Peroxidase Activity in the Developing Cotton Boll and Its Relation to Decay by Diplodia gossypina

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ABSTRACT

Extracts of healthy carpel tissues of cotton bolls contained weak polyphenol oxidase activity and a very active peroxidase. A predominant increase in activity of peroxidase over polyphenol oxidase was noted if the carpel tissues of the bolls were invaded by *Diplodia gossypina*. Electrophoresis of peroxidase preparations showed that a single form of peroxidase was present in healthy bolls of various ages and cultivars; however, two additional bands of peroxidase activity with different intensities were detected on gels of preparations from *Diplodia*-infected cotton bolls. A similar pattern, of multiple bands of peroxidase activity, was found on gels of preparations from carpel tissue of bolls infected by *Phytophthora parasitica, Colletotrichum gossypii*, and *Rhizoctonia solani*. The additional multiple forms of peroxidase in diseased tissues were the result of host response. Quantitative analyses of phenol content, reducing sugars, and peroxidase activity of healthy and *Diplodia*-infected cotton bolls of various ages, indicate that resistant ages of cotton bolls were more related to peroxidase activity than to levels of phenol content or reducing sugars in the carpel tissues.

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Among the diseases of the North American cotton crop, boll rots are either first or second in order of economic importance, according to annual national surveys by the Cotton Disease Council (10). The diversity of microorganisms involved, and the nature of the field environment under which this crop is produced, dictate the necessity for improved cultivar resistance. Previous work by Wang & Pinckard (16) demonstrated that intermediate-aged bolls, between the ages of ca. 15 to 28 days after anthesis, are resistant to invasion by *Diplodia gossypina, Rhizoctonia solani (Pellicularia filamentosa = Thanatephorus cucumeris)* and *Phytophthora parasitica. Diplodia* spore germination on the surfaces of bolls of various ages, was not a factor in the nature of the observed resistance (15). Wang & Pinckard (16) also showed that intermediate-aged bolls have thicker cuticle and wax deposits and more cutin acids during this boll-age growth period than at other times. Furthermore, four fractions of the cuticle (an ethanolic extract, cutin acids, waxes, and an aqueous extract) taken from bolls of resistant ages, had fungistatic activity against the above fungi. We have also observed that progress of decay was slowed in carpel wall tissue of intermediate-aged bolls (15). These observations indicated the presence of some form of tissue resistance within the carpel walls of intermediate-aged bolls.

Other workers have endeavored to find a parallelism between phenol content, reducing sugars, peroxidase activity, and disease resistance of different plant organs and cultivars (2, 3, 6, 8, 9, 12, 13).

Results have been conflicting. Resistance of field-grown cultivars of *Solanum tuberosum* to late blight, caused by *Phytophthora infestans*, has been indicated, with some exceptions, by the peroxidase test (6). Fehrmann & Dimond (4) have reported a striking correlation between peroxidase activity in different organs of the potato plant and resistance to *P. infestans*. No correlation between peroxidase activity and resistance to late blight was observed by Umaerus (12) in *Solanum demissum*.

The purpose of the present study was to determine the relationship, if any, of phenol content, reducing sugars and peroxidase activity of carpel tissue to observed resistance in developing cotton bolls.

MATERIALS AND METHODS.–Unless otherwise stated, surface-sterilized or autoclaved greenhouse-grown 30-day-old (from anthesis) bolls of cotton (*Gossypium hirsutum* L. 'Deltapine 16') were inoculated with *Diplodia gossypina* Cke., *Phytophthora parasitica* Dast., *Colletotrichum gossypii* South., or *Rhizoctonia solani* Kühn and incubated at 30 C for 4 to 9 days (14, 15). After various incubation periods, the infected carpel tissues were homogenized in a cooled (± 4 C) 0.1 M sodium phosphate buffer (1:2 g/ml), pH 7.5 for 3 min with a Waring Blendor. The homogenate was strained through cheesecloth, centrifuged for 20 min at 3,200 *g* and dialyzed overnight (14, 15). Noninoculated healthy and lightly surface-cut carpel tissues of bolls were carried as controls and extracts were prepared in the same manner as the extracts of infected carpel tissues.

