# In vitro Cultivation of the Entomogenous Nematode Filipjevimermis leipsandra

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*Abstract:* The mermithid nematode, *Filipjevimermis leipsandra,* was successfully cultivated to the preadult stage in Schneider's Drosophila medium supplemented with 20% fetal bovine serum. Upon transfer to a solid substrate the preadults continued to develop into ovipositing adult females. Four molts were observed. The first molt occurred in the egg. The second occurred after 6–8 days in culture during which the very thin cuticle was shed completely. The third molt occurred after 18–20 days in culture; the cuticle was retained by the third-stage nematode. This stage was considered comparable to the preadult stage that emerges from host larva, *Diabrotica spp.* The fourth molt occurred within 12 days after the preadult was transferred from the liquid medium to a solid substrate. Adult females began ovipositing viable eggs 1–3 days after the final molt. *Key words: Diabrotica balteata,* banded cucumber beetle, mermithid.

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The mermithid nematode, *Filipjevimermis leipsandra* Poinar and Welch, is a destructive parasite of the banded cucumber beetle, *Diabrotica balteata* LeConte, and other chrysomelid beetles (1,3). It is unique among mermithids in being parthenogenic (6). The potential of this nematode as a biological control agent against root-feeding insect larvae has generated the need for its mass production to obtain inoculum for field studies (2).

Currently, the nematode is being mass reared in vivo with a production capacity of about 5 million eggs per week. The process is laborious and expensive because both the host insect and the parasite must be cultured simultaneously. In vitro mass production would have significant technical and economic advantages over the in vivo system. This paper describes techniques for in vitro culture of *F. leipsandra* through its complete life cycle.

## MATERIALS AND METHODS

Gravid females of *F. leipsandra,* reared on *D. balteata* larvae, were placed in Petri dishes with about 5 ml of sterile distilled water for oviposition. Eggs were removed from the dishes with a micropipette and their surface sterilized by immersing them for 10 min in a 0.26% sodium hypochlorite solution and rinsing three times with sterile distilled water. All subsequent procedures were performed under sterile conditions in a laminar flow work hood at room temperature (25 C). The surface-sterilized eggs were transferred to a bolting cloth sieve (71-$\mu m^2$ openings) on a Baermann funnel apparatus. As eggs hatched, 25–50 juveniles were transferred to 1 ml of culture medium in 15-ml screw cap, flat-bottomed, Leighton culture tubes. Preliminary studies on nematode growth were conducted with culture media consisting of Schneider's Drosophila (SD) medium (7) supplemented with either 20% calf, lamb, horse, or fetal bovine serum, brain extract, amniotic fluid, or $\beta$-ecdysone (an insect molting hormone). The cultures were held at 25 C for 20 days. Cultures were examined daily with an inverted microscope for growth and nematode activity.

As SD plus 20% fetal bovine was observed to be the most promising for nematode growth, it was selected for more detailed study. Every 2 days for 26 days an aliquot of the nematode culture was removed from culture tubes with a sterilized pipette and the nematodes stained lightly in acid fuchsin lactophenol at room temperature overnight. The cultures were replenished with an equal amount of fresh medium. The stained nematodes were mounted in glycerine on a slide to which a ring of ZUT, slightly thicker than the nematode, had been applied in order to minimize compression of the specimens by the cover slip (4).

Because the nematodes were usually arched or coiled, measurements were made

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from photomicrographs of the specimens. Negatives were projected through a photographic enlarger, and the images were traced on paper. Lengths of the tracings were measured with a map measurer; the width at the location of the gonad rudiment was determined with a metric rule. The magnification of the images was calculated from a photograph of a stage micrometer taken and enlarged at the same magnification for conversion of the traced images to actual size.

The effects of osmotic pressure of NaCl solutions and of a solid substrate were also examined as possible relevant factors in development of the preadult to maturity.

