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In vitro observations on the infection of *Meloidogyne incognita* eggs by the zoosporic fungus *Catenaria anguillulae* Sorokin

Urs WYSS *, Britta VOSS ** and Hans-Börje JANSSON ***

* *Institut für Phytopathologie, Universität Kiel, Hermann-Rodewald-Str. 9, 2300 Kiel 1, Germany;*
** *Zentrum für Molekulare Neurobiologie, Universitätskrankenhaus Eppendorf, Martinistr. 52, 2000 Hamburg 20, Germany, and*
*** *Department of Microbial Ecology, University of Lund, Helgonavägen 5, 223 62 Lund, Sweden.*

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Summary — Zoospores of the chytridiomycetous fungus *Catenaria anguillulae*, obtained from axenic cultures on nutrient media, were added to *Meloidogyne incognita* eggs at different stages of embryonic development in diluted salt solution and studied at 25 °C, using video-enhanced light microscopy. Zoospores moved around at random for many hours without becoming attached to the eggs. However, once a zoospore encysted by coincidence and germinated in such a way that the germ tube penetrated the lipid layer, other zoospores were suddenly attracted to chemotactic substances leaking out. The embryo was killed within a few minutes following mass aggregation and encystment of the zoospores. On eggs with fully developed and motile second stage juveniles, zoospore attraction and encystment occurred in two phases, first after a zoospore had penetrated the lipid layer of the egg and second immediately after the juvenile had been killed within about one hour. Juveniles ready to hatch were not killed and those that had hatched were never attacked.

Résumé — ***Observations* in vitro *sur l'infestation des œufs de* Meloidogyne incognita *par les zoospores du champignon* Catenaria anguillulae *Sorokin*** — Des zoospores du champignon *Catenaria anguillulae* (Chytridiales) provenant de culture axénique sur milieu nutritif sont mises en présence d'œufs de *Meloidogyne incognita* à différents stades de développement embryonnaire dans une solution saline diluée maintenue à 25 °C. Les observations sont réalisées en microscopie optique assistée d'une vidéo à haute définition. Les zoospores se déplacent au hasard pendant plusieurs heures sans s'attacher aux œufs. Cependant, dès qu'une zoospore s'enkyste au contact d'un œuf et produit un tube germinatif qui pénètre dans la couche lipidique de celui-ci, d'autres zoospores sont rapidement attirées sous l'influence de substances chémotactiques diffusant dans le milieu. L'embryon est tué dans les quelques minutes qui suivent l'aggrégation en masse et l'enkystement des zoospores. Dans le cas d'œufs contenant des juvéniles de deuxième stade totalement formés et mobiles, l'attraction des zoospores et leur enkystement ont lieu en deux phases : la première après que la zoospore ait pénétré dans la couche lipidique de l'œuf et la seconde immédiatement après que le juvénile ait été tué, ce qui dure une heure environ. Les juvéniles sur le point de sortir de l'œuf et ceux déjà libérés ne sont jamais attaqués.

Key-words : Nematode parasitic fungi, *Meloidogyne*, *Catenaria*.

The chytriomycetous fungus *Catenaria anguillulae* Sorokin is a parasite of a variety of nematode species (e.g. Stirling & Platzer, 1978; Esser & Schubert, 1983; Jaffee, 1986). All published studies on this fungus have dealt with its infection of vermiform nematode stages. There are a few reports on parasitism of eggs of other animals, e.g. midges and trematodes by *Catenaria* spp. (e.g. Butler, 1928; Buckley & Clapham, 1929; Martin, 1975, 1978), but none on the parasitism of nematode eggs. Another species, *C. auxiliaris*, is a well known parasite of female cyst nematodes (e.g. Tribe, 1977). *C. anguillulae* infects nematodes with its uniflagellate zoospores that are attracted to compounds exuded from natural openings of nematodes (e.g. Barron, 1977). After adhesion and encystment, infection of the animal takes place, leading to the production of zoosporangia and zoospores. Generally the parasitic life cycle of the fungus is completed within 24 hours. In the current study details of the *in vitro* infection of eggs of the root knot nematode, *Meloidogyne incognita*, are presented.

