

Population dynamics of *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* in *in vitro* monoxenic solid culture

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Summary – Studies of the population dynamics of the entomopathogenic nematodes *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* in *in vitro* solid culture showed that inoculum size is important for optimising the final yields of infective juveniles, and the time in which these are achieved. The highest yield for *H. bacteriophora* was found with an inoculum of 10^6 infective juveniles per flask, which was ten-fold the optimal inoculum for *S. carpocapsae*. Populations of these nematodes derived from the extremes of high and low inocula ($1-2$ to 10^7 infective juveniles per flask) demonstrated differences in reproduction and population development between *H. bacteriophora* and *S. carpocapsae*. At the highest inoculum of 10^7 nematodes per flask, populations of *H. bacteriophora* doubled whereas *S. carpocapsae* halved. Inoculation of one *H. bacteriophora* infective juvenile per flask resulted in a final population of about 25×10^6 nematodes in 6 weeks. However, it was not possible to initiate population development of *S. carpocapsae* in flasks by inoculating two infective juveniles per flask. Results from this study are useful for mass production of entomopathogenic nematodes and further investigations of the detailed relationship between inoculum sizes, population development and the final infective juvenile populations of these nematodes should improve the efficiency of commercial nematode production. © Elsevier - ORSTOM

Résumé – Dynamique des populations d'*Heterorhabditis bacteriophora* et de *Steinernema carpocapsae* en élevage monoxénique *in vitro* sur milieu solide - La présente étude sur la dynamique des populations des nématodes entomopathogènes *Heterorhabditis bacteriophora* et *Steinernema carpocapsae* en élevage *in vitro* sur milieu solide montre que la taille de l'inoculum est importante pour optimiser la récolte finale des juvéniles infestants et pour déterminer leur temps de développement. Les plus fortes récoltes sont obtenues pour *H. bacteriophora* avec un inoculum de 10^6 juvéniles infestants par fiole, ce qui représente dix fois la valeur de l'inoculum optimal pour *S. carpocapsae*. Les populations issues des inoculums de valeurs extrêmes (un ou deux contre 10^7 juvéniles infestants par fiole) montrent des différences dans la reproduction et le développement des populations entre *H. bacteriophora* et *S. carpocapsae*. Un inoculum de 10^7 juvéniles infestants par fiole provoque un doublement de la population de *H. bacteriophora*, tandis qu'il diminue de moitié celle de *S. carpocapsae*. L'inoculation d'un seul juvénile infestant de *H. bacteriophora* conduit à une population finale de 25×10^6 nématodes en 6 semaines. Cependant, il n'a pas été possible d'initier le développement d'une population de *S. carpocapsae* en inoculant seulement deux juvéniles infestants par fiole. Les résultats de cette étude pourraient être utilisés pour la production en masse de nématodes entomopathogènes, et des recherches futures sur les relations intimes entre valeurs de l'inoculum, développement des populations et population finale de juvéniles infestants de ces nématodes pourraient augmenter l'efficacité de la production commerciale de ces nématodes. © Elsevier - ORSTOM

Keywords : *Heterorhabditis bacteriophora*, inoculum, *in vitro* culture, population dynamics, *Steinernema carpocapsae*.

Entomopathogenic nematodes were first reared in *in vitro* solid culture more than 60 years ago (Glaser, 1932). Since then, many studies have been carried out to mass produce these nematodes in *in vitro* solid culture (McCoy & Glaser, 1936; House *et al.*, 1965; Bedding, 1976, 1981, 1984; Hara *et al.*, 1981; Bedding *et al.*, 1991). Bedding's three-dimensional monoxenic culture has been proved to be the most successful method in solid culture (Friedman, 1990) and is currently used for commercial production of *Heterorhabditis* and *Steinernema* species. However, little is known about the population dynamics or the population development of these nematodes in *in vitro* solid culture.

