A Rapid and Sensitive Plate Assay for the Detection of Cutinase Produced by Plant Pathogenic Fungi

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ABSTRACT

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A selective procedure for the detection of cutinase production by plant pathogenic fungi has been developed. This procedure involves growing fungi on a modified Czapek-Dox mineral medium containing purified cutin as the sole source of carbon and a basic pH indicator dye. On this medium, the cutinase-producing isolates generate zones of color change in the basic indicator dye in advance of visible mycelial growth. Presumably this is caused by a lowering of the pH in the medium due to the release of a fatty acid monomer into the medium from cutin hydrolysis. This is a convenient, rapid, and sensitive assay for detecting cutinase production by fungi. Although it was originally designed to screen cutinase-deficient mutants of *Colletotrichum gloeosporioides*, the causal agent of papaya anthracnose, it also can be applied to test other fungi for cutinase production.

Additional key words: *Carica papaya*.

Cutinolytic enzymes secreted by phytopathogenic fungi play a decisive role in the infection of certain plant tissues by fungal pathogens (9). The cutinases enable pathogens to penetrate the outer layer of host tissue by degrading cutin which is the primary structural component of the cuticle.

We have focused our attention on the anthracnose disease of papaya (*Carica papaya* L.), a postharvest fruit rot caused by the deuteromycetaceous fungus, *Colletotrichum gloeosporioides* Penz. Evidence has shown that the infection of papaya fruit takes place throughout the growing season, but remains latent until the fruit is harvested and has reached the postclimacteric stage of development (3). Biochemical studies have shown that a cutin-degrading enzyme (cutinase) is secreted by the fungus when grown on cutin as the sole carbon source. This enzyme is believed to be necessary for penetration of the host's cuticle based on the observation that specific inhibition of this enzyme, either with antibodies made against the cutinase, or by chemical inhibitors, results in suppression of lesion formation (2,6); and when fruits are mechanically wounded prior to fungal inoculation, neither anticutinase nor the cutinase inhibitors suppress lesion formation.

Additional confirming evidence for the role of cutinase in the penetration of papaya fruit by the anthracnose fungus could be obtained by studying the effect of the loss of the cutinase function on the penetrating ability of *Colletotrichum*. However, a convenient rapid and direct assay for the selection of cutinase deficient mutants of the pathogen has not been available.

We describe here a plate assay for the selection of cutinase-minus (Cut^-) mutants of *C. gloeosporioides*. Although the assay was designed for the screening of Cut^- mutants, it is equally applicable for studying cutinase production by fungal pathogens in general.

MATERIALS AND METHODS

Fungi. *C. gloeosporioides, Mycosphaerella* sp., *Fusarium* sp., *Botryodiplodia theobromae, Penicillium* sp., *Phytophthora palmivora, Phomopsis* sp., and *Stemphylium lycopersici* were isolated from naturally infected papayas. Single-spore isolates were obtained and maintained on either 10% V-8 juice agar (VJA) or potato-dextrose agar (PDA). The organisms were routinely reinoculated on papaya fruits in the laboratory to maintain pathogenicity and to minimize the variation that occurs during repeated vegetative transfers.

Media. The cutinase-selective medium used for screening and testing of fungi consisted of a modified Czapek-Dox medium (14); 3.0 g $NaNO_3$, 1.0 g K_2HPO_4, 0.5 g KCl, 0.01 g $FeSO_4 \cdot 7H_2O$ and 17 g of Noble agar (Difco) in 1 L of distilled water. Papaya cutin, which served as the sole source of carbon, was purified as described previously (2), ground to a fine powder in a ball mill and added to the medium at a final concentration of 0.2%. Prior to sterilization (121 C, 20 min), the pH was adjusted with NaOH to the upper range value of the indicator dye to be tested (Table 1). Appropriate concentrations of the various dyes were added after separate autoclaving. Plates were poured containing approximately 10 ml of medium per plate.

Indicator dyes. Phenol red, cresol red, bromphenol blue, bromcresol purple, phenolphthalein, and thymol blue were purchased from Sigma Chemical Co., St. Louis, MO. The dyes were tested at various concentrations to optimize the color change resulting from a decrease in pH.

Mutagenesis. An agar plug from a culture of *C. gloeosporioides* was placed in a VJA plate and the plate incubated for 2 wk at 25 C. The plate was then flooded with sterile distilled water and the fungal conidia gently removed by scraping with a sterile loop. The suspension was filtered through two layers of sterile cheesecloth, centrifuged, and resuspended in sterile distilled water to a final concentration of approximately 10^6 spores per milliliter. The spores were then mutagenized by treating them with either ultraviolet light, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, ethylmethanesulfonate, or nitrous acid. A preliminary report on the results of mutagenic experiments has been published (5).

