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In Vitro Culture and Feeding Behavior of *Belonolaimus longicaudatus* on Excised *Zea mays* Roots

XIANG HUANG AND J. OLE BECKER[1]

Abstract: A greenhouse population of the sting nematode, *Belonolaimus longicaudatus,* obtained from an infested golf course in California's Coachella Valley, was surface-decontaminated and cultured on excised roots of *Zea mays* supported by Gamborg's B5 medium. At 26–27 °C the females laid eggs, and newly emerged juveniles of the second generation completed three molts within 29 days after egg deposition. Sixty days after inoculation with 60 females and 40 males, an average of 529 nematodes and 83 eggs were recovered from the culture. The feeding process consisted of probing, stylet penetration, ingestion, and stylet retraction. Feeding seemed to be necessary before egg deposition or molting occurred. The sting nematode was observed feeding exclusively as an ectoparasite and preferably at the region of cell division and elongation. Vigorous feeding by many nematodes usually caused discoloration of root tips and termination of growth.

Key words: Belonolaimus longicaudatus, corn root culture, feeding behavior, in vitro culture, nematode, sting nematode, *Zea mays.*

The sting nematode, *Belonolaimus* spp., is one of the most destructive agricultural nematode pathogens in the southeastern United States. A large number of plants, including many agricultural crops, turf grasses, and forage grasses, are hosts (Robbins and Barker, 1973), and the damage to hosts is often serious (Smart and Nguyen, 1991). The nematode is a quarantined pest in California. However, in 1994, *B. longicaudatus* was found to be associated with dying turfgrass in eight Coachella Valley golf course sites in California, and in two home lawns near one of the golf courses (Mundo-Ocampo et al., 1994). Due to its wide host range, there is concern about the potential spread and threat of *B. longicaudatus* to major agricultural industries in southern California's inland deserts.

Although the sting nematode has been known since the 1940s (Steiner, 1949), details of its life cycle and host-parasite interactions are unknown (Perry and Rhoades, 1982; Smart and Nguyen, 1991). This may be due partly to the difficulties encountered when culturing this ectoparasite in vitro, as well as the availability of nematicides, which have been very effective against *B. longicaudatus* (Smart and Nguyen, 1991). However, most of these pesticides are no longer available in California. The in vitro culture of plant-parasitic nematodes permits continuous observation of nematode development. Many nematodes have been cultured on various plant tissues, though only a few of them are ectoparasites (Dasgupta et al., 1970; Dolliver et al., 1962; Khera and Zuckerman, 1962; McElroy and Van Gundy, 1968; Zuckerman, 1971).

The objective of this study was to observe the feeding behavior and development of *B. longicaudatus* in monoxenic culture on excised corn roots.

MATERIALS AND METHODS

Corn root culture: Excised corn (*Zea mays* L. cv. Golden Jubilee) roots were cultured on Gamborg's B5 medium (Huettel and Rebois, 1985) in 10-cm petri dishes. Corn seeds were surface-sterilized in 75% alcohol for 3 minutes, transferred to a 15% solution (0.79% NaOCl) of commercial bleach for 15 minutes, rinsed in sterile water three times, and germinated on 1.2% water agar. Seedling root tips of at least 3-cm length were excised and transferred onto B5 media. The root cultures were incubated in darkness at 26–27 °C and inoculated with nematodes within 1 week.

Surface decontamination of nematodes and in-

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[1] Department of Nematology, University of California, Riverside, CA 92521.

E-mail: ole.becker@ucr.edu

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oculation of roots: The procedure used was a modification of a method previously described by Huang (1995). *Belonolaimus longicaudatus* was obtained from infested turf grass at Tamarisk Golf Club, Rancho Mirage, California, and cultured on bermudagrass in sandy soils in the greenhouse of the Nematode Quarantine Facilities at the University of California, Riverside. Nematodes were extracted with a combination of wet sieving and centrifugal flotation (Jenkins, 1964). The sting nematodes were handpicked into sterile osmotic water in a 3.5-cm petri dish. Under aseptic conditions and with the assistance of a dissecting microscope, adult nematodes were embedded, using a fine needle, in the center of a 10-cm petri dish containing 1.0% agar. The dishes were incubated in darkness at 26–27 °C overnight. The nematodes were considered surface-decontaminated after migrating through the agar for at least 2 centimeters. Nematodes were then aseptically transferred onto nutrient agar (beef extract 3 g, peptone 5 g, agar 15 g, distilled water 1 liter, pH 6.0) (Difco Laboratories, Detroit, MI) in Tri-Petri dishes (Lab-Tek, Naperville, IL), with one nematode in each section, and incubated in darkness for 24 hours. Nematodes in sections in which no bacterial or fungal colonies appeared were used to inoculate excised root cultures. Each of four dishes was inoculated with 60 females and 40 males, and sealed with Parafilm (American National Can Company, Neenah, WI) to reduce evaporation. The cultures were maintained in darkness at 26–27 °C. Subcultures of *B. longicaudatus* were maintained by aseptically transferring 60 females and 40 males from an old culture onto a fresh root culture plate every 8 weeks.

