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Relationship between medium composition, inoculum size, temperature and culture time in the yields of *Steinernema* and *Heterorhabditis* nematodes

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Summary — The rotatable composite design was introduced to determine the effects or interactions of medium composition, bacterial and nematode inoculum sizes, temperature and culture time on the growth and yield of *Steinernema* sp. CB2B and *Heterorhabditis bacteriophora* H06 in Bedding's sponge culture system. Nematode yields were significantly affected by medium composition, temperature and culture time but not by bacterial and nematode inoculum sizes in the coded range. Corn flour and fish meal with more lipid additions could serve as media for the ease of production process. Negative interactions between medium composition and temperature, bacterial inoculum and temperature on CB2B, and between lard additions and bacterial inoculum, lard additions and culture time on H06 were observed. The nematode growth rates decreased with increasing nematode inoculum size and the optimal inoculum size will depend on achieving maximum yield with minimum inoculum size in a said unit medium. Various culture formulas were computed and the culture management system for commercial production of nematodes was discussed.

Résumé — ***Relations entre composition du milieu, valeur de l'inoculum, température et durée d'élevage dans la production des nématodes* Steinernema *et* Heterorhabditis** — Un système tournant composite est décrit pour déterminer l'action ou les interactions de la composition du milieu, de la valeur des inoculums de bactéries et de nématodes, de la température et de la durée d'élevage sur la croissance et la production de *Steinernema* sp. CB2B et d'*Heterorhabditis bacteriophora* H06 élevés sur éponge (système de Bedding). Dans la plage explorée, la production des nématodes est significativement modifiée par la composition du milieu, la température et la durée de l'élevage mais non par la valeur des inoculums bactériens ou de nématodes. La farine de maïs et la farine de poisson enrichie en lipides peuvent être utilisées comme moyens facilitant les processus de production. Il a été observé des interactions négatives entre composition du milieu et température, inoculum bactérien et température (sur CB2B), addition de lard et inoculum bactérien, addition de lard et durée d'élevage (sur H06). Le taux de croissance du nématode décroît si la valeur de l'inoculum augmente. La valeur optimale de l'inoculum dépendra de l'obtention, pour une unité donnée du milieu, d'une production maximale par un inoculum minimal. Différentes formules d'élevage ont été calculées et il est discuté d'un système pratique d'élevage pour une production commerciale.

Key-words : *Steinernema, Heterorhabditis,* growth inoculum, culture medium.

Steinernema and *Heterorhabditis* nematodes have been widely used for the control of various soil-inhabiting pests as biocontrol agents. Extensive use of these nematodes depends on successful mass rearing. Now methods of rearing nematodes have been established by *in vivo* process in which insects (usually the greater wax moth larvae *Galleria mellonella*) serve as a medium (Dutky *et al.*, 1964) and in *in vitro* process by which nematode grows monoxenically with the symbiotic *Xenorhabdus* bacteria on sponge culture system (Bedding, 1981, 1984; Wouts, 1981) or liquid medium system (Pace *et al.*, 1984; Buecher & Popiel, 1989; Friedman, 1990).

Monoxenic mass culture is developed by inoculating the culture medium with the symbionts prior to introduction of the nematode inoculum. The effects of several environmental factors such as temperature, nutrient components (Dunphy & Webster, 1989) and inoculum size (Li *et al.*, 1987; Xu *et al.*, 1989) on the yields of nematodes were reported. However, to a commercial mass production process, the cost of nematode tode production is of critical importance. Factors that affect nematode yields should be adjusted simultaneously to be reliable, maximal harvest of infectives and at low cost. Therefore, interaction of critical factors on the yields of entomopathogenic nematodes requires to be determined.

The present study introduced the rotatable composite design, a methology useful in connection with experimental determination of response surface, to determine the importance of interactions among medium composition, temperature, bacterial and nematode inoculum sizes, and culture time on the growth and yield of nematodes; and to determine which factor combination results in the greatest yield in Bedding's sponge culture system.

