Host, temperature and media additive effects on the growth of *Bursaphelenchus seani* [1]

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SUMMARY

Bursaphelenchus seani developed and reproduced on fourteen of eighteen fungi tested, including two insect pathogenic fungi, *Beauveria bassiana* and *Ascosphaera apis*, and eight species of fungi isolated from adults and nests of its phoretic host, *Anthophora bomboides stanfordiana*. *B. seani* also developed and reproduced on alfalfa callus. Generation time (J2 to J2) for *B. seani* on the fungus, *Monilinia fructicola*, was 16.0, 7.3, 4.2, 3.1 and 3.1 days at 15, 20, 25, 30 and 33°, respectively. No development occurred at 9 and 36°. At 25°, *B. seani* dauer juveniles (JIII) were found after four weeks on cultures of *M. fructicola*. The mean number of *B. seani* produced per culture plate over time increased when potato dextrose agar (PDA) was supplemented with glycerol (115 mg/g hydrated PDA), oleic acid (10 mg/g hydrated PDA) or lactic acid (6.7 mg/g hydrated PDA). JIIIs were found in supplemented cultures within two weeks and occurred in large numbers in glycerol-treated cultures after seven weeks.

RÉSUMÉ

Effets additifs de l'hôte, de la température et du milieu sur la croissance de Bursaphelenchus seani

Bursaphelenchus seani s'est développé et reproduit sur quatorze champignons parmi les dix-huit testés, qui comprenaient deux champignons pathogènes des insectes, *Beauveria bassiana* et *Ascosphaera apis*, et huit espèces de champignons isolées à partir d'adultes et de nids de son hôte phorétique, *Anthophora bomboides stanfordiana*. Il s'est également reproduit sur des cultures de tissu de luzerne. La durée d'une génération (de J2 à J2) pour *B. seani*, sur le champignon *Monilinia fructicola* a été de 16 ; 7,3 ; 4,2 ; 3,1 et 3,1 jours à 15, 25, 30 et 33° respectivement. Il n'y a eu aucun développement à 9 et 36°. A 25°, les « dauerlarven » (JIII) ont été trouvées après quatre semaines sur des cultures de *M. fructicola*. Le nombre moyen de *B. seani* produit par boîte de culture augmentait quand le milieu à la pomme de terre glucosé (PDA) était supplémenté avec du glycerol (115 mg/g de PDA hydraté), de l'acide oléique (10 mg/g) ou de l'acide lactique (6,7 mg/g). Des JIII ont été trouvés dans les cultures supplémentées dès deux semaines et se trouvaient en grand nombre dans les cultures traitées au glycerol après sept semaines.

The nematode, *Bursaphelenchus seani*, is phoretically associated with the solitary soil-dwelling bee, *Anthophora bomboides stanfordiana* (Giblin & Kaya, 1980 ; Giblin, Kaya & Brooks, 1981 ; Giblin & Kaya, 1983). Dauer juveniles (JIII) of *B. seani* are carried in the adult bees' reproductive tract and are transmitted to new host cells during oviposition. The JIIIs molt to the propagative phase and feed on fungi that infest the bee cell. Fungal host range studies were initiated to better understand the nematode-bee-fungi relationship and to select a fungus for mass rearing of *B. seani*. The ability of the nematode to grow on alfalfa callus and the bacterium, *Escherichia coli*, was tested. The effects of temperature on the generation time of *B. seani* grown on the fungus, *Monilinia fructicola*, were observed and the effects of the addition of lactic acid, oleic acid or glycerol to the *M. fructicola* growth medium on *B. seani* were examined.

Materials and methods

HOST RANGE STUDIES

B. seani isolated from the reproductive tracts of adult *A. bomboides stanfordiana* from Sonoma County, California were subcultured on *M. fructicola* or *Botrytis cinerea* on potato dextrose agar (PDA). Monoxenic stock cultures were established by inoculating surface sterilized nematodes onto one-week-old *M. fructicola* culture. The nematodes were surface-sterilized in 1% water agar containing the fungicide, Aretan® (methoxyethyl mercury chloride), and a bactericide, dihydrostreptomycin sulfate (U. S. Biochemical Corp., Cleveland, Ohio), for 48 h (Moody, Lownsbery & Ahmed, 1973).

