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Assessing the Effects of ILeVO and VOTiVO Seed Treatments on Reproduction, Hatching, Motility, and Root Penetration of the Soybean Cyst Nematode, Heterodera glycines

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Abstract

Successful management of the soybean cyst nematode *Heterodera glycines* is limited by increased virulence of nematode populations on resistant soybean cultivars and persistence of the nematode in the soil in the absence of hosts. Seed treatments are now available for *H. glycines* management. However, it is unclear how these treatments affect specific life stages of the nematode. The objectives of this study were to assess the effects of ILeVO (with active ingredient fluopyram) and VOTiVO (with active ingredient *Bacillus firmus* I-1582) seed treatments on *H. glycines* reproduction and important processes in the nematode life cycle, such as second-stage juvenile (J2) hatching, motility, and root penetration. The effects of seed treated with formulated (ILeVO and VOTiVO) and nonformulated active ingredient (fluopyram and *B. firmus* I-1582) on *H. glycines*reproduction were conducted in a greenhouse. Nematode reproduction on plants grown from seed treated with ILeVO or technical fluopyram (active ingredient only) was reduced by 35 to 97% relative to the nontreated control, suggesting that the fluopyram active ingredient was affecting *H. glycines* directly and was not an inert ingredient in the seed treatment formulation. Hatching, motility, and root penetration experiments also were conducted using only the formulated seed treatments. Exudates collected from ILeVO-treated seed reduced J2 hatching and motility by more than 95% in laboratory assays. Exudates from radicles grown from ILeVO-treated seed reduced hatching in vitro by 48% in one run but had no significant effect in the second run compared with the nontreated control exudates. There also were no consistent effects of radicle exudates, regardless of treatment, on hatching and motility of the J2. ILeVO reduced root penetration of *H. glycines* J2 at different inoculation densities in a growth chamber experiment. VOTiVO did not affect any of the processes or life stages of the nematode studied. The results of this study indicate that the use of nematode-protectant seed treatments may be useful in controlling *H. glycines*; however, additional investigations into the precise effects of ILeVO and VOTiVO on *H. glycines* life processes and in different parts of the soil profile are necessary.

Disciplines

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Comments

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Assessing the Effects of ILeVO and VOTiVO Seed Treatments on Reproduction, Hatching, Motility, and Root Penetration of the Soybean Cyst Nematode, *Heterodera glycines*

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Abstract

Successful management of the soybean cyst nematode *Heterodera glycines* is limited by increased virulence of nematode populations on resistant soybean cultivars and persistence of the nematode in the soil in the absence of hosts. Seed treatments are now available for *H. glycines* management. However, it is unclear how these treatments affect specific life stages of the nematode. The objectives of this study were to assess the effects of ILeVO (with active ingredient fluopyram) and VOTiVO (with active ingredient *Bacillus firmus* I-1582) seed treatments on *H. glycines* reproduction and important processes in the nematode life cycle, such as second-stage juvenile (J2) hatching, motility, and root penetration. The effects of seed treated with formulated (ILeVO and VOTiVO) and nonformulated active ingredient (fluopyram and *B. firmus* I-1582) on *H. glycines* reproduction were conducted in a greenhouse. Nematode reproduction on plants grown from seed treated with ILeVO or technical fluopyram (active ingredient only) was reduced by 35 to 97% relative to the nontreated control, suggesting that the fluopyram active ingredient was affecting *H. glycines* directly and was not an inert ingredient in the seed treatment formulation. Hatching, motility, and root penetration experiments also were conducted using only the formulated seed treatments. Exudates collected from ILeVO-treated seed reduced J2 hatching and motility by more than 95% in laboratory assays. Exudates from radicles grown from ILeVO-treated seed reduced hatching in vitro by 48% in one run but had no significant effect in the second run compared with the nontreated control exudates. There also were no consistent effects of radicle exudates, regardless of treatment, on hatching and motility of the J2. ILeVO reduced root penetration of *H. glycines* J2 at different inoculation densities in a growth chamber experiment. VOTiVO did not affect any of the processes or life stages of the nematode studied. The results of this study indicate that the use of nematode-protectant seed treatments may be useful in controlling *H. glycines*; however, additional investigations into the precise effects of ILeVO and VOTiVO on *H. glycines* life processes and in different parts of the soil profile are necessary.

The soybean cyst nematode, *Heterodera glycines* Ichinohe, is the most damaging pathogen of soybean (*Glycine max* (L.) Merr.) in the United States (Allen et al. 2017). Growing nonhost crops such as maize (*Zea mays* L.), and soybean cultivars with resistance to the nematode can reduce *H. glycines* reproduction and soybean yield loss (Tylka et al. 2016). However, these management tactics have limitations. Eggs of the nematode can persist in the soil for many years, meaning that yield-suppressing densities of *H. glycines* may be present even after nonhost crops are grown several times in infested fields (Howard et al. 1998). Additionally, the incidence of virulent populations of *H. glycines* capable of reproducing on soybean cultivars containing the commonly used Plant Introduction (PI) 88788 source of resistance has increased in recent years (McCarville et al. 2017; Mitchum et al. 2007; Niblack et al. 2008). Long-term control of *H. glycines* requires incorporating additional management tactics along with nonhost crops and resistant soybean cultivars into an integrated management plan. An added management option is nematicides, which can reduce *H. glycines* population densities and increase soybean yields (Epps and Young 1981; Sasser and Uzzell 1991). The use of soil-applied nematicides often is not economical, and a number of the older products are no longer available due to environmental concerns (Ristaino and Thomas 1997; United States Environmental Protection Agency 1993). The limitations of soil-applied nematicides have led to increased interest in developing seed treatments for *H. glycines* management.

