

The monoxenic culture of *Neoplectana carpocapsae* DD 136 and *Heterorhabditis heliothidis*

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SUMMARY

A series of experiments were done to determine the optimum physical and chemical components of the medium for maximum production of *Neoplectana carpocapsae* DD136 and *Heterorhabditis heliothidis*. The associated bacteria, *Xenorhabdus nematophilus nematophilus* and *X. luminescens* of both nematode species had an optimum temperature for growth of 30°. However, maximum yields of *N. carpocapsae* DD136 and *H. heliothidis* in monoxenic culture occurred at 25° and 30° respectively. In MOPS-buffered

The entomogenous nematodes *Neoaplectana carpocapsae* and *Heterorhabditis heliothidis* are of considerable interest as biological insecticides because of their ease of culture, rapid action, environmental safety (Bedding, 1981; Morris, 1985) and chemical pesticide tolerance (Poinar, 1979). Steinernematids can be cultured, in either a host insect (Dutky, Thompson & Cantwell, 1964) or on synthetic diet. In the latter case, nematode growth and reproduction may occur axenically (Stoll, 1948; Hansen *et al.*, 1968) or in the presence of its bacterial associate *Xenorhabdus* spp. (Bedding, 1981; Wouts, 1981) i.e., monoxenically. Under natural conditions the bacterium is vectored in the nematode gut (Bird & Akhurst, 1983), and is released into the insect hemocoel where it establishes conditions favorable for nematode growth and reproduction (Poinar & Thomas, 1966).

Monoxenic mass culture techniques with the goal of producing, cost effectively, large numbers of infective

Nematodes

Dauer stages of *N. carpocapsae* DD136 strain and of *H. heliothidis* NC15 were isolated from *Galleria mellonella* larvae on White's water traps, gravity washed three times with sterile, distilled water and surface sterilized with 0.1 % Thimersol (Dunphy & Webster, 1985). After washing twice in sterile, distilled water, 0.1 ml aliquots containing 1×10^3 nematodes were added to lipid agar in Petri dishes (Wouts, 1981) that had been inoculated 24 h previously with the nematode's corresponding bacterium to produce a confluent bacterial lawn (Dunphy & Webster, 1985). The nematodes were subcultured on bacterial laden lipid agar at 21 d intervals and incubated at 25°. New monoxenic cultures were initiated every two months using dauer juveniles.

EXPERIMENTAL PROCEDURES

30 ml of lipid agar previously inoculated with bacteria were incubated at specified temperatures and pH values 24 h prior to inoculation with nematodes. Preliminary pH measurements showed no change in medium pH during this time. Nematodes were harvested after 10 d of incubation.

Nutritional study*

All experiments were conducted in 9 cm diameter Petri dishes of lipid agar. Based on nematode reproduction in specified supplemented agar, industrial media containing growth promoting nutrients were selected for their possible ability to support monoxenic nematode culture.

The industrial nutrient most conducive to nematode reproduction for a given experiment was incorporated into the basal medium which will be referred to subsequently as modified medium. The modified medium then would be used for the next nutrient test.

Oils : Both *N. carpocapsae* and *H. heliothidis* were grown on the basal medium containing sunflower oil (Safflo, Concord, Ontario) supplemented with selected fatty acids, sterols or phospholipids at 0.03 mM. Oils screened included butter, halibut oil, cod liver oil, olive oil and corn oil at 0.1 ml to 1.0 ml per 100 ml of basal medium.

Nitrogen sources : Industrial fermentation products (= complex nitrogen sources) (e.g., lactalbumin hydrolysate, fish meal, yeast extract, casein), standard microbiological media [e.g., peptone, nutrient broth (Difco)] and tissue culture media [e.g., heat inactivated fetal calf serum (GIBCO, Burlington, Ont.), amino acids from Grace's insect tissue culture medium (Grace, 1962)] were compared with the modified medium containing sunflower oil at the optimum concentration of 0.1 ml/100 ml of medium. Nutrient broth was deleted from the media supplemented with complex nitrogen sources to prevent masking the effects of the nitrogen supplements. All nitrogen sources were adjusted to give 2.5 g of total nitrogen per liter. The optimum concentration of the most effective nitrogen source was determined and subsequently, replaced the nutrient broth source of the modified medium.

Carbohydrates : The monosaccharides D-fructose, D-glucose, D-galactose and D-sorbose; the disaccharides sucrose, maltose and trehalose; and the alcohols glycerol and D-mannitol were added separately in tests to the modified media to produce 14 mM total carbon levels. The industrial carbon sources molasses, corn syrup, cornstarch, malt extract and soluble starch were screened for their ability to support monoxenic nematode culture.

