Some Biochemical Factors Associated with the Infection of Cotton Fruit by Diplodia gossypina

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ABSTRACT

Germinating spores and mycelium of *Diplodia gossypina* on the surfaces of the immature fruit of cotton, *Gossypium hirsutum* 'Deltapine 16', caused the necrosis and death of certain epidermal cells within 48 to 72 hr after inoculation. The first epidermal cells injured were the guard cells of stomata, their subsidiary cells, and multicellular epidermal hairs. These symptoms were reproduced by treating bolls with extracts of rotted carpel wall tissue, commercial polygalacturonase, cellulase, and cell-free culture filtrates of *D. gossypina* grown on synthetic media containing pectin, carboxymethylcellulose, glucose, and lecithin and on cotton boll tissue medium. Cell injury was substantially reduced after the culture filtrates were heated. Culture filtrates containing pectic enzymes and commercial polygalacturonase were the most active in producing cell necrosis. Glucose, fructose, galactose, sucrose, raffinose, and two unidentified materials were found present in the nectar and the washings from normal boll surfaces. Cellulase, cellobiase, xylanase, arabanase, proteases, and phosphatidases were found in extracts of rotted tissues. Active tissue maceration paralleled optimal conditions for polygalacturonate *trans*-eliminase activity. The fungus first predisposes the thin-walled cells surrounding the stomata to invasion by enzymatic and toxic action in advance of hyphal penetration. Exopolygalacturonase is suspected of being a dominant enzyme responsible for the primary symptoms of cotton boll rot caused by *D. gossypina*.

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Additional key words: disease resistance.

Diplodia species are common plant pathogens on many kinds of food and fiber crops, and are distributed world-wide. *Diplodia gossypina* occurs on cotton wherever the crop is grown, but is particularly damaging to bolls in the humid portion of the cotton belt of the USA (12).

Although some evidence has been presented indicating that the fungus is able to penetrate uninjured bolls (2, 22), the mode of entry has never been described. One method of penetration of *D. gossypina* into the boll is through the open stomata (5). Baehr & Pinckard (1) confirmed this, and showed that the fungus entered the open stomata of bolls and bracts and also penetrated the parenchyma of carpel sutures of bolls approaching maturity. They also showed invasion of host cells killed in advance of the invading pathogen. Such pathological action has been attributed to toxins, enzymes, and their several combinations (4, 6, 9, 16). In addition (20), we reported on some properties of pectic enzymes produced by *D. gossypina* and showed that this fungus produced pectic enzymes in vitro and in vivo in great abundance. Exopolygalacturonase was found to be important in the early colonization of the fungus on the cotton boll, and polygalacturonate *trans*-eliminase in conjunction with pectin methylesterase to be of major importance in the later stages of boll decay.

The purpose of this study is to elucidate the mode of penetration of *D. gossypina* into the uninjured boll in relation to the formation of phosphatidases, proteases, and cell-wall degrading enzymes associated with the primary symptoms and further decay of the cotton boll. A preliminary report has been published (19).

MATERIALS AND METHODS.—*Development of symptoms of Diplodia boll rot.*—Greenhouse-grown cotton plants, *Gossypium hirsutum* L. 'Deltapine 16', with bolls of 5 to 40 days of age (from anthesis) were placed under temperature and humidity conditions similar to those of midsummer. Dew formation was induced by the liberal release of steam at night. Day temperatures ranged from 25 to 35 C; and night temperatures, from 20 to 25 C. Freshly formed spores of *Diplodia gossypina* Cke., isolate 23A (20), collected from 3-week-old potato-dextrose agar (PDA) plates, were suspended in water and immediately atomized onto the bolls. Some bolls were enclosed in polyethylene bags, to retain high humidity near their surfaces during the day. Under these conditions, spore germination was efficient and germ tube growth was substantial in 10 to 12 hr.

Bolls for laboratory use were detached from the plant, and the bracts and calyx organs removed by cutting through the base of the boll above the corolla ring. After surface sterilization in 10% Clorox (0.5% sodium hypochlorite) for 10 min followed by rinses, the bolls were inoculated with nonsporebearing mycelial mats cut from PDA plates (20).

