In vitro occurrence of dispersal fourth stage juveniles in *Bursaphelenchus xylophilus* co-incubated with *Monochamus alternatus*

Nobuo Ogura¹ and Tadakazu Nakashima¹

The pinewood nematode (PWN), *Bursaphelenchus xylophilus*, was cultured on an unidentified fungus, which was related to *Contortylenchus genitalicola*, grown on potato-glucose-agar medium supplemented with yeast extract in a flattened culture bottle. *C. genitalicola* is an entomophagous and mycetophagous nematode parasitic to the Japanese pine sawyer, *Monochamus alternatus*. The mean number of dispersal 3rd stage juveniles (DSJ₃s) and the mean percentage of DSJ₃ in the populations of PWN were 24, 870 and 93.1% at 80 days' culture. Dispersal 4th stage juveniles (DSJ₄s) developed in the culture bottles when *B. xylophilus* were co-incubated with pupa or adult *M. alternatus* on the fungus. DSJ₄s (mean number, 9,105) were recovered from 9-day-old *M. alternatus* adults that had been co-incubated with *B. xylophilus* during the pupal and adult stages. The usual theory that occurrence of DSJ₄ is triggered mainly by existence of *Monochamus* cerambycid pupa and adult has been supported by the *in vitro* experiment. Jpn. J. Nematol. 32 (2), 53-59 (2002).

Key words: the pinewood nematode, the Japanese pine sawyer, *in vitro* culture.

Hatched juveniles (2nd stage juveniles) of the pinewood nematode, *Bursaphelenchus xylophilus* (Steiner et Buhrer) Nickle, have three developmental courses to adult stage (Mamiya, 1975); (1) Under conditions favorable for propagation, hatched juveniles develop into adults through propagative 3rd and 4th juvenile stages. (2) Under unfavorable conditions such as dry conditions, low temperature and lack of food, hatched juveniles develop into dispersal third stage juveniles (DSJ₃s). This stage has a round tail (Mamiya, 1975) and numerous lipid droplets within the body (Kondo and Ishibashi, 1978). The DSJ₃ molts to a propagative fourth stage juvenile then to an adult if it was given food such as *Botrytis cinerea* Pers. (3) DSJ₃, in a certain case, molts to dispersal 4th stage juvenile (DSJ₄). DSJ₄ has a dome-shaped head and subcylindrical tail with a digitate terminus sharp tail and lack of stylet. DSJ₄ enters a cerambycid vector (Mamiya and Enda, 1972). It will molt to the adult stage under favorable conditions for propagation (Mamiya, 1975). The molt from DSJ₃ to DSJ₄ is triggered by the presence of cerambycid vector such as *Monochamus carolinensis* (Olivier) (Warren and Linit, 1993; Necibi and Linit, 1998) and *M. alternatus* Hope (Maehara and Futai, 1996). The molt appeared to be related to adult eclosion in *M. carolinensis*, and so the cue (s) from the cerambycid may be volatile chemicals (Necibi and Linit, 1998). In this paper, we tried *in vitro* induction of DSJ₄ to obtain basic knowledge for developing bioassay method of the cue (s).

MATERIALS AND METHODS

¹Forestry and Forest Products Research Institute, P. O. Box 16, Tsukuba Norin-danchi, Ibaraki 305-8687, Japan.

B. xylophilus: T-4 strain isolated from dead *Pinus densifolia* Sieb. et Zucc. in Iwate prefecture, Japan (Kiyohara, Y., pers. comm.) were maintained on cultures of an unidentified fungus grown on 4 cm³ of potato-dextrose-agar (Pearlcore[®], Eiken Chemical, Japan) supplemented with yeast extract (Difco, USA) (YPDA) in culture bottles (4.0×7.5×height 2.5 cm). The unidentified fungus was isolated from a dead juvenile of *Contortylenchus genitalicola* Kosaka and Ogura which is entomophagous and mycetophagous nematode parasitic to *M. alternatus* (Ogura and Kosaka, 1991; Kosaka and Ogura, 1993). The unidentified fungus clearly differs from known fungi isolated from pine trees and/or from *M. alternatus* and does not form spores on YPDA (Tabata, M., pers. comm.). The composition of YPDA was yeast extract 0.1%, potato extract 0.4%, glucose 2.0%, agar 1.5% and distilled water (DW). The nematodes were isolated using both a sterilized Baermann funnel and sterilized DW and used for inoculum.

