Pectic Enzymes Produced by Diplodia gossypina In Vitro and In Infected Cotton Bolls

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Supported by the Cotton Producers' Institute and the Cooperative State Research Service, USDA.
Accepted for publication 22 April 1971.

ABSTRACT

Culture filtrates of *Diplodia gossypina* grown in a pectin medium, extracts of *Diplodia*-diseased cotton boll tissues, and extracts of fungal mycelia from diseased cotton boll surfaces contained a pectin methylesterase, an exopolygalacturonase, and a polygalacturonate *trans*-eliminase with pH optima of 6.5, 4.5, and 9.0, respectively. Pectin methylesterase from culture filtrates was stimulated by K^+, Na^+, Ca^{++}, Mg^{++}, and Mn^{++}, but inhibited by EDTA. Exopolygalacturonase from culture filtrates was stimulated by K^+, Na^+, Mg^{++}, Mn^{++}, Zn^{++}, and Cu^{++} but slightly inhibited by EDTA and Ca^{++}. Polygalacturonate *trans*-eliminase from all three sources was stimulated by Ca^{++}; however, this enzyme obtained from diseased tissues was inhibited by Ca^{++} when the concentration was higher than 1.4×10^{-3} M. Polygalacturonate *trans*-eliminase from culture filtrates was slightly stimulated by Na^+ and Mg^{++}, but inhibited by EDTA. All these enzymes were inductive; their activities were slightly reduced when 0.1% glucose was added to the medium for growth. Young cultures contained principally exopolygalacturonase. The activities of pectin methylesterase and polygalacturonate *trans*-eliminase increased to a maximum when mycelial growth was maximum. Exopolygalacturonase activity decreased, but pectin methylesterase and polygalacturonate *trans*-eliminase activities increased rapidly as cotton boll decay progressed. We conclude that exopolygalacturonase may play an important role in the early stages of infection, and that polygalacturonate *trans*-eliminase and pectin methylesterase become important in later stages of boll rot. Phytopathology 61:1118-1124.

Members of the genus *Diplodia* cause decay of the fruits and storage organs of many kinds of plants, and the world-wide economic importance of the genus has been well documented. *Diplodia gossypina* Cke. is one of our more important causes of cotton boll rot in the humid part of the cotton belt, and is responsible for substantial losses in yield (20, 23) and in quality of the lint (22). It develops extremely rapidly in our warm and humid climate of the cotton belt (3), especially after mechanical damage by cultivation or picker injury to unopened bolls (20).

Delgado (8) showed that the mature spores of *D. gossypina* (and other fungi) germinate on the surfaces of bolls, and their germ tubes which grow along the furrows of the wax ridges may bypass closed stomata but enter open stomata. Baehr & Pinckard (5) confirmed and extended Delgado's work. They showed that the fungus entered open stomata of bracts and the parenchyma of carpel sutures of bolls approaching maturity; the host tissues were invaded intercellularly.

Enzymes that degrade pectic substances of primary cell walls and middle lamellae are of major importance in the invasion of plant tissues by pathogenic fungi. If more were known about the production and properties of the pectic enzymes produced by *D. gossypina,* some means of blocking or delaying hyphal invasion of the host might be possible. The purpose of this study was to investigate the characteristics of pectic enzymes produced by *D. gossypina* in vitro and in vivo, and the nature of the association of pectic enzymes with boll rot of cotton. Portions of this work have been published as an abstract (27).

MATERIALS AND METHODS.—*Growth of the fungus.*—Four isolates of *D. gossypina* Cke. were used. Isolate 23A was obtained as a single-spored culture of the pathogen reisolated from a diseased cotton boll; the original isolation was made from a diseased peanut seed by R. Aycock, North Carolina, 1946. Isolate 17D was originally obtained from a diseased sweet potato root by W. J. Martin, Louisiana State University Agricultural Experiment Station. Isolate 10B was obtained from a naturally infected boll collected in a Louisiana field. Isolate 6C was originally obtained from the Mississippi Agricultural Experimental Station, Stoneville. All isolates have been in culture for several years and have remained highly pathogenic. Unless otherwise noted, isolate 23A was used throughout this investigation. The isolates were grown in a synthetic liquid medium consisting of 1.3 g L-asparagine, 1.0 g $MgSO_4 \cdot 7H_2O$, 0.5 g K_2HPO_4, 0.5 g KH_2PO_4, 0.5 g NaCl, 0.01 g $FeSO_4 \cdot 7H_2O$, and 20 g of either dextrose or pectin N.F. (Sunkist Growers, Ontario, Calif.) dissolved in 1 liter of deionized water. The medium was adjusted to pH 5.0 both before and after it was autoclaved at 121 C for 15 min at 15-lb. pressure. Flasks of 250-ml capacity containing 50 ml of medium were seeded with mycelium cut with a No. 3 cork-borer from 4-day-old cultures of *D. gossypina* grown on potato-dextrose agar (PDA) plates and incubated at 30 C. The mycelial mats were harvested, washed with deionized water, oven-dried overnight on weighed filter papers in petri dishes, cooled in a desiccator, and weighed.

