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CYTOLOGICAL CHANGES INDUCED BY THE ECTOPARASITIC NEMATODE *LONGIDORUS LATOCEPHALUS* IN TOBACCO ROOTS

by
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Summary. The migratory ectoparasitic root nematode *Longidorus latocephalus*, exposed to tobacco seedlings in aseptic agar culture, fed exclusively on the root tip. The plant response was swelling of the root apices which became transformed into terminal galls three days after nematode infection. Sections through the galls incited by *L. latocephalus* revealed the presence of a cluster of necrotic cells representing the feeding site. The drastic disturbance of these cells was represented by a general loss in distinctness of the cell membranes and the disappearance of cytoplasmic organelles. Concomitant with this change was the hypertrophy of the cells bordering the feeding area. An increased cytoplasmic density with gelled ground substance and proliferation of whorls of endoplasmic reticulum, producing lipid bodies together with dense osmiophilic inclusions in the vacuoles indicated that nematode elicitors induced an hypersensitive host response. Changes in cell structure were not restricted to cells close to the nematode but also involved, though to a lesser extent, cells away from this region. As a result, the root tip lost its normal physiology and growth efficiency.

Longidorus species are parasites mainly of herbaceous plants and are genuine root-tip feeders (Cohn and Orion, 1970). The general and externally evident reaction of a host plant to feeding by *Longidorus* spp. is the formation of a terminal root gall. *Longidorus* species have very long odontostyles which penetrate a column of cells near the root apex. The time that the nematode remains at the feeding site varies from a few minutes to several hours, depending on the species, the point of insertion of the odontostyle into the roots, and various environmental factors affecting the behaviour of the nematode (Robertson *et al.*, 1984). During ingestion, the stylet remains inserted in a single cell, but food also seems to be derived from the surrounding cells (Towle and Doncaster, 1978).

Gall formation induced by *Longidorus* feeding has been related to hypertrophy of the procambial cells in the host root tip. *Longidorus elongatus* feeding on perennial ryegrass induced an enlargement of procambial cells, whose content was gradually depleted as feeding continued until the galled tissue became necrotic and collapsed (Griffiths and Robertson, 1984).

Exposure of cereals and legumes to *L. belloi* infection (Andres *et al.*, 1989) gave a different reaction in terms of lysis that was much more extensive and a large syncytium resulted from the breakdown of the cell walls in the procambial tissue of the root tip. This structure has been defined as a "lysigenous cavity" by Bleve-Zacheo *et al.* (1979) in celery roots fed upon by *L. apulus*. The cells involved in the cavity lost their original structure and showed amorphous ground material.

Extensive cell wall dissolution along the mid-

dle lamella has also been reported in tomato root cells fed upon by *Paralongidorus buchae* (Bleve-Zacheo *et al.*, 1985). Cell wall lysis and dissolution occurring between adjoining cells was very intense and spread so rapidly that the root tip was completely destroyed within one week. It seems plausible that plant enzymes are involved in wall digestion and that the nematode secretions exaggerate a normal plant cell process. The characteristic structure of the lysigenous cavity is formed as a response to specific substances, introduced by the nematodes, that provide large volumes of cytoplasm which are tapped by the parasite.

As the process of lysis progresses, the nematode abandons its feeding site that could no longer provide sufficient nutrients and another root tip is selected as a new feeding site. The result of *Longidorus* feeding is a reduced growth of the plant to varying degrees, depending on the population density of the nematode.

The presence of *Longidorus latocephalus* Lamberti, Choleva *et* Agostinelli in the rhizosphere of tobacco showing growth retardation and chlorosis in Bulgaria (Fig. 1) prompted the investigation of the feeding behaviour of this nematode and the related host response.