Nematode-protectant seed treatments contain active ingredients (AI) that protect against plant damage caused by plant-parasitic nematodes. The mode of action of these treatments varies; some affect nematodes directly whereas others protect plants indirectly via mechanisms such as inducing plant defense responses. The advantages of using seed treatments over soil-applied pesticides includes reduced amounts of AI used per hectare, the ability to add multiple different treatments to the same seed (i.e., fungicides, nematicides, and insecticides), and reduced effects of the pesticides on nontarget organisms (Munkvold et al. 2014). The first published report of using seed treatments to control plant-parasitic nematodes was in 1960 (O'Bannon and Reynolds 1960). The AI in the seed treatments examined since the initial report have been both chemical (Gray and Soh 1989; Rodriguez-Kabana et al. 1977) and biological (Oostendorp and Sikora 1989; Zuckerman et al. 1993). Only since 2006 has nematode-protectant seed treatments become available for row crops like maize, cotton (*Gossypium hirsutum* L.), and soybean. There are currently several seed treatment options for *H. glycines* management, with more expected to come in the near future. Two current options are ILeVO (fluopyram; Bayer CropScience, Inc.) and VOTiVO (*Bacillus firmus* I-1582*;* Bayer CropScience, Inc.).

The performance of nematode-protectant seed treatments has been inconsistent in field studies, as measured by increased yields or decreased nematode reproduction (Barham et al. 2005; Tylka et al. 2015; Wheeler et al. 2013). The variable performance may be attributable to varying environmental conditions (Wheeler et al. 2013) that are largely unpredictable, making it difficult to forecast the potential economic return when using a seed treatment to manage plant-parasitic nematodes (Gaspar et al. 2014). It also is unclear how initial nematode population densities in the soil affect the performance of nematode-protectant seed treatments.

The effects of these treatments on the biology of *H. glycines* are not well understood. That is, it is unclear what life stages and processes of the nematode life cycle are affected by various nematode-protectant seed treatments and how long these treatments remain active. Determining how nematode-protectant seed treatments act on *H. glycines* will likely increase our ability to take advantage of this novel management tactic, potentially explain some of the field variability observed with these products, and direct future efforts to develop new seed treatments.

In addition to the specific effects of seed treatments on nematode biology, the movement of seed treatment AI through the seed and

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root zones of plants is not well understood, and likely varies depending on the specific product. There currently are no studies documenting the effects of seed treatments on root exudates. Faske and Starr (2007) found that abamectin seed treatment resulted in cotton root protection against *Meloidogyne incognita* but it was unclear whether this effect was due to the abamectin moving through the soil or on the root. It is possible that compounds or biological AI move on or through the root and are present at concentrations high enough for nematode protection. This hypothesis is particularly relevant for systemic compounds such as fluopyram.

The objectives of this study were to (i) assess the effects of ILeVO and VOTiVO seed treatments on reproduction of *H. glycines* under greenhouse conditions, (ii) determine the effect of seed and radicle exudates from treated seed on second-stage juvenile (J2) hatching and motility, and (iii) determine the effect of the seed treatments on *H. glycines* J2 root penetration at different inoculation densities and incubation times.

Materials and Methods

Seed treatments and source of *H. glycines* inoculum. Soybean cultivars susceptible (cultivar Williams 82) and resistant (cultivar Jack, PI 88788) to *H. glycines* were treated by Bayer CropScience personnel. Treatments included *B. firmus* I-1582 and fluopyram as either the formulated product (VOTiVO or ILeVO) or the nonformulated AI alone. The nonformulated product contained only the AI with none of the carriers or polymers typically found in formulated seed treatments. Seed were treated with *B. firmus* I-1582 or fluopyram at the commercial rate of 5 million spores/seed or 0.15 mg/seed, respectively. There were no colorants added to seed treated with formulated or nonformulated product. An HG Type 0 *H. glycines* population (Niblack et al. 2002) collected from an infested field (Fruitfield coarse sand soil, pH 7.6, and 3.9% organic matter) in Muscatine, IA, was used for the experiments and was maintained in the greenhouse on susceptible soybean (Williams 82).

Greenhouse experiments. Two separate experiments were conducted in the greenhouse with different initial population densities of *H. glycines*. One experiment used the field soil described above, with a population density of 4,000 eggs per 100 cm^3 of soil. In the other experiment, the field soil was diluted 1:3 with construction sand to create an initial *H. glycines* population density of 1,000 eggs per 100 cm^3 of soil. The 4,000 eggs per 100 cm^3 of soil will be referred to as moderate *H. glycines* population density soil and the 1,000 eggs per 100 cm^3 of soil will be referred to as the low *H. glycines* population density soil, based on current extension guidelines (Tylka 2012).

