Peroxidase and 6-Phosphogluconate Dehydrogenase in Resistant and Susceptible Cotton Infected by Meloidogyne incognita[1]

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Abstract: Assays of specific activities and electrophoretic separations of multiple forms of 6-phosphogluconate dehydrogenase and peroxidase in cotton resistant and susceptible to *Meloidogyne incognita* were conducted 6 days after inoculation. Specific activities were greater in infected than in uninfected roots and also were greater in the resistant cultivar, 'Clevewilt 6-3-5,' than in the susceptible cultivar, 'M8.' In uninfected roots, peroxidase activity was greater in Clevewilt roots than in M8 roots, but activity of 6-phosphogluconate dehydrogenase was the same. Multiple forms of peroxidase and 6-phosphogluconate dehydrogenase were separated and resolved by polyacrylamide gel electrophoresis. These experiments demonstrated the occurrence of altered metabolism upon infection and differences in enzyme activity between resistant and susceptible cultivars. *Key Words:* Resistance, root-knot nematode, biochemistry, multiple forms, specific activity.

Resistance in cotton *(Gossypium hirsutum)* to *Meloidogyne incognita* has been attributed to hypersensitivity and failure of cells to respond to larvae, which results in fewer galls (2, 23). In the cultivar 'Clevewilt,' root-knot resistance is a post-infectional phenomenon occurring at the cellular level (20) and due in part to reduction in virulence of penetrating larvae by a toxin(s) (21). These larvae also might be unable to induce the syncytia necessary for establishing a parasitic relationship due to differences in the host components essential for syncytial ontogeny or to a lack of stimulatory secretions (6).

Several investigations of enzymes of nematode-infected plants have been conducted. Peroxidase activity increased in roots of cabbage *(Brassica oleracea)* infected with *Pratylenchus penetrans* (1), whereas *Ditylenchus dipsaci* induced qualitative differences in multiple forms of peroxidase of

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Wando pea *(Pisum sativum)* (13). Qualitative differences were detected between peroxidase multiple forms of uninfected and galled stems of tomato *(Lycopersicon esculentum)* but not between uninfected and galled roots (12).

The pentose phosphate pathway is important in biosynthesis of nucleic acids and aromatic ring systems, and peroxidase catalyzes several reactions including those involved in metabolism of phenols and indoles. Therefore, we have examined the activity of 6-phosphogluconate dehydrogenase (6-PGDH, E. C. 1.1.1.43) and peroxidase (PER, E. C. 1.11.1.7) in infected root tissue of cotton resistant and susceptible to *M. incognita.*

MATERIALS AND METHODS

Two-cm segments of roots infected with 50-100 surface-sterilized larvae were obtained from 8-day-old resistant and susceptible cotton cultivars, 'Clevewilt 6-3-5 (CW)' and 'M8,' respectively (22). Uninoculated but otherwise similarly treated seedlings served as controls. Determinations of enzyme activity and electrophoretic separations of multiple forms were conducted 6 days after inoculation. During this period, in the susceptible cultivar, syncytial formation is initiated and premolting growth of the nematode occurs.

Assays of specific activities: Enzyme extracts were prepared by freezing approximately 0.5 gm of 2-cm root segments in liquid nitrogen and pulverizing them in a mortar. After partial thawing, 2.0 ml of either the TRIS-M or phosphate buffer as subsequently described were added with continued grinding. The preparation was pipetted into a syringe in which a folded 2-cm^2 piece of cheesecloth had previously been placed and then injected into a centrifuge tube and centrifuged at 10,000 *g* for 20 min at 6 C. Controls were prepared in the same manner except that autoclaved extracts were used.

Solymosy and Farkas' method was modified for the determination of 6-PGDH activity (27). Ten root segments were ground and extracted in 5.0 ml of 0.2 M tris(hydroxymethyl)aminomethane-maleate (Tris-M) buffer, pH 7.2. The reaction mixture consisted of 1.0 ml of extract; 1.0 ml of 3.96 mM 6-phosphogluconate (trisodium salt) (6-PG); 0.1 ml of 19.0 mM $MgCl_2$; 1.0 ml of 18.7 mM nicotinamide adenine dinucleotide phosphate (NADP). The change in absorbance at 340 nm was read every minute for 8 min in a Beckman DB-G spectrophotometer.

