[SHORT COMMUNICATION]

Inhibitory effect of several insecticides on *in vitro* propagation of the pinewood nematode, *Bursaphelenchus xylophilus*

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Pine wilt disease is caused by infestation of the pinewood nematode, *Bursaphelenchus xylophilus* (Steiner and Buhner) Nickle (Mamiya, 1986). One of control methods for the disease is prophylactic injection of a nematicide into the trunk of pines. Thus gamma-aminobutyric acid (GABA)-receptor agonists such as emamectin benzoate, milbemectine and nemadectin, muscle activity blockers such as morantel tartrate salt and levamisol hydrochloride, and acetylcholine esterase (AChE) inhibitor such as mesulfenfos are used for the trunk-injection (Takai et al., 2000). Because of particular goods and/or deleterious substances, these compounds are not available at common gardening shops. Fenitrothion (Sumithion®), one of popular insecticides sold at

common gardening shops, has a nematicidal activity against the white tip nematode, *Aphelenchoides besseyi* Christie (Lee, 1972). Fenitrothion showed a lethal effect on the pinewood nematode populations when used to treat pine tree in the nursery. These pines were first injected with fenitrothion and then inoculated with the nematodes (Matsuura, 1976; Matsuura and Kobayashi, 1976). Therefore, compounds lethal on the pinewood nematode may be available from among the popular insecticides used in home gardening. Compounds with potential nematicidal activity were investigated in this paper as candidates for low cost and easily obtainable trunk-injection agents.

MATERIALS AND METHODS

Nematode:

The pinewood nematode (Ka-4 strain) isolated from dead *Pinus densiflora* Sieb. and Zucc. in Kasama City, Ibaraki Prefecture, 1994 (Kiyohara, T., pers. Comm.) was used.

Test compounds:

Six organophosphorus insecticides, an organochlorine acaricide, an organophosphorus nematicide and a tetrahydropyrimidine nematicide were tested (Table 1).

Table 1. Test compounds.

Compound	Source (Trading name)	Content	Trading company
Acephate ¹	Orutoran ⁵	15%	Takeda Gardening Co., Japan
Chlorpyrifosmethyl ¹	Reldan	40%	Riken Green Co., Japan
Dicofol ²	Kelthane	40%	Takeda Gardening Co., Japan
Fenitrothion ¹	Sumithion	50%	Sumitomo Chemical Co., Japan
Malathion ¹	Malathon	50%	Takeda Gardening Co., Japan
Pirimiphosmethyl ¹	Actellic	45%	Takeda Gardening Co., Japan
Pyridaphenthion ¹	Ofunack	40%	Yashima Sangyo Co., Japan
Mesulfenfos ³	Nemanorn	50%	Nihon Bayer Agrochem Co., Japan
Morantel tartrate salt ⁴ (reagent)		ca. 100%	Sigma Chemical Co., USA

¹ organophosphorus insecticide, ² organochlorine acaricide, ³ organophosphorus nematicide, ⁴ tetrahydropyrimidine nematicide, ⁵ Orthene in English

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In vitro assay:

