Evidence for Cutinase Production by *Aspergillus flavus* and Its Possible Role in Infection of Corn Kernels

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ABSTRACT

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Aspergillus flavus can infect nondamaged corn kernels and produce aflatoxins before harvest. Experiments were conducted to determine if *A. flavus* produces cutinase and, if so, to investigate its possible role in kernel infection. Plate assays in vitro showed that *A. flavus* can grow on purified cutin as the sole carbon source and that it secretes extracellular cutinase. *A. flavus* grew better in liquid culture at pH 8 than at pH 6. Most cutinase substrate (*p*-nitrophenyl butyrate [PNB]) hydrolysis activity was in the 30% ammonium sulfate fraction. Two proteins with different PNB hydrolysis activity, designated C_1 and C_2, were isolated from *A. flavus* culture filtrates using native polyacrylamide gel electrophoresis. C_2 had greater PNB hydrolysis activity than C_1. The molecular weights for C_1 and C_2 were 36 kDa and 22 to 23 kDa, respectively. Kernels pretreated with bacterial cutinase or the 30% ammonium sulfate fraction from *A. flavus* culture filtrate supported increased levels of aflatoxin production similar to those in wounded kernels. The cutinase activity was strongly inhibited by diisopropyl fluorophosphate (DFP), a specific inhibitor of fungal cutinase, which indicated that *A. flavus* cutinase may be a serine esterase. Adding DFP to the spore suspension reduced aflatoxin production in kernels of Pioneer 3154 (susceptible) but not kernels of GT-MAS:gk (resistant). These data demonstrate that *A. flavus* secretes extracellular cutinase when growing on cutin-containing medium and suggest a possible role for cutinase in pathogenicity of *A. flavus*.

Additional keywords: mycotoxin, parasitism, *Zea mays*.

Many pathogenic fungi penetrate plants directly (20,21,24,56). The first physical barrier encountered by these pathogens is the plant cuticle, a hydrophobic structure covering the layer of epidermal cells (21,22,24). Evidence suggests release of cutinase by pathogens during early stages of plant invasion and its involvement in the penetration of this physical barrier, which indicates that cutinase may play a crucial role in pathogenicity (56). The presence of cutinase activity in fungal cultures grown on purified cutin as the sole carbon source has been reported for over 20 plant pathogens, and the role of cutinase in pathogenicity has been proposed for several host-pathogen systems (21,25,26,54,60).

Infection of corn (*Zea mays* L.) kernels by *Aspergillus flavus* Link:Fr. causes ear and kernel rot (53), resulting in reduced grain quality and, potentially, aflatoxin contamination of grain. The initial association of *A. flavus* only with storage rots led to the assumption that the fungus has no, or only limited, parasitic abilities (57). Its widespread occurrence in the field, however, suggests that *A. flavus* may be able to penetrate kernels directly (2,17,30,31,32,33,36,37,38,41,47,48).

The pericarp is the outermost layer of corn kernels and consists of several layers of cells differing in their degree of degradation and cell wall thickness (58). It affords considerable protection against invasion of the kernel by pathogens. Research shows that *A. flavus* can produce cell wall-degrading enzymes such as pectinases and that those enzymes may play a role in the invasion of cotton bolls by this fungus (7,8,10). To aid in penetration, many pathogens secrete extracellular cutinase to hydrolyze cutin, a polyester of C_{16}- to C_{18}-length hydroxy fatty acids, in plant cuticles (4,22,27,40). Much published evidence supports the hypothesis that cutinase facilitates penetration of the cuticle by plant pathogenic fungi (14,24,26,27,35,56). However, cutinase production by *A. flavus* has not been investigated.

The purpose of the present study was to determine whether *A. flavus* produces cutinase and, if so, to determine the possible role of cutinase in the *Aspergillus*-corn pathosystem. A preliminary report has been published (15).