Material and methods

NEMATODES

Monoxenic cultures of *Steinernema* sp. CB2B from Biological Control Institute, Beijing, and *Heterorhabditis*

bacteriophora H06 (= C 8406) (Poinar, 1990) from Shandong, China, were established by Han *et al.* (1990*a*) in Bedding's sponge culture (Bedding, 1984) and maintained at 25 °C. These nematode strains were chosen since they showed much potential for pest control in the field (Li, *et al.*, 1989; Yang *et al.*, 1990).

Experimental design

Rotatable composit design was used in this study. Tests were conducted with two design formulas. Four variables with each at five levels were used for CB2B (Table 1) and five variables for H06 (Table 2). The levels of each variable were coded as — 2, — 1, 0, + 1, + 2, and varied on a linear scale for medium composition, temperature and culture time variables, and on a logarithmic scale to the base 2 for inoculum size of H06. The response variable was the yields of infectives after 15 days with CB2B and the yields of infectives at the coded culture time with H06.

A total 36 flasks for each nematode strain was established, containing twelve replicates at the center point in Table 3, or ten replicates in Table 4. The variation within the replicated treatments was used in the computation error. The entire design was repeated twice.

Medium composition

Medium combinations were formulated based on the nutritional components in different coded levels for CB2B (Table 1) and H06 (Table 2) culture. 40 g of combinated nutrient components mixed with 10 g polyether polyurethane sponge was placed in 500 ml conical flasks and autoclaved at 121 °C for 40 min.

Inoculation and incubation

10 ml of a 48 h old primary phase bacterial suspension in YS broth (1 % peptone, 1 % beef extract, 0.5 % NaCl) was added to each culture flask after serially diluted to the coded levels (Tables 1, 2) with a sterilized culture of the symbiont, and incubated for 3 days at the coded levels of temperature.

10 ml of a salt-buffer (0.5 % NaCl) dilution series of infectives (Tables 1, 2), collected from 4 week old monoxenic sponge cultures, gravity washed in sterile salt-buffer, was then introduced into the flasks incubated with the symbiont.

The inoculum sizes were distributed based on the screening tests.

Harvesting and data analysis

CB2B was harvested after 15 days of incubation and H06 after each coded level of culture time. The number of infectives per flask was estimated based upon the average number of extracted infectives in three aliquot samples of 1 ml of suspension.

A BASIC computer programme based on Box (1954) and Ding (1986) was used to calculate the regression coefficients and analysis of variance of the equations for the response surfaces of yield. T-test was used to determine the statistical significance of the individual regression coefficients.

Table 1. Test variables and coded values for *Steinernema* sp. CB2B.

Variable	Level	1	2	3	4	5
	Coded value	− 2	− 1	0	1	2
Medium composition (X_1) (g/flask)						
Chicken offal		20	25	30	35	40
Mixed components *		20	15	10	5	0
H_2O		5	5	5	5	5
Sponge		5	5	5	5	5
Bacterial inoculum size (X_2)		0	4×10^7	8×10^7	12×10^7	16×10^7
Temperature (X_3) (°C)		15	19	23	27	31
Nematode inoculum size (X_4)		4.7×10^4	9.4×10^4	14.1×10^4	18.8×10^4	23.5×10^4

* Corn flour : fish meal : lard = 1:1:1.

Table 2. Test variables and coded values for *Heterorhabditis bacteriophora* H06.

Variable	Level	1	2	3	4	5
	Coded value	− 2	− 1	0	1	2
Medium composition (X_1) (g/flask)						
Lard		0	3	6	9	12
H_2O		12	9	6	3	0
Chicken offal		34	34	34	34	34
Sponge		4	4	4	4	4
Bacterial inoculum size (X_2)		3.75×10^7	7.5×10^7	1.5×10^8	3×10^8	6×10^8
Temperature (X_3) (°C)		17	21	25	29	33
Nematode inoculum size (X_4)		5×10^4	10×10^4	20×10^4	40×10^4	80×10^4
Culture time (day) (X_5)		15	20	25	30	35

Results

Nematode growth in culture flasks

CB2B grew in the temperature range 15-31 °C although poorly at 15 °C. The color in culture turned

Table 3. Observed yields of *Steinernema* sp. CB2B after 15 days in 50 g medium flasks with various treatments.

