Fundam. appl. Nematol., 1996, **19** (1), 47-52

Effect of temperature on the *in vitro* reproduction of *Aphelenchoides rutgersi*

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Accepted for publication 15 December 1994.

Summary – The effect of temperature on egg production, hatching and the life cycle from adult to adult of *Aphelenchoides rutgersi* was investigated *in vitro*. The optimum temperature for the reproduction of *A. rutgersi* was 28 °C. At this temperature, a freshly matured female deposited on average 60 eggs during the first 11 days of its reproductive period; hatching started on day 2; egg viability was about 80 % in sterile tap water and over 95 % in axenic medium; minimum development time was 6 days; and the time required for a 100 % increase in adult females was slightly more than 8 days. At 33 °C, *A. rutgersi* was unable to increase its population. As the temperature decreased below 28 °C, fewer eggs were produced, hatching started later, and both minimum and mean generation time were at least 2 days longer.

Résumé – ***Influence de la température sur la reproduction* in vitro *d'*Aphelenchoides rutgersi** – L'influence de la température sur la reproduction d'*Aphelenchoides rutgersi* en culture axénique a été étudiée. La température optimale de reproduction d'*A. rutgersi* est de 28 °C. A cette température, une femelle d'*A. rutgersi* pond en moyenne 60 œufs pendant les 11 jours qui suivent sa maturation; les juvéniles commencent à éclore dès le deuxième jour; 80 % des œufs pondus dans l'eau et plus de 95 % des œufs pondus dans le milieu axénique sont viables. A cette même température, la durée minimale de développement est de six jours, tandis que la durée moyenne de développement, mesurée comme le temps nécessaire pour doubler le nombre de femelles adultes, est de 8 jours. A 33 °C, les populations d'*A. rutgersi* n'augmentent plus. A des températures inférieures à 28 °C, les œufs pondus sont moins nombreux, les éclosions plus tardives, et les durées minimale et moyenne de développement demandent au moins 2 jours de plus.

Key-words : *Aphelenchoides rutgersi,* temperature, reproduction, axenic culture.

Aphelenchoides rutgersi was originally isolated from the rhizosphere of citrus in Orlando, California (Myers, 1967), and, after previous difficulties with its identification, it was established as a new species (Hooper & Myers, 1971). Since then, it has also been reported from eastern Europe (Katalan-Gateva & Budurova, 1979). *A. rutgersi* was first cultured axenically by Myers (1967, 1968), who, together with his coworkers at Rutgers University, also studied its amino-acid (Balasubramanian & Myers, 1971; Myers & Balasubramanian, 1973), carbohydrate (Petriello & Myers, 1971), nucleic acid/nucleotide (Thirugnanam, 1974, 1976; Thirugnanam & Myers, 1974), and heme requirements (Thirugnanam, 1974, 1976). Improved axenic media supporting continuous reproduction were reported by Buecher *et al.* (1970) and Myers *et al.* (1971).

Information on the effects of physical parameters such as temperature on the axenic culture of *A. rutgersi* has also been provided previously (Myers, 1971). Since preliminary observations indicated that our axenic *A. rutgersi* populations had different temperature requirements than those reported by Myers (1971), the effect of temperature on *i)* egg production, *ii)* hatching and *iii)* the life cycle from adult to adult was investigated.

Materials and methods

A. rutgersi cultures on *Botrytis cinerea* were obtained from R.F. Myers, Department of Plant Pathology, Rutgers University. Therefore, the nematodes originated from the same area as those used in the above cited literature. Nematodes were axenized according to previously described procedures (Myers, 1971, 1992). At the time of the experiments described here, *A. rutgersi* had been in permanent axenic culture for almost one year.

A. rutgersi was cultured axenically according to Buecher *et al.* (1970) : medium containing 2 % of soy-peptone and 3 % of yeast extract, and Myers *et al.* (1971) : supplement of chicken embryo extract, 10 % final concentration. Penicillin, streptomycin sulphate and fungizone were added in final concentrations of 500 U/ml, 500 U/ml and 1.25 µg/ml, respectively

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(Myers, 1973). Stock cultures were kept at 20 and 28 °C in the dark in 50 ml tissue culture flasks (Costar), containing 15 ml of the axenic medium, and placed horizontally to improve oxygen exchange.

