JARQ 27, 49-54 (1993)

Control of Scarabaeid Grubs with an Entomogenous Nematode, Steinernema kushidai

Nobuo OGURA
Forest Biology Division, Forestry and Forest Products Research Institute
(Tsukuba, Ibaraki, 305 Japan)

Abstract

A new species of steinernematid, *Steinernema kushidai* was recovered from a soil sample collected in Hamakita City, Shizuoka Prefecture, Japan. The nematobe showed a significant lethal effect on several species of scarabaeid grubs. Application of 1,000 infective juveniles (IJ) of the nematobe onto the soil in plastic cups where the 3rd instar larvae of the cupreous chafer were individually reared killed the larvae within a few days. In applications of the nematobes on soil in a sweet potato field, more than 100,000-200,000IJ/m^2 reduced the numbers of the sweet potatoes seriously injured by scarabaeid grubs. *S.kushidai* applied on a sweet potato field and on a nursery of the Japanese cypress persisted for at least 2 years. The nematode could be propagated *in vitro* on peptone or yeast extract-rich media.

Discipline: Insect pest
Additional key words: biological control, integrated control

Introduction

An entomogenous nematode which exerts a significant lethal effect on several species of scarabaeid grubs was isolated from a soil sample collected in Hamakita City, Shizuoka Prefecture, Japan in 1984[7]. The morphology and life cycle of the nematode were peculiar to the genus *Steinernema*. Comparison of the position of the excretory pore, shape of both spicule and gubernaculum, and body length of infective juveniles (dauer 3rd stage juveniles) of the nematode with those of other *Steinernema* spp., indicated that the nematode was a new species of *Steinernema*. Mamiya designated this nematode as *Steinernema kushidai*[8]. It is commonly called Kushidanema in Japan.

The scarabaeid grubs live in soil where they injure the roots of several plants including nursery trees, tea trees, sweet potatoes, peanuts and grasses used for planting lawns. Although several chemical pesticides are available for the control of these grubs, they are not very effective. Furthermore, these pesticides have been found to be environmental pollutants in recent years. Therefore, *S. kushidai* could be effectively used as a biological control agent.

Methods of propagaption and preservation, lethal effect on insects and field application tests of *S. kushidai* are reviewed here.

Life cycle

When the 3rd instar larvae of the cupreous chafer, *Anomala cuprea* are reared in soil containing the infective juveniles (IJ) of *S. kushidai*, the grubs die within 2 days in the earliest case (Plate 1-A). The cadavers are soft with a peculiar odor. *S. kushidai* female adults (length : ca. 3.5 mm) and male adults (length : ca. 1.5 mm) were observed in the hemocoeles of the 2 to 3-day-old cadavers (Plate 1-B). The hemocoeles of the 6 to 7-day-old cadavers were filled with IJ with a uniform size (length : ca. 0.6 mm). The IJ leave the cadavers (Plate 1-C), into soil and await the next chance of infection.

S. kushidai IJ contain Gram-negative symbiotic bacteria, *Xenorhabdus japonicus* in their intestines (Nishimura, M., personal communication) (Plate 1-

