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Red Food Coloring Stain: New, Safer Procedures for Staining Nematodes in Roots and Egg Masses on Root Surfaces

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Abstract: Acid fuchsin and phloxine B are commonly used to stain plant-parasitic nematodes in roots and egg masses on root surfaces, respectively. Both stains can be harmful to both the user and the environment and require costly waste disposal procedures. We developed safer methods to replace both stains using McCormick Schilling red food color. Eggs, juveniles, and adults of *Meloidogyne incognita* stained in roots with red food color were equally as visible as those stained with acid fuchsin. Egg masses stained with red food color appeared as bright-red spheres on the root surfaces and were highly visible even without magnification. Replacement of acid fuchsin and phloxine B with red food color for staining nematodes is safer for the user and the environment, and eliminates costly waste disposal of used stain solutions.

Key words: environmental safety, *Meloidogyne* spp., resistance, root-knot nematode, staining procedures, worker protection.

Staining of nematodes in root tissues is routine in most nematology laboratories worldwide. The ability to visualize the parasitic nematode in root tissue is essential to many areas of plant nematological research, including assessments of host plant resistance, elucidation of nematode development and life cycles, and evaluation of efficacies for biocontrol products and traditional nematicides. A number of staining procedures have been developed for use in plant nematology. However, these methods all require the use of chemicals that may pose health hazards to the individual performing the procedure and also result in waste that may harm the environment (Byrd et al., 1983; Fenner, 1962; Holbrook et al., 1983; McBeth et al., 1941; Southey, 1970). Acid fuchsin has been widely used for staining nematodes in root tissue. An early popular staining method used acid fuchsin for staining nematodes and lactophenol for destaining root tissue (McBeth et al., 1941); this method was especially hazardous because it required use of toxic phenol. Byrd et al. (1983) developed an improved method for staining nematodes in root tissue that combined components of several methods including clearing the roots with either NaOCl or H_2O_2, staining the nematodes with acid fuchsin, and destaining the roots with acidified glycerin (McBeth et al., 1941; Southards, 1965). The method developed by Byrd et al. (1983) eliminated the use of lactophenol but not acid fuchsin. Exposure to acid fuchsin may be harmful by inhalation, ingestion, or skin absorption and is irritating to mucous membranes and the upper respiratory tract, eyes, and skin (Sigma-Aldrich, 1999b). Acid fuchsin must be disposed of by incineration, if available, or through a chemical waste disposal company. Protective eyewear, chemical-resistant gloves, and a respirator approved by the National Institute for Occupational Health (NIOSH)/Mine Safety and Health Administration (MSHA) are recommended as personal safety protective equipment when using acid fuchsin.

Staining root-knot nematode (*Meloidogyne* spp.) egg masses present on root surfaces with phloxine B is another technique that is widely used in plant nematology (Fenner, 1962; Holbrook et al., 1983). In our laboratory, we have routinely used the phloxine B procedure to estimate nematode reproduction in the evaluation of vegetable crops for resistance to several species of root-knot nematodes. These large-scale evaluations generated large volumes of phloxine B waste in our laboratory. During the last decade, more stringent regulations have necessitated disposal of phloxine B (a human mutagen) by incineration (Sigma-Aldrich, 1999a). Protective eyewear, chemical-resistant gloves, and a NIOSH/MSHA-approved respirator are recommended as personal safety protective equipment when using phloxine B.

The safety and environmental constraints associated with the use of acid fuchsin and phloxine B prompted us to develop safer alternative procedures for nematode staining. Our objectives in these studies were to develop: (i) a technique for staining plant-parasitic nematodes in root tissue that would be safer than the acid fuchsin staining procedure and would not require disposal of the stain as chemical waste and (ii) a method for staining egg masses of root-knot nematodes on root surfaces that would be safer than the phloxine B method and thus eliminate the need to dispose of large quantities of phloxine B as chemical waste.

