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Immunogold Localization of Tobacco Rattle Virus Particles within *Paratrichodorus anemones*[1]

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Abstract: Unequivocal evidence of the viral nature of virus-like particles observed at the specific site of retention of tobacco rattle virus (TRV) in *Paratrichodorus* and *Trichodorus* nematodes has not previously been available. A new staining technique using safranin-O, which does not affect viral antigenicity, was used with an antiserum raised against the coat protein of TRV and prepared for use with immunogold labelling. Application of this method enabled the occurrence and localization of particles of TRV to be confirmed in the pharynx of the natural vector of the virus, *Paratrichodorus anemones,* and provided unequivocal evidence that the particles observed were TRV particles. The TRV particles were observed attached only to the cuticle lining the posterior tract of the pharyngeal lumen of the vector. Therefore, the specific site of retention of TRV particles in *P. anemones* is apparently more localized than reported to occur in other vector trichodorid species.

Key words: Cross-absorption, detection, electron microscopy, immunogold labelling, localization, nematode, *Paratrichodorus anemones,* safranin-O staining, specificity, stubby-root nematode, Trichodoridae, tobacco rattle virus, virus acquisition, virus retention.

Tobacco rattle (TRV), pea early-browning (PEBV), and pepper ringspot (PRV) viruses, which collectively comprise the tobravirus family, are naturally transmitted by several *Trichodorus* and *Paratrichodorus* species, members of the family Trichodoridae (Taylor and Brown, 1997). Vector specificity in the transmission of TRV is determined by the viral coat protein (CP) and a non-structural protein (NSP) encoded by the RNA-2 segment of the viral bipartite genome (Hernandez et al., 1997), whereas with PEBV the CP and two NSPs are involved (MacFarlane et al., 1996).

Acquisition of virus particles by vector trichodorids occurs during the ingestion phase of the nematode's feeding cycle when the contents of root cells are removed as food. The acquired virus particles bind specifically to the cuticle lining the lumen of the pharyngostome and pharynx, in the anterior end of their vectors (Brown et al., 1996; Taylor and Robertson, 1970). This binding may be facilitated by non-structural proteins acting as a "bridge" between the viruses and the site of retention in the vector nematodes (Brown and MacFarlane, 1999).

Virus transmission and subsequent infection of healthy plants occur when viruliferous nematodes feed on roots of uninfected virus-host plants. Virus particles may become detached from the site of retention when the nematode injects esophageal gland secretions into the root cell immediately prior to the commencement of feeding (Brown and MacFarlane, 1999).

Osmium tetroxide (OsO_4) is widely used to post-fix nematodes, including trichodorids, for examination by transmission electron microscopy (TEM) and was used with viruliferous *Paratrichodorus pachydermus* and *Trichodorus similis* to determine the site of retention of TRV-like particles in these vector species (Brown et al., 1996; Taylor and Robertson, 1970). However, post-fixation staining with OsO_4 inhibits the antigenic properties of virus particles, preventing serological methods from being used effectively to confirm the identity of the observed particles. Consequently, identification of the "TRV-like" particles in these vector nematodes was based on the morphology of the observed particles.

Recently, a new staining procedure has become available that allows serological methods to be applied to ultrathin sections of nematodes (Karanastasi et al., 1999). We report the successful application of this technique with an immunogold labelling

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(IGL) method to detect and unequivocally identify TRV particles at the specific site of retention in vector *P. anemones*.

Materials and Methods

Nematode extraction and virus transmission tests: Soil samples containing *P. anemones* naturally associated with TRV were collected from Holme on Spalding Moor, Yorkshire, England. The nematodes were extracted from soil samples with a modified decanting and sieving method (Brown and Boag, 1988) followed by overnight final separation in Baermann funnels. Individual trichodorids were bait-tested in 0.5-cm^3 plastic capsules (Brown et al., 1989), and after 10 days the contents of each capsule were washed into a counting dish. Nematodes were recovered, heat-killed, fixed in 1% formalin-glycerol mixture, and stored in 0.5-cm^3 capsules containing fixative. Each bait plant was placed in a compost block and allowed to grow for 3 weeks; then the root system was washed free of adhering compost and comminuted in a mortar and pestle. The resultant suspension was rubbed by finger onto leaves of *Chenopodium amaranticolor* and *C. quinoa* virus-indicator plants. After 5 to 10 days, leaves of test plants were examined for the presence of TRV symptoms (local necrotic or chlorotic lesions). Nematodes from bait plants whose root suspensions had produced TRV symptoms on the virus-indicator plants were chosen for examination with TEM.

