# The Histopathology of Alfalfa Roots Infected by *Hoplolaimus galeatus*

Oi-Cheng Ng and T. A. Chen

Former graduate student and professor, Department of Plant Pathology, Rutgers University, New Brunswick, NJ 08903.
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## ABSTRACT

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Pathological changes in axenically cultured alfalfa roots infected by a single *Hoplolaimus galeatus* were studied by light and electron microscopy. Five days after inoculation, cell damage extended far beyond the stylet; damaged cells could be traced up to 105 $\mu$m away from the sites of nematode penetration in a longitudinal direction. Damaged cells within the lesion stained more intensely than healthy cells or cells from noninfected control tissues. Mechanical damage to the cortex was caused by formation of passageway and feeding cavities by the nematode. Although cells with dense tonoplast or darkened granular contents were also observed, these changes were probably not due to mechanical damage. Hypertrophy and early division of host cells in the pericycle were observed shortly after nematode feeding began. As the pericycle cells increased in size and number, the endodermis flattened and collapsed. Electron-dense material accumulated along the tonoplast in the cells in the endodermis and pericycle of infected roots. Vascular damage included feeding cavities, lysed phloem tissue, and the association of electron-dense material with the xylem elements. Thus, both mechanical and chemical injuries are involved in the pathogenesis of *Hoplolaimus galeatus*.

The lance nematode *Hoplolaimus galeatus* (Cobb) Thorne, (syn. *H. tylenchiformis* and *H. coronatus* Sher) is a root-feeding, ecto-endoparasitic nematode. In the United States, *H. galeatus* is widely distributed on a variety of hosts, especially woody and graminaceous plants (14). Many studies associate a reduction in yield with infection by *H. galeatus* under field and greenhouse conditions (2,4,6–8,10,14,19,21,24,25). In New Jersey, high population densities of *H. galeatus* are frequently found in the soil around diseased and poorly growing plants, particularly on turf (22), but the nature of this association has not been examined and only limited studies on its pathology have been reported (10,18,20). In this study, we investigated the effect of *H. galeatus* on alfalfa in aseptic culture to observe histopathological changes occurring at the nematode feeding site and to study sequential microscopic and ultrastructural changes in infected cells and tissues during various stages of nematode infection.

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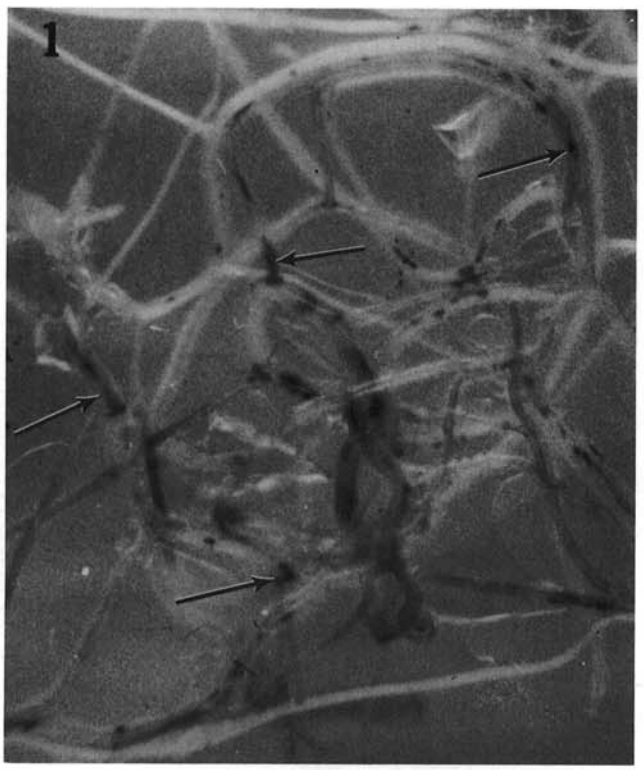
## MATERIALS AND METHODS

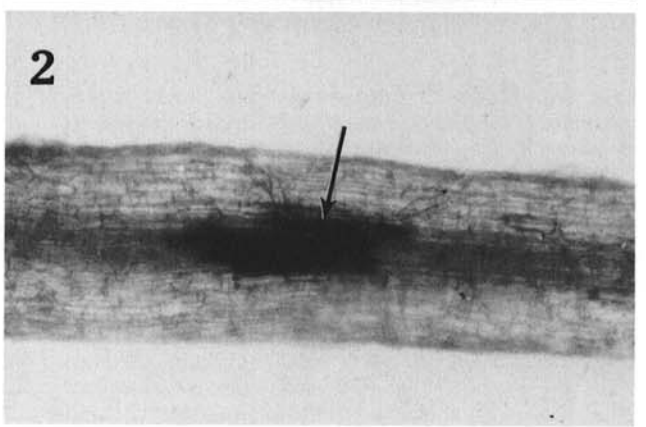
The *H. galeatus* used in our studies was collected from a turf plot at Rutgers University and maintained on alfalfa (*Medicago sativa* L.) in a greenhouse. The nematodes were extracted from the soil by a centrifugation-flotation technique (9) and surface-sterilized for 12 hr with a 100 mg/L methoxyethyl mercuric chloride (Aretan) solution containing 10 $\mu$m of fungizone, 100 units of penicillin, and 100 units of streptomycin per milliliter. They were rinsed with sterilized water three times before inoculation.

