Comparative Morphology and Enzyme Histochemistry in Root Knot Resistant and Susceptible Soybeans

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ABSTRACT

A comparison was made of certain enzyme histochemical and morphological responses of susceptible (Lee) and resistant (Delmar) soybeans to infection by *Meloidogyne incognita acrita*. The activity of certain oxidoreductive, hydrolytic, and oxidative enzymes of the susceptible variety were increased significantly primarily within the syncytium. Initially, galling response to infection was similar in both susceptible and resistant plants, i.e., slight galling was observed prior to the microscopic detection of syncytia. During the first few days after inoculation, both cultivars showed a similar, slight increase of host enzyme activity at the nematode feeding site. With further disease development, however, the responses of the two cultivars differed.

In the susceptible host, syncytia developed at the site of nematode feeding. These syncytia generally developed into extensive units that contained increased levels of enzyme activity. The syncytia usually were sufficient to support the development of the nematode to maturity.

At a time corresponding to syncytia induction in the susceptible host, the most common resistant host response was cell necrosis. Often after inducing necrosis, the nematode migrated to nonnecrotic cells and commenced feeding; these cells subsequently became necrotic. An increase in enzyme activity was seldom found in resistant-host cells associated with nematode feeding. Unlike the susceptible plant response to the nematode, the resistant plant rarely produced syncytia. In rare cases, however, where the nematode induced syncytia in the resistant plant, the syncytia contained increased levels of enzyme activity. Phytopathology 60:896-902.

Previous enzyme histochemical studies of nematode-infected soybean plants have dealt exclusively with infection of susceptible plants by the root knot nematode *Meloidogyne incognita acrita* Chit. (4, 8). In these studies, every enzyme investigated increased its activity in the host cells at the feeding site of the nematode. As the disease progressed, the increased activity became manifest within the syncytium and continued in host cells from the initiation of infection by second-stage larvae through the development of adult nematodes.

Based upon our earlier observations (4, 8) and a realization that *M. incognita acrita* was an obligate parasite attacking a susceptible host, we hypothesized that the same obligate parasite attacking a resistant host would elicit a response that would interfere with the obligate nature of the parasite. Several mechanisms could achieve this purpose. Wilski & Giebel (9) suggested a toxification mechanism involving a phenolic glucoside and a glucosidase. The nematode presumably induces or contributes β-glucosidase to a syncytium which contains a nontoxic glucoside. The hydrolysis of the glucoside yields a toxic phenolic aglycone that kills the host cells, the nematode, or both. It was our contention that the resistant host would show histochemically either no increase in enzyme activity as a result of nematode feeding, or it would be hypersensitive and produce a necrotic response to infection.

The purpose of this investigation, therefore, was to observe the histochemical and morphological responses of resistant plants to infection by *M. incognita acrita* and to compare these responses with those in susceptible plants.

MATERIALS AND METHODS.—Susceptible (Lee) and resistant (Delmar) cultivars of soybeans (*Glycine max* L.) were grown and inoculated with *Meloidogyne incognita acrita* Chit. as described previously (4). Inoculated plants of both varieties were harvested at various intervals from 2 to 36 days after inoculation. The histochemical procedures previously adapted for the demonstration of various enzymes in Lee soybeans (4, 8) were used for both the susceptible and resistant cultivars without modification. Each histochemical localization medium was prepared and divided into two equal lots; one lot was used for each cultivar. The enzymes investigated were malate dehydrogenase, glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase, alkaline phosphatase, acid phosphatase, ATPase, peroxidase, cytochrome oxidase, and esterase. The method of sectioning fresh root knot material and the procedures for the demonstration of particular enzymes have been described (4).

The selection of Delmar to represent a soybean cultivar resistant to the root knot nematode was based on an earlier report by Crittenden (1). A preliminary study was made to confirm the resistance of Delmar and to rate its resistance to infection by *M. incognita acrita* against the resistance of several other soybean cultivars. Between 23 and 49 plants of five soybean cultivars, Lee, Hill, Pickett, Dyer, and Delmar, were inoculated with measured suspensions of infective *M. incognita acrita* larvae. The inoculated plants were held for 42 days in the greenhouse. Upon harvesting, the roots were gently washed to remove adhering soil particles and blotted dry. The roots were examined and the galls removed; the number of galls and their total wt (air-dried at room temp for 48 hr) was determined for each cultivar. The total root wt (air-dried for 48 hr), less galls, was determined for each cultivar. Calculations were made in an effort to rate the cultivars for resistance to *M. incognita acrita* under our greenhouse conditions.

