Korean J. Org. Agric.
Volume 30, Number 1: 103-118, February 2022
http://dx.doi.org/10.11625/KJOA.2022.30.1.103

ISSN 1229-3571 (Print)
ISSN 2287-819X (Online)

Toxicity Assessment of *Photorhabdus temperata* Isolated from *Heterorhabditis megidis* Gwangju Strain (Nematoda: Heterorhabditidae) in Fish and Rat*

Park, Sun-Han[***,****] · Chung, Nam-Jun[****] · Choo, Young-Moo[*****] · Kim, Young-Joon[******] · Kim, Jin-Ho[**]

Heterorhabditis megidis Gwangju Strain (Nematoda: Heterorhabditidae)으로부터 분리한 *Photorhabdus temperata*의 어류 및 쥐 독성평가

박순한 · 정남준 · 추영무 · 김영준 · 김진호

Photorhabdus is a bacterial symbiont of entomopathogenic nematodes of the genus *Heterorhabditis* in the family Heterorhabditidae. *Photorhabdus* is known to have nematicidal activity in addition to insecticidal activity. *P. temperata* isolated from Korean indigenous *H. megidis* Gwangju strain also produced high control efficacy against root-knot nematode *Meloidogyne incognita* and root-lesion nematode *Pratylenchus penetrans*. *P. temperata* has drawn interest as a potential bionemati-

* This research was financially supported by the Ministry of Agriculture Food and Rural Affairs (MAFRA), Korea, under the "Development of Industrialization Technology for Crop Viruses and Disease Pests" (Ref. No. 120081-05) supervised by the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture and Forestry. We want to thank Dr. Hoyul Choo, emeritus professor of Gyeongsang National University, for his comments on the initial draft of the manuscript.

** Corresponding author, School of Ecology and Environmental System, College of Ecology and Environmental Sciences, Kyungpook National University, Gyeongbuk 37224 (kimjinho@knu.ac.kr)
Phone: +82-54-530-1202, *** - **** - ****

*** School of Ecology and Environmental System, College of Ecology and Environmental Sciences, Kyungpook National University, Gyeongbuk 37224, Republic of Korea

**** Nambo Co., Ltd., Gyeongnam 52840, Republic of Korea

***** Jinju Bioindustry Foundation, Gyeongnam 52839, Republic of Korea

****** School of Applied Biosciences, College of Agriculture and Life Sciences, Kyungpook National University, Daegu 41566, Republic of Korea

cide for the control of root-knot nematodes thereby. For the registration as an organic agricultural material, the toxicity of *P. temperata* was assessed by the acute toxicity test in carp (*Cyprinus carpio*) and acute oral and dermal toxicity tests in Sprague-Dawley rat (*Rattus norvegicus*) in compliance with the guidelines of the Rural Development Administration (RDA). In the acute toxicity test in fish, neither lethality nor abnormal responses of carp were observed. Body length and weight of carp and changes in DO concentrations and pH values were not significantly different between the treated group and the untreated control. In the acute oral and dermal toxicity tests, clinical signs, abnormal behavior, mortality, and pathological findings were not observed in all the experimental rats. The weight increment of all rats was normal. Acute toxicity results of *P. temperata* in fish and rats belonged to categories III, IV, and IV of RDA, respectively. Toxicity results of the present study indicated that *P. temperata* could be a safe and promising bionematicide against root-knot nematodes and root lesion nematode.

Key words : *acute toxicity, symbiotic bacteria, entomopathogenic nematodes, Photorhabdus temperata, Heterorhabditis megidis*

Ⅰ. Introduction

Photorhabdus temperata is an entomopathogenic bacterium symbiotically associated with nematodes of the family Heterorhabditidae (18). The natural habitat of *P. temperata* is in the intestinal lumen of entomopathogenic nematodes (EPNs) *Heterorhabditis megidis*, NC subgroup of *H. bacteriophora*, *H. zealandica*, and *H. downesi* (24). Since EPNs are highly virulent to broad insect pest hosts and safe to vertebrates, plants, and other non-target organisms, there has been tremendous research and commercial interest in EPNs in the genera *Steinernema* and *Heterorhabditis* themselves and their symbiotic bacteria, *Xenorhabdus* for *Steinernema* and *Photorhabdus* for *Heterorhabditis* (14, 15). These symbiotic bacteria, *Xenorhabdus* and *Photorhabdus* can be used as eco-friendly control agents for insect pests independently of the nematodes through their insecticidal activities (13, 30) and even as biocontrol agents for plant-parasitic nematodes through their nematicidal activities (1, 32, Park, unpublished data). As an example of nematode control by EPN-associated bacteria, *X. nematophila*, and *P. luminescens* isolated from *S. carpocapsae* and *H. bacteriophora*, respectively, reduced egg hatching of root-knot nematode *Meloidogyne javanica* (26). And *P. temperata* and *P. luminescens* isolated from *H. megidis* Gwangju strain and *H. bacteriophora* Hamyang strain, respectively, showed high control effect against *M. incognita* and root-lesion nematode *Pratylenchus penetrans* in laboratory experiment (Park, unpublished data). Apart from the importance of root-lesion