Alfalfa seeds (cultivar Dupuits No. 2) were washed for 30 min in running water then immersed for 30 sec in 75% alcohol. For surface-sterilization, the seeds were soaked for 30 min in 0.1% HgCL and washed three times with sterile distilled water. The treated seeds were then transferred to a petri dish of sterile water agar for germination. When the alfalfa seedlings were approximately 1.5 cm long, roots of seedlings were excised and transferred to 50 × 9-mm Seal-Tight petri dishes (Falcon, Oxnard, CA) containing Chen's medium (3). The dishes were incubated in a growth chamber (25 C) and the excised roots were allowed to grow for 3 wk before inoculation. About 15 nematodes were pipetted into each petri dish containing a 3-wk-old excised alfalfa root

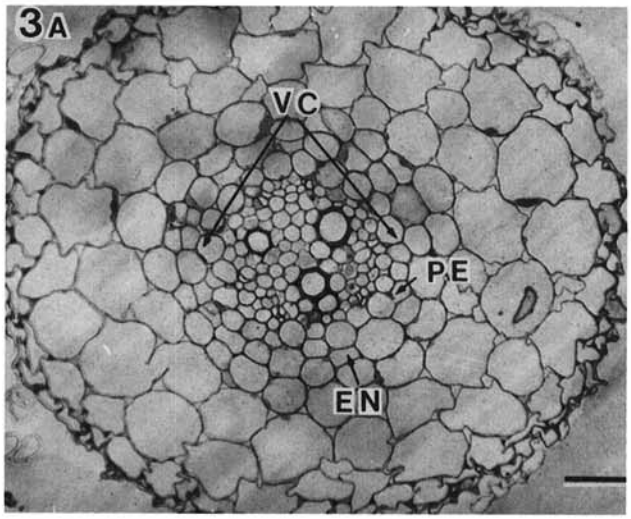
culture. Other excised alfalfa roots, also in petri dishes incubated at 25 C, were repeatedly pierced with a fine needle to simulate mechanical damage by the nematode. The cultures were observed each day under a dissecting microscope. Particular attention was paid to those root sites where only one nematode was feeding and to the mechanically probed lesions. Other controls included excised root cultures incubated at 25 C but not inoculated with nematodes or pierced with the needle.

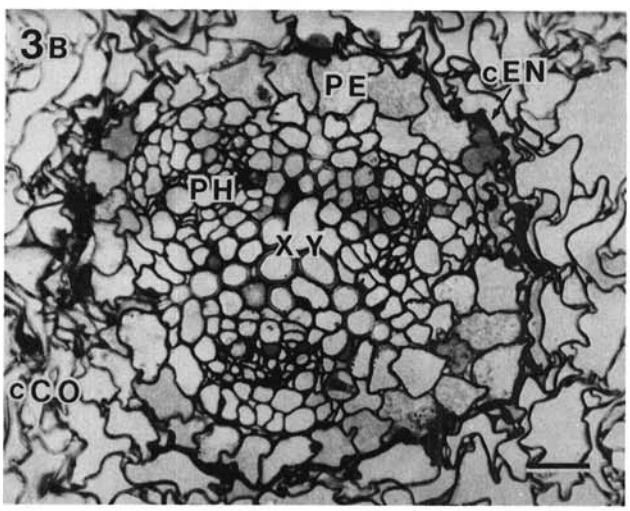
Root pieces containing the feeding sites with a single nematode still attached were excised and fixed immediately for 1 hr in 2.5% glutaraldehyde (in 0.1 M phosphate buffer solution), postfixed for 1 hr in 1% osmium tetroxide, and dehydrated in an alcohol series (50–100%). The specimens were then transferred to propylene oxide and embedded in low-viscosity Spurr's medium (23). Thin sections (60–90 nm thick) were cut with a diamond knife and mounted on a 0.5% formvar-coated slot grid (26). The sections were stained with 2% uranyl acetate and lead citrate and examined with a Seimen's 1A electron microscope operated at 80 kV. For the light-microscopic study, 0.5 $\mu$m-thick sections were cut and mounted on a glass slide. The sections were stained with toluidine blue and basic fuchsin (paragon epoxy tissue stain) on a hot plate for 1 min. To observe the histopathological changes occurring in infected roots at




**Figs. 1–2.** *Hoplolaimus galeatus* in excised alfalfa roots. Mass infection in a monoxenic culture. The nematodes fed on both young and old roots, causing dark necrotic lesions (arrows) at the feeding sites. Noninfected roots remain white. ×2. **2,** Enlargement of necrotic lesion (arrow) induced by a single *Hoplolaimus galeatus* on an excised alfalfa root. The brownish discoloration was not confined to the point of penetration, but extended along the vascular bundles. ×12.



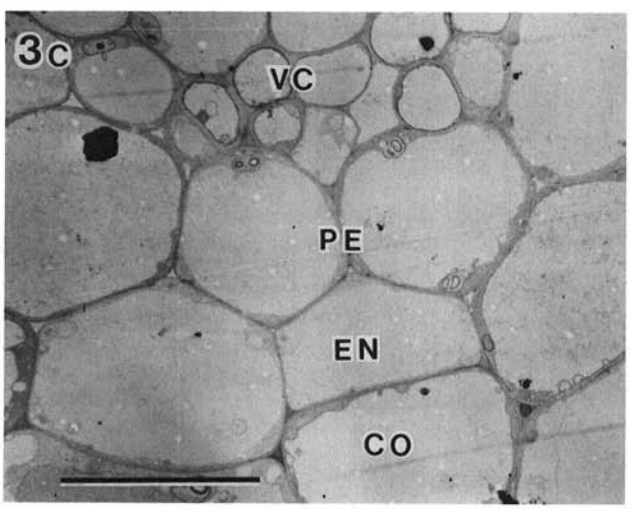




**Fig. 3.** Cross sections (0.5 $\mu$m thick) of normal uninoculated alfalfa roots showing primary and secondary growth. **A,** Young root. **B,** Secondary growth of the vascular cylinder that crushed the endodermis and cortex. The pericycle at this stage of differentiation was a single-layered tissue. **C,** Electron microscopic view of part of the vascular cylinder and cortex of a young alfalfa root. Bar = 20 $\mu$m. Symbol legend: CO, cortex; cCO, crushed cortex; EN, endodermis; cEN, crushed endodermis; PE, uniseriate pericycle; PH, phloem; VC, vascular cylinder; and XY, xylem.