Treatment level (see Table 1)					Observed yield
No.	X_1	X_2	X_3	X_4	(x10^6/flask)
1	− 1	− 1	− 1	− 1	8.04
2	− 1	− 1	− 1	1	9.00
3	− 1	− 1	1	− 1	17.12
4	− 1	− 1	1	1	17.28
5	− 1	1	− 1	− 1	11.10
6	− 1	1	− 1	1	13.80
7	− 1	1	1	− 1	17.60
8	− 1	1	1	1	18.56
9	1	− 1	− 1	− 1	0.83
10	1	− 1	− 1	1	1.43
11	1	− 1	1	− 1	5.28
12	1	− 1	1	1	6.24
13	1	1	− 1	− 1	2.34
14	1	1	− 1	1	2.65
15	1	1	1	− 1	5.28
16	1	1	1	1	5.60
17	− 2	0	0	0	1.92
18	2	0	0	0	11.84
19	0	− 2	0	0	8.48
20	0	2	0	0	10.72
21	0	0	− 2	0	0.14
22	0	0	2	0	8.64
23	0	0	0	− 2	7.68
24	0	0	0	2	8.32
25	0	0	0	0	7.68
26	0	0	0	0	12.80
27	0	0	0	0	6.32
28	0	0	0	0	12.88
29	0	0	0	0	12.16
30	0	0	0	0	6.68
31	0	0	0	0	10.88
32	0	0	0	0	9.60
33	0	0	0	0	8.32
34	0	0	0	0	6.72
35	0	0	0	0	6.36
36	0	0	0	0	12.24

Analysis of variance		Mean squares
Source	df	
Total	35	
Regression	14	28.03*
Lack of fit	10	33.18**
Error	11	7.18
Residual	21	19.56

Significant : * at 5 % level; ** at 1 % level. Pooling sums of squares for error and lack of fit terms. Residual term was used in determining significance of regression coefficients where the lack of fit term was not significant.

Table 4. Observed yields of *Heterorhabditis bacteriophora* H06 in 50 g medium flasks with various treatments.

Treatment level (see Table 2)						Observed yield
No.	X_1	X_2	X_3	X_4	X_5	(x10^6/flask)
1	1	1	1	1	1	18.30
2	1	1	1	− 1	− 1	22.80
3	1	1	− 1	1	− 1	23.40
4	1	1	− 1	− 1	1	18.30
5	1	− 1	1	1	− 1	25.50
6	1	− 1	1	− 1	1	18.30
7	1	− 1	− 1	1	1	18.90
8	1	− 1	− 1	− 1	− 1	27.60
9	− 1	1	1	1	− 1	16.50
10	− 1	1	1	− 1	1	12.00
11	− 1	1	− 1	1	1	15.30
12	− 1	1	− 1	− 1	− 1	18.00
13	− 1	− 1	1	1	1	10.80
14	− 1	− 1	1	− 1	− 1	15.60
15	− 1	− 1	− 1	1	− 1	18.00
16	− 1	− 1	− 1	− 1	1	14.10
17	2	0	0	0	0	20.40
18	− 2	0	0	0	0	1.10
19	0	2	0	0	0	20.10
20	0	− 2	0	0	0	16.20
21	0	0	2	0	0	0.05
22	0	0	− 2	0	0	0.05
23	0	0	0	2	0	24.30
24	0	0	0	− 2	0	20.40
25	0	0	0	0	2	14.10
26	0	0	0	0	− 2	12.60
27	0	0	0	0	0	16.50
28	0	0	0	0	0	24.00
29	0	0	0	0	0	18.60
30	0	0	0	0	0	21.90
31	0	0	0	0	0	22.50
32	0	0	0	0	0	16.50
33	0	0	0	0	0	22.80
34	0	0	0	0	0	18.30
35	0	0	0	0	0	15.30
36	0	0	0	0	0	19.20

Analysis of variance		Mean squares
Source	df	
Total	35	
Regression	20	52.79**
Lack of fit	6	49.54*
Error	9	9.32
Residual	15	25.41

Significant : * at 5 % level; ** at 1 % level. Pooling sums of squares for error and lack of fit terms. Residual term was used in determining significance of regression coefficients where the lack of fit terms was not significant.