nematodes, root-knot nematodes in the genus *Meloidogyne* are the most serious plant-parasitic nematodes. These nematodes are an economically important polyphagous group of highly adapted obligate plant parasites and distributed worldwide and parasitize nearly every species of higher plants (19). More than 90 *Meloidogyne* species have been described from over 3,000 plant species designated already as hosts to root-knot nematodes in the year 2003 (28). The most abundant and damaging root-knot nematodes are *M. incognita*, *M. javanica*, and *M. arenaria* from Mediterranean and tropical areas, and the temperature species *M. hapla* (17). In Korea, these four species of nematodes *M. arenaria*, *M. hapla*, *M. incognita*, and *M. javanica* have been frequently found from many economic crops and field plants including weeds, especially *M. hapla* and *M. incognita* being main and serious root knot nematodes on important crops in greenhouses and large-scale crop farming (6, 7). Root-knot nematodes are extremely serious pests that are challenging to their control. Chemical nematicides are the main tools for the control of root-knot nematodes in economic crops. However, indiscriminate and repeated application of pesticides leads to loss of biodiversity, pest resistance, and other ecological imbalance (16). Growing dissatisfaction with chemical nematicides due to their health and environmental hazards has prompted much effort towards agronomically durable, economically feasible, and environmentally safe alternatives for such chemicals. Biological control agents are such promising alternatives including symbiotic bacteria of EPNs (2). More recently, symbiotic bacteria of *H. bacteriophora*, *H. megidis*, *S. carpocapsae*, and *S. feltiae* have turned out to be effective biological control agents for pinewood nematode *Bursaphelenchus xylophilus* and root-knot nematodes *M. incognita* and *M. javanica* (24, 25, Park unpublished data). Nematicidal activity is one of the broad bioactivities of *Xenorhabdus* and *Photorhabdus*. Although both bacteria produce nematicidal and highly effective insecticidal activity, they are not hazardous to vertebrates except *P. asymbiotica* isolated from human clinical specimens in the USA and Australia (5, 10). That is, *X. nematophila*, *X. bovienii*, and *P. luminescens* isolated from *S. carpocapsae*, *S. bibionis*, and *H. bacteriophora*, respectively, produced no disease symptoms, signs of infectivity, pathogenicity, toxicity, and/or harmful effects on leghorn chicks, adult albino mice, guinea pigs, and rats (20, 21). However, there are no safety and toxicity data reported on *P. temperata* to date.

Nevertheless, the safety and nematicidal activity of these bacteria have aroused interest in the development of *P. temperata* and *P. luminescens* as bionematicides against root-knot nematodes (Park, unpublished data).

For *P. temperata* or any other organic agricultural material to be allowed as bionematicide in Korea, it has to be registered by submitting mammalian and environmental biotoxicity data to

the Rural Development Administration (RDA) (24). Many countries such as the USA and most European countries exempt the registration of EPNs (29), but information on registration of *Xenorhabdus* and *Phtorhabdus* as agropesticides is rare. Toxicity assessment of organic agricultural materials is generally made through acute toxicity tests, following the guidelines of RDA.

Therefore, acute toxicity tests in fish and acute oral and dermal toxicity tests in rats were carried out to assess the toxicity of *P. temperata* in compliance with the guidelines of RDA. The present study will contribute to the development of bionematicide with symbiotic bacteria for the biocontrol of root-knot nematodes.

Ⅱ. Materials and Methods

1. *P. temperata* and Symbiotic Nematode

The symbiotic EPN, *H. megidis* was isolated from the sawtooth oak (*Quercus acutissima*) forest of Mt. Mudeungsan in Gwangju, Gwangju Metropolitan City using the *Galleria mellonella* (L.) trapping method (8). The infective juveniles emerged from *G. mellonella* cadavers were harvested in White traps and stored at 10℃ for no more than 3 weeks before they were used. To isolate *P. temperata*, *G. mellonella* larvae were exposed to 20 infective juveniles of *H. megidis* per larva. Two days later, larvae were sterilized by dipping into 10% sodium hypochlorite and rinsed three times in sterilized distilled water. Then, hemolymph was collected from surface-sterilized *G. mellonella* cadavers with a sterilized 26G×1/2" syringe (Jung Rim Medical Industrial Co. Ltd., Korea). A drop of collected hemolymph was streaked on NBTA and cultured at 28℃ for 48 hrs. The *Photorhabdus* colonies were selected 48 hrs later and cultured on the same medium for 48 hrs. This culture was transferred into 250 mL Erlenmeyer flask containing 20 mL tryptic soy broth (TSB, Difco) and cultured at 25℃ for 48 hrs.