* Unless otherwise stated all chemical were purchased from Sigma (St. Louis, Mo, USA).

Vitamins : The modified media were individually supplemented with 0.1 or 1.0 mg/l of ascorbic acid, D-pantothenic acid, riboflavin, nicotinamide, biotin, m-inositol, choline chloride, folic acid, p-aminobenzoic acid. Also tested were combinations of these vitamins, the concentration of each vitamin being 0.1 mg/l or 1.0 mg/l. The vitamins were filter-sterilized in 0.2 µm Nalgene filters and aseptically added to the media at 45°.

Salts : The effects of selected salts were tested for *H. heliothidis* culture. Cation levels of 1.0 and 10 mM were separately added to the modified media. The pH of the media were adjusted to 6.5 prior to autoclaving.

Harvesting and data analysis : All nematode cultures were harvested 10 d post inoculation by flooding the Petri dishes with 10 ml of distilled water. Cultures were incubated at 25° for 10 min to dislodge juvenile nematode from the agar-bacterial matrix. A combination of scraping with a rubber policeman and pipetting ensured nematode dispersion (Dunphy, Rutherford & Webster, 1985). The total collective number of all stages of nematodes per dish was estimated based upon the average number of nematodes in five aliquot samples of 100 µl of suspension per dish.

A minimum of four replicates were used for each treatment. Data were analyzed using Natrella's (1972) 95 % confidence overlap procedure.

Results

OPTIMUM PHYSICAL PARAMETERS

Both *X. nematophilus nematophilus* and *X. luminescens* showed most rapid growth at the higher temperatures (Fig. 1). However, the temperature responses differed with the bacterial species in that *X. luminescens* grew more slowly and exhibited a more restricted profile than *X. nematophilus nematophilus*.

In monoxenic culture both nematode species exhibited sharp temperature optima; *N. carpocapsae* at 25° and *H. heliothidis* at 30° (Fig. 2). *H. heliothidis* did not grow at or below 15° and both species grew poorly at 35°.

X. nematophilus nematophilus was sensitive to buffer type and to pH, and only MOPS buffer permitted growth at pH 6.5 to the level of the unbuffered medium (Fig. 3). The degree of response of *X. luminescens* to pH also varied with the type of buffer. MOPS buffer resulted in a broad pH curve with optimum values of pH 6.5 to 7.5 for *X. luminescens*, and HEPES produced a sharper profile with an optimum pH of 7.0. The other buffers were extremely toxic to both bacterial species.

The least toxic buffers for both nematode species were BIS-TRIS and MOPS. The optimum pH of the monoxenic culture varied with the buffer type and the nematode-bacterium culture (Fig. 4). The greatest yields

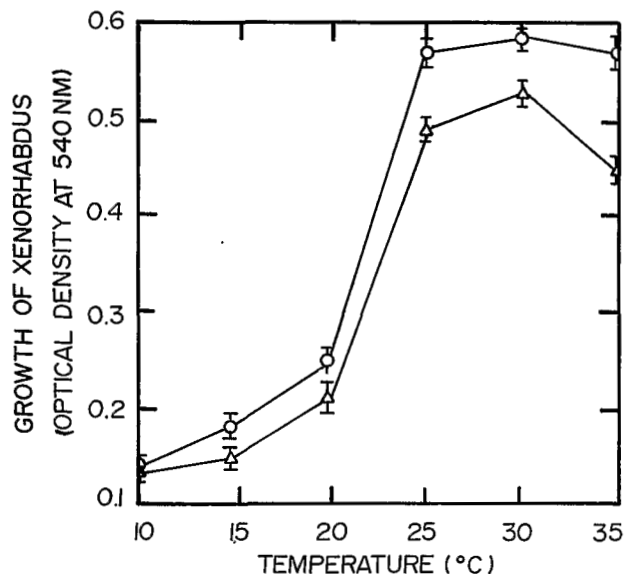


Fig. 1. Growth of *Xenorhabdus nematophilus* subsp. *Nematophilus* (○) and *X. luminescens* (△) in lipid broth at selected temperatures based on the optical density readings of the cultures at 540 nm.

of *N. carpocapsae* occurred with MOPS buffer at pH 6.0, whereas the medium containing BIS-TRIS showed greatest yield of *H. heliothidis* at pH 7.5

NUTRITIONAL STUDY

Lipids

Both sodium acetate and stearic acid enhanced the culture of *N. carpocapsae* above the basal medium (Tab. 1). Stearic acid also elevated the growth of *H. heliothidis*. The sterols and phospholipids produced *N. carpocapsae* levels comparable to those of the stimulatory fatty acids. Cholesterol elevated *H. heliothidis* levels but not to the value of stearic acid.

