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Observations on the Invasion and Endoparasitic Behavior of the Root Lesion Nematode *Pratylenchus penetrans*

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Abstract: The endoparasitic behavior of *Pratylenchus penetrans* was examined using video-enhanced contrast microscopy to observe living nematodes inside root tissue. Feeding behavior could be separated into phases of probing, cell penetration by the stylet, salivation, and food ingestion for brief and extended periods. After cell penetration, a small "salivation zone" was formed around the stylet tip. No feeding tubes were observed. Feeding and migration were interrupted by rest phases when a nematode became characteristically coiled inside a cell. Tissue damage was caused primarily by migration and extended feeding periods. Aspects of egg laying and molting are also described.

Key words: behavior, lesion nematode, *Pratylenchus penetrans*, video-enhanced microscopy.

Pratylenchus penetrans (Cobb) Chitwood & Otiefa is an economically important migratory nematode that feeds ectoparasitically on root hairs (30,31) but is primarily an endoparasite with a wide host range. Several workers have used light microscopy to examine tissues invaded by nematodes, the results of their feeding, the associated tissue damage, and host reaction (9,11,15,16,19–21). There is a paucity, however, of descriptive data on the endoparasitic behavior of *P. penetrans.*

This species enters roots primarily in the region of root hair development and, to a lesser extent, in the zone of elongation (17,19) where it feeds on cortical cells. Kurppa and Vrain (6) and Zunke (28) observed the penetration and feeding behavior and found a pattern of exploration and penetration behavior similar to that previously reported for other nematodes (1,2,21).

High resolution video-enhanced microscopy enables observations to be made of the nematode inside the root tissue which would be obscure under normal light and interference contrast microscopy. The objective of these studies was to provide information on the life cycle biology of *P. penetrans,* including penetration, migration to a feeding site, feeding, egg laying, and molting, by using high resolution video-enhanced contrast microscopy to examine living nematodes. Preliminary observations of the tissue damage have also been made using transmission electron microscopy (TEM).

MATERIALS AND METHODS

Pratylenchus penetrans was obtained from cultures supplied by R. M. Webb (Rothamsted) and J. Roessner (Giessen). Nematodes were subsequently maintained on monoxenic excised root cultures of maize (*Zea mays* L.) and rape (*Brassica napus* L. cv. Akela) grown on nutrient agar medium (8). For observations of invasion and life cycle biology, nematodes of all stages were placed in the rhizosphere of seedlings of rape, oil radish (*B. napus,* cv. Siletina), tobacco (*Nicotiana tabacum* L. cv. Samsun), and potato (*Solanum tuberosum* L. cv. Hansa) growing in aseptic nutrient agar in special observation chambers as described by Wyss and Zunke (23). Observations were made with a Reichert Polyvar microscope with differential interference contrast optics. Behavioral sequences were recorded on 2.5-cm video tapes with the aid of video-contrast enhancement (25,32) and were

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analyzed, where required, by single frame evaluation (10,26,29).

Nematodes also were examined inside tobacco roots using TEM. Root tissue from agar cultures was cut into small pieces (ca. 1–2 mm long) and fixed overnight at 5 C in 3% glutaraldehyde with 0.1 M cacodylate buffer (pH 7.1). After repeated washings, the tissue was postfixed for two changes of 1 hour at room temperature in 1% osmium tetroxide in 0.1 M cacodylate buffer. The tissue was washed in five changes (each 15 minutes) of double distilled water, infiltrated with low viscosity epoxy resin (14), polymerized, and then sectioned on a Reichert UM2 ultramicrotome. Sections were collected on coated copper grids and stained in 0.5% aqueous uranyl acetate followed by 0.2% lead citrate. Grids were examined in a Philips EM 300 microscope at 100 kV accelerating voltage.

Results

Entry into the root and migration through root tissue: In the agar nematodes moved toward a root. Although a number of nematodes migrated to the area where lateral roots emerged from the main root, to the bases of root hairs, and to the root tip, the majority of nematodes aggregated around the zone of root elongation. On contact with the root surface, nematodes rubbed their lips along the surface for a brief period then began stylet thrusts (Fig. 1a). The number and duration of stylet thrusts appeared to vary greatly, presumably related to the structure and thickness of the cell walls. Once a cell wall had been punctured, the nematode occasionally fed for a short time (Fig. 1b) before moving through the epidermal cells. Where one nematode had successfully pentrated an epidermal cell, the area became attractive to other nematodes which aggregated around the damaged cell (Fig. 1c), and several were often observed to enter through the hole. Some were observed subsequently to exit through the same hole and move to another area of the root. During aggregation, adult males and females came into contact with each other, often lying side by side with vulva and spicules touching; however, copulation was not observed.

Several nematodes might have followed the same path through the tissue (Fig. 1d). Nematodes were able to turn around in a cell and puncture the side wall (Fig. 1d). They traversed layers of cells (Fig. 1e) and caused extensive destruction of cortical tissue. During migration through tissue, individuals frequently fed on cells which they punctured (Figs. 1f, 2a); in these cases the bulb opened and closed only a few times (< 10) and no salivation was observed. Migration through the cortical tissue was accomplished by puncturing and penetrating neighboring cells. Usually this process started with stylet thrusts at a corner of a cell (Fig. 2a) followed by stylet thrusts at the opposite corner and then over the entire end wall. The nematode caused a rupture in the wall by pressing the anterior end against the weakened area; it was then able to pass through to the adjacent cell (Fig. 2b). Stylet thrusting continued even after the nematode had entered the next cell.

