Spread of *Aspergillus flavus* in Cotton Bolls, Decay of Intercarpellary Membranes, and Production of Fungal Pectinases

R. L. Brown, T. E. Cleveland, P. J. Cotty, and J. E. Mellon

Southern Regional Research Center, Food and Feed Safety Research Unit, 1100 Robert E. Lee Blvd., New Orleans, LA 70124.
Accepted for publication 12 December 1991.

ABSTRACT

Brown, R. L., Cleveland, T. E., Cotty, P. J., and Mellon, J. E. 1992. Spread of *Aspergillus flavus* in cotton bolls, decay of intercarpellary membranes, and production of fungal pectinases. Phytopathology 82:462-467.

Developing cotton bolls were wound-inoculated with high- and low-virulence strains of *Aspergillus flavus* and also with *A. nidulans* FGSC4 and were examined at maturity for disease symptoms and fungal pectinases. All inoculated bolls showed tight-lock formation (failure of mature locks to expand and fluff out), but specific strain-related differences in intercarpellary membrane decay were observed. A highly significant correlation was found between fungal spread to adjacent locules and decay of intercarpellary membranes in bolls inoculated with *A. flavus*. The high-virulence strain caused severe deterioration of intercarpellary membranes and produced three polygalacturonase activities in bolls and culture. A pectinesterase also produced by this strain in culture was not detected in inoculated bolls. The low-virulence strain caused less severe deterioration of membranes and was lacking a major *A. flavus* endopolygalacturonase, P2C. *A. nidulans* produced pectinases in culture but failed to do so in cotton bolls and caused very little damage to intercarpellary membranes. The endopolygalacturonase, P2C, was the only pectinase produced by the tested strains that was not catabolite-repressed in culture by the simple sugars common in developing cotton bolls. Results support a role for *A. flavus* pectinases in interlocule fungal spread in bolls and suggest that the lack of catabolite repression of P2C facilitates this role.

Aspergillus flavus contaminates cottonseed with high levels of aflatoxins during infestation (3). Strains of *A. flavus* that exhibit high and low virulence in cotton have been isolated and identified within *A. flavus* populations (8). These two strain types produce three pectinase activities in common, P1, P3, and pectinesterase (PE), in cotton bolls and in culture (4,10). In addition to these pectinase activities, high-virulence strains produce a polygalacturonase activity (P2C) in bolls and culture (4). The presence of these four pectinases and the absence of pectate lyase (PL) activity in *A. flavus* were verified by both activity staining in isoelectric-focusing (IEF) gels and by biochemical assays (5,10). Investigations of strain differences in virulence and pectinase production revealed a correlation between production of pectinase P2C and the strain's ability to spread between cotton locules (4).

Carbon catabolite repression of pectolytic enzymes has been investigated in a number of studies involving fungi. Repression

of pectinases by glucose or other sugars in vitro has been demonstrated in *Verticillium albo-atrum*, *Fusarium oxysporum* f. sp. *lycopersici*, *Pyrenochaeta lycopersici*, *Aspergillus niger*, *Suillus luteus*, and *Hebeloma oculatum* (7,14,15,19). A direct relationship exists among the utilization of sugars by *Rhizoctonia solani*, inhibition of disease development in cotton seedlings, and repression of pectinases (24). Similarly, it was found that increasing the sugar content of onion roots infected with *Pyrenochaeta terrestris* reduced fungal endopolygalacturonase synthesis and symptom development (pink root), whereas decreasing the sugar content exerted the opposite effect (17).

The present study sought to relate regulation of pectinase production by *A. flavus* during infection of developing cotton bolls with specific boll rot symptoms. To do so, catabolite repression of pectinase production by high- and low-virulence strains of *A. flavus* and a single strain of *A. nidulans* was studied in detail. Catabolite repression was related both to pectinase production in the high-sugar environment of developing cotton bolls and to the symptoms developed by bolls inoculated with the various fungi. Catabolite repression has been suggested to be an important regulatory determinant of pathogenicity in *Aspergillus nidulans* (11). The results presented here suggest that the failure of a single *A. flavus* pectinase to be catabolite-repressed may permit this fungus to cause decay in intercarpellary membranes of cotton bolls and to spread between cotton boll locules.

MATERIALS AND METHODS

Cultures. The *A. flavus* strains, 13 (high virulence) and 42 (low virulence), that were used in this study have been described in a previous investigation (8). These strains along with *A. nidulans* FGSC4 (Glasgow wild type) (11) were maintained at 30 C in the dark on 5:2 agar (5% V8:2% agar) (9) plates or slants. The liquid medium was the same as that used by Adye and Mateles (1), but the carbon source was varied for production of pectolytic enzymes and for testing catabolite repression. Liquid media (25 ml) contained grade 1 (7.7% methoxy content) citrus pectin (1%) (Sigma, St. Louis, MO) as the sole carbon source or pectin (1%) with one of the following sugars (5%): arabinose, fructose, raffinose, rhamnose, sucrose (Sigma); cellobiose, mannose, xylose (P-L Biochemicals, Inc., Milwawkee, WI); glucose (J. T. Baker Chem. Co., Phillipsburg, NJ); and maltose (MCB, Norwood, OH). Liquid media containing either pectin and a cottonseed extract or pectin, glucose, and a cottonseed extract were also employed. The cottonseed extract was prepared by autoclaving 100 g of cottonseed in 1 L of distilled water at 121 C (10^5 N/m^2) for 20 min and filtering through Whatman No. 1 filter paper. The extract made up 10% (v/v) of the total medium. All media were adjusted to pH 5.0, sterilized, and inoculated with 100 μl of a spore suspension (4.0 × 10^6 spores per milliliter) prepared from a 2- to 3-wk-old fungal culture. Cultures were incubated at 28 C without shaking for 4 days. Tests were repeated twice.