Polyphenol oxidase (*O*-diphenol:O_2 oxidoreductase, EC 1.10.3.1) and peroxidase (donor:H_2O_2 oxidoreductase EC 1.11.1.7) were assayed at room temperature using the method of Maxwell & Bateman (8). The reaction mixtures for polyphenol oxidase assay, contained 1.0 ml of enzyme extract, 1.0 ml of 0.1 M substrate (chlorogenic acid or catechol), and 4.0 ml of 0.1 M sodium phosphate buffer, pH 7.0. One unit of polyphenol oxidase activity was expressed as that amount of enzyme catalyzing an absorbance change of 0.01 in 5 min at 495 nm under the assay conditions used.

Unless otherwise stated, reaction mixtures for the perioxidase assay contained 1.0 ml of enzyme extract diluted 1:10 with deionized water, 1.0 ml of 0.1 M *p*-phenylenediamine, 1.0 ml of 0.03% H_2O_2 and 3 ml of 0.1 M sodium phosphate buffer, pH 6.5. One unit of peroxidase activity was expressed as the amount of enzyme which catalyzed an absorbance change of 0.01 in 5 min at 485 nm under the assay conditions used. Protein was determined as previously described (14). Specific activity is defined as unit per mg protein. Inhibitory studies were made by replacing 0.5 ml of phosphate buffer with 0.5 ml of 1.0×10^{-3} M inhibitor in the reaction mixtures.

Polyacrylamide gel (disk) electrophoresis technique was used to separate the peroxidase multiple forms in extracts of healthy, wounded, and infected cotton bolls as described by Davis (1). The enzyme extract was absorbed onto a 4.8-mm diam disk of absorbent paper (No. 740-E, Carl Schleicher & Schuell Co., Keene, New Hampshire), then placed horizontally on the sample gel of a polyacrylamide column. The separation was carried out in 0.05 Tris-glycine buffer at pH 8.3. The current was applied at 3.0 mA per column. The tracking dye was allowed to move 50 mm. After electrophoresis, peroxidase activity was visually demonstrated by a modification of the benzidine method (13). Gel columns were incubated separately in 18.0 ml of 0.5% benzidine·2HCl solution in a test tube for 30 min; 2.0 ml of 0.1% H_2O_2 was then added to the reaction medium which was incubated for 10 min. The excess substrate was washed away, and photographs of the gel columns were taken immediately. Controls for all enzymatic studies consisted of autoclaved enzyme extracts or substrate-deficient reaction mixtures.

A modification of the methods of Okasha et al. (9) was used to estimate the phenolic compounds and reducing sugars in healthy and infected carpel tissues. Ten g of fresh carpel tissue of healthy and *D. gossypina*-infected greenhouse bolls of various ages were ground in 95% ethanol with an Omni-Mixer then extracted for 24 hr with 200 ml ethanol in a Soxhlet extractor. The extracts were concentrated to a volume of 100 ml. This alcoholic extract was treated with an equal volume of saturated neutral lead acetate to precipitate the phenolic compounds. After three filtrations and resuspensions with deionized water, the filtrates were combined and treated with an excess of sodium oxalate to remove the lead ions. After filtration of the lead oxalate, the sugar solutions were condensed and brought to a volume of 200 ml. The reducing sugar was determined with the 3,5-dinitrosalicylic acid reagent (14). D-glucose was used as a standard. The precipitate of lead-phenol complex was treated with 20 ml of 2 N H_2SO_4 to regenerate the phenolic compounds, filtered and washed twice with 20 ml of deionized water. The filtrate was heated and brought to 100 ml with water. The phenol content was determined with the Folin-Ciocalteu reagent at 448 nm (7). Gallic acid was used as a standard.

RESULTS.–*Polyphenol oxidase and peroxidase activity in healthy and infected carpel tissues.*–Extracts of noninoculated healthy carpel tissues of cotton bolls contained weak polyphenol oxidase activity and active peroxidase activity. When the bolls became infected with *D. gossypina* (Table 1), only a slight increase in polyphenol oxidase activity was observed, but a great increase in peroxidase activity occurred. However, only slight activity of the two enzymes was detected when *D. gossypina* was seeded on autoclaved bolls. When chlorogenic acid was used as the substrate, polyphenol oxidase activity was higher than when catechol was used. Peroxidase activity was inhibited by 8.3×10^{-6} M reduced glutathione and also sodium diethyldithiocarbamate. However, the peroxidase activity was stimulated by 8.3×10^{-6} M sodium ethylenediaminetetracetate (EDTA).

