Theratromyxa weberi, An Amoeba Predatory on Plant-Parasitic Nematodes

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Abstract: Theratromyxa weberi, an amoeba, was isolated and cultured in the laboratory to determine the physical conditions that influence its predation on plant parasitic nematodes. In greenhouse tests, the amoeba was a poor biological control agent of the root-knot nematode, *Meloidogyne incognita. Key Words:* biological control, interaction, predation, Proteomyxida, Vampyrellidae, soil amoeba.

Upon examining *Meloidogyne incognita* (Kofoid and White) Chitwood, populations extracted from soil on a greenhouse bench, I saw amoebae engulfing *M. incognita* larvae. During later observations, I found other stages of this amoeba similar to those described by Weber et al. (11) and Paramonov (7).

Zwillenberg (13) named this organism *Theratromyxa weberi* Zwillenberg, and placed it in the Order Proteomyxida, family, Vampyrellidae. Van der Laan (6) concluded that the slow rate of spread in soil, the nonspecific attack on all nematodes and the susceptibility to desiccation made *T. weberi* an unlikely candidate for effective biological control of *Heterodera rostochiensis* Wollenweber. Nevertheless, Paramonov (7) suggested that this amoeba might control root-knot nematode species.

This paper outlines some methods for culturing the amoeba and evaluates its potential as a biological control agent of *M. incognita*.

MATERIALS AND METHODS

Prey for the amoebae were larvae of *M. incognita* and *Heterodera trifolii* Goffart, and the adults and larvae of *Aphelenchus avenae* Bastain, *Aphelenchoides rutgersi* Hooper and Myers, 1971 and *Rotylenchulus reniformis* Linford and Oliveira. *M. incognita* larvae were collected by Dropkin's method (2). Cysts of *H. trifolii* were collected by flotation and placed on nylon screens submerged in water in petri dishes. Periodically, the emerging larvae were collected by pouring the water from the dish (9). By use of Evans' methods (3), *A. avenae* and *A. rutgersi* were cultured on the fungus, *Rhizoctonia solani* Kuehn, and collected. Reniform nematodes were reared on cotton plants grown in the greenhouse and extracted from the soil using a flotation-screening method (10). Specimens of all nematode species were counted, adjusted in a water suspension to 3000/ml, and 1 ml was pipetted into each 60 X 10-mm plastic culture dish that contained amoebae in a final volume of 6 ml.

Cover slips (18-mm diam) were used as the inert substrate on which to grow amoebae. Colonized slips were removed from culture dishes with forceps and placed in water in counting dishes. At 100 X magnification, both creeping and sedentary stages were recognized and counted. When specific numbers of amoebae were needed, all stages were washed from dishes and counted, or whole or broken parts of cover slips were assembled until the required numbers of amoebae were obtained.

To establish new cultures of the amoeba, colonized slips were put in a culture dish containing four clean cover slips and a fresh suspension of nematodes. This method has been used successfully to maintain the amoebae in culture since December 1970.

Colonized cover slips were prepared for observation by cleaning one side and adding a few drops of propionic-orcein dye to the other side to stain the amoebae. After 1 hr, the amoebae were destained in acid alcohol and then dehydrated though a graded series of ethyl alcohols to absolute ethanol. Finally, the cover slip with amoebae on the bottom side was placed on a drop of Eurparil® supported on a microscope slide.

Fenwick's saline solution (4) was prepared and tested at full, 1/2, 1/4 and 0 of its original concentration to determine the most suitable medium for rearing the amoebae.

Cultures were freed from certain unwanted microorganisms by passing the cysts through rinses of sterilized Fenwick's solution. A micropipette was the best tool for transferring cysts to the dishes containing nematodes because it damaged the cysts less than either a bamboo pick or an Irwin's loop.

Cinemicrography techniques and time-lapse studies were used to determine the rate of movement of the amoeba as well as the duration of its other life stages (8).

