Relationship Between Polygalacturonase Activity and Cultural Characteristics of Verticillium Isolates Pathogenic in Cotton

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ABSTRACT

Analyses were made of the pectic enzymes produced in vitro by isolates of *Verticillium* that differed in virulence in cotton, *Gossypium hirsutum* cultivars 'Acala 4-42' and 'Deltapine Smoothleaf'. A single predominant pectic enzyme, endopolygalacturonase (PG), was detected in dialyzed culture liquids of a severe and two intermediate isolates of *V. albo-atrum* and a mild isolate of *V. nigrescens* grown in standing culture on pectin-glucose-potato broth. Regardless of virulence, culture age, pH, composition of the medium, or method of enzyme preparation, lyases, pectinmethylesterase, and other pectic hydrolases were present only in trace quantity or not detected. Among the four isolates, PG activity was inversely correlated with virulence, and when tests were expanded to include 25 additional isolates of *V. albo-atrum*, the relative activity of PG per μg protein averaged 3.09, 1.40, and 0.99 in eight mild, 13 intermediate, and eight severe isolates, respectively.

Mild isolates were principally mycelial, whereas severe isolates developed black microsclerotial colonies on potato-dextrose agar. In liquid culture, none of the 29 isolates produced microsclerotia. Mycelial variants obtained from a severe microsclerotial isolate showed a six-to-tenfold increase in PG activity, and a concomitant, but not proportional, decrease in virulence. The mycelial variants, like their severe microsclerotial parent, still caused the defoliation of infected plants, though the defoliation rate was significantly reduced.

Peroxidase and polyphenol oxidase were not detected in culture liquids of sclerotial or mycelial isolates. Polyphenol oxidase and, especially, peroxidase were present in cotton stem tissues and vascular fluids, but were unaffected by disease development or symptom severity. Preparations from healthy and *Verticillium*-infected plants exhibited only traces of PG activity. Phytopathology 60:641-646.

Although the involvement of pectic enzymes in many host-parasite interactions is uncontested, their importance in pathogenesis is often doubtful. This study was conducted to determine the importance of pectic enzymes in *Verticillium* wilt of cotton.

The symptoms induced by *Verticillium* in cotton suggest the involvement of at least two mechanisms for pathogenesis, i.e., vascular occlusion and phytotoxic activity. The wilting occasionally shown by newly infected plants is indicative of a water hardship and perhaps vascular occlusion. Although true wilting is not a prominent symptom, it is possible, in spite of contradictory evidence (4, 14), that pectic or other macerating enzymes liberate high-molecular wt fragments capable of occluding the vessels of infected plants (3, 5).

Other symptoms induced by *Verticillium* in cotton, like vascular discoloration and chlorosis and necrosis of leaves, suggest that phytotoxins might also be involved in pathogenesis. In many studies, the phytotoxicity of *Verticillium* has been demonstrated indirectly by the wilting induced in excised leaves and cuttings placed in culture filtrates. Accelerated wilting in such instances is often a nonspecific response induced by many of the components found in culture liquids. Necrosis of vascular parenchyma, while perhaps a better indication of phytotoxicity, is also often a nonspecific response. The chlorotic and necrotic leaf symptoms most characteristic of *Verticillium* infection in cotton have not yet been duplicated in tissues exposed to cell-free preparations of the pathogen. Hence, evidence also remains circumstantial for the involvement of phytotoxins in *Verticillium* wilt of cotton.

Both pectic enzymes (principally polygalacturonase) and phytotoxins have been observed in culture liquids of the *Verticillium* fungus. In certain instances, pectic enzyme activity is directly related to the phytotoxicity of culture filtrates (11, 12). As yet the phytotoxic materials involved have been characterized only partially (7, 18, 19), but it is possible that the immediate reaction products of pectic enzymes may be included among them.

More recently, lyase enzymes capable of degrading pectic substances have been characterized (1, 6). If lyases are produced by *Verticillium*, their importance in pathogenesis in cotton should be determined. An important part of this study, therefore, was concerned with the detection of both hydrolytic and *trans*-eliminative pectic enzymes in culture liquids of *Verticillium* isolates differing in virulence in cotton.

