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Effect of N-Viro soil and Amonia (NH3) on Egg Hatch of Soybean Cyst Nematode (SCN), Heterodera glycines Ichinohe

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Abstract

The soybean cyst Nematode (SCN), *Heteroderaglycines* Ichinohe, is a devastating root pathogen of soybean. Symptoms of SCN can be confused with the symptoms of nutrient deficiencies, and chemical. Infected plants may exhibit chlorosis or be stunted. Chlorosis is caused primarily due to N deficiency as a result of suppression of *Rhizobium* nodule formation by the nematode infection.NVS is a municipal biosolid product in which human pathogens killed by an alkaline stabilization process combining alkaline pH, drying, high temperature, high ammonia and salts. Clear understanding of the mechanisms of egg hatch and environmental and chemical factors that influence egg hatch must be known to develope an effective management tactics for SCN. In this study, effects of N-viro leachates extracted from varying ages of N-viro soils and the impact of different concentrations of ammonia on the egg hatch of SCN in vitro were investigated. Age of N-viro soil was positivelly correlated with percent egg hatch of scn *in vitro*. Egg hatch of SCN was suppressed by the leachates from 0 to 1 month-old NVS in which cummulative percent egg hatch was 0, and 1.48, respectivelly. Egg hatch was not affected in leachates from 3, 6, and 12 month-old NVS or in 3 mM zinc sulfate solution compared to distilled water at 24 hr. Average cumulative percent egg hatch was 3.36, 3.52, and 4.60 in leachates of 3, 6, and 12 month-old NVS, respectively, at 24 hr. All the concentrations of ammonia (0.001, 0.01, and 0.1 M) significantly ($P \leq 0.01$) suppressed emergence of J2 juveniles from SCN eggs compared to distilled water and 0.02 M phosphate buffer at 48 hr.

Keywords: Soybean Cyst Nematode (SCN), *Heteroderaglycines*, N-viro soil (NVS), egg hatch,

INTRODUCTION

Sustainable management of Soybean Cyst Nematode (SCN), *H.glycines* is difficult because it survives as eggs in its protective cyst [1].*In vitro* research to manipulate egg hatch of *H. glycines*has had much attention for decades. Several swine manure products and breakdown products, such as indole, 4-ethyl phenol, butylated hydroxytoluene, and 4-amino acetophenone at 1one mM and 3-methyl indole and 4-methyl phenol at 2 mM, were evaluated to determine the effects on eggs and J2 juveniles of *H. glycines* in airtight containers [2]. The study above showed that contact with 3-methyl phenol and indole inhibited the egg hatch, but volatiles from indole, 4-ethyl and 4-methyl phenol stimulated the hatch and body movement of J2 juveniles after the hatch. Breakdown products of secondary plant metabolites, glucosinolates such as cyanohydroxipropene and cyanohydroxipropene propionate at 10 and 100µg/mL resulted in 0.4% and 4.6% hatch of *H. glycine* eggs of that occurring in distilled water after 24 days [3]. In the work above, egg hatch of SCN was irreversibly inhibited because transfer of eggs from glucosinolate breakdown products to deionized water or 3 mM zinc sulfate did not cause any further eggs to hatch after 24 days. Another study [4] reported that several analogs of glycinoeclepin A, a natural hatching stimulus of SCN inhibited the egg hatch of SCN. Thus, they suggested that inhibition of egg hatch was due to maximum functionality of a ketodiacid.

Zinc salts have been reported to be very active hatching stimulants for SCN eggs in vitro [5] and [6]. Three mili molar concentration of zinc sulfate stimulated emergence of J2 juveniles of SCN from encysted eggs and free eggs compared to distilled water [7]. In another study, strong and moderate increase in egg hatch of SCN was observed when free eggs were incubated in zinc chloride and zinc sulfate solutions compared to distilled water [8].

N-Viro Soil (NVS) is a product of an advanced alkaline stabilization process for municipal biosolids[9]. A great majority of inorganic nitrogen in NVS is in the form of ammonium (NH_4^+), though oxidized forms such as nitrate(NO_3^-) are low. Although 99 % of an NH_3-NH_4 mixture is in the form of NH_4^+ at neutral pH or lower, above pH 11 over 98 % of the mixture is free NH_3 at equilibrium [10], [11]. Due to pH decrease and volatilization with time in NVS, free NH_3 drops rapidly. As a result, NH_3 is converted into NH_4^+ and immobilized by actively growing microorganisms [11].