The determination of PER activity was patterned after Loebenstein and Linsey's method (18). Pyrogallol was prepared immediately before use by adding fresh 0.5 M pyrogallol solution to 0.15 M sodium phosphate buffer, pH 6.0, then diluting the solution to 100 ml with distilled water. The assay procedure was as follows: extracts from 5 root segments were prepared as for 6-PGDH except that a total volume of 5.0 ml of sodium phosphate buffer was used. To 5.0 ml of the pyrogallol reagent in a colorimeter tube was added 1.0 ml of the extract. The tube was inverted to mix the contents and then inserted in the spectrophotometer (Bausch and Lomb Spectronic 20). After the galvanometer was adjusted to 0 absorbance at a wavelength of 420 nm, 1.0 ml of freshly prepared 0.29 M hydrogen peroxide was added to the tube. Changes in absorbance were read after 90 sec.

The molar extinction coefficients of the products of the reactions catalyzed by 6-PGDH and PER were used to calculate the specific activities. These were calculated as μmoles of reduced nicotinamide adenine dinucleotide phosphate (NADPH) or pyrogallin formed/min/mg protein. Protein concentrations were calculated by the method of Lowry et al. (19) using bovine albumin as the standard. The activity data in Figs. 1 and 2 are the differences obtained between activities of autoclaved and unautoclaved extracts.

Electrophoretic separation of multiple forms: Multiple forms of 6-PGDH and PER from infected and uninfected roots of both cultivars were separated by polyacrylamide gel electrophoresis (4). Extracts from 40 root segments were obtained in the same manner as for the enzyme assays except that homogenates were centrifuged at 18,000 *g* for 30 min at 6 C in 2.0 ml of Tris (0.0495 M)-glycine(0.384 M) buffer, pH 8.3. Extracts were diluted to equivalent amounts of protein as estimated by the method of Lowry et al. (19) using bovine serum albumin as the standard. Sucrose was added

to make a 20% solution, and 150 μl of extract containing 50 μg of protein was layered onto the stacking gel. A current of 2 mAmp per gel (5.0 mm diam) was applied and the sample was electrophoretically separated in an anionic [Tris(0.0495 M)-glycine(0.384 M) buffer, pH 8.3] system at 4 C in a 7.0% gel until the tracking dye moved 60 mm. Then the gels were removed and stained for enzyme activity.

The reaction mixture for detection of multiple forms of 6-PGDH contained 13.4 mM 6-PG, 0.42 mM NADP, 3.2 mM $MgCl_2$, 0.41 mM p-nitroblue tetrazolium chloride (NBT), and 0.27 mM phenazine methosulfate in 0.2 M Tris-M, pH 7.2.

Peroxidase multiple forms were located by incubating the gels in a solution containing 0.05% hydrogen peroxide and 0.5% guaiacol.

RESULTS

Activities of 6-PGDH and PER were greater in infected than in uninfected tissues (Fig. 1, 2). The largest difference in activity between infected and uninfected tissues occurred with 6-PGDH (Fig. 1). Uninfected tissues from the resistant CW and susceptible M8 roots exhibited the same 6-PGDH activity, whereas PER activity was greatest in CW (Fig. 1, 2). Activities of 6-PGDH and PER were greater in infected CW tissue than in infected M8 tissue (Fig. 1, 2).

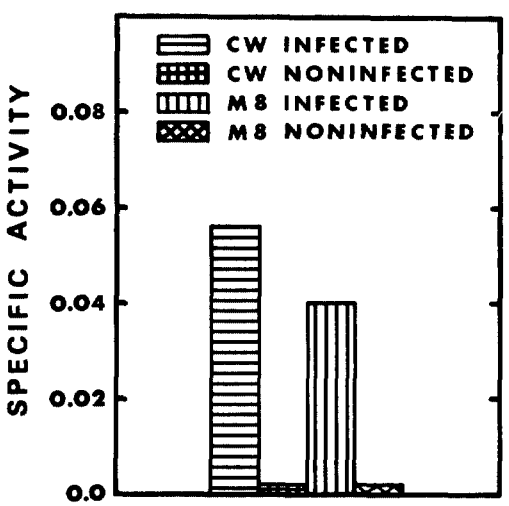

FIG. 1. Specific activity of 6-phosphogluconate dehydrogenase from roots of cotton 6 days after infection with *Meloidogyne incognita*. CW = cultivar Clevewilt 6-3-5, resistant to *M. incognita;* and M8 = cultivar susceptible.

