Cultivation of the Pine Wilt Nematode, Bursaphelenchus xylophilus, in Axenic Culture Media[1]

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Abstract: The pine wilt nematode, *Bursaphelenchus xylophilus,* has been cultured axenically in vitro on soy peptone/yeast extract or modified *Caenorhabditis* medium supplemented with cholesterol and hemoglobin. Although growth, development and reproduction were best in soy peptone/yeast extract medium, satisfactory population size increases were observed in the chemically defined *Caenorhabditis medium. Key words:* axenic culture, *Bursaphelenchus xylophilus,* in vitro culture, pine wilt disease.

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Several free-living and parasitic nematodes have been maintained in axenic culture systems, some of which are chemically defined, during at least part of their life cycle (1–6,9–13,18,20,23,26). The use of such culture systems for growth, reproduction, and development of nematodes has facilitated biochemical and physiological studies.

Bursaphelenchus xylophilus (Steiner and Buhrer, 1934) Nickle, 1970 (sym. *B. lignicolus,* Mamiya and Kiyohara, 1972) is parasitic in several pine and nonpine species (7,8,19,22) and causes rapid wilting (17). Infection is epidemic in Japan (12,17) and is wide spread in the United States (7, 21).

The symptomatology of the disease and the life cycle of *B. xylophilus* are fairly well defined (11–15,17), but the physiology and biochemistry of the nematode and the host-parasite relationship have not been elucidated.

Bursaphelenchus xylophilus is localized in the resin canals where it feeds on epithelial cells lining the canal (17). Disease symptoms become apparent, however, prior to the time that the nematode population is large enough to cause conspicious damage to the canals (17). This has led Oku et al. (21) to propose that toxic materials produced by the nematode, or by bacteria associated with the nematode, may be responsible for the disease symptom. Recent results (Bolla, unpublished observation) support this hypothesis. If a toxin is produced by *B. xylophilus,* it would be advantageous to define the metabolic precursors of toxin synthesis. Unfortunately, as is true of most plant parasitic nematodes, little is known about the intermediary metabolism of this nematode. Progress could be made toward elucidating the intermediary metabolism of plant parasitic nematodes if techniques for axenic culture on a chemically defined medium were available. Presently *B. xylophilus* is maintained in the laboratory on fungal mats (15). The reported studies were undertaken, therefore, to develop a defined axenic culture system for *B. xylophilus.*

MATERIALS AND METHODS

Axenization: A population of *B. xylophilus,* orginally isolated from pine wood chips, was established in the laboratory on *Botrytis cinerea* growing on potatoe dextrose agar (16). After the cultures were 1 month old, the nematode larvae and adults were washed from fungal cultures with sterile 0.9% saline, filtered through cheese cloth, and collected by centrifugation at 100 g for 3 min. These mixed populations of nematodes were then washed through 5–7 changes of 10 ml each of an axenization

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medium composed of 0.9% sterile NaCl containing 10 mg/ml of streptomycin sulfate, 5 mg/ml of Nystatin (prepared immediately before use), and 16,000 units/ml of penicillin-G. An incubation period of 5 min was allowed between each washing. The number of nematodes in the final wash was determined and known numbers of nematodes were transfered to the culture media.

Culture media: Two culture media have been tested. One medium consisted of 4% soy peptone (a tryptic digest of soy peptides, Sheffield Chemical Co., Nashville, Tennessee) and 1% Difco yeast extract supplemented with 50 μg/ml of cholesterol (Chol) and 50 μg/ml bovine hemoglobin (hb) (SP/YE supplemented medium). Cholesterol was solubilized as a stock solution in dimethyl sulfoxide prior to being added to the cultures. The medium was autoclaved at 15 psi for 7 min. The second medium consisted of modified *Caenorhabditis* medium (6) buffered to pH 7.4 with 0.1 M KH_2PO_4/Na_2HPO_4 and supplemented with 50 μg/ml chol and 50 μg/ml hb. Stock solutions for this medium were prepared as previously described (23) and were sterilized either by Millipore filtration or autoclaving at 15 psi for 7 min. The final medium was compounded immediately before use and buffered to pH 7.4.

Culture conditions: Two physical conditions for culture were tested. In one system, 4% agar substrates in petri dishes were overlaid with 10 ml of liquid culture medium containing 20,000 or 40,000 adults and larvae of *B. xylophilus*. The cultures were covered and incubated at 25 C. A second set of cultures were established in 50 ml tissue culture flasks containing 10 ml of liquid medium inoculated with 20,000 or 40,000 nematodes per ml from a mixed population. The flasks were tightly closed and incubated at 25 C. After 15 days in culture, a mixed population of 20,000 nematodes, containing both larvae and adults, was transferred directly to fresh 10 ml cultures. Transfers were made only from supplemented SP/YE to supplemented SP/YE or from modified, supplemented *Caenorhabditis* medium to the identical medium. Culture conditions were also kept consistent. Further subcultures were made at 15-day intervals by serial transfer. All cultures were maintained for at least 30 days.