Isolation of pectinases from culture filtrates. Filtrates were separated from mycelia by filtering through Whatman No. 1 filter paper. The filtrate was then centrifuged at 27,200 *g* to create pellets of remaining mycelia and spores. Supernatants were carefully removed, and the pellets were combined with mycelial mats for dry weight analyses. Supernatants were dialyzed against double-distilled water and concentrated 100-fold with Centricon concentrators and Centriprep microconcentrators (10,000 MW cutoff; Amicon Co., Danvers, MA). The concentrated solutions were used for protein determinations (based on the bicinchoninic acid assay, "BCA", bovine albumin standard; Pierce, Rockford, IL), IEF, and radial diffusion cup plate assays.

Inoculation and sampling of cotton bolls. Cotton plants (Delta Pine 90) were grown in a greenhouse for 21 days in 3-L pots containing a 50:50 mixture of sand and Pro-mix (Premier Brands Inc., New Rochelle, NY) (8). Plants were then fertilized weekly with 100 ml of Miracle-Gro (Sterms Miracle-Gro Products, Inc., Port Washington, NY) at 2,000 ppm. Flowers were dated at opening (9). Unopened cotton bolls were wounded once 26–28 days postanthesis by using a cork borer (3 mm diameter) to a depth of 2 mm, and the resulting plug from the carpel area was removed. The exposed lint was inoculated with a 10-μl spore suspension (4.0 × 10^6 spores per milliliter) of one of the three fungal strains. We demonstrated in a previous study (5) that wounding alone does not induce pectinase activity in developing cottons bolls. Wounded, uninoculated bolls frequently become contaminated by various saprophytes in greenhouse experiments. Therefore, bolls unwounded and uninoculated served as controls in the test described here. All bolls to be used for pectinase extractions were collected 7 days after inoculations. Bolls to be examined for fungal aggressiveness were collected after boll opening, usually 2–3 wk after inoculations. For boll examinations, each treatment was replicated six times; each replicate consisted of two bolls. Tests for both pectinase production and fungal aggressiveness were repeated twice.

Extraction of pectinases from cotton bolls. The procedure for extracting pectinases from bolls was similar to one employed in an earlier study (5). To each locule, 10 ml of 0.1 M sodium acetate buffer (pH 4.5) was added. Air was squeezed from the lint with a spatula, and the tissue was soaked in the buffer with continuous agitation for 30 min. Buffer containing the pectinases then was squeezed from the lint and seed and centrifuged for 10 min at 27,200 *g* to remove insoluble material. The supernatant was dialyzed and concentrated 100-fold with Centricon microconcentrators, and the concentrated solution was used for protein determinations, IEF, and radial diffusion assays.

Evaluation of fungal spread and aggressiveness. Mature harvested bolls were dried in a forced air oven at 60 C for 2 days and kept at room temperature in sealed plastic bags containing silica gel desiccant until evaluated. Fungal spread between locules was used as one measure of pathogenic aggression. The presence of an *A. flavus* specific, "bright greenish yellow fluorescence" (BGYF) on cotton lint was used as an indicator of *A. flavus* activity and spread (2,8). Uninoculated locules adjacent to wound-inoculated locules were examined under ultraviolet light after drying, and BGYF on the lint of each locule was rated as follows: 0 = no BGYF; 1 = BGYF on less than 25% of the lint; 2 = BGYF on 25–50% of the lint; 3 = BGYF on more than 50%

TABLE 1. Production of pectinases in cotton bolls and on autoclaved cottonseed by *Aspergillus flavus* and *A. nidulans*[a]

Isolate	Pectinase activities detected by isoelectric focusing[b]					Pectinase activities detected by radial diffusion assay (mm)[c]	
	P1	P2C	P3	PE	PL	Clear zone	Red zone
A. flavus 13	+/+	+/+	+/+	−/−	−/−	11.0/16.5	0/0
A. flavus 42	+/+	−/−	+/+	+/+	−/−	4.0/7.0	5.0/4.0
A. nidulans	−/−	−/−	−/−	−/−	−/+	0/1.5	0/0

[a] Pectinases were extracted according to procedures in text. Pectinases were subjected to radial diffusion assays to quantify total extractable pectinase activity and to isoelectric focusing (IEF) to identify individual pectolytic enzymes. + = Present, and − = absent. Numerator denotes cotton bolls; denominator denotes cottonseed.

