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Scanning electron microscopy study of infective juveniles *Heterorhabditis bacteriophora* (*Nematoda: Heterorhabditidae*)

Badania larw inwazyjnych *Heterorhabditis bacteriophora* (*Nematoda: Heterorhabditidae*) z zastosowaniem skaningowej mikroskopii elektronowej

SUMMARY

Studies were conducted on the morphology of infective larvae *Heterorhabditis bacteriophora* using a scanning electron microscopy. The purpose of the studies was to point to the morphological and morphometric characters of infective larvae of entomogenous nematodes that are useful in taxonomy and species identification. It was found out that the studies using SEM make it possible to analyze the morphological characters unvisible in an optic microscope. Morphometric properties can also be examined in SEM, and since the measurements are very accurate they can be applied in taxonomic studies and in species identification. The preparation of the material for the studies in SEM and their performance is neither more labour-consuming nor more tiresome than in optic microscopy.

STRESZCZENIE

Przeprowadzono badania nad morfologią larw inwazyjnych *Heterorhabditis bacteriophora* z wykorzystaniem skaningowego mikroskopu elektronowego. Celem badań było wskazanie użytecznych w taksonomii i identyfikacji gatunku cech morfologicznych i morfometrycznych larw inwazyjnych entomopatogenicznych nicieni. Stwierdzono, że badania przy użyciu SEM pozwalają analizować cechy morfologiczne, które nie są widoczne w mikroskopie optycznym. Cechy morfometryczne także mogą być badane w SEM, a dokonane pomiary są bardzo dokładne i możliwe do

wykorzystania w badaniach taksonomicznych i identyfikacji gatunku. Przygotowanie materiału do badań w SEM oraz ich prowadzenie nie jest bardziej pracochłonne i uciążliwe niż w mikroskopii optycznej.

K e y w o r d s: *Heterorhabditis bacteriophora*, SEM, morphology of infective juvenile, nematodes.

INTRODUCTION

Profound and multi-directional studies on entomogenous nematodes provide abundant information concerning their biology, morphology, physiology and ecology [2–4, 9]. New species and their local populations are discovered and described. Taxonomists attempt to sort out the diversity in this group of nematodes and point to the distinct features of the species already described that may be useful in their identification. The establishment of such properties is very hard at the present stage due to the natural changeability within the described species and because of various techniques applied by researchers in preparing the material for studies. At present, there is an urgent need to work out possibly simple, universal and fast methods which would provide univocal results and which would enable species identification.

The present paper is an attempt to point to the morphological and morphometric characters on the basis of the analysis of nematode infective larvae morphology using scanning electron microscopy (SEM) that would be useful in taxonomy and species identification. The present studies focused on the morphology and morphometry of infective larvae in view of the fact that the infective stage is comparable in all species of entomogenous nematodes. Besides, it can be obtained in huge quantities, with no additional contaminations. Although the morphological and morphometric characters of infective larvae of entomogenous nematodes are considered by many researchers to be very useful in taxonomy and species identification, so far they have been of little use due to the lack of sufficient studies in this field.

MATERIAL AND METHODS

The studies used infective larvae *Heterorhabditis bacteriophora* obtained as a result of infecting the caterpillars of *Galleria mellonella* L. with a preparation LARVANEM by Koppert B. V. (Holland). LARVANEM is a bio-preparation produced on the basis of infective larvae *Heterorhabditis bacteriophora* and it is also applied in Poland, in agriculture and forestry in plant protection. *Heterorhabditis bacteriophora* is the species that was described in the family *Heterorhabditidae* the earliest.

The studies used the freshly collected infective larvae (24–48 hours after leaving the host's body). The larvae were washed three times in Ringer solution [5] in order to get rid of impurities. The dead infective larvae (hot Ringer solution 60°C, 3 minutes) were then fixed in 8% glutaraldehyde (25% glutaraldehyde POCH diluted in Ringer solution) for 24 hours at the temperature of 20°C. After fixation, the suspension of infective larvae was again washed three times with Ringer solution (each turn — 15 minutes). The preserved and washed nematodes were dehydrated in a series of alcohols (ethyl alcohol POCH: 30, 50, 70, 90, 100%, each turn — 10 minutes), after which the alcohol was exchanged for amyl acetate, (POCH) (ETOH — amyl acetate: 25 : 75, 50 : 50, 75 : 25, 100% amyl acetate each turn — 10 minutes). So prepared larvae *Heterorhabditis bacteriophora* were dried at the critical point (Polaron CPD 7501, Quorum

Technologies Ltd Company), then coated with a 30 nm layer of gold-pallad (Polaron SC 7620 Mini Sputter Coater, Quorum Technologies Ltd Company) and observed in scanning electron microscope (LEO 1430VP) at the voltage of 15 kV.