Sterility of the initial culture and each subculture was determined by transfering aliquots of the culture medium to nutrient agar, potato dextrose agar, thiglycollate broth, or nutrient broth. Sterility check cultures were incubated at both room temperature and at 37 C for up to 1 month. No bacterial or fungal growth was observed either from initial cultures or from subcultures.

Cultures were evaluated by counting the nematodes in a small aliquot of the liquid culture medium on days 3, 5, 7, 15, 25, and 30 and by determining if the nematodes remained pathogenic.

Pathogenicity of *B. xylophilus* from axenic culture was tested by determining the ability of the nematode to induce wilting of 45-day-old *Pinus sylvestris* seedlings. Seedlings were infected by suspension in 2 ml of water containing a mixed population of 2,000 *B. xylophilus*. The rate of seedling wilting was determined by daily observation.

RESULTS

Figures 1 and 2 show the results obtained when *B. xylophilus* is maintained in tissue culture flasks containing 10 ml of supplemented SP/YE media or of modified supplemented *Caenorhabditis* medium. Identical results are obtained when these liquid media are used in the agar overlay system. As can be seen, growth, development, and reproduction of *B. xylophilus* is supported axenically in either medium (Figs. 1, 2). Best growth and development (based on rate of increase in population, the appearance of larval stages in the culture system, and the final population size) occurred in the supplemented SP/YE medium (Fig. 1). Population doubling time in this medium was approximately one-half that in modified and supplemented *Caenorhabditis* medium (Fig. 2). Although larval stages were present in both culture media, the population size attained in the supplemented SP/YE medium was 1.5–2-fold greater than that in the modified *Caenorhabditis* medium (Figs. 1, 2). Reestablishment of the population occurred when 20,000 adults and larvae of

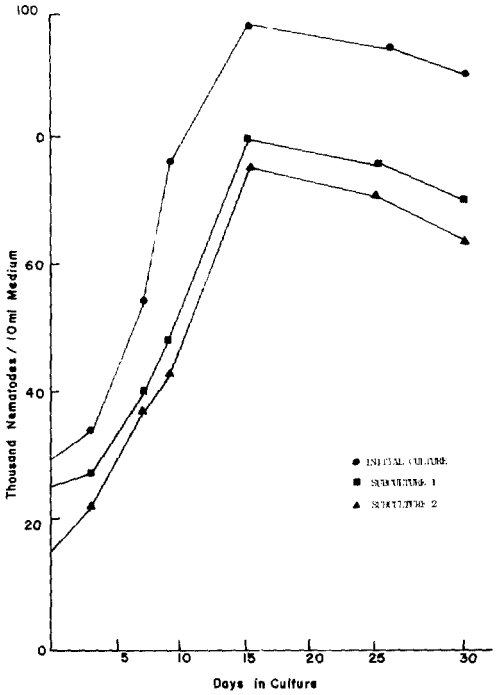

Fig. 1. Growth and development of *Bursaphelenchus xylophilus* in an axenic culture medium consisting of 4% soy peptone and 1% Difco yeast extract supplemented with 50 μg/ml of cholesterol and 50 μg/ml of bovine hemoglobin. Cultures were maintained at 25 C in tissue culture flasks. Results are given as total number of nematodes in 10 ml of culture medium.

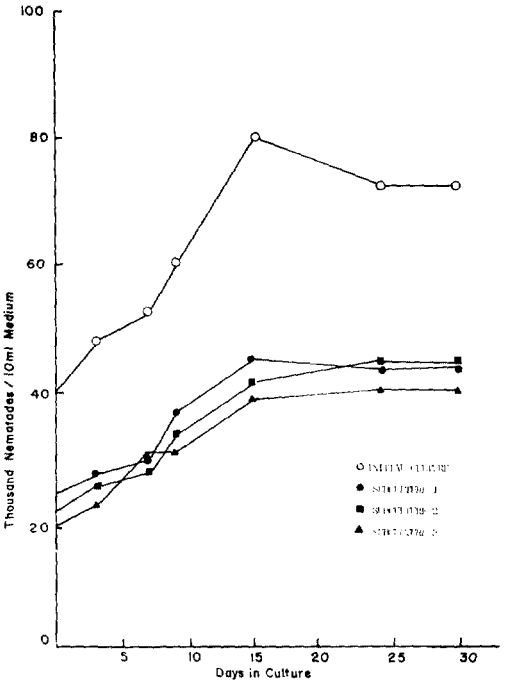

Fig. 2. Growth and development of *Bursaphelenchus xylophilus* in an axenic culture medium consisting of modified *Caenorhabditis* medium (5,23) supplemented with 50 μg/ml each of cholesterol and bovine hemoglobin. Cultures were maintained at 25 C in tissue culture flasks. Results are given as total number of nematodes in 10 ml of culture medium.