the nematode feeding site, thicker sequential cross sections were made from the necrotic lesions caused by the nematode 5 days after inoculation. The thick sections (0.3–0.5 $\mu$m) were cut at 15, 30, 45, 75, 90, and 105 $\mu$m from the penetration point and were studied by using light microscopy. The lesions induced by the nematode 1, 2, 5, and 8 days after inoculation were used to study sequential micro- and ultra-structural changes in infected cells and tissue during various stages of nematode infection. Thick and thin sections were

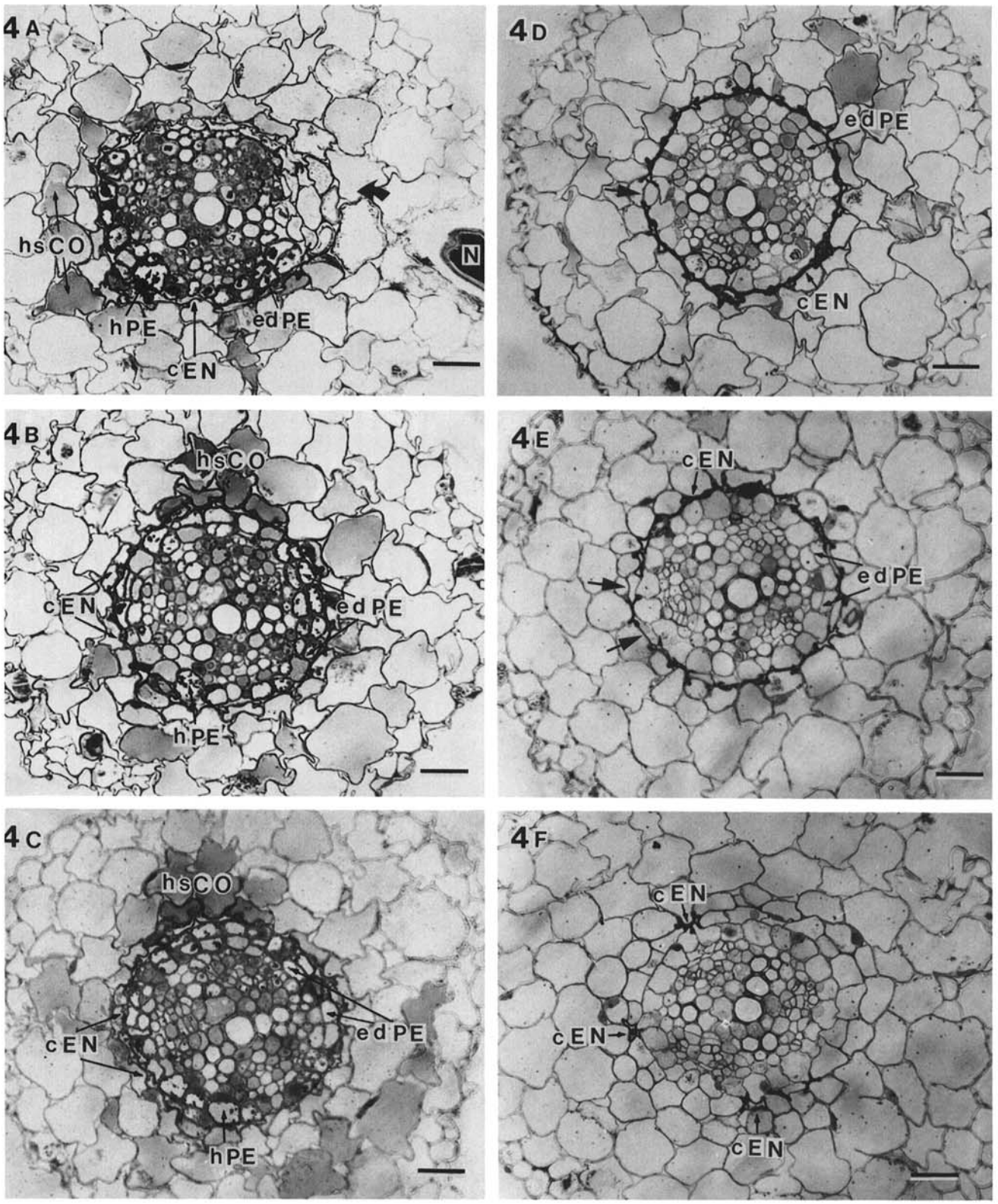



**Fig. 4.** Light microscopic view of sequential cross sections through a necrotic lesion in an alfalfa root 5 days after it was inoculated with a single *Hoplolaimus galeatus*. Elements **A to F** represent, respectively, sites 15, 30, 45, 75, 90, and 105 $\mu$m from the penetrating point of the nematode. A. Part of the nematode in the cortex and collapsed cells around the feeding area (curved arrow). **A to C,** Heavily stained vascular cylinder and several cortical cells showing early divided and hypertrophied pericycles and crushed endodermis. **D and E,** Although the majority of the endodermal cells were crushed, a few cells appear to be normal (large arrow). **F,** A few crushed endodermal cells indicate the only evidence of damages. Bar = 20 $\mu$m. Symbol legend: cEN, crushed endodermis; edPE, early divided pericycle; hPE, hypertrophied pericycle; hsCO, heavily stained cortex; and N, nematode.

made from each lesion at the penetration point of the nematode. The wide-field electron microscope technique (26) was used in this study to compare the histopathological reactions in infected alfalfa roots under low-magnification electron microscopy to those observed by light microscopy.

