Localization of Peroxidase in Rhizoctonia solani-Infected Cotton Seedlings

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ABSTRACT

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Cotton (*Gossypium hirsutum*) seedlings become less susceptible to soreshin pathogen (*Rhizoctonia solani*) with age. In noninoculated seedlings peroxidase activity was prominent in the epidermis, hypodermis, endodermis, and some vascular parenchyma cells of 5- and 11-day-old hypocotyls. Generally, activity was minor in the cell walls of the cortex. Occasionally, 11-day-old hypocotyls had peroxidase activity in the cortical cells that apparently had been crushed by secondary growth. Peroxidase activity increased in pre-existing sites of localization in response to infection by *R. solani*. New sites of increased activity were induced, first in the cell walls and later in the cytoplasm, as the disease developed; these developed near the advancing margin of the necrotic lesion and prior to tissue browning. Since phenolase activity was not detected, it was concluded that peroxidase was responsible for the oxidation of polyphenols, the major component of the brown pigment. Infection-induced sites of peroxidase activity were distributed similarly in seedlings inoculated at 5 or 11 days of age. Thus, the sites of constitutive or infection-induced peroxidase activity apparently are not related to the increased resistance of older seedlings to *R. solani*.

Additional key words: histochemistry, phenoloxidation, necrosis.

Oxidized phenols probably are the major component of the brown pigment in lesions on cotton (*Gossypium hirsutum* L.) hypocotyls infected by the soreshin pathogen (*Rhizoctonia solani* Kuhn). Oxidized phenols also are known to inhibit some cell-wall-degrading enzymes. The enzyme system responsible for the phenol oxidation has not been studied, however.

King (7) extracted an active phenolase from cotton leaves and Yunusov and Yuldashev (14) confirmed that the enzyme was present in leaves, but did not detect it in seeds or cotyledonary tissue. Wang and Pinckard (12) reported a weak phenoloxidase in cotton bolls, but this enzyme did not increase substantially in activity after infection by *Diplodia gossypina*. Herrero and Hall (3), however, observed an increase in polyphenoloxidase activity in cotton in the presence of ethylene, a fungal metabolite. In one report, Hunter (5) mentioned phenoloxidase activity in cotton hypocotyls, but later (6) referred to the enzyme as a metal-containing oxidase because it could be inhibited by cyanide. In preliminary histochemical and spectrophotometric studies, I was unable to detect phenolase (*o*- or *p*-diphenoloxidase) activity in cotton hypocotyls.

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Peroxidase catalyzes the oxidation of many phenols (10) and several workers (2, 7, 9, 10, 11, 12, 13) have reported peroxidase activity in cotton. Hunter (5) reported that oxidized catechin was a major phenol in *R. solani* lesions on cotton hypocotyls; however, I was unable to find a reference reporting the oxidation of catechin by cotton peroxidase.

Peroxidase activity in cotton apparently is altered by adverse growing conditions. The activity and the number