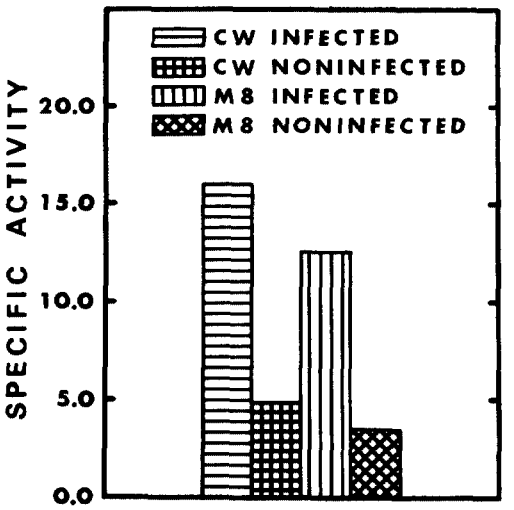

FIG. 2. Specific activity of peroxidase from roots of cotton 6 days after infection with *Meloidogyne incognita*. CW = cultivar Clevewilt 6-3-5, resistant to *M. incognita;* and M8 = cultivar susceptible.

Multiple forms of 6-PGDH were separated by polyacrylamide gel electrophoresis (Fig. 3). Bands 1 and 6, which were present in all extracts, were quite diffuse. Bands 2,

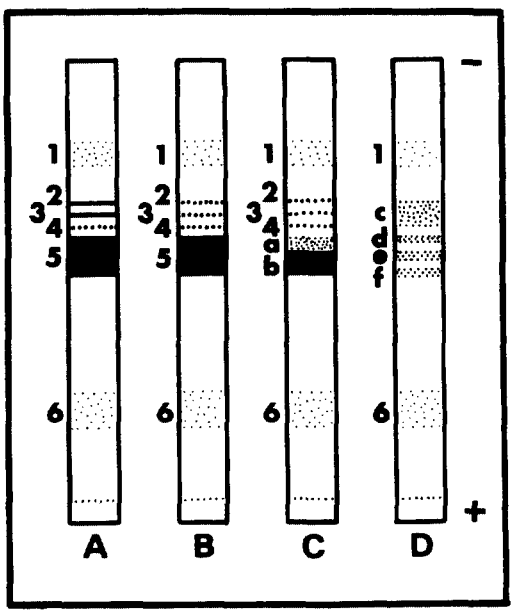

FIG. 3-(A-D). Diagram of polyacrylamide gels stained for 6-phosphogluconate dehydrogenase activity in resistant Clevewilt 6-3-5 and susceptible M8 cotton 6 days after infection with *Meloidogyne incognita*. *A)* Clevewilt infected. *B)* M8 infected. *C)* Clevewilt uninfected. *D)* M8 uninfected.

3, and 4 were obtained from infected roots of both cultivars and uninfected roots of CW (Fig. 3 A-C). A lightly stained diffuse band (c) was obtained from uninfected M8 roots (Fig. 3-D). Band 5, which was resolved in preparations from infected CW and M8 roots, was large and quite intensely stained and may have consisted of two or more forms which were not well separated during electrophoresis. Bands designated as a and b (Fig. 3-C) and d, e, and f (Fig. 3-D), which were obtained from uninfected CW and M8 roots, did not stain as intensely and migrated the same distance as band 5 from infected roots.

