Induced Salivation in Plant-Parasitic Nematodes

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ABSTRACT

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Stylet thrusting activity was induced in *Meloidogyne incognita, Heterodera schachtii,* and *Aphelenchus avenae* by treatment with toluene, xylene, catechol, resorcinol, hydroquinone, guaiacol, caffeic acid, and serotonin. *M. incognita, H. schachtii,* and *Xiphinema americanum* also produced stylet and/or amphidial exudate that was visible when the incubation medium contained one of these compounds and Coomassie Blue. Anterior extensions of the subventral esophageal glands became swollen and packed with secretory granules when the nematodes were treated with a number of the same compounds. Ultrastructure of the glands and of secretory granules in treated nematodes did not differ significantly from that of the controls. Stylet movement and exudation was induced in *M. incognita* by 40 ppm aldicarb but gland stimulation was not.

Plant-parasitic nematodes feed by inserting their buccal stylet into host cells, by modifying cellular components, and by withdrawing cell contents (5,15). Advances in video-enhanced microscopy (16) have provided valuable information regarding the processes of penetration, intracellular migration, initiation of feeding sites, and the mechanical aspects of feeding. However, the biochemical nature of the nematode secretion, believed to be responsible for host cell perturbations, is largely unknown. Poehling and Wyss (10) analyzed proteins from *Xiphinema* index and host tissues and found two acid glycoproteins in the esophageal bulb that may have been constituents of salivary granules within the dorsal gland cell.

Reddigari et al (11) isolated subcellular granules from homogenates of preparasitic juveniles (J2) of *Meloidogyne incognita* and found a structural resemblance between those granules and secretory granules of the subventral esophageal glands. The proteinaceous components of the subcellular granules were separated electrophoretically, and the amino acid content of each fraction was determined. Homology between the subcellular granules and the secretory granules of the subventral glands was not confirmed.

A more direct approach has been taken by Veech et al (*personal communication*), who removed stylet exudate from the heads of adult *Meloidogyne* spp. females with glass microneedles. Electrophoretic analysis of pooled exudates revealed the presence of at least nine major protein bands, three of which were probably glycoproteins.

As yet, however, it has not been possible to collect, directly, stylet exudate from preparasitic J2. Moreover, stylet exudate from J2 nematodes has not been observed in vitro. Because host cell alterations are evident within hours of root penetration by J2 of *M. incognita* (8), and because stylet secretions are thought to play an important role in development of feeding sites (1,11), we have examined a number of compounds for their ability to stimulate salivary secretions in *M. incognita* and other nematodes. Our investigation was based on an unpublished observation that live J2 nematodes, mounted in various dyes on microscope slides and sealed with nail varnish, frequently showed stylet thrusting activity. Mixing separate components of nail varnish into petroleum jelly, which was then used to seal the coverslips, showed that toluene was the active component. Our purpose was to determine whether additional compounds structurally similar to toluene would also induce feeding responses and to examine these responses at the histological and cellular levels of expression. A number of neurotransmitters (substances involved in cell-to-cell transmission of action potentials) were included because of their structural relationships to certain of the naturally occurring plant

derivatives, such as catechol and caffeic acid, which induced salivation. Aldicarb, a known cholinergic inhibitor, also was included.

MATERIALS AND METHODS

Eggs of *M. incognita* race 3 (7) were collected (9) and hatched (3) to obtain infective juveniles. Hatched juveniles were collected daily and rinsed with distilled water before use. Cysts of *Heterodera schachtii* were collected from infected sugar beets (*Beta vulgaris*) by centrifugal flotation (6), and hatched juveniles were collected daily by the method used for *M. incognita*. Mixed populations of *Ditylenchus dipsaci*, onion race, were obtained by incubating infected garlic (*Allium sativum*) in distilled water, and collecting the nematodes after 8–10 hr. *Aphelenchus avenae* was reared on *Rhizoctonia solani* on potato-dextrose agar and extracted for use by placing pieces of the agar in distilled water. *Scutellonema brachyurum* and *Xiphinema americanum* (broad sense) were extracted (13) from soil around the roots of *Nerium oleander*.