All the dishes were observed with a dissecting microscope and an inverted compound microscope (Carl Zeiss, Oberkochen, Germany).

Results

Reproduction of the sting nematodes: After inoculation, *B. longicaudatus* adults fed on corn roots and females began to lay eggs. Four days after egg deposition, the first-stage juveniles molted in the egg (Fig. 1A). Second-stage juveniles (J2) hatched 5 days after egg deposition. Three subsequent molts occurred within 29 days after egg deposition. An average of 529 ± 27 nematodes and 83 ± 8 eggs were counted 60 days after inoculation (Table 1). This population of *B. longicaudatus* was subcultured and maintained monoxenically for more than 10 months.

Observations on feeding behavior: After inoculation near the root tips, *B. longicaudatus* moved actively on the surface or within the nutrient agar medium. Some sting nematodes went directly to the root tip. For convenience, description of the feeding behavior was divided into four periods: probing, stylet penetration, ingestion, and stylet retraction.

After arrival at the root tip, the nematodes moved around the root tip and contacted the root surface by rubbing the lip region against the epidermal cells at different sites. During the rubbing, the stylet began probing the root, typically once at each site. The nematode arched its anterior body so as to bring the stylet down perpendicular to the root surface (Fig. 1B). The initial probing process lasted from 1 to 30 minutes, during which the nematode sometimes left one root and initiated probing in another.

After initial probing, the nematode selected a region near the root tip and the probing accelerated to the rate of 2–3 times per second for 20–30 seconds. The frequency of probing then decreased to 1–2 times per second but with increased intensity. Finally, the stylet tip was inserted into the root and penetrated slowly as the stylet moved back and forth, with the lips pressed onto the root surface. At this point the valve of the esophageal medium bulb began pulsating at a rate of about once per second. The stylet continued to penetrate deeper into the root tissue until the esophageal lumen was almost straight, allowing about half of the stylet to penetrate into the root tissue (Fig. 1C). The penetration took 3–7 minutes, and no body movement was observed to aid stylet insertion.

The nematode then became quiescent for 1–2 minutes with cessation of valve pulsa-