Peroxidase activity varied in extracts of healthy

and *Diplodia*-infected carpel tissues of cotton bolls at various ages (Table 2). Enzyme activity in healthy bolls reached its maximum as the bolls approached maturity at about 30 days of age and declined during boll dehiscence. The degree of *Diplodia* infection of cotton bolls of various ages was different; the most destructive rot was observed on bolls of about 10 and 40 days of age. Bolls 15, 20, and 30 days old were relatively more resistant. The increase of peroxidase activity in infected carpel tissues of various-aged bolls was correlated with peroxidase activity in healthy bolls of similar ages.

Peroxidase electrophoresis.—A single band of peroxidase activity was detected on gels of preparations from the carpel tissues of noninoculated healthy bolls of the cultivars 'Deltapine 16', 'Tuxtula', 'Coker 100 Wilt', 'Coker 2 Wiles', 'Lone Star', 'Northern Star', 'Pilose', 'Half & Half' (yellow anthers), and 'Frego Upland' (Fig. 1-A). No increase or change of the peroxidase band was detected when the enzyme extracts were prepared from healthy bolls of various ages (Fig. 1-B) or from carpel tissue of surface-wounded bolls. Callus tissue was formed along these cut edges. The intensity of the peroxidase reaction on the gels was not sufficiently sensitive to detect accurately the different magnitudes of peroxidase activity in bolls of various ages, but a slightly greater intensity of the peroxidase stain was found on gel columns of intermediate ages of bolls. Three regions of peroxidase activity with different intensities were detected on gels of preparations from bolls infected with *D. gossypina* (Fig. 1-C). If the bands of peroxidase activity were numbered consecutively from the most anionic ends of the gel columns, the second band of peroxidase activity from the infected carpel tissues was comparable in migration distance and intensity to the peroxidase activity on gels of preparations from noninoculated healthy carpel tissues. The first peroxidase band had much greater intensity than the third band. The maximum intensity of bands 1 and 3 was reached just before the bolls became completely rotted. Afterwards, the intensity of the two bands decreased gradually. The electrophoresis of filtrates and extracts of mycelium of *D. gossypina* grown on carpel tissue medium (15), did not reveal peroxidase activity. Electrophoresis of extracts of carpel tissues infected with *D. gossypina, P. parasitica, C. gossypii*, and *R. solani* revealed a similar pattern of peroxidase multiple forms (Fig. 1-D). The differences in intensity of peroxidase bands 1 and 3 on the gels may reflect the degree of infection of bolls by the various fungi.

Phenol content and reducing sugars in carpel tissues at various ages.—The greatest phenol content in healthy carpel tissues was found at a boll age of 5 days (Table 3) and decreased gradually as boll age increased. The reducing sugar content was low in 5-day-old bolls, but had increased rapidly to a maximum at the age of 10 days then decreased gradually (Table 3). Both phenol content and reducing sugars decreased after the bolls were infected by *D. gossypina* (Table 3). There was a greater decrease in phenol content and reducing sugars in infected carpel tissues of 30- and 40-day-old bolls than in 20-day-old bolls. It was noted that regenerated acidic phenol solution from the lead-phenol complex of healthy carpel tissues was red in color; however, the regenerated acidic phenol solution from the lead-phenol complex of infected carpel tissues was colorless.

DISCUSSION.—In our previous work (11, 15) wherein intermediate-aged bolls were found to be resistant to fungal invasion by *D. gossypina, R. solani*, and *P. parasitica*, as well as some other less well known boll-rotting organisms, we noted that boll

TABLE 1. Polyphenol oxidase and peroxidase activities in extracts of healthy and *Diplodia gossypina*-infected (autoclaved and nonautoclaved) carpel tissues of 30-day-old cotton bolls

	Enzyme activity (units)[a]		
	Peroxidase[b]	Polyphenol oxidase[c]	
Extracts		chlorogenic acid as substrate	catechol as substrate
Healthy	40	1	1
Infected			
autoclaved	4	1	1
nonautoclaved	380	5	2

[a] One unit of enzyme activity was expressed as that amount of enzyme catalyzing an absorbance change of 0.01 in 5 min at 485 nm (peroxidase), or at 495 nm (polyphenol oxidase).