Electron microscopy. Nematodes for electron microscopy were fixed in 3% glutaraldehyde in 0.06 M phosphate buffer, pH 7.3, for 90 min at 21–24 C, rinsed three times with five volumes of phosphate buffer, postfixed with 1.5% osmium tetroxide in phosphate buffer, and dehydrated in an acetone series (10% increments). Dehydrated specimens were infiltrated with Spurr's B in acetone (10% increments), and the blocks were cured at 60 C for 18–24 hr. Silver-gold sections were cut and mounted on 74-μm (pore size) copper grids. Mounted sections were stained at room temperature for 20 min in 2% aqueous uranyl acetate, poststained with Reynold's lead citrate for 10 min, and photographed on a Hitachi H-500 electron microscope at 75 kV.

Experimental methods. Chemically treated nematodes were observed on glass microscope slides at ×400–1,000 with either bright-field or interference contrast optics. Two methods of treatment were used to accommodate compounds with different physical properties: 1) toluene, xylenes, and guaiacol (10 μl), volatile substances with low solubility in water, were mixed individually on a glass slide with 100–200 mg of white petroleum jelly. A ring of the mixture was then applied to a clean glass slide, and 10 μl of the nematode suspension in 0.05% Coomassie Brilliant Blue G (Sigma Chemical Co. St. Louis, MO, stain B 1131) was placed inside the ring. A coverslip was quickly applied with slight pressure from forceps to make an airtight seal and to spread the nematode suspension until it made contact with the petroleum jelly, which contained the test chemicals. Controls consisted of nematodes, similarly mounted, with untreated petroleum jelly. For catechol, resorcinol, hydroquinone, caffeic acid, protocatechuic acid, serotonin, acetylcholine, histamine, octopamine, epinephrine or dopamine, water-soluble substances with low volatility, 10 μl of a 1.0% (w/v) solution was mixed in a 1.5-ml polyethylene microcentrifuge tube with 10 μl of nematode suspension containing 0.1% (w/v) Coomassie Brilliant Blue G. A 10-μl aliquot was then pipeted into a previously formed ring of petroleum jelly (no chemicals added) on a microscope slide and sealed with a coverslip as described previously. Propylene phenoxytol (Goldschmidt Chemical, 153 Waverly Place, New York, NY) was similarly tested at a final concentration of 0.5% (w/v). Controls consisted of nematodes in 0.05% Coomassie Brilliant Blue G. Three histological stains also were found to have an effect on *M. incognita* (N. von Mende and M. McClure, *unpublished*): eosin B, rose bengal, and acridine orange. They were tested by mixing 20 μl of a 0.1% (w/v) solution with an equal volume of nematode suspension and sealing a 10-μl portion under a coverslip on a microscope slide with petroleum jelly. Aldicarb granules (Temik 15G), obtained locally, were soaked in distilled water and the supernatant diluted to a concentration of 40 ppm active ingredient. All treatments were repeated three or four times, and hundreds of specimens per treatment were observed.

RESULTS

Depending on the species of nematode and the chemical applied, several reactions were observed (Tables 1 and 2). *Aphelenchus avenae, H. schachtii,* and *M. incognita* responded to treatment with toluene by stylet thrusting, which was first observed in those nematodes closest to the edge of the coverslip. Stylet thrusting of *A. avenae* was sporadic, rapid, and uncoordinated, whereas that of *M. incognita* appeared more regular and controlled. Frequency of thrusting by *M. incognita* was variable, averaging about one thrust per second. Frequency was not measured in the other species tested but was noticeably slower in *H. schachtii* than in *M. incognita*. Stylet thrusting by *S. brachyurum* and *D. dipsaci* was not observed. Nematodes exposed to catechol responded more rapidly, probably because they were submerged in this compound from the beginning of the experiment. *M. incognita* in catechol solutions began to thrust their stylets almost immediately, and stylet and/or amphidial exudate was observed within 5 min (Figs. 1A, 2B, 3B, and 4B). Most nematodes became quiescent or moved slowly after 90 min or more in 0.5% catechol. However, nematodes near the edges of the coverslip moved more rapidly and continued to move for up to 24 hr. Occasionally, massive accumulation of exudate was observed on the heads of treated nematodes (Fig. 4B), but the origin of the exudate was difficult to determine. Similar reactions were observed with *M. incognita* in resorcinol, caffeic acid, guaiacol, hydroquinone, and xylene. Eosin, rose bengal, and acridine orange, 0.05% (w/v), induced stylet movement and salivary secretion. Protocatechuic acid did not induce a response. Serotonin induced vigorous stylet thrusting by *M. incognita*, whereas aldicarb induced a weaker response, although both induced small amounts of stylet exudate to form (Table 2). *M. incognita* produced cephalic exudate in epinephrin, but stylet movement with this treatment was not observed. Treatment with other neurotransmitters, such as acetylcholine, did not induce stylet thrusting, and treatments with neurotransmitters or aldicarb did not stimulate the esophageal glands. *H. schachtii* became

