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TECHNIQUES AND MEDIA FOR ISOLATION, CULTURE, STORAGE, MOUNTING AND BIOASSAY OF ENTOMOPATHOGENIC STEINERNEMATID AND HETERORHABDITID NEMATODES

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There are many types of associations between nematodes and insects. Some nematodes merely use the insect for transportation (phoretic), others parasitise the insect (with effects on the insect varying from none to severe/death) and some are entomopathogenic.

The families of Steinernematidae and Heterorhabditidae are obligate entomo-pathogenic nematodes and characteristically kill their host in 24–72 hours. They are from different phyla, but apparently their similar physical environment (soil) and host choice (insects) have resulted in the almost identical life histories.

These entomopatogenic nematodes have a very broad host range, can be mass produced on artificial media and can be stored for some time. Field application can be done under fairly high pressure and together with chemicals (insecticides, fertilisers) using standard equipment. Resistance development in natural insect populations is very unlikely and they are considered safe for plants, birds and mammals. These qualities have led to a strong commercial interest in the development of nematodes as biocontrol agents or biological insecticides. Several companies are producing and selling nematodes in Europe, the US, Canada and Australia. Formulations are on clay, aliginated gel, polyacrylamide or flowable gel. Bedding (1981, 1984) developed a cheap method for culture of nematodes on offal on crumbed polyurethane sponge.

There are, of course, limitations to the usefulness of nematodes as pest control agents.

Although the host range of the nematodes is wide, each species has a preferred host range and will be less effective against other insects. Steinernematids are generally most effective against insects found near the soil surface and may be best adapted to attacking insects which feed at the soil–litter interface or on the soil surface (mole-crickets, cutworms). Heterorhabditids are more effective against insects that occur relatively deep in soil, such as the Japanese beetle, due to superior host-seeking abilities and a tendency to disperse downward.(Georgis and Hom, 1992) Steinernematids as well as heterorhabditids, have been found effective biological control agents against larvae of scarabs. (Klein, 1990).

Moisture is also a limiting factor. Irrigation before and after application and continuous moderate soil moisture are essential for nematode movement, persistence and pathogenicity (Georgis and Gaugler, 1991).

The effectiveness of the nematodes is further limited by soil texture, aeration and temperature and a wide range of biotic factors (bacteria, other nematodes, fungi).

PATHOGENESIS

Steinernematids and heterorhabditids have a symbiotic association with the bacterium *Xenorhabdus* spp. The infective third-stage juvenile, the only free-living stage of the nematode which is adapted to remain without feed for a prolonged period, carries the bacterium in its intestine. When it encounters a suitable insect it enters via

mouth, anus or spiracles and penetrates into the haemocoel (*Heterorhabditis*, with the help of a tooth, can also enter through the cuticle of more fragile insects). In the haemocoel the nematode releases the bacteria, which proliferate and cause septicaemia and death of the insect within 24–72 hours. The bacteria provide nutrients for the nematodes and inhibit the growth of many micro-organisms. The nematodes pass through several generations in the insect host and eventually emerge as infective juveniles (at 18–28°C usually after 18–20 days) carrying the bacteria to the next host (cover photo).

ISOLATION

Nematodes can be isolated from soil by using their behavioural and physical properties.

In the Baermann funnel method the nematodes are allowed to migrate through a nylon sieve that holds back the soil.

In the flotation method they are separated from the soil that precipitates faster than the (moving) nematodes.

The *Galleria* bait method (Bedding and Akhurst, 1975) is more specific for nematodes that actively seek out insects. Basically the method consists of exposing a susceptible insect, usually *Galleria mellonella* larvae, for some time to a medium containing nematodes and then collecting the nematodes from the infected (dead) insects. There are a lot of variations on this method (amount of soil and insects used, placing of insects in the soil, etc.). An additional possibility is, of course, to use the target insect instead of waxmoth larvae which should result in fewer but more specific isolates.

The three methods are described in the appendix after Curran and Heng (1992) who compared their efficacy in estimating the number of nematodes in soil samples.

MULTIPLICATION

Nematodes can be multiplied on artificial media which is quite complicated and labour intensive (see Bedding, 1981, 1984).

To multiply the nematodes *in vivo*, susceptible hosts are exposed to the nematodes (see below, Laboratory assays). Mortality of the insects is checked daily and the cadavers are transferred to a white trap (Appendix 2).

The waxmoth, *Galleria mellonella*, is extremely susceptible to nematodes (and other diseases) and the larvae are often used for multiplication of nematodes. The moth can be easily reared on a diet of cereals and honey (Appendix 3).

The number of nematodes produced in one larva is, among other things, dependent on the species of nematode, the number of nematodes (more is only better up to a certain limit), the species of insect, the size of the insect. In last instar *Galleria* the average production of infective juveniles would be 30,000–50,000 (Woodring and Kaya, 1988).

Storage

Commercially prepared 3rd instars (the free-living stage) are stored according to the manufacturers' specifications, e.g. nematodes on aliginated gel have to be kept refrigerated, nematodes formulated on crumbed sponge (Bedding, 1981, 1984) can be kept at room temperature for a few months.

Nematodes in suspension (grown *in vivo*, isolated from soil, insects or commercial formulations) can be kept for only a short time. The optimal temperature for storing nematodes in suspension is 15°C. Oxygen is a limiting factor. Bubbling air through the suspension and keeping the surface–volume ratio high will favour survival.

Examination, Mounting

Infection can be accompanied by a colour change due to the symbiotic bacteria (gray = steinernematids, reddish = heterorhabditids). Nematodes can often be seen through the host cuticle under a dissecting microscope (this can be especially obvious in the area of the spiracles). If not, and if the insect is suspected to be infected, it should be dissected in a physiological solution (0.9% NaCl). If only the first and second stage nematodes which are not free-living are present they can survive for a while in the physiological suspension. Identification of the infected tissue can help identify the nematode.

