A Nonpathogenic Mutant Strain of *Colletotrichum magna* Induces Resistance to *C. gloeosporioides* in Avocado Fruits

Dov Prusky, Stanley Freeman, Rusty J. Rodriguez, and Noel T. Keen

Department of Plant Pathology, University of California, Riverside, CA 92521 U.S.A.
Received 16 July 1991. Revision received 28 December 1993. Accepted 18 January 1994.

A mutant strain (path-1) of *Colletotrichum magna* (teleomorph *Glomerella magna*) that grew endophytically in watermelon plants was recently isolated. Unlike the wild-type isolate, the mutant did not cause disease symptoms and protected plants from disease caused by the wild-type fungus. We observed that inoculation of freshly harvested avocado fruit with path-1 inhibited subsequent decay development by the normal pathogen *Colletotrichum gloeosporioides*. *C. gloeosporioides* isolate Cg-14 and the wild-type and mutant strains of *C. magna* germinated and formed appressoria on fruit peel, but Cg-14 and the wild type formed two- to threefold more appressoria on avocado peel than path-1. Cell wall elicitors from all three *Colletotrichum* isolates induced higher levels of phenylalanine ammonia lyase activity and mRNA in avocado cell suspension cultures than untreated controls. Path-1 and path-1 co-inoculated with Cg-14 also induced higher levels of phenylalanine ammonia lyase in freshly harvested fruits than Cg-14-inoculated and untreated controls. The isolates differed, however, in the level and extent of induction of epicatechin and an antifungal diene in the peel of ripening fruits. Path-1 induced higher levels of epicatechin that lasted in ripening fruits, whereas Cg-14 induced epicatechin levels for 1 day only. It is therefore suggested that the enhanced resistance of avocado fruits to *C. gloeosporioides* by path-1 results from the induction of epicatechin, a phenol that inhibits oxidation of the antifungal diene.

Additional keywords: biological control, host defense mechanisms, inducible preformed antifungal compounds.

Freeman and Rodriguez (1993) recently isolated a mutant strain of *Colletotrichum magna* S.F. Jenkins & Winstead that endophytically colonizes watermelon and other cucurbits but does not cause detectable symptoms of disease. The mutant strain also protects watermelon plants against infection by the wild-type strain of *C. magna* and reduces seedling mortality caused by *Fusarium oxysporum* f. sp. *niveum*. The basis for this biological disease control has not been elucidated, but it may involve the activation of normal host defenses (Freeman and Rodriguez 1993). It was accordingly of interest to inoculate avocado fruits with the *C. magna* mutant strain and test effects on the postharvest decay caused by *C. gloeosporioides* (Penz.) Penz. & Sacc. in Penz. Prusky *et al.* (1983) described the presence of a preformed antifungal compound, 1-acetoxy-2-hydroxy-4-oxo-henicosa-12,15-diene, in the peel and flesh of unripe avocado fruits and demonstrated that its concentration decreases markedly during fruit ripening when decay symptoms occur. The diene compound was suggested to be the major factor accounting for resistance to decay in unripe fruit (Prusky *et al.* 1983; Prusky *et al.* 1991b). More recent results indicated that concentrations of the preformed diene could be enhanced by a series of biotic and abiotic factors, including inoculation with *C. gloeosporioides* (Prusky *et al.* 1991a). Wounding, gamma radiation, and exposure of freshly harvested fruits to 30% CO_2 also enhanced levels of the preformed antifungal diene (Prusky and Plumbley 1992). Despite an initial reduction in concentration of the diene in freshly harvested fruits, a subsequent rapid increase in the concentration occurred to regain the initial level (Karni *et al.* 1989). This suggested rapid turnover of the diene compound and led us to consider it as an inducible, preformed compound.

Levels of the preformed diene compound in CO_2-treated fruits appear to be regulated by epicatechin, a phenol that inhibits the activity of lipoxygenase, which, in turn, degrades the diene compound (Prusky *et al.* 1985). Thus, increased concentrations of epicatechin result in decreased degradation of the antifungal diene and prevention of decay. It was accordingly of interest to search for other agents that might increase epicatechin levels in avocado fruits. We report here that the mutant strain of *C. magna* infected avocado fruit peel to produce insignificant symptoms but enhanced epicatechin and diene levels and delayed decay development caused by *C. gloeosporioides*.

RESULTS

Interaction *in vitro* between Cg-14 and path-1 conidia of *Colletotrichum*.

Conidia of Cg-14 and path-1 showed 96 ± 2 and 80 ± 6% germination, respectively, on Millipore filters. When both conidial suspensions were mixed together, the percent germination of Cg-14 was similar to that of Cg-14 alone, 96 ± 1%.

Present address of D. Prusky: Department of Postharvest Science of Fresh Produce, Volcani Center Bet Dagan, 50250 Israel.

Present address of S. Freeman: Department of Plant Pathology, Volcani Center Bet Dagan, 50250 Israel.

Present address of R. J. Rodriguez: National Fisheries Research Center, Bldg 204 Naval Station, Seattle, WA 98115 U.S.A.

MPMI Vol. 7, No. 3, 1994, pp. 326-333
©1994 The American Phytopathological Society

When cultures of Cg-14 and path-1 were inoculated on the same plate, no sign of inhibition between the two strains was observed.

Decay development in avocado fruit inoculated with *Colletotrichum* isolates.

Initial symptoms of decay in avocado fruit inoculated with *C. gloeosporioides* isolate Cg-14 were observed 5 days after harvest, with lesions increasing in diameter to 17 mm at day 10 (Fig. 1). Fruits inoculated with the wild-type *C. magna* isolate showed minor symptoms of decay; lesions measured 2 mm at day 10. Fruits inoculated with path-1 showed even less symptom development; decay lesions measured 0.5 mm at day 10 (Fig. 1).

A significant delay in fruit decay development was observed regardless of whether path-1 was inoculated prior to Cg-14 inoculation or after (Fig. 2). At day 8, decay development reached 4, 0.75, and 0.5 mm in Cg-14, Cg-14 + path-1, and path-1 + Cg-14 inoculations, respectively (Fig 2). A similar experiment using the wild-type isolate of *C. magna* and Cg-14 was not performed, since the wild-type isolate produced visible decay symptoms that would interfere with the symptoms produced by Cg-14.

