

Endopolygalacturonase: Evidence Against Involvement in Verticillium Wilt of Cotton

N. T. Keen and D. C. Erwin

Assistant Professor and Professor, respectively, Department of Plant Pathology, University of California, Riverside 92502.

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ABSTRACT

Thirteen isolates of *Verticillium albo-atrum* produced extracellular endopolygalacturonase (endo-PG) inducibly in culture, but rates of production in artificial media showed no relationship to virulence on cotton plants. Enzyme synthesis in the presence of the inducer sodium polypectate was repressed by 0.1 M glucose and stimulated by 0.01 M glucose. Low activities of endo-PG were extracted from severely infected cotton plants, but the ac-

tivity recovered was not related to virulence of the isolates used. Cotton cuttings wilted when placed in dialyzed crude culture fluids, but exhibited no symptoms when placed in highly purified, apparently homogeneous preparations of *V. albo-atrum* endo-PG. These results argue against endo-PG as a factor in development of Verticillium wilt of cotton. Phytopathology 61:198-203.

Additional key words: enzymes, wilting mechanisms.

Chain-splitting pectic enzymes have been thought to play a role in symptom development in certain fungal vascular wilt diseases (4, 5, 26, 28). The evidence for such involvement is equivocal, however (6, 32), derived mainly from indirect studies with resistance (3, 7, 11, 25), histologic stains for pectic plugs in diseased plants (18, 26), correlations of pathogenicity with enzyme production (17, 22), and demonstrations of wilting of cuttings by solutions containing pectic enzymes (1, 10, 33).

If pectic enzymes are in fact responsible for a part of vascular wilt symptomatology, the design of inhibitors or repressors of the enzyme(s) involved would present a lucrative approach to disease control. The former rationale has been tested with partial success by Grossmann (11, 12). An attempt was made, therefore, to critically assess the role of chain-splitting pectic enzymes in the development of *Verticillium* wilt of cotton. We have studied the production of endopolygalacturonase (endo-PG) in culture and in cotton plants by *Verticillium albo-atrum* Reinke & Berth., and have tested the effects of highly purified preparations of *Verticillium* endo-PG on cotton seedling cuttings.

Pectic enzymes may be produced inducibly or constitutively by fungi, and production is frequently regulated by catabolite repression (15, 23). Endopolygalacturonase production by *Verticillium albo-atrum* has been considered inducible by some workers (14, 30, 31) and constitutive by others (20, 21). These conflicting reports may have resulted from isolate variation or from differences in culture conditions. Few reports have critically compared endo-PG production by *V. albo-atrum* in culture with respect to (i) the use of defined media; (ii) adequate control of medium pH during culture growth; (iii) relation of enzyme production to fungus growth; and (iv) use of wildtype (microscerotial) isolates rather than mycelial variants.

MATERIALS AND METHODS.—Isolates of *V. albo-atrum* were maintained on potato-dextrose agar (PDA) slants and transferred as single spores. The defoliating

V3H isolate has been described previously (8), and was used unless otherwise indicated. Single spore white, mycelial variants of V3H were selected on PDA and arbitrarily designated W-1, W-2, etc. Isolates T-9B, T-9W (a white variant of T-9B), and SS-4D were obtained from J. E. DeVay, and have been described (31). Isolates T-1 and SS-4M (20, 21) were obtained from H. Mussell. Conidia of all isolates were produced by growing the isolates on the buffered synthetic medium described by Malca et al. (19) (hereafter called "standard medium") containing glucose. For inoculation of plants or establishment of liquid cultures, conidia were washed with water by centrifugation and spore concn estimated turbidimetrically at 400 nm.

Media used for cultivation of the *V. albo-atrum* strains were potato-dextrose broth (PDB, Difco) (31), a modified Czapek's medium (21), and the standard medium containing various carbon sources. Unless otherwise stated, the latter medium was used in the experiments. Cultures were started by introducing 10^7 - 10^9 conidia into 50-ml DeLong flasks containing 10 ml of medium. The isolates were grown at 25 C in shaken culture (19) unless otherwise stated. Under these conditions, growth was mainly as spores. In some experiments, cultures on the standard medium with sodium polypectate (Sigma) or glucose were grown in still culture at 25 C, producing primarily mycelial growth. In other experiments, $10 \mu\text{M}$ 5-fluorodeoxyuridine (5-FUdR, Hoffmann-LaRoche) were added to shaken cultures. This chemical induced a high percentage of mycelium in shaken cultures (N. T. Keen, unpublished data).

