OBSERVATIONS ON A NOVEL HATCHING BIOASSAY FOR *GLOBODERA ROSTOCHIENSIS* USING FLUORESCENCE MICROSCOPY

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One of the first effects of stimulation by potato root diffusate (PRD) on the hatching of *Globodera rostochiensis* is a change in permeability of the lipoprotein membranes of the eggshell (Perry, Wharton & Clarke, 1982) which may involve a Ca^{2+} dependent structural alteration (Clarke & Perry, 1985). This allows leakage of solutes, principally trehalose (Clarke, Perry & Hennessy, 1978), from the egg fluid with concomitant increased hydration of the unhatched juvenile (Ellenby & Perry, 1976). The juvenile subsequently starts the behavioural sequence leading to eclosion.

The period of exposure to PRD which is required to initiate hatching of *G. rostochiensis* is very brief (Perry & Beane, 1982). Although the onset of behavioural activity usually does not start until at least three days after application of PRD (Doncaster & Shepherd, 1967), a period of 24h in PRD is sufficient for permeability changes and juvenile water uptake to have occurred in free eggs (Ellenby & Perry, 1976).

Knowledge of these initial stages in the hatching sequence has enabled us to develop a novel hatching bioassay technique for *G. rostochiensis*. It is based on the hypothesis that eggshell permeability changes and juvenile water uptake, induced by hatching agents such as PRD, may allow the passage of a selected fluorochrome through the eggshell and into the unhatched juvenile. Using fluorescence microscopy, we have examined the uptake of acridine orange by unhatched juveniles treated with four hatching and non-hatching agents on a comparative basis.

Materials and methods

Cysts of *G. rostochiensis*, Ro1, grown on potato cv. Arran Banner in pots, were taken from a single generation harvested in 1983 and stored at room temperature (20°) after extraction from the soil. Cysts were soaked in glass distilled water (GDW) for between 4 to 6 days at 20° before being cut open to release the eggs. Suspensions of eggs were rinsed in GDW before being added to 6 mm diameter glass cylinders, each with 30 µm mesh nylon netting fixed over the lower end to retain the eggs (Forrest & Perry, 1980). Each cylinder was placed in an excavated glass block containing the experimental solution and kept at 20°; solutions were changed by transferring each cylinder to a glass block containing fresh solution.

Pilot studies showed that treatment with test solutions of hatching and non-hatching agents before staining gave variable results. However, important and consistent differences emerged between treatments when acridine orange was dissolved in the test solutions, probably because the stain was carried along with water movement into the nematode by solvent drag. Acridine orange was made up to a concentration of 0.001 % in four different test solutions : two hatching agents, PRD and 10 mM zinc sulphate, and two non-hatching agents, autoclaved PRD (APRD) and GDW. PRD was obtained by the method of Fenwick (1949) and diluted with GDW 1 in 4 by volume. To give a non-hatching solution, PRD was deactivated by autoclaving at a pressure of 103.4 KPa for 20 min. Eggs were kept in solutions for different time periods (6, 12, 90 min, 6 and 24 h) all of which were long enough to intiate hatch but too short for actual eclosion to have occurred (Perry & Beane, 1982). Eggs from each time/solution combination were then rinsed in GDW and transferred from the nylon mesh into a drop of GDW on a clean glass microscope slide and covered with a cover-slip.

Slides were examined under ultra-violet light using a Leitz Wetzlar fluorescence microscope fitted with a broad band blue excitation filter (300 to 500 nm) and a supression filter allowing passage of light above 510 nm. Where the acridine orange had passed through the eggshell, the unhatched juvenile fluoresced a bright green and could easily be distinguished from non-fluorescing juveniles in eggs where there was no uptake of stain; occasional fluorescence in these eggs was restricted to the shell. Using both bright field and fluorescence microscopy, the percentage of stained, unhatched juveniles was determined based on a minimum of 100 eggs for each treatment.

As a comparison, routine hatching tests were carried out on five batches of 20 cysts for each solution. Counts of hatched juveniles were made at weekly intervals and total percentage hatch determined at the end of each trial. Solutions were changed at weekly intervals.