Free NH_3 dissolved in water (ammonia) is very toxic to most soil microorganisms [9] and aquatic animals [10]. In a factorial experiment, cations $NH4^+$, Ca^{++}, and Mg^{++} were used in combination with each of the anions NO_3^-, $SO_4^=$, and Cl^- to evaluate the effects of those inorganic ions on egg hatch of SCN [12]. In the experiment above, NH_4^+ or NO_3^- containing compounds inhibited egg hatch compared to phosphate buffer. The results of our previous experiments in the greenhouse showed that roots of soybean 'Corsoy 79' grown in soil infested with 2,000 eggs and J2 juveniles of SCNrace 3formed fewer cysts and eggs than the control plants when they were treated with 1.27 and 6.35 g NVS per 150 mL sand. These results led to further investigation of the effects of the N-Viro leachates extracted from varying ages of N-Viro soils and the impact of different concentrations of ammonia on the egg hatch of SCN *in vitro*.

MATERIALS AND METHODS

Materials. N-Viro Soil (NVS) was obtained from the Bayview N-Viro facility, Toledo, OH.

Source of SCN. Soybean Cyst Nematode, *Heteroderaglycines* race 3 was maintained on the roots of soybean 'Corsoy 79' grown in 15 in. pots in greenhouse and used as a source of eggs for hatching experiments.

Extraction of N-Viro Leachate. NVS was dumped in the field at the Waterman Farm, The Ohio State University, Columbus. The pile was 2 m long, 1 m wide, and 0.5 m high. The NVS soil cores were collected from the interior of the pile, 15 cm deep, at 4 different places, 0, 1, 3, 6, 12 months later. The NVS samples were kept at 5℃ until used.

Water extract of NVS samples were collected with a mechanical extractor (Model 24-01, Centurion International Inc., Lincoln, NE) using a 60 ml syringe [13]. Ten g of NVS was placed in the sample tube. Approximately 55 mL of water extract was collected over 12 hr. The NVS extract was kept at 5℃ to use in future experiments.

pH of NVS Extracts. pH of NVS extracts was determined with a digital pH meter (Model 430, Corning Incorporated, Science Product Division, Corning, NY) by inserting an electrode into the extracts in 100 ml plastic bottle. Due to the very limited amount of NVS leachates, 2mL/replicate, NVS leachates from the same treatments were collected and pH of the total sample was determined. However, statistical analysis was not performed on pH of NVS extracts because there were not enough samples to be analyzed.

Effect of the Leachates Collected from Various Ages of NVS. Fifty mL of soil containing cysts of SCN were removedfrom root zones of actively growing soybean 'Corsoy 79'. Soil was dumped in 5 L water in a 10 L plastic beaker. The water was stirred vigorously by hand in order to resuspend the cysts. The soil was allowed to settle in the bottom of the beaker for 10 sec. Supernatant water was poured through an 850 µm-pore (20 mesh) sieve nested over 250 µm-pore (60 mesh) sieve. The 850 µm-pore sieve was removed, and cysts collected on the 250 µm-pore sieve washed into a 250 mL glass beaker with 30 mL tap water. The cysts were rinsed into a 40 mL Ten-Broeck cyst homogenizer and crushed. The suspension was poured onto a nested 250 µm-pore (60 mesh) sieve over 75 µm-pore (200 mesh) sieve over 25 µm-pore (500 mesh) sieve, and the pestle was rinsed onto the 250 µm-pore sieve. The top sieves were removed. Eggs and J2 juveniles of SCNon the 25 µm-pore (500 mesh) sieve were washed in to a clean 250 mL glass beaker. Concentration of the suspension was standardized to 65 eggs / mL suspension by adding tap water to the suspension.

One mL aliquots containing approximately 65 eggs from continuously stirred suspension in 250 mL beaker were taken by a 5 mL pipette and poured into a small 3 cm-diameter plastic petri dishes. Eggs were allowed to settle in the bottom of the petri dishes for 10 min. Water in the dishes was removed carefully with a Pasteur pipette without disturbing the eggs on the bottom. Petri dishes were filled with 2 mL leachate of 0, 1, 3, 6, 12-month-old N-viro soil, distilled water or 3 mM zinc sulfate, pH 7. Final volumes of the treatment solutions and checks were 2mL. Treatments were replicated 5 times. There were a total of 35 petri dishes for each experiment. Petri dishes were placed into two plastic trays on tissue paper separately for each experiment. The petri dishes were kept at laboratory condition under 8/16 hrs light/dark illumination at 25℃ on a lab bench. Percent egg hatch were determined by counting the second stage juveniles using a stereomicroscope (40X) at the beginning of test, 24, 48 and 72 hr after the test was initiatedstarted. Data were reported as cumulative hatch at 0, 24, 48 and 72 hr.

