

A System for Continuous Production of Root-Knot Nematode Juveniles in Hydroponic Culture

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ABSTRACT

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A hydroponic culture system that enables ready production and retrieval of freshly hatched, infective root-knot nematode juveniles was developed. *Meloidogyne javanica*-infected tomato plants produced at least 100,000 juveniles per day for as long as 3 mo. Juveniles reinfected roots within the culture system, which possibly accounts for the extended period of

production. The hydroponically grown nematodes retained characteristic infectivity and host range. This culture system is useful when a cohort of uniform-age juveniles is required or for analyses in which high numbers of nematodes are needed.

Additional keywords: *Lycopersicon esculentum*, resistance screening.

Root-knot nematodes (*Meloidogyne* spp.) are important pathogens on many crops (7,8). Nematode juveniles of uniform age are essential for many research applications, including breeding for host resistance, nematicide screening, biochemical analyses, and biological control experiments. Because these pathogens are obligate endoparasites and require differentiated plant tissue on which to feed and reproduce, procedures to generate

a continuous supply of uniform, infective inoculum are limited. Inoculum obtained from greenhouse cultures of *Meloidogyne* spp. on host plants has many limitations. Contamination by neighboring cultures, saprozoic nematodes, bacteria, or fungi is problematic. Production of high numbers of nematodes at regular intervals requires considerable greenhouse space. In addition, because nematode yields from greenhouse cultures are often inconsistent, extra cultures must be established to ensure a reliable source of inoculum. Frequent extracting and reculturing of these organisms to maintain a continuous supply of infective juveniles

are time-consuming. Extraction procedures, particularly treatment with chemicals such as sodium hypochlorite, can reduce nematode viability and infectivity (11).

Alternatives to greenhouse nematode cultures have been developed. Gnotobiotic root-culture systems have been used to study host-parasite interactions in soil (6,13) and on nutrient agar medium (5,10). Although these monoxenic cultures are useful, they are not suitable for routine inoculum production because infective juvenile yields are low, and maintenance of these cultures is costly and time-consuming. Another culture method (2) consists of root-knot-nematode-infected plants grown in gravel contained in crocks and fertilized with a circulating nutrient solution. Juveniles were collected by draining fluid from the crocks. Although this method produced inoculum of this pathogen for an extended period, the juveniles collected were of mixed age, and the yield was low.

This paper presents a hydroponic nematode culture system that is simple to establish and maintain. The system yields, on a daily basis and for an extended period of time, high numbers of infective root-knot nematode juveniles.

MATERIALS AND METHODS

Hydroponic apparatus construction. The chamber of the apparatus (Fig. 1) was a modified 3-L polypropylene Buchner funnel (Fisher Scientific, Pittsburgh, PA). The plastic grid covering the bottom of the funnel top was removed with a hot scalpel. The funnel top and bottom were then welded together with a soldering iron. The stem of the funnel was cut 90° to the longitudinal axis, and a 15.8- to 6.4-mm reducer was welded to the end. Silicone tubing (6.4 mm, i.d.; Small Parts, Inc., Miami, FL) and a T-connector were attached to the bottom of the funnel and produced an air inlet and drain. The tubing connection to the funnel was secured with hose clamps ("snapper" type; Small Parts, Inc.), which prevented the tubing from slipping. Plastic connectors were

