ULTRASTRUCTURAL RESPONSE OF COFFEE ROOTS TO ROOT-KNOT NEMATODES, *MELOIDOGYNE EXIGUA* AND *M. MEGADORA*

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ABSTRACT

Rodrigues, A. C. F. de O., I. M. de O. Abrantes, M. T. Melillo, and T. Bleve-Zacheo. 2000. Ultrastructural response of coffee roots to root-knot nematodes, *Meloidogyne exigua* and *M. megadora*. Nematropica 30:201-210.

Histological and ultrastructural changes induced in *Coffea arabica* cv. Catuaí amarelo and Catimor (*Coffea arabica* cv. Caturra × Timor Hybrid) by two populations of *Meloidogyne exigua* and one of *M. megadora* are reported. All second-stage juveniles of both species penetrated through the rhizodermis of the root directly behind the root cap and reached the stele near the vascular elements. In Catuaí amarelo roots, cells acting as feeding sites increased their synthetic activity and became multinucleate cells. Nuclear activity and dense cytoplasm observed in cells of roots inoculated with *M. exigua* indicated a susceptible response of the coffee plants. Seedling cells fed upon by *M. megadora* showed retraction and roughening of the inner wall surface, great presence of paramural bodies and processes of autophagy within the vacuoles, all suggesting an intermediate response between susceptibility and resistance. In Catimor coffee, all *Meloidogyne* populations tested induced necrosis of injured root cells. Following minor cytoplasmatic changes, the nurse cells showed condensation of chromatin, altered carbohydrate metabolism, development of lysosomes and autophagic vacuoles. This response seems to be a hypersensitive-like response that leads to cell death six days after nematode infection.

Key words: coffee, host-parasite interactions, hypersensitivity, resistance, root-knot nematodes, susceptibility, TEM, ultrastructure.

RESUMEN

Rodrigues, A. C. F. de O., I. M. de O. Abrantes, M. T. Melillo y T. Bleve-Zacheo. 2000. Respuesta ultraestructural de las raíces de café a los nematodos agalladores de la raíz, *Meloidogyne exigua* y *M. megadora*. Nematropica 30:201-210.

Los cambios histológicos y ultraestructurales inducidos en *Coffea arabica* cv. Catuaí amarelo y en Catimor (*Coffea arabica* cv. Caturra × Timor Hibrido) por dos poblaciones de *Meloidogyne exigua* y una de *M. megadora*, son reportados. Todos los juveniles de segundo estadío de ambas especies, penetraron a través de la rizodermis de la raíz directamente detrás de la punta de la raíz y alcanzaron la estela, cerca de los elementos vasculares. En las raíces del Catuaí amarelo, las células actuando como sitios de alimentación aumentaron su actividad sintetizadora y se convirtieron en células multinucleadas. La actividad nuclear y el citoplasma denso, observado en células de raíces inoculadas con *M. exigua*, indicaron la susceptibilidad de las plantas de café. Las células de las plántulas infestadas con *M. megadora* mostraron retracción y arrugamiento de la superficie de la pared interna, gran presencia de cuerpos paramurales y procesos de autofagía dentro de las vacuolas, todo lo cual sugiere una respuesta intermedia entre la susceptibilidad y la resistencia. En el café Catimor, todas las poblaciones de *Meloidogyne* evaluadas, indujeron necrosis de las células dañadas de la raíz. Luego de cambios citoplasmáticos menores, las células portadoras mostraron condensación de la cromatina, alteración del metabolismo de carbohidratos, desarrollo de lisosomas y vacuolas autofágicas. Esta respuesta parece ser una respuesta tipo de hipersensibilidad, que conduce a la muerte celular seis días después de la infeción por el nematodo.

Palabras claves: café, interacciones parásito-hopedante, hipersensibilidad, resistencia, nematodos agalladores de la raíz, susceptibilidad, TEM, ultraestructura.

