Journal of Nematology 39(1):43–49. 2007.

Incorporation of a Fluorescent Compound by Live *Heterodera glycines*

N. E. Schroeder,[1] A. E. MacGuidwin[2]

Abstract: The incorporation of fluorescein isothiocyanate (FITC) by J2 of *Heterodera glycines,* the soybean cyst nematode, and the resulting effects on fitness were determined. Live soybean cyst nematode J2 incubated in FITC fluoresced, primarily in the intestinal region, beyond auto-fluorescence. Dissection of animals, as well as fluorescence-quenching techniques, indicated that FITC was not simply bound to the cuticle. FITC was also found to cross the egg shell. Fluorescence increased in relation to FITC concentration and incubation time. Nematodes incubated in FITC remained active and did not lose their fluorescence even after two weeks at room temperature. Fluorescence of nematodes was not stable through development. Males which developed from fluorescent juveniles did not retain the stain. Both FITC and the DMF solvent reduced the hatching rate. However, those individuals that successfully hatched remained viable and able to infect roots. Incorporation of FITC was found to occur in three other genera of nematodes. Rhodamine B isothiocyanate was also found to be incorporated by *H. glycines*.

Key words: chemical uptake, FITC, fluorescein isothiocyanate, RITC, *Heterodera glycines*, physiology, technique, behavior

The use of a chemical marker that is non-toxic and taken up by live plant-parasitic nematodes would have use for both tracking individuals as well as for examining the biology of xenobiotic uptake. Fluorescein isothiocyanate (FITC) is a hydrophobic compound (MW = 389.38) used for protein labeling. It is excited at 494 nm and emits fluorescent light at 518 nm. It has been used to map neural structure and function in *Caenorhabditis elegans* (Hedgecock et al., 1985; Shakir et al., 1993). In *C. elegans*, FITC is stable within the amphids of live nematodes for several hours, but any free FITC can be flushed from the intestine in as few as 10 minutes (Hedgecock et al., 1985). Bird (1979) used a different fluorescein conjugate, fluorescein diacetate, to demonstrate differences between live and dead nematodes. This study showed that live *Meloidogyne incognita* nematodes exposed to fluorescein diacetate showed discrete fluorescent particles in the gut, while dead nematodes showed high intensity fluorescence throughout the length of the nematode. The fluorescence seen was attributed to the action of esterases in the gut that hydrolyzed the fatty acid ester leaving the fluorescent fluorescein molecule. This research did not examine whether the compound itself was toxic to the nematode or if the nematodes could be used following staining.

FITC has been used with plant-parasitic nematodes to model the amphidial uptake of pesticides by *Globodera pallida* and *Heterodera glycines* (Winter et al., 2002). Researchers have also used FITC to demonstrate proof of principle for the ingestion of dsRNA by plant-parasitic nematodes. For example, Urwin et al. (2002) found that 10 to 15% of *G. pallida* and *H. glycines* individuals ingested FITC within four hours of exposure to the invertebrate neurotransmitter octopamine. Bakhetia et al. (2005) used this method with *Meloidogyne incognita* and reported ingestion with 95% of the nematodes.

Our objective in this study was to determine the utility of FITC for marking individuals for behavioral studies and for examining the biology of chemical uptake. We examined the dose and time-response relationships of FITC incorporation and the resulting fluorescence for live second-stage juveniles not treated with octopamine. We also examined whether accumulation of a fluorescent compound is exclusive to FITC and to *H. glycines* by testing another fluorescent compound, rhodamine B isothiocyanate, and several additional nematode genera with FITC. Finally, we tested the potential effects of FITC exposure on nematode fitness. Response variables included hatching, infectivity of treatment cohorts and second-generation effects.