Effect of temperature on egg production

In the first experiment, 50 ± 5 egg-carrying females of *A. rutgersi* were hand picked at random from a population in exponential growth phase, and transferred to 1 cm³ wells, two females per well, each containing 700 µg/l of axenic medium. In order to facilitate observations, excess debris was removed prior to addition to the wells by a single centrifugation (5000 rpm) of the axenic medium. Although no influence of this treatment was observed during short-term experiments (up to 2 weeks), population densities of cultures kept in centrifuged axenic medium for several weeks were significantly lower compared to those kept in non-centrifuged medium (Moens, unpubl.). The wells were then incubated at 16, 20, 24, 28 and 33 °C in the dark, and the number of deposited eggs and hatched juveniles was counted daily for a period of 8 days. The entire experiment was replicated three times.

In the second experiment, 30 egg-carrying females of *A. rutgersi* were hand picked from a young stock culture, transferred to 1 cm³ wells, and individuals were incubated at 28 °C as described for the first experiment. After 24 h, the females were removed and the deposited eggs allowed to develop for one week and eventually hatch. Then, 25 of these freshly matured second generation females were randomly selected, transferred individually to 1 cm³ wells and incubated at 28 °C. After 6 days, the females were transferred to new medium. Older juveniles were also removed from the experiment to prevent them from adding to the total egg production. Egg deposition was studied for a period of 11 days, with counts every 2 or 3 days. This experiment was performed once.

Effect of temperature on hatching

Thirty (± 5) newly deposited eggs with a maximum of six visible nuclei (Rowse, 1969), were hand picked at random from a young stock culture, washed five times with sterile (0.22 µm millipore filtered) tap water and transferred to a 1 cm³ well containing 650 µl of sterile tap water. Three wells each were incubated in the dark at 16, 20, 24, 28 and 33 °C. Numbers of eggs and hatched juveniles were counted daily during the first week, then every 2 days for a period of 11 more days. In order to compare egg hatch in sterile tap water with egg hatch in axenic medium, hatching of eggs deposited by the 25 freshly matured second generation females during the first 6 days of the second experiment described above, was followed for 5 days at 28 °C. Similar tests were conducted at 20 and 24 °C. This experiment was performed once.

Effect of temperature on the life cycle from adult to adult

For a description of the methods, see effect of temperature on egg production, first experiment. In addition to the number of deposited eggs and hatched juveniles, the development of these juveniles into adult females (*A. rutgersi* is a parthenogenetic species and males are extremely rare in our populations) was also observed daily for 10 to 14 days, depending on temperature. We deliberately chose not to use extra antibiotics or antimycotics in addition to the standard substances added to the axenic medium in order not to disturb in any way the normal reproduction of *A. rutgersi*. As reported previously (Myers, 1973), this strongly limits the duration of this type of experiment. To eliminate the effects of crowding on the maturation process, controls with diverse nematode inocula (one to 30 females per well) were added.

Results

Effect of temperature on egg production

In the first experiment, the highest average number of eggs was produced at 28 °C (Fig. 1). In the wells incubated at 16 °C, a microbial infection was repeatedly observed after a few days and the counting was stopped. At this temperature, the number of eggs produced during the first 2 days was lowest compared to the other temperatures, averaging slightly less than two per female. Egg production at 20 and 24 °C was comparable, except during the first 2 days when number of eggs at 20 °C was significantly lower. At 28 and 24 °C, maximal reproduction occurred during the first 24 h after transfer from the stock culture, while at 33 and 20 °C, it occurred during the second day probably as a consequence of temperature adaptation, since stock cultures were kept at 28 °C. At 33 °C, no more eggs were produced after 5 days. At all the other temperatures, eggs were deposited throughout the experiment. Maximum individual egg production within 24 h averaged 4.2, 4.4, 8.9 and 6.2 at 20, 24, 28 and 33 °C, respectively.