With Bedding's method, normally 30-50 million infective juveniles (IJ) of *Heterorhabditis* and *Steinernema* can be produced in a single 500 ml flask containing 80-100 g of chicken offal medium, but yields vary from very poor (a few million) to a record high of over 100 million per flask, and both the quality and quantity of nematodes from successive cultures can decrease significantly (Bedding, 1986). Why this is so and what the optimum conditions are for *in vitro* monoxenic culture of the nematodes still remain to be determined, but an understanding of the population dynamics of these nematodes should be helpful.

Although Bedding (1986) examined various factors that could affect nematode yields, he did not consider

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inoculum size, since all inoculation was then conducted by transferring portions of mature solid culture to initiate new cultures. Han *et al.* (1993) studied the relationship between inoculum size, temperature and time on *in vitro* production of *Steinernema carpocapsae* with emphasis on the combination effects on the final yields of IJ. With the adoption of liquid inoculation of culture trays through sterile diaphragms to facilitate mechanisation in commercial production (Bedding *et al.*, 1991), an understanding of the effect of inoculation size on population dynamics in solid culture will help to optimise the time required for, and the final yields from nematode mass production. In the present study, we examined the population development of *H. bacteriophora* and *S. carpocapsae* in *in vitro* monoxenic flask culture to determine the effects of a range of inoculation dosages on the final yields and populations of the nematodes.

Materials and methods

The Leningrad strain (A24) of *S. carpocapsae* and C1 strain of *H. bacteriophora* were used throughout the study because these nematodes represent commonly used species in the families of Steinernematidae and Heterorhabditidae. The method is a modification of the three-dimensional, monoxenic solid culture method of Bedding (1981).

PREPARATION OF CULTURE FLASKS

Fresh chicken offal, with gall bladders and gizzards removed, was homogenized in a Waring blender with 20% w/v added water (with the addition of 10% lard for the culture of *H. bacteriophora*). The resulting homogenate was evenly distributed by kneading into crumbed polythene foam at a ratio of ten parts by weight of homogenate to one part foam. Then, 80 g of this material was placed in a 500 ml flask, the necks cleaned, stopped with Steristopper bungs, and covered with aluminium foil. The flasks were autoclaved for 30 min at 121°C, allowed to cool to about 45°C and then shaken to separate the coated foam particles. After cooling to below 25°C, each flask was inoculated in a laminar flow sterile cabinet with 10 ml of a 24 h-old culture of symbiotic bacteria in yeast extract (YS) broth, shaken to distribute the inoculum, and then incubated for four days at 23°C. The symbiotic bacteria (*Xenorhabdus nematophilus* for *S. carpocapsae* and *Photorhabdus luminescens* for *H. bacteriophora*) were cultured on nutrient agar plates, and selected primary form colonies were transferred to the YS broth.

PREPARATION AND INOCULATION OF NEMATODES

Nematode inoculum was obtained from 5- to 6-week-old culture flasks containing at least 99% IJ and

previously tested and confirmed as being monoxenic. From these flasks, nematodes were extracted aseptically in a laminar flow cabinet using four stainless steel 2 L bowls, 25 cm in diameter and with 20 cm diameter sieves of 1 mm mesh, which had been autoclaved previously while wrapped in aluminium foil. The contents of two culture flasks were placed in each sieve, covered with sterile tap water and left for 2 h. The sieve was then removed, leaving extracted nematodes in the bottom of the bowls.

In each bowl, the nematodes were washed by decanting, adding 1 L of sterile water, settling for 1 h, and decanting again. The washing process was repeated four times. The nematodes from all bowls were mixed together to homogenize the inoculum. Three samples were taken from the inoculum, diluted to suitable density (about 200 nematodes per ml), and counted under the microscope. Water from the nematode suspension was checked for monoxenicity by streaking onto nutrient agar plates and culturing at 28°C for 48 h.