MATERIALS AND METHODS

Fungal culture and materials. *A. flavus* strain 13 was obtained from USDA/ARS/SRRC, New Orleans, and maintained on V8 juice-agar plates (5% V8 juice and 2% agar) at 28°C. This strain produces large quantities of aflatoxins (6,9). Conidia from 7-day-old cultures suspended in deionized water served as inoculum. Purified apple cutin and cutinase from *Pseudomonas putida* (45) were provided by P. E. Kolattukudy, Biotechnology Center, Ohio State University, Columbus. Cutinase, a crude preparation from a select strain of *Pseudomonas*, also was purchased from InterSpex Products, Inc. (Foster City, CA). All other chemicals were obtained from Sigma Chemical Company (St. Louis).

Plate assay. Cutinase production by *A. flavus* was studied using an esterase assay (23). Petri plates were filled with growth medium used by Adye and Mateles (A & M medium) (1) modified

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to include 1.5% noble agar and 50 mM potassium phosphate (pH 8). Sucrose as the sole carbon source was replaced with either 0.5% purified apple cutin, 0.3% apple cutin plus 0.3% 16-hydroxyhexadecanoic acid (cutin monomer), or 0.5% glucose. Glucose was included because it was reported to suppress cutinase production (34). Purified cutin and the cutin monomer served as inducers for cutinase production (34,39). After the medium solidified, a well (3 mm in diameter) was cut into the center of each plate, and a suspension (10 µl) of *A. flavus* conidia (10^6/ml) was pipetted into each well. Cultures were incubated at 28°C in darkness. After 5 days, fungal colonies (3 cm in diameter) had formed. Colonies were overlaid with staining agar (50 mM potassium phosphate [pH 8], 0.5 mM *p*-nitrophenyl butyrate [PNB], and 1.5% agarose). The presence of yellow color in the staining agar above the fungal colonies indicated the production of cutinase, which catalyzes production of pigmented *p*-nitrophenol from PNB (23,49).

Isolation of cutinase. *A. flavus* was grown in Erlenmeyer flasks (500 ml), each containing 100 ml of liquid A & M medium modified to include 50 mM potassium phosphate buffer at either pH 6 or 8 and 0.5 g of powdered apple cutin as the sole carbon source. The cultures were incubated with shaking at 60 rpm at 28°C in darkness for 3 weeks. Cultures then were filtered twice through Whatman No. 1 paper (Whatman International, Ltd., Maidstone, England). The filtrate was centrifuged at 10,000 × *g* for 15 min to pellet remaining mycelia and spores, and the supernatant was lyophilized. The lyophilized material was resuspended in phosphate buffer (pH 8). Proteins in the resuspended phosphate buffer were precipitated with ammonium sulfate. Powdered ammonium sulfate was added slowly with stirring on ice until 30, 50, or 75% saturation was achieved. The precipitate was collected by centrifugation at 15,000 × *g* for 15 min, dissolved in a minimal volume of phosphate buffer (pH 8), and dialyzed overnight against the same buffer at 4°C.

PNB hydrolysis activity in *A. flavus* culture filtrates and in final ammonium sulfate-precipitated fractions were monitored spectrophotometrically. The reaction mixtures (1 ml) contained 50 mM phosphate buffer (pH 8), 0.5 mM PNB, and proportional amounts of protein extract (1:1, 1:10, 1:25, and 1:50, vol/vol). Absorbance of the reaction mixture was measured at 405 nm using a Uvikon 860 spectrophotometer (Beckman Instruments, Inc., Irvine, CA) to indicate the release of *p*-nitrophenyl from PNB.

Electrophoresis. The 30% ammonium sulfate precipitation fraction from *A. flavus* culture filtrate at pH 8 and commercial cutinase from *Pseudomonas* was separated using native polyacrylamide gel electrophoresis (PAGE). Separation of proteins was carried out on a 10% running gel with a 4% stacking gel. Gels were run for 13 h at 4°C and 100 V of constant current. Gels were washed twice for 20 min each in 100 mM Tris-HCl (pH 8). Detection of the protein bands with esterase activity was accomplished using the indoxyl acetate assay (11). Indoxyl acetate (30 mg) was dissolved in 1 ml of acetone and added to 49 ml of 100 mM Tris-HCl (pH 8). Gels were incubated at room temperature in the indoxyl acetate solution with constant agitation until bands of desired intensity appeared.

