Mass Production of the Entomogenous Nematode Heterorhabditis heliothidis (Nematoda: Heterorhabditidae) on Artificial Media

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Abstract: Heterorhabditis heliothidis is reared monoxenically on an artificial medium consisting of commercially available nutrient broth, yeast extract, and vegetable oil. These components are cooked with flour and coated onto polyether polyurethane sponge, autoclaved, inoculated with a suspension of the bacterial symbiont of the nematode, and incubated at 25 C for 3 d. The bacterial garden on sponge provides an excellent rearing medium. Up to 10 million infective juveniles are produced per 250 ml rearing flask in one month. *Key words:* greater wax moth, *Galleria mellonella,* bacteria, insects, *Xenorhabdus luminescens.*

The entomogenous nematode *Heterorhabditis heliothidis* Poinar, 1976 has recently been reported in New Zealand (8) Infective juveniles of this species carry in their guts the symbiont *Xenorhabdus luminescens* Thomas and Poinar, 1979, a bacterium lethal to the nematode's hosts. Under laboratory conditions the nematode-bacterium complex kills the local insect pest species porina, *Wiseana cervinata* (Walker); greasy cutworm, *Agrotis ypsilon aneituma* (Walker); black beetle, *Heteronychus arator* (F); and tropical army worm, *Spodoptera litura* (F).

To investigate the biological control potential of the complex against these pests in the field, large numbers of infective juveniles are required. Limited numbers can be reared on a natural or alternative host (4), but large numbers for field application can be reared more economically monoxenically on artificial media; i.e., homogenized kidney and heart tissues of animals and birds (1) or dog food (5). Rearing methods based on these media, however, are laborious and generate offensive odors. Therefore, a medium, based on Difco Bacto nutrient broth, which is easier and more pleasant to work with was developed.

MATERIALS AND METHODS

Extraction and preparation of H. heliothidis: Infective *H. heliothidis* juveniles were extracted from soil by the sugar floatation method (3). The collected material was transferred to a watch glass and the *H. heliothidis* infective juveniles transferred to slides to confirm identification. The number of infective juveniles extracted from soil was usually low. To increase their number, 25–50 specimens were injected into the abdomens of greater wax moth larvae, *Galleria mellonella* (L). The inoculated larvae, incubated at room temperature in the dark, died within 48 h. After 8 d they were transferred to a White (7) water trap for the extraction of infective nematode juveniles. Nematodes were collected daily for 5–10 d. When stored in aerated water at room temperature, they survived several months.

Isolation of symbiotic bacteria: About 10 infective *H. heliothidis* juveniles were injected into the abdomen of a greater wax moth larva. After 24 h, a leg of the wax moth larva was removed and the released haemolymph was streaked on Nutrient-Agar (NA) plates containing 0.8% Difco Bacto nutrient broth and 1.2% agar. Plates were incubated at 25 C. After 3 d, characteristic bioluminescent colonies of *X. luminescens* (6) developed; these were repeatedly subcultured until a pure culture was obtained. Pure cultures were transferred to nutrient broth slants, incubated at 25 C for 3 d, stored at 6 C, and subcultured at least once a month to maintain viability.

Preparation of monoxenic H. heliothidis inoculum: As symbiotic bateria on NA plates did not support efficient nematode development, the nutrient broth content was doubled and 1% corn (maize) oil was added. Thus fortified, the medium supported a bacterial growth on which the nematodes developed as fast as on a natural host.

Plates of fortified NA medium were evenly inoculated with a suspension of *X. luminescens* in peptone water and incubated at 25 C. After 3 d, a layer of bacterial

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growth covered the plate. A small piece of agar was then removed from the center of the plate, and an aqueous suspension of infective *H. heliothidis* juveniles was placed in the resulting cavity. These juveniles moved into the bacterial growth (leaving dead specimens and contaminants behind), fed on the bacteria, and developed into adult females within 3 d.