M. alternatus: Adults were captured in Ibaraki prefecture, Japan. Larvae were aseptically reared on artificial diet at 25±1°C under 16 L: 8 D photoperiod (Kosaka and Ogura, 1990). Mature larvae were chilled at 7°C for over 3 months to break diapause and then used for experiments.

Culture of *B. xylophilus* and occurrence of DSJ₃

Four ml of YPDA was poured into culture bottles, sealed with foam silicon plugs and autoclaved (120°C, 15 min). A small block (5×5×1.3 mm) of the unidentified fungus-mycelia grown on YPDA was implanted onto YPDA in culture bottles. Three days after the implantation, a 20 µl of nematode suspension containing ca. 1,000 *B. xylophilus* (mixture of larvae and adults) was dropped on the mycelia in the culture bottles. We feared that co-incubated *M. alternatus* was covered with medium during co-incubation of *B. xylophilus* and *M. alternatus* in the following experiments. The cultures were, therefore, placed in desiccators (30×50×30 cm) to decrease moisture content of YPDA and cultured at 25±1°C. Approximate 3,000 g silica gel was placed in the desiccators to keep relative humidity below 10%. Nematodes were isolated using the Baermann funnel method for 48 hr at 25±1°C at 20, 40, 60, 80 and 100 days after inoculation. Number of replicates was 3 or 5.

Co-incubation of *B. xylophilus* and *M. alternatus* larva, pupa or adult

Three pieces of thin pulp-tissue paper (4.5×6.6 cm) that had been sterilized by autoclaving were placed on YPDA on which *B. xylophilus* had been cultured on mycelia of the unidentified fungus for 50 days. Post-diapause mature larvae, newly molted pupae or newly emerged adults of *M. alternatus* were individually placed on the tissue paper. Then the culture bottles were maintained in the desiccators at 25±1°C for 10 days. Nematodes on the surface of the larvae, pupae and adults were washed away with DW. The DW, tissue paper and YPDA from a culture bottle were put together and the nematodes were isolated using the Baermann funnel method. Adults were cut into small pieces with scissors and then placed in the Baermann funnel to isolate nematodes.

Co-incubation of *B. xylophilus* and *M. alternatus* during pupal and adult stage

Newly molted pupae were individually put into culture bottles that contained 50-day-old cultures of *B. xylophilus* and 3 pieces of thin pulp-tissue paper. They were incubated at 25±1°C in the desiccators. Pupal periods were ca. 15 days. Newly formed adults remained in the culture bottles for 0, 3, 6, 9 or 12 days. Nematodes were isolated respectively from the adults and both tissue paper and YPDA using Baermann funnel.

Calculation of nemtode numbers

Nematodes isolated using the Baermann technique were diluted with tap water to a final volume of 2, 5 or 10 ml. The number of DSJ₃s, DSJ₄s, and other stage nematodes in each of four 30 µl samples of the suspension was counted to estimate total numbers of the nematodes. Analysis of variance (ANOVA) and Tukey HSD multiple comparisons were performed using SYSTAT (SYSTAT Inc., USA). The analysis and comparisons of proportion of DSJ₃s in total nematodes were carried out using arcsine transformation of the datum.

