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Differences in Hatching of *Heterodera glycines* Egg-mass and Encysted Eggs in vitro[1]

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Abstract: Hatching studies with *Heterodera glycines* typically have been conducted with a mixture of egg-mass and encysted eggs. Laboratory research was conducted to compare hatching of *H. glycines* eggs from external egg masses with that of eggs extracted from within females and cysts (encysted eggs). Egg-mass eggs were collected by soaking infected soybean roots in 0.5% sodium hypochlorite, and encysted eggs were collected from females and cysts dislodged from the same roots with a stream of water. Eggs were incubated at 25 °C in deionized water, 3.0 mM $ZnSO_4$ solution, or one of three synthetic *H. glycines* hatch inhibitors, and hatched juveniles were counted every other day for 22 days. Samples of eggs collected at the beginning and end of all experiments were analyzed to determine extent of embryo development. Egg-mass eggs hatched more rapidly than encysted eggs during the first 16 days, but not thereafter. Throughout the experiments, hatch of egg-mass eggs in deionized water was greater than that of encysted eggs. From day 8 to day 22, egg-mass eggs were less sensitive than encysted eggs to the hatch inhibitor 2-(2′-carboxyethyl)-5-[carboxy(hydroxy)methylidenyl]cyclopentanone. A greater proportion of egg-mass eggs contained vermiform juveniles than did encysted eggs at the beginning of the experiments, but not at the end. Results indicated that *H. glycines* egg-mass and encysted eggs have different hatching behaviors that cannot be explained entirely by differences in embryological development.

Key words: ecology, egg mass, embryogeny, encysted eggs, flow cytometry, hatch assay, hatch inhibitor, hatching, *Heterodera glycines*, soybean cyst nematode.

Females of the soybean cyst nematode (*Heterodera glycines* Ichinohe) produce two types of eggs. A gelatinous egg mass, located at the posterior end of the female, contains approximately one-third of the total eggs produced (Ishibashi et al., 1973), and encysted eggs, located within the hardened body of the female or cyst, constitute the other portion. The cyst protects the enclosed eggs from adverse environmental conditions. The cyst wall also contains one or more compounds that inhibit encysted *H. glycines* eggs from hatching (Okada, 1972). Encysted eggs that become dormant inside the *H. glycines* cyst may remain viable for up to 11 years (Inagaki and Tsutsumi, 1971).

Ishibashi et al. (1973) studied the differences in soybean root penetration between *H. glycines* second-stage juveniles (J2) hatched from egg-mass eggs and encysted eggs. They found that J2 from egg-mass eggs entered the root earlier than those from encysted eggs. Also, as the duration of egg storage at −10 °C increased, infectivity of *H. glycines* J2 from egg-mass eggs decreased, whereas infectivity of J2 from encysted eggs increased (Ishibashi et al., 1973). No additional research on differences between *H. glycines* egg-mass eggs and encysted eggs has been published.

Many compounds have been used to influence the hatching of *H. glycines* eggs. Zinc ions stimulate hatching of *H. glycines* eggs in vitro (Clarke and Shepherd, 1966). Another hatch stimulant is glycinoeclepin A, a compound extracted from the roots of the kidney bean, *Phaseolus vulgaris* L. (Masamune et al., 1982). Recent attempts to synthesize glycinoeclepin A have led to the discovery of synthetic *H. glycines* hatch inhibitors (Kraus et al., 1994, 1996). Although these compounds are analogs of glycinoeclepin A, they inhibit hatching of *H. glycines* eggs at 10 to 100 µg/ml in vitro (Kraus et al., 1994, 1996).

Traditionally, collection of *H. glycines* eggs for research has not involved distinctions between egg types, and it is likely that eggs used in previous hatching studies were mix-

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tures of egg-mass eggs and encysted eggs. Koshy and Evans (1986) observed greater hatch of *H. cruciferae* Franklin egg-mass eggs than encysted eggs in oilseed rape root diffusate. The research described in this paper was conducted to assess how the hatching of egg-mass and encysted *H. glycines* eggs was affected by a known hatch stimulator and several hatch inhibitors. Our hypothesis was that hatching of *H. glycines* egg-mass and encysted eggs would differ in response to hatch stimulators and inhibitors.

Materials and Methods

Inoculum preparation: Soybean plants, *Glycine max* (L.) Merrill cv. Corsoy 79, were grown from seed in soil that contained an Iowa *H. glycines* race 3 isolate. After 30 days, plants were removed from pots, and loose soil was shaken from the root systems. Roots were soaked in tap water for 5 minutes, and soil was gently washed from the root systems. Roots were cut into approximately 2.5-cm lengths, from which eggs were recovered.