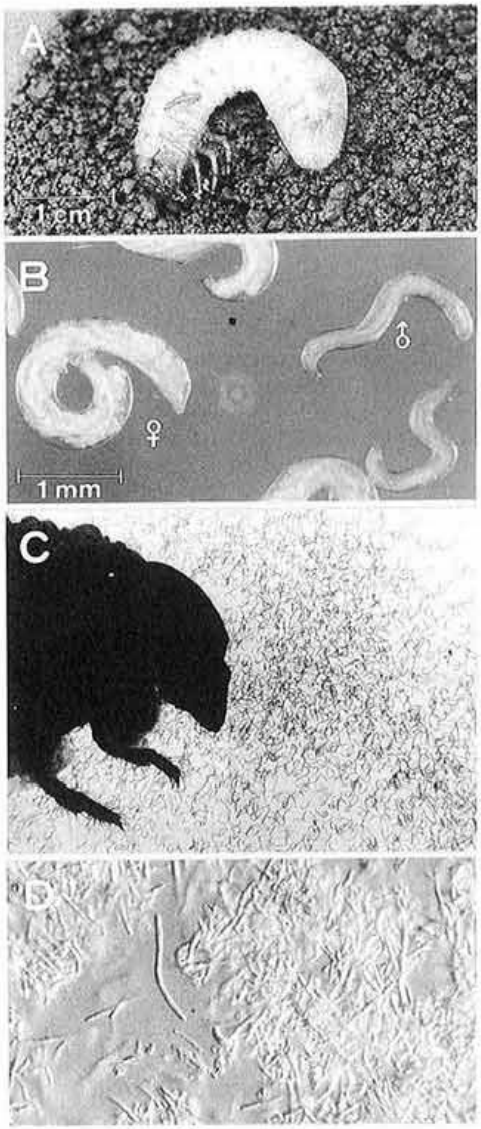

Plate 1. A : Third instar larva of *Anomala cuprea* which died after infection with *Steinernema kushidai*
B : Adult female and male of *S. kushidai*
C : Numerous infective juveniles (body length : ca. 0.6 mm) emerging from the *Anomala cuprea* cadaver
D : Symbiotic bacteria. *Xenorhabdus japonicus* on YS broth (width : ca. 0.8 μm)

D)[17]. The bacteria have two phases as other *Xenorhabdus* spp.[1]. Phase I and II colonies are blue and brown, respectively, on YS broth[3] ($NH_4H_2PO_4$: 0.05%, K_2HPO_4 : 0.05%, $MgSO_7 \cdot 7H_2O$: 0.02%, NaCl : 0.5%, yeast extract : 0.5%, agar : 1.2% in water) supplemented with 2, 3, 5,-triphenyltetrazolium chloride (0.004%) and bromothymol blue (0.0025%). Both phase I and II bacteria can be maintained on YS broth by subculture every 2 weeks at 20°C. The relationship between the phases and the virulence against host insects has not been studied. The IJ, which invade the host insects *via* the mouth, integument or anus, release the bacteria into the host hemocoele. The bacteria propagate there and the host death is due to septicemia. The IJ feed on the bacteria and the host tissues, become adults, copulate, oviposit and finally produce numerous IJ carrying bacteria in their intestines.

In vitro culture

1) Media

Symbiotic bacteria of steinernematids usually proliferate on media composed mainly of dog food or offal of domestic animals[2,4]. *X. japonicus*, however, hardly proliferates on these media and requires a medium of dog food or animal offal supplemented with hydrolyzed protein such as peptone or yeast extract. Therefore, *S. kushidai* is cultured on media supplemented with peptone or yeast extract (Table 1). Ingredients are blended, heated to 80°C for the solubilization of fat and then poured into flat culture bottles, Erlenmeyer flasks with silicon plugs or petri dishes autoclaved at 120°C for 20 min[11,12].

2) Inoculation and subculture

The IJ isolated from the host cadavers were washed through three changes of 0.1% benzethonium chloride for 5 min or through three changes of 0.1% thimerosal (Sigma Chemical Co., USA) for 20 min and then washed three times with sterilized distilled water (DW). When 100 μl of a suspension containing 500 to 1,000 surface-sterilized IJ was inoculated onto 5 ml of culture medium, the adults appeared after 4 to 5 days and after 15 days of culture there was a 200 to 400 fold increase in the numbers of IJ at 25°C.

Inoculation of 500 IJ onto 5 ml medium composed of chopped pig intestine (8.8%), peptone (1.2%) and agar (0.3%) in DW in culture bottles (4 × 7 cm base × 2.5 cm height) yielded about 70,000 IJ within 15 days at 25°C. When the cultures were then in-

Table 1. Media for *in vitro* culture of *Steinernema kushidai*[11]

Medium type	Ingredients	No. of infective juveniles produced in 5 m*l* medium
Dog food −peptone /agar	Dog food (8.8%), peptone (1.2%), agar (0.2%) in buffer[a]	344,300
Pig intestine −peptone/agar	Finely chopped pig intestine (26.4%), peptone (1.2%), agar (0.3%) in buffer[a]	244,000
SGLPY/agar	Soluble starch (0.6%), D-glucose (1.0%), lard (3.0%), peptone (1.5%), yeast extract (1.5%), agar (0.5%) in buffer[b]	469,500

a) : Sörensen phosphate buffer (33 mM KH_2PO_4 : 33 mM Na_2HPO_4=7 : 3) (pH 6.5).
b) : 33 mM Bis-Tris buffer. DW can be substituted for this buffer.

cubated at 5, 10, 15, 20 and 25°C for 6 months, most of the IJ in the culture at 5, 10, and 25°C died, while 20% of the IJ in the cultures at 20°C survived. Even at this low survival rate at 20°C, it appeared that for storage purposes *S. kushidai* could be maintained by subculture every 6 months. To initiate new cultures in order to rapidly build-up the population size, an aliquot of the old semi-liquid medium containing the IJ can be simply pipetted into fresh medium in a new culture bottle.