RESULTS

Culture of *B. xylophilus* and occurrence of DSJ₃

The mean number of surviving nematodes per culture bottle after 40 days culturing was 114,799 (SD = 25,369) (n = 5), then gradually decreased (Fig. 1). Variances increased as days in culture increased. Some nematodes survived in all bottles until the 80th day of culture. YPDA in 2 out of 5 culture bottles were dried up at 100 days' culture and all nematodes within the bottles had died. The mean number of surviving nematodes was 6,213 (n = 5, SD = 136,791) after the 100 days of culture. Mean numbers of DSJ₃ per culture bottle at 40, 60, 80 and 100 days were 33,814 (n = 5, SD = 10,746), 42,459 (n = 5, SD = 42,668), 24,869 (n = 5, SD = 29,380) and 7,638 (n = 3, SD = 13,077), respectively. The

Fig. 1. Population change in cultures of *Bursaphelenchus xylophilus* on an unidentified fungus related to *Contortylenchus genitalicola*. Solid circle; Numbers of surviving nematodes (mean ± SD) (n = 5); Open circle; Percentage of dispersal third stage juveniles (DSJ₃) (mean ± SD) (n = 5 except an indication of n = 3). Different letters in figure show a significant difference at p < 0.05 (Tukey HSD multiple comparisons).

Table 1. Numbers of *Bursaphelenchus xylophilus* DSJ₃ and DSJ₄ collected from culture bottles where *Bursaphelenchus xylophilus* and *Monochamus alternatus* were co-incubated for 10 days (n = 7 for each beetle life stage).

Beetle treatment	Nematode life stage	Mean***	SD
Mature larva	DSJ ₃	117,982	19,301
	DSJ ₄ **	158ab	159
Pupa	DSJ ₃	102,304	8,826
	DSJ ₄	1,819bc	1,860
Adult	DSJ ₃	21,720	38,761
	DSJ ₄	3,218c	2,736
Control****	DSJ ₃	104,884	24,820
	DSJ ₄	0a	0

* Dispersal third-stage juvenile

** Dispersal fourth-stage juvenile

*** Different letters show a significant difference at p < 0.05 (Tukey HSD multiple comparison)

**** Cultures with tissue paper

Table 2. Numbers of *Bursaphelenchus xylophilus* collected from culture bottles where *B. xylophilus* and *Monochamus alternatus* pupa and adult were co-incubated (n = 5 for each beetle life stage).

Co-incubation period	Nematode life stage	Mean***	SD
Pupal stage	DSJ ₃ *	98,204a	17,960
+ adult stage 0 day	DSJ ₄ **	995	1,068
Pupal stage	DSJ ₃	56,705ab	36,955
+ adult stage 3 days	DSJ ₄	2,669	1,503
Pupal stage	DSJ ₃	46,452bc	33,305
+ adult stage 6 days	DSJ ₄	13,402	11,059
Pupal stage	DSJ ₃	29,586bc	11,388
+ adult stage 9 days	DSJ ₄	11,884	8,868
Pupal stage	DSJ ₃	10,448c	8,542
+ adult stage 12 days	DSJ ₄	9,765	4,077

* Dispersal third-stage juvenile

** Dispersal fourth-stage juvenile

***Different letters show a significant difference at $p < 0.05$ (Tukey HSD multiple comparison). DSJ₃s and DSJ₄s were total of ones isolated from YPDA, tissue paper and insects.

Fig. 2. Change in numbers of dispersal fourth stage juveniles (DSJ₄s) isolated from *Monochamus alternatus* adults (mean $\pm$ SD) (n = 5). Different letters in figure show a significant difference at $p < 0.05$ (Tukey HSD multiple comparisons).

percentage of DSJ₃ increased as culture days increased. At 40, 60, 80 and 100 days of culture, mean percentage of DSJ₃ in total number of *B. xylophilus* in each culture bottle were 31.9 (n = 5, SD = 14.2), 68.6 (n = 5, SD = 38.8), 93.1 (n = 5, SD = 50.9) and 92.8 (n = 3, SD = 30.9), respectively.

