Endopolygalacturonase: Evidence for Involvement in Verticillium Wilt of Cotton

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ABSTRACT

Endopolygalacturonase (endoPG) but not exopolygalacturonase (exoPG) purified from culture fluids of *Verticillium albo-atrum* was toxic to susceptible cotton leaves when assayed in a divalent salt solution. Leaf symptoms generated by this endoPG were identical to some of the symptoms observed in susceptible cotton after infection with *V. albo-atrum*. Symptoms similar to those generated by endoPG treatment were also observed after treatment of cotton leaves with several substrate-oxidase combinations which generated hydrogen peroxide. Symptom expression in endo PG-treated leaves could be blocked by pretreatment of the leaves with catalase or reduced by pretreatment with peroxidase. Hydrogen peroxide could be detected in solutions bathing endoPG-treated leaf tissue 30 min after treatment. The above results support a hypothesis that endoPG-induced leaf damage is mediated through hydrogen peroxide appearing intramurally in endoPG-treated cotton leaves.

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Additional key words: disease physiology, pathogenesis, cell death, defoliation, *Gossypium hirsutum*.

Polygalacturonases (PG) have been shown to be important for successful pathogenesis by several phytopathogenic fungi (1, 2, 3, 13). Host repression of fungal synthesis of these enzymes has been proposed as a possible resistance mechanism in plants (2). In Verticillium wilts, however, the evidence for a precise role for PG in pathogensis is both conflicting and contradictory (5, 14, 15, 18, 22, 23, 26, 31, 33). This paper reports the conditions required to demonstrate the phytotoxicity of an endopolygalacturonase (endoPG) produced by cotton isolates of *Verticillium albo-atrum* Reinke & Berth. Evidence is also presented which suggests that the mechanism of endoPG-induced leaf damage is related to the intramural appearance of hydrogen peroxide.

MATERIALS AND METHODS.–EndoPG and exopolygalacturonase (exoPG) (EC 3.2.1.15) were purified as described previously (25) from 12 isolates and four derived hyaline mycelial variants of *V. albo-atrum* from cotton, *Gossypium hirsutum* L. (25). The fungi were grown in a liquid medium containing the following salts in g/liter: $NaNO_3$-4.0; KH_2PO_4-1.4; KCl-0.5; $MgSO_4$-0.4; and $FeSO_4$-0.025. This mixture (GB salts), containing 1% glucose, was adjusted to pH 5.0 with ammonium hydroxide prior to autoclaving. Cultures were grown for 12 days in 125 ml-Delong culture flasks containing 25 ml of medium under conditions previously described (23). Total production of endoPG and exoPG by each isolate was determined using 500-ml aliquots of culture fluids. Macromolecules were concentrated and desalted using ultrafiltration (UM-2 membrane, Amicon Corp.). The desalted macromolecules were then electrofocused for 72 hr in a linear 0 to 40% sucrose gradient containing 1% ampholine, pH 3 to 10 (25). Fractions of 2.5 ml were collected, and the PG activity peaks were located using a specific bioassay (24). The two activity peaks obtained were individually pooled and adjusted to 200 ml each with 0.02 M phosphate pH 5.0. Activity of the two fractions was measured viscometrically with activities calculated in relative viscometric units (RVU) defined as 1,000 times the reciprocal of the time (in minutes) required for a 50% reduction in viscosity of a mixture containing 5 ml of 1% sodium polypectate and 1 ml enzyme incubated at 39 C (25). Cellulase (Cx) was assayed viscometrically as above except that the substrate used was 1% carboxymethyl cellulose (Hercules Powder Co., type 7MF).

Fractions were pooled at each step during enzyme purification, desalted by ultrafiltration, and assayed for phytotoxicity in deionized water and 0.1 GB salts. Assays for phytotoxicity were performed on cotton cuttings with six to eight leaves, and also using single leaves taken from the fourth and fifth nodes of eight-leaf cotton plants. Cuttings and leaves were excised while the plants were submerged in tap water, and the cuttings or leaves were transferred directly into 5 ml of the test solutions. After taking up the test solutions, leaves and cuttings were incubated in 0.1 GB for the duration of the experiments. Leaf damage was estimated visually on a scale of 0 (healthy) to 5 (totally necrotic), with intermediate values assigned according to the apparent ratio of healthy to necrotic leaf tissue. Once the phytotoxicity of endoPG was established by the above assays, all subsequent experiments were carried out with electrophoretically homogeneous endoPG prepared as previously described (25).