## RESULTS

Under monoxenic conditions, *H. galeatus* penetrated the excised roots at various locations without any evidence for preferred sites. The nematode usually penetrated the root perpendicular to the long axis of the stele. *H. galeatus* fed as a semi-endoparasite with the anterior end of the body embedded in the root and seldomly changed its position. Brownish lesions developed at the point of entry within 24 hr. The lesions gradually extended along the vascular bundle, eventually extending several millimeters away from the feeding site (Figs. 1 and 2). In contrast, the mechanically probed control lesions on alfalfa root were confined to the point of penetration of the needle. Discoloration, if it occurred, was light yellow and never extended very far from the injury site. In the cross sections of such roots, the mechanically damaged cells could be easily detected, but the surrounding tissues appeared to be normal as described below on the uninoculated controls. The healthy root in culture remained white.

**Cross section of alfalfa root in uninoculated controls.** Alfalfa is an herbaceous dicotyledon plant with secondary growth. Two figures illustrate various stages of development of noninoculated alfalfa roots (Fig. 3). Fig. 3A illustrates a young root at the level where the first xylem elements matured. Fig. 3B shows older roots in which all the vascular elements were differentiated. The crushed endodermis and cortex in the older root sections was apparently caused by the expansion and differentiation of the xylem and phloem elements. At this stage, the periclinal divisions of the pericycle involved in secondary root growth were not observed.

**Histopathological observations.** The results of light microscopic studies of serial sections of the same lesion showed that damage occurred distant from the feeding site. Five days after inoculation, damage to the root tissues extended 105 $\mu$m from the penetrating point of the nematode (Fig. 4). Damaged cells within the vascular cylinder and cortex stained more intensely than the unaffected cells or cells from noninfected control roots. Vascular elements in the section 105 $\mu$m from the feeding site were extensively damaged (Fig. 4A). Accumulation of dense materials in the vascular elements was probably responsible for the dark staining. These dense materials also could be seen in sections 30 and 45 $\mu$m from the nematode (Fig. 4B and C). The vascular elements and the cortical cells appeared healthy in the section 75 $\mu$m from the feeding site (Fig. 4D). In all sections through the lesion, damage in the pericycle and endodermis was the most prominent feature. Hypertrophied and divided cells of the pericycle were common even in young roots. At some point, the pericycle developed into a double layer as the result of early division. This enlargement of pericycle resulted in the crushing of endodermal cells. Crushed endodermis appeared as a dark ring surrounding the vascular cyclinder (Fig. 4D). In the section 90 $\mu$m from the feeding site, most of the endodermal cells were crushed ( Fig. 4E). In the section 105 $\mu$m from the nematode, most of the cells in the pericycle were normal. Few of them, however, appeared to have commenced enlarging. When the enlargement occurred, the endodermal cells just outside of these cells were crushed (Fig. 4F). Therefore, the crushed endodermal cells were the only evidence of nematode infection found at the farthest end of the lesion.