Inoculation of bolls.—Greenhouse-grown bolls were tagged at anthesis so that their age could be determined accurately, and unless otherwise noted, 30- to 35-day-old bolls were used throughout this study. Unpublished work by students at this laboratory have shown that bolls of this age are approaching maturity and are highly susceptible to decay. The bolls were surface-sterilized with 10% Clorox (0.5% sodium hypochlorite)

for 10 min and washed twice with sterile water. They were then placed in sterile culture dishes fitted with moist filter papers, wounded, and finally inoculated with mycelia cut with a No. 3 cork borer from 4-day-old cultures of *D. gossypina,* isolate 23A, grown on PDA plates. Control bolls with surfaces similarly treated, but without inoculation, were incubated under the same conditions. Autoclaved bolls received comparable treatments. In the tests that follow, several of the other isolates were used; the results were similar to those obtained with isolate 23A.

Preparation of enzymes from culture filtrates, fungal mycelium, and diseased tissues.—Unless otherwise noted, the cultures were incubated either 7 or 10 days at 30 C, their pH values determined, and they then were strained through cheesecloth. The filtrates were centrifuged 20 min at 3,020 *g* to clear the preparation, after which the solution was dialyzed, under toluene against deionized water for about 18 hr at 4 C. Unless otherwise noted, enzyme extracts from mycelium and diseased tissues were prepared from surface-sterile bolls. After 10 days' incubation, the entire boll became covered with a thick mat of mycelium which was removed with tweezers. These mycelial mats and the decayed carpel walls were extracted separately by macerating in a cooled 0.5 N NaCl (1:3, w/v) solution for 3 to 5 min with a super Omni-Mixer and a Waring Blendor, respectively. The homogenate was strained through cheesecloth and its pH determined. Thereafter, the extracts were treated in the same manner as the culture filtrates. The enzyme extract from rotted locks was prepared similarly, except that the rotted locks were stirred and soaked in 0.5 N NaCl (1:2, w/v) for 2 hr at 4 C.

Enzyme assay methods.—Pectin methylesterase (PME) activity was determined by a modified titration method of Kertesz (12). The reaction mixture contained 20 ml of 1.5% pectin N.F. in 0.1 N NaCl solution, and 10 ml of dialyzed enzyme preparation in a 100-ml beaker in a water bath at 30 C. The pH of the reaction mixture was monitored with a pH meter. All titrations were made with 0.02 N NaOH. One unit of PME activity is defined as the amount of enzyme that under assay conditions required the addition of 1 microequivalent (μeq) of NaOH/hr to maintain the reaction mixture at pH 7.0.

Polygalacturonase (PG) activity was determined by measuring the reduction in viscosity of 1.2% sodium polypectate (Sunkist Growers, Ontario, Calif.), and also by determining increase in reducing groups liberated. Viscosity measurements were made at 30 C with a Fensk-Ostwald viscometer (size 300) (7). Unless otherwise noted, all viscosimetric measurements were made in viscometers containing 4 ml 1.2% sodium polypectate in 0.05 M citrate buffer, pH 4.5, 1 ml deionized water, and 1 ml dialyzed culture filtrate. Relative activity is expressed as the reciprocal of the time in min for 50% loss in viscosity multiplied by 10^3 (6). Reducing groups were determined by the modified dinitrosalicylic acid (DNS) method of Miller (15). D-galacturonic acid (Sigma Co.) was used as a standard. The reaction mixture, containing 4 ml 0.3% polygalacturonic acid (Sigma Co.) in 0.05 M citrate buffer at pH 4.5 and 1 ml of dialyzed culture filtrate, was incubated at 30 C for 1 hr. One unit of PG activity is defined as that amount of enzyme under assay conditions that catalyzes the release of 1 μg of D-galacturonic acid/hr.

