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Screening of Nematicides against the Lotus Root Nematode, *Hirschmanniella diversa* Sher (Tylenchida: Pratylenchidae) and the Efficacy of a Selected Nematicide under Lotus Micro-Field Conditions

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Abstract: In Japan, *Hirschmanniella diversa* is an important pest in lotus cultivation in paddy fields and only lime nitrogen is registered for its control. Therefore, additional nematicides are required to control the nematode. The objective of this study was to screen for an effective nematicide. Fourth-stage juveniles and adults of *H. diversa* sampled from a lotus field were tested in in vitro solution experiments against 37 pesticides that are registered for the pest control of crops in Japan. Carbamate-based benfuracarb, organophosphate-based fenthion, nereistoxin-based cartap hydrochloride and cyanamide showed nematicidal effects against *H. diversa*. Benfuracarb at 1 µg/mL showed a nematostatic effect on *H. diversa* in an agar plate assay. Further, *H. diversa* treated with benfuracarb did not resume activity 7 days post nematicide treatment when transferred to distilled water. Benfuracarb was tested in micro-field experiments, in which *H. diversa* density and lotus tuber damage levels were monitored. Results showed that benfuracarb reduced *H. diversa* densities in the roots during the cultivation period in 2012 and consistently reduced damage levels during a five year study period. Thus, benfuracarb is recommended as an effective nematicide to be used for *H. diversa* control in lotus cultivation.

Keywords: benfuracarb; chemical control; Indian Lotus (*Nelumbo nucifera*); lime nitrogen; nematicidal effect; nematistatic effect

1. Introduction

Plant-parasitic nematodes (PPN) cause annual estimated crop losses of US$ 125 billion [1]. Among the major groups of PPN are migratory endoparasitic nematodes, which have a destructive mode of feeding by continuously moving through the cells of root tissues, resulting in enormous tissue necrosis [2]. Species of the root lesion nematode, *Pratylenchus*, and the burrowing nematode, *Radopholus*, are important and migratory endo-parasitic nematodes occurring world-wide [2]. *Hirschmanniella* is also an endo-parasitic nematode and is well adapted to moist habitats [3]. The lotus root nematode, *Hirschmanniella diversa*, has recently been of major concern causing blackening and deformation in lotus tubers and has decreased their economic value by more than one million dollars per year, affecting

lotus tuber production in Japan [4–7]. Some farmers have given up lotus farming because of the serious and drastic damages by *H. diversa*.

Lime nitrogen (cyanamide as the active substance) is the only nematicide that is registered to control *H. diversa* in Japan [6]. The registered amount of lime nitrogen is 500–1000 kg/ha. Lime nitrogen is a nematicide and a fertilizer, since it contains 20–21% nitrogen. By applying the registered amount of lime nitrogen to a field for nematode control, 100–210 kg/ha nitrogen is released to the environment. Lotus is cultivated in paddy fields near a lake and a river due to easy access to water supply and good soil quality. The most vigorous lotus cultivation area in Japan is located around Lake Kasumigaura, the second biggest lake of Japan. Nitrogen is one of the major pollutants in waterways [8] and the government has made attempts to reduce nitrogen in Lake Kasumigaura [9]. Currently, only lime nitrogen has been used for nematode control and, thus, screening alternative nematicides is urgently needed in order to develop an effective management system.

The objectives of this study were to screen a diverse group of pesticides using in vitro assays, to select the best performing nematicide and to evaluate them in further in vitro assays and field trials. These extensive in vitro and field assays should yield an effective nematicide to be used singly or collectively with lime nitrogen or other options in integrated pest management (IPM) systems in order to manage *H. diversa* in lotus paddy fields.

2. Materials and Methods

2.1. Nematodes and Pesticides

Hirschmanniella diversa populations used in this study were collected from a lotus cultivation field in Iseki township, Ishioka city, Ibaraki Prefecture, Japan (36°08′ N, 140°19′ E). Lotus roots were collected from the lotus paddy field, and *H. diversa* individuals were extracted using the Baermann funnel method at 25 °C for 48 h [10]. *Hirschmanniella diversa* individuals were stored in a glass vial with tap water in a cold room (4.0 ± 1.0 °C) until experimentation. The pesticides used in this study are listed in Table 1. Pesticide concentrations are determined based on the active substance (a.s.) concentration (1–100 μg/mL) or the registered concentrations for nematode control in Japan.

2.1.1. In Vitro Assays: Nematicidal Effect of Pesticides on H. diversa

Vials containing *H. diversa* were moved from the cold room temperature to room temperature (25.0 ± 1.0 °C) to restore activity of the nematodes. A diluted pesticide solution (10 mL) (see Table 2 for the dilution rates) was poured into a 6-cm plastic Petri dish. Active fourth-stage juveniles and adults (20% and 80%, respectively) picked using a fine needle were released into the Petri dish containing the pesticide solutions under a stereoscopic microscope (Olympus SZH). Each pesticide treatment was replicated five times with each replicate having 15 to 30 individuals. Parafilm (American National CanTM Greenwich, CT 06836) was then used to wrap around the Petri dishes to avoid air contamination and evaporation of pesticides. The dishes were then placed in an incubator at 25.0 ± 1.0 °C in the dark. After 2 weeks treatment, all *H. diversa* individuals were transferred from dishes with pesticide solution into glass vials (diameter of 20 mm and a height of 50 mm) using a pasture pipette, and left for 1 h to have the *H. diversa* individual settle to the bottom of the glass vials. The pesticide solutions not containing the *H. diversa* individuals were emptied from the glass vial with a pasture pipette. Tap water was added to the glass vial to dilute pesticide and left for 1 h to allow the nematodes to settle to the bottom. This procedure was repeated twice to wash off the pesticide solutions thoroughly. Nematodes were then placed in an incubator at room temperature (25.0 ± 1.0 °C) for 24 h to let them recover. After 24 h, nematodes were touched by a fine needle to determine whether they were alive or dead under a microscope. Mortality rate was evaluated using Abbott's formula [11]. In addition, benfuracarb, cartap hydrochloride and cyanamide were further diluted to give different concentrations (Table 2) to determine their median lethal concentration dose (LC_{50}).