RESULTS.—Delmar resistance tests based on the number and size of galls confirmed the resistance of Delmar to infection by *M. incognita acrita*. The results (Table 1) showed Delmar to be the most resistant and Lee the most susceptible of five cultivars tested.

General.—The term "enzyme activity" as used in this report refers to the relative histochemical activities of the enzymes studied. Since what was true in terms of activity and sites of localization for one enzyme, e.g., malate dehydrogenase, was generally true for the other enzymes, e.g., alkaline phosphatase or cytochrome oxidase, no distinction was made between the various enzymes.

Prior to infection by *M. incognita acrita,* the roots of the susceptible (Lee) and resistant (Delmar) soybean cultivars are morphologically and histochemically indistinguishable. During the early stages of disease development, the pattern of enzyme distribution in the two cultivars remained similar. Infective larvae penetrated the roots of both cultivars with equal facility. After penetration, the larvae migrated through the root tissues of both varieties with very limited tissue destruction or necrosis. While in the migratory stage, the larvae apparently probed occasional cells along their path, thus inducing these cells to have a more intense enzyme localization pattern. The most notable differences in host response to disease were the induction of numerous and extensive syncytia and a progressive increase in the intensity of enzyme localization in the susceptible host, compared to necrosis and very slight syncytial development in the resistant host.

Susceptible response.—Second-stage larvae that migrated through the root tissues after penetration at or near the root apex caused little or no tissue damage. Cortical cells that were probed by the migrating nematode, especially those near the stele, often became hypertrophied but were not observed to be polynuclear. The level of enzyme activity increased slightly in the hypertrophied cortical cells; the most significant increase was found in the cortical cells nearest the stele. There was no progressive increase in enzyme activity in the affected cortical cells, hence they did not attain the level of activity observed in syncytia. The ephemeral nature of the interaction between the nematode and the affected cortical cells probably accounts for the leveling-off of activity before it attained the amount of stimulation characteristic of syncytia. Generally, the nematode "probed" the cortical cell, then resumed migration without sustained feeding. Physical or chemical irritation created by the probing of cells in the vascular region was sufficient to stimulate the affected host cells to increase their amount of enzyme activity. The relative amount of enzyme activity was interpreted from the relative intensity of the chromophore indicator of a specific enzyme.

Within 3 days after inoculation, extensive stimulation of affected host cells was evident in terms of hypertrophy, increased cytoplasmic density, multinucleation, and increased enzyme activity (Fig. 1-A). A second-stage larva, apparently undergoing a molt, was associated with these cells. Further increases in enzyme activity of affected cells were associated with more advanced stages of the disease. Figure 1-B, 5 days after inoculation, shows a nematode closely associated with hypertrophied cells. The enzyme activity in the enlarged cells was significantly higher than that observed in nonaffected cells. Furthermore, the highest level of activity in one of the enlarged cells was at the end nearest the stylet of the nematode. The high activity in the cell (dark spot) to the left of the hypertrophied

TABLE 1. Five soybean cultivars rated for resistance to *Meloidogyne incognita acrita*. The ratings (in brackets in the body of the table) are from 1, most resistant, to 5, most susceptible

Item	Cultivar				
	Lee	Dyer	Hill	Pickett	Delmar
A. No. plants examined	35	29	23	41	31
B. No. plants with galls	33	24	16	33	5
C. Total no. galls	129	58	43	97	8
D. Total root wt, g[a]	19.3	13.8	7.8	12.9	19.9
E. Total gall wt, g[a]	0.58	0.16	0.09	0.14	0.01
Avg gall wt, mg (E/C)	[5] 4.5	[4] 2.8	[3] 2.0	[2] 1.4	[1] 1.1
% Infection (B/A × 100)	[5] 94	[4] 83	[2] 70	[3] 80	[1] 16
Infection efficiency[b] $\left(\frac{1}{A/C}\right)$	[5] 3.7	[3] 2.0	[2] 1.9	[4] 2.4	[1] 0.3
Infection efficiency[c] $\left(\frac{1}{D/C}\right)$	[4] 6.6	[2] 4.2	[3] 5.5	[5] 7.7	[1] 0.4

[a] Samples air-dried for 48 hr at room temperature.
[b] Based on the no. plants.
[c] Based on the total root wt.

