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A COMPLETE SAFE AND COST EFFECTIVE METHOD FOR STAINING ROOT-KNOT NEMATODES *MELOIDOGYNE* SPP.

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Abstract

Red food coloring artificial dye Carmoisine can be used in totally safe, low cost and environment friendly nematode staining procedure. Both egg masses on the root surface and nematodes in the root tissue stained perfectly with the Carmoisine. Egg masses stained the best at higher stain concentrations (3, 4 and 5%). 30-60 min root submersion in the stain resulted in best contrast. White vinegar can be used efficiently to store egg mass bearing roots. Nematodes in the root tissue were stained the best at low stain concentrations (0.1 and 0.2%) with 5% vinegar. 70% sucrose solution acidified with 0.5% vinegar was comparable to acidified glycerin for destaining. Stained nematodes were still visible with high contrast even after being stored for 8 to 11 months at room temperature.

***Key Words*:** Food coloring dyes, Staining, *M. incognita*, Acid fuchsin, Egg mass.

Introduction

Many areas of studying and controlling approaches in nematology involve nematodes to be visible in the root tissues. Thus root clearing and staining technique is necessary to facilitate nematode visibility. The staining procedure using acid fuchsin for staining and lactophenol for destaining remained the only reliable method for several decades (McBeth *et al.*, 1941; Hooper, 1970 and 1986). The procedure also involves a pre-staining treatment to clear the root tissue from its natural pigments. Except for the sodium hypochlorite (NaOCl), several compounds including KOH, nitric acid and H_2O_2were eliminated from the pre-staining method due to their harmfulness and or requirement of extreme care (Byrd *et al.,* 1983). A brilliant study by Byrd *et al.,* (1983) was conducted to eliminate the toxic lactophenol from de-staining to be substituted by acidified glycerin. The same study addressed the precise amount of NaOCl and the effective time required to satisfactory pre-staining root clearing. Environment and health concerns enhanced several attempts to use a staining material that less toxic and much safer than the acid fuchsin. Thies *et al.,* (2002) used red food coloring stain (McCormic Schilling food dyes) to substitute both acid fuchsin in root staining and phloxin B in egg masses staining procedures. Damasceno *et al.,* (2016) stated that McCormic Schilling food dyes are unavailable in Brazil, they used Bordeaux based food dyes to stain nematodes eggs and egg masses. Food coloring dyes are varying from a country to another not only by their chemical bases but also in their availability in the markets. The food coloring dyes available in Iraq are mainly Carmoisine (in the Azorubine dye group). They have never been used or tested for nematode staining techniques. The destaining procedure on the other hand requires using HCl acidified glycerin. If those materials (glycerin and HCl)can be replaced with similar efficient materials that not only cheaper but also safer, will be of significance. Replacing the glycerin with sucrose solution at similar density level, if possible, will decrease the cost of the procedure. Whereas, using the culinary white vinegar in staining-destaining procedure will be much easier and safer than using HCl. Objectives of this study were to i) evaluate the efficacy of the available red food coloring dye in nematode staining procedures (nematodes in root tissue and egg masses attached to the root) and ii) investigate the possibility of using sucrose solution acidified

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with white vinegar to substitute HCl acidified glycerin for the distaining procedure.

Materials and Methods

Experiment general procedure

Six weeks old tomato plants cultivar 'Mermande' were inoculated with 2000 *M. incognita* eggs and J2(s) in 2 liter plastic pots. 35 days post inoculation (DPI) plants were uprooted and washed free of soil and organic material. Plants were then split into two groups, one for the egg masses staining and the other for staining root-knot nematode at different stages in the root tissue. Five concentrations (0.1, 0.2, 0.3, 0.4 and 0.5% w/v) of the staining solution were prepared by solving 1, 2, 3, 4 or 5g of the stain powder (Carmoisine food coloring stain) in 1000 ml of distilled water. In case of staining nematodes in roots, white vinegar was added at 5%, 10% or 20% (v/v) to each stain concentration while the control satin was left plain (without vinegar). Regarding the destaining procedure, sucrose solution was used instead of glycerin at three concentrations (60, 70 or 80%). The sucrose solutions were either non-acidified or acidified with white vinegar at 0.1% or 0.2% (v/v). Acidified glycerin (Byrd *et al.,* 1983) was used in the destaining as a control. The experiment was repeated using infected plants at 65 DPI. The best stain concentrations for egg masses or root nematodes staining and the best destaining solution were later tested on okra and cucumber infected plants.

