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Istituto di Nematologia Agraria, C.N.R., 70126 Bari, Italy

EARLY STAGE OF DISEASE IN FIG ROOTS INDUCED BY *XIPHINEMA INDEX* (1)

by
T. BLEVE-ZACHEO and G. ZACHEO

The ectoparasite nematode, *Xiphinema index* Thorne *et* Allen, is one of several species that damage root tips by forming galls.

Previous studies indicated that the host range of these species is restricted to a few plants, which support a rapid population increase (Weischer and Wyss, 1976; Prota *et al.*, 1977; Wyss, 1978). Direct observations on the feeding behaviour of *X. index*, in agar culture (Fisher and Raski, 1967; Cohn, 1970; Cotten, 1973), revealed that the activities of the nematode, on the host roots, consisted in exploration, cell wall perforation with the stylet, salivation and ingestion of the cell cytoplasm and, finally, withdrawal of the stylet from the feeding site (Wyss, 1977a, 1977b).

In response, the root-tips progressively swell and gradually transform into a terminal gall. Galls are strongly attractive to feeding nematodes which often aggregate at single sites (Weischer and Wyss, 1976).

Sections of galled root-tips showed modified cells, with high metabolic activities, expressed in hypertrophied and lobed nuclei and in density of the cytoplasm, rich in mitochondria and rough endoplasmic reticulum. Multinucleate cells were observed (Wyss, 1980) contiguous with the cortical cells, which were collapsed by nematode feeding.

In the present study we report the results of cytochemical re-

(1) Work partly done at S.C.R.I., Invergowrie, Dundee, Scotland, U.K.

sponse during the initial attack of *X. index* and a sequential description of structural phenomena that occur in cells of parasitized fig (*Ficus carica* L.) root tips, after removal of the nematode.

Materials and Methods

Seeds of fig were sterilized in sodium hypochlorite and germinated on damp filter paper, in a plastic Petri dish, at 20°C. After two weeks, seedlings were transferred singly into 2% agar in plastic Petri dishes; the plates were sealed with parafilm and stored at 20°C, 3000 lux for two-three days. Then they were each inoculated with 20 *X. index* females (extracted from a stock culture maintained on fig, kept in a greenhouse at 25°C), sterilized for 30 min in 0.03% NaN_3 solution and washed three times in sterile distilled water.

One day later the most active females were transferred onto new seedlings and their behaviour examined under a light microscope. For electron microscope studies, one female was put on each seedling and removed two hours after ingestion had started; the tips of the lateral roots were then excised at 0, 12 and 36 hours after the removal of the nematode. The root tips were fixed in 3% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.2 for 3 hours at 4°C, rinsed and left in the same buffer overnight. Root tips, post-fixed in 2% osmium tetroxide for 4 hours, were stained in 0.5 M uranyl acetate overnight, then dehydrated in a graded ethanol series and embedded in Spurr's resin.

For electron microscope examination of peroxidase localization, 100 μm thick sections from injured and healthy tips were fixed for 5 min, in cacodylate-buffered 3% glutaraldehyde, at 4°C. After several washes in buffer, sections were incubated for 1 hour, at 37°C, in a medium containing 10 mg of 3-3' diaminobenzidine tetrahydrochloride (DAB, Sigma) dissolved in 5 ml 0.05 M 2-methyl-1,3-propandiol buffer, pH 8. As a control, the same reaction was performed in the presence of 10 mM KCN (Novikoff *et al.*, 1972). After incubation, the sections were rinsed several times in buffer and fixed for electron microscopy, as previously described, but without staining in uranyl acetate.

Ultrathin sections were cut with a LKB ultratome III, stained in uranyl acetate and lead citrate and examined under a JEOL 100 B electron microscope.

Results

Females of *X. index*, reared in agar culture, became very active 12 hours after sterilization. They were observed to initiate feeding after a brief period of exploration (3-5 hours). However, preliminary observations had shown that nematodes selected lateral roots rather than tap roots as feeding sites; therefore for further studies the tap roots were cut off and the nematodes were deposited on the emerging lateral roots.

The root tip started to swell (Fig. 1a) eight hours after the first probe of the nematode. Root areas, already fed upon, became very attractive to other nematodes. Figure 1b shows a two day old gall with nematodes aggregated at a single site; as many as ten nematodes were observed at the same feeding site.

When the nematode fed on the initial cells, induced necrosis of the root tip and a lateral root primordium emerged, close to the necrotic area (Figs 1c, d). Four days after inoculation, feeding females produced eggs (Fig. 1e) and fifteen days later second stage larvae were observed feeding on root tips (Fig. 1f), indicating that the experimental conditions did not seriously affect normal behaviour.