After a 48- to 72-hr incubation period, the bolls were placed in a freezer overnight, removed, and gently thawed in water at 55 C. The epidermis was peeled off in strips, mounted in glycerol, and examined microscopically.

Methods of enzyme assay.—Unless otherwise noted, *D. gossypina* was grown on a synthetic medium (20) using as carbon sources glucose, pectin N.F. (Sunkist Growers, Ontario, Calif.), carboxymethylcellulose (CMC-7MP; Hercules Powder Co., Wilmington, Del.), or refined soybean

lecithin (Nutritional Biochemicals Corp.). Methods of inoculation of bolls and preparation of enzymes from liquid cultures of *D. gossypina* from mycelia grown on surfaces of rotted bolls and from rotted boll tissues are described elsewhere (20). Polygalacturonase (PG), polygalacturonate *trans*-eliminase (PGTE), and pectin methylesterase (PME) were assayed by described methods (20).

Cellulase (C_X, carboxymethylcellulase) was assayed with carboxymethylcellulose (CMC) as the substrate by a viscosimetric method (3) and a dinitrosalicylic acid (DNS) method (20). Reaction mixtures contained 5 ml of 0.6% CMC, 0.1 ml of enzyme solution, and 0.9 ml of 0.05 M citrate buffer, pH 4.5. Relative activity is expressed as the reciprocal of the time in minutes for 50% loss in viscosity multiplied by 10^3. Glucose was used as a standard. Cellobiase was considered present when cellobiose was hydrolyzed to yield glucose.

Xylanase and arabanase were assayed by a DNS method (20) and paper chromatography. Reaction mixtures contained 1 ml of enzyme extract, 4 ml of 0.1% xylan (Nutritional Biochemicals Corp.), or 0.1% araban (Matheson, Coleman & Bell) in 0.05 M citrate buffer, pH 5.0. Xylose was used as a standard. Protein was determined by the method of Waddell & Hill (18). Specific activity is defined as μg per mg protein per hr.

Phosphatidases were assayed with refined soybean lecithin as the substrate by the 'cup plate' method described by Tseng & Bateman (17). The dialyzed extract of Diplodia-rotted carpel wall tissue was lyophilized before using the cup plate assay. The lyophilized extracts were dissolved in water to give 5 mg/ml concentration, and 0.2 ml were placed in each well. The zones of substrate hydrolyses were measured after 24-hr incubation at 30 C. Phosphatidase activity was indicated by development of a white zone around the wells containing the active enzyme preparation. Autoclaved enzyme preparations served as controls. One unit of phosphatidase activity is defined as that amount of enzyme that hydrolyzes a mm^2 white zone around the well.

Proteolytic enzymes were assayed using the method described by Hancock & Millar (7). Reaction mixtures contained 1 ml of enzyme preparation and 1 ml of 1% casein (Fisher Scientific Co.) in 0.1 M phosphate buffer at pH 7.5. The reaction mixtures were incubated at 30 C for 1 hr, then precipitated with 3 ml of 5% trichloroacetic acid (TCA), after which they were held at room temperature for at least 1 hr before clearing by centrifugation. The absorbance of the supernatant fluid from the reaction mixture precipitated with TCA at 0 time was used as a blank. One unit of proteolytic activity is defined as that amount of enzyme that causes an increase in absorbance of 0.1/hr under assay conditions.

Paper chromatographic analyses of normal boll surface washings, nectar (below bracts), and reaction products of cellulase, cellobiase, xylanase, and arabanase were developed in butanol-acetic acid-H_2O (4:1:5, v/v) and isopropanol-H_2O (4:1, v/v) solvent systems and sprayed with aniline, resorcinol-HCl, and alkaline silver nitrate reagents (15).