From the above monoxenic nematode suspension, serial dilutions were made to achieve inocula of 1, 10², 10⁴, 10⁶, and 10⁷ IJ per 10 ml for *H. bacteriophora* and 2, 10², 10³, 10⁴, 10⁵, 10⁶, and 10⁷ for *S. carpocapsae*. Each dilution was counted under the microscope and adjusted three times to confirm the number of nematodes in each dilution. Where inocula of one or two nematodes were used per flask, each nematode was picked up under the microscope with a hair mounted on the tip of a needle and added to the flask in the laminar flow cabinet. Sterilized water (10 ml) was added to the flask. The nematode suspension was inoculated into flasks using 10 ml pipettes attached to a Bel-Art Pipette Pump[®]. In all, 30 flasks were inoculated with each level of inoculum, except for the inocula of one *H. bacteriophora* or two *S. carpocapsae* per flask, where 50 flasks were inoculated. All of the experimental flasks were kept at a constant temperature of 23 °C.

SAMPLING AND COUNTING OF NEMATODES

Of the 30 flasks exposed to each inoculum level, one flask was extracted each day and three more flasks were extracted each week by leaving their contents in a sieve immersed in Ringer's solution within a bowl for 3 h, with gentle agitation every 30 min. Over 95% of all nematode stages could be extracted in this way. Counts and observations were made of all nematode stages under the microscope. This involved the preparation of various dilutions to facilitate accurate counts because of widely disparate numbers. Due to the considerable time involved in accomplishing this, it was not feasible to count more than one flask from each inoculum level every day, but the three flasks from each inoculum level were examined weekly.

Results

HETERORHABDITIS BACTERIOPHORA

Population dynamics of *H. bacteriophora* from varying inocula at different times are shown in Table 1.

Even with the highest inoculum of 10^7 nematodes per flask, *H. bacteriophora* still increased its population size. However, only about half of the inoculated IJ developed into hermaphrodites after 1 week and the offspring released from these hermaphrodites formed

the final population. Only one reproductive generation occurred. With an inoculum of 10^6 IJ per flask, population development was similar to that of 10^7 inoculum although the final yields of nematodes were higher. There was mainly one reproductive generation. At the inocula of 10^5 and 10^2 nematodes per flask, there was a much greater increase of the nematode populations during the first week. The nematode populations developed over 4 and 5 weeks to reach stable high levels containing almost all IJ. With an inoculum of 10^4 per

Table 1. Population dynamics of *Heterorhabditis bacteriophora* in in vitro solid monoxenic culture with different inoculum sizes.

