Research Note

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A Technique for Determining Live Second-stage Juveniles of *Heterodera glycines*[1]

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Abstract: We developed a quick and reliable technique to distinguish live and immobile (presumed dead) *Heterodera glycines* second-stage juveniles (J2) following their treatment with microbial culture filtrates. About 50 J2 in 1 ml of culture filtrate or water were placed in wells of a 24-well tissue-culture plate. After incubation, the nematodes in the wells were observed with the aid of an inverted microscope. The J2 lay straight and their viability could not be determined by direct microscopic observation. With the addition of one or two drops (50 to 100 µl) of 1 N NaOH into the well, the live nematodes changed their body shape from straight to curled or hook-shaped after about 30 seconds. The nematodes that responded to NaOH by changing their body shape within 3 minutes were considered a live, while those nematodes that failed to respond within 3 minutes and were immobile were presumed to be dead. The technique is simple, fast, and useful for the examination of a large number of samples in which one wants to determine the effects of microbial cultural filtrates on nematodes, or in similar tests.

Key words: culture filtrate, *Heterodera glycines,* juvenile, mortality, nematode, second-stage juvenile, technique.

Various methods have been tried to distinguish between live and dead nematodes. Thorne (1939) observed that live juveniles expelled from eggs of *Heterodera* sp. straightened immediately, whereas dead ones remained curled or kinked. Staining, considered a more efficient method for distinguishing dead from live nematodes, has been widely used. The stains that have been used include iodide, acridine orange, chrysoidin, phloxine B, new blue R, Meldola blue, eosin-Y, nile blue A, rhodamine B, and $KMnO_4$ (Cairns, 1960; Hooper, 1986). Fluorescein also has been used to distinguish live and dead nematodes (Bird, 1979; Donald and Niblack, 1994; Meyer et al., 1988). These stains provide good results sometimes but not always. Staining often takes hours to several days, and reaction with the stains may depend on nematode species and cause of death. In studies on effects of fungal or bacterial filtrates on vermiform nematodes, probing nematodes with a fine needle was one method used to distinguish paralyzed or dead nematodes from active nematodes (Cayrol et al., 1986). Direct observation has been used as well (Becker et al., 1988). Ciancio (1995) used lactic acid to detect live *Meloidogyne javanica* juveniles treated with mycotoxins.

In our studies on the effects of fungal culture filtrates on *Heterodera glycines* Ichinohe second-stage juveniles (J2), we tried to distinguish paralyzed or dead nematodes from live nematodes by probing with a needle. This method proved to be tedious, insensitive, subjective, difficult to manipulate, and time-consuming. Although staining methods for separating live and dead nematodes are reported as simple (Ogiga and Estey, 1974), they proved difficult when dealing with a large number of samples. Also, the slow reaction of the nematode to stains made this method unsuitable for our purposes. In our study we needed to distinguish live nematodes from immobile (presumed dead) nematodes in a short time. For this purpose, we developed a quick and reliable technique.

Materials and Methods

Agents tested: Heterodera glycines J2 hatched from eggs in a solution of 4 mM $ZnCl_2$ for 24 hours were used. About 50 J2 in 1 ml of