Both the formulated (VOTiVO and ILeVO) and nonformulated (*B. firmus* I-1582 and fluopyram) seed treatments were tested. Experiments were set up as a complete factorial and arranged in a randomized complete block design. Factors were soybean cultivar (Williams 82 and Jack) and seed treatment (VOTiVO, *B. firmus* I-1582, ILeVO, fluopyram, and nontreated control). Seed were planted in plastic cone-tainers (Stuewe and Sons, Inc.) containing 150 cm^3 of soil. To regulate temperature, cone-tainers were placed in buckets filled with construction sand and incubated in a greenhouse water bath set to 27°C. Each bucket served as a block and contained all cultivar–seed treatment combinations. Plants were watered every 2 to 3 days. Thirty days after planting, *H. glycines* females were dislodged from roots using a stream of water and were collected on a 250-μm-pore sieve. Roots were dried in an oven at 70°C for at least 24 h and their weights recorded. Females were counted on a AZ100 Multizoom microscope (Nikon Corp.) at ×10 magnification before they were crushed with a motorized rubber stopper (Faghihi and Ferris 2000) to release the eggs, which were collected on a 25-μm-pore sieve. The eggs were stained with acid fuchsin (Niblack et al. 1993) and counted under the same microscope at ×20 magnification. Each of the two different experiments contained seven replications of each cultivar–seed treatment combination, and each experiment was conducted twice.

Collection of seed and radicle exudates. Seed and radicle exudates were collected from treated (VOTiVO and ILeVO) and nontreated susceptible soybean seed (Williams 82) for J2 hatching and motility experiments. A method adapted from other studies using seed and radicle exudates was used (Riga et al. 2005; Zhao et al. 2000). The previously published methods collected seed or radicle exudates in sterile water at room temperature. In our studies, exudates were collected by placing seed in a 50-ml beaker with a ratio of one seed per 5 ml of sterile, distilled water. Radicle exudates were collected similarly by germinating seed on 1.5% water agar for 3 days at 25°C, then placing the radicles (5 to 7 cm in length) of intact seedlings in a 50-ml beaker with a ratio of one radicle per 5 ml of sterile water. The seed and seed coat were suspended on cheesecloth and did not touch the water during radicle exudate collection. The beakers were agitated on a platform shaker for 1 h at 100 rpm at room temperature (approximately 23°C), and exudates were filtered through 30-μm-pore nylon mesh after collection to remove debris. To compare the effects of exudate sterility on *H. glycines* in the J2 hatching and juvenile motility experiments, half of the exudates were filter sterilized by passing the solutions through a 0.22-μm-pore filter. The filtering was done primarily to determine if the presence or absence of *B. firmus* I-1582 cells in the VOTiVO exudates affected hatching. All exudates were stored at 4°C and used within a week of collection.

Hatching experiments. *H. glycines* females and cysts (dead females) were collected from 4- to 8-week-old *H. glycines*-infected soybean (Williams 82) by dislodging the nematodes from roots using a stream of water and collecting them on a 250-μm-pore sieve. The females and cysts were crushed with a motorized rubber stopper as described previously, and eggs inside of the females and cysts were collected on a 25-μm-pore sieve. Eggs were further separated from soil debris using sucrose centrifugation (Jenkins 1964) and rinsed three times with sterile, distilled water before use in hatch studies.

The effects of seed and radicle exudates were studied in separate experiments. Sterile, distilled water and 5 mM zinc sulfate served as negative and positive controls, respectively (Tefft and Bone 1984), in both experiments. The experiments were set up in a factorial design and included the factors sterility and exudate. Each experimental unit consisted of an average of 232 ± 53 (mean ± standard deviation) *H. glycines* eggs on a 30-μm-pore microsieve constructed from nylon mesh (Elko Filtering Co.) and plastic test tube caps (Wong et al. 1993). The sieves containing the eggs were incubated in 3 ml of exudates in 3-cm-diameter Petri dishes. The hatching experiments were set up inside of an incubator at 25°C. J2 that hatched and migrated down through the sieves into the liquid in the bottom of the dishes were counted at 3, 7, and 14 days under a microscope at ×20 magnification, and microsieves were transferred to Petri dishes with fresh solutions at 3 and 7 days. Eggs and J2 remaining on microsieves were counted at day 14. Cumulative percent hatch was determined by dividing the number of hatched juveniles by the number of hatched juveniles plus the remaining unhatched eggs and then multiplying by 100. There were four replications of each seed treatment–sterility combination and the two controls (sterile water and 5 mM $ZnSO_4$), and the experiments were each conducted twice.

Motility experiments. The effects of seed and radicle exudates on *H. glycines* J2 motility were tested in separate experiments. The experiments were set up in a factorial design. Sterile, distilled water served as a negative control. Factors were sterility (water control was sterile only) and seed or radicle exudates. An average of 36 ± 16 (mean ± standard deviation) *H. glycines* J2 that were hatched in sterile, distilled water within 48 h were incubated in 500 μl of treatment solution in a 1-cm-diameter watch glass. The experiments were set up in an incubator at 25°C. The J2 were visually rated as motile or nonmotile after 2 and 24 h of incubation in treatment solutions using a microscope at ×20 magnification. A J2 was considered nonmotile if it did not move after being touched with a thin wire probe (Faske and Starr 2007; Schroeder and MacGuidwin 2010). There were four replications of each seed treatment–sterility combination and the sterile water control, and the experiments were conducted twice.