Four ml of medium composed of yeast extract 0.5%, potato extract (Difco, Co., USA) 0.4% and glucose (Wako Chemical Co., Japan) 2% in distilled water (DW) was poured into culture bottle (3×4 cm base $\times$ height 2 cm). The culture bottles were plugged with foamed silicon plugs and autoclaved at 120°C for 15 min and cooled at room temperature. Concentrations of Orutoran®, Reldan®, Kelthane®, Sumithion®, Malathon®, Actellic®, Ofunack® and Nemanorn® were adjusted for use by dilution with sterilized DW. Morantel tartrate salt was solved in sterilized DW. An aliquot of the solution (2 – 128 μ l) was pipetted into the autoclaved medium and then the culture bottles were shaken for 1 min. to make medium supplemented with acephate, chlorpyrifosmethyl, dicofol, fenitrothion, malathion, pirimiphosmethyl, pyridaphenthion, mesulfenfos or morantel tartrate salt at approximate 0.25 – 32.00 ppm. A small piece of potato dextrose agar (ca. $4 \times 4 \times 4$ mm), on which *Botrytis cinerea* Persoon had grown, and 20 μ l of the pinewood nematode suspension containing approximate 1,000 pinewood nematodes (mixture of juveniles and adults) were inoculated onto the medium. The culture bottles were kept at 25°C for 10 days. Then nematodes were isolated using Baermann funnel at 25°C for 48 hr and diluted for counting with DW. The final dilution volume was either 2, 5 or 10 ml. The numbers of nematodes in each of four 30 μ l samples of the suspension was counted and the total number of the nematodes growing on the experimental and control cultures was estimated. Each experiment for each compound and for each control was replicated three times.

Analysis of variance (ANOVA) and Tukey HSD multiple comparison were performed using SYSTAT (SYSTAT Inc., USA). Regression equations of nematode numbers were calculated using KALEIDA GRAPH (SYNERGY SOFT-

WARE Inc., USA).

RESULTS AND DISCUSSION

Average number of nematodes after 10 days culture (number of 10 day-nematodes) at 32 ppm was 43,926.0 for acephate, 0.0 for chlorpyrifosmethyl, 18,448.0 for dicofol, 0.0 for fenitrothion, 3,103.3 for malathion, 0.0 for pirimiphosmethyl, 0.0 for pyridaphenthion, 3.3 for mesulfenfos and 21.3 for morantel tartrate salt. Average number of 10 day-nematodes in control was 66,340.7. Solvents of the gardening insecticides and acaricide used here were composed of surfactants and organic solvents. Low concentration, for example 32 ppm, of these substances may not have any lethal effect against the pinewood nematode. *B. cinerea* grew in all cultures. This result, therefore, indicates that not only trunk-injection agents such as mesulfenfos and morantel tartrate salt but also several insecticides such as chlorpyrifosmethyl, fenitrothion, pirimiphosmethyl and pyridaphenthion show nematocidal activity at concentrations at least as low as 32 ppm.

The average numbers of 10 day-nematodes present in media supplemented with chlorpyrifosmethyl, fenitrothion, pirimiphosmethyl, pyridaphenthion, mesulfenfos or morantel tartrate salt at 0.25 – 16.0 ppm is shown in Table 2. There were compound-dependent differences at 0.25 – 2.0 ppm (ANOVA: factor, compound including control; df=6, 14; $F=19.7$; $p < 0.001$ at 0.25 ppm: df=6,14; $F=69.1$; $p < 0.001$ at 0.5 ppm: df=6,14; $F=81.8$; $p < 0.001$ at 1.0 ppm: df=6,14; $F=81.8$; $p < 0.001$ at 2.0 ppm). Numbers of 10 day-nematodes in media with chlorpyrifosmethyl, fenitrothion, pirimiphosmethyl or pyridaphenthion at 0.25 and 0.5 ppm were significantly fewer than that in media with mesulfenfos or morantel tartrate salt (Tukey HSD, $p < 0.05$). There were also compound dependent differences at 4.0 ppm (ANOVA: factor, com-

Table 2. Effect on in vitro propagation of *Bursaphelenchus xylophilus* of insecticides used in gardening and of trunk-injection nematicides.