Brief feeding: Although juvenile stages, especially the second stage (J2), were observed to attack cortical cells of large roots, they were more often found feeding in or on small lateral roots (Fig. 2c, d). The duration of brief feeding varied according to the stage of nematode development; J2 were observed to feed for up to 5 minutes, whereas other stages fed for periods of up to 10 minutes. Extended feeding occurred for much longer periods, often over many hours. The contrast between brief and extended feeding was more obvious when related to visible changes induced in the plant cell.

At the onset of brief feeding, a small salivation zone appeared around the stylet tip (Fig. 2d) and the cell contents did not change, although the rate of cytoplasmic streaming increased slightly. The cell rarely died during brief feeding; however, when the nematode resumed migration (Fig. 2e), each cell through which the nematode passed died rapidly and TEM showed bro-

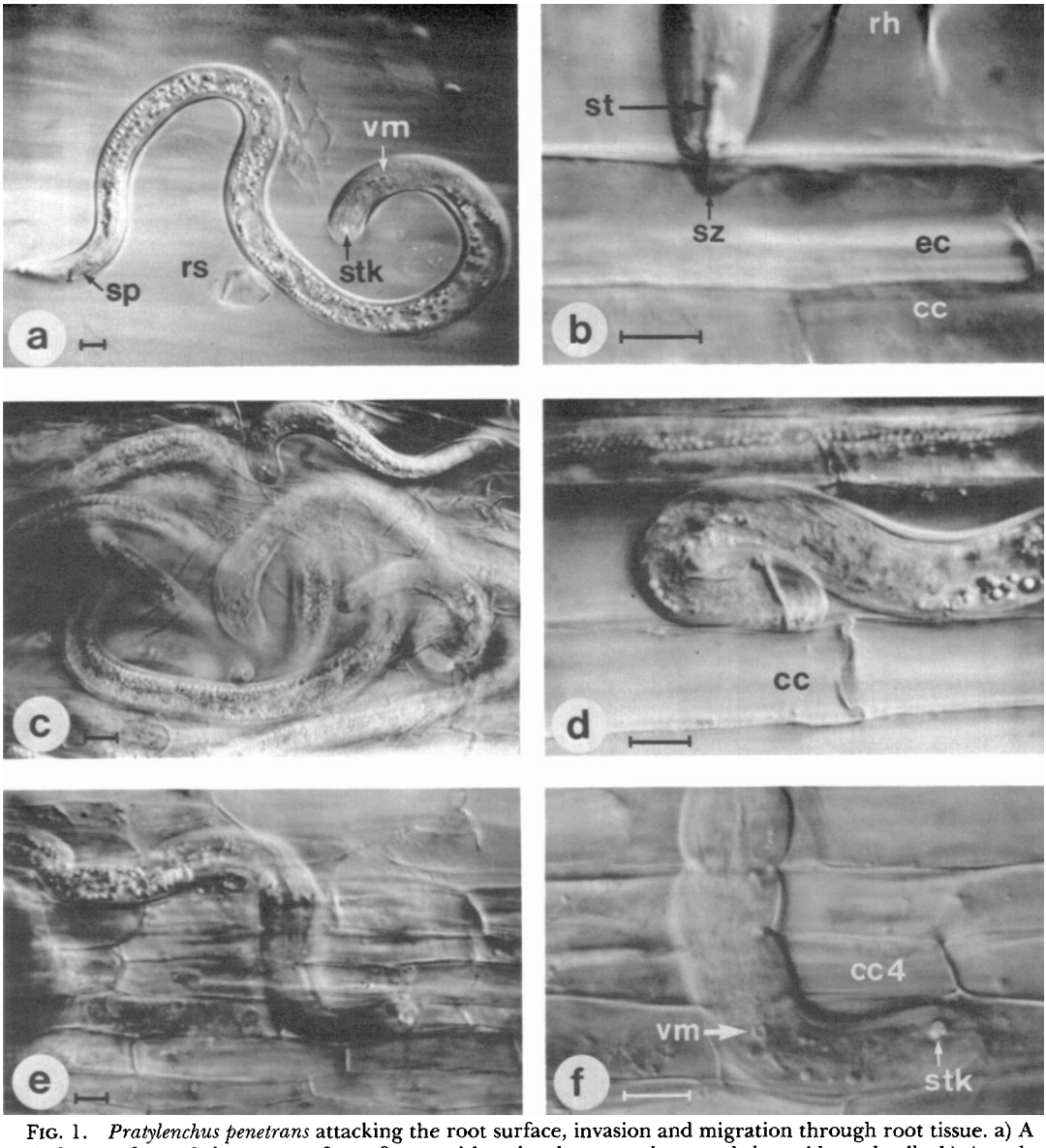


FIG. 1. *Pratylenchus penetrans* attacking the root surface, invasion and migration through root tissue. a) A male has perforated the root surface of rape with stylet thrusts and entered the epidermal cells. b) A male after feeding on a tobacco epidermal cell for 12 minutes and 13 seconds; the stylet tip is surrounded by a small salivation zone. c) Several nematodes of different stages aggregated around a hole where another nematode had penetrated the tobacco root surface a few seconds before. d) Different stages of *P. penetrans* within a tobacco cortex cell; the male started with stylet thrusts to perforate the cortex cell in the next layer. e) During migration a male passed through cortex cells of potato. f) Before penetrating the next cortex cell, nematodes frequently fed for brief periods. During feeding, the open valve of the median bulb pumped a few times, indicating food intake.