Egg mass staining method

Root systems of *M. incognita* infected plants were subjected to egg masses staining procedure. After removing from soil, each root system was thoroughly washed, blotted dry and placed in a 200-ml beaker with 100 ml of the stain solution (prepared as formerly described). After 15 minutes of soaking in stain, roots were rinsed in tap water and blotted dry. Egg masses were observed, counted and photographed under the stereomicroscope at 2X magnification.

Staining nematodes in root tissue

Infected roots were gently washed free of soil, cut into 3-5cm pieces and stained using methods described by Daykin and Hussey (1985) and improved by Hussey (1990) with some modifications. Roots were cleared in a 100 ml beaker filled with 50 ml tap water and 20 ml of 0.5% NaOCl (1.5% NaOCl) for 5 minutes for small young roots (35 DPI) or for 10 minutes in case of older roots (65 DPI). Roots were occasionally agitated while soaking. The roots were then rinsed for 1 minute and soaked for 20 minutes in tap water to remove NaOCl residues. Roots were transferred to the stain solutions, 20 root systems were used for each stain concentration to be five root systems for each stain type (with or without vinegar). The root-solution was heated and removed from heating source (hot plate) when just started to boil, allowed to cool to room temperature, and rinsed with tap water. Stained roots of each stain type were divided into four portions, one portion for each destaining solution (previously mentioned). In 100-ml beakers, the root-destatining solution was heated to boil, removed and left to cool to room temperature. The distaining solution was discarded and sufficient amount of the 70% sucrose solution was added to each root beaker which was stored for observation. By the aid of a dissecting microscope at 2X magnification, the stained nematodes in the root tissue were observed and the staining-destaining methods were evaluated.

Results and Discussion

Egg masses on the root surface were stained the best at higher stain concentrations (3, 4 and 5%). The 1% and 2% also stained the egg masses, but the contrast was very low. Older egg masses stained firmly and they were darker and much easier to detect than younger ones (Fig. 1A). It was found that submersion the roots in the stain for 30-60 min resulted in better contrast between the egg masses and the root surface. The 3% stain was the best in staining large coalesced galls of infected okra roots compared to the 4 and 5% concentrations in which the stain was excessive affecting the differentiation quality. Saving the stained roots with their attached egg masses in the white vinegar did not affect the contrast between them and the root surface nor reduce stain quality of eggs (Fig. 1B).

I case of staining nematode in the root tissue, the two low concentrations (0.1 and 0.2%) without or with 5% vinegar resulted in the highest contrast between the root tissue and the nematodes at different stages especially where destained with 70% non-acidified or 0.5% vinegar acidified sucrose solution (Fig. 1 C and D). The later solution was comparable to acidified glycerin giving fine results in terms of clear root, bright visible nematodes and high contrast.

The other high concentrations of the stain regardless to adding vinegar could stain the nematode and the roots firmly, but the roots could not be destained sufficiently with any of the destaining solutions including acidified glycerin. Generally, the younger infected roots can be stained and destained easier with less care than older roots and the results from the first will be better and more accurate than from the later. Surprisingly, the stained specimens were still showing high acceptable contrast