A variety of fungi were isolated into pure culture and identified from the inner cell surfaces, provisions and body surfaces of *A. bomboides stanfordiana*

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from Bodega Head State Park, Sonoma County and Point Reyes National Seashore, Marin County, California (Tab. 1). In addition, certain saprophytic fungi and plant and insect pathogenic fungi were included in the host range studies. Petri dishes (100 mm × 15 mm) with 15 ml of PDA were inoculated with a small block of fungal mycelia (except *Metarhizium anisopliae* and *Beauveria bassiana* which were inoculated by streaking conidia). The fungi were incubated for one week before being inoculated with *B. seani*. Nematodes were extracted from stock cultures on a Baermann funnel, surface-sterilized and counted. Surface-sterilization for the host range studies and medium additive experiments was done in two steps ; first as described above (Moody, Lownsbery & Ahmed, 1973) and sterilized again as described by Hara, Lindegren and Kaya (1981) for 3 h. Three hundred nematodes were pipetted onto an individual fungal culture, or uncontaminated PDA or nutrient agar (NA) plate (control). The number of nematodes was counted from three randomly selected Petri dish cultures at one week intervals for four weeks. Nematodes were extracted from individual plates for 3 h on a Baermann funnel and the total number of nematodes/plate was estimated by counting a 1 ml aliquot sample of a known volume of the extract.

The bacterium, *E. coli*, was assayed for its suitability as a host for *B. seani*. *E. coli* was streaked onto NA plates and cultured for three days before inoculation with 300 surface-sterilized nematodes. Culture time, extraction and counting procedures were as described above. Alfalfa callus was also tested for its suitability as a host for *B. seani*. Alfalfa seeds, *Medicago sativa* cv. Moapa, were surface sterilized in concentrated H_2SO_4 for 30 mn and rinsed with sterile distilled water (Tamura & Mamiya, 1975). Seeds were germinated on sterile filter paper, soaked with sterilized water, in Petri dishes. Two germinated seedlings were aseptically transferred to individual Petri dishes (100 mm × 15 mm) containing a growth medium (Reidel, Foster & Mai, 1973). The callus was incubated for seventeen days before being inoculated with 100 surface-sterilized nematodes. Alfalfa cultures were harvested and nematode populations counted 5, 7 and 10 weeks after inoculation as described above. All tests were conducted at 25 ± 1°. A host is defined as an organism capable of sustaining the growth and reproduction of *B. seani*.

Temperature and generation time of *B. seani*

B. seani eggs were obtained from one-week-old *M. fructicola*/*B. seani* cultures and washed in distilled water. Six to ten newly-hatched nematodes were collected and added to individual culture wells of a multiwell tissue culture plate containing *M. fructicola* mycelia. Cultures were incubated at each of seven temperatures (± 1°) : 9, 15, 20, 25, 30, 33 and 36°. Nematodes were collected individually from three randomly selected culture wells for each temperature at 6-24 h intervals until J2s of the next generation were first observed. Two trials were done at 9, 15, 20, 25, 30 and 33 and one trial for 36°. If development was not observed within three weeks, the cultures were transferred to 25° and incubated for one week and checked for development. Generation time is defined as the time from J2 eclosion to J2 eclosion of the following generation.

Medium additives and population growth of *B. seani*

Glycerol (115 mg/g hydrated PDA) or oleic acid (10 mg/g hydrated PDA) were added to PDA prior to autoclaving at 15 p.s.i. at 110° for 20 mn. Lactic acid (6.7 mg/g hydrated PDA) was added aseptically to PDA after autoclaving. Petri dishes (100 mm × 15 mm) with *ca* 15 ml of supplemented PDA or plain PDA (control) were inocultated with *M. fructicola* mycelia and incubated. After one week the cultures were inoculated with 300 surface-sterilized *B. seani*/plate and incubated at 25°. Two groups of three randomly chosen plates were harvested and counted, as previously described, at 2, 4, 7 and 12 week intervals. Nematodes from each of the different treatments or control cohorts for a particular week were pooled after harvesting and counted. Nematodes were heat-killed and stained in 45% acetic orcein for *ca* 24 h (Hirschmann, 1962). A random sample of 100 stained nematodes were examined to quantify the appearance of JIIIs, as determined by the development of the gonad and other morphological features (Giblin & Kaya, 1983) in treated *vs.* control cultures. Mean numbers of *B. seani* for this experiment were statistically compared by a Kruskal-Wallis test (non-parametric, single factor analysis of variance) and separation of means was done with the Wilcoxon and Wilcox test (non-parametric multiple range comparison test).

