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Evaluation of nematicides for *Meloidogyne enterolobii* management in sweetpotato

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Abstract

Sweetpotato is an important crop whose roots are consumed by people worldwide. *Meloidogyne enterolobii* stands out as a highly deleterious variant among the species of root-knot nematode that causes significant damage in sweetpotato. In the present study, the activity of four nematicides against *M. enterolobii* was assessed both *in vitro* and in growth cabinet experiments. After 48 hours of exposure, fluopyram and cyclobutrifluram had a greater negative effect on the motility of *M. enterolobii* second-stage juveniles (J2s) compared to fluensulfone and hymexazol, with respective median effective concentration (EC_{50}) values of 0.204, 0.423, 22.335 and 216.622 mg L^{-1}. When *M. enterolobii* eggs were incubated for 72 hours at the highest concentration of each nematicides, the inhibitory hatching effect of cyclobutrifluram (2.5 mg L^{-1}), fluopyram (1.25 mg L^{-1}) and fluensulfone (80 mg L^{-1}) surpassed 85%, whereas hymexazol (640 mg L^{-1}) was only 67%. Similar results were observed in growth cabinet experiments as well. The disease index (DI) and gall index (GI) were significantly decreased by all four nematicides compared to the control. However, the application of hymexazol did not yield a statistically significant difference in the egg masses index compared to the control, a finding which may be attributed to its potentially limited penetrability through the eggshell barrier. Overall, this study has demonstrated that all four nematicides effectively suppress *M. enterolobii* in sweetpotato, and this is the first report on the nematicidal activity of cyclobutrifluram and hymexazol against *M. enterolobii*.

Keywords

cyclobutrifluram, hymexazol, fluopyram, fluensulfone, *Meloidogyne enterolobii*, sweetpotato

1. Introduction

Sweetpotato (*Ipomoea batatas* (L.) Lam., family Convolvulaceae) is widely cultivated in tropical and warm temperate climates and is the seventh most widely cultivated food crop in the world and the sixth most widely cultivated food crop in China, but its production is limited by various biotic constraints including plant parasitic nematodes (8,11). *Meloidogyne enterolobii* (syn. *mayaguensis*) stands out as a highly deleterious variant among the species of root-knot nematode (RKN) that causes significant damage in sweetpotato (11,17,21,37). This is largely attributable to the damage inflicted upon sweetpotato tubers by nematode infection, resulting in reduced crop quality and diminished marketability (21).

In the United States, *M. enterolobii* was first reported in Puerto Rico in 1988 (27). It has since spread and has been reported in North and South Carolina (5,29,39). More recently, *M. enterolobii* was

found in eight North Carolina counties: Johnston, Harnett, Sampson, Wayne, Greene, Wilson, Nash and Columbus (39,35,32). Sweetpotato production areas in the Carolinas have been particularly hard hit by the introduction of *M. enterolobii*, which has spread relatively quickly in sweetpotato production areas (30). In 2014, *M. enterolobii* on sweetpotato was reported in Guangdong Province, China (11). Subsequently, in 2022, root-knot nematode infections of sweetpotato by *M. enterolobii* was also reported in Guangxi province, China (15). Guangxi Province is the largest sweetpotato producer in south China and the third top producing region in the whole country. Thus, *M. enterolobii*, an important emerging nematode, is a growing threat to sweetpotato agriculture both in the United States and China.

Historically, broad spectrum soil fumigants (e.g. methyl bromide), organophosphates (e.g. aldicarb) and carbamates (e.g. oxamyl) have been used to control various soilborne pests that threaten crop productivity, including plant parasitic nematodes (40). Driven by the phase-out of environmentally and human-health hazardous traditional chemistries, the past 15 years have witnessed the development of innovative nematicides with significantly reduced toxicity to vertebrates (36). These include fluopyram, fluensulfone and cyclobutrifluram, which have a trifluoro (3-F) group in the chemical structure (36).