The assay method used for measuring the activity of polygalacturonate *trans*-eliminase (PGTE) involved a standard viscosimetric technique (7) and two tests which measured the reaction products, a direct spectrophotometric measurement of increasing absorbance at 230 nm caused by unsaturated bonds formed during the depolymerization of polygalacturonic acid (11), and a thiobarbituric acid (TBA) test for the unsaturated products (4). The reaction mixture for the TBA test contained 5 ml of 0.8% polygalacturonic acid (or pectin) in 0.05 M Tris [tris (hydroxymethyl) amino methane]-HCl buffer (pH 8.5), 1 ml 0.05 M $CaCl_2$, and 2 ml dialyzed enzyme preparation. The mixture was incubated at 30 C for 2 hr. After incubation, 1.0 ml 9.0% $ZnSO_4 \cdot 7H_2O$ and 1.0 ml 0.5 N NaOH were added to stop enzyme action and to precipitate enzyme protein and excess substrate. The mixture was vigorously mixed, then centrifuged at 12,100 *g* for 15 min. Clarified reaction mixtures (1:10 dilution) were used in the TBA test. One unit of PGTE activity is defined as that amount of enzyme which effected an increase of 0.01 in absorbance at 550 nm/hr under the specified assay conditions of the TBA test. In all enzyme assays, identical substrate-buffer mixtures containing heat-inactivated dialyzed enzyme preparation served as controls. Protein was determined by the method of Waddell & Hill (26). Specific activity is defined as unit per mg protein.

Reaction products analysis.—Reaction products were examined by descending chromatography on Whatman No. 1 paper. Solvent systems used were 60 mg sodium formate dissolved in a solution mixture of 85 ml 77% ethanol and 15 ml 88% formic acid (18), ethylacetate-acetic acid-water (2:1:2, v/v) (11), and *n*-butanol-acetic acid-water (50:12:25, v/v) (21). The chromatograms were irrigated for 18-24 hr at room temperature, dried, then examined under ultraviolet light before development with aniline reagent (24).

RESULTS.—*Pectic enzymes in culture filtrate.*—The culture filtrate of *D. gossypina,* isolate 23A, grown in a pectin medium for 7 days contained pectic enzymes that hydrolyzed glycosidic linkages of sodium polypectate and pectin (Fig. 1). The enzyme degraded sodium polypectate at an optimal pH value around 4.5; activity decreased rapidly as pH of the reaction mixtures increased over 5.0. Similar results were demonstrated by measuring the release of reducing groups (Table 1). The pattern of sodium polypectate degradation changed when a 10-day-old culture filtrate was used for the viscosity analysis; in addition to the first optimal activity at pH 4.5, a second peak of activity appeared above pH 9.0.

When pectin was incubated with a 7-day-old culture filtrate, it showed two peaks of enzyme activity, one