INTRODUCTION

Coffee (*Coffea arabica* L.) is a very important world crop in which nematodes, especially root-knot nematodes, are widely distributed and cause great losses to farmers and to a country's economy (Campos *et al.*, 1990). So far, there have been reports of fifteen species of *Meloidogyne* parasitizing coffee (Campos *et al.*, 1990; Carneiro *et al.*, 1996; Decker and Fuentes, 1989; Eisenback *et al.*, 1994; López and Salazar, 1989). Some of the species are restricted in distribution and there remains the possibility, due to earlier misidentification, of finding new species among the known populations (Carneiro *et al.*, 1996).

The cellular response, at ultrastructural level, following root-knot nematode invasion of different hosts and non-hosts has been studied extensively (*e.g.,* Bleve-Zacheo *et al.*, 1998; Endo, 1987; Paulson and Webster, 1970). However, little is known about the relationship between *Meloidogyne* and *Coffea*, and the characterization of ultrastructural changes induced by these nematodes in coffee plants is completely lacking. Mendes *et al.* (1977), Negrón and Acosta (1987) and Vovlas and Di Vito (1991) characterized the reaction of *C. arabica* cv. Mundo Novo to *M. exigua*, of *C. arabica* cv. Borbon to *M. incognita* and of *C. arabica* to *M. incognita* and *M. exigua*, based on light microscopy studies. Mendes *et al.* (1977) and Negrón and Acosta (1987) found two to five giant cells with thickened walls, numerous nuclei and dense cytoplasm with a granular appearance in the vascular system of coffee roots of both cultivars inoculated with both populations, while Vovlas and Di Vito (1991) found several undersized giant cells around heads of *M. incognita* juveniles, in contrast to well developed large multinucleate giant cells induced by *M. exigua*.

Studies on the pathogenic variation within and among species of *Meloidogyne* on coffee plants showed that *Coffea arabica* cv. Catuaí amarelo was susceptible to *M. exigua* (Moraes *et al.*, 1973) and to *M. megadora* (Abrantes *et al.*, 1995) and that Catimor (*C. arabica* cv. Caturra × Timor Hybrid) was either resistant or susceptible to *M. exigua* (Morera and López, 1987; Rodrigues, unpub.), depending on the degree of resistance of parental lines. To date, no information about the host-parasite relationship between Catimor and *M. megadora* has been published, although this species causes great losses in coffee fields in República Democrática de São Tomé and Príncipe (Abrantes *et al.*, 1995).

This study was undertaken to compare the cellular reactions occurring on roots of *C. arabica* cv. Catuaí amarelo and Catimor to *M. exigua* and *M. megadora* infection. The results reported here represent the first report on ultrastructural changes following root-knot nematode infection in coffee roots.

MATERIALS AND METHODS

Second-stage juveniles (J2) of two populations of *M. exigua* Goeldi from Brazil and Nicaragua, and one of *M. megadora* Whitehead from República Democrática de São Tomé and Príncipe, were hatched from egg masses dissected from galled roots of infected tomato (*Lycopersicon esculentum* Mill.) cv. Santa Cruz and *C. arabica* cv. Catuaí amarelo plants for *M. exigua* and *M.*

megadora, respectively. Coffee seedlings of *Coffea arabica* cv. Catuaí amarelo and Catimor (*Coffea arabica* cv. Caturra × Timor Hybrid) were grown individually in 5 cm diameter clay pots containing sterilized quartz sand (particle size 0.1-0.3 mm). When root initials appeared, each seedling was inoculated with 100 J2 of one or the other species. Fifty plants were used for each population and were grown in growth chambers at 27°C, with a 12h photoperiod at 5000 lux and relative humidity of 70%. Plants were watered, when needed, with Hoagland's nutrient solution.