Fluopyram was first developed for crop use as a fungicide and was shown later to be effective against plant parasitic nematodes. Fluopyram can inhibit the function of succinate dehydrogenase, an enzyme that is essential to the mitochondrial respiratory chain (6,31). Fluensulfone was registered by the US Environmental Protection Agency (EPA) for use on cucurbit and fruiting vegetables in 2014 (20). Fluensulfone was shown to reduce root infection and plant parasitic nematodes penetration into plants (24,26). This compound is a unique nematicide with a mode of action distinguished from anticholinesterases and macrocyclic lactone (18,23). Cyclobutrifluram strongly affects the survival and fertility rates of *Caenorhabditis elegans* by decreasing the number of germ cells (12). Furthermore, it has been proven by a genetic approach that cyclobutrifluram also inhibits the function of the mitochondrial succinate dehydrogenase complex (12). However, research on its effectiveness against root knot nematodes has not been reported. Hymexazol is used as a broad-spectrum fungicide for treating soil-borne diseases due to its high efficiency and low cost (19). It was also used to control complex infestations of plant pathogenic nematodes and fungi (9). However, just like fluopyram which started out as a fungicide, it is unclear whether hymexazol also have an inhibitory effect on the growth and development of plant parasitic nematodes.

Synthetic non-fumigant nematicides have shown considerable efficacy in glasshouse and field trials (33,34,38), but different plant parasitic nematodes do not share equal sensitivity to these compounds. For instance, *in vitro* incubation studies with fluensulfone have shown similar results, with *Aphelenchoides besseyi*, *A. fragariae*, *Bursaphelenchus xylophilus* and *Ditylenchus dipsaci* showing tolerance to fluensulfone exposure, while *Pratylenchus penetrans* and *Xiphinema index* showed sensitivity to this compound (22). This research aims to assess the efficacy of four nematicides in controlling *M. enterolobii* on sweetpotato, and to explore the potential of introducing new nematicides for managing the nematode infections in sweetpotato.

2. Materials and Methods

2.1. Nematodes

The population of nematode used in this study was originally isolated from black nightshade (*Solanum nigrum*) in Lufeng county (22°55′57.44″N, 115°33′10.31″E), Guangdong Province, China (7). Based on the morphological, molecular analyses of mtDNA cytochrome c oxidase I (COI) and D2-D3 regions of 28S rDNA and detection using species-specific primers, the nematode was identified as *M. enterolobii*. *M. enterolobii* was maintained on potted sweetpotato (*Ipomoea batatas* (L.) Lam 'Long 9') plants in a growth cabinet for three months prior to use. Eggs of *M. enterolobii* were extracted from infected roots of sweetpotato with 0.5% sodium hypochlorite solution(Hussey & Barker, 1973). The whole infested root systems were put into 500 mL screw-top flasks and shaken in 0.5% sodium hypochlorite solution for 5 min prior to being washed with water over a No. 500 sieve (25 µm) and transferred to the beaker with about 30 mL of water. To obtain second-stage juveniles, the egg suspension was poured over a Baermann funnel and incubated in sterile water for 5 days at 28°C. Emerging J2s were collected daily by a sieve, and only those collected on the fifth day of incubation were used in the experiments.

2.2. Chemicals

Cyclobutrifluram [45% suspension concentrate, SC] was obtained from Syngenta group China and

Fluopyram [41.7% suspension concentrate, SC] was provided by Bayer Crop Science, Greater China. Fluensulfone [40.0% emulsifiable concentrate, EC] was obtained from ADAMA Agricultural Solutions Ltd, Beijing, China. Hymexazol [70.0% water power, WP] was obtained from Weifang Huanuo Biotechnology Co., Ltd., Weifang, China.