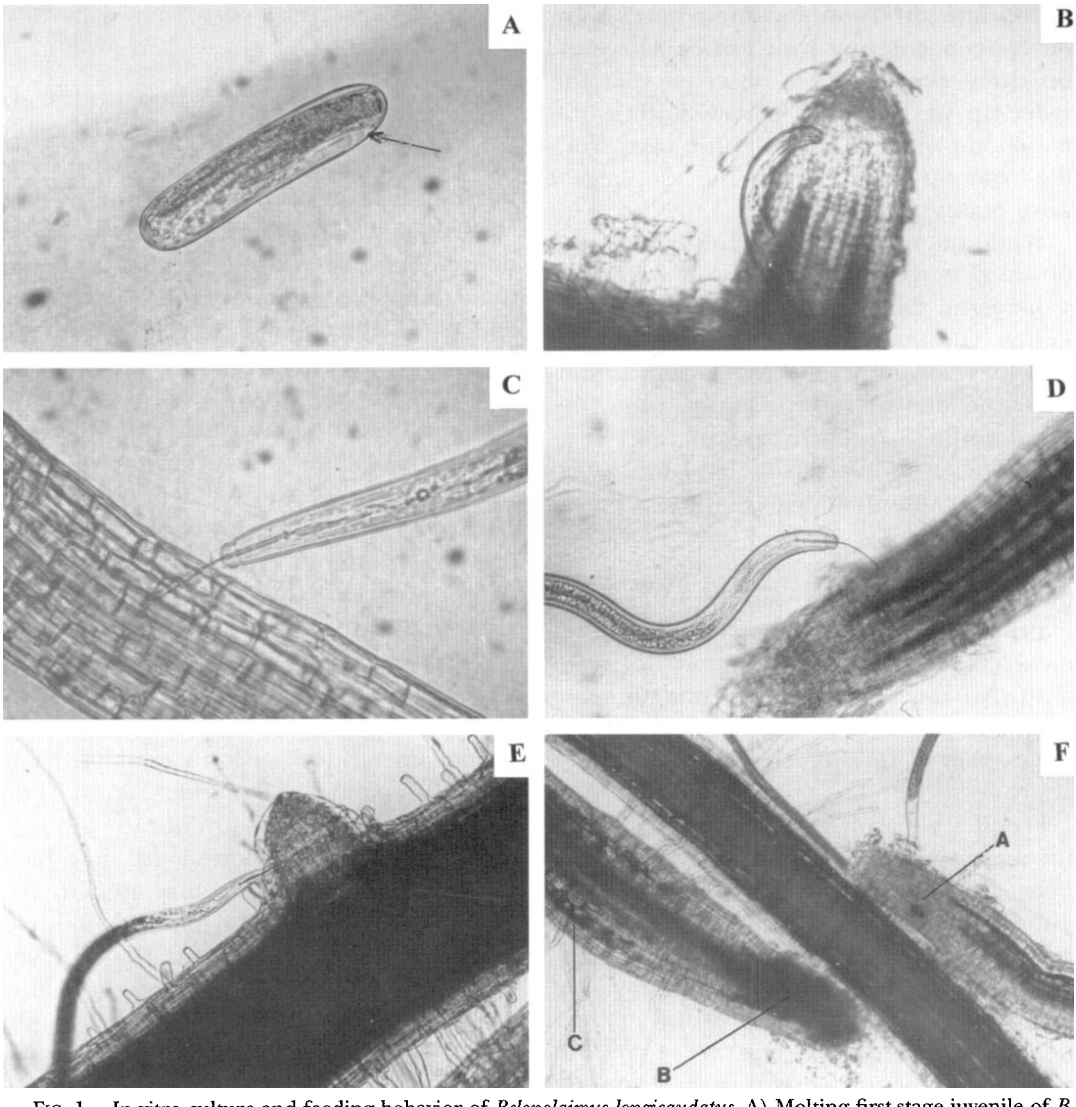

FIG. 1. In vitro culture and feeding behavior of *Belonolaimus longicaudatus*. A) Molting first-stage juvenile of *B. longicaudatus* in egg (×210), arrow = old cuticle. B) Adult rubbing lip region against root surface during probing (×105). C) Nearly straight esophageal lumen following stylet penetration (×210). D) *B. longicaudatus* adult removing stylet from root (×105). E) *B. longicaudatus* feeding on a root initial (×71). F) Parasitized corn roots (×35). Brown lesion that developed at the feeding site (A); brown root tip that stopped growing in response to feeding by many nematodes (B); swollen root behind lesion (C).

tion, followed by resumed valve pulsation at the rate of about 3–6 times per second, which remained constant till the end of feeding. Presumably the ingestion of plant nutrients began when valve pulsation resumed. Feeding in this manner usually lasted from 1 minute to 12 hours but occasionally occurred for more than 48 hours. During the ingestion period, the nematode body remained stationary. The nematodes fed strictly as ectoparasites with the stylet in-

TABLE 1. Reproduction of *B. longicaudatus* on excised corn roots.

Days after inoculation	Number of nematodes and eggs present				
	Females	Males	Juveniles	Eggs	Total
0	60	40	0	0	100
60	95 ± 6	94 ± 4	340 ± 17	83 ± 8	612 ± 35

Data are means of four replicates (± SE).

serted into the root, and their heads were never seen entering root tissues. However, on three occasions, we observed that the stylet tip was partially extruded while the nematode remained 50–100 µm away from the root tip surface, with the esophageal valve pulsating slowly.

When the nematode was finished feeding or was disturbed by vibration or the microscope light, the valve gradually stopped pulsating. The nematode withdrew its body to pull its stylet out of the root within 5 minutes, and then retracted the stylet into the body within 5 seconds to 3 minutes (Fig. 1D). The stylet was flexible and was sometimes bent almost 90 degrees without breaking.

Adequate feeding usually resulted in the nematode's intestine being full of globular material. After stylet retraction, juveniles moved away from the root and began to molt. No molting or mating was observed during the feeding process. Eggs formed in the uteri of mature females either during or after feeding.