Materials and methods

Seeds of tobacco (*Nicotiana tabacum* L.) cv. Melmek 812 were surface-sterilised by immersion for 2 min in 70% ethanol, 4 min in 5% calcium hypochlorite, followed by rinsing three times in sterile water. The seeds were then transferred to 9 cm plastic Petri dishes, containing 7 ml nutrient medium comprising 0.1% Gamborg's B5 vitamin solution and 2% sucrose. The plants were kept at 25 °C and a 16 h light regime throughout the experiment. Specimens (females and juveniles) of *L. latocephalus* obtained from a tobacco field at Petrič, Bulgaria, were sterilised for 30 min in 0.03% NaN_3 solution and washed three times in sterile distilled water. Batches of 20 nematodes were transferred to five day old tobacco seedlings in an aqueous suspension and their behaviour examined under a light microscope.

For electron microscopy studies, swollen root tips were fixed in 2% paraformaldehyde and 2% glutaraldehyde in 0.05M cacodylate buffer (pH 7.2) for 2h, rinsed 4 times in the same buffer and post-fixed in a solution of 2% osmium tetroxide in cacodylate buffer for 2h at 4 °C. They were dehydrated through an ethanol series, substituted by propylene oxide and then infiltrated with Spurr's resin. The specimens were transferred into flat moulds and the resin was polymerized at 60 °C for 24h. Ultra-thin sections were made with a Reichert (Leica) Ultracut E, mounted on formvar coated 100 mesh grids and stained with a saturated ethanolic solution of uranyl acetate followed by lead citrate. Specimens were examined in a Philips 400 T transmission electron microscope.

Results

Root response

When the seedlings grown in axenic conditions were infected with females and/or third and fourth stage juveniles, lateral roots were usually selected for primary attack (Fig. 2a). *Longidorus latocephalus* showed a distinct preference for the undifferentiated root tissue. Several hours after the first attack the root usually started to swell at the root tip. Once initiated, root-tip swelling continued for several days and suppression of meristematic activity sometimes was irreversible. Cell elongation was inhibited, but cell differentiation was not affected and the formation of lateral root primordia was not impeded. Root proliferation continued under the influence of nematode feeding. New root tips were always attacked and they soon ceased growing (Fig. 2b). Older galls (6-7 days) showed extensive necrotic areas or had collapsed (Fig. 2b). Growth of seedlings inoculated with 20

nematodes was suppressed, and stunting and leaf discoloration were evident (Fig. 2a).

Cell response

Sections through the apical region of unattacked, well grown root tips of tobacco seedlings displayed characteristic feature of meristematic cells. All cells were uninucleate and contained relatively large, more or less spherical nuclei embedded in dense cytoplasm.

The nuclear content included a nucleolus that exhibited the nucleolar organiser, representing regions of the genetic material for ribosomal RNA. The chromatin in the nucleoplasm was present mostly in a dispersed form rather than as heterochromatin, indicating intensive transcription of genes as would be expected in an actively growing and dividing cell (Fig. 3a). In the micrograph a cell in anaphase with parts of several chromatid arms demonstrates that processes of mitosis were in progress.

Vacuoles in the cytoplasm were few. They occurred in a densely particulate cytoplasm, each particle being a ribosome. Adjacent cells communicated through the intervening wall plasmodesmata (Fig. 3a). Apart from ribosomes also studding the outer membrane of the nuclear envelope, other components of the cytoplasm were mitochondria, proplastids, endoplasmic reticulum cisternae and Golgi bodies (Fig. 3b).

Considerable cellular alterations were observed in the parasitized swollen root tips (Fig. 3c). Sites of nematode feeding were indicated

Fig. 1 - A Tobacco field at Petrič, Bulgaria, with a patch of declining plants in soil heavily infested by *Longidorus latocephalus*.