Root penetration experiment. The experiment was conducted in a growth chamber (16 h of light and 8 h of darkness, 25°C) using *H. glycines*-susceptible soybean (Williams 82) grown from seed that were nontreated or treated with formulated seed treatments (VOTiVO

and ILeVO). A complete factorial treatment arrangement and a randomized complete block design were used in this experiment. Factors included seed treatment, inoculation density, and incubation time. Seed were planted in a pasteurized, 2:1 sand/soil mixture in conetainers placed in buckets filled with construction sand. A sand-filled bucket containing a cone with each of the 12 treatment–inoculation density–incubation time combinations served as a block. Five days after planting, seedlings were inoculated with either 100 or 500 J2 (hatched in water within 3 days) by placing 500 µl of J2 in a water suspension into 3-cm-deep holes located 1 cm from the base of the seedling. Soybean inoculated with *H. glycines* J2 were incubated for 1 or 3 days before being carefully washed and stained with acid fuchsin (Byrd et al. 1983). Roots were examined under the microscope (×20 magnification), and J2 that had penetrated roots were counted. There were five replications of each seed treatment–inoculation density–incubation time combination, and the experiment was conducted twice.

Statistical analyses. Statistical analyses were conducted in SAS (version 9.4; SAS Institute, Inc.) using PROC GLIMMIX. For the statistical models, the following factors were considered fixed effects: seed treatment (all experiments), cultivar (greenhouse experiment), sterility (hatching and motility experiments), inoculation density (root penetration experiments), and block (greenhouse and root penetration experiments). The factor block was nested in run, and the factor run was considered a random factor. An analysis of variance using the normal distribution was conducted for each experiment, and main and interactive effects were analyzed for significance. The control solutions (water and $ZnSO_4$ for the hatching experiments and water for the motility experiments) were not included with the exudate solutions in the initial two-factor analyses for the hatching and motility experiments because the control solutions were sterile only (and, thus, the sterility factor could not be tested with the control solutions). If the sterility factor was not significant, the data from the sterile and nonsterile exudates were combined for analysis, with the additional sterile control solutions as a single factor experiment with unbalanced numbers of replications. For the experiments with unbalanced number of replications across treatments (hatching and motility studies), a Kenward-Roger adjustment was used to adjust the degrees of freedom. Data from staining times of 1 and 3 days after inoculation (DAI) were analyzed separately in the root penetration experiment. For all experiments, the separation of treatment means was done using Tukey's honestly significant difference test ($\alpha = 0.05$).

Results

Greenhouse experiments. The experiment with moderate *H. glycines* population density soil (4,000 eggs per 100 cm^3 of soil) and the experiment with the low *H. glycines* density soil (1,000 eggs per 100 cm^3 of soil) each were conducted twice and the data were analyzed separately. There was a significant interactive effect of seed treatment and cultivar for the majority of the response variables measured (females per root, eggs per root, root weight, eggs per gram of root, and females per gram of root); therefore, data from each cultivar were analyzed separately. Across all experiments, the number of females per root on the resistant cultivar (Jack) were 10% or less of the susceptible variety (Williams 82) (Tables 1 and 2).

For the moderate *H. glycines* population density soil experiment, the seed treatment factor significantly affected three of the five measured variables in the susceptible cultivar (Table 1). In contrast, no variables were significantly affected by seed treatment in resistant Jack. The roots of susceptible soybean grown from seed treated with ILeVO had 35% fewer females relative to the nontreated control (Table 1). There was no significant difference between the nonformulated fluopyram and the nontreated control in terms of the number of females. However, both ILeVO and fluopyram reduced the number of eggs per root compared with the nontreated control. Also,

Table 1. Reproduction of *Heterodera glycines* on soybean after 30 days using field soil (4,000 eggs per 100 cm^3 of soil)[z]

Cultivar, treatment	Females per root	Eggs per root	Root weight (g)	Females per gram of root	Eggs per gram of root
Williams 82 (susceptible)					
VOTiVO	476 a	106,973 a	0.46 a	1,122 a	261,388 a
Bacillus firmus	376 abc	84,100 ab	0.41 ab	1,063 a	249,367 a
ILeVO	263 c	51,214 c	0.31 bc	1,043 a	205,943 a
Fluopyram	286 bc	62,659 bc	0.32 c	1,127 a	260,099 a
Nontreated	404 ab	93,432 a	0.46 a	1,020 a	245,142 a
Jack (resistant)					
VOTiVO	47 a	6,558 a	0.38 a	143 a	22,529 a
B. firmus	40 a	7,573 a	0.36 a	130 a	22,272 a
ILeVO	36 a	4,901 a	0.33 a	112 a	15,108 a
Fluopyram	34 a	6,100 a	0.37 a	90 a	16,062 a
Nontreated	38 a	5,593 a	0.38 a	102 a	14,838 a

[z] Values are least-squared means of 14 replications over two experimental runs. The two soybean cultivars tested were analyzed separately. Different letters in the same column for each cultivar represent significant differences according to Tukey's honestly significant difference test ($\alpha = 0.05$).

Table 2. Reproduction of *Heterodera glycines* on soybean after 30 days using a diluted field soil (1,000 eggs per 100 cm^3 of soil)[z]

Cultivar, treatment	Females per root	Eggs per root	Root weight (g)	Females per gram of root	Eggs per gram of root
Williams 82 (susceptible)					
VOTiVO	200 a	53,664 a	0.24 ab	821 a	218,543 a
Bacillus firmus	209 a	58,300 a	0.28 a	767 a	212,784 a
ILeVO	7 b	1,414 b	0.22 b	32 b	6,759 b
Fluopyram	18 b	4,579 b	0.23 ab	94 b	24,006 b
Nontreated	199 a	49,250 a	0.29 a	753 a	183,385 a
Jack (resistant)					
VOTiVO	9 ab	1,543 a	0.26 ab	35 a	6,172 a
B. firmus	12 a	1,907 a	0.27 a	42 a	6,856 a
ILeVO	2 c	579 b	0.21 b	7 b	2,636 b
Fluopyram	3 bc	593 b	0.23 ab	15 ab	2,668 b
Nontreated	8 abc	1,171 a	0.25 b	35 a	4,841 ab