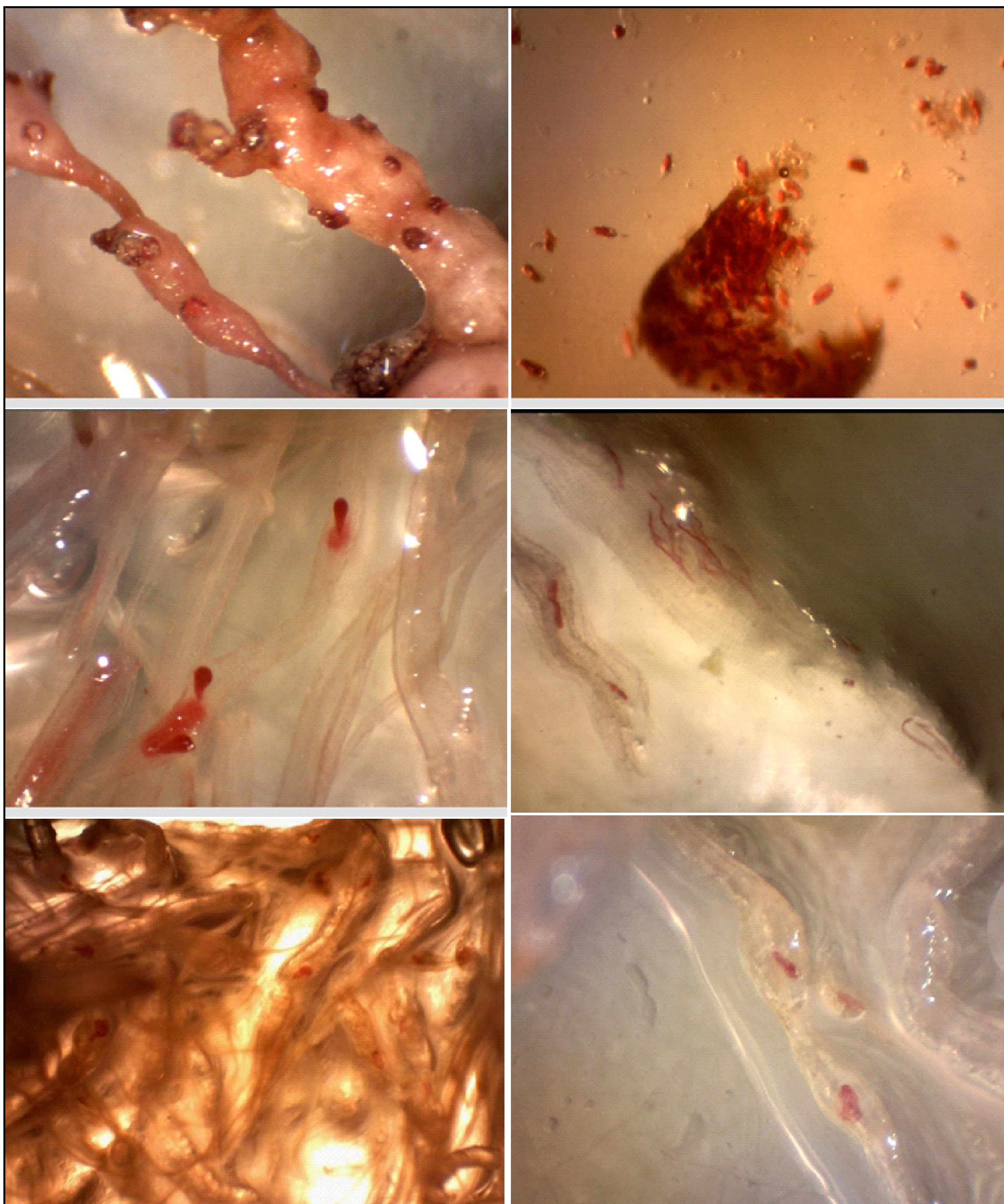

Fig. 1: Root-knot nematode egg masses, eggs and other life stages in different plant's roots stained with red food coloring (carmoisine) and destained with 70% sucrose acidified with 0.5% white vinegar. A) *Meloidogyne javanica* egg masses on okra roots. B)*M. javanica*stained eggs within an egg mass. C and D) *M. incognita* infected tomato roots showing stained nematodes at different stages. E and F) *M. javanica* infected okra and tomato roots showing acceptable contrast between nematodes and root tissue after 9 months of storing at room temperature.

between the nematodes and the root tissue even after being stored more than ten months at room temperature.

This staining procedure using food coloring dyes (Thies *et al.*, 2002; Damasceno *et al.*, 2016) and sucrose for the destaining can be applied in high throughput experimentation especially when screening for resistance or for educational activities such as nematology classes, training and workshops. In contrast to the classic staining technique involving the acid fochsin, phloxin B and HCl acidified glycerin (Byrd *et al.*, 1983), this food coloring-sucrose staining technique addressed in this study is comparably easier, safer and cost effective.

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