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Full Length Article

Toxicity of Cyhalodiamide against *Bursaphelenchus xylophilus* and its Preventive Effect in the Forest

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Abstract

In order to examine the toxicity and preventive effect of cyhalodiamide to *Bursaphelenchus xylophilus*, the toxicity and reproduce of *B. xylophilus* were studied by dip method. In addition, the residue of different tree height after injection of cyhalodiamide was tested by HPLC and analysed the effect of preventive treatment of cyhalodiamide to *B. xylophilus*. Results showed that the LC_{50} of cyhalodiamide after a 24 h treatment was 0.019 mg/L that could poison and inhibit the population of *B. xylophilus*. The medicament could be quickly transported from the base to top of the pine by injecting of cyhalodiamide after three months the dosages of cyhalodiamide of different tree height were significantly different and the pharmaceutical content of southern sides of tree was higher than other direction (8.674 mg/L at 4 m). Moreover, cyhalodiamide showed good results of prevention test in the forest. These results provided new agents and theoretical basis for the control of pine wilt disease. © 2017 Friends Science Publishers

Keywords: Cyhalodiamide; *Bursaphelenchus xylophilus*; Toxicity measurement; Trunk injection; Prevention effect

Introduction

Pine wilt disease (PWD) is an internationally recognized devastating quarantine disease. Its pathogen is *Bursaphelenchus xylophilus* (Bolla *et al*., 1986; Vicente *et al*., 2016) with features of easy spread fast incidence difficult prevention and cure etc. PWD is distributed all over the world the most serious area covers China, Japan, South Korea and other countries (Shin *et al*., 2004; Gruffudd *et al*., 2016). China has expanded to more than 170 cities and counties in 15 provinces, causing huge losses to the national economy and forest ecosystems (Shi *et al*., 2013; Yang *et al*., 2013). At present, the prevention and treatment of PWD mainly uses chemical method as the main comprehensive measure the nematicide on the market includes avermectin, emamectin benzoate and other pesticides and has good prevention effect (Takai *et al*., 2000; 2001; 2003; 2004; Sousa *et al*., 2013; Fettig *et al*., 2014). However, the long-term use of emamectin benzoate could lead to the emergence of *B. xylophilus* resistance and the decrease of control effect (Zhao *et al*., 2006; Kang *et al*., 2013). Therefore, the prevention and treatment of PWD urgently requires the application of new and effective medicament.

Cyhalodiamide is a new generation of phthalic diamidepesticide is researched and developed by China (Zhu *et al*., 2011; Liang *et al*., 2015) is a research hotspot in recent years (Tohnishi *et al*., 2005). Studies have shown that it has strong insecticidal activity against rice-stem borer, plutella xylostella and other lepidoptera pests (Xing *et al*., 2013) and has good promotion and application in agricultural production. At present, there have been no reports on the application of cyhalodiamide in forestry diseases and pests control. In order to extend the application of cyhalodiamide, the toxicity of cyhalodiamide to *B. xylophilus* was determined by dip method, and the effect of cyhalodiamide to *B. xylophilus* reproduction was investigated by toxicity regression analysis. The distribution of cyhalodiamide in *Pinus massoniana* and the in-forest efficacy test were determined based on trunk injection technique to provide new agents and technical support for the prevention and cure of PWD.

Materials and Methods

Test Agent

A 4.8% cyhalodiamide EC and liquid preparations (50 mL/bottle) supplied by Jiangxi Danong Chemical Co., Ltd., were used.

Test *B. xylophilus*

The dead trunk of *Pinus massoniana* infected with PWD in Xingan County Jiangxi Province was selected and the nematode was isolated by the Bellman funnel method, which was identified as *B. xylophilus*. Then it was cultured

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on *Botrytis cinerea* (25ºC, 6–7 d) the nematodes were isolated, nematode suspension (100 pcs/mL) and nematode suspension (20–30 thousand pcs/mL) were prepared for toxicity measurement and inoculation prevention test, respectively.

Test Tree Species

The selected test site was a hilly red soil mountainous area with clay and the tested tree species were 10–15 a healthy (the health of the trees was determined by the pine resin flow) *P. massoniana* with heights of about 8 m.