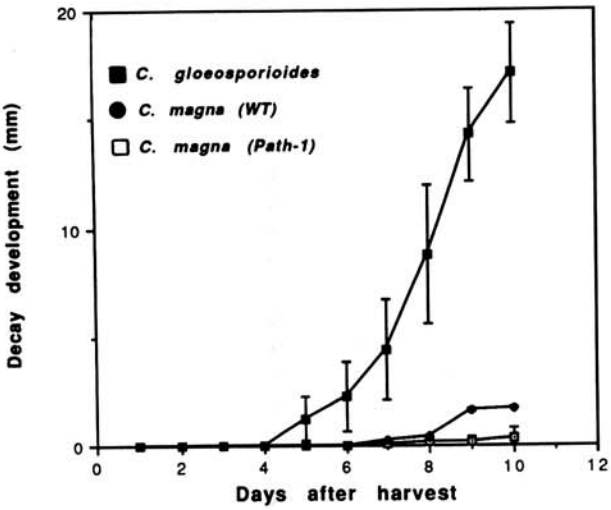

Fig. 1. Effect of *Colletotrichum gloeosporioides* (isolate Cg-14), and the wild-type (WT) and nonpathogenic mutant (path-1) isolates of *C. magna* on decay development on avocado fruit. Fruit were spot-inoculated with 1–2-µl suspensions of each isolate (2×10^6 conidia/ml) at harvesttime. Decay development was determined by measuring the diameter (in millimeters) of lesion formation. Vertical lines on the graph denote standard errors from 60 replicates.

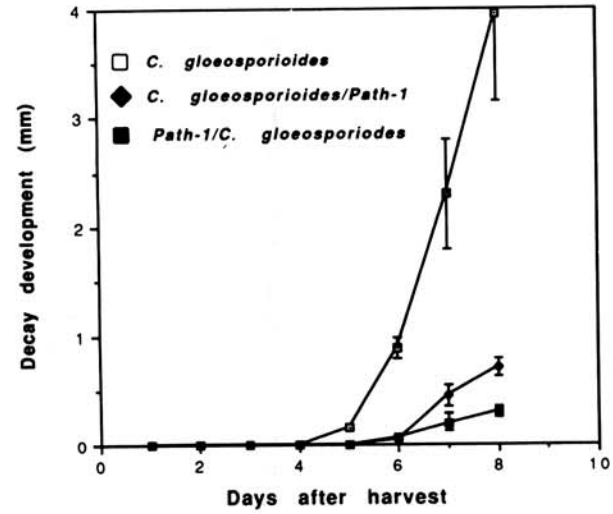

Fig. 2. Effect of the nonpathogenic mutant (path-1) of *Colletotrichum magna* on decay development caused by *C. gloeosporioides*, isolate Cg-14. Upon harvesting, fruit were spot-inoculated with Cg-14 (1–2-µl of 2×10^6 conidia/ml) alone or with Cg-14 followed 24 hr later by whole fruit immersion in conidial suspensions (2–3 × 10^6 conidia/ml) of path-1 (*C. gloeosporioides*/path-1). Fruit were also inoculated upon harvest by being dipped in conidial suspensions of path-1 and challenged 24 hr later by spot inoculation with Cg-14 (*C. gloeosporioides*/path-1). Decay development was determined by measuring the diameter (in millimeters) of lesion formation. Vertical lines on the graph denote standard errors from 60 replicates.

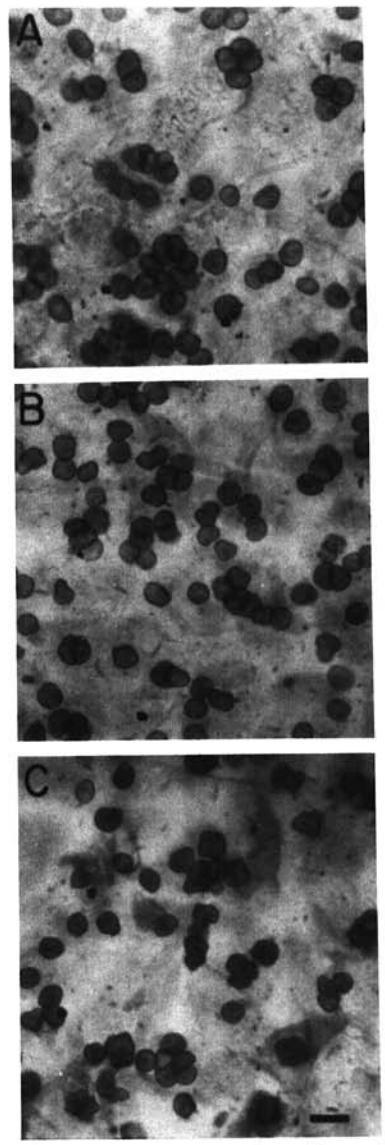

Fig. 3. Appressorial formation on avocado cv. Fuerte fruit peel by **A,** *Colletotrichum gloeosporioides* isolate Cg-14, and **B,** the wild-type and **C,** nonpathogenic mutant of *C. magna*. Note the infection peg observed from the upper part of the appressorium in all the different isolates. Magnification bar = 20 µm.

Appressorium formation by *Colletotrichum* isolates on avocado peel.

Isolate Cg-14 of *C. gloeosporioides* and the wild-type and path-1 isolates of *C. magna* all formed appressoria on the peel of avocado fruit (Fig. 3). Isolates Cg-14 and wild-type *C. magna* formed two- to threefold more appressoria on fruit than path-1 (approximately 120 vs. 40–50 per field). When conidia were germinated on isolated avocado wax, the percentage that formed appressoria was 93, 83, and 65% for Cg-14, wild-type, and path-1, respectively.

