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Improved Nematode Extraction from Carrot Disk Culture[1]

DAVID T. KAPLAN AND ERIC L. DAVIS[2]

Abstract: *Radopholus* spp. were reared in carrot tissue culture via established procedures, with slight modification. Several plant tissue maceration enzymes and flotation media (salts and sucrose) were evaluated with regard to nematode toxicity and extraction efficiency. Best extraction of viable nematodes and eggs was attained when carrot tissue infested with *Radopholus citrophilus* or *R. similis* was macerated with a mixture of 0.50% driselase and 0.50% cellulysin, w/v each, with 2.5 ml of enzyme solution based for each gram of carrot tissue. Maceration slurries containing carrot tissue and nematodes were maintained in open flasks on a rotary shaker (175 rpm) at 26 C for 24 hours. Nematodes and eggs were extracted from resultant culture slurries by flotation with $MgSO_4$-$7H_2O$ (sp gr 1.1). A protocol is presented to extract large quantities of viable burrowing nematodes and their eggs from carrot disk cultures.
Key words: burrowing nematode, egg, enzyme, migratory endoparasite, nematode culture, *Radopholus citrophilus, R. similis.*

The burrowing nematode sibling species, *Radopholus citrophilus* Huettel, Dickson & Kaplan and *Radopholus similis* (Cobb) Thorne (5), cause significant damage to a broad range of plants (12). Improving management strategies to limit crop losses attributed to burrowing nematodes has been a priority for many years (11,12). For this and many other plant-parasitic nematodes, aseptic, monoxenic culture on excised plant tissue (3,8–11,14) presents a possibility for production of larger numbers of nematodes than conventional greenhouse nematode-culture techniques. However, even though large numbers of nematodes and eggs may be present in the cultures, it is often difficult to extract them from culture tissues.

In 1967, O'Bannon and Taylor (14) cultured burrowing nematodes on carrot tissue excised from taproots and cut into disks. This technique was modified (11), and a detailed procedure for preparing carrot disk cultures (3) and for nematode surface sterilization is available (8). Extraction of nematodes from carrot disks depended upon migration of the nematodes. We determined, however, that significant numbers of burrowing nematode adults, juveniles, and eggs present in carrot disk tissue were not extracted by existing techniques (unpubl. data).

Our objective was to improve culture efficiency by improving extraction of viable nematodes and eggs from carrot disk culture. This paper provides a protocol for the use of commercial enzyme preparations and flotation media to extract burrowing nematodes from carrot disk culture. In addition, the technique was used to determine the length of the life cycle of the burrowing nematode in carrot disk culture.

MATERIALS AND METHODS

Carrot tissue: Carrot disk cultures were established and maintained as previously described (3,10,11,14) but with several modifications. Carrots were surface sterilized by dipping them in 95% ethanol and igniting them. The outer tissues were removed with a sterile scalpel and the carrot was cut into 8-mm-thick disks. Four disks (ca. 6.5 g fresh weight) were placed in culture tubes (15.0 × 2.5 cm) and closed with plastic caps (Kapputs, Bellco Glass, Vineland, NJ). Carrot disks were inoculated with nematodes when initial signs of callus formation were apparent on the disk surfaces, generally 3–4 weeks after the disks were placed in the tubes. Inoculated cultures

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[2] Research Plant Pathologist, and Research Associate, respectively, U.S. Department of Agriculture, ARS, 2120 Camden Road, Orlando, FL 32803.

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were maintained in an incubator at 26 C (± 1).

Nematode inoculum: Nematode inoculum was produced on carrot disk cultures. Consisting of mixed life-cycle stages, it was collected by rinsing carrot disk and culture tube surfaces with sterile distilled water. Nematodes were surface sterilized for all experiments by a modification of the procedure of Krusberg and Sardanelli (8). Nematode suspensions were placed onto a glass chromatography column (2.2 × 49 cm). The column was filled with a mixture of 3-mm-d and 6-mm-d glass beads supported by a platform of fiberglass window screen and an antibiotic mixture (0.5% gentamycin, 0.5% tetracycline/oxytetracycline HCl, 0.0005% chlorhexidine digluconate in tap water) and was fitted with a nalgene stopcock. Surface-sterilized nematodes were aseptically rinsed free of sterilant. A subsample of sterile nematodes was quantified using a dissecting microscope (40 ×), and the carrot disks were inoculated by pipetting 20.0 liters of nematode suspension on the surface.

