Nematol. medit. (1997), 25: 141-149

Department of Biology, Imperial College of Science, Technology and Medicine, Silwood Park, Ascot, Berkshire SL5 7PY, United Kingdom

HATCHING IN THE POTATO CYST NEMATODE *GLOBODERA ROSTOCHIENSIS* INDICATING DIAPAUSE AS A CAUSE OF VARIABILITY IN EMERGENCE

by

Z. MUHAMMAD [1] and A. A. F. EVANS

Summary. Over a period of 12 months "new" cysts (extracted soon after maturity on host roots) and "old" cysts of *Globodera rostochiensis* (stored for one calendar year outdoors in a gravel plunge) were hatched in potato root diffusate. Diapause was evident in "new" cysts in early autumn, winter, spring and mid-summer but was absent in late autumn and summer. By contrast when "old" cysts were hatched, emergence was about 90% in all periods. It is suggested that diapause persists in "new" cysts, but is overcome when cysts are stored for 12 months in the soil outdoors. A comparison of infectivity of juveniles obtained in the periods when eggs have overcome their diapause failed to show any differences. Also when the number of eggs in "new" and "old" cysts were compared, the number of eggs in "old" cysts exceeded the number of eggs in "new" cysts. It is suggested that hatching in "old" cysts is due to absence of diapause. The presence of large numbers of eggs in "old" cysts even after 12 months storage outdoors in the soil failed to support the theories of hatching, microorganism induced hatching or persistence of hatching factors in the soil.

Most species of plant parasitic nematodes will hatch from eggs shortly after being placed in favourable conditions of moisture, temperature and aeration. The ability of viable eggs of *Globodera rostochiensis* to persist more than eight years in soil (Franklin, 1937) is shared by *Heterodera* spp. whose eggs survived both summer heat (> 40° C) (Thomason and Fife, 1962) and winter freezing (Krusberg and Sardanelli, 1989). A similar situation was recorded for *Meloidogyne javanica* which remained in soil, even after a 4-year fallow (Martin, 1967).

Suggested expllanations for such observations include the production of hatching inhibitors (Ellenby, 1946), oxygen deficit (Wallace, 1959), seasonality (Calam *et al.*, 1949) and nematode genotyope (El-Shatoury, 1978). Shepherd and Cox (1967) and Oostenbrink (1967) interpreted evidence from their hatching experiments as indicating a diapause comparable to that found in insects. However, Hominick *et al.* (1985) criticised most previous studies because of the absence of data on the history of the cysts: host and host growth conditions, cyst extraction date and storage conditions, hatching conditions and hatching medium. They accordingly proposed a standardized protocol to define as many variables as possible and concluded that their experiments offered evidence for a diapause in their population of *G. rostochiensis*. Similar incidence of diapause has also been reported in *G. pallida* (Muhammad, 1994).

[1] Present address: Department of Biological Sciences,University of Maiduguri, P.M.B. 1069, MAIDUGURI, NIGERIA.

This paper reports attempts to further elucidate diapause in *G. rostochiensis*, in line with earlier works of Hominick *et al.* (1985), using the same population of cysts of *G. rostochiensis* and similar procedures in the production of potato root diffusate (PRD), but with modifications in the hatching protocols and a clear definition of the cysts used.

Materials and methods

In the course of the experiment a "nematode-response" (where cyst are set to hatch and counting continued until hatching ability was depleted before another batch of cysts are set to hatch) hatching protocol was used as opposed to the "calendar-hatching" (where cysts are set to hatch at pre-fixed two months interval) protocols of Hominick *et al.* (1985). Also production of "new" and "old" cysts was directed to elucidating the diapause in *G. rostochiensis*. "New" cysts were those extracted soon after their maturity on host roots, and were hypothesized to be those exhibiting diapause, while "old" cysts were those that were stored for one calendar year outdoors in a gravel plunge and were hypothesized to be those that had overcome their diapause.

In accordance with the strict protocol adovcated by Hominick *et al.* (1985) a detailed account of the origin and treatment of nematode and plant material is presented.