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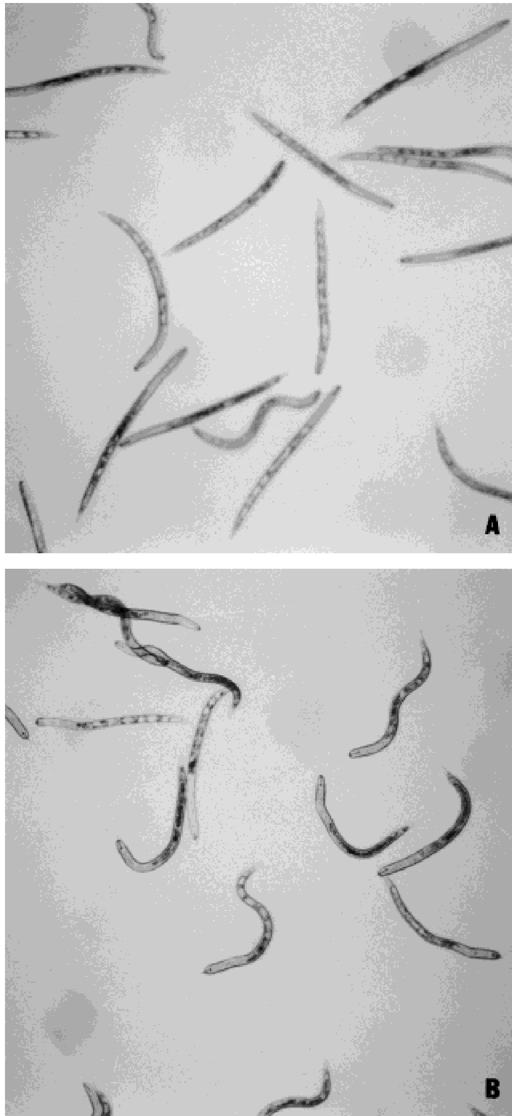

FIG. 1. Change of body shape of *Heterodera glycines* second-stage juveniles (J2) tested with NaOH. The J2 were stored at room temperature (23–24 °C) for 25 days. A) Before adding NaOH, most J2 lay straight and, whether they were alive or not, could not be determined by direct microscopic observation. B) Two minutes after 100 µl of 1 N NaOH were added into 1 ml of water. The live J2 changed their body shape to curled or hook-shaped.

water were placed in each well of a 24-well tissue-culture plate and incubated for 3 days to 1 week at room temperature (23–24 °C). After the incubation periods, most of the J2 lay straight in water and their viability could not be determined by direct observation. The chemical agents tested to impose a response were 95% ethyl alcohol, 37% formaldehyde, 1 N HCl, 1 N NaOH, 5% NaOCl (bleach), and white distilled vinegar (CH_3COOH, 5% acidity). An initial volume of 10 µl of each of the agents was added to 1 ml of water, in a well, and the amount of each agent was increased until a strong response of the J2 was observed. The mobility or contraction response of the nematodes was examined with the aid of an inverted microscope at magnifications of ×40 to ×100.

Optimal concentration of NaOH for distinguishing live nematodes: Second-stage juveniles that hatched within 24 hours were placed in 1 ml of water in wells of tissue-culture plates and incubated for 5 or 12 days at room temperature. A volume of 1 to 200 µl of 1 N NaOH was added to each well, and each treatment was replicated four times. The number of J2 that responded to the NaOH was counted within 2 minutes. Regression analysis was performed to determine the relationship between the number of live J2 detected and the concentrations of NaOH tested.

Comparison of NaOH method with other methods: Second-stage juveniles hatched within 24 hours were collected. The J2 were incubated for various periods under the following conditions: (i) stored for 2 weeks at room temperature (23–24 °C) after hatching, (ii) incubated in sandy soil in a greenhouse for 1 week, (iii) treated with yeast culture filtrate for 8 hours, (iv) treated with yeast culture filtrate for 48 hours, and (v) treated with culture filtrate of *Paecilomyces lilacinus* Samson for 8 hours. About 50 J2 were placed in the wells of a tissue-culture plate containing either 1 ml of water or culture filtrate per well. Live or immobile status of J2 was determined with each of the following five methods: (i) for direct observation, the water in the well was agitated with a pipet, and mobile and immobile J2 were counted; (ii) nematodes were probed with a fine needle, and those responding to the probing and the total number of nematodes were counted; (iii) one drop of 1 N NaOH was added; (iv) one drop of 5% NaOCl was added; and (v) one drop of a 5% phloxine B solution was added to 1 ml of water in the well with J2, and the percentage of J2