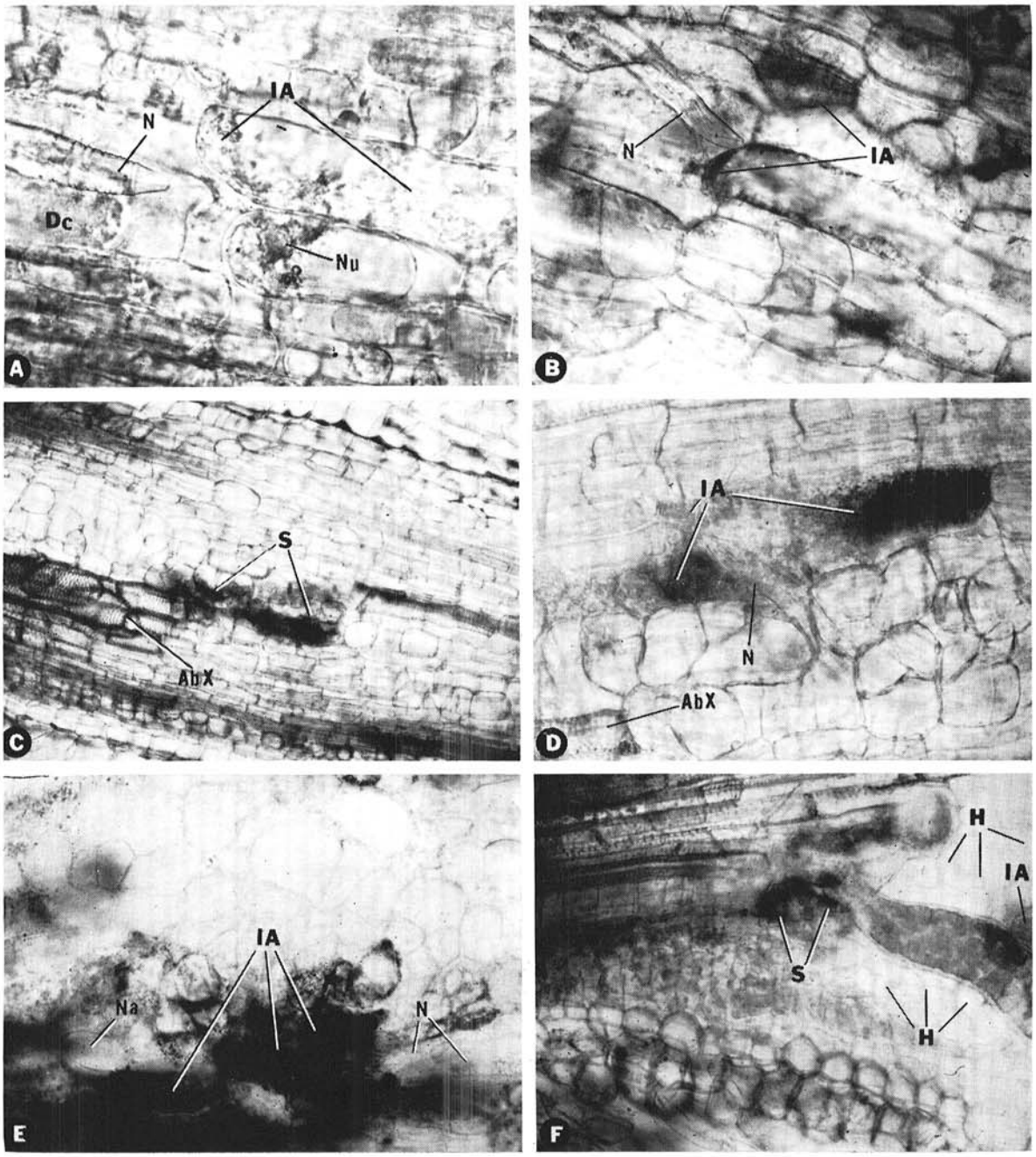

Fig. 1. The histochemical localization of various enzymes in a susceptible cultivar (Lee) of soybean (*Glycine max,* at different stages of infection by the root knot nematode(*Meloidogyne incognita acrita*). **A)** A section 3 days after inoculation showing a nematode (N) associated with hypertrophied cells containing very dense cytoplasm (DC), an enlarged nucleus (Nu), and increased enzyme activity (IA) (malate dehydrogenase). **B)** High enzyme activity (IA) was localized in a portion of a cell associated with the lip region of the nematode (N). The cell to the left of the nematode, also showing increased enzyme activity (IA), was probably a former feeding site of the nematode. The section was made 5 days after inoculation (malate dehydrogenase). **C)** A more advanced stage of the disease, 8 days after inoculation, shows a large increase in enzyme activity localized within the syncytium (S). An additional effect of the infection is the development of abnormal xylem (AbX) (isocitrate dehydrogenase). **D)** Although older, 14 days after inoculation, the stage of development of this infection is not as advanced as the previously illustrated section. The nematode (N) does not appear to have enlarged very much beyond the early second stage. Affected cells, however, show sufficient increase in enzyme activity to be easily recognized (IA); abnormal xylem (AbX) is present (isocitrate dehydrogenase). **E)** An extensive infection, induced by at least two nematodes (N, Na), shows a very large increase in enzyme activity (IA). The section was made 15 days after inoculation (glucose-6-phosphate dehydrogenase). **F)** An advanced larva is shown in a section 36 days after inoculation. The lip region of the nematode is associated with a syncytium (S) having increased enzyme activity. The major portion of the nematode body is associated with hypertrophied and hyperplastic cortical tissue (H). A small area of increased enzyme activity (IA) is shown in the proliferated tissue (malate dehydrogenase).