[z] Values are least-squared means of 14 replications over two experimental runs. The two soybean cultivars tested were analyzed separately. Different letters in the same column for each cultivar represent significant differences according to Tukey's honestly significant difference test ($\alpha = 0.05$).

formulated ILeVO and nonformulated fluopyram significantly lowered root weights compared with the nontreated control in the susceptible cultivar but not the resistant cultivar. There was no significant difference between the nontreated control and VOTiVO (formulated *B. firmus* I-1582) or nonformulated *B. firmus* I-1582 seed treatments for nematode reproduction or root weights in either cultivar (Table 1).

In the experiment where the low *H. glycines* population density was used, there were significant differences due to the ILeVO seed treatment in both susceptible and resistant cultivars (Table 2). The roots of susceptible soybean grown from seed treated with ILeVO (formulated fluopyram) and nonformulated fluopyram had 96 and 91% fewer females, respectively, compared with the nontreated control, and there was a similar trend for eggs per root. ILeVO also reduced females per root in resistant soybean by 75% compared with the nontreated control but the number of females per root on plants grown from seed treated with technical fluopyram was not significantly different from the nontreated control. Both ILeVO and fluopyram significantly reduced the number of eggs per root by about 50%. There were reduced root weights for both cultivars tested with ILeVO and nonformulated fluopyram. Even with reduced root weights, fluopyram seed treatments significantly reduced the number of females and eggs per gram of root by more than 90% in the susceptible cultivar. However, ILeVO reduced only the number of females per gram of root in the resistant cultivar, and there were no significant effects of fluopyram on females or eggs per gram of root in the resistant cultivar. There was no effect of VOTiVO or *B. firmus* I-1582 seed treatments relative to the nontreated control on any of the variables measured for either cultivar (Table 2).

Hatching experiments. There was no significant difference in hatching between sterile and nonsterile seed or radicle exudates (data not shown); therefore, data from sterile and nonsterile exudates were combined, and the control solutions then were analyzed with the exudates. Both seed and radicle exudates had a significant effect on *H. glycines* hatching. There was no effect of exudates collected from VOTiVO-treated seed on hatching but exudates from ILeVO-treated seed significantly reduced hatching by 98% compared with hatching in exudates from nontreated seed (Fig. 1A). Regardless of treatment, seed exudates did not significantly stimulate hatching compared with the water control (Fig. 1A).

There was a significant interaction between the effects of seed treatment and experimental run with radicle exudates; therefore, data from the two runs were analyzed separately. Overall, hatching of *H. glycines* in radicle exudates and controls was higher in the first run compared with the second run. Radicles grown from VOTiVO-treated seed did not significantly affect hatching relative to the nontreated control in either run. In the first run, ILeVO radicle exudates reduced nematode hatching by 48% compared with the nontreated control but this did not occur in the second run. Also, exudates from radicles across all treatments significantly stimulated hatching relative to the water control in the first run but not in the second (Fig. 1B).

Motility experiments. There was no significant difference in motility of J2 between sterile and nonsterile seed or radicle exudates (data not shown); therefore, the data were combined and the water control was added for analysis for both sets of experiments. Seed and radicle exudates did not stimulate J2 motility relative to the water control (Fig. 2). Exudates from seed treated with ILeVO significantly

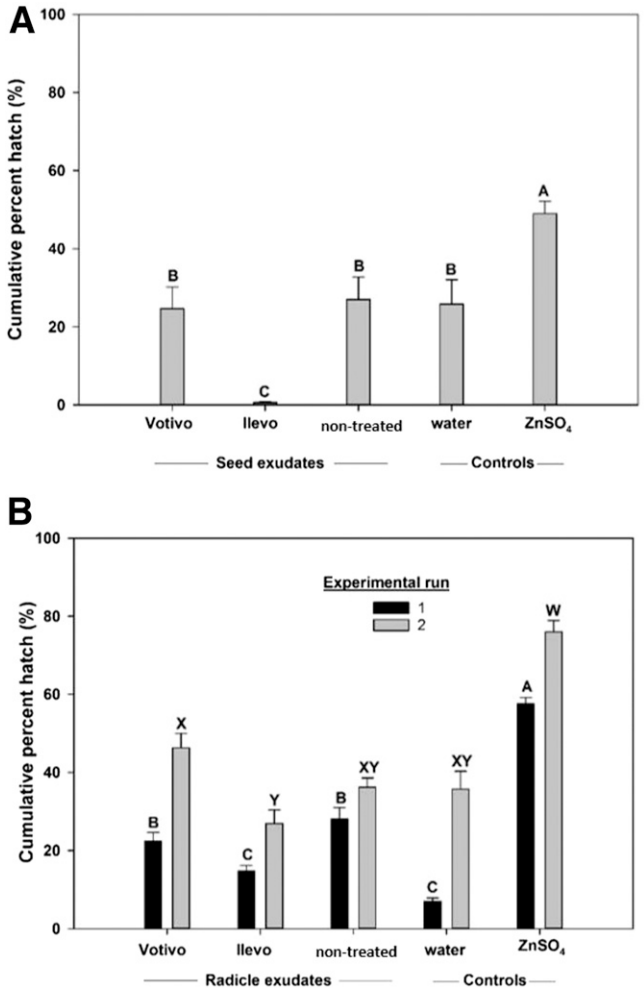

Fig. 1. Effect of **A,** seed and **B,** radicle exudates on cumulative percent hatching of *Heterodera glycines* juveniles (mean ± standard error of the mean) over 14 days. There were 16 replications for seed exudates over two runs and 8 replications per run for the radicle exudates.