Collection of egg-mass eggs: The procedure used to collect *H. glycines* egg-mass eggs was a modification of the technique of Hussey and Barker (1973). Root pieces were soaked, without agitation, for 1 minute in 0.5% sodium hypochlorite (NaClO) solution. The solution and root segments were poured over stacked 710-, 250-, 75-, and 25-µm-pore sieves and gently rinsed with tap water. The 710- and 250-µm-pore sieves were set aside for collection of encysted eggs. The 75- and 25-µm-pore sieves were rinsed a second time with tap water, and the egg-mass eggs were collected from the 25-µm-pore sieve.

Collection of encysted eggs: Cysts and females were dislodged from the root pieces remaining on the 710- and 250-µm-pore sieves with a high-pressure stream of water. The cysts and females were crushed with a motorized pestle (Niblack et al., 1993) to release the encysted eggs, which were collected on a 25-µm-pore sieve.

In vitro hatching experiments: All eggs were separated from debris by centrifugation in a sucrose solution (454 g/liter) at 220*g* for 1 minute (Jenkins, 1964). Eggs were surface-disinfested by immersion in 0.5% chlorohexidine diacetate (Sigma Chemical, St. Louis, MO) (Acedo and Dropkin, 1982) for 15 minutes followed by rinsing several times with sterile deionized water adjusted to pH 7.0 with 1 M HCl or 1 M KOH. A sample of each type of eggs was fixed in 3% paraformaldehyde solution for 2 to 3 days at 26 °C. Fixed eggs were stored at 4 °C for subsequent analysis of embryonic development.

The in vitro hatching units and experimental design used were as described by Behm et al. (1995), except that the units were incubated for 22 days and different incubation solutions were used. Approximately 4,000 egg-mass or encysted eggs were dispensed onto 20-mm-diam., 25-µm-pore microsieves, and the microsieves were incubated in plastic trays containing 12 ml of the treatment solution. Treatment solutions consisted of synthetic analogs of glycinoeclepin A each at 100µg/ml: 2-(2′-carboxyethyl)-5-[carboxy(hydroxy) methylidenyl] cyclopentanone (SJV2.241), 2-(1-ethoxycarbonyl-1-hydroxymethylene) cyclopentanone (SJV5.558), and 2-hydroxymethylenecyclopentanone (SJV5.636). The positive control solution was 3.0 mM $ZnSO_4$, and the neutral control solution was sterile deionized water adjusted to pH 7.0 with 1 M HCl or 1 M KOH. Microsieves were transferred every other day to new trays containing fresh solutions, and the hatched *H. glycines* J2 were counted at each transfer. Unhatched eggs remaining on the microsieves after 22 days also were counted, and all J2 counts were converted to cumulative percent hatch. Unhatched eggs then were combined by treatment solution and egg source, and fixed in 3% paraformaldehyde for 2 to 3 days at 26 °C. Fixed eggs were stored at 4 °C for subsequent analysis of embryonic development.

A factorial treatment arrangement within a randomized complete block design of five-fold replication with two egg sources and five treatment solutions was used for the experiments. The complete experiment was

performed three times. The means from each experiment were averaged and treated as single data points in a three-replication experiment, where each replication equaled one complete experiment.

Analysis of embryonic development: Embryonic development of fixed *H. glycines* eggs was determined with a Coulter EPICS 752 flow cytometer to measure changes in egg size and opacity (Tylka et al., 1993). Subsequently, maturity indices were calculated for each egg sample by dividing the number of mature eggs (those containing vermiform juveniles) by the total number of eggs in the sample.

In addition to fixed eggs from the in vitro hatch experiments, seven additional replications of egg-mass and encysted eggs were collected for analysis of embryonic development. The collection, fixation, storage, and analysis procedures were the same as mentioned above.

Data analyses: The hatch percent means were subjected to analysis of variance (ANOVA), by day, to determine egg source and treatment solution main effects (Cochran and Cox, 1957). When interactions were detected, the data were sorted by treatment solution, then egg source, and analyzed again with ANOVA. A least significant difference was calculated for each day. Maturity indices were analyzed with ANOVA to determine significant main and interaction effects of egg source and solution treatment.

Results

In vitro hatching experiments: Data from both sources of eggs were combined in each of the five solution treatments to determine solution main effects. Throughout the experiments, hatch of eggs was greatest in $ZnSO_4$ solution. By day 22, hatch in $ZnSO_4$ reached 37.6%, whereas hatch of eggs in water reached only 12.1% (Fig. 1). All three synthetic hatch inhibitors suppressed hatching to below 7%. Overall hatch was 6.2% in SJV2.241, 3.3% in SJV5.558, and 0.9% in SJV5.636 (Fig. 1).