[b] Reaction mixtures contained 1.0 ml of 1:10 dilution of enzyme extract, 1.0 ml of 0.1 M *p*-phenylenediamine, 1.0 ml of 0.03% H_2O_2, and 3.0 ml of 0.1 M sodium phosphate buffer, pH 6.5.

[c] Reaction mixtures contained 1.0 ml of enzyme extract, 1.0 ml of 0.1 M substrate, and 4.0 ml of 0.1 M sodium phosphate buffer, pH 7.0.

TABLE 2. Peroxidase activity in extracts of healthy and *Diplodia gossypina*-infected carpel tissues of cotton bolls of various ages

Boll ages (days)	Specific activity[a] (units/mg)	
	Healthy[b]	Infected
10	8	38
15	39	283
20	80	613
30	135	876
40	62	304

[a] Reaction mixtures contained 1.0 ml enzyme extract, (1:10 dilution of infected tissue extract), 1.0 ml of 0.1 M *p*-phenylenediamine, 1.0 ml 0.03% H_2O_2 and 3.0 ml of 0.1 M sodium phosphate buffer, pH 6.5. One unit of peroxidase activity was expressed as that amount of enzyme catalyzing an absorbance change of 0.01 in 5 min at 485 nm. Specific activity was defined as unit/mg protein.

[b] Healthy carpel tissues were obtained from freshly detached greenhouse cotton bolls. Infected carpel tissues were prepared from a 7-day-old diseased boll.

resistance was related to cuticle thickness and to its chemical composition. Additionally, we also observed that the growth of fungal mycelia on the carpel walls of intermediate-aged bolls was much slower than on the carpel walls of younger or older bolls. The nature of the resistance encountered in the cultivar Deltapine 16, if better understood, might lead to improved over-all resistance.

