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A Rapid and Simple Method for Staining Lipid in Fixed Nematodes[1]

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Abstract: A method is described for staining lipid in fourth-stage dispersal juvenile nematodes fixed with formal-acetic fixative (FA4:1). *Bursaphelenchus xylophilus* fourth-stage dispersal juveniles were fixed with hot FA4:1 for 24 hours, excess fixative was removed, and a solution of saturated oil red O in 96% ethanol added and allowed to sit for 25 minutes at 60 C. Excess oil red O was removed, nematodes were washed twice with 70% ethanol, and were processed to pure glycerin. Lipid droplets within the nematodes were viewed by light microscopy and appeared as dark red spheres of various sizes. Computerized image analysis was used to quantify lipid droplet area.

Key words: Bursaphelenchus xylophilus, pine wilt, pinewood nematode, oil red O, lipid, staining.

Lipid is essential for nematode survival and is the major source of energy for the dauerlarvae or infective stages of virtually all animal and plant-parasitic nematodes (1,3). The fate of lipid under various conditions and the rate of its utilization are of importance in understanding nematode ecology, behavior, and physiology. Energy utilization, metabolic rates, and aging progress can be determined from the lipid content of nematodes (1,10).

Bursaphelenchus xylophilus (Steiner and Buhrer, 1934) Nickle, 1970 is the causal agent of pine wilt (4). The fourth-stage dispersal juvenile of *B. xylophilus* is a nonfeeding stage that is morphologically and physiologically distinct from other life stages. It contains a large amount of lipid that is utilized as an energy source (5,6). Fourth-stage dispersal juveniles are carried from infected pines to new hosts by cerambycid beetles, *Monochamus* spp.(7). We are interested in the relationship between lipid content and exit behavior of dispersal juveniles from beetle vectors. This paper reports on a lipid-staining technique (modification of Croll (2) and Seinhorst (8)) that facilitates quantification of lipid content in single or a small number of nematodes.

The technique was modified from the work of Croll (2). In Croll's study, the nonfeeding infective stage of the hook worm *Ancylostoma tubaeforme* (Zeder), a vertebrate parasite, was alive in water when stained with oil red O and processed to glycerin by a method modified from Seinhorst (8). The nematodes were then analyzed with a scanning microdensitometer at 517 nm. Our method is for fixed specimens.

MATERIALS AND METHODS

Fourth-stage dispersal juveniles were obtained from newly emerged adult *Monochamus carolinensis* (Olivier) beetles that had developed in jack pine, *Pinus banksiana* Lamb., logs infected with *B. xylophilus*. Nematodes were collected from adult beetles using a modified Baermann technique (9) and were transferred to wells of a 24-well plastic tissue culture plate (Falcon #3047, Becton Dickenson, Lincoln Park, NJ). Most of the water was removed from each well with a Pasteur pipette under a dissecting microscope to prevent removal of nematodes. Following a standard method for fixing nematodes, 2–3 ml of hot (90 C) formal-acetic fixative 4:1 (FA4:1; 10 parts formalin (40% formaldehyde), 1 part glacial acetic acid, 89 parts distilled water) was quickly added to each well (9). The tissue culture plate was covered, kept

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at room temperature for 24 hours, and then most of the fixative was removed from each well with a Pasteur pipette under magnification, as described above.

For our staining method, a saturated solution of oil red O (no. O-0625, Sigma Chemical Co., St. Louis, MO) in ethanol was made by adding 1–3 g of oil red O powder to 100 ml of 96% ethanol (Croll (2) used 70% ethanol) and was stirred for 20 minutes. The solution was then filtered through a vacuum filter apparatus using a Whatman no. 2 paper filter.

Each well received 1.5 ml of the saturated oil red O solution, and the plate was covered and held at 60 C for 25 minutes in an oven (2). Upon removal from the oven, most of the oil red O solution was removed from each well with a Pasteur pipette and 2 ml of 70% ethanol was added to each well. After sufficient time had elapsed for the nematodes to settle to the bottom of each well (about 10 minutes), the excess ethanol was removed with a Pasteur pipette. The ethanol wash was repeated a second time (modified from distilled water washes as described in Croll (2)).

Instead of adding a 50/50 (V:V) water-glycerin solution to the wells and allowing the water to evaporate as described by Croll (2), we processed the nematodes to glycerin in the wells of the tissue culture plate following a modification of the quick method of Seinhorst (8,9). Two ml of "Seinhorst I" solution (20 parts 96% ethanol, 1 part glycerin, 79 parts distilled water) at room temperature (21 C) was added to each well. The uncovered tissue culture plate was placed in a closed vessel containing a small volume of 96% ethanol. This vessel was placed in an oven at 35–40 C for 24 hours. The excess liquid in each well was removed with a Pasteur pipette, and 2 ml of a modified "Seinhorst II" solution (10 parts glycerin, 90 parts 96% ethanol) at room temperature (21 C) was added. The uncovered tissue culture plate, not in the closed vessel, was returned to the oven until all ethanol had evaporated, by which time the stained nematodes were then in pure glycerin and ready to mount. The nematodes were mounted in drops of pure glycerin on standard microscope slides using the wax ring method (9) and viewed with the use of transmitted light microscopy.

Results and Discussion

Once the nematodes were in pure glycerin, the lipid droplets appeared as dark red spheres of various sizes and were quite obvious when the nematodes were mounted and viewed under light microscopy (Fig. 1). The lipid droplets within the nematodes did not appear to be red or appeared to be stained very lightly red while going through the process. This was usually an artifact of processing.

