SEM Observations on the Intratracheal Existence and Cuticle
Surface of the Pine Wood Nematode, *Bursaphelenchus*
xylophilus, Associated with the Cerambycid Beetle,
Monochamus carolinensis

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Scanning electron microscopic observations were made to reveal the mode of existence of the pine wood nematode, *Bursaphelenchus xylophilus*, in the tracheae of adult cerambycid beetle, *Monochamus carolinensis*, and the cuticle surface property of the dispersal (dauer) 4th stage larvae (L_{IVS}) of the nematode. About 80% of all L_{IVS} were recovered from the thoracic tracheae and the rest from the tracheae of the abdomen, head, and legs. Most L_{IVS} orientated in the same direction in the tracheae, but some were reversed. The head of L_{IVS} was hemispherical with a small oral aperture, poorly developed amphid aperture, and no labial papillae. Many particles were observed on the sticky cuticle surface, but no bacteria were observed on the nematode specimens examined under SEM.

The pine wood nematode, *Bursaphelenchus xylophilus*, is associated with insects during the course of its life cycle. The dispersal (dauer) 4th stage larvae (L_{IVS}) are carried from dead to healthy pine trees mostly by the cerambycid beetle, *Monochamus* spp., and less frequently by other insects (MAMIYA and ENDA, 1972; LINIT et al., 1983). Not only are the insects contaminated by the nematodes, but the nematodes isolated from the dead pine trees are known to be contaminated with bacteria (TAMURA and MAMIYA, 1973). OKU and his colleagues (1980) proposed a double vector theory in which they stressed the importance of the toxin-producing bacteria, *Pseudomonas* sp., as a causal agent of pine wilt disease which is still devastating pine forests in Japan (MAMIYA, 1983) and less severely damaging pine trees in the USA (DROPKIN et al., 1981).

In the present investigation, SEM observations were made on the nematodes in the tracheae of adult cerambycid beetle, *Monochamus carolinensis*, and on the cuticle surface property of L_{IVS} of *B. xylophilus*. The results of the insect-nematode-bacteria association will be further discussed. Some of the results presented in detail here were the subject of a previous report (KONDO et al., 1982).

MATERIALS AND METHODS

Insect and nematode. Fifty-five dead Scotch pine, *Pinus sylvestris*, were collected at Christmas tree plantations in Missouri, USA in March, 1981, about 6 months after the

appearance of yellow brown needles, a typical disease symptom. The trees were 8 to 11 years old, with a mean diameter of 6.4 cm at the base of the stem. All branches were cut off and the remaining stems (1.5 to 2.0 m long) were kept in an outdoor screen cage to allow the adult *M. carolinensis* to emerge from early April to late June. Within one day after emergence, the beetles were fixed and processed for SEM observation or used for recovering L_IVs of *B. xylophilus* by immersing the chopped insect body in shallow water. Nematodes swimming out within 24 hr were used for SEM observation.

Scanning electron microscopy. The method employed in preparing materials for SEM was essentially that developed for soft biological materials (KELLEY et al., 1973). The materials were pre-fixed in 2% OsO₄ for 2 days at 5°C, thoroughly rinsed with distilled water, and treated with a saturated water solution of thiocarbonylhydrazide for 45 min at room temperature. After rinsing, they were post-fixed with 1% OsO₄ for 30 min at room temperature, and then dehydrated through a graded series of ethanol: 20, 40, 60, 80, 90, 100, 100, 100%, 2 hr in each with swirling. The dehydrated materials were critical point dried, mounted on specimen holders with conductive tape, coated with gold for 2 min by ion sputter, and observed under JSM-35 operated at 20 kV.

The materials prepared for investigating the intra-tracheal existence and cuticle surface of L_IVs were as follows:

1. L_IVs in the tracheae of head, thorax, abdomen, legs, and wings of adult *M. carolinensis* prepared as described above.
2. L_IVs in the thoracic tracheae dissected longitudinally along the dorso-ventral line just before the critical point drying.
3. L_IVs with or without four rinsings with distilled water before pre-fixation in 2% OsO₄.
4. L_IVs which were allowed to move out from the cut end of the thoracic tracheae placed on a clean cover glass strip, fixed overnight at 3–5°C by vapor of OsO₄, dried in a desiccator, and ion-sputter coated.