Materials and Methods

Nematode inoculum: Heterodera glycines were collected from a field in south-eastern Wisconsin and maintained in a growth chamber on susceptible soybean cultivar McCall at 28°C and 12 hr light. Whole cysts were extracted using a procedure modified from Jenkins (1964). Monoxenic cultures of *H. glycines* used for hatching assays were established on root explants in STW media and maintained in the dark at 25°C (Lauritis et al., 1983). *H. glycines* J2 used for FITC incorporation studies were obtained by incubating eggs on 25-µm-pore filters (Sefar American Inc., Depew, NY) in 3 mM $ZnCl_2$ to induce hatch. *Pratylenchus penetrans* and *Ditylenchus destructor* were maintained on axenic soybean root cultures (Lauritius et al., 1983). *Caenorhabditis elegans* strain HIM-5, obtained from the lab of Dr. M. Barr, was cultured on NGM media (Brenner, 1974). Dauer larvae were isolated by soaking plates in 2% SDS for 15 min followed by selection of individual dauers. Infective juveniles of *Steinernema carpocapsae*, obtained from the lab of Dr. H. Goodrich-Blair, were recovered from *Galleria mellonella* larvae and stored in deionized H_2O at 4°C for no more than 1 wk prior to use.

Exposure Protocol: Fluorescein isothiocyanate and rho-

Received for publication May 31, 2006.

[1] Graduate student and [2]Professor, Department of Plant Pathology, University of Wisconsin – Madison, Madison, WI 53706.

This research was in partial fulfillment of the first author's Ph.D. dissertation and was supported by Hatch Funds project WIS04900.

E-Mail: aem@plantpath.wisc.edu

This paper was edited by Andrea Skantar.

damine B isothiocyanate (RITC, MW = 536.08) were purchased commercially (Sigma Co., St. Louis, MO). Stock solutions of 5 mg/ml FITC, made by dissolving in dimethylformamide (DMF), were stored at 4°C for no more than 1 wk. Ten actively moving *H. glycines* J2 were placed in 0.1 mg/ml FITC in deionized water with 2% DMF or in deionized water alone and incubated overnight in the dark at 25°C. Nematodes were then washed three times in deionized water to remove excess compound. A subset of juveniles hatched in water was also tested to control for any effects of $ZnCl_2$ on fluorescence. To demonstrate the internal nature of the stain, a subsample of the exposed juveniles was incubated in a solution of 0.4 mg/ml trypan blue (Sigma Co.), a fluorescence quencher, for 5 min before being transferred back to deionized water (Loike and Silverstein, 1983). To further confirm internal accumulation as opposed to cuticular binding, several juveniles were bisected with an endodontic file (Henry Schein Co., Melville, NY) and examined for the release of fluorescent material. This experiment was repeated five times. Incorporation of RITC was tested using the same concentration and methods as FITC and repeated five times.

To study the ability of FITC to cross the egg shell, eggs containing *H. glycines* J2 were exposed to 0.1 mg/ml FITC overnight. These eggs were then removed from FITC and washed three times as before. Eggs were then incubated in 3 mM $ZnCl_2$ to induce hatch in the absence of FITC. Hatched J2 were examined for fluorescence and compared with J2 never exposed to FITC. This experiment was repeated twice.

We determined the stability of the fluorescence within *H. glycines*. Individual juveniles previously incubated in FITC were placed in deionized water, stored at room temperature and compared for fluorescence over 2 wk with individuals not exposed to FITC.

Testing the response of other nematode genera to FITC was performed at the same concentration as for *H. glycines*. Trials were performed using an average of 10 actively moving individuals per trial. Nematodes were examined for the accumulation of FITC and compared for fluorescence to the negative control. The numbers of trials conducted were as follows: Five with mixed stages of *P. penetrans;* four with the dauer stage of *C. elegans;* three with mixed stages of *D. destructor;* and two trials with infective juveniles of *S. carpocapsae*.

Dose-response experiments: Five active *H. glycines* J2 were incubated for 15 hr in the dark at 25°C in one of the following FITC concentrations: 0, 0.001, 0.003, 0.006, 0.01, or 0.1 mg/ml FITC with 2% DMF. Following incubation, nematodes were washed three times with deionized water. Nematodes from each treatment were examined for fluorescence at ×200 magnification and photographed using brightfield (0.1-sec exposure) and fluorescent (2.05-sec exposure) illumination. Nematodes were mounted immediately prior to imaging and gently heated to relax. Fluorescent images of individual nematodes were captured at the same focal plane immediately following the brightfield image. The experiment was repeated twice.

Time-response experiment: Five active *H. glycines* J2 were incubated in 0.1 mg/ml FITC with 2% DMF in the dark at 25°C for the following time periods: 0 min, 10 min, 1 hr, 3 hr, 5 hr and 17 hr. Following exposure to FITC, nematodes were washed three times in deionized water. Imaging was identical to the dose-response experiment. The experiment was repeated twice.