Enzyme assay. Eight fungal pathogens of papaya, including *Colletotrichum*, were grown on VJA plates and 3-mm-diameter agar plugs taken from the edge of advancing hyphae were transferred to the cutinase-selective medium containing phenol red as the indicator dye. At the same time, a spore suspension of each fungus was inoculated into a liquid mineral medium with papaya cutin as the sole carbon source (2). The culture filtrates were obtained following a 2-wk incubation period and tested for esterase activity in a spectrophotometric assay which utilizes a model

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substrate (p-nitrophenyl butyrate) instead of cutin for the detection of esterase activity in fungal culture filtrates (2).

RESULTS

When two of the pH indicator dyes, phenol red and cresol red, were incorporated into the medium, yellow zones were exhibited in advance of fungal growth from agar plugs of *C. gloeosporioides* transferred to the dye-containing medium (Fig. 1). This was presumably due to the release of fatty acids caused by cutin hydrolysis resulting in lowering of the pH. The dye concentration giving the sharpest contrast was 0.5% for both dyes. Zones could be easily observed 40 hr following transfer. Bromphenol blue, bromcresol purple, and phenolphthalein did not show any detectable color changes.

Each dye was tested with 0.2% glucose substituted for cutin in the medium and inoculated with the wild-type *C. gloeosporioides* to determine if these dyes had any deleterious effects on fungal growth. Growth of the pathogen in the dye-supplemented medium was the same as in the controls, and there was also no color change in the medium, thus excluding the possibility that color change in cutin-containing media was due to other acidic molecules produced by the fungus.

The general utility of this method was established by testing other pathogenic fungi of papaya including *Fusarium* sp., *Mycosphaerella* sp., *B. theobromae, P. palmivora, Phomopsis* sp., *Penicillium* sp., and *S. lycopersici*. There was a direct correlation between esterase activity in the culture filtrates and production of zones of color change in the selective medium (Table 2). *S. lycopersici*, a weak fungal pathogen (1), which does not easily infect papaya even when the fruit is artificially wounded, showed no esterase activity in the culture filtrates and neither exhibited growth nor caused a color change on the selective medium.

In another experiment, purified cutinase from *C. gloeosporioides* (25 μl) was placed in wells (3 mm) in the pH indicator dye medium in a series of dilutions (1–8 μg). Zones appeared within 5 hr and the diameter of the zones was directly correlated to concentrations of enzyme at 1–2 μg (Fig. 2). Thus, this assay can be used as a qualitative as well as a quantitative assay, and may be useful in monitoring cutinase activity during purification procedures.

TABLE 1. Growth of, and cutinase production by, *Colletotrichum gloeosporioides* on media containing different pH indicator dyes

Indicator	pH range	Color change	Rating[a]
Phenol red	8.4–6.8	Pink red to yellow	++
Cresol red	8.8–7.2	Violet red to yellow	++
Bromphenol blue	7.6–6.0	Blue to yellow	+
Bromcresol purple	6.8–5.2	Purple to yellow	+
Phenolphthalein	10.0–8.3	Red to colorless	+
Thymol blue	2.8–1.2	Orange to red	–

[a]Symbols: ++ = growth of *C. gloeosporioides* and detectable color change on selective medium, + = growth of *C. gloeosporioides* but no detectable color change on selective medium, – = no growth.

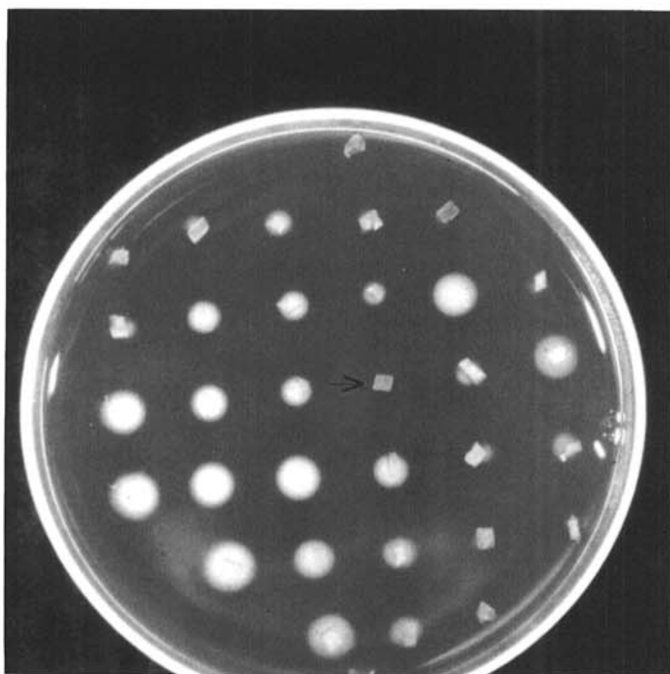

Fig. 1. Cutinase-selective medium using phenol red as the indicator dye. Plate was inoculated with single spores of mutagenized survivors. Zones indicate cutinase activity. The arrow points to a possible cutinase-negative phenotype. Photograph was taken 40 hr after transfers.