Inoculum size**	Days of culture	Number of nematodes of various stages per flask ($\times 1000$)*						
		Eggs	1st instar larvae	2nd instar larvae	3rd instar larvae / infective juveniles	4th instar larvae	Males	Hermaphrodite/females
10^7	7	200 \pm 32	170 \pm 25	0	2070 \pm 267	700 \pm 76	0	4300 \pm 356
	14	0	0	870 \pm 105	18 200 \pm 1091	2800 \pm 320	0	1400 \pm 167
	21	0	0	0	33 800 \pm 1768	370 \pm 41	630 \pm 84	930 \pm 103
	28	0	0	0	23 200 \pm 1275	70 \pm 12	0	270 \pm 28
	35	0	0	0	21 530 \pm 769	0	0	0
10^6	7	133 \pm 21	212 \pm 22	1042 \pm 165	313 \pm 34	221 \pm 29	83 \pm 16	571 \pm 64
	14	0	0	200 \pm 27	22 067 \pm 1516	800 \pm 13	333 \pm 45	1333 \pm 161
	21	0	0	0	39 866 \pm 2035	1200 \pm 144	400 \pm 39	2533 \pm 273
	28	0	0	0	38 667 \pm 1783	133 \pm 23	133 \pm 16	800 \pm 68
	35	0	0	0	35 933 \pm 1290	0	0	0
10^4	7	5 \pm 1	13 \pm 2	88 \pm 11	80 \pm 9	97 \pm 102	39 \pm 3	107 \pm 10
	14	0	0	33 \pm 4	6633 \pm 579	33 \pm 23	33 \pm 4	570 \pm 33
	21	0	0	1367 \pm 218	18 167 \pm 1043	733 \pm 87	33 \pm 4	1367 \pm 116
	28	0	0	567 \pm 74	25 533 \pm 1458	967 \pm 76	100 \pm 12	1433 \pm 107
	35	0	0	0	28 133 \pm 1214	0	0	0
10^2	7	0	1 \pm 0.3	6 \pm 0.5	3 \pm 0.5	5 \pm 0.5	2 \pm 0.5	1 \pm 0.5
	14	0	1 \pm 0.2	12 \pm 1	212 \pm 24	10 \pm 1	14 \pm 2	75 \pm 6
	21	0	20 \pm 2	1713 \pm 219	1153 \pm 176	227 \pm 20	80 \pm 4	547 \pm 47
	28	0	0	3767 \pm 336	20 066 \pm 1501	967 \pm 112	33 \pm 3	967 \pm 82
	35	0	0	266 \pm 22	28 267 \pm 1334	67 \pm 6	0	0
	42	0	0	0	28 200 \pm 1146	0	0	0
1***	21	8 \pm 2	13 \pm 2	19 \pm 3	25 \pm 3	8 \pm 0.7	8 \pm 1	22 \pm 2
	28	0	0	1483 \pm 219	5817 \pm 616	600 \pm 57	17 \pm 2	533 \pm 46
	35	0	0	533 \pm 48	24 267 \pm 1639	200 \pm 23	0	0
	42	0	0	0	25 155 \pm 1274	0	0	0

* Number of nematodes = mean \pm standard error of the mean, n=3.

** Number of infective juveniles per flask.

*** Because of the very small number of nematodes and large amount of unconsumed food in 7 and 14 days, counts are unreliable.

