Spore Germination of Diplodia gossypina in the Presence of Carbohydrates and Phenolic Compounds in Relation to Boll Rot of Cotton

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ABSTRACT

Black boll rot of cotton (*Gossypium hirsutum*) caused by *Diplodia gossypina* is transmitted by spores extruded from pycnidia, the ostioles of which protrude through the epidermis of the carpel walls. The emerging spores are hyaline and nonseptate. These spores became dark colored and 1-septate about 6 hr after discharge. Maximum spore production occurred on bolls over 40 days of age (from anthesis) but before sutural dehiscence. Young, nonseptate, hyaline spores germinated more rapidly and with higher percentage than older 1-septate spores. Optimal conditions for germination occurred with approximately week-old spores at 30 C and at pH 6.5 to 7.0 in 1.0×10^{-3} M citrate-phosphate buffer. Germination was stimulated by boll surface washings and leachates of dewaxed bolls but inhibited by extracts of cuticular fractions. Spore germination was stimulated by 12 of 14 carbohydrates tested and inhibited by all of the 11 phenolic compounds tested. Polygalacturonase was released during spore germination in the presence of Na-polypectate, polygalacturonic acid, pectin, xylan, CM-cellulose, araban, and boll leachates.

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Diplodia gossypina Cke. causes black boll rot of cotton in the humid area of the cotton belt and is responsible for substantial losses in yield and quality of lint. In previous studies from this laboratory (11, 15, 16), it was established that spore suspensions applied to squares, blossoms, and fruits of various ages caused blossoms and young bolls to fall and/or decay and older fruit to rot. Intermediate-aged bolls appeared to be resistant to infection by *Diplodia*. Fungal mycelium on the surface of immature bolls caused the necrosis and death of certain epidermal cells within 48 to 72 hr after inoculation. Nectar residues consisting of glucose, fructose, galactose, sucrose, raffinose, and two unidentified materials were found to be generally present in boll washings. Several of these materials stimulated mycelial growth in culture and induced pectic enzyme production, whereas certain extracts of the boll cuticle inhibited mycelial growth. Inasmuch as these results have a practical bearing on the nature of boll rot resistance and disease escape, the purpose of this study was to investigate the effects of carbohydrates, phenolic compounds, boll surface washings, dewaxed boll leachates and cuticular fractions on spore germination of *D. gossypina* and enzyme release.

MATERIALS AND METHODS.–*Source of pycnospores.*–A single isolate of *Diplodia gossypina* Cke., identified as No. 23A, was used. This isolate was obtained as a single-spore culture of the pathogen reisolated from a diseased cotton boll (*Gossypium hirsutum* L.); the original isolation was made from a diseased peanut seed by R. Aycock, North Carolina, 1946 (1). It was used in our previous reports (6, 11, 13, 14) and appears to be very stable. Its pathogenicity was no different from that of recent isolates, and, additionally, we found no significant differences between repeated experiments. It has been deposited with the American Type Culture Collection as accession No. 22644. An abundant supply of fresh spores were obtained as needed from sporulating pycnidia (Fig. 1). These were produced by inoculation of previously surface-sterilized cotton bolls 40-50 days after anthesis incubation of them in individual sterile, moist jars (4-oz) under fluorescent lights at 25 C for 1-2 weeks. A fresh supply of spores of known age (within 1-2 hr) became available after passing a sterile swab across the pycnidia, removing