Nematode extraction: The relative yields of nematodes and eggs from carrot disk cultures were compared for three extraction methods: enzymatic maceration of carrot tissue; incubation in 5 mM sodium acetate, pH 5.8; and incubation in a closed container similar to Young's jar extraction technique (17). Carrot tissue from 30–40-day-old burrowing nematode cultures was removed from culture tubes and chopped with a hand-operated chopper. Six-gram subsamples were placed in each of 30 Erhlenmeyer flasks (125-ml capacity). Ten flasks were covered with parafilm without adding any solution; 10 flasks received 15 ml of 5 mM sodium acetate, pH 5.8; and 10 flasks received 15 ml of a mixture of 0.50% driselase (a crude powder from basidiomycetes containing laminarinase, xylanase, and cellulase [Plenum Scientific Research, Hackensack, NJ]) and 0.50% Cellulysin cellulase derived from *Trichoderma viride* (Calbiochem, La Jolla, CA) in 5 mM sodium acetate, pH 5.8. Before use, the enzyme solution was centrifuged at 2,000 *g* for 1 minute to remove precipitate. All flasks were placed on a rotary shaker at 175 rpm at 26 C ± 2 for 16 hours after which 15 ml of 5 mM sodium acetate, pH 5.8, was added to each of the flasks that had not previously received any solution. Each flask was then covered and shaken vigorously with a Vortex Genie (70% of full speed) for 10 seconds. The slurry from each flask was poured individually through a sieve with 1-mm-d pores. Debris on the sieve was discarded after rinsing with 5 mM sodium acetate, pH 5.8.

The effluent from the sieve was decanted into 40-ml conical glass centrifuge tubes which were centrifuged at 125 *g* for 3 minutes. The supernatant was discarded and the pellet was resuspended in the 3.0–5.0 ml of liquid with a Vortex Genie (50% of full speed) for 10 seconds. Then the total volume of each tube was increased to 30 ml with 5 mM sodium acetate, pH 5.8, and centrifuged at 125 *g* for 3 minutes. The supernatant was removed and the resultant pellet was resuspended. This was repeated until the supernatant was clear (usually two or three times). Then the pellet was resuspended in a total volume of 15 ml of 5 mM sodium acetate, pH 5.8, using a Vortex Genie (50% of full speed). A syringe, fitted with a 10-cm-long cannulus, was used to deliver 10 ml of $ZnSO_4$ (sp gr 1.1) beneath the nematode–carrot cell suspension. A specific gravity of 1.1 was selected on the basis of previous findings involving *Pratylenchus vulnus* Jensen & Allen, 1951 (16). The tubes were immediately centrifuged at 2,000 *g* for 3 minutes. The band at the interface between the buffer and the salt solution was removed with a Pasteur pipet. The nematodes were rinsed three times with 5 mM sodium acetate, pH 5.8, by centrifugation at 125 *g* for 3 minutes. The number of females, juveniles, males, and eggs was estimated from a 1.0-ml aliquot with a Hawksley slide and a dissecting microscope (40 ×), and data were expressed as nematodes per gram. The experiment was conducted twice for each nematode species.

To determine relative efficiency and ef-

fect on nematode viability of various flotation solutions, 80 g of chopped carrot tissue from stock *R. citrophilus* cultures was divided into two 40-g subsamples. One of the subsamples was placed on a shaker (175 rpm) in open flasks containing 100 ml of a mixture of 0.75% driselase and 0.75% Cellulysin cellulase, w/v, in 5 mM sodium acetate solution, pH = 5.8, at 25 C ± 2 for 6 hours. Following maceration, the suspension was mixed and 5-ml aliquots were transferred into 40-ml conical centrifuge tubes; each aliquot was processed to remove enzymes, low-weight carrot debris, and pigments.