Bars = 10 μm; cc = cortex cell, cc4 = fourth layer of cortex cell, ec = epidermal cell, rh = root hair, rs = root surface, sp = spicule, st = stylet, stk = stylet knob, sz = salivation zone, vm = valve of the median bulb.

ken cell walls with cut corners and the hypertrophied nucleus with a granular appearance (Fig. 2f). Older juveniles and adults attacked the cell walls from various angles (Fig. 3a). Often the cytoplasm from the adjacent cell wall was connected to the stylet for some seconds (Fig. 3b).

Extended feeding: The onset of extended

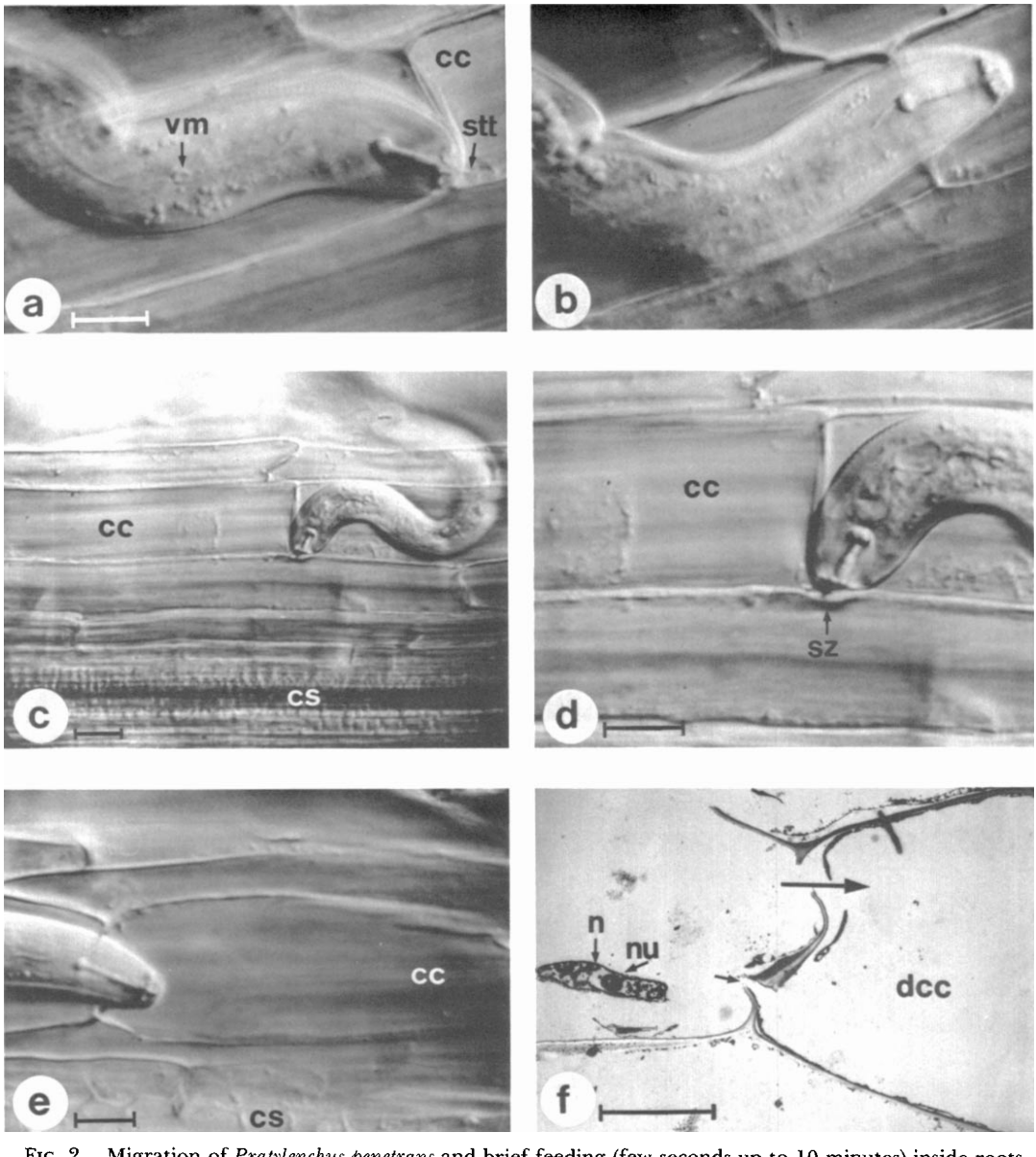


FIG. 2. Migration of *Pratylenchus penetrans* and brief feeding (few seconds up to 10 minutes) inside roots of different host plants. a) Brief food uptake by a J4 interrupted by stylet thrust before penetrating the next oil-radish cortex cell. b) Nematode from 2a penetrating the next cortex cell. c) A J2 that invaded a lateral potato root. It fed for almost 5 minutes on a cortex cell near the central stele. d) The salivation zone of a J2 was always quite small, relative to older stages, during brief feeding. e) A male penetrating into the next cortex cell parallel to the central stele. f) Penetration path of a nematode (arrows) through cortex cell walls. Nucleus is coagulated.