The effectiveness of the amoeba as a biological control agent was tested by placing cover slips colonized with approximately 600 amoebae in 60 X 10-mm culture dishes and covering them with 10 ml of moist sand. About 3000 *M. incognita* larvae were pipetted onto the surface of the sand. After regular time intervals, the sand and nematodes were washed into beakers, and the surviving nematodes were separated from the sand by flotation. The supernatant containing most of the nematodes was decanted onto a filter disk in a Büchner funnel. The disk with adhering nematodes was inverted into a counting dish containing water, and the nematodes were counted after they had settled.

Tomato seedlings (*Lycopersicon esculentum* Mill. 'Rutgers') were used as indicator plants in a bioassay to determine the efficacy of the amoeba as a biological control agent. Moist sand or soil infested with 4000 *M. incognita* larvae was mixed and put into 7.6-cm plastic pots. The amoeboid stages of *T. weberi* on cover slips were buried in the sand or soil at the rate of 0, 600, 1200 and 2400 amoebae/pot. Tomato seedlings were transplanted into some test containers at the same time that the nematodes were added; in other tests, seedlings were transplanted 3 and 10 days after nematode infestation. These last two time periods allowed time for the amoebae to prey

1 CREEPING STAGE

6 RESTING STAGE

LIFE CYCLE

2 AMOEBA ATTACHED TO LARVAE

5 EXCYSTMENT

3 LARVA BEING INGESTED

4 LARVA COILED IN A DIGESTIVE CYST

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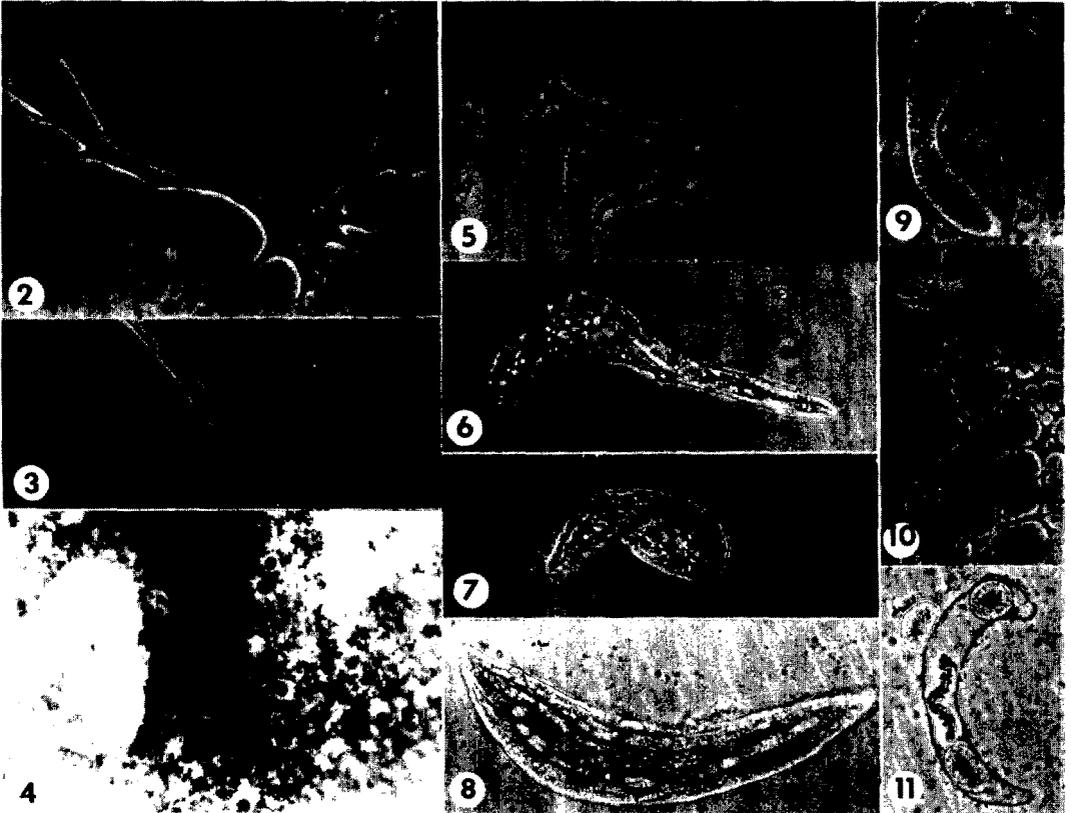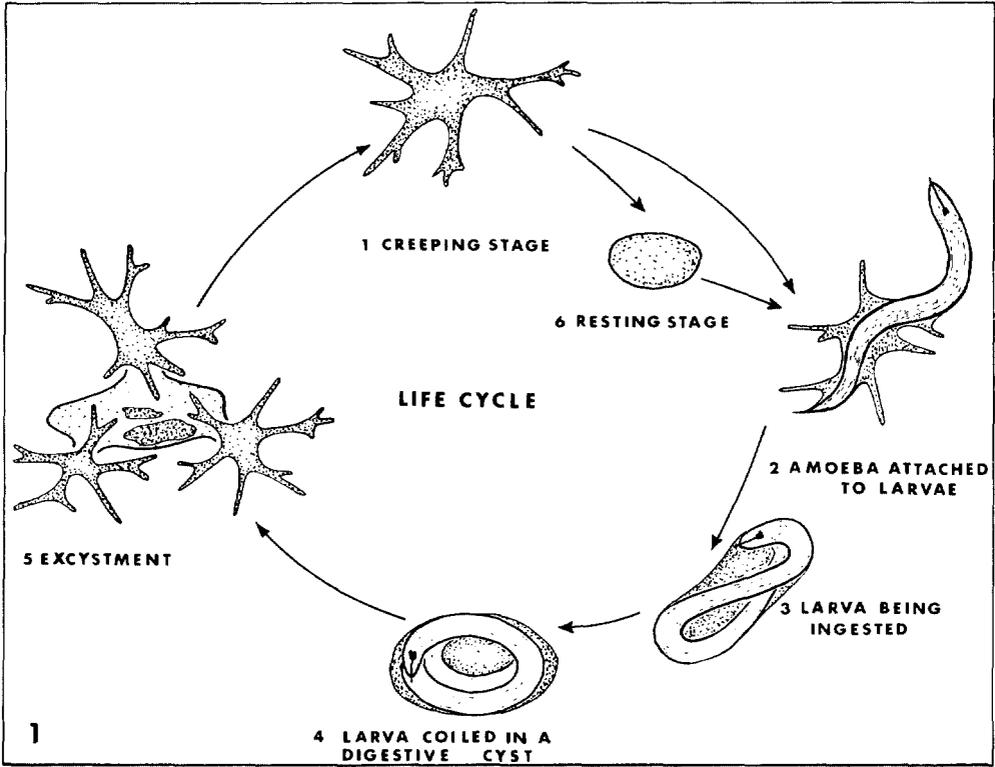
on the nematodes. Seedlings were watered with Hoagland's solution (5). Four weeks after transplanting, the tomato plants were removed from the pots and the washed roots rated for nematode disease severity using Daulton and Nusbaum's method (1).