Virus identification: Total RNA was isolated from *C. amaranticolor* virus-indicator plants showing virus symptoms. Virus identity was confirmed with RT-PCR using primers complementary to sequences identified on the 5′ region of TRV strains PaY4 and TpO3 (MacFarlane, 1996).

Transmission electron microscopy: Individual nematode specimens were processed for TEM with a modification of a fixation and embedding schedule developed by Roberts (1994). Specimens were placed in gluteraldehyde (5% in PIPES [Piperazine-NN′-bis-2-ethanesulphoric acid] buffer) for 18 hours to ensure adequate fixation, washed in 0.2 M PIPES buffer (2 times, 60 minutes each) and distilled water (60 minutes), stained for 30 minutes in 0.1% aqueous safranin-O, and finally repeatedly washed in distilled water to remove excess stain. After embedding in 1% aqueous agar, the specimens were dehydrated in 100% ethanol (2 times, 30 minutes each) and propylene oxide (30 minutes), infiltrated with a 1:1 propylene oxide-Araldite epoxy-resin mixture for 24 hours, and finally individually embedded in Araldite epoxy-resin blocks (Karanastasi et al., 1999).

A consecutive series of sections of *P. anemones* specimens, from the head region to the posterior end of the esophageal bulb, were cut with glass knives on a Reichert ultramicrotome. Series of five groups of 5 to 7 ultrathin sections (ca. 100 nm) were cut from the regions indicated in Figure 1 from each specimen and collected on plastic (Pyroxylin)-coated grids. Representative grids from each region were stained with uranyl acetate, followed by lead citrate, and examined with a JEOL 1200EX microscope.

A second series of sections from the various regions was subjected to IGL with a polyclonal antiserum raised against the coat protein of TRV strain N5. This antiserum, which cross-reacts with TRV strain PaY4 but not with TpO3 (Ploeg et al., 1992, unpub. data), was diluted in IGL buffer (4 µl in 176 µl) and cross-absorbed with a suspension made by micro-comminuting a group of 20 non-viruliferous *P. anemones* in 20 µl of distilled water with carborundum powder. In previous studies with plant and insect tissues, cross-absorption reduced the amount of background non-specific labelling of non-viral protein structures (Da Rocha et al., 1986; Fasseas et al., 1989; Roberts, 1994). Grids containing the sections were floated for 1 hour on 20-µl drops of IGL buffer (0.05 M PIPES, pH 6.8, containing 0.5% BSA, 0.5% Tween-20, and 0.02% sodium azide). They were then removed, drained, and transferred to 20-µl drops of the cross-absorbed, 1:50 diluted TRV antiserum. The grids were incubated for 18 hours on the antiserum and then removed, drained, and washed (2 times, 10 minutes each) on 430-µl drops of IGL buffer in microtitre plates. Af-

