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A Nondestructive Method of Determining *Bursaphelenchus xylophilus* Infestation of *Monochamus* spp. Vectors[1]

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Abstract: Pine wilt is caused by the nematode *Bursaphelenchus xylophilus*, which is transported to host trees in the trachea of *Monochamus* spp. (Coleoptera: Cerambycidae). The study of the relationship between the nematode and its beetle vectors has been hampered by the inability to estimate nematode presence or density within live beetles. This report describes a rapid method for estimating nematode load within live *M. carolinensis* and *M. alternatus* by visual examination of the atrium of the first abdominal spiracle. Visual estimates of nematode numbers correlated highly with actual nematode numbers. This method is a timesaving technique for determining relative numbers of *B. xylophilus* in pine wilt research.

Key words: Bursaphelenchus xylophilus, Monochamus carolinensis, Monochamus alternatus, nematode, trachea, phoresy, pine wilt.

Pine wilt, a serious disease threat to native pine forests in Japan and to exotic pine species in North America, is caused by the nematode *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle, which is transported to host trees by cerambycid beetles in the genus *Monochamus* (Coleoptera: Cerambycidae) (5,7). *Bursaphelenchus xylophilus* fourth-stage dispersal juveniles are carried phoretically in the tracheae of beetles to live pines where beetles feed (3,6) and to dead or dying pines where beetles mate and oviposit (1,2). Individual beetles may carry more than 100,000 dispersal juveniles. The mean nematode load within a population, however, is usually less than 10,000 per beetle, with many individuals carrying no or very few nematodes (5). Considerable research has been conducted on the relationship between beetle vectors and *B. xylophilus*. In these studies beetles were destroyed in the process of estimating the number of dispersal juveniles carried because it was not possible to determine the number of dispersal juveniles carried by live beetles. This led to inefficient use of beetles in experimental procedures because beetles could not be placed in treatment groups based upon nematode load before their use in experimentation. We describe here a fast and nondestructive method for categorizing the number of dispersal juveniles carried by live beetles.

MATERIALS AND METHODS

Two species of *Monochamus* were used to evaluate this method. *Monochamus alternatus* Hope from Taiwan was obtained through the Forestry and Forest Products Research Institute, Tsukuba, Japan. *Monochamus carolinensis* (Olivier) was collected from Scots pine, *Pinus sylvestris* L., grown near Ashland, Missouri. Both species were reared in the laboratory as described by Linit (4), with the following modifications to obtain nematode-infested beetles. The wood-staining fungus, *Ophiostoma minus* (Hedgc.) H. & P. Sydow was grown for 10 days on 4.5% malt extract agar in petri dishes. Jack pine, *P. banksiana* Lamb., logs were inoculated with the fungus and the agar into two 1.3-cm-d holes drilled through the bark and 6 cm into the wood on opposite sides of the log, about 5–10 cm from each end. The inoculation holes were sealed with styrofoam plugs and covered with a thin layer of petroleum jelly. Seven days later, two 1.3-cm-d holes were drilled opposite from the previous inoculation points, and about 25,000 nematodes of all life stages were suspended in 2 ml of distilled water and pipetted into each hole and sealed as above. The nematodes were reared on *Botrytis cinerea* Pers. grown on potato dextrose agar (9). Inoculated logs were subject to beetle oviposition for 4

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days in one of two large screen cages, each containing approximately 50 beetles of one species. Logs were then placed in an incubator at 28 C, 70% RH, and adult *M. carolinensis* or *M. alternatus* were collected upon emergence (about 8 weeks later). Sixty beetles of each sex from the two species were examined for the presence of dispersal juveniles using two new techniques and a previously described method (3).

The first technique utilized a beetle restraining block. A beetle was clamped onto a small block of wood with a flexible plastic "yoke" around its neck (Fig. 1A). Particularly active beetles were slowed by cooling at 4 C for 5 minutes. The restraining block was fashioned from a piece of poplar wood about 5.5 cm (l) × 2.0 cm (w) × 2.0 cm (h) with a 15° angle cut along the longer side. A slit, about ⅔ the depth of the wood, was cut down the middle of the block with a coping saw to accept the plastic "C"-shaped yoke, cut from the white plastic lid of a food container. A small flap was left at the inside top of the opening cut into the plastic to hold the beetle securely.