Cells, observed under the electron microscope, two hours after nematodes had fed on them, showed no pathological modifications. The cells displayed typical features of meristematic cells without intercellular spaces, dense cytoplasm, rich in organelles such as mitochondria and plastids, not completely differentiated; nuclei showed normal cytokinesis, numerous small vacuoles were present in the cytoplasm. The cells of the central cylinder and the cortex were distinguishable (Fig. 2).

Holes were observed in the cytoplasm of cells that had been perforated by the stylet, and were delimited or contained electron dense material (Fig. 2, Inset). Cells at the feeding site, fixed 12 hours after nematode feeding, showed considerable changes: plasmolysis was evident on cell walls and the cytoplasm appeared to be coagulated and more electron-dense, while all cellular components appeared normal with intact membranes and no changes in shape or size (Fig. 3, Inset). Tissue, immediately adjacent to parasitized cells, also showed plasmolysis on the cell walls and electron dense material associated with the plasma membranes; nuclei appeared to be undergoing rapid mitosis (Fig. 3). Cells of this tissue sometimes were binucleate and

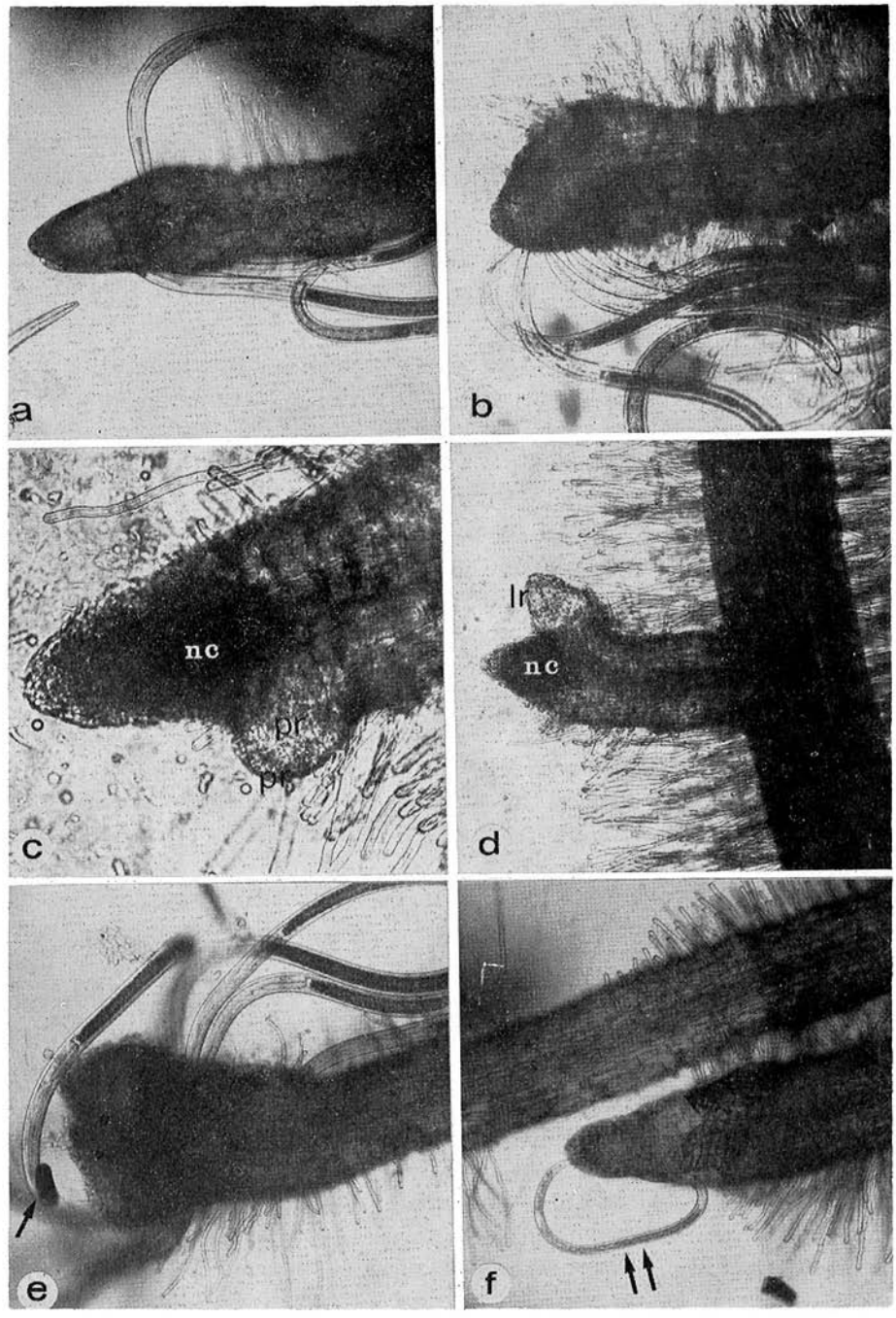

Fig. 1 - Response of fig root tips to feeding of *Xiphinema index*. a) 8 hour old gall on a lateral root tip, where two nematodes are feeding. Nematode stylets are inserted into the tip cells. b) Two day old gall where 5 nematodes are feeding at the same site. c, d) Very young lateral roots, where nematode feeding induced necrosis of the initial cells. Primordium (pr) and new lateral root (lr) close to the necrosis (nc) can be observed. e) 4 day old gall, with four feeding nematodes. An egg was deposited near the feeding site (arrow). f) Second stage larva (double arrow) feeding on root tip.