Tissue maceration.—Rings of carpel wall tissue were cut from carpel walls of 30-day-old bolls with No. 1 and 2 cork borers. The rings were used as substrates for studying enzymatic tissue maceration by extracts of diseased tissue or culture filtrates. Reaction mixtures contained 5 ml of enzyme solution, 2 ml of citrate buffer (0.05 M, pH 4.5) or Tris buffer [tris (hydroxymethyl) amino methane]-HCl (0.05 M, pH 9.0), 1 ml of $CaCl_2$ (0.01 M) or H_2O and 0.01 ml of Merthiolate (Eli Lilly &

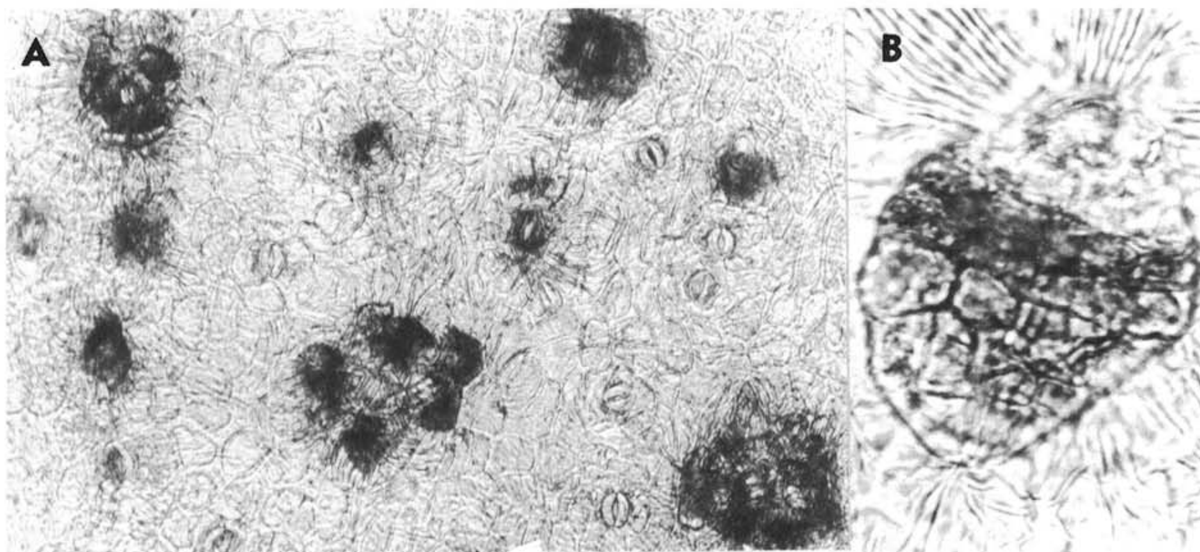

Fig. 1. Epidermal strips showing symptoms of Diplodia boll rot of cotton resulting from spore germination after 48 hr under greenhouse conditions. **A)** Necrosis of guard cells and subsidiary cells around the stomata of a 20-day-old boll (X 100). **B)** An infected multicellular epidermal hair of a 10-day-old boll (X 200).

Co., Indianapolis, Ind.), and eight pieces of carpel wall rings. The controls also contained Merthiolate.

Because Orgell (11) showed that plant cuticle could be isolated with pectic enzymes, extracts of diseased carpel walls and culture filtrates were tested for their ability to separate cuticle from epidermal tissue. Epidermal strips of carpel walls were easily peeled from the frozen bolls after gently thawing in warm water.

RESULTS.–*Development of primary symptoms of Diplodia boll rot.*–Upon removal of the sparse mat of mycelium resulting from germination of the spores on the surface of inoculated greenhouse-grown bolls, and before hyphal penetration had progressed noticeably, yellow flecks of necrotic epidermal tissue beneath the mats were observed on bolls of the several ages used. Microscopic examination of epidermal strips taken from the area revealed the yellow flecks to consist of affected guard cells, their subsidiary cells, and adjacent epidermal cells (Fig. 1-A). On 5- and 10-day-old bolls, the multicellular epidermal hairs were also affected (Fig. 1-B). No mycelial invasion of cell walls or tissues was observed, indicating that toxic action was proceeding in advance of hyphal penetration. Within a few hours, increasing numbers of affected cells were observed forming large, sunken groups of brown tissue which broke away from the epidermis when attempts were made to strip it off. Similar symptoms of toxic action were observed on inoculated, detached bolls held in moist chambers at 30 C for 48 hr. Bolls 15 to 25 days old were less affected than those 5 to 10 and 30 to 40 days of age, indicating that some form of resistance to toxic action occurred within the 15- to 25-day age group. Control bolls sprayed with sterile water remained normal in appearance.

