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RESPONSES OF SOYBEAN CYST NEMATODE *HETERODERA GLYCINES* TO MACROELEMENT AND MICROELEMENT COMPOUNDS

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Abstract

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A variety of macroelement and microelement compounds were tested against the soybean cyst nematode (SCN), *Heterodera glicines*, *in vitro* and *in vivo* on soybean. *In vitro*, KH_2PO_4 was the best compound to reduce the number of surviving J2 and achieve the lowest value of LC50 (0.01M) and LC90 (0.03M) among the macroelement treatments. Almost all the tested compounds significantly inhibited the hatching of SCN, except for $ZnSO_4 \cdot 7H_2O$. Among the microelement treatments, $FeCl_3 \cdot 6H_2O$ was the best one to inhibit J2 survival with the lowest value of LC50 (0.0004M) and LC90 (0.001M). All the tested compounds significantly inhibited the hatching of SCN and all inhibitions were over 90%. All treatments improved the growth of root and the application of $(NH_4)_2HPO_4$ gave the best root growth (dry and fresh weighs). In glasshouse, all treatments were significantly effective in reducing the reproduction of SCN. $FeCl_3 \cdot 6H_2O$ was the best treatment for suppressing cyst production and achieving the highest percentage of nematode inhibition (72.54%).

Key words: compound, macroelement, microelement, soybean cyst nematode (SCN), inhibition

Introduction

The soybean cyst nematode (SCN), *Heterodera glycines*, is an important pest of soybeans all around the world. As a typical soil-borne pest, the small plant-parasitic roundworm attacks roots of soybeans. Conditions of planting soil, such as ions, pH value, temperature, humidity and agrotype, influence the infecting activity of SCN directly (Tetft, 1982; Alston and Schmitt, 1988; Hill, 1989; Singh and Sharma, 1995; Xing, 2002). Many years ago, experiments on the usage of chemical compounds to improve plant growth and decrease nematode populations have been widely developed. The efficacy of certain compounds on plant nematodes, especially those containing nitrogen, has been largely studied by many researchers (Rodriguez-Kabana, 1986; Oka, 2002). For a long time, planting resistant cultivars are a major method to control SCN in China. However, losing resistance, lower yield and land use limit are becoming serious problems. So to develop effective, economical and safe controlling methods are necessarily expected.

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It is our long-term goal to understand the effect of soil compound fertilizer on SCN and find out the best crop management strategies to prevent and control SCN. Based on this background, the aim of this study was to determine the responses of SCN survival and hatch to the compounds from chemical fertilizers, and identify lethal concentrations of those effective compounds.

A solution-exposure assay was used to determine the direct sensitivity of SCN to common necessary macroelements, including $NaNO_3$, $NaNO_2$, Urea NH_4Cl, $(NH_4)_2HPO_4$, KCl, $Ca_2HPO_4 \cdot 12H_2O$, KH_2PO_4, $Na_2HPO_4 \cdot 12H_2O$, $MgCl_2 \cdot 6H_2O$ and $CaCl_2 \cdot 2H_2O$, and microelements, incluing $Na_2B_4O_7 \cdot 10H_2O$, $Na_2Mo_4O_4 \cdot 2H_2O$, $ZnCl_2$, $ZnSO_4 \cdot 7H_2O$,$FeCl_3 \cdot 6H_2O$, $FeSO_4 \cdot 7H_2O$ and $CuCl_2 \cdot 2H_2O$. Nitrogen resources are elements that have been studied extensively. Mineral fertilizer application has been long recognized to inhabit the population densities of plant-parasitic nematodes, offering the potential option in nematode controlling and management (Coyne et al., 2004). Cadmium, copper, lead and zinc decreased *Steinernema feltiae* pathogenicity against the reproduction of tested insects (Jaworska, 2002). Castro (1990) suggested that some of simple inorganic salts of ions, such as K^+, NH^{4+}, Cs^+, NO^{3-} and Cl^-, which are strongly repellent to infective second-stage larvae of *Meloidogyne incognita*, might be a new method of plant protection (Castro and Belser, 1990). In our study, we intended to compare the effectiveness of different chemical compounds of both macroelements and microelements and identify the best candidates to control the outbreak of SCN.