The industrial lipids most conducive to increased reproductive levels were Tween 60 (0.03 mM), sunflower oil (0.1 ml/100 ml) and Tween 80 (0.03 mM). *N.*

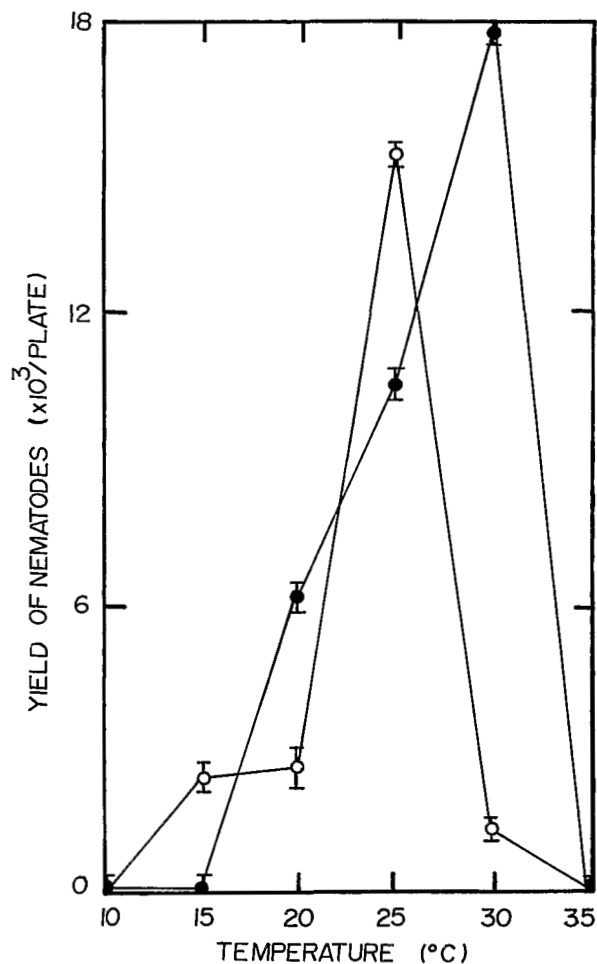


Fig. 2. Population increase of *Neoaplectana carpocapsae* DD136 (○) and *Heterorhabditis heliothidis* (●) in monoxenic culture on lipid agar at selected temperatures.

Nitrogen sources

The modified medium for both nematode species during these tests consisted of lipid agar with the lipid supplement appropriate for the nematode species and minus the nutrient broth. The latter was done to prevent masking of the nitrogen supplementation effects.

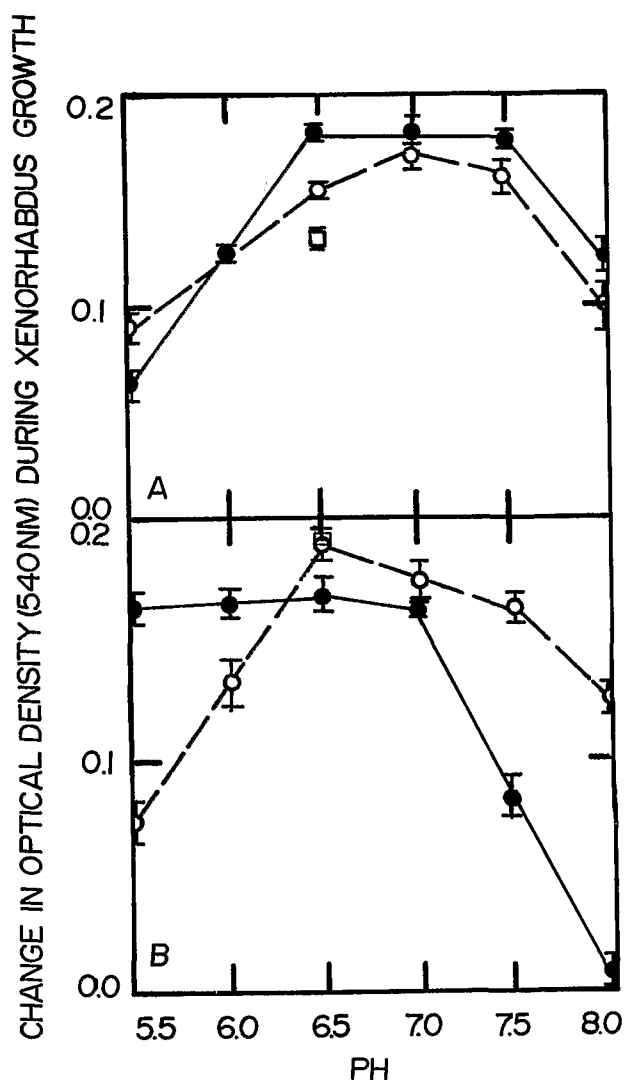


Fig. 3. Growth of *Xenorhabdus luminescens* (A) and *Xenorhabdus nematophilus* subsp. *nematophilus* (B) in lipid broth at selected pH values with MOPS (○), HEPES (●), (□) unbuffered control medium. Growth was based on optical density readings at 540 nm.

soyatone (soybean meal digest). The optimum concentration of tryptone and soyatone for *N. carpocapsae* cultures was 10 g/l and 15 g/l, respectively (Fig. 5). However, these digests did not support nematode yields comparable with that of tryptic soy broth.