Cell kill assays were performed as described by Tribe (32), using potato tuber and cucumber pericarp tissues. Tissue sections were incubated in endoPG solution, then immersed in a plasmolyzing solution containing neutral red for 20 min. The sections were then washed with 1 M KNO_3 (21), and cell viability was estimated visually with a value of 5 being assigned to sections in which all of the cells retained the red dye (viable), and a value of 0 assigned to sections in which no cells retained the dye (21). Intermediate values were assigned according to the

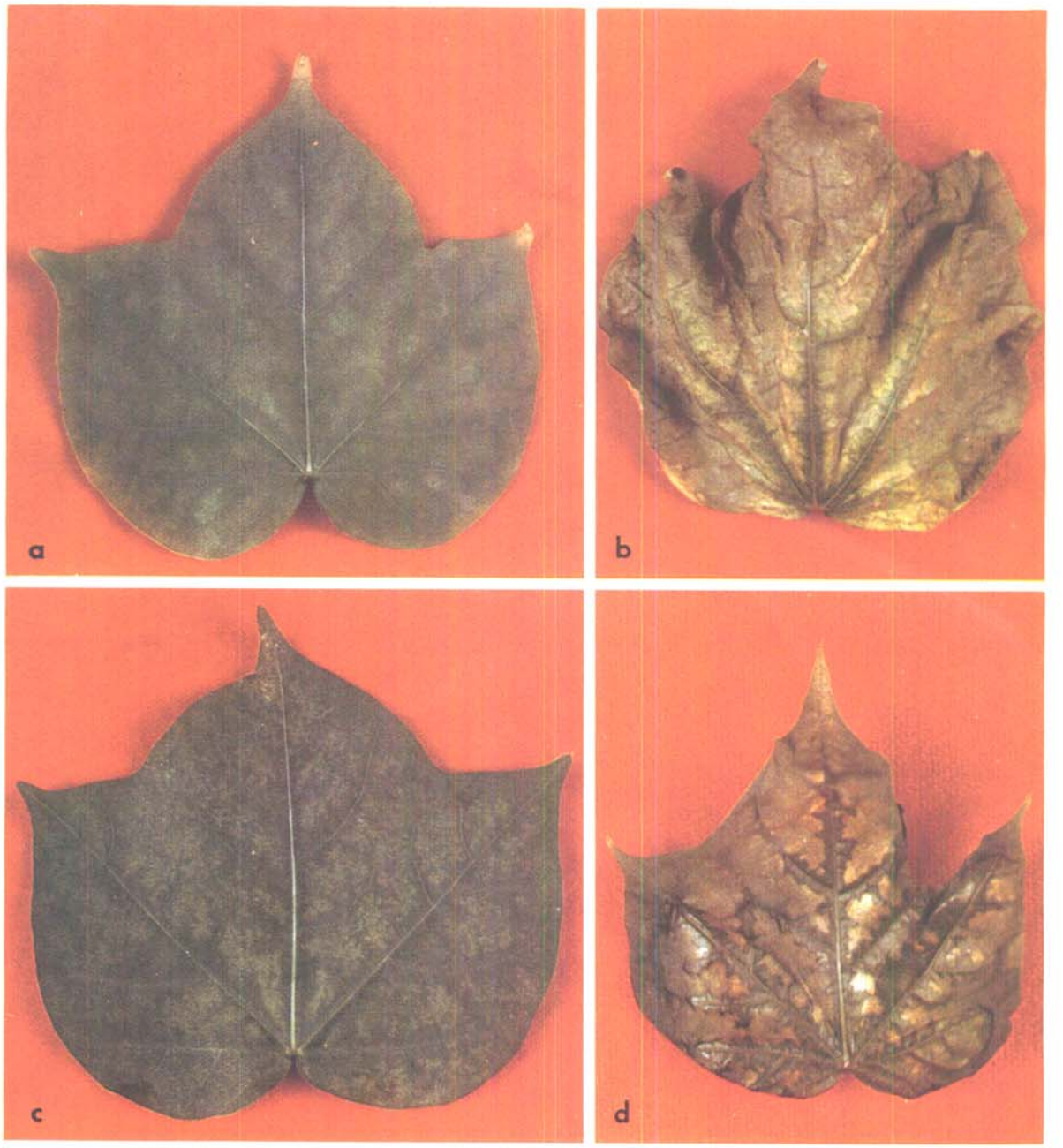


Fig. 2. Foliar symptoms in cotton. **a)** *Verticillium*-infected, 7 days after inoculation. **b)** *Verticillium*-infected, 12 days after inoculation. **c)** Endopolygalacturonase (endoPG) treated, 24 hr after treatment. **d)** EndoPG treated, 72 hr after treatment.

apparent ratio of stained to unstained cells in the tissue sections. Tissue maceration was determined by measuring the weight loss of five cylinders of tissue 5 mm in diam and 5 mm in length after incubation in endoPG solutions (24).

To measure hydrogen peroxide generated in treated leaf tissue, five 1.5-cm discs of tissue from mature cotton leaves were placed in 10 ml of 0.1 GB with or without 3.5 μg of purified endoPG. The discs were infiltrated under vacuum for 2 min, then incubated at 23 C. At intervals, 1-ml aliquots of this bathing solution were removed and added to 9 ml of a 6 mM solution of *o*-dianisidine in 0.01 phosphate buffer, pH 7, containing 2,000 units of horseradish peroxidase. After 10-min incubation at 23 C, 0.2 ml of 4 N HCl was added, and 5 min later the absorbance was read at 460 nm. Calculations of hydrogen peroxide concentration were based on the stoichiometric oxidation of *o*-dianisidine, using a molar absorbancy index of 1.13×10^4 cm^{-1} for the oxidized dye (Worthington Enzymes and Enzyme Reagents Catalog, Worthington Biochemical Corp., Freehold, N. J.).