TABLE 1. Effects of catechol and toluene on plant-parasitic nematodes

Nematode	Reaction	
	Catechol[a]	Toluene[b]
Aphelenchus avenae	None. Nemas immobilized within 2 hr	Rapid stylet thrusting begins within 30 min
Ditylenchus dipsaci	None. Nemas immobilized within 45 min	None
Heterodera schachtii	Nemas quiescent after 90 min, 10–20% with sporadic stylet movement. Some after 1–2 hr with stylet exudate	Regular stylet thrusting after 30 min. Some nemas with stylet exudate
Meloidogyne incognita	Stylet thrusting of some J2 begins almost immediately. Stylet and amphidial exudate within 5 min	Stylet thrusting begins within 10 min in nemas near edge of coverslip. Stylet and amphidial exudate follow. Some nemas still active after 24 hr
Scutellonema brachyurum	None. Nemas immobilized within 45 min	None. Nemas immobilized in 3 hr
Xiphinema americanum	Nemas immobilized in 5 min with stylets extended. Some with stylet exudate after 2 hr	None. Some nemas active up to 3 hr

[a] Catechol (0.5%) in distilled H_2O containing 0.05% Coomassie Blue.
[b] Toluene (10 μl) in about 150 mg of petroleum jelly used to seal microscope slide coverslip.

lethargic after 90 min in catechol, and movement was generally restricted to the head and stylet. Stylet extension occurred after 90–120 min, and a small amount of exudate was observed emanating from the ventral side of the stylet tip (Fig. 3A). The stylet was then slowly retracted, and the exudate deposited on the edge of the mouth. A few J2 (< 10%) nematodes began to produce stylet exudate soon after treatment with catechol and continued for up to 3 hr, resulting in accumulation of exudate on the head (Fig. 4D).

Stylet movement of *Xiphinema* was restricted to irreversible stylet extension when adult females or juveniles were placed in catechol. Of those with extended stylets, 15–20% produced stylet