Similar but more pronounced toxic action was noted when mycelial mats, grown on PDA, were placed on boll surfaces without injury to the epidermis and incubated at 30 C for 72 hr. The degree of epidermal necrosis associated with the primary symptoms was clearly related to boll age (Fig. 2). Young bolls, 5 to 12 days old, had more of their surface affected than did bolls 15 to 25 days old. Bolls 28 to 40 days old were more affected during the 48- to 72-hr period than were bolls of intermediate age. Coalescense of affected cells again resulted in sunken areas from which the epidermis could not be removed without breaking it. On bolls 45 days old where the sutures of the capsule had opened, exposing the underlying tissue, mycelial penetration of the parenchyma beneath the sutures resulted in a rapid and obscuring decay.

Development of symptoms with culture filtrates.–Cultural filtrates in which the mycelium had been removed by filtration and centrifugation also produced the primary symptoms of Diplodia infection as described above. These filtrates were obtained from synthetic media with carbon sources of pectin, CMC, glucose, and lecithin. Symptoms were also produced from culture filtrates of cotton boll tissue medium, extract of rotted boll tissue, commercial polygalacturonase, and cellulase. Typical

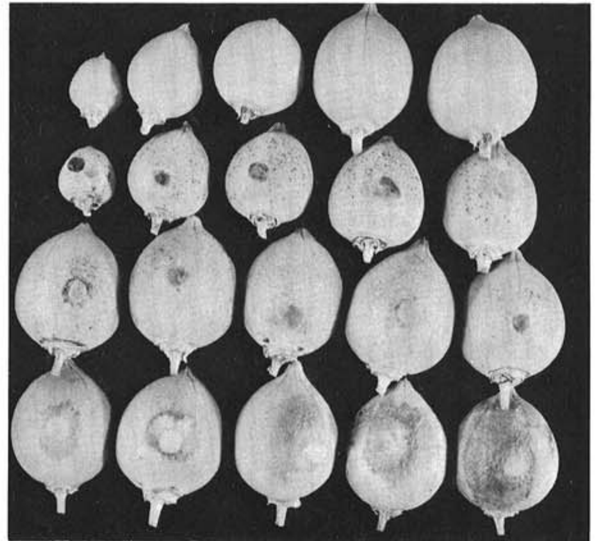

Fig. 2. Symptoms on cotton bolls of various ages inoculated with mycelium of *Diplodia gossypina* and incubated at 30 C for 3 days. From top to bottom, left to right: 1st row, control bolls 5, 7, 10, 21, and 35 days old; 2nd row, inoculated bolls 5, 7, 9, 11, and 13 days old; 3rd row, inoculated bolls 15, 17, 20, 25, and 28 days old; 4th row, inoculated bolls 30, 32, 35, 40, and 45 days old.

necrotic symptoms were not produced on sterile synthetic media (composed of the various carbon sources), normal tissue extract, phosphate buffer (pH 4.5), Tris buffer plus Ca^{++} at pH 8.5, water, and heat-inactivated commercial polygalacturonase and cellulase. Heat-inactivated culture filtrates reduced the symptoms substantially, but not completely. No significant difference was noted between treatments with dialyzed and undialyzed culture filtrates. Among all the treatments used, commercial polygalacturonase and culture filtrates of *D. gossypina* grown on pectin medium were the most active (Fig. 3). Commercial cellulase and culture filtrates from the lecithin medium were least active. Five-day-old culture filtrates grown on pectin medium were more active at pH 4.5 than at pH 8.0 (plus Ca^{++}). Assays for enzymatic activity of the above filtrates showed that 1,750 μg/ml of exoPG and 35 units/ml of PGTE were present. In CMC medium, 84 units/ml of cellulase were present, and in the lecithin medium 1.0 unit/ml of phosphatidase activity was noted.