RESULTS

Intra-tracheal existence

The L_IVs of *B. xylophilus* invaded the tracheal system of adult *M. carolinensis* mainly through the largest spiracles on the metathoracic body region (Fig. 1A). When cutting was made on the large tracheae running on both sides of the beetle and on smaller tracheae branching out under the spiracles, many L_IVs emerged from the cut ends (Fig. 1B). Most L_IVs orientated in the same direction in the tracheae with a few nematodes in the opposite direction (Fig. 1C). Fewer L_IVs were observed in the abdominal tracheae (Fig. 2A). Some nematodes were reversed in the tracheae. A few L_IVs were recovered from the tracheae which ran from the thorax to the head of the beetle (Fig. 2B). The nematodes were also observed in the legs (Fig. 2C) and a few of them reached the tibia. Of all nematodes recovered from 15 beetles, an average of 82.8% were from the thorax, 14% from the abdomen, 0.7% from the head, and 2.5% from the appendages including legs, wings and antennae (data omitted).

Property of cuticle surface

To investigate the possibility that the toxin-producing bacteria are efficiently carried by the nematode from dead to healthy pine trees, SEM observations were

Fig. 1. The dispersal 4th stage larvae (LIVs) of the pine wood nematode, *B. xylophilus*, on the thorax of the adult cerambycid beetle, *M. carolinensis*. A: LIVs (Ne) invading the tracheae through the 1st abdominal spiracle on the metathoracic body region of the beetle. B: mass of LIVs emerging from the cut end of the tracheae (Tr) directly connected with the metathoracic spiracles. C: LIVs emerging from the cut end of the thoracic tracheae. Arrows indicate heads; body ends without arrows are tails.

Fig. 2. LIVs (Ne) in the tracheae of abdomen (A), neck (B), and leg (C). A: LIVs in the tracheae (Tr) of abdomen. Note some nematodes reversed (arrows). B: LIVs in the tracheae surrounding the gut (Gu) of the insect. One trachea contained a few LIVs and the other none. C: LIVs emerging from the cut end of the tracheae (Tr) in the femur (Fe) of leg.

made on the cuticle surface properties of LIVs, the developmental stage carried by the insect vectors. The LIVs had no functional mouth nor other orifices through which the bacteria might be able to get into the nematodes. The diameter of the oral aperture on the hemispherical head was less than $0.1 \mu\text{m}$ (Fig. 3A). Amphid apertures were almost closed and rarely exuded such substances as observed on those of the propagative form of *B. xylophilus* (unpublished data).

The body surface of LIVs was rather smooth but sticky, so that some nematodes

adhered to the wall of the glass container used for the specimen preparation. When the L_IYs were fixed without pre-rinsing, small particles were found on the cuticle but no bacteria were on any of the materials examined (Fig. 3B). These particles were mostly removed when the nematodes were rinsed with distilled water before pre-fixation (Fig. 3A).

In order to decrease the possibility that bacteria on the cuticle are washed off

Fig. 3. Cuticle surface of L_IYs. A: head of the L_IYs with an oral aperture (Oa) and amphid aperture (Aa). Nematodes were rinsed with distilled water before pre-fixation with 2% OsO₄. B: cuticle surface at the mid body part of L_IYs which were not rinsed with distilled water before pre-fixation with 2% OsO₄. Note the particles attached to the sticky cuticle surface. An: annulation, Lf: lateral field. C: L_IYs (Ne) fixed with OsO₄ gas and then processed for SEM observation without being passed through any fluid. Tr: trachea. D: enlarged view of the cuticle surface of the L_IYs shown in (C).

Fig. 4. A bundle of L_IYs (Ne) in the thoracic tracheae. The nematodes in the tracheae were cut just before the critical point drying. A: L_IYs closely packed in the tracheae between the thoracic muscles (Mu). B: enlarged view of the L_IYs shown in (A). Note the clean surface of the nematode cuticle excepting the particles which may have attached when the tracheae were cut.

Fig. 5. Bacteria on the mites (Mi). A: many bacteria on the dorsum of a mite on the abdomen of *M. carolinensis*. Note the clean cuticle surface of *B. xylophilus* (Ne) near the abdominal spiracle (Sp). B: enlarged view of various bacteria on the mite shown in (A).

during fixation and dehydration, the thorax of the beetle was cut longitudinally just before the critical point drying. Many nematodes were closely packed in almost all thoracic tracheae (Fig. 4A). The cuticle surface of these nematodes was contaminated with particles, though no bacteria were found (Fig. 4B). Even when the nematodes were fixed by OsO_4 gas and then processed without passing them through liquid-like fixative and dehydrating solution, the cuticle surface was free from bacteria, though the cuticle itself was considerably shrunk (Figs. 3C and 3D).

