Cotton Boll Cuticle, a Potential Factor in Boll Rot Resistance

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ABSTRACT

The cuticle of various-aged cotton bolls was removed and separated into waxes, cutin acids, and ethanol-soluble, water-soluble, and final residue fractions. The first four fractions were fungistatic to nine species of fungi frequently associated with boll rots. *Penicillium spinulosum* was not affected by any of the four fractions. The ethanol-soluble fraction had the greatest fungistatic activity; the water-soluble fraction, the least. Cuticle thickness, and quantity of waxes and cutin acids were related to boll age, being greatest for intermediate-aged bolls as they approached full size. Because all four fractions of the cuticle were fungistatic, we conclude that the quantities of the various fractions present in the thicker cuticle are partly responsible for the previously observed resistance of intermediate-aged bolls to fungal penetration.

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Diplodia gossypina is capable of rotting the bolls of many varieties of cotton (*Gossypium hirsutum* L.) (24); the pathogen is most destructive to bolls younger than 15 days and older than 28 days (20, 22). Intermediate-aged bolls are resistant also to *Rhizoctonia solani* and *Phytophthora parasitica* Wang & Pinckard (*unpublished data*). Pinckard and associates (16, 17, 18) have shown that in the Mississippi Delta area, cotton boll rot is caused by at least six species of parasitic microorganisms and an indefinite number of nonparasitic types present on the boll surface. These microorganisms, mostly fungi, are capable of entering the boll either directly, or through natural openings as the boll approaches maturity (16, 17).

Sciumbato (20) and Wang & Pinckard (22) showed that fruit buds are highly susceptible to invasion by *D. gossypina* from before anthesis to about 7 days after anthesis. These organs then become increasingly resistant from this age to onset of capsule dehiscence.

Diplodia spores germinated readily on boll surfaces of all ages, and spore germination was stimulated by boll surface washings (22). It was apparent that resistance of the intermediate-aged bolls was not the result of inhibition of spore germination. It seemed likely that resistance to *Diplodia* infection by intermediate-aged bolls might be related to cuticle development. The developing cuticle might act as a physical barrier that inhibits fungus penetration, or it might contain substances that inhibit the growth of the fungus, or both factors might be operative (11). The major chemical components of plant cuticles are waxes and cutin (1, 8, 14, 15), the latter being a polyester of fatty and hydroxyfatty acids. The waxes, which are embedded within the cuticular membrane and exuded over its surface, consist of long-chain alcohols, ketones, hydrocarbons, fatty and hydroxyfatty acids, and esters. Other components include cellulose and pectin, which occur in the membrane where it merges into the outer walls of the epidermal cells (2). Polyphenolic compounds are also occasionally found in the membrane, but it is not clear whether they occur freely or are chemically bound to other cuticular components (2, 3, 5, 9). In addition, Huelin (10) found a minor fraction of sugars and amino acids in hydrolysates of apple cutin. Cuticle as used in this paper refers to the cuticular membrane, which was isolated enzymatically from epidermal strips of cotton bolls of known age.

The purpose of this study was to investigate the fungistatic properties of several fractions of the cuticle of cotton bolls for factors that might be important in boll rot resistance, and also to obtain a quantitative estimation of the cuticle, waxes, and cutin acids of various-aged bolls.

MATERIALS AND METHODS.—*Removal of cotton boll cuticle.*—Greenhouse-grown cotton bolls, 15 to 25 days from anthesis, were detached from plants and kept frozen until used. Epidermal strips were removed after they were thawed in warm water (23). The cuticle was then removed from these strips with a 1% pectinase and a 1% cellulase (Nutritional Biochemicals Corp.) solution in 0.05 M phosphate buffer, pH 5.0.