Deltapine Smooth Leaf (DPL) cotton plants were grown in the greenhouse in steamed U.C. mix and inoculated at 21-60 days after planting with spores of *V. albo-atrum* (10^7 /ml) by the modified stem puncture method (8). The disease severity index was estimated at 12-20 days after inoculation on an arbitrary 0-4 scale based on foliar symptoms.

Verticillium albo-atrum liquid cultures were centri-

fused for 2 min at 4,000 g, and the culture supernatants retained for endopolygalacturonase assay. The pelleted hyphae and spores were washed once or twice with distilled water, and the fungus dried at 75 C and weighed.

Cortical tissues were stripped from stems and petioles of noninfected and *Verticillium*-infected cotton plants and from cuttings exposed to purified enzyme solutions. The vascular cylinders were minced with a razor blade and then ground for 30 sec at 0 C in a Sorvall Omnimixer at full speed with 4 ml/g fresh wt of 0.02 M potassium phosphate, pH 6.5, containing 0.2 M KCl, 1 mM dithiothreitol, and 4.5% (w/v) of insoluble polyvinylpyrrolidone (Polyclar AT, General Aniline Film Corp.). The extracts were filtered through cheesecloth, centrifuged for 10 min at 20,000 g at 1 C, and the supernatant fluids finally dialyzed against several changes of 5 mM potassium phosphate, pH 6.5 at 1 C.

Endopolygalacturonase (EC 3.2.1.15) activity was assayed viscosimetrically (29). One endo-PG unit catalyzes the liberation of 1 μ mole galacturonic acid equivalents/min from sodium pectate at pH 6.5 and 35 C (29). All data from experiments with culture supernatants were expressed as specific activity, defined as endo-PG units per mg dry wt of fungus. Activities in plant extracts were expressed as endo-PG units/ml extract.

Ultracentrifugally, electrophoretically, and chromatographically homogeneous preparations of endo-PG from the V3H and W-6 isolates were obtained as described elsewhere (29), and had specific activities in excess of 2,000 units/mg protein.

Cuttings of cotton seedlings (1-3 leaf stage) were made under water and the cuttings equilibrated for 60 min in water before being placed in various concn of active or inactivated (121 C, 10 min) purified endo-PG or dialyzed (water at 1 C) culture supernatants. The cuttings were maintained under light banks, and supplied water after they had taken up the enzyme solutions. Cuttings were observed for symptom development, and in some cases the portion of the stem below the initial liquid level was removed and the upper stem segment extracted for endo-PG.

RESULTS.—Production of endo-PG in culture.—All isolates produced greater specific endo-PG activities on the standard pectate medium than on standard-glucose (Table 1), although SS-4D and T9-W exhibited higher constitutive levels than the other isolates. Endo-PG production by the tested isolates differed considerably on the various media, but no consistent relationship existed between virulence and enzyme production on any medium (Table 1).

The V3H and SS-4D isolates produced similar endo-PG activities on buffered medium containing sodium pectate when grown as spores or mycelium + spores in shaken culture or as mycelium in standing culture (Table 2). The SS-4D isolate, however, produced 3 to 10 times higher endo-PG activities than V3H on glucose, regardless of whether growth was as mycelium or spores. Both isolates produced more enzyme in standing than in shaken glucose cultures.

Sodium pectate, galacturonic acid, and pectin as carbon sources resulted in the highest endo-PG specific activities in V3H cultures (Table 3), and glucose and glucose + alanine produced the lowest activity. Stimulation of endo-PG production was noted when glucose was supplemented with glucosamine or when starch was used as a sole carbon source.

The V3H isolate and seven white, mycelial variants produced more endo-PG on pectate than on glucose medium (Table 4). Although some of the variants produced a few microsclerotia at the center of aging PDA colonies, all were predominantly mycelial. The white variants exhibited variable enzyme production on pectate, but they all produced higher constitutive levels on glucose than did the wild type isolate. Two variants, W-4 and W-6, produced constitutive levels nearly as high as the induced level of the wild type isolate. Considerable variation in virulence was observed between the white variants, and W-2 and W-4 produced symptoms more rapidly on cotton plants than the wild type isolate. No over-all relationship was apparent between the rate of enzyme production by the variants in culture and their virulence to cotton plants (Table 4).