2. Formulation of *P. temperata* for Acute Toxicity Tests

P. temperata cultured in TSB at 25℃ for 48 hrs was transferred into 500 ml Erlenmeyer flask containing 300 mL modified nutrient broth (MNB) (g/liter: egg yolk 0.625, cholesterol 0.025, lecithin 0.125, sweet whey powder 2.5, NaCl 2.5, KH_2PO_4 0.625, yeast 1, peptone 3.75) and cultured at 25℃ initially. Mass production was made in fermenter by transferring culture into a gradually larger fermenter as follows; 300 mL of culture was transferred into 30 L optimized

MNB in a 50-liter fermenter and cultured for 48 hrs. Again, 30 L of culture was transferred into a 3,000 L culture medium in a 5,000-liter fermenter and cultured at antibiotic and optimal culture conditions for 48 hrs. Then, 3,000 L of culture received 1.5% Na-alginate and 1.0% starch as microcapsule substance and stabilizer, respectively. Finally, 3,000 L of microcapsule formulation culture was diluted with the same amount of 3,000 L of water containing efficacy enhancer and penetrant at the ratio of 50:50 to quantify. This formulation was used in the acute toxicity test in fish and acute oral and dermal toxicity tests in rats.

3. Acute Toxicity Test of *P. temperata* in Fish

The healthy carp (*Cyprinus carpio*) was used in the acute toxicity tests of *P. temperata* based on the nominal concentration of main ingredient input ratio because OECD recommended 2-4 cm long carp or 1-2 cm long Japanese rice fish (*Oryzias latipes*) for toxicity test in fish (24). Thus, the acute toxicity test was conducted in carp, following the guidelines of RDA and OECD as environmental biotoxicity testing (21, 22).

Because lethality of carp was not observed at the limit test concentration of 10.0 mg/L in the preliminary test, a fixed concentration of 10.0 mg/L of *P. temperata* product was treated to acclimated healthy carp in the acute toxicity (23). The test was performed in the 24 L (38.0 x Ø 30.0 cm) glassware with ten carps at the Fish Experimental Room of the Korea Bio-safety Institute KBSI) (Eumseong, Chungbuk province) for 96 hrs. Before the test, carps were acclimated in a 80 L cuboid water tank (30×60×45 cm) in the Fish Breeding Room, Good Laboratory Practice (GLP) Research Building of KBSI for 7 days. The tank was continuously aerated to keep 80% or more of the saturated concentration of dissolved oxygen. More than 110% of needed carps were maintained at 20-24℃ under a 16 hr-light and 8 hr- dark cycle. The carp were fed once a day and fasted for 24 hours before the test. The carp was provided with one-week purified groundwater, which was filtered successively through 1.0, 0.5, and 0.1 μm filters. The same number of carps was used in the negative and positive control groups. Negative control group received groundwater, whereas positive control group was treated with pentachlorophenol sodium salt (C_6Cl_5NaO) (Kanto Chemical, Japan Lot. 30602312) at the rate of 0.050, 0.071, 0.100, 0.141, and 0.200 mg/L (common ratio 1.41) (23).

Body abnormality, abnormal swimming, hemorrhage, and mortality were observed at times 3, 24, 48, 72, and 96 hrs after treatment. Mortality was decided by stopping gill breath and motionlessness when touched with a glass rod. The length and weight of all the tested carps were measured. Water quality, water temperature, pH, and dissolved oxygen concentration (DO)

were examined every day during the test. The pH and DO were measured with Orion 4 star (Thermo Scientific, USA). Hardness of carp was measured by taking a sample from the negative control group before the carp exposure. Water temperature was 20-24℃ both in the control group (negative and positive) and treated group. The pH values were 6.91-7.71 in the control group and 6.81-7.83 in the treated group (Table 2). The dissolved oxygen was over 60% of the saturated concentration, i.e., 94.0-110.5% in the control group and 67.0-112.2% in the treated group during the test (Table 2). Statistical analysis was not made as the test was terminated at the limit test concentration of 10.0 mg/L.

Acute toxicity values were expressed as LC_{50}, following the criteria of the Globally Harmonized Classification System (GHS) (23, 27).

4. Acute Oral and Dermal Toxicity Tests of *P. temperata* in Rat

For the acute oral and dermal toxicity tests of *P. temperata* as mammalian toxicity testing, the Sprague-Dawley rats (*Rattus norvegicus*) were used. The rats were obtained from the Hanrim Laboratory Animal Research Institute (Hwaseong, Gyeonggi province). The female rats were used in the acute oral toxicity test, whereas female and male rats were used in the acute dermal toxicity test. The tests were also performed at the Korea Bio-safety Institute (KBSI) (Eumseong, Chungbuk province).

The acute oral toxicity test was conducted in female rats in compliance with the guidelines of RDA (23). The female rats were acclimated in a polycarbonate cage (26×42×18 cm) for a week before the test. The acclimation and test were made under the environmentally controlled conditions of 23±2℃, 50±10% relative humidity, 12- hour light (from 7:00 am to 7:00 pm), and dark cycle, and illuminance of 200-300 lux. Rats were fed with a certified rodent diet (Cargill Agri Purina, Inc., Korea) and filtered groundwater was provided. Rats were fasted for 12 hours before administration and fed again from 3 hours after administration. The administration was made in two steps with eight-week-old female rats; the weights of rats were 193.0~197.5 g in step 1 and 203.0~207.5 g in step 2. Three healthy rats were included in each test unit. Each rat was marked with picric acid on the body, while cages contained three rats with different tags. Oral administration was made at a fixed dose of 2,000 mg/kg body weight (BW) both in the 1st and 2nd steps because no lethality was observed for 72 hours in the 1st step. The dose was administered to the rats based on their body weight. The control received distilled water. Then, general toxic symptoms and death cases were examined for two weeks; at 30 min, 1, 2, 3, and 4 hrs after administration on the 1st day and once a day from 2nd to 14th day. Every rat was

weighed just before administration and on the 1st, 3rd, 7th, and 14th day after administration. All the survived rats were anesthetized with CO_2 gas at the end of the test and performed necropsy (23). Acute toxicity values were expressed as LD_{50} following the criteria of the GHS (23, 27).