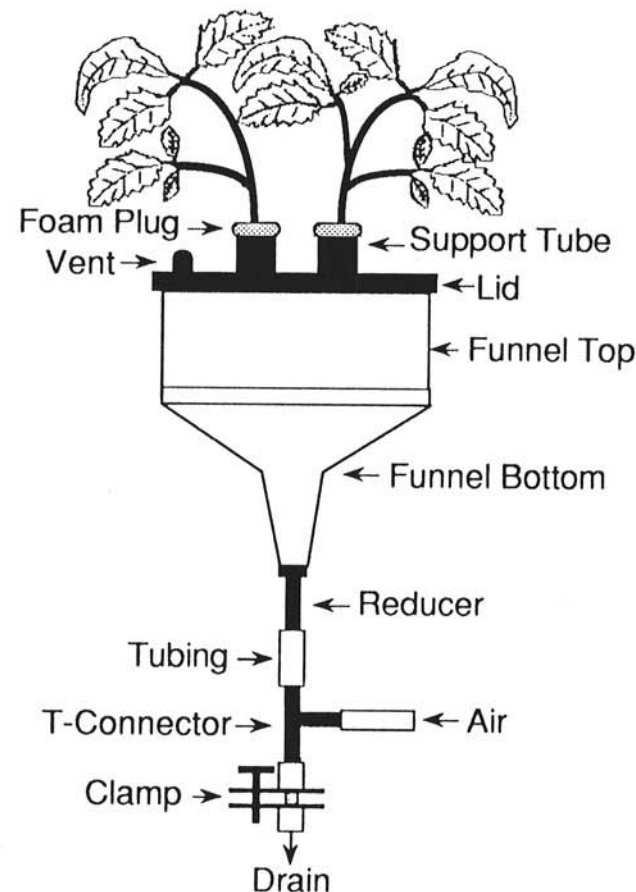


Fig. 1. Hydroponic culture apparatus design.

obtained from PGC Scientific (Gaithersburg, MD). Air flow was generated from a laboratory air outlet. An Acro-50 air filter (Gelman, Inc., Ann Arbor, MI) attached to the air line removed airborne contaminants. The air line (approximately 5 mm, i.d.) was attached to a valve that controlled air flow. Both air line and valve were obtained from an aquarium supply store. The air line was attached to the silicon tubing of the air inlet by a plastic reducer.

The chamber lid was a 15-cm-diameter black acrylic disk. Two 3.8-cm-diameter holes near the center and one 1.58-cm-diameter hole 2.5 cm from the edge were drilled into the disk. A 3.2-cm strip of 3.8-cm (i.d.) acrylic tubing that was welded onto each hole with acrylic solvent-glue created a plant support tube. A 15.8- to 6.4-mm reducer glued into the 1.58-cm hole functioned as a vent. The base of a black-painted plastic test tube formed a loose-fitting cap for the vent. The lid was sealed to the chamber with black silicon glue. After the glue was dry, a scalpel was used to separate the lid, with the attached silicon seal, from the chamber. The hardened silicone formed a lip that held the lid in place and minimized light penetration.

Nutrient solution. To prepare sterile nutrient solution for hydroponic cultures, we autoclaved the following stock solutions separately: $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 653 g/L; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 399 g/L; KNO_3 , 184 g/L. The remaining stock solutions were individually filter-sterilized (0.22- μm filter): $\text{NH}_4\text{H}_2\text{PO}_4$, 108 g/L; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 10 g plus 72 ml of 500 mM EDTA (pH 8.0) per liter; and micronutrients (per liter: $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 1.81 g; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.1 g; $\text{ZnSO}_4 \cdot 5\text{H}_2\text{O}$, 0.22 g; H_3BO_3 , 2.86 g; $\text{H}_2\text{MoO}_4 \cdot \text{H}_2\text{O}$, 0.02 g). Polypropylene carboys (20 L) were autoclaved and filled with 20 L of deionized water that had been filter-sterilized through a reusable 0.22- μm capsule filter (VWR Scientific, Philadelphia, PA). The stock solutions were added to the carboy: $\text{Ca}(\text{NO}_3)_2$, 10 ml; MgSO_4 , 10 ml; KNO_3 , 30 ml; $\text{NH}_4\text{H}_2\text{PO}_4$, 10 ml; Fe/EDTA, 10 ml; micronutrients, 10 ml. Sterile nutrient solution was stored at room temperature.

Plant growth and inoculation. Tomato seeds, *Lycopersicon esculentum* Mill. 'UC82', were surface-disinfected for 15 min in 0.53% NaOCl and then rinsed four times in sterile water. Drain holes were punched in the bottom of a 1-L ethanol-cleaned styrofoam cup, which was then lined with autoclaved 88- μm nylon mesh (Small Parts, Inc.). The cup was filled with autoclaved river sand, and three seeds were placed in each cup. Cups were placed in a growth chamber at 25 C with a 14-h light period and were watered daily with sterile nutrient solution. Seedlings were thinned to one per cup after 1 wk and were inoculated 4-6 wk later. Inoculum for the initial culture consisted of approximately 3,000 *Meloidogyne javanica* (Treub) Chitwood second-stage juveniles (J2) that were collected from excised root cultures and placed on a Baermann funnel. A second culture was initiated with egg masses hand-picked from roots of a greenhouse pot culture and placed into an antibiotic solution (Pen-Strep-Fungizone solution from ICN Biomedicals, Inc., Costa Mesa, CA). After 4-5 h, two to three egg masses were pipetted into two small holes in the sand at the base of the tomato plant.