TABLE 1. Growth and production of endopolygalacturonase by *Verticillium albo-atrum* isolates on various media.^a

Isolate	Disease index ^c	Medium ^b							
		Potato-dextrose broth		Czapek's medium		Standard glucose		Standard pectate	
		U/mg ^d	mg dry wt fungus	U/mg ^d	mg dry wt fungus	U/mg ^d	mg dry wt fungus	U/mg ^d	mg dry wt fungus
SS-4M	2	<0.001	71	0.01	26	0.006	102	0.50	16
SS-4D	2	0.15	47	0.07	14	0.27	103	0.84	14
V3H	4	0.006	81	0.05	14	0.005	114	1.00	18
T-9B	4	0.01	73	0.05	17	0.01	111	0.67	12
T-9W	3	0.09	74	0.30	30	0.07	103	0.47	17
T-1	4	0.02	78	0.27	16	0.02	113	1.25	12

^a Data average of 3 replicates; cultures were grown 5 days in shaken culture.

^b Final medium pH values = 6.2-7.1 for potato-dextrose broth; 6.2-6.3 for standard + pectate; 6.2-6.5 for standard + glucose; and 7.6-8.3 for Czapek's medium.

^c Visual estimate of disease severity with 0 = no symptoms to 4 = severe symptoms.

^d Endopolygalacturonase units (U) per mg dry wt fungus.

TABLE 2. Growth and endopolygalacturonase production by *Verticillium albo-atrum* isolates V3H and SS-4D under various culture conditions^a

Carbon source	Culture conditions ^b	Predominant morphologic form	V3H		SS-4D	
			U/mg ^c	mg dry wt	U/mg ^c	mg dry wt
Glucose	Standing	Mycelium	0.30	25	1.00	51
Glucose	Shaken	Spores	0.062	44	0.52	49
Glucose + 5-FUdR ^d	Shaken	Mycelium + spores	0.031	44	0.38	51
Polypectate	Standing	Mycelium	1.66	13	1.33	17
Polypectate	Shaken	Spores	2.03	17	0.84	18
Polypectate + 5-FUdR ^d	Shaken	Mycelium + spores	1.26	19	1.27	18

^a Data averages of three replicate cultures.

^b The fungus was grown for 5 days on standard medium containing 1% (w/v) glucose or sodium polypectate in standing or shaken culture.

^c Endopolygalacturonase units (U) per mg dry wt fungus.

^d Cultures supplemented with 10 μ M 5-fluorodeoxyuridine (FUdR).

Endo-PG production by the V3H isolate on sodium polypectate was not repressed by supplemental glucose until the glucose concn reached 0.1 M (Fig. 1). Enzyme production was also rapid in these cultures after 4 days, presumably due to exhaustion of glucose. The rate of enzyme production was stimulated by supplemental glucose at 0.01 M.

Production of endo-PG in Verticillium-infected cotton stems.—Endopolygalacturonase activity was not detected in extracts from stems of noninoculated cotton plants (Table 5). Barely detectable activities were often, but not always, recovered from severely infected plants. Activity was not detected in extracts from infected plants in earlier stages of infection. Controls in which purified endo-PG was processed through the tissue extraction procedure with or without stem tissue showed recoveries of 30-90% initial activity. Only 0.5-4.0% of the supplied endo-PG activity was recovered from stem segments of cuttings exposed to purified enzyme solutions (Table 5).

Cuttings exposed to purified endo-PG.—There were no external foliar symptoms up to 4 days on cotton cut-

TABLE 3. Growth and endopolygalacturonase production by the V3H isolate of *Verticillium albo-atrum* on various carbon sources^a

Carbon source ^b	U/mg dry wt ^c	mg dry wt fungus
2.5% Glucose	0.031	108
2.5% Glucose + 0.07 M alanine	0.016	138
2.5% Glucose + 0.1 M glucosamine	0.078	109
1% Sodium polypectate	1.25	21
1% Pectin	0.28	38
1% Pectin + 0.07 M alanine	0.23	50
2.5% Starch	0.094	57
1% Galacturonic acid	0.98	17

^a Data are averages of 3 replicate cultures; cultures were grown 7 days in shaken culture.

^b All cultures were supplied the standard medium with the noted carbon sources.

^c Endopolygalacturonase units (U) per mg dry wt fungus.

tings that were allowed to take up purified *V. albo-atrum* endo-PG preparations. When high enzyme activities were supplied (from 10-300 units/plant), considerable maceration of cortical tissues of the submerged stem portions occurred, but no external foliar symptoms and no vascular browning were noted, and no significant differences in water uptake relative to the control plants occurred. But cuttings placed in crude dialyzed culture fluids of *V. albo-atrum* exhibited wilting after 4-12 hr.

