Four types of dormancy exhibited by eggs of *Heterodera schachtii*

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SUMMARY

Cysts of *Heterodera schachtii* from sugarbeet plants grown in a greenhouse and lathhouse were used for hatching studies directly after harvesting or after storage in moist soil for 3 or 6 months. Hatching was initiated by exposing cysts for various times to different temperatures, to different concentrations of sugarbeet root diffusate and to root diffusates from other host and non-host plants. Four types of egg hatch were observed in *H. schachtii* : 1) eggs that hatch rapidly in water; 2) eggs that hatch rapidly in host root diffusate; 3) eggs that hatch over a long period in water; and 4) eggs that hatch over a long period in host root diffusate. Additionally,

Banyer and Fisher (1971) reported that some eggs were induced into dormancy by a period of warmth following cold temperatures. Oostenbrink (1967) observed a seasonal decline in the hatchability of *H. schachtii* eggs, and concluded that there was a clear and distinct diapause. In discussing the facultative diapause involved in the seasonal dormancy of eggs of *Heterodera* and *Globodera* spp., Evans and Perry (1976) noted that second-stage juveniles within eggs are dormant prior to receiving the hatch stimulus. They considered this dormancy to be obligate quiescence. Although the two types of dormancy described for *Heterodera* spp. account for some observations of egg hatch, they do not explain all hatching mechanisms observed for this genus. For example, a proportion of eggs will hatch in water, while others respond only to host root diffusate. The present study was undertaken to investigate and categorize additional types of dormancy occurring in eggs of *H. schachtii*.

Materials and methods

Forty sugarbeet plants (*Beta vulgaris* L. cv. US H-11) were germinated in sandy loam in pots of *ca* 1400 ml capacity, and grown in a greenhouse for 1 month before being divided into two groups. One group of ten plants was used to produce root diffusate at weekly intervals; the other group of 30 plants was inoculated with 1000 second-stage juveniles of *H. schachtii* per plant. Two weeks after inoculation, 10 of the 30 plants were moved to a lathhouse, and cysts were harvested 6 months later in December under low temperature conditions. Cysts from plants grown in the greenhouse were harvested in September following development under high temperature and high light conditions. Mature cysts filled with eggs were selected for egg hatch experiments.

Ten plant species, which included both hosts and non-hosts of *H. schachtii*, were grown in a greenhouse. The host plants were brussels sprouts (*Brassica oleracea* L. var. *gemmifera* cv. dwarf improved), cauliflower (*Brassica oleracea* L. var. *botrytis* cv. early snowball), mustard (*Brassica juncea* L. var. *crispifolia* cv. giant curled), cabbage (*Brassica oleracea* L. var. *capitata* cv. Danish ball head), and turnip (*Brassica rapa* L. cv. unknown); the non-hosts were potato (*Solanum tuberosum* L. cv. Russet Burbank), onion (*Allium cepa* L. cv. white bunching), barley (*Hordeum vulgare* L. cv. TL75-790), alfalfa (*Medicago sativa* L. cv. Phytoe), and oat (*Avena sativa* L. cv. Montezuma). After 2 months, root diffusate was collected from all plants by using the method of Fenwick (1949) : sufficient water was added to each pot to saturate the soil; as water began to run from the pots, an additional 50 ml of water was added and the diffusate collected. This solution was considered to be 100 % diffusate concentration. Fresh diffusate was collected at weekly intervals and stored at 5 °C.

Egg hatch tests were conducted in small hatch chambers. The chambers consisted of a doughnut-shaped stryrofoam float with a 4 cm outer diam and a 1 cm central hole through which a 3-mm length of polyethylene tubing was inserted. The lower end of the tube was covered with nylon gauze screen of aperture 0.24 mm such that the screen was submerged when hatch chambers were floated in water. Fifteen *H. schachtii* cysts were placed on each screen and the position of the tube adjusted so that cysts were covered with a thin layer of water. The hatch chambers were floated on hatching solutions (either distilled water or root diffusate) in 5-cm diam Petri dishes. The hatch chambers were transferred to containers of fresh hatching solution at weekly intervals, which also minimized contaminant growth. Juveniles that emerged into the solution during the previous week were counted.