Co-incubation of *B. xylophilus* and *M. alternatus* larva, pupa or adult

Mean numbers of DSJ₃s and DSJ₄s recovered from the culture bottles, where *B. xylophilus* and larvae, pupae and adult of *M. alternatus* had been co-incubated, are showed in Table 1. The mean numbers of DSJ₄s in culture bottles with larva, pupa and adult were 158, 1,819 and 3,218, respectively. The mean number of DSJ₄s isolated from adults was 1,150 out of 3,218. There was a stage-dependent difference in the number of DSJ₄s (ANOVA, factor: stage, $F = 5.9$, $df = 3, 24$; $p = 0.004$). There was, however, no significant difference in the numbers of DSJ₄s in the culture bottles between larva and tissue paper-control (Tukey HSD, $p = 0.998$).

Co-incubation of *B. xylophilus* and *M. alternatus* during pupal and adult stage

The mean numbers of DSJ₃s and DSJ₄s recovered from cultures (*M. alternatus* adults, tissue paper and YPDA) at 0, 3, 6, 9 and 12 days after emergence are showed in Table 2. The range in both DSJ₃ and DSJ₄ numbers was very large. There was an incubation period-dependent difference in the numbers of DSJ₃ (ANOVA, factor: incubation period, $F = 10.8$, $df = 4, 20$; $p = 0.000$). The numbers decreased as the incubation period increased. There was no incubation period-dependent difference in the numbers of DSJ₄s (ANOVA, factor: incubation period, $F = 2.8$, $df = 4, 20$; $p = 0.052$). The mean numbers of DSJ₄s

isolated from adult beetles at 9 and 12 days after emergence were 9, 105 (SD = 6, 553) and 8, 208 (SD = 3757), respectively. These were significantly greater than at 0 day (mean number; 995, SD; 1, 068) (Tukey HSD, $p < 0.05$). The mean numbers of DSJ₄s at 9 days was larger than ones at 0 and 3 days (Tukey HSD, $p = 0.031$) (Fig. 2).

DISCUSSION

The proportion of DSJ₃s in the total nematode population increased as moisture content decreased in pine seedlings (Suzuki and Kiyohara, 1978) and in pine chips (Tomminen et al., 1991). A similar trend, although not statistically significant, was shown in pine bolts in which *Ophiostoma minus* (Hedgcock) H. et P. Sydow, *B. xylophilus* and *M. carolinensis* were co-incubated (Warren and Linit, 1993). Correlation between moisture content and the percentage of DSJ₃ to total nematodes, however, was not found in pine bolts into which *Trichoderma* sp. and *B. xylophilus* had been inoculated (Maehara and Futai, 1996). Ishibashi and Kondo (1977) cultured 10 female and 10 male adults of *B. xylophilus* on *Botrytis cinerea* in test tubes (18×180 mm) containing 10 ml potato-sucrose-agar medium at 20°C without humidity control. In their experiment, the nematode population reached ca. 1×10^5 after 30 days and then decreased to 10^2 after 180 days. DSJ₃ began to appear after 90 days and constituted more than 95% of the surviving population (less than 10^3) after 270 days.

We placed cultures in a desiccator to decrease moisture content of YPDA to prevent co-incubated *M. alternatus* from being covered with medium. We obtained ca. 1×10^4 – 1×10^5 DSJ₃s per culture bottle after 80 days' culture when *M. alternatus* was not co-incubated. Nevertheless, YPDA in culture bottles with *M. alternatus* adult was bitten and trampled down by the adult. This may be one of the reasons that numbers of DSJ₃s isolated from cultures with the adult decreased as incubation periods prolonged. The other reasons for decrease in numbers of DSJ₃s is occurrence of DSJ₄ and may be death due to failure of molting from DSJ₃ to DSJ₄. It was not obvious whether or not the dry condition was good for *in vitro* increase of DSJ₃s.