Bars = 10 μm; cc = cortex cell, cs = central stele, dcc = dead cortex cell, n = nucleus, nu = nucleolus, stt = stylet tip, sz = salivation zone, vm = valve of the median bulb.

feeding was marked by a period of salivation of ca. 2 minutes which resulted in a salivation zone around the stylet tip (Fig. 3c). Feeding tubes were never observed. During the salivation period, granules from the dorsal esophageal gland flowed down the gland duct to the ampulla which opens just behind the stylet knobs. The median bulb did not pump during salivation. After salivation, feeding commenced and often

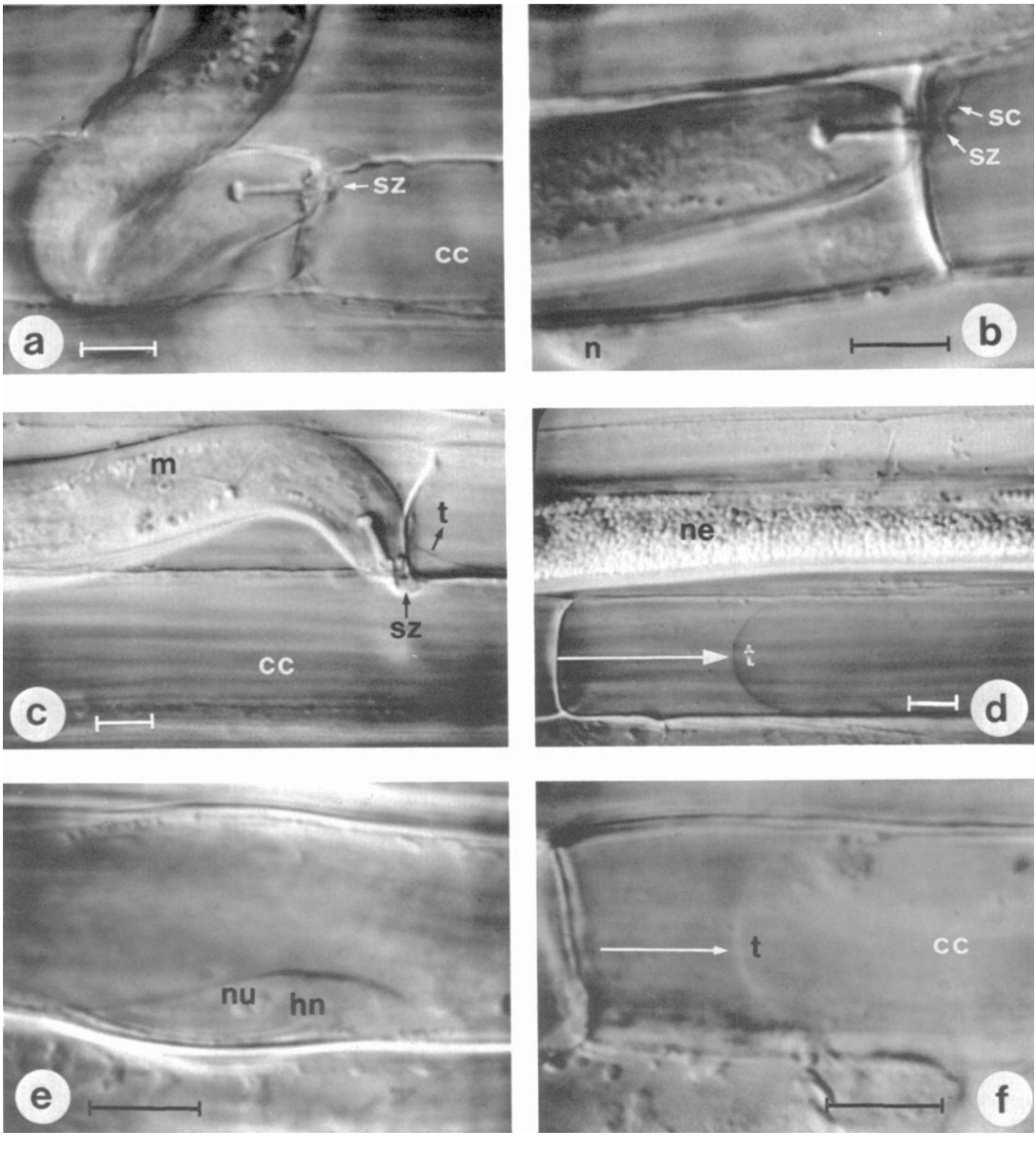


FIG. 3. Extended feeding (10 minutes up to several hours) of *Pratylenchus penetrans* on a single cortex cell. a) This J4 fed on this cortex cell for 14 minutes and 15 seconds. b) This male fed on a cortex cell for 12 minutes and 38 seconds. The nucleus of the cortex cell was not affected during feeding by the nematode. c) A male fed more than 4 hours without interruption. After 2 hours of pumping the median bulb, the salivation zone was still the same size. No direct effect on the cell was noted, except that the tonoplast of the adjacent cortex cell shrank. d) Four hours after starting observation of the feeding nematode, the tonoplast of the parasitized cortex cell progressively decreased in size toward the feeding site. e) Extended feeding on potato cortex cells caused hypertrophy of the nucleus. f) The tonoplast of a potato cortex cell decreased in size toward the feeding site during an extended feeding period.

Bars = 10 μm; cc = cortex cell, hn = hypertrophy of the nucleus, m = median bulb, n = nucleus, ne = nematode, nu = nucleolus, sc = cytoplasm-like strands, sz = salivation zone, t = tonoplast.

continued for many hours. The median bulb contracted three or four times per second (mean of 40 individuals), but some individuals observed over a 1-hour period pumped at rates of 10 contractions per second (n = 5) and others at only 2 contractions per second (n = 4). Defecation occurred every 2–4 minutes without interrupting feeding.