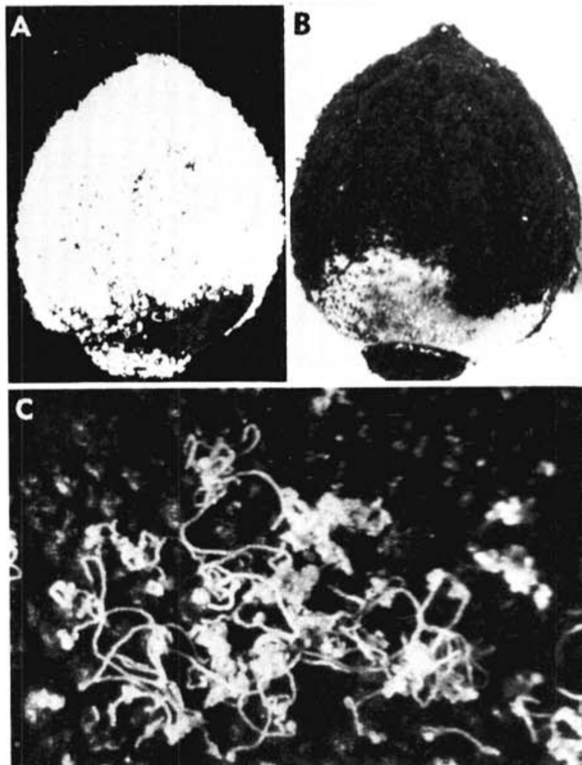

Fig. 1. A) Hyaline, single-celled spores discharged on surface of cotton boll. **B)** Black, two-celled spores on surface of cotton boll. **C)** Hyaline, single-celled spores were discharged in cirri on the surface of a cotton boll.

the previous crop. The first spores formed thereafter were single-celled, hyaline, and were extruded in white cirri (Fig. 1-A, C). The spore walls slowly assumed a gray-brown color after 2-3 hr, becoming black and 1-septate after 6 hr. The spore tendrils were removed by means of tweezers, pressed between sterile cover slips and suspended in sterile deionized water to make a suspension of 4 to 5 $\times$ 10^4 spores/ml. Although it is customary to wash spores produced in artificial media to remove media residue the spores employed in this study were collected from the surface of the boll on which they were produced a few minutes earlier. Because preliminary washing in deionized water had no obvious effect on percentage germination, we dispensed with washing in this study.

Unless otherwise stated, 0.5 ml of spore suspension and 0.5 ml of germination media (carbohydrates, phenolics, buffer, boll washings, etc.) were mixed. Two drops of the mixture were transferred onto a glass slide, placed in moist paper-lined petri dishes and incubated for 6 hr at 30 C. Results are reported as the average percentage germination in five high power microscopic fields at $\times$ 430 magnification.

The carbohydrates, phenolic compounds, and buffers used were obtained from either the Sigma Corporation or the Nutritional and Biochemical Corporation. The sodium citrate-phosphate buffer of various ionic concentrations and pH values was prepared according to Gomori (8). Boll washings were obtained by bathing the surfaces of normal greenhouse-grown bolls ('Deltapine 16') with deionized water (boll/ml). Boll leachates were obtained by first soaking washed bolls in hexane for 1 min to remove surface waxes, then collecting the droplets formed on the boll surface after storage for several hours at 30 C (in sterile containers) and under saturated moisture conditions. The short wash in hexane, removed wax from epidermal cells, but did not prevent exudation or leaching of cell sap. The response was very much like the exudates produced by *Diplodia* spore germination in the early stages of boll infection.

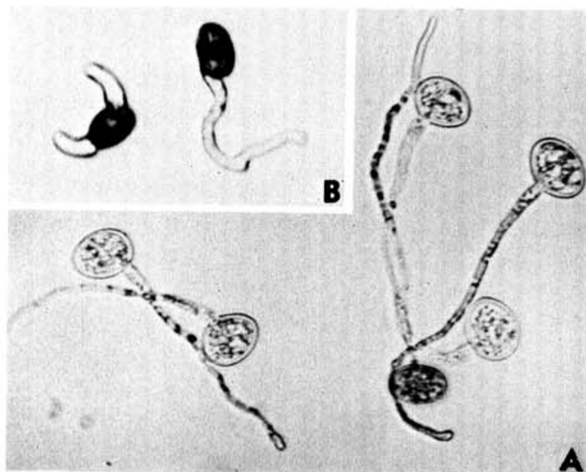

Fig. 2. A) Hyaline, single-celled spores germinating in water after 3 hr at 30 C. **B)** Black, two-celled spores germinating with one or two germ tubes in water after 3 hr at 30 C.