The infective larvae were measured using the program LEO-32 (Annotation). Results of the measurements were expressed in the form of the following morphometric characters:

L — body length,

EP — distance from anterior end to excretory pore,

T — tail length (distance from the beginning of anus to the end of the body),

L/EP — body length/distance from anterior end to excretory pore,

L/T — body length/tail length (distance from the beginning of anus to the end of the body),

EP/T — distance from anterior to excretory pore/tail length (distance from the beginning of anus to the end of the body).

RESULTS

MORPHOMETRIC CHARACTERS

Using a relatively small enlargement in SEM (within the range between 350–800×) it is possible to establish the length of nematode infective larvae precisely. It was found out that the mean length of infective larvae (L) is 458.41 μm (429.80–497.90) (Tab. 1, Phot. 1).

With a much greater range of enlargements (3000×) measurements were made of the distance from the anterior end to excretory pore (EP) and from the beginning of anus to the end of the body (T). The mean values of those measurements were 88.48 (82.16–93.87) and 73.18 μm (65.57–78.93), respectively (Tab. 1, Phot. 2 and 3).

Based on the measurements, the coefficients L/EP, L/T and EP/T were calculated. It was found out that their mean values were: 5.18 (4.88–5.38), 6.27 (5.88–6.92), 1.21 (1.13–1.33), respectively.

Table 1. Measurements of infective juvenile *Heterorhabditis bacteriophora* (number of specimens measured = 29)

No.	L (in μm)	EP (in μm)	T (in μm)	L/EP	L/T	EP/T
1	2	3	4	5	7	
1	482.60	92.93	73.55	5.19	6.56	1.26
2	444.50	86.70	74.59	5.13	5.96	1.16
3	436.80	87.07	70.88	5.02	6.16	1.23
4	469.40	88.85	77.29	5.28	6.07	1.15
5	473.90	91.21	72.55	5.20	6.53	1.26
6	459.00	86.95	71.36	5.28	6.43	1.22

Table 1 — contd

1	2	3	4	5	6	7
7	458.00	87.74	71.68	5.22	6.39	1.22
8	441.30	84.56	73.66	5.22	5.99	1.15
9	452.10	91.07	73.65	4.96	6.14	1.24
10	453.70	87.16	65.57	5.21	6.92	1.33
11	453.60	86.87	73.13	5.22	6.20	1.19
12	434.40	82.57	71.34	5.26	6.09	1.16
13	472.40	90.60	73.90	5.21	6.39	1.23
14	441.90	82.16	72.77	5.38	6.07	1.13
15	470.80	87.70	75.56	5.37	6.23	1.16
16	429.80	88.10	70.53	4.88	6.09	1.25
17	440.50	85.62	74.89	5.14	5.88	1.14
18	497.90	93.87	78.93	5.30	6.31	1.19
19	454.50	89.91	70.80	5.06	6.42	1.27
20	490.50	93.03	77.88	5.27	6.30	1.19
21	438.80	89.75	68.00	4.89	6.45	1.32
22	467.70	90.17	72.56	5.19	6.45	1.24
23	454.40	90.22	72.64	5.04	6.26	1.24
24	469.60	90.70	73.45	5.18	6.39	1.23
25	444.50	86.70	74.59	5.13	5.96	1.16
26	456.50	86.45	74.88	5.28	6.10	1.15
27	470.40	88.68	74.76	5.30	6.29	1.19
28	459.70	87.94	72.61	5.23	6.33	1.21
29	474.80	90.64	74.34	5.24	6.39	1.22
Min $\bar{X}$	458.41	88.48	73.18	5.18	6.27	1.21
Range	429.80–497.90	82.16–93.87	65.57–78.93	4.88–5.38	5.88–6.92	1.13–1.33
Standard deviation SD	17.06	2.85	2.71	0.13	0.22	0.05

MORPHOLOGICAL CHARACTERS

The use of greater enlagements (5000–15000×) in SEM showed that infective larvae have a very characteristic head region (Phot. 4). The triangular shape of the stomal opening, the shape and location of labial papillae and the location of amphidia on the head sides are distinctly visible. The cuticle covering the head section has a characteristic sculpture in the form of rings. Further on to the end of the body the cuticle forms squared fields and lists. Their structure is very well seen in SEM pictures (Phot. 4, 5, 8).