cell was probably a result of earlier probing by the nematode.

By 8 days after inoculation, the syncytium developed into an easily recognized unit. Reactions for the localization of the various enzymes showed that the syncytium supported extremely high levels of activity; furthermore, the activity was strictly confined within the limits of the syncytium (Fig. 1-C). Sections cut in chronological order did not always show a corresponding advance in the stage of the disease. In a section 14 days after inoculation (Fig. 1-D), the affected cells showed highly localized increased enzyme activity, but the development of the syncytium seemed limited for the age of the infection. Congruously, the nematode associated with the syncytium appeared limited in development. This suggested that the larva did not become sedentary and establish a syncytium for several days after penetrating the host. Figure 1-E illustrates a 15-day-old infection site. At least two nematodes were associated with the extensive syncytium that showed high enzyme activity. In contrast to the high activity within the syncytium, there is a region of hyperplasia with very low activity. An advanced larval stage of the nematode is shown in a section 36 days after inoculation (Fig. 1-F). The lip region of the nematode was associated with the syncytium, and the body of the nematode extends into the proliferated cortical tissue. Only a small portion of the syncytium was found in this section; most of the syncytium was located in a serial section above or below the plane illustrated. The hyperplastic cortical tissue around the body of the nematode probably contributes to the typical shape of a root knot gall.

