Invasiveness of *Aspergillus flavus* Isolates in Wounded Cotton Bolls is Associated with Production of a Specific Fungal Polygalacturonase

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We gratefully acknowledge the excellent technical assistance of Christopher J. Foell and Edwin J. Bowers.
Accepted for publication 12 September 1990.

ABSTRACT

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.

Aspergillus flavus produces several pectolytic enzymes during invasion of cotton bolls. To investigate the importance of pectinases in this process, pectinase production by 16 isolates of *A. flavus*, with known ability to spread between cotton boll locules and rot them, was quantified by a cup-plate assay. Aggressiveness of isolates during the infection process was correlated with their ability to secrete pectinase(s) on sterilized cottonseed, on pectin-containing liquid media, and in living host tissues. Four isolates with reduced ability to spread through boll tissues and rot them lacked a major endopolygalacturonase activity that always was present in highly aggressive isolates. Thus, certain pectolytic enzymes produced by *A. flavus* during host infection contribute to fungal aggressiveness.

Pectinases produced by plant pathogens (4,8,9,11,15–17,21) often are involved directly in plant tissue maceration during disease development and in rendering plant cell walls more susceptible to attack by other cell wall-degrading enzymes (5,14). Pectinases also may be important for invasion and rotting of cotton bolls by the aflatoxigenic mold, *Aspergillus flavus* Link ex. Fries (7). This fungus requires a wound (3) or natural opening (18) to invade plant tissues, but after initial infection by *A. flavus*, pectinases might be important for establishment of this fungus in locular tissues (7). We have shown that *A. flavus* produces at least three pectinase activities that increased with time during infection of wound-inoculated locules, and we have theorized that these enzymes might be important for growth through the lint and into the cottonseed (7), where aflatoxins are produced (3).

Recently, several diverse isolates of *A. flavus* were shown to vary in their ability to rot bolls and to spread between cotton locules (10). This suggested that isolates of *A. flavus* with varying aggressiveness are present in field environments. In other plant-fungus interactions, investigators have reported "natural" variation in pathogenicity of native fungal pathogens, and furthermore, pathogenicity of the isolates was correlated with their variable ability to produce specific pathogenicity-related enzymes. For example, high pathogenicity of native isolates of *Fusarium solani* f. sp. *pisi* infecting pea (25) and of *F. s.* f. sp. *phaseoli* infecting bean (24) was correlated with the ability of individual isolates to produce phytoalexin-detoxifying enzymes.

We have speculated that the varying aggressiveness observed in native isolates of *A. flavus* that invade cotton bolls (10) might be due to a differential ability of the various isolates to produce pathogenicity-related enzymes of an unknown nature. Results of a recent preliminary report (6) indicated that *A. flavus* pectinases might be the pathogenicity-related enzymes involved in aggressiveness of fungal isolates. The purpose of the present study was to investigate the hypothesis that the ability of various native isolates of *A. flavus* to infect cotton bolls is related to the ability of the isolates to secrete certain pectolytic enzymes during the infection process.

MATERIALS AND METHODS

Cultures. Isolates of *A. flavus* and their maintenance have been described (10). A liquid medium (PLM) for production of pectolytic enzymes was used to culture all fungal isolates. The medium was similar to that employed in a previous investigation (1), except that the carbon source was changed (7) to include 1% (w/v) citrus pectin (grade I, 7.7% methoxyl content, Sigma Chemical Co., St. Louis, MO) and a cottonseed infusion (made by autoclaving 100 g of cottonseed in 1 L of deionized water and then filtering through Whatman No. 4 paper) was added to the medium at a concentration of 10% (v/v). Cultures were initiated by inoculation of 25-ml aliquots of PLM in 50-ml Erlenmeyer flasks with about 10^5 conidia. Cultures were shaken (150 rpm) for 3 days at 28 C to produce mycelia and extracellular pectinases.