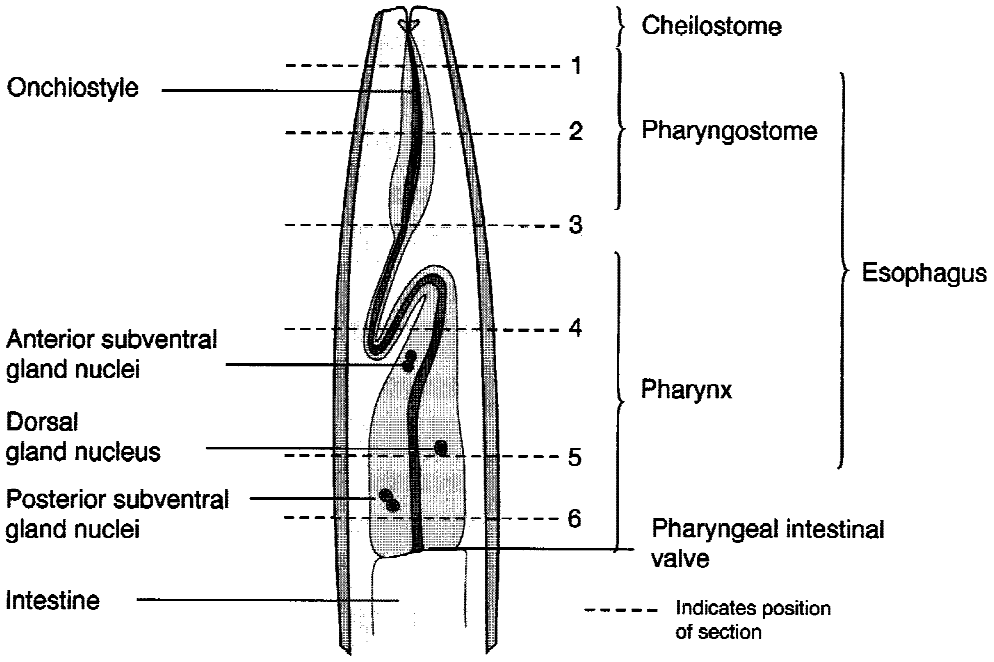

FIG. 1. Diagram of the anterior anatomy of a trichodorid nematode indicating positions from which ultrathin sections were obtained for examination by TEM.

ter draining, the grids were placed on 20-µl drops of gold-labelled (gold particles with 15-nm diam.), goat anti-rabbit immunoglobulin G (IgG) (Amersham International plc, England) diluted in IGL buffer (1:50). After 6 hours the grids were sequentially washed on 0.05 M PIPES and distilled water for 10 minutes during each treatment and then drained, dried, and subsequently stained with uranyl acetate and lead citrate (Roberts, 1994). Ultrathin sections of the esophageal regions in which virus-like particles were not present were used as negative controls. These particle-free controls were compared with sections containing virus particles to determine the specificity and sensitivity of the method.

RESULTS

No TRV particles were present in ultrathin sections from the anterior and central regions of the pharyngostome (positions 1 and 2 in Fig. 1) (Fig. 2A,B), or from the anterior end of the pharyngeal lumen immediately posterior to the pharyngostome (position 3 in Fig. 1) (Fig. 2C). Virus-like particles were observed in sections cut at the point where the esophageal lumen formed a loop (position 4 in Fig. 1) (Fig. 3). However, "TRV-like" particles were present only in the lumen of the posterior region of the loop immediately adjacent to the anterior end of the pharyngeal bulb, and were not present in the lumen of the anterior region of the loop (Fig. 3). Virus-like particles were present in sections cut from the midpoint of the pharyngeal bulb (position 5 in Fig. 1) (Fig. 4) but were absent from sections cut from the extreme posterior section of the bulb (position 6 in Fig. 1) (Fig. 5) in the region between the posterior subventral glands and the pharyngeal-intestinal valve (Fig. 1).

Immunogold labelling revealed a specific association of colloidal gold particles on ultrathin sections with virus–like particles (Fig. 4), but gold particles did not label areas on sections in which virus-like particles were not present (Figs. 2A,B,C;5).

