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Distinguishing between Viable and Nonviable *Heterodera glycines* Eggs[1]

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Abstract: Greenhouse tests were conducted to determine the persistence of nonviable *Heterodera glycines* eggs in soil. Laboratory and greenhouse tests were conducted to determine the usefulness of the vital stains acridine orange and tetrazolium red for differentiating viable and nonviable eggs of *H. glycines*. Nonviable (boiled) egg preparations were compared with preparations that were not boiled for their persistence in methyl bromide-fumigated soil. Boiled eggs persisted longer (>200 days) than untreated eggs, perhaps due to disinfestation of the egg suspensions by boiling. Neither stain was a good indicator of egg viability as inferred from infectivity of juveniles in a bioassay of the same egg preparations exposed to the stains.

Key words: biological control, egg viability, *Heterodera glycines*, nematode, soybean cyst nematode, viability stain.

Soybean management decisions are based on *Heterodera glycines* egg counts per volume of soil; egg hatch and infectivity are assumed to be 100%. Insecticides and nematicides may kill eggs, but egg death cannot be determined unless the contents are visibly damaged. Fungal biological control agents in eggs can be detected only after mycelium has proliferated within the egg. Differentiation between viable and nonviable eggs is needed to evaluate the effectiveness of biological control agents for managing soybean cyst nematode, *H. glycines*.

Various stains have been used to determine *H. glycines* egg viability after exposure to potential biological control organisms (3). The goal of this research was to develop a method for determination of egg death before visible damage or fungal proliferation occurred. The specific objectives were: i) to determine how long nonviable eggs persist in the soil reservoir under laboratory conditions; and ii) to compare staining with tetrazolium chloride and acridine orange as a predictor of egg viability.

Materials and Methods

Detection of nonviable eggs in soil: Soil samples were collected from a field infested with *H. glycines*. Eggs were mechanically extracted from cysts collected with a semi-automatic elutriator (5). Two egg preparations were made: one boiled for 30 seconds and the other unboiled (control). Aliquots of 1,000 eggs, either boiled or control, were pipetted into each of 42 arbitrarily chosen polyvinylchloride tubes (2.5-cm-d × 20 cm long) containing 100 cm^3 sandy loam soil (74% sand, 16% silt, 10% clay) previously fumigated with methyl bromide. The tubes were placed upright in lots of 21 in 1-liter plastic beakers with soil in the bottoms. The tubes were kept in a laboratory (18–24 C) and the soil surface was watered periodically with tap water.

A subset of tubes was processed with a semi-automatic elutriator every 2 weeks to recover eggs. A subsample (43%) of the water passing through the elutriator was collected in buckets and then passed through a sieve with 28-μm openings (500 mesh) to recover the eggs (5). Egg suspensions were separated by sucrose centrifugation (2) before being stained and counted. Each treatment was replicated four times every 2 weeks for 2 months.

The experiment was repeated once. In the repetition, eight tubes were processed monthly until eggs were no longer recovered. When no eggs were detected, tubes were processed every day for 3 days to confirm that no eggs were being recovered.

Relationship between staining and infectivity: Tetrazolium chloride (10 ppm) and acridine orange (10 ppm) were compared for

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use in a quick assay to determine *H. glycines* egg viability. Tetrazolium red (2,3,5-triphenyltetrazolium chloride) is a vital stain used for seed viability testing (4). A dark pink pigment (formazan) indicates a reaction of tetrazolium molecules with hydrogen atoms released by dehydrogenases involved in respiration (4). Lack of fluorescence following staining with acridine orange indicates viable eggs (3); fluorescence may indicate either egg death (3) or nearness to hatching (6).

Eggs were extracted from a greenhouse population of *H. glycines* as previously described (5). A 30 mM solution of $ZnSO_4$ (0.3 ml) was added to certain egg suspensions before staining to determine if this hatching stimulator (8) increased respiration and improved detection of viable eggs by tetrazolium chloride staining. Aliquots of ca. 100 eggs were stained with either tetrazolium red (4) or acridine orange (3) with or without $ZnSO_4$ for 24 hours at room temperature (22–25 C) before observation. Controls for this experiment included unstained eggs and eggs boiled and stained with tetrazolium red or acridine orange. Either acridine orange (0.3 ml) or tetrazolium chloride (0.3 ml) was added to a glass vial along with 0.3 ml tap water containing *H. glycines* eggs. Eggs were transferred to a hemocytometer for counting (7). Fluorescence was observed with an Olympus microscope with a BH2-RFL attachment (Olympus, 4 Nevada Dr., Lake Success, NY) and bright field microscopy was used to determine the total number of eggs present in a field of view. There were six replications of each treatment.