Three days after nematode inoculation, all plants were removed from their pots and their roots were washed free of sand. Half of them were returned to uninfested sand for three additional days. Root tip segments (collected three days after inoculation) and portions of infected roots (collected six days after inoculation) were fixed in buffered 3% glutaraldehyde (0.05 M sodium cacodylate buffer, pH 7.2) at 4°C for 4h, rinsed overnight in the same buffer and post-fixed in 2% osmium tetroxide in the same buffer for 4h at 4°C, stained overnight in the dark, at room temperature, in 0.5% uranyl acetate, dehydrated in an ascending series to absolute ethanol and embedded in Spurr's resin. Sections, 2 µm thick, were cut with a LKB ultratome IV, stained with toluidine blue and observed under the light microscope to verify nematode location. Ultrathin sections (200 Å) were then cut from the same area, stained with 3% aqueous uranyl acetate and 0.5% lead citrate, and examined at 80 KV in a Philips 400T electron microscope (Bleve-Zacheo *et al.*, 1995).

RESULTS

Microscopic observations showed that J2 of both nematode species penetrated through the rhizodermis of the root by breaking cell walls directly behind the root cap. J2 migrated within the cortex, and feeding nematodes were found in the stele near the vascular cylinder. Although subsequent migration of the juveniles was mostly intercellular, numerous cells were also mechanically broken down in both Catuaí amarelo and in Catimor roots. One of the first indications of nematode presence was a slight swelling of the root apices and induction of giant cells in cv. Catuaí amarelo, and necrosis involving cortical and vascular tissues in Catimor.

Coffea arabica *cv. Catuaí amarelo response to two populations of* M. exigua *second-stage juveniles*: The cellular events associated with J2 feeding, three days after inoculation, show a parenchymatic cell adjacent to a developing xylem element selected as a feeding site (Fig. 1A). Nuclei in affected parenchyma cells became highly irregular in shape (Fig. 1B) and some were undergoing or had already undergone apparently normal division (Fig. 1C, D). In some cells it was possible to observe the alignment of vesicles for cell plate formation (Fig. 1C). However, a complete cell plate was never observed in these cells and a binucleate cell was the result (Fig. 1D). The cytoplasm was dense and endoplasmic reticulum, ribosomes, Golgi vesicles, mitochondria and plastids with starch grains were fairly abundant. Ribosomes occurred either as free particles in the cytoplasm or as polysomes on the surface of endoplasmic reticulum. Numerous vacuoles, possibly indicating a high rate of ribosome synthesis, were also present in the nucleoli (Fig. 1E). Both plastids and mitochondria occasionally assumed irregular configurations and were in division (Fig. 1C, D). Cytokinesis without cell division has also been found to involve cells of the pericycle, six days after nematode infection (Fig. 1E). Giant cells had dense cytoplasm rich in organelles, indicating