In the second experiment, the average reproduction of freshly matured females was considerably higher than among randomly collected females from cultures in exponential growth phase. The mean number of eggs produced over a 9 day period was 55, compared to 36 in the first test (Fig. 2). There were large differences between individual females, with total number of progeny per female ranging from 28 to 99 over an 11 day period. Therefore, the most productive female deposited on average nine eggs per day with hardly any decline in egg production towards the end of the experiment, suggesting a considerably higher total reproductive capacity for *A. rutgersi*.

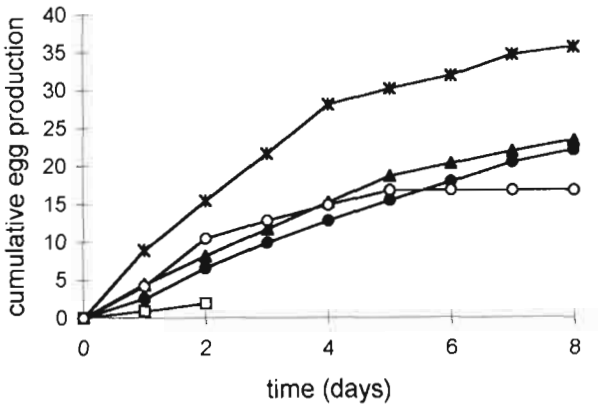

Fig. 1. *Effect of temperature on the average number of eggs produced by a single female of* Aphelenchoides rutgersi, *randomly sampled from a stock culture. Each average represents the mean of 150 ± 15 females (□ 16 °C, ● 20 °C, ▲ 24 °C, * 28 °C, ○ 33 °C).*

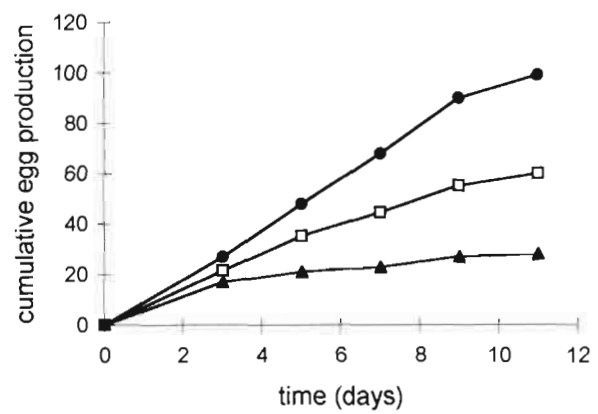

Fig. 2. *Number of eggs produced by freshly matured females of* Aphelenchoides rutgersi *at 28°C. Each point (□) represents the mean of 25 females; Highest (●) and lowest (▲) reproduction are data from the individual females producing the highest and lowest number of eggs, respectively.*

Effect of temperature on hatching

In sterile tap water, egg hatch started at day 2 at 24, 28 and 33 °C, and at day 3 at 16 and 20 °C (Fig. 3). At 16, 20, 24 and 28 °C, 50 % or more of the eggs had hatched after 7, 5, 3 and 2 days, respectively. Almost maximal hatch (more than 90 % of total hatch) was attained after 14, 7, 5 and 4 days at 16, 20, 24 and 28 °C, respectively. Egg viability was about 80 % at the latter three temperatures, compared to almost 70 % at the former. At 33 °C, no further hatching was observed after 3 days, and egg viability never exceeded 47 %.

Egg viability in axenic medium was considerably higher than in tap water. Four days after deposition – when at 28 °C maximal hatch is nearly reached – 95 % of eggs had hatched. Similar results were obtained at 24 and 20 °C.

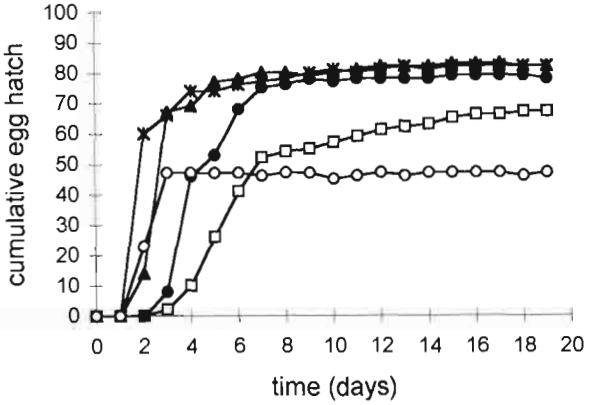

Fig. 3. *Effect of temperature on the average percentage of cumulative egg hatch of freshly deposited* Aphelenchoides rutgersi *ova in tap water. Each average represents the mean of at least 80 eggs (□ 16 °C, ● 20 °C, ▲ 24 °C, * 28 °C, ○ 33 °C).*