Fluorescent imaging: All nematodes were examined initially for fluorescence with a Leica MZ FLIII fluorescent stereomicroscope with mercury-vapor lamp (W. Nuhsbaum Inc., McHenry, IL) and "41025 PSTN GFP"(excitation: 470 ± 40 nm, emission: 515 ± 30 nm) or "41002 TRITC (Rhodamine):Dil" (excitation: 535 ± 50 nm, emission: 610 ± 75 nm)filter sets (Chroma Technology, Rockingham, VT)for FITC or RITC, respectively. Images were taken using an Olympus fluorescent compound microscope with mercury-vapor lamp and "Olympus NIB" (excitation: 480 ± 10 nm, emission: 515 nm long pass) or "Omega Optical Quad #XF1045" (excitation: 560 ± 15 nm, emission: 605 ± 20 nm) filter sets for FITC and RITC, respectively. Images were captured with an attached camera and MagnaFire Sp software (Optronics, Goleta, CA) in 8-bit grayscale TIFF format. Comparison images of nematodes were taken at equivalent exposure lengths.

Due to the difficulties in precisely quantifying fluorescent intensity with micrograph images, we developed an alternative measure for the dose and time-response assays using the NIH software ImageJ (http://rsb.info.nih.gov/ij/). This measure was defined as the percent area of a nematode's body with fluorescence above a given threshold level based on the 8-bit/256 grayscale. The particular threshold level chosen for a given experiment was the average threshold needed to eliminate all background and auto-fluorescence in the negative control images. This determination was based on a software-established pixel count. The threshold level varied between experiments but was kept constant for any individual experiment. The number of fluorescent pixels for a given nematode was established by first adjusting the fluorescent images to the determined threshold level. A binary function was then utilized, such that pixels were defined as either fluorescent or non-fluorescent. The total pixel area for each nematode was determined using the software "polygon tool" to outline the body of the nematode in the brightfield image. The number of fluorescent pixels for a given nematode was then divided by the total pixel area. This resulted in a percent fluorescence for each individual. All percent data were asine (sqrt) transformed for statistical comparison; untransformed data are given in figures. Comparisons were made using ANOVA and Fisher's LSD for comparison among means. Minitab

Statistical Software was used for this and all other data analysis (Minitab Inc., State College, PA).

Hatching assay: Four whole, tanned cysts from monoxenic cultures were placed in hatching chambers using a system modified from Wong et al. (1993) consisting of 25-µm nylon mesh (Sefer America Co., Depew, NY) held between 2.3-cm × 1.9-cm and 2.0-cm × 1.9-cm cylinders formed from test tube caps (Fisher Scientific Co., Pittsburgh, PA). Hatching chambers were placed on inverted 50-ml centrifuge tube caps that served as reservoirs. Chambers were incubated with either 0.1 mg/ml FITC dissolved in 2% DMF in 3 mM $ZnCl_2$, 2% DMF in 3 mM $ZnCl_2$, or 3 mM $ZnCl_2$ alone. Chambers were arranged in a completely randomized manner and stored at 25°C. Every 72 hr, reservoirs were emptied into test tubes and fresh solutions were added for approximately 5 wk. Hatched juveniles were counted after each time point. Subsamples of hatched juveniles were examined for fluorescence. At the end of the assay, cysts were removed from the chambers, crushed open and the remaining eggs counted. From this we calculated a percent hatch for each of 15 replicates. Data were analyzed by comparison of the cumulative percent hatch for each treatment using one-way ANOVA with Fischer's LSD for comparison between treatments. The experiment was conducted three times.

Infection assay: Second-stage juveniles were collected by hatching eggs from pot cultures in 3 mM $ZnCl_2$ with or without the addition of 0.1 mg/ml FITC. A subsample of juveniles from each treatment was examined for fluorescence. The inoculum was stored at 4°C for no more than 1 wk prior to infestation. The effect of FITC on nematode development was monitored in situ by visually inspecting the roots of nematode-infected soybean cultivar McCall grown in transparent compact disc jewel cases. The cases, maintained in upright positions, were modified by making an opening to allow plant emergence and covered with brown paper when not being inspected. The growth medium was a 1:1 mixture (v/v) of quartz sand and vermiculite. Plants were maintained in a growth chamber with 12 hr light at 28°C, watered daily and fertilized weekly with Hoagland's solution.

