

SCIENTIFIC NOTE

## Root-knot nematode staining with artificial food dyes

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### HIGHLIGHTS

- Egg masses were stained with ponceau 4R food dyes at different concentrations.
- Ponceau 4R, at 1.0%, can substitute phloxine B for egg mass staining.
- Ponceau 4R, at 4.0%, can substitute acid fuchsin for egg staining.
- Ponceau food dyes can replace acid fuchsin and phloxine B for root-knot nematode staining and be reused.

**ABSTRACT:** The dyes used for staining nematode egg masses and eggs can cause environmental hazards and human health concerns. The aim of this study was to evaluate different artificial food dyes, as a replacement for acid fuchsin and phloxine B, for staining eggs and egg masses of *Meloidogyne javanica* from infected tomato plants. Five different combinations of dyes were tested at five concentrations. For control treatments, infected roots were stained with acid fuchsin and phloxine B for coloring eggs and egg masses, respectively. Egg staining with the dyes ponceau 4R with red 40 and ponceau 4R with brilliant blue, at the concentration of 4%, showed results similar to those obtained with acid fuchsin. However, egg masses were effectively stained with ponceau 4R food dyes with red 40 and with ponceau 4R with brilliant blue at all tested concentrations. Ponceau food coloring dyes gave a good contrast for nematode egg masses and egg counting, do not present human health and environmental pollution problems, and can be reused, with less waste being generated in the laboratory. These dyes are low-cost products that are available at most supermarkets, and can efficiently substitute acid fuchsin and phloxine B for staining eggs and egg masses of *M. javanica*.

**Keywords:** aniline, acid fuchsin, phloxine B, *Meloidogyne javanica*, nematode eggs, egg masses.

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### INTRODUCTION

In most plant nematology laboratories the usual methods for nematode staining in plant roots involve chemicals that are hazardous to the environment and also to human health<sup>[1,2]</sup>. Fuchsin, which is commonly used for nematode staining, is considered a hazardous substance that can cause skin, eye and respiratory tract irritation. In addition, when it reaches the gastrointestinal tract, fuchsin can lead to nausea, vomiting, and diarrhea. Long-term exposure to this dye can result in blood, liver, spleen, thyroid, and nervous system damage and cause migraine, dizziness, lethargy and muscular contraction<sup>[3]</sup>. The use of phloxine B, also for nematode staining, generates high volumes of waste materials. In fact, several authors have pointed out the need to replace acid fuchsin and phloxine B in plant-parasitic nematode staining procedures with substances that are effective and also present fewer risks to the environment and to laboratory workers<sup>[1,4]</sup>.



A methodology to detect nematodes in plant roots, with the use of lactophenol for tissue clearing and acid fuchsin for nematode staining was described by McBeth et al.<sup>[5]</sup>, and modified by Byrd et al.<sup>[6]</sup>. These latter authors added sodium hypochlorite (NaOCl) or hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) to the staining procedure, which allowed them to eliminate the use of phenol, which is very toxic, but they maintained the use of acid fuchsin. Thies et al.<sup>[4]</sup> developed a non-toxic method for staining egg masses, juveniles and adult nematodes of *Meloidogyne incognita* (Kofoid & White) Chitwood, by substituting acid fuchsin and phloxin B with the use of food coloring dyes (McCormick Schilling red food color). According to these authors, the method with these dyes stained the nematodes and egg masses as efficiently as the method with acid fuchsin. However, this dye is rarely found in markets. On the other hand, Rocha et al.<sup>[1]</sup> showed that bordeaux based dyes, used normally in the food industry for juices, were effective in staining eggs, egg masses, juveniles and females of *M. incognita*. These dyes can be easily found in markets and are not expensive.

In Brazil, only a few artificial dyes are allowed as food additives, such as sunset yellow, brilliant blue FCF, bordeaux S or amaranth, erythrosine, indigo carmine, ponceau 4R, tartrazine and red 17<sup>[7]</sup>. These dyes present high stability (light, oxygen, heat, and pH), uniform color, high tinctorial quality, as well as low production cost<sup>[8]</sup>.