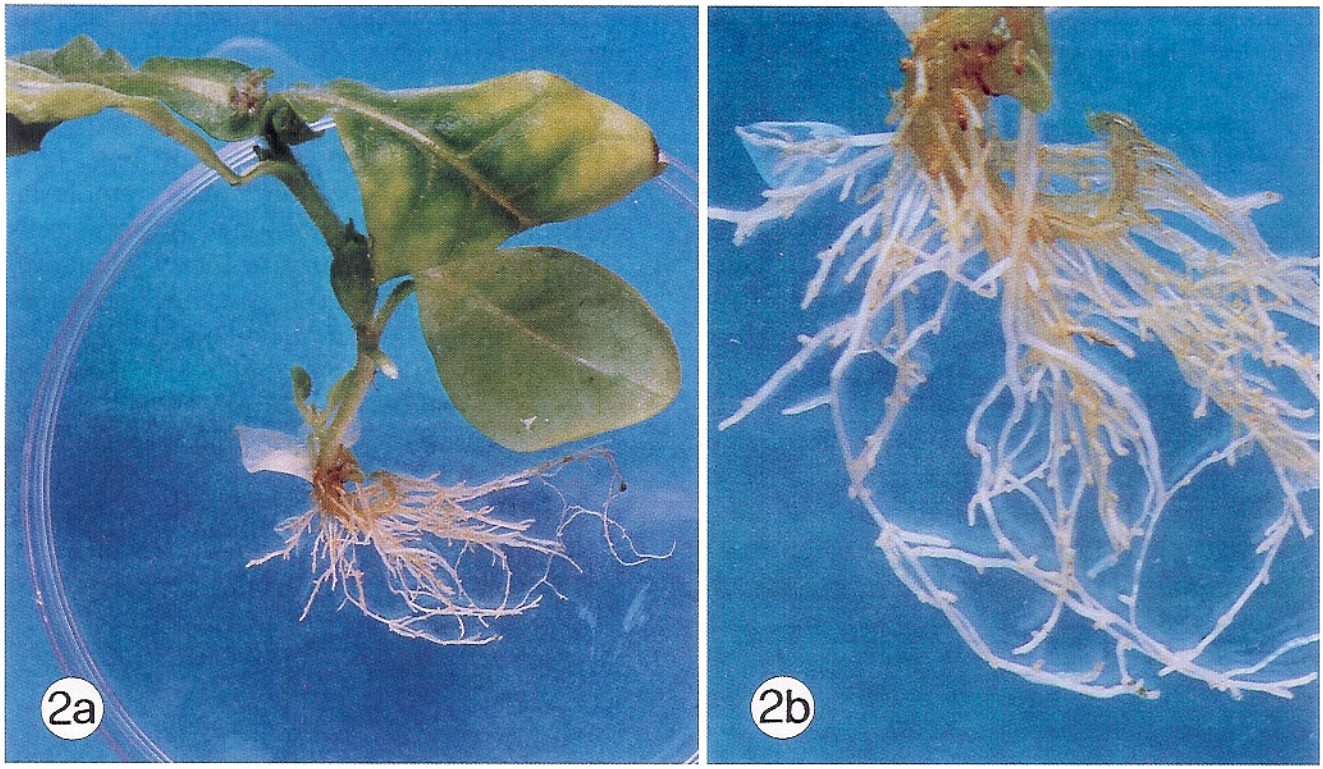

Fig. 2 - Response of a tobacco seedling to feeding by *L. latocephalus* in axenic culture: a) root system shows severe reduced growth concomitant with stunting and leaf discoloration; b) root system showing swelling of most of root tips, as a response to nematode feeding; some galled root tips are necrotic.

by the presence of several necrotic or partially empty cells with degraded cytoplasmic content. These necrotic cells resulted either from direct injury caused by the nematode odontostyle or from secretory products that had been injected. The nematode presumably ingested nutrients from the cytosol of its food cells through its stylet. As a consequence, a cluster of meristematic cells died and their cytoplasmic structure completely deranged; pycnotic nuclei, mitochondria and other organelles were no longer recognisable, and extensive vacuoles fused with each other. The electron dense remains of the cytoplasm appeared to be constituted by aggregates of membranes and ribosomes (Fig. 3d). These cells maintained their shape and there were no signs of wall breakdown. Indeed, a process of build-up rather than an expected breakdown was evident from finger-like ingrowths stretching out along all the cell walls (Fig. 3d). The cells surrounding the area where cell contents had been removed were about two times larger than corresponding cells in uninfected root-tips (Fig. 3d). This means that the root swelling is the result of hypertrophy of the root cells.