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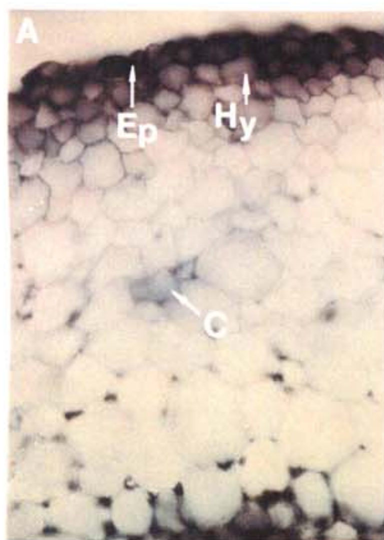
Fig. 1-(A to I). Peroxidase and brown pigment localization in cotton seedling hypocotyls noninoculated (A-C) and inoculated (D-I) with *Rhizoctonia solani*. Sites of peroxidase activity show blue color roughly proportional to enzyme activity. **A**) Peroxidase activity in noninoculated epidermis (Ep) of hypocotyls, lesser activity in the hypodermis (Hy), and only slight activity in the walls of some mid- and outer cortical cells (C). **B**) Peroxidase activity in the endodermis (En) and some vascular cells and slight activity in one or two layers of cortical cells immediately adjacent to the endodermis. **C**) Occasional 11-day-old noninoculated seedlings with high peroxidase activity in cortical cells that appeared crushed. The crushed cells probably result from the deposition of secondary vascular tissue. **D**) Increased peroxidase activity 12 hours after inoculation was localized in the walls of the hypodermis (Hy) beneath the fungus (Fu). **E**) Portions of the epidermis and hypodermis with brown pigment (probably oxidized catechin-type phenols) largely restricted to the cell walls. Intense peroxidase activity in the walls of cortical cells adjacent to the browned tissue. **F**) A later stage of disease with additional cells with brown pigment in both walls and cytoplasm of the mid- and outer cortical cells. Peroxidase activity evident in both the walls and cytoplasm (arrow) of the cortical cells near the periphery of the brown-pigmented tissue. **G**) Epidermis and hypodermis are heavily impregnated with the brown pigment. A layer of cortical cells with abundant peroxidase activity and separated from the browned tissue by a layer of cells (arrow) devoid of both the brown substance and peroxidase. **H**) A section from the hypocotyl 5 mm above the top edge of the external lesion with many mid-cortical cells impregnated with the brown substance and with high peroxidase activity localized in the cortical cells on either side of the browned tissue, but separated from it by a layer of cells (arrows) devoid of both the enzyme and the brown pigment. **I**) Advanced stage of disease development with all tissues either heavily impregnated with the brown substance or with high peroxidase activity; the browned cortical cells appear crushed, but cells with high peroxidase activity appear intact.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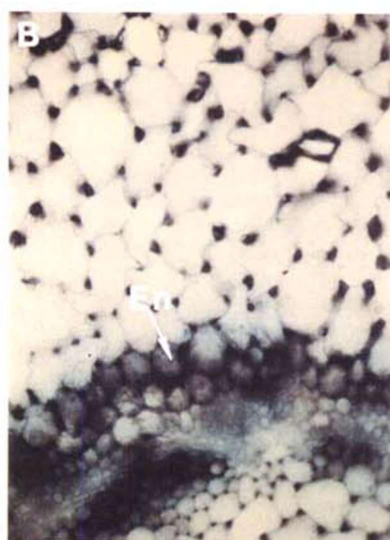
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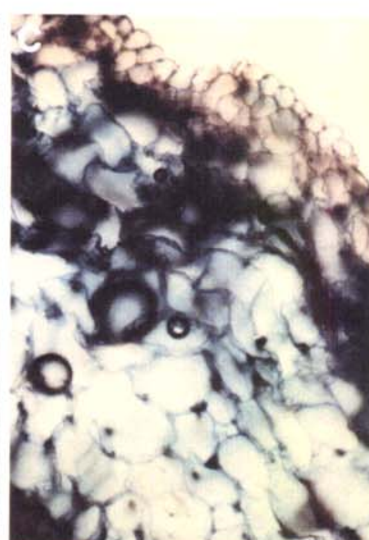
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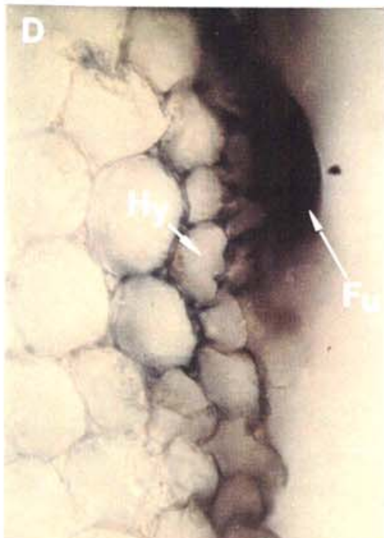
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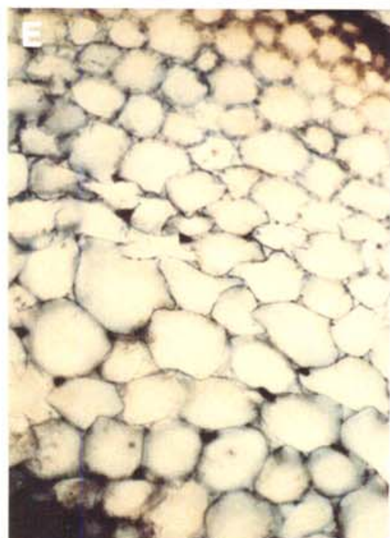
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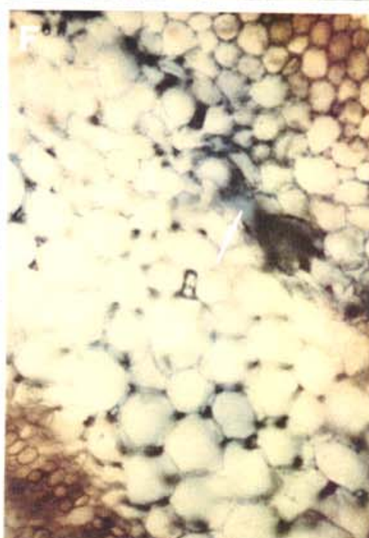
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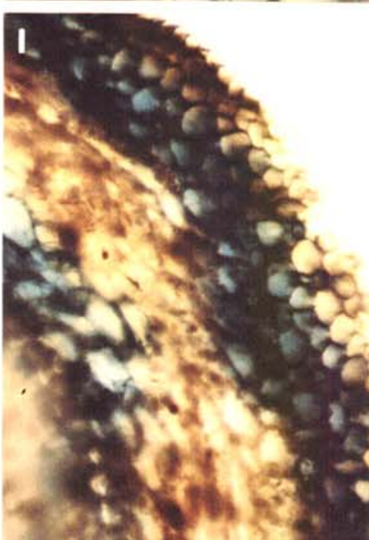
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of peroxidase isozymes increased in bolls infected by *D. gossypina* (12). Ethylene, a metabolite of many fungi (1), also induced increases in peroxidase activity and isozyme content (1, 9). Some cotton peroxidase isozymes also have been shown to occur only in certain tissues (13). Strand and Mussell (11) showed that endopolygalacturonase from *Verticillium dahliae* solubilized a cell-wall-bound fraction of cotton peroxidase.