Mites and bacteria association

Mites (unidentified) were inhabiting the ventral sides of the abdomen, thorax, and on the abdominal dorsum under elytra of adult *M. carolinensis*. The number of mites recovered per insect was from 2 to 61, averaging 22 (data omitted). Most of them were more or less contaminated with bacteria which were attached to their anterior and posterior body parts (Fig. 5A). They were also detected in the interspace between these body parts and on the legs. The mites did not associate with a specific bacterium but were contaminated with various bacteria with different morphology (Fig. 5B). Bacteria were not found on the L_IVs of *B. xylophilus* on the insects which carried bacteria-contaminated mites (Fig. 5A).

DISCUSSION

The dispersal (dauer) 4th stage larvae (L_IVs) of the pine wood nematode, *Bursaphelenchus xylophilus*, are carried from dead to healthy pine trees with the aid of insects (MAMIYA and ENDA, 1972; MORIMOTO and IWASAKI, 1972). Of all known vectors in Japan, *Monochamus alternatus* is the most efficient, carrying ca. 15,000 L_IVs per insect on an average (MAMIYA and ENDA, 1972). In Missouri, USA, *M. carolinensis* was the most efficient vector and the average number of L_IVs recovered was ca. 19,000 per insect (LINIT et al., 1983).

The present SEM observations clearly showed the mode of existence of L_IVs in the tracheae of adult *M. carolinensis*. As in *M. alternatus*, the L_IVs invaded primarily through the large spiracles on the metathoracic body region of *M. carolinensis* and distributed mostly in the thoracic tracheae. The L_IVs were closely packed in the tracheae of the thorax and loosely in tracheae of the abdomen and appendages. Most of them orientated in the same direction, though some were reversed in the tracheae. The head of L_IVs apparently lacks the labial papillae and the amphid aperture does not emit such substances as seen on the propagative form of *B. xylophilus*. These structural characteristics seemed to show that the chemoreceptor of L_IVs was less functional than that of the propagative form. To determine the mechanism by which the L_IVs detect the spiracles of newly emerged adult *Monochamus* in the pupal chamber in dead pine trees and leave it during the maturation feeding on healthy pine trees, further physiological and ultrastructural investigations are required.

The association between nematode and bacteria is widely known (PITCHER and CROSSE, 1958; HAWN, 1963, 1971; GUPTA and SWARUP, 1972). For instance, a bacterium, *Corynebacterium* sp., specifically attaches to the cuticle of a nematode, *Anguina agrostis*, by means of a sticky slime material (capsule) around the bacterial cells (BIRD, 1981). Several species of bacteria have been isolated from the propagative form of the pine wood nematode: *Brevibacterium* sp., *Pseudomonas* sp., and *Bacillus* sp. (TAMURA and MAMIYA, 1973). OKU and his colleagues (1980) proposed a double vector theory: the insect carries the pine wood nematodes harboring the toxin-producing bacteria, *Pseudomonas* sp. However, the intimate association between the nematode and bacteria should be clarified as to the participation of toxin-producing bacteria in the disease incidence.

Under natural conditions, the developmental stage of the pine wood nematode carried by the insect from dead to healthy pine trees is limited to the L_IVs (MAMIYA and ENDA, 1972; MORIMOTO and IWASAKI, 1972). The oral aperture of the L_IVs was less than 0.1 μm in inner diameter, too narrow for the bacteria to enter the nematode body. Neither prominent cuticle invasion nor outgrowth was observed on the body surface of L_IVs, so that the smooth but sticky cuticle surface was the only possible site to which the bacteria may attach. The sticky substance on the nematode cuticle was water soluble because a surface wash with distilled water made the body clean. As shown under SEM, many particles were found on the cuticle, however, no bacteria were found on any nematodes examined in this experiment. None were found even on the cuticle of L_IVs fixed by OsO₄ gas and then subjected to the procedure without passing through liquid which may have washed them off. On the other hand, the inoculation of bacteria-free nematodes caused the pine wilt disease (KONDO et al., 1982; TAMURA, 1983). These results show that the L_IVs do not carry many toxin-producing bacteria and that the bacteria are not required to be transmitted by the insects in induction of the disease.

Various kinds of mesostigmatid mites are known to associate with the cerambycid beetle, *M. alternatus* (ISHIKAWA, 1977a, 1977b; TAMURA and ENDA, 1980). In the present investigation, many mites were contaminated with bacteria, but the L_IVs processed exactly the same way for SEM observation were not. However, the contribution of the bacteria on mites is not essential for the disease incidence, because the disease develops on nematode-inoculated pine trees without mite participation.

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