Toxicity Measurement

In this study, dip method was used that is nematode suspension + pesticide direct contact method. 4.8% cyhalodiamide EC was diluted to 500 times, 1,000 times, 1,500 times, 2,000 times and 2,500 times and transferred into 1.5 mL centrifuge tubes with 100 nematodes placed in a 25℃ constant temperature incubator. After 24 h the survival and death count of *B. xylophilus* were checked under the microscope to calculate the lethality rate (refer to the Zhang and Zhao, 2013) and SPSS software was used for toxicity regression analysis. With water treatment as the control each treatment was set up for five replicates.

Mortality of control= number of dead insects (control)/number of test insects (control).

Corrected mortality = (Average mortality of treatment mortality of control)/(1-mortality of control).

Influence of Cyhalodiamide on *B. xylophilus* Growth

100 *B. xylophilus* adults (50♀+50♂) were randomly picked and soaked in cyhalodiamide (10^{-5} mg/L) for 5 h then washed three times with sterile water and then added to PDA medium with *Botrytis cinerea*, placed at 25℃ constant temperature for raising until the *B. cinerea* in a petri dish were eaten up then stop culturing. The *B. xylophilus*'s feeding of *B. cinerea* on different treated PDA plates and the numbers of nematodes treated with different treatments were observed. With water treatment as the control each treatment set up five replicates.

Concentration Testing of Cyhalodiamide in *Pinus Massoniana* Trunk

Cyhalodiamide liquid preparation (50 mL/bottle) was injected to 10–15 a *P. massoniana* in mid-March 2015. A hole was drilled from the stem at a height of 0.5 m from the ground (hole depth of 6–8 cm) the perforation angle was 45 ° obliquely downward and a bottle was injected. The scissors were used to cut the tip of the vial and the head of the vial was inserted into the injection hole the bottom of the vial was punctured using a pick needle. At the end of July, the trunk sections (10 cm in thickness) and the tip shoots (near 8 m) at the height of 0 m, 2 m, 4 m and 6 m of *P. massoniana* were collected separately according to the quarter-circle of the east, west, south and north. After drying, the trunk sections were crushed and methanol was used as the solvent. The cyhalodiamide was extracted and concentrated in vacuo and the concentration of cyhalodiamide in the concentrated solution was determined by high performance liquid chromatography (HPLC). The specific method was described in Takai *et al.* (2004) with slight modification.

Prevention Test of Cyhalodiamide against PWD

50 mL and 100 mL of 4.8% cyhalodiamide liquid was injected into 10–15 a *P. massoniana* trunk respectively *B. xylophilus* (20-30 thousand pcs/tree) was inoculated after two weeks and one month. Then the incidence of pine tree and the number of *B. xylophilus* in dead pine trees were observed and statistically analyzed to evaluate the prevention effect, while the control treatment trees were inoculated with *B. xylophilus*, but not injected.

Results

Toxicity Measurement of Cyhalodiamide to *B. xylophilus*

As shown in Table 1, cyhalodiamide has a strong toxic effect on *B. xylophilus*. The lethality rate of the nematode was significantly different with different concentration of medicament and it increases with the increase of medicament concentration. When 4.8% cyhalodiamide EC was diluted by 1,500 times the lethality rate of nematodes reached 70.41%; when diluted by 500 times lethality rate was as high as 100%. The results of toxicity analysis showed that the toxicity regression equation of cyhalodiamide to *B. xylophilus* calculated by dip method was Y=20.4344+ 3.2692x and the semi-lethal concentration was 0.019 mg/L. Cyhalodiamide was much better than the lethal effect of common nematicides on the market.

Toxicity Measurement of Cyhalodiamide to *B. xylophilus*

Fig. 1 showed that cyhalodiamide had a significant effect on the reproduction of *B. xylophilus*. When the *Botrytis cinerea* in the control petri dish was completely consumed by *B. xylophilus* (Fig. 1B) the feeding area of *Botrytis cinerea* by namatode of cyhalodiamide-treated in medium was still small (Fig. 1A). Separated *B. xylophilus* in the petri dish the number of *B. xylophilus* in the control treatment (CK) was 22 and 215 pcs/dish and the number of *B. xylophilus* after 5 h of cyhalodiamide treatment was only 296 pcs/dish (Fig. 2) indicating that cyhalodiamide has a strong inhibitory effect to the population of *B. xylophilus*.