RESULTS

At room temperature (21 C), the life cycle of the amoeba, starting from the creeping stage, was about 23 hr (Fig. 1). During the creeping or trophic stage, the amoeba was granular in appearance (Fig. 2) and when fixed and stained showed a multinucleate structure (Fig. 4). Its rate of movement was about 70 μm/min. Numerous very fine reticulopodia were observed at its advancing margin (Fig. 3), and narrow arms of protoplasm, some 350-μm long, extended from the trailing margin. These arms flowed back into the main body when stretched to the point that they lost contact with the culture dish.

When a live nematode contacted an amoeba (Fig. 5), it usually pulled the amoeba from the dish bottom. The amoeba then flowed over the nematode and ingested its entire body. All species of plant nematodes tested were ingested in this manner.

After an amoeba covered a nematode, there was an infolding, which resulted in a smaller cyst (Fig. 6, 7). This process took about 2 hr. The resulting cyst was usually vacuolated (Fig. 8), but within the next several hours it became more dense (Fig. 9). Generally, cyst size and shape gave a good indication of the number of nematodes ingested. Within 23 hr, the protoplasm within a digestive cyst cleaved, and over a period of 15-20 min some four to ten amoeba emerged (Fig. 10). What may be the undigested nematode cuticle remained within the cyst shell (Fig. 11).