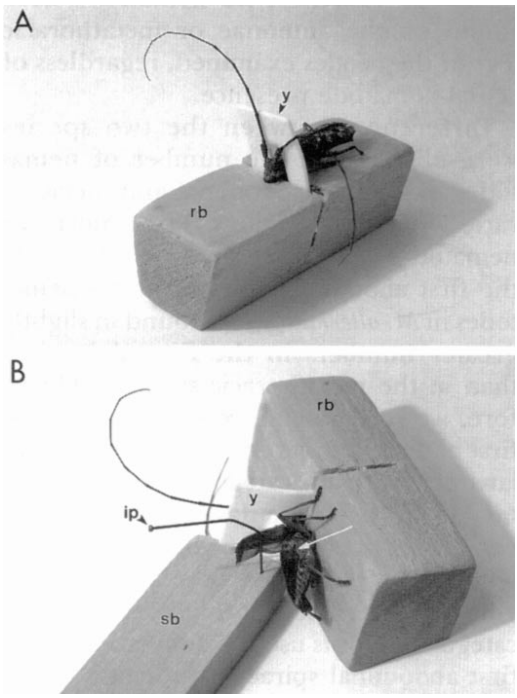

FIG. 1. A) Beetle positioned in the restraining block before spreading the elytra and exposing the first abdominal spiracle. B) Restraining block laid on the angle side while the insect pin in the spreading block holds back the right wing to expose the first abdominal spiracle, the position of which is indicated by the white arrow. ip = insect pin; rb = restraining block; sb = spreading block; y = plastic yoke.

Once a beetle was secured in the yoke, the restraining block was laid on the angle side under a dissecting microscope so that one side of the beetle faced upward. The elytra and hindwing were opened and held away from the body with a bent insect pin stuck into a small flat piece of wood. The pin was slipped under the terminal margin of the elytra, slid under the hindwing to the base of the wing, then pulled away from the body (Fig. 1B). Either the right or left side of the beetle was used, depending on the preference of the examiner. Only one side of each beetle was examined. The first abdominal spiracle, a fringed slit posterior to and angled away from the wing base, was opened carefully with the tips of a fine pair of forceps to expose the atrium. Great care was taken not to puncture the body of the insect with the forceps. The atrium was examined at ×160 magnification for the presence of dispersal juveniles. The metathoracic spiracle was also examined in all beetles.

The second technique to examine the spiracular cavities did not utilize the wooden restraining block. The adult beetle was given a small piece of paper to bite and grasp and the examiner restrained the beetle by holding its head between the thumb and index finger so that the beetle's abdomen was distal from the examiner's hand. The beetle was held on its side under a dissecting microscope. The elytra and hindwing were spread and held away from the body with a bent insect pin, and the spiracles were opened and examined as described above. With practice, this technique becomes more rapid than the previous one.

The two techniques differed only in the method of beetle restraint, not in the method of spiracular cavity examination, and did not effect categorization of beetles. Most beetles used in this study were restrained using the hand-held technique.

Beetles were categorized based on obser-

vation of dispersal juveniles within the spiracular atrium. Beetles in which the tracheal opening were clearly visible at the base of the atrium and in which no dispersal juveniles were observed (Fig. 2A) were assigned to category 0. If no nematodes were observed within the atrium, the anal portion of the hindwing that normally overlays the first abdominal spiracle was examined. If nematodes were observed on the hindwing (but not within the spiracular cavity), the beetle was assigned to category 1. Beetles were also assigned to category 1 if dispersal juveniles were observed within the atrium or elsewhere but the tracheal openings remained visible (Fig. 2B). Beetles in which the tracheal openings were indistinct or not visible because of the presence of dispersal juveniles or in which the atrium was completely occluded by a large mass of dispersal juveniles were assigned to category 2 (Fig. 2C,D).

Kobayashi et al. (3) described a method of estimating nematode population densities by examination of the terminal segments of the antennae or metathoracic leg. A modification of this method was evaluated as well. Before spiracle inspection, the antenna and metathoracic leg were examined visually under the dissecting microscope (×160) for the presence of nematodes on the cuticle. Following examination of the antenna, leg, and spiracles, each beetle was macerated and nematodes collected for 24 hours using the Baermann funnel technique.

Correlation analysis (8) was used to examine the relationship between category number and the number of dispersal juveniles collected from individual beetles following maceration. Correlations were conducted using all beetles, each species alone, each sex alone, and species-sex combinations.