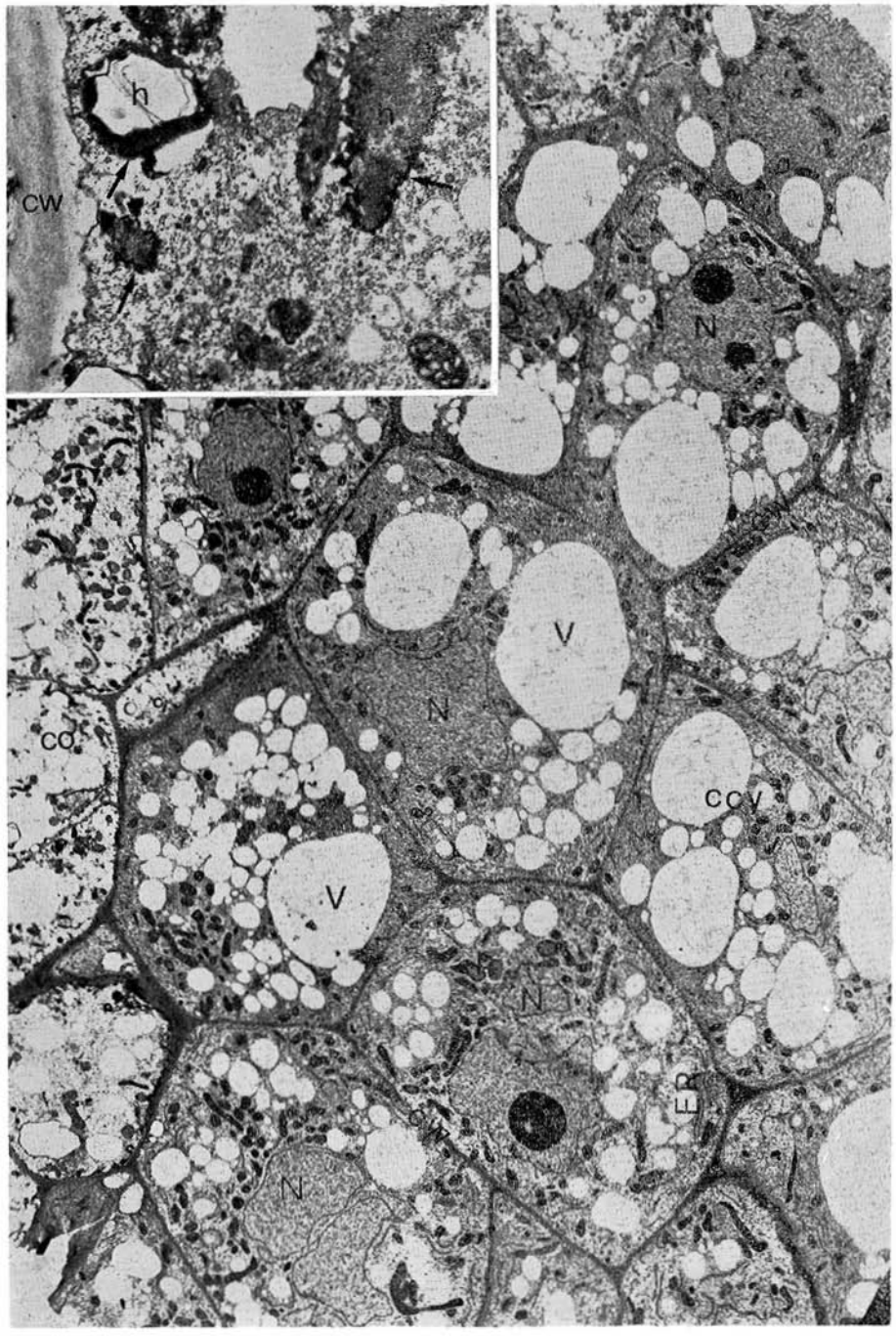

Fig. 2 - Meristematic cells fed upon for 2 hours by a nematode. Two kinds of cells are detectable: cortex (co) and central cylinder (ccy); cw = cell wall; N = nucleus; ER = endoplasmic reticulum; p = plastids; V = vacuole ($\times$ 3,000). Inset: holes (h) induced by nematode stylet in the cytoplasm, surrounded or full of electron dense deposit (arrow). ($\times$ 16,000).