Isolation of pectinases from culture filtrates. Mycelia were separated from the culture filtrates by vacuum filtration through Whatman No. 4 filter paper. Mycelia were dried at 60 C to obtain mycelial dry weights. Filtrates were centrifuged at 10,000 *g* for 10 min, and a portion of each supernatant (about 1.5 ml) was stored at 4 C with about 0.1 ml of $CHCl_3$ as a preservative. A portion of each supernatant (0.1 ml) was reserved for pectinase assays by the cup-plate method (12). The remainder of each supernatant (1.4 ml) was dialyzed (12,000 molecular-weight cutoff) for 4 hr against 2 L of 1% glycine and then concentrated about 10-fold by overlaying dialysis bags with crystalline sucrose as previously reported (7). Concentrated pectinase activities were analyzed by isoelectric focusing (IEF) (7).

Inoculation of cotton bolls and extraction of fungal pectinases from locular tissues. To determine if the same pattern of pectinase production occurred in developing cotton bolls as in fungal culture, pectinase production by selected fungal isolates was monitored in inoculated bolls. Two highly aggressive isolates, 13 and 36, and two isolates with low aggressiveness, 12 and 25, were used in boll inoculations. Cotton plants (cultivar Delta Pine 90) were grown in the greenhouse, and two locules of each unopened boll were wound inoculated as described previously (10). To extract fungal pectinases produced in inoculated bolls, the contents of inoculated locules were removed and extracted in 10 ml of 0.1 M acetate buffer (pH 5.0) as previously reported (7). Locule extracts were dialyzed and concentrated 10-fold as described above and reserved for pectinase assays and IEF.

Inoculation of sterilized cottonseed and extraction of pectinase activity from seed tissues. Cottonseed from Delta Pine 90 were autoclaved 15 min at 121 C and dried in an oven for 24 hr at 60 C. About 15 g of sterile, dried cottonseed in sterile 9-cm petri plates was inoculated with about 10^7 fungal conidia of each of the 16 *A. flavus* isolates in 3 ml of sterile deionized water (20% final seed moisture content). Plates were sealed with Parafilm

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to prevent drying and incubated at 32 C for 7 days. Seed were transferred to test tubes containing 5 ml of 0.1 M sodium acetate buffer and allowed to soak with occasional agitation for 16 hr at 4 C. Seed extracts containing pectinase activity were transferred to microfuge tubes and centrifuged at 10,000 *g*, and supernatants (1 ml) were removed and stored with 0.1 ml of $CHCl_3$ at 4 C for eventual use in pectinase assays.

Pectinase assays. Cup-plate assays (12) were used to quantify pectinase activity in concentrated culture filtrates, seed extracts, and extracts of bolls infected with *A. flavus*. Cup-plate assays were modified slightly in the present study; assays were initiated by placing about 60 μl of pectinase-containing preparations in cylindrical wells (5 mm in diameter and 3 mm deep) cut from a pectin (0.5%)-agarose (1.0%) layer containing 0.1 M sodium acetate, pH 5.0, in 9-cm petri plates. Plates were covered, incubated at 37 C for 24 hr, and then stained 20 min with a solution of 0.05% ruthenium red (Sigma Chemical Co.). Plates then were destained in deionized water for 30 min, and radii (minus the radius of the well) of unstained clear zones (indicating pectolytic activity) were measured. One pectinase unit was defined as the amount of pectinase activity resulting in a clear zone with a 1-mm radius under the conditions of the standard cup-plate assay (above); when mycelial dry weight could be quantitated (in PLM cultures), pectinase units were expressed per gram of mycelial dry weight.

The presence of previously described (7) pectolytic activities P1, P2c, and P3 in culture filtrates and in inoculated bolls was determined by IEF and a pectin-agarose overlay technique (7,23). Overlaid gels were incubated 30 min at 37 C, and overlays were removed and stained with ruthenium red to visualize zones of pectolytic activity.

Statistical analysis. Pearson product-moment correlations were calculated for relationships between isolate aggressiveness as previously determined (10) and ability to produce pectinase activity on various substrates. Analyses were performed with the Statistical Analysis System (SAS Institute, Inc., Cary, NC).