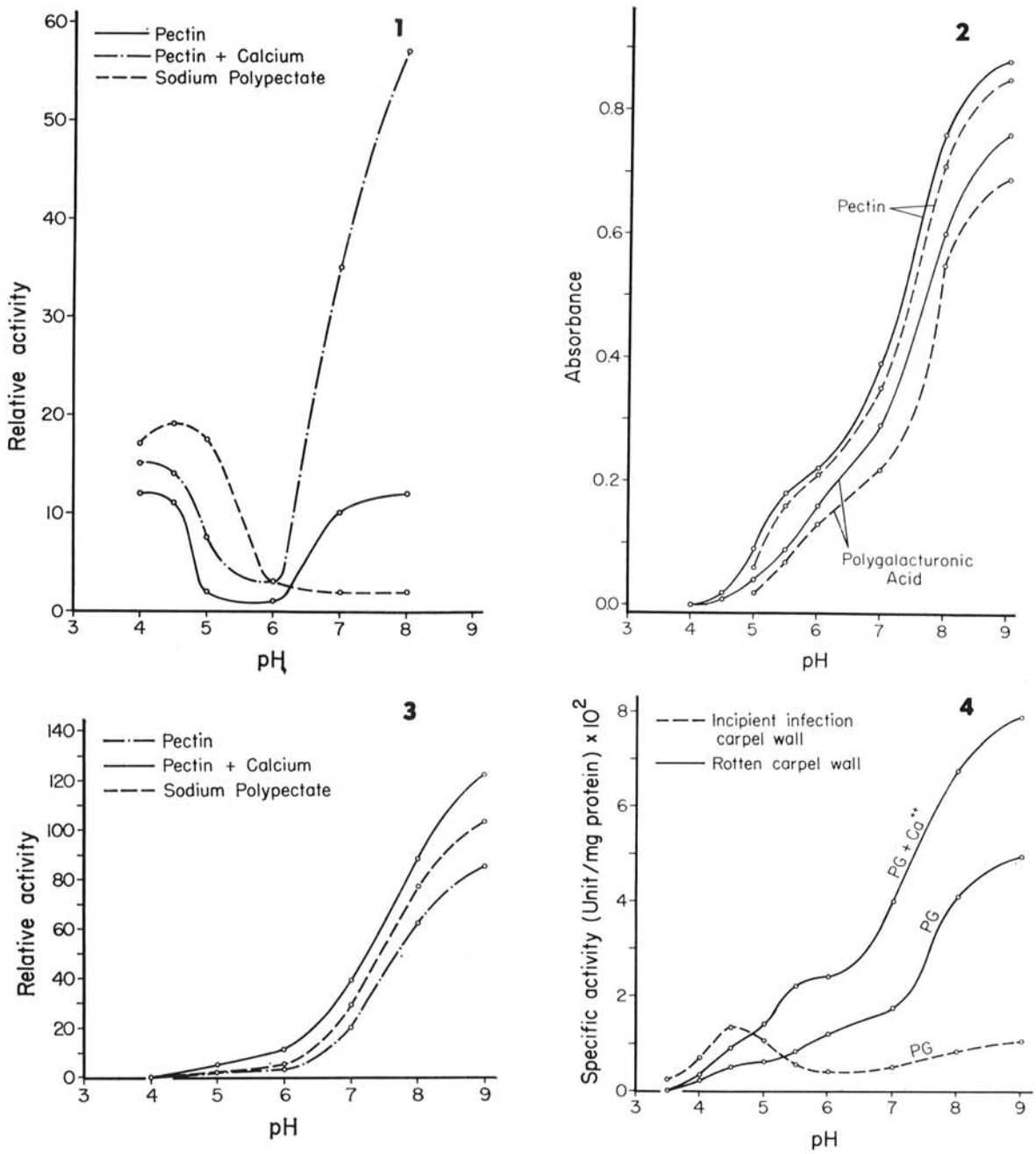

Fig. 1-4. **1)** Relative loss of activity measured by the loss in viscosity of sodium polypectate and pectin N.F. with or without Ca++ at various pH values when incubated with a dialyzed culture filtrate of *Diplodia gossypina* grown in a pectin medium at 30 C for 7 days. Relative activity is expressed as the reciprocal of the time in minutes for 50% loss in viscosity multiplied by 10^3. **2)** The effect of pH on polygalacturonate *trans*-eliminase activity in a 10-day-old culture filtrate of *D. gossypina*. Solid lines represent the absorbance at 550 nm with thiobarbituric acid method. Broken lines represent the direct measurement of reaction products formation at 230 nm. Tris [tris(hydroxymethyl) amino methane]-citrate buffer (0.05 M) at various pH values were used for preparing the substrates (polygalacturonic acid and pectin). **3)** Relative loss of activity measured by the loss in viscosity of sodium polypectate and pectin N.F. with or without Ca++ at various pH values when incubated with a dialyzed extract of *Diplodia*-rotted carpel walls of cotton bolls. **4)** Pectolytic enzyme activity in carpel walls of cotton bolls at the stage of incipient infection or when rotted by *D. gossypina* as a function of pH as measured by dinitrosalicylic acid method. Reaction mixture contained 3 ml of 0.3% polygalacturonic acid in 0.05 M Tris-citrate buffer at various pH values, 1 ml Tris-citrate buffer (or 0.01 M $CaCl_2$), and 1 ml of dialzed enzyme extract incubated at 30 C for 2 hr.