Table 1. Nematicides used and their mode of action and registered application rates in Japan.

Mode of Action [1]	IRACcode	Common Name of Active Substance (a.s.)	Formulation [2]	a.s. Percentage (%)	Registrated Concentration of a.s. (ha^{-1}) in Japan [3]	Maunufacturing Corporation [4]	Target Pest [5]
Carbamete	1A	Benfuracarb	G	8.0	4 g/5 L rice nursery soil [6]	OAT Agrio Co., Ltd.	Nematode
		Alanycarb	WP	40.0	1.5–1.6 kg	OAT Agrio Co., Ltd.	Lepidoptera
		Carbaryl	G	5.0	1.5−3.0 kg	Sanmei Chemical Co., Ltd.	Lepidoptera
		Methomyl	WP	45.0	0.7–2.7 kg	Corteva Agriscience	Lepidoptera
		Carbosulfan	G	3.0	1.5−9 kg	I.S.K Bioscience K. K.	Nematode
		Oxamyl	G	0.8	0.5−4.0 kg	Mitsui Chemicals Agro, Inc.	Nematode
Organophosphate	1B	Fenthion	G	5.0	1.0–2.0 kg	Bayer Cropscience K. K.	Coleoptera
		Fenitrothion	EC	40.0	1.0–1.5 kg	Sumitomo Chemical Co., Ltd.	Hemiptera
		Trichlorfon	EC	50.0	1.3–3.0 L	Nippon Soda Co., Ltd.	Lepidoptera
		Imicyafos	G	1.5	2.3–7.5 kg	Agro-Kanesho Co., Ltd.	Nematode
		Fosthiazate	G	1.5	1.5–4.5 kg	I.S.K Bioscience K. K.	Nematode
		Acephate	G	5.0	1.5–6.0 kg	Arysta LifeScience Corp.	Lepidoptera
		Isoxathion	MGF	3.0	1.8−2.7 kg	Nippon Soda Co., Ltd.	Hemiptera
Phenylpyrazole	2B	Fipronil	FL	5.0	0.05–0.08 L	BASF Japan Ltd.	Hemiptera
Pyrethroid	3A	Pyrethrins	EC	3.0	0.06–0.09 L	Dainihon Jochugiku Co.,Ltd.	Lepidoptera
		Etofenprox	G	1.5	0.3–1.4 kg	Mitsui Chemicals Agro, Inc.	Coleoptera
		Silafluofen	WP	20.0	0.3 kg	Bayer Cropscience K. K.	Hemiptera
Neonicotinoid	4A	Tiamethoxam	G	0.5	0.2−0.5 kg	Syngenta Japan K. K.	Hemiptera
		Imidacloprid	WP	10.0	0.2–0.3 kg	Bayer Cropscience K. K.	Hemiptera
		Dinotefuran	G	1.0	0.3−2.0 kg	Agro-Kanesho Co., Ltd.	Hemiptera
		Clothianidin	G	0.5	0.2–0.5 kg	Sumitomo Chemical Co., Ltd.	Hemiptera
		Thiacloprid	WP	30.0	0.2–0.5 kg	Bayer Cropscience K. K.	Hemiptera
		Nitenpyram	WP	10.0	0.1–0.2 kg	Kyoyu Agri Co., Ltd.	Hemiptera
		Acetamiprid	G	2.0	0.6–1.2 kg	Nihon Nohyaku Co., Ltd.	Hemiptera
Avermectin and milbemycin	6	Abamectin	EC	1.8	1.5–6.06 L	Syngenta Japan K. K.	Hemiptera
		Emamectin benzoate	EC	1.0	1.5–3.06 L	Syngenta Japan K. K.	Lepidoptera
		Milbemectin	EC	1.0	1.5–3.06 L	Mitsui Chemicals Agro, Inc.	Acariformes
Miscellaneous non -specific (Multi-site) inhibitor	8	Metam sodium	L	33.0	132.0–198.0 L	Nippon Soda Co., Ltd.	Nematode
		DD	EC	97.0	145.5–388.0 L	Agro-Kanesho Co., Ltd.	Nematode
		DCIP	G	30.0	90.0 kg	Sumitomo Chemical Co., Ltd.	Nematode
Nereistoxin	14	Cartap hydrochloride	WP	75.0	0.8–4.5 kg	Sumitomo Chemical Co., Ltd.	Nematode
		Tiocyclam	WP	50.0	0.8–1.5 kg	Mitsui Chemicals Agro, Inc.	Hemiptera
Tetronic and tetramic acid derivative	23	Spirotetramat	FL	22.4	0.2–1.3 L	Bayer Cropscience K. K.	Hemiptera
		Spiromesifen	FL	30.0	0.2–1.8 L	Bayer Cropscience K. K.	Acariformes
Uncategorized	-	Fluopyram	G	0.5	1.0 kg	Bayer Cropscience K. K.	Nematode
		Cyanamide	G,D	27.5	55.0–275.0 kg	Katakura & Co-op Agri Corp.	Nematode
		Morantel tartrate	L	12.5	13.8–162.5 ml/pine wood [6]	Nichino Ryokka Co., Ltd.	Nematode

[1.] Subgroups defined by the Insecticide Resistant Action Committee (IRAC). Uncategorized pesticides are specifically not defined by IRAC, [2] G: granule, WP: wettable powder, L: liquid formulation, EC: emulsifiable concentrate, FL: flowable, MGF: microgranule fine, D: dust formulation, [3] Registered rate during 2010 and 2011, when this study was performed. Benfuracarb and morantel tartrate registrations were not kg/ha exceptionally. [4] Some products are manufactured by multiple companies and the names listed here are of the major producing manufacturers. [5] Targeted agriculturally important pests by the specific pesticide. In the case where the pesticide was effective against multiple pests including the nematodes, the nematode was listed ahead. [6] Benfuracarb and morantel tartrate registrations were not registered in kg or L per ha.