Effect of Ammonia (NH_3) on Egg Hatch of SCN. Ammonia solutions, 0.001, 0.01, and 0.1 M and 0.02 M phosphate buffer solution and 3 mM ZnSO4 solution were prepared in advance.

Eggs and J2 juveniles of SCN were extracted from 200 mL soil infested with cysts as described above. Concentration of eggs in the 200 mL tap water was adjusted to 80 eggs /mL suspension. One mL from continuously stirred egg suspension was pipetted into a 3 cm diameter plastic petri dish. The eggs were allowed to settle in the bottom of the petri dish for about 20 min. One mL tap water in the dishes were removed and replaced by 2 mL solution of the treatments 0.1, 0.01, 0.001 M ammonia, 0.02 M phosphate buffer, 3mM $ZnSO_4$ and distilled H_2O with a Pasteur pipette. There were seven replicates of each treatment and positive and negative controls, 3mM zinc sulfate and distilled water, respectively.

Eggs were counted immediately in each replicate. Emerged J2 juveniles of SCN were counted at time 0, 24, 48 and 72 hrs. Each treatment had approximately 50-100 eggs per hatching dish. Data were reported as cumulative hatch at 0, 24, 48 and 72 hr.

ANOVA was performed on the means of each treatment and checks. Treatment means were separated by Fisher's Pairwise Comparison Test.

RESULT

pH of NVS Extracts. pH of leachates from 0 and 1 mo-old NVS declined from 13 and 12.39 to 8.16 and 8.34, respectively (Table 1). Change between initial pH and final pH of leachates from 3, 6, and 12 mo-old NVS was less than one pH level (pH initial, 8.18, 8.06 and 8.08 and pH final, 8.90, 8.86 and 8.83, respectively) at 72 hr. PH of 3 mM zinc sulfate in 0,02 M phosphate buffer was the most consistent and did not change significantly at the beginning and at the end of the experiment (pH initial, 7.00 and pH final 7.11) although pH of distilled water showed a 0.92 unit increase at the end of experiment (Table 1).

Table 1.pH of leachates of 0,1, 3, 6, and 12 mo-old N-Viro soil and positive and negative control solutions determined at 0 hr and 72 hr (3mM $ZnSO^4$ and distilled water,-respectively).

NVS leachates[a]	pH Initial (0 hr)[b]	pH Final (72 hr)
0	13.00	8.16
1	12.39	8.34
3	8.18	8.92
6	8.06	8.87
12	8.08	8.85
distilled H_2O	7.03	7.95
3 mM $ZnSO_4$	7.00	7.11

[a]Water extract of 0, 1, 3, 6, and 12 mo-old NVS samples were collected with a mechanical extractor (Model 24-01, Centurion International Inc., Lincoln, NE) using a 60 ml syringe (Jaynes and Bigham, 1986) on 5 Jan. 2000.

[b]pH of NVS extracts were determined with a digital pH meter (Model 430, Corning Incorporated, Science Product Division, Corning, NY 14831) by inserting an electrode into the extracts in 100 mL plastic bottle at the beginning (0 hr) and end of the experiment (72 hr).

Effect of the LeachatesLeachates Collected from Various Ages of N-Viro Soil. Age of N-Viro soil was positively correlated with percent egg hatch of SCN *in vitro* (Table 2) ($P \leq 0.05$). Egg hatch of SCN was suppressed by the leachates from 0 and 1 mo-old NVS in which cumulative percent egg hatch was 0, and 1.48, respectively. Egg hatch was not affected in leachates from 3, 6, and 12 mo-old NVS or in 3 mM zinc sulfate solution compared to distilled water at 24 hr(Table 2). Average cumulative percent egg hatch was

3.36, 3.52, and 4.60 in leachates of 3, 6, and 12 mo-old NVS, respectively, and 3.16 and 5.02 in distilled water and in 3 mM zinc sulfate, repectively, at 24 hr.

Egg hatch was suppressed in leachates from 0 and 1 mo-old NVS but was not affected in leachate from 3, 6, 12 mo-old NVS compared to distilled water at 48 hr. However, egg hatch was significantly stimulated by 3 mM zinc sulfate solution at 48 hr. Cumulative percent egg hatch was 0, 1.64, 3.55, 5.02, and 5.56 in leachates from 0, 1, 3, 6, and 12 mo-old NVS, respectively. It was 3.29 and 6.32 in distilled water and 3mM zinc sulfate solution, respectively, at 48 hr (Table 2).