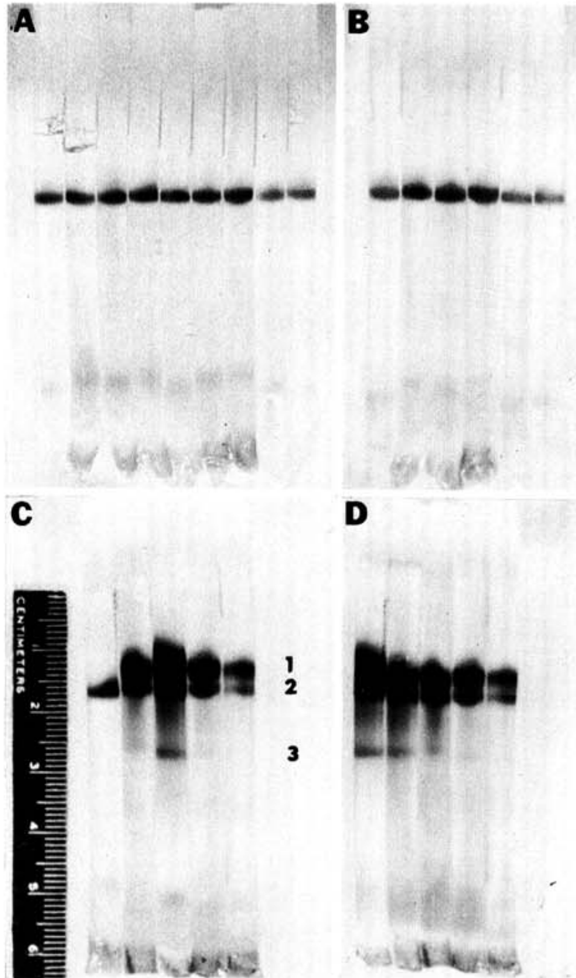

Fig. 1. Peroxidase activity on electrophoretic gels of inoculated and noninoculated cotton boll preparations. **A)** Extracts of noninoculated carpel tissues of cotton bolls of *Gossypium hirsutum* cultivars. Left to right: 'Deltapine 16'; 'Tuxtula'; 'Coker 100 Wilt'; 'Coker 2 Wiles'; 'Lone Star'; 'Northern Star'; 'Pilose'; 'Half & Half' (yellow anthers); 'Frego Upland'. **B)** Extracts of noninoculated carpel tissues of cotton bolls of Deltapine 16 at various ages. Left to right: 15, 20, 25, 30, 35, 40 days old. **C)** Extracts of *Diplodia gossypina*-infected bolls. Left to right: noninoculated, 4, 5, 7, 9 days after inoculation with *Diplodia*. The bolls were completely rotted after about 5 days. **D)** Extracts of carpel tissues from cotton bolls infected by various fungi after 5 days at 30 C. Left to right: *Diplodia gossypina, Phytophthora parasitica, Colletotrichum gossypii, Fusarium* sp., and *Rhizoctonia solani*.

TABLE 3. Quantitative analysis of the total content of phenolic compounds and reducing sugars in carpel tissues of healthy and *Diplodia gossypina*-infected cotton bolls at various ages

Boll ages[a] (days)	Phenolic compounds[b] (mmole/gm fresh wt)		Reducing sugars[c] (mmole/gm fresh wt)	
	Healthy	Infected	Healthy	Infected
5	43		0.8	
10	23		1.8	
15	22		1.7	
20	20	14	1.7	0.9
25	17		1.5	
30	15	3	1.2	0.4
40	12	1	0.5	trace

[a] Greenhouse-grown bolls were tagged at anthesis so that their age could be determined accurately. Healthy carpel tissue obtained from freshly detached greenhouse cotton bolls. Infected carpel tissue prepared from 7-day-old diseased bolls.

[b] Phenolic compounds were determined with a Folin-Ciocalteu reagent at 448 nm.

[c] Reducing sugars were determined with a 3,5-dinitrosalicylic acid reagent at 575 nm.

Inasmuch as a single band of peroxidase activity was found on gels of preparations of several widely different varieties of bolls (Fig. 1-A) and various-aged bolls of the cultivar Deltapine 16 (Fig. 1-B) we conclude that detection of peroxidase band by electrophoresis cannot be used for indicating the presence of resistance in cotton boll carpel tissue. Two new multiple forms of peroxidase with different reactivity with the benzidine stain were extracted from *Diplodia*-infected bolls (Fig. 1-C). A similar pattern of peroxidase multiple forms was found in carpel tissue extracts of bolls infected by *P. parasitica, C. gossypii*, and *R. solani* (Fig. 1-D). Additionally, electrophoresis of filtrates and mycelial extracts of *D. gossypina* grown on carpel tissue medium, or on autoclaved bolls, did not reveal any peroxidase activity, although weak activity was detected in mycelial extracts, but not filtrates, if the activity was measured by a colorimetric method. It is probable that two multiple forms of peroxidase were produced by host response to fungal invasion. The rapid appearance of peroxidase multiple forms could be the result of de novo synthesis of isoenzymes (3, 5). It is interesting to note that peroxidase band 2 △ infected carpel tissues and the peroxidase showed identical electrophoretic mobility and benzidine reactivity on gels, from healthy carpel tissues (Fig. 1-C).

Phenolic compounds and reducing sugars are reported to generally accumulate in diseased tissues (2, 9). However, our results show that maximum levels of these materials occurred in the carpel walls of 5- and 10-day-old bolls and that, upon invasion of these tissues by *D. gossypina*, these materials decreased rapidly. There appears to be little if any relation between phenolic compounds and reducing sugars to boll rot resistance. Bolls of intermediate age

lost their resistance to *Diplodia* after the tissues were killed with heat or ethanol (S. C. Wang, *unpublished*). It is apparent that the participation of oxidative metabolism of phenolics is more important than the dynamic phenolic constituents of the boll in boll rot resistance. Peroxidase activity in extracts of healthy bolls increased as the bolls approached maturity at about 30 days of age. Enzyme activity declined after boll dehiscence. These results indicate that the degree of disease resistance in carpel tissues of intermediate-aged bolls is probably caused by a higher "potentiality" (active metabolic activity) in cotton bolls for a fast host response reaction to overcome the infection. This correlation of peroxidase activity with resistant ages of bolls may be of some assistance in selecting cotton cultivars for boll rot resistance.

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