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A rapid method for separation of adults in a mixed population of *Steinernema carpocapsae* (Nematoda : Steinernematidae)

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Summary – Separation of a large number of viable nematodes is essential for physiological studies on *Steinernema carpocapsae*. In this paper, we describe a fast and useful method for the separation of adults of this nematode species. This method relies on the determination of the density of males and females by applying a modified version of Stokes' law. Adults were separated in a discontinuous sucrose gradient at 10 and 20 %. Males and females separated by this method were 95 % viable.

Résumé – ***Méthode rapide de séparation des adultes à partir d'une population brute de* Steinernema carpocapsae *(Nematoda : Steinernematidae)*** – La sélection d'un grand nombre de nématodes viables est une condition impérative pour les études sur la physiologie de *Steinernema carpocapsae*. Est décrite ici une méthode rapide et pratique permettant la séparation des mâles et des femelles de ce nématode à partir d'une suspension contenant une population brute. Pour l'utilisation de cette méthode, la densité des mâles et des femelles est déterminée par application de la loi de Stokes modifiée. Les adultes sont séparés dans un gradient discontinu de saccharose s'étageant de 10 et 20 %. Quatre-vingt-quinze pour cent des mâles et des femelles isolés grâce à cette méthode restent viables.

Key-words : Gradient, separation, *Steinernema carpocapsae,* sucrose.

The entomopathogenic nematodes of the genus *Steinernema* have received much attention in recent years because of their biological control potential against a wide range of insect pests (Laumond *et al.*, 1979; Klein, 1990). However, the commercial exploitation of this bioinsecticide has been limited by the inability to produce them in large numbers and by their inconsistent efficacy for controlling crop pests in the field. To improve mass production and mode of action of these nematodes, a better knowledge of their developmental biology, physiology and genetics is required (Gaugler *et al.*, 1989; Fodor *et al.*, 1990; Glazer, 1992; Lunau *et al.*, 1993). These studies, which are done in synchronous cultures, require that large numbers of females and males be separated from a mixed population.

In the present study, we described a technique for separation of nematodes simply by determining the density of adult nematodes by using an expression that results from Stokes' law after measurement of the sedimentation rate of each sex in solutions with different physical properties.

Material and methods

NEMATODE CULTURE

Steinernema carpocapse strain Az 20 was reared in fortified lipid agar (FLA) and in liver homogenate (LH), after bioconversion by the symbiotic bacteria of the nematode *Xenorhabdus nematophilus nematophilus* Az 20 strain (Xnn Az 20).

FLA was prepared with 1.6 % nutrient broth, 1 % sunflower oil, and 1.2 % bacteriological agar. 10 ml portions of medium were poured into Pertri dishes (9 cm diam.), inoculated with 1 ml of bacterial suspension of Xnn Az 20, phase 1, in exponential growth, swirled to ensure uniform bacterial dispersion over the agar surface, and incubated at 30 °C during 24 h. After this time, 1000 infective juveniles (IJs) of *S. carpocapsae* (Az 20) previously surface-sterilized in (1 %) merthiolate for 3 h were added to each Petri dish.

LH was prepared by thoroughly mixing 70 % liver homogenate, 10 % sunflower oil, and 20 % water on a sponge matrix according to Bedding (1984). The medium was autoclaved at 110 °C for 30 min, and distributed in 250 ml Erlenmeyer flasks (25 g of medium/flask). Each flask was inoculated with 1 ml of bacterial suspension of Xnn Az 20 phase 1, in exponential growth, and incubated at 30 °C during 24 h. Afterwards, 2000 IJs of *S. carpocapsae* (Az 20) previously surface-sterilized in 1 % merthiolate for 3 h were added to each Erlenmeyer.

BACTERIAL GROWTH

The primary phase of Xnn Az 20 was isolated from a drop of insect hemolymph (Poinar & Thomas, 1966) previously inoculated with 10 surface-sterilized IJs of *S. carpocapsae* (Az 20) and stored in 17 % glycerol/nutrient broth (v/v) at – 18 °C (Akhurst, 1980).

Bacterial growth was achieved by transferring a loopful of the stored culture to 10 ml of 3 % tryptic soy

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broth supplemented with 0.5 % yeast extract. The culture was incubated at 30 °C on a 150-rpm shaker for 24 h.

Nematode measurement

First generation adults, coming from FLA and LH, were placed in Tyrode solution (TS) (Marchal *et al.*, 1982) at 80 °C for immobilization and immediately measured. Each adult was measured using a stereomicroscope with a micrometric ocular. Fifty measurements of each generation for each sex were performed.