Amoebae moved randomly after excystment. Those failing to contact nematodes became more spherical in shape, and finally in 3-4 hr collapsed inward to form the resting or hypnocyst having an approximate diameter of 40 μm.

Three to 4 days after the water was changed and a fresh suspension of nematodes was added to a culture dish, amoebae began emerging singly from hypnocysts. Fewer than 5% of the hypnocysts excysted, and most remained dormant even though they gave a positive pink color reaction in a 0.1% solution of 2,3,5-triphenyltetrazolium chloride, an indication of viability.

Weber et al. (11) reported that 5-mm diam wells drilled in clear plastic blocks were suitable containers for culturing and observing the amoeba. They found glass containers, microscope slides and hanging drop methods were less satisfactory, and concluded that the slightly roughened surface of the wells aided amoeboid movement and was largely responsible for their successful use. They observed the anastomosing of several amoebae into a protoplasmic network that could engulf a hundred or more larvae at one time.

In my tests, anastomosing was not observed either in plastic petri dishes or on cover slips. Tests were conducted to determine the effectiveness of the Falcon Microtest Plates No. 3034® in stimulating anastomosis. A few digestive cysts and approximately 100 nematodes were added to each of 60 2-mm diam wells. When several trophic forms emerged simultaneously from cysts and were in close proximity to one another and to nematodes, the amoebae failed to form a large protoplasmic network. The crowding of the amoebae and nematodes resulted in only slightly larger digestive cysts, each containing from 6 to 10 nematodes. The amoeboid isolate retained its individuality during the trophic stage.

Fenwick's solution prolonged nematode viability and enhanced predation by the amoeba during the 2-week exposure period in some of the tests. The concentration of the solution influenced the predator-prey relationship (Fig. 12-A). At full strength, the solution stopped reproduction of the amoebae even though viable nematodes were present. In the 1:4-strength solution, the amoebae reproduced rapidly and were more numerous at the end of the experiment than in either the 1:2 dilution, or the distilled water check. The

FIG. 1-11. Stages in the life cycle of *Theratromyxa weberi.* 1. Life cycle of *T. weberi.* 2. The amoeba's trophic stage. 3. Numerous reticulopodia on the advancing arm of an amoeba. 4. Stained preparation of an amoeba showing several nuclei (× 1600). 5. *M. incognita* larvae ensnared by an amoeba. 7. Larva enfolded by an amoeba. 8. An early digestive cyst showing vacuoles and several trapped larvae. 9. A dense digestive cyst just prior to excystment. 10. Excystment of a digestive cyst. 11. Shell of digestive cyst containing the remains of nematode cuticle and two hypnocysts. A third cyst is on the outside wall.

relationship was much the same when Hoagland's solution was used instead of Fenwick's solution.

Temperature influenced the rate at which amoebae reproduced. At constant temperatures of 9, 14, 19, 24 and 29 C the amoebae preyed on the larvae and reproduced (Fig. 13-A). The maximum numbers of the amoebae were obtained at 19 C over the 14-day test period. Populations of prey decreased most rapidly at 24 and 29 C where ingestion of larvae was most rapid, and their numbers were exhausted on the 9th day (Fig. 13-B).

Both van der Laan (6) and Zwillenberg (13) suggested that light be excluded as a necessary condition for culturing the amoeba. This procedure was found helpful, but not essential.

At 22 C and while exposed to 2690 lx (250 ft-c) of light, the amoebae ingested nematodes and reproduced. When light was excluded at the same temperature, more nematodes were attacked and digested (Fig. 12-B).

Predatory activity of *T. weberi* was also influenced by changes in pH of the medium. In a 0.01 M trismaleate buffer solution, amoeboid activity as measured by predation on nematodes was suppressed at pH 5.7, while at pH 8.3, activity exceeded that of the check treatment (Fig. 12-C).

Predation in sand culture during 18 days at a constant temperature of 19 C showed that numbers of nematode larvae fell to 21% of that of the check (Fig. 12-D).