Table 2. Nematicidal effect of pesticides on *Hirschmanniella diversa*.

Mode of Action [1]	Pesticide	Concentration of Pesticide (μg/mL)	Corrected Mortality (%) [2]
Carbamete	Benfuracarb	100	100
		20	98
		10	100
		5	49
		1	17
		0.1	2
	Alanycarb	100	100
		10	0
		1	0
	Carbaryl	100	88
		1	0
	Methomyl	100	77
		10	0
	Carbosulfan	10	100
	Oxamyl	100	83
Organophosphate	Fenthion	100	100
		10	100
		1	82
	Fenitrothion	100	100
		10	13
		1	5
	Trichlorfon	100	100
	Imicyafos	100	83
		10	69
		1	0
	Fosthiazate	100	71
		1	0
	Acephate	10	13
	Isoxathion	2.21	10
Phenylpyrazoles	Fipronil	22	2
Pyrethroid	Pyrethrins	60	52
	Etofenprox	0.15	0
	Silafluofen	100	0
Neonicotinoid	Tiamethoxam	0.1	16
	Imidacloprid	100	0
	Dinotefuran	0.2	0
	Clothianidin	0.1	0
	Thiacloprid	150	3
	Nitenpyram	100	0
	Acetamiprid	0.4	0
Avermectins and milbemycins	Abamectin	36	20
	Emamectin benzoate	5	69
	Milbemectin	10	40
Miscellaneous non -specific (Multisite) Inhibitor	Metam sodium	108	3
		72	2
		48	0
		24	0
	DD	38.8	2
		77.6	11
	DCIP	10	4
Nereistoxin	Cartap hydrochloride	1500	100
		750	100
		250	100
		50	81
		25	67
		8	8
	Tiocyclam	1,000	98
		500	100
Tetronic and tetramic acid derivative	Spirotetramat	11.2	17
	Spiromesifen	600	11
		150	5
Uncategorized	Fluopyram	0.2	4
	Cyanamide	4.5	100
		3.6	100
		2.25	98
		1.8	89
		0.9	39
		0.45	18
	Morantel tartrate	100	55
		10	0

[1] Subgroups defined by Insecticide Resistant Action Committee (IRAC). Uncategorized pesticides are specifically not defined by IRAC. [2] Corrected Mortality was calculated using Abbott's formula [11]. Nematodes were dipped in pesticide solution for two weeks (n = 5).

2.1.2. In Vitro Assay: Nematistatic Effect of Nematicides on H. diversa

Hirschmanniella diversa spends its entire life cycle in paddy fields [7], where irrigation is frequently done. It is possible that nematodes may resume activity as the nematicide concentrations are reduced, or they may move to sites in a paddy field where less or no nematicides are present. Therefore, the nematistatic effect of selected nematicides was examined. *H. diversa* were removed from cold storage as mentioned above and kept in room temperature for 30 min to recover activity. Agar (10 mL, 1%) containing different concentrations of nematicides was prepared in a 6-cm diameter Petri dish. Agar containing only distilled water was used as the control treatment. As with the nematicidal test described above, active fourth-stage juveniles or adult *H. diversa* individuals (about 50 individuals. The ratio of juveniles and adults was similar to the nematicidal effect test) were picked and released into the center of the Petri dish (marked X in Figure 1). The Petri dishes were wrapped with Parafilm and placed in the incubator under optimal conditions (25.0 ± 1.0 °C). Five replicates were performed for each treatment.

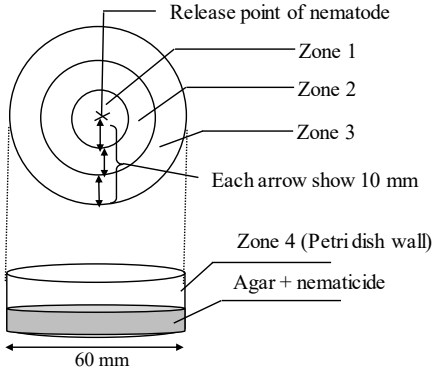

Figure 1. In vitro agar plate chemotaxis assay showing the separation of zones to determine the nematistatic effect of test compounds on *Hirschmanniella diversa*. Top view (upper) and side view (lower) of a 60-mm diameter Petri dish.

The agar medium in the Petri dish was divided into four zones as shown in Figure 1. After 36 h, the numbers of *H. diversa* individuals in each of the four zones were counted under the stereoscopic microscope and the ratio for the number of *H. diversa* individuals in each zone was calculated. In addition, to determine whether nematodes treated with pesticides would recuperate, nematodes in the agar plates of zones 1 to 3 were picked with a fine needle and transferred to distilled water and incubated in laboratory conditions (25.0 ± 1.0 °C). *Hirschmanniella diversa* individuals in zone 4 were all dead for desiccation, therefore were not picked for further experimentation. After 24 h, the nematodes were evaluated if they were alive by touching nematodes with a fine needle.

2.2. Field Assay: Effect of Benfuracarb on H. diversa Under a Micro-Field Condition

Field studies were conducted for five years; in 2012 and from 2014 to 2017. From the results of the in vitro assays, benfuracarb was selected for further tests in micro-field conditions. Containers with a volume of 1.98 m^3 (1.62 m width × 1.02 m length × 1.20 m height) were used to simulate lotus paddy field conditions. The containers were filled to about 0.7 m in height with silty clay soil collected from a water channel near the lotus paddy field in Tamuramati township, Tsuchiura city, Ibaraki Prefecture, Japan (36°04′ N, 140°14 E). Residues of lotus and soil infected with *H. diversa* (300–400 individuals/20 g lotus root) were also collected from the same field as mentioned in 2.1 and added at 30 kg/container on 8 May 2012 and 24 May 2012.