The acute dermal toxicity test on female and male rats was also conducted under the guidelines of RDA (23). Eight-week-old female and male rats were used in the test. The weight of female rats was 218.5~225.0 g and that of male rats 260.0~270.0 g. The rats were acclimated in a polycarbonate cage (26×42×18 cm) for a week before the test. The acclimation, feeding, and testing were as described above. Five healthy rats were in each test unit. Rats and cages were marked as described above. Ten rats in total, five females and five males were used, which were randomly selected and assigned. *P. temperata* was treated with a single dose of 4,000 mg/kg BW. Every rat was weighed just before treatment. One day before treatment, each rat was clipped to a size of 5×6 cm with an electric clipper (Joas Co., Namyangju, Gyeonggi, Korea) and administered onto 4×4 cm size. Gauze containing given dosage was wrapped with 3 M™ Coban™ self-adherent wrap (3M Health Care Ltd., USA). After 24 hrs, gauze was removed and the treated part was cleaned and dried up with distilled water and medical cotton wool, in turn. Then, clinical signs and lethality were investigated for two weeks. Examination and weight measurement were performed as described above. Since no general toxic symptoms and lethality were observed, a necropsy was not performed. In addition, statistics were not processed because all the tested rats were alive. Acute toxicity values were expressed as LD_{50} following the criteria of the GHS (23, 27).

Ⅲ. Results

1. Toxicity of *P. temperata* in Carp

The carp exposed to 10 mg/L of *P. temperata* showed neither lethality nor abnormal behavior during the experiment (Table 1). Mean body length and weight of carp were not different between the untreated control and *P. temperata* treatment, which were 4.26±0.18 cm and 0.89±0.13 g in the untreated control and 4.45±0.29 cm and 1.05±0.24 g in the *P. temperata* treatment. Changes in DO concentrations and pH values were also not significantly different between the untreated control and the treatment (Table 2). LC_{50} was not determined because a single concentration was applied based on nominal concentration and mortality was not observed.

Thus, LC_{50} values of *P. temperata* were more than 10.0 mg/L (Table 3). This value in fish has corresponded to category III, ≥2.0 mg/L for pesticide products.

Table 1. Acute toxicity of *Photorhabdus temperata* to freshwater carp *Cyprinus carpio*

Treatment	Conc. (mg/L)[a]	No. of fish	Exposure time (hours)									
			3		24		48		72		96	
			AR	CM	AR	CM	AR	CM	AR	CM	AR	CM
Control	0	10	NO	0 (10)[b]	NO	0 (10)	NO	0 (10)	NO	0 (10)	NO	0 (10)
P. temperata	10	10	NO	0 (10)	NO	0 (10)	NO	0 (10)	NO	0 (10)	NO	0 (10)

Note: [a] based on nominal concentration of main ingredient input ratio.
[b] Number in the parenthesis is numbers of alive fish.
AR, abnormal response; Loss of equilibrium, fish behaviour at the water surface and at the bottom, hemorrhage, and vertebral deformation were observed.
CM, cumulative mortality
NO, normal

Table 2. Changes in water pH and DO concentration during the acute toxicity test of *Photorhabdus temperata* in freshwater fish *Cyprinus carpio*

Treatment	Conc. (mg/L)[a]	No. of fish	Exposure time (hours)									
			3		24		48		72		96	
			pH	DO	pH	DO	pH	DO	pH	DO	pH	DO
Control	0	10	7.71	102.1	7.34	94.5	6.96	94.0	7.32	103.7	7.51	110.5
P. temperata	10	10	7.83	97.1	7.30	89.6	6.81	75.2	6.97	67.0	7.18	111.2

Note: [a] based on nominal concentration of main ingredient input ratio.
DO, dissolved oxygen concentration; %sat

Table 3. LC_{50} values of *Photorhabdus temperata* during the experimental time

Substance	LC_{50} (mg/L) with time (hours)	
	48	96
Control	NA[b]	NA
PCP-Na salt[a] (15-KET-PC001)	0.08P[c]	0.075
P. temperata	>10.0[d]	>10.0

Note: [a] duration of reference substance (pentachlorophenol sodium salt, Kanto Chemical, Japan lot 30602312) test from June 22, 2015 to June 26, 2015.
[b] not applicable
[c] active ingredient
[d] main base material input ratio

2. Oral Toxicity of *P. temperata* in Rat

There were no adverse effects on rats exposed to 2,000 mg/kg BW of *P. temperata* in the acute oral toxicity test. Clinical signs, abnormal behavior, and lethality were not observed during the two-week observation period (Table 4). The weight increment of rats was normal (Table 5). No pathological findings were observed from all the experimental rats in steps 1 and 2 at necropsy. Oral toxicity results of *P. temperata* based on LD_{50} value, $\geq$2,000 mg/kg BW was corresponded to the categories 5 of GHS and OECD and IV of RDA for acute oral toxicity.