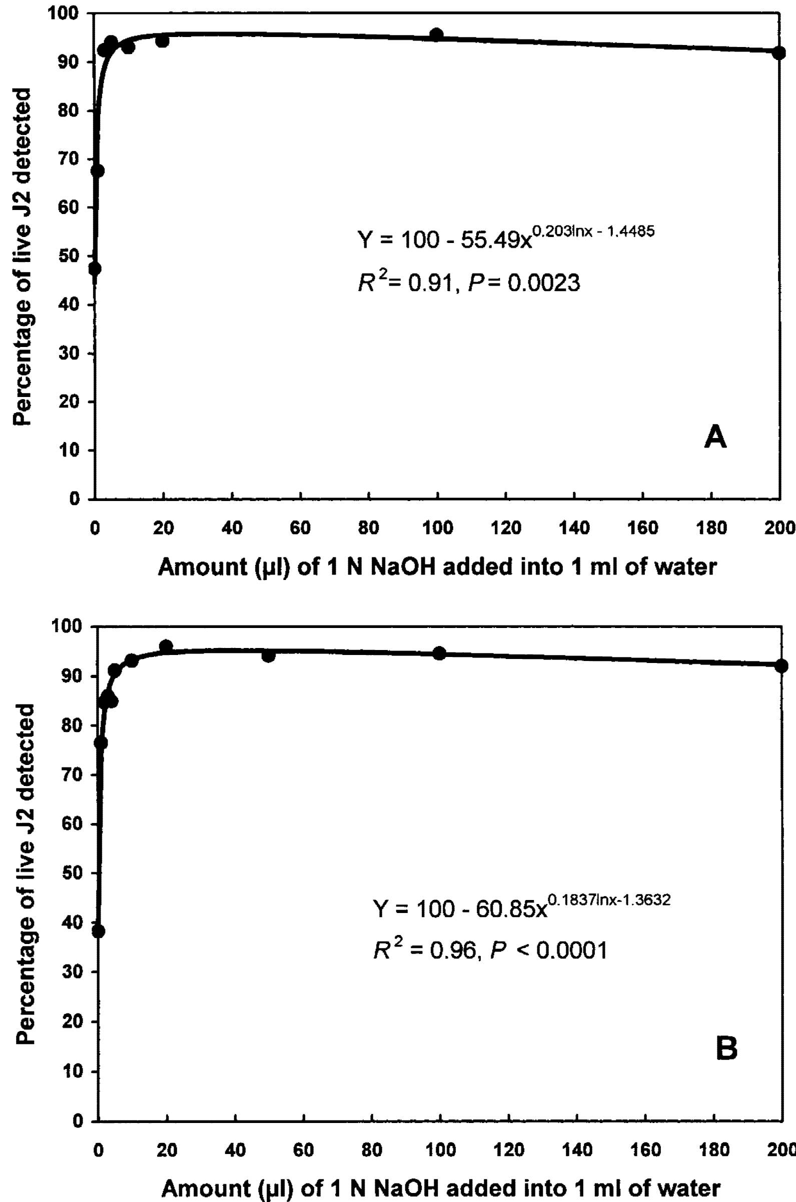

FIG. 2. Percentage of live second-stage juveniles (J2) of *Heterodera glycines* detected with NaOH treatment. A) J2 stored for 5 days at room temperature (23–24 °C) after hatching. B) J2 stored for 12 days at room temperature after hatching.

stained red was determined after 3 hours of incubation at room temperature.

Results and Discussions

Among the agents tested, NaOH and NaOCl gave the best response. Nematode response to these agents occurred rapidly, generally within 30 seconds, and appeared uniform among the J2 within each well when the agent volume added to 1 ml of water was 20 to 200 µl. The J2 changed their body shape from straight to curled or hook-shaped, whereas, the shape of immobile nematodes did not change (Fig. 1). This curled body shape lasted more than 10 minutes, which was long enough for counting. After about 10 minutes, the nematodes were apparently dead and became straight. NaOCl caused disruption of the J2 cuticle after

TABLE 1. Percentage of live or dead second-stage juveniles (J2) of *Heterodera glycines* detected by different methods.