Table 1: The lethal effect of cyhalodiamide on *Bursaphelenchus xylophilus*

4.8% Cyhalodiamide EC/dilution multiple	Adjusted mortality /%	Toxicity regression equation	Semi-lethal concentration LC50/(mg/L)	95% Confidence interval
500	100±0.0	y=20.4344+3.2692x	0.019	0.0122-0.0236
1000	90.92±7.92			
1500	70.41±10.29			
2000	66.17±17.28			
2500	47.63±6.33			
CK	0±2.94			

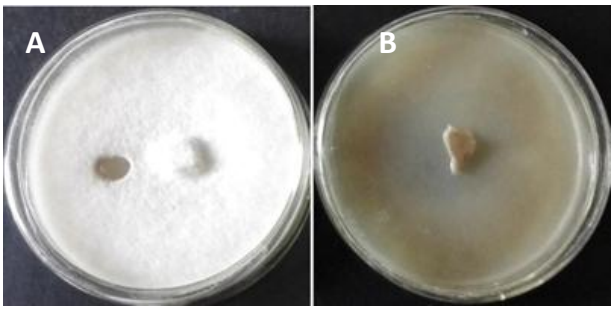

Fig. 1: The acquisition feeding results of *Bursaphelenchus xylophilus* after cyhalodiamide treatment
A: cyhalodiamide treatment; B: control treatment

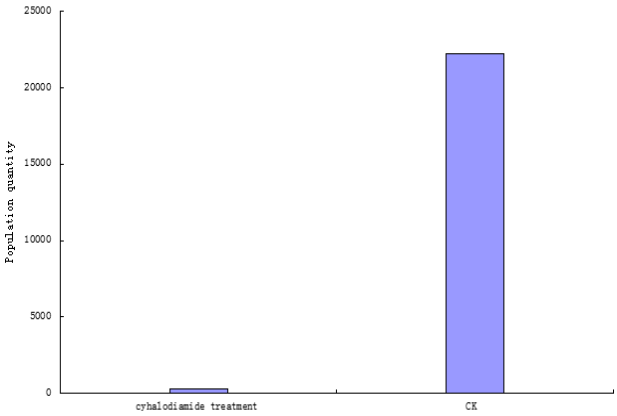

Fig. 2: The populationnumber of *Bursaphelenchus xylophilus* after cyhalodiamide treatment

Distribution of Cyhalodiamide in Pine Trunk after Injection

The trunk sections (thickness of 10 cm) were cut at the height of 0 m, 2 m, 4 m and 6 m in three months after the injection of 4.8% cyhalodiamide preparations in the *P. massoniana* trunk at the height of 0.5 m. The contents of cyhalodiamide in trunk section and top tip of different height sections were determined by HPLC. The results showed that *P. massoniana* wood chip treated by 20 mg of cyhalodiamide had a peak on the HPLC chromatogram after methanol extraction (Fig. 3b) but no peak on the chromatogram of the *P. massoniana* wood chip without cyhalodiamide treatment (Fig. 3c) indicating that the concentration of cyhalodiamide in *P. massoniana* can be determined by HPLC. The mass concentration of cyhalodiamide was linearly correlated with the peak area the regression equation was y=8.4780x+8.8107 and the correlation coefficient was 0.9994, which satisfied the need of quantitative analysis. The fortified recovery of cyhalodiamide was in 84.35–94.58% the relative standard deviation is in 7.82–9.37% and the lowest detection concentration is 0.01 mg/L.