MATERIALS AND METHODS.—*Collection of isolates and virulence tests.*—Isolations were made from field-grown cotton plants showing symptoms of *Verticillium* wilt and occasionally from other hosts in California. Twenty-eight isolates of *V. albo-atrum* Reinke & Berth. and one isolate of *V. nigrescens* Pethybr., which was identified according to the criteria of Isaac (9, 10), were isolated on potato-dextrose agar (PDA). Single conidia from each *Verticillium* colony were transferred to PDA slants, and only resultant colonies that resembled the parent were retained. The single-cell cultures were maintained thereafter by periodic mass

transfer to fresh PDA slants. This mass-transfer procedure avoided undue morphological alterations in succeeding cultures.

The isolates were classified as either white, gray, or black. White isolates were wholly mycelial, black isolates appeared wholly microsclerotial, and gray isolates produced both hyaline mycelium and black microsclerotia.

Each isolate was tested for virulence in greenhouse-grown cotton plants, *Gossypium hirsutum* L. 'Acala 4-42' and 'Deltapine Smoothleaf'. Fresh conidial suspensions of each isolate washed from 10-day-old cultures on PDA were used as inocula. The suspensions were adjusted with distilled water to 10^6 viable conidia/ml based on hemocytometer counts or optical-density measurements at 540 mμ. Plants were inoculated either by dipping their roots into the suspension or by introducing approximately 0.05 ml of the suspension into hypocotyls with a dissecting needle. Control plants were treated similarly, but with water or an autoclaved suspension of conidia.

Usually 6-10 plants, 8-12 weeks old, of each variety were used for each isolate and for each method of inoculation. Disease symptoms were observed at intervals after inoculation, and the isolates were classified as mild (slight stunting and little or no leaf symptoms), intermediate (increased stunting, prominent epinasty, and interveinal chlorosis and necrosis of leaves), and severe (defoliation and, in some instances, death in addition to the stunting and leaf symptoms).

Growth of isolates and methods of enzyme preparation.—To determine the ability of the isolates to produce pectic enzymes, a number of liquid media and several methods for preparing enzymes in culture liquids were evaluated. The following techniques were found most useful.

All isolates were grown at 22-23 C on 30 ml of stationary medium in 125-ml Erlenmeyer flasks. The medium contained 1% glucose, 1% pectin (Sunkist Growers, Ontario, California), and the broth from 200 g of potatoes/liter of distilled water. The medium, autoclaved for 10 min at 121 C, was inoculated with mycelial discs of each isolate. The pH value of the unbuffered medium (4.5) was not altered appreciably after 14 days of fungal growth. During this period, all isolates produced abundant mycelia and conidia, but few or no microsclerotia.

After 10-14 days of incubation, duplicate cultures were combined in tared centrifuge tubes and centrifuged for 20 min at 20,000 *g* at 2 C. After centrifugation, the supernatant containing extracellular enzymes was dialyzed overnight against distilled water at 2 C and used directly for enzyme tests. Protein in duplicate 1-ml aliquots of the dialyzed culture liquids was estimated by the method of Lowry et al. (13), and a standard curve prepared from bovine serum albumin. Fungal pellets remaining in the tubes were dried at 80 C to a constant wt for dry-wt measurements. Fungal dry wt in 10-day-old cultures was 80-100 mg flask.

Enzyme assays.—Pectic enzymes were assayed viscometrically at 25 C with No. 300 Ostwald-Fenske viscometers. Reaction mixtures contained 5 ml of 1.1% pectin or sodium polypectate substrates in 0.1 M phosphate buffer at pH 5.5, or in 0.1 M Tris [tris(hydroxymethyl)amino methane] at pH 7, 8, or 9, and 2 ml of dialyzed enzyme or an appropriate dilution thereof. Reaction mixtures were also subjected to reducing-sugar tests (16), chromatography (2), thio-barbituric acid tests (17), and ultraviolet absorption analysis at 230 mμ to detect ketodeoxygalacturonic acid. Pectinmethylesterase was assayed by a titrimetric method with 0.01 N sodium hydroxide to determine the change in milli-equivalents of acid in reaction mixtures at pH 4.5, 5.5, and 6.5.

Tests for other enzymes included the ascorbic acid-benzidine reaction for peroxidase (8) and oxygen electrode assays for polyphenol oxidase using catechol, caffeic acid, tyrosine, and chlorogenic acid substrates. All enzyme assays included boiled-enzyme or water controls.

The above techniques were also used to measure polyphenol oxidase, peroxidase, and pectic enzyme activity in extracts of cotton stems and in tracheal fluids collected with suction from decapitated stems of plants grown in the field or greenhouse.