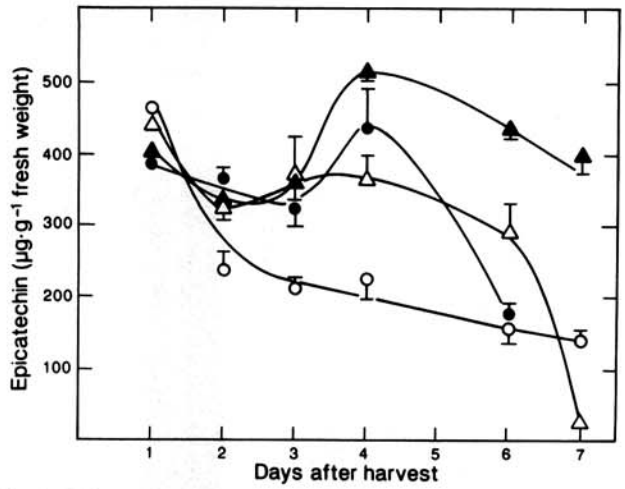

Fig. 4. Effect of *Colletotrichum* inoculation on the concentration of epicatechin in avocado cv. Fuerte peel. Fruit were dip-inoculated upon harvesting in conidial suspensions (2×10^6 conidia/ml) of *C. gloeosporioides* isolate Cg-14 (●—●), or wild-type (WT) (Δ—Δ) or nonpathogenic mutant (path-1) (▲—▲) of *C. magna,* or dipped in water (O—O). The peel was removed, and epicatechin concentration (µg/g fresh weight) was determined for inoculated and noninoculated fruit. Vertical lines on the graph denote standard errors of three replications.

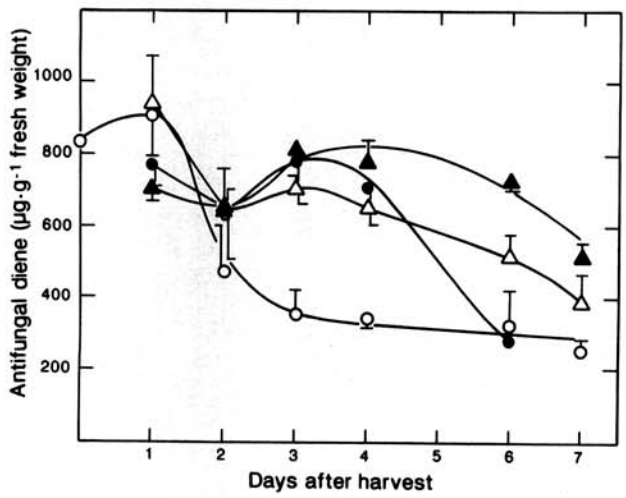

Fig. 5. Effect of *Colletotrichum* inoculation on concentration of the antifungal diene in peel of avocado cv. Fuerte from the University of California, Riverside. Fruit were dip-inoculated upon harvesting in conidial suspensions (2×10^6 conidia/ml) of *Colletotrichum gloeosporioides* Cg-14 (●—●), or wild-type (WT) (Δ—Δ) or nonpathogenic mutant (path-1) (▲—▲) of *C. magna,* or dipped in water (O—O). The peel was removed, and diene concentration (µg/g fresh weight) was determined for inoculated and noninoculated fruit. Vertical lines on the graph denote standard errors of three replications.

Effect of *Colletotrichum* isolates on epicatechin and diene levels in avocado peel.

The epicatechin concentration in inoculated fruit peel 1 day after harvest was 470 µg/g fresh weight, and the level decreased during fruit ripening to 150 µg/g fresh weight. In all *Colletotrichum*-challenged fruits, however, an increase in the epicatechin level was observed, starting 3 days after inoculation (Fig. 4). At the sixth day after harvest, the epicatechin

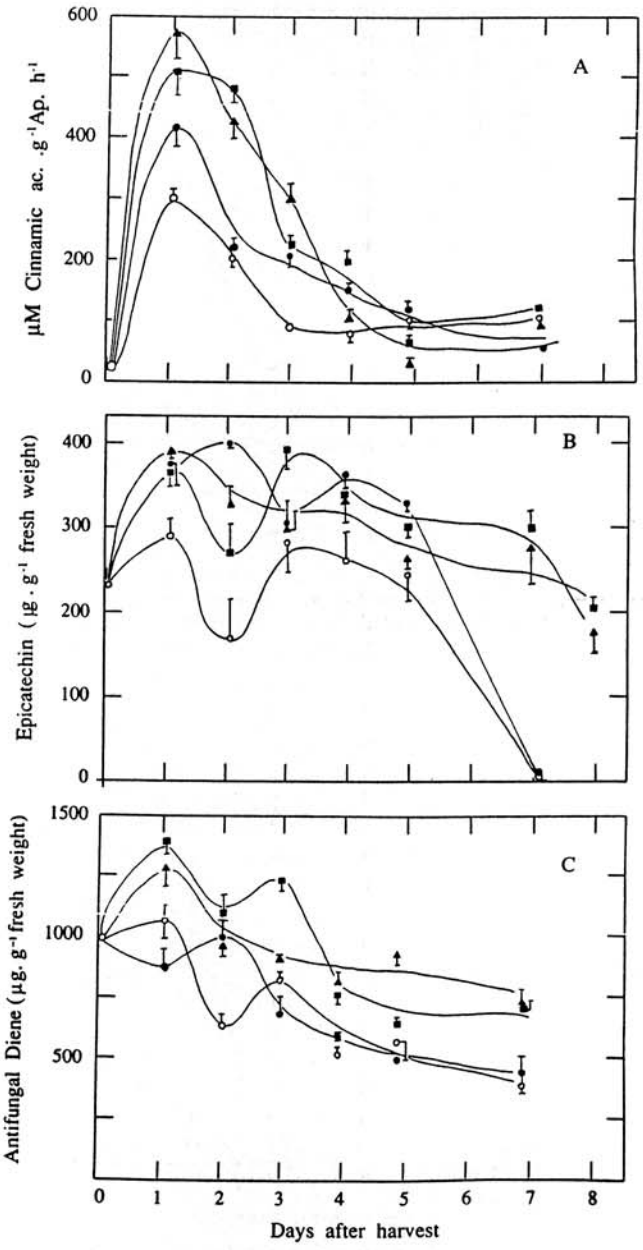

Fig. 6. Effect of co-inoculation of path-1 and Cg-14 on **A,** phenylalanine ammonia-lyase activity, **B,** concentration of epicatechin, and **C,** the antifungal diene in peel of avocado cv. Fuerte from Ayanot, Israel. Fruit were dip-inoculated upon harvesting in conidial suspensions (2×10^6 conidia/ml) of *Colletotrichum gloeosporioides* isolate Cg-14 (●—●), Cg-14 co-inoculated with the nonpathogenic mutant (path-1) (■—■) of *C. magna*, path-1 (▲—▲), or dipped in water (O—O). The peel was removed, and diene concentration (µg/g fresh weight) was determined for inoculated and noninoculated fruit. Vertical lines on the graph denote standard errors of three replications.