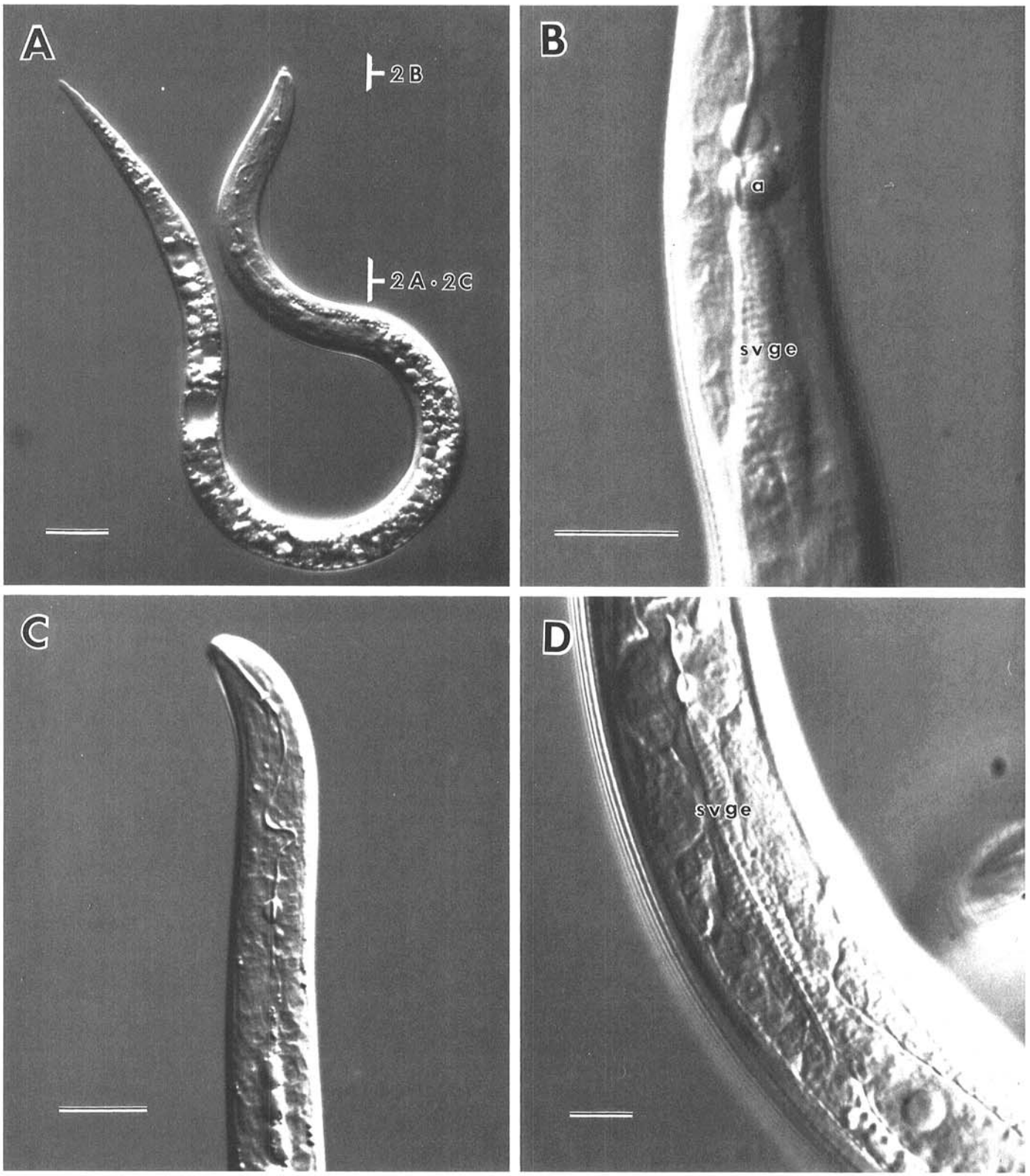

Fig. 1. Interference contrast micrographs of nematodes treated with elicitors of salivary responses. **A,** *Meloidogyne incognita* J2 after 3 hr in 0.5% resorcinol (without stain). Note stylet exudate at the oral aperture and the swollen ampullae of the subventral glands. Numbers and letters refer to the areas represented in Figure 2. **B,** *M. incognita* after 90 min in 0.5% catechol with 0.05% Coomassie Blue. Anterior extensions of the subventral gland (svge) and their ampullae (a) are swollen and packed with secretory granules. **C,** *M. incognita*, untreated control. **D,** Dorsal esophageal gland and anterior extensions of the subventral glands (svge), with secretory granules, in *Heterodera schachtii* treated for 90 min with 0.5% catechol and 0.05% Coomassie Blue. Scale bars = 10 μm.

exudate, which originated near the stylet tip on the dorsal side (Fig. 3C). Unstained exudate from *M. incognita* was observed in 0.5% resorcinol without Coomassie Blue (Fig. 1A). Coomassie Blue, alone, did not induce exudation from any of the tested nematode species.

Subventral esophageal gland extensions and ampullae of *M. incognita* became swollen and packed with secretory granules when J2 nematodes were exposed to catechol (Fig. 1B), resorcinol (Fig. 1A), hydroquinone, and propylene phenoxytol (Fig. 4A). Gland extensions and ampullae of untreated *M. incognita* (Fig. 1C) contained only a few secretory granules, and the ampullae were not dilated. Stylet movement of *M. incognita* was positively correlated with stimulation of the subventral glands, and J2 nematodes without glandular stimulation seldom exhibited stylet