Cysts of *G. rostochiensis* (Wollenw.) Mulvey *et* Stone (RO1, personal communication, Dr. J.M.S. Forrest) were of an isolate that had been continuously cultured outdoors for several years on potato cv. Pentland Crown at the Scottish Crop Research Institute, Invergowrie, UK. They were received in late February 1987, after being harvested in 1985, extracted from soil and stored at room temperature in the dark.

At Silwood Park, Ascot, UK, the cysts were counted randomly into batches of 100 and stored in glass vials in the dark at 20° C.

Certified seed potato (*Solanum tuberosum* L.) tubers, cv Pentland Crown were obtained from commercial sources and sprouted at 5-8 °C. Thirty 18 cm plastic pots with the drainage holes covered with fine muslin cloth were filled with sterilized loam: sand (2:1). A sprouted potato tuber was planted in each pot and kept for three days in a glasshouse at 18-34 °C.

On 28th April 1987, thirty batches of 100 cysts of *G. rostochiensis* were soaked in sterilized tap water for seven days in the dark at 20 °C. On 5th May 1987, each pot containing a sprouting potato tuber was inoculated with one batch of presoaked cysts of *G. rostochiensis.* Pots were kept outdoors in a gravel plunge and watered when necessary throughout the growing period.

About three months later, as the potato foliage became senescent, the 30 pots were removed from the gravel plunge. The soil and the roots were emptied into a large tray and dried slowly and in the dark in a warm-air chamber.

After five days, the dry contents of 15 pots were extracted using a Fenwick can. The float containing the cysts was again dried in the dark in a warm air chamber. When the debris and the cysts were completely dry, after three days, cysts were hand-picked from the debris and randomly counted into batches of 50 and stored in glass vials at 20 °C in the dark. These cysts were referred to as "new" cysts.

The soil from the remaining 15 pots was sieved to remove all traces of living potato roots or tubers, while retaining all cysts. Soil was then replaced in the pots which were returned to the gravel plunge outdoors at the same time as the "new" cysts began their storage at 20 °C in the dark. The pots were left for 365 days and then cysts in each pot were extracted with a Fenwick can on 4th September 1988. Cysts were dried, hand-picked and counted into batches of 50 and stored in glass vials at 20 °C in the dark from 8th October 1988. These cysts were referred to as "old" cysts. In the absence of any living potato tissue to allow reproduction, the

"old" cysts are presumed to be similar to the "new" cysts, with the only difference being one of age and storage conditions.

On May 5th, 1987 potato root diffusate from cv. Pentland Crown was produced and stored according to the protocols of Hominick *et al.* (1985).

Hatching was done at 20 °C according to a "nematode-response" protocol (Fig. 1). "New" cysts were stored in batches of 50 cysts in the dark (Fig. 1, S1). Four replicates of 50 cysts each were hatched as required. During the experiments, each batch of cysts was kept on a nylon hatching sieve (45 µm), in 1 ml of either sterilized tap water or potato root diffusate in a well of a 24-well Linbro culture plate. The plates were individually wrapped in a black plastic sheet to limit exposure to light; while changing the medium care was taken to minimise exposure of the cysts to light.

The first "new" cysts were set to hatch in October, 1987 by soaking in sterilized tap water for two weeks (Fig. 1, H1) then hatched in potato root diffusate (Fig. 1, H2). Emerging juveniles were counted weekly until fewer than 100 juveniles/replicate/week had emerged. Hatching was then concluded for these cysts which were rinsed in sterilized tap water, dried and stored on their hatching sieves at 20 °C in the dark for a period of twelve months (Fig. 1, S3). A new batch of cysts was chosen at random and set to hatch immediately. This protocol was continued for one calendar year (Table I).

"New" cysts had a second hatching after each replicate had experienced twelve months of dry storage at 20 °C (Table I). Cysts were first soaked in sterilized tap water for one week (Fig. 1, H3) and then hatched in potato root diffusate (Fig. 1, H4). Emerging juveniles were counted weekly until fewer than ten juveniles/replicate/week had emerged.