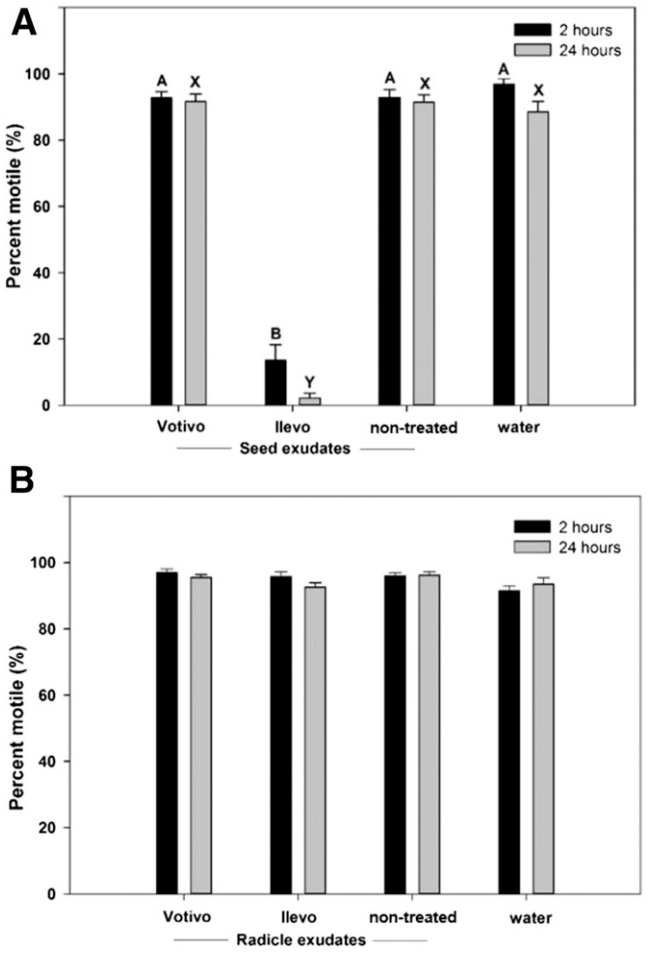

Fig. 2. Effect of **A,** seed and **B,** radicle exudates on motility of *Heterodera glycines* juveniles (mean ± standard error of the mean) after 2 and 24 h of exposure. There were 16 replications for the exudates and eight replications for the water-only control solution over two runs. Bars of the same color with different letters are significantly different from one another according to Tukey's honest-significant-difference test ($\alpha = 0.05$).

reduced J2 motility by 85 and 98% after 2 and 24 h of incubation, respectively, compared with the nontreated control (Fig. 2A). In contrast, exudates collected from seed treated with VOTiVO did not affect J2 motility (Fig. 2A). Motility of *H. glycines* J2 incubated in ILeVO or VOTiVO or nontreated radicle exudates was no different from each other or the water control (Fig. 2B).

Root penetration experiment. Half of the soybean roots were stained and the J2 were counted 1 DAI, and the remaining roots were stained and J2s counted 3 DAI. Data from the two time points were analyzed separately. There also was a significant interactive effect between seed treatment and *H. glycines* inoculation density for both time points; therefore, the two inoculation density treatments were analyzed separately (data not shown). At 1 DAI, there was no significant difference in root penetration detected between ILeVO and VOTiVO and the nontreated control at the lower inoculation density (100 J2/plant) but there was a significant, 79% decrease in the number of J2 infecting roots in the ILeVO treatment compared with the nontreated control for the higher inoculation density (500 J2/plant) (Fig. 3A). At 3 DAI, there were 61 and 79% fewer penetrated J2 in the ILeVO treatment at the lower and higher inoculation density, respectively, compared with the nontreated control (Fig. 3B). There was no significant effect of VOTiVO on *H. glycines* J2 root penetration compared with the nontreated control or ILeVO at any inoculation density or time point (Fig. 3).

Discussion

ILeVO significantly reduced *H. glycines* reproduction, hatching, motility, and root penetration. In the greenhouse experiments, nonformulated seed treatments containing only the AI without the inert ingredients found in formulated seed treatments were tested along with formulated products. It was necessary to conduct experiments with AI-only treatments as well as the formulated products because it had not been shown in published reports that the fungicide AI of ILeVO, named fluopyram, was responsible for nematode suppression. It also is important to note that, although soybean seeds are typically treated with multiple products (fungicides, insecticides, and nematicides), the seed used in our experiments were only treated with the nematode products; namely, ILeVO and VOTiVO. ILeVO (formulated fluopyram) and nonformulated fluopyram had similar effects in both sets of greenhouse experiments. Both ILeVO and nonformulated fluopyram reduced the number of females and eggs per root in our experiments, particularly on the susceptible cultivar. Our results also suggest that initial nematode population density may be an important factor to consider when evaluating seed treatments. It is unclear why ILeVO reduced *H. glycines* more at a lower initial nematode population density compared with a moderate nematode population density. One possibility is that the amount of fluopyram applied to the seed was not enough to act on a critical number of nematodes at moderate *H. glycines* population densities. Also, it is possible that the increased sand content of the soil (used to dilute the field soil in our experiments) allowed for greater movement of the fluopyram or improved the efficacy of fluopyram in some other way. Because we tested different population densities in separate experiments, it is not possible to make conclusive statements on interactions between ILeVO and *H. glycines* population density. Additional seed treatment research where soybean is inoculated with different amounts of *H. glycines* in a single experiment would be necessary to better understand this potential interaction.