The dense bands from the 30% ammonium sulfate fraction were cut from the native gel. Slices (3 to 5 mm) of each band were frozen in liquid nitrogen and ground to a fine powder using mortar and pestle. The protein was extracted from the powder in phosphate buffer (pH 8). PNB hydrolysis activity of the extracted protein, with or without 0.005 mM diisopropyl fluorophosphate (DFP), was tested spectrophotometrically as described above. DFP, a known inhibitor of serine esterase (35,49), was included to test if the cutinase produced by *A. flavus* was a serine esterase. The molecular weights of the proteins extracted from native gel were determined using sodium dodecyl sulfate (SDS)-PAGE. Purified cutinase from *P. putida*, commercial cutinase from *Pseudomonas*, and the 30% ammonium sulfate fraction from *A. flavus* were used as controls. Electrophoresis was done according to Laemmli (28) using a 14% polyacrylamide separating gel with a 4% stacking gel. Protein standards and corresponding molecular weights were bovine serum albumin (66,000), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), trypsin inhibitor (20,000), and alpha-lactalbumin (14,200). The gels were stained 30 min with 0.25% Coomassie brilliant blue in 50% methanol:10% acetic acid, and destained in 10% acetic acid:40% methanol overnight.

Kernel inoculation. The corn genotypes Pioneer 3154 (susceptible) and GT-MAS:gk (resistant) were selected based on results from a previous study (16). Intact kernels were surface-sterilized in sodium hypochlorite (0.75%) for 5 min, rinsed in three changes of sterile, distilled water, and air-dried. Kernels were left intact or wounded in endosperm to a depth of 1 mm using a 20-gauge needle. Intact and wounded kernels of both genotypes were immersed in either commercial bacterial cutinase (100 µg/ml) in phosphate buffer (pH 8) or in the 30% ammonium sulfate fraction (diluted 1:10) from *A. flavus* culture filtrate. Controls were immersed in phosphate buffer (pH 8) alone. All kernels were incubated 6 h at room temperature before inoculation. Kernels then were immersed in a suspension of *A. flavus* conidia (10^6/ml) and removed immediately. Five kernels of each genotype, which constituted one experimental unit, were placed in a single cell (35 mm in diameter, 20 mm in height) of a six-cell culture dish (Costar Corp., Cambridge, MA). Four dishes then were sealed in a plastic food container (180 mm × 150 mm × 90 mm; NAKGEBE; Nalge Company, Rochester, NY) that contained 100 ml of water to maintain high humidity. Each treatment was replicated 12 times. The experiment was conducted twice. The inoculated kernels were incubated at 28°C in darkness for 7 days. Following this, kernels were dried in a forced-air oven at 60°C for 2 days to stop fungal growth and aflatoxin production. Levels of aflatoxin B_1 were determined using official methods of the American Oil Chemists' Society (3) with modifications (16). Aflatoxin was identified using thin-layer chromatography and quantified directly on plates using a scanning densitometer with a fluorometry attachment (Model CS-930; Shimadzu Scientific Instruments, Inc., Tokyo).

In a second study, DFP, a potent inhibitor of fungal cutinases (35,49), was added to suspensions of *A. flavus* conidia (10^6/ml) to yield final DFP concentrations of 0, 0.01, and 0.1 mM. Intact kernels of both genotypes were immersed in these amended conidia suspensions. Inoculation and incubation techniques were as described. Each treatment was replicated 12 times, and the experiment was conducted twice. Aflatoxin B_1 levels were measured as described.

Toxicity of DFP to *A. flavus* was tested on kernels and on plates. Petri dishes containing A & M medium and kernels of both genotypes were inoculated with *A. flavus* conidia suspensions (10 µl) amended with DFP at the concentrations described above and incubated at 28°C in darkness. Spore germination and hyphae growth on kernel surfaces and A & M medium were observed using a stereo microscope.

Statistical analysis. Before analysis, aflatoxin B_1 data were subjected to $\log_{10}(X + 1)$ transformation to equalize variances. Mean separations were accomplished using least significant difference. All statistical procedures were performed using the SAS system (43,44).

RESULTS

Production of cutinase. *A. flavus* grew on A & M medium amended with purified apple cutin or cutin plus cutin monomer 16-hydroxyhexadecanoic acid as the sole carbon source (Fig. 1). Colonies filled the culture dishes (9 cm in diameter) after 7 days (Fig. 1A), but cutin plus cutin monomer 16-hydroxyhexadecanoic acid supported more fungal sporulation than did cutin alone.