Considering the harmful effects on human health and the environment, and the high cost of the dyes used for staining nematode eggs and egg masses, this study aimed to evaluate a technique with artificial food dyes for staining egg masses and eggs of *Meloidogyne javanica* (Treb) Chitwood in tomato roots.

## MATERIAL AND METHODS

### Staining of egg masses of *Meloidogyne javanica*

Tomato plants (*Solanum lycopersicum*) cv. Santa Cruz Kada were transplanted 20 days after seed germination, to plastic pots containing 2 kg of a mixture of sterilized soil and sand in a 1:1 volume proportion. Plants were then inoculated with 2,000 second stage juveniles (J2) of *M. javanica* and grown in a greenhouse with daily irrigation. After 40 days, the root system was rinsed with tap water to remove soil. *M. javanica* egg masses in the roots of the tomato plants were stained with the following commercial food dyes: 1 - ponceau 4R with red 40; 2 - ponceau 4R with brilliant blue; 3 - erythrosine with bordeaux and brilliant blue; 4 - tartrazine with sunset yellow, and 5 - tartrazine with brilliant blue (Table 1). Solutions at the concentrations of 1.0, 2.0, 3.0, 4.0 and 5.0% (v/v) were prepared in 1000 mL of water, for each commercial dye.

Egg masses were stained according to the method described by Rocha et al.<sup>[1]</sup> with some modifications. The entire tomato root system was immersed for 15 min at room temperature (28 ± 2 °C), in a 1000 mL beaker containing the staining solution, at different concentrations. The roots were then transferred to a beaker with tap water for 10 min to remove the excess of dye and dried on paper towels. Control treatment went through the same procedure stained with phloxine B at 0.0015% for 15 min<sup>[9]</sup>.

**Table 1.** Artificial food dyes tested for staining egg masses and eggs of *Meloidogyne javanica* in tomato roots.

Code	Commercial name in Brazil	Color	Food Dyes	Formula
1	Mix Coralim vermelho morango <sup>®</sup>	Red	Ponceau 4R with red 40	Ponceau 4R - C <sub>20</sub> H <sub>11</sub> N <sub>2</sub> Na <sub>3</sub> O <sub>10</sub> S <sub>2</sub> Red 40 - C <sub>18</sub> H <sub>14</sub> N <sub>2</sub> Na <sub>2</sub> O <sub>8</sub> S <sub>2</sub>
2	Mix Coralim roxo batata <sup>®</sup>	Purple	Ponceau 4R with brilliant blue	Ponceau 4R - C <sub>20</sub> H <sub>11</sub> N <sub>2</sub> Na <sub>3</sub> O <sub>10</sub> S <sub>2</sub> Brilliant blue - C <sub>37</sub> H <sub>34</sub> N <sub>2</sub> Na <sub>2</sub> O <sub>9</sub> S <sub>2</sub>
3	Mix Coralim pink <sup>®</sup>	Pink	Erythrosine with bordeaux and brilliant blue	Erythrosine - C <sub>20</sub> H <sub>11</sub> N <sub>2</sub> Na <sub>3</sub> O <sub>10</sub> S <sub>2</sub> Bordeaux - C <sub>20</sub> H <sub>11</sub> N <sub>2</sub> Na <sub>3</sub> O <sub>10</sub> S <sub>2</sub> Azul brilhante - C <sub>37</sub> H <sub>34</sub> N <sub>2</sub> Na <sub>2</sub> O <sub>9</sub> S <sub>2</sub>
4	Mix Coralim amarelo gema <sup>®</sup>	Orange	Tartrazine with sunset yellow	Tartrazine - C <sub>16</sub> H <sub>9</sub> N <sub>4</sub> Na <sub>3</sub> O <sub>9</sub> S <sub>2</sub> Sunset yellow - C <sub>16</sub> H <sub>10</sub> N <sub>2</sub> Na <sub>2</sub> O <sub>7</sub> S <sub>2</sub>
5	Mix Coralim verde hortelã <sup>®</sup>	Green	Tartrazine with brilliant blue FCF	Tartrazine - C <sub>16</sub> H <sub>9</sub> N <sub>4</sub> Na <sub>3</sub> O <sub>9</sub> S <sub>2</sub> Brilliant blue - C <sub>37</sub> H <sub>34</sub> N <sub>2</sub> Na <sub>2</sub> O <sub>9</sub> S <sub>2</sub>

