1 **Avicta and Clariva affect the biology of the soybean cyst nematode, *Heterodera glycines***

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11 **Abstract**

12 Nematicidal seed treatments are a relatively new strategy for managing plant-parasitic

13 nematodes in row crops. Two such seed treatments, Avicta® (abamectin) and Clariva® (*Pasteuria*

14 *nishizawae*), are marketed by Syngenta® for use against *Heterodera glycines* in soybean

15 production in the upper Midwest. The specific effects of these seed treatments on the biology of

16 the nematode have not been previously reported. The effects of Avicta and Clariva on *H.*

17 *glycines* hatching, movement, attraction, penetration, development, and reproduction were

18 determined in controlled-environment experiments. Avicta inhibited juvenile movement and

19 penetration at the seed depth and 3 cm below the seed. Clariva inhibited juvenile movement and

20 penetration 3 and 5 cm below the seed and nematode development within the roots of young

21 plants. Both seed treatments affected nematodes in 10- and 20-day-old plants, but effects were

22 not detected on nematodes developing in older plants (30 and 60 days) with larger root systems.

23 These results provide details of the specific mechanisms of early season protection provided by

24 Avicta and Clariva seed treatments.

25 **Introduction**

1   Continued improvement of yield potential through optimization of traits and technology

2   has made seeds the most valuable agricultural input for the soybean farmer. With such a large

3   investment in soybean seed, the agriculture industry has turned its focus to protecting the seed

4   and young seedling against early season diseases and insect damage (Munkvold 2009).

5   Fungicidal, insecticidal, and nematicidal seed treatments are an efficient and cost-effective

6   means to deliver protection against pests and pathogens at the time of planting. Advantages of

7   this approach include a reduced amount of chemical applied per field, continuous early season

8   delivery of chemical within the soil, and fewer non-target effects, among others. The global seed

9   treatment market has grown from $1 billion in 2002 to an estimated $4 billion in 2017

10   (Munkvold et al. 2014).

11   Traditional soil-applied granular and fumigant nematicides are environmentally

12   hazardous and cost prohibitive for use in large-scale row cropping (Monfort et al. 2006, Zasada

13   et al. 2010). Nematicidal seed treatments are a welcomed new tool for nematode management in

14   soybean production. In the upper Midwest, the soybean cyst nematode, *Heterodera glycines*

15   Ichinohe, is the principal yield-reducing pathogen of soybeans (Allen et al. 2017). Until a decade

16   ago, the main means of managing *H. glycines* was rotation of non-host crops and *H. glycines*-

17   resistant soybean varieties (Niblack and Tylka 2012). Syngenta Crop Protection (Greensboro,

18   NC) has developed two nematicidal soybean seed treatments, Avicta[®] and Clariva[®], for

19   managing *H. glycines*.

20   Abamectin, the active ingredient of Avicta, is a small molecule that was known to have

21   nematicidal effectivity as early as the 1980s (Sasser et al. 1982, Garabedian and Van Gundy

22   1983, Nordmeyer and Dickson 1985). Due to the low water solubility and short half-life of

23   abamectin (Putter et al. 1981, Bull et al 1984), the compound received little attention in

1   agriculture until it was formulated as a seed treatment. As a broad-spectrum chemical

2   nematicide, abamectin has been shown to have activity against *Pratylenchus zeae, Meloidogyne*

3   *incognita, Heterodera schachtii,* and *Rotylenchulus reniformis* (Faske and Starr 2006, Cabrera et

4   al. 2009, da Silva et al. 2016).

5   Clariva is a biologically based seed treatment containing spores of *Pasteuria nishizawae,*

6   an obligate, endospore-forming bacterial parasite of *H. glycines* (Sayre et al. 1991). For many

7   years it was not possible to produce large quantities of *Pasteuria* for use in fields for nematode

8   biocontrol. But in 2004, Pasteuria Biosciences succeeded in developing a large-scale

9   fermentation process for *Pasteuria* species (Wilson and Jackson 2013), and subsequently,

10   Syngenta Crop Protection developed and launched the seed treatment Clariva in 2014.

11   Field-based studies of nematicidal seed treatments are useful to assess if the products

12   affect yields and nematode population densities and to what extent (Barham et al. 2005, Vitti et

13   al. 2014, Tylka et al. 2015, Bissonnette et al. 2018). But such field studies do not reveal

14   specifically how or when a treatment works. To assess the specific benefits of a seed treatment,

15   controlled-environment experiments are necessary. In this paper, our objective was to develop a

16   combination of greenhouse and laboratory assays to study nematode biology. We then used these

17   methods to determine the effects of Avicta and Clariva on specific activities and life stages of *H.*

18   *glycines*.

19   **Materials and Methods**

20   We conducted experiments to assess the effects of Avicta and Clariva seed treatments on

21   nematode hatching, movement, attraction, root penetration, development within roots, and

22   reproduction. The first two methods described immediately below explain how *H. glycines* eggs,

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1   second-stage juveniles (J2s), and leachates from soil planted with treated seeds were collected.

2   Each subsequent section describes the experiments conducted to test the effects of Avicta and

3   Clariva seed treatments on an individual life stage or process of *H. glycines.* All seed used in the

4   experiments was produced and harvested in 2015. Untreated seed of the varieties used in the

5   experiments were sent to Syngenta personnel in Stanton, MN, where each seed was treated with

6   approximately 0.1mg of abamectin in Avicta 500 FS solution or 1×10^{7} spores of Clariva PN

7   solution. The control solutions used in the hatching, movement analysis, and juvenile attraction

8   experiments consisted of formulated Avicta and Clariva that were received and first opened in

9   2015.

10   **Nematode egg and juvenile preparation.** Populations of *H. glycines* used in laboratory and

11   greenhouse experiments were maintained in greenhouse culture pots on *H. glycines*-susceptible

12   soybeans, *Glycine max* cv Williams 82, grown in Fruitfield coarse sand collected from

13   Muscatine, IA, naturally infested with *H. glycines* (HG type 2.5.7). Eggs and second-stage

14   juveniles (J2s) were collected from adult *H. glycines* females exposed on the surface of 30-day-

15   old soybean roots. The adult females were dislodged from soybean roots placed on a 850-μm-

16   pore sieve nested over a 250-μm-pore sieve by spraying the roots with a stream of tap water. The

17   adult females collected on the 250-μm-pore sieve were separated from soil and root debris

18   through sucrose centrifugation using a 1,362 g/L sucrose solution (Jenkins 1964). Eggs were

19   released by crushing the females using a rotating rubber stopper on the surface of a 250-μm-pore

20   sieve (Faghihi and Ferris 2000). The eggs then were collected on a 25-μm-pore sieve nested

21   under a 75-μm-pore sieve. A second round of sucrose centrifugation with 454 g/L sucrose/water

22   solution further separated the eggs from debris (Jenkins 1964). The eggs were rinsed with water

1   on a 25-μm-pore sieve to remove the sucrose solution and then were used in experiments or were

2   set up for collection of hatched J2s.