Overlay of 5-day-old cultures with staining agar containing PNB resulted in the production of the characteristic yellow pigment, which suggests that cutinase was produced by *A. flavus* (Fig. 1B) to catalyze production of pigmented *p*-nitrophenol from PNB. When glucose was provided as sole carbon source, *A. flavus* grew well, but failed to produce the yellow pigment when overlaid with PNB (data not shown).

The mycelial mat produced by *A. flavus* in liquid A & M medium containing cutin as the sole carbon source was markedly larger at pH 8 than at pH 6 (data not shown). PNB hydrolysis activity was most evident in the 30% ammonium sulfate precipitation fraction from pH 8 culture filtrate (A_{405} = 1.75 min^{-1} ml^{-1}) (Fig. 2). Much less PNB hydrolysis activity was observed for the 30% ammonium sulfate fraction from pH 6 culture filtrate (A_{405} = 0.14 min^{-1} ml^{-1}) (Fig. 2). Minor color reactions also were detected for both the 30 to 50% and >75% fractions from pH 8 cultures and

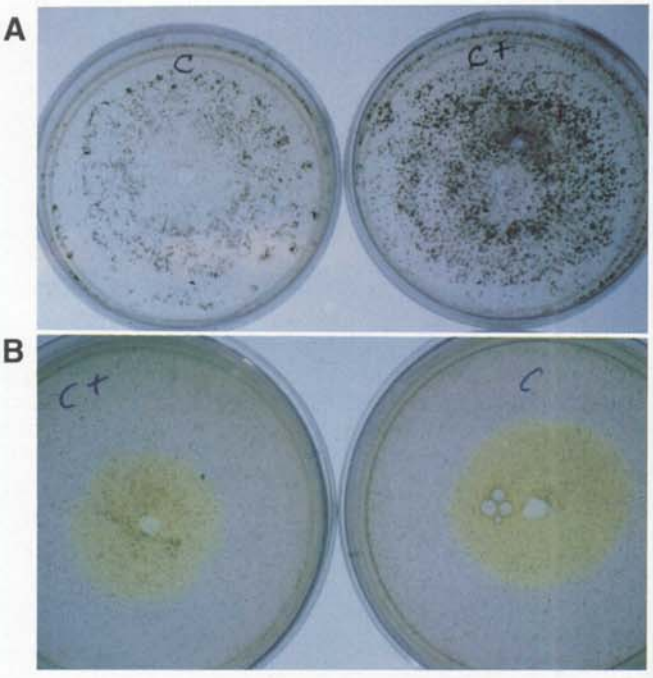

Fig. 1. A, *Aspergillus flavus* growth (after 7 days) on A & M medium with 0.5% purified apple cutin (C) or 0.3% cutin plus 0.3% 16-hydroxyhexadecanoic acid (cutin monomer) (C+) as the sole carbon source. **B,** Colonies (after 5 days) overlaid with agarose containing *p*-nitrophenyl butyrate (PNB) (0.5 mM). Yellow pigment in agarose above colonies indicates PNB hydrolysis activity and, thereby, induction of cutinase production.

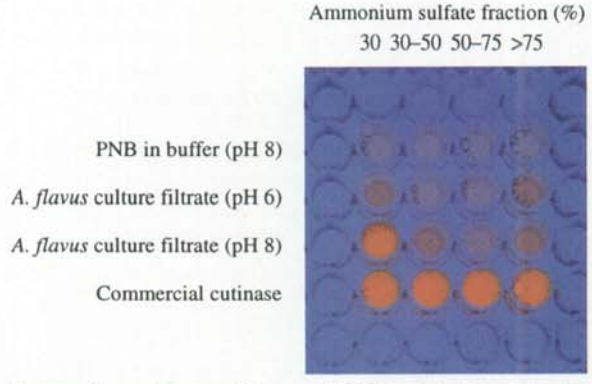

Fig. 2. Assay for *p*-nitrophenyl butyrate (PNB) hydrolysis activity. From top to bottom, rows contained PNB substrate in phosphate buffer (pH 8), *Aspergillus flavus* culture filtrate (pH 6), *A. flavus* culture filtrate (pH 8), and commercial cutinase from *Pseudomonas*. Only in the second and third rows did the columns contain ammonium sulfate precipitate fractions of, from left to right, 30, 30 to 50, 50 to 75, and > 75%.