Materials and Methods

Staining nematodes in root tissue: Roots of pepper (*Capsicum annuum* L.) infected with *Meloidogyne incognita* (Kofoid & White) were washed, blotted dry, cut into 1 to 2-cm-pieces, placed into plastic histological tissue-staining capsules, and submerged in ca. 100 ml 1.5% NaOCl (71.4 ml tap H_2O + 28.6 ml Clorox chlorine bleach) in a 200-ml beaker. (Small whole root systems weighing ≤10 g were left intact for processing and were not placed in staining capsules.) The roots were agitated in the NaOCl solution for 4 minutes, placed in a

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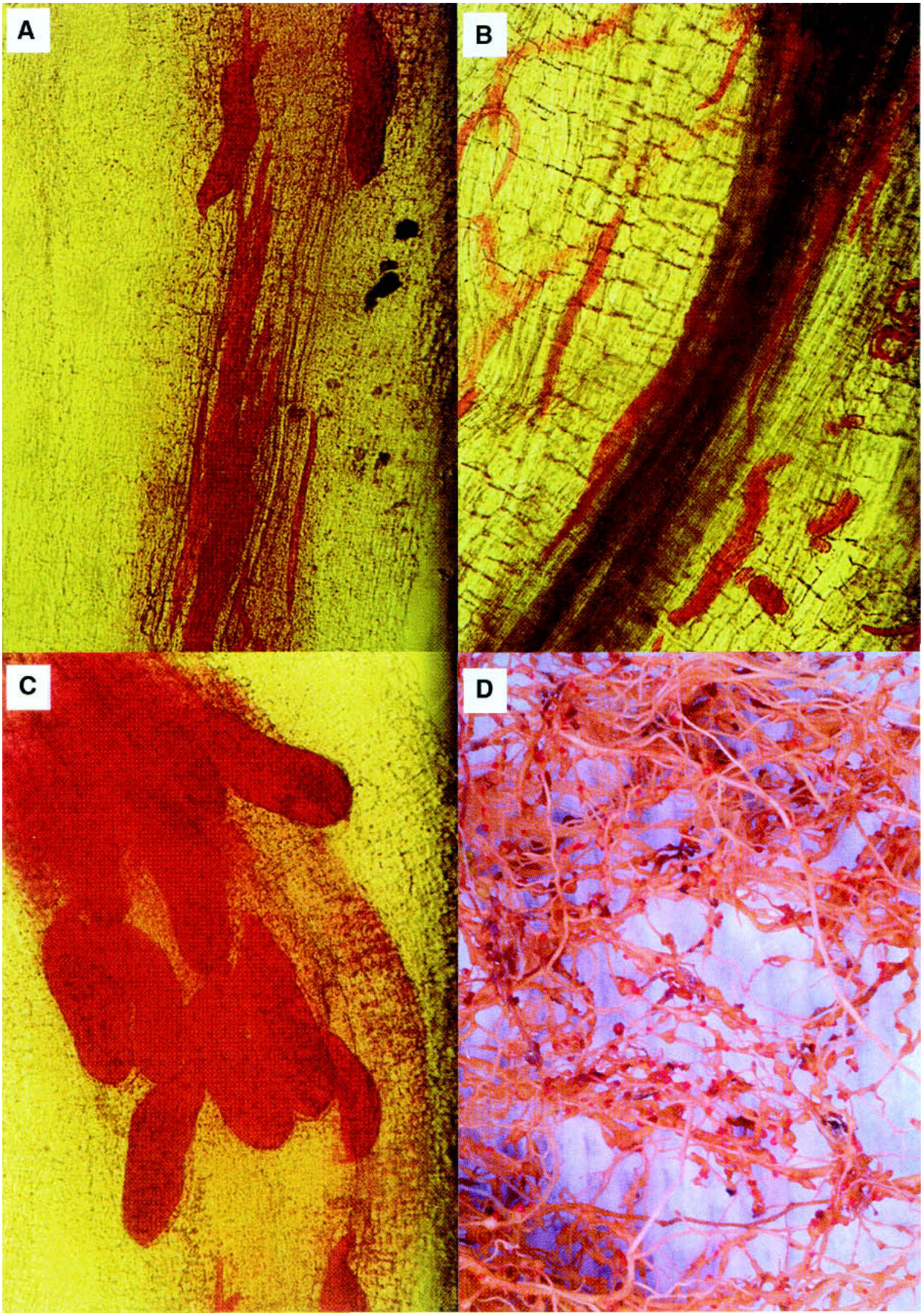