3   To collect J2s, eggs were placed on a 30-μm-pore sieve and submerged in a layer of tap

4   water at 25°C for 72 hours (Wong et al. 1993). Hatched J2s moved through the pore openings in

5   the sieve into the water below. To obtain active J2s for *in vitro* experiments, hatched J2s that

6   were collected after 72 hours were placed on a 30-μm-pore sieve again and set in a layer of tap

7   water. After one to two hours, the J2s that moved through the sieves were collected in the water

8   below and used for experiments.

9   **Collection of soil leachate.** In a polystyrene 6-well microplate (Thermo Fisher Scientific Inc.,

10   Waltham, MA), a 30-μm-pore sieve containing 6.5 g of sterile 1:1 sand/soil mixture was placed

11   in each well. Next, one seed of *H. glycines*-susceptible soybean (cv Williams 82) treated with

12   either Avicta or Clariva was planted in the soil in each sieve in the 6-well plate. Untreated seed

13   and unplanted (fallow) soil were included as controls. Sterile distilled water (2 ml) was added to

14   each sieve. The plates were covered with lids and placed in an incubator for 24 hours at 25°C in

15   the dark. Next, 2.5 ml of sterile distilled water was added to each sieve, and the leachate that

16   flowed through the sieve was collected in the well below. Soil leachates from five wells with the

17   same treatment were collected and combined into a sterile 50-ml conical tube and stored at 4°C

18   until used. New leachate was collected for each run of the experiments described below.

19   **Hatching experiments.** In a sterile, polystyrene 6-well microplate, 3 ml of a single treatment

20   solution were added to each well. Treatment solutions consisted of 500 μl of a soil leachate

21   (described above) combined with 2.5 ml of sterile distilled water. Control treatments were 0.1

22   mg of abamectin in Avicta solution, 1×10^{7} spores of Clariva PN solution, sterile distilled water,

23   and 5.5mM $ZnSO_4$, all made to a final volume of 3 ml with sterile distilled water. Next, a 30-

1   μm-pore sieve was added to the two left-most wells in each well plate. Each row of the 6-well

2   plate served as an experimental unit. Between 300 and 400 *H. glycines* eggs were added to each

3   sieve. The plates were covered with a lid, sealed with Parafilm®, and incubated in the dark at

4   25°C. The sieves (containing the unhatched eggs) were transferred to the middle wells of the

5   plate after three days and to the right-most wells after seven days. After each transfer, the plates

6   were re-covered, sealed with Parafilm, and incubated in the dark at 25°C. On the 14th day, the

7   remaining contents of each sieve were washed out with water into a clean, empty well of a new

8   plate. The number of J2s that hatched at days 3, 7, and 14 were counted. The number of

9   unhatched eggs from the remnants washed from the sieve on day 14 also was counted.

10 Cumulative percentage hatch was calculated by dividing the total number of hatched juveniles

11 (on days 3, 7, and 14 combined) by the total number of nematodes in the experimental unit

12 (hatched juveniles and unhatched eggs). The experiment was conducted twice with four

13 replications per treatment per run.

14 **Movement analysis experiments**. Active J2s in 100 μl of water (collected as described above)

15 were incubated in a solution of 500 ml of soil leachate (collected as described above) diluted

16 with 500 ml of sterile distilled water for two hours in 1.5 ml microcentrifuge tubes in the dark at

17 25°C. Control treatments consisted of 1 ml solutions of 0.1mg of abamectin in Avicta solution,

18 1×10^7 spores of Clariva PN solution, sterile distilled water, and heat-killed J2s in sterile distilled

19 water.

20   To isolate the J2s from the incubation solutions, each treatment was spun at 2,000x *g* for

21 three minutes in a mini centrifuge (Thermo Fisher Scientific Inc., Waltham, MA) to concentrate

22 the nematodes in a pellet. The top 500 ml of treatment solution supernatant were removed and

23 500 ml of 454 g/L sucrose solution were added. Each tube was vortexed to resuspend the

1   nematodes with the sucrose solution, then spun again at 2,000x *g* for three minutes. Clean

2   nematodes were contained in the sucrose supernatant, and debris from the soil leachates were

3   contained in the pellet. The top 500 ml supernatant were removed, added to a new 1.5 ml

4   microcentrifuge tube, and mixed with 500 ml of sterile distilled water.

5   Nematodes incubated in leachates of soil planted with treated seeds and in control

6   solutions were analyzed using motion analysis of individual nematodes described by Jensen et al.

7   2018. J2s in 30 ml of suspension from the treatments described above were placed on a

8   microscope slide with coverslip and observed with overhead light microscopy (Leica LED 5000

9   light attached to Leica M205 C stereoscope) at 63x magnification (6.3x magnification, 10x

10   eyepiece, and 1x camera). Images were taken of an individual J2 every 0.1 seconds for 60

11   seconds with a high-speed camera (QICAM 12-bit color fast 1394). Then the images were

12   converted into a video, and individual J2s were subjected to movement analysis using a second,

13   custom MATLAB program that calculated the speed and change in curvature of tracking points

14   along the nematode body. For each treatment, 8-15 J2s were analyzed at the anterior-most

15   tracking point in each of two experimental runs.

16   **Juvenile attraction experiments**. Active J2s (15 to 60) were added to the center nematode entry

17   port of a microfluidic chemotaxis chip (Supplementary Fig. 1) filled with sterile distilled water,

18   as described by Beeman et al. (2018). Soil leachate (30 μl), collected as described above, was

19   added to a treatment port of a randomly assigned chip (1-14), lane (1-4), and side (left or right)

20   combination. Sterile distilled water was used as the control in the opposite treatment port for

21   each lane. Control solutions tested included 0.1 mg of abamectin in Avicta solution, $1x10^7$ spores

22   of Clariva PN solution, 500 mM $CaCl_2$, and 500 mM KNO_3. Also sterile distilled water was

23   added to both ports of one lane in each chip to make sure there was no bias for movement of

1 nematodes to one side of the chip or the other. Once the soil leachate treatments and control

2 solutions were added, the chips were placed in an inverted, square, 10 cm petri dish, sealed with

3 Parafilm, then incubated at 25°C in the dark.

4   After 24 hours, the chips were examined under a stereoscope, and the number of J2s in

5 each side resting chamber (treatment or control) and in the center nematode entry port was

6 counted. The number of J2s in each side of the lane was divided by the total number of J2s in all

7 three areas of the lane to determine percentage. Juveniles found in the treatment resting chamber

8 were considered attracted to the treatment whereas juveniles in the control resting chamber were

9 considered repelled by the treatment. The percentage of juveniles in the treatment resting

10 chamber was compared to the percentage of juveniles in the control resting chamber to determine

11 if there was a significant treatment effect. There were five to six replications per treatment per

12 run, and the experiment was conducted twice.