ILeVO reduced root weights in most of our greenhouse experiments. However, roots grown from seed treated with fluopyram (nonformulated ILeVO) did not have significantly reduced root weights compared with the nontreated control. The root weight reductions that were detected were in 30-day greenhouse experiments and were possibly associated with the phytotoxic effects of fluopyram, which are reported to not result in long-term stunting or yield loss in one soybean study (Wise et al. 2015). We did not conduct experiments for longer periods of time to determine if the reduced root weights would have persisted.

Exudates collected from seed treated with ILeVO inhibited both *H. glycines* hatching and motility as much as 98%. Exudates collected from radicles grown from ILeVO-treated seed reduced hatching in only one experimental run and did not affect motility of the J2. These results suggest that fluopyram is possibly most concentrated around the seed, and a small amount may move either on or through the emerging radicle. The variability of the data from the hatching studies using ILeVO radicle exudates do not allow us to make strong conclusions on its effect on *H. glycines* hatch. But our results suggest that nematodes near the seed would likely be the most affected by the fluopyram seed treatment. However, movement of fluopyram from the seed would presumably be downward through the soil, possibly explaining why we observed reduced *H. glycines* root penetration in soybean grown from ILeVO-treated seed.

Fluopyram belongs to the family of systemic fungicides known as the succinate-dehydrogenase inhibitors and is used to control fungal diseases in a number of crops (Ishii et al. 2011; Vitale et al. 2016). Formulated as ILeVO, it is one of the few labeled seed treatments with activity on the causal pathogen of soybean sudden death syndrome (SDS), *Fusarium virguliforme* (Kandel et al. 2016). Interestingly, research in the last few years has documented nematicidal or nematistatic properties of fluopyram (Broeksma et al. 2014; Faske and Hurd 2015). Zaworski (2014) found that ILeVO reduced *H. glycines* reproduction under greenhouse conditions in some experiments but not in others. Additionally, Faske and Hurd (2015) reported that fluopyram inhibited motility of *M. incognita* and *Rotylenchulus reniformis* but that the inhibition was largely reversible after rinsing the nematodes with water, suggesting a nematistatic effect. Faske and Hurd (2015) also reported that brief exposure of both nematode species to fluopyram resulted in reduced root infection of tomato. Jones et al. (2017) reported reduced *M. incognita* damage to lima bean when fluopyram was applied to the soil before planting. Our results suggest that the effects of ILeVO on *H. glycines* are similar to effects of the product on *M. incognita* and *R. reniformis*. It also is possible

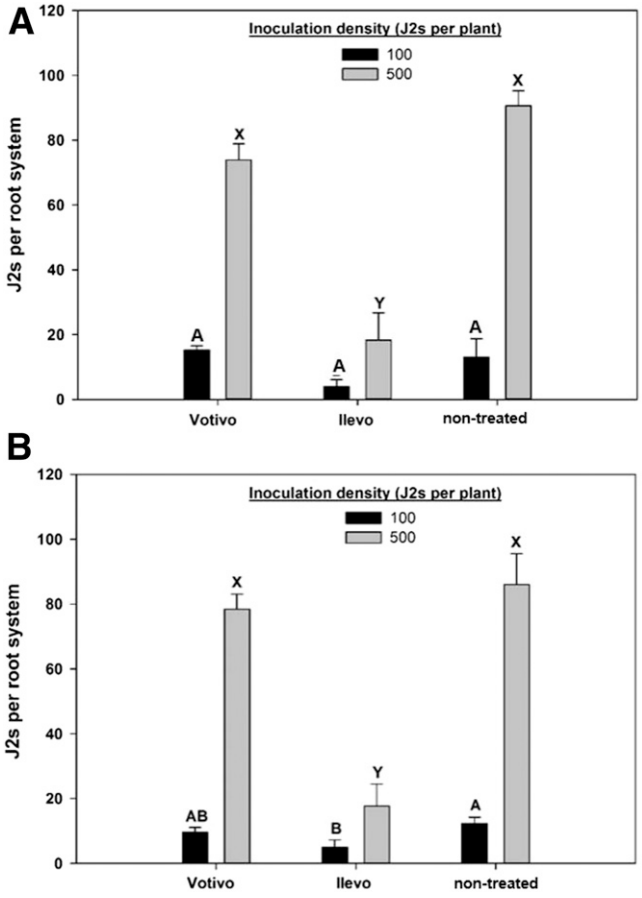

Fig. 3. Effect of seed treatments on root penetration of *Heterodera glycines* juveniles (mean ± standard error of the mean) at **A,** 1 and **B,** 3 days after inoculation. There were 10 replications for each treatment over two runs. Bars of the same color with different letters are significantly different from one another according to Tukey's honest-significant-difference test ($\alpha = 0.05$).

that fluopyram induces plant defenses, resulting in nematode protection, but this hypothesis has not been studied thus far.