The cell response and feeding behavior of *P. penetrans* was the same for all hosts examined. The tonoplast of rape (Fig. 3d) and potato (Fig. 3f) cortical cells progressively decreased in size during feeding. The plant cell nucleus gradually hypertrophied during and after extended feeding periods (Fig. 3e) and vacuole-like structures occasionally were observed in the cytoplasm. Extended feeding periods always resulted in cell death, often hours after the nematode had ceased feeding and moved away. Invaded tobacco tissue viewed with TEM illustrated the contrast between damaged and intact cells (Fig. 4c, d) and showed the shrunken tonoplast (Fig. 4c).

Egg laying: After extended feeding periods of several hours, adult females deposited eggs inside the cortical tissue or, if they had moved out of the root after feeding, near the root surface. Outside the root, the eggs were laid along the length of the root; inside the root, considerably more effort was required to squeeze each egg into restricted spaces between cortical cells and other eggs.

Egg laying by one female was observed over a period of 3 days (Fig. 5). After the eggs had passed the spermatheca, movement of the tail and internal tissues forced the egg backward to the vulva (Fig. 5a, b). During this period the vulva repeatedly opened and closed becoming increasingly dilated. The tip of the egg then protruded from the vulva (Fig. 5c); where egg laying was blocked by other eggs in the cortical cell, the female tail moved forward to allow the egg to be deposited in a less constricted area (Fig. 5d). Being readily deformable, the eggshell was squeezed through the vulval opening with the contents of the egg flowing from the unlaid part of the egg through to the pole of the egg outside the vagina (Fig. 5d–h). When half of the egg had emerged through the vulva, the remaining portion was expelled rapidly (Fig. 5i), presumably with the assistance of internal pressure and (or) muscular contraction. The whole process, from initial appearance of the egg (Fig. 5c) to the completion of deposition (Fig. 5j), took nearly 2.5 minutes. The rate of egg laying at 23 C by this female was one egg per day.

Molting: Pratylenchus penetrans molted in the soil or inside the root cortex. Different stages molted inside the cortical cells. The individuals became immobile and then contracted as the new cuticle separated from the old. The amphids detached from the anterior end. The old cuticle with the remains of the stylet was clearly larger than the enclosed nematode (Fig. 6a). Subsequently, the molted individual expanded. The escape from the old cuticle was not observed, and the absence of molted cuticle shells inside or outside the root indicated that enzymic action may be involved in dissolution of the cuticle.

Rest phase: Migration and feeding by all stages of *P. penetrans* is frequently interrupted by rest phases. Nematodes coiled inside cells remained quiescent for many hours (Fig. 6b, c); only small body movements occasionally were seen. At the end of the rest phase, the nematode either resumed migration or started to feed (Fig. 6d).

Discussion

The small size of *P. penetrans* makes observations of behavior inside the root difficult even with video-enhanced microscopy. The plants chosen for observation were good hosts of *P. penetrans* and had relatively transparent roots compared with other hosts such as maize. More than 500 nematodes were observed in order to obtain a film record of the life cycle biology of *P. penetrans* outside and inside the root (31). Ectoparasitic feeding behavior of *P. penetrans* on root hairs has been described previously (30).