13 **Experiments to measure penetration of juveniles at discrete soil depths**. Falcon 50 ml

14 conical centrifuge tubes (Fisher Scientific, Waltham, MA) were inverted to stand on the cap, the

15 conical tips of the tubes were removed, and three 3.175-mm diameter holes were drilled into the

16 sides at 2, 5, and 7 cm from the top. Once fabricated, the tubes were filled with approximately 50

17 cm^3 of sterile 1:1 sand/soil. One seed of *H. glycines*-susceptible soybean cv Williams 82 treated

18 with Avicta or Clariva seed treatment was planted per tube, about 2 cm deep. Untreated Williams

19 82 and untreated *H. glycines*-resistant soybean cv Jack were planted in other tubes as controls.

20   Each tube was watered initially with 10ml of tap water. The tubes were arbitrarily

21 arranged in a plastic box then placed in a growth chamber with 16-hour day length, at 50 percent

22 humidity, and at 25°C. Each tube received 5 ml of tap water each day for days two through

23 seven. On the sixth day, approximately 200 active J2s suspended in 150 μl water were pipetted

1 through the pre-drilled holes to the center of the soil column at one of three soil depths (2, 5, or 7

2 cm), and the tubes were incubated for an additional 24 hours. On the seventh day, the plants were

3 removed from the tubes, and the roots washed free of soil and patted dry. The roots were

4 separated from the stems at the soil line. The mass of each root system was recorded, roots were

5 frozen for 12 hours at -20°C, and then thawed.

6 To isolate the penetrated J2s, each thawed root system was cut into 1.5-cm-long pieces

7 and macerated with 75 ml of tap water using a household blender on high for 30 seconds, similar

8 to what was described by Ruan et al. 2012. The macerated mixture was poured through a 250-

9 μm-pore sieve nested over a 75-μm-pore sieve nested over a 25-μm-pore sieve. The root debris

10 in the 250-μm-pore sieve were macerated further by pushing the material through the sieve with

11 a rotating rubber stopper (Faghihi and Ferris 2000). The macerated root mixture was flushed

12 with water through the middle 75-μm-pore sieve, and the released J2s were collected on the

13 bottom 25-μm-pore sieve.

14 The number of penetrated J2s was counted via microscopic observation and compared

15 among treatments on a per gram of fresh root basis. A proportion was calculated as the number

16 of penetrated nematodes of a treatment at a set location, for a single replication, per gram of root,

17 divided by the average number of penetrated nematodes at that same location of the untreated

18 Williams 82 control per gram of root, over all replications. After this, the proportion of all

19 untreated Williams 82 treatments was set to 1.0, as previously explained by Halbrendt et al.

20 1992. Three to four replications were planted for each treated seed and control seed for each run

21 of the experiment, and the experiment was conducted twice.

22 **Experiments to measure total juvenile penetration and development.** Cone-tainers with a

23 volume of 150 cm^3 were filled sandy soil naturally infested with *H. glycines* with an initial egg

1   density of 1,500 eggs per 100 cm^3. One seed of Williams 82 treated with Avicta or Clariva seed

2   treatments was planted in each Cone-tainer, about 2 cm deep. Seeds of untreated Williams 82 or

3   untreated Jack were planted as controls. Plants were grown in a growth chamber in 16-hour day

4   length, at 50 percent humidity, at 25°C, and watered with 20 ml of tap water every 2 days for 10,

5   20, or 30 days.

6   On the designated sample dates, plants were removed from the Cone-tainers and their

7   root systems were carefully dipped in water to remove soil. The stems were removed from the

8   roots at the soil line and discarded, the roots were patted dry, and root mass was recorded. The

9   30-day-old roots were placed on a 850-μm-pore sieve nested over a 250-μm-pore sieve and were

10   sprayed with water to dislodge adult females that were exposed on the root surface. The females

11   were stored in labeled 50 ml conical tubes. Individual root systems were frozen for 12 hours at -

12   20°C then thawed and cut into 1.5-cm-long segments, added to 75 ml of tap water in a household

13   blender, and pulsed on high 10 times then blended on high for 30 seconds. The macerated

14   mixture was poured through a stack of sieves with pore sizes 850, 250, 150, 75, 45, and 25 μm.

15   Almost all of the nematodes collected on each individual sieve were of a single developmental

16   stage because the *H. glycines* life stages are of different sizes. (de Boer et al. 1999, Graney and

17   Miller 1982, Raski 1949). Specifically, adult females were captured on the 250-μm-pore sieve,

18   fourth-stage juveniles on the 150-μm-pore sieve, third-stage juveniles on the 75- and 45-μm-pore

19   sieves, and second-stage juveniles on the 25-μm-pore sieve. The presence of root debris similar

20   in size to third-stage juveniles made it necessary to collect nematodes of that developmental

21   stage on two sieves.

22   The top 850-μm-pore sieve was flushed with water 10 times, removed from the stack, and

23   its contents discarded. The second sieve, with 250 μm pores, was flushed with water 10 times

1   and the contents were transferred to a 50-ml conical tube. Each subsequent sieve was flushed

2   with water 10 times, then removed from the stack, and its contents transferred to a labeled

3   conical tube. Overall, the nematodes contained within a root were collected in five different

4   tubes based on the sieves on which the macerated mixture was captured. The nematodes obtained

5   from each sieve were then counted under a stereoscope with overhead lighting. Adult females

6   previously collected from 30-day-old roots were combined with the contents recovered from the

7   250-μm-pore sieve of the same root system, and these adult female nematodes were counted.

8   Some of the adult vermiform males may have left the roots and moved into the soil for

9   mating, therefore it was not possible to get a complete count of the number of adult males, and

10 consequently, males were excluded from the tallies. The number of nematodes in a specific life

11 stage was divided by the total number of nematodes found in the entire root system to give a

12 percent distribution of each developmental stage. These percentages then were converted to a

13 proportion, given as the percentage of nematodes in a given life stage, for a single replication

14 (root system), divided by the average percentage of nematodes in the same life stage for all

15 replications of the untreated Williams 82 control. The proportion for all untreated Williams 82

16 treatments was set to 1.0, as previously explained by Halbrendt et al. 1992. Five replications

17 were planted for each treatment and control in each run of the experiment, and the experiment

18 was conducted twice.