The growing medium was then infested with 400 J2 when seedlings were at the cotyledon stage. The two treatments were replicated eight times, and the cases arranged in a randomized design. Females were first observed through the jewel cases after 14 d. At this time, all life stages of *H. glycines* were destructively sampled from plants. Cysts and egg masses were crushed (Faghihi and Ferris, 2000) to recover eggs. Infection was estimated by the number of adults (male and female) recovered. Data were analyzed using Student's *t*-tests. The experiment was conducted twice.

Second generation effects: Because the solvent used, DMF, is a known teratogen, we investigated possible effects upon the offspring of nematodes exposed to FITC. To do this we infested soil with eggs collected from the previous infection assay. Each jewel case received 600 ± 25 eggs. Treatments included eggs from parents either exposed or not exposed to FITC with eight replicates per treatment. In addition, one jewel case of noninfested soil was included as a control. After 23 d, females were observed through jewel cases. Nematodes were then recovered, and data collected as described previously. This experiment was conducted twice.

Results

Incorporation of FITC by nematodes: All *H. glycines* individuals exposed to FITC showed levels of fluorescence visibly greater than normal auto-fluorescence (Fig. 1A,B). The fluorescence was concentrated in the cephalic and intestinal regions (Fig. 1A). The amphids and nerve rings fluoresced in many individuals. The presence of $ZnCl_2$ in the hatching solution did not affect fluorescence. Nematodes incubated in trypan blue following exposure to FITC did not show a reduction in fluorescence. Nematodes exposed to FITC that were subsequently bisected expelled fluorescent material from the incision point. Nematodes that were exposed to FITC as eggs but allowed to hatch in the absence of FITC also showed fluorescence greater than auto-fluorescence. Individuals exposed to FITC that were subsequently stored in water at room temperature did not lose fluorescence over a period of 2 wk.

The response of *H. glycines* to RITC was similar as to FITC (Fig. 2A). All juveniles incubated in RITC showed fluorescence. No auto-fluorescence was seen in negative controls at the wavelengths appropriate for RITC. Fluorescence from RITC was more extensive in the anterior of the nematode than with FITC. Similar to FITC, exposed nematodes that were subsequently bisected expelled fluorescent material from the incision point (Fig 2B). No difference in mortality, determined by movement, was seen between nematodes exposed to RITC and the control group.

Variation in the incorporation of FITC between species was marked. Ninety-five percent of *P. penetrans* examined showed fluorescence greater than the negative controls. *Pratylenchus penetrans* showed a similar distribution of fluorescence to *H. glycines* in the intestinal region; however, no fluorescence was ever observed anterior to the intestine (Fig. 1C,D). Seventy-three percent of *C. elegans* dauers examined showed fluorescence beyond the negative controls. The fluorescence in the non-feeding *C. elegans* dauers was seen throughout the length of the nematode (Fig. 1E,F). All *Steinernema carpocapsae* examined exhibited an increase in fluorescence. However, the increase was only observed in the anterior portion of the intestinal region (Fig. 1G,H). *Ditylenchus destructor* showed no increase in fluorescence upon exposure to FITC (data not shown).