The salts—$(NH_4)_2SO_4$, K_2SO_4, Na_2SO_4, NaCl, $MgSO_4$-$7H_2O$, $CaCl_2$, $ZnSO_4$ (sp gr 1.1) were evaluated as flotation solutions as described with $ZnSO_4$. As a control, nematodes and eggs were extracted from nematode–carrot cell suspensions following maceration without the use of a flotation medium by pouring the slurry through a 106-μm-pore sieve (to remove carrot debris) and then a 25-μm-pore sieve. Nematodes and eggs for all treatments were resuspended in 5 mM sodium acetate, pH 5.8, and quantified with the aid of a dissecting microscope (40×).

Nematodes and eggs were extracted from the second 40-g batch of carrot tissue without macerating enzymes to determine if the macerating enzymes influenced nematode viability. Nematodes were retrieved by vigorously shaking the chopped tissue in 5 mM Na acetate solution, pH 5.8, for 1 minute. The slurry was then poured through a 1-mm-pore sieve to remove large pieces of tissue and then centrifuged at 175 *g* for 3 minutes. Nematodes and eggs in the pellet were subsequently extracted by flotation.

Eggs and nematodes extracted with each flotation medium (with or without macerating enzymes) were quantified with a Hawksley slide on a dissecting microscope (40×). To ascertain the effect of extraction technique on infectivity, penetration of soybean (*Glycine max* (L.) Merr. cv Centennial) roots extracted by each method was monitored (1). Data were expressed as number of nematodes per root segment. The experiment was repeated twice.

The influence of flotation media (with or without macerating enzymes) on egg hatch was determined with eggs pooled from each treatment. Eggs were separated from vermiform stages with a 25-μm-pore sieve. Then they were rinsed with distilled water and quantified with a Hawksley slide at 40× with an inverted microscope. Volumes of each of the treatments were modified until the final concentration of eggs was 100 eggs/100-μl aliquot. Eight 100-μl aliquots were removed from each treatment and placed in individual wells of a 96-well microtiter plate. The number of unhatched eggs per well was counted and recorded at the initiation of the experiment and again on days 1, 4, and 8. The experiment was repeated twice.

To determine length of the nematode life cycle in carrot disk cultures, culture tissues were macerated with 0.5% driselase and 0.5% cellulysin for 24 hours. Nematodes and eggs were extracted with $MgSO_4$-$7H_2O$ as a flotation medium, and eggs were separated from vermiform stages with a 25-μm-pore sieve. The resultant suspension of eggs was placed on a modified Baermann funnel (pan), and vermiforms were collected on a daily basis until the nematodes collected consisted of second-stage juveniles. The juveniles were surface sterilized by incubation in 15 ml of 0.5% gentamycin and 0.1% chlorhexidine antibiotic solution for 4 hours, mixing occasionally. The juveniles were then centrifuged (125 *g*) for 3 minutes, and the nematode pellet was transferred aseptically to sterile conical centrifuge tubes. The nematodes were then incubated in 5 ml of 0.03% NaN_3 for 15 minutes and rinsed three times with sterile distilled water followed by centrifugation (125 *g*). The resultant nematode pellet was diluted with sterile 0.5 mM sodium acetate, pH 5.8, a subsample was taken, and the titer of nematodes was adjusted to 5,000 nematodes/ml sterile distilled water. Eleven culture tubes containing 1-month-old carrot disks exhibiting callus were inoculated with

2,500 nematodes in 0.5 ml water. An aliquot of the nematode inoculum was used to estimate the life cycle stages at day 0. The cultures were incubated at 25 C ±1, and two cultures were macerated at days 2, 5, 9, 12, 15, and 18. Morphometrics and reproductive organ development were observed in 30 nematodes and compared to life cycle stages described by van Weerdt (15). The experiment was conducted twice.

Results

Macerating enzymes increased the total numbers of nematodes and eggs extracted from carrot disk cultures by 650% and 300% when compared with incubation or aeration, respectively, of carrot disk tissue for a 24-hour period (Fig. 1).

Experiments were conducted using 5 mM sodium acetate, pH 5.8, because it was determined to provide optimal enzyme and nematode activity when compared with phosphate or Tris buffered saline or with 5 mM sodium acetate with salts (3.5 or 7.0 mM $CaCl_2$ and 36 or 72 mM NaCl). In addition, a pH no higher than 5.8 was critical to separation of nematodes and eggs from carrot debris using flotation media (unpubl. data).