### *Meloidogyne javanica* egg staining

For *M. javanica* egg staining, tomato roots were rinsed with tap water, chopped in a blender for 20 s, as described by Hussey & Barker<sup>[10]</sup>, and modified by Boneti & Ferraz<sup>[11]</sup>. The root water suspension was poured through a set of two sieves, with decreasing pore size from top to bottom 200 mesh and 500 mesh, respectively. The eggs collected in the 500-mesh sieve were transferred with water to a 100 mL beaker and 1 mL was transferred to test tubes containing 20 mL of the staining solution. The following dyes were tested: 1 - ponceau 4R with red 40; 2 - ponceau 4R with brilliant blue; 3 - erythrosine with bordeaux and brilliant blue; 4 - tartrazine with sunset yellow, and 5 - tartrazine with brilliant blue (Table 1). The concentrations were: 1.0, 2.0, 3.0, 4.0 and 5.0% (v/v).

Test tubes were incubated at room temperature ( $28 \pm 2$  °C) for 24 h and their contents were then poured into a 500-mesh sieve, and rinsed with water, in order to remove the excess of staining solution. The eggs were then transferred to a 50 mL beaker with water. For the control treatment, the eggs were stained in a beaker with 30 mL of water and 1 mL of acid fuchsin dye solution (0.35 g of acid fuchsin, 25 mL of acetic acid, and 75 mL of distilled water), at room temperature, for 24 h<sup>[12]</sup>.

Stained eggs were transferred to a Peters chamber and observed under a light microscope with the 10X objective, for comparison with those stained with acid fuchsin (control treatment).

## RESULTS AND DISCUSSION

### Staining of *Meloidogyne javanica* egg masses

Intense coloring of the egg masses was observed for the control treatment phloxine B. Egg masses stained red with dye 1 (ponceau 4R with red 40) and stained purple with dye 2 (ponceau 4R with brilliant blue), for all tested concentrations (Figure 1a, b). Homogeneous and bright colors were obtained with both dyes 1 and 2, at concentration of 1.0%, similar to the control treatment (Figure 1f). For these treatments, only the egg masses were efficiently stained with a uniform and stable color, with high contrast between the color of the egg masses and the natural color of the root tissue. The roots were not affected by the dyes, and their tissues remained with the natural color regardless of the concentration of the dyes tested. In addition, for these dyes, egg mass coloring with the higher tested concentrations (2, 3, 4 and 5%) was similar to the coloring results with 1%, with a high contrast between the egg masses' color and the roots' natural color. Therefore, the lowest concentration of these dyes (1%) is recommended. According to Rocha et al.<sup>[1]</sup> a strong contrast between the egg masses and the root tissue is necessary for adequate egg mass visualization and counting. Egg masses stained with dye 1 preserved the staining color even after rinsing the roots with water three days later. However, when the other dyes were used, egg masses returned to their natural color after a few hours.

Egg masses were stained pink with dye 3 (erythrosine with bordeaux and brilliant blue) at all tested concentrations. However, for the solution at 5.0%, the root tissue was also stained and there was no contrast between the egg masses and roots (Figure 1c). Rocha et al.<sup>[1]</sup> reported the inefficiency of bordeaux with indigotine blue (Q.refresko® grapes), because both egg masses and root tissues were stained, thus reducing the contrast between roots and egg masses.

Dye 4 (tartrazine with sunset yellow) only stained egg masses at 5.0% concentration (Figure 1d), giving them a light yellow color, without contrast between egg masses and roots. This dye was not tested for egg staining because of this lack of contrast with the roots. Egg masses stained with dye 5 (tartrazine with brilliant blue) were colored green only at the highest concentration (Figure 1e).