2.3. Effect of nematicides on nematode motility

To determine the effect of nematicide concentration on J2s motility of *M. enterolobii*, a 24-well microplate motility assay was performed. In the preliminary phase, we first conducted a pre-experiment to determine the approximate range of EC_{50} for each chemical. Based on the result of the preliminary experiment, we designed distinct application rates for each chemical accordingly. Plate wells were filled 1ml aqueous solutions of cyclobutrifluram at 2.5, 1.25, 0.63, 0.31 and 0.16 mg L^{-1}, Fluopyram at 1.25, 0.63, 0.31, 0.16 and 0.08mgL^{-1}, Fluensulfone at 80, 40, 20, 10 and 5 mgL^{-1}, and Hymexazol at 640, 320, 160, 80 and 40 mg L^{-1}, or tap water (control; PH 7.3). Approximately 100 J2s suspended in 10 µL of tap water were introduced into each well. At 48 h post inoculation, 25 µL of mol L^{-1} sodium hydroxide (NaOH) was added to the appropriate wells as an irritant to distinguish between motile and immotile nematodes (8). Percent motility was recorded 30 seconds after the addition of the NaOH using a stereoscope (Nikon SMZ745). Each treatment was replicated three times, and all experiments were conducted twice.

2.4. Effect of nematicides on egg inhibitory hatching rate

To determine the effect of nematicide concentration and exposure time on eggs hatching of *M. enterolobii,* a 24-well microplate egg hatching assay was performed. The treatment concentration of cyclobutrifluram was 2.5, 0.63 and 0.16 mg L^{-1}; Fluopyram was 1.25, 0.31 and 0.08 mg L^{-1}, Fluensulfone was 80, 20 and 5 mg L^{-1}, and Hymexazol was 640, 160 and 40 mg L^{-1}. Eggs were exposed to tap water (PH 7.3) as the control. Approximately 100 eggs suspended in 10 µL of tap water were introduced into each well. At 24, 48 or 72 h post inoculation, the number of hatched nematodes in each treatment was observed and counted by a stereomicroscope. Each treatment was replicated three times, and all experiments were conducted twice. Inhibitory hatching rate (IHR) was calculated for each nematicide treatment using the following formula:

$$IHR = \frac{\text{number of hatched nematodes in control} - \text{number of hatched nematodes in treament}}{\text{number of hatched nematodes in control}} \times 100\%$$

2.5. Growth cabinet nematicide efficacy trial on sweetpotato

A growth cabinet experiment was conducted to assess the impact of applying nematicides at the recommended dosage on the reproductive capacity of *M. enterolobii* infecting sweet potato root systems. In simple terms, 20 cm wide square pots were filled with 1.5 L of dry heat sterilized sandy loam soil, which was augmented with organic peat. The control group consisted of pots inoculated with *M. enterolobii* but not treated with any nematicides. Three days after the sweetpotato seedlings ('Long 9') were transplanted, nematodes were inoculated into the potted soil. Each pot was inoculated with 1000 J2s of *M. enterolobii.* Nematicide treatments were applied to each pot except for fluensulfone, which was applied 3 days prior to planting to avoid phytotoxicity issues. Each treatment was as follows: (1) Cyclobutrifluram (0.03 mL pot^{-1}), (2) Fluopyram (0.03 mL pot^{-1}), (3) Fluensulfone (0.2 mL pot^{-1}), (4) Hymexazol (1 mg pot^{-1}), and (5) $Nema^{+}$ (nematodes with no nematicide). Each treatment consisted of three replicates, each containing six sweetpotato plants, with one 15 cm tall sweetpotato seedling per pot. All experiments were conducted twice. Pots were watered as necessary throughout the experiment to maintain adequate soil moisture. After 50 days of growth in a temperature-controlled growth cabinet set at 28±1°C with 12 hours of light per day, the experiment was terminated.