In contrast to the observations of Kurp-

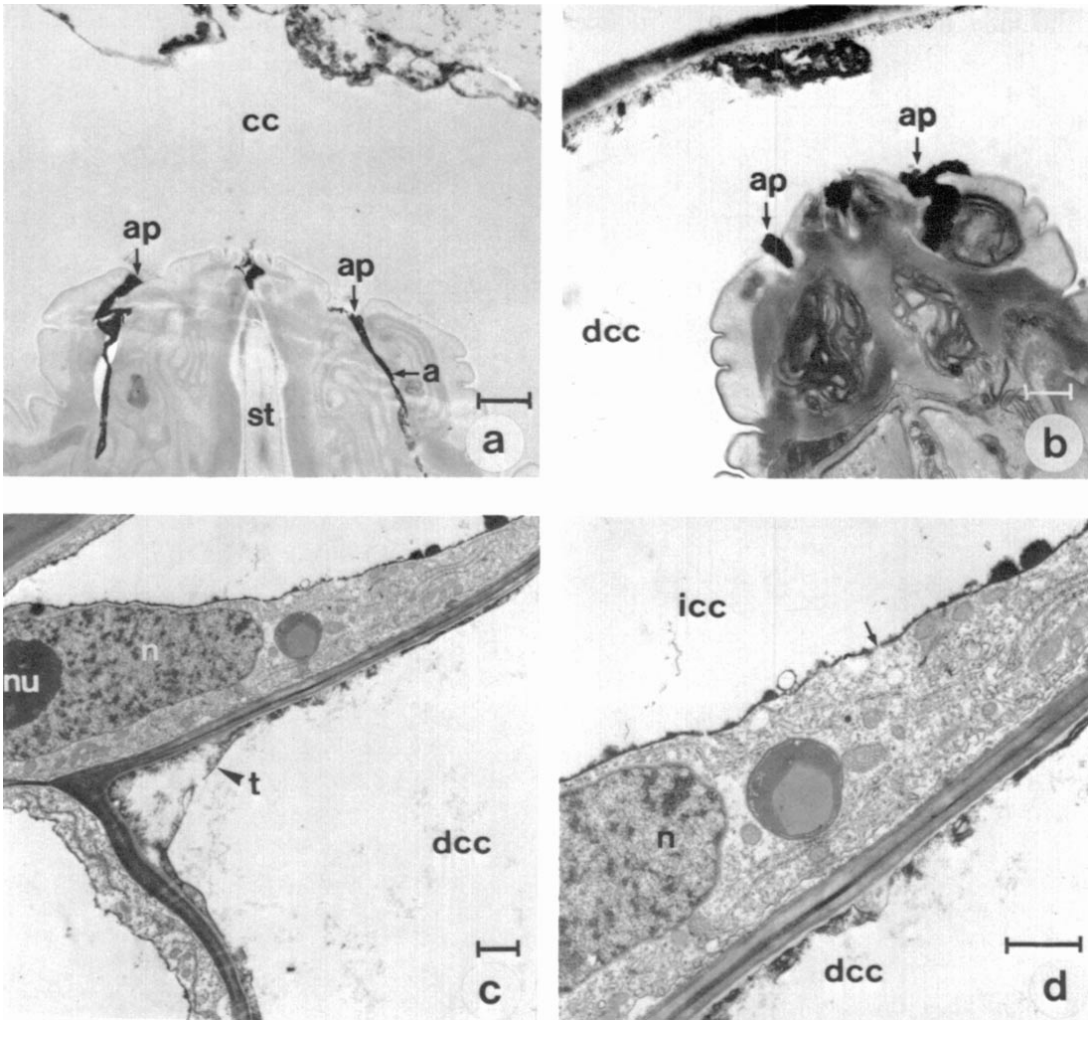
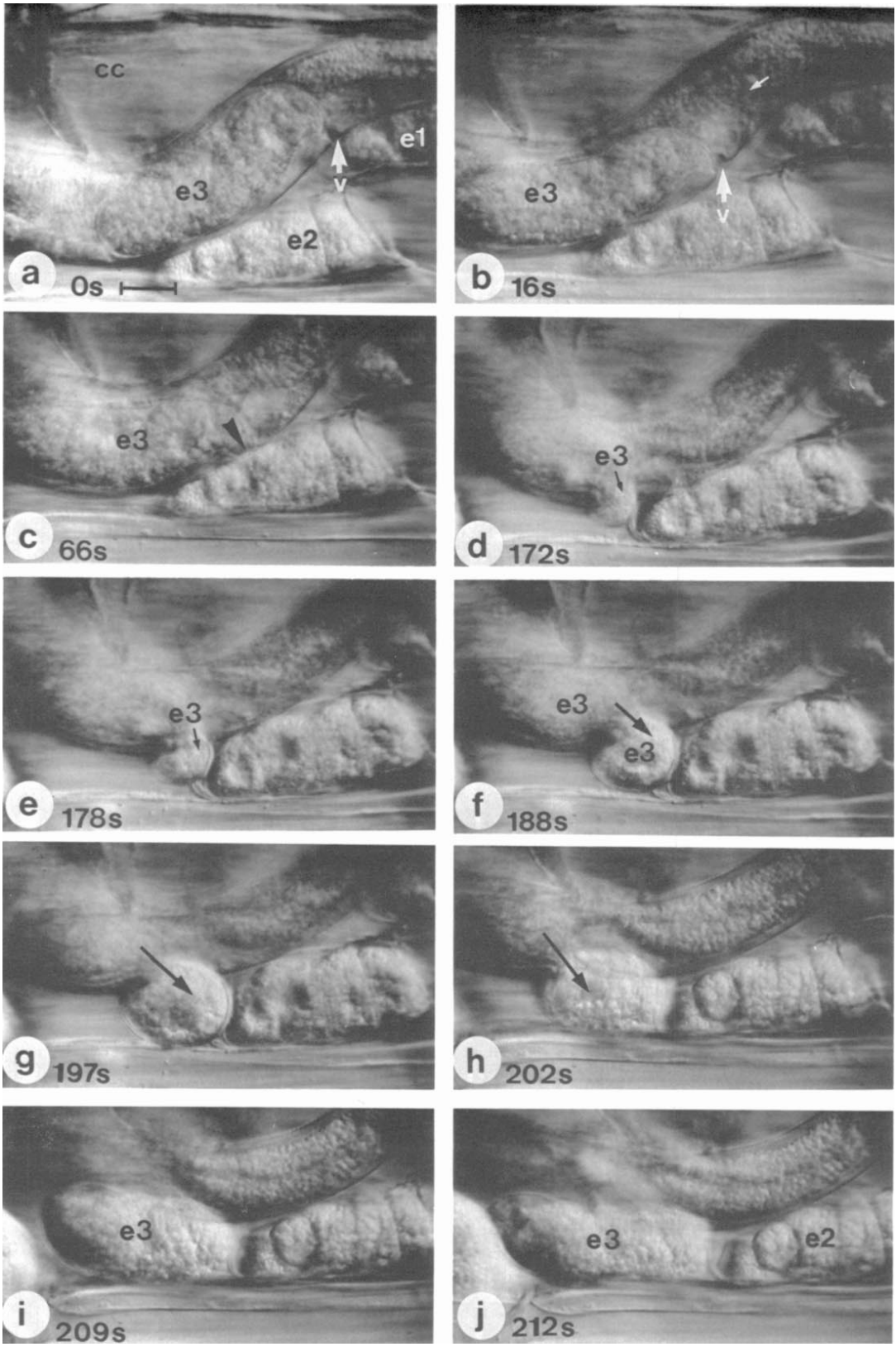


FIG. 4. Orientation of *Pratylenchus penetrans* inside tobacco cortex cells. a, b) TEM of unstained (a) and stained (b) nematodes showing strong osmiophilic reactions within the amphidian canals and amphidial pores. c) Coagulated cytoplasm and pieces of tonoplast within a dead cortex cell. d) Comparison between an intact cortex cell, with a nucleus and a cytoplasm area bounded by the tonoplast (arrow), and a dead cortex cell showing coagulated cytoplasm.