Three greenhouse population egg lots collected for the staining test were divided and used for the greenhouse infectivity test. Egg suspensions (5 ml) were added to *H. glycines*-susceptible (cv. Pioneer 9391) soybean seed in 10-cm-diam. pots containing sandy loam soil as described above. Each pot contained five seeds and the seedlings were grown for 14 days postemergence. The soybean seedlings were carefully removed from the soil and the roots stained with acid fuchsin to determine the number of juveniles infecting plants (1).

Results and Discussion

Eggs were recovered from equivalent numbers of tubes regardless of whether the eggs were boiled or untreated for the 2-month duration of the first trial (data not presented). In both trials, egg counts decreased rapidly during the first month but were recovered for 6 months in the second trial (Fig. 1). The range in number of eggs recovered per tube was 0–504 eggs. Boiled eggs were recovered longer than untreated eggs, but untreated eggs were recovered in higher numbers. The greater persistence of boiled eggs was unexpected but may be explained by two factors: first, the soil was fumigated and, thus, few microorganisms were present to degrade the boiled eggs; and second, the untreated eggs may have been infected with microorganisms that degrade eggs and egg shells.

The number of viable eggs determined by tetrazolium red was different than when eggs were stained with acridine orange (Table 1). Tetrazolium red staining consistently resulted in a low percentage of eggs stained pink and sometimes a pink color was not observed even though tests with acridine orange indicated that eggs were viable. Evidently soybean cyst nema-

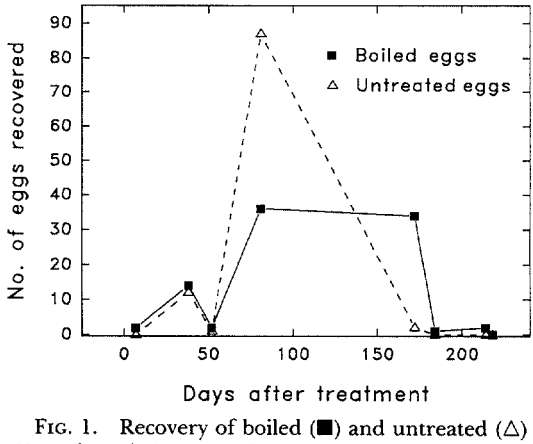

FIG. 1. Recovery of boiled (■) and untreated (△) *Heterodera glycines* eggs from fumigated sandy loam soil contained in polyvinylchloride tubes in the laboratory.

TABLE 1. Percentage viable *Heterodera glycines* eggs (±standard deviation) estimated by biological stains.

Trial	Sample size (eggs/ml)	Tetrazolium red† (%)	Acridine orange‡ (%)	Infectivity§ (%)
1	7,530	0	67.0 ± 5.8	24.2
2	3,460	0	96.0 ± 2.2	100.0
3	19,860	7.5 ± 2.4	75.0 ± 6.1	44.8

Values are means of three replications.
† Percentage of eggs appearing pink after staining with tetrazolium chloride for 24 hours.
‡ Percentage of eggs appearing normal (nonfluorescing) upon observation with fluorescence microscopy after staining for 24 hours with acridine orange.
§ Percentage of juveniles penetrating soybean roots after hatching from the same egg lot.

tode eggshells are impervious to tetrazolium red or the eggs are metabolically inactive. Addition of $ZnSO_4$ did not increase the number of eggs stained with tetrazolium red.

The number of viable eggs as estimated by acridine orange stain was compared with the number of *H. glycines* that actually penetrated the soybean roots (Table 1). The number of nematodes in the roots indicates that the acridine orange staining assay overestimated the number of viable eggs in trials 1 and 3 and confirms that egg viability and juvenile infectivity are not equivalent.

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