Finally, after the completion of hatching, cysts were individually broken open and the numbers of viable eggs (i.e. those eggs containing unhatched coiled second stage juveniles) counted in each batch of cysts. Juveniles which were freed in the process of breaking the cysts were counted as viable unhatched eggs, while empty eggs were disregarded. Emergence was calculated as a percentage of the total hatched and unhatched viable eggs from each replicate.

The same protocol (Fig. 1) and strict calendar (Table I) was followed to hatch "old" cysts, the only difference being that these cysts were first stored for twelve months outdoors and then for various periods at 20 °C in the dark (Fig. 1, S2). When emergence was fewer than 100 juveniles/replicate/week, the cysts were broken open and the number of viable unhatched eggs determined as before. Because of lack of time cysts were not stored and hatched for a second time as with "new" cysts.

A sample of juveniles emerging immediately from "new" cysts was taken during peak emergence in any given hatching period for an infectivity test. The infectivity test used three-week old tomato (*Lycopersicon esculentum* Mill.) plants cv. Moneymaker planted into 9 cm plastic pots filled with a loam: sand mixture (1:1). After three days, when plants were fully estabiliched in the potting medium, four replicates of the plants were each inoculated with approximately 1000 one-week old juveniles. Pots were kept at 20 °C in a controlled temperature room with 16 hours light and eight hours dark regime for two weeks, during which plants were watered lightly when necessary with a complete nutrient solution. At the end of the two week period, plant roots were gently lifted, washed, and stained in 0.05% acid fuchsin in lacto-glycerol (Bridge *et al.*, 1982). The number of stained nematodes was recorded and finally scored as percentage infectivity.

A chi squared test was used (the statistical package: "Statistix" from NH Analytical Software, St. Paul, MN 55117, USA) to asses whether the hatching curves in "new" and "old" cysts differed significantly from one another in any hatching period, using the hatching curves of "old" cysts as the "expected" data. The total