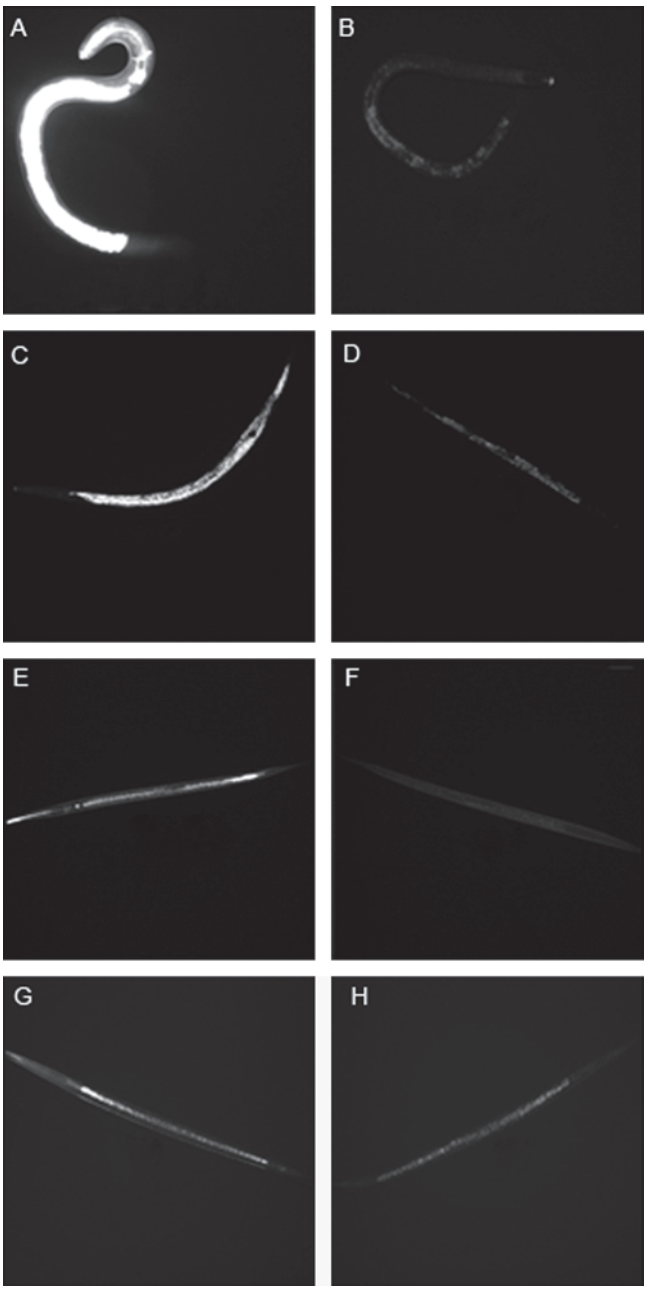

FIG. 1. Fluorescent micrographs of nematodes exposed (left column) or not exposed (right column) to 0.1 mg/ml FITC overnight followed by examination at ×200 magnification. Note that individuals not exposed to FITC show autofluorescence at wavelengths appropriate for FITC. Species and exposure times are as follows: (A,B) *H. glycines*, 2.075 sec; (C,D) *P. penetrans*, 650 msec; (E,F) *C. elegans* dauer larvae, 1.037 sec; (G,H) *S. carpocapsae*, 2.075 sec.

Dose/Time studies using H. glycines: Accumulation of FITC by *H. glycines* as quantified by the percent of the nematode's body that fluoresced beyond normal autofluorescence was positively correlated with the concentration of FITC (Fig. 3). Significant differences were found between concentrations ($P = 0.003$). A regression line was fit to the log (dose + 0.0001) transformed data ($y = 2.93x + 24.6$, $R^2 = 0.425$). Additional experiments (not shown) found no increase in fluorescent intensity from 0.1 mg/ml to 1 mg/ml.