Table 4. Mortality and clinical signs of rats in the acute oral toxicity test of *Photorhabdus temperata* for 14 days

Group	Dose (mg/kg BW)	Sex	Individual number	Mortality	Days after administration					
					0	3	6	9	12	14
1	2000	Female	1	0	NAD	NAD	NAD	NAD	NAD	NAD
			2	0	NAD	NAD	NAD	NAD	NAD	NAD
			3	0	NAD	NAD	NAD	NAD	NAD	NAD
2	2000	Female	1	0	NAD	NAD	NAD	NAD	NAD	NAD
			2	0	NAD	NAD	NAD	NAD	NAD	NAD
			3	0	NAD	NAD	NAD	NAD	NAD	NAD

Note: NAD, no abnormality detected.
Mortality was observed during all the experimental period for 14 days, but presented only the results every three days because all the experimental rats were alive.

Table 5. Body weight change of rat during the acute oral test of *Photorhabdus temperata*

Group	Dose (mg/kg BW)	Sex	Individual number	Days after administration				
				0	3	7	14	Gain
1	2000	Female	1	197.0	221.5	231.0	261.5	64.5
			2	197.5	218.5	228.0	244.5	47.0
			3	193.0	220.5	231.5	251.5	58.5
Mean ± SD				195.8±2.5	220.2±1.5	230.2±1.9	252.5±8.5	56.7±7.3
2	2000	Female	1	203.0	218.0	232.5	260.0	57.0
			2	207.5	214.5	224.5	247.5	40.0
			3	220.5	242.5	271.5	302.5	82.0
Mean ± SD				210.3±9.1	225.0±15.3	242.8±25.1	270.0±28.8	59.7±17.2

Note: Weight of rats was observed during all the experimental period for 14 days, but presented only the results of 0, 3, 7, 14 days.

3. Dermal Toxicity of *P. temperata* in Rat

The rats exposed to 4,000 mg/kg BW of *P. temperata* have not shown any clinical signs, lethality, and abnormality in the acute dermal toxicity test (Table 6). Weight of female and male rats was increased normally (Table 7). No pathological findings were observed from all the experimental rats in the steps 1 and 2. Dermal toxicity results of *P. temperata* based on LD_{50} value ≥4,000 mg/kg BW has corresponded to the categories 5 of GHS and OECD and IV of RDA for acute dermal toxicity.

Table 6. Mortality and clinical signs of rats in the acute dermal toxicity test of *Photorhabdus temperata* for 14 days

Group	Dose (mg/kg BW)	Sex	Individual number	Mortality	Days after administration					
					0	3	6	9	12	14
1	4000	Female	1	0	NAD	NAD	NAD	NAD	NAD	NAD
			2	0	NAD	NAD	NAD	NAD	NAD	NAD
			3	0	NAD	NAD	NAD	NAD	NAD	NAD
			4	0	NAD	NAD	NAD	NAD	NAD	NAD
			5	0	NAD	NAD	NAD	NAD	NAD	NAD
2	4000	Male	1	0	NAD	NAD	NAD	NAD	NAD	NAD
			2	0	NAD	NAD	NAD	NAD	NAD	NAD
			3	0	NAD	NAD	NAD	NAD	NAD	NAD
			4	0	NAD	NAD	NAD	NAD	NAD	NAD
			5	0	NAD	NAD	NAD	NAD	NAD	NAD

Note: NAD, no abnormality detected.
Mortality was observed during all the experimental period for 14 days, but presented only the results every three days because all the experimental rats were alive and clinical signs were not observed.

Table 7. Body weight change of rat during the acute dermal test of *Photorhabdus temperata* for 14 days

Group	Dose (mg/kg BW)	Sex	Individual number	Days after administration				
				0	3	7	14	Gain
1	4000	Female	1	224.0	228.5	236.0	249.0	25.0
			2	219.5	222.5	249.5	279.5	60.0

Group	Dose (mg/kg BW)	Sex	Individual number	Days after administration				
				0	3	7	14	Gain
1	4000	Female	3	225.0	229.0	235.0	250.0	25.0
			4	219.0	225.0	259.0	300.0	81.0
			5	218.5	222.5	236.5	253.5	35.0
Mean ± SD				221.2±3.1	225.5±3.1	243.2±10.6	266.4±22.6	45.2±22.0
2	4000	Male	1	266.0	273.0	296.5	333.0	67.0
			2	265.0	269.0	304.5	346.5	81.5
			3	266.5	279.5	309.5	355.0	88.5
			4	260.0	274.5	300.5	342.0	82.0
			5	270.0	288.0	309.0	345.5	75.5
Mean ± SD				265.5±3.6	276.8±7.3	304.0±5.6	367.0±8.0	78.9±7.2

Note: Weight of rats was observed during all the experimental period for 14 days, but presented only the results of 0, 3, 7, 14 days.