The meristematic cells bordering the nutritional cells also responded to the nematode with development of convoluted membrane structures, deposits of electron-dense material in the vacuoles, that were either a mass of small vacuoles or fused in one large vacuole occupying about one third of the cell, and extensive separation of the plasma membrane from the wall (Fig. 3c). Detailed analysis of these cells showed that there was secondary wall thickening and the presence of vesicular membranes

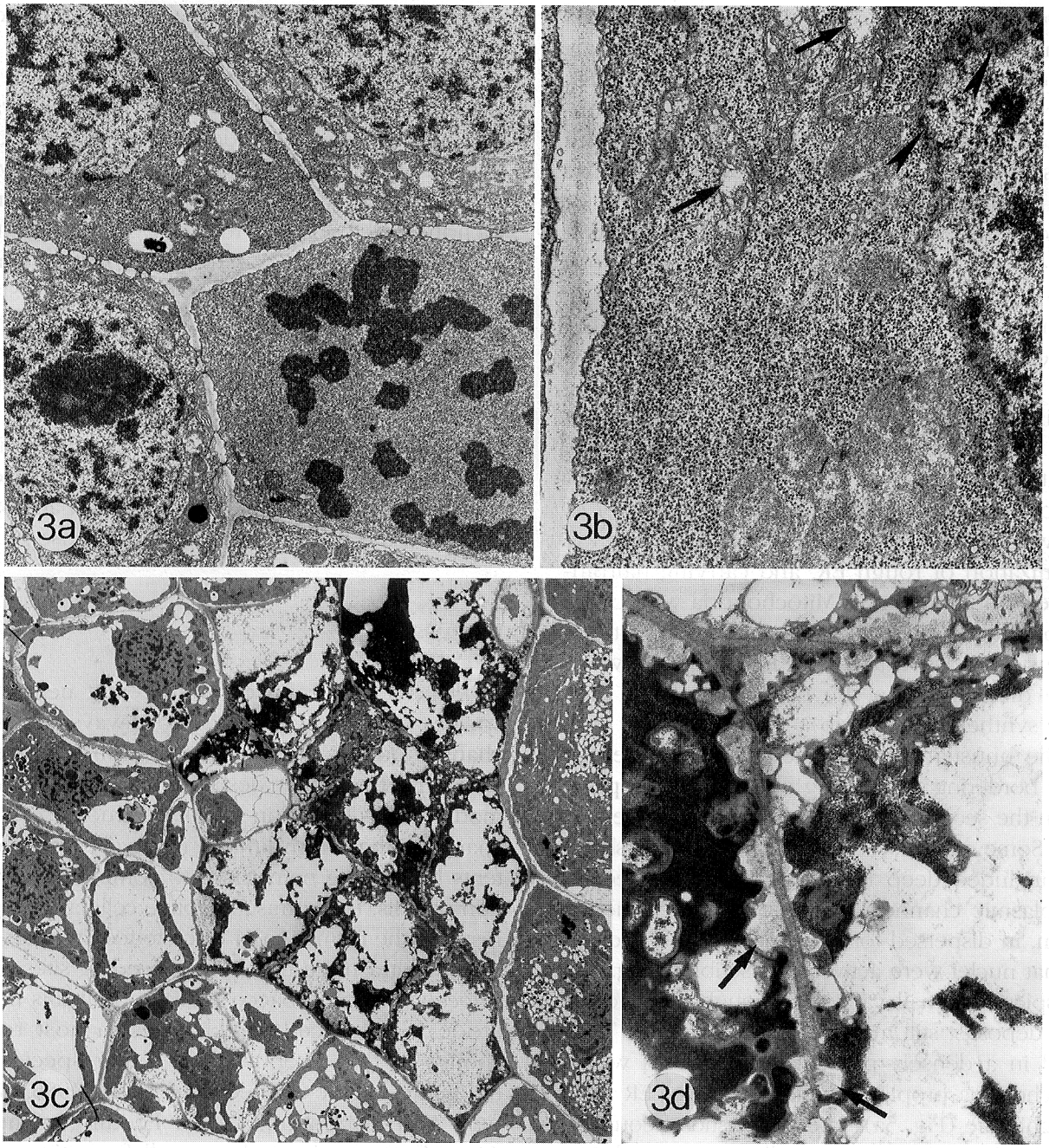

Fig. 3 - a) Longitudinal section through an unattacked root tip, showing meristematic cells. The ground cytoplasm is dense with very few scattered vacuoles. Nuclei, at different phases, show either a large vacuolated nucleolus and heterochromatin in condensed form (telophase), or chromosomes (anaphase). Note the frequent plasmodesmata along the cell wall. X 9,000; b) meristematic cell structure showing numerous ribosomes, mitochondria with mitochondrial DNA (arrow), proplastids, and profiles of ER in an unattacked root. Note the nuclear pores along the double nuclear membrane (head arrow). X 20,500; c) longitudinal section through a swelling root tip. A series of necrotic cells, representing the feeding site, are almost empty with only remains of cytoplasmic content. No wall breakdown is detectable in the cell walls of the feeding area. Cells bordering the feeding area are hypertrophied. X 1,600; d) finger-like wall ingrowths (arrow) on the walls between cells fed upon by the nematode. x 16,500.

between the wall and the disconnected plasma membrane (Fig. 4a). Unusual concentric arrangements of endoplasmic reticulum (ER), underlying conspicuous Golgi stacks with numerous vesicles at their margins, together with ribosomes and mitochondria constituted the bulk of the cytoplasm. Lipid droplets, with or without an electron–translucent core, had a surface skin of lipid molecules oriented into a monolayer in contact with the cytoplasm. The synthesis of lipid droplets seemed to be related to the concentric arrays of ER. The 12 to 20 parallel cisternae were closely stacked, confining the intervening cytoplasm to very thin layers (Fig. 4b, c). The characteristic of these cisternae was that they gradually lost ribosomes on their cytoplasmic surface starting from the exterior of the whorls, the inner part still maintaining the organization of rough ER, and carrying hairpin-shaped polyribosomes. Mitochondria with circular profiles or with branched configurations were strictly associated with lipid bodies, which were discharged in the cytoplasm once they were synthetized (Fig. 4b).