level of path-1-inoculated fruit was 1.5 times higher than that of wild-type *C. magna*-inoculated fruit and threefold higher than that of Cg-14-inoculated or noninoculated control fruit peels. The decrease in epicatechin levels of path-1-inoculated fruit at later times was also more gradual than in Cg-14 inoculated fruit (Fig. 4).

The diene level in path-1 inoculated fruit was induced to a higher concentration compared with the other inoculation treatments or the noninoculated control (Fig 5). At day 6, the diene level in peel inoculated with path-1 was 1.5-fold that of the *C. magna* wild-type inoculation and 2.5-fold that of Cg-14-inoculated or noninoculated control peels (Fig. 5). The decrease in diene level of path-1 inoculated peel was also more gradual than that of the Cg-14 treatment.

Effect of co-inoculation of Cg-14 and path-1 on phenylalanine ammonia-lyase, epicatechin, and diene levels in avocado peel.

Phenylalanine ammonia-lyase (PAL) activity was measured in avocado peel inoculated with Cg-14, path-1, and a combination of both isolates. Elevated levels of PAL activity were detected (as cinnamic acid, see Materials and Methods) during the first 1 to 3 days after inoculation (Fig. 6A). One day after inoculation, fruits inoculated with path-1 and path-1 + Cg-14 exhibited PAL activities 60–80% higher than untreated controls, while an increase of only 34% activity was observed in Cg-14-inoculated fruits.

The epicatechin concentration in noninoculated fruits 1 day after harvest was 260 µg/g fresh weight. The level decreased for 1 day, which was followed by a similar increase to initial concentration. This rapid decrease is a postharvest response of early-harvested fruits (Prusky *et al.* 1990). The level of epicatechin decreased during ripening to 15 µg/ml fresh weight. In all the *Colletotrichum*-challenged fruits, an increase in the epicatechin level was observed, starting 1 day after inoculation (Fig. 6B). At the seventh day, the epicatechin level of path-1 and path-1 + Cg-14 inoculated fruits was almost 7 times higher than that of Cg-14 and noninoculated fruits. The decrease of epicatechin in path-1 and co-inoculated fruits was slower than that of Cg-14 and noninoculated fruits.

The diene level in fruits inoculated with path-1 and path-1 + Cg-14 was higher 1 day after inoculation than in Cg-14-inoculated or the noninoculated control (Fig. 6C). On day 2, the diene level of Cg-14-inoculated fruits increased significantly compared with that of the control, but from day 3 and during fruit ripening, a similar decrease was observed in the diene levels of Cg-14-inoculated and noninoculated peels. The decrease in diene level of peel inoculated with path-1 and path-1 + Cg-14 was also more gradual than that of the Cg-14 treatment alone.

Effect of *Colletotrichum* elicitors on expression and activity of PAL in avocado cell cultures.

PAL expression and activity were measured in avocado cell cultures that were treated with elicitors originating from the cell walls of isolates Cg-14 of *C. gloeosporioides* or the wild-type and path-1 isolates of *C. magna*. Total RNA was extracted from elicitor-treated and untreated avocado cell cultures and probed with a cloned PAL gene from avocado to determine mRNA expression. Northern blot analysis showed enhanced levels of PAL mRNA in all *Colletotrichum* elicitor-treated cell cultures compared with basal levels of expression in the untreated control (Fig. 7A). Elevated levels of PAL activity were also detected in all cultures treated with the three *Colletotrichum* wall elicitors (Fig. 7B). The elicitor-treated cultures exhibited PAL activities 30–60% higher than the untreated control, consistent with the PAL mRNA results.

DISCUSSION

Freeman and Rodriguez (1993) showed that path-1, a nonpathogenic mutant of *C. magna,* grows through cucurbits as