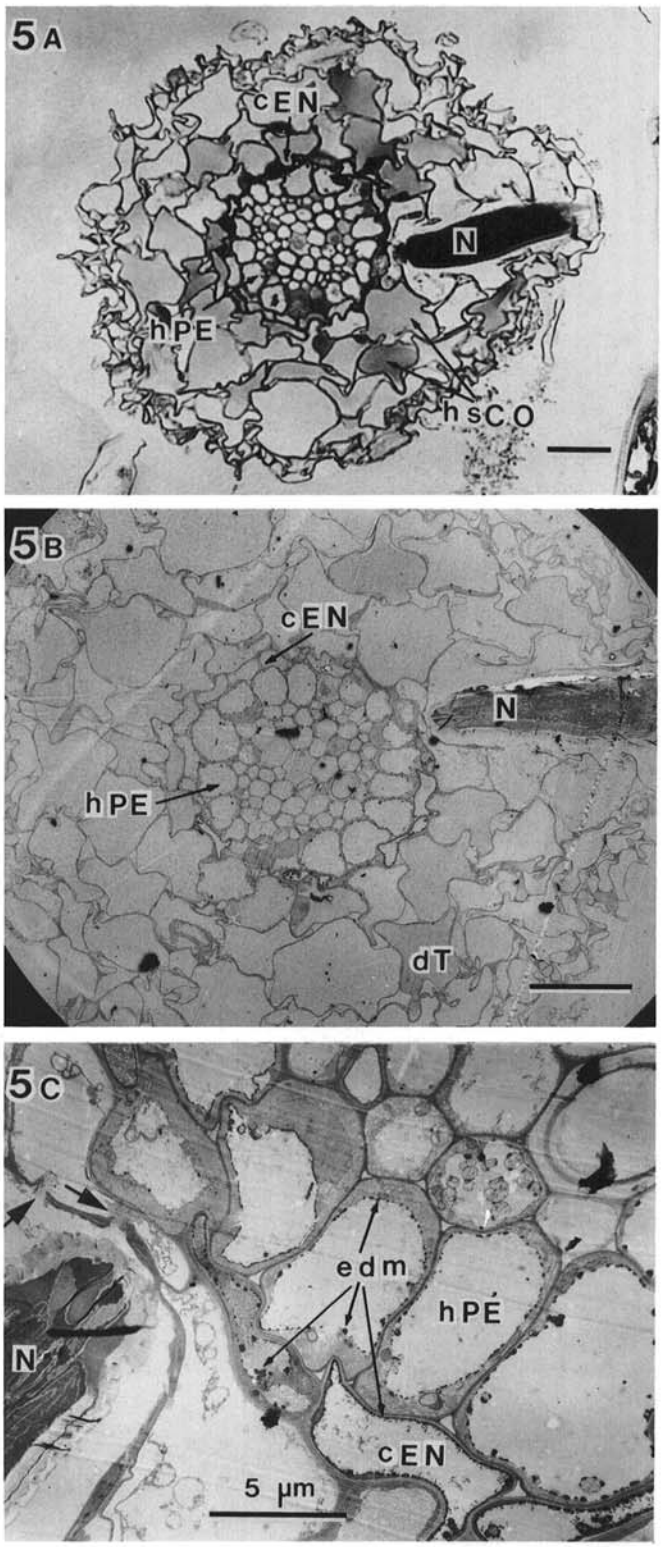



**Fig. 5.** Cross sections through a necrotic lesion in an alfalfa root, 1 day after inoculation with a single *Hoplolaimus galeatus*, showing the nematode feeding on the inner cortical cells. **A,** Light microscopic view (5-$\mu$m thick) showing heavily stained cortical cells, crushed endodermis, and hypertrophied cells in the pericycle. **B,** Wide-field electron microscopic view showing the nematode in the cortex tissue. Cells around the nematode apparently had been fed upon because the walls were broken and the cytoplasms were absent. Cortical cells with dense tonoplasts, crushed endodermis, and hypertrophied pericycles can be seen not only near the nematode but some distance away from the feeding site. **C,** Close-up of feeding site showing punctured cell walls (large arrows), enlarged pericycle cells, and crushed endodermal cells. Electron-dense materials can be seen accumulated along the tonoplast of the hypertrophied pericycle and the deformed endodermis. Bar = 20 $\mu$m. Symbol legend: cEN, crushed endodermis; dT, dense tonoplast; edm, electron-dense materials; hsCO, heavily stained cortical cells; hPE, hypertrophied pericycle; and N, nematode.

Light and electron microscopic studies were made of changes occurring 1, 2, 5, and 8 days after inoculation. For the purpose of a direct comparative study between light- and wide-field electron microscopy, the same root, lesion, and feeding site were used and are shown in Figs. 5–8. In infected roots, after probing on the surface of a given cell, the nematode penetrated the same cell and began to feed in sequence on adjacent cells. The progressive penetration by the nematode induced the collapse of cell walls and the leakage of cell contents along the penetrating stylet and body wall (Figs. 5–8). Nematodes fed on the innermost cortical cells 1 and 2 days after inoculation (Figs. 5 and 6). After 5 days, the nematode fed on the vascular elements (Fig. 7). Most *H. galeatus* remained in the same feeding site for several days; however, 8 days after inoculation, the nematodes shifted to different feeding sites in the same lesion. As a result of prolonged feeding and movement of the nematodes, cavities were formed in the cortex and the vascular bundle. Fig. 8 shows a nematode at a feeding site at which the nematode was apparently capable of moving its head freely within the area and probing with its stylet into several cells. Cells in the cortex and vascular cylinder at the feeding site were altered by nematode feeding as evidenced by ruptured cell walls and accumulation of the dark, granular cell contents. Although walls of cells not directly probed by the nematode were intact, the contents were often lysed.

At the initial stage of infection (1–2 days after the inoculation), the cells in the pericycle appeared to be abnormally enlarged against the endodermis. Early cell division in the pericycle could not be found (Figs. 5 and 6). However, 5 days after inoculation, both hypertrophy and early cell division of pericycle appeared in a diseased vascular bundle (Fig. 7). At 5–8 days after inoculation, some of the cells in the pericycle were lysed due to nematode feeding (Figs. 7A and 8B). A characteristic of the altered cells in the pericycle was the presence of electron-dense materials on the tonoplast and enlarged nuclei (Figs. 5C, 7A, and 8B). At various stages of infection, the prominent feature of abnormal pericycle occurred not only near the nematode head but many cells distant. During the first day of infection, electron-dense materials accumulated along the tonoplast of the squeezed endodermal cells (Fig. 5C). In the latter infection (5–8 days after inoculation), endodermal cells were crushed and lysed (Figs. 7B and 8B). The collapsed endodermal cells occurred not only near the feeding site but around the vascular bundles (Figs. 7B and 8B). Some of the innermost cortical cells around the endodermis were also crushed by the abnormal pericycle.

The area of damaged cortical cells in the diseased root was greater when the nematode fed for a longer period of time. One and 2 days after inoculation, damaged cortical cells were restricted to areas around the penetration path and to a few cells in the vascular bundle (Figs. 5 and 6). However, by the eighth day such damages were more extensive. At this time, there were more lysed cells in the vascular cylinder than earlier sampling periods.

The fine structure of the cortical cells in the feeding area of the nematode was altered. In general, five types of cellular changes were observed. The most severely damaged cells were completely broken and the disorganized contents consisted of dense granules, small vesicles, ruptured membranes, and amorphous electron-dense materials (Fig. 7B). In others, cortical cells surrounding the path of the nematode had broken cell walls and contained no protoplasm. The adjacent cells not in contact with the nematode either lacked protoplasm or the cytoplasm was dark and granular (Fig. 8B). Cortical cells further away from the nematode feeding site were slightly affected. As observed by electron microscopy, these cells contained cytoplasm, but amorphous dense material was present in the vacuole (Fig. 8). In some cases, the intercellular spaces of the cortex were filled with an amorphous dense material (Fig. 6B), which might have been the disorganized protoplasm from the collapsed cells. In the primary phloem, cells with disrupted protoplasts were often observed (Fig. 8). The xylem of the diseased root was probably the last tissue to exhibit a response to the feeding of the nematode. The affected xylem elements also accumulated dense materials which filled the entire cell lumen (Fig. 8).

## DISCUSSION

In some cases, it was not determined whether nematodes were primary causal agents for lesion formation because experiments were carried out under non-aseptic conditions. In this study, *H. galeatus* induced necrotic lesions on alfalfa roots in the absence of other microorganisms. The lesions were not confined to the point of nematode entry but extended along the vascular bundles several millimeters beyond the feeding site. Five days after inoculation, various degrees of cell damage could be traced 105 $\mu$m from the penetration point of a single nematode (13).