Table 5. Regression coefficients and their T-test values for *Steinernema* sp. CB2B yield equation.

Source	Coefficient	coded levels	T-test values
Linear	b_0	9.39	12.13
	b_1	− 2.63	4.80*
	b_2	0.67	1.23
	b_3	2.53	4.63*
	b_4	0.34	0.63
Interaction	b_{12}	− 0.47	0.70
	b_{13}	− 0.84	1.26
	b_{14}	− 0.16	0.24
	b_{23}	− 0.59	0.88
	b_{24}	0.10	0.15
	b_{34}	− 0.14	0.20
Quadratic	b_{11}	− 0.35	0.73
	b_{22}	0.33	0.70
	b_{33}	− 0.97	2.05
	b_{44}	− 0.07	0.14

* Significant at 1 % level.

Table 6. Regression coefficients and their T-test values for *Heterorhabditis bacteriophora* H06 yield equation.

Source	Coefficient	coded levels	T-test values
Linear	b_0	18.78	19.73
	b_1	3.81	6.11**
	b_2	0.15	0.24
	b_3	− 0.58	0.92
	b_4	0.33	0.52
	b_5	− 1.60	2.57*
Interaction	b_{12}	− 0.67	0.88
	b_{13}	0.45	0.59
	b_{14}	− 0.11	0.15
	b_{15}	− 0.60	0.79
	b_{23}	0.19	0.25
	b_{24}	0.30	0.39
	b_{25}	0.49	0.64
	b_{34}	0.30	0.39
	b_{35}	− 0.04	0.05
	b_{45}	0.08	0.10
Quadratic	b_{11}	− 1.02	1.90
	b_{22}	0.83	1.53
	b_{33}	− 3.70	6.85**
	b_{44}	1.88	3.48**
	b_{55}	− 0.37	0.69

Significant : * at 5 % level; ** at 1 % level.

orange in all treatments but green in that with the medium at the second level at 27 °C. As the symbiont of CB2B produced an uncommon pH-sensitive pigment, i.e. it was green when pH > 8.0 and orange to red when pH < 7.0 (Cao & Han, 1990), the change of color indicated a pH value in culture. In the test treatments yields of infectives ranged from 0.14×10^6 to 18.56×10^6 with highest infectives of 0.464×10^6 per g medium obtained at the No. 8 treatment.

Gravid females of H06 were present at 17 °C or 33 °C, but development was poor and fewer infectives formed, and increasing mortality occurred. The cultures with the medium containing the highest lard level were yellow (pH < 7.0) and those with no lard addition dark red (pH > 8.0). Yield range was from 0.05×10^6 to 27.6×10^6. The highest yield of 0.69×10^6 per g medium at No. 8 treatment indicated that with adequate combinations of culture factors nematode growth can be enhanced considerably.

Equations for the response surfaces of yield

The observed yields of CB2B and H06 are separately shown in Tables 3 and 4. The data was from one experiment as exploratory. The replicated experiments demonstrated the same yield patterns. The second-order multiple regression equations on the basis of the coded levels for the response surfaces of yields were established by Box (1954) and Ding (1986). The regression coefficients and their statistical significance of T-test are given in Tables 5 and 6. Although significant lack of fit term revealed the existence of a certain amount of bias in CB2B yield equation, significant regression trends (linear and quadratic effects) and relatively high multiple-regression coefficients ($R = 0.6989$ for CB2B yield equation; $R = 0.8572$ for H06 yield equation) indicated that both equations used might account for most of the variability due to treatments.

Yields and test factors

The magnitude of the interations between factors response to the nematode yields was evaluated based on the size of the regression coefficients (Tables 5, 6). The regression equation resulted in statistically significant negative first-order effect for medium composition, positive effect for temperature on CB2B yield, and negative effect for culture time, temperature, positive for medium composition on H06 yield, but non-significant effect for bacterial and nematode inoculum on the yield of both nematode strains in the test scale.

A comparison of the b value showed that in sponge culture system, medium composition, temperature and culture time should be carefully concerned and adjusted adequately to each nematode strain for maximal yields as they represented the largest main effects, and that two genera of nematodes differed in their adaptation to culture conditions.