Cellulase (C_X) and cellobiase in extracts of diseased tissues and mycelia.–Dialyzed extracts of noninoculated carpel walls and contents of seed chambers (locks) of cotton contained neither C_X nor cellobiase. Dialyzed extracts of Diplodia-rotted carpel walls and locks incubated with CMC revealed a very high C_X activity, 1,670 and 1,500 units/mg, respectively, when assayed viscosimetrically. The enzyme was also present in extracts of mycelia (880 units/mg), but was lower than in extracts of decaying carpel walls and locks. When C_X was assayed viscosimetrically as a function of pH, a similarly

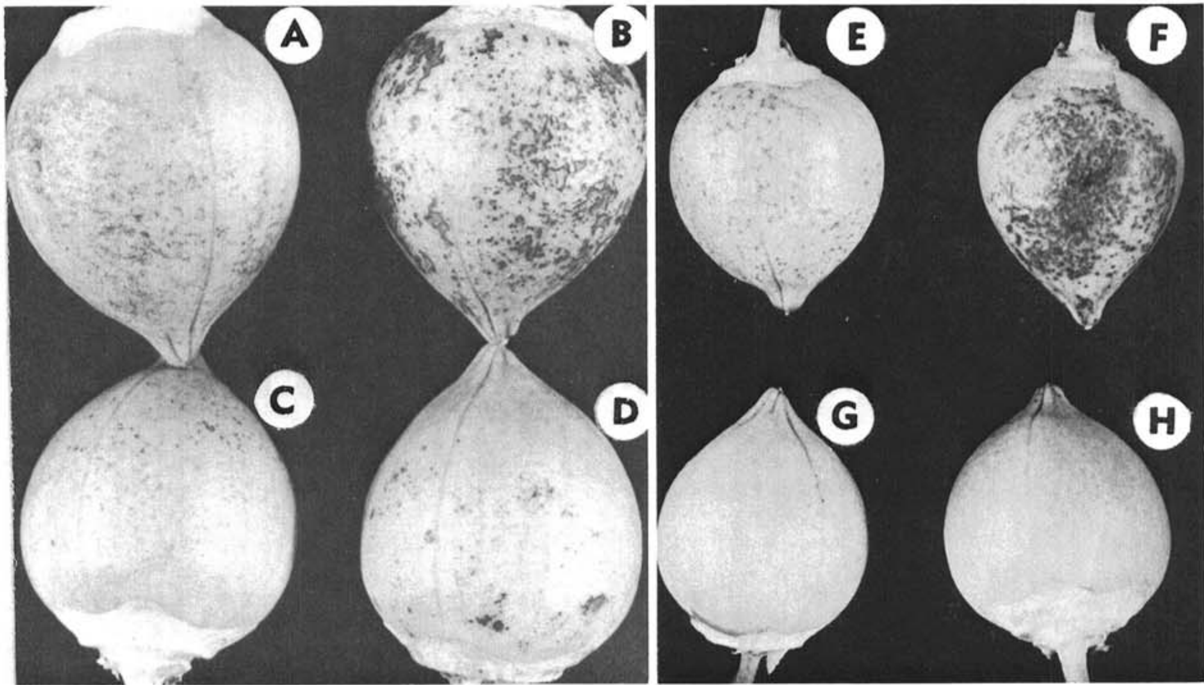

Fig. 3. Symptoms of Diplodia boll rot of cotton induced by enzymatic action after 20 hr contact with 30-day-old bolls; (Left) 5-day-old culture filtrate of *Diplodia gossypina* grown in pectin medium (A) at pH 8.5 plus Ca^{++}; (B) at pH 4.5; (C) heat-inactivated culture filtrate at pH 8.5; (D) heat-inactivated culture filtrate at pH 4.5. (Right) (E) high cellulase concentration; (F) high polygalacturonase concentration; (G) heat-inactivated cellulase; (H) heat-inactivated polygalacturonase.

broad pH optimum at pH 4.5 to 5.5 was found in extracts of rotted carpel walls and locks as well as mycelia. As the rot progressed, C_X activity increased, having ca. 3 times higher specific activity in extracts of rotted carpel walls 10 days after inoculation than from extracts of rotted carpel walls 4 days after inoculation. Paper chromatographic analyses of reaction products of CMC with extracts of rotted carpel walls and locks, as well as mycelia, after 24-hr incubation at 30 C were mainly glucose and traces of cellobiose. Cellobiase was considered to be present when glucose was detected chromatographically in reaction mixtures of all three enzyme preparations and with cellobiose after 24-hr incubation at 30 C.