A disease measurement was obtained in the pot experiments. The first was disease incidence, which was the number of diseased plants out of the total number of plants in each replicate and was reported on a percentage basis. The sweetpotato seedlings whose root systems exhibited visible galls were identified as diseased plants. The root gall index (GI) was calculated as the number of root galls on an individual plant divided by the total weight of the plant roots, and then multiplied by 100. The egg mass index (EMI) was calculated as the number of egg masses on an individual plant divided by the total weight of the plant roots, and then multiplying the result by 100. The GI and EMI can effectively reflect the nematode's reproduction status in plant and the severity of damage to the plant. Counting egg masses was

performed using a stereoscope. A disease severity was determined by rating the roots on each plant on a 0 to 5 scale (Zhou et al., 2016), in which 0= less than 10% root-knot; 1= very slight root-knot, between 11%-20%; 2= obvious root-knot, percentage was between 21%-50%; 3, percentage was between 51%-80% root-knot; 4, percentage was between 81%-90% root-knot; 5= root knots exceeded 91%. A disease index (DI), whose values ranged from 0 to 100 was calculated for each nematicide treatment using the following formula:

$$DI = \frac{\sum(\text{the number of diseased plants in the pot} \times \text{rating } i)}{\text{total number of plants in the pot} \times 5} \times 100$$

Where *i* is 1-5.

2.6. Data analysis

Analysis of variance was performed on data from individual experiments using IBM® SPSS® Statistics (Version 20). Data were subjected to analysis of variance (ANOVA) and following significant results, means were separated according to the Student's Protected LSD tests. A significance level of $\alpha = 0.05$ was used in all analyses. Median effective concentration (EC_{50}) values for each nematicide were calculated by probit analysis of the nematodes' motility after 48h of incubation.

3. Results

3.1. *In vitro* nematicidal activity of four nematicides on nematode motility

Non-motile J2s of *M. enterolobii* did not recover their motility after NaOH was add to the wells, indicating that cyclobutrifluram, fluopyram, fluensulfone and hymexazol were nematicidal. The EC_{50} (48h) for cyclobutrifluram, fluopyram, fluensulfone and hymexazol was 0.423, 0.204, 22.335 and 216.622 mg L^{-1}, respectively (Table 1). Based on the J2s motility assays, J2s of *M. enterolobii* were more sensitive to fluopyram and cyclobutrifluram than fluensulfone and hymexazol.

3.2. Effect of nematicides on egg inhibitory hatching rate

After incubating *M. enterolobii* eggs in each of the nematicide treatments, the inhibitory hatching rate of eggs increased over time at the same concentration, and it also increased with concentrations at same time point (Fig. 1). Incubating *M. enterolobii* eggs in each nematicide concentration for 24 hours resulted in inhibitory hatching rate of 67.19%, 72.53%, 76.39% and 49.90% for cyclobutrifluram (2.5 mg L^{-1}), fluopyram (1.25 mg L^{-1}), fluensulfone (80 mg L^{-1}) and hymexazol (640 mg L^{-1}), respectively. After incubating *M. enterolobii* eggs in each nematicide concentration for 48 hours, inhibitory hatching rates were observed as follows: cyclobutrifluram (2.5 mg L^{-1})- 88.28%, fluopyram (1.25mg L^{-1})- 83.81%, fluensulfone (80 mg L^{-1})- 88.08% and hymexazol (640 mg L^{-1})- 63.51%. Following an incubation period of 72 hours, inhibitory hatching rates were recorded as cyclobutrifluram (2.5 mg L^{-1})-90.73%, fluopyram (1.25mg L^{-1})- 96.78%, fluensulfone (80 mg L^{-1})-87.45% and hymexazol (640 mg L^{-1})-67.39%. The highest concentration among all tested nematicides after 72 hours of exposure showed that cyclobutrifluram, fluopyram and fluensulfone achieved an inhibitory hatching effect exceeding 85%, while hymexazol only reached a rate of 67%.

Table 1: Toxicity of Cyclobutrifluram, Fluopyram, Fluensulfone, Hymexazol to second stage juveniles of *M. enterolobii* (48h).