RESULTS.—Initial studies of pectic-enzyme production by *Verticillium* involved four representative isolates, 68, 53, SS4, and T9, which differed in both virulence in cotton and culture type on PDA. Isolate 68, classed as mild, was a mycelial form of *V. nigrescens*; isolates 53 and SS4, classed as intermediate, were predominantly microsclerotial; and isolate T9, classed as severe, was a microsclerotial form of *V. albo-atrum*.

Assays of 10-day-old standing cultures grown on pectin-glucose-potato broth detected a single major pectic enzyme, endopolygalacturonase (PG), in the culture liquids of each isolate. Other pectic hydrolases, lyases, and pectinmethylesterase were either present in only trace quantity or not detected. By day 10, standing liquid cultures of the four isolates contained approximately 80% of the PG activity present at 20 days, but at no time during this period were other pectic enzymes detected in more than trace quantity.

Since the isolates appeared capable of producing only PG under these cultural conditions, several procedural modifications were explored that might affect the spectrum of pectic enzymes produced by the fungi or detected in the culture liquids. The isolates were grown on a medium of pectin, glucose, and inorganic salts buffered at either pH 5, 7, or 9. Such modifications, and others such as omitting pectin from the media, resulted only in reducing total PG activity in culture liquids. All four isolates produced PG in high constitutive quantities, but total PG activity was increased as much as 50% by the presence of pectin in the medium.

PG was also the main pectic enzyme detected in shake cultures and in culture liquids in which the fungal growth was homogenized. Similarly, only trace activity for pectic enzymes other than PG was found in culture liquids subjected to ammonium sulfate precipitation or fractionation on Sephadex G-75.

The PG produced by each isolate was similar in sub-

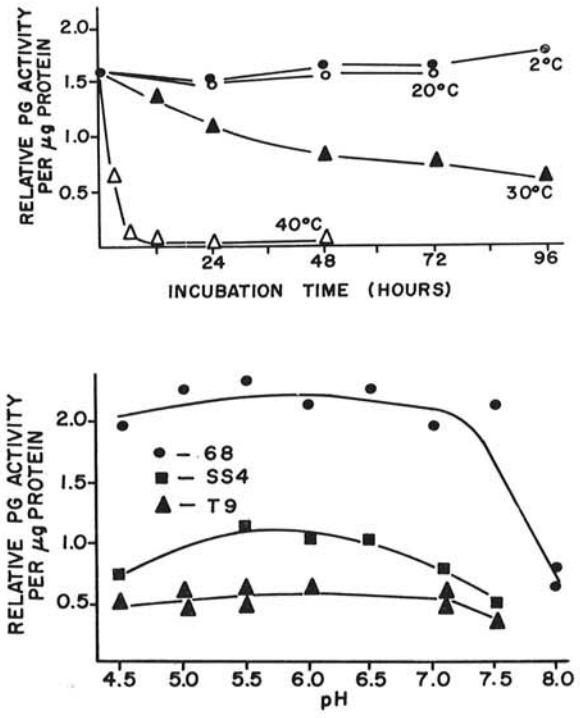

Fig. 1-2. **1)** Heat stability of endopolygalacturonase in dialyzed culture liquids from *Verticillium nigrescens* (isolate 68). Relative activity expressed as 1,000 times the reciprocal of the time required for a 50% reduction in viscosity. **2)** Effect of pH on reactivity of endopolygalacturonase from mild (68), intermediate (SS4), and severe (T9) isolates of *Verticillium*. Relative activity expressed as 1,000 times the reciprocal of the time required for a 50% reduction in viscosity.

strate specificity, pH optimum, heat stability, and nature of reaction products. All culture liquids readily reduced the viscosity of sodium polypectate substrates, but showed reduced activity against pectin. The activity of PG from *Verticillium*, like that of commercial PG (Sigma Chemical Co.), was reduced 30-60% in the presence of the pectin substrate (estimated to be 80% methylated).

PG in dialyzed culture liquids was stable for several days at room temperature, but at 30 C lost activity slowly (Fig. 1). At 40 C, activity was reduced to 50% in 3-5 hr. Viscosity reduction of sodium polypectate substrates was maximum in reaction mixtures buffered between pH 4.5 and 6.0. Total activity was sharply reduced above pH 6.5, however (Fig. 2).