[b] Polygalacturonases P1, P2C, and P3 were previously identified as activities of *A. flavus* by Cleveland and McCormick (5), and pectate lyase (PL) of *A. nidulans* was identified previously by Dean and Timberlake (11). Pectinesterase (PE) activity in *A. flavus* was identified by Cotty et al (10).

[c] Pectinase activity was determined by measuring the width (mm) of clear zones (for P1, P2C, P3, and PL) and/or red (for PE) zones.

of the lint.

Intercarpellary membranes adjacent to inoculated locules and membranes from uninoculated control bolls were examined microscopically to determine the effects of fungal infection. Membranes were rated as follows: 0 = no symptoms; 1 = discolored on surface next to inoculated locule; 2 = same as 1 plus slight discoloration on surface away from inoculated locule; 3 = highly discolored on both surfaces; 4 = same as 3 plus pin-hole tears; 5 = same as 3 plus tears of 1 mm or larger.

Inoculation of autoclaved cottonseed and extraction of pectinases. Petri dishes (10 cm), each containing 5 g of sterile cottonseed, were inoculated with 1 ml of spore suspension (4.0 × 10^6 spores per milliliter). Each dish was sealed with Parafilm and incubated at 28 C for 7 days. After incubation, 2 ml of 0.1 M acetate buffer (pH 4.6) was added to each 5-g sample, which was then placed in a flask and shaken vigorously on a shaker for 1 h. The flask contents were filtered through Whatman No. 1 filter paper, and the filtrate was dialyzed and concentrated in the manner described above for liquid cultures. The concentrate was used for IEF and radial diffusion assays. Tests were repeated twice.

Radial diffusion assays. The procedure used was basically the same as that used in a previous investigation (5). Petri plates (10 cm) were each filled with 10 ml of a medium composed of 1% agarose (Calbiochem Corp., La Jolla, CA) and 0.5% grade 1 citrus pectin (Sigma) in 0.1 M sodium acetate. The medium was adjusted to pH 4.8 for polygalacturonase and PE with 1.0 M sodium hydroxide. A 50 mM Tris-hydrochloride medium containing 1.5 mM $CaCl_2$, 1% agarose, and 0.5% pectin was adjusted to pH 8.5 with 1.0 M sodium hydroxide for PL detection. After each medium solidified, a 3-mm-diameter well was cut in the center with a cork borer, and the agar plug was removed. Into each well, 35 μl of the test solution was pipetted, and the plates were covered, sealed with Parafilm, and incubated at 37 C for 16 h. Plates were then stained with ruthenium red (Sigma) for 30 min and afterwards rinsed with deionized water. The width of clear (polygalacturonase or PL) (4,5) and red zones (PE) around wells was measured to estimate enzymatic activity (10). When both red and clear zones appeared on the same plate, the red zone (red zones appear on the outside of clear zones) width was measured from the endpoint of the clear zone.

IEF and pectin-agarose overlay technique. The IEF procedure used was the same as that previously employed (21), except that the pH of the gel was determined directly with a flat pH electrode. After IEF was performed, gels were immediately assayed for activity by using pectin-agarose overlays (5,23). Differential detection of pectolytic enzymes was obtained by varying buffers in some overlays (23). Polygalacturonase (P1, P2C, and P3) (5) and PE were assayed in 50 mM potassium acetate, pH 4.8, containing 10 mM ethylene-diaminetetraacetic acid (EDTA). PL was assayed in 50 mM Tris-hydrochloride, pH 8.5, containing 1.5 mM $CaCl_2$. Overlays remained in contact with gels for 30 min at 37 C. After-

wards, they were removed, stained with ruthenium red for 30 min, destained in distilled water for 15 min, and air-dried overnight. Clear (polygalacturonase or PL) or red (PE) bands on the overlay indicated pectinase activities in the IEF gel. The isoelectric points (pIs) of the individual bands were determined from the pH value at which pectinases reached their pI on IEF gels.

Statistical analysis. Significant differences among intercarpellary membrane ratings were determined with the Mann-Whitney U test. Pearson product moment correlations were calculated for relationships between intercarpellary membrane damage and the amount of BGYF present on lint.

RESULTS

Production of pectinases in wound-inoculated cotton bolls and on autoclaved cottonseed. Pectinase activities were detected by the radial diffusion assay in locular extracts taken from single locules wound-inoculated with A. flavus strains 13 or 42 (Table 1). No pectinase activity was detected in extracts of lint and seed from locules of uninoculated bolls or in locular extracts from locules wound-inoculated with A. nidulans. Resolution of pectinases by IEF and pectin-agarose overlays indicated the presence of previously reported polygalacturonases (5) in extracts of locules inoculated with either A. flavus strain. Strain 42 also showed PE activity. No pectinases were detected by IEF and pectin-agarose overlays at either pH 4.8 or 8.5 in locules inoculated with A. nidulans.