Materials and Methods

Culturing *Heterodera glycines*

Cysts of SCN were collected since the late autumn to October (2008) from a susceptible soybean cultivar (Liao NO. 10, 11) in the experimental field of Plant Nematology Lab of Shenyang Agriculture University. Samples were collected from the 0-20 cm depth at five locations. The surrounding soybean roots of soil were dug from each field and placed in plastic bags for transporting back to laboratory facilities.

Collected soil were washed through a 250 μm aperture sieve, then soil on the sieve were collected into the tube (50 ml) with water and Kaolin clay, and then were centrifugated (2000 r/min) for 3 min. Cysts in precipitation were collected by floatation in 75% sugar solution, then hand-picked under a stereomicroscope. SCN was cultured on soybean plants (*Glycine max* (L.) Merri) and grown in a glasshouse of 25-30^{0}C. J2 hatched 3-4 days later after cysts were placed in sterile deionized water. For these cultures, 2-week-old soybean seedlings were inoculated with SCN J2 and cysts were collected 35-40 days later. The collection method is the same as above.

SCN cysts were surface-sterilized in 0.5% sodium hypochlorite solution for 3 min. Then cysts were rinsed 3 times by sterile water. Sterilized cysts were placed in Petri dish with sterile deionized water, and then put into self-made hatching pond in sterile deionized water under 25^{0}C. After several days, fresh active J2 were collected for following tests.

In vitro Experiments

J2 viability:

The sensitivity of SCN J2 to the increasing concentrations of fertilizer sources, that is, plants necessary macroelements and microelements was examined *in vitro* experiments. The tested macroelement and microelement compounds were showed in Table 1. Tested compounds are all analytical reagent. All tested solutions were made up by deionized water, and were sterilized by autoclave for 30 min.

Using pipettor (2-20 μl), 20μl water with 50 J2 were added to each concentration of all tested solutions (1ml) in 1.5 ml polyethylene micro-centrifuge tubes, which were washed by deionized water and were sterilized by autoclave. Each treatment had three replicates, and sterilized deionized water was used in comparison with the test group.

After incubation in the assay solutions in the dark for 24h at 25^{0}C, nematodes were transferred to culture dish by pipettor. Dead SCN J2 was counted under a stereomicroscope. We used a uniform standard to estimate whether J2 were dead. Nematodes that

Table 1

The microelement and macroelement compounds and associated concentrations used for the analysis

Macroelement compounds and used concentrations	
$NaNO_3$	0.05M, 0.1M, 0.2M, 0.3M, 0.5M, 0.8M, 1M
$NaNO_2$	0.01M, 0.02M, 0.03M, 0.04M, 0.05M, 0.1M
Urea	0.01M, 0.03M, 0.05M, 0.08M, 0.1M, 0.3M, 0.5M
NH_4Cl	0.05M, 0.1M, 0.2M, 0.3M, 0.5M, 0.8M, 1M
$(NH_4)_2HPO_4$	0.01M, 0.03M, 0.05M, 0.08M, 0.1M, 0.3M, 0.5M
KCl	0.05M, 0.1M, 0.2M, 0.3M, 0.5M, 1M
$Na_2HPO_4 \cdot 12H_2O$	0.05M, 0.1M, 0.2M, 0.3M, 0.5M, 1M
KH_2PO_4	0.002M, 0.005M, 0.01M, 0.02M, 0.03M
$MgCl_2 \cdot 6H_2O$	0.02M, 0.05M, 0.1M, 0.2M, 0.3M, 0.5M, 1M
$CaCl_2 \cdot 2H_2O$	0.05M, 0.1M, 0.2M, 0.3M, 0.5M
Microelement compounds and used concentrations	
$Na_2B_4O_7 \cdot 10H_2O$	0.02M, 0.05M, 0.08M, 0.1M, 0.15M, 0.2M
$Na_2Mo_4O_4 \cdot 2H_2O$	0.05M, 0.08M, 0.1M, 0.15M, 0.2M, 0.3M, 0.4M, 0.5M
$ZnCl_2$	0.02M, 0.03M, 0.05M, 0.08M, 0.1M, 0.2M
$ZnSO_4 \cdot 7H_2O$	0.01M, 0.03M, 0.05M, 0.08M, 0.1M
$FeCl_3 \cdot 6H_2O$	0.0001M, 0.0002M, 0.0005M, 0.0008M, 0.001M, 0.002M
$FeSO_4 \cdot 7H_2O$	0.05M, 0.1M, 0.2M, 0.3M, 0.4M, 0.5M
$CuCl_2 \cdot 2H_2O$	0.0005M, 0.001M, 0.002M, 0.003M, 0.004M, 0.005M, 0.01M

were acting or moving were treated alive; however, those motionless ones were not always dead, because of their habit of mimic death. Motionless J2 were touched by eyelash-made needle continuously more than 30s. If J2 was active after being touched, it was alive, whereas dead. In generally, SCN J2 with straightly stiff body was dead.