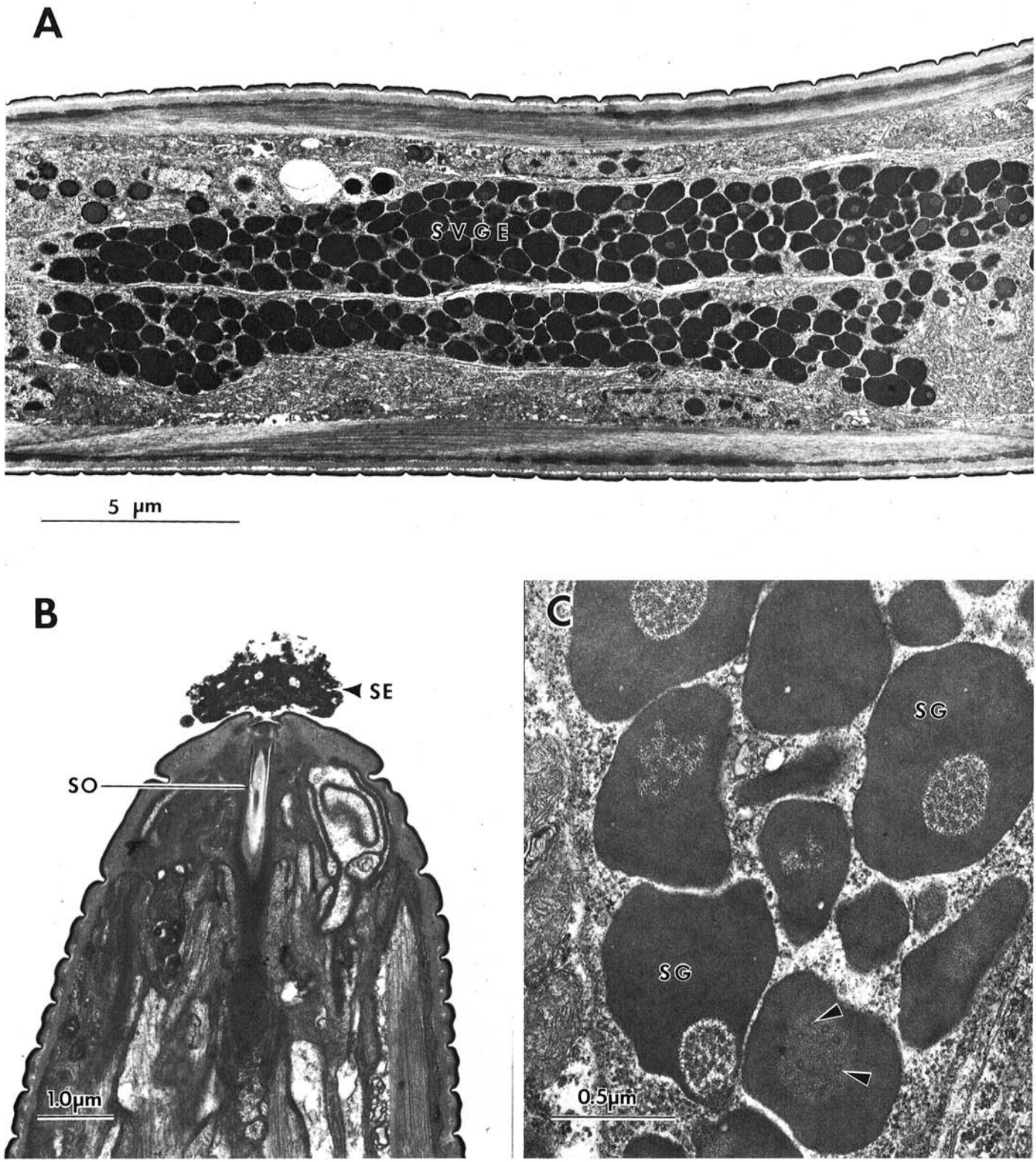

Fig. 2. Transmission electron micrographs of *Meloidogyne incognita* (see Fig. 1A). **A,** Parasagittal section through the anterior subventral gland extensions (svge) of a J2 treated with toluene. Gland extensions are swollen and packed with secretory granules. **B,** Parasagittal section through the head of a J2 treated for 90 min with 0.5% catechol. Stylet exudate (se) that had emanated from the stylet orifice (so) collected at the oral aperture. **C,** Details of the secretory granules (sg) in an untreated J2. Granules are membrane-bound and contain a core with minute vesicles or spherical bodies (arrows).

movement. Fine structure of the glands and their secretory granules in treated *M. incognita* (Fig. 2A) did not differ from that of untreated controls (Fig. 2C). In both cases, the granules were membrane-bound and contained regions that stained differentially. The core material of some granules was observed close to the outer membrane through which core materials appeared to discharge (Fig. 2C). The core itself differed in density between granules and contained, in some cases, minute vesicles or spherical bodies (Fig. 2C).