For all subsequent cultures, 5- to 7-wk-old plants were first inoculated on the surface of the sand with 3,000-7,000 J2 produced in hydroponic culture. Inoculation was repeated 5 days later, when nematodes were pipetted approximately 10 cm below the sand surface. Inoculations were repeated at 5-day intervals by alternating surface and deep inoculations until 15,000-20,000 J2 had been applied.

Hydroponic nematode cultures. Five to 6 days after the final inoculation, the styrofoam cups with infected plants were cut in half longitudinally, and the roots were immersed in deionized water to remove the sand. Sand was gently and thoroughly removed to avoid plugging the tubing in the hydroponic apparatus. The chamber was filled with sterile nutrient solution. We wrapped roots in a plastic sheet to compress and protect them and inserted them through the support tube (Fig. 1). Once the plant was inserted through the lid, the plastic sheet was removed, and the lid with the inserted plants was placed onto the funnel. A foam plug (45 mm wide) with a 6.4-mm-diameter hole down the axis and a

longitudinal cut along one edge was wrapped around the stem and wedged into the plant support tube to prevent light and contaminants from entering the chamber and to support the plant. Air flow was adjusted to a gentle bubbling (600–1,000 ml/min). Funnels were placed under fluorescent and incandescent lights (14-h light period) at 25 C and were filled daily with sterile nutrient solution, which replaced water lost by transpiration. At 3- to 4-day intervals, the nutrient solution was changed by filling the funnel, allowing solution to bubble for 5 min, draining the funnel completely, and then refilling it with sterile nutrient solution. Plants were pruned, and flowers were removed as needed.

Collection of nematodes. To collect J2, funnels were filled with sterile nutrient solution, air was bubbled for 5 min for agitation, the solution was drained into an appropriate container, and the funnel was refilled. The drained solution was passed through a 245- μm -pore sieve that removed large root pieces and then was concentrated on a 25- μm -pore sieve. To remove small root pieces and nematode eggs, we allowed J2 to migrate for 6 h through 10 layers of paper tissue (Kimwipes, Fisher Scientific, Pittsburgh, PA) placed on a wire screen that was attached to the top of a 500-ml beaker filled with nutrient solution. Active juveniles moved through the tissue and into the beaker and were concentrated by collection on a 25- μm -mesh screen or by centrifugation.

To quantitate nematode production, we determined the number of J2 in the collected nutrient solution by counting three 1-ml aliquots. The number of J2 collected was divided by the number of hours between refilling and draining the funnel (never longer than 72 h) and multiplied by 24 to standardize nematode production per day.

Host range studies. The North Carolina Differential Host-Range test was conducted as previously described (4); seeds were obtained from the North Carolina State University Department of Plant Pathology. Host plants were grown in sand in a greenhouse in 1-L styrofoam cups filled with river sand. One-month-old plants were inoculated by pipetting 3,000–4,000 hydroponically grown J2 onto the surface of the sand. Three replicates of each host-differential plant were inoculated. After 2 mo, the sand was washed off the root system, and galling was assessed.

Nematode-resistant and -susceptible tomato seeds were obtained from PetoSeed, Woodland, CA (VFNT cherry, Rossol, Roma) or SunSeed, Hollister, CA (UC82). The necrotic reaction characteristic of the resistance response was assessed as previously described (2).

RESULTS

Nematode production. Two tomato plants were each inoculated with approximately 20,000 J2 as described in Materials and Methods. Plants were established in funnels 24 days after the first inoculation. Production of J2 was monitored for approximately 3 mo (Fig. 2). High numbers of J2 (more than 100,000 J2 each day) were produced beginning approximately 2 wk after funnel cultures were initiated. Nematode reproduction reached a maximum yield of 320,000 or 520,000 J2 collected in a 24-h period between 50 and 60 days after funnel setup. J2 production then decreased slowly over the next 20 days. Two additional hydroponic nematode cultures were established on each of two separate occasions. Production of J2 was monitored for these funnels (not shown). Yields over time were similar to those shown in Figure 2. At least 100,000 J2 were recovered from each culture in each 24-h period tested for 2–3 mo.

A random sample of roots was removed from a 2-mo-old culture and stained with acid fuchsin (1) to determine if the J2 that hatched in the hydroponic system infected roots in the chamber. Third- and fourth-stage nematodes were observed in the stained roots and provided evidence that the J2 reinfected roots within the hydroponic system.

