulmonarius* when ABTS was used as a substrate (8). The activity of *Colletotrichum* laccase was also affected by the presence of copper (39). The addition of 2 mM CuSO₄ to the culture medium or to the assay mixture increased the activity four- or fivefold. *C. gloeosporioides*, similarly to the ectomycorrhizal fungus *Suillus granulatus* (12), was able to metabolize several different phenolic compounds that are substrates for *Colletotrichum* laccase. *Colletotrichum* laccase reduced the concentration of catechin and epicatechin efficiently (98.9 and 87.5%, respectively), and coumaric acid less efficiently (37%), but could only marginally metabolize caffeic acid. It is possible that this substrate specificity may contribute to the differential host range of *Colletotrichum* spp.

Fungal laccases play a significant role in plant pathogenicity by detoxification of antifungal compounds and tannins in the host

environment (1,19,26,37). In *C. gloeosporioides*, a series of experiments suggest the involvement of fungal laccase in the activation of quiescent infections: (i) secretion of laccase was detected during fruit decay development accompanied by highest laccase activity; (ii) purified laccase from decayed tissue rapidly metabolized epicatechin in vitro, while no laccase activity was detected in healthy fruits; (iii) inhibition of laccase by EDTA and thioglycolic acid blocked decay development in avocado fruits, similar to the effect reported for laccase from *Pleurotus eryngii* (24); and (iv) *C. gloeosporioides* isolates that showed reduced capability to metabolize epicatechin were not able to colonize ripe fruits. On the contrary, Mexican isolates that metabolized epicatechin more rapidly showed early decay symptoms in unripe fruits. The mechanism underlying the differential secretion of laccases by the pathogenic versus the reduced pathogenicity isolates remains unknown.

The DNA fragment that was isolated by degenerate primer PCR was clearly related to fungal laccases (9,18,40,45). Southern blot of *C. gloeosporioides* DNA suggested one and possibly two sequences with homology to the *cglac1* probe. It should be noted that two laccase genes were identified in *B. cinerea* and the difference in their DNA sequence and function was substantial (38).

Interestingly, the enzyme activity as determined by the metabolism of epicatechin by *C. gloeosporioides* was highest at pH 5.4 and the level of transcript expression of *cglac1* in the presence of epicatechin increased as the pH increased from 5.1 to 5.6. This effect of pH increase on the enhancement of transcript expression of *cglac1* was similar to that reported for *pelB* encoding pectate lyase from *C. gloeosporioides* (47). This would imply that the alkalization that results from fruit ripening and ammonia accumulation by *C. gloeosporioides* (34) induced the activation of *cglac1* expression, its secretion, and the consequent degradation of the flavonoid epicatechin. All these results, taken together, support the importance of laccase activity as a factor that induces susceptibility in ripening fruits by modulating the level of epicatechin, the decline of the AFD, and consequently, the activation of quiescent infections by *C. gloeosporioides* in ripening fruits.

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