Materials and methods

Catenaria anguillulae isolate C 11/1 was isolated with *Meloidogyne incognita* as bait from a Florida soil, as described elsewhere (Voss & Wyss, 1990). It was grown axenically on corn meal agar (CMA, Difco) at 25 ± 1 °C in the dark and subcultured monthly. Zoospores were obtained by flooding a 2-3 week old culture (9 cm Petri dish) with 5 ml of diluted salt solution (Machlis, 1953). They were then concentrated to about 1×10^6 per ml by centrifugation at 600 *g* for 10 min.

M. incognita was reared monoxenically on cucumber roots (*Cucumis sativus* cv. Hoffmanns Vollendung) on B 5 agar (Huettel & Rebois, 1985). Egg masses were removed by hand and treated with 10 % sodium hypochlorite (about 1 % active chlorine) for 3 min (Voss, 1988). After several washings in sterile water the eggs were transferred to the zoospore suspension in a total volume of 2 ml (approx. 5×10^5 spores and

500 eggs/ml) in 15 ml conical centrifuge tubes in an upright position.

After the eggs had settled, 10 µl fluid were removed from the bottom of the tubes and pipetted onto the center of a 8 cm diameter cover slip (0.13 mm thick), surrounded by a 3 cm rectangle of cut glass slides (1 mm thick), smeared at the sides, top and bottom with a thin layer of vaseline. A second identical cover slip was placed on the rectangle so that it just touched the drop. The observation chamber thus formed was immediately inverted. Most eggs settled to the bottom of the fluid column, but some stayed just below the coverslip. Parasitism of these eggs by zoospores was then observed at high magnification, using high resolution video-enhanced contrast light microscopy (Wyss & Zunke, 1986). The observation chambers allowed continuous observation for several days until all eggs were parasitized. The temperature of the microscope stage was kept constant at 25 ± 1 °C.

Results

Zoospores of *C. anguillulae* moved around at random for many hours, sometimes for up to two days, without adhering to the egg's surface. When they adhered by coincidence, they retracted their flagellum, encysted and started to germinate. The germ tube was not always directed towards the egg's surface and hence grew away from it (Fig. 1 A, B). Infection was also not successful when the germ tube had penetrated the chitinous layer of the eggshell but then grew along the lipid layer and reemerged through the eggshell (Fig. 1 C, D). In most cases, however, zoospore encystment resulted in infection. The chitinous layer of the eggshell was penetrated within a few minutes, but the lipid layer opposed considerable resistance, especially at the poles, where it was thickest (Fig. 1 E). The germ tube started to swell at the point of contact with this layer (Fig. 1 F) and up to 40 min elapsed until it was penetrated, as indicated by the attraction of other zoospores (Fig. 1 G, H). Released chemotactic substances caused hundreds of zoospores to aggregate at the infection site. Many of them encysted, forming a cluster (Fig. 1 I) and the embryo was killed within 3-4 min, with its contents becoming rapidly disorganized (Fig. 1 J-L).

Zoospore attraction decreased after about 15 min, but quite often a second attraction phase occurred within one hour, with zoospores aggregating on different sites along the eggshell that enclosed the dead embryo. But even when hundreds of zoospores swarmed around the source of stimulation, none of them was seen to adhere to the surface of an immediately adjacent unaffected egg, as shown in Fig. 2 A. Egg No. 2 was here attacked 9 h later, at a time when the parasitized egg No. 1 was already packed with differentiating zoosporangia (Fig. 2 B), out of which evacuation tubes started to emerge 2 h later (Fig. 2 C).

In mature eggs, containing moving J2, the infection process, leading to the death of the J2, was different. Again a single penetrating spore (Fig. 2 D, E) was able to evoke an initial attack by many zoospores that were attracted to chemotactic substances leaking out after the lipid layer had been injured. This layer was now usually thinner than in embryonating eggs and hence offered less resistance. The aggregating zoospores formed a cluster of encysted zoospores (Fig. 2 F), but the juvenile was not quickly killed. It continued to move for up to about 80 min while germ tubes of the aggregated spores grew inside the egg (Fig. 2 G, H). Cuticle penetration by the fungus was never observed, but movement of the juvenile gradually slowed down. As the first signs of J2 disintegration near the site of infection became vaguely visible, a second wave of zoospore attraction was initiated. This wave was extremely strong so that the egg was sometimes tossed around by the swarm of attracted zoospores, with hundreds finally encysting around the egg (Fig. 2 I, J).