Carbohydrates

Compared with the modified media, the medium together with one of the supplements D-galactose, D-fructose, D-glucose and glycerol supported significant *N. carpocapsae* reproduction and D-glucose, D-sorbitose and D-mannitol significantly greater *H. helio-*

Table 1

Effect of different lipids (0.03 mM) on the production of *Neoplectana carpocapsae* and *Heterorhabditis heliothidis* in monoxenic culture after incubation for 10 d

Lipid	Total yield of nematodes ($\times 10^2$ /plate) ^a	
	<i>N. carpocapsae</i>	<i>H. heliothidis</i>
	DD136	
FATTY ACIDS		
Oleic acid	63.5 \pm 1.8	—
Stearic acid	109.3 \pm 2.3	5.0 \pm 1.6
Palmitic acid	28.3 \pm 1.2	—
Palmitoleic acid	95.3 \pm 2.2	—
Myristic acid	67.0 \pm 1.8	—
Linoleic	57.3 \pm 1.7	—
Pentadecaneic acid	34.4 \pm 1.3	—
Sodium acetate	153.0 \pm 2.8	—
STEROLS		
Cholesterol	109.3 \pm 2.3	38.1 \pm 1.3
B-sitosterol	129.4 \pm 2.5	—
PHOSPHOLIPIDS		
Phosphatidylcholine	146.0 \pm 2.7	—
Acetylcholine	165.0 \pm 2.9	—
Lipid agar basal medium	90.0 \pm 2.1	27.3 \pm 1.2

^a Mean \pm standard error of the mean; N = 5.

^b Not determined.

thidis reproduction in culture (Tab. 4). Trehalose did not increase the number of individuals of these species in culture.

Corn syrup was the preferred industrial carbon source for the culture of both nematode species (Tab. 5). The optimum corn syrup concentration for *N. carpocapsae* and *H. heliothidis* was 4 g/l and 10 g/l, respectively (Fig. 7). *H. heliothidis*, unlike *N. carpocapsae*, grew well in monoxenic culture containing soluble starch.

Vitamins

Individual and pooled vitamins ranging in concentration from 0.1–1.0 mg/l were not conducive to elevating the reproduction of *N. carpocapsae* or *H. heliothidis* (data not shown). Ascorbic acid, m-inositol, choline chloride, riboflavin, folic acid and nicotinamide were toxic to both nematode species at the concentration of 1.0 mg/l.

Salts

Magnesium chloride (10 mM), potassium chloride (10 mM) and potassium nitrate (1 mM) enhanced *H. heliothidis* production (Tab. 6). Manganese chloride,