The cuticular fractions from 20- to 30-day-old bolls, their waxes, cutin acids, and ethanol-soluble fraction were prepared as previously described (13). The fractions were dissolved in a small amount of ethyl ether or ethanol (1 mg/ml) on a drop-size area of a glass slide. After the solvent evaporated, a film of waxes, cutin acids and ethanol-soluble residue remained. Spore suspensions applied to these residues were incubated in moist chambers at 30 C for 6 hr. Control slides with spore suspensions applied to ether and ethanol residues were carried along for comparison.

To study the enzyme activity in the germination filtrate, spore suspensions of *Diplodia* were incubated for 6 hr at 30 C in 5.0 $\times$ 10^{-3} M sodium phosphate buffer, pH 7.0, containing 0.05% pectin (or Na-polypectate, polygalacturonic acid, xylan, CM-cellulose, or araban). The germination filtrates were made available for enzyme assay after removal of the germinating spores by filtration through several layers of cheesecloth and centrifugation as previously described (14). Polygalacturonase activity was measured by the reducing power release (14). The reaction mixture contained 4.0 ml of spore germination filtrate and 1.0 ml of 1% polygalacturonic acid in 1.0 M sodium phosphate buffer, pH 4.5, and incubated at 30 C for 20 hr. The controls remained sterile, indicating that the enzyme activity resulted from spore germination and not from microbial activity. Nonspore germination media and autoclaved germination filtrate were carried as controls. One unit of enzyme activity is defined as that amount of enzyme catalyzing the release of 1 μg of D-galacturonic acid in 20 hr under assay conditions. Protein concentration was determined by the method of Waddell & Hill (12). Specific activity is defined as units per mg of protein.

RESULTS.–*Pycnospore formation*.–Several exploratory experiments made earlier (11) resulted in poor pycnidial development on potato-dextrose agar, V-8 juice, cotton boll carpel tissue agar, and autoclaved cotton bolls when incubated at 25 and 30 C. However, an abundant supply of both single and two-celled pycnospores of known age were readily obtained from the surface of previously inoculated bolls in 1 to 2 weeks. Discharge of an abundant uniform supply of freshly formed single-celled hyaline spores occurred within 0.5 hr from bolls such as that illustrated in Fig. 1-A. The walls of these spores changed to a gray-brown color after about 2 hr; a septum began to form and after about 6 hr, the nonseptate spores became dark walled and appeared to be two-celled. It would be interesting to know whether the septum is continuous; and whether the spore becomes truly two-celled as it ages. The spores were discharged from the pycnidia in rope-like masses (Fig. 1-C).

TABLE 1. Percentage germination and length of germ tubes of *Diplodia gossypina* two-celled spores in the presence of various carbohydrate media at 30 C

Medium used (0.05%)	Time			
	3 hr		6 hr	
	Germ tube length (μ)[a]	Germination (%)[b]	Germ tube length (μ)[a]	Germination (%)[b]
Deionized water	10	20	50	76
D-xylose	25	30	60	70
L-arabinose	25	30	60	70
D-glucose	50	80	120	92
D-galactose	25	76	60	90
D-fructose	25	80	120	95
Sucrose	50	90	150	95
Melibiose	10	10	60	60
Raffinose	25	85	130	95
Pectin	0	0	50	90
Na-polypectate	25	30	100	85
CM-cellulose	10	10	100	66
Xylan	25	20	100	66
Araban	25	20	50	60

[a] Average length of germ tubes per microscopic field (× 430).
[b] Average % germination in five microscopic fields (× 430).