Chromatograms of reaction mixtures and reducing-group tests clearly indicated the hydrolytic and random attack of the PG enzyme. In certain instances, the viscosity of the substrate was reduced as much as 90% in 5 min, yet little or no increase in reducing power or in mono-, di- or tri-galacturonic acid units in reaction mixtures was detected after 4 hr of reaction time. Substantial but inconsistent increases in the reducing power of reaction mixtures were detected after 10 hr of reaction time and, especially, after 24 hr. It is conceivable that such belated increases in reducing power were due to microbial contamination of the reaction mixtures, though no attempts were made to verify this supposition.

In all the preliminary tests, PG activity was greatest in culture liquids from the mild, mycelial 68 isolate, and least in culture liquids from the severe microsclerotial T9 isolate. In a typical experiment, the PG activities in equal quantities of dialyzed culture liquids

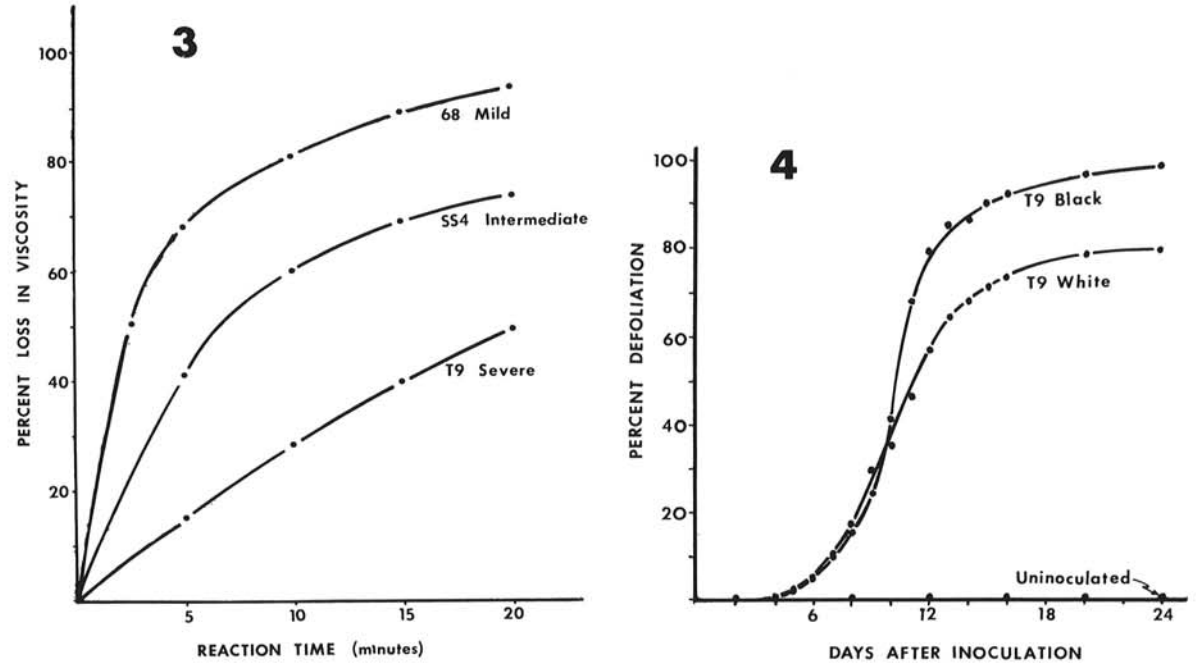

Fig. 3-4. **3)** Rate of viscosity loss of 1.1% sodium polypectate at pH 5.5 in the presence of 1 ml of dialyzed culture liquid from *Verticillium* isolates 68, SS4, and T9. **4)** Rate of defoliation of Acala cotton plants infected with black parental-type progeny and white variant progeny from the severe T9 isolate.

from isolates 68, SS4, and T9, respectively, reduced the viscosity of sodium polypectate by 50% in 2.5 min, 7 min, and 21 min (Fig. 3). Such viscosity losses, when expressed in terms of total protein in the culture liquids or in terms of total dry wt of the fungal culture, likewise support an inverse relationship between PG production and virulence (Table 1).

In light of previous findings linking pectic enzyme production directly with virulence (10, 11), the preliminary indication of an inverse relationship was unexpected. To test the relationship between PG production and virulence further, enzyme assays were expanded to include 25 additional isolates of *V. albo-atrum*. On two separate occasions, pectic enzymes were screened and PG determined in eight mild, 13 intermediate, and eight severe isolates. All 29 isolates, regardless of culture type or virulence, produced PG as a single major pectic enzyme. Relative PG activity per μg protein averaged 3.3 ± 2.2 for mild, 1.3 ± 0.5 for intermediate, and 0.9 ± 0.2 for severe isolates (Table 2). Occasional isolates, however, did not support the inverse relationship between PG production and virulence. Isolate 39 was mild in cotton, yet resembled intermediate isolates in both culture type and PG production. Conversely, isolate 33 was intermediate in cotton but in culture resembled mild isolates in gross appearance and in PG production.