## RESULTS

The only successful cultivation of *F. leipsandra* in vitro to a stage equivalent to that of the in vivo preadult was obtained in SD medium containing 20% fetal bovine serum. After 20 days in SD medium supplemented with calf or lamb serum, juveniles developed only to a stage comparable to a 4-day-old nematode cultured in vivo. Supplements of horse serum, β-ecdysone, amniotic fluid, or brain extract had no stimulatory effect on nematode development (Table 1).

*Growth in SD plus fetal bovine medium:* Detailed observations of nematode growth in SD plus 20% fetal bovine serum disclosed that newly hatched juveniles (Fig. 1A) transferred from water to the medium continued their normal rapid serpentine movement for 1–2 days and then changed to a slow undulatory motion. The onset of growth was identified by the appearance of a rippled cuticle within 2–4 days (Figs. 1B, C). During the first 4 days, the length of the worms decreased but the width increased. For 6 days the worms grew little in length (Fig. 1D); thereafter, the length increased steadily, almost doubling from the 6th to the 10th day (Fig. 1D-F). The stylet was prominent during this period, but it did not change in length. The trophosome increased in size, and the stichocytes became enlarged and very distinct. The oval gonad rudiment of the juvenile grew from 18 μm to 32 μm and assumed a lunar shape when viewed laterally (Fig. 1E). The length of the nematode increased greatly between 12 and 14 days (Fig. 1G, H), tripling within a 48-h period (Table 2). During this period the gonad rudiment began to differentiate also, appearing peanut shaped as it elongated anteriorly and posteriorly (Fig. G). Periodic pulsations in the anterior pharynx were ob-

Table 1. The effect of various growth supplements* incorporated in Schneider's Drosophila medium on in vitro cultivation of *Filipjevimermis leipsandra* for 20 days.

| Supplement | Relative activity† |
|---|---|
| Fetal bovine | ++++ |
| Calf (heat inactivated) | +++ |
| Calf | +++ |
| Lamb | +++ |
| Porcine | ++ |
| New borne bovine | ++ |
| Amniotic fluid | – |
| Brain extract | – |
| Horse | – |
| β-ecdysone | – |

*Prepared as a 20% solution in Schneider's Drosophila medium.

†Based on gonad growth and/or nematode growth:

– = no gonad growth.
\+ = gonad semilunar shaped-nematode < .5 mm long.
++ = gonad semilunar and nematode 0.5–2 mm long.
+++ = gonad semilunar and nematode 2–5 mm long.
++++ = ovary outstretched > 5 mm long.

Table 2. Growth of *Filipjevimermis leipsandra* in Schneider's Drosophila medium supplemented with 20% fetal bovine.

| Days in culture | n | Length (mm) ± S.D. | | With (mm) ± S. D. | |
|---|---|---|---|---|---|
| 0 | 10 | .528 | .016 | .017 | .0004 |
| 2 | 13 | .403 | .040 | .016 | .0005 |
| 4 | 17 | .470 | .049 | .027 | .004 |
| 6 | 22 | .549 | .058 | .037 | .006 |
| 10 | 21 | .865 | .155 | .063 | .012 |
| 12 | 27 | 1.158 | .264 | .089 | .015 |
| 14 | 22 | 3.327 | .114 | .114 | .016 |
| 16 | 20 | 4.230 | 1.390 | .112 | .022 |
| 18 | 21 | 6.320 | 2.100 | .151 | .036 |
| 20 | 14 | 8.800 | 3.550 | .152 | .032 |
| 22 | 12 | 7.437 | 3.790 | .133 | .046 |
| 24 | 5 | 8.500 | 5.900 | .188 | .037 |
| 26 | 14 | 10.600 | 4.728 | .158 | .029 |
| 66 | 27 | 12.519 | 4.09 | .184 | .034 |