FIG. 1. A–C) *Meloidogyne incognita* eggs and nematodes at other developmental stages stained with red food color in pepper (*Capsicum annuum*) roots. D) Egg masses of *M. incognita* stained with red food color on the surface of pepper (*C. chinense*) roots.

kitchen strainer, rinsed under running water for 30 seconds, and submerged in ca. 150 ml tap H_2O for 15 minutes. The staining capsule containing the roots was then placed in a 100-ml beaker containing a 12.5%, 33.3%, or 50% (v/v) solution of McCormick Schilling red food color (ingredients: H_2O, propylene glycol, FD&C Reds 40 and 3, and propyparaben; available in 1-quart bottles from McCormick & Co., Inc., Hunt Valley, MD) in distilled H_2O. The red food color solution containing the roots was brought to a boil, boiled for 30 seconds, and allowed to cool to room temperature. The roots were then rinsed with tap water, swirled for 15 seconds in acidified glycerin (40 ml glycerin + 5 drops 5N HCl) that had been heated to ca. 40 °C (not boiled), and removed from the glycerin. The roots were allowed to cool to room temperature and then mounted between two 5 × 12-cm glass slides for observation of the nematodes under a stereomicroscope.

Egg-mass staining method: Whole, intact root systems of pepper (*C. chinense* Jacq.) plants infected with *M. incognita* were removed from the soil, washed, and blotted dry. The dry root system was placed in a 500-ml beaker containing a 10% or 20% (v/v) solution of McCormick Schilling red food color for 15 minutes, after which the roots were rinsed in tap water and blotted dry. Egg masses were observed either directly or under a ×20 magnifier light.

Results and Discussion

Staining nematodes in root tissue: We developed a modification of an acid fuchsin staining method (Byrd et al., 1983) that uses red food color for staining nematodes in root tissue. In our experiments, the 12.5% (v/v) solution of McCormick Schilling red food color in distilled H_2O provided the highest contrast for staining all stages of plant-parasitic nematodes in root tissue (Fig. 1, A–C). The 33.3% and 50% solutions of red food color stained the nematodes and the roots deeply and equally, making it difficult to differentiate the nematodes from the plant tissue. We also discovered that nematodes can be adequately stained by placing roots in 12.5% red food color for 15 to 30 minutes at room temperature. This variation in the procedure is useful for staining seedling roots because boiling seedling root systems in the stain often damages the fragile secondary and tertiary roots. Acidified glycerin was preheated to 40 °C and the roots then briefly swirled in the glycerin because we observed that placing the roots in the acidified glycerin and then heating to boiling (Byrd et al., 1983) caused excessive destaining of nematodes and also caused the roots to disintegrate. The substitution of red food color for acid fuchsin when staining nematodes eliminates hazards to the individual performing the technique and also eliminates any need for costly chemical waste disposal.

Egg-mass staining method: Egg masses of *Meloidogyne* spp. present on the root surfaces were stained bright red after submersion of roots in a 20% solution of red food color for 15 minutes (Fig. 1,D). Egg masses stained using a 10% (v/v) solution of red food color were not as visible as those stained with a 20% solution. The use of red food color stains the egg masses as brightly as staining with phloxine B; this improved method eliminates hazards to the user and the need for disposal of large quantities of phloxine B waste.

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