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Melanization of *Steinernema feltiae* Filipjev and *S. kraussei* Steiner in larvae of *Otiorhynchus sulcatus* (F.)

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Summary – The present study showed that late instar (L 4-6) *Otiorhynchus sulcatus* can encapsulate and melanize invasive juveniles (I J) of *Steinernema feltiae* and *S. kraussei* that enter their digestive tracts. Dissection of *O. sulcatus* larvae, exposed at 9 °C to nematode isolates found in the Swiss Alps, revealed up to nine melanized I J in the mid-gut region. Encapsulation of I J occurred exclusively in insect larvae that they died from the nematode treatment. The observed immune response in *O. sulcatus* larvae is therefore unimportant for the infectivity of *S. kraussei* and *S. feltiae* at 9 °C. The potential influence of temperature on both the host's immune system and the bacterium/nematode complex is discussed.

Résumé - Mélanisation de* Steinernema feltiae *Filipjev et* S. kraussei *Steiner à l'intérieur des larves d'*Otiorhynchus sulcatus *(F.). – La présente étude démontre que les derniers stades instars (L 4-6) d'*Otiorhynchus sulcatus* sont capables d'encapsuler et de mélaniser les juvéniles infestants (I J) de *Steinernema feltiae* et *S. kraussei* ayant pénétré dans leur tractus digestif. Lors de dissections de larves d'*O. sulcatus* exposées, à 9 °C, à des isolats de nématodes provenant des Alpes Suisses, le nombre d'I J mélanisés trouvés dans l'intestin moyen peut s'élever jusqu'à neuf. L'encapsulation des I J ne se produit que dans les larves d'insectes mortes à la suite du traitement par les nématodes. L'immuno-réaction observée, à 9 °C, chez les larves d'*O. sulcatus* ne joue donc aucun rôle dans l'infestivité de *S. kraussei* et *S. feltiae*. L'influence possible de la température tant sur le système immunitaire de l'hôte que sur le complexe bactéries/nématode est discutée.

Key-words : Melanisation, encapsulation, *Steinernema*, black vine weevil, immune system.

Insects have evolved an efficient immune system to control invading micro-organisms and parasites. The major defence mechanisms of insects against attacks by parasitic nematodes involve cellular and humoral factors. While humoral (i.e., cell-free) encapsulation seems to be restricted to certain Diptera (Götz & Boman, 1985), cellular defence reactions are found in several insect orders. A common type of cellular response to entomopathogenic nematodes is encapsulation and melanin formation. Although entomopathogenic nematodes were frequently used to control the larvae of *Otiorhynchus sulcatus* (F.) (Moorhouse *et al.*, 1992), encapsulation of nematodes is not reported in the literature. This paper shows melanotic encapsulation of infective juveniles (I J) of *Steinernema feltiae* (Filipjev) and *S. kraussei* (Steiner) in late instar larvae of the black vine weevil.

Material and methods

Late instar larvae (L 4-6) of *O. sulcatus* were exposed in potted strawberries to twenty nematode isolates found in the Swiss Alps; i.e. one isolate of *Heterorhabditis* sp. (NW European type), six *S. feltiae*, twelve *S. kraussei*, and one *Steinernema* sp. 1 [a species closely related to *S. intermedia* (Poinar)]. All steinernematids were identified by morphometric characters (I J and first generation males and females) and by analysis of the restriction fragment length patterns (performed by A. Reid, GB-Ascot). The bioassays were performed at approximately 9 °C at two concentrations (2000 or 4000 nematodes per dm^3 of soil). After 10 or 20 days, the insect larvae were recovered, washed, and incubated on wet filter paper at 20 °C. Mortality was assessed after 3 and 8 days. Both dead and surviving larvae were dissected.

Results

Late instar larvae of *O. sulcatus* were susceptible to all isolates (except *Heterorhabditis* sp.). Encapsulation of I J was observed with *S. kraussei* (in 6 % of 68 infected insect larvae) and with *S. feltiae* (21 % of 19), and occurred exclusively in larvae that had died from the nematode treatment. Only one *O. sulcatus* larva (out of 527) survived nematode infection. Its hind-gut was filled with a brown mass of presumably melanized material, which loosely enclosed a dead juvenile of *S. kraussei*.