The selection of driselase and Cellulysin cellulase for use in these studies was based on results of experiments that compared the ability of macerase, driselase, and Cellulysin cellulase in various combinations and concentrations to macerate carrot disk tissue. Pectinol 59L (Genicor, San Francisco, CA) and mechanical maceration (Sorvall Omnimixer) were also considered but did not yield adequate maceration and (or) nematodes and eggs in good condition. A solution of driselase plus Cellulysin cellulase produced the most efficient and complete maceration at concentrations of 0.5% for 16–21 hours or 0.75% for 4–6 hours at a ratio of 2.5:1 (milliliters of enzyme solution to grams of carrot disk tissue). In addition, leaving flasks open throughout the tissue maceration improved nematode viability (unpubl. data).

Magnesium sulfate and zinc sulfate (sp gr 1.1) yielded the largest numbers of nematodes and eggs from carrot disk macerate (Fig. 2). In addition, the flotation medium influenced the amount of carrot disk slurry from which nematodes could be effectively extracted. The largest quantities of slurry could be extracted with $MgSO_4$ or $ZnSO_4$ (sp gr 1.1) in a single centrifuge tube. In contrast, sucrose (1.25 M) was not suitable for use as a flotation medium because nematodes could be extracted only from a quantity of slurry equivalent to 6.0 g of carrot disk (fresh weight) in a single centrifuge tube (unpubl. data).

Burrowing nematode infection (in vitro) was greatest when nematodes were extracted from enzymatically macerated carrot disk tissue using $MgSO_4$ as a flotation medium (Fig. 3). Hatch of eggs extracted from enzymatically macerated carrot disk tissue was faster than hatch of eggs extracted without enzymes (Fig. 4). Egg hatch was adversely affected by $ZnSO_4$.

The resultant protocol for extraction of burrowing nematodes from carrot disk tissue is as follows: Burrowing nematode-infected carrot tissue is removed from culture tubes, weighed, and chopped with a hand-operated chopper. A mixture of 0.50% driselase and 0.5% Cellulysin cellulase (w/v) in 5 mM sodium acetate, pH 5.8, with volume equal to 2.5 times the total tissue weight is centrifuged at 2,000 *g* for 1 minute to remove precipitate. The enzyme solution is added to the chopped carrots, and the uncovered vessel is placed on a rotary shaker at 175 rpm at 25 C ± 2 for 16 hours. The flasks are then covered and shaken vigorously for 10 seconds. The slurry is poured through a 1-mm-pore sieve and the debris on the sieve is rinsed with 5 mM sodium acetate, pH 5.8. The effluent from the sieve is decanted into 40-ml glass conical centrifuge tubes and centrifuged at 125 *g* for 3 minutes. The supernatant is discarded and the pellet is resuspended in the small amount of supernatant remaining in each tube with a Vortex Genie (50% of full speed) for 10 seconds. The pellet is repeatedly washed with 30 ml of 5 mM sodium acetate, pH 5.8, and centrifuged at 125 *g* for 3 minutes until the supernatant