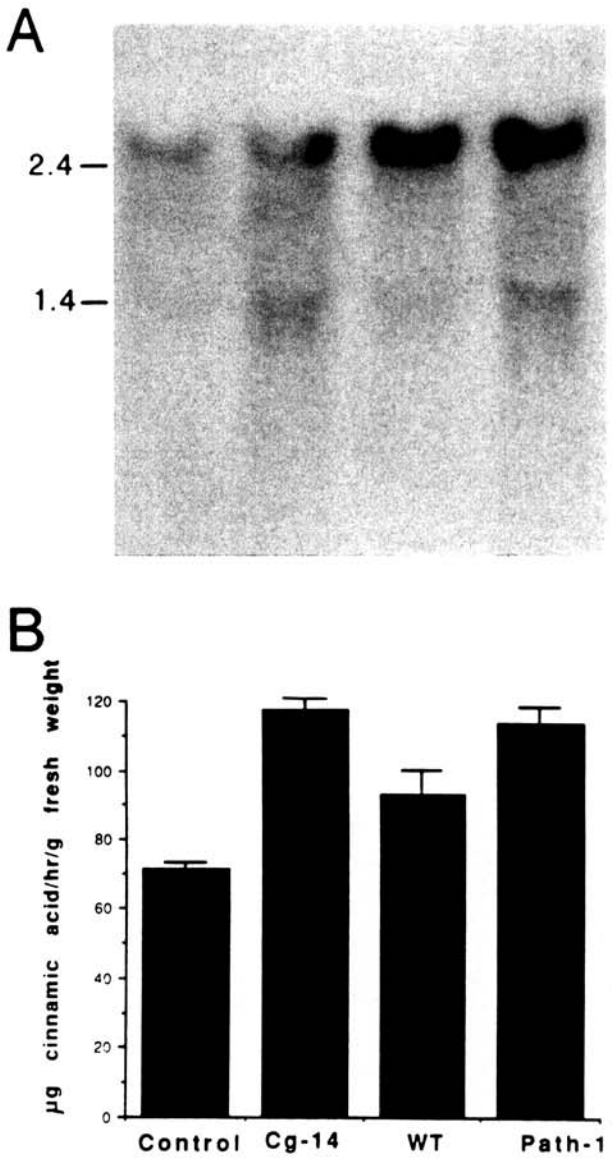

Fig. 7. Effect of *Colletotrichum* cell wall elicitors on **A,** mRNA levels and **B,** activity of phenylalanine ammonia-lyase (PAL) in avocado cell suspension cultures. Suspension cultures were treated with elicitors from *C. gloeosporioides* isolate Cg-14, or wild-type (WT) or nonpathogenic mutant (path-1) of *C. magna* and compared with untreated controls. PAL mRNA was assessed by Northern blot analysis using a PAL avocado probe hybridized to total RNA of elicitor-treated or untreated cell cultures. PAL activity was determined spectrophotometrically based on the production of cinnamic acid (measured in in µg/hr per gram fresh weight). Vertical lines on the graph denote standard errors of three replications.

an endophyte and retains the wild-type host range but protects plants from disease caused by a wild-type isolate of *C. magna*. Inoculation of freshly harvested avocado fruits with path-1 also enhanced the antifungal diene to higher concentrations and for longer periods of time relative to inoculation with the wild-type isolate of *C. magna* or isolate Cg-14 of *C. gloeosporioides*. In contrast to isolate Cg-14, which produced extensive decay development, wild-type *C. magna* produced visible symptoms and path-1 did not cause significant symptoms of decay on avocado fruits.

When the initial steps of infection of the three fungal isolates were compared, all germinated and produced appressoria on the peel of intact fruits and on wax removed from avocados. Appressoria with infection pegs were observed on the cuticle of fruit inoculated with the three isolates. This is a key point for induction, since without this initial infection process of peel penetration no induction should occur. The induction of increased levels of the diene appears to occur following the initial infection. Concentration of the antifungal diene in avocado peel is thought to be modulated by lipoxygenase, which is, in turn, regulated by the natural inhibitor, epicatechin (Prusky *et al.* 1985). The involvement of epicatechin in the regulation of lipoxygenase activity was demonstrated broadly in the past under several elicitor conditions and consequently on avocado fruit resistance to postharvest decay (Karni *et al.* 1989; Prusky *et al.* 1985, 1988, 1991a). Path-1 caused a 2.5-fold increase in the concentration of epicatechin in avocado peel, and this higher level was maintained even in ripe fruits. Consistent with earlier work, concentrations of epicatechin 6–7 days after inoculation with path-1 showed almost two to three times the concentration of epicatechin that is needed for inhibition of lipoxygenase (Karni *et al.* 1989; Prusky *et al.* 1985). Epicatechin appears to be a major factor associated with decreased degradation of the antifungal diene and delayed decay development. The increase in epicatechin concentration was shown to be nonspecific and caused by several factors: fungal infection, wounding, gamma radiation, and 30% CO_2 treatments. The fact that the three different strains breach the fruit cuticle via the infection peg (Fig. 3) is a key factor for initiation of the induction process. Postharvest pathogens such as *Diplodia natalensis* and *Penicillium digitatum* that cannot penetrate the fruit peel did not enhance higher levels of the antifungal diene (Prusky *et al.* 1990). The differential degree of induction by the *Colletotrichum* strains should be related at least to two factors: intrinsic factors of the isolates' cell wall components and/or other active inducing factor(s) produced by the different strains.

The induction of PAL, a key enzyme of the phenylpropanoid pathway, during the increase in concentration of epicatechin by CO_2 was described recently (Prusky *et al.* 1991a). No possibility exists, however, to test the crude extracts of elicitors on whole fruits. For that purpose, a cell suspension culture was used to show that nonspecific elicitor activity that was extracted from cell walls of three *Colletotrichum* isolates induced higher levels of PAL. It is not clear, however, whether the cell wall elicitor that enhanced PAL activity for 1–2 days after inoculation accounts for increased epicatechin levels in path-1-inoculated and co-inoculated avocado fruits. Autoclaved extracts of cell walls induced significantly higher levels of PAL activity and mRNA transcripts. However, the high level of epicatechin that is still present in path-1-inoculated ripe fruit suggests that path-1 may continuously stimulate host defenses during penetration of unripe and ripening fruit, whereas the Cg-14 isolate and wild-type isolate of *C. magna* stimulated the host reaction only for a limited time and only in unripe fruit. It has similarly been suggested that path-1 "primes" the defense mechanism in watermelon, thus avoiding a delay in the defense response following challenge inoculation by wild-type *C. magna* (Freeman and Rodriguez 1993).

The search for biological control strategies for diseases of fruits and vegetables following harvest has intensified in recent years, particularly with the withdrawal of several pesticides (Wilson and Chalutz 1989). Most biocontrol strategies are directed towards wound pathogens and involve the use of antagonistic bacterial strains, some of them producing antibiotics or yeasts that compete successfully for nutrients, overgrow the pathogen, and produce lytic enzymes that affect germinating fungal hyphae (Wilson and Chalutz 1989; Droby *et al.* 1989; Chalutz and Wilson 1990; Wisniewski *et al.* 1991). The search for biocontrol agents against pathogens causing quiescent infections has been more difficult, since the infecting hyphae are protected from microorganisms once the pathogen has penetrated the plant cuticle. Thus, few reports describe the biological control of quiescent infections in general and in avocado fruits in particular (Korsten and Kotze 1992; Jeffries and Koomen 1992; Koomen and Jeffries 1993). However, treatment of pepper fruit with a polysaccharide from the walls of *Glomerella cingulata* protected against another postharvest pathogen, *Botrytis cinerea,* raising the possibility that vegetables and fruits might be protected (Adikaram *et al.* 1988). In the work described here, anthracnose symptoms were inhibited by challenge with path-1 applied either 24 hr before or 24 hr after *C. gloeosporioides* inoculation. Furthermore, the isolates were not affected when both were germinated or cultured together *in vitro,* indicating that no interaction occurs between them. Instead, the nonpathogenic mutant appeared to inhibit decay development by activating the defense mechanism in avocado fruits. The utilization of such mutants as biocontrol agents therefore represents a promising avenue for the control of postharvest diseases and may have considerable impact on future agricultural practices.