TABLE 1. Degradation of sodium polypectate and pectin N.F. at various pH values by 7-day-old culture filtrate of *Diplodia gossypina* as measured by dinitrosalicylic acid method[a]

Approximate pH	Reducing groups (units/ml filtrate)		
	Sodium polypectate	Pectin	Pectin + Ca++
4.0	4,250	2,150	2,250
4.5	4,750	2,150	2,250
5.0	4,550	950	2,500
7.0	750	750	4,050
8.0	500	2,050	4,850

[a] The reaction mixture, containing 3 ml of 0.3% substrate in 0.05 M citrate or Tris[tris(hydroxymethyl)amino methane]-HCl buffers at indicated pH values, 1 ml buffer (or 0.01 M $CaCl_2$), and 1 ml of dialyzed culture filtrate, was incubated at 30 C for 1 hr. One unit of enzyme activity is defined as that amount of enzyme under assay conditions that catalyzed the release of 1 μg of D-galacturonic acid/hr.

around pH 4.0 and one above pH 8.0 (Fig. 1). Enzyme activity was stimulated slightly by the addition of 0.01 M $CaCl_2$ to the reaction mixture if the pH was below 6.0, but was greatly stimulated above 6.0. The pattern of pectin degradation by a 10-day-old culture filtrate was similar to that obtained with a 7-day-old culture filtrate, except that enzyme activity increased slightly below pH 5.0 but a great deal above 5.0.

The ultraviolet absorption spectra of reaction products of polygalacturonic acid, incubated with a dialyzed 10-day-old culture filtrate of *D. gossypina,* showed an increasing absorption at 230 nm. The ultraviolet absorption maximum near 230-235 nm is characteristic of unsaturated compounds produced from pectic substances by heating (2) or by *trans*-eliminase activity (1). A scan of the reaction products of polygalacturonic acid with TBA reagent exhibited an absorption maximum of 550 nm. Spectra of TBA reaction products confirmed the formation of unsaturated galacturonyl products resulting from the *trans*-eliminative activity of *D. gossypina* in the culture filtrate.

Culture filtrates of *D. gossypina* (isolates 23A, 17D, 10B, and 6C) grown in a modified Czapek-Dox broth (CDB, with L-asparagine as the nitrogen source) did not contain measurable quantities of PME, PG, or PGTE. However, all four isolates of *D. gossypina* produced highly active PME, PG, and PGTE, when pectin was the carbon source. The relative enzyme activity of PME, PG, and PGTE in 10-day-old culture filtrate of isolate 23A decreased 20, 39, and 25%, respectively, when 0.1% glucose was added to the growth medium (pectin).

Properties of pectic enzymes from culture filtrates.—PME was stimulated by cations Ca^{++}, Mg^{++}, Mn^{++}, Na^{+}, and K^{+} (3.3×10^{-3} M). Enzyme activity increased more in the presence of divalent cations than monovalent cations. The addition of EDTA (3.3×10^{-3} M) greatly decreased PME activity. Cations may have combined with the carboxyl groups released during enzyme action to prevent the formation of inactive enzyme-carboxyl complexes (13). Optimal pH for PME activity was ca. 6.5.

PG was stimulated by such cations as Mg^{++}, Mn^{++}, Zn^{++}, Na^{+}, K^{+} (2×10^{-2} M), and Cu^{++} (4×10^{-3} M), although Ca^{++} decreased the activity. PG activity decreased rapidly and was proportioned to increase in Ca^{++} concentration in the reaction mixture up to, but not higher than, 1×10^{-3} M. A similar inhibition of fungal PG activity by Ca^{++} has been reported by other workers (4, 19). PG was slightly inhibited by EDTA (2×10^{-2} M). The optimal pH for PG activity was at pH 4.5 (Table 1). This optimal pH for PG activity agreed with the loss in viscosity of sodium polypectate.

The *trans*-eliminase produced by *D. gossypina* reacted with pectin, pectic acid (Nutritional Biochemicals Corp., Ohio), polygalacturonic acid, and sodium polypectate; the absorbance of reaction products at 230 nm was 0.85, 0.63, 0.58, and 0.25, respectively. The *trans*-eliminative activity of pectin and polygalacturonic acid was very low below pH 5.0, although enzyme activity increased rapidly above pH 5.0. The enzyme activity in the presence of polygalacturonic acid and pectin increased as the pH of the reaction mixture increased to 9.0 (Fig. 2).

Direct spectrophotometric measurement of *trans*-eliminase activity at 230 nm with pectin as the substrate showed linear activity to increase from zero absorbance to 0.05 during an 8-min period. When NaCl (8.0×10^{-2} M), $MgCl_2$ (2.0×10^{-2} M), and $CaCl_2$ (2.0×10^{-3} M) were added, the activity increased linearly from an absorbance of 0.00 to 0.10, 0.13, and 0.29, respectively, during an 8-min period. Enzyme activity was completely inhibited in the presence of EDTA (2.0×10^{-2} M). Ca^{++} stimulation of *trans*-eliminase activity was different in the presence of polygalacturonic acid and pectin. Enzyme activity increased rapidly when assayed with polygalacturonic acid and 1.4×10^{-3} M Ca^{++} was optimal. However, enzyme activity increased slowly when assayed with pectin and 7.1×10^{-3} M Ca^{++} was optimal.