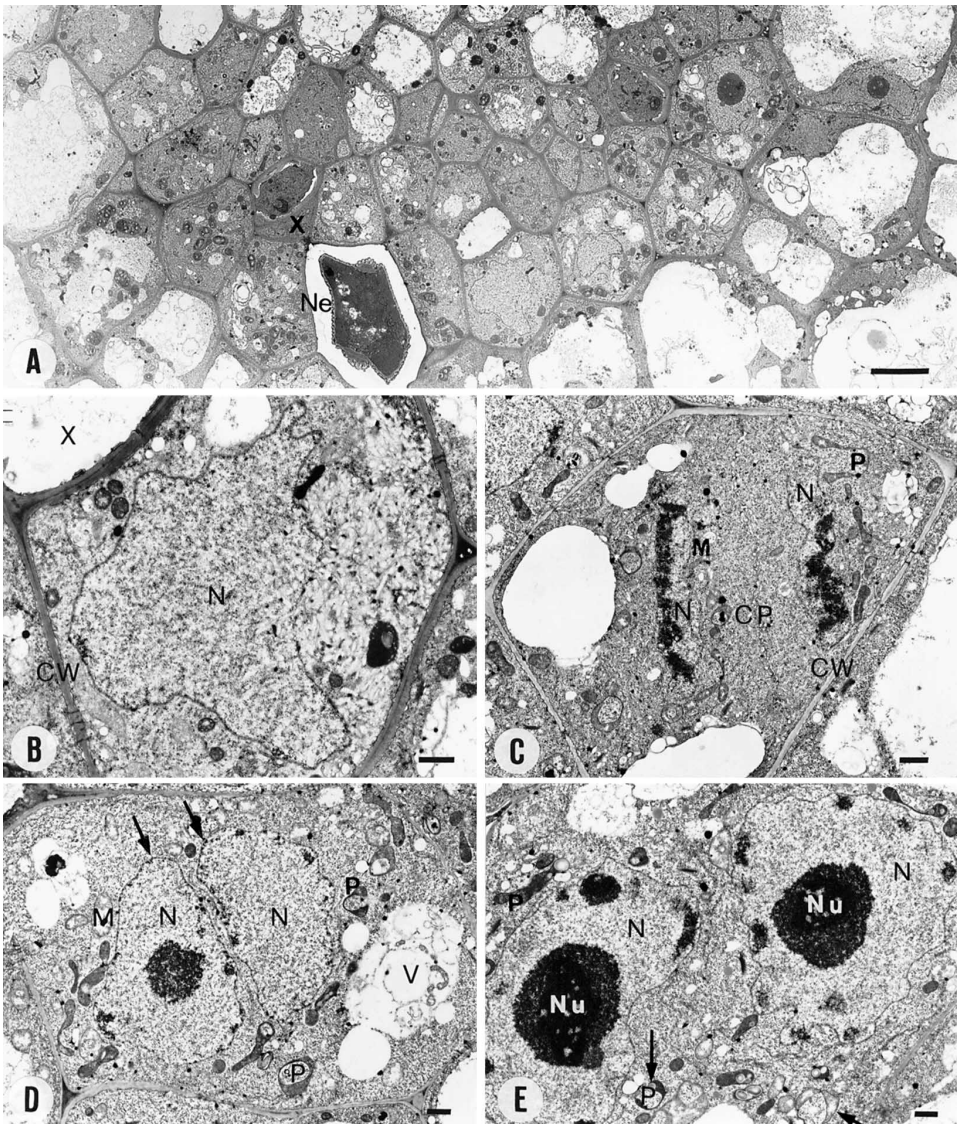

Fig. 1. Micrographs of cross sections of Catuaí amarelo coffee roots infected by *M. exigua*. A) Root tip, showing a juvenile located at a parenchyma cell of differentiating xylem elements, three days after inoculation. Note the typical meristematic appearance of all the cells. B) Irregularly shaped nucleus of a parenchyma cell near nematode's head, three days after inoculation. C) Meristematic cell adjacent to the feeding site, three days after inoculation. Arrangement of vesicles and microtubules on the cell plate appears normal as do the migration and arrangement of chromosomes. Cell plate did not form cell wall. D) Binucleate cell, three days after inoculation. Ribosomes, mitochondria and proplastids in division, and small vacuoles are present in the cytoplasm together with a divided nucleus (arrows). E) Part of a multinucleate pericycle cell, six days after nematode inoculation, showing dense cytoplasm rich in organelles. Some proplastids (arrows) are in division and contain starch granules. Nuclei are fairly irregular in shape and show clumps of electron-dense chromatin along the nuclear membrane, while nucleoli are segregated into granular and fibrillar regions and are vacuolized. CP-cell plate; CW-cell wall; M-mitochondrion; N-nucleus; Ne-nematode; Nu-nucleolus; P-plastid; V-vacuole; X-xylem vessel. Scale bars = 10 μm.

active metabolism, but also showed segregation into fibrillar and granular components in their nucleoli (Fig. 1E).