MATERIALS AND METHODS

Fruit and fungal inoculation procedures.

Avocado fruits (*Persea americana* Miller var. *drymifolia* (Schldl. and Cham.) S. F. Blake 'Fuerte') were obtained from an orchard at the University of California, Riverside, and from an orchard at Ayanot, Nes Tziona, Israel. Cell suspension cultures of avocado were maintained on Murashige and Skoog basal media containing 0.5 μg/ml picloram (D. Prusky, H. Hamdan, and N. T. Keen, unpublished). Cell suspensions obtained 3–4 days after culture transfer were used for the experiments. A single spore isolate of *C. gloeosporioides* isolate Cg-14, obtained from decayed avocado fruits, and two isolates of *C. magna* (teleomorph: *Glomerella magna*)—a wild type (obtained from S. Brown and O. C. Yoder, Cornell University, Ithaca, NY) and a mutant (path-1) derived by ultraviolet treatment (Freeman and Rodriguez 1992)—were

used to inoculate fruits in all experiments. Spot inoculation was carried out by placing 1–2-µl water suspensions of conidia (2×10^6/ml) on the fruit surface. Total fruit inoculation was carried out by dipping the fruit for 4–5 sec in the water suspension of conidia. When a co-inoculation experiment was carried out, fruit was dipped first in path-1, dried for 30 min under a sterile hood, and immersed in Cg-14. Fruit were then maintained under high humidity at 25° C for 24 hr (Prusky *et al.* 1991b). Decay developed as fruit became ripe and soft. Darkening of the peel (lesion development) in a zone 5-mm diameter or greater was considered symptomatic of disease. The effect of preinoculation of path-1 on decay development caused by isolate Cg-14 of *C. gloeosporioides* was determined after dipping fruit in 2×10^6 conidia/ml, maintaining the fruit under high humidity at 25° C for 20 hr, and spot inoculating with isolate Cg-14. In some cases, path-1 was inoculated after spot inoculation with isolate Cg-14. Spot inoculation was done at six points, three on each side along the longitudinal axis of the fruit. Ten fruit were inoculated per treatment (for a total of 60 inoculation spots per treatment), and the experiments were repeated four times.

To compare germination of the different strains, conidia from a 7- to 10-day culture were collected in 2 ml of water. The conidia were filtered through Miracloth, washed once by centrifugation, and brought to the required concentration. Two hundred conidia of either Cg-14 or path-1 were spotted onto a 13-mm-diameter Millipore filter disks (0.45-µm pore size). A mixture of conidia of Cg-14 and path-1 was spotted under similar conditions. The disks were incubated over a glass slide in a moist chamber at 25° C overnight, and their development was terminated by staining with cotton blue in lactophenol.

Numbers of appressoria formed on avocado peels were determined by counting in five separate microscopic fields from at least five different fruits. One thousand conidia were spotted at infection sites, and 24 hr after inoculation slices of approximately 0.5 mm or thinner were taken with a razor blade from the inoculation spots and stained with cotton blue in lactophenol. Appressoria were easily observed microscopically but not all conidia could be clearly seen.

For appressoria quantitation on avocado wax, fruits were dewaxed by dipping them for 1–2 min in hexane (Prusky *et al.* 1991b). This extract was concentrated and dried, weighed, and redissolved in hexane before use in the bioassay. The number of appressoria formed from germinated conidia was determined on Millipore disks covered with 5 mg of avocado peel extract. The wax extracts were spotted onto a 13-mm-diameter Millipore filter (0.45-µm pore size), and similar amounts of the solvent only were placed on a control filter. After drying the hexane, 10 µl of a suspension of *Colletotrichum* conidia (10^6 conidia/ml) was placed on the disks. The filters were incubated in a moist chamber at 25° C overnight, and their development was terminated by staining with cotton blue in lactophenol.

Cell walls of *Colletotrichum* isolates and elicitor preparation.

Conidia obtained from single-spore cultures were used to inoculate 500-ml Erlenmeyer flasks containing 200 ml of potato dextrose broth. The flasks were incubated at 27° C with continuous agitation at 125 rpm. After 7 days' growth, the mycelium was harvested by filtration on Whatman no. 1 paper and washed with 50 ml of 10 mM Tris-HCl buffer, pH 6.5. For cell disruption, 6 g (fresh weight) of freshly grown or frozen mycelia was resuspended in 30 ml of water and mixed with 37 g of dry glass beads (0.45–0.5 mm) and agitated at maximum speed in a Braun MSK homogenizer for three periods of 45 sec each with cooling by flowing CO_2 (Bartnicki-Garcia *et al.* 1978). The broken cell walls were sedimented in centrifuge tubes by centrifugation at 1,000 *g* for 5 min and washed six times with 10 ml of distilled water. The residual walls were lyophilized, resuspended in distilled water (20 mg/ml), and autoclaved for 30 min. The autoclaved suspension was centrifuged at 5,000 *g* for 10 min at 4° C, and the supernatant was used as a cell wall elicitor.

Two hundred microliters of the cell wall elicitor preparation was added to 20 ml of cell suspension 3–4 days after transferring the culture. The cells were harvested at various times by filtration on Whatman no. 1 filter paper, washed with 10 ml of water, and dried for 3 min under vacuum.

Extraction and quantitative analyses of the antifungal diene and epicatechin.