Chemicals	Regression	Slope SE[a]	EC_{50} (mg L^{-1})	95% confidence interval	*P*[b]	χ^2
Cyclobutrifluram	0.425+1.137X	0.144	0.423	0.318~0.537	0.318	3.521
Fluopyram	0.76+1.101X	0.132	0.204	0.157~0.256	0.191	4.748
Fluensulfone	–1.408+1.044X	0.189	22.335	17.393~28.908	0.351	3.273
Hymexazol	–3.664+1.569X	0.152	216.622	182.532~259.864	0.989	0.123

[a] slope standard error.
[b] *P*-value.

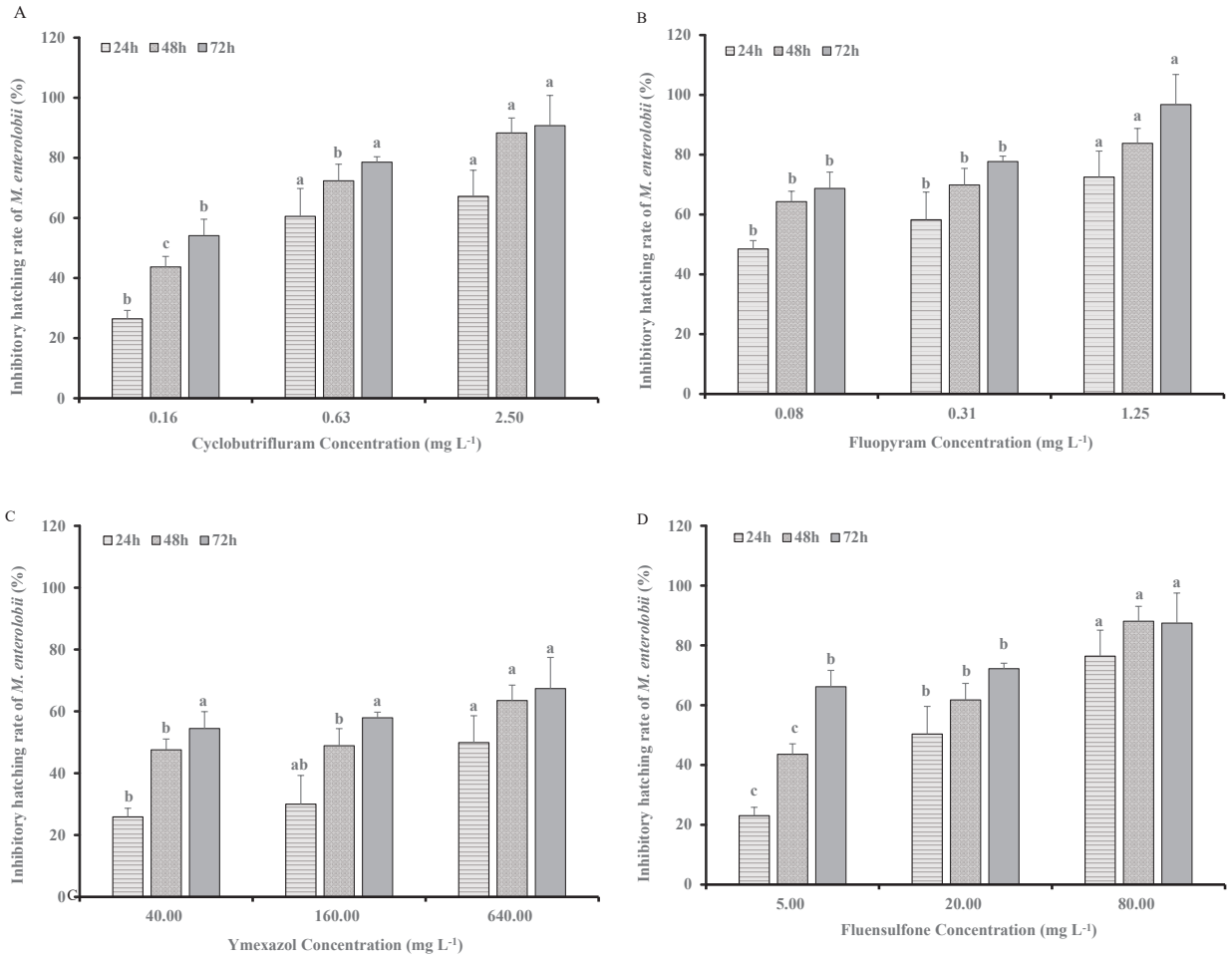

Figure 1: Inhibitory hatching rate of *Meloidogyne enterolobii* after 24h, 48h, and 72h of exposure to varying concentrations (A) cyclobutrifluram, (B) fluopyram, (C) hymexazol, and (D) fluensulfone in a 24-well microplate egg hatch assay. Bar values represent means ± standard error of three replicates. Treatments within the same experiment, connected by the same letter, were not significantly different according to the Protected LSD ($\alpha = 0.05$).

3.3. Growth cabinet nematicide efficacy trial on sweetpotato

In the growth cabinet studies conducted on sweetpotato infected with *M. enterolobii*, all the nematicide treatments exhibited lower disease incidence compared to the control, except for hymexazol. Among these treatments, fluopyram demonstrated the lowest disease incidence (50%), which was significantly different from the control (100%) (Table 2). However, each nematicide treatment resulted in a significantly lower DI of sweetpotato compared to the control. The ascending order of DI under each treatment was as follows: fluopyram (7), cyclobutrifluram (13), fluensulfone (25) and hymexazol (38). Cyclobutrifluram and fluopyram showed significantly difference from the other treatments. Similarly, the GI of sweetpotato under each nematicides was significantly lower than that of the control. The ascending order of GI was as follows: fluopyram (64), cyclobutrifluram (69), fluensulfone (157) and hymexazol (238). Cyclobutrifluram and fluopyram were significantly different from other treatments. The EMI of sweetpotato in each of the nematicide treatments was significantly lower than the control except for hymexazol. The EMIs of sweetpotato under each treatment were fluopyram (36), cyclobutrifluram (51), fluensulfone (150) and hymexazol (238) in ascending order, with cyclobutrifluram and fluopyram being significantly different from other treatments.