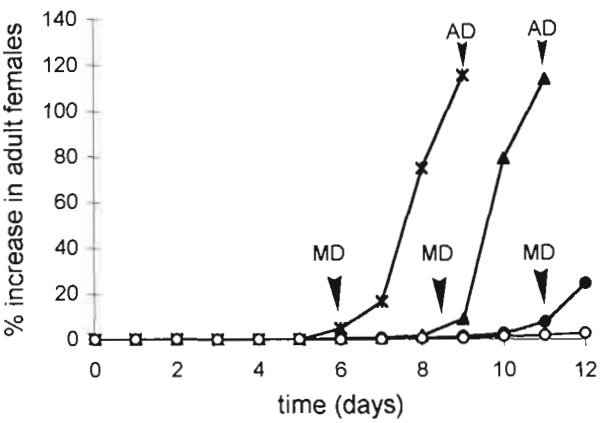

Fig. 4. *Effect of temperature on the minimum (MD) and mean (AD) development times – as determined from percent increase in adult females – of* Aphelenchoides rutgersi *in axenic culture. Experiments started with 150 ± 15 females (● 20 °C, ▲ 24 °C, * 28 °C, □ 33 °C).*

Effect of temperature on the life cycle from adult to adult

In view of previous discussions on the precise meaning of the terms minimum and mean generation time, we have used minimum and mean development time to describe the time necessary for a 5 and 100 % increase in adult females, respectively.

Minimum development times were 6, between 8 and 9, and 11 days at 28, 24 and 20 °C, respectively (Fig. 4). Mean development time was slightly more than 8 days at 28 °C, between 10 and 11 days at 24 °C and an estimated 13 to 14 days at 20 °C. At 33 °C, no egg-carrying

second generation females were observed and although some offspring individuals with a visible vulva were observed towards the end of the experiment, a 5 % increase in adult females was never reached. Moreover, mortality rates at this temperature were significantly higher than in the controls, while there were no significant differences with respect to this parameter in the 16 to 28 °C range (data not shown). No effects of crowding on development times could be observed in the controls with varying nematode inocula.

Discussion

Egg production, hatching, and length of the life cycle of *A. rutgersi* are strongly influenced by temperature. The optimum temperature for the development of *A. rutgersi* was 28 °C, which is high compared with an optimum of 23 °C as reported by Myers (1971). Several factors, abiotic and/or inherent to the different axenic growth media used, may be responsible for this discrepancy. Since the temperature in our stock cultures had only recently been changed from 20 to 28 °C, it is unlikely that an adaptation phenomenon was responsible for our results. Moreover, upon comparison of reproduction by nematodes sampled from stock cultures kept at 20 and 28 °C, almost identical data were obtained. Adaptation was, however, considered a possible cause for the temperature optimum of 23 °C as found by Myers (1971). An optimum temperature of 28 °C is also high compared to the available information on other *Aphelenchoides* species (Table 1).

Table 1. *Optimum temperature for the reproduction of five species of* Aphelenchoides *spp.*

Species	Optimum temperature	Reference
A. besseyi	23-30 °C	Huang *et al.* (1972)
	20-28 °C	Wang *et al.* (1993)
A. composticola	20 °C*	Younes (1969)
		Cayrol (1967)
A. fragariae	≤ 18°C*	Strümpel (1967)
A. hamatus	20-24 °C	Rossner & Nagel (1984)
A. ritzemabosi	14-23 °C	Wallace (1960)
		French & Barraclough (1961)

* Optimum temperature estimated by the authors of the present study on the basis of temperature *vs* generation time data in the work cited.

