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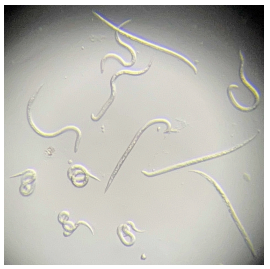
# *In vitro* compound toxicity protocol for nematodes



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**We use this protocol and it's working**

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## Abstract

Nematode bioassays form a useful approach to evaluate the toxicity of chemical compounds of interest. Such evaluations are important for confirming a compound's effectiveness and the duration thereof. In medicine, it is essential to know the minimally effective dose of a drug compound to supply so that overdosing may be prevented. In agriculture, nematode bioassays are crucial tools for conducting efficacy and safety assessments before any soil application of novel chemicals, thereby avoiding the build-up of hazardous waste. Nematodes are known as bioindicators of soil health status and can be very sensitive to soil modifications. Changes in soil composition and structure will affect the types and species of nematodes that inhabit a patch of soil, making them useful indicators for testing the effects of distinct compounds. Bioassays allow observation of changes in nematode behavior as well as changes in their mobility and mortality. *Caenorhabditis elegans*, the model nematode, has been used to observe its responses to exposure to different compounds. Other bioassays have employed plant-parasitic nematodes such as stubby root nematodes (e.g., *Trichodorus* and *Paratrichodorus* spp.) and root-knot nematodes (*Meloidogyne* spp.).
*In vitro* assays can be used to perform rapid analyses in the laboratory. These tests can help reveal the molecular and cellular mechanisms of a compound’s toxicity through changes in nematode behavior, mobility, and death when working with juveniles. When working with adult nematodes, effects on nematode egg hatch can also be analyzed.

## Image Attribution

Juveniles of the second stage of *Meloidogyne incognita* displaying coiled phenotype and death phenotype (straight) after 72 hours of exposure to 15 mM of ouabain octahydrate.

## Guidelines

This protocol has been developed to evaluate *in vitro* the effect of a purified compound on second-stage juveniles (J2s) of the southern root-knot nematode, *Meloidogyne incognita*.

## Materials

Purified compound (in this example, ouabain octahydrate (Millipore Sigma, Cat. No. O3125) was used)
Sucrose solution 35%
Bleach 10%
$H_2O$ (running water)
Scale
Glass dish with a mesh insert
Microsyracuse watch glasses
Hooks to pick up nematodes
Glass dish with a mesh insert
1,000 mL micropipette
Tips for 1,000 mL micropipette
Safety wash bottle
Plastic beaker
Tissue paper
Second-stage juveniles (J2s) of *Meloidogyne incognita* (in this example, nematodes were obtained from an infected tomato (*Solanum lycopersicum*) plant.

## Safety warnings

Remember to take precautions before working with bleach, such as wearing closed-toe shoes, appropriate clothing, protective goggles, and gloves.

## Before start

Before beginning, be aware that the materials and the compound to use are available.

During the 10% bleach preparation, consider modifying the bleach concentration for the cleaning based on the level of the active ingredient of the bleach brand used.

Consider preparing the stock solution of the compound you will use in advance. Prepare aliquots of the stock, keep them separated, and have one on hand to use.

## PREPARATION OF THE LABORATORY SPACE

1 Clean around the bench or workspace with 70% ethanol and lay tissue paper near the work area.

## PROCEDURE

2 Extract nematode eggs from infected plant roots and prepare egg suspensions in 15-mL Falcon tubes (for a protocol, see Godinez-Vidal et al., 2024). In this example, we use population Project 77 (pathotype/race 3) of *M. incognita*.

3 Prepare 100 mL of 35% sucrose solution by dissolving 35 g of sucrose in 60 mL $H_2O$ and then adjusting to 100 mL.

4 Spin the egg suspensions at 2,000 rpm for 5 min at room temperature.

5 Decant the supernatant in the plastic beaker. Keep the pellet.

6 Fill Falcon tubes to 13.5 mL with the 35% sucrose solution. Mix with a glass stirring rod and let the tubes stand for 2 min.