Ⅳ. Discussion

Photorhabdus and *Xenorhabdus* are symbiotic bacteria of EPNs in the genera *Heterorhabditis* and *Steinernema*, respectively, which encode a multitude of insecticidal toxins, thus being excellent sources of novel insecticidal agents (13, 14). *P. temperata*, a symbiotic bacterium of *H. megidis*, *H. bacteriophora*, *H. downesi*, and *H. zealandica* has both insecticidal activity and nematicidal activity against root-lesion nematode *P. penetrans* and root-knot nematode *M. incognita* (3, 25, 30, Park, unpublished data). Nematicidal activity is one of the broad bioactivities of *Xenorhabdus* and *Photorhabdus*.

The nematicidal activity of *Photorhabdus* has aroused interest in the development of commercial bionematicide including *P. temperata*. However, they have to be registered by submitting the toxicity data to RDA like other organic agricultural materials. *Photorhabdus* and *Xenorhabdus* have been known as non-hazardous organic materials. For example, *X. nematophila*, *X. bovienii*, and *P. luminescens* isolated from *S. carpocapsae*, *S. bibionis*, and *H. bacteriophora*, respectively, have not produced any disease symptoms, signs of infectivity, pathogenicity, toxicity, and/or harmful effects on leghorn chicks, adult albino mice, guinea pigs, and rats (13, 20).

Nevertheless, toxicity tests on *P. temperata* have to be performed to register this bacterium as bionematicidal material because there are no toxicity data reported on *P. temperata* to date.

After all every organic agricultural material has to be registered by submitting toxicity data in Korea (23). There are some toxicity studies on organic agricultural materials in Korea. Lee *et al*. (2018) has carried out toxicity and safety evaluation of 1,590 items of organic agricultural materials registered in Korea, but toxicity tests of any *Photorhabdus* and *Xenorhabdus* have not been made to date. Therefore, when the toxicity of *P. temperata* was assessed by acute toxicity test in carp and acute oral and dermal toxicity tests in Sprague-Dawley rats, no adverse results were obtained from all the acute toxicity tests of the present study. That is, the carp exposed to 10 mg/L of *P. temperata* showed neither lethality nor abnormal behavior during the experiment. No adverse effects were also observed from the acute oral toxicity test. Any clinical signs, abnormal behavior, and lethality were not observed during the two-week observation period. In addition, no pathological findings were observed from all the experimental rats. In the acute dermal toxicity test, clinical signs, lethality, and abnormality were not observed. The weight of rats increased normally both in acute oral and dermal toxicity tests.

Acute toxicity results of *P. temperata* in fish (10.0 mg/L both in 48 hrs and 96 hrs) belonged to category III. The acute fish toxicity of organic agricultural materials is classified into three categories I (<0.5 mg/L), II (0.5-0.2 mg/L), and III (≥2.0 mg/L) based on LC_{50} (mg/L, 48 hrs and 96 hrs) (23, 27). Accordingly, *P. temperata* belonged to safe material and thus can be developed as potential bionematicide for root-knot nematodes. Although an acute toxicity test of *P. temperata* in fish was not made, there were some toxicity assessments of other bioinsecticides and/or bionematicides in fish. Their effects on fish in the environmental biotoxicity test were variable depending on materials or concentrations.

For example, *Bacillus thurigiensis* var. *kenyae* (Btk) was not toxic to freshwater fish, western mosquitofish (*Gambusia affinis*). The 200-1,000 mg/L (2.5×10^7 spores/mg) of wettable powder formulation of Btk showed no abnormal behavior and mortality (17). Bt *kurstaki* and Bt *israelensis* also did not produce any adverse effects Zebrafish (*Danio rerio*) and Nile tilapia (*Oreochromis niloticus*) although the increased frequency of necrotic cells was found in the necrosis-apoptosis study on peripheral erythrocytes of *O. niloticus*. LC_{50} of both strains to these fish species were higher than 5×10^6 spores/ml (12).

In another toxicity test of two Malaysian indigenous Bt products in Nile tilapia (*Tilapi nilotica* = *O. niloticus*) with four concentrations, 64 mg, 128 mg, 256 mg, and 512 mg/L, Terakil-1, a wettable powder and Teracon-1, a protein concentrate showed no significant difference in mortality of *T. nilotica* at a dose of 128 mg/L as compared to the lowest concentration, 64 mg/L in the ecotoxicity tests. Each product led to 30% and 12.5% corrected mortality of *T. nilotica* at 128 mg/L at four days after treatment, respectively, but LC_{50} of Terakil-1 and Teracon-1 were

included in practically non-toxic (100-1,000 mg/L) according to the Rating scheme used by the United States Fish and Wildlife Services for aquatic toxicity. In general, Bt has been turned out to be practically non-toxic to fish from several toxicity tests in different species of fish.