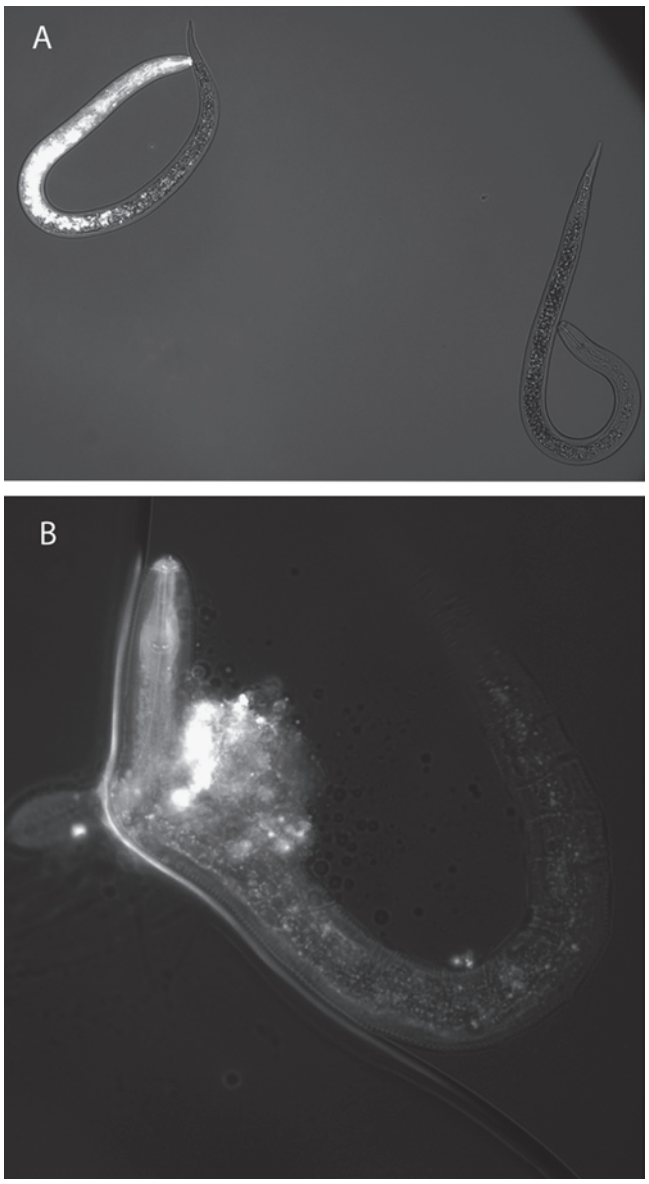

FIG. 2. Fluorescent micrographs with minimal brightfield light: (A) *H. glycines* exposed (left) and not exposed (right) to RITC for 15 hours followed by examination at ×200 magnification and 351 msec exposure time; (B) *H. glycines* J2 exposed to RITC and subsequently bisected showing expulsion of fluorescent contents at ×400 magnification and 351 msec exposure time.

Accumulation of FITC by *H. glycines* was positively correlated with time. There were significant differences between incubation times ($P < 0.001$) (Fig. 4). A regression line was fit to log (time+1) transformed data ($y = 17.536x - 2.4628$, $R^2 = 0.6345$). No further increase in fluorescence was observed after 3 hr.

Response of H. glycines to FITC: Exposure to 0.1 mg/ml FITC and to the DMF solvent alone reduced the cumulative hatching rates compared with the control group ($P < 0.001$, Fisher's LSD = 11.12). Regression lines were fit to the log (days) transformed data as follows: Control, $y = 17.524x - 6.4885$ ($R^2 = 0.326$); FITC and DMF, $y = 5.8716x - 8.3293$ ($R^2 = 0.249$); DMF alone, y =

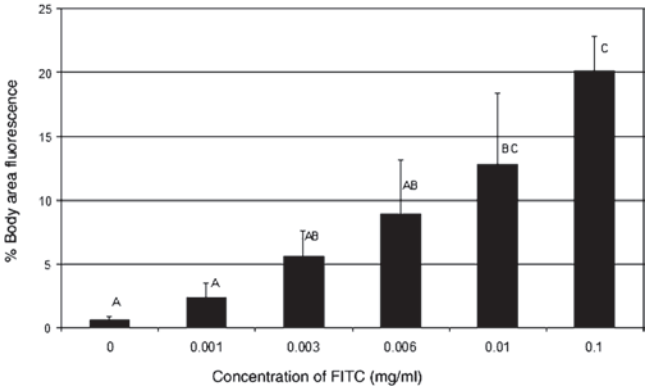

FIG. 3. Dose-response of active *H. glycines* J2 exposed overnight to varying concentrations of FITC. The percent fluorescence ± sem of five replicates was calculated by dividing the number of fluorescent pixels by the total number of pixels for individual nematodes in each treatment. Letters represent significant differences based on Fischer's LSD $\alpha = 0.05$.

12.484x – 17.943 ($R^2 = 0.322$) (Fig. 5). Exposure to 0.1 mg/ml FITC during the hatching process caused no adverse effects on infection and development. More adults ($P = 0.056$) were recovered from the FITC treatment compared with the control group (Table 1). By 14 d, egg production had commenced in both treatments. Recovered males showed no fluorescence. The offspring of nematodes exposed to FITC had higher rates ($P = 0.018$) of infection and development than offspring produced by nematodes not exposed to FITC (Table 1).