Bars = 1 μm; ac = amphidial canal, ap = amphidial pore, cc = cortex cell, dcc = dead cortex cell, icc = intact cortex cell, n = nucleus, nu = nucleolus, st = stylet, t = tonoplast.

pa and Vrain (6) on strawberry roots, most *P. penetrans* aggregated and penetrated around and above the zone of root elongation of all hosts used. Some individuals fed on and penetrated the root tip and sites where lateral roots emerged. *Hemicycliophora* spp. also feed on root tips (7). The basic behavior pattern of *P. penetrans,* i.e., exploring and selecting a penetration site and entering the root, was similar to that described by Doncaster and Seymour (2). Migration and feeding seemed to be restricted to the cortical cells; no individuals were observed feeding on the endodermis or the central stele. Townshend (16) also found that the endodermis was not invaded by *P. penetrans,* although it was the first tissue to become discolored, perhaps reflecting a high concentration of phenolic substances. This contrasts with the behavior of *Heterodera schachtii* which migrated, without feeding, through the cortical tissue to the central stele where a feeding site was initiated (26).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Migration through cortical tissue caused extensive damage. Whereas *Meloidogyne* spp. move intracellularly and intercellularly (Zunke, unpubl.), *P. penetrans* almost always moved through cells by breaking down the cell walls. This resulted in death of cells along the route followed by the nematode. Damaged tissue caused by migration of *Globodera rostochiensis* and *G. pallida* to a feeding site has been visualized by fluorescence microscopy as defined tracks (12). However, species of cyst nematodes do not feed on cortical tissue, whereas *P. penetrans* feeds on cortex cells causing additional tissue damage.

Although brief feeding rarely resulted in cell death, extended feeding caused the tonoplast to shrink, and the nucleus gradually hypertrophied and became granular and cell death occurred, often after the nematode moved away. Extended feeding occasionally affected cells not being fed upon directly; thus, the tonoplast of a cell adjacent to the one being fed on became shrunken too (Fig. 3c). Root tissue was also damaged when the nematode left the root (Fig. 6e), and numerous dead epidermal cells could be found on the surface of the root (Fig. 6f). Thus, in addition to the extensive tissue damage caused by nematode migration and extended feeding, the movement of nematodes into and out of the root provides entry points for other pathogenic organisms, including bacteria (6).

Aggregations of *P. penetrans* frequently were found in cortical tissue associated with extensive localized tissue necrosis. Some nematodes in these aggregations were feeding, some were molting, but most were quiescent in cells. Males and females were found together, but copulation was not observed. More information is needed about the importance of nematode density in these aspects of the life cycle behavior and whether a survival mechanism is associated with the aggregation inside roots (4).

The amphids are presumed to be chemosensory organs involved in orientation of the nematode (18). The amphids of *P. penetrans* fixed inside the roots contain osmiophilic material (Fig. 4a, b), whereas nematodes fixed outside root tissue have very little osmiophilic material in their amphids (18). Such material may be cell derived and may assist the nematode in orientation and sensing which cells to use as a food source. Further electron microscopy is needed to study amphidial changes that occur during invasion.

Pratylenchus penetrans shows no regular rhythm of salivation, feeding, and defecation as has been observed in *Heterodera schachtii* (26). Feeding tubes have been associated with salivation in *H. schachtii* (24); *G. rostochiensis, G. pallida,* and *Meloidogyne* spp. (13); *Helicotylenchus* spp. (5); and *Trichodorus* spp. (22), but feeding tubes were not formed at any time during salivation by *P. penetrans.*

During extended feeding, the usual rate of pumping of the median bulb of *P. penetrans* (three or four contractions per second) is slower than the pumping rate of *H. schachtii* (5–7 contractions per second) (26). Food uptake by *P. penetrans* is limited by the small size of the nematode and the associated salivation zone and by the small size of the pump chamber of the median bulb which may also dictate the slow rate of pumping.

Compared with *H. schachtii* (26), the salivation zone formed around the stylet tip of *P. penetrans* is very small and probably reflects the small body size and volume of the dorsal esophageal gland cell volume

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FIG. 5. Egg laying of *Pratylenchus penetrans* inside the third layer of potato cortex cells at 23 C. a–j) No food uptake was observed during the egg laying process. d–j) In approximately 2 minutes the egg passed the vulva and flowed out. Length of arrow indicates the speed of egg laying. Numbers with s are the numbers of seconds that elapsed during the process.

Bars = 10 μm; cc = cortex cell, e1 = egg laid 2 days before e3, e2 = egg laid 1 day before e3, e3 = third egg laid, v = vulva.