3) Propagation for field application tests

Inoculation of 1,000 to 2,000 IJ to 3rd instar larvae of *A. cuprea* yielded about 300,000 IJ after 10 days[7]. Inoculation of 10,000 IJ onto 60 m*l* of the dog food-peptone/agar medium in petri dishes (12 cm diameter×3 cm height) yielded about 3,000,000 IJ after 20 days. These IJ can be used for field tests.

Preservation

Ninety-five percent of the IJ in DW survived for 100 days at 10 to 15°C (DW : 0.7 cm depth), while at 5°C more than 90% died within 20 days[7]. To investigate other methods of preservation of the nematode populations, 250,000 IJ were mixed in 3 kg of wet loam soil or 630,000 IJ were mixed in wet bark compost. These populations were incubated at 25°C for 10 months after which the soil or compost was put separately into plastic cups (7 cm diameter×5 cm depth). Effective survival of the nematodes was then assayed as 2nd instar larvae of *A. cuprea* were reared individually in each cup. In the cup containing the wet loam/nematode inoculum, 10 out of 10 grubs died and in the cup containing the bark compost/nematode inoculum, 8 out of 10 grubs died[7]. Thus *S. kushidai* IJ could be preserved in DW for 100 days at 10 to 15°C or in wet soil for 10 months without losing their lethal effects on grubs.

Lethal effect

S. kushidai showed a lethal effect on the 3rd instar larvae of *Heptophylla picea* at temperatures between 17 and 35°C (Table 2)[7]. *S. kushidai* was highly lethal to the 2nd to 3rd instar larvae of *A. cuprea* (Table 3) but displayed a negligible lethal effect on prepupal-yellowish larvae of *A. cuprea*[7]. Adults of some scarabaeid species were also infected with this nematode (Ogura, unpublished). Several species of scarabaeid grubs, *Popillia japonica, Anomala rufocuprea, Heptophylla picea, Maladera*

Table 2. Effect of temperature on lethality of *S. kushidai* to 3rd instar larvae of *Heptophylla picea*[7]

	Temperature (°C)	No. of larvae inoculated	Mortality (%)	Days until death (Mean±SD)
Application of infective juveniles	15	10	0	−
	17	10	40	10.7±3.2
	20	10	80	7.8±6.5
	25	10	100	5.3±3.4
	30	10	100	3.9±1.7
	35	10	100	1.1±0.3
No treatment	30	10	90	12.0±2.3
	35	10	100	3.0±1.6

Third instar larvae of *H. picea* were reared individually in 25 g of soil in 9 cm petri dishes. One thousand infective *S. kushidai* juveniles (in 0.1 m*l*) were inoculated into each dish.

Table 3. Lethal effect of *S. kushidai* on *Anomala cuprea* grubs[7]

No. of IJ applied	Instar of grubs tested	No. of grubs tested	Mortality (%)	Days until death (Mean±SD)
50	1	10	60	15.3± 8.5
	2	10	30	12.0±10.1
	3	10	80	7.6± 3.1
100	1	10	70	14.9±13.6
	2	10	90	11.4± 9.6
	3	10	70	8.1± 4.9
200	1	10	90	11.0± 6.0
	2	10	100	6.2± 0.4
	3	10	90	7.1± 3.4
500	2	5	100	3.8± 0.4
	3	5	100	3.6± 0.5
1.000	2	5	100	4.6± 1.8
	3	5	80	3.5± 0.6

First to third instar larvae of *A. cuprea* were reared individually in 25 g of soil in 9 cm perti dishes. An infective juvenile suspension (0.1 m*l*) was pipetted onto each dish.

