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Endomigratory feeding behaviour of *Mesocriconema xenoplax* parasitizing walnut (*Juglans regia* L.)

Aurelio CIANCIO* and Gaetano GRASSO**

**Istituto di Nematologia Agraria Applicata ai Vegetali, C.N.R., Via Amendola 165/A, 70126 Bari, Italy, and **Tecnopolis Novus Ortus, Str. Prov. Casamassima km 2, Valenzano, 70010 Bari, Italy.*

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Summary –The parasitic feeding behaviour of *Mesocriconema xenoplax* on roots of walnut (*Juglans regia* L.) was investigated with Scanning Electron Microscopy. Roots of cv. Bleggiana seedlings showed lesions produced by the nematode penetration through the first epidermis layers and the cortex, with associated cell disruption. In the lesions were seen epidermis and cortex fragments that had been removed or detached by nematodes during their migrations between the two root layers. Specimens of *M. xenoplax* were frequently observed deeply inserted inside roots and an extensive area of epidermis erosion was also visible surrounding the lesions. Eggs were observed in the roots, where they were deposited beneath the epidermis. Root penetration by nematodes and their migrations under the epidermis were considered to be the primary cause of root damage. The feeding of *M. xenoplax* was found to be a more complex process than regular ectoparasitism and was characterized as an ecto-endoparasitic behaviour. The implications of this behaviour for nematode dispersal and host selection for resistance are discussed.

Résumé – *Comportement nutritionnel endomigratoire de* Mesocriconema xenoplax *parasitant le noyer* (Juglans regia *L.*) – Le comportement parasitaire et nutritionnel de *Mesocriconema xenoplax* sur les racines du noyer (*Juglans regia* L.) a été étudié en microscopie électronique à balayage. Les racines de jeunes plants de noyer cv. Bleggiana montrent des lésions causées par la pénétration du nématode dans les premières couches de l'épiderme et du cortex avec destruction des cellules associées. Des fragments d'épiderme et de cortex sont soulevés ou détachés par la migration des nématodes entre deux couches du tissu racinaire. Des individus de *M. xenoplax* sont fréquemment observés profondément insérés dans les racines où une zone étendue d'érosion épidermique est également visible autour des lésions. Des œufs ont été observés déposés sous l'épiderme des racines. La pénétration des nématodes et leur migration sous l'épiderme sont considérées comme la cause primaire des dommages racinaires. La prise de nourriture de *M. xenoplax* apparaît plus complexe que celle d'un véritable ectoparasite et plutôt caractérisée par un comportement ecto-endoparasitaire. Les implications de ce comportement sur la dispersion des nématodes par le matériel de propagation et sur la sélection de sources de résistance chez l'hôte sont discutées.

Key-words: endoparasitism, host response, lesion, *Mesocriconema*, ring nematode, walnut.

Mesocriconema xenoplax (Raski) Loof & De Grisse (= *Criconemella xenoplax* [Raski] Luc & Raski) is a major nematode pest that causes significant yield losses to intensive tree cultivation (Lownsbery *et al.*, 1978; Nyczepir *et al.*, 1993). When parasitizing peach trees, *M. xenoplax* predisposes plants to bacterial canker (*Pseudomonas syringae pv. syringae*) and cold injury. Bacterial canker is associated with a deadly syndrome known as "peach tree short-life complex" (Weaver *et al.*, 1974; Nyczepir *et al.*, 1983; Reilly *et al.*, 1986; Nyczepir, 1990) that can dramatically affect production by inducing premature plant death, which constitutes a problem in orchard replanting (Zehr *et al.*, 1982). *Mesocriconema xenoplax* is a polyphagous species that has been reported parasitizing several tree crops and non-woody plants worldwide (Seshadri, 1964; Bird & Ramsdell, 1985; Zehr *et al.*, 1986, 1990).

The feeding behaviour of *M. xenoplax* is generally described as ectoparasitic, although different authors observed and reported specimens embedded in root lesions on susceptible host plants (Sher, 1959; Westcott & Hussey, 1992). Long term *in vitro* studies, however, showed that this nematode can feed continuously as an ectoparasite during lengthy periods on single nourishing cells. Furthermore, *M. xenoplax* was also observed in association with root lesions that were considered to occur *in vitro* as the result of natural events (Westcott & Hussey, 1992).