The following experiments were conducted using the methodology described above :

Effect of sugarbeet root diffusate and distilled water on *H. schachtii* egg hatch

a) H. schachtii eggs from cysts produced in the greenhouse were incubated in distilled water and in sugarbeet root diffusate for 8 weeks. The number of eggs hatching in both solutions was measured at daily intervals for 1 week and at weekly intervals for 7 weeks.

b) A determination of cumulative egg hatch in distilled water and root diffusate was also conducted over a longer period (6 months) with hatch determinations made at 2-week intervals.

c) Hatching of *H. schachtii* eggs from cysts produced in the greenhouse in a constant environment was compared to egg hatch from cysts produced under cool-season lathhouse conditions.

Cysts from both the greenhouse and lathhouse were selected for the same apparent physiological age (light yellow coloration, few juveniles in the eggs). Each of these experiments was repeated three times and the results combined. For experiments *a* and *c*, described above, all cysts were dissected after 7 weeks; eggshells and unhatched eggs were counted.

Effect of exposure time and concentration of host root diffusate on *H. schachtii* egg hatch

H. schachtii eggs were exposed to sugarbeet root diffusate for different times (10 min, 1 day, 3 days, 7 days), using distilled water as a control. Incubation intervals in root diffusate were repeated once a week for 7 weeks. In each treatment where exposure time was not continuous, cysts were washed three times with distilled water following incubation in root diffusate, and transferred to Petri dishes of distilled water for the remainder of the week. Hatch chambers were incubated at 20 °C and the number of juveniles hatching from eggs in the cysts was determined at weekly intervals. The hatch rate of *H. schachtii* eggs in different concentrations of sugar-

beet root diffusate (100 %, 75 %, 50 %, 10 %, 5 %, 1 %, 0.1 %, 0 %) was also determined; the cumulative hatch over 7 weeks at 20 °C was recorded.

EFFECT OF STORAGE OF *H. SCHACHTII* CYSTS PRIOR TO EGG HATCH

H. schachtii cysts were stored in moist sand at 10 °C for 3 or 6 months prior to incubation at 20 °C in distilled water or sugarbeet root diffusate. The number of eggs hatching in both solutions was determined.

EFFECT OF EXPOSURE OF *H. SCHACHTII* EGGS TO HOST AND NON-HOST ROOT DIFFUSATE

H. schachtii eggs were exposed to diffusates from host and non-host plants. Hatch chambers were transferred to fresh diffusate at weekly intervals, and the hatched juveniles were counted. Cysts were dissected after 7 weeks to determine the number of eggshells and unhatched eggs.

DETERMINATION OF VIABILITY OF *H. SCHACHTII* JUVENILES REMAINING IN EGGS

The state of juveniles remaining in eggs after exposure to water or sugarbeet root diffusate for 7 weeks in hatching chambers, and in newly-harvested cysts from sugarbeet roots, was determined by removing the eggs from the cysts and breaking the eggshell to liberate the juvenile. Approximately 1000 juveniles from new and old cysts were observed microscopically at regular intervals for periods of 24 h or more.

ESTABLISHMENT OF NORMATIVE TIME REQUIRED FOR *H. SCHACHTII* EGG HATCH

We define the "*normative time*" for egg hatch as the time required for eggs to develop and hatch when they are not in a dormant state and have not been subjected to stress. To establish the normative time for egg hatch, eggs from mature white cysts were removed, separated by soaking in 0.4 % bleach solution for 30 s, and then washed repeatedly with distilled water. The eggs were suspended in a hanging drop under a coverslip over a BPI watch glass and observed microscopically. Five eggs were placed in each drop; hatch solutions were sugarbeet root diffusate and distilled water. The treatments were replicated seven times, and the experiment was repeated three times.