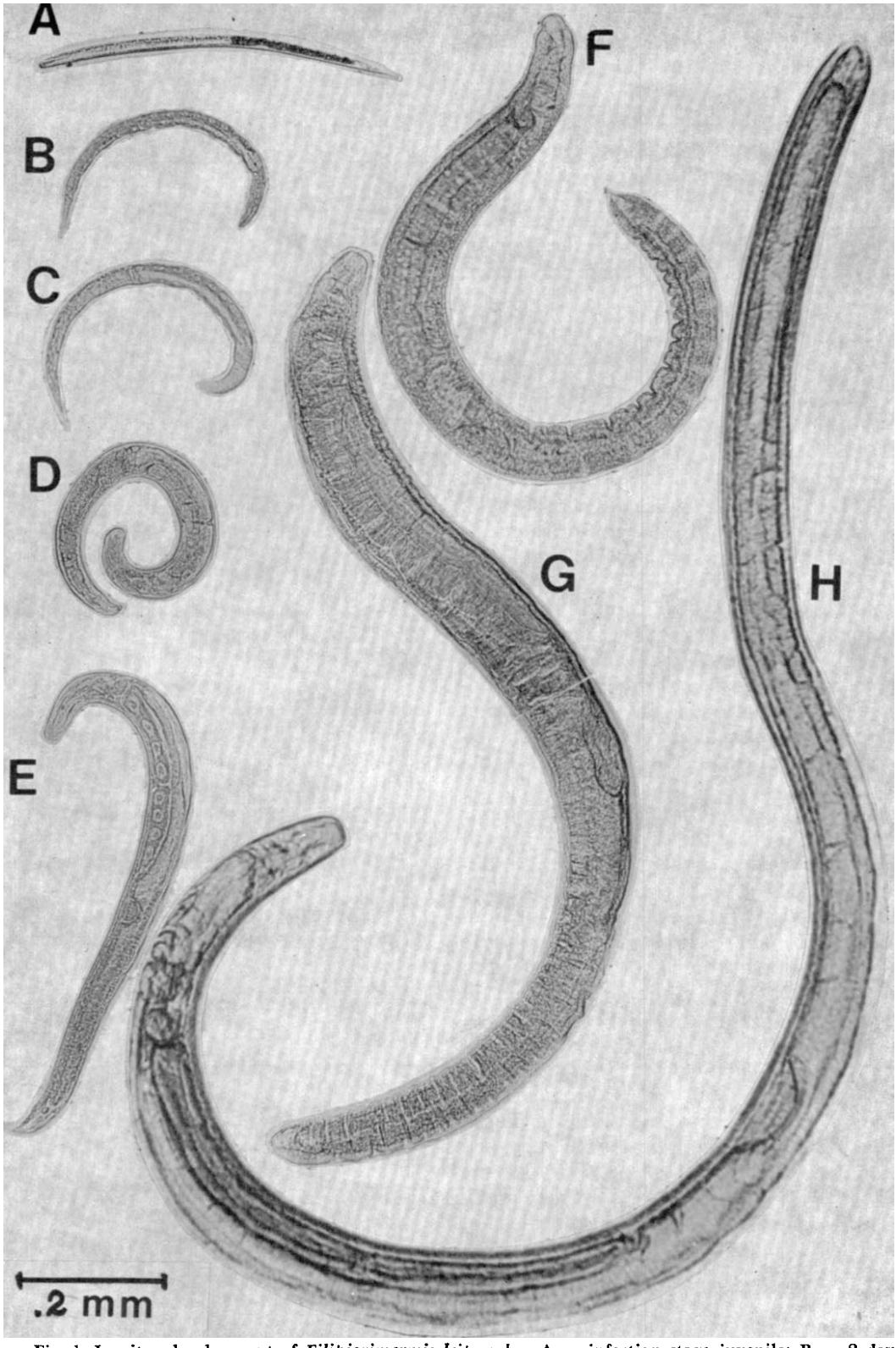


Fig. 1. In vitro development of *Filipjevimermis leipsandra*. A = infection stage juvenile; B = 2 days; C = 4 days; D = 6 days; E = 8 days; F = 10 days; G = 12 days; H = 14 days in culture.

served, which may indicate active feeding although the stylet remained retracted.