Before encystment the zoospores squeezed themselves as usual into the aggregation clusters or moved away again after a few seconds. Forward progression along the surface of the egg was typically in an amoeboid fashion (Fig. 2 K) and once a suitable site for encystment was found, the flagellum was retracted within 20 seconds by a clockwise rotation of the protoplast. A short peg on the encysted zoospores indicated the site of flagellum retraction (Fig. 2 L).

Eggs containing juveniles ready to hatch, were also attacked by many zoospores in the same manner. However, in nearly all cases the juveniles survived, even when many hours elapsed until they finally ruptured the egg shell. The long germ tubes that grew out of the encysted zoospores (Fig. 3 A) did not affect the nematodes. Fig. 3 B shows an empty eggshell with attached empty zoospores and their proliferating germ tubes and rhizoids. Hatched J2 were never attacked, even when the density of zoospores moving around was very high.

Within 3 h after the lethal infection of embryos or juveniles, a thallus with zoosporangial primordia was clearly visible (Fig. 3 C). The zoosporangia became gradually filled with lipid-like globules that increased in size (Fig. 3 D-F). The first evacuation tubes were formed 10 to 12 h after infection (Fig. 3 G). Fig. 3 H shows a parasitized egg, 13 h after the initial zoospore attack. Eggs were then packed with zoosporangia that had formed evacuation tubes and were in the process of zoosopore differentiation. Up to about 500 tubes / egg were counted. The number of zoospores produced varied between 8 and 59, depending on the size of the zoosporangium (Fig. 3 I); these values based on only a few observations.

Before zoospore differentiation the lipid-like globules decreased in size and vacuoles were formed, which disappeared again about 30 min prior to the release of the zoospores. Final differentiation was very rapid