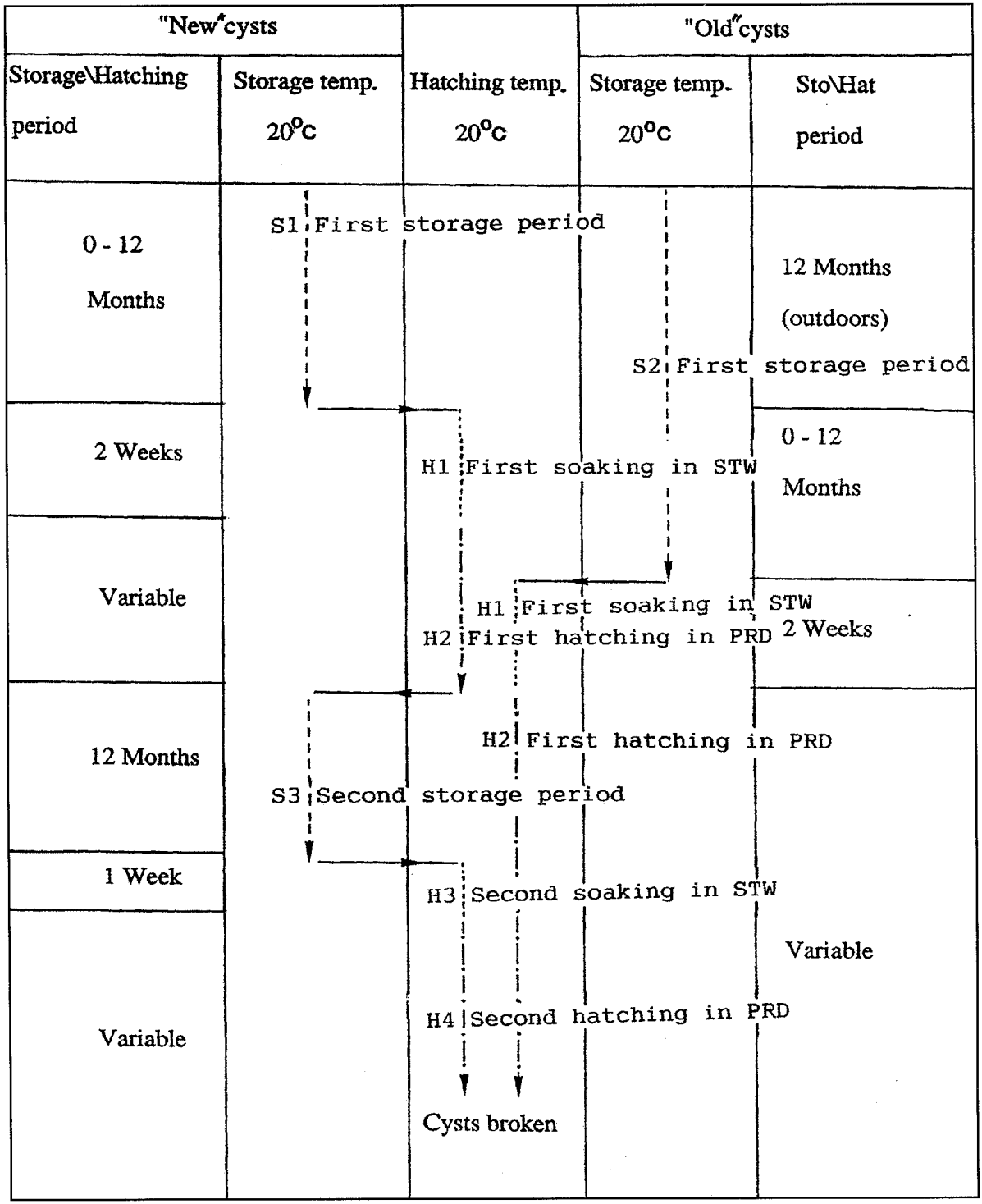

Fig. 1 - "Nematode response" hatching protocol for "new" (cysts extracted soon after maturity on host roots) and "old" cysts (cysts stored for one calendar year outdoors in a gravel plunge) of *Globodera rostochiensis* incubated dry at 20 °C and hatched (after soaking in sterilized tap water, STW) at 20 °C in potato root diffusate (PRD) over a period of one and two calendar year(s).

TABLE I - *Calendar of hatching of "new" and "old" cysts of* Globodera rostochiensis *and total number of eggs in "new" and "old" cysts.*

Hatching period	Calendar of hatching			Total number of eggs in batches of 50 cysts (means and SD)	
	"New" cysts		"Old" cysts	"New" cysts	"Old" cysts
	First hatch	Second hatch	First hatch		
October	5:10:87	5:10:88	5:10:88	14949±840	27733±2309*
November	23:11:87	23:11:88	23:11:88	20178±1577	24619±3688
January	11:1:88	11:1:89	11:1:89	16623±1396	32678±2417*
February	15:2:88	15:2:89	15:2:89	21872±1782	35048±3096*
April	4:4:88	4:4:89	4:4:89	22291±4002	30445±2574
May	21:5:88	21:5:89	21:5:89	17082±591	22317±1346*
June	6:6:88	6:6:89	6:6:89	19457±2810	23549±1498
July	4:7:88	4:7:89	4:7:89	17903±2187	21419±3800
August	22:8:88	22:8:89	22:8:89	21263±1272	18633±431

* Significant difference between number of eggs in "new" and "old" cysts at $P<0.05$.

number of eggs in "new" and "old" cysts were also tested with one way analysis of variance (ANOVA) to assess whether there were significant differences between their contents during any hatching period. Percentages were angularly transformed (arch-sin) before analysis.

Results

The cumulative percentage emergence of juveniles from "new" and "old" cysts is shown in Fig. 2. Nine batches of "new" cysts were hatched in twelve months by following the "nematode-response" protocol. The cumulative percentage emergence in "new" cysts increased (Fig. 2) from less than 50% in January and April to more than 80% in November. Most hatching periods yielded 50-70% hatch. The peak rate of emergence was always noted in the second and third week of hatching in potato root diffusate, except in November and May where there were two peaks of emergence. The shortest emergence period was six weeks during the April hatch, while the longest was 13 weeks during the October hatch. Most emergence occurred in seven to nine weeks.