19 **Female maturity and reproduction experiments.** Small and large Cone-tainers (150 cm^3 and

20 600 cm^3, respectively) were filled with Fruitfield coarse sand collected from Muscatine, IA, that

21 was naturally infested with *H. glycines* with an initial egg population density of 1,500 eggs per

22 100 cm^3 of soil. One seed of either Jack or Williams 82, treated with either Avicta or Clariva,

23 was planted about 2 cm deep. Untreated Jack and Williams 82 were planted as controls. One

1   Cone-tainer of each treatment was arbitrarily placed in an 11-L bucket. Small and large Cone-

2   tainers were planted in separate buckets. Construction sand was added to the fill the spaces

3   around the Cone-tainers. The buckets were placed in a water bath kept at 25°C inside a

4   greenhouse.

5   Each Cone-tainer was watered with tap water and the soil was kept moist every day for

6   the first five days, then every two days until the end of the experiment. Soybean plants were

7   grown in small Cone-tainers for 30 days and large Cone-tainers for 60 days. After 30 days, plants

8   were removed from the small Cone-tainers and the roots were carefully dipped in water to

9   remove soil adhered to the roots. Each root system was placed onto a 850-μm-pore sieve nested

10   over a 250-μm-pore sieve. With a stream of water, adult females exposed on the root surface

11   were removed from the root and collected on the 250-μm-pore sieve. These females were stored

12   at 4°C until counted. Root systems were separated from stems at the soil line and patted dry, and

13   the mass of each root system was recorded.

14   After 60 days, the contents of the large Cone-tainers were emptied one by one into clean

15   11-L buckets. Each bucket was filled with 1L of water and the root systems were carefully

16   dipped repeatedly to remove soil adhered to the roots. Females were removed from roots as

17   described above, stored in labeled containers, and the mass of each root system recorded. Cysts,

18   the dead *H. glycines* females, were extracted from the soil in the bucket. The soil-water mixture

19   in the bucket was stirred clockwise at a constant speed for 20 seconds then let sit for 20 seconds.

20   Next, the suspension was poured through a 850-μm-pore size nested over a 250-μm-pore sieve

21   (Gerdemann 1955). Another 1L of tap water was added to the bucket and the process was

22   repeated once more. Cysts from the soil were collected from the 250-μm-pore sieve and stored at

23   4°C until counted.

1   Using a stereoscope with overhead lighting, the number of females and cysts (for the 60-

2   day experiment) were counted for each plant. Next, the females and cysts were crushed using a

3   rotating rubber stopper (Faghihi and Ferris 2000) to release the eggs. Eggs collected on a 25-μm-

4   pore sieve were stained acid fuchsin (Niblack et al. 1993) and counted.

5   The number of females and eggs from a single replication was divided by the mass of its

6   root system to standardize nematode numbers for differences in root growth across replications.

7   The number of females per gram of root, number of eggs per gram of root, and number of eggs

8   per female per gram of root were compared for all treatments and controls. For both the 30- and

9   60-day experiments, there were six replications of each treatment per run with two experimental

10   runs.

11   **Data analysis.** All statistical analyses were completed using JMP Pro 13 (SAS Institute, Cary,

12   NC). Analysis of variance (ANOVA, P=0.05) with a fit model analysis was used to determine

13   significance of effects of treatment solutions for hatching, movement analysis, total juvenile

14   penetration, and reproduction experiments. Treatment and experimental run were included in the

15   analysis when experimental run was significant in the effect tests (this includes movement

16   analysis, total juvenile penetration, and reproduction experiments). Significant treatment means

17   were separated using Tukey's honestly significant difference test (HSD, α=0.05). The means

18   tested included percent hatch for the hatching experiment; speed and change in curvature for

19   movement analysis experiments; nematodes per gram of root for total juvenile penetration

20   experiments; and variety and treatment for reproduction experiments. A paired T-test was used to

21   determine significant differences (P<0.05) between percent attraction or repulsion to a given

22   treatment in attraction experiments. Proportions for both the spatial penetration and juvenile

1   development experiments were compared against the null hypothesis (proportion is equal to 1.0)

2   through a T-test with α=0.05.

3   **Results**

4   **Hatching experiments.** Overall, an average of two percent of the juveniles hatched in all

5   treatments by day 3. Hatching increased in many, but not all, treatments by day 7. Similar hatch

6   was observed on day 14 for all treatments except $ZnSO_4$ (Figure 1A). Cumulative percentage

7   hatch was greatest in $ZnSO_4$, at 40 percent, and was lowest in Avicta solution, at 5 percent

8   (Figure 1B). There were no significant differences in cumulative percent hatch of juveniles from

9   eggs incubated in leachates of soil planted with treated or untreated seeds compared to the fallow

10   and water controls.

11   **Movement analysis experiments.** The movement of the anterior region of individual *H.*

12   *glycines* J2s was analyzed for two parameters, speed and change in curvature (Figure 2). The

13   average speed of J2s incubated in water was 1.67 μm/sec and 1.39 μm/sec in leachate of fallow

14   soil. There were no significant differences in J2 speed of movement in leachates of soil planted

15   with untreated Williams 82, fallow soil, and the water control. Speed of movement of the J2s

16   was significantly less in leachates of soil planted with seeds treated with either Avicta or Clariva

17   than the water control. Similar treatment effects occurred with change in curvature. The average

18   change in curvature for J2s incubated in water was 0.0011 μm^{-1} and in leachate of fallow soil

19   was 0.00081 μm^{-1}. There were no significant differences in change in curvature of J2s between

20   those in the water control and in leachates of soil planted with untreated Williams 82 seed and

21   fallow soil, but change in curvature was significantly less in leachates of soil planted with seeds

22   treated with Avicta and Clariva compared to the water control.

1   **Juvenile attraction experiments.** Overall, 8 to 13 percent of the juveniles in the experiment

2   moved either towards or away from the treatments. There was significant attraction of J2s

3   towards the KNO_3 positive control, at 15 percent. Also, there was significant repulsion of J2s

4   from the $CaCl_2$ negative control, at 7 percent. There were similar numbers of nematodes in the

5   left and right sides of chips when water was used in both ports of the same lane. And there were

6   no significant differences in J2 attraction or repulsion for any of the soil leachate treatments or

7   control solutions, with eight percent or less movement of J2s towards or away from any specific

8   treatment (Figure 3).