To convey a three dimensional image to the enzyme distribution pattern of infected roots, transverse sections are necessary. Figure 2-A illustrates the hypertrophied cortical tissue around the body of the nematode. Because of the orientation of the nematode in the root, transverse sections generally result in an excising of the nematode. Usually when this happens the internal contents of the nematode are dispersed over the area. Although emptied of a part of the internal contents, the body outline of the nematode was evident in the mass of hypertrophied root tissue. Stimulated enzyme

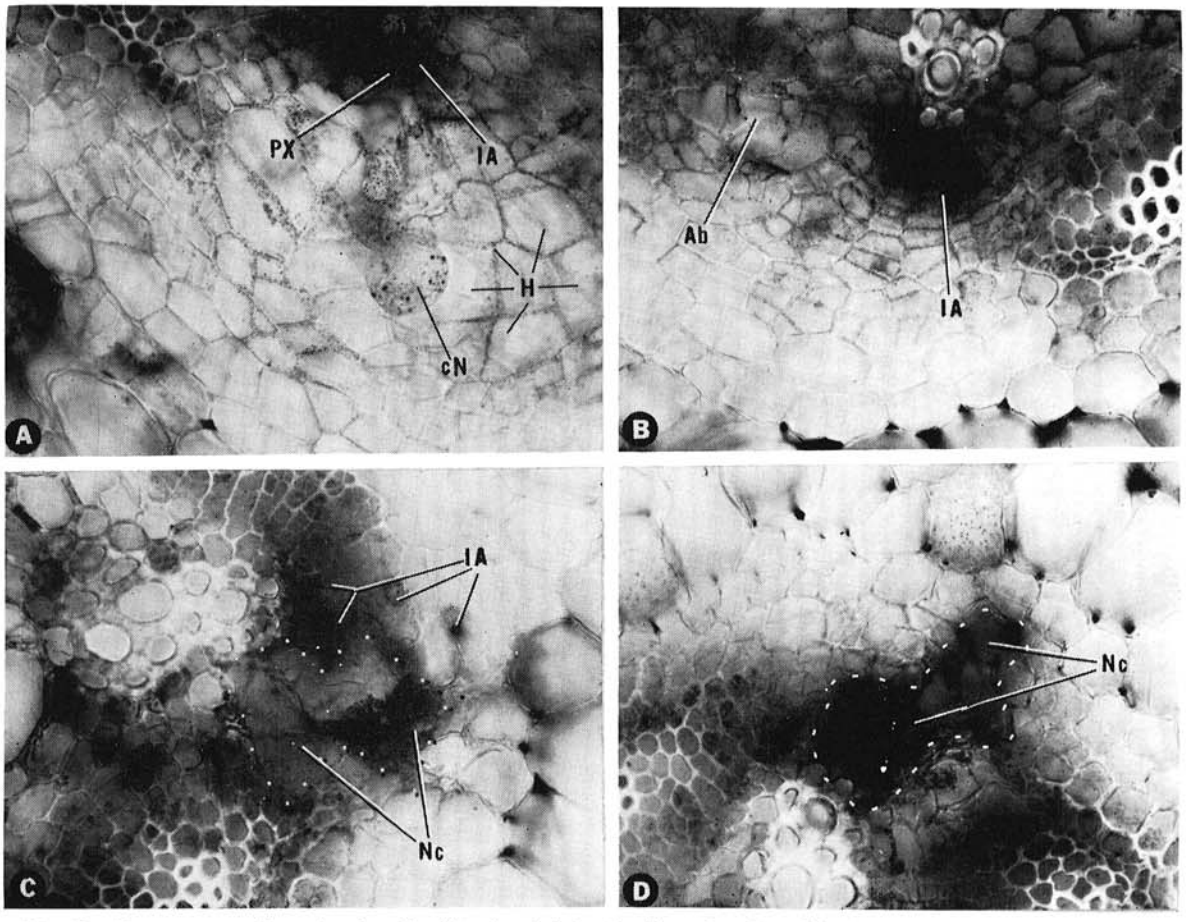

Fig. 2. Transverse sections showing the histochemical localization of malate dehydrogenase in a susceptible cultivar (Lee) *A* and *B* and a resistant cultivar (Delmar) *C* and *D* of soybean (*Glycine max,* infected by the root knot nematode (*Meloidogyne incognita acrita*). **A)** Increased enzyme activity (IA) and hypertrophy (H) associated with a nematode (cN) are indicated. The greatest increase in activity was localized near the protoxylem (Px). The nematode was excised during sectioning. **B)** A serial section of the previous figure. A large increase in activity (IA) was observed at the protoxylem. Some abnormal tissue (Ab) is indicated. **C)** A section made slightly above the nematode shows increased activity (IA) and necrosis (Nc). **D)** A serial section of the previous figure shows only necrosis (Nc).

activity as a result of nematode feeding was most pronounced at the protoxylem pole. Another section (Fig. 2-B), taken from the same root but somewhat removed from the site of the nematode, showed relatively high enzyme activity at the protoxylem pole; however, the activity of the hypertrophied tissue appeared to be less than the activity observed in the previous plane of section.