Dry storage of "new" cysts at 20 °C for twelve months after first hatch and their subsequent second hatching in Potato root diffusate gave a negligible additional increase in the cumulative hatch of the first hatching period.

When "old" cysts were hatched, emergence was rapid reaching about 90% in all periods except August which had about 75% emergence. The longest period of emergence for "old" cysts was ten weeks in October and the shortest five weeks in November. Hatching curves in "new" and "old" cysts were significantly different in October, January, February, April, May and July $P<0.05$, Fig. 2). However, hatching curves in November, June and August were not significantly different. Infectivity of juveniles emerging from eggs of "new" cysts were not significantly different. However, infectivity ranged from 2.5% in October to 7% in August (Fig. 3).

More eggs were found in "old" cysts than in "new" cysts ($P<0.05$, Table I) at the end of all hatching periods except August.

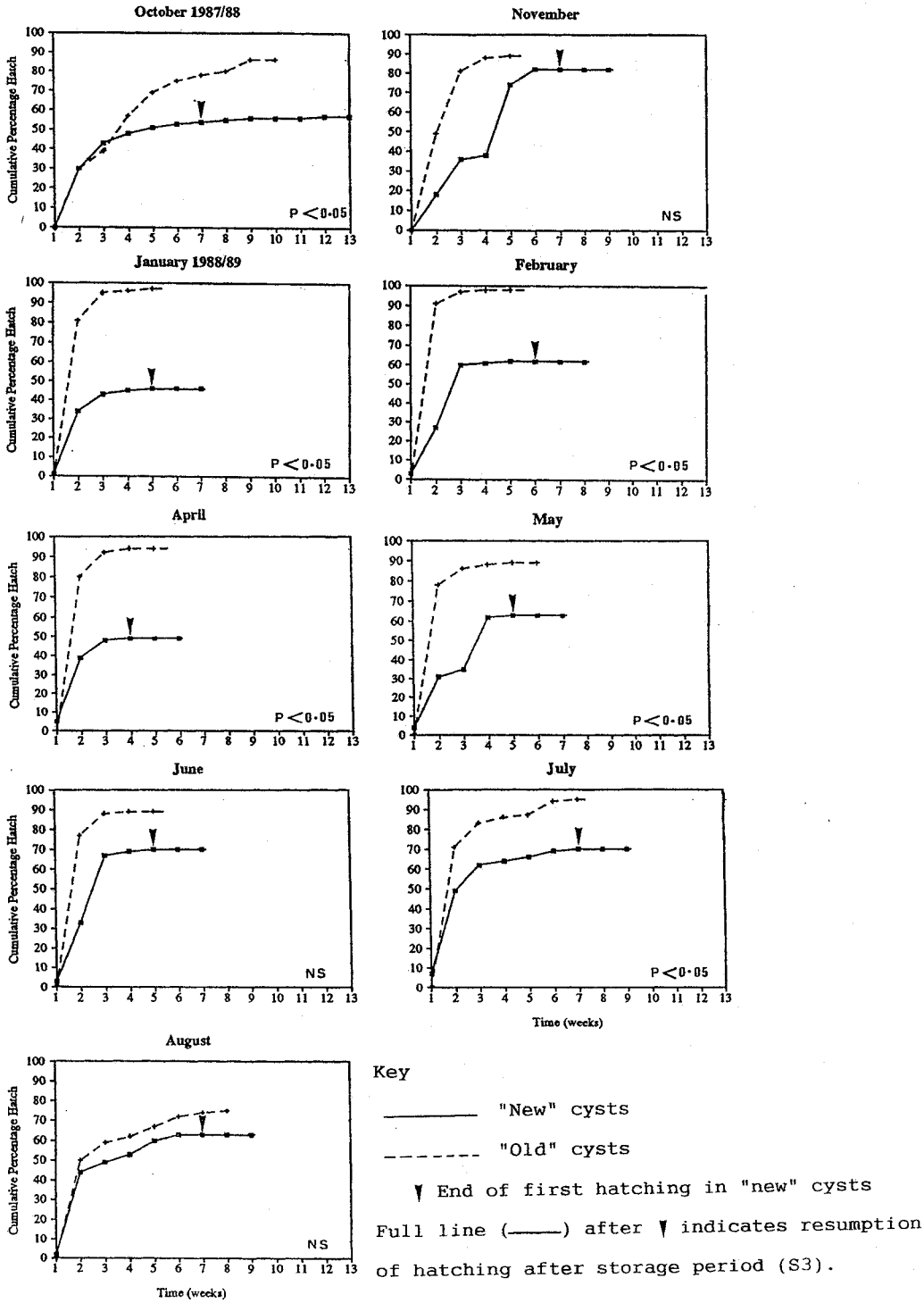

Fig. 2 - Cumulative percentage hatch of "new" (cysts extracted soon after maturity on host roots) and "old" cysts (cysts stored for one calendar year outdoors in a gravel plunge) of *G. rostochiensis* stored dry at 20 °C for various periods before hatching in potato root diffusate at 20 °C over a hatching cycle of one and two calendar year(s). P<0.05 indicates significant difference between hatching curves of "new" and "old" cysts, while NS indicates non significant difference at P<0.05.