9   **Experiments to measure penetration of juveniles at discrete soil depths.** Of the

10   approximately 200 J2s introduced into the soil at each depth, 7 to 13.5 percent penetrated the

11   roots of the untreated Williams 82 control. The average number of nematodes in the roots of the

12   untreated control, by depth, was 14, 18, and 27 for 2, 5, and 7 cm, respectively. The highest

13   number of J2s that penetrated a single plant was 59, and some plants had no nematodes

14   penetrated. Significantly fewer J2s penetrated roots of seedlings grown from Avicta- or Clariva-

15   treated seeds than roots of untreated Williams 82 when inoculated at the seed depth, which was 2

16   cm below the soil surface (Figure 4). Similar significant reductions in penetration were observed

17   when J2s were inoculated at 5 cm deep, which was 3 cm below the seed. At 7 cm deep, or 5 cm

18   below the seed, there were significantly fewer J2s in roots of seedlings grown from Clariva-

19   treated seeds relative to those in the untreated Williams 82 roots, but not so for the Avicta

20   treatment.

21   **Experiments to measure total juvenile penetration and development.** On average there were

22   245, 397, and 334 nematodes per gram of root in plants harvested at 10, 20, and 30 days,

23   respectively. There were no differences in the total number of nematodes or the number of

1   nematodes per gram of root among treatments (data not shown). That is, the total number of

2   nematodes per root were not significantly different among Williams 82 treated with Avicta,

3   Clariva, or left untreated, or untreated Jack.

4   The proportion of *H. glycines* of estimated developmental stages in roots grown from

5   treated seed was compared to that in untreated Williams 82 at days 10, 20 and 30. After 10 days,

6   the average number of nematodes on the untreated control at the J2 and J3 life stages was 87 and

7   156, respectively. There were significantly more (greater proportion) J2s present and

8   significantly fewer J3s on Clariva-treated plants compared to the untreated control (Figure 5A).

9   There were no differences in nematode development in Avicta-treated plants compared to the

10   untreated control 10 days after planting. At 20 days, the average number of nematodes in the J2,

11   J3, J4, and adult female stages for the untreated control was 20, 457, 147, and 63, respectively,

12   and there were significantly more J3s and significantly fewer adult females in Avicta- and

13   Clariva-treated plants compared to the untreated control (Figure 5B). After 30 days, the average

14   number of nematodes at the J2, J3, J4, and adult female stages was 54, 122, 84, and 242,

15   respectively, in the untreated control. There were significantly fewer J2s in both Avicta- and

16   Clariva-treated plants than in untreated Williams 82, but similar proportions of nematodes at

17   later life stages (Figure 5C). Overall, slower development was observed in Avicta-treated roots at

18   20 days and in Clariva-treated roots 10 and 20 days after planting. Differences in proportions of

19   nematodes in different developmental stages were not detected 30 days after planting.

20   **Female maturity and reproduction experiments.** There were significant differences in

21   numbers of *H. glycines* adult females and eggs between Jack (*H. glycines*-resistant) and Williams

22   82 (susceptible) varieties; therefore, each variety was analyzed separately. The average number

23   of adult females on the untreated Williams 82 control at 30 and 60 days after planting was 136

1   and 397 females per gram of root, respectively. There were no differences in number of females

2   per gram of root in either Avicta or Clariva treatments for either variety at 30 or 60 days (data

3   not shown). The average number of adult females per gram of root on 30-day-old plants was 116

4   and 119 for Avicta and Clariva treatments on Williams 82, respectively. After 60 days, the

5   average number of adult females was 472 and 427 for Avicta and Clariva treatments on Williams

6   82, respectively.

7   When standardized to plant root mass, the average number of eggs per gram of root on

8   the untreated Williams 82 control was 16,232 and 79,648 for 30 and 60 days, respectively. There

9   were no differences in the number of eggs per gram of root between treatments for either variety

10   at 30 and 60 days (data not shown). The average number of eggs after 30 days on Williams 82

11   was 17,730 and 18,323 for Avicta and Clariva treatments, respectively. After 60 days, the

12   average number of eggs per gram of root was 82,139 and 68,595 on Avicta and Clariva-treated

13   Williams 82, respectively. The number of eggs per female per gram of root mass for the

14   untreated Williams 82 control was 62 and 53 at 30 and 60 days, respectively. There were no

15   differences among treatments for either variety at 30 and 60 days.

16   **Discussion**

17   The seed treatment Avicta significantly affected the most vulnerable life stage of *H.*

18   *glycines*, the J2, which mostly exists outside the protection of the soybean root. Significant

19   effects were observed on J2 movement and penetration at several soil depths. In previous

20   experiments, we found that *H. glycines* was sensitive to abamectin at concentrations of 1 to 10

21   µg/ml (Jensen et al. 2018). A similar concentration of 1.56 µg/ml was reported to paralyze *M.*

22   *incognita* (Faske and Starr 2006). When J2s were incubated in leachate from soil planted with

1 Avicta-treated seed, movement was significantly inhibited. This inhibition likely also occurred

2 when J2s were inoculated on seedlings grown from Avicta-treated seed. Significantly fewer J2s

3 penetrated roots when the nematodes were added near or 3 cm below the planted seed compared

4 to roots untreated Williams 82. However, at 5 cm below the planted seed, there was no

5 difference in J2 penetration on Avicta-treated plants compared to roots grown from untreated

6 seed. These results are consistent with results of penetration of *M. incognita* and *R. reniformis* on

7 roots of abamectin-treated cotton (Faske and Starr 2006). Because abamectin has low solubility

8 in water and high affinity for organic matter (Wislocki et al. 1989), it is probable that as a seed

9 treatment, most of the active ingredient will remain near or with the seed coat. It is likely that

10 only small amounts of abamectin move with roots deep in the soil, providing minimal protection

11 from *H. glycines*.

12 If the effects of Avicta are limited to the upper 3 cm or so of root systems, this may

13 explain why minimal or no effects were observed in experiments where Avicta-treated plants

14 were grown for 30 days or more. Although the penetrated nematodes developed slower in treated

15 versus non-treated plants 20 days after planting, delayed juvenile development was not detected

16 at 30 days. Avicta also had no effect on the number of adult females or eggs produced on plants

17 grown for 30 and 60 days in our experiments. The 30- and 60-day time frames were enough

18 time for one and two generations of nematodes to occur, respectively. Given the short half-life,

19 high binding-affinity for soil particles and limited mobility of abamectin, it is possible that

20 Avicta did not protect larger root systems of plants 30 days or older under our experimental

21 conditions.