Mesocriconema xenoplax was frequently found in the rhizosphere of walnut (*Juglans regia* L.) in Italy (Ciancio *et al.*, 1996). The damage caused by this nematode on walnuts (*Juglans* spp.) is extremely severe. Artificially infested walnut seedlings showed reduced development and growth, with extensive root necrotic areas and phloem lesions (Lownsbery *et al.*, 1978). Considering the economic impact and the worldwide distribution of this nematode pest, it was considered worthwhile to clarify the mechanisms of plant damage. For this purpose, we investigated the feeding behaviour of *M. xenoplax* on walnut roots at the ultrastructural level, and the results are herein presented.

Materials and methods

Seedlings of walnut (*Juglans regia* L.) cv. Bleggiana were obtained from a national grower and found by light microscopy and soil examination to be naturally infested by a population of *M. xenoplax*. Five plants were maintained outdoor in 20 cm diameter plastic pots with an organic sandy soil mixture. Nematode densities were assessed during 1 year at 6 months intervals, using the sieving and decanting technique with 710 μm and 45 μm sieves. Plants heights and trunk diameters were measured 1 year after transplantation. For examinations with Scanning Electron Microscopy (SEM), root fragments 0.5-2 cm long were fixed in red lactophenol under low magnification light microscopy and subsequently dehydrated in a 2.5 % formalin-5 % glycerol solution by the slow method at room temperature. Six fragments were subsequently observed under low magnification light microscopy and mounted on a metal stub for SEM. Excess glycerol was then removed with a tissue paper tip and the roots were gold covered in vacuum for examination with a Stereoscan 360 Cambridge SEM at 5 kV.

For sectioning parasitized roots, fragments were fixed in FAA, dehydrated through an ethanol-TBA series and subsequently infiltrated with paraffin. Sections were stained with safranin and fast green, then observed with a Leitz Orthoplan light microscope at 125 × (Southey, 1970).

Results

The ultrastructural studies were made on a highly susceptible seedling of *J. regia* cv. Bleggiana, which consistently supported *M. xenoplax* population densities higher than those on the other seedlings. Population density of 27-30 *M. xenoplax* per cm^3 of soil were observed, which was 5.4-11 times higher than the mean population densities of the other seedlings. When extracting roots for SEM examinations, more than 1800 *M. xenoplax* per g of roots were recovered from this seedling in the sieved suspension, after gentle root washing. The nematode density measured in December for all seedlings was $9.5 \pm 10 \times 10^3$ nematodes per dm^3 of soil. The highly susceptible seedling also had fewer lateral shoots, but no significant difference in plant height (86 ± 6.9 cm) and trunk basal diameter (3.1 ± 0.2 cm) was observed among seedlings.

Fragments of secondary roots observed with SEM showed that *M. xenoplax* feeding was highly destructive and caused severe damage in epidermis and cortex root layers (Fig. 1). The nematodes were capable of penetrating into the most external root tissues, and lesions 0.2-0.3 mm long were also observed associated with *M. xenoplax* specimens embedded into the root (Fig. 1 A-F). These lesions appeared as holes or pits in the epidermis (Fig. 1 C, E, F), or as more elongated and deeper grooves and incisures (Fig. 1A, B). Inside the lesions, the nematodes were observed firmly inserted and oriented toward the central root layers (Fig. 1 D; 2 A,B), or oriented longitudinally in a direction parallel to the root axis (Fig. 1 C, E, F). In some cases, the lesions were smaller than the nematode body diameter and may have been produced by specimens of *M. xenoplax* migrating into the cortex and beneath the epidermis. Some ruptures were obviously produced by the pressure of the nematode body on epidermal cells. In these areas, the outermost cells were raised and parts of the nematode body were visible through the corresponding openings (Fig. 1 C, E).