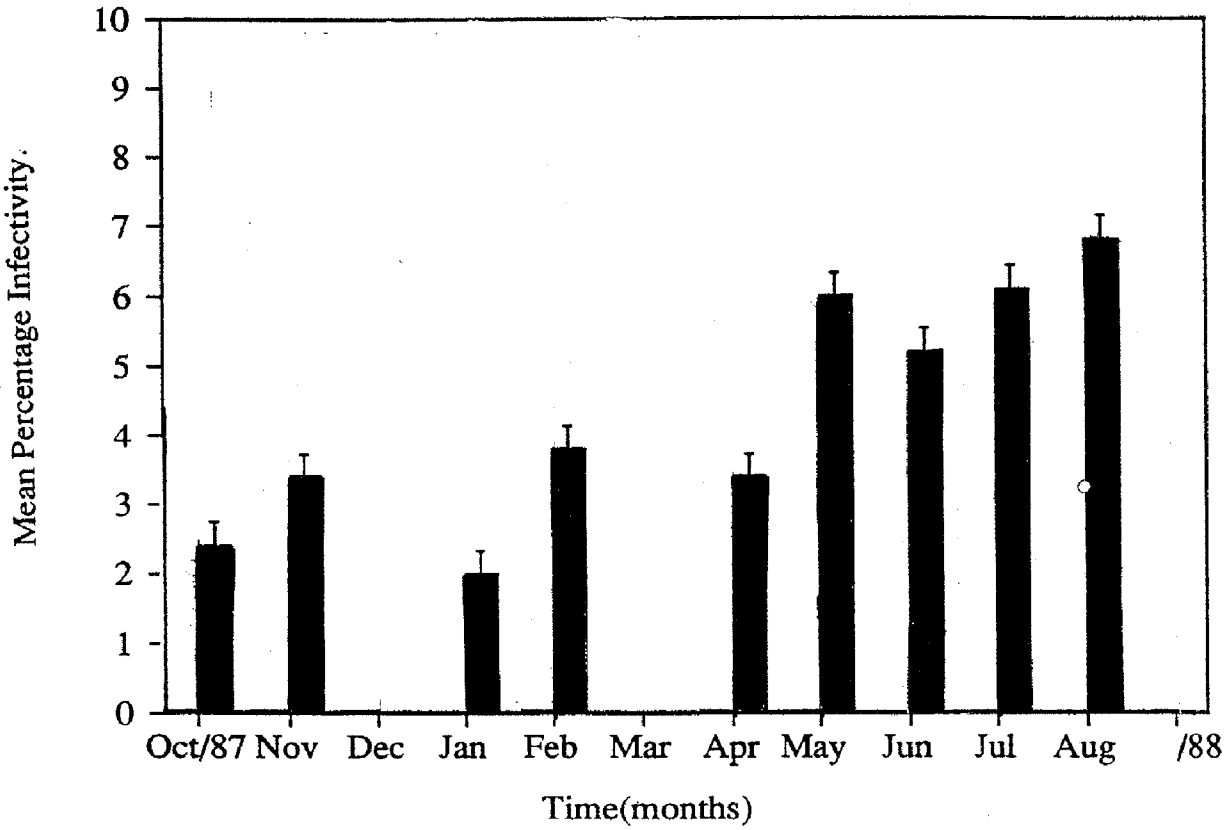

Fig. 3 - Infectivity of *G. rostochiensis* on tomato cv. Moneymaker over a hatching cycle of twelve months. Second stage juveniles (J2) were from "new" cysts (cysts extracted soon after maturity on host roots) and inoculation was done with about 1000 one-week old J2/plant with four replicates. Lines above bars indicate standard error of the mean.

Discussion

This work differs from previous studies on the hatch of *G. rostochiensis* egg in two ways. First, the use of the "nematode-response" protocol and second, the sequential use of the same batch of cysts over two seasons to explore their innate propensity to hatch. The "nematode-response" protocol was begun in October 1987 and only when the hatching ability of the first batch of cysts was depleted was a second batch set to hatch. This was continued for a year, after which batches of "old" cysts were hatched exactly twelve months after the "new" cysts. Significant differences between the two batches were seen in October, January, February, April, May and July, which seasonally corresponds to early autumn, winter, spring and mid-summer; but no differences were seen in November, June and August corresponding to late autumn and summer. These results clearly indicate a different emergence pattern between "new" and "old" cysts lending support to the hypothesis that "new" cysts experienced a form of dormancy which had been lost from the "old" cysts during their year of storage outdoors.

The type of dormancy shown in our experiment suggests a facultative diapause (Evans, 1987) which is present in early autumn, winter,

spring and mid-summer; but absent in late autumn and summer. However, these results differ in detail from those of Hominick *et al.* (1985) who reported reduced hatch (diapause) in autumn and early winter and absent in spring and summer. There are many possible reasons for these differences which may include differences in hatching periods, storage conditions and seasonal differences. The summer of 1987 during which cysts used in this work were raised, was wetter and warmer (according to records of mean temperature and rainfall at Silwood Park, Ascot, UK, 1947 to 1990) than the summer of 1988 in which cysts were hatched and may have influenced the extent of the diapause observed in the population of *G. rostochiensis* studied.