Hatch in $ZnSO_4$ solution was greater ($P \leq 0.05$) than hatch in all other solution treatments (Fig. 1) throughout the experiments, but hatch in water was not different ($P > 0.05$) from that in SJV5.558 and SJV2.241 through day 6 and day 10, respectively. After day 10, hatch in water became greater ($P \leq 0.05$) than that in the synthetic hatch inhibitors. From day 2 to 22, hatch in SJV2.241 and SJV5.558 did not differ ($P > 0.05$); however, hatch in SJV2.241 was greater ($P \leq 0.05$) than hatch in SJV5.636 throughout the experiments. Hatch in SJV5.636, the most effective synthetic inhibitor tested, was consistently lower ($P \leq 0.05$) than hatch in water and SJV2.241, but not different ($P > 0.05$) from hatch in SJV5.558.

Data from all solution treatments were combined to determine egg source main effects. Hatch of egg-mass eggs was consistently greater ($P \leq 0.05$) than hatch of encysted eggs from day 2 through day 18 (Fig. 2). Hatch of egg-mass eggs ranged from 1.7% to 13.3%, whereas that of encysted eggs ranged from 0.8% to 10.8% during the experiments. From day 20 to day 22, hatch of the two egg sources was not different ($P >$

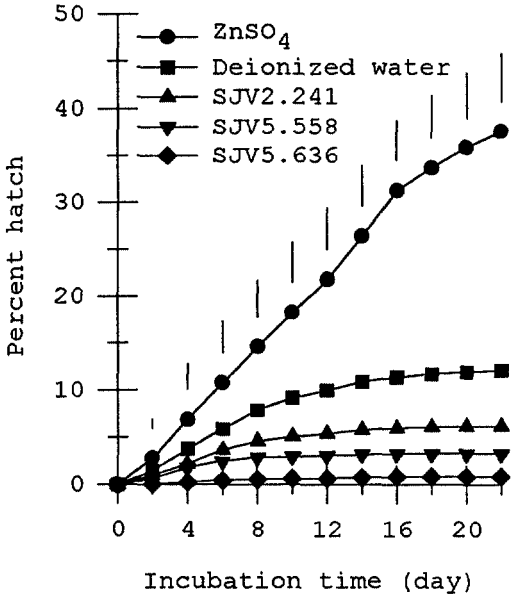

FIG. 1. Main effects of solution treatment on cumulative percent hatch. Egg-mass eggs and encysted eggs of *Heterodera glycines* were incubated in treatment solutions for 22 days. The error bars represent the least significant differences ($P = 0.05$).

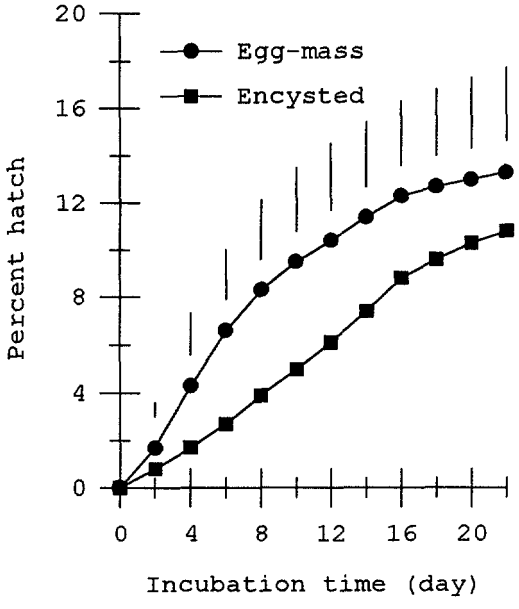

FIG. 2. Main effects of egg source on cumulative percent hatch. Egg-mass eggs and encysted eggs of *Heterodera glycines* were incubated in treatment solutions for 22 days. The error bars represent the least significant differences ($P = 0.05$).