Subventral gland extensions of untreated *H. schachtii* (Fig. 1D), *S. brachyurum,* and *D. dipsaci* (Fig. 4C) generally contained secretory granules. The number of granules was not increased and ampullae were not distended by any treatment. Secretory granules were not observed in the dorsal gland ampulla of *M. incognita* but often were present in the dorsal gland ampulla of untreated *H. schachtii.*

DISCUSSION

Plant cell modifications induced by parasitic nematodes result when the cell is penetrated by the nematode stylet and feeding begins. It is presumed that cellular changes are initiated and controlled by salivary secretions that originate in the esophageal glands. The secretions most likely are released from secretory granules, which are synthesized in the basal portion of the glands and transported to the stylet via the esophageal lumen during feeding (16). Histochemical and physical/chemical analyses of granules from J2 of *Meloidogyne* (1,11) and of stylet exudate from adult females of *Meloidogyne* (2; J. Veech, *personal communication*) have shown that salivary secretions contain proteins, but the precise chemical and functional properties of these materials are not known.

Efforts to extend current understanding of the means by which nematodes affect host cells have been hampered by lack of a method for obtaining stylet exudate from J2 nematodes. Indeed, preparasitic J2 are not known to produce stylet exudate in vitro. However, induction of salivary secretions by chemical elicitors should provide a method for obtaining quantities suitable for analysis.

It is not known if the secreted material induced by treating *Meloidogyne* with toluene and other substances originates from the dorsal or subventral glands. However, the accumulation of secretory granules in the ampullae of the subventral glands, and the positive correlation between granule accumulation and stylet thrusting, suggest that the secretions may have their origins in the subventral glands. The paucity of secretory granules in the dorsal gland extension, including the ampulla, also indicated that the dorsal gland did not contribute significantly to the induced secretions.

In contrast, a more prominent role for the dorsal gland of *H. schachtii* can be surmised from our observations of secretory granules in the dorsal gland ampulla and analysis of video films (16), which clearly illustrate a constriction of the posterior metacorpal muscles during feeding, a function that would preclude anterior movement of subventral gland secretions. Because *M.*

TABLE 2. Responses of *Meloidogyne incognita* J2 to neurotransmitters[a] and a cholinesterase-inhibiting nematicide[b]

Compound	Response			
	Body movement[c]	Stylet movement	Stylet amph. exudate	Esophageal gland stimulation
Acetyl choline	Yes	No	No	No
Dopamine	Yes	No	No	No
Epinephrin	Yes	No	Yes	No
Histamine	Yes	No	No	No
Octopamine	Yes	No	No	No
Serotonin	Yes	Yes	Yes	No
Aldicarb	Yes	Yes	Yes	No

[a] Compounds at 0.5%, w/v, containing 0.05% Coomassie Blue. Tests conducted on glass microslides sealed with petroleum jelly.
[b] Aldicarb (40 ppm).
[c] After 18 hr.