Results and discussion

The percentage of fluorescing juveniles increased with

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increase in time in PRD/acridine orange and $ZnSO_4$/acridine orange solutions, to reach a maximum after 24 h. This is likely to be a reflection of the time taken for trehalose leakage and associated juvenile hydration to reach a maximum in free eggs (Ellenby & Perry, 1976). Twenty-four hours was also the optimum period tested for differentiation between inactive solutions and those having hatching activity.

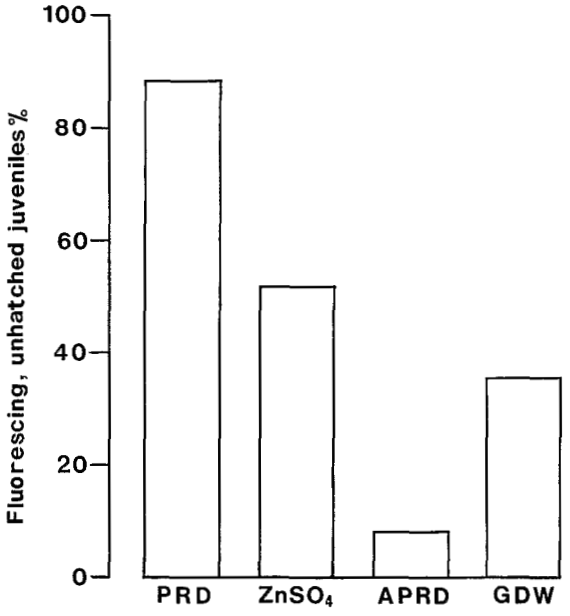

Fig. 1. The percentage of fluorescing, unhatched juveniles of *G. rostochiensis* after 24 h in 0.001 % acridine orange in potato root diffusate (PRD), $ZnSO_4$, autoclaved PRD (APRD) and glass distilled water (GDW).

Results of percentage juveniles stained after 24 h exposure to the experimental solutions are given in Figure 1. The percentage of juveniles fluorescing after PRD/acridine orange treatment (88 %) was greater than in any other treatment and correlated well with the 80 % hatch obtained from routine hatching tests. PRD itself fluoresces (Clarke, 1968) but this did not occur with the excitation/supression filters used for acridine orange detection. However, if fluorescence is associated with the hatching factors in PRD, then the intensity of this fluorescence may form the basis of a separate assay for PRD hatching activity.

Clarke and Shepherd (1966) classed $ZnSO_4$ as an " active" hatching agent with a hatch rating of 49 (root diffusate = 100). In the present tests, the percentage of juveniles fluorescing after $ZnSO_4$/acridine orange treatment was 52 % (Fig. 1), virtually the same as the 51 % hatch obtained in hatching tests. The percentage of juveniles hatching in both non-hatching solutions (APRD and GDW) was negligible and in neither treatment did it exceed 10 %. The percentage of juveniles fluorescing after APRD/acridine orange treatment was similarly low (8 %) but the percentage fluorescing in GDW (36 %) did not correlate as closely, although both values were considerably less than when PRD was used (Fig. 1).

Routine hatching bioassays are time consuming and require large numbers of cysts. Their replacement by a test taking only hours to complete and using free eggs is desirable and our work indicates that a hatching bioassay system using fluorescence microscopy is feasible for *G. rostochiensis*. Using standard diffusate, the technique could be used to assess the hatching potential of various populations of *G. rostochiensis;* it may also be useful in a primary screen to evaluate the hatching activity of novel compounds, although the value for percentage fluorescing juveniles in GDW may be too great to identify with confidence any hatching agents with a hatch rating less than 50.

Picrolonic acid could not be tested as, in solutions with acridine orange, precipitates were formed. The use of rhodamine overcomes this problem but, although similar treatment differences were found using this stain, the percentages of stained, unhatched juveniles were consistently smaller. This may be because rhodamine has a greater molecular weight (479) than acridine orange; the molecular weight of acridine orange (370) is very close to that of trehalose (378) which is able to pass through an eggshell whose permeability characteristics have been altered by PRD. We are currently examining alternative fluorochromes for use in the bioassay and determining whether the technique is applicable to other cyst nematodes.

ACKNOWLEDGEMENT

We thank the Nuffield Foundation for financial support.

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Accepté pour publication le 26 février 1986.