The unusual biosynthetic activity of the cell layer bordering the feeding area was also apparent in the second layer of cells but to a lesser extent. Some nuclei were highly irregular in shape and included deep invaginations penetrated by cytoplasmic channels. Chromatin in the nucleoplasm, in dispersed form as euchromatin, indicated that nuclei were actively transcribing (Fig. 5a). Conspicuous small vacuoles, though presence of dark deposits, still maintained cell turgidity. They layed in a densely particulate cytoplasm where mitochondria proplastids and profiles of ER were recognisable (Fig. 5a). The proplastids frequently contained starch grains, usually round in shape, and in addition there was an accumulation of material that was extremely electron dense (Fig. 5b). This occurred in distended intra-thylakoid compartments not in the stroma, where plastoglobuli can be found. In other material it has been found that this type of accumulation can be digested by treatment with the lipid–and protein–digesting enzymes lipase and pronase. It may therefore contain a lipoprotein.

Phenolic material may also be present in plastids. Dense deposits were seen in the vacuoles, and there is evidence that plastids can extrude phenol-containing droplets to the cytoplasm and vacuoles. Moreover, ring-shaped mitochondria have been found together with the common cylindrical to ellipsoid forms. Their unusual profiles probably resulted when a shallow dishshaped mitochondrion was sectioned across the dish so that only the rim was contained within the section. The two layers of the mitochondrial envelope were visible at both inner and outer faces of the ring (Fig. 5b). Mitochondria and plastids were tightly appressed, indicating that they have more than an accessory role in the cell's physiological response.

Discussion

Plant cells parasitized by different nematode species are modified in diverse ways. Cellular changes due to nematode parasitism have been categorized as either destructive or adaptive cell modifications (Dropkin 1979). Destructive cellular changes range from limited removal of cell contents by feeding nematodes to complete destruction of cells. In contrast, adaptive cellular changes result in the formation of discrete feeding sites.

Our ultrastructural study of feeding sites of *L. latocephalus* on tobacco revealed that this nematode induces cellular changes in host roots similar to those reported for other species of the genus (Bleve-Zacheo *et al.*, 1979, 1985; Griffiths and Robertson, 1984). Feeding of most *Longidorus* species induces gall formation with hypertrophy of the procambial cells in the host root tip. These nematodes are considered to induce destructive cellular changes by completely removing the contents of root cells until the galled tissues become necrotic and collapse.