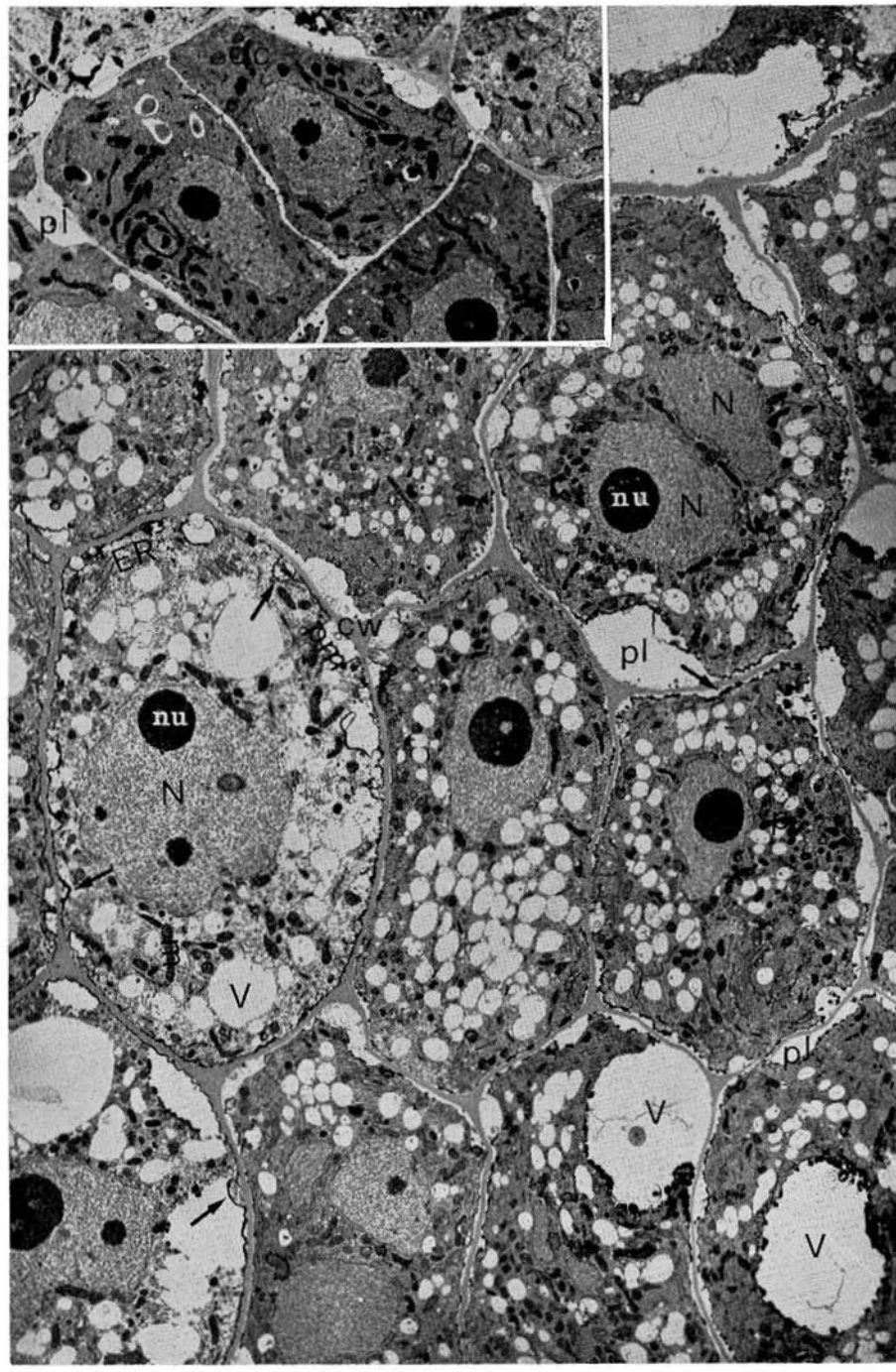

Fig. 3 - Cells of root tip 12 hours after nematode attack. Inset: collapsed cells (cc) at the feeding site; cytoplasm is electron dense, all cellular components are distinguishable (× 3,800). On modified cells, adjacent to the feeding site, are present plasmolysis (pl) on the cell wall (cw) and electron dense deposit (arrow) on the plasma membrane (pm). Cytoplasm is rich in vacuoles (v) mitochondria (m) plastids (p) and RER (ER). Nuclei (N) with hypertrophic nucleoli (nu) are present (× 3,200).

failed cytodieresis was evident from the presence of opposite stubs on the cell wall (Fig. 4).