Results

EFFECT OF SUGARBEET ROOT DIFFUSATE AND DISTILLED WATER ON *H. SCHACHTII* EGG HATCH

a) A substantial number of *H. schachtii* eggs hatched rapidly when placed in hatching chambers in water or sugarbeet root diffusate (Fig. 1). Within 1 week, approximately 50 % of the eggs hatched in water and about 55 % in root diffusate. After the first week, the rate of egg hatch in water did not differ significantly from 0; however, in root diffusate, egg hatch continued at a constant, but reduced rate. After 7 weeks, significantly fewer eggs of *H. schachtii* remained unhatched in cysts when incubated in root diffusate at 20 °C than in water (Table 1). There was a higher frequency of cysts containing few unhatched eggs after 7 weeks of incubation at 20 °C in sugarbeet root diffusate than in water (Fig. 2). The mode proportion of unhatched eggs in cysts was 19 % after incubation in root diffusate and 43 % after incubation in distilled water (Fig. 2).

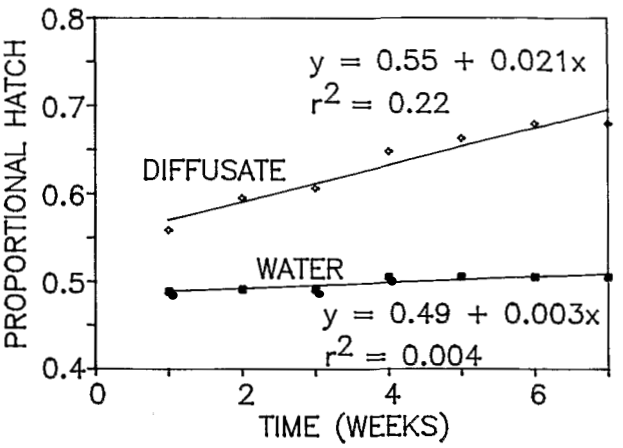

Fig. 1. Proportion of *Heterodera schachtii* eggs hatching in water and sugarbeet root diffusate at 20 °C over 7 weeks.

b) Proportional egg hatch in cysts after a 7-month period revealed slow and continued hatch both in water and root diffusate (Fig. 3). The hatch rate in diffusate over this period was approximately twice that in water. Although the long-term hatch in both water and diffusate was well described by linear models (Fig. 3), the hatching process was irregular; no hatching occurred in some weeks, while in others, periods of mass hatching

Table 1

Mean proportion of *Heterodera schachtii* eggs remaining unhatched after 7 weeks incubation in water or sugarbeet root diffusate at 20 °C.

Experiment	*Water*	*Diffusate*	*t*
1-a	0.583	0.339	0.065**
1-b	0.504	0.232	0.090**
1-c	0.543	0.339	0.039**

** $P \leq 0.01$.

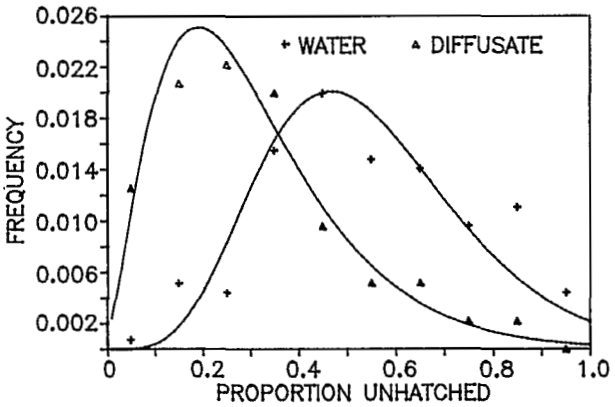

Fig. 2. Frequency of unhatched eggs in *Heterodera schachtii* cysts after 7 weeks incubation at 20 °C in water and sugarbeet root diffusate.