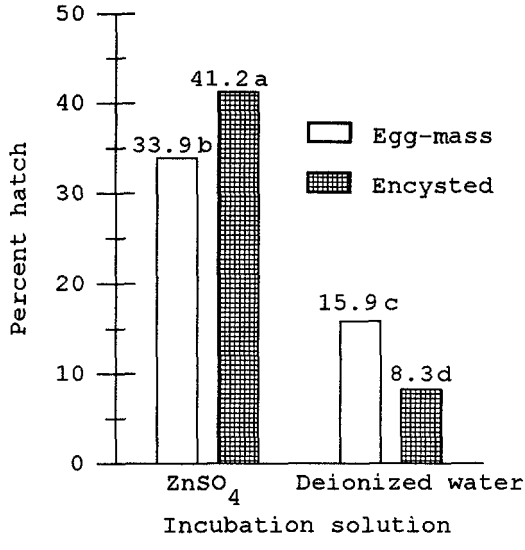

FIG. 3. Percent hatch of *Heterodera glycines* egg-mass and encysted eggs in 3.0 mM $ZnSO_4$ solution and in deionized water. Data shown are total cumulative hatch after 22 days and are the means of three experiments. Values on bars followed by the same letter are not significantly different according to Fisher's least significant difference test ($P = 0.05$).

0.05). From day 2 to day 8, hatch of egg-mass eggs increased at a greater rate than hatch of encysted eggs (Fig. 2).

Solution treatments and source of eggs were analyzed together to determine interactions between the two factors. Hatch of egg-mass eggs in $ZnSO_4$ was similar to that of encysted eggs in $ZnSO_4$ until day 22 (data not shown). At day 22, hatch of egg-mass eggs in $ZnSO_4$ was less ($P \leq 0.05$) than hatch of encysted eggs (Fig. 3). Throughout the experiments, hatch of egg-mass eggs in deionized water was greater ($P \leq 0.05$) than that of encysted eggs. By the end of the experiments, hatch of egg-mass eggs in deionized water was approximately 100% greater ($P \leq 0.05$) than that of encysted eggs (Fig. 3).

The synthetic inhibitors suppressed hatching of egg-mass and encysted eggs to less than 10% (Fig. 4). Throughout the experiments, the hatch of egg-mass and encysted eggs in SJV5.558 and SJV5.636 did not differ ($P > 0.05$) (Fig. 4). From day 2 to day 6, hatch of egg-mass and encysted eggs in SJV2.241 was similar; however, from day 8

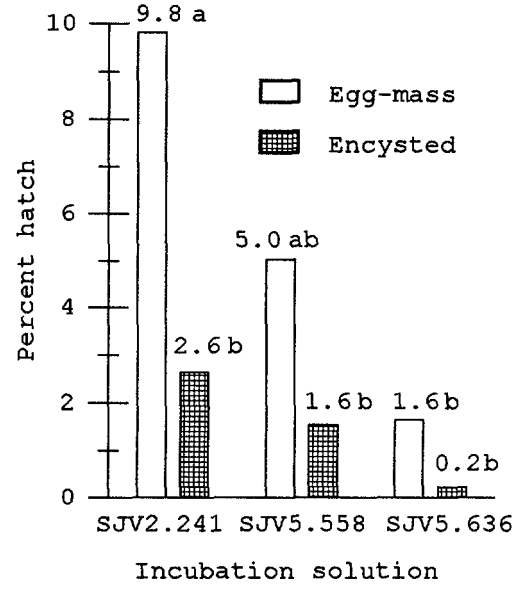

FIG. 4. Percent hatch of *Heterodera glycines* egg-mass and encysted eggs in three synthetic hatch inhibitors (SJV2.241, SJV5.558, SJV5.636). Bars represent total cumulative hatch after 22 days, and values are the means of three experiments. Values on bars followed by the same letter are not significantly different according to Fisher's least significant difference test ($P = 0.05$).

to day 22, hatch of egg-mass eggs in SJV2.241 was greater ($P \leq 0.05$) than hatch of encysted eggs (Fig. 4).

Analysis of embryonic development: Results of flow cytometer analyses indicated that, at the beginning of the experiments, approximately 73% of egg-mass eggs and 60% of encysted eggs contained vermiform juveniles (Table 1); this difference was not significant ($P > 0.05$) when only data from the hatch experiments were analyzed. However, analysis of the additional seven replications of egg-mass and encysted egg samples revealed that egg-mass eggs were more mature ($P \leq 0.05$) than encysted eggs (Table 2).

At termination of the experiments, the egg-mass and encysted eggs consisted of 90% or more mature eggs, with no differences between egg source (Table 1). Eggs from both sources became more mature ($P \leq 0.05$) over the course of the experiments (Table 1). Egg maturity at termination of the experiments was analyzed by treatment solution to determine solution treatment effects, but no differences ($P > 0.05$) were detected in maturity index between treatment solutions (Table 1).

TABLE 1. Maturity indices of *Heterodera glycines* egg-mass eggs and encysted eggs before and after incubation in selected solution treatments for 22 days.