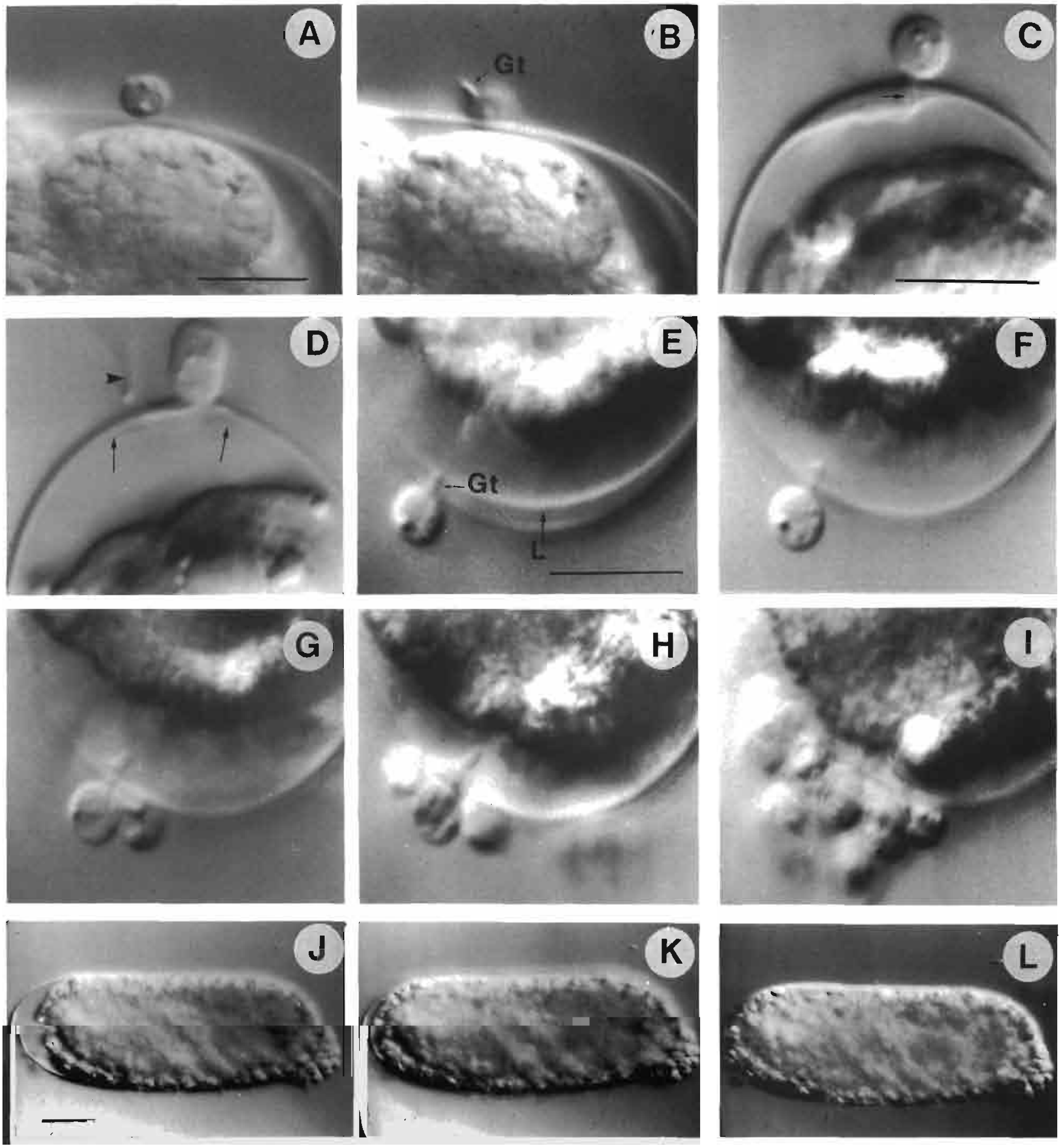

Fig. 1. *Catenaria anguillulae* attacking embryonating eggs of *Meloidogyne incognita.* A : Encysted zoospore on eggshell; B : Germ tube (Gt) growing away, 11 min after A; C : Germ tube (arrow) growing along lipid layer; D : Germ tube (arrowhead) reemerged through chitinous layer of eggshell, 5 h after C, only part of germ tube shown; lipid layer of eggshell marked by arrows; E : Germ tube (Gt) of encysted zoospore in contact with lipid layer (L) of an egg with embryo in gastrula stage; F : 17 min after E, tip of germ tube swollen; G : A second zoospore has encysted, 13 min after F; H : Four encysted zoospores at infection site, 7 min after G; I : Zoospores now aggregating at infection site, 3 min after H; J : Same egg, 4 min after I; K : Disintegrated embryo, 6 min after J; L : Disintegrated embryo, 1 min after K *(Bars = 10 µm).*