TABLE 1. Pectinase production on sterilized cottonseed and in liquid culture and aggressiveness during invasion of cotton bolls by various isolates of *Aspergillus flavus*

		Pectinase units[a]		Measures of aggressiveness	
Experiment	Isolate	In seed	In culture (per gram dry weight of mycelia)	BGYF[b]	IL/UL[c]
I	36	8.9	185	0.79	0.45
	13	7.1	305	1.11	0.47
	6	7.4	317	0.93	0.49
	11	7.8	224	0.79	0.53
	42	4.4	104	0	0.55
	3	7.6	195	0.30	0.57
	12	3.4	175	0.26	0.57
	25	4.4	95	0	0.62
II	16	9.9	261	1.18	0.52
	41	7.8	203	0.88	0.55
	54	6.4	255	1.24	0.56
	60	8.6	198	0.88	0.56
	70	8.0	184	0.84	0.60
	65	8.0	182	0.64	0.61
	58	4.5	52	0.19	0.63
	66	8.2	179	0	0.70

[a] One pectinase unit is the amount of pectinase resulting in a 1-mm zone of clearing during cup-plate assays.

[b] Fungally induced bright greenish yellow fluorescence (BGYF) (2,20) on lint of locules adjacent to wound-inoculated locules; degree of fluorescence was used as an indication of fungal spread through boll tissues (10).

[c] Dry weight of inoculated locules/dry weight of uninoculated locules (10). This is an estimate of the ability of fungal isolates to degrade or impede development of locular tissues compared with uninoculated controls.

RESULTS

A. flavus isolates 12, 25, 42, and 58, with reduced ability to spread through and rot cotton bolls (10), consistently yielded pectinase activity that was lower than that of more aggressive isolates assayed in this study, either when growing on cottonseed or in a liquid medium (Table 1). Also, isolates 12 and 25 with low aggressiveness showed greatly reduced ability to produce pectinase activity (less than 2-mm radius of clearing on cup-plate assays) in inoculated cotton bolls relative to aggressive isolates 13 and 36 (greater than 7-mm radius of clearing on cup-plate assays) (data not shown).

The ability of *A. flavus* isolates to spread through or rot boll tissues and their ability to produce pectinase activity in culture or on sterilized cottonseed (Table 1) were statistically correlated. The strongest correlations were observed between levels of bright greenish yellow fluorescence (BGYF) in uninoculated cotton boll locules (adjacent to inoculated locules), an assay of fungal spread (10), and levels of fungal pectinase activity produced in liquid culture (Table 2, Fig. 1). Correlations between the amount of reduction in dry weight of inoculated locules relative to uninoculated locules (IL/UL) and pectinase-producing ability by isolates growing in liquid culture were not significant in either experiment alone; however, when data from both experiments were combined, a significant correlation existed (Table 2). Statistically significant correlations also were observed between fungal aggressiveness and ability to produce pectinase on sterilized cottonseed (Table 2), but these correlations generally were not as great as those observed between fungal aggressiveness and levels of pectinase produced in liquid culture.

To assess levels of individual pectinases produced by *A. flavus* isolates in liquid culture or in infected bolls, the pectinase activities were separated by IEF, and their levels were determined visually on pectin-agarose overlays (Table 3, Fig. 2). Isolates 36, 13, 6, 11, 3, 16, 41, 54, 60, 70, 65, and 66 produced the same three pectinases, P1, P2c, and P3, in liquid culture as observed in a previous study (7). However, isolates 12, 25, 42, and 58 did not produce detectable quantities of polygalacturonase P2c when growing in liquid culture (Fig. 2, Table 3).

Two of the *A. flavus* isolates with low aggressiveness (12 and 25) did not produce polygalacturonase P2c but produced pectinases P1 and P3 in cotton locules, whereas two highly aggressive isolates (13 and 36) produced all three pectinases in infected cotton bolls (Table 3). Thus, the same *A. flavus* pectinases observed in inoculated liquid culture were obtained from inoculated bolls.

TABLE 2. Correlation coefficients and probabilities of correlations between ability of an isolate to produce pectinase activity and isolate aggressiveness assayed by the BGYF[a] and IL/UL[b] methods

	Experiment I		Experiment II		Combined experiments	
Correlation	*r*[c]	*P*	*r*	*P*	*r*	*P*
BGYF vs. culture pectinase[d]	0.91	0.002	0.77	0.027	0.79	0.003
BGYF vs. seed pectinase	0.74	0.003	0.37	0.370	0.60	0.014
IL/UL vs. culture pectinase	−0.68	0.060	−0.57	0.140	−0.59	0.020
IL/UL vs. seed pectinase	−0.71	0.048	−0.33	0.429	−0.27	0.318

[a] Bright greenish yellow fluorescence.