In the areas surrounding the lesions, fragments of epidermis were frequently removed by the nematodes (Fig. 1 A,B,F), or detached because of their movements and/or repeated entry (Fig. 1 D). Some specimens of *M. xenoplax* were caught emerging from the cortex cells (Fig. 2 A,B), or penetrating into this tissue, or partially emerging from the cells, nested inside the lesion and covered by a raised epidermis fragment (Fig. 1 F).

When the epidermis was partially or totally separated from the roots, imbedded specimens of *M. xenoplax* were observed among cortical cells (Fig. 2 A, B). Occasionally, eggs were found in clusters adhering to the cortex close to the lesions (Fig. 2 E).

Areas of extensive root damage and epidermal erosion were also visible with low magnification light microscopy, often associated with *M. xenoplax* specimens inserted into the roots (Fig. 2 C). In transverse sections, the root cavities were seen to be empty holes extending to the central vascular system and cambial zone (Fig. 2 D). Epidermis layers not directly affected by the destructive feeding of nematodes were indirectly affected by the reduction of the functions of the surrounding root tissues and by the associated cortex disruption.

Discussion

These ultrastructural observations confirmed that damage induced by *M. xenoplax* on walnut is characterized by a severe destruction of the epidermis and cortex layers. This agrees with previous observations on the dramatic effects of this nematode on walnut and other plants (Lownsbery *et al.*, 1978). *In vitro* tests on clover and carnation have demonstrated the relation that exists between the damage caused by *M. xenoplax* and the disorganization of the transport pathway between specialized feeding cells and adja-