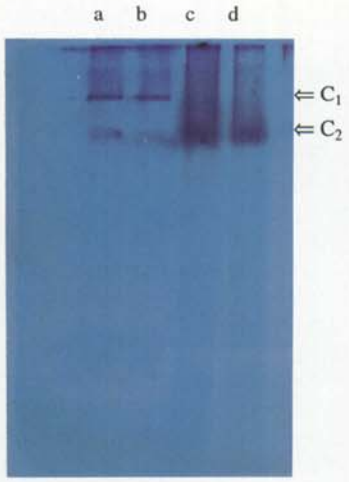

Fig. 3. Native gel electrophoresis of 30% ammonium sulfate fraction from *Aspergillus flavus* culture filtrate (pH 8). Bands were visualized using indoxyl acetate. Lanes a and b: commercial cutinase from *Pseudomonas* sp.; and lanes c and d: 30 µl and 15 µl, respectively, from *A. flavus* filtrate.

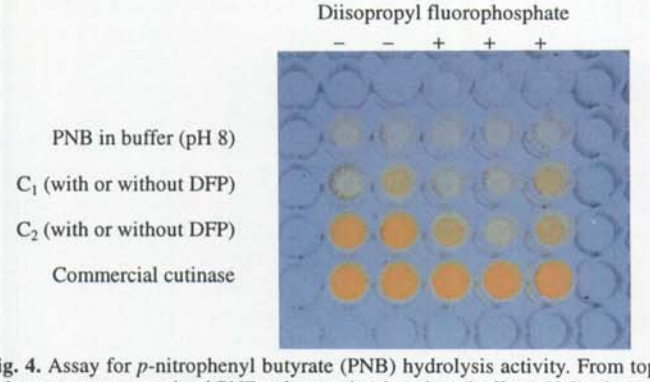

Fig. 4. Assay for *p*-nitrophenyl butyrate (PNB) hydrolysis activity. From top to bottom, rows contained PNB substrate in phosphate buffer (pH 8); C_1 and C_2 from native polyacrylamide gel (Fig. 3); and commercial cutinase from *Pseudomonas*. For C_1 and C_2, from left to right, the first and second wells were without the cutinase inhibitor diisopropyl fluorophosphate (DFP) and the last three wells contained DFP.

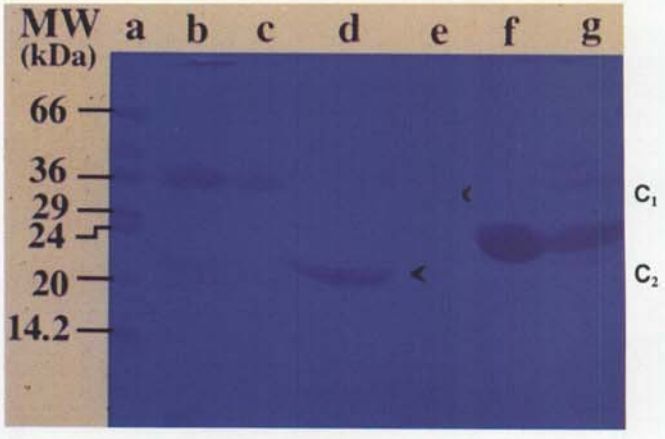

Fig. 5. SDS-PAGE of liquid culture filtrates of *Aspergillus flavus* grown on A & M medium containing cutin as sole carbon source. Lane a: molecular weight markers; lanes b and c: 30 µl and 15 µl, respectively, of 30% ammonium sulfate fraction from *A. flavus* liquid culture filtrate at pH 8; lane d: C_2 (22 to 23 kDa); lane e: C_1 (36 kDa); lane f: purified cutinase (30 kDa) from *Pseudomonas putida*; and lane g: commercial *Pseudomonas* cutinase.

the >75% fraction from pH 6 cultures (Fig. 2). Strong color reactions were detected in wells containing commercial bacterial cutinase, but not the buffer alone (Fig. 2).