As shown in Fig. 4 the content of cyhalodiamide preparation can be detected in different heights of sapwood of *P. massoniana* after three months, and there is a big difference in different heights and directions (E, S, N and W) indicating that cyhalodiamide has a good systemic uptake in *P. massoniana* trunk, which can be transferred from the bottom of the trunk to the upper part in a short time. The medicament mainly concentrates at 2 m, 4 m and 6 m of sapwood above the injection point, with mean concentration of 1.25, 4.35 and 0.31 mg/L all significantly higher than cyhalodiamide LC_{50} (0.019 mg/L). The sapwood E and S content was significantly higher than the sapwood W and N the content of cyhalodiamide in the southern sides of tree was higher than that in the other three directions the content at the height of 4 m in S direction was as high as 8.674 mg/L and the content of shoot tip reached 0.298 mg/L indicating that cyhalodiamide injection could effectively inhibit the growth and reproduction of namatode in the tree, and had strong toxic effect on namatode, so as to effectively protect *P. massoniana* against PWD within the the forest area.

Prevention Test of Cyhalodiamide against PWD

Table 2 showed that the cyhalodiamide had a certain effect on PWD prevention. The test trees were 10–15a *P. massoniana* at the dosage of 50 mL and 100 mL *B. xylophilus* was inoculated 15 days or 30 days after injection. Compared with the control group the average survival rate of *P. massoniana* was increased by 40% and the average survival rate of *P. massoniana* increased by 66% the difference was significant. The results showed that *P. massoniana* at the tree age could significantly improve the survival rate under the two doses of 4.8% cyhalodiamide, which was due to the rapid transport of cyhalodiamide into the shoots and the inhibition of nematode reproduction and a good preventive role.

Table 2: The result of the prevention test of cyhalodiamide against Pine Wilt Disease

Dose of 4.8% cyhalodiamide	Time of inoculation	Treated trees	Survival		Survival rate (%)
			Healthy trees	Dead trees	
50 mL	15 days after injection	15	11	4	73.3
	30 days after injection	15	10	5	66.7
100 mL	15 days after injection	15	15	0	100.0
	30 days after injection	15	14	1	93.3
CK	Consistent with the treat	10	3	7	30.0

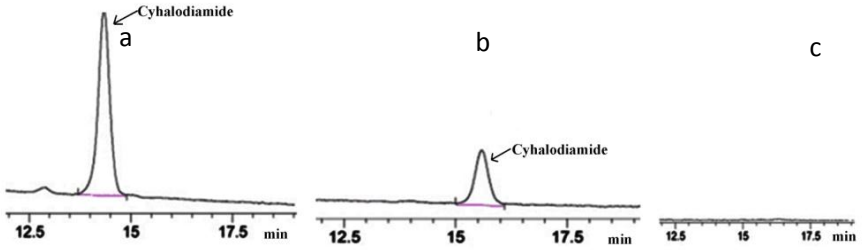

Fig. 3: The cyhalodiamide in the tree by chromatographic detection: (a) 20 ng of cyhalodiamide standard; (b) methanol extract of 20 mg of Pine wood chips treated with cyhalodiamide after the cleanup procedure; (c) methanol extract of 20 mg of Pine wood chips untreated with cyhalodiamide after the cleanup procedure

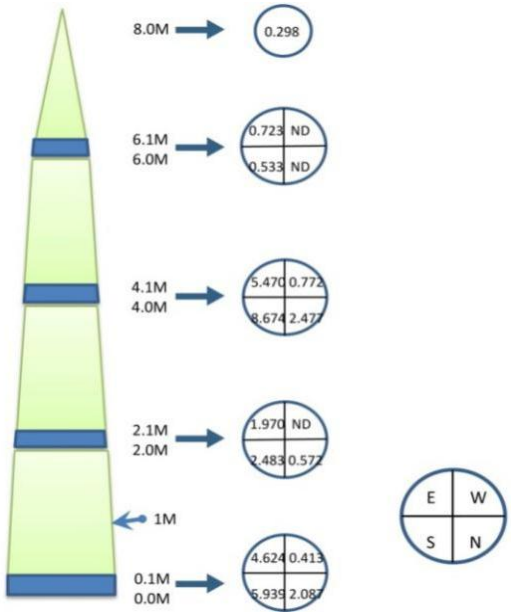

Fig. 4: Conduction distribution of cyhalodiamide in the pine tree

ND= not detected (below 0.01 mg/L, the limit of detection); E: East, W: West, S: South, N: North