The plant response is undoubtedly dependent on specific interaction with the parasite

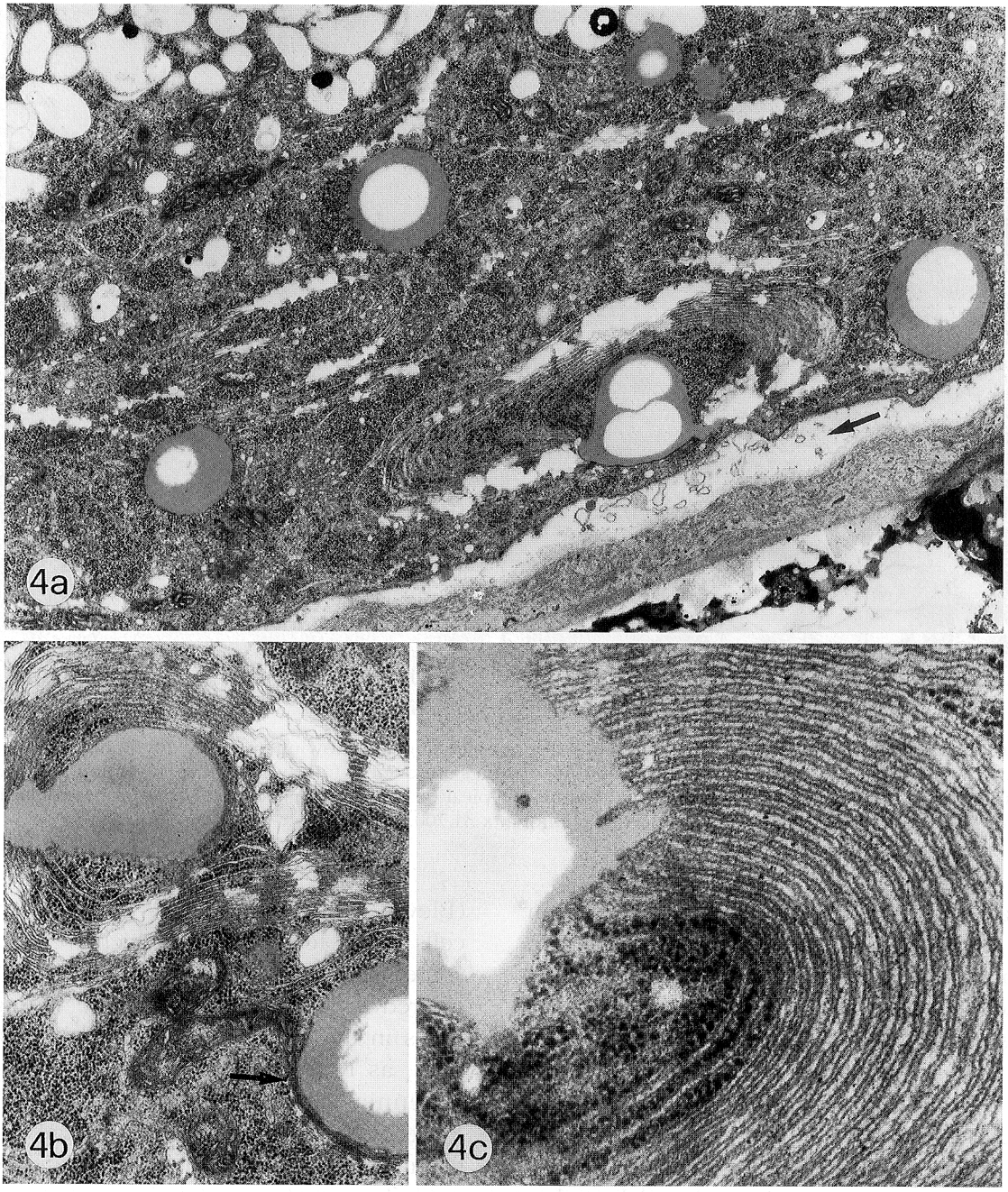

Fig. 4. a) Micrograph showing secondary wall apposition and plasma membrane disconnection due to development of vesicles (arrow) in a cell contiguous with the feeding area. The densely particulate appearance of the cytoplasm, whose ground substance seems to be gelled, results from the accumulation of ribosomes dissociated from the endoplasmic reticulum. Whorls of ER associated with Golgi stacks indicate secretory activities of the cell. Numerous small vacuoles are assembled in the centre of the cell. x 15,000; b) high magnification of unusual regular arrangement of the ER. Numerous parallel cisternae closely stacked and partially deranged delimit a portion of cytoplasm where fatty bodies are produced. Elongated mitochondria (arrow) are strictly related to lipid bodies free in the cytoplasm. x 26,500; c) a large system of smooth ER channels with flattened cisternae incorporating an irregular lipid body indicate that fats could be either secretory or membrane deterioration products. x 75,000.