DISCUSSION

We have shown that fluorescent compounds are incorporated in a dose- and time-dependent manner by *H. glycines* without exogenously applied neurotransmitters. In our study, the intensity of fluorescence varied among individuals; however, 100% of *H. glycines* J2 incubated in 0.1 mg/ml FITC showed an increase in fluorescence beyond auto-fluorescence. Variation also existed between species, with three of the four other genera tested showing increased but distinct patterns of fluorescence compared to the control groups. All of the life-stages chosen for this study were non-feeding, suggesting that active ingestion is not necessary for incorporation of FITC in these nematodes. Our results are most similar to those of Bird (1979), who found that live *M. incognita* incubated in fluorescein diacetate show distinct fluorescent particles in the gut.

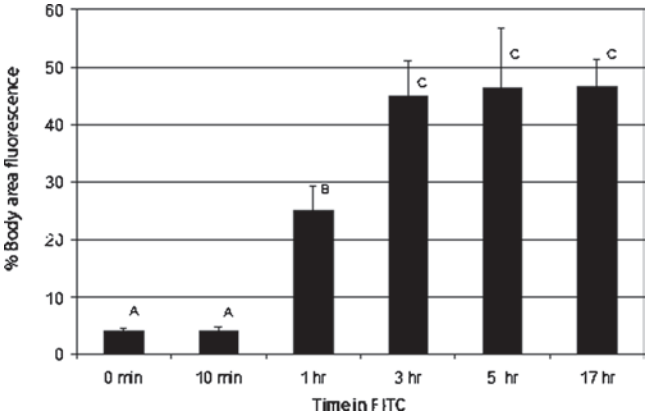

FIG. 4. Time-response of active *H. glycines* J2 exposed to 0.1 mg/ml FITC for varying time periods. Percent fluorescence ± sem of five replicates was calculated as in the dose-response assay. Letters represent significant differences based on Fisher's LSD $\alpha = 0.05$.

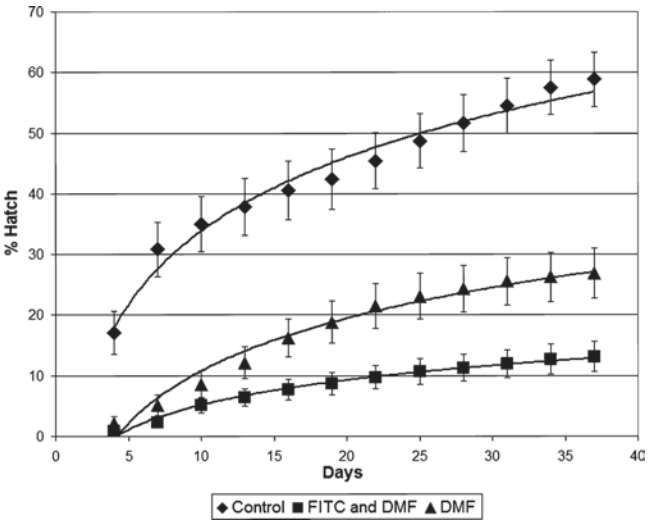

FIG. 5. Hatching curves of *H. glycines* exposed to either 3 mM ZnCl, 0.1 mg/ml FITC in 2% DMF and 3 mM ZnCl, or 2% DMF and 3 mM $ZnCl_2$ alone. Juveniles were counted every 72 hours. Points represent means ± standard error. Regression lines were fit to the log (days) transformed data as follows: Control, y = 17.524x – 6.4885 ($R^2 = 0.326$); FITC and DMF, y = 5.8716x – 8.3293 ($R^2 = 0.249$); DMF alone, y = 12.484x – 17.943 ($R^2 = 0.322$).

The internal location of FITC was shown both by our use of a fluorescent quencher and the expulsion of fluorescent material upon bisection of the nematodes. The precise mode of entry was not examined in this study. Several possibilities for chemical entry exist within the nematode. Various studies have found significant ingestion of FITC and a corresponding fluorescence in the pharyngeal lumen when nematodes were exposed to octopamine (Urwin et al. 2002; Bakhetia et al., 2005). We did not observe fluorescence in the pharyngeal region as would be likely if ingestion were the

TABLE 1. Effect of FITC applied during hatch on infection in the exposed generation and possible teratogenic effects on infection by the second generation. Eggs were hatched in 0.1 mg/ml FITC dissolved in 2% DMF with 3 mM $ZnCl_2$ or in 3 mM $ZnCl_2$ alone (control). Soybeans were infested with 400 hatched juveniles. After 14 days, adults and eggs were recovered. New plants were then infested with 600 eggs collected from the previous assay. After 23 days, adults were recovered. Numbers represent means ± sem.