Density determination

Male and female densities were determined using an expression obtained by applying Stokes' law to the sedimentation rates of the nematodes in Tyrode and sucrose solutions (5 %). Application of Stokes' law to each solution gives :

a) for the Tyrode solution :

$$S_1 = \frac{gD^2(\rho_n - \rho_1)}{18\,\mu_1} \quad (1)$$

b) for the sucrose solution :

$$S_2 = \frac{gD^2(\rho_n - \rho_2)}{18\,\mu_2} \quad (2)$$

where : S_1 = sedimentation rate in Tyrode solution; g = gravity acceleration; D = nematode diameter; P_n = adult nematode density; ρ_1 = Tyrode solution density; μ_1 = viscosity of Tyrode solution; S_2 = sedimentation rate in sucrose solution (5 %); ρ_2 = sucrose solution density (5 %); μ_2 = viscosity of sucrose solution (5 %).

As the nematode average diameter *(D)* and nematode densities (ρ_n) are unknown, the use of two Stokes equations will be resolved to eliminate the unknown diameter. Isolating the diameter on the first side of each equation gives :

$$D^2 = \frac{18\,S_1\mu_1}{g(\rho_n - \rho_1)} \quad (3)$$

and

$$D^2 = \frac{18\,S_2\mu_2}{g(\rho_n - \rho_2)} \quad (4)$$

Equating the second sides of (3) and (4) and simplifying gives :

$$\frac{S_1\mu_1}{\rho_n - \rho_1} = \frac{S_2\mu_2}{\rho_n - \rho_2} \quad (5)$$

Working on equation (5) gives the following expression for ρ_n :

$$\rho_n = \frac{\mu_2\rho_1 S_2 - \mu_1\rho_2 S_1}{\mu_2 S_2 - \mu_1 S_1} \quad (6)$$

where : ρ_n = adult nematode density; μ_2 = viscosity of sucrose solution (5 %); ρ_1 = Tyrode solution density; S_2 = sedimentation rate in sucrose solution (5 %); μ_1 = viscosity of Tyrode solution; ρ_2 = sucrose solution density (5 %); S_1 =sedimentation rate in Tyrode solution.

The density of each solution was determined using a PAAR DMA 6000 densimeter at 25 °C with air and water as calibration standards. Viscosities were measured using a capillary viscosimeter (Cannon Fenske ASTM - D 2515). The capillary constant of the viscosimeter was determined using water and glycerol (50 %) as calibrants.

The time *(t)* an adult took to travel a defined distance (d = 10 cm) inside a test-tube was recorded, and the sedimentation rate *(S)* was calculated through the expression :

$$S = \frac{d}{t} \quad (7)$$

Fifty replicates were done for each sex.

Adult separation

Males and females were separated in a discontinuous sucrose gradient. Sucrose solutions of decreasing density 25, 20, 15 and 10 (v/w) respectively, were carefully layered in centrifuge tubes with a pipette. One ml of Tyrode solution with a mixed population containing 1000 adults was then added to the top of each gradient by tilting the centrifuge tube at 45 °C and allowing the nematode suspension to drip down the side of the tube wall. The tubes then was centrifuged at 3000 rpm for 15 min. Ten replicates were done for statistical analysis. Once separation had been achieved, males and females were recovered using a Pasteur pipette. The adult stages were then washed three times by resuspending them in Tyrode solution and counted using a stereomicroscope. The viability was verified by checking for nematode movements and response to mechanical stimuli.

Data analysis

Data from size measurements and adult separation were subjected to analysis of variance.

Results

Male and female densities

Male and female densities were 1.076 and 1.035 respectively when cultured in FLA (Table 1). Densities were calculated using a modification of Stokes' expression (equation 6) after determination of the sedimentation rates. Male sedimentation rates in Tyrode solution and sucrose solution (5 %) were 0.177 cm s^{-1} and

0.132 cm s^{-1} respectively. Female sedimentation rates in Tyrode solution and sucrose solution (5 %) were 0.372 cm s^{-1} and 0.206 cm s^{-1} respectively.

The density determined for *S. carpocapsae* adult is not dependent on the length in each sex; length and density of males and females reared in FLA and in LH are not significantly different ($P \leq 0.05$) (Table 2).

Separation of males and females

The difference of densities allows separation of males and females from a mixed population in a discontinuous sucrose gradient (Fig. 1). Most of the females (91.4 %) concentrate in the 10 % band with a density of 1.0351; likewise the majority of males (97.1 %) accumulate in the 20 % band with a density of 1.0761. A high degree of purity is achieved in each of the two bands (Table 3). This separation rate is obtained with adults coming both from FLA and LH. The viability of males and females separated by this method is 95 %.

Discussion

The difference in densities between males and females leads to a simple, rapid and efficient method to separate large quantities of males and females from a mixed population. This method consists of centrifugation of a mixed population in a discontinuous sucrose gradient containing two bands with the same density as the nematodes, separated by an intermediate density band. Centrifugation in sucrose gradients, continuous or discontinuous, is a technique largely used for the separation of macromolecules and viruses (Brakke, 1957; Anderson & Clive, 1967; Arora *et al.*, 1973), and in the synchronization of yeast cultures (Lieblová & Streiblová, 1964; Mitchison & Vincent, 1965; Baldwin & Kubitschek, 1984).