Fractionation of cuticle.—Unless otherwise noted, the dry cuticle (300 mg) was refluxed with chloroform for 1 hr to remove surface and embedded waxes. The residue of cutin was then saponified with 0.5 N ethanolic KOH by refluxing in a Soxhlet apparatus for 24 hr. The insoluble residue was separated by filtration, then washed with 95% ethanol; the washings were combined with the filtrate. The insoluble residue was placed in boiling water for 5 min to give H_2O-soluble fraction and H_2O-insoluble fraction. The combined alkaline ethanolic filtrate and washings were acidified and evaporated to dryness. This dried material was extracted first with petroleum ether, which gave a colorless cutin acid fraction, then with absolute ethanol, which gave a brownish ethanol-soluble fraction. The residue that remained after both extractions was a colorless salt.

Quantitative analyses of cuticles, waxes, and cutin acids from various-aged cotton bolls.—Thirty to 50 discs (1 cm^2) were cut from boll cuticles. The discs were dried overnight at 40 C and weighed after being cooled in a desiccator. Waxes were obtained by refluxing the dry cuticular discs with chloroform in a Soxhlet apparatus for 1 hr, after which they were dried and weighed. The resulting wax-free cutin was saponified with 0.5 N ethanolic KOH by refluxing for 24 hr. The cutin acids were extracted from the alkaline ethanolic filtrate with petroleum ether and determined by the method of Heinen & De Vries (6). Stearic acid was used as a standard.

Fungistatic studies of cuticular fractions.—Unless otherwise noted, waxes, cutin acids, and ethanol-soluble and H_2O-soluble fractions obtained from 300 mg of dry cuticle were each incorporated into 500 ml of potato-dextrose agar (PDA) which made 25 plates for fungistatic studies. Waxes, cutin acids, and ethanol-soluble fractions were dissolved in 5 ml of petroleum ether or ethanol before being mixed in liquid PDA in 1-liter beakers. The PDA controls also contained 5 ml of ether or ethanol. The pH of the PDA mixtures and controls was adjusted to ca. 6.0. The beakers were placed in a boiling water bath to evaporate the petroleum ether and ethanol. Sufficient deionized water was added to give a final volume of 500 ml. The H_2O-soluble fraction was also mixed with PDA, to give a final volume of 500 ml, and adjusted to pH 6.0. All the PDA mixtures and controls were autoclaved at 121 C for 15 min. These preparations resulted in ca. 0.07 mg of waxes, 0.15 mg of cutin acids, 0.19 mg of ethanol-soluble fraction, and 0.12 mg of H_2O-soluble fraction/ml of PDA medium. With the same procedures, the cuticular fractions were incorporated in oatmeal agar plates for bioassay with *P. parasitica*.

The fungi used for testing the fungistatic activity of cuticular fractions were pathogenic to 30-day-old cotton bolls. They were *D. gossypina* Cke., *R. solani* Kuehn, *Rhizopus stolonifer* (Ehrenb. ex Fr.) Lind, *Aspergillus* sp., *Fusarium* sp., *Colletotrichum gossypii* South., *P. parasitica* Dast., *Alternaria* sp., *Penicillium spinulosum* Thom, and *Pestalotia* sp. The fungi were grown on PDA or oatmeal agar plates. The diameter (mm) of fungal growth was measured after 24-, 48-, and 72-hr incubation at 30 C.

Paper chromatographic analyses.—Ethanol-soluble, H_2O-soluble, and H_2O-insoluble fractions and their acid-hydrolysates were examined for amino acids, carbohydrates, and phenolic compounds by descending chromatography on Whatman No. 1 paper. *n*-Butanol-acetic acid-water (4:1:5, v/v) was used as the developing solvent. Ninhydrin, aniline, and diazotized sulfanilic acid reagents were used to reveal amino acids, carbohydrates, and phenolic compounds, respectively (4).

Quantitative analyses of boll surface waxes of different varieties.—Twenty commercial varieties of cotton were grown at the Northeast Branch Agricultural Experiment Station, St. Joseph, La. Twenty 15-day-old bolls of each variety were randomly collected in late August 1971. We obtained

the surface waxes of bolls by washing each boll in 200 ml petroleum ether for 1 min. The ether solutions were filtered, air-dried, and weighed. The surface areas of the bolls were estimated by the prolate spheroid surface formula (7). The average values of major and minor axes of 20 bolls were used to calculate the surface area per boll. The data on surface wax content of different varieties were analyzed, using Duncan's multiple range test (21).