Xylanase and arabanase in extracts of diseased tissues.—When extracts of rotted carpel walls and locks were incubated with xylan or araban at pH 5.0 for 24 hr and 30 C, traces of either xylose or arabinose were detected chromatographically in extracts of carpel walls but not in extracts of locks. After 2-hr incubation of reaction mixtures at 30 C, the specific activity for xylanase and arabanase in extracts of carpel walls assayed colorimetrically was 1,700 and 540 μg/mg protein per hr, respectively. It is apparent that extracts of rotted carpel walls contain higher xylanase activity than arabanase activity.

Phosphatidases and proteases in extracts of diseased tissues.—Phosphatidases and proteases were produced in diseased tissues but not in healthy tissues. After 5 days of incubation, activities of phosphatidases and proteases were 1.0 and 0.05 units/mg of lyophilized extract, respectively. Enzyme activities increased to 4 and 1.13 units/mg of lyophilized extract after a 10-day incubation period when bolls were completely rotted. The optimal pH for phosphatidase activity was found to be about pH 4 to 5 when assayed at pH values of 4.0 to 8.0.

Tissue degradation by extracts of Diplodia-rotted carpel walls.—Extracts of diseased tissue degraded healthy carpel wall tissue rapidly; however, extracts of healthy carpel walls did not degrade such tissue. The rate of maceration was greater in alkaline conditions and was stimulated by the addition of Ca^{++}. Similar degradation of carpel wall tissue was accomplished with 7-day-old culture filtrates of *D. gossypina* grown on a pectin medium. The rate of degradation of carpel wall tissues by culture filtrates was very low at pH 4.5 both in the presence and absence of Ca^{++}, but increased greatly in the presence of Ca^{++} at pH 8.5. When epidermal strips were incubated with the culture filtrate at 30 C for 48 hr and buffered with Ca^{++} at pH 8.5, the clean cuticle was released from epidermal tissue.

Carbohydrates present in boll surface washings and nectar.—Paper chromatographic analyses showed the carbohydrates present in normal boll surface washings to be similar to those found from nectar. These were glucose, fructose, galactose, sucrose, raffinose, and two unknowns which developed more slowly than raffinose and which were revealed by

alkaline silver nitrate reagent but not resorcinol-HCl reagent.

DISCUSSION.–If moisture conditions are suitable, one- or two-celled mature pycnospores of *D. gossypina* may be extruded from pycnidia in large numbers. Freshly produced spores germinate in the presence of water; one germ tube is produced from the single-celled hyaline spores, and sometimes two germ tubes, from the two-celled pigmented spores. The germ tube becomes attached to the cuticle of the cotton boll host, and is not easily removed by washing (14). In these early stages of spore germination, cells of the boll epidermis adjacent to the germ tubes changed color and appeared to be affected by the advancing mycelium, although penetration had not occurred. The first cells affected were the guard and accessory cells, the cuticle of which was devoid of ridges and seemed to be thinner (13) than that of cells some distance away from the stomata (Fig. 1). Fungal injury to guard cells and accessory cells resulted in the formation of surface droplets from the host cells. This material undoubtedly provided additional nutrients for hyphal growth, and probably increased the concentration of cell wall-degrading enzymes (21). During this process, the cuticle became more fragile. These conditions would seem to favor hyphal growth and development, and provide additional fungal penetration of the cuticle as well as movement through stomatal apertures and into the interior of the capsule wall. Progress of hyphal penetration and growth from the surface of the boll into the epidermal cells and substomatal cavities was dependent on boll surface humidity and its duration. If the surface humidity was insufficient to protect the germ tubes from desiccation, permitting them to take up liquid nutrients before they could reach the host tissue, ability to colonize the host was lost. Under these conditions the affected host cells coalesced, became a chocolate brown color, and resembled the so-called nonaggressive stage of the chocolate spot disease of bean as reported by Wilson (23).

Under field conditions, the upper surfaces of nearly mature bolls exposed to abundant sunlight often show numerous chocolate spots locally known as sun-scald. It appears that these spots start with the breakdown of the thin-walled cells surrounding the stomata which may be permanently open and therefore unable to control loss of cell water.