Detailed description and illustration of the response of the susceptible cultivar to root knot infection have been reported previously (4, 8).

Resistant response.—As in the susceptible cultivar of soybean, *M. incognita acrita* larvae produced little or no tissue damage to the resistant host during migration in the host tissues. Occasional cortical cells along the path of the migrating larvae had increased enzyme activity. Like the susceptible cultivar, the activity in the affected cortical cells did not attain the level of enzyme activity observed in syncytia.

Figure 3-A shows a second-stage larva in a section made from a resistant root 3 days after inoculation. The three sites of increased enzyme activity along the body of the nematode were probably induced by the larva as it fed on these cells prior to reaching the present feeding site. Up to this point, the responses of the two cultivars have been similar.

With further disease development, a necrotic response was typical in the resistant host. Both cortical and vascular cells became necrotic in response to nematode feeding. Necrosis began as a localized response involving only a few cells; however, host cells in the vicinity of the necrosis often showed increased enzyme activity (Fig. 3-B). As the disease developed, necrosis became more widespread. Nematodes suspected of inducing the necrotic response were seldom observed directly associated with necrotic cells; usually they appeared to be migrating away from the necrotic area. In Fig. 3-C it was assumed that the nematode first attacked the cells in the area designated NC_1; after inducing necrosis in that area, the larva migrated to the area NC_2. When the necrotic response was induced in that area, the nematode moved to another area, IA, and induced an increase in enzyme activity. Since the nematode also migrated away from that area, it was assumed that those cells with increased enzyme activity would eventually become necrotic.

Although host necrosis and inhibition of nematode development was a common occurrence in the resistant roots invaded by *M. incognita acrita,* a few larvae were able to parasitize root tissues with little or no associated necrosis. Figure 3-D represents a section of a 15-day infection. The larva was in contact with a hypertrophied cell that showed increased enzyme activity. Since the larva in this figure had not enlarged much beyond the infective stage, it was difficult to determine whether or not the area with high activity would develop into a syncytium. The activated cell might subsequently become necrotic, in which case the nematode would resume migration. Figures 3-E and 3-F, 15 and 36 days after inoculation, respectively, show that it is possible for *M. incognita acrita* larvae to establish a syncytium in resistant roots. Furthermore, the presence of adult females with egg masses in roots tested for determining the resistance of Delmar indicates that extensive syncytia are formed. Consequently, if a syncytium is produced in the roots of a resistant host, it is impossible both anatomically and histochemically to distinguish it from a syncytium in the susceptible host. However, when the numbers of syncytia in susceptible and resistant plants were compared, fewer instances were found where nematodes were successful in establishing syncytia in the resistant host.

Transverse sections 7 days after inoculation (Fig. 2-C, D) of the infected resistant cultivar complete the anatomical picture of the resistant response to infection. Both sections were cut from the same infected root, but several mm apart. Necrosis is present in both sections, but only one section, probably the most proximal to the nematode, showed increased enzyme activity. We assume that the cells showing increased activity (Fig. 2-C) would have become necrotic if the response to infection had continued a few more days without interruption.

Movement of the nematode lip region as described by Linford (6) for isolated nematodes was observed in infected roots. Active nematodes were observed several hr after incubating the root sections in the enzyme localization medium. Apparently the cuticular coat prevents appreciable penetration of the enzyme localization medium into the nematode. Except when cut in the process of sectioning, the larvae showed no enzyme activity with the histochemical procedures used.

Discussion.—During the early stages of larval penetration and migration, differences in enzyme localization in host tissues of susceptible and resistant plants are negligible. Changes in the pattern of enzyme localization in the inoculated susceptible cultivar (Lee) have been documented in detail in earlier papers (4, 8). To insure comparability of age, type of inoculum, environmental condition, histochemical procedures, etc., between the two cultivars, the susceptible plants were studied again in relation to the response of the resistant cultivar (Delmar).