J2 hatch: 20 plump cysts in each self-made hatching pond were immersed in tested compounds with concentration of LC50 tested through the *in vitro* experiment, in Petri dish (6 cm) containing 10 ml teat solution in 25^0C thermo tank. The number of hatched J2 was investigated under a stereomicroscope everyday, and the total amount of hatched J2 within 20 days was recorded. The same solution of each treatment was changed everyday and each treatment had three replicates.

Glasshouse Experiments

Plump and healthy seeds of the soybean cultivar Liao NO.11 were selected to process pregermination with water in Petri dish. After 7 days, seedlings with germ were transplanted into 14 cm diameter. Clay pots were filled with about 1kg (dry weight) soil collected from the test field of Plant Nematology Lab of Shenyang Agriculture University. 500g soil contained about 50 cysts, in other words, soil of each treatment contained 100 cysts before inoculation. Additionally, 30 cysts were inoculated into soil of each treatment. $4g \cdot kg^{-1}$ urea, $9g \cdot kg^{-1}$ $(NH_4)_2HPO_4$, $1g \cdot kg^{-1}$ KH_2PO_4 and $0.11g \cdot kg^{-1}$ $FeCl_3 \cdot H_2O$ solution (100 ml) were

Table 2
Influence of macroelement compounds on SCN J2

Treatment	LC50 (M)	LC90 (M)
$NaNO_3$	0.31 ± 0.0474^{e}	1.07 ± 0.317^{f}
$NaNO_2$	0.04 ± 0.0006^{b}	0.06 ± 0.002^{ab}
Urea	0.07 ± 0.0015^{c}	0.23 ± 0.003^{bc}
NH_4Cl	0.30 ± 0.0051^{e}	0.78 ± 0.010^{e}
$(NH_4)_2HPO_4$	0.07 ± 0.0023^{c}	0.21 ± 0.018^{abc}
KCl	0.30 ± 0.0151^{e}	0.91 ± 0.083^{ef}
KH_2PO_4	0.01 ± 0.0006^{a}	0.03 ± 0.004^{a}
$Na_2HPO_4{\cdot}12H_2O$	0.19 ± 0.0079^{d}	0.41 ± 0.006^{cd}
$MgCl_2{\cdot}6H_2O$	0.1863 ± 0.0127^{d}	0.565 ± 0.082^{d}
$CaCl_2{\cdot}2H_2O$	0.2307 ± 0.0085^{e}	0.530 ± 0.024^{d}

Means followed by the different letter(s) within a column in each block are significantly different ($p\leq 0.05$) according to Ducan's One-Way ANOVA.

mixed with *H. glycines* - infested soil, respectively. Soil of the control group was added with nothing. Treated soil were well-mixed and kept in house temperature for 3 days, then soybean seeds were planted into clay pots. According to the results of *in vitro experiments*, urea, $(NH_4)_2HPO_4$, KH_2PO_4 and $FeCl_3{\cdot}6H_2O$ were selected to be the tested candidates. Each treatment was replicated three times, including untreated inoculated pots served as the control groups. This experiment was completed in a glasshouse at 30 ± 5^0C and all received similar watering treatments.

35 days after inoculation, plants were removed from pots and numbers of white cysts on roots and data on roots growth were documented.

Data Analysis

Inhibition and Change was calculated as:

- Inhibition (%) = (total in control – total in treatment)/total in control×100%

- Change (%) = (total in treatment – total in control)/total in control×100%

LC50 and LC90 value were determined by probit regression analysis. One-way ANOVA was used to test for the differences between compounds. The statistical computer software, SPSS for window 15.0 was used to all statistical analyses.