The viability of adults recovered by this process is identical to that obtained with a needle-carrier (un-

Table 1. *Densities of adults of* Steinernema carpocapsae *(Az 20).*

	Sedimentation velocity (cm s^{-1})		Density
SEX	TS	SS	
Male	0.177	0.132	1.076
Female	0.372	0.206	1.035

TS = Tyrode solution; SS = sucrose solution.

Table 2. *Length and density of adults of* Steinernema carpocapsae *(Az 20) coming from different media.*

MEDIUM	MALES		FEMALES	
	Length*	Density	Length*	Density
FLA	1650 ± 320 *a*	1.076	4615 ± 759 *a*	1.035
HL	1840 ± 210 *a*	1.074	4767 ± 848 *a*	1.040

* In µm. Average ± SD; n = 50 for each sex and each medium. Means with the same letters in the same column are not significantly different ($P \leq 0.05$).

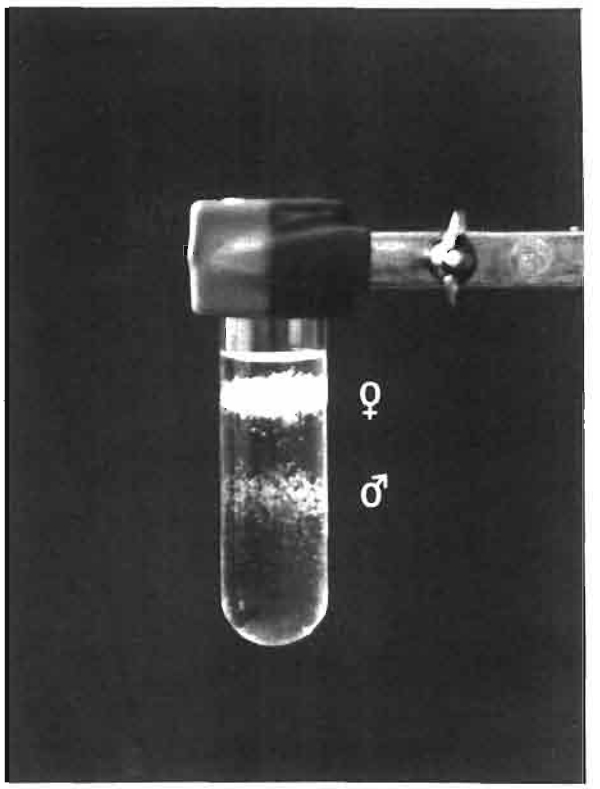

Fig. 1. *Distribution of males and females of* Steinernema carpocapsae *(Az 20) by the bands of the discontinuous sucrose gradient.*

Table 3. *Distribution of males and females of* Steinernema carpocapsae (Az 20) *at each band of the discontinuous sucrose gradient.*

GRADIENT BAND	MALES		FEMALES	
	Average ± SD	%/band	Average ± SD	%/band
10	47 ± 5.56 *a*	8.6	499 ± 10.30 *a*	91.4
15	0 ± 00 *b*	0	0 ± 0 *b*	0
20	433 ± 11.84 *c*	97.1	13 ± 2.62 *c*	2.9
25	8 ± 3.92 *b*	100	0 ± 0 *b*	0

Means with the same letters in the same column are not significantly different ($P \leq 0.05$) (ANOVA).

publ.). Therefore this method will allow large quantities of different stages of each sex to be obtained. However, attention has to be paid to the time that adults remain in the sucrose solution as it has a very high osmotic pressure even at low concentration, so that the adults must be washed in Tyrode solution as soon as the centrifugation is finished to avoid nematode plasmolysis.

The density determinations of adults were done by the application of the modified Stokes' law which involves determination of the sedimentation rates. The determination of these rates by the sedimentation method is controversial as other factors besides cell size can affect the sedimentation rate, such as cell shape, surface roughness and membrane deformability. However, measurements of the sedimentation rates of sheep erythrocytes in a buffered step gradient gave values that agreed well which calculated values (Sharp, 1988). In the case of the nematode, size, as well as shape, are the two main factors that influence the sedimentation rate. The effect of the size is quite obvious, since, as may be seen by nematode size measurements, they have a wide range of dimensions. For nematode shape, it must be considered that the Stokes' law expression for terminal settling velocities was originally obtained for spherical particles. For non-spherical particles, as is the case for nematodes, the drag forces are dependent on the relative orientation of the particles to the direction of flow. We indeed have observed that nematodes do not always have the same orientation when falling. However, in most cases, males and females fall oriented in the same way. So, we have considered that, by measuring the sedimentation rates of a large number of nematodes, the above described influences were minimized.

The determination of nematode density will also be useful in liquid culture where contact between the sexes must be favored. For example, in bioreactors the mixing patterns are controlled, not only by the agitation power, but also by the physical properties of the liquid phase, namely density and viscosity, and of the solid phase (density, particle size, and concentration) (Coulson & Richardson, 1991). In the bioreactor the nematode will constitute a non-homogen solid phase where two sexes with different sizes and densities coexists.

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