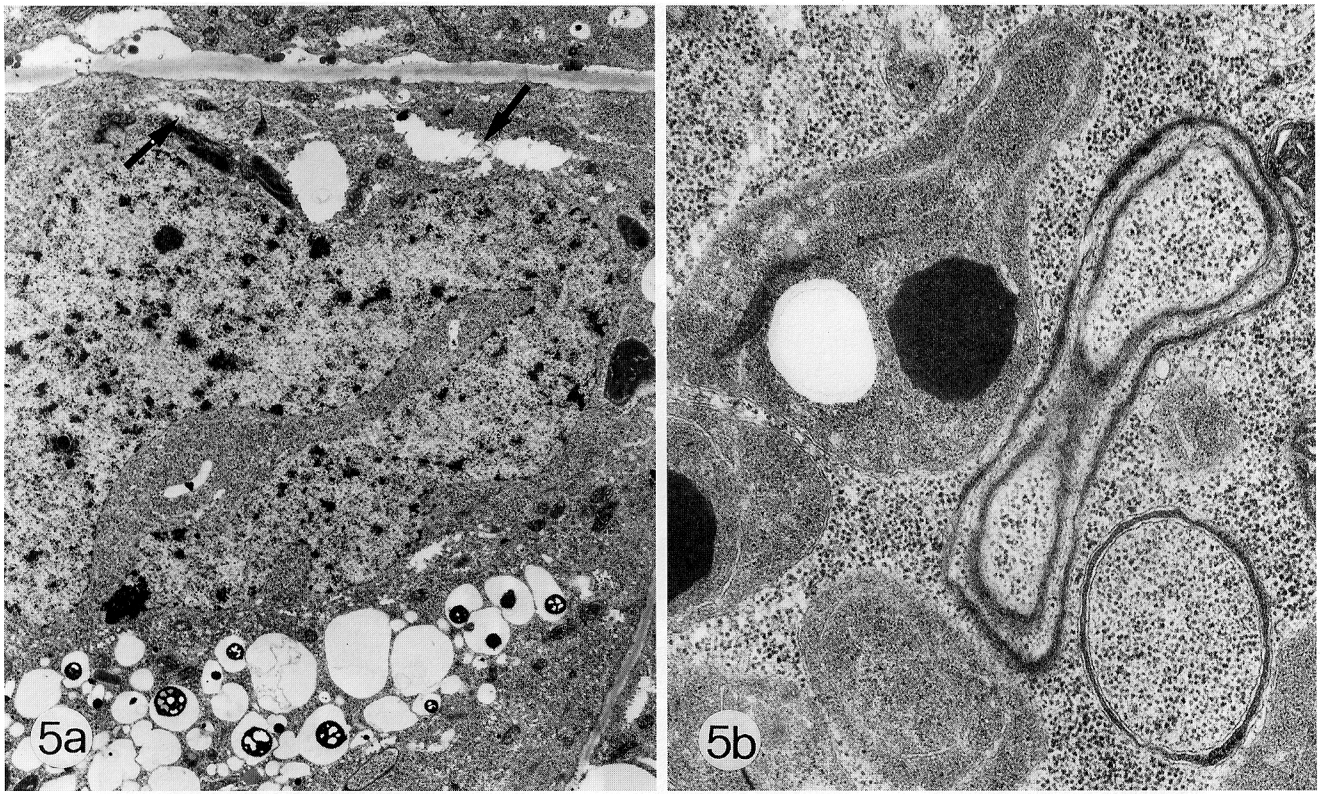

Fig. 5. a) Micrograph of a cell located at the 2nd layer from the feeding site showing an irregularly branched nucleus, small vacuoles in a dense cytoplasm where some damaged membranes are evident (arrow). x 5,400; b) portion of a meristematic cell distant from the feeding site. The cytoplasmic feature is typical of the meristematic tissue apart from proplastids containing starch and protein bodies and ring-shaped mitochondria. x 31,700.

and is associated with the injection and diffusion within the root cells of secretions from the nematode's oesophageal gland. The characteristic structure of the feeding area is formed as a response to specific substances, introduced by the nematode, that provide large volumes of metabolites that are utilized by the parasite as a food source. Thus, the nutritional cells detected in tobacco roots may be compared in their function to the lysigenous cavity induced by other longidorid and tylenchid ectoparasitic nematodes (Zacheo and Bleve-Zacheo, 1995). *Longidorus latocephalus* did not induce cell wall lysis and breakdown of feeding cells in tobacco root tips as in the case of *L. belloi* (Andres *et al.*, 1989), *L. apulus* and *P. buchae* (Bleve-Zacheo *et al.*, 1979, 1985), and *Hemicycliophora typica* (Bleve-Zacheo *et al.*, 1987). The content of the cells in the feeding area, which were located at some distance from the odontostyle tip, are considered to flow to the nematode through small holes in the cell walls produced by a localized lysis, as reported for *L. elongatus* feeding on perennial ryegrass (Robertson *et al.*, 1984). Moreover, transfer cell-like wall ingrowths which occur only on cells at the feeding sites of *L. latocephalus* may be regarded as a transport pathway upon a symplastic hinterland in a secretory-absorptive situation. On the other hand, a secretory system has been induced by the parasite around the feeding area. It is demonstrated by the presence of labyrinths of rough *versus* smooth ER. The role of the labyrinths of smooth ER in these cells is obscure as smooth ER is