Pectic enzyme activity in extracts of diseased boll tissues and fungal mycelium.—The dialyzed extract of healthy noninoculated carpel walls and locks of cotton contained neither exo- nor endopolygalacturonase nor polygalacturonate *trans*-eliminase, although traces (if any) of pectin methylesterase were sometimes present. However, the dialyzed extract of *Diplodia*-rotted carpel walls of cotton not only had high pectin methylesterase, but also caused rapid loss in viscosity with both sodium polypectate and pectin in alkaline conditions (Fig. 3). The loss in viscosity with sodium polypectate was faster than with pectin. The addition of Ca^{++} slightly increased the loss in viscosity with pectin. A spectrophotometric scan of the reaction products of polygalacturonic acid revealed an absorption maximum at 230 nm. The absorption spectrum of reaction products with TBA revealed an absorption maximum at 550 nm. These are characteristics of PGTE activity (1). The extract of rotted locks showed PME and PGTE activity, although it was lower than in rotted

carpel walls. The optimal pH for activity of PME and PGTE from both rotted carpel walls and locks was 6.5 and 9.0, respectively. These pH optima were found to be the same as those of PME and PGTE from mycelial extracts. PGTE was stimulated slightly by Ca++ when the concentration was below 1.4×10^{-3} M. However, at Ca++ concentrations beyond this level, the activity was inhibited. The optimum Ca++ concentration for PGTE activity was 0.7×10^{-3} M. When the *trans*-eliminase was assayed in the presence of pectin as the substrate, the activity was stimulated by Ca++ with an optimal concentration of 7.1×10^{-3} M. PGTE was inhibited by 14.3×10^{-3} M of EDTA.

Four days after bolls were inoculated with *Diplodia* and kept at 30 C, visual symptoms of cotton boll rot advanced rapidly until ca. 0.25 of the boll turned a brownish-black color under a surface mycelial mat. This diseased portion of carpel walls was extracted and assayed colorimetrically for pectic enzyme activity at various pH values. Two peaks of activity were found, one at pH 4.5 and one at 9.0 (Fig. 4). The first peak was caused by exoPG activity and the second peak by PGTE. ExoPG was inhibited by Ca++ (2.0×10^{-2} M), and EDTA (2.0×10^{-2} M). After a 10-day incubation period, the boll completely rotted; at this stage of decay, the surface mycelium was removed, and the rotted black carpel walls were extracted and assayed for pectic enzyme activity at various pH values. Pectic enzyme activity was a function of pH values, and was stimulated by Ca++ (Fig. 4). Release of reducing groups in the extracts of rotted carpel walls decreased slightly under acidic conditions, but increased greatly under alkaline conditions. It was apparent that both exoPG and PGTE were present in the extracts of diseased carpel walls. ExoPG was more active in the early stages of infection, but the activity of PGTE increased greatly as the rot progressed. The pH changed from 5.3 in healthy carpel walls through 6.8 during early stages of rot to 7.9 in the final stages of decay as determined for 20 g tissues ground with 50 ml of deionized water.

PGTE activity was detected in extracts of both rotted surface-sterilized and autoclaved carpel walls, but the activity in extracts of rotted, surface-sterilized carpel walls was double that found in autoclaved carpel walls.

Release of pectic enzymes during the growth phase.—During the first 4 days of growth of *D. gossypina* on pectin medium, the pH decreased slightly (initial pH = 5.0), then increased gradually to 8.4. Mycelial dry weight increased gradually, reached a maximum after 7 days, then decreased slightly (Fig. 5-B). Activities of pectic enzymes during the growth phase of the fungus (period of increase in dry weight) are shown in Fig. 5-A. The measurement of reducing groups for PG activity under assay conditions was not influenced by the presence of PGTE activity in the culture filtrate, because PGTE activity was not detected in the reaction mixture at pH 4.5 in the absence of Ca++. PG activity increased rapidly and in direct proportion to increase of mycelial weight up to a maximum at 7 days. During this period, the pH of the culture remained below 7.0. PG activity decreased rapidly after maximum growth, whereas the pH of the culture increased to above 7.0. PGTE activity was not detected after 4 days. PME activity was very low at 4 days, and increased slowly during the next 6 days. PGTE and PME activities reached maxima after about 13 days of growth. At this time, pH of the culture was above 7.0. Similar results were obtained with isolate 10B.