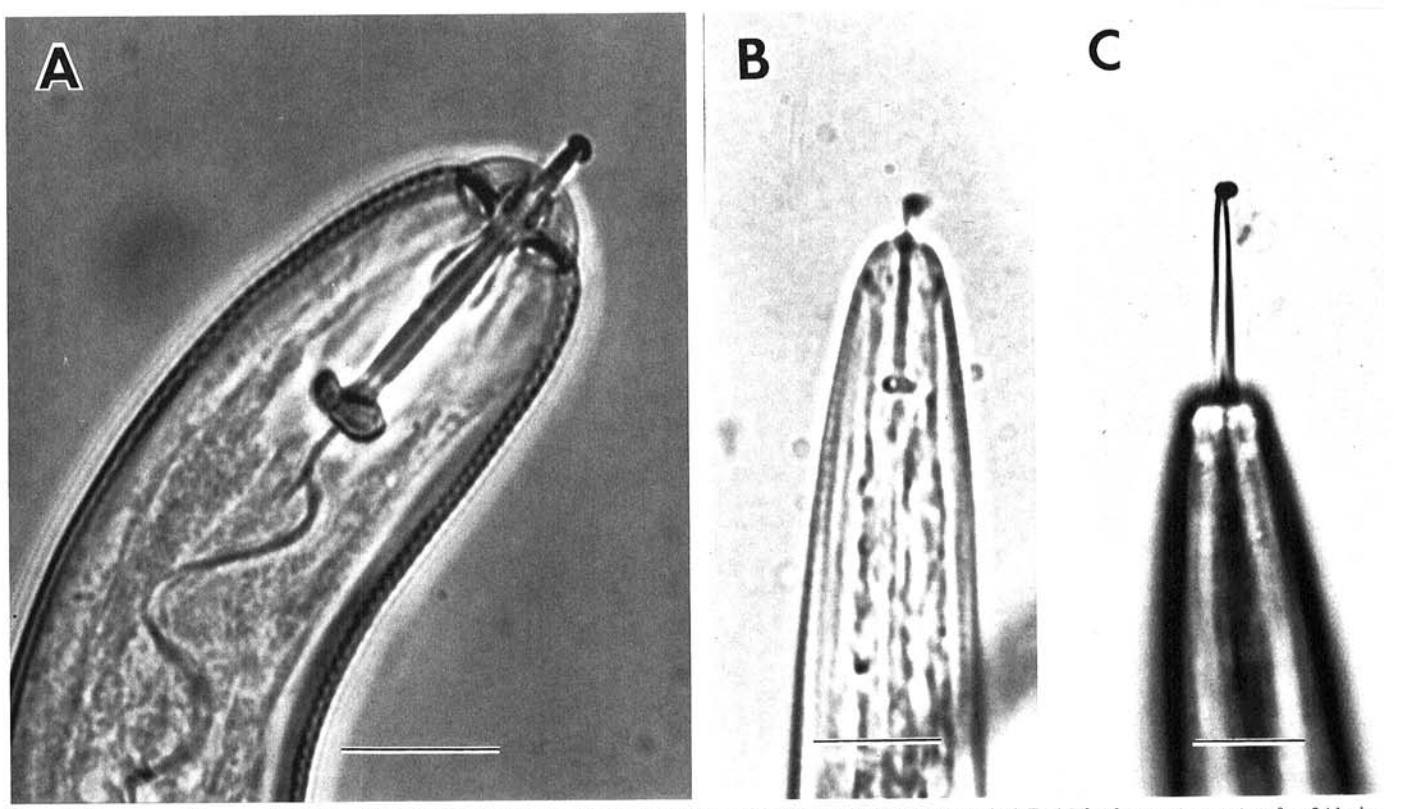

Fig. 3. Salivary secretions on stylet tips of treated nematodes. **A,** *Heterodera schachtii* after 90 min in 0.5% catechol. **B,** *Meloidogyne incognita* after 24 hr in 0.5% resorcinol. **C,** *Xiphinema americanum* after 2 hr in 0.5% catechol. All treatments included 0.05% Coomassie Blue. Scale bars = 10 μm.

incognita and *H. schachtii* differ in their host range, mode of host penetration, migration through host tissues, and initiation of feeding sites, differences in the origin and composition of their salivary secretions also can be expected.

Stylet thrusting of nematodes in distilled water is not common. It has been induced by treatment with aldicarb, a carbamoyloxime nematicide, which is thought to act on muscle cell transduction processes of the stylet protractors (14). We have confirmed the effect of aldicarb on stylet movement and have observed minute amounts of stylet exudate on stylets of treated *M. incognita* J2. Serotonin was the only other neuroregulatory material tested that induced stylet movement and/or stylet exudate. Negative