Pectinases of A. flavus strains 13 and 42 were detected in extracts from autoclaved cottonseed inoculated with these respective strains by the radial diffusion assay (Table 1). Extracts from autoclaved cottonseed inoculated with strain 42 showed a red zone in this assay. Radial diffusion assays demonstrated very slight pectinase activity in extracts of cottonseed inoculated with A. nidulans. The IEF and pectin-agarose overlays detected the presence of all previously described A. flavus pectinases except PE in extracts from cottonseed inoculated with strain 13 and all except P2C in seed inoculated with strain 42. One band was present in pH 8.5 overlays of extracts from seed inoculated with A. nidulans, indicating the presence of the previously reported PL (11).

Growth of A. flavus strains and of A. nidulans in culture media. Figure 1 illustrates the growth of the three isolates in media containing pectin as the sole carbon source and in some pectin-containing media with an additional carbon source. A. flavus strains 13 and 42 grew similarly on all carbon sources, although strain 13 generally grew more. For these two isolates, growth on carbon sources (pectin alone or pectin + glucose, + sucrose, + fructose, + rhamnose, and + xylose) as shown in Figure 1 was similar to growth on pectin and certain other carbon sources not illustrated in this figure. The observed similarities were glucose ~ glucose + cottonseed extract; sucrose ~ mannose; fructose ~ maltose; rhamnose ~ arabinose; and pectin ~ pectin + cottonseed extract. Growth in pectin + cellobiose was nearly the same for both strains and comparable to growth of strain 42 in pectin + rhamnose. Growth in pectin + raffinose was also nearly the same for both and comparable to growth of strain 13 in pectin + rhamnose. A. nidulans exhibited poorer growth than strains 13 and 42 in all carbon sources except glucose. For A. nidulans, growth on carbon sources, as shown in Figure 1, was similar to growth on pectin and other carbon sources not shown in this figure. The observed similarities were glucose ~ glucose + cottonseed extract; sucrose ~ raffinose ~ maltose; fructose ~ mannose; rhamnose ~ arabinose ~ cellobiose; and pectin ~ pectin + cottonseed extract. Growth of A. nidulans on pectin + xylose was poorer than on pectin alone and was the poorest growth displayed by any isolate in culture media.

Production of pectinases in culture media. All culture filtrates of strain 13 produced clear zones in the radial diffusion assay, which indicates pectinase activity (Table 2). The largest clear zone was produced by pectinase activity in the filtrate obtained from pectin-containing media with no additional carbon source. Analy-
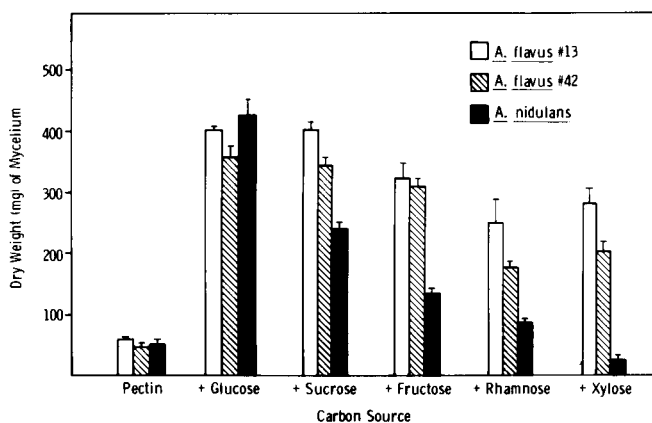

Fig. 1. Dry weight of mycelium of *Aspergillus flavus* strains 13 and 42 and of *A. nidulans* grown in media supplemented with selected sugars.

TABLE 2. Production of pectinases in culture by *Aspergillus flavus* strains 13 and 42[a]

	Pectinase activities detected by isoelectric focusing				Pectinase activities detected by radial diffusion assay (mm)[c]	
Carbon source[b]	P1	P2C	P3	PE	Clear zone	Red zone
Pectin	+/+	+/−	+/+	+/+	15.0/3.0	0/5.5
+ extract	+/+	+/−	+/+	+/+	13.0/5.0	0/2.0
+ glucose	−/−	+/−	−/−	−/−	10.0/0	0/0
+ glucose + extract	−/−	+/−	−/−	−/−	10.0/0	0/0
+ other sugars	−/−	+/−	−/−	−/−	10.0/0	0/0

[a] Pectinases were extracted according to procedures in text. Pectinases were subjected to radial diffusion assays to quantify total extractable pectinase activity and to isoelectric focusing (IEF) to identify individual pectolytic enzymes. + = Present, and − = absent. Numerator denotes strain 13; denominator denotes strain 42.

[b] All treatments contained 1% pectin. All culture treatments except two (pectin and pectin + extract) contained an additional carbon source at a level of 5%. Extract added to pectin and to pectin and glucose treatments was from cottonseed and made up 10% of the medium. In addition to glucose, other sugars tested were arabinose, cellobiose, fructose, maltose, mannose, raffinose, rhamnose, sucrose, and xylose.