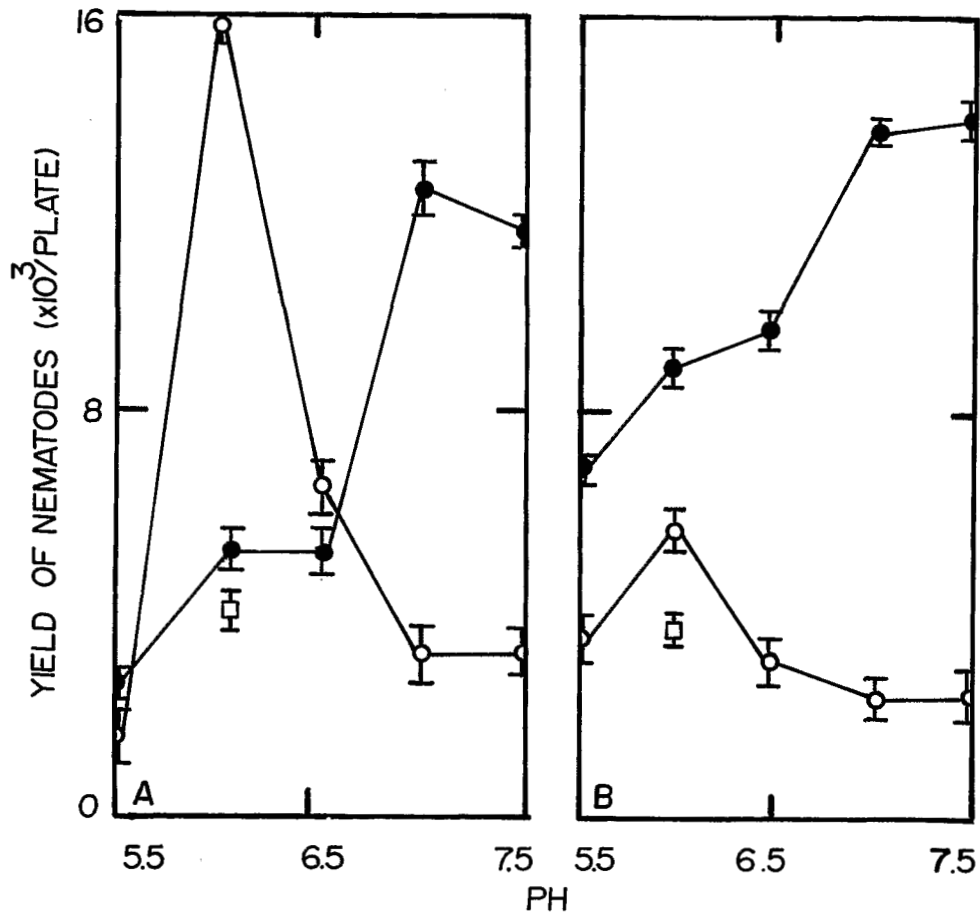


Fig. 4. Effect of MOPS (○) and BIS-TRIS (●) buffer on the growth of *Neoplectana carpocapsae* DD136 (A) and *Heterorhabditis heliothidis* (B) in monoxenic culture at their optimum temperature. (□) unbuffered control medium.

ferric chloride and sodium bicarbonate were apparently detrimental to the nematode cultures.

Media comparison

and the optimum temperature of 30° for *H. heliothidis* and *X. luminescens* is shown. Dunphy and Webster (1986) reported limited and good passaging of *H. heliothidis* and *Neoplectana* DD136 through *C. gl.*

Table 2

Effect of industrial lipids on the production of *Neoaplectana carpocapsae* and *Heterorhabditis heliothidis* in monoxenic culture after incubation for 10 d

Component	Concentration	Total nematode yield ($\times 10^3$ /plate) ^a	
		<i>N. carpocapsae</i>	<i>H. heliothidis</i>
Tween 40	0.03 mM ^b	94.0 \pm 2.8	— ^d
Tween 60	0.03 mM	145.7 \pm 3.4	86.3 \pm 2.7
Tween 80	0.03 mM	174.0 \pm 3.8	23.0 \pm 1.4
Butter	0.1 ml ^c	31.8 \pm 1.6	31.3 \pm 2.3
	1.0 ml	11.2 \pm 1.0	—
Halibut oil	0.1 ml	42.7 \pm 1.9	—
	0.2 ml	—	31.9 \pm 1.6
	0.4 ml	—	29.7 \pm 1.6
	0.8 ml	—	38.6 \pm 1.6
	1.0 ml	91.5 \pm 2.8	—
Cod liver oil	0.1 ml	93.6 \pm 2.8	29.0 \pm 1.6
	0.5 ml	—	42.1 \pm 1.9
	1.0 ml	54.8 \pm 2.1	24.3 \pm 1.4
Olive oil	0.1 ml	13.1 \pm 1.0	18.0 \pm 1.2
	0.5 ml	—	23.0 \pm 1.4
	1.0 ml	11.7 \pm 1.0	22.1 \pm 1.4
Corn oil	0.1 ml	—	5.1 \pm 0.6
	0.5 ml	—	82.1 \pm 2.6
	1.0 ml	—	9.5 \pm 0.9
Lipid agar modified medium with sunflower oil	0.1 ml	294.1 \pm 5.0	—
	1.0 ml	90.0 \pm 2.7	27.3 \pm 1.5

^a Mean \pm standard error of the mean; N = 4.

^b Total fatty acids supplied by Tween compounds.

^c Volume of oil added to make 100 ml of medium.

^d Not determined.

quantitative differences in their responses to many of the nutrients. This may reflect species differences in : *i*) nematode metabolism, and/or *ii*) bacterial metabolism and/or *iii*) differences in the nematode-bacterium metabolic association. Thus, despite the significance of these experiments, it is difficult to draw unequivocal conclusions on nematode nutrition using monoxenic culture.