Coffea arabica *cv. Catuaí amarelo response to* M. megadora *second-stage juveniles*: Sections through meristematic cells, three days after *M. megadora* inoculation, showed that nuclei were usually more regular in shape than those in cells fed on by *M. exigua*. Clumps of electron-dense chromatin were present along the nuclear membrane and nucleoli, with vacuoles, occupied a large part of the nucleus (Fig. 2A). The cytoplasm had the typical density of meristematic cells, with mitochondria, rough endoplasmic reticulum, and ribosomes occurring either as free particles in the cytoplasm or as polysomes. Plastids contained unusually large starch grains (Fig. 2A). Cell walls underwent a thickening process. The initial changes in the primary walls were the roughening of the inner wall surfaces and, in some places, invaginations of the plasma membrane towards the protoplast (Fig. 2B). Many vesicles of different shape and electron density appeared as paramural bodies moving towards the plasma membrane (Fig. 2B). Six days after nematode inoculation, large portions of the cytoplasm in the giant cells appeared to be subjected to lytic processes and unusual membranous structures included cell organelles, but most of the cell was still well preserved (Fig. 2C).

*Catimor (*C. arabica *cv. Caturra × Timor Hybrid) response to second-stage juveniles of two populations of* M. exigua *and to* M. megadora: Sections of infected roots taken three days after inoculation showed necrosis of cells adjacent to parenchyma cells of xylem vessels (Fig. 3A). Most of these cells, probably damaged by mechanical force exerted by nematodes during penetration, had the cytoplasm reduced to a boundary layer of dense material in which some organelles were still detectable (Fig. 3A). Cells of the pericycle had irregular-shaped nuclei and large vacuoles (Fig. 3B). The cytoplasm around the nuclei showed mitochondria with very few and disorganized cristae and plastids containing starch grains and proteinaceous bodies (Fig. 3B). No giant cells were observed in roots infected with either nematode species but some hypertrophy was seen. Death of cells that have been fed upon had already occurred six days after inoculation, as demonstrated by the amorphous remains of the cytoplasm (Fig. 3C). The resistant reaction involved the stelar cells, which passed through stages of membrane degradation (Fig. 3D) and appearance of autophagic vacuoles (Fig. 3E).

DISCUSSION

The morphology of the cells acting as the feeding site or located near the feeding site is different from other cells; differences include the nucleus, the number and shape of the different organelles present in the cytoplasm, and the cell wall. According to Paulson and Webster (1970), lobing of nuclei, proliferation of cytoplasm and organelles, and development of irregularly thickened cell walls, as observed in coffee cv. Catuaí amarelo roots inoculated with all populations, are related to increased metabolic activity. The occurrence of nuclear lobing, and segregation of chromatin and vacuoles in the nucleolus, detected in cells of Catuaí amarelo inoculated with *M. exigua*, has also been related to changes in post-transcriptional processing of ribosomal precursors (Rose *et al.*, 1972). Fernández-Gómez *et al.* (1972) and Kim *et al.* (1978) observed similar reactions in nucleoli of meristematic root tip cells of *Allium cepa* treated with adenosine-3' deoxyriboside and in expanding primary leaves of *Phaseolus vulgaris* inoculated with bean golden mosaic virus (BGMV). These nucleolar changes usually occur in giant cells of susceptible