present in a variety of plant glands secreting fats, oils, and fragrant essential oils. But, at least, noticeable lipid synthesis has been observed in these meristematic cells, that are not gland cells. Smooth ER secretes fatty acids that accumulate to form lipid droplets. Special enzymes can flip lipid from the cytoplasmic face of the ER membrane to the luminal face, correcting the imbalance that arises from asymmetrical synthesis. In this way the surface area of the ER membrane increases. Expanses of membranes may then be mobilized to the other system in the form of vesicles to the plasma membrane, or lipid synthesis can be picked out by lipid transfer enzymes and moved as individual molecules to sites of utilization (Gunning and Steer, 1996). But the development of a hypersensitive-like reaction enclosing nurse cells, such as the lysigenous cavity, seems to be an impediment to the nematode utilizing this food source. Indeed, because of the salivary components of the parasite there is no single mechanism involved in overproduction of metabolic products for nematode needs. A series of biochemical reactions inducing the process of the physiological cell death as a host response can be initiated. Hypersensitive reaction of tobacco root cells is exhibited by secondary wall apposition; it may be directed to regulate nematode damage, and cessation of cytoplasmic streaming as supposed by the feature of the gelled cytoplasm in the cells bordering the feeding area. Clearly, the cessation of cytoplasmic movements is a consistent event preceding the collapse of host tissue in host-fungus interaction (Goodman and Novacky, 1994).

Another possible explanation of the lipid-like accretions that appear in many membranes is that they are signs of membrane derangement. Moreover, subsequent dissolution of the lipid bilayer and the coalescence of lipids into ultrastructural "pools", reveals that the plant reacts to nematode elicitors through oxidation of membrane lipids by oxygen radicals. Lipid peroxidation is a consequence of conditions in which membrane deterioration results from the oxidative perturbation of cell membranes (Thompson *et al.*, 1987).

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