DISCUSSION

Earlier reports of the occurrence of "TRV-like" particles in the pharyngostome and

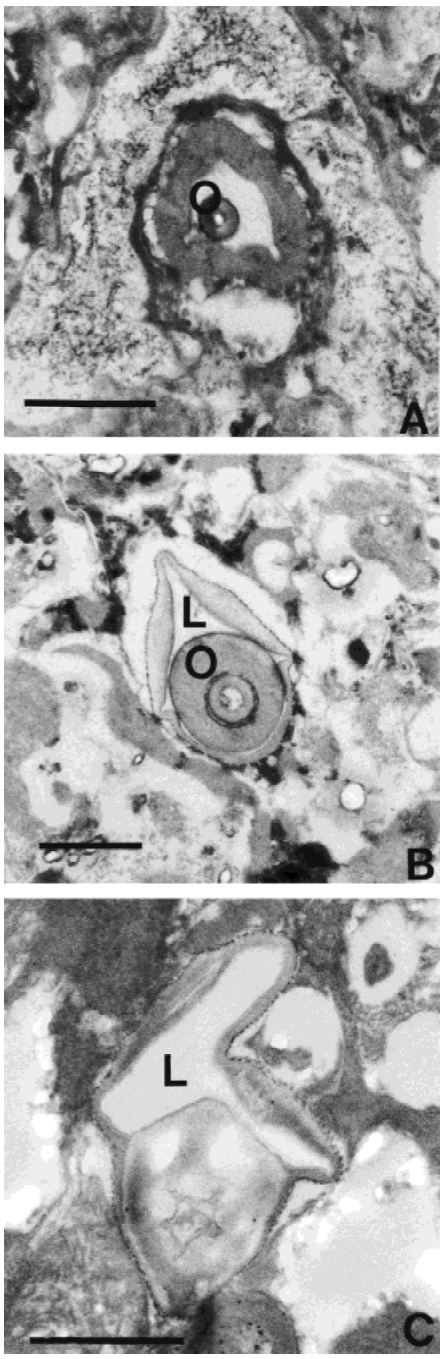

FIG. 2. TEM of transverse sections of the pharyngostome and pharynx of *Paratrichodorus anemones,* subjected to gold labelling to detect tobacco rattle virus particles. Virus particles are absent, and there is no aggregation of colloidal gold particles. A): Anterior end of pharyngostome (position 1 in Fig 1). B): Mid-section of pharyngostome (position 2 in Fig. 1). C): Anterior end of the pharyngeal lumen immediately posterior to the pharyngostome (position 3 in Fig. 1). O: onchiostyle, L: lumen. Scale bars = 1 µm.

pharynx of trichodorid nematodes involved the use of specimens post-fixed with osmium tetroxide (OsO_4) and stained with uranyl acetate and lead citrate. Identification of the "TRV-like" particles was based on their morphology (Taylor and Robertson, 1970; Brown et al., 1996). The use of OsO_4 can severely diminish viral antigenicity; thus, serological assays such as IGL could not be performed on ultrathin sections cut from these nematodes. Substituting OsO_4 post-fixation with safranin-O staining enables specimens to be readily located in the epoxy resin block prior to sectioning, permits recognition of morphological detail, and appears not to affect viral antigenicity (Prior et al., 1999; Karanastasi et al., 1999). In our study, safranin-O was successfully used with IGL, with a polyclonal antiserum prepared specifically against the CP of TRV strain N5, to provide, for the first time, unequivocal evidence of the presence of TRV particles in a vector trichodorid species. Immunogold labelling confirmed the identity of TRV particles associated with the cuticle lining the posterior region of the pharyngeal tract, particularly the lining of the lumen in the pharyngeal bulb, in viruliferous *P. anemones.* In these nematodes, virus particles were not present in the anterior region of the pharyngeal tract or the pharyngostome, and were not present in the most posterior region of the pharyngeal bulb.

Several trichodorid species have the ability to transmit more than one strain of TRV, and some even transmit two different tobraviruses (Taylor and Brown, 1997). Brown and MacFarlane (1999) suggested that complementarity of transmission by these species could involve specific retention of the different viruses or virus strains at different sites within the nematode's feeding apparatus. Comparison of our results, in which virus particles are retained only in the posterior region of the pharyngeal tract, with those of previous studies, in which they are retained throughout the length of the pharyngeal tract (Taylor and Robertson, 1970, 1975; Brown et al., 1996), provides the first evidence that differences can occur in the