In nine separate greenhouse tests, the
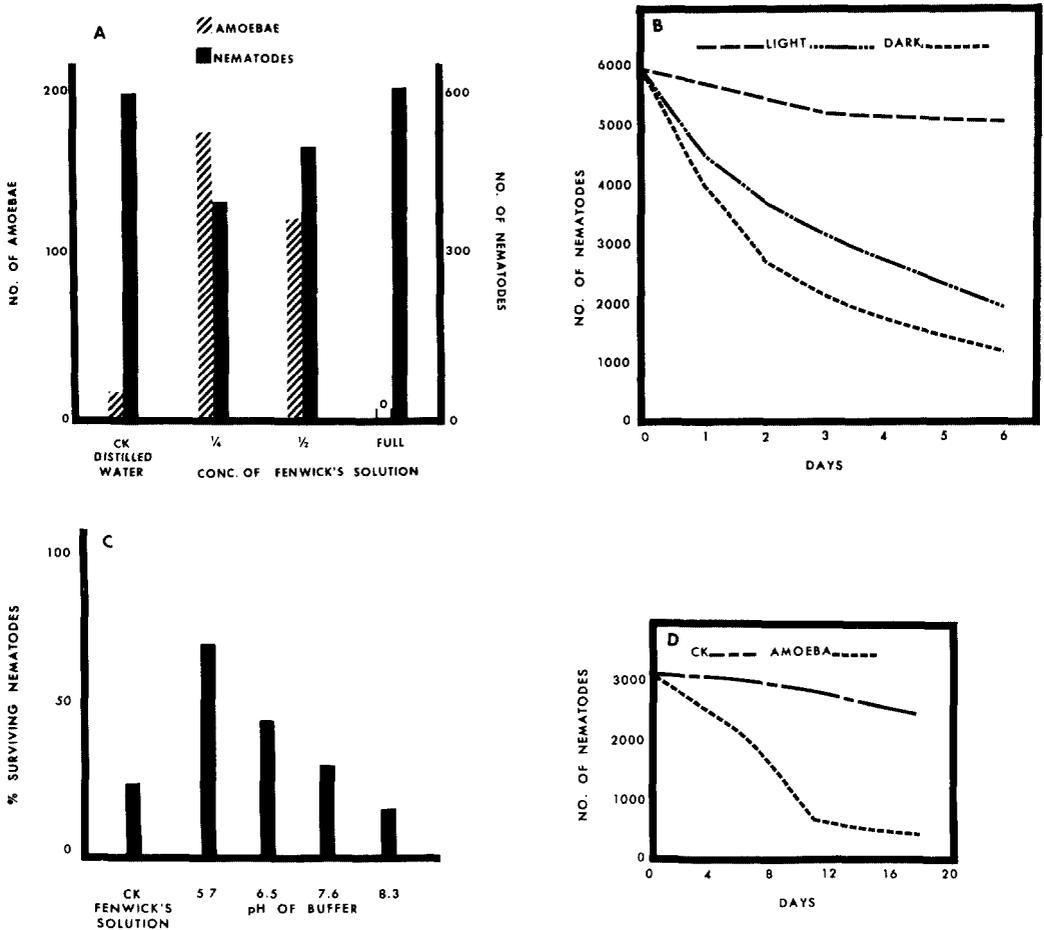
FIG. 12. Predatory activity of *Theratromyxa weberi* in different environments. A) Influence of the concentration of Fenwick's solution on the prey-predator relationship between *Meloidogyne incognita* larvae and *T. weberi*. B) Decreased predatory activity of amoebae when exposed to 250 ft-c of light at 22 C. C) Influence of pH on the predatory activity. D) Predatory activity of amoebae in moist sand on a population of *Aphelenchoides rutgersi*.

predation of amoebae on *M. incognita* larvae and subsequent reduction in nematode populations were measured using as an index the severity of galling on tomato roots. In all tests, the galling index of tomato roots grown in amoeba- and nematode-amended soil was not significantly lower than that of plants grown in soil infested only with nematodes.

No amoebae could be reisolated after they were added directly to sand or soil. In other tests where the amoebae had been added to soil on cover slips, a few were reisolated intact on those slips incubated in culture dishes with nematodes. The hatching of a few cysts indicated survival.