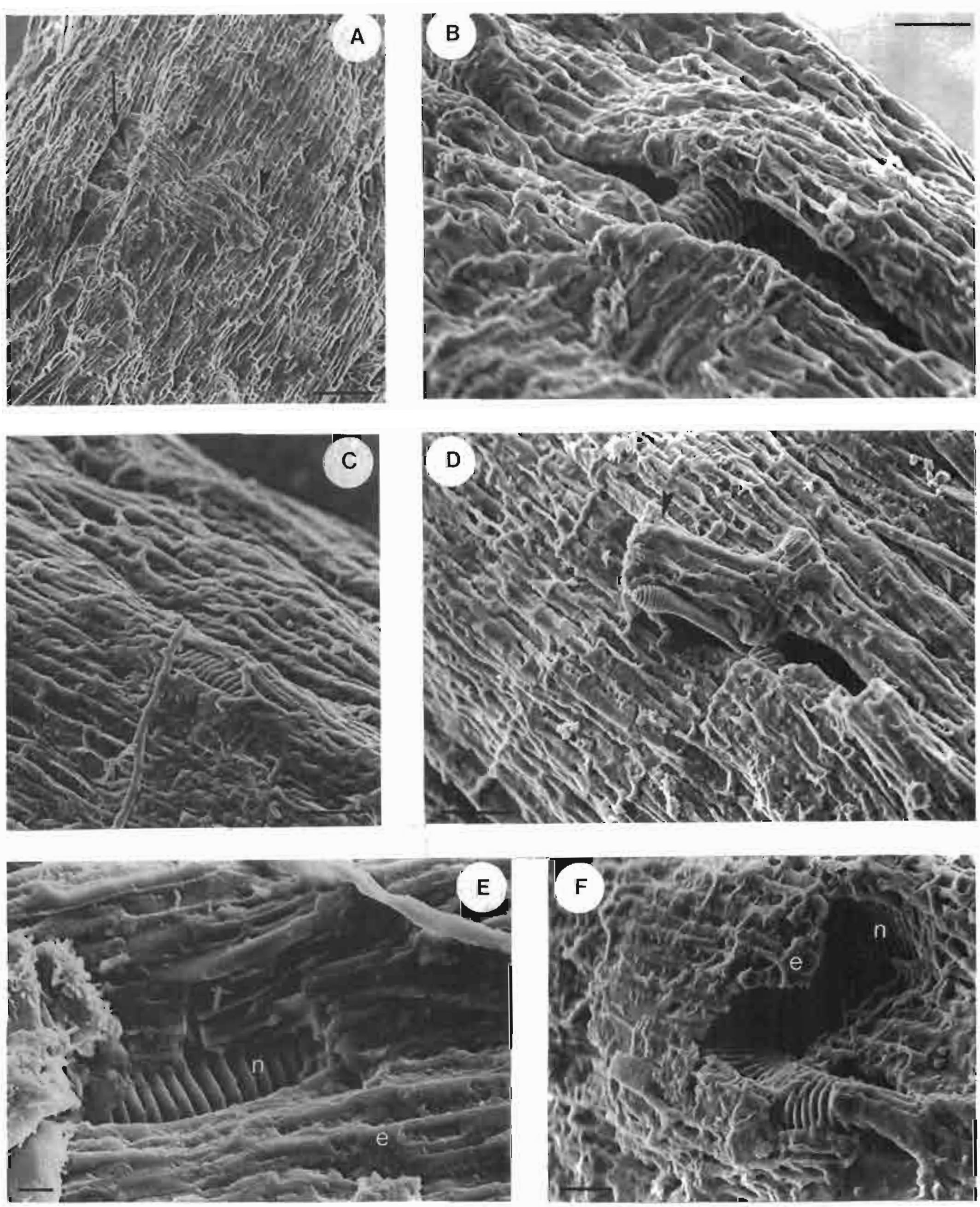

Fig. 1. *Scanning electron microscopy photographs of roots of walnut cv. Bleggiana showing lesions associated with parasitism by* Mesocriconema xenoplax. *A: Lesion (arrow) in the epidermal root layer with associated nematode lying underneath and adjacent detached epidermis fragment (arrow heads); B: Higher magnification SEM photograph of the same lesion showing the nematode migrating beneath the epidermis and lifting the outermost cell layer; C: 20 μm wide lesion (arrow head) induced on the root surface by the pressure exercised by the nematode visible through the hole and migrating beneath the first epidermis cell layer; D: Lesion produced by* M. xenoplax *embedded in the root and by its subsequent local migration, which resulted in the formation of a microcavity and in the raising of an adjacent epidermis fragment (arrow head); E: 15-17 μm wide lesion produced by a female of* M. xenoplax *(n) that penetrated into the root and migrated beneath the epidermal root layer (e); F: Specimen of* M. xenoplax *(n) emerging from the root cortex and nested between disrupted cortex and partially removed epidermis (e) cells. (Scale bars: A = 100 μm; B-D = 50 μm; E = 10 μm; F = 25 μm).*