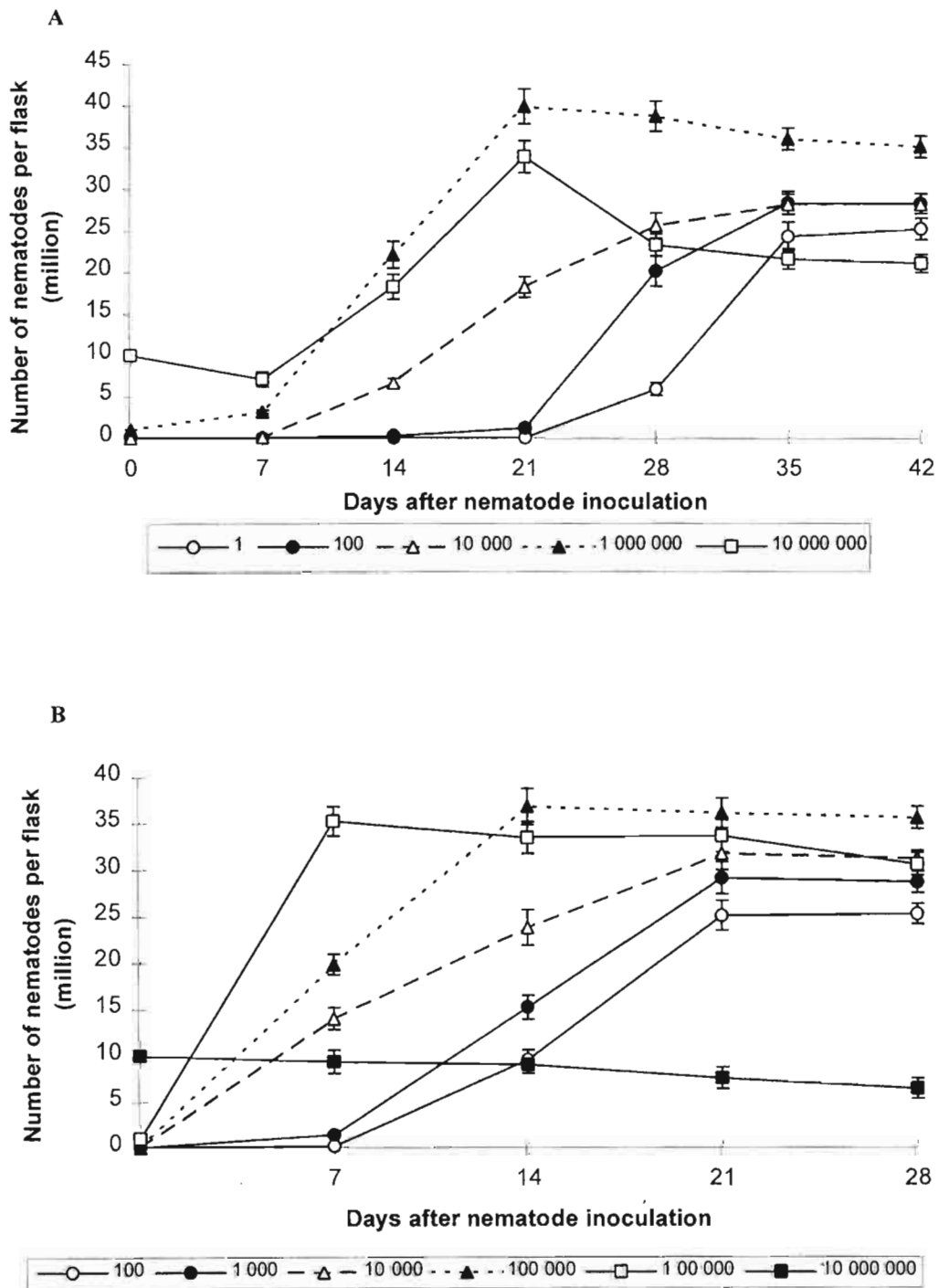


Fig 1. Population development of *Heterorhabditis bacteriophora* C1 (A) and *Steinernema carpocapsae* A24 (B) in in vitro solid monoxenic culture with different inocula.

flask, two reproductive generations contributed to the nematode population, whereas at least three reproductive generations occurred with the inoculum of 10^2 nematodes per flask over a period of 5 weeks.

Although during the first 2 weeks it was not possible to monitor the early population development at the lowest inoculum of one nematode per flask (because of the large amount of unconsumed food material and small number of nematodes), considerable numbers of nematodes were observed 3 weeks after nematode inoculation. The nematode populations developed to their maximum yields of about 25×10^6 IJ per flask after 5 weeks. It was difficult to estimate the number of reproductive generations with this minimal inoculum.

A comparison of the general populations of *H. bacteriophora* initiated by different nematode inocula is shown at Fig. 1A.

STEINERNEMA CARPOCAPSAE

Table 2 shows the population dynamics of *S. carpocapsae* with different inocula at different times.

With all inocula, except the two infective juveniles per flask, nematode populations developed to the final stages, containing nearly pure IJ in three weeks. At all levels of inoculation, exsheathment of IJ and subsequent development proceeded within 24 h. Within 48 h, 80% of the nematodes became adults with all inocula except with the 10^7 inoculum, where only 12% were adults after 48 h and 80% adults after three

Table 2. Population dynamics of *Steinernema carpocapsae* in in vitro solid monoxenic culture with different inoculum sizes.