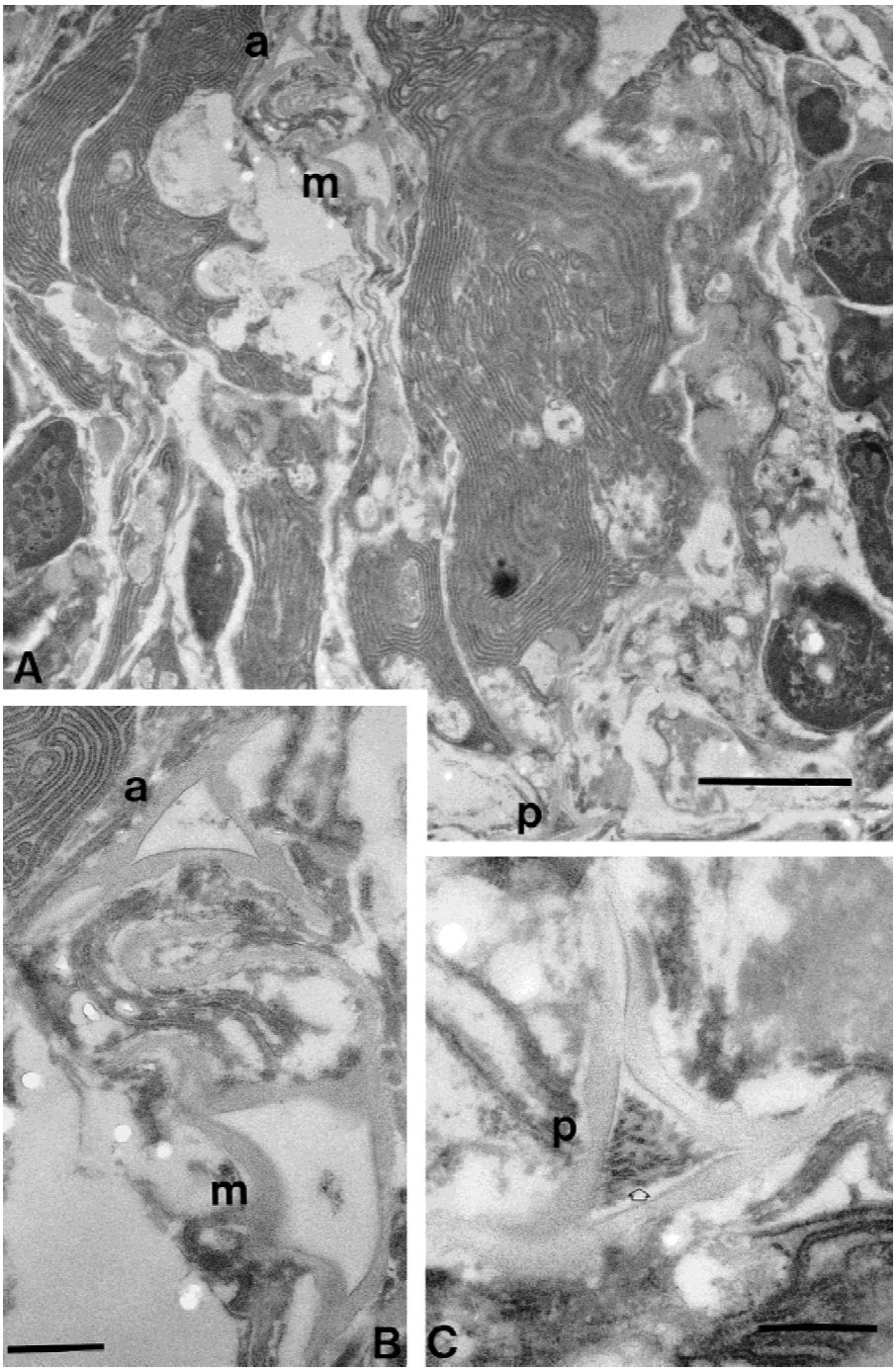

FIG. 3. TEM of a transverse section (position 4 in Fig. 1) across the pharyngeal loop adjacent to the anterior end of the pharyngeal bulb of *Paratrichodorus anemones* A): Entire micrograph showing three sections of the lumen (a: anterior section; m: midsection; p: posterior section). Tobravirus-like particles are present only in the lumen of the posterior section. B): Magnified view of the anterior and midsections of lumen shown in A; C): Magnified view of the posterior section (arrow indicates a virus particle). Scale bars: A = 2 μm; B = 500 nm; C = 200 nm.

site of retention of viruses in their associated vectors.