Results

Host range studies

Eight species of fungi, isolated from the inner cell surface, provisions and bodies of *A. bomboides stanfordiana* served as hosts for *B. seani* (Tab. 1). However, the fungi that supported the highest nematode populations were not from bee cells. For

example, the plant pathogenic fungi, *M. fructicola*, *B. cinerea* and *B. allii*, and the insect pathogenic fungi, *B. bassiana* and *Ascosphaera apis*, sustained excellent growth and reproduction. *B. seani* did not reproduce or reproduced poorly on the fungi, *Ceratocystis allantospora*, *M. anisopliae*, *Trichoderma* sp. and *Graphium penicilloides*, and the bacterium, *E. coli*. Alfalfa callus was an adequate host, although peak populations were not observed until seven weeks after inoculation. The largest average nematode populations were observed on fungi two weeks after inoculation, except *Fusarium* sp. which was observed at three weeks.

Table 1

Mean population numbers of *Bursaphelenchus seani* on various fungi, *Escherichia coli* and alfalfa callus

Food source	*Highest population mean*	*S.D.*
1. *Monilinia fructicola*	167 400	9 525
2. *Beauveria bassiana*	158 600	58 742
3. *Ascosphaera apis*	52 740	26 205
4. *Botrytis cinerea*	32 910	7 433
5. *Botrytis allii*	31 200	6 373
6. *Chaetomium* sp. (1)	29 540	10 266
7. *Fusarium* sp. (1)	14 530	10 172
8. *Torula* sp. (2)	13 120	6 044
9. *Agraphium novum*	11 280	5 204
10. *Penicillium* sp. (1)	10 591	5 658
11. *Oidiodendron* sp. (1)	10 283	6 586
12. *Fusarium solani* (1)	8 320	3 288
13. Unknown Zygomycete (1)	7 212	909
14. *Medicago sativa* var. Moapa (Alfalfa callus)	6 360	2 100
15. *Fusarium oxysporum* (1)	4 040	1 667
16. *Ceratocystis allanospora*	565	420
17. *Metarhizium anisopliae*	390	173
18. *Trichoderma* sp.	220	125
19. *Escherichia coli* (Bacterium)	150	31
20. *Graphium penicilloides*	75	85
21. PDA	75	40

(1) Fungus isolated from bee cell of *Anthophora bomboides stanfordiana*.

(2) Fungus isolated from adult *A. bomboides stanfordiana*.

Effects of temperature on generation time of *B. seani*

Nematodes that were stored at 9° for three weeks did not develop, but went through one generation within one week after being transferred to 25°. Nematodes did not survive incubation at 36° for three weeks. The generation times of *B. seani* cultured at 15, 20, 25, 30 and 33° were 384 h (16 days), 175 h (7.3 days), 101 h (4.2 days), 74 h (3.1 days) and 74 h (3.1 days), respectively.

Effect of additives to PDA on population growth of *B. seani*

Table 2 summarizes the effects of media additives on the mean number of *B. seani* produced per culture plate at 25°. *B. seani* grown on unsupplemented media was not significantly different when compared to glycerol and oleic acid supplemented cultures during week 2. After two weeks, supplemented cultures were significantly more productive than unsupplemented (control) cultures.

JIIIs of *B. seani* were found in supplemented cultures at four weeks, but not found in control cultures until seven weeks after inoculation (Tab. 3). There were similar percentages of JIIIs per plate in control and glycerol supplemented plates for weeks 7 and 12, although the number of JIIIs per plate in glycerol supplemented cultures was much higher than in controls or the other treated media.

During weeks 4 and 7, in glycerol supplemented cultures, a large number of J2 nematodes were observed that were larger than normal J2s. At the J2-JIII intermolt these nematodes were significantly larger than the normal J2-J3 intermolt (Giblin & Kaya, 1983).

Discussion

B. seani feeds on a wide variety of fungi for growth and reproduction. All fungi isolated from *A. bomboides stanfordiana* adults and cells as well as the insect pathogenic fungi, *A. apis* (pathogenic to the honey bee, *Apis mellifera*) and *B. bassiana* (pathogenic to the sweat bee, *Augochlora pura* and many other insects) (Batra, Batra & Bohart, 1971) are good hosts for *B. seani*. Some of the fungi isolated from the cells of *A. bomboides stanfordiana*, such as *F. solani* and *F. oxysporum* are suspected facultative pathogens of bees (Batra, Batra & Bohart, 1971). Thus, *B. seani* is similar to other bursaphelenchs which also have relatively wide fungal host ranges (Townsend, 1964 ; McGawley, Jones & Birchfield, 1980).