Symptoms of infection were readily reproduced by culture filtrates of *D. gossypina* known to contain pectic enzymes. They were also readily reproduced by preparations of commercial polygalacturonase. A poor expression of symptoms was obtained from culture filtrates containing cellulase and phosphatidases. A preparation of commercial cellulase also gave a poor expression of symptoms. Although *D. gossypina* grew well on media containing either pectin or glucose, culture filtrates applied to the bolls resulted in a good expression of symptoms only with the culture filtrate from pectin medium. By means of heat-inactivation of commercial polygalacturonase, cellulase, and culture filtrates containing these and other enzymes, we conclude that the necrosis of epidermal tissues of the cotton boll by the hyphae of *Diplodia* is caused by cell wall-degrading enzymes, principally pectic enzymes (4), and possibly enhanced by the presence of heat-stable toxins. Other investigators have reported similar results with other materials. Hancock et al. (8) induced leaf spot on onion with sprays containing polymethylgalacturonase and cellulase. Deverall & Wood (6) suggested that chocolate spot of bean was caused by interaction of pathogen, pectic enzymes, and host phenolase. We have found no mention of the role of pectic enzymes in predisposing host epidermal cells to penetration by the germ tubes, although Wilson (23) reported that stomatal guard cells and multicellular epidermal hairs were often deeply pigmented during the "nonaggressive" stage of the chocolate spot disease in bean. In Diplodia boll rot of cotton, the primary symptoms of infection of the uninjured host began around the open stomata which remain permanently open as the age of the boll increases (13). As the boll ripens and more stomata remain permanently open, splits appear in the sutures of the capsule, and the thin-walled guard cells apparently are the first in the epidermis to lose their permeability. At this stage of maturity, the boll epidermis loses any resistance it may have had to microbial invasion because of the numerous open stomata and splits in the sutural parenchyma (1, 13).

Washings from the surfaces of nonfungal bearing bolls revealed the presence of carbohydrates similar to those found in boll nectar (10) and which were found to be readily utilized by *Diplodia*. ExoPG was not detected during *Diplodia* spore germination in the presence of the washing as well as its component sugars. However, exoPG was found during *Diplodia* spore germination in the presence of boll leachates and certain polysaccharides (21).

Sciumbato's report (14) that bolls from 5 to 15 days old are more suceptible to Diplodia rot than those 15 to 28 days old was confirmed. Younger bolls appear to have more functional stomata per unit area (13), and consequently a greater number of thin-walled guard cells and accessory cells. Bolls of the middle class of 15 to 28 days old have less numbers of functional stomata per unit area and thicker cuticle (21).

After the fungus enters the subepidermal host tissues, destruction of the boll is very rapid. At 30 C, under favorable growing conditions, the speed of germ tube elongation may be as much as 10 to 15 μ/hr. A characteristic of Diplodia boll rot is complete destruction of carpel wall tissues, leaving only the lignified framework of the vascular system. Since the extraction of Diplodia-infected tissues contained high concentrations of pectic enzymes, high cellulase activity, and weak cellobiase, xylanase, arabanase, proteases, and phosphatidases activity, it seems likely that the former group of enzymes is mainly responsible for the tissue degradation of Diplodia boll rot of cotton. A comparison of maceration activity of normal healthy carpel wall tissue with extracts of decayed tissues and with culture filtrates of *Diplodia*

showed extensive maceration activity at pH 8.5 in the presence of calcium. These conditions are identical for polygalacturonate *trans*-eliminase activity (20). Under acid conditions, tissue maceration activity was low although cellulase, cellobiase, polygalacturonase, xylanase, arabanase, and phosphatidases were active in acid pH ranges.

We conclude that penetration of the cotton fruit by *D. gossypina* begins with the softening of the cuticle in the vicinity of the thin cuticular surfaces of the guard and accessory cells of the stomata and multicellular hairs. The excretion of enzymes, particularly pectic, and the possible excretion of toxic substances by the germinating spore and rapidly growing hyphal tips predispose the outer walls of the epidermis to invasion by *D. gossypina*. Once penetration has been effected, polygalacturonate *trans*-eliminase participates with other cell-wall degrading enzymes, proteases, phosphatidases, and possibly toxins enabling the fungus to use and destroy carpel wall tissues extensively for its nourishment in the final destruction of the boll.

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