Brownish discoloration of plant roots was probably due to the

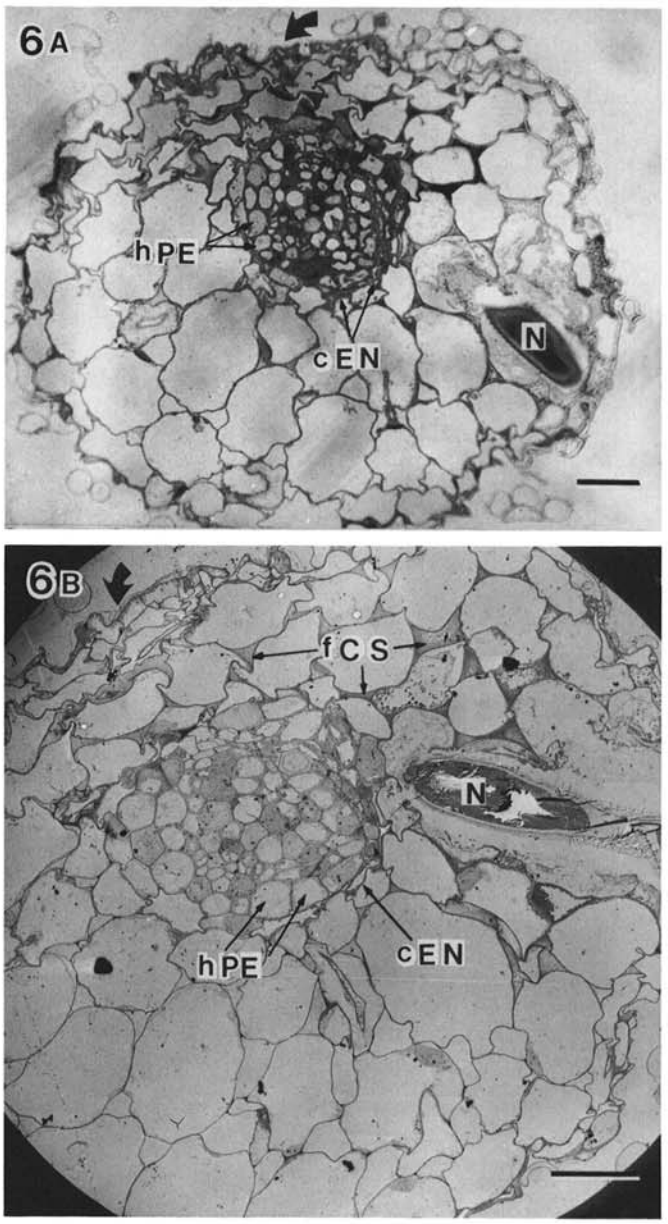



**Fig. 6.** Cross sections of a necrotic lesion in an alfalfa root, 2 days after inoculation with a single *Hoplolaimus galeatus*, showing extensive tissue damage induced by the nematode. The collapsed epidermal and cortical tissues with heavily stained intercellular spaces on the upper sides of the sections (curved arrows) had been fed upon by the nematode which subsequently moved to the opposite side of the root. **A,** Light microscopic view showing also the enlarged pericycles and crushed endodermis. **B,** Wide-field electron microscopic view showing the path of nematode penetration. Cortical cells were broken and the intercellular spaces were filled with amorphous materials. Bar = 20 $\mu$m. Symbol legend: cEN, crushed endodermis; fCS, filled intercellular spaces; hPE, hypertrophied pericycle; and N, nematode.

extreme reaction to nematode infection by the endodermal cells. In our study, the endodermis was the only abnormal tissue beyond the feeding site. Previous studies showed that browning of the infected is a result of the accumulation of phenolic compounds (1,11,12,15,16). Since the Casparian strip of an endodermal cell is the region where deposition of phenolic compounds occurs (5,16), the increased stainability of the endodermis was probably associated with an increased amount of phenolic substances.

The hypertrophy and early division of cells of the pericycle associated with the feeding of other nematodes reported by other workers were also found in this study. The abnormal growth of pericycle in the lesion did not occur in noninfected roots; thus, it was apparently a result of nematode infection.

*Hoplolaimus* spp. are primarily cortex feeders (8,17,18,20). However, a preference for phloem tissue was reported for *H. galeatus* (10). In this study, *H. galeatus* fed at various sites on the