Cumulative hatch was used to assess hatch (rather than rate of initial emergence) as was suggested by Clarke and Perry (1977). "New" cysts showed slower initial hatching than "old" cysts at each hatch, except shortly after cyst collection in October and in the following August when diapause was probably completed (Fig. 2).

A second hatching of "new" cysts in Potato root diffusate after a storage period of twelve months did not increase emergence greatly in any of the hatching periods. The "new" cysts may require further environmental stimulation after hatch in root diffusate unlike the control cysts hatched in the second year of the study of Hominick *et al.* (1985) which showed no evidence of diapause. However, the differences noted between total number of eggs in "new" and "old" cysts were often considerable and may in part account for the poor hatch from "new" cysts in the second year. It seems possible that a larger proportion of eggs in "new" cysts failed to survive the period after their first hatch than did those of the "old" cysts stored outdoors in the soil and the reasons for this are unknown. However, the presence of higher number of eggs in "old" cysts than in "new" cysts failed to support the theories of spontaneous hatching (den Ouden, 1960), micro-organism induced hatching (Elleny and Smith, 1967; Stelter and Sager, 1987) and persistence of hatching factors in the soil (Perry *et al.*, 1981).

Infectivity tests showed no significant difference, but only minor fluctuations during winter. These results support Storey (1984) who reported no difference in infectivity in cysts unhatched for one, four or seven year.

Finally, it must be emphasized that this work was done with a population of *G. rostochiensis* known to show poor hatch through winter months; there may be many populations which do not show this feature to the same extent. Nevertheless, the work described lends support to the proposal that there is a dormancy in the eggs of this population, best described as a facultative diapause (Evans, 1987) acting in the first year after cyst production.