[c] Pectinase activity was determined by measuring the width (mm) of clear and/or red zones.
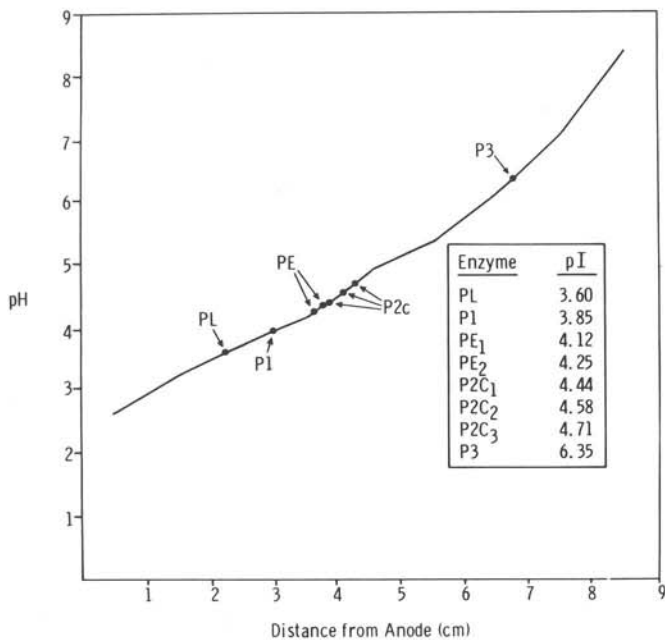

Fig. 2. A pH profile of a "typical" isoelectric-focusing (IEF) gel illustrating isoelectric points (pIs) of pectinases produced by *Aspergillus flavus* and *A. nidulans*. The pIs were determined by the IEF and pectin-agarose overlay technique as described in the text.

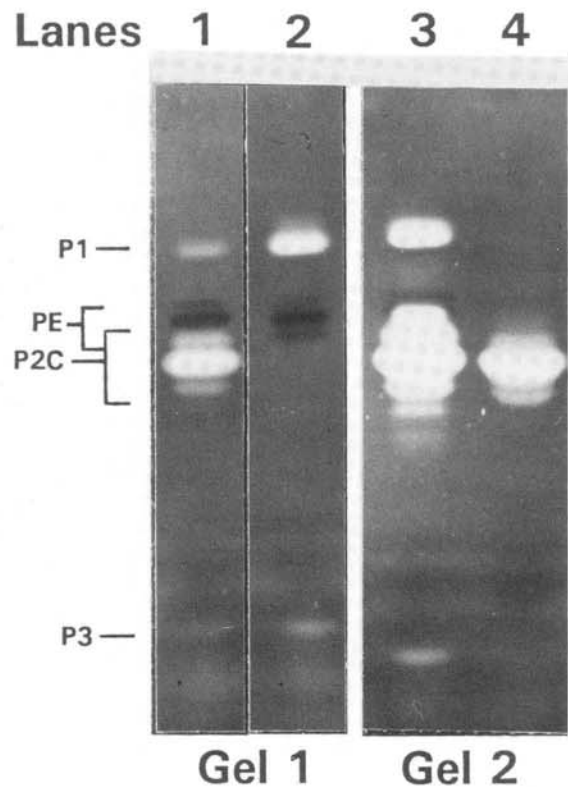| Enzyme	pI
PL	3.60
P1	3.85
PE$_1$	4.12
PE$_2$	4.25
P2C$_1$	4.44
P2C$_2$	4.58
P2C$_3$	4.71
P3	6.35

Fig. 3. Pectinases produced by *Aspergillus flavus* and resolved by the isoelectric focusing (IEF) and pectin-agarose overlay technique. Gels 1 and 2 were run at different times; consequently, positions of pectinase bands on the two gels vary slightly. Gel 1: lane 1 = strain 13 and lane 2 = strain 42 (both grown in a medium containing pectin as the only carbon source). Gel 2: lane 3 = strain 13 grown in pectin medium only (P2C partially masks PE) and lane 4 = strain 13 grown in a medium containing both pectin and glucose. Protein concentrations were as follows: lane 1 = 2.0 μg/μl; lane 2 = 4.1 μg/μl; lane 3 = 0.79 μg/μl; and lane 4 = 1.08 μg/μl.

sis of strain 13 pectinases by IEF (Fig. 2; Fig. 3, lanes 1,3) indicated the presence of the same pectinases in the filtrates of cultures containing either pectin as a sole carbon source or pectin + cottonseed extract. Strain 13 cultures grown in media with additional sugars contained only P2C (Fig. 3, lane 4).

The radial diffusion assay detected pectinase activity in filtrates of strain 42 only when that strain was grown in media containing pectin without additional sugars (Table 2). Both clear zones that resulted from polygalacturonase activities and red zones bordering the clear zones that resulted from PE activity were detected by this assay. Bands on pectin-agarose overlays of IEF gels indicated the presence of all *A. flavus* pectinases, except P2C, in filtrates of strain 42 cultures grown in pectin (Fig. 3, lane 2) or in pectin + cottonseed extract. No pectinases were present on overlays of strain 42 cultures grown on media with sugar supplements.

Pectinase activity was detected by the radial diffusion assay in filtrates from *A. nidulans* cultures grown in pectin or pectin + cottonseed extract; addition of cottonseed extract to pectin-containing media was observed to enhance pectinase production (Table 3). No activity was seen in filtrates from cultures grown in media with supplemental sugars. Resolution of pectinase bands by overlays of IEF gels indicated the presence of PL in filtrates from *A. nidulans* cultures grown with pectin and pectin + cottonseed extract. PL was also detected by IEF in some *A. nidulans*

filtrates with supplemental sugars that were pectinase-negative in the radial diffusion assay.