As the second-order terms in the equation were

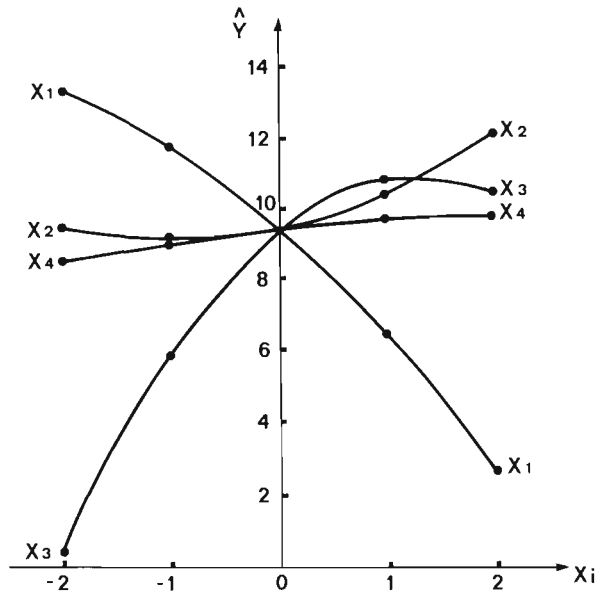

Fig. 1. Predicted response curves for main effects of test factors on *Steinernema* sp. CB2B yield ($\times$ 10^6/flask) as calculated from the reduced unifactor regression equations. Medium composition (X_1), bacterial inoculum (X_2), temperature (X_3) and nematode inoculum (X_4).

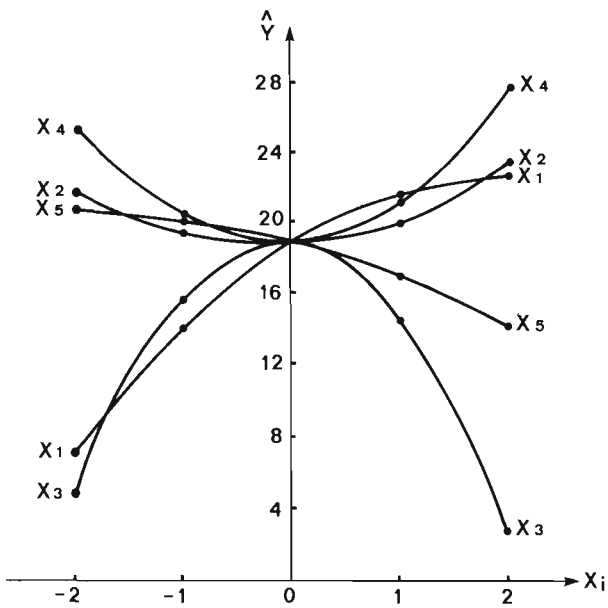

Fig. 2. Predicted response curves for main effects of test factors on *Heterorhabditis bacteriophora* H06 yield ($\times$ 10^6/flask) as calculated from the reduced unifactor regression equations. Medium composition (X_1), bacterial inoculum (X_2), temperature (X_3) and nematode inoculum (X_4) and culture time (X_5).

centralized and satisfied the orthogonality, the regression coefficients are independent of each other, which made it possible to construct a series of unifactor equations by in turn setting all values equal to zero except one varied for different levels in the yield equation. These reduced equations may represent a series of response curves of unifactor experiments. As shown in Figures 1 and 2, a decrease in chicken offal content and increase in mixed content enhanced CB2B yield; an increase in lard content resulted in increasing H06 yield. The temperature responses differed with the nematode strains in that H06 grew more poorly at the higher temperature than CB2B. The optimum temperature was 27 °C for CB2B and 25 °C for H06. Sizes of bacterial and nematode inoculum showed non-significant effects on the final yields of two nematode strains. Increasing the culture time did not enhance H06 yields of infectives because of increasing mortality in culture.

The interaction of two-factor on yields

The coefficients in Tables 5 and 6 showed the existence of interactions between test factors on nematode yield.

Greatest CB2B yields were expected with higher mixed components at the high temperature. The effects of bacterium inoculum on CB2B yields were decreased with the increase of temperature. The bacterial inoculum size and culture time could be reduced by more lard additions.