Electrophoresis. The 30% ammonium sulfate fraction from *A. flavus* culture at pH 8 was separated using native PAGE (Fig. 3). The indoxyl acetate assay indicated a single minor band and a single major band, designated C_1 and C_2, respectively (Fig. 3). For comparison, the commercial bacterial cutinase was also displayed on the native gel (Fig. 3). Assay of gel slices containing C_1 or C_2 for PNB hydrolase activity indicated that this was much stronger for C_2 than for C_1 (Fig. 4). Incorporation of the inhibitor DFP into reaction mixtures of C_1 and C_2 (Fig. 4) resulted in the reduction of PNB hydrolysis activity. C_2 PNB hydrolysis activity was significantly reduced ($A_{405} = 0.05$ min^{-1} ml^{-1}), indicating that the cutinase (C_2) produced by *A. flavus* may be a serine esterase.

Electrophoresis on SDS-PAGE of C_1 and C_2, along with both purified and commercial bacterial cutinases and the 30% ammonium sulfate fraction, showed the molecular weights for C_1 and C_2 to be 36 kDa and 22 to 23 kDa, respectively (Fig. 5). The 30% ammonium sulfate fraction mixture from *A. flavus* liquid culture filtrate showed bands corresponding to C_1 and C_2, as well as some minor bands. The molecular weight of cutinase from *P. putida* was 30 kDa, as reported (42). Commercial bacterial cutinase had a major band at 30 kDa and several minor bands (Fig. 5).

Kernel inoculation. Aflatoxin levels were lower in resistant GT-MAS:gk than in susceptible Pioneer 3154 (Fig. 6). Wounding kernels, which bypassed the pericarp (16), resulted in increased aflatoxin levels for both genotypes (Fig. 6). Treatment of nonwounded kernels with commercial bacterial cutinase or the 30% ammonium sulfate fraction from *A. flavus* culture also resulted in increased aflatoxin levels (Fig. 6). These levels were similar to those obtained in wounded kernels that did not receive any cutinase treatment. Cutinase treatment increased *A. flavus* growth and sporulation (data not shown) in comparison with treatment without cutinase.

Aflatoxin levels in kernels of Pioneer 3154 were reduced significantly when DFP was mixed with *A. flavus* conidia before inoculation (Fig. 7). Aflatoxin levels were similar, regardless of DFP concentration. Aflatoxin levels in kernels of GT-MAS:gk were very low and were not changed by DFP treatment (Fig. 7). Fungal growth, as evidenced by conidia production on kernel surfaces, also was reduced by DFP treatment in a manner similar to that in Figure 7 (data not shown). Microscopic examinations showed that spore germination and hyphae growth on A & M medium and on kernels were not influenced by DFP at any tested concentration (data not shown).

DISCUSSION

Preharvest infection of corn kernels by *A. flavus* and subsequent aflatoxin production can result from several mechanisms. The consistent association of insect damage with fungus sporulation and aflatoxin production (57) suggests that insects play a major role in aflatoxin contamination of corn kernels in the field. Insect feeding damage provides infection courts for the fungus, thus bypassing defense mechanisms associated with an intact kernel pericarp (16). Artificial removal of pericarp wax and cutin layers dramatically decreased resistance to kernel invasion by *A. flavus* resulting in high aflatoxin levels (16). *A. flavus* also can colonize silks and developing kernels in the absence of insect damage (37). However, reports (2,17,18,29,32,38) of undamaged kernels containing aflatoxin suggest that direct penetration of corn kernels by *A. flavus* may be more important than once thought. Previous studies showed that *A. flavus* produces pectinases, which aid in the colonization of cotton bolls (7,8,10). The present results indicate that *A. flavus* produced cutinase, an enzyme crucial for direct penetration of corn kernels.