Characteristics of *A. flavus* and *A. nidulans* pectinases. *A. flavus* pectinases P1 and P3 (Fig. 3) and *A. nidulans* PL (Fig. 2) each resolved into a single band on IEF gel and pectin-agarose overlays. PE usually resolved into two bands (Figs. 2,3) but occasionally resolved in isolate 42 as three bands (Fig. 3, lane 2). Pectinase P2C resolved in most IEF and overlay assays as a three-band complex (Fig. 3, lanes 1,4), although additional bands in this region of the gel were sometimes observed (Fig. 3, lane 3). The pIs for P2C bands and for PE bands are very similar (Fig. 2),

and P2C can be seen partially masking PE on some gel overlays (Fig. 3, lane 3).

Fungal spread and aggressiveness. Tight locks were induced in inoculated cotton bolls by *A. flavus* strains 13 and 42 and by *A. nidulans*. Significant differences in decay (see rating scale for quantitating damage in Materials and Methods) of intercarpellary membranes were found among these three fungi ($P = 0.005$) by the Mann-Whitney U test. Strain 13 was the most virulent at decaying the membrane with an average (av) rating of 4.5. Membranes inoculated with strain 42 had significantly lower ratings (av = 2.1), as did membranes inoculated with *A. nidulans* (av = 1.0). Damage to the intercarpellary membranes was highly correlated with the amount of BGYF present on lint of uninoculated locules for strains 13 and 42 ($r = 0.91$; $P = 0.001$) (Fig. 4). Intercarpellary membranes of locules inoculated with *A. nidulans* developed only superficial discoloration. *A. flavus* strain 42 caused discoloration throughout the membranes and small tears in membrane fibers, whereas strain 13, in addition to causing discoloration, caused large tears.

DISCUSSION

Both *A. flavus* strains, 13 and 42, induced tight locks in inoculated cotton bolls and caused damage to intercarpellary membranes. The severity of membrane damage caused by strain 13 was significantly greater than that caused by 42 as evidenced by relatively large tears in membranes bordering locules that were inoculated with this strain. It has been suggested that this membrane may play a role in limiting fungal spread to uninfected locules (9). The relatively low level of BGYF (indicating the presence of *A. flavus*) in locules adjacent to those inoculated with the less virulent strain 42 and the relatively minor membrane decay support this concept.

In the present investigation, production of pectinases by strains 13 and 42 was examined on several additional substrates than those used in previous studies (4). The patterns of pectinases produced here are in general agreement with those previously observed in vivo and in vitro. Pectinase P2C, the major polygalacturonase activity produced by *A. flavus*, is consistently produced only by the high-virulence strain in vivo and in vitro on all carbon sources, and this activity may provide strain 13

TABLE 3. Production of pectate lyase (PL) in culture by *Aspergillus nidulans*

Carbon source[a]	PL activity detected by isoelectric focusing[b]	PL activity detected by radial diffusion assay (mm)[c]
Pectin	+	3.0
Pectin +		
extract	+	5.5
glucose	−	0
glucose + extract	−	0
arabinose	+	0
cellobiose	−	0
fructose	+	0
maltose	−	0
mannose	+	0
raffinose	+	0
rhamnose	−	0
sucrose	+	0
xylose	−	0

[a] All treatments contained 1% pectin. All culture treatments except one (containing pectin alone) contained an additional carbon source at a level of 5%. Extract added to pectin and to pectin and glucose treatments was from cottonseed and made up 10% of the medium.
[b] PL activity in *A. nidulans* was previously identified by Dean and Timberlake (11). Enzyme was extracted according to procedures in text and subjected to radial diffusion assays to quantify total extractable pectinase activity and to isoelectric focusing (IEF) to identify individual pectolytic enzymes. + = Present, − = absent.
[c] *A. nidulans* culture filtrates produced a clear zone when pectinase was present; no red zone (pectinesterase) was evident in radial diffusion assays.
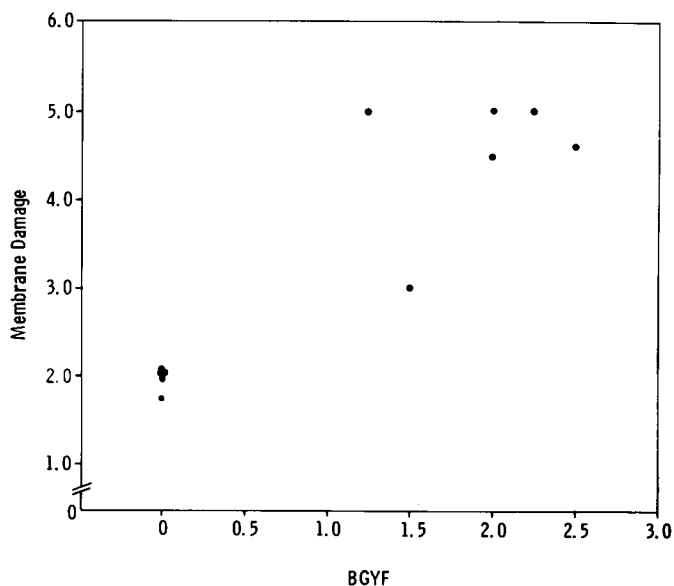
with greater ability than strain 42 (which consistently does not produce P2C) to decay intercarpellary membranes and spread to adjacent locules.