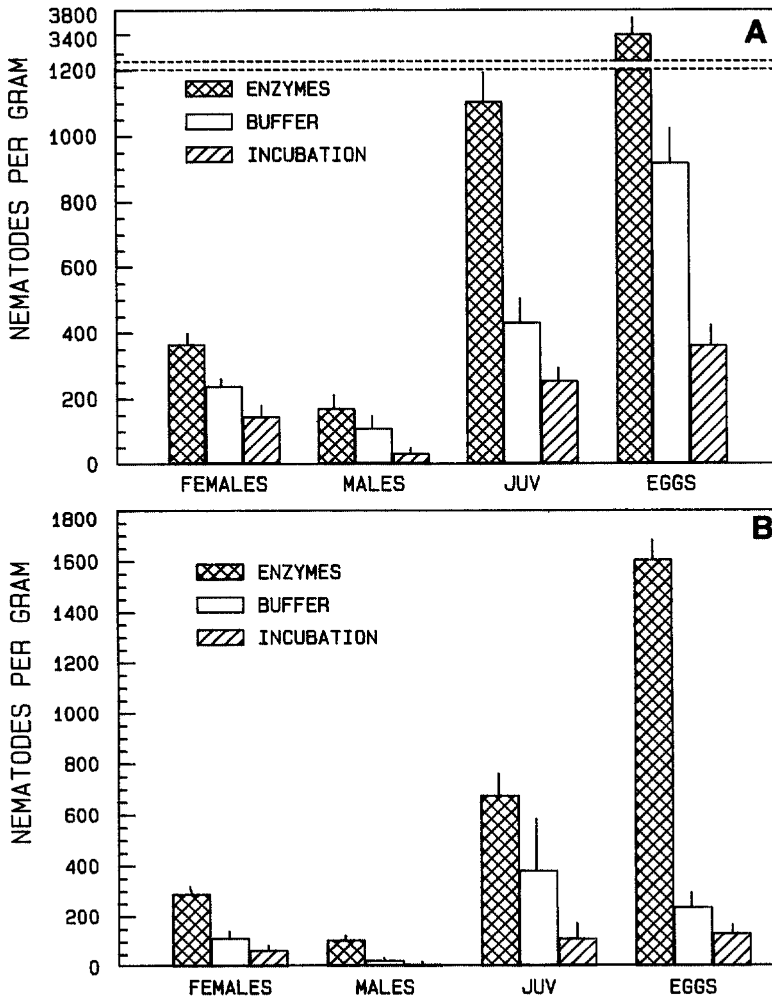

FIG. 1. Numbers of burrowing nematodes per gram (fresh weight) of carrot disk culture tissue as related to extraction technique. Enzymes = 0.5% driselase + 0.5% Cellulysin cellulase in 5 mM sodium acetate, pH 5.8; buffer = 5 mM sodium acetate, pH 5.8; incubation = chopped tissue incubated in closed flask without any solution added. A) *Radopholus similis.* B) *R. citrophilus.* Standard deviation indicated by lines extending from top of bars.

is clear (usually two or three times). Then the pellet is resuspended in a total volume of 15 ml of 5 mM sodium acetate, pH 5.8, with a Vortex Genie (50% of full speed). A syringe, fitted with a 10-cm-long stainless steel cannulus, is used to deliver 10 ml of $MgSO_4$ (sp gr = 1.1, or 225.9 g per liter) beneath the nematode–carrot cell suspension. The tubes are then centrifuged at 2,000 *g* for 3 minutes. The band at the interface between the buffer and the salt solution is removed with a Pasteur pipet. The nematodes are rinsed three times with 5 mM sodium acetate, pH 5.8, by centrifugation at 125 *g* for 3 minutes. The extraction efficiency for this procedure was determined to be 87% for females, 82% for males, 64% for juveniles and 45% for eggs (unpubl. data).

Using this extraction method, fewer than 9 days were required for development of adult males and females from second-stage juveniles; eggs were detectable after 9 days, and a new generation of second-stage juveniles occurred within 16–18 days of inoculation at 25 C. Cultures inoculated with

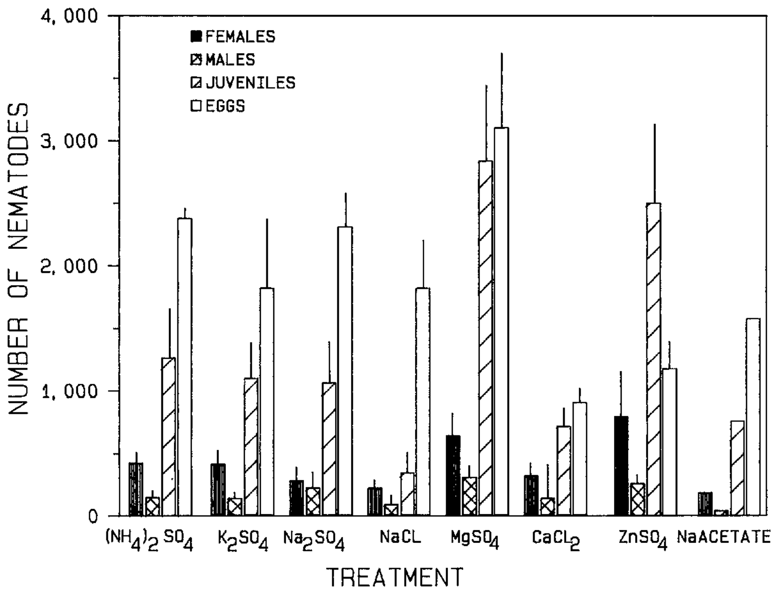

FIG. 2. Numbers of burrowing nematode (*Radopholus citrophilus*) adult females and males, juveniles, and eggs extracted from subsamples of carrot disk macerate as related to the use of flotation media (sp gr 1.1). Standard deviation indicated by lines extending from top of bars.