In the 2012 study, benfuracarb (5% granule) were applied at rates of 10 kg/ha and 20 kg/ha. Fosthiazate (1.5% granule) was used as a reference (positive control), since fosthiazate is the most commonly used nematicide on other crops in Japan. Fosthiazate was applied at a rate of 6 kg/ha and

untreated containers were prepared as the control treatment (negative control). Benfuracarb 10 kg/ha, 20 kg/ha and fosthiazate 6 kg/ha were prepared at a rate of 16.5 g, 33.0 g and 9.9 g per container (per 1.65 m^2 area), respectively. The nematicides were then added to the containers on 31 May 2012 and thoroughly mixed by hand. Six to eight *H. diversa* free lotus tubers 'Fukudaruma' were planted in the containers. Water levels from the surface to the filled soil were kept at a dept of 10 to15 cm and refilled whenever the water levels depreciated. After one month, more than 15 g of roots were uprooted from three points in one container and the nematodes were extracted from 5 g of root in triplicates using the Baermann funnel method (25 °C, 48 h). Sampling of roots was conducted on 7 June, 28 June, 12 July, 15 August, 11 September and 12 October 2012. On 12 October, three to five root systems with more than three tubers were harvested per container and the damage index (see Figure 2 for schematic representation) was assessed per tuber based on the damage level as indexed from 0 to 4: 0: Not damaged; 1: slightly damaged with little small black spots caused by *H. diversa* (less than 3 mm in diameter) on the tuber surface but having no effect on the commercial value; 2: many small black spots but having no effect on the commercial value; 3: many small and large black spots (more than 3 mm in diameter) with deformed tuber surfaces resulting in 20% reduction in marketable quality and 4: critically damaged tubers, highly deformed with many big black spots, thus >95% reduction in marketable quality. The damage index was calculated using the formula: $(A \times 0 + B \times 1 + C \times 2 + D \times 3 + E \times 4)/(4 \times N) \times 100$, where A, B, C, and D were the number of tubers with damage levels of 0, 1, 2, 3, and 4, respectively.

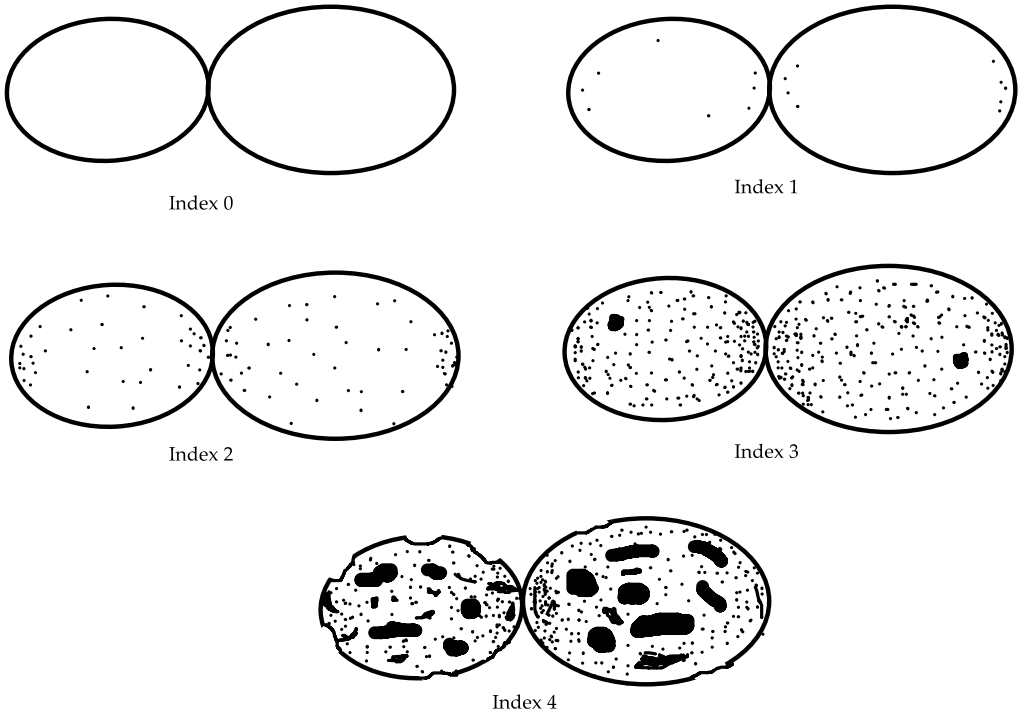

Figure 2. Schematic representation of the lotus tuber damaged by *Hirschmanniella diversa* as indexed from 0 to 4. 0: Not damaged, 1: Slightly damaged, little small black spots by *H. diversa* (less than 3 mm in diameter) on the tuber surface. No effect on the commercial value, 2: Many small black spots. Decreased commercial value, 3: Many small and big black spots (more than 3 mm in diameter). Deformed tuber surfaces, 20% reduction in marketable quality. 4: Critical damage to tubers. Highly deformed, many big black spots, >95% reduction in marketable quality.

To confirm the field data in the 2012 study, field studies were repeated at the optimized dosage of benfuracarb (8% granule) at the field rate of 12 kg/ha (19.8 g per container, 1.65 m^2 area) during 2014 to 2017. The benfuracarb formulation was changed from 5% to 8% because the formulation content was reduced and 8% granules sink easily in paddy fields due to their high specific gravity. The benfuracarb

application, lotus planting and assessment of lotus damage index were conducted on 12 June, 12 June and 15 October of 2014, 8 May, 8 May and 29 September of 2015, 20 June, 20 June and 20 September of 2016, 25 May, 25 May and 5 October of 2017, respectively. The soil in the containers were not renewed throughout the study period, but the containers were randomly rotated with each of the treatments conducted every year to average the initial nematode density.