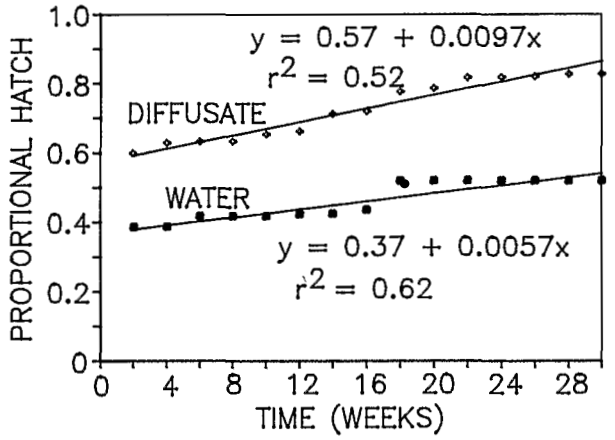

Fig. 3. Proportion of *Heterodera schachtii* eggs hatching in water and sugarbeet root diffusate at 20 °C over 30 weeks.

were observed as shown by the individual data points in Fig. 3. Although hatching periods of eggs incubated in water and diffusate occurred simultaneously at least once, there was no discernible change in incubation conditions or methodology associated with periods of egg hatch. At the end of this experiment, significantly fewer eggs remained unhatched in cysts incubated in root diffusate than in water (Table 1).

c) More juveniles hatched from eggs in cysts produced under relatively constant temperature conditions in the greenhouse than from cysts obtained in fluctuating and cool season conditions in the lathhouse (Fig. 4). In both cases, the hatch rate was greater in sugarbeet root diffusate than in water; however, the pattern of hatch varied in cysts obtained from the two sources. In cysts from the greenhouse, a period of rapid hatch occurred during the first week, followed by gradual, continued hatch, as observed in experiment *a* (Figs 1, 4). Again, significantly fewer eggs remained unhatched after incubation in root diffusate than after incubation in water (Table 1). In cysts from plants grown in the lathhouse, however, egg hatch in the first week was 14 % in water and 18 % in sugarbeet root diffusate. The slopes of the regression lines indicate that the hatch rate over subsequent weeks was greater than that for eggs obtained from the greenhouse.

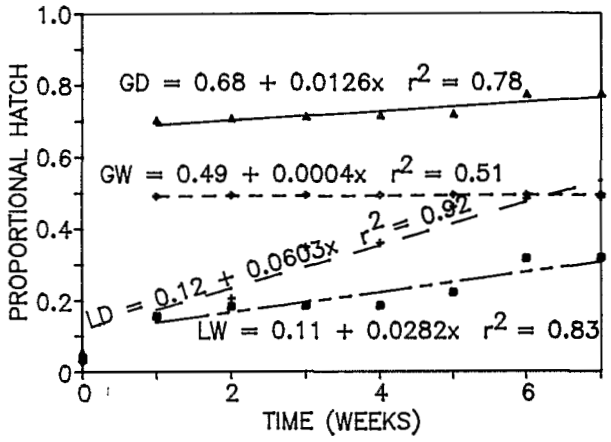

Fig. 4. Proportion of *Heterodera schachtii* eggs hatching after population development under controlled temperature (greenhouse) or fluctuating temperature (lathhouse) conditions when incubated for 7 weeks at 20 °C in water or sugarbeet root diffusate (GD : greenhouse, diffusate; GW : greenhouse, water; LD : lathhouse, diffusate; LW : lathhouse, water).

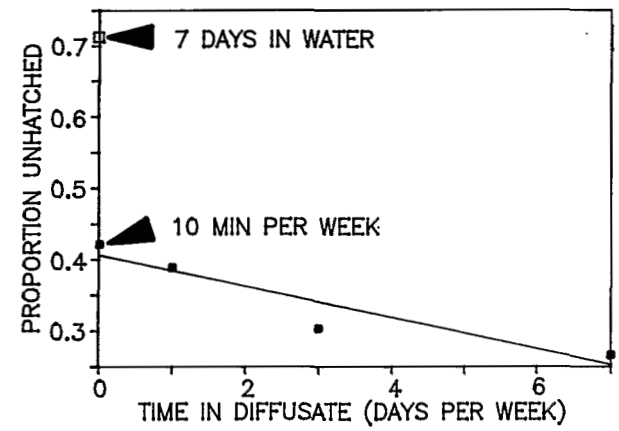

Fig. 5. Proportion of *Heterodera schachtii* eggs remaining unhatched after 7 weeks incubation at 20 °C in water or for different weekly periods of exposure to sugarbeet root diffusate.