Table 4. Lethal feffect of *S. kushidai* on various species of insects and other animals[7]

Animals tested	No. of animals tested	No. of IJ applied/ container	Mortality (%)
Arthropoda: Insecta			
Spodoptera litura larvae	20	1,290	80
Acanthoplusia agnata larvae	20	1,260	65
Galleria mellonera larvae[a]	200	250	64
	200	500	82
	200	1,000	93
Promachus yesonics larvae	5	2,700	0
Arthropoda: Diplopoda			
Parafontaria laminata armigera	10	5,000	30
Annelida: Oligochaeta			
Earth worm (species: unknown)	10	5,000	0

The test organisms were reared in a 9 cm petri dish or in plastic cups (7 cm diameter×5 cm depth).
Mortality was determined 5 days after application.
a) : Ogura (unpublished observation) : 1 m*l* IJ suspension was poured onto a filter paper in each petri dish where then 10 last instar larvae were reared at 25°C.

japonica, Protaetia orientalis and the horned beetle, *Xylotrups dichotomus* were also killed by *S. kushidai*[7]. Effects of this nematode on animals in other orders, classes and phyla are shown in Table 4.

Application tests

1) Laboratory application test

An aliquot of IJ suspension containing 6,000 IJ/m*l*, obtained from infested *A. cuprea*, was applied onto soil in plastic containers (53 × 33 cm × 18 cm height) in which 25 *A. cuprea*-3rd instar larvae were reared. The final nematode concentrations of 1,800 IJ (10,000 IJ/m²), 18,000 IJ (100,000IJ/m²) and 180,000 IJ (1,000,000 IJ/m²) killed 25, 84 and 96% of the larvae, respectively[7].

2) Field application tests

To develop test plots at the Forestry and Forest Products Research Institute in Kukizaki, Ibaraki Pref., vinyl chloride (VC) plates (40 cm width) were sunk to a 30 cm depth in the soil and enclosed in a 1 m² area as shown in Plate 2. Thereafter 2-year-old seedlings of the Japanese cypress, *Chamaecyparis obtusa*, were planted in each plot, and 100,000, 500,000 and 1,000,000 IJ of *S. kushidai* in 2,000 m*l* tap water (IJ : obtained from infested *A. cuprea*) were inoculated onto the plot, and mixed into the soil. Thereafter the plot was covered with a lawn. After 30 days, 40 *A. cuprea*-2nd instar larvae were released into each plot. Eighteen days after this release, all the surviving grubs were recovered. They were reared individually in the laboratory, during which nearly 100% of the grubs recovered from each plot died. To test the persistence of the nematodes in soil, 25 *A. cuprea*-2nd instar larvae were released onto the plots 1 and 2 years after the initial application of *S. kushidai*. Again all the grubs recovered and those reared in the same way died during rearing, suggesting that *S. kushidai* can persist for at least 2 years in the field[6].

Rearing of *S. kushidai* on SGLPY medium supplemented with 0.04% neutral red yielded IJ containing the dye in their intestines. In November, 2 million of neutral-red-labeled IJ were sprayed into the bottom of the hole (30×30 cm and 15 cm depth)

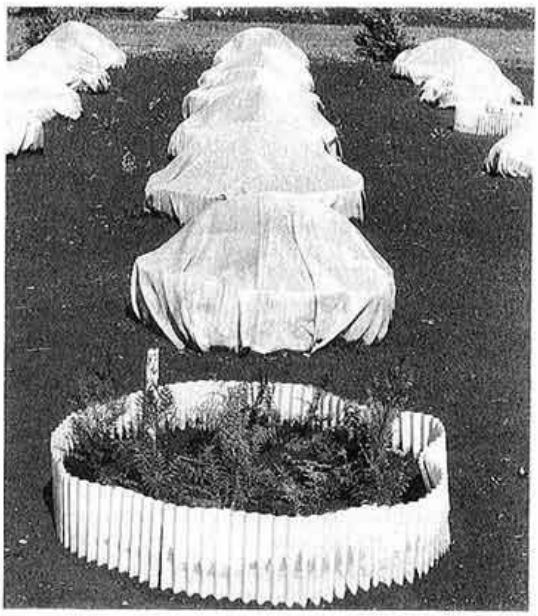

Plate 2. Plots (ca. 1.2 m diameter) used for *S. kushidai* application at Forestry and Forest Products Research Institute

dug in the experimental nursery. The hole was refilled with the soil that had been dug out. To demonstrate that *S. kushidai* could overwinter in the field, the soil was removed from the hole 6 months later. We were able to isolate active, viable labeled IJ from this soil, indicating that the applied IJ were able to hibernate in the field[10].