DISCUSSION.—The data indicate that endo-PG from *V. albo-atrum* is not involved in symptom production: (i) no relation existed between virulence of a number of isolates and their endo-PG production in culture;

TABLE 4. Endopolygalacturonase production and disease indices of several white, mycelium variants of the V3H isolate of *Verticillium albo-atrum*^a

Isolate	U/mg dry wt fungus ^b			Colony appearance ^d
	2.5% Glucose	1% Poly-pectate	Disease index ^c	
V3H	0.016	1.65	4	Microsclerotial
W-1	0.11	2.54	2	Few microsclerotia
W-2	0.078	2.28	4	Few microsclerotia
W-3	0.11	1.91	4	Very few microsclerotia
W-4	0.84	3.28	4	Mycelial, no microsclerotia
W-5	0.078	1.45	4	Mycelial, no microsclerotia
W-6	1.12	3.12	2	Mycelial, no microsclerotia
W-7	0.094	1.27	1	Few microsclerotia

^a Data averages of three replicates; cultures harvested after 4 days.

^b Endopolygalacturonase units (U) per mg dry wt fungus; dry wt of the cultures ranged from 59-69 mg (glucose) and 21-28 mg (polypectate); final medium pH values, 6.2-6.5.

^c Visual estimate of disease severity with 0 = no symptoms to 4 = severe symptoms.

^d On potato-dextrose agar after 7-14 days at 25 C.

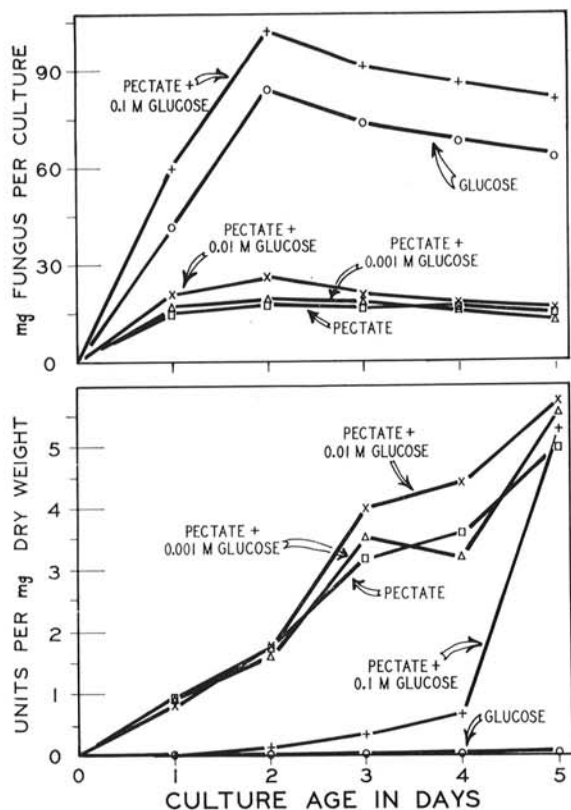


Fig. 1. Fungus dry wt (upper) and specific endopolygalacturonase activity (lower) of *Verticillium albo-atrum* cultures grown on 0.1 M glucose, 1% sodium polypectate, or 1% sodium polypectate supplemented with various concn of glucose.

(ii) little or no endo-PG activity was recovered from infected cotton stems and showed no relationship to the virulence of the fungus isolate inoculated; (iii) no symptoms were produced on cuttings exposed to homogeneous preparations of the *V. albo-atrum* endo-PG. The wilting of cuttings by dialyzed, crude culture fluids appears to be associated with a lipopolysaccharide complex (N. T. Keen & Margaret Long, unpublished data). Although endo-PG is the predominant pectic enzyme liberated by *V. albo-atrum* (25, 27, 28, 29), it is doubtful that this enzyme has a role in the production of wilt symptoms in cotton plants. The *V. albo-atrum* endo-PG effectively macerates plant tissue (29), and therefore could play a role in direct root penetration and passage through cortical tissues by the fungus (9). The general lack of evidence relating this enzyme with the virulence of *V. albo-atrum*, however, discourages the rationale that endo-PG inhibitors or repressors may effect disease control.

Although polyvinylpyrrolidone and dithiothreitol were used during extraction to minimize enzyme inactivation by phenolic compounds, and 0.2 M KCl was added to reduce enzyme adsorption to host components, extremely low endo-PG activities were recovered from *Verticillium*-infected cotton plants (Table 5). In control extractions, 30-80% recoveries resulted when puri-

TABLE 5. Endopolygalacturonase activities extracted from cotton stems which were noninoculated, inoculated with various isolates of *Verticillium albo-atrum*, or allowed to take up purified *Verticillium* endopolygalacturonase solutions^a