61–446 nematodes (mixed life cycle stages), yielded 14–87 × 10^3, 20–127 × 10^3, and 56–296 × 10^3 adults, juveniles, and eggs, respectively, at the time of culture breakdown. Population densities can reach 75 × 10^3 nematodes and eggs per gram of fresh weight carrot tissue 50 days after inoculation with 375 nematodes (unpubl. data).

DISCUSSION

The use of macerating enzymes on carrot disk cultures provides a relatively easy, inexpensive, and rapid method for extracting burrowing nematodes from cultures. The yield of eggs of this migratory endoparasite is much better with this method than with other culture and extraction techniques (3,10,11,14,17). The protocol presented is now routinely used in our laboratory. The concentration of macerating enzymes may be increased from 0.50% to 0.75% which reduces the time required for maceration from 16–24 hours to 4–6 hours without apparent adverse effect on nematodes. The total cost for enzymes was $0.11 per gram of carrot disk fresh weight at 0.5% enzyme concentrations.

The burrowing nematode appears to be ideally suited for culture on excised carrot disk. Cultures remain in good condition for 45–65 days following inoculation before they begin to deteriorate. The life cycle of *Radopholus* spp. in the carrot disk system is relatively short among plant-parasitic nematodes and is shorter than the 18–20 days reported for burrowing nematodes in citrus roots (2). At this rate, as many as four generations of *Radopholus* spp. may develop in a single carrot disk culture.

Moody et al. (11) indicated that carrots used in carrot disk culture should be freshly harvested from the field and with foliage intact just before use. In our experiments, however, culture longevity and contamination rates did not differ between cultures from carrots harvested directly from the field and those from carrots purchased in the grocery store (unpubl. data). If carrot disk culture of nematodes depended upon freshly harvested carrots, the culture technique would be impractical because fresh carrots are difficult to obtain most of the year. The condition of the carrots is of greater importance. Carrots that are badly bruised or cut should be avoided. At certain times (late winter and early spring),