Table 2: Control efficiency of four kinds of nematicides on sweetpotatos infected by *Meloidogyne enterolobii* under greenhouse conditions.

Treatment	Disease Incidence (%)	DI[a] (0-100)	GI[b]	EMI[c]
Cyclobutrifluram	78 ± 5.3[d] ab	13 ± 0.8d	69 ± 5.0d	51 ± 1.5c
Fluopyram	50 ± 19.5b	7 ± 2.7d	64 ± 2.2d	36 ± 9.6c
Fluensulfone	83 ± 0.0ab	25 ± 1.6c	157 ± 10.0c	150 ± 11.0b
Hymexazol	100 ± 0.0a	38 ± 2.4b	238 ± 21.1b	238 ± 20.7a
Control	100 ± 0.0a	46 ± 4.2a	313 ± 19.6a	256 ± 26.4a

[a-c] DI, disease index; GI, gall index; EMI, egg masses index.
[d] Values are means ± standard error. Any means within the same column not connected by the same letter are considered significantly different according to Protected LSD ($\alpha = 0.05$).

4. Discussion

M. enterolobii is one of the species of root knot nematode that is a growing threat to sweetpotato agriculture in China and the United States. Combinations of management, including crop rotation and use of resistant cultivars, are not completely efficient and searching for new nematicides constantly is needed (4). Assessing nematicidal activity using plant bioassays evaluates more points of possible nematode inhibition, whereas *in vitro* assays only evaluate a small component of the infection process (e.g. hatching or motility). In the present study, we demonstrated that cyclobutrifluram had high nematicidal activity against *M. enterolobii in vitro*, and application of cyclobutrifluram significantly reduced DI and nematode reproduction in sweetpotato plants infected by the parasites. Meanwhile, we also found that hymexazol had nematicidal activity against *M. enterolobii*, but the effect was significantly lower than cyclobutrifluram, fluopyram and fluensulfone. The current study is the first report of the nematicidal activity of cyclobutrifluram and hymexazol against *M. enterolobii* in sweetpotato.