Characterization of juveniles. Juveniles produced from the cultures were examined under a dissecting microscope. In a 24-h cohort, more than 95% of the J2 collected were active. Intestinal vacuoles indicative of starvation (9) were generally not observed. Responses of test plants in the standard North Carolina

Differential Host-Range test (4) to hydroponically grown *M. javanica* were as expected. Nematode-resistant (VFNT cherry, Rossol) and susceptible (Roma, UC82) tomato plants were inoculated with the cultured juveniles. No galls were observed on resistant plants in greenhouse tests, whereas susceptible varieties were heavily galled. In laboratory assays (2), the localized necrotic reaction typical of the resistant phenotype was observed in resistant but not in susceptible plants.

DISCUSSION

High levels of J2 were produced in six funnels under the conditions described. These cultures produced useful levels of nematode inoculum over 9-mo. Several factors for optimizing the quantity and quality of J2 produced were considered in designing the hydroponic culture system. Lack of oxygen inhibits egg hatching (12), so the hydroponic culture system was designed to provide continuous aeration of nematodes and plant roots. The location of the air inlet at the bottom of the chamber keeps the juveniles suspended in solution. Aseptic conditions used when practical during the establishment of the cultures minimized the levels of contaminating organisms in the system. The system design collects nematode cohorts of known age. The average age of the juveniles collected is controlled by the time between draining and refilling the funnel and subsequent collection of J2.

Extended production of J2 from the funnels was unexpected. A root-knot nematode adult female typically produces eggs for approximately 30 days at 25 C (3); therefore, production of J2 should decline significantly by 2 mo after inoculation, unless J2 reinfest the plant. Our observation that third- and fourth-stage juveniles were present in 2-mo-old cultures indicates that reinfection occurs. The multiple inoculations with J2 over time may also extend the productive life of the funnel. In addition, a female's life span may be extended in this system.

The high numbers of J2 used to inoculate plants for the hydroponic culture would severely stress or perhaps kill the host in a greenhouse pot culture. However, hydroponic root systems appeared to tolerate the large numbers of *M. javanica* females. In the hydroponic system, nutrients are always available for proliferation of new roots. Reinfection probably occurs at a lower level than in greenhouse cultures and does not appear to stress the plant. In contrast, massive reinfection in greenhouse cultures may result in severe decline in plant health.

Additional variables that could further increase nematode production remain to be tested. For example, the use of plants that tolerate higher numbers of adult root-knot females than do tomato plants could increase J2 yields. Infection of a larger root system or inoculation techniques that cause a more uniform infection of the root system are additional possibilities. However, use of

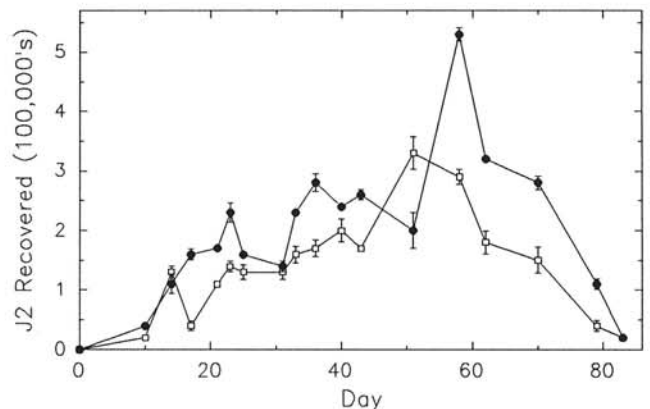


Fig. 2. *Meloidogyne javanica* stage-two juvenile (J2) production in hydroponic culture. Plants were established in funnels on day 0. The black circles (funnel 1) and white squares (funnel 2) represent the number of J2 collected in a 24-h period on the day shown. Three subsamples were counted from each funnel at each sampling time. Error bars represent the SE from the mean.

a larger root system would require a culture chamber of greater volume.

The hydroponic culture system described here is inexpensive, requires little space, and is capable of producing millions of juveniles per day if multiple funnels are used. The system should be applicable to most other *Meloidogyne* spp. Properties of the hydroponically produced J2 are typical, so the technique is useful for breeding and host resistance screening. Additional uses of this system include testing of biological control agents and potential nematicides, production of nematode biomass for molecular studies, and production of uniform-age cohorts for demographic analyses.

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