Tween 60 and 80 enhanced *N. carpocapsae*, and Tween 60 elevated *H. heliothidis* yields. However, *X. nematophilus nematophilus* does not produce Tween 80 active lipase (Akhurst, 1986), whereas *X. luminescens* does (Thomas & Poinar, 1979), which suggests that bacterial lipase is not a prerequisite for nematode production. Although lipase has not been detected in

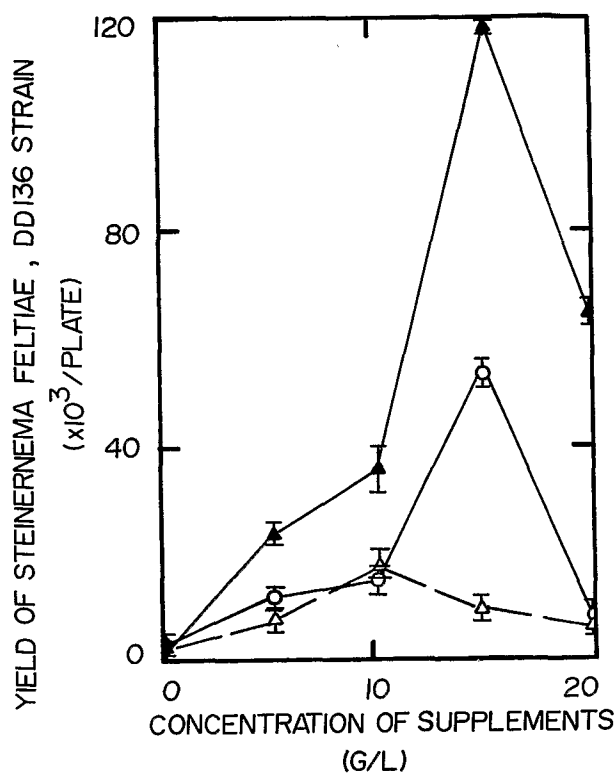


Fig. 5. Population increase of *Neoaplectana carpocapsae* on modified medium containing the nitrogen supplements tryptic soy broth (▲), soyatone (○) and tryptone (△).

possible the Tween effect may reflect different lipase types or different mechanisms of lipid uptake between *N. carpocapsae* and *H. heliothidis*. In consonance with Dutky, Robbins and Thompson (1967), cholesterol and the plant sterol β -sitosterol were shown to enhance *N. carpocapsae* production. Cholesterol elevated the yield of *H. heliothidis*.

The basal medium was shown to contain more nutrient broth than was required for nematode reproduction of both species. Tryptic soy broth enhanced *N. carpocapsae* production but neither tryptone nor soyatone either individually or together in levels reflecting their tryptic soy broth concentrations produced nematode yields comparable to those on tryptic soy broth. Consequently, other dietary components in tryptic broth supplements must enhance *N. carpocapsae* culture.

Yeast extract stimulated the reproduction of both nematode species. The effect is not attributed to the vitamin content of yeast extract because (1) autoclaving the medium would have destroyed the vitamins and (2) individual and pooled vitamin supplements did not elevate nematode yields. The vitamin supplements were tested at concentrations spanning those encountered in yeast extract. Jackson and Suddiqui (1965) and Jackson

Table 3

Effect of different nitrogen sources on the production of *Neoplectana carpocapsae* and *Heterorhabditis heliothidis* in monoxenic culture after 10 d incubation

Nitrogen source ^a	Total nematode yield ($\times 10^3$ /plate) ^b	
	<i>N. carpocapsae</i>	<i>H. heliothidis</i>
Grace's amino acids ^c	8.3 ± 0.8	—
Fetal calf serum	23.9 ± 1.4	—
L-broth ^d	8.5 ± 0.8	—
Tryptic soy broth	588.0 ± 7.0	3.0 ± 0.5
Casein	62.7 ± 2.3	—
Casamino acids (vitamin free)	96.8 ± 2.8	6.5 ± 0.7
Lactalbumin hydrolysate	141.0 ± 3.4	4.2 ± 0.6
Liver	52.7 ± 2.1	—
Fishmeal	123.0 ± 3.2	—
Peptone	186.6 ± 3.9	2.5 ± 0.4
Proteose peptone	—	5.5 ± 0.7
Albumin	129.3 ± 3.2	—
Yeast extract	241.5 ± 4.5	242.0 ± 4.5
Nutrient broth	166.3 ± 3.7	137.2 ± 3.4
Basal medium ^e	168.1 ± 3.7	124.3 ± 3.2

^a Total nitrogen level = 2.5 g/L.

^b Mean ± standard error of the mean, N = 4.

^c From Grace, T.C.C. 1962. Nature 195 : 788-789.

^d From Dillon, J.-A., Nasin, A. and E. R. Nestmann, 1985, Recombinant, DNA Methodology, John Wiley and Sons, N.Y., p. 82.

^e For : *N. carpocapsae* : 2.3 g nutrient agar plus 0.1 ml sunflower oil/100 ml medium.