Condition	U/ml extract ^b
Noninoculated plant	<0.002
Enzyme control; 10.0 U/ml ^c	8.3
V3H-inoculated plants	<0.002-0.005
SS-4M-inoculated plants	<0.002-0.003
SS-4D-inoculated plants	<0.002
T-1-inoculated plants	<0.002
T-9B-inoculated plants	<0.002-0.007
Noninoculated plant + 0.16 U purified endo-PG/ml ^d	0.086
V3H-inoculated plants + 0.16 U purified endo-PG/ml ^d	0.050
Cuttings exposed to H ₂ O ^e	<0.002
Cuttings allowed to take up 31 U-purified endo-PG ^e	0.24
Cuttings allowed to take up 500 U-purified endo-PG ^e	19.8

^a All plants harvested at 15 days after inoculation; all data represent means of 3-6 replicate extractions and assays.

^b Endopolygalacturonase units (U) per ml of extract or solution of purified enzyme.

^c Purified endo-PG added to extraction solution only and run through extraction procedure and dialysis.

^d Purified endo-PG added to extraction solution with noted stem segments and run through extraction procedure.

^e Cotton seedlings were allowed to take up 10 ml H₂O or purified endo-PG and then supplied water for 30 hr; then the submerged stem segments were discarded and the remaining stems extracted.

fied enzyme was added to plant tissue as an internal standard. These facts indicate that endo-PG production by the fungus in infected host tissues is exceedingly low, thus confirming similar data obtained by Wiese et al. (31).

Endopolygalacturonase production by *V. albo-atrum* in culture was greatly affected by medium composition. Confirming published reports (14, 30, 31), all isolates produced greater activities on sodium polypectate than on glucose, both in standing and shaken culture. Thus, endo-PG production appeared to be inducible. Mussell & Green (20, 21), however, concluded that endo-PG production by *V. albo-atrum* was constitutive. This discrepancy is believed due to their use of Czapek's medium. Since Czapek's medium is poorly buffered and contains sodium nitrate, the pH of cultures generally shifts to the alkaline range. Growth of *V. albo-atrum* is known to be reduced at high pH values (19), and the *Verticillium* endo-PG is less stable at alkaline pH values (29). When we used media which prevented large pH shifts, no difficulty was experienced in demonstrating inducibility of the endo-PG.

Wiese et al. (31) found an inverse relationship between the virulence of isolates T-9B and SS-4D and their endo-PG production on potato-dextrose broth. On the contrary, Mussell & Green (20, 21) suggested a direct relationship, using the defoliating T-1 isolate and SS-4M grown on Czapek's medium. The data in Table 1 confirm both these reports. Although the two SS-4 cultures originated from the same isolate, large differences in enzyme production occurred between the sub-

cultures used by Wiese et al. (31) and Mussell & Green (20, 21). This and the different media used by the two groups rationalized the difference in conclusions. Our data showed that when a number of isolates were examined, no consistent relationship existed between virulence and enzyme production in culture.

In confirmation of literature reports, starch resulted in greater enzyme production in culture than did glucose (13), and glucosamine stimulated enzyme production on glucose (24). Contradictory to the report by Booth (2), alanine did not stimulate enzyme production by our isolates, and repressed production on pectin (Table 3).

No appreciable difference was observed in endo-PG production in shaken or unshaken polypectate cultures (Table 2), but shaken glucose cultures produced 2 to 20 times less activity than unshaken cultures. This may have been due to an effect of aeration rather than to the different morphogenetic forms of the fungus. Shaken glucose cultures supplemented with 5-FUDR contained a high proportion of mycelium (Table 2), but produced no more endo-PG than nonsupplemented cultures which grew primarily as spores.

Endo-PG production on sodium polypectate by *V. albo-atrum* was repressed only by relatively high (0.1 M) concn of supplemental glucose (Fig. 1), while lower (0.01 M) glucose concn stimulated production. These findings are similar to results obtained with other phytopathogenic fungi (15, 23). In contrast to the relative insensitivity of endo-PG synthesis to glucose repression, production of β -galactosidase by *Verticillium albo-atrum* was severely repressed by relatively low hexose levels in the presence of the inducer lactose (16).

The virulence of a series of white, mycelial variants of the V3H isolate varied from extremely low to as high or higher than the parent isolate, but showed no relationship to endo-PG production in culture. As reported by other workers (31), generally less virulent, mycelial *Verticillium* isolates and the nondefoliating type isolates exhibited considerably higher constitutive endo-PG levels than the highly virulent microsclerotial isolates (Tables 1, 4). Whether this apparent tendency of the microsclerotial isolates to exercise more stringent regulatory control of enzyme production is of importance to the saprophytic or parasitic behavior of the fungus is not known.

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