B. xylophilus were subcultured to fresh medium of the same type as used for the initial culture (Figs. 1, 2). This was true whether the agar overlay or the tissue culture flask system was used. The rate of subculture population increase is dependent on the liquid culture medium used and is more rapid in SP/YE medium.

A decrease in population size in the liquid phase of the agar overlay system was observed on culture day 7. This decrease occurred when either SP/YE or modified *Caenorhabditis* medium was used, and was a result of a migration of the nematodes under the agar.

Population size increased through day 15 in both the initial culture and subcultures of *B. xylophilus* maintained on supplemented SP/YE; however, from day 15 to day 30 a slight but significant decrease was observed (Fig. 1). This decrease was not observed when modified *Caenorhabditis* medium was used; i.e., the population size increased to day 15 and then did not change significantly to day 30 (Fig. 2).

Bursaphenenchus xylophilus (2,000) recovered at any time from the initial culture or from subcultures are infective to 45-day-old *P. sylvestris* seedlings and cause complete wilting of the seedlings within 3–5 days of infection. This is the rate of wilting induced in these seedlings by 2,000 *B. xylophilus* recovered directly from culture on mats of *B. cinerea*. To date *B. xylophilus* has been maintained through 25 subcultures over a period of 13 months on modified supplemented *Caenorhabditis* medium with no change in pathogenicity to 45-day-old *P. sylvestris* seedlings.

DISCUSSION

As an initial approach to the axenic in vitro cultivation of *B. xylophilus,* two culture media which have been successfully used for axenic cultivation of several species of nematodes (1–6,18) have been investi-

gated. The media were supplemented with hemoglobin and cholesterol, since some nematodes are incapable of de novo synthesis of these factors which appear to be essential for growth and development (1, 9,23,24).

It is evident from the present studies that both media tested support growth and development of *B. xylophilus*. Better growth and development was supported on the chemically undefined SP/YE medium. This might suggest that this nematode, like other stylet bearing nematodes (25), requires a source of soluble protein or peptides as supplied in the SP/YE medium. Modified *Caenorhabditis* medium contains only amino acids and precipitated bovine hemoglobin. This medium does, however, support a significant growth and development of *B. xylophilus* and has the advantage of being chemically defined (23).

The reestablishment of the population following subculture to fresh medium is additional evidence that these media support axenic growth and development. Retained pathogenicity of *B. xylophilus* from subculture also supports the success of the culture system.

A decrease in population size was consistently observed after 15 days of initial culture in SP/YE medium and after 15 days in each subsequent subculture in this medium. In addition, population size remained unchanged after 15 days culture in modified *Caenorhabditis* medium. There are several possible reasons for these changes in population growth rate. The nutrients in the medium may become depleted and unable to support the large population; or the accumulation of metabolic end products may alter the suitability of the medium for continued maintainance of the nematode; or metabolites may create a toxic environment or lower the pH of the medium to a harmful level. A change in pH brought about by excretion of acidic metabolites would be most significant in the unbuffered SP/YE medium. We have observed a decrease of pH from pH 6.8 in freshly innoculated SP/YE medium to pH 5.2 over 15 days of culture. This is not the only reason for the changes in population dynamics, since daily adjustment of pH with 100 m*M* NaOH does not extend the actively increasing phase of the cultures. Additionally, the pH of the buffered modified *Caenorhabditis* medium does not change during 15 days of culture. It is unlikely that the decrease of population growth rate represents some physiological or biochemical change resulting from maintaining the nematodes in the in vitro culture system since transfer of the nematodes, at a lower population density, to fresh medium results in a relatively rapid reestablishment of population size. A similar decrease in population size has been reported in natural infections of *B. xylophilus* just before and after death of the tree (12,13,17), and in 6-month-old fungal culture prior to dauer larvae development (Dropkin, personal communication).

Although the in virto population doubling time is greater than when *B. xylophilus* is in its natural habitat or when it is maintained on *B. cinerea* (population doubling occurs in 5–7 days [12]), these culture media do support significant growth and development of this nematode. These should, therefore, be helpful in obtaining axenic *B. xylophilus* populations for fundamental studies on metabolism and phytotoxin production and to elucidate the relationship of the nematode and other organisms associated with the infection.

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