Results and Discussion

Twenty-five beetles were placed in category 0, 17 in category 1, and 18 in category 2. Of those assigned to category 0, 22 beetles carried no dispersal juveniles and three beetles carried 1, 5, and 10, respectively (mean ± SD; 0.64 ± 2.2). Beetles assigned to category 1 carried 15,418 ± 14,248 (range = 200–50,519) dispersal juveniles, and those in category 2 carried a mean of 52,803 ± 24,414 (range = 24,250–112,400). Fifty percent of *M. alternatus* in category 1 contained beetles with over 18,000 dispersal juveniles, whereas only one of five *M. carolinensis* carried more than 13,000. All beetles of both species assigned to category 2 carried over 24,000 dispersal juveniles. There was a significant, positive correlation between assigned category and the numbers of dispersal juveniles carried by beetles, and this relationship held for all species-sex combinations (Table 1). Results of correlation analyses suggest that visual inspection of the spiracles is a good gross indicator of total dispersal juveniles load.

The modified method of Kobayashi et al. (3) was not effective for determining nematode presence; no nematodes were found on the antennae or metathoracic legs of the beetles examined, regardless of actual nematode presence.

Differences between the two species were observed in the number of nematodes in the first abdominal and metathoracic spiracular openings. The majority of nematodes in *M. carolinensis* were found in the first abdominal spiracle, while nematodes in *M. alternatus* were found in slightly greater numbers in the first abdominal than in the metathoracic spiracle. Therefore, we recommend examining both the first abdominal and metathoracic spiracular openings with categorizing *M. alternatus*. If both spiracular openings have nematodes present, regardless of the number, the beetle should be placed in category 2. Otherwise, the higher of the two categorizations is used. Examination of the first abdominal spiracular opening is sufficient in *M. carolinensis*.

We propose a tri-level system for the classification of *Monochamus* beetles. Beetles with no nematodes visible on the hindwing or in the spiracular atrium such that the tracheal openings at the base of the atrium are clearly visible are placed in category 0. These beetles carry few (<100) or