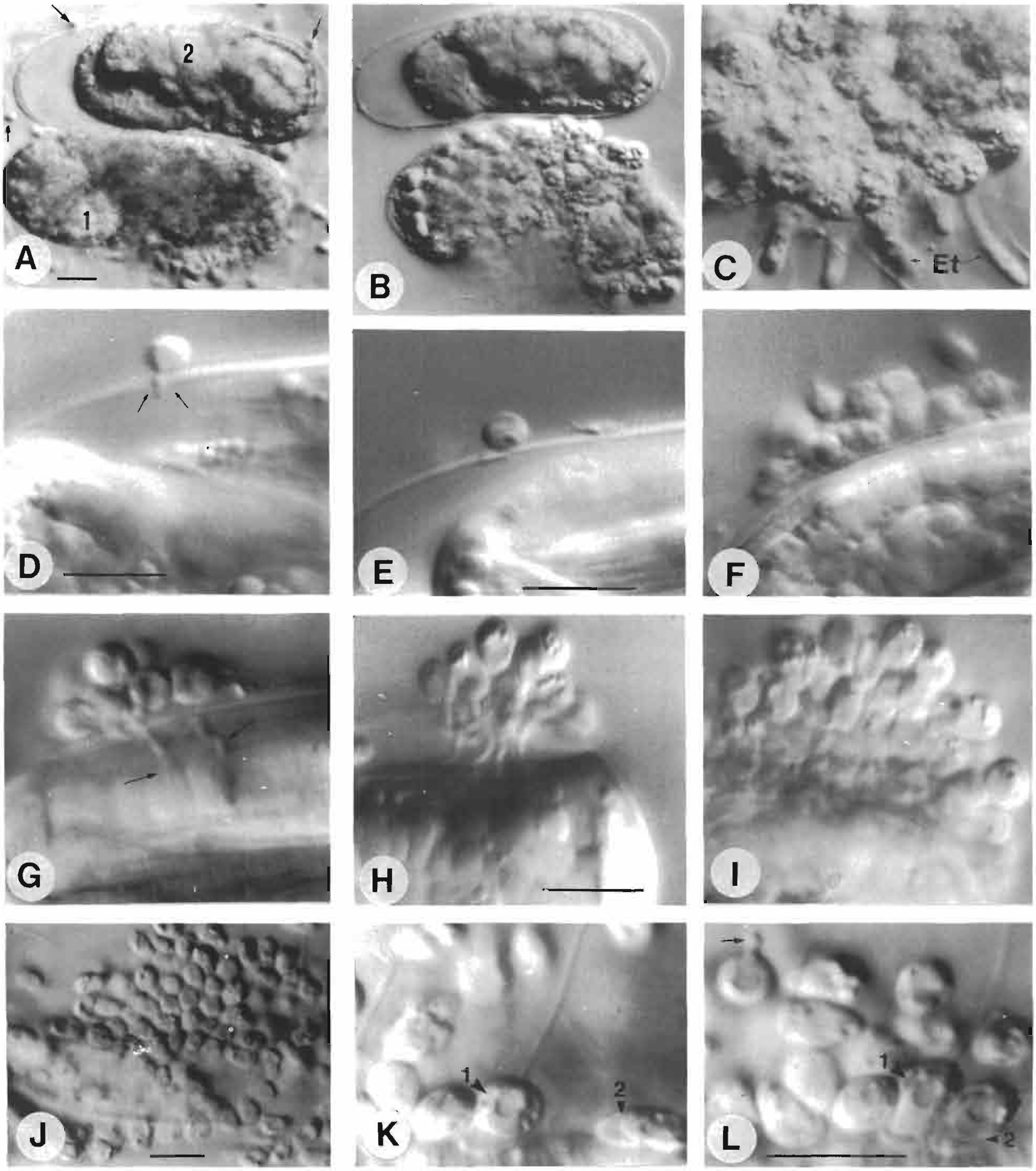

Fig. 2. *Catenaria anguillulae* attacking eggs of *Meloidogyne incognita*, with embryo still developing (A-C) and with developed J2-juveniles (D-L) — A : Disintegrated egg (No. 1), 45 min after massive zoospore attack. Egg. No. 2, in gastrula stage, surrounded by swarm of zoospores, with those marked by arrow not encysting; B : Egg No. 1 now packed with differentiating zoosporangia. Egg No. 2 unaffected, in early tadpole stage, 8 h 30 min after A; C : Parasitized egg No. 1 enlarged, with evacuation tubes (Et) growing out, 2 h 30 min after B; D : Zoospore penetrating eggshell, lipid layer indented (arrows) at point of contact with germ tube; E : Another egg, in same stage as D, i.e. with J2 moving inside egg. Encysted zoospore penetrating egg shell; F : Cluster of aggregating and encysting zoospores, 10 min after E; G : Germ tubes (arrows) growing inside egg, 40 min after F; J2 still moving; H : Another egg, in same stage as G. Cluster of germ tubes inside egg, with J2 still moving; I : Cluster of encysted zoospores, 50 min after H, dead juvenile in process of disintegration; J : Another egg, in similar stage as I, massive aggregation and encystment of zoospores; K : Same egg, zoospores No. 1 and 2 moving in an amoeboid fashion along egg shell; L : 40 s later; No. 2 ready to encyst next to Nr. 1. Arrow points to peg of encysted zoospore, few seconds after flagellum retraction *(Bars = 10 µm).*