Results

Influence of compounds on SCN J2 survival

Data in Table 2 indicated that among the compounds containing N-P-K and other macroelements (Mg and Ca), KH_2PO_4 was the best treatment for reducing the number of surviving J2 and achieving the lowest value of LC50 (0.01M) and LC90 (0.03M), *in vitro*. $NaNO_3$ was the least effective treatment. LC50 of compounds containing major elements were sequentially in the order

$KH_2PO_4 < NaNO_2 < (NH_4)_2HPO_4 =$ Urea $< MgCl_2{\cdot}6H_2O < Na_2HPO_4{\cdot}12H_2O < CaCl_2{\cdot}2H_2O < NH_4Cl = KCl < NaNO_3$.

LC90 of macroelement compounds were sequentially in the order

$KH_2PO_4 < NaNO_2 < (NH_4)_2HPO_4 <$ Urea $< Na_2HPO_4{\cdot}2H_2O < CaCl_2{\cdot}2H_2O < MgCl_2{\cdot}6H_2O < NH_4Cl < KCl < NaNO_3$.

Table 3
Influence of microelement compounds on SCN J2

Treatment	LC50 (5%) (M)	LC90 (5%) (M)
$Na_2B_4O_7 \cdot 10H_2O$	0.0920 ± 0.0053^{c}	0.141 ± 0.019^{c}
$Na_2Mo_4O_4 \cdot 2H_2O$	0.2227 ± 0.0097^{e}	0.569 ± 0.016^{d}
$ZnCl_2$	0.0453 ± 0.0006^{b}	0.080 ± 0.004^{b}
$ZnSO_4 \cdot 7H_2O$	0.0423 ± 0.0006^{b}	0.120 ± 0.006^{bc}
$FeCl_3 \cdot 6H_2O$	0.0004 ± 0.0000^{a}	0.001 ± 0.000^{a}
$FeSO_4 \cdot 7H_2O$	0.2880 ± 0.0026^{f}	0.552 ± 0.024^{d}
$CuCl_2 \cdot 2H_2O$	0.0080 ± 0.0104^{a}	0.005 ± 0.000^{a}

Means followed by the different letter(s) within a column in each block are significantly different ($p \leq 0.05$) according to Ducan's One-Way ANOVA.

In microelements compounds (B, Mo, Zn, Fe, and Cu) (Table 3), $FeCl_3 \cdot 6H_2O$ was the best treatment for reducing the number of surviving J2 and achieved the lowest value of LC50 (0.0004M) and LC90 (0.001M). LC50 of compounds containing microelements were sequentially in the order

$FeCl_3 \cdot 6H_2O < CuCl_2 \cdot 2H_2O < ZnSO_4 \cdot 7H_2O < ZnCl_2$ $Na_2B_4O_7 \cdot 10H_2O < Na_2Mo_4O_4 \cdot 2H_2O < FeSO_4 \cdot 7H_2O$. LC90 of compounds containing microelements were sequentially in the order

$FeCl_3 \cdot 6H_2O < CuCl_2 \cdot 2H_2O < ZnCl_2 < ZnSO_4 \cdot 7H_2O < Na_2B_4O_7 \cdot 10H_2O < FeSO_4 \cdot 7H_2O < Na_2Mo_4O_4 \cdot 2H_2O$.

Influence of compounds on hatching of SCN

Except for $ZnSO_4 \cdot 7H_2O$, all other tested compounds significantly inhibited the hatching of SCN at their concentration of LC50. $ZnSO_4 \cdot 7H_2O$ didn't inhibit hatching of SCN, and the number of hatched J2 was almost the same as that of control group. Inhibition efficiency of most compounds can reach over 90% and $NaNO_2$ the best one for inhibiting hatching with 100% efficiency. $ZnCl_2$ was least effective with efficiency 71.36% (Table 4).

Reproduction of SCN and soybean root growth with effective compounds

Compared with water treated control, urea significantly inhibited the germination of soybean seeds, while other three tested compounds had no effects on that (Table 5). All treatments inhibited bud growth. Except for urea, other treatments promoted the growth of soybean radical to different extents. In contrast, urea resulted in an adverse effect, seriously inhibiting the growth of radical.