The larvae of *O. sulcatus* responded similarly to invading *S. feltiae* and *S. kraussei*. Dissection revealed melanized I J in the mid-gut region (e.g. Fig. 1 A, C). Up to eight I J of *S. feltiae* or nine *S. kraussei* became melanized in a single dead weevil larva. Under the light microscope the layer enclosing the nematodes was brown to black in colour, and is therefore considered to be melanin. 54 % of the melanized nematodes (n = 33) were completely

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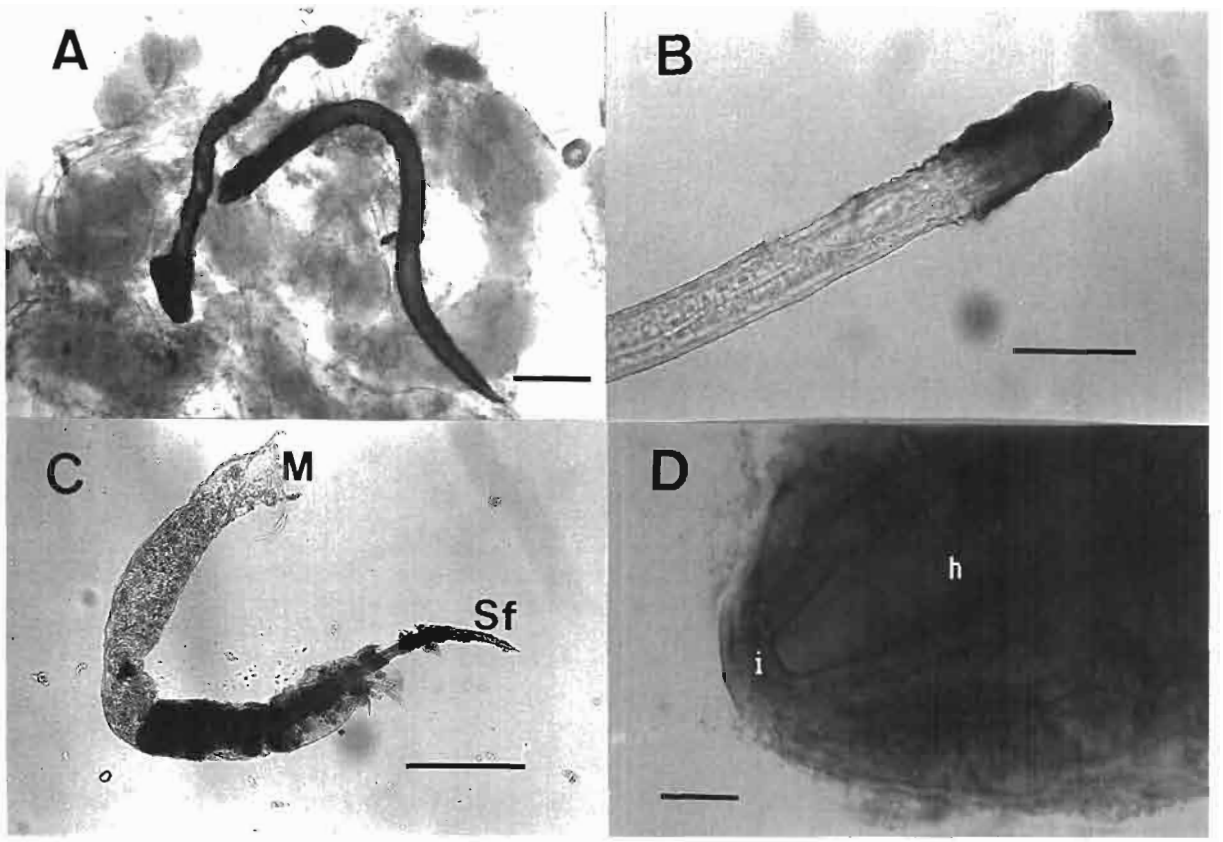

Fig. 1. *Encapsulated steinernematids in late instar larvae of* Otiorhynchus sulcatus *photographed using a light microscope. A : Two completely encapsulated, immobilised infective juveniles (If) of* Steinernema kraussei *in the mid-gut region; B : A partially melanized If of* S. kraussei *with a deposit of melanin around its head; C : An encapsulated If of* S. feltiae *(Sf) in an opened Malpighian tube (M); D : A close-up of the nematode shown in (C), the capsule shows an impression (i) of the nematode's head, h : position of the head.* (Scale bars : A, C = 250 μm; B = 50 μm; D = 10 μm.)

sealed by this material (Fig. 1 A, C) that forms a negative of the nematode's body (Fig. 1 D). In other specimens, only the posterior and/or the anterior ends of the larvae were covered (Fig. 1 B).

Non melanized specimens carried *Xenorhabdus bovienii* in their vesicles. In contrast, nematodes found in the same insect with only their heads melanized had empty intestinal vesicles, but rod like bacteria (2-4 μm long) attached around the mouth and behind the head capsule. Bacteria of the same shape and size occurred also in the Malpighian tubules of weevil larvae infected by either *S. kraussei* or *S. feltiae*.

Discussion

Recognition of entomopathogenic nematodes by the immune system of insects is reported for Coleoptera (e.g. Thurston *et al.*, 1994), Diptera (e.g. Ehlers & Gerwien, 1993) and Blattodea (Zervos & Webster, 1989). Findings of the present study show that late instar of *O. sulcatus* can encapsulate and melanize invasive juveniles of *S. feltiae* and *S. kraussei* that enter their digestive tracts. However, at least some of these nematodes managed to release their bacteria before being melanized. The nematode shown in Fig. 1 B, for example, has already released its symbiotic bacterium, as indicated by the empty intestinal vesicle and the presence of numerous bacteria behind the head capsule and around the mouth. These bacteria were similar to *X. bovienii* photographed by Haukeland (1993) on *S. feltiae*.