Thus, because phenols are oxidized in soreshin lesions in the absence of phenoloxidase activity, I investigated the role of peroxidase in *R. solani*-infected cotton. This paper reports the histochemical localization of peroxidase (E.C.1.11.1.7) in cotton hypocotyls infected by *R. solani*. Since older cotton seedlings are less susceptible to *R. solani* (6), seedlings of two ages were inoculated and alterations in the sites of peroxidase localization were examined for their relationship with the onset of browning in soreshin infections.

MATERIALS AND METHODS

Cultivar Deltapine 16 cottonseeds were germinated on moist paper towels in the dark at 30 C for 72 hours. Ten seedlings, arranged with the cotyledons along the edge of a towel, then were covered with a second moist towel and the towels rolled into a cylinder. The cylinder was covered with waxed paper and placed in a beaker; distilled water was added to a depth of 2 cm. The seedlings were maintained in a controlled environment with 14-hour days at 28 C and 10-hour nights at 18 C until harvested.

Rhizoctonia solani (isolate 63 SD 2) was grown in the medium described by Hunter (6), and inoculum was prepared by grinding 1 g fresh weight of mycelial mat from 7-day-old cultures in 75 ml of distilled H_2O. Seedlings were inoculated 5 or 11 days after seeding by pipetting 0.05 ml of inoculum onto the hypocotyl of each seedling in the paper roll.

Seedlings were harvested and examined at up to 7 days after inoculation. The seedlings were washed in tap water and blotted dry. The soreshin lesions and adjacent tissues were excised from the hypocotyls and mounted in dry split pith sticks. Fresh transverse sections, 50- to 100-μm thick, were cut with a sliding microtome, and transferred to either 0.4% formaldehyde neutralized with calcium (to aid in the stabilization of the reaction at the benzidine-blue stage), or cold (4 C) distilled water, for up to 5 minutes. The sections then were incubated at room temperature for 2 minutes in a peroxidase localization reagent (5.0 ml 0.05 M 2-N-morpholino ethanesulfonic acid, pH 5.0; 0.1 ml saturated benzidine-HCl; and 0.1% H_2O_2). Controls consisted of comparable tissue sections, incubated in the peroxidase reagent minus H_2O_2 or heat-killed (boiled in distilled H_2O for 10 minutes) prior to incubation in the complete reagent. After removal from the reagent, sections were washed in tap water, mounted wet on microscope slides, observed under the microscope, and photographed.