A number of amoebae culture dishes were stored at 19 C and periodically sampled over a 1-year period to determine cyst survival without nematodes. After storage, the plates were drained to remove any inhibitory products and to reduce salt accumulation. A fresh suspension of nematodes was added, and after 1 week the plates were examined for the presence of active trophic stages of the amoeba. Viable amoebae were found in plates that had been stored for 8 months. In plates that were allowed to dry, amoebae never excysted, nor did they give a positive reaction in solutions of 2, 3, 5-triphenyltetrazolium chloride.

DISCUSSION

Some of the cultural similarities between this amoeba and previous descriptions of *T. weberi* are the appearance and size of the trophic stage and cysts, the manner of locomotion, the diversity of nematode prey, the formation of digestive and resting cysts and the process of excystment. These similarities are sufficient evidence that the organism is *T. weberi*.

However, there is one significant cultural difference between the organism used in my study and *T. weberi*. The spore-bearing cysts as described by Zwillenberg (13) were not observed. Both digestive and resting cysts upon excysting, gave rise to trophic amoebae. However, occasionally some peculiar bodies were observed within a few cysts. These structures could be a phase in the life cycle, but were more likely a fungus or protozoan parasite of nematodes that were engulfed during encystment. Since other organisms, including amoeboid species of the limax type and a *Cochliopodium* sp., were found occasionally in the *T. weberi* cultures, the full life cycle was not
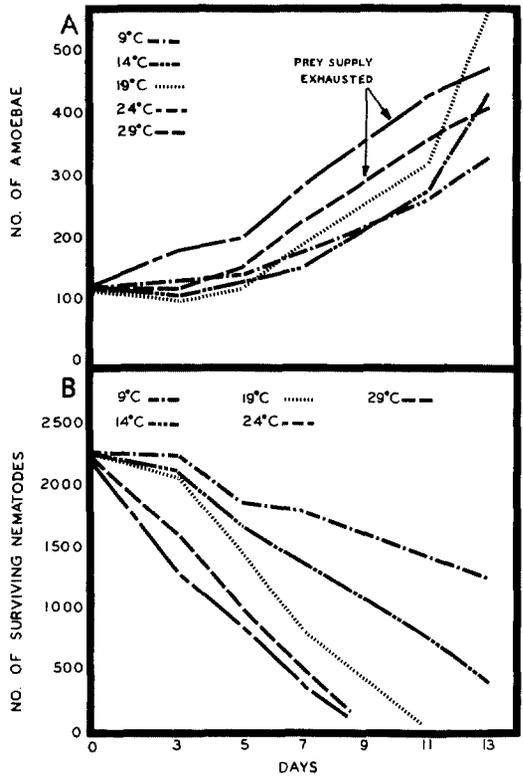
FIG. 13. The influence of temperature on the prey-predator relationship between the amoeba and larvae of the root-knot nematode. A) The increasing amoeba populations at the various temperatures; B) The declining populations of prey nematodes.

determinable. The complete cycle will require monoxenic culture of the amoeba.

Physiological differences between this isolate and previously described amoebae were observed. The present isolate reproduced at temperature ranges above 15 C, which was reported previously as being detrimental to *T. weberi* (6, 13). The high temperatures of the greenhouse may be responsible for the selection of this high-temperature variant. The adaption of this amoeba to soil temperatures favorable for many crop plants enhanced this organism's chances of being an effective biological control agent. This single physiological difference prompted the investigation.

Several amoebae of this isolate failed to anastomose and form a large network. This was a departure from the description of Weber et al. (11), but agreed with the findings of Winslow and Williams (12). However, the possibility remains that networks might form under different laboratory conditions.

In greenhouse tests, the amoebae were ineffective in controlling root-knot nematode disease of tomatoes. Apparently, the primary reason is that trophic stages were not present initially in sufficient numbers to reduce nematode populations significantly before the infective larvae penetrated the roots. Unfortunately, culture plates yield mostly the digestive and resting cysts. These stages cannot attack nematodes until they excyst. Resting cysts were the most numerous, but were very slow to excyst. Conditions governing excystment are not known. Consequently, there is no known method for inducing the simultaneous release of many trophic amoebae and enhancing the possibilities of biological control.

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