Pycnospore germination.—Both types of spores germinated readily in water after 2-6 hr (Fig. 2-A, B). A sucrose concentration of 0.5 - 1.0% stimulated formation of two germ tubes. As the 1-septate spores aged, percentage germination and speed of germination decreased. Sixty to 100% germinated during the first week after discharge while 40 to 60% germinated during the second week.

Effect of ionic concentration, pH, and temperature.—Using approximately 7-day-old spores in a germination study in water and in sodium citrate-phosphate buffer at concentrations of 0.0, 1.0×10^{-3} M, 5.0×10^{-3} M, 1.0×10^{-2} M, 5.0×10^{-2} M, and 1.0×10^{-1} M, percent germination was 50, 62, 56, 33, 18, and 12, respectively. The low concentrations of citrate-phosphate buffer (1.0×10^{-3} to 5.0×10^{-3} M) slightly stimulated germination while the higher concentration (5.0×10^{-2} M) inhibited germination. At a citrate-phosphate buffer concentration of 1.0×10^{-3} M, no germination occurred below pH 5.0 or above 8.0. Optimum pH for germination was around 6.5 to 7.0. Percentage of germination under optimum pH and citrate-phosphate buffer concentrations at 25 C and 30 C, was 34 and 57, respectively. No germination occurred at 35 C, which indicates that this species of fungus is not *D. natalensis* (1, 4, 5).

Effect of carbohydrates.—Percentage germination and length of germ tubes were stimulated in the presence of sucrose, raffinose, D-fructose, D-glucose, D-galactose, Na-polypectate, and pectin (Table 1). No significant effect on percentage germination was evident, although a slight increase in length of germ tubes was noted in the presence of D-xylose, L-arabinose, CM-cellulose, xylan, and araban. A decrease in germination was noted in melibiose, while no germination was observed in mannitol. Several of the hexose sugars that stimulate spore germination and growth of germ tubes are normally present in boll nectar, which is produced in abundance by many commercial varieties of cotton (7, 15). Recently, nectarless types have been developed, and, theoretically at least, these may be less prone to *Diplodia* boll rot than the standard nectary bearing cottons, other factors being equal.

Effect of phenolic compounds.—Spore germination in 1.0×10^{-3} M sodium phosphate buffer, pH 7.0 at 30 C was 62%. Upon the addition of 1.0×10^{-4} M resorcinol or pyrogallol, germination was reduced to 20 and 30%, respectively; in the presence of 1.0×10^{-4} M chlorogenic, tannic, D (−) quinic, gallic, quinolinic, or caffeic acids, germination was reduced to 10-20%; in the presence of 1.0×10^{-4} M catechol, catechin, or coumarin, germination was reduced to less than 10%. Malformed (stout) germ tubes were noted in the latter preparations. Should these materials be present in the carpel walls of the fruit, they would (presumably) have a bearing upon infection and penetration of *D. gossypina*, and should be explored further in a search for cotton cultivars resistant to *Diplodia* boll rot.

Effect of boll surface washings, boll leachates, and fractions of the cuticle.—Spore germination in deionized water was 30 and 62% during 3- and 6-hr germination periods, respectively. In boll washings and boll leachates, germination was 55 and 43%, respectively, during 3-hr germination periods. During 6-hr incubation periods in the presence of cuticular fractions, waxes, cutin acids and ethanol-soluble materials, germination was 43, 55, and 10%, respectively. These results suggest that the carpel walls of the cultivar Deltapine 16 used in this study

contain fungitoxic substances inhibitory to germinating spores of *D. gossypina*. Further studies using other varieties of cotton and other boll-rotting fungi should prove of practical value to breeders of boll rot resistant cottons.

Polygalacturonase activity.–Polygalacturonase was detected in the media when spores germinated in the presence of Na-polypectate, polygalacturonic acid, pectin, xylan, CM-cellulose, and araban by measuring the reducing power in the assay media (Table 2). Inasmuch as no activity was detected by means of the viscosimetric method (13), it is apparent that no endopolygalacturonase activity was present. Polygalacturonase activity was greatest in the Na-polypectate medium and decreased progressively in the presence of polygalacturonic acid, pectin, xylan, CM-cellulose, and araban. Polygalacturonase was also detected when *Diplodia* spores germinated in boll leachate media, but not in boll surface washings from normal bolls.