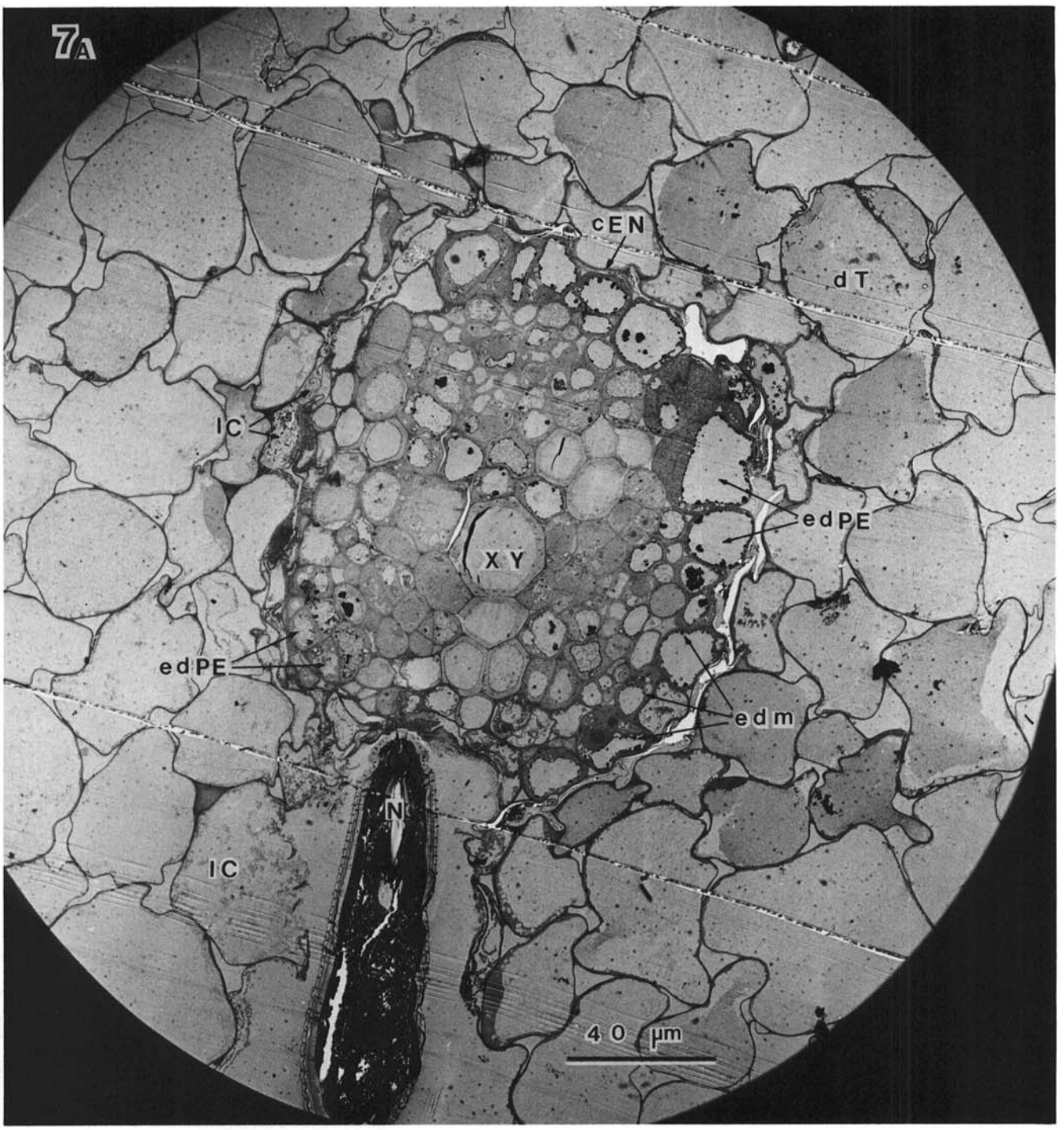
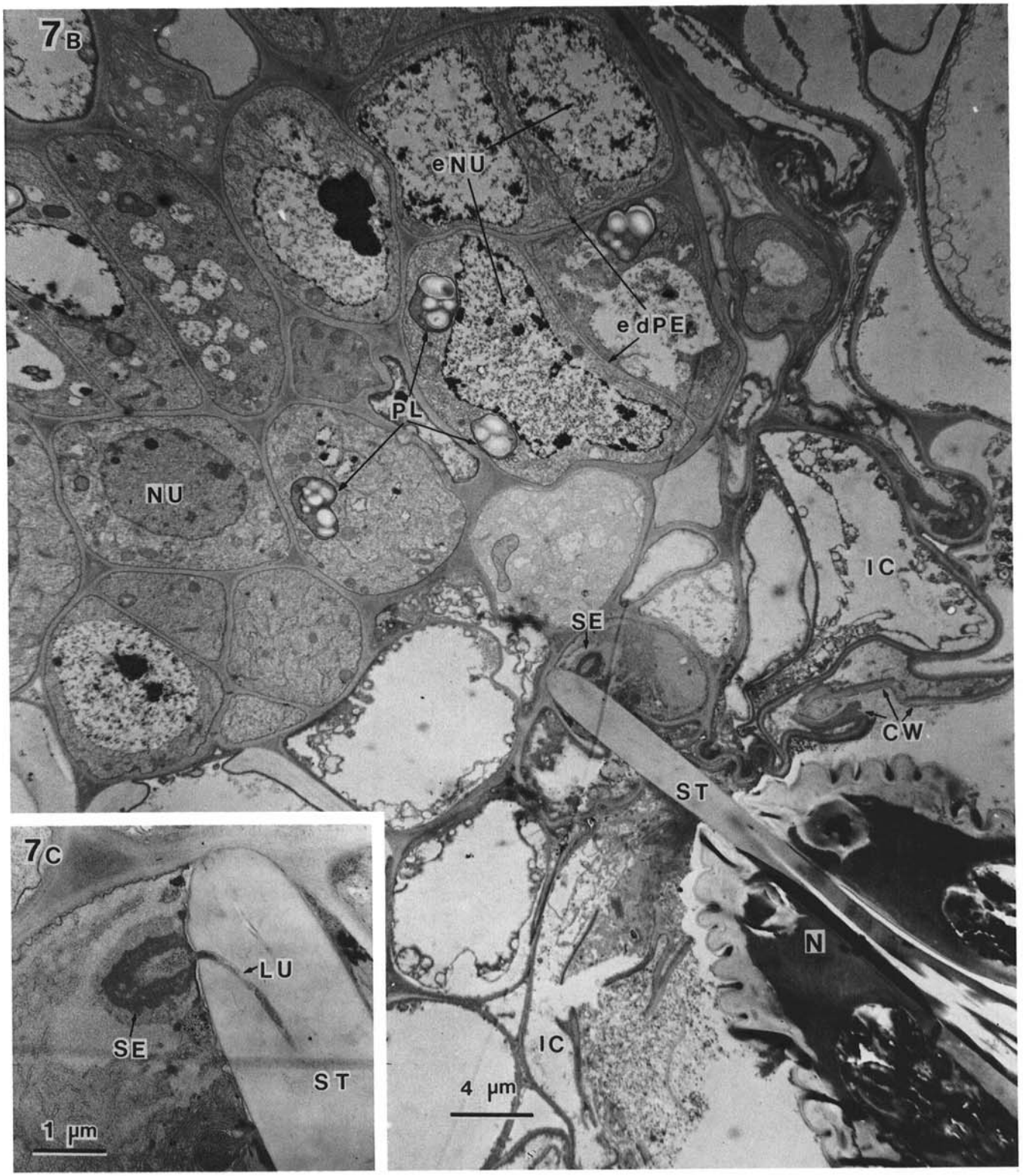