Virulence indices for each of the fungal isolates were obtained by inoculating 8-week-old cultivar M8 cotton with 10^5 spores in 0.01 ml H_2O by stem puncture (4). Inoculated plants were maintained in an environmental chamber programmed for 24 C days and 20 C nights on a 16-hr photoperiod with 1,500 ft-c of light supplied by mixed incandescent and fluorescent light. Fourteen days after inoculation, plants were rated on a scale of 0 for no symptoms to 5 for death of the plants. Eight plants were inoculated with each isolate.

The glucose oxidase, galactose oxidase, amino acid oxidase, catalase, and peroxidase used were the highest quality available from Worthington Biochemical Corp.

RESULTS.—All isolates of *V. albo-atrum* tested produced both exoPG and endoPG when cultured on the GB salts medium containing glucose (Table 1). Isolates of the more virulent, defoliating pathotype (30, 33) consistently produced more endoPG than isolates of the less virulent, nondefoliating pathotype. Although the amount of endoPG produced correlated well with the pathotype of the isolate, it did not correlate directly with the virulence indices of the individual isolates within each pathotype.

During purification of these two PG, phytotoxicity was consistently associated with PG activity (Fig. 1). When the two PG were separated by electrofocusing, phytotoxicity was found associated with only the endoPG (Fig. 1-c). Comparison of the toxicity of purified endoPG applied to cotton leaves in water and 0.1 GB demonstrated that toxicity of this enzyme was enhanced when it was applied in a salt solution. Viscometric assays indicated that none of the salts in the GB mixture had any effect on enzymatic activity of the purified endoPG when assayed at the concentrations used in the leaf tests.