22 We found Avicta seed treatment to have no effect on hatching of *H. glycines*. That is,

23 hatching in leachates of soil planted with Avicta-treated seed was not significantly different than

1   hatching in the water control. However, the 10 percent hatch observed for *H. glycines* eggs in

2   0.1mg of abamectin in Avicta solution was comparable to the 20 percent hatch observed for

3   *Meloidogyne arenaria* eggs in 0.1mg of abamectin B1 (Cayrol et al. 1993). The inability of

4   abamectin to completely inhibit nematode hatching may be explained by the egg shell of *H.*

5   *glycines* acting as a barrier, protecting developing juveniles from the effects of abamectin, or

6   perhaps the developing juveniles in the eggs may not yet have fully developed nervous systems

7   for the abamectin to affect. Also, perhaps the leachates we used were too dilute, leading to no

8   observed effects.

9   Clariva contains spores of *P. nishizawae* that attach to the cuticle of the nematode J2, so

10   it was not unexpected that we saw no effect of Clariva seed treatment on hatching. Like Avicta,

11   Clariva seed treatment significantly affected *H. glycines* J2 movement, penetration success of J2

12   near or 3 and 5 cm below the treated seeds, and development in young plants 10 and 20 days

13   after planting. The observed decrease in J2 movement after incubation in leachate of soil in

14   which Clariva-treated seeds were planted implies that spore attachment may interfere with

15   nematode mobility. A decrease in J2 mobility also may explain why significantly fewer

16   nematodes penetrated roots grown from Clariva-treated seeds compared to roots from untreated

17   Williams 82 seeds at all three depths studied (0, 3, and 5 cm below the planted seed). The fact

18   that Clariva reduced nematode penetration at 5 cm below where the treated seed was planted in

19   the soil suggests that the active ingredient may be mobile in the soil. If so, *P. nishizawae* would

20   encounter more nematodes to infect than if it were immobile. The observed decrease in rate of

21   development of *H. glycines* in young plants 10 and 20 days old is most likely due to the

22   penetrated J2s being infected with *P. nishizawae*.

1   There was no difference in the rate of development of *H. glycines* in roots of plants

2   grown from Clariva-treated seeds compared to the untreated control at 30 days after planting or

3   later. Likewise, there was no observed difference in the total number of penetrated juveniles on

4   whole plants, number of adult females, or number of eggs in these older plants. The lack of

5   effects of Clariva seed treatment in root systems of older plants in our experiments may be

6   attributed to several factors. Environmental conditions in our experiments may have affected the

7   development of *P. nishizawae* within the nematode. Also, not all *H. glycines* hatch at the same

8   time (Niblack et al. 2006), so perhaps a disproportionately high number of Clariva spores

9   attached to the first hatched J2s entering roots, leaving less of an impact on J2s that did not hatch

10   right away. These later-hatching J2s may have been successful at parasitizing the roots.

11   Antagonistic effects of nematicidal seed treatments that repel nematodes from roots could

12   add another layer of protection. In experiments using microfluidic chemotaxis chips, we found

13   that Avicta and Clariva seed treatments neither attracted or repelled *H. glycines* J2s. There was

14   lower overall movement of the J2s in our attraction experiments (8 to 13 percent) than Beeman et

15   al. (2016) reported (32 to 52 percent). So it is possible some treatment effects may have been

16   missed due to lower movement of the J2s in our studies. Still, J2s in the control treatments in our

17   experiments were significantly attracted to KNO_3 and significantly repelled from $CaCl_2$ just as

18   reported by Beeman et al. (2016), indicating that our assays were valid. It is likely that Avicta

19   may not diffuse through the microfluidic filters into the lanes of the chemotaxis chips given the

20   insolubility of abamectin in water, resulting in no effect. And spores of *P. nishizawae* are on

21   average 1.9 μm wide (Atibalentija et al. 2004) and most likely did not pass through the 1 μm

22   width of the filters from the treatment ports into the resting chambers of the microfluidic chips to

1   affect the J2. Additionally, it is unknown if spores of *P. nishizawae* secrete any exudates that

2   may have been able to diffuse into the lanes of the chemotaxis chip.

3   Conceptually there are at least three possible scenarios that explain how nematode-

4   protectant seed treatments may adversely affect nematode biology: 1) they may reduce the

5   number of nematodes that enter a root but not affect nematode development after infection, 2)

6   they may affect nematode development within a root but not the number of nematodes that enter

7   a root, or 3) they may affect both nematode penetration and development. Our experiments

8   suggest that Avicta was most effective at reducing the number of nematodes that penetrated roots

9   of younger plants, but had no effect on development (scenario 1 above). Clariva reduced the

10 number of nematodes that entered into the roots and also affected development up to 20 days

11 after planting (scenario 3 above). Neither seed treatment, however, affected the nematode in

12 older plants with larger root systems. To fully understand the effectiveness of Avicta and Clariva

13 seed treatments, more research is needed on the interactions with different environments and the

14 presence of other pathogens.

20

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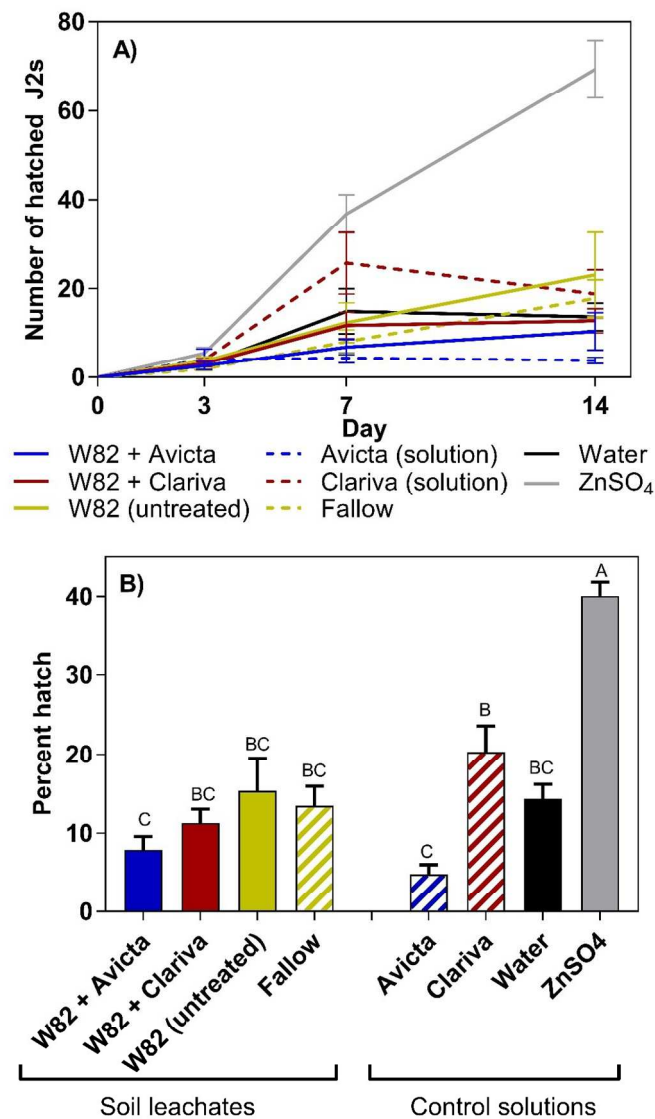28 DOI: 10.1146/annurev-phyto-073009-114425