2.3. Statistical Analysis

All statistical analyses were performed using EZR [12], which is a graphical user interface for R [13] Ver. 3.6.1 (The R Foundation for Statistical Computing, Vienna, Austria, Ver. 3.6.1).

3. Results

3.1. In Vitro Assays

3.1.1. Nematicidal Effect on H. diversa

Pesticides belonging to phenylpyrazole, pyrethroid, neonicotinoid, avermectin and milbemycin, tetronic and tetramic acid derivatives and multisite inhibitor showed little or no nematicidal effect (less than 70% mortality at any concentration) on *H. diversa* (Table 2). On the other hand, most pesticides that were categorized as carbamate, organophosphates and nereistoxin showed high *H. diversa* mortality: carbamates benfuracarb (10 to 100 μg/mL a.s.), alanycarb (100 μg/mL a.s.), carbryl (100 μg/mL a.s.), carbosulfan (10 μg/mL a.s.) and oxyamyl (100 μg/mL a.s.), organophosphates fenthion (1–100 μg/mL a.s.), fenitrothion (100 μg/mL a.s.), trichlorfon (100 μg/mL a.s.) and imicyafos (100 μg/mL a.s.), nereistoxins cartap hydrochloride (50 to 1500 μg/mL a.s.) and tiocyclam (500–1000 μg/mL a.s.), and cyanamide (1.8 to 4.5 μg/mL a.s.) showed mortality ranging from 80% to 100%. The LC_{50} values of benfuracarb, cartap hydrochloride and cyanamide were 5.46 μg/mL, 20.8 μg/mL and cyanamide at 0.89 μg/mL, respectively (Probit analysis, $p < 0.05$).

3.1.2. Nematistatic Effect on H. diversa

More than 90% of *H. diversa* individuals stayed at the release point, zone 1, in the tests on selected nematicides, except for cartap hydrochloride, at the higher to middle concentrations (Table 3). Even in the lowest concentration, above 50% of *H. diversa* stayed at zone 1 in these nematicides and the ratios were significantly higher than those in the control treatment ($p < 0.05$; Tukey HSD test).

When the nematodes were removed from the agar plates containing nematicides and placed in water, >85% of *H. diversa* resumed their activity in oxyamil and cadusafos at all the dosages (Table 4). In cartap hydrochloride, fosthiazate and imicyafos, nematodes resumed their activity depending on the dosages, e.g., >15% of *H. diversa* resumed their activity at 25 to 60 μg/mL, although >98% of *H. diversa* did not at >600 μg/mL. On the other hand, all the nematodes collected from 10 μg/mL and 100 μg/mL showed no recovery in activity in benfuracarb, although individuals collected from 1 μg/mL resumed their activity. These results indicated that benfracarb had both nematistatic and nematicidal effects.

Table 3. Nematistatic effect of nematicides on *Hirschmanniella diversa* in agar plate.

Nematicide	Concentration of Active Substance (μg/mL)	Total No.of Tested *H. diversa*	Abundunce Ratio of *H. diversa* (%) [1]			
			Zone 1 ± SD [2]	Zone 2 ± SD	Zone 3 ± SD	Zone 4 ± SD
Benfuracarb	100	230	100.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
	10	227	99.5 ± 1.0 a	0.5 ± 1.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
	1	272	74.3 ± 6.8 b	11.5 ± 3.6 b	12.3 ± 3.7 b	1.9 ± 2.7 a
Control	-	329	14.3 ± 10.9 c	19.8 ± 7.5 c	0.0 ± 0.0 a	66.0 ± 17.1 b
Cartaphydrochloride	2500	358	99.3 ± 1.0 a	0.7 ± 1.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
	250	223	63.5 ± 10.2 b	20.7 ± 6.2 b	15.7 ± 9.6 b	0.0 ± 0.0 a
	25	255	24.8 ± 6.2 c	18.8 ± 6.3 b	18.6 ± 7.4 b	37.8 ± 15.3 b
Control	-	329	14.3 ± 10.9 c	19.8 ±7.5 b	0.0 ± 0.0 a	66.0 ± 17.1 c
Fosthiazate	600	249	100.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
	60	239	92.7 ± 3.6 a	3.4 ± 2.5 a	3.8 ± 1.9 b	0.0 ± 0.0 a
	6	215	66.5 ± 6.9 b	22.4 ± 6.9 b	9.6 ± 1.7 c	1.5 ± 1.1 a
Control	-	329	14.3 ± 10.9 c	19.8 ± 7.5 b	0.0 ± 0.0 a	66.0 ± 17.1 b
Oxamyl	100	253	100.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
	10	221	99.1 ± 1.2 a	0.9 ± 1.2 a	0.0 ± 0.0 a	0.0 ±0.0 a
	1	186	85.6 ± 4.8 b	12.2 ± 5.4 b	1.1 ± 1.5 b	1.0 ± 2.3 a
Control	-	245	10.2 ± 6.9 c	3.9 ± 3.6 a	4.1 ± 2.8 b	81.8 ± 8.8 b
Cadusafos	100	186	97.0 ± 3.2 a	3.0 ± 3.2 a	0.0 ± 0.0 a	0.0 ± 0.0 a
	10	196	90.5 ± 7.5 a	8.4 ± 5.6 a	1.1 ± 2.4 a	0.0 ± 0.0 a
	1	192	55.4 ± 9.3 b	28.3 ± 11.8 b	13.2 ± 7.4 b	3.1 ± 2.0 a
Control	-	245	10.2 ± 6.9 c	3.9 ± 3.6 a	4.1 ± 2.8 a	81.8 ± 8.8 b
Imicyafos	3000	236	100.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
	600	217	99.4 ± 1.3 a	0.6 ± 1.3 a	0.0 ± 0.0 a	0.0 ± 0.0 a
	60	220	96.5 ± 5.5 ab	0.0 ± 0.0 a	0.9 ± 1.2 ab	2.6 ± 5.8 a
	6	229	91.9 ± 3.8 b	3.8 ± 1.5 b	4.3 ± 4.6 b	0.0 ±0.0 a
Control	-	236	5.6 ± 4.1 c	6.9 ± 2.9 b	4.6 ± 3.3 b	82.8 ± 4.7 b