Inoculum sizes**	Days of culture	Number of nematodes of various stages per flask ($\times 1000$)*						
		Eggs	1st instar larvae	2nd instar larvae	3rd instar larvae / infective juveniles	4th instar larvae	Males	Females
10^7	7	0	0	603 ± 76	1330 ± 206	350 ± 42	3260 ± 414	3730 ± 268
	14	0	0	300 ± 35	4950 ± 397	150 ± 23	2060 ± 217	1520 ± 135
	21	0	0	0	7517 ± 463	0	0	0
	28	0	0	0	6360 ± 518	0	0	0
10^6	7	0	7662 ± 733	$11\ 773 \pm 2086$	$14\ 933 \pm 1657$	350 ± 41	240 ± 33	265 ± 19
	14	0	0	4836 ± 513	$27\ 952 \pm 1744$	150 ± 25	160 ± 21	252 ± 16
	21	0	0	0	$33\ 623 \pm 1659$	0	0	0
	28	0	0	0	$30\ 583 \pm 1243$	0	0	0
10^5	7	1425 ± 213	2382 ± 257	8200 ± 635	6814 ± 773	460 ± 52	205 ± 32	265 ± 22
	14	0	635 ± 66	4900 ± 518	$30\ 395 \pm 1974$	350 ± 41	220 ± 25	252 ± 19
	21	0	0	0	$36\ 086 \pm 1685$	50 ± 6	0	0
	28	0	0	0	$35\ 554 \pm 1242$	0	0	0
10^4	7	2143 ± 257	3247 ± 315	7124 ± 789	533 ± 65	480 ± 52	212 ± 28	236 ± 21
	14	0	755 ± 564	4902 ± 536	$17\ 095 \pm 1565$	550 ± 65	220 ± 26	252 ± 19
	21	0	0	0	$31\ 641 \pm 1647$	100 ± 12	0	0
	28	0	0	0	$31\ 244 \pm 915$	0	0	0
10^3	7	851 ± 91	457 ± 55	80 ± 64	0	0	0.5 ± 0.2	1 ± 0.2
	14	742 ± 86	841 ± 79	3700 ± 452	9255 ± 868	450 ± 53	120 ± 15	125 ± 10
	21	0	0	0	$28\ 903 \pm 1673$	100 ± 12	0	50 ± 3
	28	0	0	0	$28\ 724 \pm 1126$	0	0	0
10^2	7	85 ± 7	42 ± 5	25 ± 3	0	0	0.3 ± 0.1	0.5 ± 0.2
	14	556 ± 62	4021 ± 561	1709 ± 216	2284 ± 357	650 ± 72	120 ± 18	125 ± 12
	21	0	0	0	$24\ 582 \pm 1598$	250 ± 33	90 ± 11	150 ± 12
	28	0	0	0	$25\ 254 \pm 1060$	0	0	0

* Number of nematodes = mean \pm standard error of the mean, $n=3$.

** Number of infective juveniles per flask.

days. The number of first generation adult nematodes remained approximately constant from the second to the fifth day after inoculation, but declined significantly from the sixth day, except with the 10^7 inoculum where numbers remained relatively constant until the eighth day. Although up to 80% of the initial inoculum of IJ developed into adults, the sizes of females were markedly smaller than those of all other inocula and most of them appeared to be malnourished and did not produce offspring. No eggs were found and those (about 30%) that did reproduce produced only a few juveniles (1-3 per female) which emerged from their dead parents as second-instar juveniles. None of the nematodes developed into a second generation. There was no increase in the final yields with this highest inoculum.

The differences in the general population development of *S. carpocapsae* with various inocula are shown in Fig. 1B.

Discussion

Results from the highest inoculum of 10^7 IJ per flask showed that the population dynamics of *H. bacteriophora* and *S. carpocapsae* are rather different. The population development and the final yield of IJ of *H. bacteriophora* at this high inoculum indicates that the hermaphrodites may make better use of the limited food resources than *S. carpocapsae*. Malnutrition, low-productive or non-productive females and a reduction of the final populations suggests that the lack of available food, possibly together with other factors, inhibited the development and reproduction of *S. carpocapsae* at the highest inoculum of 10^7 IJ.