Figure 1. Hatching of *H. glycines* in leachates of soil planted with Avicta- and Clariva-treated and untreated Williams 82 (W82) soybean seeds, leachate of unplanted soil, and control solutions. The number of second-stage juveniles hatched was counted at 3, 7, and 14 days (A), and cumulative percentage hatch was calculated at day 14 (B). Letters above each bar indicates differences in cumulative percentage hatch according to Tukey's honestly significant difference test (α=0.05).┼

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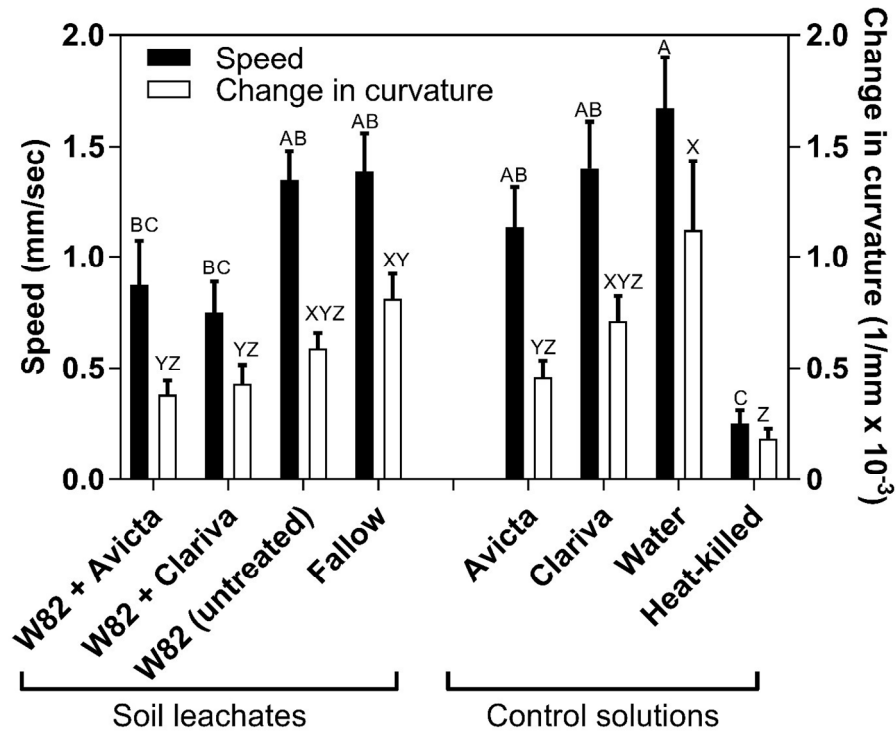
Figure 2. Movement of *H. glycines* second-stage juveniles (J2s) after exposure to leachates of soil planted with nematicide-treated soybean seeds was measured by speed (black bars) and change in curvature (white bars) over 60 seconds of observation. The speed was analyzed separate from the change in curvature. Letters above each bar indicate differences among speed (ABC) or change in curvature (XYZ) according to Tukey's honestly significant difference test (α=0.05).