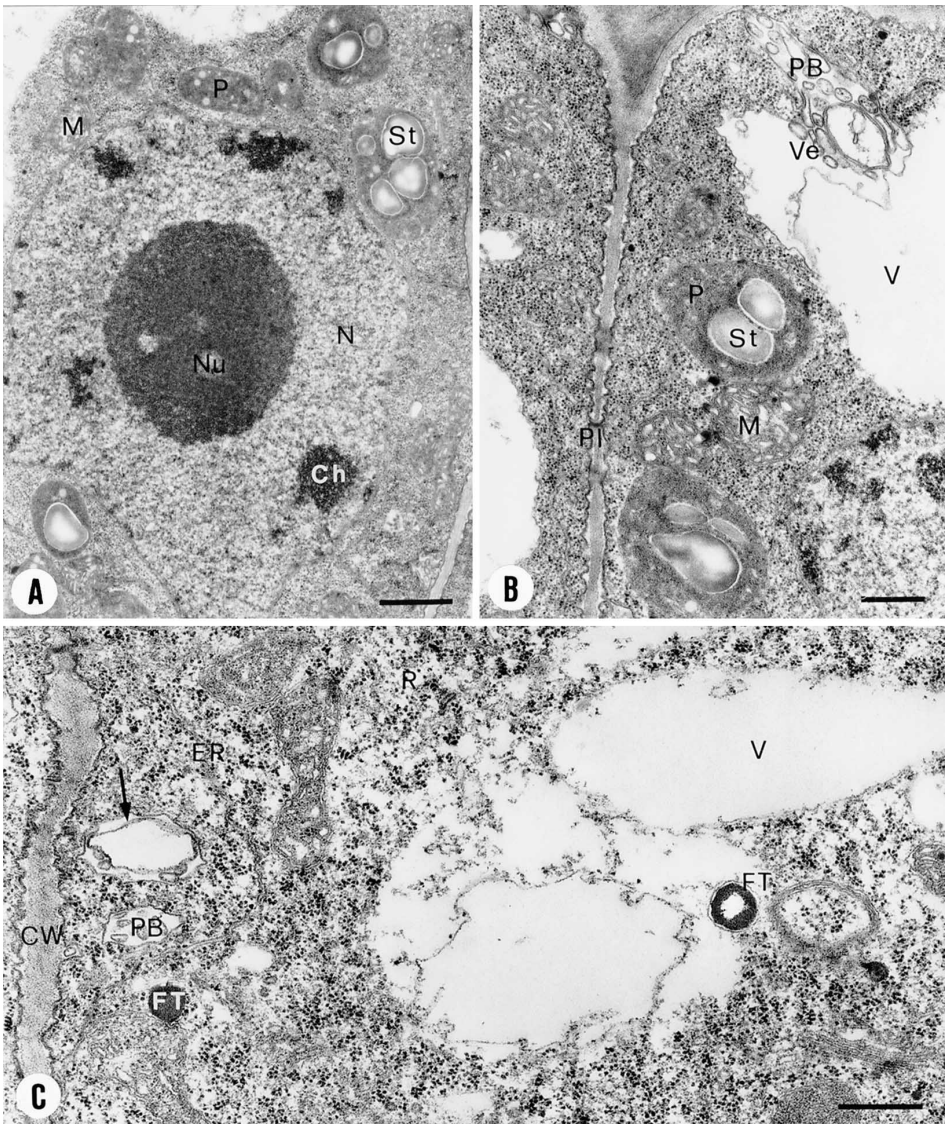

Fig. 2. Cross sections through Catuaí amarelo coffee roots infected by *M. megadora*. A) Part of a cell fed on by *M. megadora*, three days after inoculation. Nucleus with a more regular shape than in cells fed upon by *M. exigua*. Plastids contain large starch grains. Mitochondria, ribosomes and endoplasmic reticulum are fairly abundant. B) Cells of the feeding site, six days after inoculation. Intercellular spaces have already undergone secondary wall apposition. The dense cytoplasm, rich in ribosomes, mitochondria and endoplasmic reticulum indicates an active metabolism. The plasma membrane has a rough outline and paramural bodies with vesicles of different sizes and contents are associated with the plasma membrane and lytic vacuoles. Note plasmodesmata at the wall of adjoining cells. C) Giant cell, six days after nematode inoculation. An electron-dense structure (feeding tube?) lies in a portion of digested cytoplasm where remains of membranes are still detectable. Other parts of cell organelles are sequestered by a row of ring-shaped membranes (arrow), indicating a progressive lytic process. Paramural bodies are scattered along the cell wall. Ch-chromatin; CW-cell wall; ER-endoplasmic reticulum; FT-feeding tube; M-mitochondrion; N-nucleus; Nu-nucleolus; P-plastid; PB-paramural body; Pl-plasmodesma; R-ribosomes; St-starch grain; V-vacuole; Ve-vesicles. Scale bars =10 μm in A, and 5 μm in B and C.