	Adults recovered	2nd Generation adults recovered
control	66 ± 5	25 ± 3
FITC	93 ± 12	34 ± 2
P-value	0.056	0.018

method of entry. Kimber et al. (2007) recently found RNAi to be possible even in the absence of octopamine. Although they did not demonstrate a particular mode of entry, their results suggest the successful uptake of dsRNA without exogenous neurotransmitter. Alternative modes of entry include diffusion across the cuticle and uptake through the amphids. Previous kinetic studies have shown that small nonpolar molecules can be taken up by plant-parasitic nematodes across the cuticle (Marks et al., 1968; Castro and Thomason, 1973). However, transcuticular uptake does not explain the selective presence of fluorescence in certain regions of the nematode's body. Winter et al. (2002) found uptake of FITC in chemoreceptive neurons of *H. glycines*. This was also found occasionally in our study with *H. glycines* (Fig. 1A); however, neuronal staining does not appear necessary for fluorescence in the intestinal region. In fact, neuronal fluorescence was never seen with *P. penetrans* despite consistent fluorescence in the intestinal region.

Rhodamine B isothiocyanate, a less hydrophobic compound, also accumulated in *H. glycines* (Fig. 2A). Its presence in the anterior portion of the nematode may suggest that differences in hydrophobicity contribute to the final distribution of the compound in the nematode.

The hatching rate of *H. glycines* was reduced by exposure to 0.1 mg/ml FITC in 2% DMF. Part of the reduction in hatch from FITC exposure is due to the DMF solvent (Fig. 5). However, it appears that FITC itself also contributes to an additional reduction in hatch. We did not test whether removing the eggs from FITC causes a resumption of normal hatching levels. *Heterodeta glycines* exposed to FITC as eggs and then allowed to hatch in the absence of FITC fluoresced beyond auto-fluorescence, suggesting that FITC can cross the egg shell. This differs from work done by Blair et al. (1999) in which acridine orange was found not to cross the egg shell barrier. It has been shown that prior to hatch there is a change in egg shell permeability (Jones et al., 1998). It is possible that the nematodes examined in our study were from eggs that had already undergone this change in egg shell permeability.

Juveniles that hatched in FITC showed no morphological or functional defects compared to those hatched without FITC present. Surprisingly, individuals exposed to FITC were better able to infect than individuals not exposed to FITC ($P = 0.056$). We speculate that this may be due to a selection process in which less fit individuals are removed from the hatching process due to FITC. This is supported by our data indicating a reduction in the percent hatch due to FITC exposure. It is further supported by our second-generation assay. Those individuals whose parents had been exposed to FITC, despite never having been exposed themselves, were better at completing their life cycle than offspring from parents not exposed to FITC ($P = 0.018$).

Entomologists mark insects with paint to study individual behavior. The lack of an exoskeleton precludes this possibility in nematodes. However, an internal marker such as FITC would have a similar effect and therefore lessen the need to constantly follow an individual subject. It would also create the possibility of "release and catch" studies to examine the dispersal of individual nematodes in a natural environment. Use of different fluorescent compounds with varying excitation and emission wavelengths would allow multiple treatments of individuals within a single species.

Previous studies have utilized radiolabeled compounds to measure xenobiotic uptake in nematodes (Marks et al., 1968; Castro and Thomason, 1973; Djian and Pijarowski, 1996). However, radiolabeling can only be used with plant-parasitic nematodes at a population level or as autoradiography requiring the fixation of the specimen. Both intra- and interspecific variation in fluorescence has been noted in this study. Given this intraspecific variation and the lack of toxicity after hatching, FITC may be appropriate for studying differences between individuals in the basic biological mechanisms of xenobiotic interactions. Future studies will include closer examination of the mode of entry as well as further examination of the individual variation seen in FITC-based fluorescence.

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