A. flavus colonization of internal tissue and aflatoxin production in corn kernels were studied (5,19,48). Resistance of corn to aflatoxin production was directly related to resistance to fungal colonization (5). In the present study, the addition of supplemental cutinase and *A. flavus* cultural filtrate (30% ammonium sulfate fraction) affected fungal growth and aflatoxin production by *A. flavus* in corn kernels. Aflatoxin levels in intact kernels of both resistant and susceptible corn genotypes were increased by pretreatment with cutinase to levels similar to those in wounded kernels. This suggests that resistance to aflatoxin production in resistant genotype GT-MAS:gk is due, in part, to the cutin layer of the intact pericarp. This agrees with other findings that attribute resistance in this genotype to both pericarp (cutin and wax) and subpericarp factors (possibly biochemical in nature) (16). The addition of exogenous cutinase may have removed portions of the cutin barrier and resulted in elevated infection and aflatoxin levels for GT-MAS:gk. Although the lack of resistance in Pioneer 3154 may be because of lower levels of cutin and wax factors, the supplemental cutinase also increased the levels of infection and aflatoxin in this genotype.

All fungal cutinases purified thus far are serine esterases and, therefore, are inhibited by DFP (21,24,35). The addition of DFP to *A. flavus* inoculum resulted in reduced fungal sporulation and aflatoxin production in kernels. This suggests that the cutinase

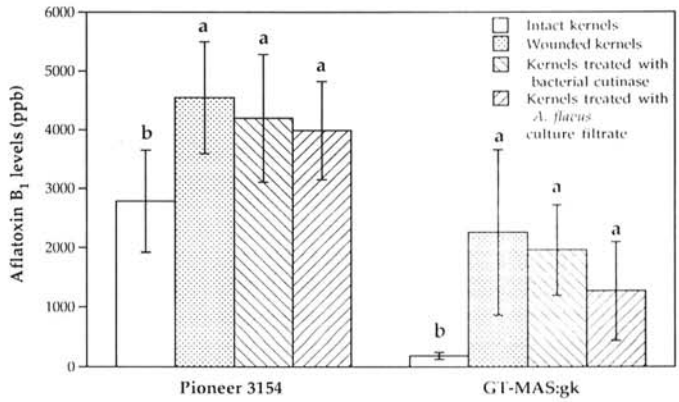

Fig. 6. Aflatoxin B_1 production by *Aspergillus flavus* in susceptible (Pioneer 3154) and resistant (GT-MAS:gk) corn kernels. Within genotypes, letters above bars indicate significant ($P \leq 0.05$) differences according to least significant difference. Vertical lines delimit standard errors. Treatment means are averages from two experiments with 12 replicates each.

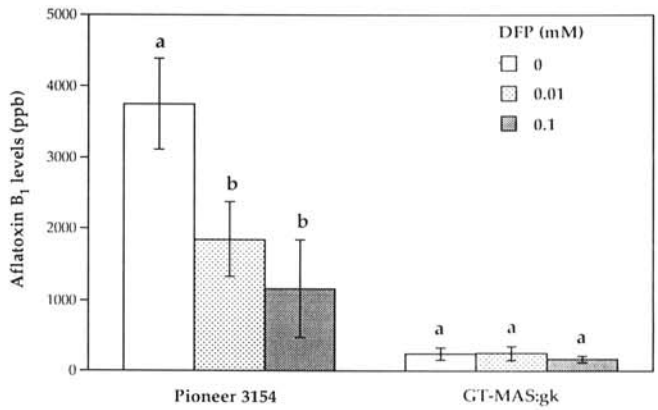

Fig. 7. Aflatoxin B_1 production by *Aspergillus flavus* in kernels of susceptible (Pioneer 3154) and resistant (GT-MAS:gk) corn genotypes inoculated with conidia suspensions that were not treated or mixed with cutinase inhibitor diisopropyl fluorophosphate (DFP) at different concentrations. Within genotypes, letters above bars indicate significant ($P \leq 0.05$) differences according to least significant difference. Vertical lines delimit standard errors. Treatment means are averages from two experiments with 12 replicates each.

produced by *A. flavus* may be a serine esterase. Lower levels of aflatoxin in kernels treated with DFP were not because of a general toxicity of this chemical to the fungus. DFP virtually prevented infection and fungal growth by *A. flavus*, as evidenced by less fungal sporulation on inoculated kernel surfaces. DFP reduced cutinase activity and, consequently, aflatoxin production in kernels of Pioneer 3154. This suggests a role for cutinase in the infection and colonization of susceptible Pioneer 3154 kernels. That DFP had no effect on aflatoxin levels in GT-MAS:gk kernels suggests that cutinase production by germinating spores was insufficient to penetrate the physical pericarp barrier formed in this genotype (16). Alternatively, GT-MAS:gk has a thicker wax layer (55) that may act as a physical barrier to block cutinase activity. The findings of this study suggest that the secretion and accumulation of the enzymes may allow establishment of *A. flavus* in intact kernels of susceptible genotypes in the field.