**Fig. 7.** Cross section through the necrotic lesion in an alfalfa root, 5 days after inoculation with a single *Hoplolaimus galeatus*, showing the head of nematode against the vascular cylinder and feeding on a cell of the vascular tissue. **A,** Wide-field electron microscopic view showing lysed cells around the nematode, cortical cells with dense tonoplasts, crushed endodermis, and early divided and hypertrophied pericycle cells. **B and C** on facing page. **B,** Electron microscopic view showing the act of feeding by *H. galeatus*. The stylet of the nematode reached 13 µm into the cell and possible nematode secretion accumulated at the stylet opening. Early divided pericycle cells with much enlarged nuclei were adjacent to the damaged cells. **C,** Enlarged nematode stylet showing secretion. Symbol legend: cEN, crushed endodermis; CW, cell wall; edm, electron dense material; dT, dense tonoplast; edPE, early dividing pericycle; eNU, enlarged nuclei; lc, lysed cells; LU, stylet lumen; N, nematode; NU, nucleus; PL, plastids; SE, secretion; ST, stylet; and XY, xylem.




roots and on both the cortex and the vascular bundles of excised alfalfa roots. Thus, the nematode behaved as a semi-endoparasite. Light microscopy of *H. galeatus* on cotton and pine (10,18,20) indicated that cortical cell damage, such as the formation of feeding cavities and emptied cell contents, were associated with the nematode penetration passages. Feeding cavities also were observed in the vascular bundles of the infected roots. Such symptoms also were confirmed in this study on alfalfa roots. The tendency of the nematode to move about in the root tissue searching for new feeding sites appeared to be more destructive than feeding alone. Thus, mechanical injury was also involved in the alfalfa-lance nematode interaction. In addition to mechanical damage, chemical injury by the nematode to the root tissue must have occurred. Damage observed in root tissues removed from the nematode feeding sites strongly supports this hypothesis. Death of these cells may have resulted from the release of toxic products diffused from feeding sites where enzymes were excreted from the nematode. Therefore, *H. galeatus* is a primary pathogen of alfalfa.

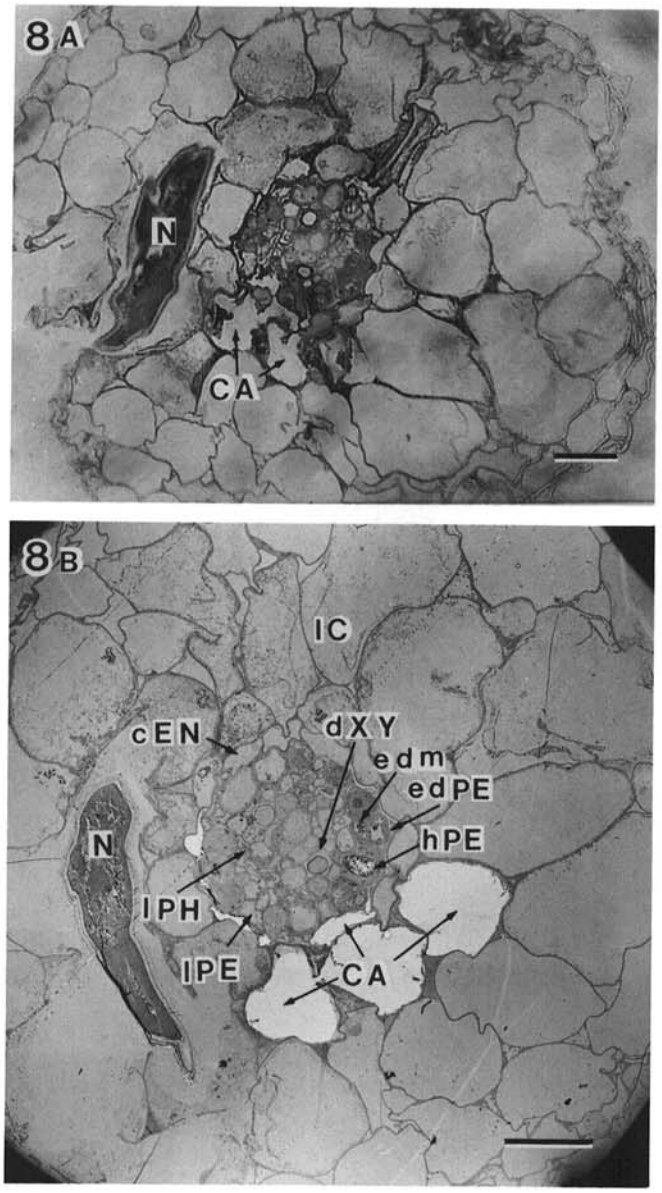



**Fig. 8.** Cross sections through the necrotic lesion in an alfalfa root, 8 days after inoculation with a single *Hoplolaimus galeatus*, showing extensive tissue damage induced by the nematode. **A,** Light microscopic view showing large cavities in the cortex as well as the vascular cylinder. **B,** Wide-field electron microscopic view showing large cavities in the cortex and granular cytoplasm of the cortical cells as well as in the vascular tissues. Early divided and hypertrophied pericycles, crushed endodermis, and accumulated dense materials in the xylem were also clearly shown. Bar = 20 $\mu$m. Symbol legend: CA, cavity; cEN, crushed endodermis; dXY, dense materials in xylem; edm, electron dense materials; edPE, early divided pericycle; hPE, hypertrophied pericycle; lc, lysed cells; lPE, lysed pericycle; and lPH, lysed phloem tissue.

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