However, biopesticide neem (*Azadirachata indica*) influenced ethological responses of snake-headed fish (*Channa gachua*) depending on exposure time and concentration, which have insecticidal and nematicidal activity (9). The fish showed erratic swimming, loss of equilibrium, and hyperactivity with the advancement of exposure time and concentration. Thus, kinds of nematicidal organic materials, concentration, and/or exposure time may be the main factors affecting fish.

In acute oral and dermal toxicity tests of *P. temperat*a as mammalian toxicity testing, *P. temperat*a also turned out to be non-toxic organic agricultural material which belonged to the category IV (low) of RDA toxicity classification criteria, $\geq$ 2,000 mg/kg BW in acute oral and $\geq$ 4,000 mg/kg BW in acute dermal. *P. temperat*a produced no adverse effects on Sprague-Dawley rats both in acute oral and dermal toxicity tests. Any clinical signs, abnormal behavior, and lethality were not observed with normal weight increment during the two-week observation period. In addition, no pathological findings were observed from all the experimental rats in steps 1 and 2 at necropsy in acute oral toxicity.

The safety data of some biological nematicides were also made through the acute oral toxicity tests except the present study. For example, Btk did not produce any mortality, gross behavioral changes, either immediately or during the 21-day observation period in the oral and dermal toxicity studies in albino rats like the effect of Bt on fish (17).

The administration of an active agent and final product of biological nematicide HeberNem which composed basically of *Tsukamurella paurometabola* did not cause any pathogenic or toxic reactions to Sprague-Dawley rat such as mortality, toxic signs, or behavioral change of rats (31).

In the treatment of 4,000 mg/kg BW of *P. temperata*, clinical signs, lethality, and abnormality were not observed in the acute dermal toxicity test. The weights of female and male rats were normally increased.

Consequently, the toxicity assessment of *P. temperata* through acute toxicity test in fish and acute oral and dermal toxicity tests in rats demonstrated that *P. temperata* could be an eco-friendly alternative bionematicide as a potentially non-toxic product.

Therefore, the present toxicity assessment study of *P. temperata* is expected to make a significant contribution to root-knot nematode control and to the development of commercial production of EPN bacteria.

[Submitted, January, 18, 2022; Revised, February, 7, 2022; Accepted, February, 10, 2022]

References

1. Aatif H. M., N. Javed, S. A. Khan, and S. Ahmad. 2012. Virulence of *Xenorhabdus* and *Photorhabdus* bacteria and their toxins against juvenile's immobilization of *Meloidogyne incognita*. Pak. J. Phytopathol. 24: 170-174.
2. Abd-Elgawad M. M. M. 2016. Biological control agents of plant-parasitic nematodes. Egyptian Journal of Biological Pest Control. 26: 423-429.
3. Ahn J. Y., J. Y. Lee, E. J. Yang, Y. J. Lee, K. B. Koo, K. S. Song, and K. Y. Lee. 2013. Mosquitocidal activity of anthraquinones isolated from symbiotic bacteria *Photorhabdus* of entomopathogenic nematode. Journal of Asia-Pacific Entomology. 16: 317-320.
4. Beiro O., G. Garcia, O. Carballo, Y. Ramirez, A. Valdivieso, and L. A. Dios. 2006. A study of acute dermal toxicity for HeberNem in rats. Biotecnologia Aplicada. 23: 43-45.
5. Boemare N, C. Laumond, and H. Mauleon. 1996. The entomopathogenic nematode-bacterium complex: biology, life cycle and vertebrate safety. Biocontrol Science and Technology. 28: 333-346.
6. Choi Y. E. and H. Y. Choo. 1978. A study on the root-knot nematodes (*Meloidogyne* spp.) affecting economic crops in Korea. Korea J. Pl. Prot. 17: 89-98.
7. Choo H. Y. and Y. E. Choi. 1979. A study on the plant parasitic nematodes (Tylenchida) in Jeju province. Kor. J. Pl. Prot. 18: 169-175.
8. Choo H. Y., H. K. Kaya, and S. P. Stock. 1995. Isolation of entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) from Korea. Japan. J. Nematol. 25: 44-51.
9. Dhara K. and S. R. Karmakar. 2016. Acute toxicity of neem (*Azadirachta indica* A. Juss) leaf extracts to snake headed fish, *Channa gachua* (Ham.) with special reference to their ethological responses and some haematological parameters. International Journal of Life-Science Scientific Research. 2: 552-558.
10. Gerrard J., N. Waterfield, R. Vohra, and R. French-Constant. 2004. Human infection with *Photorhabdus asymbiotica*: an emerging bacterial pathogen. Microbes and Infection. 6: 229-237.
11. GHS. 2016. Acute toxicity. www.chemsafetypro.com>acute_ld50_lc50.
12. Grisolia C. K., E. C. Oliveiro-Filho, F. R. Ramos, M. C. Lopes, D. H. F. Muniz, and R. G. Monnerat. 2009. Acute toxicity and cytotoxicity of *Bacillus thuringiensis* and *Bacillus*

sphaericus strains on fish and mouse bone marrow. Ecotoxicology. 18: 22-26.