Ishibashi and Kondo (1977) showed that proportion of DSJ₄s to *B. xylophilus* juveniles collected from the pupal chambers with full grown larval, early pupal, mid-pupal, late pupal and adult *M. alternatus* individual were 11.5, 29.6, 84.0, 97.5 and 98.8%, respectively. They suggested that the molt from DSJ₃ to DSJ₄ might be induced by the non-residual matter emitted from the pupating *M. alternatus*. In co-incubation (60 to 70 days) of *B. xylophilus* and *M. carolinensis* using pine bolts (40 cm long) infested with *O. minus*, mean numbers of DSJ₄s recovered from larvae, pupae and teneral (callow) adults were 25.3, 1, 619.5 and 6, 858.4, respectively (Warren and Linit, 1993). DSJ₄s occurred and boarded *M. alternatus* adults in co-incubation of *B. xylophilus* and *M. alternatus* using small pine wood blocks (25×25×50 mm) in which *Trichoderma* spp. or *O. minus* had been inoculated (Maehara and Futai, 1996). They demonstrated that *M. alternatus* was essential for the occurrence of DSJ₄. Almost similar results were obtained from co-incubation of *B. xylophilus* and *M. alternatus*, using small pine bolts (75 mm long) infested with *O. minus* (Aikawa et al., 1997; Aikawa and Togashi, 1997). Forty-eight hr co-incubation of *B. xylophilus* and *M. carolinensis* in artificial galleries made in pine disks (7 cm thick) in which DSJ₃s had been present showed that DSJ₄s were recovered from early pupae, late pupae, callow adults and adults but not from larvae (Necibi and Linit, 1998). They proposed that a genus-specific substance (s) associated with *Monochamus* adult eclosion trigger the DSJ₃ to DSJ₄ molt. In the present 10 days' *in vitro* co-incubation of *B. xylophilus* and *M. alternatus*, numbers of DSJ₄s were large when co-

incubated with adults and to a lesser extent with pupae. DSJ₄s also appeared in co-incubation with full-grown larvae, though there was no significant difference between the numbers and ones in control. This indicates that *M. alternatus* pupa and adult affect occurrence of DSJ₄ and also that even larva may have the same effect if co-incubation period is very long. When DSJ₄s recovered from *M. alternatus* adults were kept on 1.0% water agar at 20°C, 12.5% of the DSJ₄s survived for 100 days (Ishibashi and Kondo, 1977). The pupal period of *M. alternatus* at 20°C was approximately 20 days (Enda, 1976). Therefore, a part of DSJ₄s that occurred beside *M. alternatus* larvae may reside on the adults developing from the larvae.

Occurrences of DSJ₄ under insect-free condition were observed in wood chips (Tomminen et al., 1991) and in culture of *B. xylophilus* on *B. cinerea* growing on potato-glucose-agar medium supplemented with glycerol (Kiyohara and Bolla, 1990). In this paper, none of DSJ₄ was isolated from cultures without *M. alternatus*.

We conclude that occurrence of DSJ₄ is triggered mainly by existence of *Monochamus* cerambycid pupa and adult as Necibi and Linit (1998) indicated.

ACKNOWLEDGEMENT

We thank Prof. M. J. Linit, University of Missouri-Columbia, for reviewing an earlier version of the manuscript.

LITERATURE CITED

- Aikawa, T., Maehara, N., Futai, K. and Togashi, K. (1997) A simple method for loading adult *Monochamus alternatus* (Coleoptera: Cerambycidae) with *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae). Applied Entomology and Zoology 32, 341-346.
- Aikawa, T. and Togashi, K. (1997) An effect of inoculum quantity of *Bursaphelenchus xylophilus* (Nematodal Aphelenchoididae) on the nematode load of *Monochamus alternatus* (Coleoptera: Cerambycida) in laboratory. Japanese Journal of Nematology 27, 14-21.
- Enda, N. (1976) Life cycle of the Japanese pine sawyer, *Monochamus alternatus*. Forest Pest 25, 182-185. (in Japanese)
- Ishibashi, N. and Kondo, E. (1977) Occurrence and survival of the dispersal forms of pine wood nematode, *Bursaphelenchus lignicolus* Mamiya and Kiyohara. Applied Entomology and Zoology 12, 293-302.
- Kiyohara, T. and Bolla, R. I. (1990) Pathogenic variability among populations of the pinewood nematode, *Bursaphelenchus xylophilus*. Forest Science 36, 1061-1076.
- Kondo, E. and Ishibashi, N. (1978) Ultrastructural differences between the propagative and dispersal forms in pine wood nematode, *Bursaphelenchus lignicolus*, with reference to survival. Applied Entomology and Zoology 13, 1-11.
- Kosaka, H. and Ogura, N. (1990) Rearing of the Japanese pine sawyer, *Monochamus alternatus* (Coleoptera: Cerambycidae) on artificial diets. Applied Entomology and Zoology 25, 532-534.
- Kosaka, H. and Ogura, N. (1993) *Contortylenchus genitalicola* n. sp. (Tylenchida: Allantonematidae) from the Japanese pine sawyer, *Monochamus alternatus* (Coleoptera: Cerambycidae). Applied Entomology and Zoology 28, 423-432.
- Maehara, N. and Futai, K. (1996) Factors affecting both the numbers of the pinewood nematode, *Bursaphe-*