DISCUSSION.–The spore germination experiments, which were repeated twice, showed some variation in percentage germination and length of germ tubes. We attributed the variation to small differences in ages of spores collected on the boll surface from the ostioles. Percentage germination and length of germ tubes in the several carbohydrate media used, were quite similar and were greater in the presence of sucrose, raffinose, fructose, glucose, galactose, pectin, and Na-polypectate than in the other carbohydrate media or in deionized water. We have previously shown that germinating spores and mycelial growth of *D. gossypina* on the surface of both young and old bolls caused necrosis and death of certain epidermal cells within 48 to 72 hr after inoculation (13). Culture filtrates containing pectic enzymes as well as commercial polygalacturonase were more active in producing cell necrosis than were other cell wall degrading enzymes. Additionally, it was noted that the thin-walled accessory cells of the boll stomata were the first to be eroded. Polygalacturonase was found during spore germination and early growth of mycelium in the presence of pectic substances and other polysaccharides, but never in the presence of the hexose sugars. Polygalacturonase release was not detected during the 6-hr germination period in the presence of boll washings. Although our boll washings contained two unknowns in addition to the hexose sugars, the two were presumably polysaccharides which were probably not in sufficient concentration to induce the formation of exopolygalacturonase in vitro.

The release of polygalacturonase during germination of the conidia of *Botrytis cinerea* and *Geotrichum candidum* has been reported (2, 3). Barash et al. (3) have also reported leachates from safflower blossoms stimulated germination of conidia of *B. cinerea* and release of polygalacturonase. Polygalacturonase was the principle carbohydrase detected in inoculated flower heads (3). Polysaccharides and pectic materials were probably present in these leachates.

Under field conditions in Louisiana, and the humid portion of the cotton belt, *D. gossypina* may cause a very destructive boll rot if an abundance of inoculum develops in our fields. The surface of the cotton boll produced under field conditions has been found to carry an enormous microbial flora (10) which is presumably supported by debris, nectarial residues, pollen, boll exudates, etc., adhering to the surface more or less by chance and which may change during the life of the boll. We have noted, for example, that Diplodia boll rot appears to be more destructive following periods of heavy dew formation than following heavy rains. The cleansing action of the rain probably accounts for this observation. Elimination of boll nectaries through breeding and selection should be of practical value in the development of less susceptible varieties, although Pinckard & Baehr (10) noted that, even though mycelial growth collected around boll nectaries, deep penetration of nectarial tissue was never observed. Further study of the cuticular fractions of the boll inhibitory to spore germination and mycelial development (9, 13, 16) may lead to useful information about the resistance of cotton to black boll rot.

TABLE 2. Liberation of polygalacturonase activity during *Diplodia gossypina* spore germination

Germination filtrate	Enzyme activity[a] (units/ml)	Protein[b] ($\times 10^3$ mg/ml)	Specific activity[c] ($\times 10^{-2}$ units/hr-mg)
Deionized water	0	0.7	00.0
Na-polypectate	420	6.5	32.0
Polygalacturonic acid	120	5.3	11.0
Pectin	90	5.3	8.5
Xylan	70	3.9	9.0
CM-cellulose	50	2.4	10.4
Araban	10	2.4	20.8

[a] The reaction mixture contained 4 ml of spore germination filtrate and 1 ml of 1% polygalacturonic acid in 1.0 M sodium phosphate buffer, pH 4.5, and was incubated at 30 C for 20 hr. One unit of enzyme catalyzing the release of 1 μg of D-galacturonic acid in 20 hr under assay conditions.

[b] Protein concentration per ml of germination filtrate.

[c] Units/hr-mg of protein in germination filtrate.

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