13. Hinchliffe S. J., M. C. Hares, A. J. Dowling, and R. H. French-Constant. 2010. Insecticidal toxins from the *Photorhabdu*s and *Xenorhabdus* bacteria. The Open Toxicology Journal. 3: 83-100.
14. Kaya H. K. and R. Gaugler. 1993. Entomopathogenic nematodes. Annu. Rev. Entomol. 38: 181-206.
15. Kaya H. K., M. M. Aguillera. A. Alumai, H. Y. Choo, M. de la Torre, A. Fodor, S. Ganguly, S. Hazir, T. Lakatos, A. Pye, M. Wilson, S. Yamanaka, H. Yang, and R. U. Ehlers. 2006. Status of entomopathogenic nematodes and their symbiotic bacteria from selected countries or regions of the world. Biological Control. 38: 134-155.
16. Kumar S., A. K. Sharma, S. S. Rawat, D. K. Jain, and S. Ghosh. 2013. Use of pesticides in agriculture and livestock animals and its impact on environment of India. Asian Journal of Environmental Science. 8: 51-57.
17. Meher S. M., S. L. Bodhankar, Arunkumar, J. N. Dhuley, D. J. Khodape, and S. R. Zaik. 2002. Toxicity studies of microbial insecticide *Bacillus thuringiensis* var. *kenyae* in rats, rabbits, and fish. International Journal of Toxicology. 21: 99-105.
18. Meslet-Cladiere L., A. Pimenta, E. Duchaud, B. Holland, and M. A. Blight. 2004. In vivo expression of mannose-resistant fimbriae of *Photorhabdus temperata* K122 during insect infection. Journal of Bacteriology. 186: 611-622.

19 Moens M., R. N. Perry, and J. L. Starr. 2009. *Meloidogyne* species – a diverse group of novel and important plant parasites, pp. 1-17. *In* Perry RN, Moens M, and Starr, JL. (eds.), Root-knot nematodes. CAB International, Wallingford, UK.

20. Obendorf D. L., R. J. Akhurst, and L. A. Miller. 1983. Non-susceptibility of mammals to the entomopathogenic bacterium *X. nematophilus*. Environmental Entomology. 12: 368-370.
21. OECD. 1992. Guideline for testing of chemicals 203; fish, acute toxicity test.
22. Poinar G. O., G. M. Thomas, S. B. Presser, and J. L. Hardy. 1982. Inoculation of entomogenous nematodes, *Neoaplectana* and *Heterorhabditis*, and their associated bacteria, *Xenorhabdus* spp., into chicks and mice. Environ. Entomol. 11: 137-138.
23. RDA. 2015. Criteria for registration of pesticides and active substances. RDA, Korea
24. RDA. 2016. Criteria for registration of pesticides and active substances. RDA, Korea
25. Sajnaga E. and W. Kazimierczak. 2020. Evolution and taxonomy of nematode-associated entomopathogenic bacteria of the genera *Xenorhabdus* and *Photorhabdus*: an overview. Symbiosis. 80: 1-13.
26. Sayedain F. S., M. Ahmadzadeh, R. Talaei-Hassanloui, M. Olia, and H. B. Bode. 2019.

Nematicidal effect of cell-free culture filtrates of EPN-symbiotic bacteria on *Meloidogyne javanica*. Biological Control of Pests & Plant Diseases. 8: 17-26.

27. Sensitization D. 2004. Chemical hazard classification and labeling: comparison of opp requirements and the GHS. www.epa.gov>files>documents>ghscriteria-summary 22pp.
28. Tigano M. S., R. M. D. G. Carneiro, A. Jeyaprakash, D. W. Dickson, and B. J. Adams. 2005. Phylogeny of *Meloidogyne* spp. Based on 18S rDNA and the intergenic region of mitochondrial DNA sequences. Nematology. 7: 851-862.
29. Tofangsazi N., S. P. Arthurs, and R. M. Giblin-Davis. 2018. Entomopathogenic nematodes. UF/IFAS.
30. Ullah I., A. L. Khan, L. Ali, R. Khan, M. Waqas, J. Hussain, I. J. Lee, and J. H. Shin. 2015. Benzaldehyde as an insecticidal, antimicrobial, and antioxidant compound produced by *Photorhabdus temperata* M1021. Journal of Microbiology. 53: 127-133.
31. Velazco L. A., D. B. Fernandez, O. B. Castro, D. K. Cosme, L. Y. R. Nunez, C. V. Silva, N. M. Garcia, J. F. S. de la Morena, L. L. M. Caceres, T. R. A. Izquierdo, T. J. S. Alba, O. Carballo, J. M. Campos, and C. Borroto. 2012. Study of oral acute toxicity/pathogenicity of the *Tsukamurella paurometabola* active agent of the biological nematicide C-924 (Heber-nem R). Advances in Pharmacoepidemiology & Drug Safety. 1: 1-6.
32. Webster J. M., G. Chen, K. Hu, and J. Li. 2002. Bacterial metabolites, *In* Gaugler, R. (ed.), Entomopathogenic nematology. CABI Publishing, New York, NY, USA.