RESULTS.—*Analysis of boll cuticle.*—The surface of the cotton boll was covered with a cuticle of varying thickness which increased in depth as the boll age increased up to 17-20 days old (Fig. 2). In addition, steep ridges of cutin presenting a corrugated pattern were characteristic (Fig. 1-A, B). The guard cells and parts of adjacent accessory cells had a much thinner cuticle than did the normal epidermal cells (Fig. 1-A).

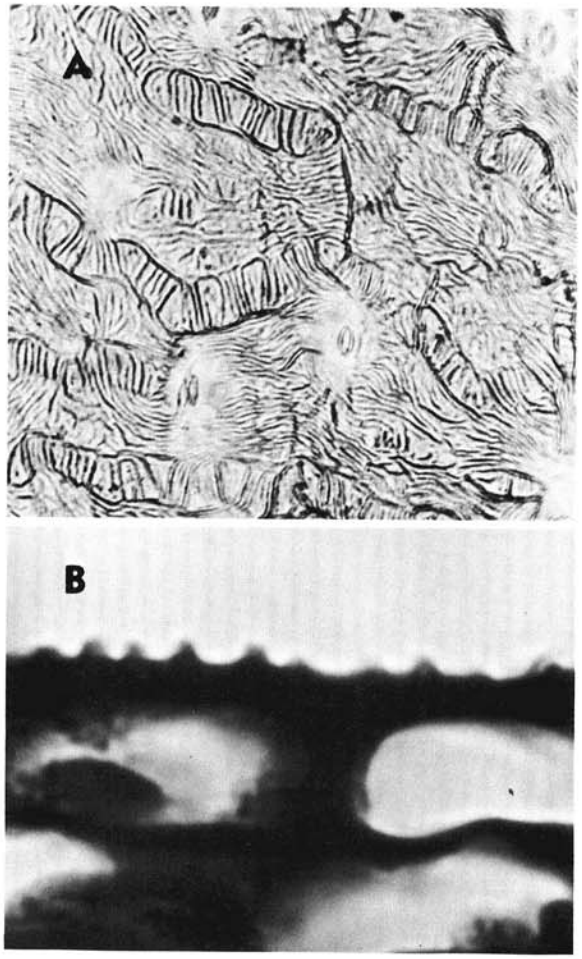

Fig. 1. A) Surface view of the cuticle of an intermediate-aged cotton boll (Deltapine 16) showing the thickened ridge pattern and thinner cuticle around the stomata which remain permanently open at maturity (× 100). The line distance was approximately 5 μ. The cuticle was isolated with a mixture of pectinase and cellulase solution, and stained with Sudan IV. **B)** Transverse section of the carpel wall of an intermediate-aged cotton boll (Deltapine 16) showing a transverse view of the cuticle surface ridges. Section was stained with safranin (× 860).

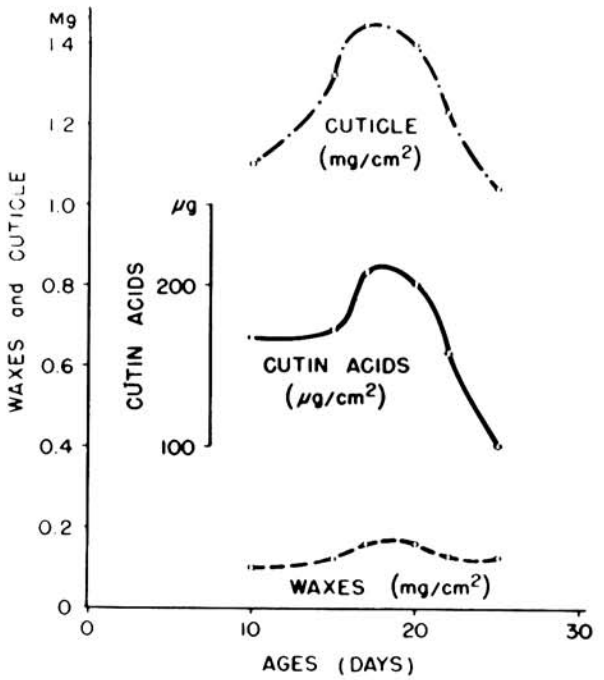

Fig. 2. Average dry weight per cm² of cuticle, waxes, and cutin acids of cotton bolls of stated ages grown in our greenhouse during April and May 1971. Cutin acids were estimated by the Cu-diethyldithiocarbamate complex method of Heinen & De Vries (6) after saponification of the cutin membrane.