The specific binding of TRV particles to the cuticle lining the posterior pharynx of *P. anemones,* but not to the anterior pharynx or the pharyngostome, provides evidence of a

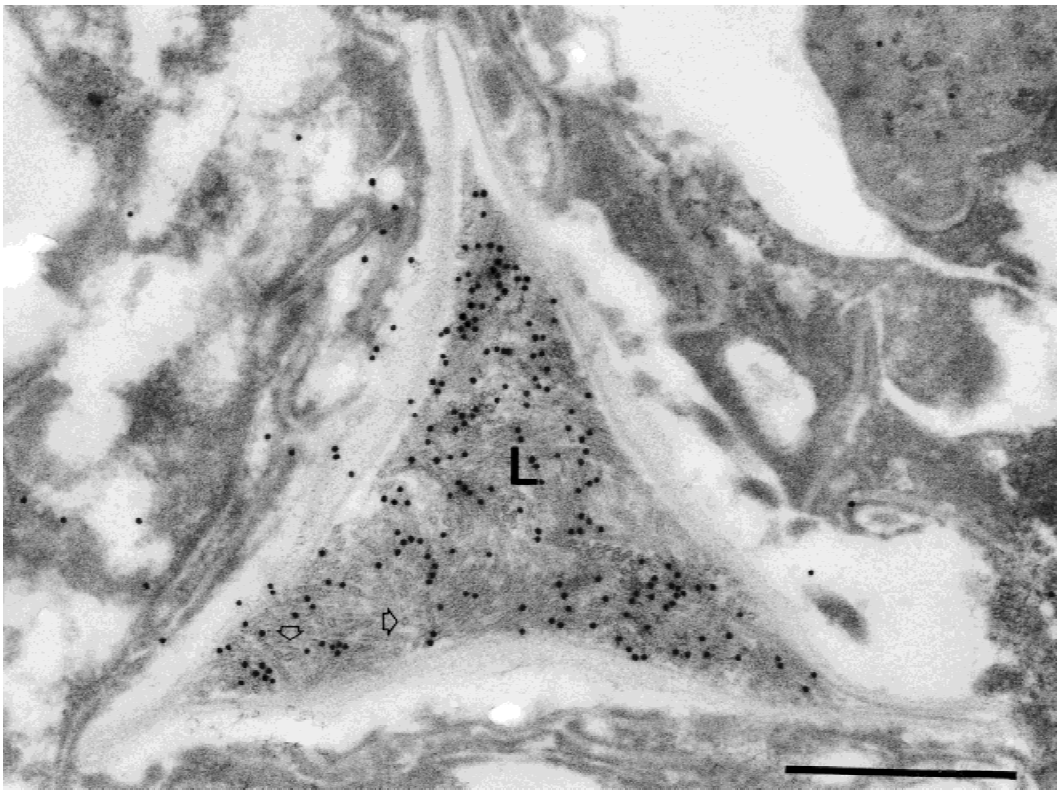

FIG. 4. TEM of a transverse section across the midsection of the pharyngeal bulb (position 5 in Fig. 1) of a *Paratrichodorus anemones,* subjected to gold labelling to detect tobacco rattle virus particles. Virus-like particles can be seen in the lumen, and colloidal gold particles are aggregated in this area providing unequivocal evidence that the particles are tobacco rattle virus capsids (arrows indicate virus particles). Scale bar = 1 μm.

difference between these regions. Three sets of glands with extensive ducts are present in the pharyngeal bulb, and the gland ducts open into, and immediately anterior to, the bulb (Taylor and Brown, 1997). Secretions produced by these glands probably cause particles to be released from the site of retention (Brown and MacFarlane, 1999). As these secretions are likely to be most abundant in the region where we observed TRV particles in *P. anemones,* it is possible that they may also have a role in the specific retention of TRV particles in this vector species.