The population development of these two species with various inoculum levels also revealed specific differences in the length of time required to develop into their final stages containing only IJ. With *S. carpocapsae*, nematode populations developed into their final stages in 3 to 4 weeks with a wide range of initial inocula, from 10^2 to 10^7 nematodes per flask. However, the length of time required for the populations of *H. bacteriophora* to develop into the final stages varied from 5 to 6 weeks depending on the initial inoculum. At the highest inoculum level of 10^7 nematodes per flask, it still took 5 weeks for the nematode populations to develop to the stage containing only IJ, while populations initiated with the inocula below 10^6 per flask took up to 6 weeks to reach the final stages.

With an inoculum of only one infective juvenile per flask, *H. bacteriophora* developed as successfully in the *in vitro* solid culture system as did it in *G. mellonella* larvae (Wang & Bedding, 1996). Although it was not possible to determine exactly how many generations were involved, at least five adult generations developed compared with three generations in *G. mellonella* larvae. In the first 3 weeks the nematode popula-

tion (mainly the number of adults) underwent a gradual increase to reach over 10^5 nematodes, of which about 70% were adults (compared to the population in *G. mellonella* larvae which developed to the final stage containing 15×10^3 IJ within 3 weeks). The population of IJ increased to its maximum levels of about 25×10^6 per flask after 5 weeks with its biggest increase (about 80% of the total number) during the fourth week after nematode inoculation. This result also showed that the available food and symbiotic bacteria were sufficient for continuous reproduction and increase of population over a period of 5 weeks.

Attempts to initiate nematode populations of *S. carpocapsae* from a single reproductive female culture failed. Of the 50 flasks inoculated with two IJ, none of them resulted in successful reproduction. This might be because the relatively large surface area in the 80 g of sponge coated medium prevented the nematodes from finding a partner of the opposite sex with which to copulate.

The final IJ yields of *S. carpocapsae* from inocula ranging from 10^2 to 10^6 nematodes in *in vitro* solid culture were from 25×10^6 to 36×10^6 per flask. This contrasts with the work of Sandner and Stanuszek (1971) on the *in vivo* culture of this nematode, in which initial levels of nematode inocula made a considerable difference to final yields (10^5 - 4×10^5 IJ per insect). This could have been the result of several factors. *G. mellonella* larvae almost certainly provide a much better medium for nematode growth and reproduction than do artificial media and give final yields of up to 3×10^6 infective juveniles per gram (Sandner & Stanuszek, 1971) compared to a maximum of about 1×10^6 per gram with artificial media. It is suggested that there may be a much greater difference between the culture medium available to the first generation of adults and subsequent generations in *G. mellonella* larvae, as compared to *in vitro* culture. Which generation contributes most to the final population would therefore make more difference to total production of nematodes in *G. mellonella* larvae than it would in *in vitro* culture. The maximum production of offspring within *G. mellonella* (8×10^2 - 10^3 juveniles per parent) (Wang, 1993) is much higher than that in *in vitro* culture. Oxygenation, CO₂ accumulation, water availability, and maintenance of symbiotic bacteria in primary form are other differences between *in vivo* and *in vitro* culture.

One result of this work, which has already been of benefit in the mass production of the entomopathogenic nematodes is the knowledge that nematodes harvested from one single flask can be used to inoculate and provide adequate yields for over 3500 daughter flasks or 175 culture trays when this is necessary. This is at least 300 or 400 times fewer nematodes than the inocula commonly used for commercial produc-

tion. The introduction of lower initial nematode inocula facilitates mechanisation of inoculation when combined with recent developments in injection of liquid inoculum into trays (Bedding *et al.*, 1991). However, final yields and the time taken to reach these are also important, so that inocula of one million nematodes per flask for *H. bacteriophora* and 10^5 per flask for *S. carpocapsae* are optimal. Then, one flask of nematodes provides inocula for 40 daughter flasks for *H. bacteriophora* and for 350 daughter flasks for *S. carpocapsae*. The final size of inoculum to be used by industry should be evaluated commercially and would probably require further experimentation, *e.g.*, to determine what happens at inoculum levels between 10^5 and 10^6 per flask for *H. bacteriophora* and between 10^4 and 10^5 for *S. carpocapsae*.

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