Enzyme extracts from cotton hypocotyls were prepared to determine if peroxidase could oxidize catechin and if such activity changed as seedlings aged from 6-16 days or during disease development. Hypocotyl tissue was homogenized in 0.05 M borate buffer pH 7.5 (4.0 ml/g fresh weight). The homogenate was filtered and the filtrate centrifuged at 20,000 *g* for 20 minutes; the supernatant liquid was the enzyme extract. A reaction mixture was prepared that consisted of 2.7 ml 0.05 M phosphate buffer (pH 6.5), 0.1 ml 0.03 M H_2O_2, 0.1 ml catechin (21.7 mg/ml methanol), and 0.1 ml of enzyme extract. The activity, read against a reference of the same composition, but with 0.1 ml H_2O in place of the H_2O_2 was recorded as the change in absorbance at 425 nm $\times$ minute^{-1} $\times$ mg protein^{-1}.

RESULTS

With one exception, the sites of peroxidase localization in noninoculated cotton hypocotyls were similar in both 5- and 11-day-old seedlings. Peroxidase activity was most prominent in the epidermis and hypodermis (Fig. 1-A) and the endodermis and vascular parenchyma (Fig. 1-B), but generally was very low in the cortex (Fig. 1-A, B) and progressively decreased in intensity inward from the hypodermis to near the endodermis. However, there was some activity in one or two rows of cortical cells adjacent to the endodermis (Fig. 1-B). This low cortical peroxidase activity appeared to be concentrated in either the cell walls or the outer portion of the cytoplasm appressed to the cell walls. Occasionally, there was intense peroxidase activity in portions of the cortex of some 11-day-old seedlings (Fig. 1-C). This activity was distributed uniformly throughout the cell and was associated only with cortical cells that appeared to have been crushed by secondary growth.

Sequential changes in the sites of peroxidase localization in *R. solani*-inoculated cotton hypocotyls are shown in Fig. 1-D to I. The figures are arranged in an order of increasing disease development which did not always correspond with increase in time after inoculation.

The presence of a large central vacuole surrounded by a thin layer of cytoplasm in cells of many tissues involved in this study make it difficult to state precisely whether localization is in the cytoplasm or the cell wall.

Within 12 hours after inoculation, peroxidase activity had increased in the outer tangential walls of hypodermal cells directly below the mycelium (Fig. 1-D). At an early stage of penetration the host cells had not turned brown, but within one day after inoculation the epidermis and outer cortex had begun to turn brown (Fig. 1-E). The brown pigment appeared to be localized in the cell walls, but not in the cytoplasm. Concomitantly, peroxidase activity increased in the walls of cortical cells near the brown cells of the hypodermis. As the disease progressed (Fig. 1-F), additional host cells became brown. Portions of the epidermis and outer cortex had substantial browning in both the walls and the cytoplasm. At this stage of disease development, peroxidase activity was high in the cortical cell walls near the browned tissue and low peroxidase activity was evident in the cortical cytoplasm, but it was not localized in particular cytoplasmic organelles. By this time, typical external soreshin lesions were apparent and much of the epidermis and hypodermis were heavily impregnated with the brown pigment (Fig. 1-G), which was deposited in both the walls and cytoplasm, but the walls were darker. A layer of cortical cells near the browned tissue had intense peroxidase activity in the cytoplasm and walls. During

the early stages of disease development, the cortical cells with prominent peroxidase activity were separated from the browned cells by at least one layer of cells devoid of peroxidase activity and the brown pigment (Fig. 1-F, G, H). Sections from 5 mm above the external lesion had intense browning in the mid-cortex (Fig. 1-H). Peroxidase activity was apparent on both sides of the browned mid-cortex and was prominent in both the walls and cytoplasm. In the most advanced stage of disease in which fresh sections could be cut (Fig. 1-I), host-plant cells either were infiltrated heavily by the brown pigment or had very intense peroxidase activity. The peroxidase activity was uniformly distributed through both the cell walls and the cytoplasm. The cortical cells with only peroxidase activity appeared structurally intact, whereas cortical cells with brown pigment appeared crushed. By 6 days after inoculation of 5-day-old seedlings, virtually all of the hypocotyl in the area of the lesion was reduced to a wet brown rot. I was unable to cut sections of this tissue. However, when a piece of this tissue was immersed in the peroxidase reagent, a blue color developed, which indicated that some peroxidase activity remained. Peroxidase localization was not observed in control sections.