The application of $FeCl_3 \cdot 6H_2O$ gave the best root growth (fresh weights), followed by $(NH_4)_2HPO_4$. Moreover, $(NH_4)_2HPO_4$ was the best treatment to improve dry weight of root. In general, the respective application of the four tested compounds was much more beneficial to soybean root growth at seedling stage. In glasshouse trial, we selected macroelement fertilizers which were applied as the fertilization in soybean field. $FeCl_3 \cdot 6H_2O$ was tested, because iron is an essential nutrient required by both host legume and bradyrhizobia for a range of physiological and biochemical processes. For nodule initiation of legume, iron is required either in the external solution or within the root (Tang et al., 1990).

Data in Table 6 indicated that all treatments were significantly effective in reducing the reproduction of SCN. $FeCl_3 \cdot 6H_2O$ treatment achieved the highest percentage of nematode inhibition (72.54%), followed by KH_2PO_4, $(NH_4)_2HPO_4$ and Urea. Thus $FeCl_3 \cdot 6H_2O$ was the best one for suppressing cyst production.

Table 4
Influence of effective compounds on hatching of eggs in cysts

Treatment	Hatched J2	Inhibition, %
$NaNO_3$	59.33±7.51f	90.77
$NaNO_2$	0.00±0.00k	100
Urea	11.67±1.53ij	98.18
NH_4Cl	36.33±7.57g	94.35
$(NH_4)_2HPO_4$	97.33±5.13c	84.85
KCl	65.00±6.56ef	89.88
KH_2PO_4	35.67±3.21g	94.45
$Na_2HPO_4 \cdot 12H_2O$	102.67±9.02c	84.02
$MgCl_2 \cdot 6H_2O$	75.33±8.50de	88.28
$CaCl_2 \cdot 2H_2O$	80.67±13.20d	87.45
$Na_2B_4O_7 \cdot 10H_2O$	21.67±1.53hj	96.63
$Na_2Mo_4O_4 \cdot 2H_2O$	15.00±5.29ij	97.67
$ZnCl_2$	184.00±4.00b	71.36
$ZnSO_4 \cdot 7H_2O$	648.00±8.00a	-0.83
$FeCl_3 \cdot 6H_2O$	21.00±2.65hj	96.73
$FeSO_4 \cdot 7H_2O$	5.00±2.00jk	99.22
$CuCl_2 \cdot 2H_2O$	27.67±3.51gh	95.7
CK	642.67±6.66a	-

Means followed by the different letter(s) within a column in each block are significantly different ($p \leq 0.05$) according to Ducan's One-Way ANOVA.

Discussion

Although there are some papers about the compound effect on nematode, this study is still one representative only based on the nematicidal nutrient compounds of plants. N-P-K, other macroelement and microelement fertilizers in soil may influence plant nutrient supply and cause many diseases (Ghorbani et al., 2008). The most commonly studied nutrient element in soil in relation to plant diseases is nitrogen. Soil phosphorus and potassium are also associated with disease development (Sullivan, 2001; Ghorbani et al., 2008). Contacting nematicidal action of chemical nutrient compounds of plant-parasitic nematodes has been reported (Agrios, 1997; Oka and Pivonia, 2002).

For SCN J2, the effective concentrations of tested compounds were different, LC50 of KH_2PO_4 and $FeCl_3 \cdot 6H_2O$ achieved the lowest among the tested macroelement and microelement compounds, respectively. The mechanism on the toxicity of these compounds is not clear at all. The toxic ammonia to organisms is attributed to its deleterious effect on the plasma membrane and its high pH. Free ammonia is known to cross the cell membrane and change the pH of cytoplasm. In contrast to ammonia, the nematicidal activity of nitrite is enhanced under the acid conditions; when nitrous acid (HNO_2) is dominate over its ionized form NO_2^-. Soils fertilized with NH_4-N can reduce the nematode damages on the soybean and bean by *Heterodera glycines* and *M.incognita* respectively (Oka and Pivonia, 2002). Generally, NO_2^-

Table 5

Influence of effective compounds on germination of seed and growth of soybean root