In the resistant cultivar, *M. incognita acrita* larvae usually did not induce the host response that results in the formation of extensive syncytia capable of supporting nematode growth and development. Localized sites of increased enzyme activity, however, lead to some degree of cellular enlargement and increased cytoplasmic density in the resistant variety. The apparent failure of these stimulated areas to continue a response to nematode feeding resulted in a deterioration of the cellular contents.

When only a few nematodes penetrate a given area, trails of necrotic cells are left by the migrating larvae as they move from one feeding site to another. When several larvae penetrate a given area, broad regions of host necrosis are produced and underdeveloped larvae are common. Necrosis of infected tissue is the most pronounced differential response of the resistant cultivar. The fact that only rarely were larvae observed to be directly associated with the necrotic tissue suggests that the nematode detects something in the infected host prior to observable necrosis. Possibly, larvae

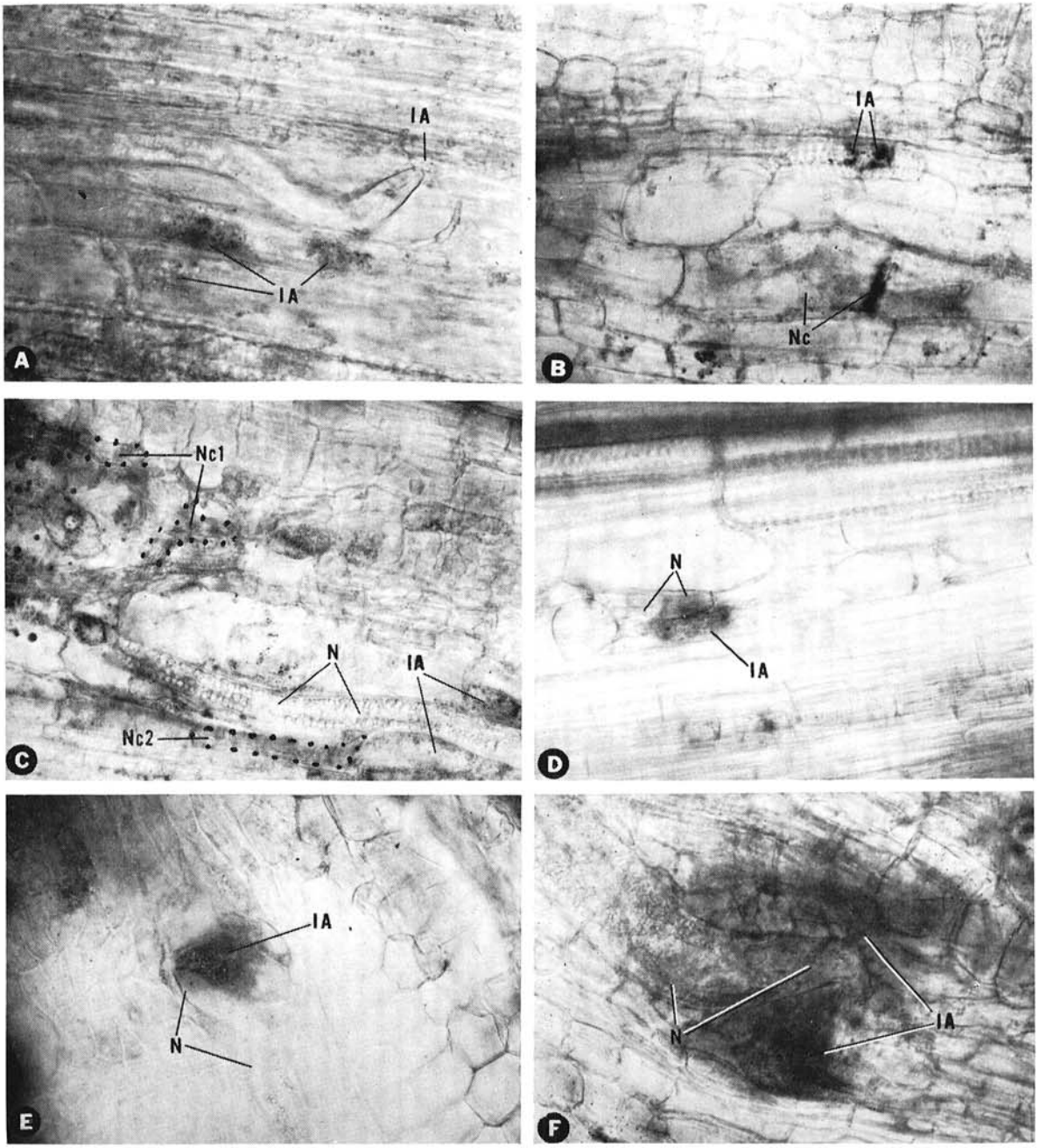

Fig. 3. The histochemical localization of various enzymes in a resistant cultivar (Delmar) of soybean (*Glycine max*) at different stages of infection by the root knot nematode (*Meloidogyne incognita acrita*). **A)** An early stage of infection, 3 days after inoculation, showing sites of increased enzyme activity (IA) in host cells at the lip region and along the body of the nematode. The increased activity in the host cells along the body of the nematode represent areas where the nematode probably probed during migration (malate dehydrogenase). **B)** Slight necrosis (Nc) and only a few cells with increased enzyme activity (IA) were observed in this section 7 days after inoculation (glucose-6-phosphate dehydrogenase). **C)** A 7-day-old infection showing a portion of a somewhat enlarged larva. The areas on either side of the nematode (N) showing increased enzyme activity (IA) are the result of probing by the larva during its migration away from the necrotic areas (Nc_1, Nc_2) (malate dehydrogenase). **D)** On rare occasions, a resistant root was found that did not become necrotic as a result of infection. At 15 days after inoculation, this section did not show any necrosis; increased enzyme activity (IA) typical of the susceptible response was associated with the lip region of the nematode (N) (glucose-6-phosphate dehydrogenase). **E)** Another section at 15 days after inoculation shows intense activity (IA) associated with a relatively large nematode (N) (malate dehydrogenase). **F)** At 36 days after inoculation, an advanced larval stage is associated with a well developed syncytium having increased enzyme activity (malate dehydrogenase).