For closer examination the nematodes can be put on a slide and examined with a light microscope. Living nematodes move a lot and are hard to observe, they can be heat killed by passing the slide a few times through a flame or by adding hot solution (see Appendix 4). Dead nematodes characteristically straighten out.

The preparation of permanent mounts is simple but takes several weeks (Appendix 4).

Counting, Quality Check

The exact number of living/surviving nematodes in the suspension can be counted under a dissecting microscope for low concentrations. For high concentrations the number of nematodes in the stock is estimated, e.g., the nematodes are counted in one ml of the stock solution spread on a slide or petri dish (it can be helpful to draw or scratch a grid into the bottom); the count is repeated 3 times and the number of nematodes is estimated from the average. The suspension can then be diluted or concentrated by letting the nematodes settle or centrifuging.

The percentage survival is an indication of the quality of the suspension. If survival is low the remaining nematodes may not be in good shape, their reserves may be low or they may have lost the symbiotic bacteria. A better indication of the quality is the percentage of nematodes with bacteria. This can be examined by decapitating a number of nematodes using a piece of razor blade or a thin injection needle (this will make the gut spill out), fixing the slide by heating and staining with gram stain (no counter stain needed). The *Xenorhabdus* can be seen as typical rods attached to the oesophagus.

Laboratory Assays

Petri dish assay : Insects are kept individually in inverted plastic petri dishes (9 cm diameter) lined with filter paper. Various nematode doses (10, 100, 1000...nematodes/insect) are added in 2 ml of water. The assays are kept in the dark at 26–27°C and checked daily. Dead insects are dissected to ascertain infection.

This method is very artificial but useful to ascertain susceptibility of the insect and pathogenicity of the nematodes. A more realistic assay is to expose the insects to soil with a certain number of nematodes added (e.g. equivalent to 10^4, 10^5 and/or $10^6/m^2$ or per kg). The containers should be kept out of direct sunlight at 24–32°C and checked regularly for 1–3 weeks.

For field tests large amounts of nematodes are needed that can (only) be produced on artificial media. There are some requirements which can be major obstacles when working in remote areas. Generally the nematodes should be protected from temperature extremes during transportation. The product is diluted on the spot and applied during early morning or late evening. To enhance chances of survival for the nematodes pre- and post-application irrigation is usually advisable (*see also* Georgis and Gaugler, 1991).

APPENDIX

1. Methods for isolation of nematodes
The (modified) Baermann funnel, the flotation method and the *Galleria* bait.
(after Curran and Heng (1992) who compared the effectivity of these methods to estimate the number of nematodes in soil samples)

Modified Baermann funnel
1. 80 g soil on tissue paper supported by nylon mesh in polystyrene container (6 cm deep, 9 cm diameter)
2. Add 100 ml water
3. Leave for 24 hours at 23°C

The nematodes migrate through the mesh and end up in the water in the polystyrene container. The nematode suspension can be poured off.

Flotation method
1. 80 g soil in 1 litre beaker
2. Add 200–300 ml water
3. Stir vigorously
4. Let settle for 30 sec
5. Pour supernatant in 1 litre polypropylene centrifuge-bottle
6. Repeat this 3 times
7. Centrifuge, 1,000 rpm (250 *g*) 4 min
8. Pour the supernatant in 100 ml beaker
9. Add 100 ml 20% NaCl to the soil pellet, mix
10. Centrifuge 250 *g* 2 minutes
11. Combine supernatants
12. Repeat
13. Pool supernatants
14. pour through 63 µm pore (240-mesh) wire sieve

Live bait
1. 80 g soil
2. Put five late 3rd instar *Galleria mellonella* on surface
3. Remove after 24 hours, wash
4. Repeat with 5 larvae, remove after 72 hours, wash
5. Repeat
6. Incubate larvae 23°C, 75% RH, 4 days

2. White trap
A petri dish with some water and a watch glass (concave side up) in the centre over which a filter paper is placed. The insects are put on the edge of this glass in such a way that the nematodes hatching from the cadavers can reach the water easily, but most of the debris will stay out of the fluid. The water is changed daily and the nematodes are washed before storing them at 15°C.

3. *Galleria* rearing medium (Poinar, 1975)

distilled water	100 ml
honey	100 ml
glycerol	100 ml
vitamins (Decca Visol)	5 ml
Mix well. Add to:	
Pablum or Gerbers mixed cereal	1200 ml

The honey can be warmed to make it more liquid. The ingredients can be adjusted to what is locally available. We reared lesser waxmoth using a variant of the recipe (vitamins = children's multivitamins, cereal = cheapest breakfast cereal available). The medium is inoculated with *Galleria* larvae and/or adults. Wax paper strips are provided for oviposition. These can periodically be introduced to boxes with fresh medium.

4. Mounting (after Poinar and Thomas, 1984)

Permanent mounts can be prepared as follows:

1. Heat kill the nematodes by adding hot physiological solution
2. Transfer to fixative, preferably TAF (7 ml 40% formalin, 2 ml triethanolamine, 91 ml distilled water)
or 3% formalin (possibly add equal amount of 6% formalin to the nematodes in the solution)
or 70% alcohol
3. After one week transfer to glycerin solution (70 ml 95% ethanol, 5 ml glycerin, 25 ml water) for 2 weeks at room temperature in a partly covered small container.
4. Mount on slide
    - transfer to drop of glycerin on slide
    - add 3 supports to avoid squashing the nematodes
    - push the nematodes to the bottom of the drop, arrange
    - cover, seal with nail polish, wax, synthetic resin

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