Feeding behavior occurred more often at the agar interphase with the bottom of the petri dish than on the surface of the medium. Sting nematodes most often fed at the region of cell division or elongation (Fig. 1E). In a few instances involving young roots, feeding was observed at the maturation zone close to the elongation region. No major differences were observed among feeding behaviors of females, males, or juveniles, and feeding occurred at each developmental stage starting with J2, and before egg deposition.

Brown lesions typically appeared at the feeding site 12 to 24 hours after initiation of feeding, and sometimes the root also became slightly swollen behind the lesion. Further attacks on the same root occurred only at lesion-free sites. If attacked by many nematodes at the same time, the whole root tip turned black, tissue became disrupted, and the root tip stopped growing (Fig. 1F). Multiple feedings on primary root tips also resulted in the occurrence of lateral roots and proliferation of root hairs near the feeding sites.

Discussion

As ectoparasites, sting nematodes are more difficult to culture than endoparasites because they spend their life in the nutrient medium instead of inside the root. All suitable biological, chemical, and physical conditions must be provided for all stages of nematodes (Dasgupta et al., 1970; McElroy and Van Gundy, 1968). In pot cultures, reproduction of *B. longicaudatus* can be significantly influenced by soil type, particle size, temperature, and moisture (Robbins and Barker, 1974), and similar factors will affect the nematodes in nutrient agar media. This research, which is the first report of the successful continuous culture of sting nematodes on excised corn roots, has significant application for in-depth studies on the biology, physiology, and behavior of these nematodes.

The production of axenic nematodes is one of the critical steps in establishing in vitro plant-parasitic nematode culture. Many disinfectants such as antibiotics, hibitane diacetate, mercuric chloride, etc. have been employed to surface-sterilize nematodes. However, some of them are either inefficient or toxic to the nematodes (Zuckerman, 1971). In preliminary studies, after sting nematodes were treated with a combination of 1% penicillin and 1% streptomycin solution, heavy contamination still occurred. Meanwhile, 0.01% or 0.001% mercuric chloride killed sting nematodes (unpubl.) even though this chemical was effective in axenizing *Aphelenchoides ritzemabosi* or cyst nematodes (Dolliver et al., 1962; Huettel and Rebois, 1985). Therefore, we used a physical means to surface-decontaminate the sting nematodes, which proved to be effective and did not adversely effect nematodes. The percentage of contaminated nematodes after treatment ranged from 0 to 8.8%. By discarding the contaminated nematodes and only inoculating with clean ones, sterility of the inoculum was maintained. Another advantage of this technique was that inoculum level and sex ratio were precisely controlled. Using this

method, we also succeeded in axenizing J2 of *Meloidogyne incognita,* males and J2 of *Heterodera schachtii,* and adults and juveniles of *Longidorus africanus* (unpubl.).

Observations on the feeding behavior of *B. longicaudatus* revealed that feeding seemed to be necessary for each molt and for egg deposition. Therefore, sting nematodes need to initiate feeding many times during their life cycle, especially for females, leading to serious damage to the host. Christie et al. (1952) reported that sting nematodes fed at root tips as well as along the root sides, and occasionally specimens were found within root tissues. However, in our observations of the cultured nematodes, *B. longicaudatus* fed strictly as an ectoparasite at or near root tips with only the stylet inserted into the root tissues. Feeding along root sides occurred only on newly initiated lateral root buds, showing a specialized relationship with type of host tissue fed upon.

When feeding, *B. longicaudatus* females were not seen to mate with males or to lay eggs, in contrast to observations of the ectoparasite *Helicotylenchus multicinctus* (Orion and Bar-Eyal, 1995). Unlike another ectoparasite, *Hemicycliophora arenaria* (McElroy and Van Gundy, 1968), *B. longicaudatus* never appeared to twist its body about the axis of the stylet to retract it after feeding.

Observations on reproduction of the sting nematode on corn root explants revealed that the nematode completed its life cycle in about 1 month, which is similar in length to observation from field experiments (Smart and Nguyen, 1991). Detailed studies of the life cycle are currently under way.

In vitro culture provides the opportunity to observe nematodes more closely in terms of behavior and interaction with the host. Furthermore, in vitro culture is more suitable for subtle manipulation of the chemical and physical environment than soil experiments. Manipulation of host tissue, medium components, temperature, osmotic pressure, pH, and other growth conditions in the culture of *B. longicaudatus* might lead to a better understanding of the host-parasite relationship and may contribute to improved management strategies for this pest.

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