- lenchus xylophilus* (Nematoda: Aphelenchoididae), carried by the Japanese pine sawyer, *Monochamus alternatus* (Coleoptera: Cerambycidae), and the nematode's life history. Applied Entomology and Zoology 31, 443-452.
- Mamiya, Y. (1975) The life history of the pine wood nematode, *Bursaphelenchus lignicolus*. Japanese Journal of Nematology 5, 16-25. (in Japanese with English summary)
- Mamiya, Y. and Enda, N. (1972) Transmission of *Bursaphelenchus lignicolus* (Nematoda; Aphelenchoididae) by *Monochamus alternatus* (Coleoptera: Cerambycidae). Nematologica 18, 159-162.
- Necibi, S. and Linit, M. J. (1998) Effect of *Monochamus carolinensis* on *Bursaphelenchus xylophilus* dispersal stage formation. Journal of Nematology 30, 246-254.
- Ogura, N. and Kosaka, H. (1991) Biology of a tylenchid nematode parasitic on the Japanese pine sawyer, *Monochamus alternatus*. Nematologica 37, 455-469.
- Suzuki, K. and Kiyohara, T. (1978) Influence of water stress on development of pine wilting disease caused by *Bursaphelenchus lignicolus*. European Journal of Forest Pathology 8, 97-107.
- Tomminen, J., Halik, S. and Berglahl, D. R. (1991) Incubation temperature and time effects on life stages of *Bursaphelenchus xylophilus* in wood chips. Journal of Nematology 23, 477-484.
- Warren, J. E. and Linit, M. J. (1993) Effect of *Monochamus carolinensis* on the life history of the pinewood nematode, *Bursaphelenchus xylophilus*. Journal of Nematology 25, 703-709.

Received September 11, 2002.

和文摘要

平底角型培養瓶を用いたマツノザイセンチュウとマツノマダラカミキリの共存培養と分散型Ⅳ期幼虫の生起

小倉 信夫・中島 忠一

平底角型培養瓶を用いて、酵母抽出物を添加した PDA 培地上で *Contortylenchus genitalicola* (マツノマダラカミキリの生殖器に寄生する菌食性昆虫寄生線虫) 関連の未同定菌とマツノザイセンチュウを培養した。80日間の培養では、分散型Ⅲ期幼虫の培養瓶当たりの平均頭数は24,869頭で、分散型Ⅲ期幼虫の全線虫頭数に占める割合の平均は93.1%であった。この未同定菌とマツノザイセンチュウを培養した平底角型培養瓶にマツノマダラカミキリの蛹あるいは成虫を入れて共存培養すると、分散型Ⅳ期幼虫が生じ、その頭数は対照区と有意な差があった。全蛹期間と成虫期の9日間をマツノザイセンチュウと共存培養したマツノマダラカミキリ成虫からは、平均9,105頭の分散型Ⅳ期幼虫が分離された。分散型Ⅳ期幼虫の生起には *Monochamus* 属カミキリの蛹および成虫が関わっているという従来の説は、培養瓶を用いた実験でも支持された。