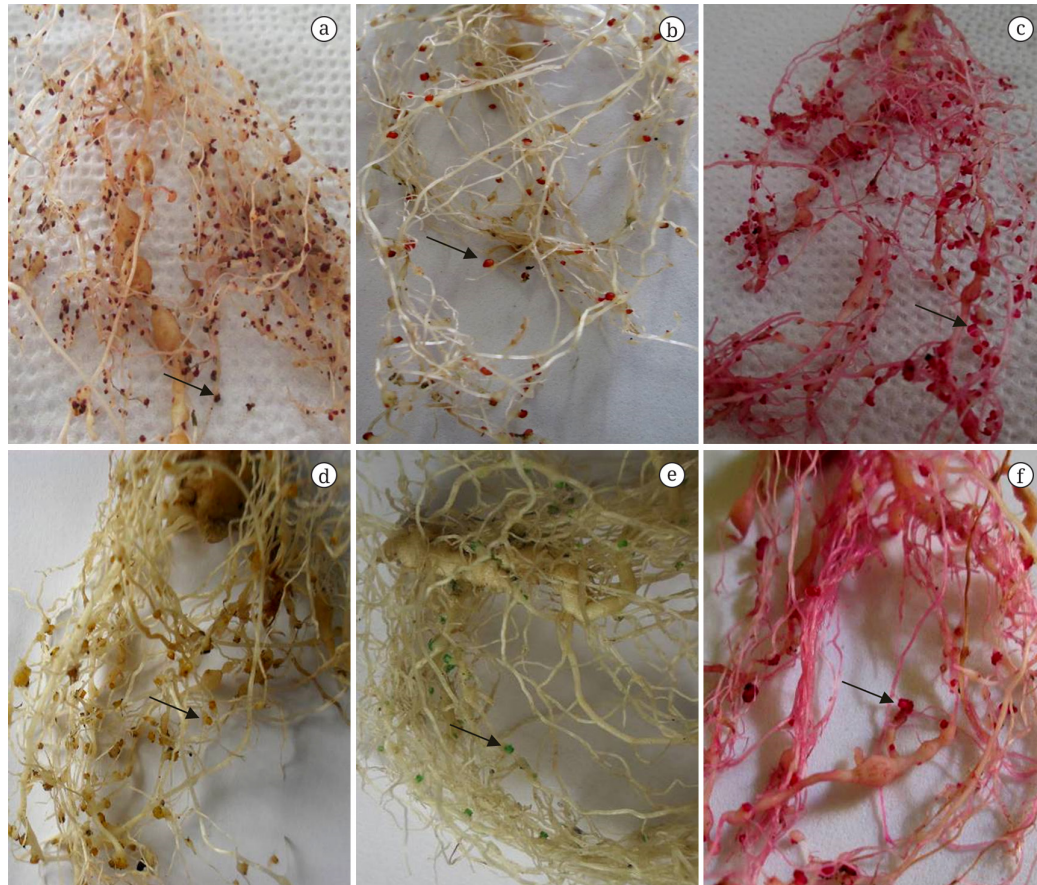
### *Meloidogyne javanica* egg staining

For egg staining, dyes 1 (ponceau 4R with red 40) and 2 (ponceau 4R with brilliant blue), at concentration of 4.0%, stained with the same intensity as obtained with acid fuchsin (Figure 2a, b). However, eggs did not stain with dyes 3 (erythrosine with bordeaux and brilliant blue), 4 (tartrazine with sunset yellow) and 5 (tartrazine with brilliant blue FCF) (Figure 2c).