It is interesting that, under mild acidic conditions (pH 6), *A. flavus* showed less growth and markedly less PNB hydrolysis activity than under mild alkaline conditions (pH 8). This suggests a preferred pH for cutin utilization by *A. flavus*. Köller and Parker (25) noted that all cutinases with alkaline pH optima for cutin hydrolysis have been purified from stem-infecting pathogens, whereas cutinases with slightly acidic optima were derived from pathogens primarily infecting leaves. Trail and Köller (54) presented evidence to support the hypothesis that two different forms of cutinase are involved in the expression of tissue specificity. Cutin hydrolysis was optimal at pH 6.5 for the leaf pathogen *Cochliobolus heterostrophus* and at pH 8.5 for the stem-infecting pathogen *Rhizoctonia solani*. The pH profile observed for *Alternaria brassicicola*, a pathogen that infects both leaves and stems, exhibited two distinct optima (pH 7 and 9) (54). Cutin hydrolysis by cutinase from *Venturia inaequalis*, the causal agent of apple scab, was optimal at pH 6 (25). However, the hypothetical pH optimum-tissue specificity relationship has exceptions (51). The evidence presented here suggests that a relationship between cutin hydrolysis activity and pH may exist for the *A. flavus*-corn pathosystem, but the relationship between pH optimum and tissue specificity remains to be determined.

Previous studies of cutinases from necrotrophic fungi have addressed the induction of enzyme synthesis (59), the mechanism of induction (34), and the role of such enzymes in plant-pathogen interactions (13,21,22,46,54). Studies involving monospecific antisera to cutinase, the cutinase-inhibitor DFP, and transformation of the papaya wound pathogen *Mycosphaerella* sp. with the cutinase gene have demonstrated the importance of this enzyme to fungi that penetrate their hosts directly (12,14,35). In contrast, results from other investigations question the importance of cutinase to fungal penetration (27,42,50,52,60). Isolates of *Fusarium solani* f. sp. *pisi* and *Alternaria brassicicola* did not lose pathogenicity when the cutinase gene was disrupted (50,51,56). The only effect of gene disruption noted was a dramatic reduction in the saprophytic growth of the mutants on media containing cutin as carbon source. Further studies using *Alternaria brassicicola* as a model system also showed that different cutinase isozymes were expressed during saprophytic and pathogenic growth (27,60). The need is clear for continued studies on the role of cutinase in fungal penetration.

Our report is the first to demonstrate that *A. flavus* can produce cutinase(s) (or nonspecific esterases or both) that may be involved in infection of intact corn kernels in the field (36,38,48). This conclusion was based on the observed substantial saprophytic growth by *A. flavus* on cutinase-induction medium (containing cutin as the sole carbon source) and on PNB (the model substrate) hydrolysis activity. In addition, treatment of kernel surfaces with *A. flavus* culture filtrate significantly increased the susceptibility of kernels to *A. flavus* invasion, whereas treatment with DFP reduced fungal invasion and aflatoxin levels. Although cutinase is known to catalyze hydrolysis of PNB by a mechanism similar to that involved in cutin hydrolysis (23), such activity is not necessarily proof of cutinase activity because of numerous other lipases or nonspecific esterases that may exist. Final verification of these hydrolytic activities, possibly involved in the degradation of cutin-containing composite materials on the kernel surface, will depend on obtaining highly purified enzymes and testing for activity in the presence of specific substrates. Such studies also could identify the specificity of C_1 and C_2 in cutin hydrolysis. Future work, as well, should focus on pathogenicity tests relating to pathogenic rather than saprophytic stages of the pathogen (27,60), in order to address the question of cutinase involvement in direct penetration of corn kernels by *A. flavus*.

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