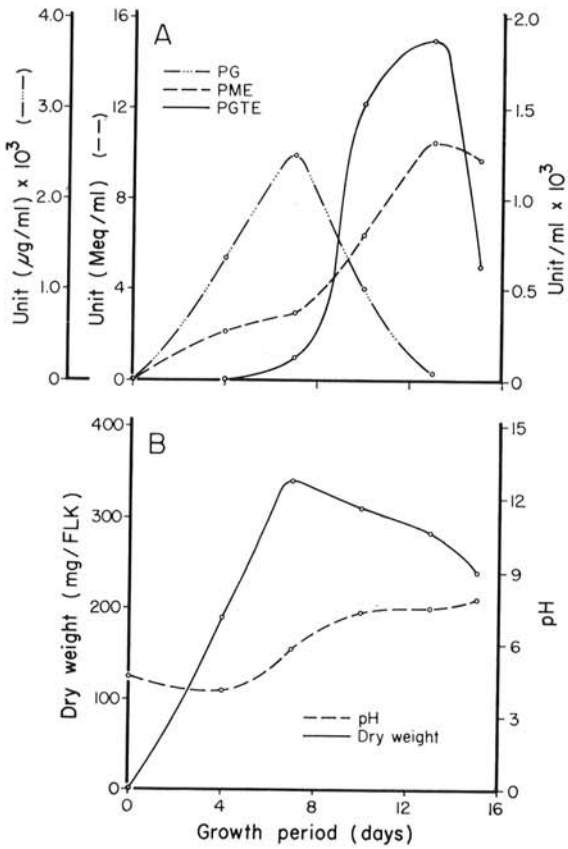

Fig. 5. A) Comparison of the activity of pectin methylesterase (PME), polygalacturonase (PG), and polygalacturonate *trans*-eliminase (PGTE) in culture filtrates of *Diplodia gossypina*, isolate 23A, grown in a pectin medium for 15 days at 30 C. PME activity was determined by the titration method. PG activity was determined by measuring the reducing groups released under the assay condition. PGTE activity was determined by measuring the reaction with thiobarbituric acid. **B)** Mycelial dry weight and pH of the culture filtrates of *D. gossypina*, when grown in a pectin medium at 30 C. The values of mycelial dry weight and pH were the average of three flasks.

Chromatographic analyses of the reaction products.—The ethanol-formate solvent system was used. No reaction products could be detected when substrates were incubated with boiled culture filtrates. The reaction products obtained with pectin and polygalacturonic acid as substrates, incubated at 30 C for 24 hr, were similar. A single reaction product was obtained when pectin or polygalacturonic acid was incubated with a 7- or 10-day-old culture filtrate of *D. gossypina* at pH

4.5. The orange color of the spot and its R_F were equivalent to that obtained with the D-galacturonic acid. Two reaction products were detected as spots on the chromatogram when either pectin or polygalacturonic acid and Ca++ was incubated with a 10-day-old culture filtrate at pH 8.5. One compound corresponded to D-galacturonic acid, the other, with an R_{GA} of 1.25, was revealed only upon exposure to ultraviolet light before application of the spray. Similar results were obtained by irrigating the chromatograms in an ethyl-acetate-acetic acid-water solvent system after 4, 15, 24, and 48 hr of incubation of culture filtrates and substrates at 30 C, with the exception that spot intensity increased as the incubation time increased. Oligogalacturonides and unsaturated oligogalacturonides apparently were not produced (10, 18). In an attempt to resolve the compound that comprised the large spot detected with ultraviolet light, *n*-butanol-acetic acid-water solvent also was used for irrigation. This system made possible resolution of D-galacturonic acid and two other compounds; one, under ultraviolet light, appeared as a large spot with an R_{GA} of 1.91, the other as a small spot with an R_{GA} of 1.59. These spots were obtained with reaction mixtures of 10-day-old culture filtrate and pectin or polygalacturonic acid with Ca++ at pH 8.5, after 24-hr incubation at 30 C. The enzymatic products obtained from extracts of diseased boll tissues and fungal mycelia were similar to those found in 10-day-old culture filtrates of *Diplodia*.