Although a primary concern of this study was the relationship between pectic enzyme production and virulence in cotton, it became obvious that pectic enzyme production was associated more closely with the mycelial habit of growth on PDA (Table 2). Without exception, the isolates which produced PG most abundantly in liquid culture were those that developed into wholly or predominantly mycelial cultures on PDA. So consistent was this relationship that in many cases the production of PG in liquid culture could be predicted from the isolate's gross appearance on the solid medium.

Because of the close association between PG production and the mycelial habit of growth, the inverse relationship between PG production and virulence may have been coincidental. But both relationships were further supported when assays were expanded to include mycelial variant cultures.

Mycelial variants are frequently found among a small percentage of the progeny developed from single conidia of microsclerotial isolates. In this particular instance, 100 conidia of the severe, microsclerotial T9 isolate were transferred to separate PDA slants. Of the 100 conidia, four developed into wholly mycelial colonies, and 96 developed colonies which in gross appearance were indistinguishable from their microsclerotial parent. The black progeny were also identical to the parent culture in both virulence in cotton and PG production in liquid culture. The relationship between PG production and the mycelial habit of growth was apparent in the variant cultures, which showed a 6-fold increase in PG production. Relative PG activity per μg protein respectively averaged 4.3 ±

TABLE 1. Relative activity of endopolygalacturonase in liquid cultures of *Verticillium* species[a,b]

Isolate	PG/mg mycelium (dry wt)	PG/μg protein
V. albo-atrum		
T9 severe	12.0	0.7
SS4 intermediate	22.0	0.9
53 intermediate	25.6	1.1
V. nigrescens		
68 mild	111.1	1.8

[a] Activity expressed as 1,000 times the reciprocal of the time required for a 50% reduction in substrate viscosity.

[b] Cultures were grown in potato broth with 1% glucose and 1% citrus pectin. Each value is based on PG activity in the combined culture liquids of two 13-day-old stationary cultures.

TABLE 2. Comparison of culture type on PDA and polygalacturonase production in liquid culture among isolates of *Verticillium* that differ in virulence in cotton

Virulence class	Isolate[a]	Culture type[b]	Polygalacturonase production[c]
Mild	50	White-mycelial	5.5
	88[d]	Gray-mycelial	5.0
	74	White-mycelial	4.6
	P2	Gray-mycelial	3.2
	68[d]	Gray-mycelial	2.5
	49	Gray-mycelial	2.4
	42	Gray-mycelial	2.3
	39	Black-sclerotial	1.4
Intermediate	31	Gray-sclerotial	1.8
	33	Gray-mycelial	1.5
	30	Black-sclerotial	1.4
	52	Black-sclerotial	1.4
	65	Black-sclerotial	1.4
	32	Gray-sclerotial	1.3
	SS4	Black-sclerotial	1.3
	53	Black-sclerotial	1.3
	70	Black-sclerotial	1.2
	34	Gray-sclerotial	1.0
	14	Black-sclerotial	0.9
	17	Gray-sclerotial	0.9
	85[d]	Gray-sclerotial	0.8
Severe	13	Black-sclerotial	1.1
	75	Black-sclerotial	1.1
	78	Black-sclerotial	1.0
	84	Black-sclerotial	0.9
	10	Black-sclerotial	0.8
	T9	Black-sclerotial	0.7
	36	Black-sclerotial	0.7
	79	Black-sclerotial	0.6

[a] All isolates were identified as *V. albo-atrum* except isolate 68, which is *V. nigrescens*.

[b] Gross appearance on PDA was either white, black, or gray. Both mycelium and sclerotia were abundant in the gray colonies, and the predominance of each is indicated by either the mycelial or sclerotial designation.

[c] Relative activity per μg protein in 1 ml of culture liquids. Relative activity is expressed as 1,000 times the reciprocal of the time required to reduce the viscosity of the sodium polypectate substrate by 50%.

[d] Isolates 88, 68, and 85, respectively, from peanut, pigweed, and tomato. All other isolates from cotton.

0.5 for the four white progeny and 0.7 for four black progeny.