Four to five multiple forms of peroxidase were resolved from preparations of infected and uninfected roots (Fig. 4). Band 4, which was obtained from all preparations, was diffuse and obscure. Band 2 was resolved in the preparations from infected roots of both cultivars (Fig. 4-A, B). Band 0 was obtained only from infected roots of M8 (Fig. 4-B). Band 3 was common to all extracts. Band 1, resolved in preparations from infected roots (Fig. 4-A, B), migrated similarly to bands a and b, which were present in preparations from the uninfected roots of both cultivars (Fig. 4-C, D).

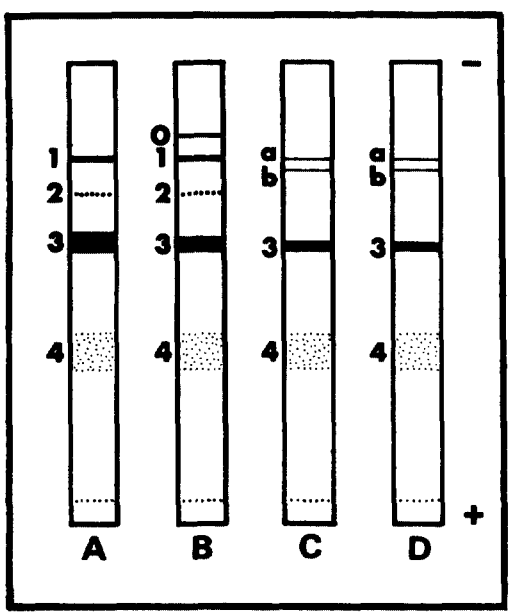

FIG. 4-(A-D). Diagram of polyacrylamide gels stained for peroxidase activity in resistant Clevewilt 6-3-5 and susceptible M8 cotton 6 days after infection with *Meloidogyne incognita*. *A)* Clevewilt infected. *B)* M8 infected. *C)* Clevewilt uninfected. *D)* M8 uninfected.

DISCUSSION

Our data show that infection of CW and M8 cultivars by *M. incognita* increases the activity of 6-PGDH. Such increases in the activities of the dehydrogenases of the pentose phosphate pathway (PPP) have been demonstrated in plants infected by pathogens and upon wounding (5, 7, 25, 26). Glucose catabolism via the PPP supplies NADPH, ribose-5-phosphate, and erythrose-4-phosphate. NADPH is an important source of reducing power in biosynthesis. Ribose-5-phosphate functions in the formation of nucleic acids, while erythrose-4-phosphate is important in the formation of aromatic ring systems such as those contained in aromatic amino acids, phenols, and indoles. Nucleic acids, free amino acids, protein, and nucleotides have been shown to increase in tomato infected by *M. incognita incognita* and *M. incognita acrita* (24). Seventeen free amino acids increased in CW and M8 cotton 6 days after inoculation with *M. incognita* (16). Among the free amino acids which increased were the aromatic amino acids phenylalanine, tyrosine, and tryptophan. The roles of such compounds in the response of plants to nematode infection have been reviewed (9).

Many peroxidase isozymes are known in the higher plants, but their isozyme patterns are difficult to interpret. This difficulty is due in part to the catalytic properties of the different isozymes (17). We found in these tests an increase in activity of peroxidase in both CW and M8 roots upon infection. Other studies have also demonstrated an increase in activity and in numbers of multiple forms as a result of disease, injury, or exposure to ethylene (10, 11). Resistance of different potato organs to infection by *Phytophthora infestans* has been positively correlated with the amount of peroxidase activity (8). Further, resistance to race 56 of *Puccinia graminis* in wheat (controlled by the Sr 6 and Sr 11 alleles) resulted in higher peroxidase activity (3). In that case, however, the increased activity was believed not to be the cause of resistance but the result of the resistance mechanism.

One or more of the peroxidase forms we isolated from infected roots may have a nematode origin or were activated by nematode feeding. These may function in giant-cell initiation and/or maintenance, although the detection of peroxidase isozymes of *M. incognita* appears to be host-dependent (14). There are several reactions catalyzed by peroxidase, including those involved in the metabolism of phenols and growth regulators (15). Further knowledge of the substrate specificity of the multiple forms we isolated and time-lapse studies would form a basis for understanding the role of peroxidase in the resistant mechanism, and the host-parasite interaction of cotton and *M. incognita*.

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