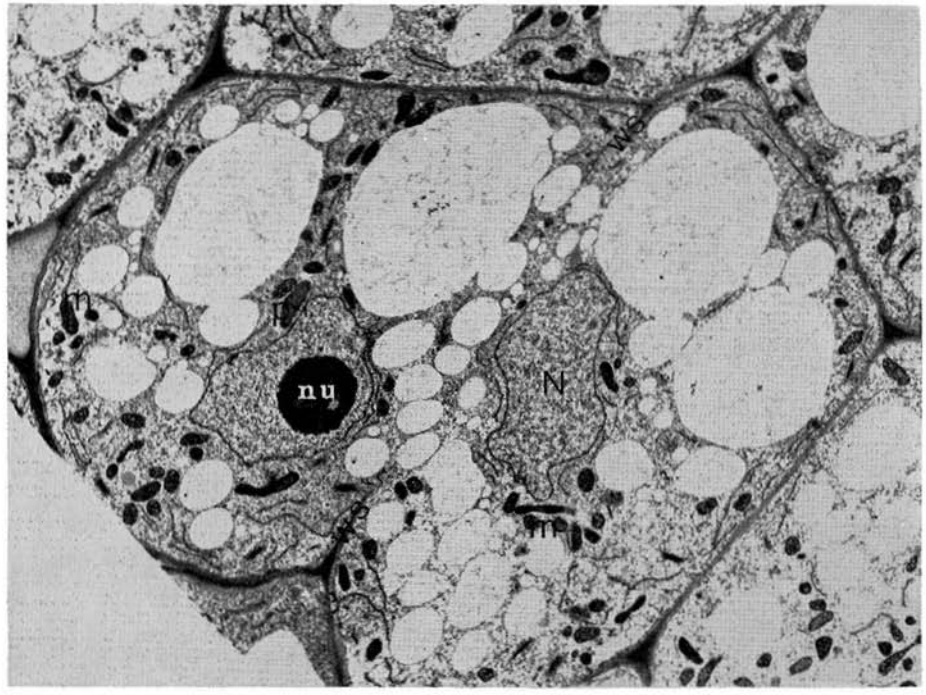

Fig. 4 - Binucleate cells, 12 hours after nematode attack with opposite wall stubs (ws) (× 4,800).

Sections on root tip swellings 36 hours after feeding revealed collapsed cells with the plasma membrane extensively disconnected from the cell wall. Collapsed cells are destined to become necrotic cells.

Neighbouring cells showed plasmolysis but the cytoplasm maintained its activity and structure (Fig. 5); only nuclei revealed high metabolic activity, indicated by the invagination of the nuclear envelope, which was sometimes amoeboid, and the presence of more than one nucleolus (Fig. 6a). Repeated mitosis without formation of new cell plates was common in these cells. Multinucleate cells, which had the feature of meristematic cell (such as cytoplasm actively synthesizing, small vacuoles, numerous mitochondria, plastids and rough endoplasmic reticulum) were present. Plasmolysis was not always evident on the cell wall, while electron dense deposits were associated with the plasma membrane (Fig. 6b).