The females, having developed on a pure culture of *X. luminescens,* usually did not contain other bacteria. To check that they were monoxenic, 20 specimens were each transferred to a fresh fortified NA plate. After 3 d the developing bacteria became visible around the females, and after 5 d they had developed into large colonies in which the females had produced offspring. To test the purity of the bacteria, half of each culture was transferred to peptone water and shaken for about 1 h. The nematodes were then allowed to settle, and a small sample of the bacterial suspension was streaked out on NA plates. The plates were incubated at 25 C and evaluated 3 d later. Plates without contaminants originated from colony halves with monoxenic nematodes. The undisturbed matching halves were used as inoculum for mass production.

Mass culture method: The medium consisted of 0.44 g Difco Bacto nutrient broth, 0.16 g Difco Bacto yeast extract, 7.2 g soy flour, 5.2 g corn oil, and 27 g water. The nutrient broth and yeast extract were dissolved in water and together with the corn oil and soy flour cooked into a smooth roux. To create a large surface area, the roux was squeezed thoroughly into 9 g of damp polyurethane foam chips (personal communication, Bedding). The impregnated chips were placed in a 250-ml Erlenmeyer flask and autoclaved for 15 min at 100 KPa. To prepare the flask for mass production, 1 ml of a suspension of *X. luminescens* in peptone water was evenly distributed over the foam chips in the culture flask 3 d before the introduction of the monoxenic nematode culture. After introduction of the nematodes, the flasks were incubated in the dark at 25 C for 5 wk. The contents of the flask were then ready for extraction or to inoculate additional culture flasks.

For the extraction of infective juveniles, the contents of the flask were spread out on a sieve (40 mesh) in a dish filled with water. The nematodes moved out of the sponge into the water, passed through the sieve, and accumulated on the bottom of the dish. After about 2 h they were transferred to a 2–1 beaker and cleaned by decanting the supernatant at hourly intervals after the nematodes settled. This process was repeated at least three times. Cleaned suspensions were stored in 250-ml aerated Erlenmeyer flasks at room temperature.

RESULTS

Three days after inoculation with the bacterial suspension, the culture flasks were uniformly colonized. When nematodes (mostly feeding stages) from the monoxenic culture on fortified NA plates were introduced to the flasks they spread out over the bacteria in an irregular manner. However, when nematode infested sponge pieces from old culture flasks were used as inoculum, the nematodes (mostly infective juveniles) quickly and uniformly colonized the flasks; these flasks usually produced larger yields than flasks infested with nematodes from nutrient agar cultures. In either case, after 2 wk, females could be observed adhering to the inside of the flask and progeny of these females developed into infective juveniles after about 3 wk. The population was ready for extraction after 4 wk. Each flask yielded about 10 million infective juveniles.

DISCUSSION

Traditionally, insect parasitic nematodes were obtained from infected insects in field collections. More recently they were obtained through a trap host (2). In either case, the nematodes have to remain in the host until they develop into infective juveniles. Infective juvenile nematodes extracted from soil, as in the method described here, can be used for further propagation immediately.

For mass production on artificial media, the nematodes must be cleaned of contaminating bacteria. As some of these bacteria are located in the gut, only feeding stages can be successfully cleaned. In the method described here, the nematodes were cleaned by allowing infective juveniles to feed and

develop on a pure culture of the bacterium *X. luminescens*. This bacterium replaces the contaminants in the gut of the nematode. Defecated contaminants are prevented from developing by the activity of the bacterial culture on the plate. Without further cleaning the resulting females usually produce monoxenic populations. Once a monoxenic population is established, it can be maintained by transfer to fresh culture flasks at monthly intervals.

The medium can be adapted to availability of ingredients. The soy flour, which serves as a binding agent, can be replaced by ground millet, wheat germ, or fine cornmeal; gluten flour is toxic. Agar is not a suitable binding agent because during sterilization in the autoclave it liquifies and drips out of the sponge. Agar works well in plates provided less than 1% oil is used in the medium. If more is used, a continuous layer of oil is formed on the surface of the plate and bacterial growth is prevented. The oil component of the medium is not restricted to corn oil. Other oils such as soybean oil, safflower oil, sunflower oil, and animal fats are suitable alternatives. In addition, butter can be used with some success.

When large numbers of infective juveniles are required, the contents of one flask are distributed over 10–15 flasks; each flask produces 10 million infective juveniles in 4 wk. The cost of the medium to produce one million infective nematode juveniles with the method described here is less than U.S. $0.01.

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