feeding on a host cell are able to detect the prenecrotic response, and thus they resume their migration until they encounter another host cell upon which to feed. The newly parasitized host cell may initially respond like a susceptible cell, i.e., by showing increased enzyme activity; however, this response is usually replaced by necrosis. As an obligate parasite, *M. incognita acrita* larvae must maintain a feeding site on living host cells or perish. Hence, the process of parasitizing a host cell, the induction of host necrosis, and the resumption of larval migration, may be repeated until the nematode perishes. Death of the larvae may result from starvation or toxification from necrotic host cells. The length of time that larvae survive under these conditions is difficult to assess. However, the limited gall size, the reduced number of sites with increased enzyme activity associated with nematode feeding, and the abundance of necrotic tissue in the resistant host indicate that the larvae usually do not develop to any great extent.

The successes some larvae have in establishing syncytia and developing into mature adults in resistant hosts have been encountered elsewhere. Goplen et al. (5) recognized that *M. incognita acrita* larvae could occur in different physiological races where one race was virulent and another race avirulent to a given host. Riggs & Winstead (7) showed how the various physiological races of *M. incognita acrita* could develop. It is therefore conceivable that the few larvae that successfully completed a life cycle on the resistant host were physiologically different from the larvae that perished. This possibility, however, was not checked. On the other hand, it is possible that the resistant-plant cells that ultimately gave rise to a syncytium were physiologically different than the resistant-plant cells that resisted syncytium formation.

Resistance associated with a necrotic host response to nematode feeding has been reported by other workers who used a number of host plants and various species of the root knot nematode (2). In some cases, however, localized host necrosis is not associated with host resistance. Second-stage larvae of the soybean cyst nematode cause a great deal of host necrosis in both susceptible and resistant cultivars as a result of their intercellular migration through the root (3).

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