Cytochemical reaction on attacked tissue revealed positive DAB

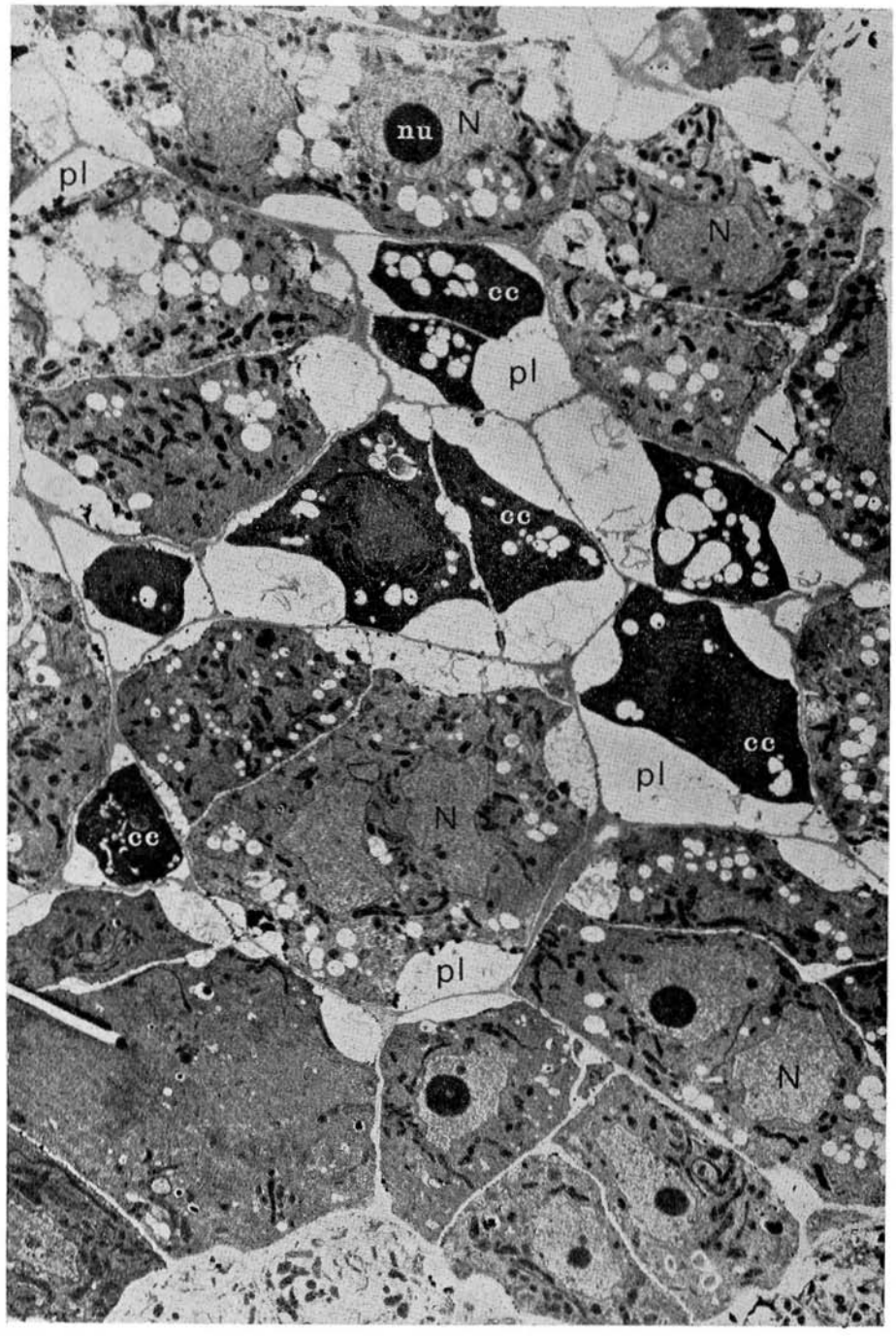

Fig. 5 - Feeding site 36 hours after nematode attack. Numerous collapsed cells (cc) in very poor conditions, with dark cytoplasm, disconnected from the cell wall, are present. Neighbouring cells show wide plasmolysis (pl), electron dense deposit on the plasma membrane (arrow), cytoplasm rich in organelles and lobed nuclei ($\times$ 3,500).