[1] Individuals of *H. diversa* were released onto agar medium with difference concentrations of nematicide and abundance ratio was evaluated after 48 h. Abundance ratio = numbers of *H. diversa* found in each zone to total numbers of released *H. diversa*. [2] Each zone designed in the plastic plate is described at Figure 1. Different letters within the same column and the same nematicide tests including control indicate significant differences (n = 5) (Tukey HSD test; $p < 0.05$).

Table 4. Number of *Hirschmanniella diversa* individuals resuming activities in distilled water after treatment with different concentrations of nematicides.

Nematicide	Concentration of a.s. (μg/ml)	Total Number of Tested *H. divesa* (individuals)	Number of Individuals that Resumed Their Activity ± SD (%) [1]
Benfuracarb	100	200	0.0 ± 0.0 a
	10	200	0.0 ± 0.0 a
	1	139	97.9 ± 2.3 b
Cartaphydrochloride	2500	159	0.0 ± 0.0 a
	250	106	22.4 ± 11.2 b
	25	67	83.9 ± 15.5 c
Fosthiazate	600	216	1.0 ± 1.4 a
	60	150	73.2 ± 3.8 b
	6	118	100.0 ± 0.0 c
Oxyamil	100	111	96.5 ± 3.5 a
	10	140	99.0 ± 2.3 a
	1	121	100.0 ± 0.0 b
Cadusafos	100	130	88.3 ± 7.6 a
	10	159	90.5 ± 4.4 a
	1	110	100.0 ± 0.0 b
Imisyafos	3000	207	1.6 ± 2.3 a
	600	214	4.3 ± 2.6 a
	60	203	15.9 ± 12.7 a
	6	184	85.9 ± 11.5 b

[1] (%) = number of individuals resumed activity to total number of tested *H. diversa*. Different letters in the same nematicides indicate significant differences (n = 5) (Tukey HSD test; $p < 0.05$).

3.2. Field Assays

3.2.1. Effect of Benfuracarb on H. diversa under Micro-Field Conditions

The density of *H. diversa* in lotus roots followed a pattern peaking around mid-August in the control treatment. This pattern was the same as that observed in conventional lotus cultivation [7]. Population dynamics in the fosthiazate treatment resembled that in the control plot and there were no significant differences between the two treatments during the study period, except for 12 July and 12 October. Benfracab application at rates of 20 kg/ha and 10 kg/ha significantly reduced the population of *H. diversa* compared to the control treatment (Figure 3) ($p < 0.05$; Tukey HSD test). Furthermore, benfuracarb and fosthiazate applications showed no phytotoxicity effects to the lotus plants (data not shown).

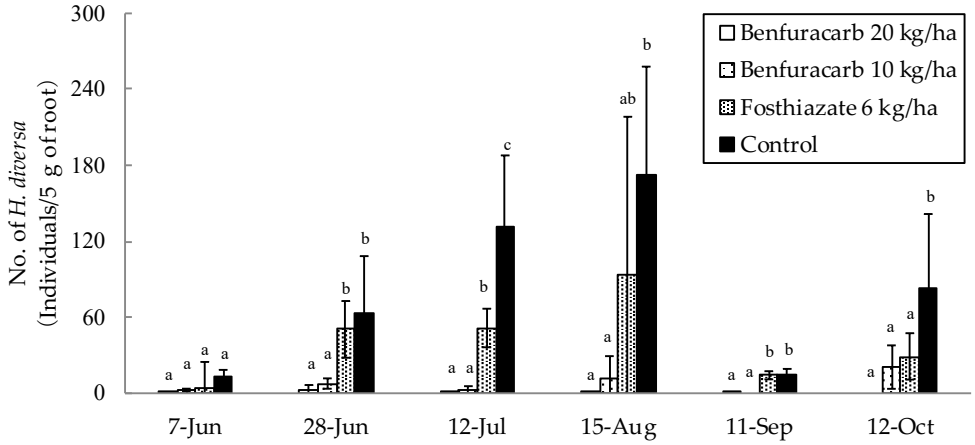

Figure 3. Population dynamics of *Hirschmanniella diversa* in the lotus root after treatment with different nematicides in the 2012 micro-field experiment. Bars indicate standard deviation ($n = 3$). Different letters in the same days indicate significant difference (Tukey HSD test; $p < 0.05$).

The damage index was significantly lower in the treatment with benfuracarb at rates of 20 kg/ha and 10 kg/ha compared to that in the control treatment (Figure 4) ($p < 0.05$; Kruskall–Wallis test). By contrast, no significant difference was observed in damage level between the fosthiazate and control treatments (Figure 4).

Table 5. Effect of benfuracarb on damage to lotus tubers caused by *Hirschmanniella diversa* in micro-field experiments.