DISCUSSION

This is the first report of a convenient and rapid assay to detect cutinase production by fungi. The fact that *C. gloeosporioides* cutinase is a basic enzyme (2) with a pH optimum near 10 and

TABLE 2. Enzyme activity of fungal pathogens of papaya

Isolate	Esterase activity[a]	Cutinase activity[b]
Colletotrichum gloeosporioides	+	+
Botryodiplodia theobromae	+	+
Fusarium sp.	+	+
Mycosphaerella sp.	+	+
Penicillium sp.	–	–
Phomopsis sp.	+	+
Phytophthora palmivora	+	+
Stemphylium lycopersici	–	–

[a]As determined spectrophotometrically at 405 nm using culture filtrates and p-nitrophenyl butyrate as substrate. Symbols: + = activity, and – = no activity.
[b]As determined by growth and color change on cutinase-selective medium.

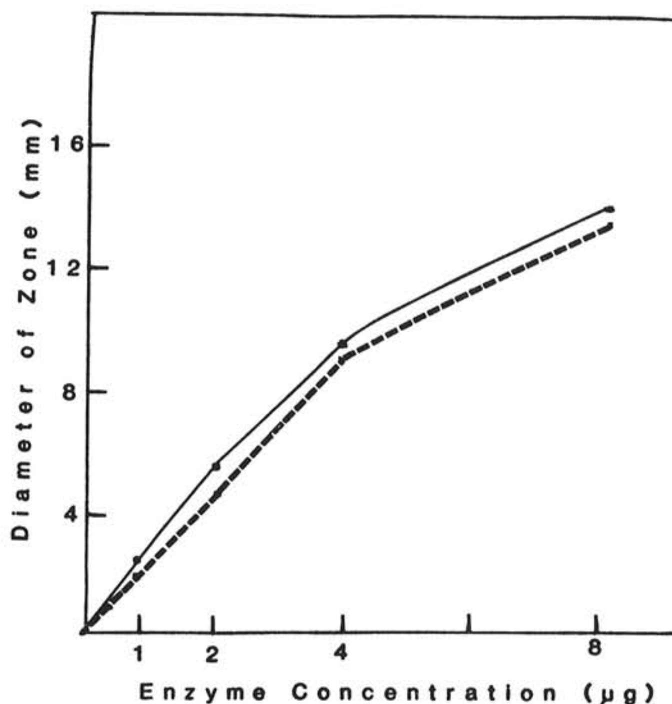

Fig. 2. Diameter of zone of color change with respect to enzyme concentration of enzyme. Purified *Colletotrichum gloeosporioides* cutinase (1 mg/ml) was dissolved in modified Czapek-Dox buffer, pH 8.5, and 20-μl aliquots were placed in the wells at the various concentrations of enzyme. Results were recorded 5 hr following addition of cutinase. The solid line represents data from trial 1 and the dashed line represents data from trial 2.

negligible activity below pH 7.5 led us to the idea of testing basic pH indicator dyes for a selective medium. The major monomer of papaya cutin, 9,16-dihydroxypalmitic acid, is released during cutin hydrolysis (15) and presumably lowers the pH of the medium causing a color change in the basic dye. The development of such an assay is essential for mutagenesis studies, since thousands of fungal spores needed to be screened in order to obtain a mutant of interest. This procedure has many advantages over existing methods of assaying for cutinase. Cutin is chemically inert and hydrolysis in liquid media has usually been detected with long incubation periods (8–10 days) when model substrates are used to measure enzyme activity (11). Tritiated cutin has also been used as a substrate; however, radioactive labeling is cumbersome and time consuming. With our assay reported here, results can be observed within 36–48 hr, and it is rapid enough for mass screening of Cut^- mutants. This assay can easily accommodate 40 individual isolates per plate.

Other screening methods were tried, particularly ones that proved useful for other fungal degradative enzymes. These included clearing assays for cellulase, and pectinase plate cup, or enzyme assays (7,10,12,13,16); all proved to be inadequate, either due to the insolubility of cutin or the long (3 wk) incubation time required for detection. Assays based on using antibodies made against cutinase in an attempt to detect enzyme production in solid media were also unsuccessful.

Although this method has been developed primarily to screen Cut^- mutants, it can also be used for examining cutinase production by other fungi. Data in Table 2 show that, except for *Penicillium* sp. and *S. lycopersici*, all other pathogens tested in the plate assay were cutinase positive. They also elaborated cutinase when grown in liquid culture with cutin as the sole carbon source. However, only *C. gloeosporioides* can infect intact papaya fruit. The other pathogens, which are considered to be wound pathogens (8), do not. Recently, from one of these fungi (*Mycosphaerella* sp.) (4), we purified a cutinolytic enzyme which is biochemically similar to the cutinase from *Colletotrichum*. The inability of these pathogens to infect unwounded papaya tissue suggests that the papaya fruit surface may regulate cutinase production. However, we cannot rule out the possibility that papaya fruit surfaces contain inhibitors that prevent spore germination of these pathogens on inoculated papaya surfaces.

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