Mass Culturing of Ditylenchus dipsaci to Yield Large Quantities of Inoculum[1]

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Abstract: Methods are described for rearing large quantities of *Ditylenchus dipsaci* on alfalfa tissues. Nematodes and alfalfa seed were disinfected and nematodes were reared in quantities sufficient to provide a continuous supply of inoculum for our alfalfa-breeding program. Nematodes reproduced best in darkness at 20-25 C. Cultures reached maximum numbers in 3-6 wk. *Key Words:* tissue culture, alfalfa, stem nematode, *Medicago sativa*.

Studies of host resistance to plant parasitic nematodes require large numbers of the specific nematode in question. Most breeding programs have depended on field infestations or greenhouse cultures as primary sources of nematode inoculum. During the past 3 yr, in our search for stem-nematode-resistant alfalfa, *Medicago sativa* L., adapted to the irrigated regions of the Pacific Northwest, we have reared in culture, and have used for inoculations, more than 300 million stem nematodes, *Ditylenchus dipsaci* (Kühn) Filipjev. Rearing this nematode in tissue culture is not new (4, 5, 6, 7), but the practical production of large numbers of the nematode in monoxenic culture for use in a resistance breeding program has not previously received attention.

We initially followed, with some success, the procedures described in the literature for isolating and culturing pure populations of nematodes (3, 4, 5, 6). However, we were not

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able to produce enough contamination-free cultures containing the large numbers of *D. dipsaci* we required. Refinements of existing techniques, including the isolation and incubation of cultures, were necessary before large numbers of stem nematodes could be obtained on a reliable and continuing basis.

DISINFESTATION AND REARING

The procedures described represent the culmination of results from 79 trial-and-error attempts to develop workable methods for freeing large numbers of seeds and nematodes from bacterial and fungal contaminants. Presentation of results from each test would require too much space to be included here.

Culture medium: Nematodes were reared on alfalfa tissue culture. The tissue culture medium was White's standard nutrient solution (8) modified by deleting KNO_3, $CuSO_4$, and MoO_3 and adding 150 ml coconut milk, 1 ppm napthalene acetic acid, and 60 μg 2,4-D per liter. Agar concn was reduced to 1%. French square widemouth bottles (120 ml) containing 19 ml of medium were loosely capped and autoclaved (120 C at 1.0 atm pressure) for 15 min. The bottles then were laid flat for the agar to cool and solidify.

Disinfestation solution: An antibiotic stock solution was prepared to disinfest both alfalfa seed and stem nematodes. Ampicillin trihydrate (250 mg) and furazolidone (100 mg) were mixed in 100 ml sterile distilled water. Oxytetracycline hydrochloride, streptomycin sulfate, and captan at 250 mg each were mixed in 200 ml sterile distilled water. The mixtures were combined, and water was added to bring the volume to 500 ml. This stock was prepared fresh for each use, because it lost effectiveness when stored, even at 4 C, for more than two days.

Tissue preparation: All alfalfa tissue cultures were prepared from high quality alfalfa seed. (We have used cultivars DuPuits, Vernal, and Team.) The seeds were well scarified and only those retained by a 16-mesh (pore size, 1.19 mm) Tyler sieve were used.

About 10 g of seed were placed in a 120-ml sterile French square bottle. The bottle was filled with heated tap water (90 C) and shaken intermittently for 1 min. The hot water was decanted, and 50-60 ml of half-strength antibiotic stock solution was added. After 30 min, the antibiotic solution was decanted, and the seeds were covered with 1:500 $HgCl_2$ for 2 min. The seeds then were rinsed 3 times with

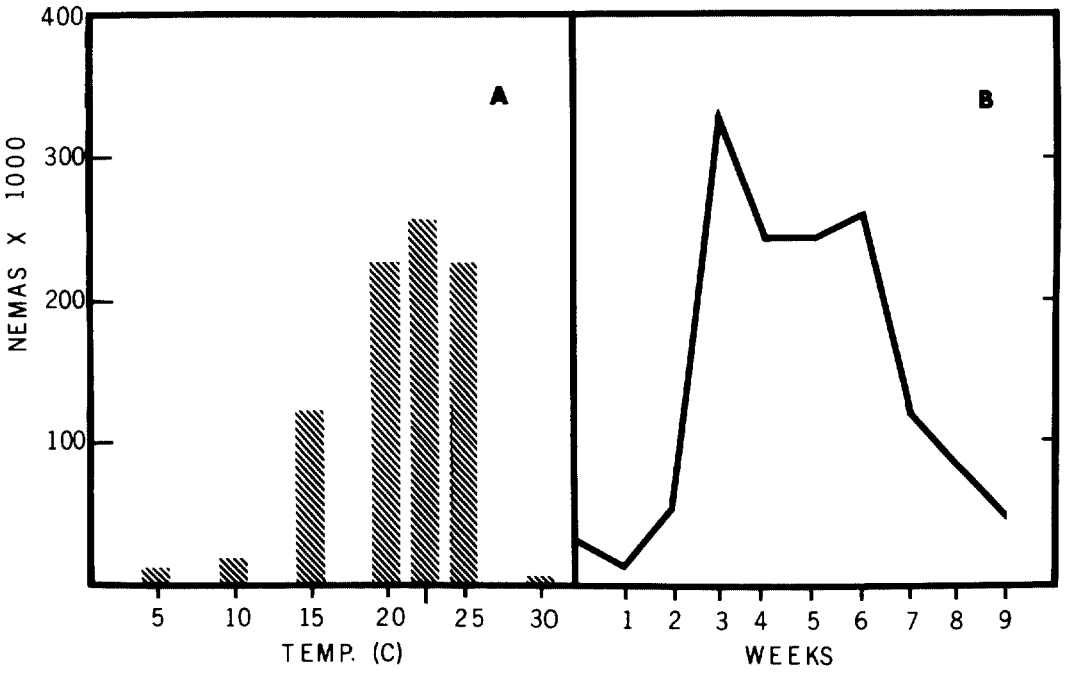

FIG. 1-(A, B). *Ditylenchus dipsaci* propagation on alfalfa tissue cultures. **A)** Effects of incubation temp on reproduction [LSD ($P = 0.05$) = 24,000]. **B)** Effect of incubation period on numbers [LSD ($P = 0.05$) = 56,000].