Method[a]	Percent live J2				Percent dead J2 phloxine B[f]
	Direct observation[b]	Probing[c]	NaOCl[d]	NaOH[e]	
A	16 c	23 b	92 a	90 a	11
B	0 a	0 a	0 a	1.6 a	100
C	38 c	45 bc	55 b	81 a	3
D	0 a	0 a	0 a	0 a	5
E	0 a	0 a	0 a	0 a	0

[a] A: J2 stored for 2 weeks at room temperature after hatching; B: J2 incubated in sandy soil in a greenhouse for 1 week; C: J2 treated with a yeast culture filtrate for 8 hours; D: J2 treated with a yeast culture filtrate for 48 hours; E: J2 treated with a culture filtrate of *Paecilomyces lilacinus* for 8 hours.

[b] Water in the tissue culture plate wells was agitated with a needle, and mobile and immobile J2 were counted.

[c] Nematodes were probed with a fine needle; those responding to the probing and total number of nematodes were counted.

[d] One drop of 1 N NaOH was added to 1 ml of water containing J2 in wells of a 24-well tissue-culture plate; J2 responding to the agent and total J2 were counted.

[e] One drop of 5% NaOCl was added.

[f] One drop of 5% phloxine B solution was added to 1 ml of water in wells with J2, and the percentage of J2 stained was determined after 3 hours of incubation.

Data are means of four (E), five (B), or six (A, C, D) replicates. Means followed by a common letter in a row were not different according to Duncan's multiple-range test ($P \leq 0.05$).

about 10 minutes. The nematode response to the other agents was either too slow, not uniform among individuals, or without significant visual change of body shape depending on concentrations used. In some cases, the agent's effects were too fast to allow accurate counting. For example, when 50 µm of 1 N HCl was added, the J2 changed body shape within 30 seconds but became straight again in 1 minute.

The maximum percentage of live J2 detected occurred at a concentration of about 35 µl of 1 N NaOH added into 1 ml of water (Fig. 2). Above that concentration, there was little decrease in the percentage of live J2 detected, although higher concentrations resulted in a faster response of the nematodes. One or two drops (50–100 µl) of 1 N NaOH was found to be adequate for determining nematode movement.

Percentages of live J2 detected with NaOH and NaOCl were similar for those samples stored at room temperature for 2 weeks, while direct observation and probing provided for detection of only a small portion of the live nematodes (Table 1). Treatment with phloxine B showed that about 89% of the J2 were unstained and thus presumed alive, which was similar to the percentage of live J2 detected by NaOH or NaOCl. Almost all J2 incubated in soil in the greenhouse for 1 week were immobile, and all of these were stained with Phloxine B (presumed dead). For the nematode sample treated with yeast culture filtrate for 8 hours, NaOH was better for detecting live J2 (81%) than NaOCl, probing, or direct observation; only 3% were stained by phloxine B and presumed dead. When the J2 were treated with yeast culture filtrate for 48 hours or *P. lilacinus* culture filtrate for 8 hours, no live J2 were detected by the first four methods; however, only 5% or 0% were stained by phloxine B, respectively.

The NaOH method was used to detect live *H. glycines* J2 treated with various fungal culture filtrates (Chen et al., 2000). The method is simple, fast, and useful in the study of effects of microbial culture filtrates on nematodes. One sample of about 50 J2 could be counted in 2 minutes. The time for nematodes to respond to NaOH apparently depends on their condition. Consequently, to test the effects of culture filtrates on *H. glycines* we arbitrarily set a response time of 2 to 3 minutes; if a nematode did not respond within this time frame, it was considered immobile or dead.

We tested only a limited variety of compounds. It may be possible to find better compounds that would give a stronger response and a slower killing process. It is not

known whether the mechanism involved is the response of the nematode to the toxic action of NaOH or simply to the pH change. The pH of 50 µl of 1 N NaOH added into 1 ml of water was about 12.3. The pH of 50 µl of 5% NaOCl added into 1 ml of water was about 11. Ciancio (1995) detected live nematodes by their movement and contractions induced by pH change when a drop of 4% lactic acid was added into 500 µl of test solution. Compared to lactic acid, NaOH appeared to be a stronger, faster, and more useful stimulant.

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