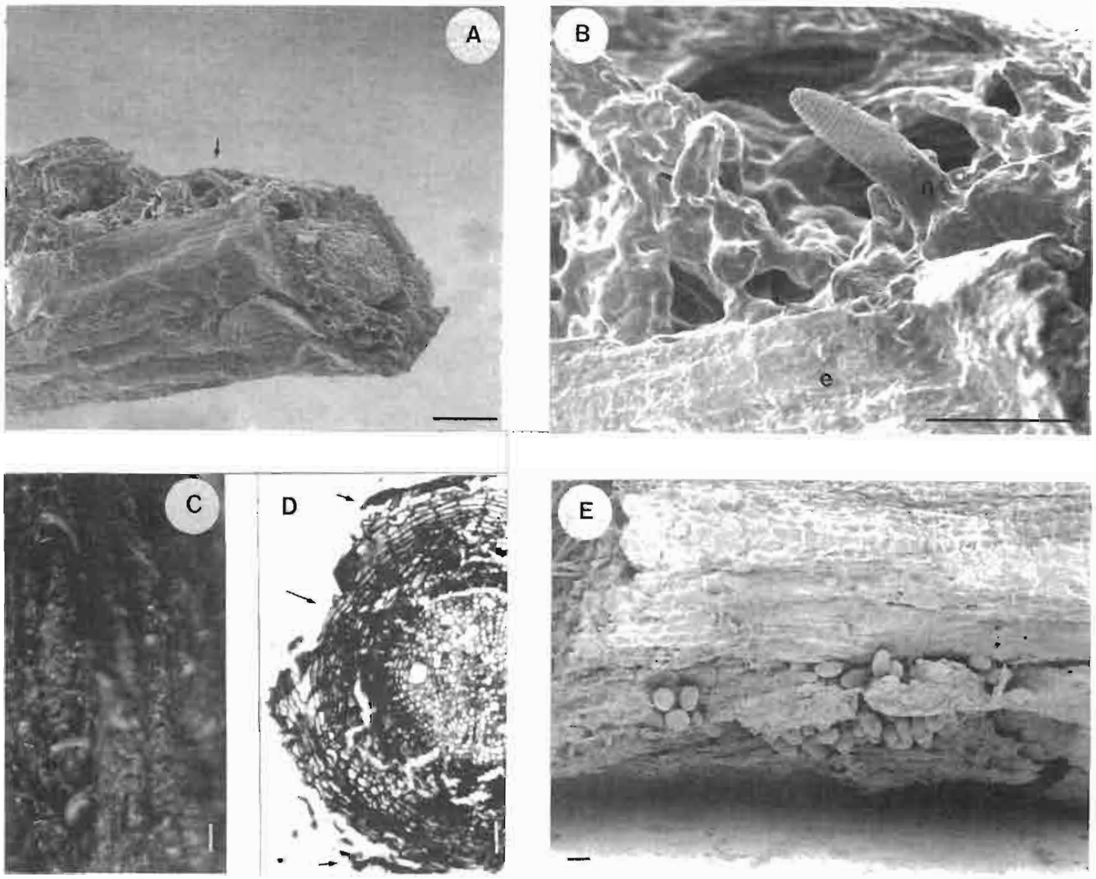

Fig. 2. Scanning electron and light microscopy photographs of roots of walnut cv. Bleggiana parasitized by Mesocriconema xenoplax. *A: Juvenile of* M. xenoplax *(arrow) partially embedded into the cortex after the root epidermis was peeled off; B: Side view of the same specimen as in A, showing some fragments of epidermis layer (e), the nematode (n), and the associated cortex cell disorganization; C: Light microscopy photograph of a walnut root lesion produced by* M. xenoplax, *showing nematodes nested and inserted into the cortex (arrows); D: Transverse root section showing the cortex lesion extending to the cambial zone (arrow) and fragments of epidermis (smaller arrows); E: Clusters of* M. xenoplax *eggs deposited onto and adhering to lesionated cortex areas beneath the epidermal layers and made visible by manual removal of the epidermis. (Scale bars: A, C, D = 100 μm; B = 50 μm; E = 20 μm).*

cent tissues (Hussey *et al.*, 1992 *a*). The cortex was the primary parasitised tissue and the subepidermal cells penetrated by the nematode stylet showed a high content of callose, which was considered to be a wound cell response (Hussey *et al.*, 1992 *b*). In walnut also, the cortex was the preferential feeding tissue. However, the epidermal cell layer was mechanically destroyed and removed by the nematode penetration, probably because it was more robust and unsuitable for feeding. In the root regions affected by nematode attacks, the damages and the reduction of the functions seemed to be a direct consequence of cell destruction and parasite migration within the surrounding tissues rather than a consequence of stylet penetration and/or cell content consumption.

The feeding behaviour of *M. xenoplax* was different from the normal ectoparasitic behaviour reported and described for tylenchid and dorylaim nematodes and characterized by a labial contact with the external root surface followed by stylet penetration of cells (Wyss, 1981). Root invasion by *M. xenoplax* was previously observed and reported (Sher, 1959; Doncaster, 1981; Westcott & Hussey, 1992), as were other related pre-feeding actions, *i.e.*, surface root rubbing and head bumping (Westcott & Hussey, 1992). In some cases, nematode penetration was interpreted as an opportunistic feeding inside "natural cracks" occurring on roots growing *in vitro* (Westcott & Hussey, 1992). However, these authors observed and reported a continuous ectoparasitic feeding that can be part of a more complex and articulated parasitic behaviour. Our SEM observations confirmed the pits and root lesions already reported and showed the preference of *M. xenoplax* for the inner cortex cells. The photographs showed lesions that were smaller (< 50 %) than the average body diameter of this species (Orton Williams, 1972), which indicates there was an alternative entrance site for the specimens seen through the lesions. These wounds were produced by the lifting of the outermost cells, which proves that the parasite developed and migrated between the epidermal and cortex layers.