H. heliothidis : 2.3 g nutrient agar plus 0.5 ml cold liver oil/100 ml medium.

^f Not determined.

biotin in the axenic culture of *Steinernema glaseri*. Thus, our results do not show that nematodes do not require vitamins but rather that the bacteria probably act as a vitamin source for the nematodes.

There was no discernible correlation between stimulatory carbohydrate and bacterial fermentation ability suggesting that the sugar effects are complex. It was surprising that trehalose did not increase nematode yields because trehalose is a major hemolymph sugar (Wyatt, 1967) and readily fermented by *Xenorhabdus* species (Akhurst, 1983).

Pye and Burman (1981) reported positive chemotaxis by *N. carpocapsae* to Cl⁻, Mg²⁺, Ca²⁺ and CO²⁻ ions at 75 mM levels as a possible host locating mechanism. The present study is the first to establish the effects of salts on heterorhabditid growth and reproduction and their potential importance in mass production media. Thus, it will be possible to ensure nutrients with inhi-

YIELD OF HETERORHABDITIS HELIOTHIDIS

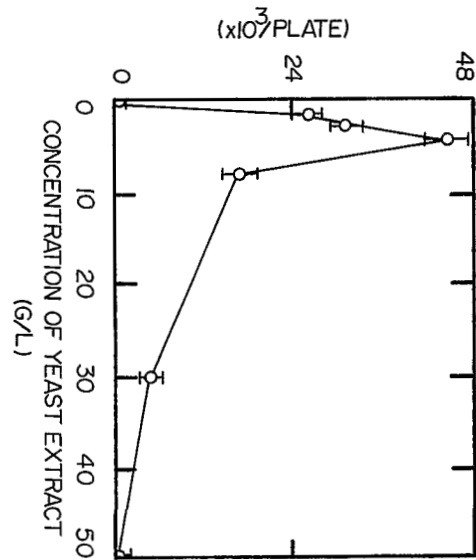


Fig. 6. Effect of yeast extract concentrations on the reproduction of *Heterorhabditis heliothidis* in lipid agar.

Table 4

Effect of different carbon sources on the yield of monoxenic cultures of *Neoplectana carpocapsae* and *Heterorhabditis heliothidis* after incubation for 10 d

Compound ^a	Total nematode yield ($\times 10^3$ /plate) ^b	
	<i>N. carpocapsae</i>	<i>H. heliothidis</i>
D-glucose	63.6 ± 2.3	35.5 ± 1.7
D-fructose	70.5 ± 2.4	11.0 ± 1.0
D-galactose	160.0 ± 3.6	— ^d
D-mannose	33.2 ± 1.6	17.1 ± 1.2
D-sorbose	—	45.0 ± 1.9
Sucrose	—	3.0 ± 0.5
Trehalose	41.0 ± 1.8	6.0 ± 0.7
Maltose	—	6.2 ± 0.7
D-mannitol	39.2 ± 1.8	23.5 ± 1.4
Glycerol	133.8 ± 3.3	—
Modified medium ^c	38.5 ± 1.8	11.0 ± 0.9

^a Total carbon added equalled 14 mM.

^b Mean ± standard error of the mean, N = 4.

^c Modified medium for : *N. carpocapsae* : 2.3 g nutrient agar + 0.1 ml sunflower oil 1.5 g soya tone/100 ml medium; *H. heliothidis* : 2.3 g nutrient agar + 0.5 ml cod liver oil + 0.5 g yeast extract/100 ml medium.

^d Not determined.

Table 5

Effects of different industrial carbon sources on the yield of *Neoaplectana carpocapsae* and *Heterorhabditis heliothidis* in monoxenic culture after incubation for 10 d

Compound ^a	Total nematode yield ($\times 10^3$ /plate) ^b	
	<i>N. carpocapsae</i>	<i>H. heliothidis</i>
Molasses	93.2 \pm 2.8	65.5 \pm 2.3
Corn syrup	172.8 \pm 3.8	148.0 \pm 3.5
Corn starch	44.0 \pm 1.9	_____ ^d
Malt extract	21.0 \pm 1.3	_____
Soluble starch	20.2 \pm 1.3	129.5 \pm 3.2
Modified medium ^c	38.5 \pm 1.8	11.0 \pm 1.0

^aTotal carbon supplied = 14 mM.

^bMeans \pm standard errors of the mean, N = 4.

^cModified medium for : *N. carpocapsae* : 2.3 g nutrient agar + 0.1 ml sunflower oil + 1.5 g soyatone/100 ml medium; *H. heliothidis* : 2.3 g nutrient agar + 0.5 ml cod liver oil + 0.5 g yeast extract/100 ml medium.

^dNot determined.