Two molts were identified during the culture period. The nematode's second molt (the first occurs in the egg) was observed after 6–8 days in culture. The second-stage cuticle was shed completely and could be seen readily under phase contrast microscopy. The third molt occurred between 18 and 20 days. However, the cuticle was not cast off but was retained around the third-stage worm. At this time the gonad rudiment had differentiated into a didelphic ovary and the vulval apparatus became discernible. This structure often was distinguished by a slight bulge in the ventral mid section of the body. We have designated this stage of development as the preadult stage, equivalent to the stage that emerges from the insect larva. Nematodes in culture reached a length of 10.6 mm ± 4.7 within 26 days. Incubation for 66 days increased their length to 12.5 mm ± 4.1. Periodic growth of the developing nematodes is shown in Table 2. However, while in the liquid medium the preadult nematodes did not advance to the adult stage. The in vitro system apparently lacked a stimulus for triggering the mechanism by which the nematode could complete its life cycle.

In nature the preadult nematode leaves the host and sheds two cuticles in the soil after the final molt before oviposition occurs (6). When in vivo nematodes are transferred to water they retain motility, molt within 2 wk, and begin ovipositing 1–2 days later. However, when preadults from in vitro cultures were transferred to water, motility stopped immediately and the nematodes ruptured. It appeared that the in vitro cultivated nematodes were unable to osmoregulate in a non-isotonic solution.

*Effect of osmotic pressure on preadult development:* The osmotic pressure of Coleoptera larvae hemolymph lies between 1.14 and 2.2 NaCl equivalent (percent wt/volume) (5). Two preadult specimens were transferred to 0.1, 0.2, 0.3, 0.4, and 0.5 M NaCl solutions (0.58 NaCl to 2.90 NaCl equivalents). In 0.1 M NaCl the nematodes did not rupture and remained motile. In 0.2 and 0.3 M solutions the nematodes became quiescent and failed to molt. Upon transfer to water after 3 wk the nematodes did not rupture but still did not molt. NaCl solutions more concentrated than 0.3 M were lethal to the nematodes.

*Growth as affected by solid substrate:* During development of cultural methods for rearing the nematode in vivo, a sand-blasting aggregate, Starblast (DuPont), was substituted for the soil substrate for preadults emerging from parasitized insect larvae (Creighton and Fassuliotis, unpublished). This material is predominantly aluminum oxide and has a dark mahogany color facilitating observation of the white nematode against the dark background.

An experiment was initiated to determine whether in vitro-reared preadults could be pre-conditioned to molt by transferring them to Starblast. Fifty 47-day-old preadults were transferred to 20-ml vials containing 2 cc of Starblast with 12% moisture. After 2, 5, 7, and 12 days in Starblast, 4–6 nematodes were transferred to water in individual wells of multiwell tissue culture plates.

When preadults were transferred from the culture medium to Starblast some coiled up and lay motionless on the surface of the aggregate while others first migrated to the sides of the vial before coiling up. Nematodes transferred from the Starblast to water before 7 days did not rupture or molt and were inactive. After 7 days in Starblast, 20% molted within 5 days after they were transferred to water; after 12 days, 50% molted in water within 2 days. Since most of the nematodes that had been in Starblast for 12 days had commenced to molt, the remaining 30 nematodes were transferred to water after 14 days in culture. By this time most (73%) had already molted in the Starblast, and the remainder molted 1–2 days later. Molted females began ovipositing 1–3 days after the fourth molt (Table 3). The shed cuticle of the third molt was thick and contained the tail projection of the third-stage juvenile (6). The shed cuticle of the fourth molt was extremely thin and remained within the third-stage cuticle. There was no projection on the terminus of the fourth-stage cuticle. Some females, however, did not oviposit even though they appeared gravid. Eggs layed by the in vitro-reared females were viable, and juveniles hatched after incubation in water for about 2 wk at