A. nidulans induced tight-lock formation in developing bolls, even though it is commonly classified as a saprophyte. However, the failure of *A. nidulans* to produce the same pectinases in bolls as in pectin-containing media may illustrate that elaboration of pectinases is not required to produce the tight-lock symptom. This would be consistent with findings of other studies that assessed the importance of fungal pectinases to symptom expression in cotton and tomato plants (12,18,22). The ability of fungal strains to spread between locules is apparently related to ability to decay the intercarpellary membrane. This conclusion is supported by the correlation observed between interlocule spread and membrane decay in bolls inoculated with *A. flavus* strains 13 and 42. Furthermore, tight-lock formation in locules inoculated with *A. nidulans* indicated that growth of this fungus occurred in inoculated locules. Thus, the failure of *A. nidulans* to cause either discoloration on the opposite side (from the inoculated locule) of the intercarpellary membranes or membrane tears may have resulted from the lack of pectinase production by *A. nidulans* in bolls inoculated with this fungus. This also points to a role for pectinases in fungal spread. The in vitro detection of PL in some filtrates of *A. nidulans* cultures grown in pectin-sugar media and the lack of detection in others may reflect different rates of depletion of various sugars during incubation. In PL-containing filtrates, this enzyme may have been induced after sugars had been depleted. Observed variations in PL detection between IEF-overlay and radial diffusion assays may be the result of sensitivity differences between these two assays. The IEF technique is apparently more sensitive.

A previous study suggested that catabolic repression of *A. nidulans* pectinases may be responsible for its weak pathogenic ability (11). In support of this theory, *A. nidulans* had low virulence and did not produce pectinases in immature boll locules. However, locules wound-inoculated with *A. nidulans* showed clear symptoms, suggesting that *A. nidulans* produces other virulence factors in wound-inoculated locules.

In the present study, pectinase P2C produced by *A. flavus* strain 13 was the only pectinase not catabolically repressed in culture media containing both pectin and sugar. This enzyme, previously shown to be constitutive (5), appears as a complex on IEF gels of inoculated boll extracts and culture filtrates and thus may be composed of several isozymes (5). On the basis of

Fig. 4. Correlation of membrane ratings with "bright greenish yellow fluorescence" (BGYF) ratings. Both ratings are measurements of fungal virulence (see text). Membranes and lint analyzed were from cotton boll locules inoculated with either *Aspergillus flavus* strain 13 or 42.

the performance of 16 strains, the ability to produce pectinase P2C has been shown to be a common characteristic of highly virulent *A. flavus* strains (4,8). The ability of strain 13 to consistently produce a major polygalacturonase activity (P2C) in culture and in bolls under no apparent catabolite repression may give this strain higher virulence over strains that produce catabolically regulated pectinases. Reduced virulence has previously been associated with catabolite repression in *R. solani* (24). With *R. solani*, a correlation was demonstrated among the ability of this fungus to grow in glucose and other sugars, the inhibition of disease development on cotton seedlings, and the repression of pectinases. A similar correlation occurred in the present study. The growth of all three aspergilli studied here was greater in media containing supplemental sugars than in media with either pectin as the sole carbon source or pectin + cottonseed extract, despite the fact that supplemental sugars repressed all pectinases except P2C.

An apparent anomaly in the present study was the observed production of pectinases P1, P3, and PE by *A. flavus* (all catabolically repressed by the sugars tested) in the high-sugar environment of immature boll tissues. The reducible sugar content in susceptible bolls ranges from approximately 1 to 2% of the dry weight, and the content of the liquid surrounding the lint is probably much higher (6,13,16,20). Pectinase production in bolls may indicate that *A. flavus* pectinases are less sensitive to catabolite repression than are pectolytic activities produced by *A. nidulans*. However, *A. flavus* strains grew better than *A. nidulans* in vitro on carbon sources commonly found in cotton boll tissues (i.e., sucrose). Thus, *A. flavus* may deplete sugars in local environments more rapidly than *A. nidulans* and subsequently release repression and permit induction of pectinases.

Strain 13 did not produce detectable PE in cotton bolls or on autoclaved cottonseed but did produce this enzyme in culture. Strain 42, however, produced PE on all substrates. Strain 13 may produce PE in the prior two cases at levels below our limit of detection. Furthermore, *A. flavus* PE has nearly the same pI as P2C (Fig. 2), and strain 13 produces very high P2C levels. Therefore, high levels of P2C may tend to mask PE during both activity assays of IEF gels and radial diffusion plates (10).