Discussion

Cyhalodiamide is a new generation of phthalic diamide pesticide, which is researched and developed by China, with strong insecticidal activity against rice-stem borer, *Plutella xylostella*, cotton bollworm and other lepidoptera pests and has significant control effect (Tohnishi *et al*., 2005; Xing *et al*., 2013). It has high efficiency, low toxicity, non-cross-resistance to existing insecticides etc. Such medicament acts on the insects' ryania receptors, causing sustained release of calcium ions leading to insect death (Tohnishi *et al*., 2005). In recent years, a number of effective nematicides such as emamectin benzoate 24 h LC_{50} of 0.23 mg/L (Jia, 2014) rotenone acetone 24 h LC_{50} of 2.8 mg/L (Weng *et al*., 2005) α- terthienyl LC_{50} of 1.89 mg/L (Zhang and Zhao, 2013) showed high insecticidal activities. In this experiment, the living environment of *B. xylophilus* in *P. massoniana* system was simulated by dip method, so *B. xylophilus* could be contacted with medicament sufficiently. The toxicity regression analysis showed that cyhalodiamide had a 24 h semi-lethal concentration (LC_{50}) of 0.019 mg/L. Compared with the above nematicides, cyhalodiamide showed a stronger lethal effect to *B. xylophilus*, the effect deserves further attention. This experiment also showed that, cyhalodiamide has a strong inhibitory effect on the population of *B. xylophilus*, the number of *B. xylophilus* treated with cyhalodiamide was 296 pcs/dish after 5 h, while the number of namatode in the control was 22,215 pcs/dish

At present, analytical methods for the determination of flubendiamide in fruits and vegetables and their environmental samples have been reported, mainly are HPLC-UV (Paramasivam and Banerjee, 2011; Takkar *et al*., 2012) and UPLC-MS /MS (Caboni *et al*., 2008; Zhu and Zhao, 2011). However, there are no reports on the analytical methods and their residual characteristics in biological samples. The results of this study indicate that HPLC is a viable method for the determination of cyhalodiamide residue in *P. massoniana*, which can be used for reference in the future. The content of cyhalodiamide in *P. massoniana* has a big difference in different heights and

different directions all significantly higher than cyhalodiamide LC_{50} (0.019 mg/L) and the content of cyhalodiamide in the southern sides of tree was higher than that in the other three directions. The results of this study were in agreement with the results of Takai *et al*. (2004), where the pharmaceutical content of the trunk at 4 m in the S direction was 8.674 mg/L, but the content of medicament at the trunk bottom was also high probably related to the site of injection time and type of additives and we need to filter and optimize the additives.

In this experiment, 10–15 a *P. massoniana* was used as the test tree and the prevention test results of the pine tree in the forest have prevention and treatment of PWD by cyhalodiamide injection. Compared with the control group the average survival rate of *P. massoniana* with a dose of 50 mL was increased by 40% and the average survival rate of *P. massoniana* with a dose of 100 mL increased by 66% the difference was significant. The results showed that cyhalodiamide could effectively kill *B. xylophilus* and eliminate the potential sources of infection, so it could effectively control some PWD potential pines in the pine forest (i.e., trees that are out of the symptoms or morbidity in the same year or even the following year). However, the preventive effect for pine trees with greater age and diameter at breast height (especially ancient pine trees over a hundred years old) the effect of cyhalodiamide on the egg hatching of *Monochamus alternatus* should be further clarified. The results of this study will provide theoretical basis and practical guidance for the prevention and cure of PWD in ancient pine trees in scenic spots and parks etc.

Conclusion

Inoculated the nematode 15 days or 30 days after cyhalodiamide injection for *P. massoniana*, this experimental result indicated that the average survival rate of the pine with a dose of 50 mL was increased by 40% and the average survival rate of the pine with a dose of 100 mL increased by 66%. Thus, the trunk injection of cyhalodiamide into *P. massoniana* is considered to be a effective control measure for pine wood nematode.

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