Avocado fruits were immersed in conidial suspensions of Cg-14, wild-type, or path-1 isolates after harvest. Epicatechin and diene levels were determined in inoculated and non-inoculated peels every 24 hr over a 7-day period. Avocado peel (10 g fresh weight) was homogenized in 95% ethanol in an Omni-mixer (Sorvall) at full speed for 3–4 min. No changes in fruit weight were ever recorded as a result of fruit inoculation. The extract was halved, with one portion used for the extraction of epicatechin and the other for diene. The diene was purified from the extract according to the modified method described by Prusky *et al.* (1991) and analyzed by a rabbit high-performance liquid chromatography (HPLC) procedure, with 50-µl samples.

Epicatechin was purified from the second half of the peel extract by the method described by Prusky *et al.* (1991) with modifications. Efficiency of the extractions is almost 100% and was demonstrated in former publications (Prusky *et al.* 1985, 1988). The organic fractions were dissolved in 8 ml of 25% ethyl acetate in methylene chloride, and a 4-ml sample was further purified by flash chromatography. A Becton Dickinson syringe (5 ml) was filled to maximum capacity with approximately 1.5 g of 60–200 mesh silica gel and equilibrated with 10 ml of methylene chloride. Inactive material was eluted by washing with 8 ml of methylene chloride and 8 ml of 30% ethyl acetate in methylene chloride. The active fraction was then eluted by washing the column twice with 20 ml of ethyl acetate and dried under a flow of nitrogen. Epicatechin was determined by HPLC analysis of 20-µl samples taken from the concentrated epicatechin fraction dissolved in 1 ml of 100% EtOH. Calculations of the diene and epicatechin concentrations were based on comparison of the HPLC peak areas with those of standards. Average values of three different extractions are presented.

Extraction of enzymes and PAL activity.

Avocado cell suspensions (0.5–0.9 g fresh weight) were ground in a mortar with 2 ml of 10 mM Tris-HCl buffer, pH 7, and 2% Triton X-100. The homogenate was centrifuged at 16,000 × *g* (10 min at 4° C) in a microcentrifuge and used as

the enzyme source. The reaction mixture consisted of 6 μmol of L-phenylalanine, 500 μmol of Tris-HCl, pH 8.0, and 100 μl of enzyme extract in a final volume of 1 ml. The reaction was incubated at 37° C for 60 min. PAL was assayed with a Beckman DU-65 spectrophotometer by measuring the amount of *trans*-cinnamic acid formed at 290 nm according to the method of Beaudoin-Eagan and Thorpe (1985).

Extraction of the enzyme from avocado peel was done essentially as described by Lisker *et al.* (1983). Avocado peel (20 g) was ground in a Waring blender for 1 min with 200 ml of acetone at −20° C. The blended peel was filtered through Whatman no. 1 filter paper, and the powder was blended again with acetone. This step was repeated three times. The acetone powder was then air-dried at room temperature overnight, collected, and stored at −20° C until used. For the PAL enzyme assay, 500 mg of the dry acetone was added to 20 ml of cold 0.1 M borate buffer, pH 8.8, and stirred for 2 hr at 4° C. The suspension was centrifuged at 10,000 × *g* at 4° C for 20 min. The supernatant was used as the enzyme source. The reaction mixture contained 2 ml of enzyme preparation, 2.5 ml of 0.1 M borate buffer, pH 8.8., and 1.0 ml of 0.05 M L-phenylalanine. Tubes containing the reaction mixture were incubated for 1 hr at 40° C. The reaction was terminated by the addition of 0.1 ml of 5 N HCl. Then, 7 ml of diethyl ether (spectroscopic grade, Merck) was added to the reaction mixture and thoroughly mixed in a Vortex apparatus. Since a thick gel was formed in the upper ether fraction, the mixture was centrifuged to separate phases at 5,000 × *g* for 3 min, and 2.0 ml of the clear ether supernatant was used for direct measurements of the cinnamic acid formed at 269 nm.

RNA extraction from cell cultures and determination of PAL expression.

Avocado cells were harvested, weighed, rapidly frozen in liquid nitrogen and freeze-dried for 2 days. RNA was extracted by a mini-prep procedure modified from Callahan *et al.* (1989). The extraction was done in 1.5-ml microcentrifuge tubes. Freeze-dried cells were ground to a fine powder with liquid nitrogen in a mortar and pestle, and approximately 100 mg was suspended immediately in 1 ml of freshly prepared extraction buffer: 100 mM Tris-HCl, pH 8.0; 100 mM NaCl; 1% sodium dodecyl sulfate (SDS); 1% polyvinylpyrrolidone (PVP-40); 1% β-mercaptoethanol, and 100 μg/ml of proteinase K. The suspension was mixed by vortexing and centrifuged for 5 min at 17,000 × *g* at room temperature. The aqueous phase was sequentially extracted with an equal volume of phenol, phenol/chloroform (1:1), and chloroform. A tenth volume of 3 M NaOAc, pH 5.2, was added to the final aqueous phase; the pH was checked to be in the 5–6 range, and NaCl and SDS were added to final concentrations of 0.5 M and 0.1%, respectively. The suspension was left on ice for 2 hr and then centrifuged at 17,000 × *g* for 15 min at 4° C to pellet polysaccharides. The resulting supernatant was saved, and lithium chloride was added to a final concentration of 2.0 M, incubated overnight at 4° C and then centrifuged at 17,000 × *g* at 4° C for 15 min. The resulting pellet containing the total RNA was air-dried, dissolved in 50–100 μl of diethyl pyrocarbonate-treated water, and quantified by measuring absorbance at 260 nm.

For Northern blot analyses, total RNA was electrophoresed in a 10 × 15 cm, 1.2% dimethylsulfoxide-glyoxal gel at 35 V for approximately 3 hr and blotted onto nylon membranes as described in Maniatis *et al.* (1982). Blots were prehybridized overnight in prehybridization mix with 40% formamide at 42° C followed by overnight hybridization at 42° C. A polymerase chain reaction (PCR)-amplified sequence of PAL obtained from avocado genomic DNA with primers designed from a consensus sequence of several PAL genes was used as a probe (H. Hamdan, personal communication). A single expected PCR product of 950 bp was subsequently cloned into the *Sma*I site of pUC18 and was confirmed by DNA sequencing to contain PAL sequences. The cloned DNA was double-digested with *Eco*RI and *Bam*HI, and a 750 bp fragment was radiolabeled by random-prime labeling and used for the hybridization experiments (Maniatis et al. 1982).