Table 3. The effect of an aluminum oxide solid substrate (Starblast) on the induction of molting of in vitro-reared *Filipjevimermis leipsandra* preadults.*

| Days in Starblast | No. of nematodes observed | % Molted in | | Days for onset of molt in water | Days after molt to oviposition | % ovipositing |
|---|---|---|---|---|---|---|
| | | Starblast | Water | | | |
| 2 | 6 | 0 | 0 | ... | ... | 0 |
| 5 | 5 | 0 | 0 | ... | ... | 0 |
| 7 | 5 | 0 | 20 | 5 | ... | 0 |
| 12 | 4 | 25 | 25 | 2 | 2 | 50 |
| 14 | 30 | 73 | 23 | 1-2 | 1-3 | 33 |

*Nematodes reared for 25 days on 20% fetal bovine serum in Schneider's Drosophila medium.

25 C.

Nematodes obtained from in vitro-cultivation were usually shorter than those recovered from field-infested *D. balteata* larvae 12.5 ± 4.1 mm vs. 31.3 ± 14 mm). The maximum worm length achieved from in vitro was less than the average length commonly observed in vivo, but was larger than the smallest worms recovered from in vivo. A direct correlation was found with the number of eggs that were layed and length of female (1).

## DISCUSSION

The preparasitic juvenile nematode penetrates the cuticle of the insect larva with the aid of its stylet and enters the hemocoele. It migrates to the cerebral or neural ganglia where nourishment is provided. Growth is rapid and in about 2 wk the nematode breaks through the neural lamella into the hemocoele where it continues to grow. The nematode emerges from the host as a preadult about 90 times larger than when it entered. During egress, the host's cuticle is ruptured and the host is killed. In the soil, the preadult nematode molts to the adult female stage within 2–3 wk and shortly thereafter begins to deposit eggs. Whereas the mermithids could be readily reared within the *Diabrotica* larva in the laboratory, in vitro cultivation can be used for studying nutritional, environmental, and other requirements of the nematode. This is the first report of the successful rearing of any mermithid species through the complete life cycle under axenic conditions in vitro. Initial use of sodium hypochlorite to surface sterilize the eggs and subsequent handling of all materials under sterile conditions rendered nematode cultures free from contaminating organisms.

The critical organic supplement for in vitro cultivation of the juvenile *F. leipsandra* to the preadult stage was the inclusion of fetal bovine serum in Schneider's Drosophila medium. However, the completion of the life cycle depended on transfer of the nematode to a solid substrate which preconditioned them and triggered the mechanism necessary for the final molt to the adult stage.

That adults of *F. leipsandra* developed from juveniles in vitro indicated that growth and maturation are possible without the benefit of the host insect larvae. Three molts, in addition to the first that occurs in the egg, were observed in vitro. The cuticle of the second stage is shed while the cuticle of the third stage is retained. Development to the preadult stage in vitro took about 1 wk longer than in vivo (20–26 vs. 12–22 days).

Variability of nematode size in vivo can be accounted for by competition among individuals in multi-infected insect host larvae. Usually insects harboring 1 or 2 nematodes release extremely large specimens, the largest recorded being 60 mm. As many as 23 mermithids emerged from one insect larva (1), but these were underdeveloped and did not molt when transferred to water. However, the large variability in length of nematodes obtained from in vitro cultivation within the same culture tube is puzzling since the nematodes were active juveniles of similar age growing under similar conditions.

Factors that may influence the whole

spectrum of nematode development in vitro include population density, toxic metabolite accumulation in the culture medium, nutritional deficiencies of the medium, pH, and aeration. Some of these aspects are currently being investigated.

We feel that with further experimentation on media constituents and methods of handling cultures, larger ovipositing females can be obtained. In vitro culture of the parasitic nematode will greatly increase the feasibility of providing the enormous numbers of juvenile nematodes and/or eggs that will be required for large-scale field application to control *Diabrotica* larvae in the soil.

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