sterile distilled water, followed by a second rinse in half-strength antibiotic solution. The antibiotic solution was decanted, the bottles loosely capped and laid flat, and the seeds incubated at room temp for 24-36 h. During this period, the bottles were periodically rotated from side to side to hold the moisture level near optimum for germination. When the radicles were 1-2 mm in length, the germinated seeds were given a third half-strength antibiotic solution rinse, and about 200 seeds were transferred aseptically to the modified White's nutrient medium in each culture bottle. After 7 days of incubation at room temp, the tissue cultures were inoculated with stem nematodes.

Nematode preparation: Nematodes were originally isolated following procedures outlined by Faulkner and Darling (3). In mass culturing, nematodes were extracted from the callus tissue cultures following a modification of the method of Christie and Perry (2). The contents of 12 culture bottles were washed onto Kimwipe (Type 900L) tissue on a 20-cm diam sieve (pore size, 0.5 mm) set in a 30-cm diam plastic wash basin. Tap water was added to cover the plant tissue and decanted twice daily for 48 h. The extracted nematodes were collected in a 1 or 2-liter flask and were stored in the refrigerator. Nematodes were rinsed by siphoning off the supernatant (after the nematodes had settled) and replacing it with tap water, before they were used for screening host resistance or as inoculum for further increase.

Nematode numbers were estimated by thoroughly agitating water suspensions, removing three 1-ml aliquots, and counting each as described by Altman (1). Aliquots from highly concd suspensions were diluted before counts were made.

For inoculating new cultures, a 1-liter Erlenmeyer flask containing a tap water suspension of 1-1.5 million nematodes was placed in a slant position for about 2 h to allow the nematodes to settle. Water was siphoned off to conc the nematodes into about 100 ml volume. An equal volume of 1:500 $HgCl_2$ was added, and the flask was gently agitated for 15 min. Sterile distilled water (700 ml) was added, and after a 2-h settling period, 800 ml of the supernatant was siphoned off. Rinsing was repeated twice, and the nematodes were again concd to a 100-ml suspension. An equal volume of antibiotic stock solution was added. The nematode suspension then was diluted with half-strength antibiotic solution to bring the final concn to 7,000-10,000 nematodes per ml. Three ml of nematode suspension (21,000-30,000 nematodes) were dispensed onto the alfalfa tissue in each new culture bottle using a sterile automatic pipette (Cornwall continuous pipetting syringe). All manipulations were carried out in a transfer chamber under standard aseptic conditions.

TEMPERATURE, LIGHT, AND INCUBATION PERIOD

Experiments were conducted to test the effects of incubation period, temperature, and light on nematode reproduction in tissue cultures.

Temperature: To test the effect of temp on nematode reproduction, 14 cultures each were inoculated with 32,000 stem nematodes and incubated for 25 days at 5, 10, 15, 20, 22.5, 25, or 30 C (± 1 C). Optimum temp ranged from 20-25 C, with a slight peak occurring at 22.5 C (Fig. 1-A). The nematodes did not reproduce well at 5 or 10 C and apparently could not reproduce at 30 C.

Light: Three replications of six cultures were inoculated with 26,000 stem nematodes each and incubated at room temp either under Gro-lux lights [2,152 lx (200 ft-c)] or in darkness. After 34 days, nematode numbers averaged 84,600 from cultures reared in darkness, whereas those exposed to light averaged 62,000. A difference of 18,500 nematodes was required to show significance at the 5% level.

Incubation period: To find the optimum incubation period for maximum nematode yields, 119 cultures of alfalfa tissue were inoculated with 29,000 nematodes each and incubated in the dark at 22-24 C. Nematodes were extracted and counted from 10 cultures each week throughout a 9-wk period. Peak numbers were reached at 3 and 6 wk after inoculation (Fig. 1-B), suggesting two completed life cycles within the cultures. Nematode numbers decreased rapidly after 6 wk. Most of the extracted nematodes were third- or fourth-stage larvae following 5-6 wk incubation. At this time a large percentage had left the plant tissues and collected in masses along the walls of the culture bottles below and above the agar medium.

DISCUSSION

The disinfectants used were selected because their combined activity retarded all bacterial and fungal contaminants encountered, and they did not significantly reduce plant tissue growth or nematode reproduction in cultures. Deletion of one or more of these materials, or of any of the procedural steps, usually resulted in a marked increase in contamination. Following the methods outlined, we now prepare and inoculate approximately 5,000 cultures each year and encounter only 1-3% contamination by bacteria and/or fungi.

Numbers of active nematodes extracted from culture decreased rapidly after 6 wk. Several factors could account for the relatively short culture life of nematodes in our studies. Of these, the rapid depletion of food supply when large amounts of inoculum are used, coupled with rapid nematode reproduction, is the most obvious. Accumulation of waste products could also be responsible.

The procedures outlined herein were developed for producing large numbers of stem nematodes in a short period of time for our alfalfa screening and evaluation programs. For that purpose they have proven to be quite effective.

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