The location of the excretory pore in relation to the ”squared field” of the cuticle sculpture can be easily determined (Phot. 5). The analysis of over 30 SEM

pictures showed that the excretory pore lies in the final region of the squared field or just behind it.

The cuticle of the final section of the infective larva is richly sculptured (Phot. 6). The shape and number of the cuticular lines in front of the excretory pore are very characteristic and can be easily analyzed (Phot. 7). Having analyzed more than 30 SEM pictures it was found out that three lists of cuticles reach the front part of the excretory pore.

Details of the cuticle sculpture in different regions of the body of the larvae require separate analysis. The arrangement of circular lines, squared fields and longitudinal cuticles in the front section of the body seem especially characteristic (Phot. 8).

DISCUSSION

So far, no thorough studies have been conducted on the morphology of the infective larvae of Nguyen nematodes. That is why these properties have never been used in taxonomy or species identification [1 and 4]. Observation and analysis of the majority of morphological and biometric properties is not possible with the use of light microscopes. Scanning electron microscopy offers much greater ranges of enlargements and enormous distribution as compared to light microscopy. The use of computer software of the microscope allows fast measurements and digital analysis of the picture.

The preparation of the material for studies may initially pose some problems since the methods of preserving, drying and coating of the material have to be established experimentally. Nevertheless, we consider the methods used in SEM as less labour consuming and less tiresome than the optic microscopy methods applied in the morphological and biometric studies on nematode larvae.

The usefulness of infective larvae in species identification of nematodes has been appreciated by a number of researchers, however, due to the technical problems associated with studying the morphology and biometry of infective larvae, mainly the morphological and biometric properties of males and females of the fist generation of nematodes as well as the genetic data have been used in taxonomy [1]. Using the SEM research techniques, the properties that are completely inaccessible by means of light microscopy can be discovered, precisely characterized and compared [6–8].

A comparison of measurements results concerning infective larvae performed by means of light microscopy [9] and by means of SEM proves that although different results referring to the dimensions of the larvae bodies are achieved, the calculated coefficients are very similar. The coefficient values are respec-

tively: for optic microscopy: L/EP = 5.48, L/T = 6.26, EP/T = 1.14, and for SEM: L/EP = 5.18, L/T = 6.27, EP/T = 1.21. One of the basic factors affecting differentiation of the results can refer to completely different techniques of preservation and further preparation of the material.

However, one has to remember the effect of various factors operating before and independently of the research techniques that can have a decisive effect on the final results. These factors include for example the conditions of nematodes culture (species and developmental stage of the host, temperature and moisture), the number of passages from nematode isolation, the age of infective larvae and methods of their storage. An obligation to describe the conditions in which the studies are performed, the methods of preservation and decolorization of the material has been neglected quite frequently. This makes interpretation of the results, especially the morphometric ones, hard and in extreme cases, impossible.

REFERENCES

1. Adams B. J., Nguyen K. B. 2002. Taxonomy and systematics. In: Entomopathogenic Nematology. Gaugler R. (ed.). CABI Publishing, CAB International.
2. Gaugler R., Kaya H. K. (eds.) 1990. Entomopathogenic Nematodes in Biological Control. CRC Press.
3. Gaugler R. (ed.). Entomopathogenic Nematology. CABI Publishing, CAB International 2002.
4. Lacey L. A., Kaya H. K. (eds.) 2000. Field Manual of Techniques in Invertebrate Pathology: Application and Evaluation of Pathogens for Control of Insects and Other Invertebrate Pests. Kluwer Academic Publishers.
5. Lacey L. A (ed.) 1997. Manual of Techniques in Insect Pathology. Academic Press.
6. Mracek Z., Bednarek A. 1992. Cuticular structures of J2 and J3 of *Heterorhabditis*. Nematologica 38: 386–390.
7. Mracek Z., Weiser J., Arteaga E. 1984. Scanning electron microscope study of *Heterorhabditis heliothidis* (*Nematoda: Heterorhabditidae*). Nematologica 30: 112–114.
8. Nguyen K. B., Smart G. C., Jr. 1995. Scanning electron microscope studies of *Steinernema glaseri* (*Nematoda: Steinernematidae*). Nematologica 41: 183–190.
9. Poinar G. O., Jr. 1975. Description and biology of a new insect parasitic rhabditoid, *Heterorhabditisbacteriophora* n. gen. n. sp. (*Rhabditida; Heterorhabditidae n. fam.*). Nematologica 21: 463–470.