Yield equation simulation

625 ($= 5^4$) culture formulas for CB2B and 3125 ($= 5^5$) for H06 were obtained by computer modeling in the test variable range $-2 \leqslant Xi \leqslant 2$.

Under the conditions of these experiments, the greatest yield of 21.02 $\times$ 10^6 per flask would be expected at the first level of medium composition, fifth level of bacterial and nematode inoculum size, fourth level of temperature for CB2B; and 42.9 $\times$ 10^6 infectives per flask at the fifth level of lard, first level of both inoculum sizes, middle level of temperature and first level of culture time for H06.

Discussion

Several critical parameters to Bedding's sponge culture system are medium composition, temperature, inoculum size and culture time. How to optimize these parameters for maximum yield is a desirable issue to nematode mass production commercial process. The present paper reported the effects and interaction of these parameters on the yield of *Steinernema* and *Heterorhabditis* nematodes. The data is a step towards this direction. The use of a rotatable design has certain advantage in this type of study since the design covers a wide range of variables and levels with a small number of treatments and characterizes the results through the multiple, second-order regression equations.

Medium composition was a key factor in nematode culture. High proportion of mixed dry ingredients supported the greatest growth of CB2B, which furnish information on further selection of culture medium. In pratice the commonly used chicken offal medium appeared to be laborious, generate offensive odors and be difficult to store. Therefore, a medium based on dry ingredients such as fish meal and corn flour, which is easier to handle and work with, shows much potential for improved industrial use. Lipid additions enhanced nematode growth, which confirms earlier reports (Bedding, 1981; Xu *et al.*, 1989; Dunphy & Webster, 1989; Han *et al.*, 1990*b*).

Temperature plays a crucial role in both CB2B and H06 nematode development. The temperature of 27 °C supported optimal production for CB2B and 25 °C for H06. Dunphy and Webster (1989) reported the optimum temperature of 30 °C for *H. heliothidis* on lipid agar. Temperature adaptation of nematodes for maximum production seems related to specified species or strains and cultural system.

With bacterial inoculum sizes ranging from sixteen times (folds) H06 yields were not pronouncedly different; and in the presence or absence of the bacterial inoculum CB2B culture yielded similar infectives. These may indicate that in the monoxenic culture the initial bacterial inoculum was not a limiting factor for nematode growth as bacterium grew much more rapidly and could provide enough nutrients available for the initial nematode growth.

In culture flasks with nematode inoculum sizes ranging from sixteen times H06 yields were also not significantly different after 25 days. Based on this result, the infectives of 20×10^6 from a single culture flask can be subcultured into 400 flasks at the lowest level of inoculum size, rather than into 25 flasks at the highest level of inoculum size. This suggested that nematode culture cost reduction can be obtained by decreasing the inoculum size and stimulating the nematode growth rates.

In vitro population dynamic studies demonstrated that increased nematode population densities and decreased bacterial nutrient promote infective juvenile formation in the presence of a possible nematode pheromone (Popiel *et al.*, 1989). The nematode developmental response to varying nematode inoculum size described here also showed that nematode growth rates decreased with increasing inoculum size. The final yields, however, are also correlated with nematode inoculum size range. Screening experiments indicated that nematode inoculum sizes of above 10^6 or below 10^3 were neither reliable nor available for greater formation of CB2B and H06 infectives; Significant yield differences were obtained in *S. glaseri* 85 011 (isolated from China) culture with nematode inoculum size range of 2304 times (48-112 000 infectives per flask with 50 g sponge medium) (Han *et al.*, unpubl.). So the optimal inoculum size will depend on achieving maximum yield with minimum inoculum size in a said unit medium.

Various culture formulas are obtained by modeling the yield equation with different levels of the variables and commercial operation in culture system becomes possible. The maximal yields of infectives can be obtained by determining the optimum culture conditions. Nevertheless, nematode production is a cost-expending process, in which except for the capital and labor, medium composition, inoculum size, culture time, temperature control and nematode yield determine the production costs. The economy of scale therefore is important in this process. The determination of such an economically effective culture formula will make a significant contribution to the commercial development.

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