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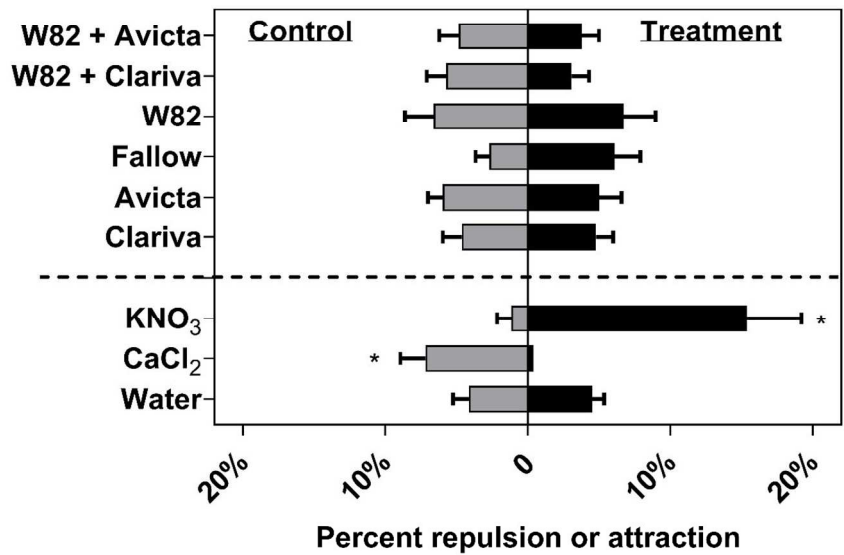
Figure 3. Percentage of *H. glycines* second-stage juveniles (J2) that moved towards (attraction) or away from (repulsion) leachates of soil planted with nematicide-treated soybean seeds. Experiments in chips were validated by observing movement in response to leachates of soil planted with untreated seed (W82) and unplanted soil (fallow), KNO3 (positive control), and CaCl2 (negative control). Black bars pointing right indicate the percentage of nematodes in a population that were attracted to a given treatment. Grey bars pointing left indicate the percentage of nematodes in a population that were repelled by that treatment. An asterisk next to a bar indicates significant attraction to or repulsion from the treatment according to a paired T-test (α=0.05).
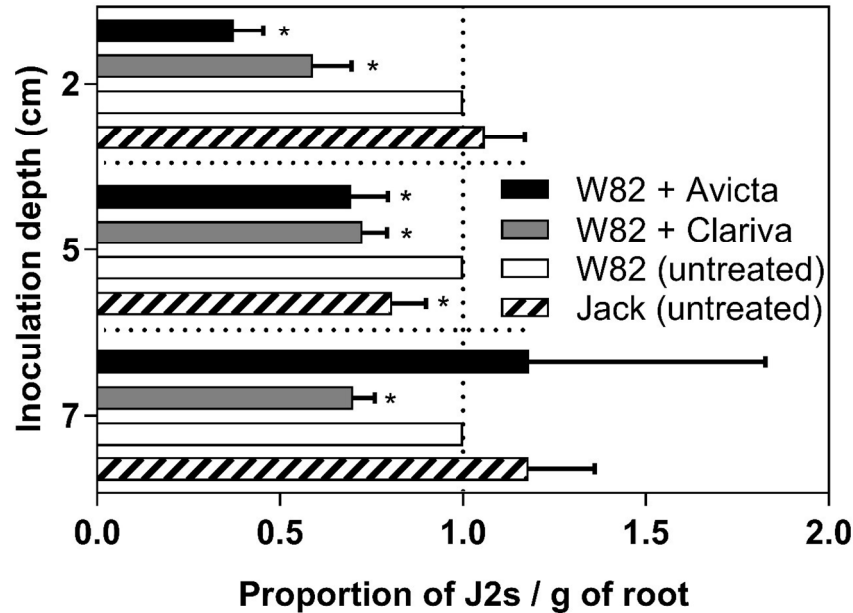
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Figure 4. The number of penetrated *H. glycines* second-stage juveniles (J2s) on seedlings grown from a nematicide-treated seed, was counted 24 hours after inoculation at depths of 2, 5, and 7 cm below the soil line. Treatments tested were Avicta- and Clariva-treated Williams 82 seeds denoted as W81 + Avicta, W82 + Clariva, respectively. Control treatments included seeds of untreated Williams 82 (W82 untreated) and Jack (Jack untreated). This number was then compared to the average number of penetrated J2s on seedlings of untreated Williams 82, yielding a proportion of penetrated J2s. The proportion of J2s at each depth was calculated by dividing the number of J2s per treatment, per gram of root by the average number of penetrated J2s per gram of root of the untreated Williams 82 control. Asterisks indicate a treatment is significantly different from the null hypothesis that the proportion is equal to 1 (the same as the untreated Williams 82 control) according to a T-test with α=0.05.
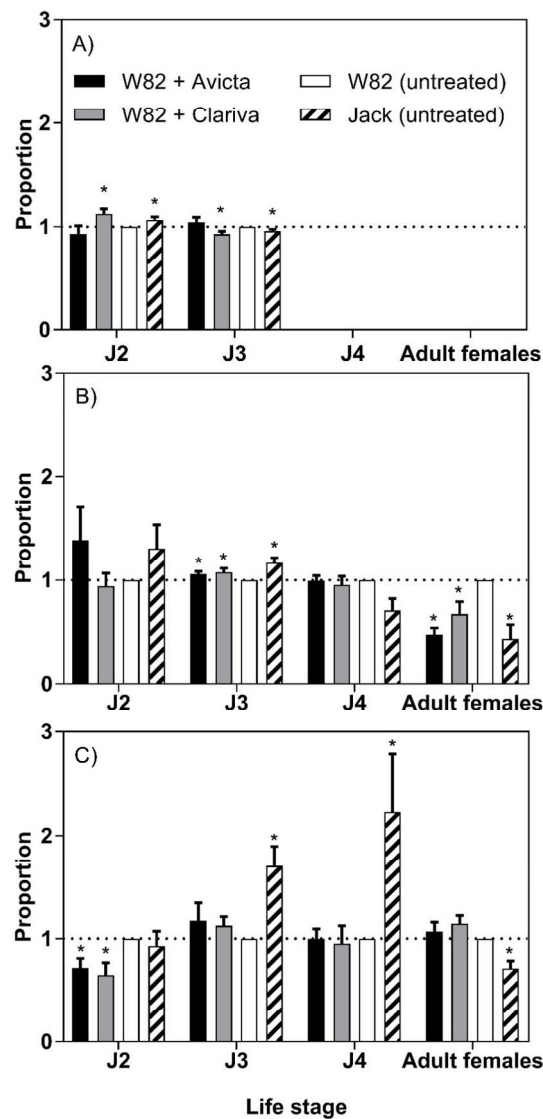
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Figure 5. The proportion of *H. glycines* in each developmental stage in roots of plants grown from nematicide-treated and untreated seeds at 10 (A), 20 (B), and 30 (C) days after planting. The developmental stages identified were second-stage juveniles (J2), third-stage juveniles (J3), fourth-stage juveniles (J4), and adult females. The number of nematodes in each developmental stage was compared to the untreated Williams 82 control, yielding a proportion of nematodes in that stage, for that treatment. The proportion was calculated by dividing the percentage of nematodes in a given developmental stage by the average percentage of nematodes in that same stage on the untreated Williams 82 control. Asterisks indicate a treatment is significantly different from the null hypothesis that the proportion is equal to 1 (the same as the untreated Williams 82 control) according to a T-test with $\alpha=0.05$.

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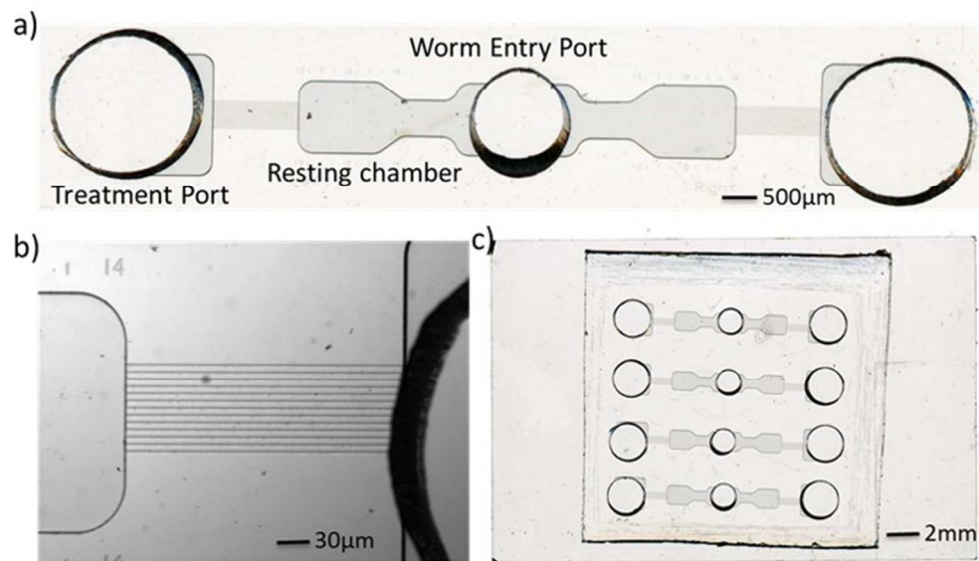
Images of the fabricated microfluidic chip for chemotaxis experiments. A, Each device comprises a worm entry port, resting chambers, filters, treatment port, and control port. B, Magnified image of the filters that permit chemical diffusion but restrict the nematodes to the resting chambers. C, Image of the final fabricated chip comprising four parallel devices and bonded to a glass slide.

128x74mm (149 x 149 DPI)