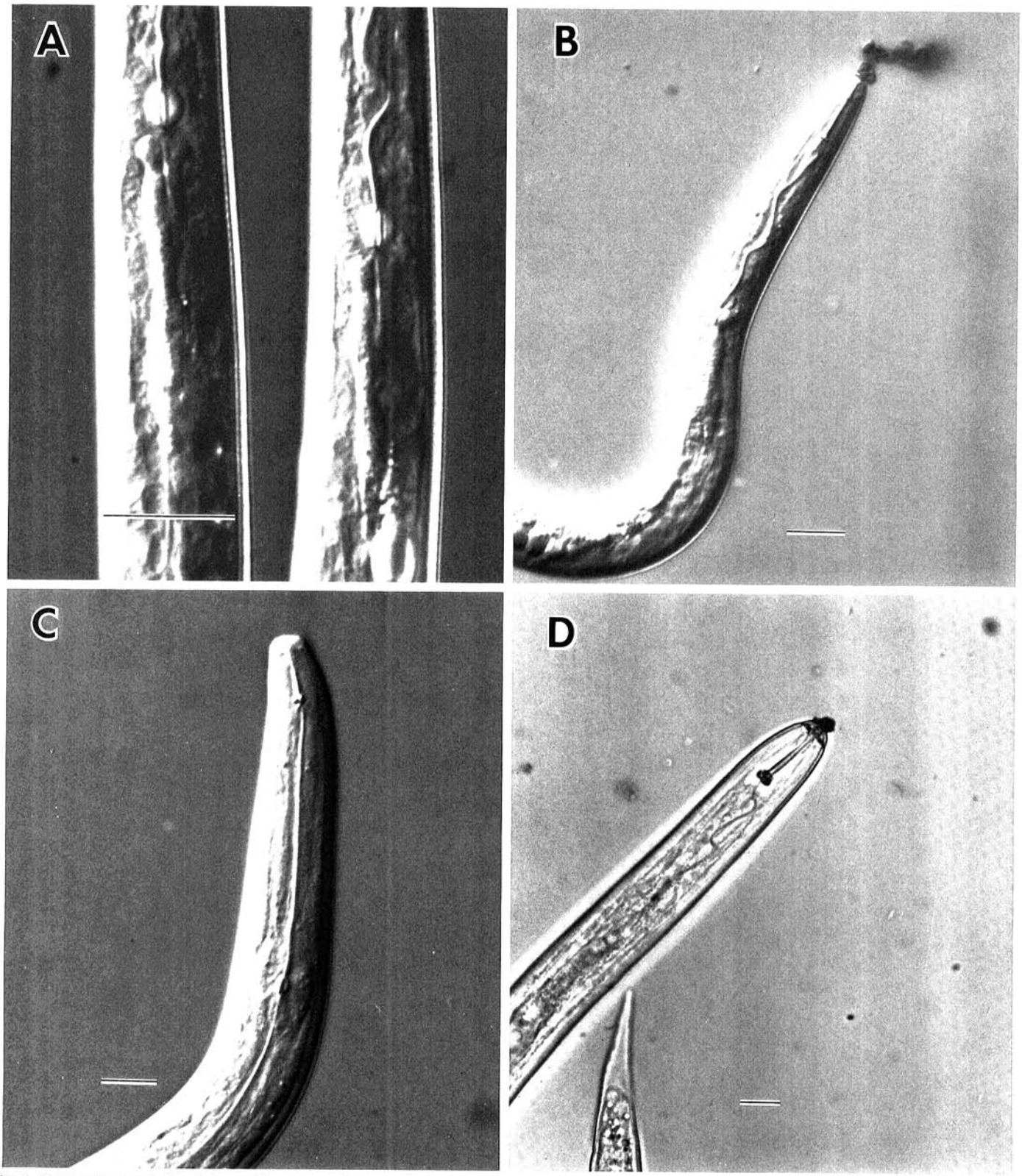

Fig. 4. Nematodes treated to induce salivary secretions or stimulation of the esophageal glands. **A,** *Meloidogyne incognita* after 1 hr in propylene phenoxytol. Subventral gland extensions and ampullae are swollen and packed with secretory granules. **B,** Massive exudation from the head of *M. incognita* in 0.5% catechol for 1 hr. **C,** *Ditylenchus dipsaci* after 1 hr in 0.5% catechol. **D,** Accumulation of stylet exudate on the head of *Heterodera schachtii* after 90 min in 0.5% catechol. Treatments in B, C, and D included 0.05% Coomassie Blue. Scale bars = 10 μm.

responses, however, were difficult to evaluate because it is not known if these substances were capable of transport across nematode membranes to putative receptor sites. In some cases, it was difficult to determine the origin of exudates that formed on the heads of the nematodes. Unless the stylet was extended, with exudate at its tip, or unless secretion of the exudate could be observed emanating from the oral aperture, it is possible that some, or all, of the exudate could have originated from the amphids. About 20% of J2 nematodes treated with epinephrin produced exudate, but the amphidial canals were stained, which would suggest secretory activity.

None of the neurotransmitters we tested stimulated the esophageal glands of *M. incognita*, an indication of a different mode of action for epinephrin and serotonin than for the catecholllike compounds. Nevertheless, plant derivatives of dihydroxy benzene (catechol, caffeic acid, hydroquinone, etc.) share structural homologies and metabolic pathways with known neurotransmitters (4) and a neurohormonal mechanism could be postulated for those materials to which nematodes respond with feeding activities. Catechol, for example, is known to exist in the roots and root exudate of *Eragrostis curvula* in concentrations capable of reducing infections of *Meloidogyne* on susceptible hosts (12). Caffeic acid and other coumarins are widely distributed in the plant kingdom, and these substances may play a regulatory role in nematode feeding and pathogenesis.

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