More recently, cyclobutrifluram has been developed as a targeted solution for plant parasitic nematodes. Its chemical structure is similar to fluopyram and fluensulfone (36). Cyclobutrifluram effectively reduced the survival rates of *C. elegans* by inhibiting the function of the mitochondrial succinate dehydrogenase complex (12). In field management applications for plant parasitic nematodes, cyclobutrifluram significantly decreased the number of nematodes per gram of soybean roots and leads to a yield increase in certain cultivars compared to treatments with only abamectin (28). Moreover, cyclobutrifluram did not exhibit any phytotoxic effects on soybean seeds and seedlings (28). The aforementioned outcomes align with the findings derived from our independent research. Results from motility and inhibitory hatching bioassays indicated that cyclobutrifluram negatively affected J2s and eggs of *M. enterolobii. In vitro*, cyclobutrifluram had a high toxicity to J2s of *M. enterolobii* with a median effective concentration (EC_{50}) of 0.423 mg L^{-1} after 48h of exposure. The highest concentration of cyclobutrifluram used in incubating *M. enterolobii* eggs for 72 hours resulted in an inhibitory hatching effect exceeding 90%. During the growth cabinet experiments involving sweetpotato subjected to *M. enterolobii*, cyclobutrifluram significantly reduced the DI, GI and EMI compared to the control.

Of the four nematicides tested, fluopyram had a stronger negative impact on *M. enterolobii* compared to fluensulfone. These findings align with previous research by Watson (37). However, a contrasting result was reported by Alam (2) in a growth cabinet experiment, where oxamyl and fluensulfone were found to be the most effective nematicides in suppressing nematode eggs hatching, followed by fluopyram. This discrepancy could be attributed to varying sensitivity of geographically distinct populations of the same species towards the same nematicide. Notably, Oka and Saroya (24) observed a more than 10-fold difference in median lethal concentration (LC_{50}) among the *M. incognita* populations after 17 hours of exposure to fluensulfone.

Hymexazol was employed for management of complex infestations caused by plant pathogenic nematodes and fungi (9). In light of this, we postulated that hymexazol could hold potential as a nematicide. Also noteworthy is the fact that hymexazol showed nematicidal efficacy against *M. enterolobii*. This was

not the first time that a fungicide was investigated for its effect on plant parasitic nematodes (9). Carbendazim was reported to possess some nematicidal activity (14) and pentachloronitrobenzene was proven to suppress *M. incognita* (1). However, the effectiveness of hymexazol against *M. enterolobii* was comparatively lower than other fluorinated nematicides, particularly in terms of its effect on inhibiting egg hatching rates. When *M. enterolobii* eggs were incubated with the highest concentration of 640 mg L^{-1} hymexazol for 72 hours, the inhibitory hatching effect reached only 67%. In growth cabinet experiments, no significant change in the EMI was observed when hymexazol was used. This may be attributed to its potentially limited penetrability through the eggshell barrier.

Prolonged use of a single nematicide can result in resistance of nematode, which poses challenges in managing root knot nematode (16). To promote the healthy development of sweetpotato agriculture, it is important to diversify the nematicides used to manage *M. enterolobii*. In this study, fluorinated nematicides and hymexazol have shown the potential to suppress *M. enterolobii* on sweetpotato when applied at the recommended rate. Further studies should be conducted to investigate the efficacy of nematicides for management of *M. enterolobii* on sweetpotato under field conditions, as well as to explore the molecular mechanisms of cyclobutrifluram and hymexazol in suppressing *M. enterolobii.*

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