Year	Nematicide [1]	No. of Investigated Lotus Tubers	No. of Tubers in Each Damage Index [2]					Damage Index [3]
			0	1	2	3	4	
2014	Benfuracarb	42	5	22	12	3	0	32.7a
	Control	123	0	15	93	15	0	50.0b
2015	Benfuracarb	28	13	11	3	1	0	17.9a
	Control	30	1	8	8	13	0	52.5b
2016	Benfuracarb	37	31	6	0	0	0	4.1a
	Control	36	17	8	11	0	0	20.8b
2017	Benfuracarb	55	34	15	6	0	0	12.3a
	Control	61	11	17	18	14	1	40.6b

[1] Benfuracarb applied amount was 12 kg/ha in each year ($n = 3$). [2] Damage index: 0: Not damaged, 1: Slightly damaged, little small black spots by *H. diversa* (less than 3 mm in diameter) on the tuber surface. No effect on the commercial value, 2: Many small black spots. Decreased commercial value, 3: Many small and big black spots (more than 3 mm in diameter). Deformed tuber surfaces, 20% reduction in marketable quality. 4: Critical damage to tubers. Highly deformed, many big black spots, >95% reduction in marketable quality. [3] $(A \times 0 + B \times 1 + C \times 2 + D \times 3 + E \times 4)/(4 \times N) \times 100$. A, B, C, and D: No. of tubers with the damage level 0, 1, 2, 3, and 4, respectively. Different letters after damage index in the same years indicate significant difference (Mann–Whitney U test; $p < 0.05$).

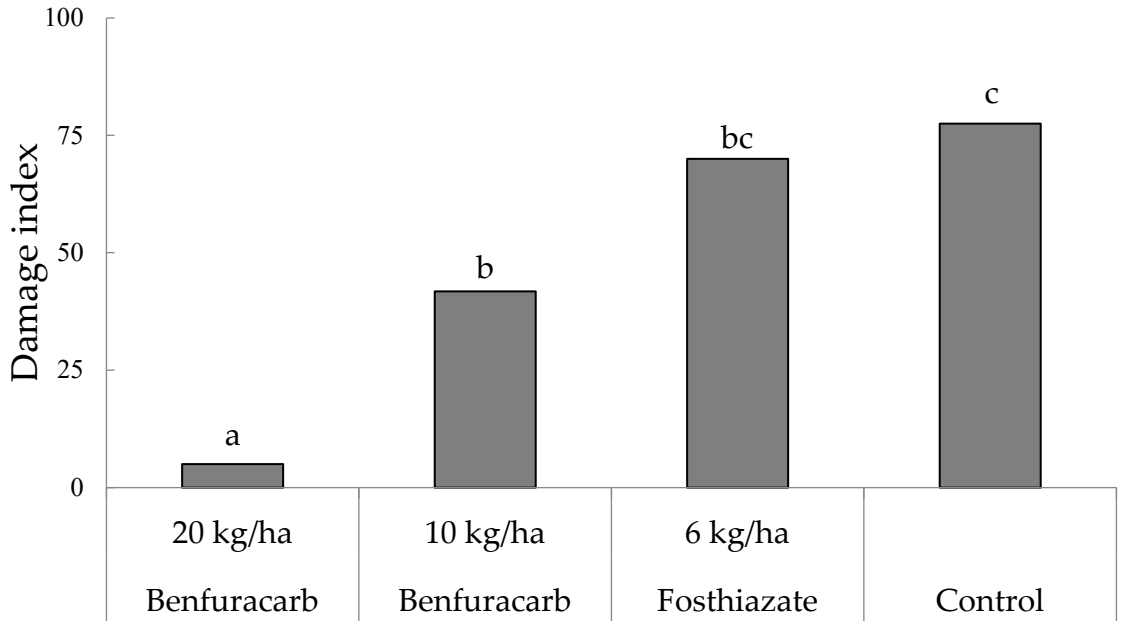

Figure 4. Effect of two nematicides, benfuracarb at 10 and 20 kg ha^{-1} and fosthiazate at 6 kg ha^{-1}, on damage to lotus tubers caused by *Hirschmanniella diversa* in the 2012 micro-field experiment (n = 3). The damage index was evaluated based on Table 5. Different letters indicate significant differences among the treatments (Kruskall–Wallis test; $p < 0.05$).

3.2.2. Effect of Benfuracarb on *H. diversa* in Consecutive Annual Studies

The damage index of lotus tubers was consistently lower in benfuracarb treatment by 17% to 32% than in the control treatment and there were significant differences in the damage index in every year (Table 5) ($p < 0.05$; Mann–Whitney U test). In 2016 and 2017, relatively high numbers of uninfected tubers were observed as a result of benfuracarb applications yielding a gradual decrease in the *H. diversa* population.

4. Discussion

Rice root nematode *Hirschmanniella oryzae* is of the same genus as that of *H. diversa* and is a major root pest of rice [14]. *Hirschmanniella oryzae* and *H. diversa* inhabit waterlogged soil conditions (paddy fields) during their whole life cycles and parasite wetland crops such as rice and lotus, respectively. Their ecology and lifecycle have been reported to be similar in nature [7]. Previous studies have revealed that nematicide application of carbamate improved the control against *H. oryzae* [14–16]. There are, however, few to no studies so far documenting the surveying of nematicides that could be effective in management of *H. diversa*. Therefore, the prospect of chemical control against *H. diversa* under paddy field conditions was examined mainly using non-fumigant nematicides, including pyrethroid and neonicotinoid, that have not been tested previously as nematicides. In addition, fumigant nematicides were also tested against *H. diversa*, although they are not feasible for use in paddy fields. In the in vitro tests on the nematicidal effect, some pesticides showed mortality of >70% at near the practical registered concentrations ranging from 1 to 10 µg/mL, while nereistoxin showed such high mortality only at concentrations greater than 50 µg/mL. Pesticides that showed high mortality were categorized into carbamate, organophosphate, and some other pesticides in which their mode of action (MoA) is still not defined, depending on the MoA classification [17]. Abamectin, an avermectin and milbemycin pesticide showed low mortality (20%) at a high concentration (36 µg/mL), in contrast to the low LC_{50} values of 1.56 µg/mL and 32.9 µg/mL reported for *Meloidoyne incognita* and *Rotylenchulus reniformis*, respectively [18]. Even though abamectin is a registered nematicide in some countries [19], it proved to be not effective against *H. diversa*. *Meloidoyne incognita* and *R. reniformis* were exposed to pesticides for 24 h whereas in our study, *H. diversa* were exposed for two weeks. Differences in exposer periods