Tests with the individual salts contained in the GB mixture demonstrated that divalent cations were responsible for the enhanced phytotoxicity observed when purified endoPG was applied in the salt mixture (Table 2). Once this was established, further tests

TABLE 1. Production of endopolygalacturonase (endoPG) and exopolygalacturonase (exoPG) by 16 isolates of *Verticillium albo-atrum* from cotton when grown on a glucose-salts medium

Isolate[a]	Virulence[c] index	PG activity, RVU[d] endoPG	exoPG
	Defoliating pathotype		
T-9	4.7	924	280
138	4.4	884	152
V3HW	4.5	751	80
103	4.1	742	126
T-1W	4.9	784	134
T-9-10W	4.5	666	45
V3H	4.5	638	175
117	4.7	606	75
T-1	4.9	595	204
T-9-1-1 [b]	0.2	89	46
	Nondefoliating pathotype		
116	2.7	370	16
V22W	2.5	131	181
V9	2.4	105	276
V22	2.6	92	144
SS-4	2.1	55	21
111	1.5	37	46

[a] Isolates with the suffix W were hyaline mycelial variants; all others were pigmented wild types.

[b] An avirulent mutant obtained from T-9.

[c] Virulence index: a subjective scale of symptom severity 14 days after inoculation, ranging from 0 for no symptoms to 5 for death of the plants.

[d] RVU = Relative Viscometric Units defined as 1,000 times the reciprocal of the time required for 1 50% reduction in viscosity of a mixture of 5 ml substrate and 1 ml enzyme incubated at 39 C.

TABLE 2. Effects of 5 mM salts on symptom expression observed in cotton leaves 72 hr after treatment with 25 μg of purified endopolygalacturonase (endoPG) or exopolygalacturonase (exoPG)

Salt	Leaf damage index[a] +endoPG	-endoPG	+exoPG	-exoPG
H_2O	1.2	0.1	0.1	0.1
KNO_3	1.2	0.0	0.0	0.0
$NaNO_3$	1.2	0.0	0.0	0.0
$Ca(NO_3)_2$	2.5	0.0	0.2	0.0
$Mn(NO_3)_2$	3.2	0.0	0.2	0.0
$Mg(NO_3)_2$	4.8	0.0	0.1	0.0
0.1 GB[b]	4.7	0.0	0.1	0.0

[a] A subjective scale for leaf damage ranging from 0 for no damage to 5 for total leaf necrosis, with intermediate values assigned according to the apparent ratio of healthy to necrotic leaf tissue.

[b] 0.1 GB = a salt mixture containing the following in g/liter: $NaNO_3$-0.4; KH_2PO_4-0.14; KCl-0.05; $MgSO_4$-0.04; and $FeSO_4$-0.0025.