A greater suppression of emergence of J2 juveniles of SCN was observed in leachate from 0 than in leachate from 1 and mo-old NVS compared to distilled water at 72 hr. Leachates of 6 and 12 mo-old NVS significantly stimulated egg hatch compared to distilled water at 72 hr. Three mili molar zinc sulfate solution caused the greatest stimulation of egg hatch compared to distilled water at 72 hr. Leachates of 0, 1, 3, 6, and 12 mo-old NVS, and distilled water and 3 mM zinc sulfate solution resulted in 0, 2.57, 4.74, 7.47, 7.58, 4.32 and 9.02 % cumulative egg hatch at 72 hr (Table 2).

Table 2. Average cumulative hatch (%) of SCN eggs incubated in leachates of 0, 1, 3, 6, and 12 month-old N-Viro (NVS) soil, distilled water and $ZnSO_4$ solution at 25 °C.

Treatments	Average Cumulative Egg Hatch (%)[a]			
NVS leachates	0	24	48	72 hr[b]
0	0a	0a	0a	0a
1	0a	1.48a	1.64a	1.81b
3	0a	3.36 b	3.55 b	3.96 b
6	0a	3.52 b	5.02 b	5.70 e
12	0a	4.60b	5.56 b	5.57 d
distilled H_2O	0a	3.16 b	3.29b	3.59 c
3 mM $ZnSO_4$	0a	5.02 b	6.32 c	7.03 e
$LSD_{(0.05)}$	0	1.89	2.22	1.53

[a] Average cumulative egg hatch (%) was calculated by adding J2 juvenile numbers at that particular time dividing it by the total number of eggs at the beginning of the experiment for each replicate and multiplying it by 100. Then, average percent cumulative egg hatch for each replicate was added up and divided by number of replicates for each experiment.

[b] Number of emerged J2 juveniles of SCN was counted under a stereomicroscope at 40 X at 0, 24, 48 and 72 hr and recorded.

Effect of Ammonia (NH_3) on Egg Hatch. Egg hatch of SCN was not suppressed by 0.001, 0.01 and 0.1 M ammonia solutions at 24 hr (Table 3). However, egg hatch of SCN was significantly stimulated in 3 mM zinc sulfate solution compared to that in distilled water and in 0.02 M phosphate buffer solution at 24 hr. Cumulative egg hatch was 1.68, 2.11, and 1.47 in 0.001, 0.01 and 0.1 M NH3 solutions. It was 1.98, 1.85, and 4.55 in distilled water, 0.02 M phosphate buffer and 3 mM zinc sulfate solution, respectively, at 24 hr (Table 3).

All the concentrations of ammonia (0.001, 0.01, and 0.1 M) significantly (*P≤0.01*) suppressed emergence of J2 juveniles from SCNeggs compared to distilled water and 0.02 M phosphate buffer at 48 hr. Emergence of J2 juveniles increased significantly (*P≤0.01*) in 3 mM zinc sulfate compared to that in distilled water and in 0.02 M phosphate buffer at 48 hr. Cumulative percent egg hatch was 2.58, 2.55, 1.90, 3.43, 3.19, and 5.87 in 0.001, 0.01 and 0.1 M ammonia, distilled water, 0.02 M phosphate buffer and 3 mM zinc sulfate solution, respectively, at 48 hr (Table 3).

The greatest suppression of emergence of J2 juveniles occurred in 0.01 and 0.1 M ammonia solution in 0.02 M phosphate buffer although a greater suppression of egg hatch was observed in 0.001 M ammonia solution compared to distilled water and 0.02 M phosphate buffer solution at 72 hr. Egg hatch of SCN was significantly (*P≤0.01*) induced by 3 mM zinc sulfate solution compared to distilled water and 0.02 M phosphate buffer at 72 hr. Cumulative egg hatch at 72 hr was 3.91, 3.50, 2.23, 5.54, 5.46, 8.93 in 0.001, 0.01 and 0.1 M ammonia solutions and in distilled water, 0.02 M phosphate buffer and in 3 mM zinc sulfate solution, respectively, at 72 hr (Table 3).

Table 3. Average cumulative hatch (%) of SCN eggs incubated in 0.1, 0.01, 0.001 M concentrations of ammonia, 3 mM $ZnSO_4$ and distilled water at 25 °C.