Treatment	Germination of seed					Growth of root			
	Germination, %	Length of bud, cm	Change, %	Length of radicle, cm	Change, %	Fresh weight, g	Change, %	Dry weight, g	Change, %
Urea	54.67a	5.52a	-34.48	0.12a	-96.34	1.20bc	62.16	0.27cd	35
$(NH_4)2HPO_4$	92.67b	6.30b	-33.65	3.61c	6.65	1.29c	74.32	0.28d	40
KH_2PO_4	92.67b	7.24c	-13.97	4.07d	3.96	1.19b	60.81	0.24bc	20
$FeCl_3 \cdot 6H_2O$	93.33b	7.61d	-9.58	4.52e	20.77	1.42d	91.89	0.22ab	10
CK	92.67b	8.42e	-	3.37b	34.22	0.74a	-	0.20a	-

Means followed by the different letter(s) within a column in each block are significantly different ($p \leq 0.05$) according to Ducan's One-Way ANOVA.

Table 6

Influence of effective compounds on reproduction of SCN

Treatment	Cysts/pot	Inhibition, %
Urea	282d	18.5
$(NH_4)2HPO_4$	252c	27.17
KH_2PO_4	213b	38.44
$FeCl_3 \cdot 6H_2O$	95a	72.54
CK	346e	-

Means followed by the different letter(s) within a column in each block are significantly different ($p \leq 0.05$) according to Ducan's One-Way ANOVA.

was considered the most toxic to nematodes among the macroelement compounds according to our in vitro results, which was consistent with the work of Mario (2004). There are uncertainties on the mechanism that how NO_2^- can be generated from the nitrous acid (HNO_2) with the effect of hydronium ions. Extremely low levels of HNO_2 can kill plant-parasitic nematodes (Oka et al., 1993) and other soil organisms (Tenuta and Lazarovits, 2002). Thus we did not select $NaNO_2$ in the glasshouse trial.

In the present study, $(NH_4)_2HPO_4$ and urea were significantly effective in reducing the reproduction of SCN in glasshouse trial. This is in agreement with the conclusion of that $(NH_4)_2HPO_4$ and urea showed nematicidal activities to *Meloidogyne javanica* (Oka and Pivonia, 2002). Urea has often been used in nematode control experiments and its nematocide activity has been confirmed (Rodriguez-Kabana and King, 1980).

Studies with other elements such as calcium, magnesium, iron, zinc and other micronutrients showed similar relationships between their levels in the soil and their susceptibility or resistance to certain diseases. The (Ca+Mg)/K ratio appears to play a role in several crops to root-knot nematode (*Meloidogyne incognita*) (Bains et al., 1984). However, some researchers thought that KCl and $CaCl_2$ did not affect the survival of nematode (Mario and Howard, 2004).

For SCN reproduction in the glasshouse, $FeCl_3 \cdot 6H_2O$ significantly reduced the number of cysts,

and gave the best soybean root growth. Positive metal ions such as Fe^{3+}, Cu^{2+}, and Mn^{2+} remarkably inhibit the hatching of SCN eggs, and they appeared to inhibit the germination of soybean seed and growth of soybean roots (Xing et al., 2002).

The hatching study demonstrated that at the concentration of LC50, only $ZnSO_4$ didn't inhibit the hatching of SCN. This is because that $ZnSO_4$ stimulated the hatching of SCN *in vitro*, while $ZnCl_2$ solution inhibited the hatching of SCN (Behm, 1995). Besides, Rademacher (1933) firstly found that $CaCl_2$ might stimulate the hatch of *H. schachtii*. Wallace (1956) reported that Ca^{2+}, Mg^{2+}, K^+, Na^+ could stimulate the hatch of *H. schachtii*, while Ca^{2+}, Mg^{2+} stimulated hatch of *H. schachtii* slightly (Rademacher and Schmidt, 1933; Wallace, 1956).. It seems that SCN hatching depends on the concentration of the tested compound. Under the tested concentrations of present study, almost all the compounds inhibited hatching.

The present results showed that a low concentration of urea, $(NH_4)_2HPO_4$, KH_2PO_4 and $FeCl_3 \cdot 6H_2O$ could cause death of J2 i*n vitro*, and these compounds significantly inhibited SCN reproduction in the glasshouse. Still we don't know whether these compounds can be suitable for controlling SCN. Field experiments for testing their efficiencies in practice are needed to design and carry out. In addition, the application of these compounds may have negative impacts on the environment. Further studies are warranted to examine the environmental impacts and the effect of soil conditions on the nematicidal activity of these compounds.

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