Test compound	Average number of nematodes after 10 days cultures (n=3)						
	ppm						
	0.25	0.5	1.0	2.0	4.0	8.0	16.0
Chlorpyrifosmethyl	636.0a	75.3a	13.3a	7.7a	2.3a	1.0a	0.0a
Fenitrothion	4,333.3a	630.3a	35.3a	2.0a	0.0a	0.0a	0.0a
Pirimiphosmethyl	8,400.0a	1,059.0a	204.7a	64.7a	2.0a	0.0a	0.0a
Pyridaphenthion	257.3a	30.3a	13.3a	8.7a	1.7a	0.3a	0.0a
Mesulfenfos	37,416.7b	14,049.7b	549.0ab	313.7a	75.0b	56.3a	35.3a
Morantel tartrate salt	43,208.3bc	16,446.0c	7,541.7b	3,633.3a	764.7b	192.7b	205.0b
Control	68,291.7c	68,291.7c	68,291.7c	68,291.7b	*	*	*

Different letters in the same vertical column show significant difference at $p < 0.05$ (Tukey HSD).

* Same as controls for 0.25 – 2.0 ppm

Table 3. Regression equations of *Bursaphelenchus xylophilus* number when the nematodes were cultured for 10 days in the medium supplemented with test compound (x , ppm; y , number of nematodes).

Test compound	Ppm used for calculation (n=3 at each ppm)	Exponential equation	r	x (ppm)* at $y=1,000$
Chlorpyrifosmethyl	0.25, 0.5, 1.0	$y = 1553 \times e^{(-5.0715 x)}$	-0.97564	0.09
Fenitrothion	0.25, 0.5, 1.0	$y = 17724 \times e^{(-6.3720 x)}$	-0.89261	0.45
Pirimiphosmethyl	0.25, 0.5, 1.0	$y = 18002 \times e^{(-4.6853 x)}$	-0.91736	0.62
Pyridaphenthion	0.25, 0.5, 1.0	$y = 359 \times e^{(-3.5351 x)}$	-0.84507	<0.25**
Mesulfenfos	0.5, 1.0, 2.0	$y = 17372 \times e^{(-2.2301 x)}$	-0.93283	1.28
Morantel tartrate salt	1.0, 2.0, 4.0	$y = 15140 \times e^{(-0.7508 x)}$	-0.89368	3.58

* Estimated using regression equations except to pyridaphenthion.

** Calculated numerical value is -0.3 since nematode numbers at 0.25 ppm were very small.

pound; $df=5, 12$; $F=7217.8$; $p < 0.001$). Numbers of 10-day nematodes in media with chlorpyrifosmethyl, fenitrothion, pirimiphosmethyl or pyridaphenthion were significantly fewer than that in media with mesulfenfos or morantel tartrate salt (Tukey HSD, $p < 0.05$). These results indicate that inhibitory effect on the in vitro nematode propagation of chlorpyrifosmethyl, fenitrothion, pirimiphosmethyl or pyridaphenthion are stronger than that of mesulfenfos or morantel tartrate salt.

Depending on increase in concentration of the individual compound, the number of 10 day-nematodes decreased exponentially until the number was closer to zero (Table 3). Estimated concentrations, at which number of the 10 day-

nematodes was the same as the number of inoculated nematodes (1,000), are approximate 0.09, 0.45, 0.62, <0.25, 1.28 and 3.58 ppm for chlorpyrifosmethyl, fenitrothion, pirimiphosmethyl, pyridaphenthion, mesulfenfos and morantel tartrate salt, respectively. Nematicidal activity in each compound should occur at a concentration higher than each concentration effective *in vitro*. Consequently, chlorpyrifosmethyl, fenitrothion, pirimiphosmethyl and pyridaphenthion are candidates for trunk-injection agent against the pinewood nematode. Assay using pine tree must be conducted as the next step.

Reldan® (chlorpyrifosmethyl emulsion), Sumithion® (fenitrothion emulsion), Ofunack® (pyridaphenthion emulsion) and Green T-7.5

emulsion® (prothiofos emulsion) (Izutsuya chemical Industry Co., Japan) are used as prophylactic insecticide against the Japanese pine sawyer, *Monochamus alternatus* Hope, a vector of the pinewood nematode. Prothiofos also has lethal effect on the pinewood nematode (Takai et al., 2000). It is very interesting that such prophylactic insecticides have lethal effect on not only the vector but also the pinewood nematode.

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