EFFECT OF EXPOSURE TIME AND CONCENTRATION OF HOST ROOT DIFFUSATE ON *H. SCHACHTII* EGG HATCH

After continuous incubation in distilled water (no exposure to host root diffusate), 28 % of *H. schachtii* eggs hatched; however, after only 10 min of exposure per week to root diffusate, approximately 60 % of the eggs hatched during the 7 weeks of this experiment (Fig. 5). As exposure times to root diffusate increased, the proportion of unhatched eggs remaining after 7 weeks decreased to almost 25 % for eggs continuously incubated in diffusate (Fig. 5).

When the concentration of host root diffusate was decreased from 100 % to 75 %, the proportion of eggs that hatched over 7 weeks decreased by approximately 20 % (Fig. 6). When the concentration was reduced to 10 %, the proportion of eggs that hatched was not significantly different from that in water.

EFFECT OF STORAGE OF *H. SCHACHTII* CYSTS PRIOR TO HATCH

When cysts were stored for 3 months at 10 °C and incubated in water or root diffusate at 20 °C, significantly more eggs hatched after 7 weeks in root diffusate than in water. When cysts were stored for 6 months, the difference between egg hatch in water and in root diffusate was not significant, although hatching was somewhat greater in diffusate (Fig. 7). A substantial number of the eggs were dead after 6 months storage at 10 °C, although the majority survived storage for 3 months at the same temperature.

EFFECT OF EXPOSURE OF *H. SCHACHTII* EGGS TO HOST AND NON-HOST DIFFUSATE

When eggs were incubated in root diffusate of non-

juveniles dissected from eggs removed from cysts incubated in root diffusate for 7 weeks. The non-moving juveniles from these cysts appeared healthy, intact, and not starved. The inactive juveniles could not be stained with 0.05 % New Blue R solution, indicating that they were alive (Shepherd, 1962). More than half of these inactive juveniles became active within 3 days.

Establishment of normative time required for *H. schachtii* egg hatch

In both host root diffusate and in distilled water, a proportion of eggs developed from multiple-cell to second-stage and then hatched immediately. In others, hatch occurred 3-4 days after the second-stage juvenile was formed, or the eggs remained at the second stage for several weeks before hatching. We consider the normative time for egg hatch to be represented by those eggs that develop from the multiple-cell stage to the second-stage juvenile and hatch without delay, rather than those that remain in the second stage for some period of time prior to hatching.

Discussion

Our experiments suggest that there are several forms of dormancy in eggs of *H. schachtii*, some of which have not been reported. We confirmed a seasonal facultative diapause (Fig. 4); additionally, we characterized several types of delay in egg hatch in cysts produced at any time during the growing season (Fig. 9) :

1) About 40-50 % of *H. schachtii* eggs will hatch rapidly in water at 20 °C, with no other environmental stimulus, if they have not been subjected to any physical stress. We assume that these eggs are non-dormant.

2) An additional 10 % of the eggs hatch rapidly when exposed to host root diffusate. Due to the speed of the response, we consider these eggs to meet criteria for obligate quiescence.

3) About 10 % of the eggs that do not hatch in water during the first week will hatch in water over a longer period. This phenomenon has been described as delayed egg hatch (Evans, 1987), but could also be considered a time-mediated obligate diapause.

4) Approximately 30 % of the eggs hatch over a long period of exposure to host root diffusate. We consider these eggs to be in a state of host-mediated obligate diapause.