Treatments	Average Cumulative Egg Hatch (%)[a]			
	0	24	48	72 (hr)[b]
0.001 mM NH_3	0a	1.68a	2.58a	3.91b
0.01 mM NH_3	0a	2.11a	2.55a	3.50a
0.1 mM NH_3	0a	1.47a	1.90a	2.23a
Distilled H_2O	0a	1.98a	3.43b	5.54c
0.02 M Phosphate Buffer	0a	1.85a	3.19b	5.46c
3 mM $ZnSO_4$	0a	4.55b	5.87c	8.93d
$LSD_{(0.05)}$	0	1.02	1.27	1.54

[a] Average cumulative egg hatch (%) was calculated by adding the total egg hatch at that particular time dividing it by the total number of eggs at the beginning of the experiment for each replicate and multiplying it by 100. Then, average egg hatch for each replicate was added up and dived by number of replicates for each experiment.

[b] Number of emerged J2 juveniles of SCN was counted under a stereomicroscope at 40 X at 0, 24, 48 and 72 hr and recorded.

DISCUSSION

In this study, egg hatch of SCN was suppressed by leachates from 0, and 1 mo-old NVS at 24, 48, and 72 hr. In another study, 0 (fresh), 1, 3, 6 mo-old NVS contained 194.2, 46.6, 8.0 and 2.3mg/L ammonia, although no ammonia was detected in leachate of 12 mo-old NVS [11]. Anhydrous ammonia is very toxic to soil microorganisms and animals [10]. since un-ionized ammonia crosses cell membranes freely and causes irreversible death. Because leachates from 0 and 1 mo-old NVS has relatively higher amounts of ammonia than the leachates of 3 mo-old or older NVS, it is possible that high ammonia concentration caused the death of J2 juveniles inside eggs and thus inhibited egg hatch. There is also a small possibility that high pH of leachates from 0 and 1 mo-old NVS, 13 and 12.39, respectively, might be accounted for suppression of egg hatch at 24, 48, and 72 hr in vitro in the experiments.

Although egg hatch was not affected by leachates of 1, 3, 6, 12 mo-old NVS, and 3 mM zinc sulfate, it was suppressed by leachates of 0 and 1 mo-old NVS at 24 hr (Table 2). Egg hatch of SCN was suppressed by leachates from 0 and 1 mo-old NVS although leachates from 3, 6 and 12 mo-old NVS did not have any effect on egg hatch compared to distilled water at 48 hr. Zinc sulfate at 3 mM concentration significant increase in egg hatch at 48 hr.

NVS has a relatively high Zn content (500 mg/L). In general, at higher pH, solubility of Zn is limited by absorption on oxides and aluminao silicates, and precipitate as Zn oxide, hydroxide, and hydrocarbonate. Becauseleachates

from 0 and 1 mo-old NVS had relatively high pH (13 and 12.39, respectively) Zn cannot be detected. More Zn can be detected at lower pH. Relatively higher mobility of Zn in leachates of 6 and 12 mo-old NVS possibly hatched more SCN eggs since zinc is a natural hatching stimulus for SCN.

All three different concentration of ammonia (0.001, 0.01 and 0.1 M) did not have any effect on egg hatch of SCN compared to distilled water and 0.02 M phosphate buffer, although 3 mM zinc sulfate significantly increased egg hatch at 24 hr. Ammonia solutions at 0.001, 0.01 and 0.1 M concentrations caused an equal suppression of egg hatch compared to distilled water and 0.02 M phosphate buffer at 48 hr albeit 3 mM zinc sulfate solution significantly induced egg hatch of SCN at 48 hr.

At 72 hr., ammonia at 0.1 and 0.01M concentrations caused a greater suppression of egg hatch than at 0.001 M concentration compared to distilled water and 0.02 M phosphate buffer although 3 mM zinc sulfate significantly stimulated egg hatch of SCN at 72 hr.

All the three concentrations of ammonia were found to be suppressive to SCN eggs at 48 and 72 hr*in vitro*. Toxicity of anhydrous ammonia to microorganisms and animals has been reported (Warren, 1962). In a factorial experiment, Lehman et al. (1971) used cations $NH4^{+}$, Ca^{++}, and Mg^{++} in combination with each of the anions $NO3^{-}$, $SO4^{=}$, and Cl^{-} to evaluate the effects of those inorganic ions on egg hatch of SCN, *H. glycines*. In that experiment, NH_4^+ or NO_3^- containing compounds significantly inhibited egg hatch compared to phosphate buffer. Inhibition of egg hatch of SCN in three different ammonia concentrations in our*in vitro* experiments agrees with results of [12].

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