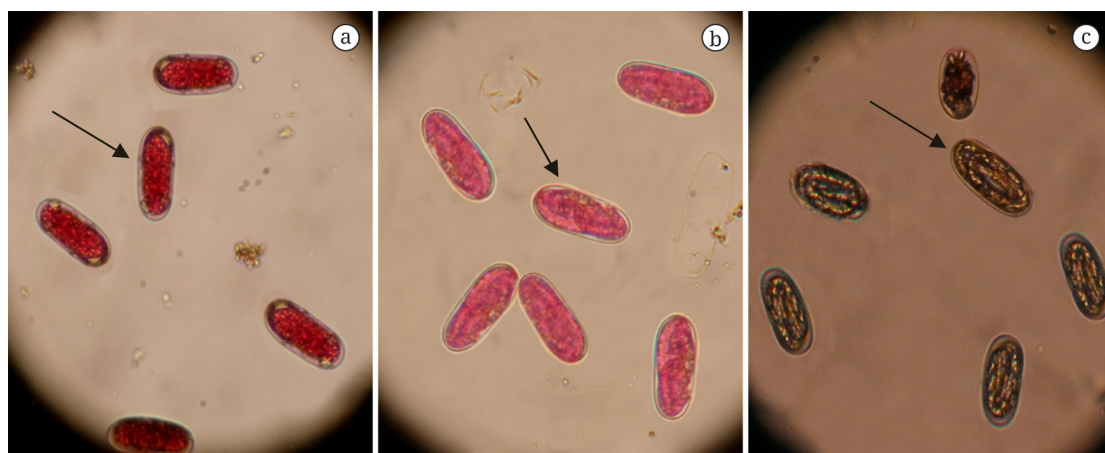
Rocha et al.<sup>[1]</sup> developed a staining method which consisted of a mixture of juice dyes for staining *M. incognita* eggs, egg masses, juveniles and adults, and found that only the dyes containing bordeaux were efficient. These dyes are easier to purchase in common supermarkets and are less expensive than acid fuchsin and phloxine B. Thies et al.<sup>[4]</sup> used meat coloring, i.e. McCormick Schilling red food color, instead of phloxine B and acid fuchsin to stain egg masses and juveniles of *M. incognita* in pepper roots (*Capsicum annuum* L.). These authors stained egg masses and juveniles for 15 minutes, with



concentrations of 10% to 20% and 12.5%, 33% and 50%, respectively. The meat dye at the concentration of 20% stained *M. incognita* egg masses in a similar manner as did phloxine B. *Meloidogyne incognita* juveniles stained with this dye at 12.5%. Although the meat coloring dye offered good results, it is difficult to purchase in local markets.



**Figure 1.** Egg masses of *Meloidogyne javanica* in tomato roots stained with different food dyes. Tomato plants grown for 40 days in plastic pots under greenhouse conditions. (a) Stained with dye 1 (ponceau 4R with red 40) - concentration of 1.0%; (b) stained with dye 2 (ponceau 4R with brilliant blue) - concentration of 1.0%; (c) stained with dye 3 (erythrosine with bordeaux and brilliant blue) - concentration of 5.0%; (d) stained with dye 4 (tartrazine with sunset yellow) - concentration of 5.0%; (e) stained with dye 5 (tartrazine with brilliant blue) - concentration of 5.0% and (f) stained with phloxine B. The arrows indicate the stained egg masses.



**Figure 2.** Egg of *Meloidogyne javanica* in tomato roots stained with different food dyes. Tomato plants were grown for 40 days in plastic pots under greenhouse conditions. (a) stained with dye 1 (ponceau 4R with red 40) - concentration of 4.0%; (b) stained with acid fuchsin, and (c) eggs that did not stain with dye 3 (erythrosine with bordeaux and brilliant blue). The arrows indicate the stained eggs.

Food dyes 1 and 2, both with ponceau 4R, gave good staining contrast and durability, and can be reused for staining purposes, with less waste being generated.

## CONCLUSIONS

Dye 1 (ponceau 4R with red 40) and dye 2 (ponceau 4R with brilliant blue) are effective for staining *M. javanica* egg masses and eggs. These dyes can substitute phloxine B and acid fuchsin used in the standard staining methods for egg masses and eggs of root-knot nematodes, respectively. Moreover, these two dyes are easily found on the market. In addition, they do not cause human health hazards and can be reused for staining procedures, with less waste being generated at the laboratories.

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