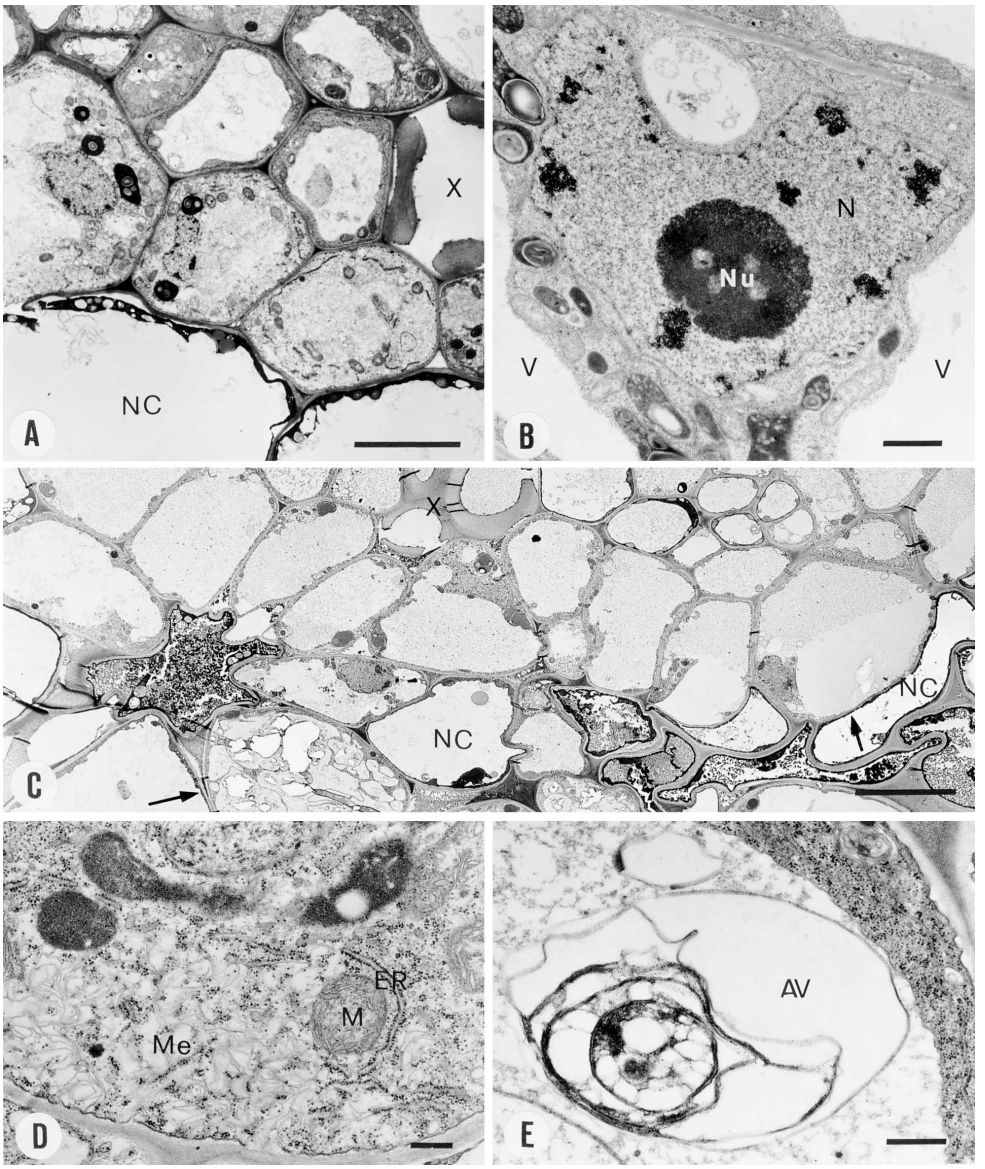

Fig. 3. Cross sections through Catimor coffee roots infected by *Meloidogyne* spp. A) Pericycle cells, three days after *M. exigua* inoculation. Cytoplasm of these cells is reduced to a necrotic boundary layer near the cell wall. B) Parenchyma xylem cell, three days after *M. exigua* inoculation. The nucleus has an ameboid shape and the nucleolus is vacuolized. The cytoplasm, still well preserved, is rich in mitochondria, ribosomes, endoplasmic reticulum, and plastids containing either starch granules or proteinaceous bodies. Large vacuoles occupy most of the cell. C) Root tip infected by *M. megadora*, six days after inoculation. The row of necrotic cells indicates the site of nematode penetration. Some hypertrophic cells (arrows) together with other cells in the stele show completely disorganized cytoplasm. D) Cytoplasmic disorganization of *M. megadora* infected cells appears to involve the degradation of membranes. E) Lytic process of autophagic vacuoles in cells of the stele, located on the opposite site from a *M. megadora* juvenile. AV-autophagic vacuole; ER-endoplasmic reticulum; M-mitochondrion; Me-membranes; N-nucleus; NC-necrotic cells; Nu-nucleolus; V-vacuole; X-xylem vessel. Scale bars = 5 μm in A, D and E, and 10 μm in B and C.

hosts and may be related to overproduction of specific proteins or mRNAs during nematode infection (Fenoll *et al.*, 1997).

Increased synthetic activity of the cytoplasm of Catuaí amarelo cells acting as feeding sites, resulting in numerous ribosomes, rough endoplasmic reticulum, mitochondria and plastids with starch grains is likely to supply the nematode with nutrients. Apparently, *M. megadora* affects some metabolic activities that potentiate cell defenses. The reaction of injured cells is expressed by the presence of vesicular and membranous structures associated with the plasma membrane. Paramural bodies have been reported in syncytia (Rice *et al.*, 1985) and giant cells (Bleve-Zacheo *et al.*, 1982, 1998). It has been suggested that both paramural bodies and multivesicular bodies could be related to cell wall deposition and thickening and may also be involved in the endocytotic pathway including degradative processes (Golinoswki *et al.