Acknowledgement. We wish to thank Dr. J.M.S. Forrest for providing cysts for this work, Dr. W. M. Hominick and Dr. R. N. Perry for their valuable suggestions. Funds for this work were provided by the University of Maiduguri and Association of Commonwealth Universities.

Literature cited

BRIDGE J., PAGE S. L. J. and JORDAN S. M., 1982. An improved method for staining nematodes in roots. *In* Rothamsted Experimental Station, Report for 1971, Part I. Lawes Agricultural Trust, Harpenden, Herts, UK: pp. 171.

CALAM C. T., RAISTRICK H. and TODD A. R., 1949. The potato eelworm hatching factor I. The preparation of concentrates of the hatching factor and a method of bioassay. *Biochem. J.*, *45*: 513-519.

CLARKE A. J. and PERRY R. N., 1977. Hatching of cyst nematodes. *Nematologica*, *23*: 350-368.

DEN OUDEN H., 1960. Periodicity in spontaneous hatching of *Heterodera rostochiensis* in the soil. *Nematologica* suppl., *11*: 101-105.

ELLENBY C., 1946. Ecology of the eelworm cyst. *Nature*, *157*: 451-452.

ELLENBY C. and SMITH L., 1967. Soil leachings and the hatching of larvae of the potato-root eelworm, *Heterodera rostochiensis* Wollenweber. *Ann. appl. Biol.*, *59*: 283-288.

EL-SHATOURY H., 1978. Genetic control of dormancy in the potato cyst nematode. *Experientia*, *34*: 448-449.

EVANS A. A. F., 1987. Diapause in nematodes as a Survival Strategy, pp. 180-187. *In*: Veech J. A. and Dickson D. W. (Eds). *Vistas on nematology: A Commemoration of the twenty-fifth anniversary of the society of nematologists.* Society of Nematologists Inc. Maryland, USA.

FRANKLIN M. T., 1937. The effect on the cyst contents of *Heterodera Schachtii* on the cultivation of maize on potato sick land. *J. Helminthol., 15*: 61-68.

HOMINICK W. M., FORREST J. M. and EVANS A. A. F., 1985. Diapause in *Globodera rostochiensis* and variability in hatching trials. *Nematologica, 31*: 159-170.

KRUSBERG L. R. and SARDANELLI S., 1989. Survival of *Heterodera zeae* in soil in the field and in the laboratory. *J. Nematol., 21*: 347-355.

MARTIN G. C., 1967. Longevity of *Meloidogyne javanica* under conditions of bare fallow in Rhodesia. *Rhodesia agric. J., 64*: 112-114.

MUHAMMAD Z., 1994. Diapause in the nematode *Globodera pallida*. *Eur. J. Plant Path., 100*: 413-423.

OOSTENBRINK M., 1967. Studies on the emergence of encysted *Heterodera* larvae. *Mededelingen Rijksfaculteit Landbouw-Wetenschappen Gent., 32*: 503-539.

PERRY R. N., HODGES J. A. and BEANE J., 1981. Hatching of *Globodera rostochiensis* in response to potato root diffusate persisting in soil. *Nematologica, 27*: 349-352.

SHEPHERD A. M. and COX P. M., 1967. Observations on periodicity of hatching of eggs of the potato cyst nematode, *Heterodera rostochiensis* Woll. *Ann. appl. Biol., 60*: 143-150.

STELTER H. and SAGER I., 1987. Der Larvenschlupt von *Globodera rostochiensis* (Wollenweber, 1923). Pathotyp I, in Bodenablaufwasser. *Archiv. fur Phytopathol. und Pflanzenschutz, 23*: 69-76.

STOREY R. M. J., 1984. The relationship between neutral lipid reserves and infectivity for hatched and dormant juveniles of *Globodera* spp. *Ann. appl. Biol., 104*: 511-520.

THOMASON I. J. and FIFE D., 1962. The effect of temperature on development and survival of *Heterodera schachtii*. *Nematologica, 7*: 139-145.

WALLACE H. R., 1959. Further observations on some factors influencing the emergence of larvae from cysts of the beet eelworm *Heterodera schachtii* Schmidt. *Nematologica, 4*: 245-252.

Accepted for publication on 22 April 1997.