Table 7

Media supporting the monoxenic culture of *Neoaplectana carpocapsae* DD136 and *Heterorhabditis heliothidis*

Lipid agar medium for both nematode species	
Nutrient agar	23.0 g/l
Nutrient broth	8.0 g/l
Safflow oil	11 mL/l
<i>N. carpocapsae</i> culture medium	
Corn syrup	4 g/l
Soyatone	15 g/l
Sunflower oil	1 mL/l
Agar	15 g/l
<i>H. heliothidis</i> culture medium	
Corn syrup	10 g/l
Yeast extract	5 g/l
Nutrient agar	23 g/l
Cod liver oil	5 mL/l
MgCl ₂ 6 H ₂ O	2 g/l

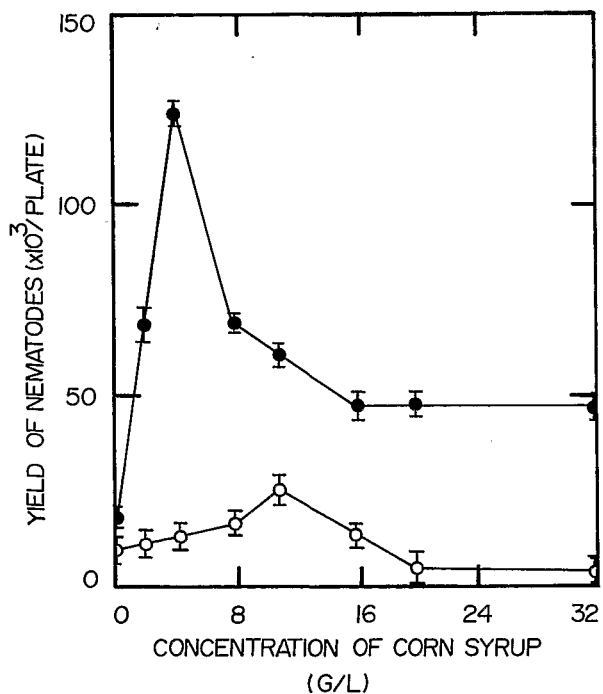


Fig. 7. Effect of various concentrations of corn syrup on the production of *Neoaplectana carpocapsae* DD136 (●) and *Heterorhabditis heliothidis* (○) on their respective culture media.

Table 8

Total yield of *Neoaplectana carpocapsae* and *Heterorhabditis heliothidis* after incubation for 10 d on different media

Medium	<i>N. carpocapsae</i>	<i>H. heliothidis</i>
Lipid agar	2,872 \pm 27/plate	6,983 \pm 42/plate
<i>N. carpocapsae</i> medium	29,750 \pm 86/plate	_____ ^b
<i>H. heliothidis</i> medium	_____ ^b	31,000 \pm 88/plate

^aMean \pm standard error of the mean, N = 4.

^bNot determined.

bitory salts or at inhibitory concentrations are not included in media formulation and that stimulatory salts are included to maximize nematode yields.

The data arising from this research enables the more reliable production and greater yields of all stages of *N. carpocapsae* and *H. heliothidis* in laboratory culture *in vitro* and is a step towards improved mass industrial production.

Table 6
Total Yield of *Heterorhabditis heliothidis* in monoxenic culture supplement
with different salts after incubation for 10 d

Salt	Concentration ^b (mM)	Nematode Yield ^a ($\times 10^2$ /plate)	Salt	Concentration (mM)	Nematode Yield ^a ($\times 10^2$ /plate)
Na ₂ CO ₃	1	50.0 ± 2.0	CaCl ₂	1	68.8 ± 2.4
	10	158.8 ± 3.6		10	43.7 ± 1.9
NaH ₂ PO ₄	1	53.8 ± 2.1	CaSO ₄	1	100.8 ± 2.9
	10	8.0 ± 0.8		10	97.2 ± 2.8
NaHCO ₃	1	16.0 ± 1.2	FeCl ₃	1	0 ± 0
	10	6.5 ± 0.7		10	0 ± 0
MgSO ₄ · 7 H ₂ O	1	180.0 ± 3.9	KNO ₃	1	200.0 ± 4.1
	10	118.8 ± 3.1		10	103.8 ± 2.9
MgCl ₂ · 6 H ₂ O	1	123.0 ± 3.2	KCl	1	78.0 ± 2.6
	10	217.5 ± 4.2		10	196.2 ± 4.0
MnCl ₂	1	5.0 ± 0.6	Modified medium ^c		145.0 ± 3.5
	10	12.5 ± 1.0			

^a Mean ± standard error of the mean, N = 4.

^b The level of the cation.

^c Modified medium : 2.3 g nutrient agar + 0.5 ml cod liver oil + 0.5 g. yeast extract/100 ml medium.

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