Chemical methods of quantifying lipid content often require large numbers of specimens, and they are impractical for single or a few nematodes. The method presented here is excellent for examining lipid in a very small number of specimens because individuals can be tracked under a microscope throughout the process. Use of a 24-well tissue culture plate also allows a large number of specimens or groups of specimens to be processed at one time and, at the end of the processing, provides convenient long-term storage. Specimen information can be recorded on the lid. This technique produces preserved specimens that can be re-examined later. Nematodes already in glycerin appear unharmed after going through part of the process a second, third, or fourth time.

The intensity and precision of the staining for lipids have allowed us to quantify lipid content in individual nematodes. Images of nematodes under transmitted light microscopy were recorded by a video camera attached to a video capture board in a personal computer and analyzed with MOCHA (Jandel Scientific, San Raphael, CA), an image analysis program that can distinguish colors and levels of density. Lipid droplet area and whole body area were quantified, and percentage lipid area (lipid per body) was used as a measure of lipid content for fourth-stage dispersal juveniles under various treatment regimes.

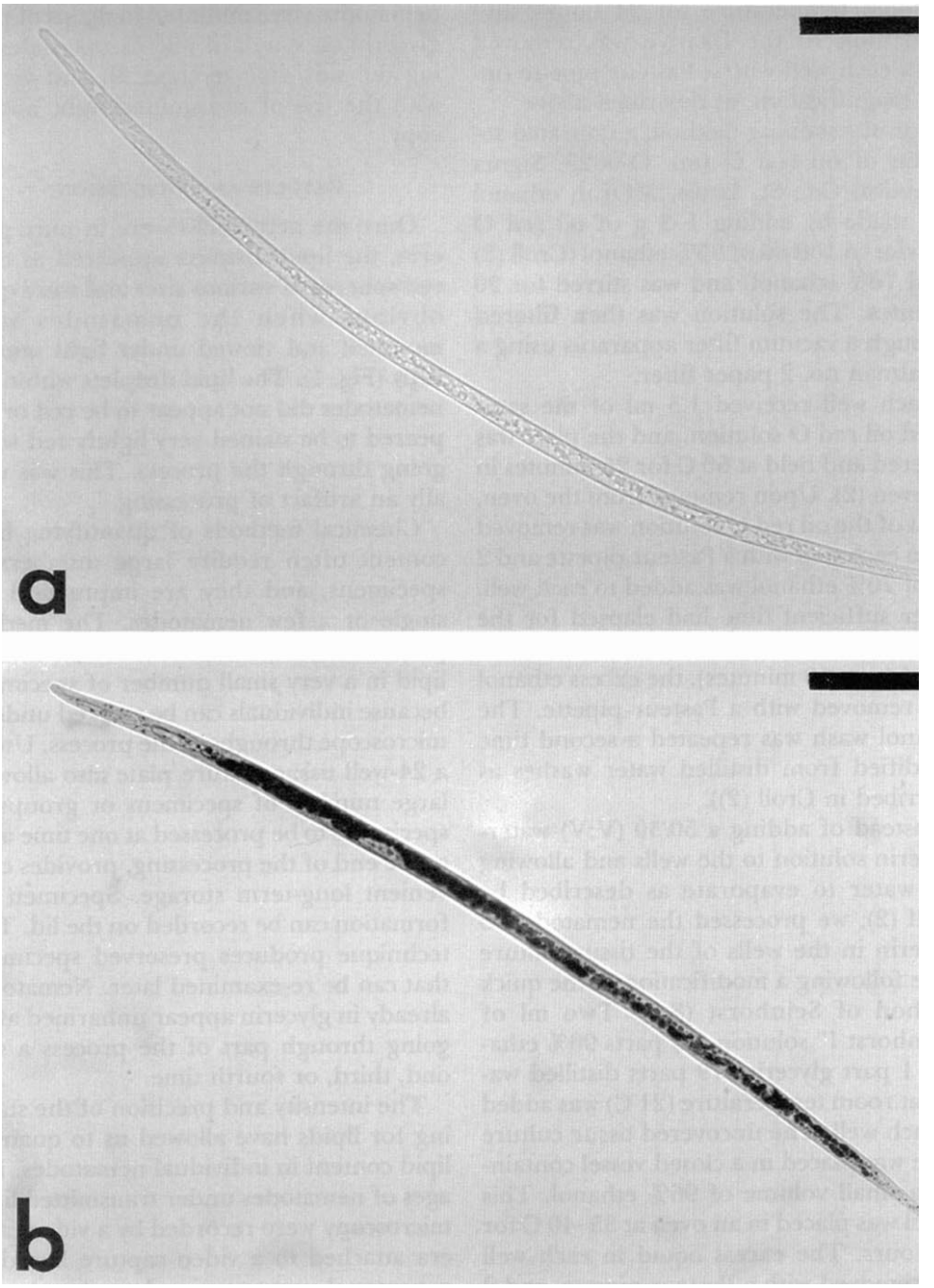

FIG. 1. Photomicrographs of A) an unstained *Bursaphelenchus xylophilus* fourth-stage dispersal juvenile and B) an oil red O-stained fourth-stage dispersal juvenile. Red-stained lipid droplets fill much of the body of the nematode and appear black in the photomicrograph. Head region in A and B at upper left. Scale bar = 0.1 mm.

The technique presented here works very well for staining fourth-stage dispersal juveniles of the nematode *B. xylophilus* and should work equally well with other fixed nematode species that contain lipid droplets. The method is convenient, rapid, and consistent in the uniformity and density of staining provided.

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