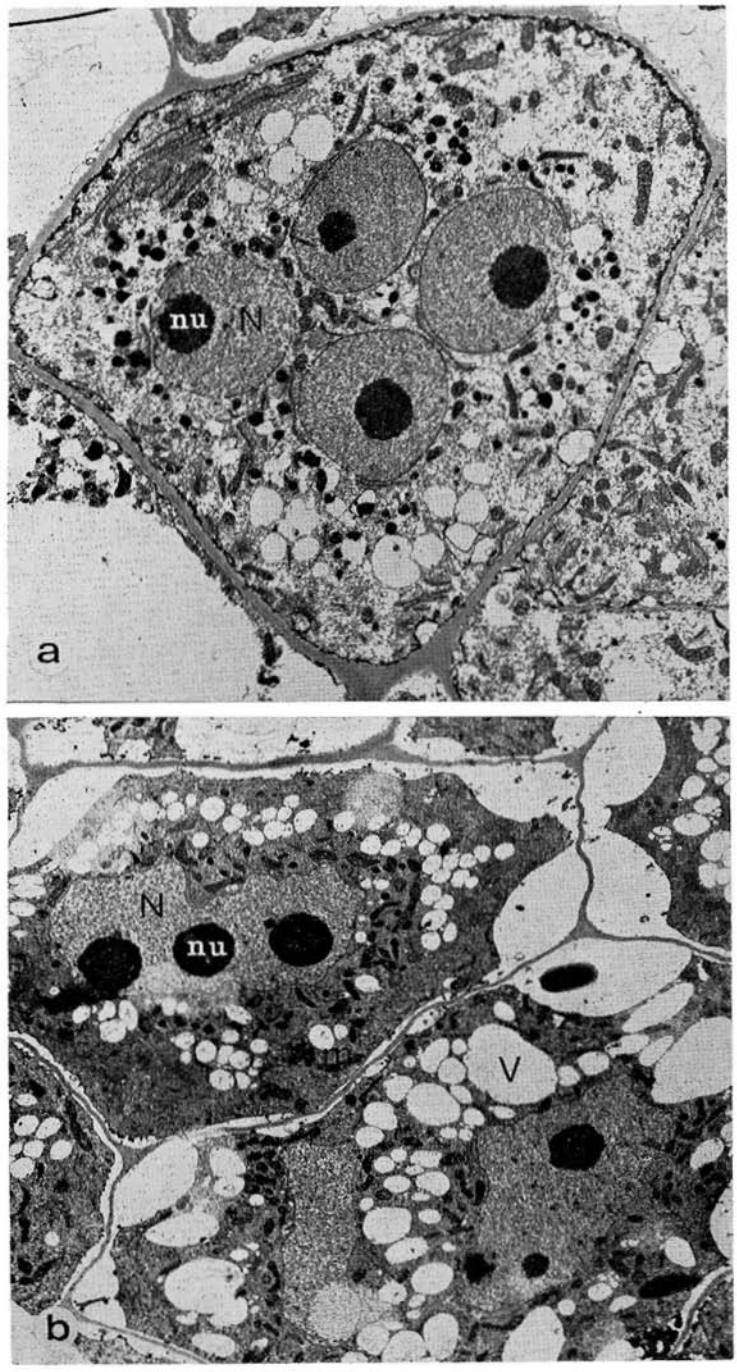

Fig. 6 - Cells as in Fig. 5. High metabolic activity of nuclei is detectable. a) Multinucleate cell, showing 4 clearly distinguishable nuclei (× 4,500). b) Nucleus with three nucleoli (× 3,000).

staining and was detectable particularly in mitochondria, vacuoles and the cell wall (Fig. 7c). The specificity of the reaction for peroxidases is further supported by the fact that incubation with KCN gave a complete inhibition of DAB staining and the mitochondrial membranes appeared completely unstained (Fig. 7a). DAB incubation of healthy root tips gave a positive reaction on the external membranes of mitochondria, but less than in attacked tissue (Fig. 7b).

Discussion

A short feeding period is sufficient for *X. index* to induce cessation of root elongation and the formation of swellings on fig root tips. It is evident that these symptoms are a direct result of nematode feeding and once initiated, continue after removal of the nematode.

Our observations on the feeding behaviour of *X. index*, in aseptic culture, agree with most of the earlier data (Wyss, 1977); moreover we observed, in our experimental conditions, that the nematode quickly initiates feeding and selects lateral roots in preference to tap roots. Soon after the commencement of feeding cells that were breached by the stylet became clearly modified; electron dense deposits, surrounding the holes, made by the stylet or present in the cell cytoplasm could be interpreted as nematode secretions. However, the host-cytoplasm may also have been independently involved because 12 hours after feeding electron dense material was found associated with the plasma membrane in cells not directly injured by the nematode.

The cells, directly involved in the feeding process, after 12 hours, showed a rapid increase in electron density of the cytoplasm, which degenerated after 36 hours. This kind of reaction, in which a limited number of cells are rapidly killed, is called a hypersensitive reaction (Wheeler, 1975) and show similarities to the degeneration of cells that occur as a response to *Meloidogyne incognita* infection in resistant tomato roots (Paulson and Webster, 1972; Bleve-Zacheo *et al.*, 1982). In fact, the necrotic area is restricted to a few cells and does not spread to the adjacent ones, which show high metabolic activity. Thirty six hours after nematode feeding, the cytoplasm remained characteristic of meristematic cells, apart from the considerably enlarged lobed nuclei, with hypertrophied nucleoli and irregular cytokinesis.