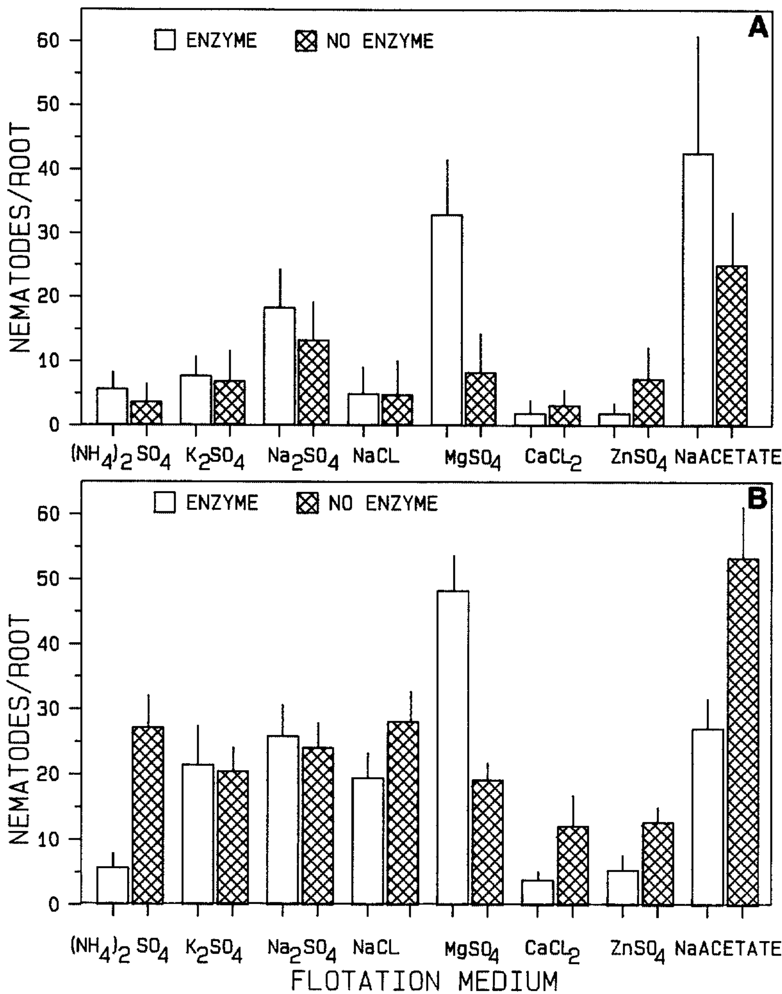

FIG. 3. Effect of flotation medium (sp gr 1.1) on infection of soybean roots (in vitro) by burrowing nematodes extracted from carrot disk tissue with enzyme (0.5% driselase + 0.5% Cellulysin cellulase) or without enzyme (mechanical maceration). A) *Radopholus citrophilus*. B) *R. similis*. Standard deviation indicated by lines extending from top of bars.

internal contaminants may be common in carrots. This becomes rate-limiting for the development of large numbers of nematode cultures. However, waste is minimized if disks are not inoculated until callus is observed on the disk surface.

Future research should focus on the production of a synthetic medium to produce plant-parasitic nematodes and improved methods to produce bulk quantities of sterile plant tissues suitable for nematode culture. Extraction methodology reported in this paper will be applicable to existing alfalfa callus culture techniques for lesion nematodes (7) and to callus culture systems for the burrowing nematode presently in development in our laboratory.

The capability to rear and extract large quantities of burrowing nematodes should facilitate the use of *Radopholus* spp. as biological models. Burrowing nematodes appear to be well suited for use as biological models because they have a relatively small number of chromosomes (4), sibling species (5), and biotypes (6) and because host and nonhost plant germplasm is available (12,13). Furthermore, the described extraction technique provides scientists with

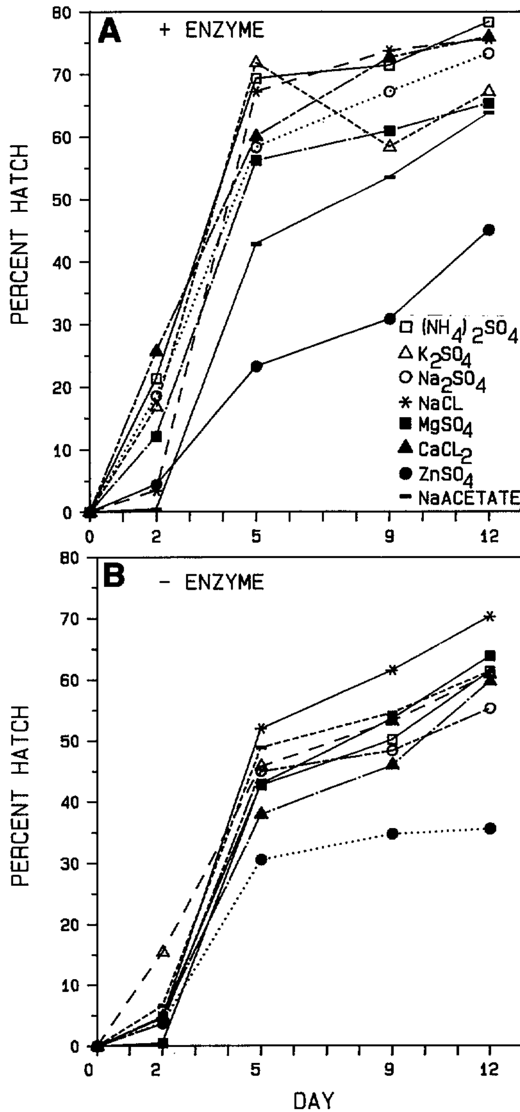

Fig. 4. Effect of flotation medium (sp gr 1.1) on cumulative percent hatch of eggs of *Radopholus citrophilus.* A) Eggs released from carrot disk tissue by macerating enzymes (0.5% driselase + 0.5% Cellulysin cellulase). B) Eggs released by mechanical chopping of carrot disk tissue.

the first opportunity to obtain large quantities of eggs produced by a migratory plant-parasitic nematode.

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