LITERATURE CITED

1. Adye, J., and Mateles, R. I. 1964. Incorporation of labelled compounds into aflatoxins. Biochim. Biophys. Acta 86:418-420.
2. Ashworth, L. J., Jr., and McMeans, J. L. 1966. Association of *Aspergillus flavus* and aflatoxins with a greenish yellow fluorescence of cotton seed. Phytopathology 56:1104-1105.
3. Ashworth, L. J., Jr., Rice, R. E., McMeans, J. L., and Brown, C. M. 1971. The relationship of insects to infection of cotton bolls by *Aspergillus flavus*. Phytopathology 61:488-493.
4. Cleveland, T. E., and Cotty, P. J. 1991. Invasiveness of *Aspergillus flavus* isolates in wounded cotton bolls is associated with production of a specific fungal polygalacturonase. Phytopathology 81:155-158.
5. Cleveland, T. E., and McCormick, S. P. 1987. Identification of pectinases in cotton bolls infected with *Aspergillus flavus*. Phytopathology 77:1498-1503.
6. Compton, J., and Haver, F. E., Jr. 1940. Studies on the developing cotton fiber. II. Identification and estimation of the reducing sugars. Contrib. Boyce Thompson Inst. 11:281-290.
7. Cooper, R. M., and Wood, R. K. S. 1975. Regulation of synthesis of cell wall degrading enzymes by *Verticillium albo-atrum* and *Fusarium oxysporum* f. sp. *lycopersici*. Physiol. Plant Pathol. 5:135-156.
8. Cotty, P. J. 1989. Virulence and cultural characteristics of two *Aspergillus flavus* strains pathogenic on cotton. Phytopathology 79:808-814.
9. Cotty, P. J. 1989. Effect of cultivar and boll age on aflatoxin in cottonseed after inoculation with *Aspergillus flavus* at simulated exit holes of the pink bollworm. Plant Dis. 73:489-492.
10. Cotty, P. J., Cleveland, T. E., Brown, R. L., and Mellon, J. E. 1990. Variation in polygalacturonase production among *Aspergillus flavus* isolates. Appl. Environ. Microbiol. 56:3885-3887.
11. Dean, R. A., and Timberlake, W. E. 1989. Production of cell wall-degrading enzymes by *Aspergillus nidulans*: A model system for fungal pathogenesis of plants. Plant Cell 1:265-273.
12. Durrands, P. K., and Cooper, R. M. 1988. The role of pectinases in vascular wilt disease as determined by defined mutants of *Verticillium albo-atrum*. Physiol. Mol. Plant Pathol. 32:363-371.
13. Elsner, O., Hani, J., and Lubenevskaya. 1983. The sugar content in cotton lint of growing bolls. Coton Fibres Trop. 38:223-225.
14. Giltrap, N. J., and Lewis, D. H. 1982. Catabolite repression of the synthesis of pectin-degrading enzymes by *Suillus luteus* (L.Ex Fr.) S. F. Gray and *Hebelone oculatum* Bruchet. New Phytol. 90:485-493.
15. Goodenough, P. W., and Kempton, R. J. 1974. Regulation of the synthesis of an extracellular polygalacturonase by *Pyrenochaeta lycopersici* when grown in media with limited carbon sources. Phytopathol. Z. 81:78-84.
16. Grindley, D. N. 1950. Changes in composition of cottonseed during development and ripening. J. Sci. Food Agric. 1:147-151.
17. Horton, J. C., and Keen, N. T. 1966. Sugar repression of endopolygalacturonose and cellulase synthesis during pathogenesis by *Pyrenochaeta terrestris* as a resistance mechanism in onion pink root. Phytopathology 56:908-916.
18. Howell, C. R. 1976. Use of enzyme-deficient mutants of *Verticillium dahliae* to assess the importance of pectolytic enzymes in symptom expression of Verticillium wilt of cotton. Physiol. Plant Pathol. 9:279-283.
19. Maldonado, M. C., Strasser de Saad, A. M., and Callieri, D. 1989. Catabolite repression of the synthesis of inducible polygalacturonase and pectinesterase by *Aspergillus niger* sp. Curr. Microbiol. 18:303-306.
20. Meinert, M. C., and Delmar, D. P. 1977. Changes in biochemical composition of the cell wall of the cotton fiber during development. Plant Physiol. 59:1088-1097.
21. Mellon, J. E., and Lee, L. S. 1985. Elicitation of cotton isoperoxidases by *Aspergillus flavus* and other fungi pathogenic to cotton. Physiol. Plant Pathol. 27:281-288.
22. Puhalla, J. E., and Howell, C. R. 1975. Significance of endo-polygalacturonase activity to symptom expression of Verticillium wilt in cotton, assessed by the use of mutants of *Verticillium dahliae*. Kleb. Physiol. Plant Pathol. 7:147-152.
23. Reid, J. L., and Collmer, A. 1985. Activity stain of rapid characterization of pectic enzymes in isoelectric focusing and sodium dodecyl sulfate-polyacrylamide gels. Appl. Environ. Microbiol. 50:615-622.
24. Weinhold, A. R., and Bowman, T. 1974. Repression of virulence in *Rhizoctonia soloni* by glucose and 3-O-methyl glucose. Phytopathology 64:985-990.