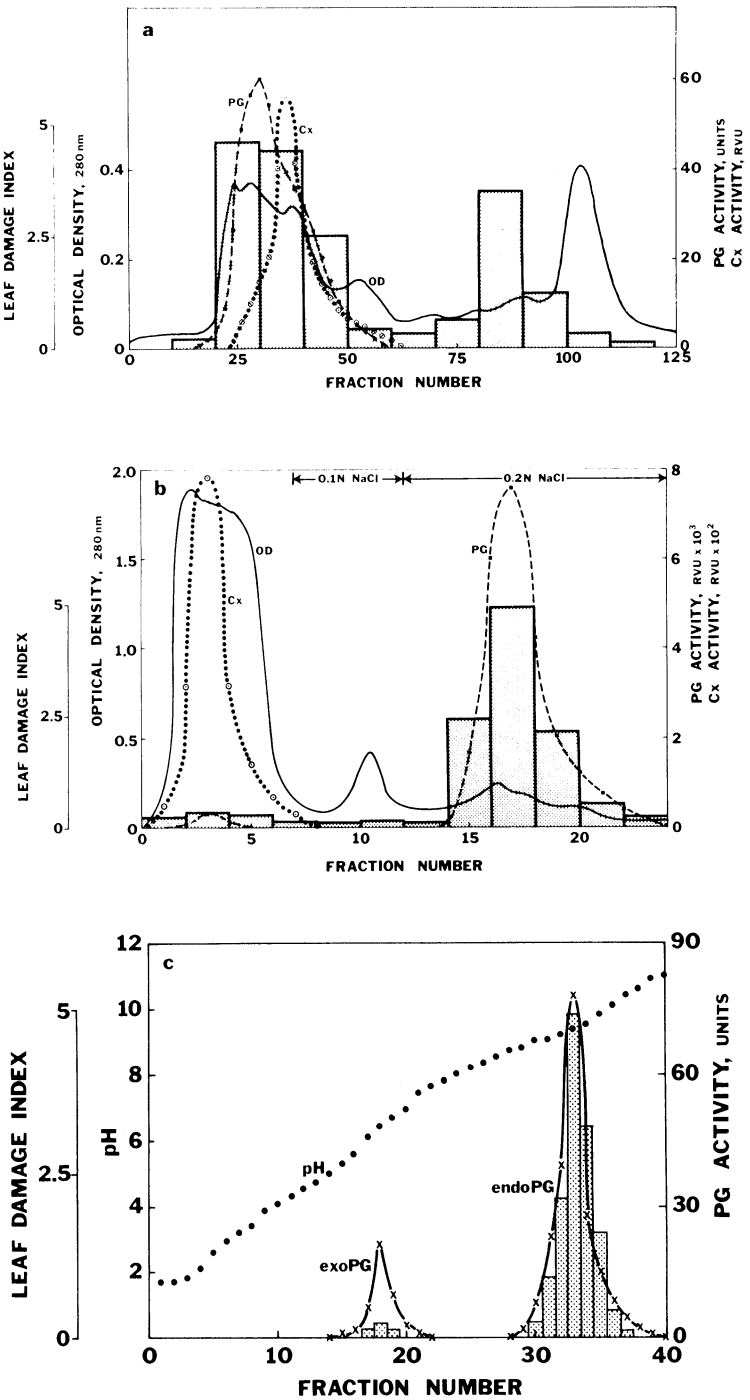


Fig. 1. Phytotoxicity to cotton leaves (shaded areas) observed in fractions obtained during purification of two polygalacturonases (PG) from *Verticillium albo-atrum*. a) Chromatography of culture fluids on Sephadex G-75. b) Chromatography of pooled PG and cellulase (Cx) fractions from (a) on Carboxymethyl Sephadex C-25. c) Electrofocusing of PG activity peak from Carboxymethyl Sephadex C-25 showing separation of exopolygalacturonase (exoPG) from endopolygalacturonase (endoPG). OD = optical density at 280 nm.

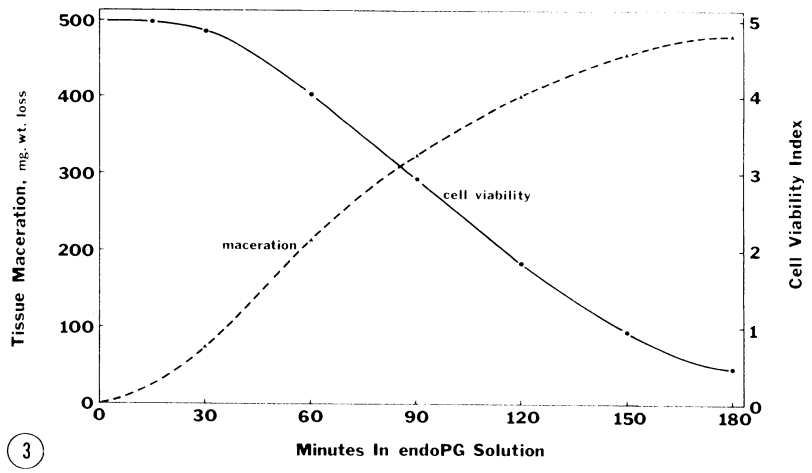
were carried out by application of the purified endoPG in 5 mM magnesium nitrate.

Symptoms appearing in cotton leaves treated with purified endoPG mimicked the progressive interveinal necrosis characteristics of Verticillium wilt of cotton (Fig. 2). Similar foliar symptoms resulted when purified endoPG was applied to cotton cuttings, and, as occurs in Verticillium wilt, mature leaves frequently abscised prior to the appearance of leaf tissue damage.

After treatment with purified endoPG, both cucumber pericarp and potato tuber tissue lost the capacity to retain neutral red dye, indicating a loss of cell viability (21, 32). Cell death was always preceded by detectable maceration of the tissues (Fig. 3).