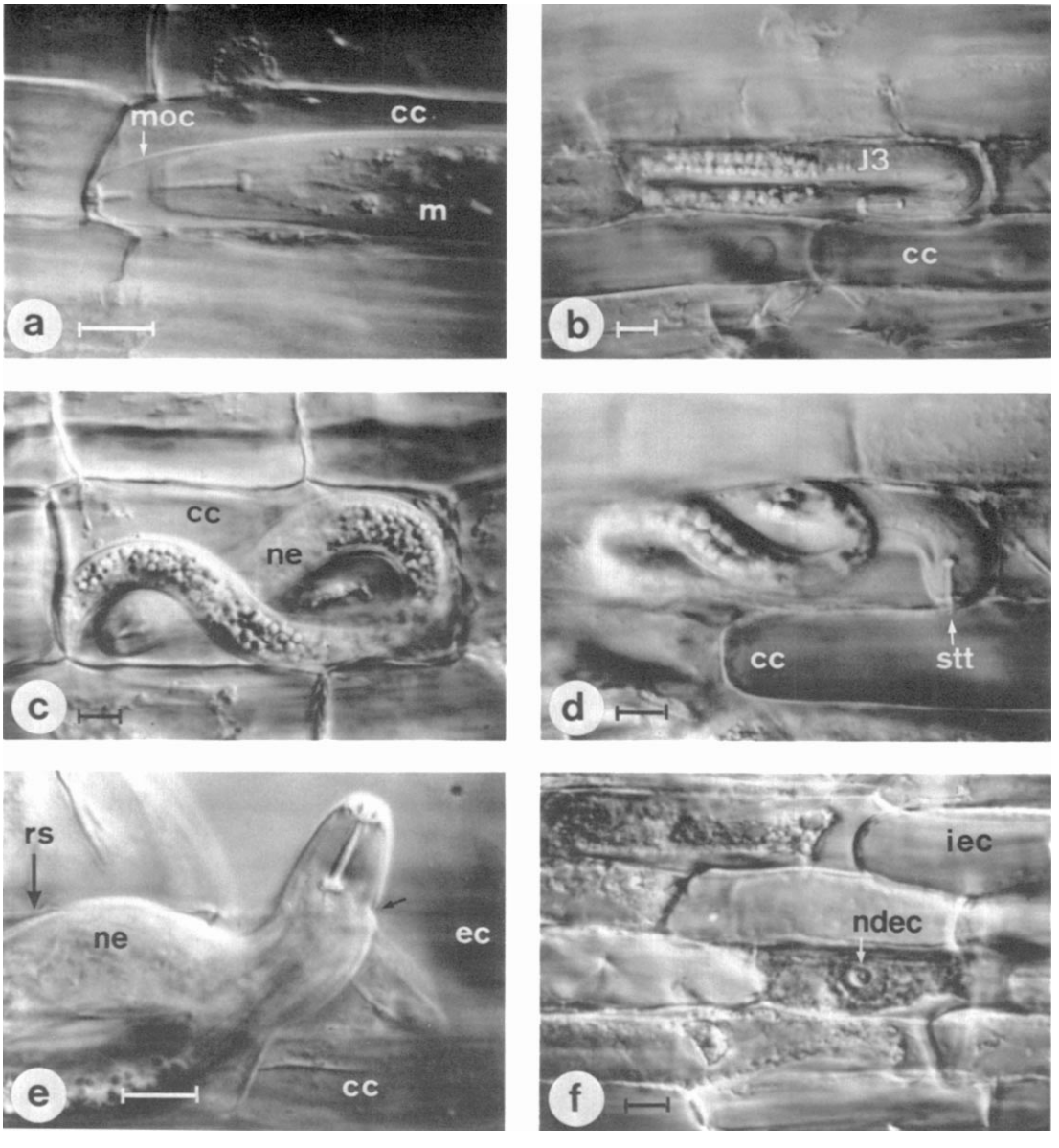

FIG. 6. *Pratylenchus penetrans* in rape and potato root tissue. a) Molting of a juvenile from J3 to J4 stage within a rape cortex cell. b) Rest phase of a J3 for a period of about 5 hours within a rape cortex cell. c) First movements of a J4 before starting feeding from the next rape cortex cell. d) Feeding on a rape cortex cell after 3 hours of a rest phase; a J4 just before a salivation period with stylet tip already inserted into the cortex cell. e) Nematode leaving a potato root. The epidermal cells and cortex cells were severely damaged. Arrow indicates head pushed through the root surface. f) Surface view of damaged epidermal cells of a potato root caused by the invasion and migration of *P. penetrans,* with coagulated cytoplasm and dead nucleus surrounded by some intact epidermal cells.

Bars = 10 μm; cc = cortex cell, ec = epidermal cell, iec = intact epidermal cell, m = median bulb, moc = old molted cuticle, ndec = dead nucleus, ne = nematode, rs = root surface, stt = stylet tip.

that can discharge limited amounts of saliva. The dorsal gland of *P. penetrans* is of a similar size to the subventral glands of the esophagus, whereas in *H. schachtii* the dorsal gland is considerably larger than the subventral glands and contains many secretory granules (27).

The egg laying sequences in *P. penetrans*

were similar to those of *Aphelenchoides blastophthorus* (3). The female of *P. penetrans* was able to position eggs carefully in a restricted space inside the root, suggesting sensory perception of the area surrounding the vulva. Hours before egg laying, the vulva regularly opened and closed, and the frequency increased as the onset of egg laying approached. The egg was rapidly expelled when half the egg had emerged, but it was unclear whether this was caused by muscular contraction of the body wall, internal hydrostatic pressure, or contraction of the vulval musculature. Observations suggested, however, that contractions of the body wall were less likely to play a role, as there was no sinusoidal wave movement caused by alternative contractions of the muscle blocks as found in *A. blastophthorus* (3).

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