A single spot equivalent to D-galacturonic acid was detected for culture filtrates of *D. gossypina* grown in a pectin medium for 3 and 6 days. No spots were observed under ultraviolet light. Neither D-galacturonic acid nor the ultraviolet-reacting compound was detected in the cultures after 10 and 13 days of growth.

DISCUSSION.—Pectic enzymes produced in culture filtrates of *Diplodia gossypina* grown in a pectin medium, in extracts of *Diplodia*-diseased cotton boll tissues, and in extracts of fungal mycelia included a pectin methylesterase, an exopolygalacturonase, and a polygalacturonate *trans*-eliminase. The similarities in properties, in heat stabilities (*unpublished data*), in reaction products, and in kinds of pectic enzymes produced in culture filtrates, in mycelium, and in diseased tissues suggest that the pectic enzymes present in diseased tissues are of fungal origin.

Chromatographic analysis indicated that only D-galacturonic acid accumulated in the 3- and 6-day-old cultures when the fungus was grown in a pectin medium. In addition, a single reaction product, identified as D-galacturonic acid, was detected when polygalacturonic acid or pectin was incubated with a dialyzed culture filtrate, extract of mycelia, or extract of diseased tissues at pH 4.5. Moreover, there was comparatively little loss of viscosity, but high reducing power, released when sodium polypectate was incubated with a dialyzed culture filtrate under acidic conditions. The optimal pH for loss in viscosity and release of reducing power was also found to be 4.5. These results indicate that the enzyme attacked terminal glycosidic linkages of polypectate and pectin, and therefore is an exopolygalacturonase. Comparatively little viscosity loss with sodium polypectate as a substrate at pH 4.5, together with the absence of oligogalacturonides in reaction products or in culture filtrates, indicated that there was no endopolygalacturonase activity.

Albersheim et al. (1), in 1960, first reported that glycosidic linkages of pectic substances could be degraded to unsaturated galacturonyl units by a cleavage of the linkage at C-4 with simultaneous elimination of H+ from C-5. The significance of this reaction has been made increasingly evident by reports of the presence of *trans*-eliminases in bacteria (14, 16, 17, 21, 25) and phytopathogenic fungi (4, 6, 9, 11, 19). Based on reaction mixtures absorbing maximally at 230 nm, a positive thiobarbituric acid reaction, and calcium stimulation of activity, it was concluded that a *trans*-eliminase was present in the culture filtrates, in mycelium, and in diseased boll tissues. Because alkaline pH levels were optimal for attack of polygalacturonic acid and pectin, the pH optimum for *Diplodia trans*-eliminase resembles more the polygalacturonate *trans*-eliminases of bacteria (16, 25) and some pathogenic fungi (4, 6, 19) than the pectin *trans*-eliminase of *Aspergillus* (9). Purification studies should be conducted to determine if the *trans*-eliminase from *D. gossypina* is actually a single enzyme capable of attacking pectin and polygalacturonic acid, because many *trans*-eliminases from pathogenic fungi (6, 11) and bacteria (25) apparently degrade both substrates; however, some *trans*-eliminases reported from bacteria (14, 17) and from one pathogenic fungus (19) have been found more active on polygalacturonic acid than on pectin.

The types of pectic enzyme that predominated in cultures of *D. gossypina* changed during the growth period. Young cultures contained principally exopolygalacturonase, but old cultures contained primarily polygalacturonate *trans*-eliminase and pectin methylesterase; these changes apparently were associated with a pH change during growth of the fungus. The extracts of diseased cotton boll tissues contained principally polygalacturonate *trans*-eliminase and pectin methylesterase, and the enzyme activity increased as boll decay progressed. In contrast, exopolygalacturonase was more active during early decay of the carpel walls; its activity decreased as boll rot increased. The sharp increase in pH of diseased tissue extracts shortly after inoculation, which may be associated with increasing calcium concentration in diseased tissues (11), is favorable for *trans*-eliminase activity but unfavorable for exopolygalacturonase activity. We, therefore, conclude that exopolygalacturonase may play an important role in the early stages of *Diplodia* penetration and colonization of the boll; however, polygalacturonate *trans*-eliminase, in conjunction with pectin methylesterase, is apparently responsible for the high rate of decay which follows.

Inasmuch as the several isolates used gave similar results although they were from widely divergent sources and cultured over a long period of time, we conclude *Diplodia gossypina* to be a very stable species.

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