Of the various types of egg dormancy, a seasonally-mediated facultative diapause has been reported for *G. rostochiensis* (Evans, 1987), but has not been defined as explicitly for *H. schachtii* (Oostenbrink, 1967). The rapid response to host root diffusate, which we feel meets the criteria for host-mediated obligate quiescence, is also well documented (Shepherd & Cox, 1967; Evans & Perry, 1976). The longer-term hatch of eggs in water over time (time-mediated obligate diapause or delayed egg hatch) and in host root diffusate (host-mediated obligate diapause) has not been clearly documented in previous studies. Our experiments suggest that, within the same cysts, there are eggs that are non-dormant or facultatively quiescent, in a state of host-mediated obligate quiescence, and in at least two forms of diapause, one host-mediated and the other time-mediated.

We also considered the role of host root diffusate as a hatching factor; since up to 40-50 % of *H. schachtii* eggs hatched in distilled water, in the absence of root diffusate, we decided that diffusate is not a basic requirement for all eggs to hatch. However, from experiments 2 and 4, the root diffusate played an important

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Heterodera schachtii Eggs

egg hatch (Evans & Perry, 1976; Evans, 1987). We intend to convey that the eggs in this condition are not stimulated to emerge by any other factor than passage of time. We have attempted to stimulate hatch of eggs in this condition using several methods, including chilling treatments and chemical stimulation. For example, 3 mM zinc chloride solution stimulated 83.7 % hatch within 7 weeks, while host root diffusate only stimulated 66.6 % and water 47.5 % hatch in the same period. Even the zinc chloride, however, required more than 1 week to be effective, indicating an egg diapause rather than quiescence. We have been unable to stimulate 100 % egg hatch within periods as long as 7 months. Hatch of nematode eggs over a long period, apparently independent of any external stimuli, has also been reported for *Meloidogyne* (Martin, 1967; Ogunforowa & Evans, 1977*a, b;* Ferris, Du Vernay & Small, 1978; de Guiran, 1979). We conducted preliminary experiments with five species of *Meloidogyne — M. incognita, M. javanica, M. hapla, M. arenaria* and *M. chitwoodi* — and found approximately 10 % of eggs for all species in diapause. They could not be stimulated to hatch by chilling treatments, but hatched gradually over prolonged periods of time. Also, in our studies with *H. schachtii,* we dissected several thousand eggs that had been subjected to hatching stimuli for up to 7 weeks. Of the juveniles liberated from these eggs, 1-2 % moved immediately in root diffusate, but the activity of the remainder increased very slowly over time, suggesting diapause, rather than an immobilizing effect of the eggshell.

Perry, Clarke and Hennessy (1980) indicated that juveniles may be held in quiescence by the high osmotic pressure associated with trehalose concentrations in the egg fluid. The trehalose concentration is maintained by the integrity of the lipoprotein membrane of the egg. The lipoprotein membrane may be degraded by fungi, allowing trehalose leakage and removal of the osmotic stress, followed by hatch of the juveniles (Perry & Trett, 1986). Such a mechanism could explain the " time-mediated obligate diapause " observed in our long-term hatch studies. However, hatch solutions were changed at weekly intervals in those studies, and we did not observe obvious fungal contamination.

The biological clock mechanisms of insects are well documented (Saunders, 1976), including observations that certain insects enter a state of dormancy as days shorten and winter approaches. Perhaps there is a similar biological clock governing the hatch rate of eggs that are deemed to be in time-mediated diapause, resulting in distribution of the hatch of these eggs over time.

The four kinds of dormancy described for eggs of *H. schachtii* in this paper together confer strong survival value for this nematodes species. They provide for opportunistic, rapid infection of host plants when present, delayed infection of those plants surviving, and long-term survival of the species in the absence of a host. It is interesting that all forms of dormancy, except the temperature-mediated seasonal diapause, occur in cysts of every generation throughout a single growing season. The control mechanisms of these processes stimulate many questions and suggest a need for additional research.

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