Endoparasitic feeding by *M. xenoplax* appears to be integrated with ectoparasitism and probably depends on the structure and strength of the host roots. Extensive necrotic root lesions were reported on walnut plants artificially inoculated with increasing *M. xenoplax* numbers, but not on control plants without nematodes (Lownsbery *et al.*, 1978). Although the assessment of a more stringent cause-effect relationship between *M. xenoplax* and root lesions would require a different inoculation protocol, the hypothesis of an opportunistic feeding occurring within natural cracks produced by other unknown factors, even *in vitro*, appears unsupported. Considering the damages herein observed and the evidence for endoparasitism, a complex non-sedentary ecto-endoparasitic behaviour, varying according to host type and root structure, would seem to be a more plausible behaviour for this species. Similar behaviour patterns were reported for some *Merlinius* spp. that fed either as true ectoparasites or as semi-endoparasites with their heads embedded within the more external root tissues (Bridge & Hague, 1974), and for other tylenchid species, including *M. xenoplax* and *Criconemoides* spp., that were partially embedded in cortical root cells during feeding (Doncaster, 1981; Wyss, 1981).

The damage in walnut roots was heightened by the local migration of nematodes through the various cell layers and beneath the epidermis, which produced the pits and the lesions observed. Migration seemed to be related to cell death and to the heavy mechanical damage caused by the nematodes rather than to plant response, *i.e.*, the occurrence of a local necrotic reaction. The possibility of a similar reaction occurring at the cell level can be excluded, since any initial nematode feeding would be impossible. The exhaustion of nourishing cells and tissues affected by feeding is most probably the factor that triggers a local endomigratory behaviour toward more favourable areas, which causes additional root lesions and damage. This type of destructive migratory behaviour appears to be necessary for allowing the nematode to feed on other intact and available root areas after the death of parasitized cells. In this regard, the indented thick cuticle of these nematodes is well adapted to direct penetration and rupture of the root surface. SEM photographs illustrate that the typical criconematid organization of the cuticle of *M. xenoplax* and its strength are capable of sustaining the body pressure required for intrusion (Doncaster, 1981), and help the succession of compression and elongation of the body during penetration by providing anchorage within the cell walls during disruption (Fig. 1 C, D; 2 A).

The presence of *M. xenoplax* inside host roots has some practical implications since it may favour the persistence of the parasite in the field and its dispersal through infested propagation material. The presence of nematodes and eggs beneath the epidermis represents a risk for growers and producers, and it may justify more stringent quarantine procedures and/or plantation and seedling control. At present, commercial walnut seedlings are sold free from soil. This procedure was considered sufficient to avoid any nematode dispersal and field introduction at planting.

In spite of the different nematode density levels observed in infested seedlings, there were little differences in total height and/or other growth parameters of plants. A response similar to that reported on other tree plants exposed to increasing densities of other nematode pests in pots (Di Vito *et al.*, 1988; Vovlas & Di Vito, 1991) was not observed. The low impact of the root damage on the walnut growth parameters can be related to the plants capacity of balancing the nematode attacks through an increased production of adventitious and secondary rootlets and/or the absence of a linear relationship between the damage induced by *M. xenoplax* and the growth rate of the plant.

Finally, the differences in *M. xenoplax* reproductive rates observed among infested seedlings may result from a natural host variability regarding nematode susceptibility. Several secondary metabolites (phenolic compounds, non-structural carbohydrates, serotonine, tannins) are known to occur in walnut during rhizogenesis (Claudot *et al.*, 1993; Jay-Allemand *et al.*, 1993; Charpentier *et al.*, 1994). These compounds may be involved in a wound response mechanism similar to that observed in roots of peach as a reaction to parasitism (Olien *et al.*, 1995). This provides a possible approach for the search for suitable sources of resistance and/or other defence mechanisms in walnut.

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