The averaged data of three experiments showed that the cuticle of 15- to 25-day-old bolls consisted of ca. 12% waxes (surface embedded) and 88% cutin (cutin membrane). After saponification, the cutin was then separated into four fractions. It yielded an average value of 26.3% cutin acids, 32.8% ethanol-soluble fractions, 20.8% water-soluble fraction, and 8.1% water-insoluble residue in three experiments. The cutin acids were colorless, waxlike materials. The ethanol-soluble fraction was pigmented and became brownish in color when alkaline, but precipitated when acidified; it gave a blue color with the Folin-Ciocalteu phenol reagent. The ethanol-soluble fraction was further separated into three unidentified compounds with R_F values of 0.95, 0.90, and 0.86. They were not equivalent to authentic standards, chlorogenic, caffeic, tannic, gallic, and pyrogallic acids. The spots with R_F values of 0.95 and 0.86 were revealed only under ultraviolet light; the third spot gave a dark orange color with diazotized sulfanilic acid. After acid hydrolysis, the water-insoluble residue contained a sugar component which was identified by paper chromatography as arabinose. No amino acids were detected in the cuticular fractions.

The heaviest, and possibly the thickest, cuticle

was obtained from bolls 17 - 20 days old (Fig. 2). At this stage of boll development, the average weight of waxes and cutin acids per cm^2 was also greater than in younger or in older bolls. Because of difficulty in obtaining 1-cm^2 samples of cuticle from 30- to 40-day-old bolls, a different method of sampling was necessary. Three 20-mg portions of dry cutin, with waxes removed, were taken from bolls of ages 10-20 and 30-40 days. The cutin acid yield after saponification for 4 hr was 157.3 μg/mg of dry cutin from the 10- to 20-day-old bolls, and 78.5 μg/mg of dry cutin from 30- to 40-day-old fruit. These results show that the amount of cutin acid decreased substantially as the age of the fruit increased over 30 days.

Quantitative analyses of boll surface waxes of different varieties.—The surface wax content of cotton bolls varied with varieties; the differences were statistically significant, based on the mean weight of wax content per cm^2 of surface. They may be grouped into high wax varieties such as: Deltapine 16 (30.00 μg); Deltapine 6225 (22.94 μg); Bayou 70 (22.33 μg); Delcot 277 (21.63 μg); La. Frego Bract 2 (21.13 μg); and Stoneville 7A (19.43 μg); intermediate wax varieties: La. Okra Leaf 2 (18.47 μg); Stoneville 603 (18.17 μg); Coker 417 (18.01 μg); Stardel (17.98 μg); Coker 201 (17.58 μg); Hy-Bee 200A (17.18 μg); Glandless O-B-1 (17.02 μg); Stoneville 213 (16.64 μg); Acala SJ-1 (16.52 μg); and McNair 1032B (15.83 μg); and low wax varieties: Paymaster 111 (14.98 μg); Deltapine 45A (15.46 μg); Coker 310 (14.17 μg); and Dixie King II (12.08 μg). The surface wax content of the highest wax variety, Deltapine 16, was approximately twice that of the lowest wax variety, Dixie King II.

Fungitoxic activity of the cuticular fractions of the cotton boll.—The ethanol-soluble fraction was generally more fungistatic than the other fractions; it inhibited the growth of the nine organisms used 20-65% (Table 1). Waxes and cutin acids gave approximately the same level of inhibition (10-40%). The water-soluble fraction was the least inhibitory (only 5-10%). The inhibitory action was greatest in the early stages of fungal growth, decreasing as the cultures aged. *P. spinulosum*, a soil-inhabiting organism known to degrade cuticle and, so far as we know, not involved in cotton boll rot, was apparently unaffected by any of the four fractions of the cuticle.