To date, there have been few published field studies examining the effect of ILeVO on *H. glycines* or other plant-parasitic nematodes. ILeVO suppressed SDS symptoms and increased soybean yields in multistate field studies but the effect of the seed treatment on *H. glycines* was not studied (Kandel et al. 2016). A recent field trial evaluating the effect of ILeVO on *M. incognita* found no effect of the seed treatment on nematode population densities or soybean yield (Hurd et al. 2015). However, additional studies across diverse environments are needed to better determine the effect of ILeVO on *H. glycines*.

Our experiments demonstrate that ILeVO has activity on *H. glycines* under controlled conditions. However, the limited time and spatial conditions of our experiments may have produced significant effects that may not be relevant under field conditions. For example, the cone-tainers used in the greenhouse and root-penetration experiments could restrict the fluopyram in the root zone in a way that would provide for greater root protection than would occur in the field. It is possible for a compound to show strong activity against a plant-parasitic nematode under controlled conditions, yet provide little or inconsistent protection in the field. For example, abamectin was reported to be nematicidal for *M. incognita* and *R. reniformis* at concentrations as low as 1 μg/ml in laboratory experiments (Faske and Starr 2006) but the same compound did not reduce plant-parasitic nematode symptoms on cotton roots (Faske and Starr 2007) or increase yields when used as a seed treatment (Faske 2006).

We observed no consistent effects of VOTiVO on *H. glycines* in any of the experiments conducted. *B. firmus* I-1582 (the AI of VOTiVO) reportedly colonizes growing root tissue and provides a barrier between nematodes and roots. Schrimsher (2013) reported a reduction in *H. glycines* J2 hatching and motility when exposed to cells of *B. firmus* I-1582 in vitro. Additional split-root experiments suggested that the bacterium induces systemic resistance in the plant. The bacterium reduced *R. reniformis* reproduction on cotton, but only at higher rates of *B. firmus* (Castillo et al. 2013). In contrast, a number of field trials have reported no effect of VOTiVO on yields or on population densities of a number of different species of plant-parasitic nematodes (Jackson and Behn 2010; Land et al. 2014; Musil et al. 2014). The inconsistency in the nematode-protectant properties of VOTiVO may be due to several factors, including competition from other soil microbes, less-than-optimal environmental conditions (such as soil pH or moisture), or experimental design. Our experiments used only seed-applied VOTiVO and were limited to early soybean growth. Longer experiments may be necessary for the bacterium to grow and begin affecting the nematodes. For example, Crow (2014) found that *B. firmus* I-1582 significantly reduced *Belonolaimus longicaudatus* population densities only after 2 months after application of the bacteria, indicating that there may be a lag time before *B. firmus* I-1582 is effective in controlling plant-parasitic nematodes. Sterility did not alter the effect of VOTiVO seed and radicle exudates in the hatching and motility experiments, indicating that the lack of activity was not due to the presence or absence of *B. firmus* I-1582 cells. Additional experiments for longer periods of time or in altered environmental conditions could provide a clearer picture of how and when VOTiVO provides nematode protection.

There are a few important considerations to be made regarding our methods and results in the hatching and motility experiments. First, ILeVO radicle exudates reduced hatching modestly in one experimental run and not in another. In addition, radicle exudates stimulated hatching compared with the water in only one run, and the overall rate of hatching in the second run was much higher than the first. The variability associated with the radicle exudate hatching experiment may be potentially explained by the effects of environmental conditions on the soybeans grown to produce *H. glycines* eggs. Hominick et al. (1985) found considerable variation in the response of the potato cyst nematode *Globodera rostochiensis* to potato root exudates and attributed these effects to environmental conditions such as photoperiod during development of the nematodes on the plants. Stimulation of *H. glycines* hatch in response to soybean exudates also has been reported to be variable across experimental runs (Charlson et al. 2008). Because we repeated experiments over time, the *H. glycines* eggs used in our studies were from soybean plants grown under greenhouse conditions at different times of the year. It is possible that our inconsistent results are due to environmental variability in the greenhouse we used to culture *H. glycines*. Although the effects of ILeVO seed exudates on *H. glycines* was highly consistent across runs, our data do not allow for firm conclusions to be drawn regarding the effects of radicle exudates.

Second, our motility studies relied on using a wire probe to assess motility of individual J2. However, assessing response of nematodes to drops of NaOH or Na_2CO_3 has been reported to be a more accurate method of assessing immobility of nematode (Chen and Dickson 2000; Xiang and Lawrence 2016). It is possible that the immobilized nematodes we observed in the ILeVO seed exudate would still respond to NaOH or Na_2CO_3. Thus, it is important to note that the immobilization of J2 in ILeVO seed exudates in our study may not necessarily indicate nematode mortality.

Although seed treatments likely cannot provide season-long protection against plant-parasitic nematodes, decreases in nematode infection and reproduction in early seedling development have been associated with increased yields and reduced plant damage. For example, Huang and Ploeg (2001) found that delaying *Longidorus africanus* infestation by 10 days resulted in increased biomass in both lettuce and carrot. Additionally, Shane and Barker (1986) found that older soybean plants are less sensitive to *M. incognita* infection than young ones. These studies suggest that nematode population densities may be suppressed and plant damage reduced if seed treatments can temporarily reduce or eliminate *H. glycines* infection in young soybean plants. However, the effects and interactions among nematode population densities, environment, and other pathogens may hinder efforts to provide a consistent benefit of seed treatments to soybean growers. Additional studies examining the effects of VOTiVO, ILeVO, and other seed treatments in the field and on the biology of *H. glycines* are necessary for the full potential of this management tool to be understood and appreciated.

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