When hydrogen peroxide was applied directly to cotton leaf tissue, rapid necrosis resulted, but without the pattern of progressive interveinal necrosis typical of Verticillium wilt. However, when leaves were treated first with a substrate, then with the specific oxidase for that substrate, necrotic symptoms identical to those caused by the purified endoPG were always produced (Table 3). On the other hand, treatment of cotton leaves with 1,000 units of peroxidase prior to application of endoPG greatly reduced symptom expression, whereas treatment with 1,000 units of catalase prior to endoPG treatment totally blocked the appearance of symptoms in treated leaves (Table 3). When applied individually to cotton leaves, none of the substrates or oxidases used in these experiments caused visible symptoms. Also, none of the substrates or enzymes used in these tests had any effect on the enzymatic activity of purified endoPG as measured viscometrically, nor did any of the enzymes used in these tests contain detectable PG activity.

Hydrogen peroxide, as measured by the enzymatic oxidation of *o*-dianisidine, was detected in bathing



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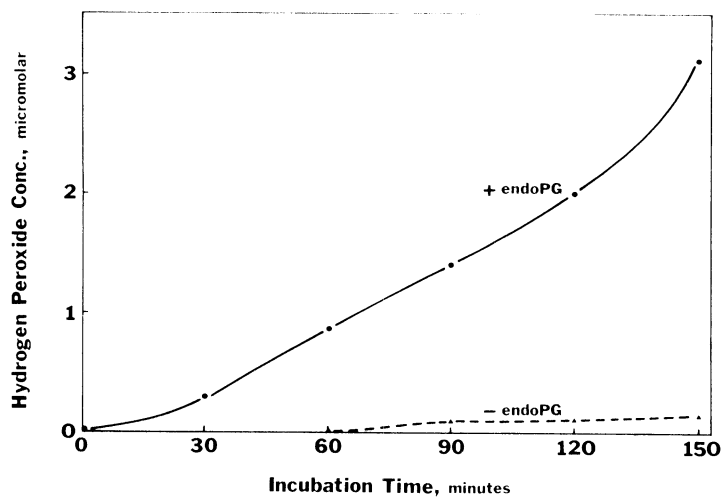

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Fig. 3-4. 3) Maceration and cell death observed in potato tuber tissue after treatment with 25 μg purified endopolygalacturonase (endoPG). **4)** Hydrogen peroxide evolved by cotton leaf tissue after treatment with 3.5 μg purified endoPG.

TABLE 3. Symptom expression by cotton leaves 72 hr after treatment with various enzymes and substrates applied in 5 mM $Mg(NO_3)_2$

Pretreatment	Treatment	Leaf damage index[e]
	Glucose[a]	0.0
	Galactose[a]	0.0
	Phenylalanine[a]	0.0
	Catalase[b]	0.0
	Peroxidase[b]	0.0
	Glucose oxidase[c]	0.0
	Galactose oxidase[c]	0.0
	Amino acid oxidase[c]	0.0
Glucose[a]	Glucose oxidase[c]	3.8
Galactose[a]	Galactose oxidase[c]	3.2
Phenylalanine[a]	Amino acid oxidase[c]	4.7
	EndoPG[d]	4.8
Catalase[b]	EndoPG[d]	0.2
Peroxidase[b]	EndoPG[d]	2.4

[a] 25 mM.
[b] 1,000 units.
[c] 10 units.
[d] 25 μg.
[e] A subjective scale for leaf damage ranging from 0 for no damage to 5 for total leaf necrosis, with intermediate values assigned according to the apparent ratio of healthy to necrotic leaf tissue.

solutions over endoPG-treated leaf tissue 30 min after infiltration of the tissue with endoPG (Fig. 4). The levels of hydrogen peroxide detected in the bathing solutions continued to rise throughout these experiments. Leaf tissue which was not treated with endoPG did not generate hydrogen peroxide until 90 min after infiltration with the salt solution, and the levels of hydrogen peroxide detected in these treatments did not show appreciable increases during the experiments.

DISCUSSION.—The levels of endoPG produced by cotton isolates of *V. albo-atrum* when grown on the GB medium with a glucose carbon source tend to correlate with the pathotype of the isolate. These data on endoPG production in vitro substantiate and expand the report of Talboys & Busch (31) that PG production by *V. albo-atrum* growing on a glucose-based medium (constitutive production) tends to correlate with the pathotype but not directly with the virulence of each isolate within a pathotype.