At Ibaraki Agricultural Experiment Station (Mito city, Ibaraki Pref.), VC plates (30 cm width) were buried to ca. a 20 cm depth and enclosed in a 1 m^2 area in the lawn-covered house. Four sweet potato, *Ipomoea batatas* plants were planted in each plot. Aliquots of IJ suspensions containing 100,000 IJ in 500 m*l* tap water were sprayed onto each plot with a sprinkling can in June or July. The IJ were then mixed with the soil. IJ were obtained from infested *A. cuprea*. Six or 46 days later, 20 *A. cuprea*-1st instar larvae were released into each plot. The sweet potatoes were harvested in November and injury to the potatoes caused by the grubs was examined. Injury in the plot inoculated with 100,000 IJ did not differ from that in the control plot to which fenthion (MPP) granules (9 g/m^2) had been applied but it was far less severe than the injury in the untreated control plot[16].

In Kagoshima Pref. in southern Japan, several scarabaeid grubs, *Anomala rufocuprea, A. daimiana, A. cuprea, A. albopilosa, Blitopertha orientalis* occur in sweet potato-fields where they severely infest the sweet potato roots, mainly in August. To test the efficiency of *S. kushidai* in the control of these insects at Kagoshima Agricultural Experiment Station, Ohsumi Branch (Kushira, Kagoshima Pref.), VC plates (30 cm width) were buried to a 20 cm depth in an experimental field and enclosed in a 2.5 $\times$5 m square. Each plot was sprayed with 200,000 IJ/m^2 (IJ : obtained from *in vitro* culture) using a spraying can in June, 1989. The plots were plowed in preparation for the planting of sweet potatoes. Two ridges (ca. 80 cm$\times$5 m) were made in each plot and 32 sweet potato plants were planted 2 days after the application of the nematodes at a 30 cm spacing. After the sweet potatoes reached maturity, they were harvested and traces of grub feeding on their surface were compared among the plots. Application of 200,000 IJ/m^2 significantly reduced the numbers of the sweet potatoes seriously damaged by the grubs[13]. In a second experiment in 1990, 6,000, 12,000, and 24,000 IJ in 15 m*l* tap water (IJ: obtained from *in vitro* culture) were injected into the soil near each sweet potato root (ca. 20 cm depth) in August. These juvenile numbers were equivalent to an application of 25,000, 50,000 and 100,000 IJ/m^2, respectively. Injury of the sweet potatoes by the grubs was reduced significantly in the plots to which 24,000 IJ/root had been applied. Inoculation of a smaller number, however, had little effect on the grubs. At the time of harvest, samples of soil from each plot were put into plastic cups (7 cm diameter$\times$5 cm height). Individual 3rd instar larvae of *A. rufocuprea* were reared in cups with soil. In the soil samples taken from the plots where 6,000, 12,000 and 24,000 IJ/root had been applied, 23.3, 40.0 and 66.7% of the grubs died, respectively, due to *S. kushidai* infection. It is possible that the density of *S. kushidai* in the soil increased due to *S. kushidai* infestation in naturally occurring grubs[14]. *S. kushidai* also was detected from these plots 2 years after the initial application (Ôya, S. personal communication).

Conclusion

From 1930's to 1940's in the eastern part of the USA, the Japanese beetle, *Popillia japonica*, was successfully controlled with *Steinernma glaseri*[15]. Lethal effects on scarabaeid grubs of several entomopathogenic nematodes introduced from abroad have been tested in Japan during the last decade. When 5,000 IJ (in 0.5 m*l* water) of *S. carpocapsae* (DD 136 and Mexican strain), *S. glaseri*, *S. feltiae* and *Heterorhabditis* sp. were inoculated into bark compost cultures in which *A. rufocuprea* grubs were individually reared, a significant lethal effect was detected. These nematodes killed approximately 30-87% of the grubs within 5 days at 25°C[5]. Aliquots of an IJ suspension containing 3,500 IJ in 1 m*l* water of *S. intermedia*, *S. carpocapsae* (Mexican strain), *S. feltiae*, *S. glaseri* and *H. heliothidis* were inoculated onto filter paper in the petri dishes (9 cm diameter×1.8 cm height) where the 2nd or 3rd instar larvae of *A. cuprea* were placed individually. In this case these species were ineffective against the grubs. The same dose of IJ of *S. kushidai*, however, killed 90% of the grubs in the same period under the same culture conditions[9]. These results indicate that *S. kushidai* is more lethal to scarabaeid grubs than other steinernematid and heterorhabditid species. Field application tests also clearly showed that *S. kushidai* is a powerful biological control agent for the management of scarabaeid grub infestation. Methods of massproduction of *S. kushidai* on a commercial basis have recently been investigated by several researchers.

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(Received for publication, Feb. 3, 1993)