DISCUSSION.—Although many factors are known to be involved in plant disease resistance, the cuticle is one which may act as a barrier to penetration by means of its physical and chemical composition (11, 14). The results of previous studies show that boll resistance is directly related to boll age (20, 22). The work reported herein suggests the possibility that cuticle thickness and chemical composition together are partially responsible for this observed resistance to fungal penetration. Waxes and cutin were deposited rapidly in young bolls until they were about 17 days old, after which the bolls became resistant. Maximum size of bolls was reached about 20-25 days after anthesis. As the bolls aged, the levels of waxes and cutin gradually decreased, and the bolls increased in susceptibility to fungal penetration.

A review of the literature revealed very little information on the fungistatic properties of cuticular components to potential fungal invaders. Compounds such as benzoxazolinones, coumarin derivatives, alkaloids, proteins, and phenolic materials toxic to fungi have been reported from bark, peel, or similar

TABLE 1. Percentage inhibition of growth of nine cotton boll-rotting pathogens by fractions of the cuticle of Deltapine 16 greenhouse-grown bolls

Organisms	Growth inhibition (%) by fractions[a] after 24 or 48 hr							
	Waxes		Cutin acids		Ethanol-soluble		Water-soluble	
	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr
Diplodia gossypina	28	11	33	11	55	44	12	5
Rhizoctonia solani	20	11	25	11	65	57	15	5
Rhizopus stolonifer	35		42		64		0	
Aspergillus sp.		37		41		45		15
Fusarium sp.		28		28		35		11
Colletotrichum gossypii		22		22		42		10
Phytophthora parasitica		27		27		31		10
Alternaria sp.		9		9		22		9
Pestalotia sp.		16		16		22		11

[a] The fractions (waxes, 0.07 mg/ml; cutin acids, 0.15 mg/ml; ethanol-soluble, 0.19 mg/ml; and water-soluble, 0.12 mg/ml) were mixed with potato-dextrose or oatmeal agar (for *P. parasitica* only), and the diameter of growth in test plates was compared with the control plates; growth is stated as average percentage of fungal inhibition in three replications.

surface tissues by Martin (11). The inhibitory effect of waxes and cutin on germination of conidia of a powdery mildew, *Podosphaera leucotricha*, and on mycelial growth of *Gloeosporium limetticola* have been reported by Martin et al. (12, 13) and Roberts & Martin (19). We found that four cuticular fractions (waxes, cutin acids, ethanol-soluble, and water-soluble fractions) were inhibitory to mycelial growth of several common boll-rotting pathogens but not to *Penicillium spinulosum*, a cutin-degrading fungus of the soil (14). Efforts were made to assay cuticular enzymes in *D. gossypina, R. solani, P. parasitica*, and *C. gossypii* using the isolated cotton boll cuticle as a substrate (14). No significant enzymatic activity was detected. Additionally, we found none of the boll-rotting fungi able to utilize isolated cotton boll cuticle as a sole source of carbon (*unpublished data*). It would seem that if the harshness of the procedure used to separate the components of the cuticle were responsible for inhibition of growth of the parasitic species, it would also be expected to result in an inhibition of *P. spinulosum*.

Previous work (23) has shown that the accessory cells of the stomata have thinner cuticle than surrounding epidermal cells, and that fungal penetration commonly occurs through these weaker areas. We assume, therefore, that resistance of boll cuticle to fungal penetration is both physical and chemical in nature.

A wide variation in surface wax content of 15-day-old bolls occurred among 20 common varieties; however, no attempt was made to relate varietal wax content to disease incidence in the field because of other variables beyond our control.

An interesting observation among growers is that DPL 16 (a high wax variety) is more resistant to rot than is Dixie King II (a low wax variety).

A further study of the cuticle of cotton varieties and genetic lines may lead to improved resistance to boll rot, a disease that cost Louisiana growers an estimated 18% loss of yield in 1971 (18), and an average loss of ca. 10% during the past 11 years.

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