therefore disqualifies the use of LC_{50} values to compare the effectiveness of pesticides between the nematode species. However, given the environment in which *H. diversa* subsists (paddy fields) implies that 24 h of exposure is inadequate to determine the effectiveness of a pesticide. Even then, longer exposure (2 weeks as shown in our study) eliminates any underestimations or overestimations in determining a lethal dose for a particular pesticide. Thus, the LC_{50} value for *H. diversa* may not be underestimated compared with the LC_{50} values for *M. inocognita* and *R. reniformis*. Furthermore, oxyamyl, carbamate, imicyafos, fosthiazate and both organophosphates, are registered in many countries against major nematodes, such as *Meloidogyne* spp. and *Pratylenchus* spp., and the fosthiazate LC_{90} value for *Meloidogyne* spp. and *Pratylenchus* spp. was as low as 0.5–1.0 µg/mL [20]. However, mortality to *H. diversa* in the three nematicides ranged between 71% and 83% even at an extremely high dosage (100 µg/mL), suggesting that *H. diversa* may be resistant to the above nematicides.

Metam sodium and DD, which are classed as multisite inhibitors, showed lower mortality even though these two compounds are fumigants. Spirotetramat also showed lower mortality. This is because it is known to be effective in inhibiting nematode hatch and is effective after changing to the spirotetramat-enol form in plant tissue [21,22]. Thus, alternative tests are needed for evaluation of these chemicals.

Fenthion gave a mortality greater than 80% even at 1 µg/mL, which can be effective in *H. diversa* control in the field. It used to be registered for pest control for rice paddy fields, but due to the high toxicity to aquatic invertebrates and birds [23], its registration was withdrawn in 2011 in Japan, when the in vitro assays were completed. Thus, fenthion was not used in subsequent experiments despite its high mortality against *H. diversa*.

The cultivation period for lotus is long and lotus tubers are harvested 150–300 days after transplanting. Since the timing of nematicide application is only before transplanting, it is important to evaluate whether nematicide-treated nematodes can recover and resume activity. To examine this aspect, nematistatic effect was evaluated. Benfuracarb and cartap hydrochloride were selected based on their high nematicidal effect at low concentrations against *H. diversa*. In addition, they are already registered in rice paddy fields against pests, including the white tip nematode *Aphelenchoides besseyi*, and registration for use with lotus is likely to be more straightforward compared with other pesticides. Oxamyl, cadusafos, fosthiazate and imicyafos were tested because they are major nematicides used in Japan. The results showed that cartap hydrochloride had the lowest nematistatic effect than the other tested pesticides, although its nematicidal effect was high. After exposure to oxamyl, cadusafos and fosthiazate, *H. diversa* resumed its activity soon after the removal of the nematicides, suggesting that the usage of these pesticides against *H. diversa* in lotus paddy fields may not be practical. Cartap hydrochloride and imicyafos showed promising trends; however, their nematistatic effects were far less than befuracarb.

Based on the LC_{50} values, which were estimated based on data in Table 2, the amounts of chemicals for field usage were calculated with a conjecture that as the active substances reach the water and soil depth of 30 cm, the active substance dissolves and spreads thus; the formulation amounts for benfuracarb granule (a.s. 8%), cartap hydrochloride wettable powder (a.s. 75%) and cyanamide granule (a.s. 27.5%) was calculated and prepared for application as 301 kg/ha, 84 kg/ha and 1000 kg/ha, respectively. Since other formulations of cartap hydrochloride granule (a.s 4.4%), instead of 75% wettable powder is used in rice paddy fields, application of 1565 kg/ha of cartap hydrochloride granule would be needed to achieve the same a.s. concentration. The application at this rate in actual field conditions would be labor intensive and expensive, in addition to its registration. Thus, the use of cartap hydrochloride to lotus fields is not feasible.

Lime nitrogen (a.s. cyanamide) is an important nematicide of *H. diversa*. However, in Japan, when 1000 kg/ha lime nitrogen, the maximum registered amount, is applied to a lotus paddy field, the predicted concentration of cyanamide is 400 µg/mL and this value is near the water pollution Predicted Environmental Concentration (PEC), of 670 µg/mL [24]. Consequently, it is impossible to register cyanamide as a nematicide in lotus paddy fields.

Benfuracarb showed promising trends in the in vitro assays and now needs further tests to establish its role against *H. diversa* in the field. In the micro-field experiments, benfuracarb showed significant nematicidal effects, since the number of nematodes and the degree of damage were consistently reduced over the five years of study. Therefore, benfuracarb is practical and realistic for use in *H. diversa* control under paddy field conditions. In the 2012 field experiment, benfuracarb was applied at a rate of 10 kg/ha or 20 kg/ha for control of *H. diversa*. In the 2014 to 2017 field experiments, benfuracarb was applied at a rate of 12 kg/ha and still gave effective nematicidal and nematistatic effects. In future work, we plan to conduct tests under actual field conditions where floodwater level fluctuations will be taken into consideration. Examination of a possible synergy effect with cyanamide and benfuracarb is also planned.

Author Contributions: M.T. conceptualized; M.T., M.G. and M.S. conducted the experiments and collected field data; M.T. wrote the paper; M.T., D.W., R.N.P. and K.T. reviewed, edited, and finalized the paper. All authors have read and agreed to the published version of the manuscript.

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