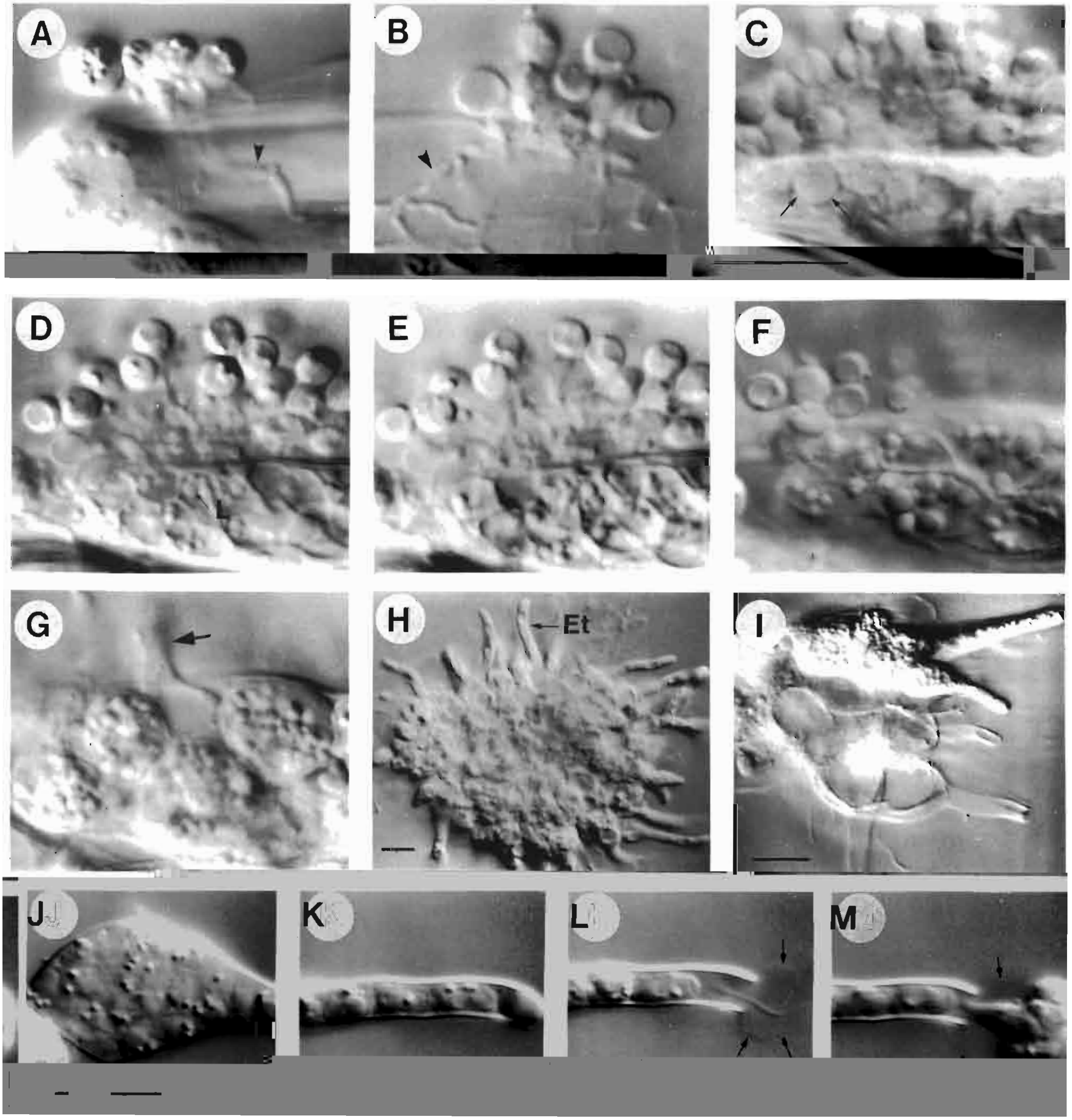

Fig. 3. *Catenaria anguillulae* attacking eggs of *Meloidogyne incognita*. A : Germinated zoospores on eggshell of J2 ready to hatch. One of long germ tubes marked by pointer; B : Another egg, J2 now hatched; germ tubes (arrowhead) and rhizoids (arrows) from cluster of encysted zoospores in empty egg. — C-G : Zoosporangium differentiation in a dead J2 inside egg. — C : 3 h after massive zoospore aggregation and encystment; one of zoosporangium primordia marked by arrows; D : Several encysted zoospores now empty, zoosporangium primordia with lipid-like globules, 80 min after C; E : Lipid-like globules in zoosporangia larger, 45 min after D; F : Lipid-like globules more enlarged, 145 min after E; G : First evacuation tube (pointer) formed, 4 h after F; H : Parasitized egg with numerous evacuation tubes, *ca* 13 h after initial zoospore attack; I : Empty and still filled zoosporangia, *ca* 14 h after initial zoospore attack; J : Zoosporangium with zoospores in final process of differentiation; K : Differentiated zoospores forced forward through evacuation tube, 102 s after J; L : Terminal membrane of tube (arrows) being expanded, 55 s after K; M : Expanding membrane (arrows) engulfes zoospores before final release, 4 s after L *(Bars = 10 µm).*