ACKNOWLEDGMENTS

We thank F. Guillemet (UCR) and Ilana Kobiler (Volcani Center, Israel) for valuable help. This research was partially supported by grants from BARD (Binational United States–Israel Agricultural Research and Development Fund), including a BARD postdoctoral fellowship (SI-0103-89) awarded to SF, and a joint NSF, DOE, USDA grant 92-37310-7821 and USDA grant 9200655 awarded to RJR.

LITERATURE CITED

Adikaram, N. K. B., Brown, A. E., and Swinburne, T. R. 1988. Phytoalexin induction as a factor in the protection of *Capsicum annum* L. fruits against infection by *Botrytis cinerea* Pers. J. Phytopathol. 122:267-273.

Bartnicki-Garcia, S., Bracker, C. E., Reyes, E, and Ruiz-Herrera, J. 1978. Isolation of chitosomes from taxonomically diverse fungi and synthesis of chitin microfibrils *in vitro*. Exp. Mycol. 2:173-192.

Beaudoin-Eagan, L. D., and Thorpe, T. A. 1985. Tyrosine and phenylalanine ammonia lyase activities during shoot initiation in tobacco callus cultures. Plant Physiol. 78:438-441.

Callahan, A., Morgens, P., and Walton, E. 1989. Isolation and in vitro translation of RNAs from developing peach fruit. HortScience 24:356-358.

Chalutz, E., and Wilson, C. L. 1990. Postharvest biocontrol of green and blue mold and sour rot of citrus fruit by *Debaryomyces hansenii*. Plant Dis. 74:134-137.

Droby, S., Chalutz, E., Wilson, C. L., and Wisniewski, M. 1989. Characterization of the biocontrol activity of *Debaryomyces hansenii* in the control of *Penicillium digitatum* on grapefruit. Can. J. Microbiol. 35:794-800

Freeman, S., and Rodriguez, R. J. 1992. A rapid, reliable bioassay for pathogenicity of *Colletotrichum magna* on cucurbits and its use in screening for nonpathogenic mutants. Plant Dis. 76:901-905.

Freeman, S., and Rodriguez, R. J. 1993. Genetic conversion of a fungal plant pathogen to a nonpathogenic, endophytic mutualist. Science 260:75-78.

Jeffries, P., and Koomen, I. 1992. Strategies and prospects for biological control of diseases caused by *Colletorichum*. Pages 337-357 in: *Colletotrichum*: Biology, Pathology and Control. J. A. Bailey and M. J. Jeger, eds. C.A.B. International, Wallingford, U.K.

Karni, L., Prusky, D., Kobiler, I., Bar-Shira, E., and Kobiler, I. 1989. Involvement of epicatechin in the regulation of lipoxygenase activity during activation of quiescent *Colletotrichum gloeosporioides* infections of ripening avocado fruits. Physiol. Mol. Plant Pathol. 35:367-374.

Koomen, I., and Jeffries, P. 1993. Effect of antagonistic microorganisms on the post-harvest development of *Colletotrichum gloeosporioides* on mango. Plant Pathol. 42:230-237.

Korsten, L., and Kotze, J. M. 1992. Postharvest biological control of avocado postharvest diseases. Proc. World Avocado Congr. 2:473-477.

Lisker, N., Cohen, L., Chalutz, E., and Fuchs, Y. 1983. Fungal infections suppress ethylene-induced phenylalanine ammonia-lyase activity in grapefruit. Physiol. Plant Pathol. 22:331-338.

Maniatis, T. A., Frisch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold

Spring Harbor, NY.
Prusky, D., Karni, L., Kobiler, I., and Plumbley, R. A. 1990. Induction of the antifungal diene in unripe avocado fruits: Effect of inoculation with *Colletotrichum gloeosporioides*. Physiol. Mol. Plant Pathol. 37:425-435.
Prusky, D., Keen, N. T., and Eaks, I. 1983. Further evidence for the involvement of preformed antifungal compound in the latency of *Colletotrichum gloeosporioides* on unripe avocado fruits. Physiol. Plant Pathol. 22:189-198.
Prusky, D., Kobiler, I., and Jacoby, B. 1988. Involvement of epicatechin in cultivar susceptibility of avocado fruits to *Colletotrichum gloeosporioides* after harvest. J. Phytopathol. 123:140-146.
Prusky, D., Kobiler, I., Jacoby, B. Sims, J. J., and Midland, S. L. 1985. Effect of inhibitors of lipoxygenase activity and its possible relation to latency of *Colletotrichum gloeosporioides* in unripe avocado fruits. Physiol Mol. Plant Pathol. 27:269-279.
Prusky, D., and Plumbley, R. A. 1992. Quiescent infections of *Colletotrichum* in tropical and subtropical fruits. Pages 289-307 in: *Colletotrichum*: Biology, Pathology and Control. J. A. Bailey and M. J. Jeger, eds. C. A. B. International, Wallingford, U.K .
Prusky, D., Plumbley, R. A., and Kobiler, I. 1991a. Modulation of natural resistance of avocado fruits to *Colletotrichum gloeosporioides* by CO_2 treatment. Physiol. Mol. Plant Pathol. 39:325-334.
Prusky, D., Plumbley, R. A., and Kobiler, I. 1991b. The relationship between antifungal diene levels and fungal inhibition during quiescent infection of unripe avocado fruits by *Colletotrichum gloeosporioides*. Plant Pathol. 40:45-52.
Wilson, C. L., and Chalutz, E. 1989. Postharvest biological control of *Penicillium* rots of citrus with antagonistic yeasts and bacteria. Sci. Hort. 40:105-112.
Wisniewski, M., Biles, C., Droby, S., McLaughlin, R., Wilson, C., and Chalutz, E. 1991. Mode of action of the postharvest biocontrol yeast *Pichia guilliermondii*. I. Characterization of attachment to *Botrytis cinerea*. Physiol. Mol. Plant Pathol. 39:245-258.