The absence of TRV particles from the most posterior region of the pharyngeal bulb may be because the cuticle lining of the lumen in this region differs from that in the remainder of the pharyngeal bulb, thus preventing TRV particles from adhering. Alternatively, when the nematode completes its feeding it may produce secretions from the posterior subventral glands that are moved posteriorly to "clean" the pharyngeal lumen of any food detritus, and simultaneously release and carry any retained particles into the nematode intestine.

Our study confirms the advantage of using safranin-O stain as compared with conventional OsO_4 post-fixation in nematode investigations. The stain enables serological techniques, such as IGL, to be applied to ultrathin sections. Antisera produced against nematode secretions that are involved in nematode-plant interactions (Davis et al., 1994; Hussey, 1989; Hussey et al., 1990, 1994; Ray et al., 1994) could be used to investigate the localization and pathways of these secretions through the nematodes. Also, safranin-O is fluorescent and can therefore be used as a post-sectioning stain, enabling sections cut from nematodes

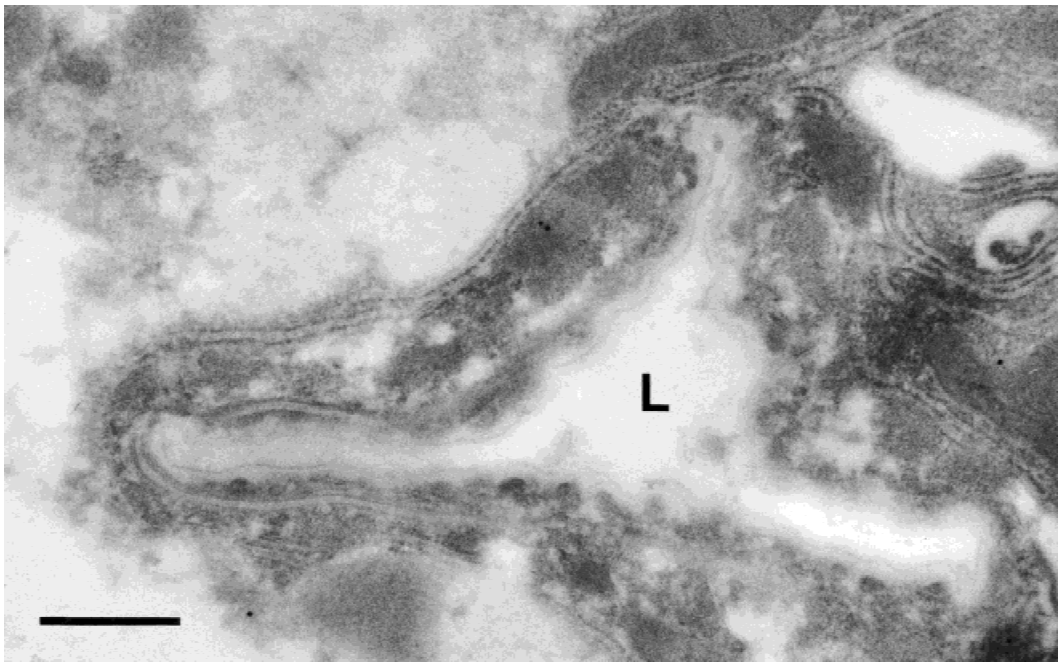

FIG. 5. Micrograph of a transverse section cut across the posterior section of the pharyngeal bulb (position 6 in Fig. 1) of a *Paratrichodorus anemones,* subjected to gold labelling to detect tobacco rattle virus particles. Virus particles are absent, and there is no aggregation of colloidal gold particles. Scale bar = 500 nm.

to be examined using confocal laser microscopy (Karanastasi et al., 1999).

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