The hypocotyl became brown and disintegrated to a wet pulp more slowly in seedlings inoculated with *R. solani* at 11 days than at 5 days; otherwise, the responses of the host at the two ages were similar.

Data in Table 1 show that peroxidase can oxidize catechin and that peroxidase activity per milligram protein increased as seedlings aged from 6-16 days. Peroxidase activity also increased more rapidly in *R. solani*-inoculated tissue than in noninoculated tissue.

DISCUSSION

Catechin is the major phenol in cotton (5, 6) and my spectrophotometric data indicate that peroxidase can oxidize it. Mace and Howell (8) reported the presence of catechin and related phenols in cotton roots in most of the tissues in which I detected peroxidase in hypocotyls; i.e., hypodermis, endodermis, and vascular parenchyma. Hunter (5) reported catechin in the cotton hypocotyl in many of the same tissues. Apparently, the enzyme and substrate are kept from interacting until cellular or membrane integrity is disrupted.

The occasional high peroxidase activity observed in the cortex of 11-day-old noninoculated seedlings appeared to be associated with cambial activity. Apparently some seedlings had either earlier or more rapid secondary growth than others. The cortex in seedlings with secondary growth was crushed at an earlier age than in other seedlings. Peroxidase activity was prominent in cortical cells that appeared crushed, but usually was weak in nondisrupted cells. The role of such increased peroxidase activity in seedling resistance to *R. solani* has not been assessed. If peroxidase is involved in the decrease in susceptibility of older seedlings, the initiation of secondary growth by the cambium would seem to be the major event leading to the increase in enzyme activity and the concomitant decrease in susceptibility.

In soreshin lesions, *R. solani* may secrete materials that induce release of cell-wall-bound peroxidase. Soon after the fungus encountered the host, peroxidase activity increased. This increase first was observed in host cell walls before the pathogen penetrated and was followed by the deposition of brown substances (possibly oxidized catechin-type phenols) in the cell wall, and subsequently by necrosis of the cell. Hunter (6) reported an increase in endopolygalacturonase in *R. solani*-infected cotton. Strand and Mussell (11) reported that this enzyme from *V. dahliae* released cell-wall-bound peroxidase in cotton leaves. However, peroxidase also may be induced in response to infection (2).

In much of the cortex, substrate (catechin) for the browning reaction is absent (5). However, Howell et al. (4) reported that *V. dahliae*-infected cotton leaves synthesized catechin-type phenols in response to infection. Hypocotyls similarly might be induced to form polyphenols in the cortex upon infection by *R. solani*. Substrates then would be available to the cortical peroxidase and browning could result.

Cotton seedlings became less susceptible to infection by *R. solani* as they matured. However, no histochemical differences were noted between the responses of seedlings inoculated at 5 or 11 days of age. Quantitative differences in increases of peroxidase activity at the two ages may have gone undetected. Peroxidase appears to mediate the phenol oxidation that causes tissue browning. The enzyme is anatomically located and induced at the proper time to accomplish this function.

TABLE 1. Peroxidase oxidation[a] of catechin; enzyme extracted from noninoculated and *Rhizoctonia solani*-inoculated cotton hypocotyls

Seedling age (days)	Noninoculated	Inoculated at 5 days of age	Inoculated at 11 days of age
6	.061	.069	...
7	.057	.200	...
8	.061	.343	...
9	.080	.267	...
10	.153	.552	...
11	...	...	...
12	.198	...	.123
13	.290	...	.440
14	.157	...	.514
15	.165	...	.616
16	.236	...	.655

[a]Activity expressed as change in absorbance at 425 nm $\times$ minute^{-1} $\times$ mg protein^{-1}.

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