The association of leaf damage with PG activity during purification of the two PG from *V. albo-atrum*, and the toxicity of the fractions containing endoPG but not those containing exoPG after electrofocusing, leave little doubt that this endoPG is toxic to cotton tissue. The first documented report of Verticillium wilt of cotton in the United States (12) described foliar symptoms in severely infected cotton as "... a mosaic pattern of rust colored dead areas with yellowish margins, lying between narrow strips of green, bordering the principal veins". The leaf symptoms obtained by application of purified endoPG to mature cotton leaves in 5 mM magnesium nitrate were very similar to this description (Fig. 2-b, d). Symptoms appearing on younger leaves after treatments were less distinct, but led to eventual death of leaf tissue, and were similar to the symptoms described by Presley (28) for leaves on young three- to five-leaf cotton plants infected with *V. albo-atrum*. The endoPG produced by *V. albo-atrum* did not cause chlorosis, wilting, or interveinal flaccidity, other characteristic symptoms of this disease (16).

The work reported here demonstrates that endoPG-induced leaf damage in cotton is accompanied by the appearance of hydrogen peroxide; that substrate-oxidase systems capable of generating hydrogen peroxide cause the same type of leaf damage as endoPG; and that addition of enzymes which break down hydrogen peroxide reduce or prevent the appearance of tissue damage induced by endoPG treatment of cotton leaf tissue. Thus, there appears to be a causal relationship between endoPG action, appearance of hydrogen peroxide, and the onset of tissue damage in endoPG-treated leaves. This work does not elucidate the source of the hydrogen peroxide appearing in treated leaves. However, Lund & Mapson (19) have demonstrated that bacterial pectolytic enzymes, acting on the intramural areas of cauliflower tissue, release a series of enzymes capable of generating both hydrogen peroxide and ethylene. The endoPG of *V. albo-atrum* may affect the intramural areas of cotton leaf tissue in a similar manner, since the size of this enzyme precludes its facile passage through cell membranes but not its lateral movement from vascular elements into adjacent intramural regions (6, 7).

The work of Lund & Mapson (19) also suggests a plausible explanation for the defoliation observed after cotton cuttings were treated with endoPG. This endoPG-induced abscission may reflect the capacity of mature leaf tissue to generate ethylene through an intramural enzyme system similar to the one observed by Lund & Mapson (19). On the other hand, this leaf drop may simply reflect the formation of a separation layer in the abscission zone through pectolytic dissolution of abscission zone pectins (20).

The requirement for a divalent cation in the generation of endoPG-induced leaf damage is, at present, an enigma, since this endoPG is neither activated nor inhibited by these levels of cations when assayed in vitro (25). However, anomalous results when comparing the in vivo and in vitro effects of divalent cations on pectolytic enzyme activity have been reported from other systems (27). This cation requirement does, however, help explain the recently reported failure to generate foliar symptoms with a purified endoPG from *V. albo-atrum* (15). Divalent salts interact with hydrogen peroxide in the nonenzymatic degradation of plant polymers (9, 11, 17), and divalent salts have also been reported to shift the enzymatic specificity of peroxidases (17, 29). Possibly, divalent cations function in conjunction with materials released through endoPG action. They may, for example, play a direct role in regulating the appearance or site of reaction of hydrogen peroxide.

The absence of toxicity in the purified exoPG from *V. albo-atrum*, and the reports of phytotoxicity associated with other endo-acting enzymes (10, 19, 21, 32), imply that cytotoxicity may be a specific property of endo-acting pectolytic enzymes, and not simply a response to the hydrolytic degradation of pectic substances. Also, the toxic response observed with potato tuber and cucumber pericarp tissue after treatment with the purified endoPG from *V. albo-atrum* suggests that the toxicity associated with endo-acting pectolytic enzymes may be expected as a general response of plant tissues susceptible to pectolytic maceration.

This paper, and other recent reports (8, 10, 19, 21, 32), emphasize the importance of the intramural areas of plant tissues as potentially being the first site of active biochemical conflict between host and pathogen. Further studies into the role of hydrogen peroxide and other materials generated intramurally during pathogensis should be a profitable approach to a greater understanding of host-parasite interactions.

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