Associated with this increase in PG production in the variant cultures was a concomitant decrease in virulence. Cotton plants inoculated by stem-puncture with equivalent quantities of viable conidia from either the black or white progeny began losing leaves after 5 days, and bore other equivalent symptoms up to 10 days after inoculation. Both progeny types were classified as severe since both induced defoliation. After day 10, however, the rate of defoliation induced by the white progeny was reduced and significantly different (1% level) from the rate of defoliation induced by the black progeny (Fig. 4). After 24 days, plants inoculated with the black progeny or the parent isolate were approaching complete defoliation, whereas those inoculated with the white progeny were approaching 80% defoliation.

To compare enzyme production in vitro with that in the infected host, tracheal fluids and extracts of stem tissues from healthy or *Verticillium*-infected cotton plants were studied. All diseased plants tested were root-dip-inoculated with either the SS4 or T9 isolate, and bore visible disease symptoms at assay. Tracheal fluids were collected from plants decapitated 7.5-15 cm above the soil level. The fluids were collected in sterile glass tubes attached to the plant stumps by short, thick rubber tubes. Assisted by suction and root pressure, fluids rose in the tubes at an average rate of 0.1 ml/hr and were usually collected at 3-hr intervals. All collected fluids were immediately transferred to an ice bath, passed through a bacterial filter, and dialyzed at 2 C against distilled water. None of the techniques used to assay culture liquids demonstrated pectic enzyme activity in tracheal fluids thus prepared.

Freshly cut stem tissues from healthy or diseased plants were used in three additional attempts to measure the activity of pectic enzymes in cotton tissues. The stem tissues were ground in a blender or in a mortar at 2 C in three times their wt of either 0.1 M NaCl, 0.1 M phosphate buffer at pH 5.0, or water, and were centrifuged. After centrifugation of the ground tissues, all supernatants were dialyzed or subjected to ammonium sulfate precipitation. In such preparations, however, only traces of PG were detected, and no other pectic enzymes were apparent.

Since oxidative enzymes have often been implicated in physiological darkening reactions, polyphenol oxidase and peroxidase were assayed in culture liquids and in cotton tissues. Regardless of the capacity of the individual isolates to produce microsclerotia, polyphenol oxidase and peroxidase were not detected in any of the culture liquids. This was true even when microsclerotia developed in liquid cultures incubated at room temperature for 30-40 days. Cotton stem extracts and tracheal fluids, on the other hand, contained measurable polyphenol oxidase and peroxidase activity. However, neither enzyme appeared altered in any of the preparations from diseased plants, even though vascular discoloration was apparent.

DISCUSSION.—Although high PG activity was associated with mild isolates and with the reduced virulence of mycelial variants, it is unlikely that the enzyme plays a major role in the pathogenesis of *Verticillium* in cotton. Its importance in pathogenesis is questionable, since three of 38 isolates did not support the inverse relationship between PG production and virulence, and since the enzyme did not appear to be more prevalent in *Verticillium*-infected plants than in healthy plants. Furthermore, in mycelial variants PG activity was increased to the level produced by mild isolates, and although virulence was decreased, it was not proportionally decreased. Our findings, however, do not eliminate the possibility that PG functions in some way to reduce symptom severity. The association of high PG activity with mild isolates and with the reduced virulence of mycelial variants cannot be considered unimportant. While it is premature to postulate a role for PG in *Verticillium* wilt of cotton, it is tempting to suppose that its reaction products in some way inhibit the production and/or activity of phytotoxins thought to be involved in pathogenesis.

Since no pectic enzymes other than PG were detected in significant quantity in culture liquids of *Verticillium* or in infected cotton tissues, their importance in the host-parasite interaction remains dubious. Because the *trans*-eliminases were not detected in infected plants, our original assumption that their reaction products might act as phytotoxins could not be substantiated either.

Although no mechanism for phytotoxin formation in *Verticillium* wilt of cotton was discerned in this study, new insights were developed regarding the association of high PG activity with reduced virulence and especially with the mycelial habit. The inverse relationship between PG production and virulence observed in the present study is at odds with certain previous reports of a direct relationship (11, 12, 15). The close association between PG production and the mycelial habit, however, could easily account for erroneous interpretations in studies that did not consider the culture type of each isolate.

In the present study, even though the growth of all the isolates in liquid culture was extremely uniform in both quantity and morphological type, PG levels in culture liquids were always indicative of each isolate's mycelial habit on PDA. Incompatible mechanisms for PG and microsclerotia production may exist in *Verticillium*.

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