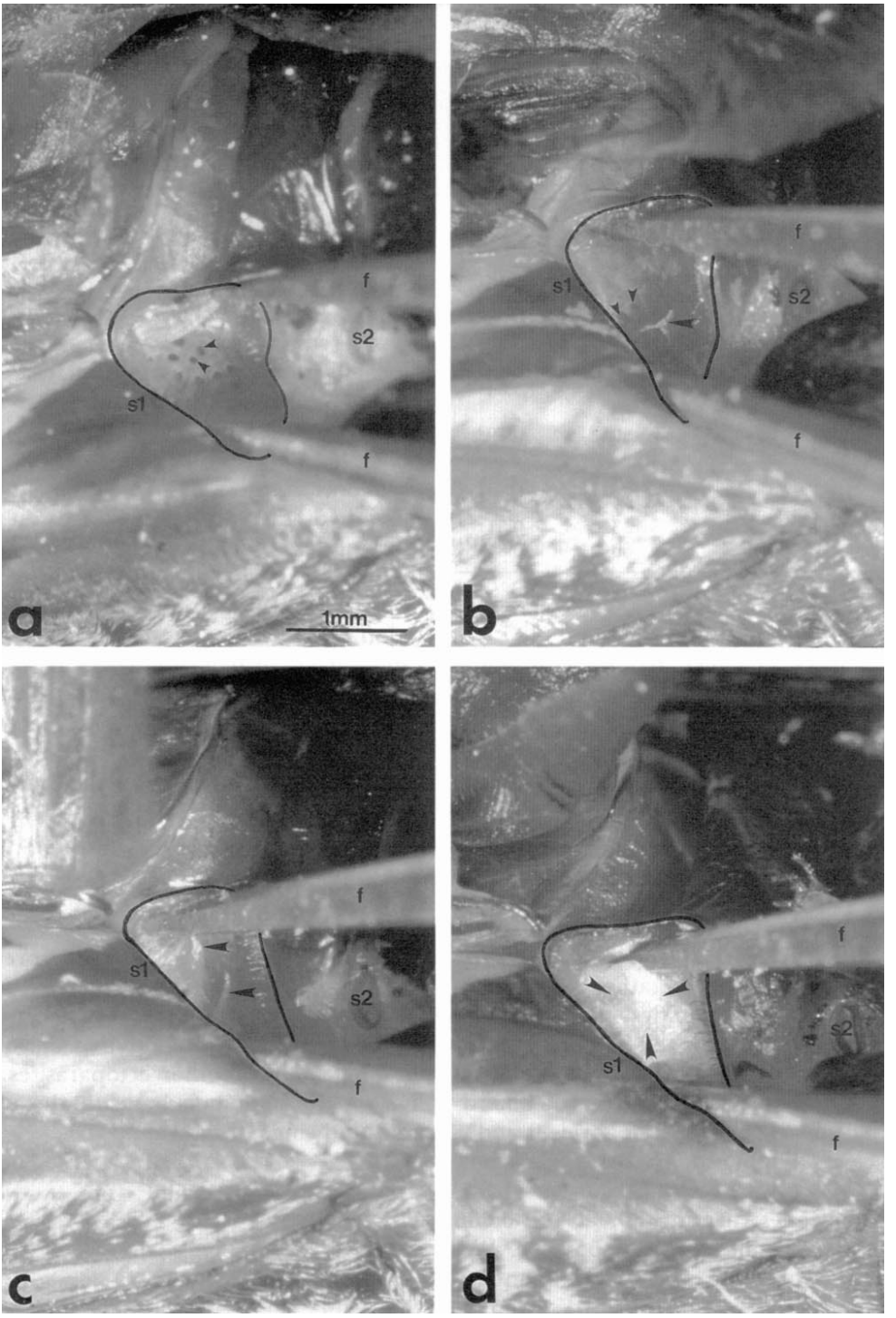

FIG. 2. Spiracular cavities of the first abdominal spiracles (outlined in black) spread open with forceps to show nematode presence or absence. The anterior of the insect is to the left and the dorsal surface is towards the top in each photograph. A) Tracheal openings clearly visible at the base of the spiracular cavity (small arrowheads) and no dispersal juveniles observed: category 0. B) Tracheal openings visible (small arrowheads) but dispersal juveniles observed within the cavity (large arrowhead): category 1. C) Tracheal openings indistinct or not visible within the cavity, dispersal juveniles easily visible (large arrowheads): category 2. D) Cavity completely occluded by a large ball of dispersal juveniles (large arrowheads): category 2. f = forceps; s1 = first abdominal spiracle (outlined in black); s2 = second abdominal spiracle.

TABLE 1. Correlations of visual assessment of nematode load category with actual numbers of nematodes as determined by Baermann funnel extraction for different species and sexes of *Monochamus*.

Comparison group	*n*	Correlation coefficient (*r*)
M. carolinensis female beetles	17	0.82
M. carolinensis male beetles	13	0.89
M. alternatus female beetles	16	0.82
M. alternatus male beetles	14	0.79
M. carolinensis both sexes combined	30	0.84
M. alternatus both sexes combined	30	0.79
Male beetles both species combined	27	0.84
Female beetles both species combined	33	0.81
All beetles	60	0.81

All correlations were significant at $\alpha = 0.05$.

no dispersal juveniles. Beetles with nematodes present on the hindwing or in the spiracular atrium such that the tracheal openings remain visible are placed in category 1. These beetles carry a moderate number (10,000–30,000) of dispersal juveniles. Beetles with abundant nematodes in the spiracular atrium such that the tracheal openings are occluded are placed in category 2. *Monochamus alternatus* beetles are also placed in category 2 if both the first abdominal and metathoracic spiracular atria have nematodes. These beetles carry a great number (20,000 or more) of dispersal juveniles.

In our sample of 60 beetles, the boundary between beetles in categories 0 and 1 was clear. The boundary between categories 1 and 2 was less so. Accurate separation of beetles carrying 20,000 to 30,000 dispersal juveniles was not possible. We, therefore, have an overlapping boundary between these categories. Fifty-seven of sixty beetles were correctly categorized using the proposed criteria. Three beetles placed in category 1 carried between 32,000 and 50,000 dispersal juveniles. This method is fairly reliable for the segregation of beetles based upon the presence or virtual absence of *B. xylophilus* dispersal juveniles and for the classification of beetles, based upon the number of dispersal juveniles carried, into three categories; less than 100, moderate, and high.

Though the presence of a very small number of nematodes in a small percentage of the beetles placed in category 0 does not allow the categorization of these beetles as nematode-free, this category still allows beetles to be eliminated from studies requiring infested beetles. In past experiments requiring large numbers of dispersal juveniles or infested beetles, a great deal of time and numerous beetles have been wasted due to the unknown infestation level. Category 0 beetles can often be treated as uninfested. For example, transmission of dispersal juveniles by beetles carrying less than 100 nematodes did not differ from transmission by uninfested beetles (2,6,10). A gross estimate of infestation level can also be useful in properly randomizing or blocking beetles across experimental treatments to reduce variance.

Though awkward at first, this method is rapid and nondestructive. Though all beetles used in this study were killed immediately following inspection, over 100 beetles used in other studies have been observed following the inspection process and appeared to function normally (i.e., flight, feeding, and mating). This method may be valuable in the study of other vectored or parasitic organisms that are carried in or live in insect trachea. Use of the restraining block and yolk provides a means of examining mites or other ectoparasites that live on the abdomen beneath the elytra and hindwings of insects.

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