7 Add ~1.5 mL water to the top of each tube using a safety wash bottle (slowly drip the water along the wall of the tube to create a top layer of water). Use a scale to balance the tubes (the eggs will float to the water layer) (Note 1).

8 Spin the tubes at 1,200 rpm for 5 min at room temperature.

9 Harvest the egg-containing layer (white layer) and transfer the eggs into a new Falcon tube. Do this by using a 1,000 mL micropipette and performing the pipetting with a spinning motion (Fig. 1).

10 Immediately dilute the sucrose by filling the tube containing the eggs to the 15-mL marker with fresh water using a safety wash bottle and then spin the tube at 2,000 rpm for 5 min.

11 Decant the water/sucrose mixture, keep the pellet, and refill the tube with water (Note 2).

12 Immediately fill the tube containing the eggs to the 15-mL marker with fresh water using a safety wash bottle and spin at 2,000 rpm for 5 min. Rinse the eggs twice.

13 Clean the eggs with 10% bleach by gently inverting the tube (rather than shaking) for 5 min.

14 Rinse the eggs twice with running water (Fig. 2).

15 Hatch the eggs by setting them up in a glass dish with a mesh insert and tissue paper.

16 Incubate the eggs in the dark at 28°C for five days. Then, quantify the J2 nematodes that hatched from the eggs.

17 Prepare the compound; in this example, ouabain was dissolved in water, and a 100 mM stock solution was prepared (Note 3).

18 After hatching, count the nematodes and set up 100 J2s into the microsyracuse watch glasses containing solutions with the desired concentrations of the compound to be tested. In this example, ouabain concentrations of 0 mM (water control), 5 mM, 10 mM, and 15 mM were tested.

19 The trial starts when the nematodes are added to the compound solutions (exposure of the nematode starts then). Incubate the nematodes at 28ºC in dark conditions.

20 Observe changes in the behavior of the nematodes and evaluate the compound's effects on J2s after 24, 48, and 72 hours of exposure.

21 Take images of the nematodes. In this example, a Leica Microscope was used with objective 10X/0.5 Plan M and a Nikon DS Camera Head. Nikon NIS Elements Imaging Software can be used to process images.

22 Confirm effects of the compound by removing the solutions from the microsyracuse watch glasses and replacing it with fresh water.

23 Observe the recovery of the nematodes at 24 hours after adding fresh water. Quantifications of any nematostatic or nematicidal effects can now be made.

## NOTES

24 1- This step needs to be performed quickly to avoid too much osmotic pressure on eggs.

2- Note that eggs can be re-extracted by repeating the sucrose and water resuspension steps until the white layer disappears. For this, refill the tube with 13.5mL sucrose solution and add 1.5mL water. Re-spin at 1,200 rpm and harvest the remaining eggs.

3- Be aware that the compounds to be tested should be dissolved in water to avoid any interference effects from other compounds, such as solvents (methanol, ethanol, propanol, EDTA, SDS, etc.) on the nematodes. If it is necessary to dissolve a stock of a compound in a solvent other than water, then the compounds should be dissolved in the lowest amount of solvent possible, and the resulting solution can then be adjusted with water to prepare the stock solution. In this case, a control solution at the same solvent:water ratio (but without the compound of interest) should be included in the assay. This second control (in addition to water control) will be helpful in verifying whether the solvent might have interactive effects with the compound on the nematodes.

## Protocol references

Mwangi NG, Stevens M, Wright AJD, Edwards SG, Hare MC, and Back MA. 2024. Sensitivity of stubby root nematodes (*Trichodorus* and *Paratrichodorus* spp.) to isothiocyanates associated with Brassicaceae in an *in vitro* assay. Nematology 26:2. DOI: 10.1163/15685411-bja10302

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