*, 1996). The presence of autophagic vacuoles associated with paramural bodies could indicate that the cells are destined to die because of an inability to respond appropriately to nematode signals. In the same way, starch storage in the plastids suggests an active response of host cells to signals of invading pathogens and the occurrence of inadequate nutrients for the nematode. The reaction of Catuaí amarelo coffee roots to *M. megadora* could represent an intermediate response between susceptibility and hypersensitivity. The giant cells induced by *M. megadora* in Catuaí amarelo roots were similar to giant cells described as type 2 by Dropkin and Nelson (1960) and by Pedrosa *et al.* (1996) in one susceptible and two resistant soybean genotypes infected with *M. arenaria* race 1. These type 2 cells are usually associated with poorly developed nematodes and have abundant inclusions of various forms.

The early Catimor coffee cell response to all *Meloidogyne* populations, expressed by necrotic cells around and in the feeding site indicates traits of genetic resistance in this plant hybrid. Injured coffee roots apparently react to prevent the cells from acting as a constant food sink and to stop nematode damage and progression in the roots as well as to repair or compensate for the damage. A similar reaction was observed in potato roots of cvs Diamant, Anosta and Morag, resistant to cyst nematode *Globodera rostochiensis* pathotype Ro1, which has been considered as a primary hypersensitive-like response (Bleve-Zacheo *et al.*, 1990). Resistance reactions are frequently accompanied by the hypersensitive death of cells in the immediate vicinity of the nematode, either during migration or after establishment of the feeding site. Therefore, the response of Catimor coffee to *Meloidogyne* populations can be considered as a hypersensitive host reaction. Although we did not establish the exact interval between penetration of the nematode and initiation of the hypersensitive reaction, it seems likely that the process in Catimor roots starts when the nematode reaches its feeding site. Expression of a strong response is first detected within the cytoplasm of the initial feeding site, immediately after nematode invasion, and progresses to the prevention of feeding site formation through the necrosis of surrounding cells. As a result, growth of the plant is not particularly influenced and the efficiency of nematode reproduction is nil (Barker, 1993).

The results of our study suggest that differences in morphological characteristics of feeding sites are related to different degrees of resistance and susceptibility of coffee plants. These morphological differences can be used in breeding programs for selection of coffee plants less susceptible to *Meloidogyne* populations.

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