Table 2

Effects of supplements to PDA on population numbers of *Bursaphelenchus seani* grown on the fungus, *Monilinia fructicola* at 25°

Week	*Control (PDA)* ($\bar{X}$ ± S.D.)	*Glycerol* (1) ($\bar{X}$ ± S.D.)	*Oleic acid* (2) ($\bar{X}$ ± S.D.)	*Lactic acid* (3) ($\bar{X}$ ± S.D.)
2	175 991 ± 27 994 aA (4)	226 327 ± 74 052 aA	250 306 ± 92 365 aA	119 889 ± 36 281 aB
4	76 500 ± 12 718 bA	290 250 ± 64 814 bB	204 675 ± 49 134 aB	120 150 ± 20 299 aC
7	12 750 ± 5 473 cA	108 615 ± 59 749 cB	51 900 ± 40 078 bB	77 775 ± 21 509 bB
12	1 227 ± 748 dA	162 410 ± 102 040 acB	———————— (5)	21 671 ± 15 707 cC

(1) 115 mg/g hydrated PDA. (2) 10 mg/g hydrated PDA. (3) 6.7 mg/g hydrated PDA. (4) Mean of two replicates with three plates per replicate. Means followed by different lower case letters in a column and by different upper case letter in a row are significantly different ($p < 0.01$) according to the Wilcoxon and Wilcox test. (5) Data not included due to contamination. $\bar{X}$ = mean number of *B. seani* per Petri dish culture (100 mm × 15 mm).

Table 3

Appearance of dauer juveniles (JIII) of *Bursaphelenchus seani* on cultures of *Monilinia fructicola* at 25°

Week	*Control (PDA)*		*Glycerol* (1)		*Oleic Acid* (2)		*Lactic Acid* (3)	
	%	($\bar{X}$)	%	($\bar{X}$)	%	($\bar{X}$)	%	($\bar{X}$)
2	0	(0)	0	(0)	0	(0)	0	(0)
4	0	(0)	3	(8 708)	1	(2 000)	1	(1 200)
7	10	(1 270)	15	(16 292)	5	(2 476)	2	(1 556)
12	29	(356)	27	(43 850)	————	(4)	3	(590)

(1) 115 mg/g hydrated PDA. (2) 10 mg/g hydrated PDA. (3) 6.7 mg/g hydrated PDA. (4) No data.

B. seani has a fast generation time under laboratory conditions and when present in large numbers (> 4 000) causes visible damage to cultures of host fungi. Similarly, *B. xylophilus* inhibits mycelial growth and sporulation of the fungus, *Gliocladium* sp. (McGawley, Jones & Birchfield, 1980). Perhaps nematode feeding damage to insect pathogenic fungi in *A. bomboides stanfordiana* cells is selectively advantageous to bees associated with *B. seani*.

Although *B. seani* is a mycophagous nematode, it can utilize plant tissue such as alfalfa callus under special conditions. However, it grows slowly on alfalfa callus. These data are similar to those obtained for *B. xylophilus* (Tamura & Mamiya, 1975). In nature, *B. seani* is probably restricted to fungi as a food source.

The media-additive experiments demonstrated that glycerol, oleic acid and lactic acid are capable of altering either the reproductive rates and/or the survivability of *B. seani* in culture. Interestingly, glycerol-supplemented agar produced greater nematode yields per plate over time than unsupplemented controls or the other additives tried. Also, glycerol-supplemented cultures produced larger numbers of JIIIs of *B. seani* than unsupplemented controls or the other treatments. Thus, *M. fructicola* grown

on PDA supplemented with glycerol can be useful for mass-culturing of *B. seani* and/or for the production of large numbers of JIIIs.

Fungi infesting the cells of *A. bomboides stanfordiana* have been found in the provisions and on the bee cell walls (Giblin, Kaya & Brooks, 1981). The source of nutrition for these fungi is not known and probably varies depending upon the species of fungus. Females of some species in the genus *Anthophora* (*i.e.*, *A. abrupta* and *A. bomboides*) are known to supplement the provisions of and line their cells with glycerides from their Dufour's gland (Norden *et al.*, 1980). Accordingly, components in the cell lining and provisions may influence the physiology and ultimately the nutritive value of the fungus for nematode growth. Dauer juvenile (JIII) production in *B. seani* may be correlated with the presence of bee-produced glycerides or fatty acids.

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