	Sampling time		
	Initial	Final	
	(Maturity index)		LSD[a]
Egg source:			
Egg mass	72.9	94.6	10.5
Encysted	60.2	95.2	15.6
LSD[a]	NS	NS	
Solution treatment:			
Deionized water		95.2	
$ZnSO_4$		93.7	
SJV2.241		94.6	
SJV5.558		94.7	
SJV5.636		96.3	
LSD[a]		NS	

Maturity indices represent percent mature eggs in population. Values are the averages of means from three experiments.

[a] Least significant difference (LSD) values are based on $P = 0.05$; NS = not significant according to ANOVA.

TABLE 2. Maturity indices of *Heterodera glycines* egg-mass eggs and encysted eggs collected from infected soybean roots.

	Maturity index
Egg source:	
Egg mass	46.5
Encysted	35.1
LSD[a]	7.5

Maturity indices represent percent mature eggs in population. Values are the mean of seven replications.

[a] Least significant difference (LSD) values are based on $P = 0.05$.

DISCUSSION

Using the technique described above, we were able to obtain relatively homogeneous populations of egg-mass and encysted *H. glycines* eggs. A higher proportion of egg-mass eggs contained vermiform juveniles than did encysted eggs immediately after the eggs were collected from the females and cysts (at the beginning of the experiments), but embryos in egg-mass and encysted eggs matured similarly over the course of the 3-week-long hatching experiments. When these homogeneous *H. glycines* egg-mass and encysted egg populations were incubated in known hatch stimulators and hatch inhibitors, differences in hatching were detected. Encysted eggs were more responsive or sensitive to zinc sulfate and the hatch inhibitor SJV2.241 than egg-mass eggs and may have greater sensitivity to other chemical stimuli (negative or positive), including host root diffusates. However, such a difference in response of *H. glycines* egg-mass and encysted eggs to host root diffusates would be opposite to the reported response of *H. cruciferae* eggs to host root diffusates (Koshy and Evans, 1986).

There are several possible explanations for the differences in hatching of encysted *H. glycines* eggs relative to egg-mass eggs. Differences in embryo development of egg-mass and encysted eggs likely explain, in part, differences in overall hatching of the two types of eggs. As noted above, egg-mass eggs had a 12% greater proportion of mature eggs than that of encysted eggs at the beginning of the experiments, and,

coincidentally, overall hatching of egg-mass eggs was significantly greater than that of encysted eggs through the first two-thirds of each experiment. Near the end of the experiments, overall hatching of egg-mass and encysted eggs was similar, and 95% of both types of eggs contained vermiform juveniles.

Reduced hatch of encysted *H. glycines* eggs relative to egg-mass eggs in deionized water could involve dormancy, as Ishibashi et al. (1973) reported that a proportion of *H. glycines* eggs become dormant within the cyst. However, dormancy is not a likely explanation for our results. Yen et al. (1995) indicated that dormancy is induced by temperature and signals from the host plant. The cultures used in our experiments had been grown in a greenhouse for several years and would be similar to the greenhouse population of Yen et al. (1995), which did not exhibit dormancy. Also, one would expect dormant eggs not to hatch regardless of the incubation conditions; in our studies, more than 40% of the encysted eggs hatched in zinc sulfate, but did so at a slower rate than egg-mass eggs.

Differences in hatching of egg-mass and encysted eggs in deionized water may have been due to physical damage to the encysted eggs during the extraction procedure. Hussey and Barker (1973) reported reduced hatching of *Meloidogyne incognita* eggs when they were extracted with a mechanical procedure. However, as with dormancy, one would expect physically damaged eggs not to hatch regardless of the incubation conditions, which was not the case in our experiments.

Intrinsic differences in response to hatching stimuli of eggs laid in the egg mass vs. those retained within the body of the *H. glycines* female and cyst also may explain hatching differences. This hypothesis is supported by the fact that we observed differences between hatching of egg-mass and encysted eggs with zinc sulfate and SJV2.241, but not with the other two hatch inhibitors. If encysted eggs were more damaged or more dormant than egg-mass eggs, one would expect similar results from all treatment solutions.

The mechanisms of *H. glycines* egg hatch inhibition of the synthetic hatch inhibitors used in this research have not been determined. Two of the inhibitors tested affected hatching of egg-mass and encysted eggs similarly, and one, SJV2.241, inhibited hatching of encysted eggs more than egg-mass eggs. Such differential results may indicate different mechanisms of action or different physical properties of the inhibitors.

Results of this work support our original hypothesis that hatching of *H. glycines* egg-mass and encysted eggs is different. Additional research is needed to confirm the observed differences in hatching of *H. glycines* eggs in natural environments. Results of experiments conducted in natural environments will provide important information on the basic biology and ecology of *H. glycines*.

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