[b] Dry weight of inoculated locules/dry weight of uninoculated locules.

[c] *r* and *P* are the correlation coefficient and probability level of the correlation, respectively.

[d] One pectinase unit is the amount of pectinase resulting in a 1-mm zone of clearing during cup-plate assays. A scatter plot of data for BGYF vs. culture pectinase, showing the best statistical correlation of all the comparisons, is shown in Figure 1.

DISCUSSION

A. flavus was shown previously to produce large quantities of pectinases during invasion of cotton bolls (7). In the current study, we found a strong correlation between pectinase-producing ability and the aggressiveness of isolates of *A. flavus* during invasion of cotton bolls. Aggressiveness of each isolate was quantified by measuring two parameters: ability to deteriorate or rot developing cotton locules, and ability to spread from inoculated locules to adjacent locules (Table 1, Fig. 1). Polygalacturonase-producing ability in vitro was most highly correlated with the ability of an isolate to spread between locules, resulting in production of BGYF (2); this fluorescent compound results from conversion of the fungal product, kojic acid, to BGYF by cotton lint peroxidases (20). Because the presence of BGYF is an indicator of the fungus-lint association, the compound is a useful measure of fungal spread in cotton bolls. In the present investigation, the association of the ability of a fungal isolate to produce BGYF in locules adjacent to inoculated locules with the ability of an isolate to produce polygalacturonase suggested that high polygalacturonase-producing isolates have an increased capacity to spread between locules and thus to infect lint and cottonseed throughout the boll.

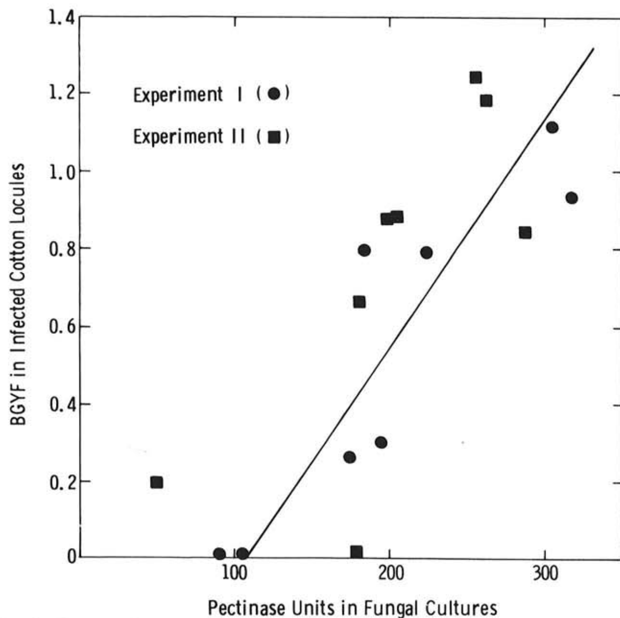

Fig. 1. Correlation between the ability of *Aspergillus flavus* isolates to spread between locules in infected bolls (assayed by the bright greenish yellow fluorescence [BGYF] method) and their ability to produce pectinase activity in a liquid medium. Pectinase units are expressed per gram of dry weight of mycelia. ● = isolates representing Experiment I; ■ = isolates representing Experiment II.

TABLE 3. Pectinases of *Aspergillus flavus* isolates in culture filtrates and in inoculated bolls

Specific pectinase	Specific pectinase detected in culture filtrates/inoculated bolls[a]					
	25[b]	42	58	12	13	36
P1	+/+	+/+	+/na	+/na	+/+	+/+
P2c	−/−	−/−	−/na	−/na	+/+	+/+
P3	+/+	+/+	+/na	+/na	+/+	+/+

[a] Individual pectinases detected by isoelectric focusing (IEF). + indicates that pectinase was detected; − indicates that pectinase was not detected; "na" indicates that the preparation was not assayed. The numerator corresponds to pectinase activity produced in culture filtrate; the denominator corresponds to activity produced in inoculated bolls.