In view of the results of the present study, 28 °C is proposed as the optimal temperature for short-term laboratory experiments with *A. rutgersi*, since at this temperature, effects on a nematode population containing all life stages and on individual nematodes during an entire maturation process can be determined using only an 8 day incubation period. For other purposes, particularly for maintaining stock cultures, lower temperatures down to 20 °C are also favorable, since they also support good reproduction and cultures can therefore be kept longer. Upon comparison of data on egg hatch and development time, it appears that differences in development times between 20 and 24 °C can be almost entirely attributed to the longer embryonic period at the former temperature. In contrast, the difference between 24 and 28 °C is more influenced by the shorter post-embryonic development time at the latter temperature.

An optimal mean development time of 8 days for *A. rutgersi* also exceeds the generation time estimated to be between 4 and 5 days, based on interpretation of the growth curve of a reproducing culture (Myers, 1973). Development times for *A. rutgersi* as obtained in this experiment do, however, compare well to those mentioned for other *Aphelenchoides* species (Table 2). The temperature *vs* development time relationship as obtained for *A. besseyi* is in close comparison with our values for *A. rutgersi*, in so far that we might tentatively suggest to accept for *A. rutgersi* the same generation time at 16 °C found for the former species : about 24 days (Huang *et al.*, 1972). Nevertheless, *A. besseyi* did not show the same distinct temperature optimum as *A. rutgersi* at 28 °C, and still matured rapidly at 35 °C, although, like *A. rutgersi*, it was incapable of increasing its population at this temperature (Huang *et al.*, 1972; Lin *et al.*, 1992; Wang *et al.*, 1993).

Table 2. *Minimum generation times for five species of* Aphelenchoides.

Species	Generation time (days)	T (°C)	Reference
A. besseyi	8 ± 2	35	Huang *et al.* (1972)
A. composticola	7.5	20	Younes (1969)
A. fragariae	10-11	18	Strümpel (1967)
A. ritzemabosi	10-14	14-23	Wallace (1960) French & Barraclough (1961)
A. sacchari	13	*	Janowicz (1978)

* Stands for room temperature.

The reproductive potential of the parthenogenetic *A. rutgersi* was lower than that of *A. sacchari* : females of the latter species deposited 166-296 eggs during a 28 to 30 day period (Janowicz, 1978). Therefore, only the best performing females from our experiments compare with these results, as can be extrapolated from Fig. 2, when assuming that the reproductive periods for both species have the same length. In contrast, females of *A. fragariae* deposited only 32 eggs over an entire repro-

ductive period at 18 °C (Strümpel, 1967). Then again, it must be stressed that comparisons between figures obtained from experiments at different culture levels should be treated with due care as data for both species were obtained from monoxenic cultures.

This may be true for the aspect of temperature dependence as well. *A. rutgersi* apparently differs from other *Aphelenchoides* species in having a distinct temperature optimum. The different temperature optimum for Myers's (1971) and our population is all the more surprising since both originate from the same place. This may be due in part to culture selection of individuals with traits which are not necessarily characteristic for the entire population. Up to now, the only studies in which a distinct temperature optimum was found for an *Aphelenchoides* species, deal with axenically cultured *A. rutgersi,* and the differences between both optima for this species may reflect to a considerable degree the variability of individual traits within *A. rutgersi.* If so, a more general comparison of published data on different *Aphelenchoides* species shows *A. rutgersi* and *A. besseyi* to thrive best at temperatures well above 20 and up to 30 °C, whereas other species prefer temperatures around or below 20 °C (Table 1). Optimal generation times are between 7 and less than 14 days among the different *Aphelenchoides* species studied. Data shown in Table 2 indicate that they may be generally shorter in species with a higher temperature optimum.

During the present study, incomplete *endotokia matricida* was observed on three separate occasions. Each of these cases consisted of the intra-uterine development of an embryo to a J2. However, the juveniles were incapable of freeing from the eggshells. There were no indications that these cases were the result of stress factors (cf. Martin & Riedel, 1982). Similar observations on *endotokia matricida* were made frequently with older females of *A sacchari* (Janowicz, 1978) and *A. fragariae* (Luc *et al.,* 1979).

Acknowledgements

The first author acknowledges a grant from the Belgian National Fund for Scientific Research. Gaetan Borgonie and Frederik Leyns are thanked for stimulating discussions and Greta Arnaut for practical support in the laboratory, respectively.

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