Two distinct degrees of melanin deposition were noted. Fully encapsulated larvae showed no activity, even when the capsules were ruptured. These nematodes were presumably dead. In contrast, partially melanized nematodes were all still alive. This suggests that encapsulation starts around the nematode's anus and/or head where the released bacteria come first into contact with the host's defence system. Mechanisms involved in capsule formation are outlined by Götz and Boman (1985).

The main entry points into *O. sulcatus* larvae are through the mouth and anus. To reach the insect's hemocoel, the I J must penetrate the gut wall or they pass along the lumen of the hind-gut and enter the Malpighian tubules. Both partially and completely melanized nematodes occur in the mid-gut and the Malpighian tubules. Only one (atypically) melanized specimen was found in the hind-gut. This suggests the absence of immune response in the hind-gut.

The presence of melanized specimens in the mid-gut and in the Malpighian tubules is difficult to interpret. On the one hand, cellular encapsulation in the alimentary tract (Fig. 1 C) is unlikely since hemocytes are lacking. On the other hand, cell-free encapsulation seems to be restricted to certain dipterans (Götz & Boman, 1985). One explanation could be that the Malpighian tubules are pierced by I J that try to push their heads through the cuticle. This would allow access of hemocytes followed by melanin deposition on the nematode's head. Analysis of the cell types using an electron microscope would be necessary to draw general conclusions about the nature (i.e., cellular or humoral) of the capsules.

Interactions of the nematode and its symbiotic bacteria with the host's defence system are poorly understood. Dunphy and Webster (1987) suggested that chemical properties of the nematode's (i.e., *S. carpocapsae*) epicuticle prevent encapsulation by the hemocytes of the host. In *O. sulcatus* larvae, however, the juveniles of both *S. kraussei* and *S. feltiae* and/or their symbiotic bacteria were recognised as non-self. Since encapsulation by *O. sulcatus* was rare in the present study and is not reported in the literature, encapsulation is presumably suppressed under most circumstances. Suppression of the encapsulation process may be influenced by temperature effects on both the host's immune system and the bacterium/nematode complex. At temperatures favourable for bacterial growth, *X. nematophilus*, for example, can suppress the cellular response by destroying the hemocytes of the nematode's host (Dunphy & Webster, 1984). Most control programs against *O. sulcatus* were performed at relatively high temperatures (> 15 °C). This allows rapid growth of the symbiotic bacteria, and, subsequently, inhibits encapsulation of the nematode. At low temperatures, however, growth of the symbiotic bacteria is considerably reduced (Gwynn & Richardson, 1994). The defence system of an insect larva may then overwhelm bacterial multiplication by rapid encapsulation of the nematode immediately after release of the symbiotic bacteria. One would thus expect the immune system to be most efficient at temperatures below optimal growth of the bacterial symbiont, provided that the temperature profiles for the growth-rates of *Xenorhabdus* spp. are similar to those reported by Gwynn and Richardson (1994). Since a host's immune system will have a lower threshold temperature for activity, its temperature range for optimal efficiency against specific nematode-bacteria complexes is relatively narrow.

Supposing the nematode is not recognised as non-self until its bacterium is released, the critical factor in the host's defence response would be the speed of encapsulation as compared with the speed of bacterial multiplication. To be effective as a biological insecticide, the I J should retain their bacteria until they encounter an environment and a temperature conducive to rapid bacterial growth. Presumably, the hind-gut of *O. sulcatus* larvae represents a rather unfavourable environment for bacterial release (e.g. defecation can remove both bacteria and nematodes). This hypothesis agrees with findings of the present study, i.e. no bacterial release and no melanization of I J in the hind-gut. Retention of symbiotic bacteria in the vesicle, i.e., 2-5 h postparasitism (Dunphy & Thurston, 1990), allows an invasive nematode to pass the lumen of the hind-gut without being recognised as non-self.

Only one late instar *O. sulcatus* specimen (out on more than 500) was found in which melanization of the nematode presumably prevented the death of the insect. In all the other singly or multiply-infected *O. sulcatus* larvae, the infective nematodes killed their host. This is in contrast to findings of Pye and Burman (1978) and of Ehlers and Gerwien (1993), showing that encapsulation of nematodes is important for the survival of *Hylobius abietis* and *Tipula paludosa* larvae, respectively. The observed immune response in *O. sulcatus* larvae is thus unimportant for the infectivity of *S. kraussei* and *S. feltiae* at 9 °C, as well as in most control programs. However, under specific environmental conditions (e.g. soil temperatures < 9 °C) the infectivity of certain nematode/bacteria complexes may be temperated by encapsulation.

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