[b] Numbers are strain designations. Pectinase activity profiles observed after IEF consisted of pectinases P1, P2c, and P3 in highly aggressive isolates 13 and 36. These profiles were similar to those of isolates 6, 11, 3, 16, 41, 54, 60, 70, 65, and 66 (not shown). Isolates 25, 42, 58, and 12 were low-aggression isolates.

Four isolates of *A. flavus* with the lowest pectinase-producing ability in culture and very low levels of aggressiveness during invasion of cotton bolls lacked detectable levels of a major endo-polymethylgalacturonase activity (P2c) (Fig. 2). This polygalacturonase (P2c), as well as pectinases P1 and P3, had been identified previously (7) in cultures and in bolls inoculated with an aggressive isolate of *A. flavus*. All three were present in culture filtrates of all highly aggressive isolates. However, isolate 66 deviated from the above pattern of results because it demonstrated very low aggressiveness during invasion of bolls (Table 1) but was capable of producing polygalacturonase P2c. Isolate 66 may lack the ability to express some other fungal trait (besides fungal pectinases) that is required for fungal aggressiveness in cotton bolls.

Production or lack of production of P2c by fungal strains is probably the primary determining factor in the strong correlation between pectinase production in culture and the ability to spread from infected locules to adjacent locules (Fig. 1). The relationship between isolate aggressiveness and the ability of isolates to produce P2c suggests that this pectinase is an important determinant of aggressiveness during infection of cotton bolls by *A. flavus*.

Polygalacturonase P2c may consist of a complex of pectolytic isozymes that could be only partially resolved by IEF in this study and in a previous investigation (7). This hypothesis is supported by the discovery of fungal isolates 12, 25, 42, and 58, which lack the ability to produce P2c. These four isolates lack the ability to produce all of the two or more activities associated with P2c, suggesting that this polygalacturonase consists of two or more genetically related isozymes.

The correlation between pectinase production and the ability of an isolate to spread from wound-inoculated locules to adjacent locules and the indication that the presence or absence of polygalacturonase P2c was the main factor determining the observed correlations imply that P2c is important for fungal spread into adjacent locules. The relatively low correlation between the ability to deteriorate locules and pectinase production may indicate that P2c is relatively less important in the deterioration of inoculated locules.

It has been clearly demonstrated that pectinases produced by several plant pathogenic bacteria function as pathogenicity and/or virulence factors (19,22). However, the role of pectinases in plant diseases incited by fungal pathogens is less clear. In *Verticillium*-induced disease syndromes, it was found that pectinases do not serve as pathogenicity factors, and their function as virulence factors either has been discounted (16) or only partially demonstrated (13).

The results of the present investigation suggested a significant role for certain pectinases of *A. flavus* in fungal aggressiveness. A very strong relationship between fungal spread/colonization and production of a specific pectinase (P2c) was observed; *A. flavus* isolates lacking polygalacturonase P2c had greatly reduced ability to spread between cotton locules. Therefore, *A. flavus* and other fungal-wound pathogens may be similar to certain soft-rotting bacteria (19,22) in having a significant dependence on pectinases during infection of plant tissues.

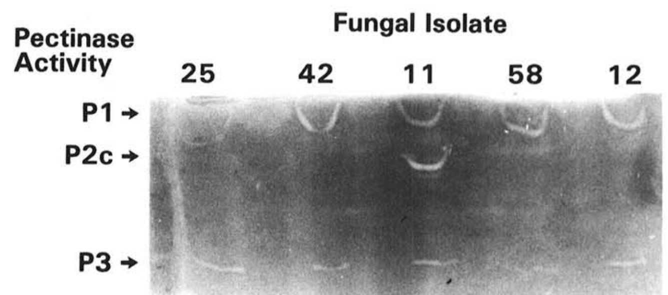

Fig. 2. Isoelectric focusing of pectinases produced by aggressive isolate 11 and four low-aggression isolates of *A. flavus* (isolates 12, 25, 42, and 58). Arrows show positions of pectinases P1, P2c, and P3.

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