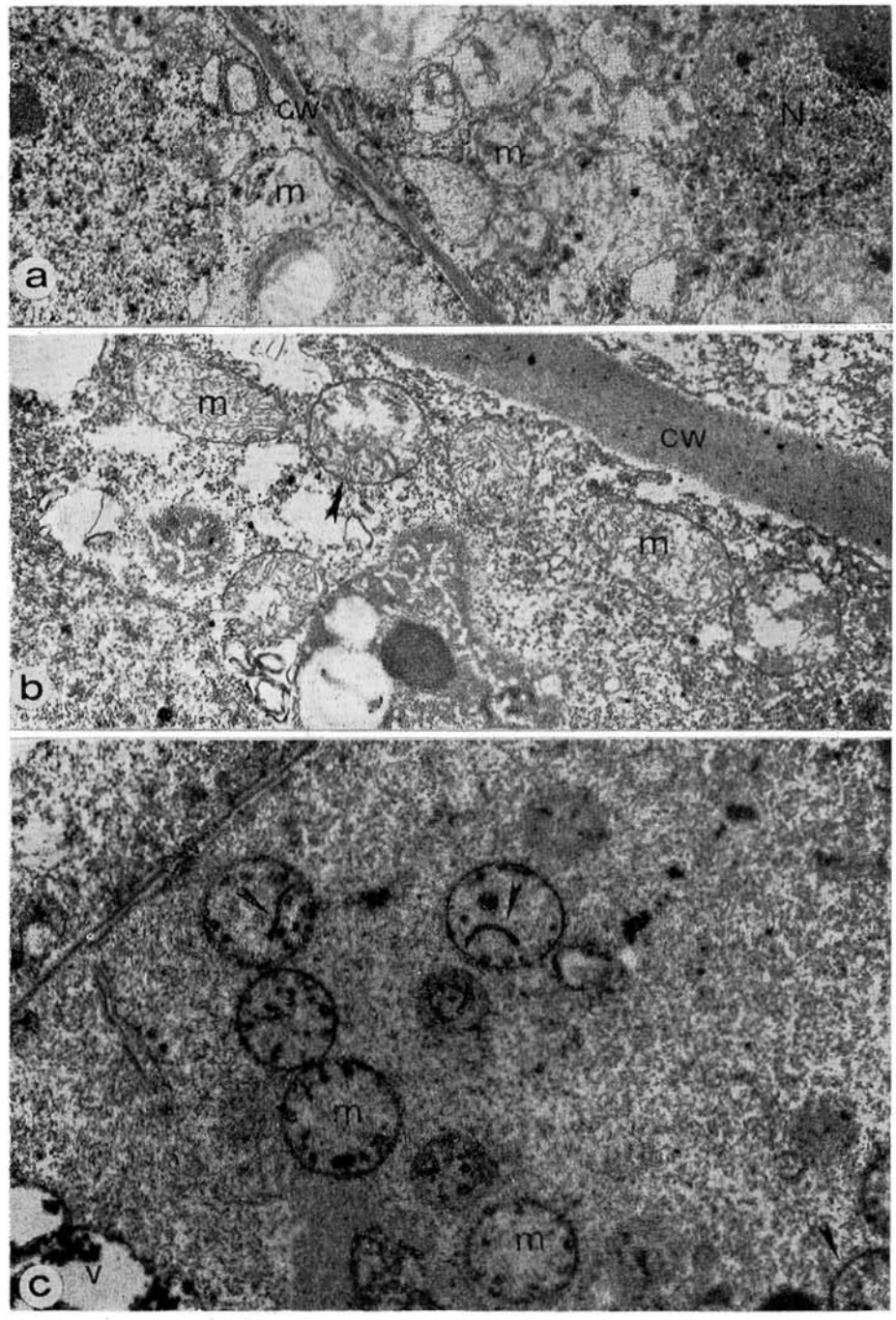

Fig. 7 - Cytochemical localization of peroxidase enzymes in infested and healthy root tips. a) DAB reaction on attacked tissues, in presence of KCN. No staining is detectable. b) DAB reaction on healthy cells. Positive staining is present in the cytoplasm and on the external mitochondrial membranes (arrow). c) Attacked tissue with positive DAB reaction; intense staining is visible on mitochondria (arrow) in the cytoplasm and in the vacuoles ($\times$ 19,500).

Little is known about the biochemical trigger that induces necrotic and modified cells in root tips of host plants of *X. index*. The cytochemical localization of DAB sensitive sites in tissue affected by nematode feeding, demonstrates a high oxidase activity associated with the infection (Hussey and Krusberg, 1970; Acedo and Rohde, 1971; Noel and McClure, 1978; Giebel, 1982). Our observations showed increased peroxidase activity in the cells, after *X. index* feeding and it was particularly bound to mitochondrial membranes. Although the association of high peroxidase activity with disease has been widely observed in many plants, its precise biological function is still unknown. Noel and McClure (1978) reported that peroxidases isolated from infected roots may have had a nematode origin or were activated by nematode feeding. Peroxidases, present in infested fig cells, could be activated by nematode feeding. Elucidation of the role of peroxidase in the host-ectoparasite nematode interaction awaits further investigations and it is hoped they will provide an answer to this intriguing problem.

SUMMARY

Tip swellings became evident 8 hours after the first feeding action of *Xiphinema index* on fig root, grown in aseptic culture. The gall continued to grow after nematode removal. Root tips were studied under the electron microscope 2, 12 and 36 hours after the beginning of attack. Cells, 2 hours later, showed holes, caused by the stylet of the nematodes, but not pathological modifications. Distinct cellular alterations were noticed after 12 hours - collapse of cells, directly fed upon by the nematode, surrounded by cells with actively synthesizing cytoplasm, containing hypertrophic nuclei. Severe damage was detected in the cells of the feeding site, 36 hours after feeding; neighbouring cells were plasmolysed but the cytoplasm did not seem to be seriously affected. Nuclei were lobed with enlarged nucleoli. Multinucleate cells were also found. Cytochemical analysis showed more intense DAB staining in tissues associated with the nematode feeding than in those that were unaffected.

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