

Video Article

High and Low Throughput Screens with Root-knot Nematodes *Meloidogyne* spp.

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Abstract

Root-knot nematodes (genus *Meloidogyne*) are obligate plant parasites. They are extremely polyphagous and considered one of the most economically important plant parasitic nematodes. The microscopic second-stage juvenile (J2), molted once in the egg, is the infective stage. The J2s hatch from the eggs, move freely in the soil within a film of water, and locate root tips of suitable plant species. After penetrating the plant root, they migrate towards the vascular cylinder where they establish a feeding site and initiate feeding using their stylets. The multicellular feeding site is comprised of several enlarged multinuclear cells called 'giant cells' which are formed from cells that underwent karyokinesis (repeated mitosis) without cytokinesis. Neighboring pericycle cells divide and enlarge in size giving rise to a typical gall or root knot, the characteristic symptom of root-knot nematode infection. Once feeding is initiated, J2s become sedentary and undergo three additional molts to become adults. The adult female lays 150-250 eggs in a gelatinous matrix on or below the surface of the root. From the eggs new infective J2s hatch and start a new cycle. The root-knot nematode life cycle is completed in 4-6 weeks at 26-28°C.

Here we present the traditional protocol to infect plants, grown in pots, with root-knot nematodes and two methods for high-throughput assays. The first high-throughput method is used for plants with small seeds such as tomato while the second is for plants with large seeds such as cowpea and common bean. Large seeds support extended seedling growth with minimal nutrient supplement. The first high throughput assay utilizes seedlings grown in sand in trays while in the second assay plants are grown in pouches in the absence of soil. The seedling growth pouch is made of a 15.5 x 12.5cm paper wick, folded at the top to form a 2-cm-deep trough in which the seed or seedling is placed. The paper wick is contained inside a transparent plastic pouch. These growth pouches allow direct observation of nematode infection symptoms, galling of roots and egg mass production, under the surface of a transparent pouch. Both methods allow the use of the screened plants, after phenotyping, for crossing or seed production. An additional advantage of the use of growth pouches is the small space requirement because pouches are stored in plastic hanging folders arranged in racks.

Video Link

The video component of this article can be found at <http://www.jove.com/video/3629/>

Protocol

1. Tomato Seedling Growth in Pots and Trays

1. For pot assays, plant tomato seeds in a single pot in an organic-rich soil such as Sunshine mix. Maintain in a greenhouse at 22-28°C. After germination, fertilize once a week with Miracle-Gro.
2. About two weeks after germination, at the two true-leaf stage, transplant individually the seedlings into pots (10 cm diameter and 17 cm deep) filled with sterile sandy soil containing 90% sand and 10% organic mix. Add Osmocote slow release fertilizer and maintain in a greenhouse at 22-28°C for two weeks. Continue to fertilize plants once a week with Miracle-Gro.
3. For high-throughput screens, plant seeds directly in trays in sandy soil, cover the tray with plastic wrap until germination and maintain as above. After germination, add Osmocote slow release fertilizer and fertilize once a week with Miracle-Gro.

2. Cowpea Growth in Seedling Pouches

1. Seeds are either germinated in a Petri dish, lined with several layers of Kimwipe paper, and transferred singly to pouches or placed directly into the paper groove of the pouch and germinated.
2. Place the pouches in a plastic hanging file folder, two per folder, and arrange the folders in a rack in a vertical position. Place the rack in a controlled-environment chamber maintained at a temperature of 25-28 °C and 16 h light / 8 h dark cycle. Racks also can be maintained in a greenhouse, but require a foil or stiff paper cover placed over the rack on both sides of the plant stems, to reduce potential for fungal contamination.
3. Water the pouches once or twice per day with reverse osmosis water. About 10 to 14 days after seeding, when an adequate root system with tertiary root tips has developed (Figure 1), the seedlings are ready for inoculation.

3. Extraction of Nematode Eggs



Extraction of eggs from infected roots is modified from a protocol developed by Hussey and Barker (1973).

1. Three to four days before nematode inoculation, extract root-knot nematode eggs from infected tomato roots. Before starting egg extraction, wash the work area thoroughly with hot water to avoid contamination. Also wash in hot water a blender, two buckets, a wire mesh support, a rubber mallet, three sieves of 425, 90 and 25 μm aperture, a graduated cylinder and scissors.
2. Stack the sieves from top to bottom in the following order: 425, 90 and 25 μm aperture. The eggs will be collected on the 25 μm sieve at the bottom. Put the sieves on a wire mesh supported in a sink. Put a bucket under the wire mesh to collect the run-through solution.
3. Cut the tops of the infected plant(s), used as the source of the inoculum, and discard. Carefully remove the plant from the pot. Wash the roots by dipping in a plastic bucket full of water. Rinse the roots further under running water until the soil particles are washed away from the roots.
4. Cut the roots with scissors into small pieces. Dispose of the taproot.
5. Put the chopped roots from a single plant in a large plastic jar with a lid, add just enough 10% bleach solution to cover the roots and close the lid.
6. Shake the jar containing the roots for 2 min.
7. Open the jar and pour the roots onto the top sieve and wash with a hose attached to a misting nozzle. Wash well until all the bleach smell is gone. Use a mallet to tap the sides of the sieves to avoid clogging the sieve pores.
8. Remove the top sieve and rinse the debris on the second sieve.
9. Remove the second sieve and collect the fine debris, which includes the eggs, from the last sieve. With a gentle stream from a water bottle, move the debris to one side of the sieve. Collect the debris and eggs into a clean beaker with minimum amount of water.
10. Sieve once more the water collected in the bucket through the 25 μm sieve to collect any eggs that may have escaped. Rinse well with water and collect in the same beaker.
11. Discard the plant debris from the top sieve and wash thoroughly all three sieves with pressurized water. Do not touch the mesh part of the sieves as it may distort the pore size.

4. Hatching Nematode Eggs

1. Line a clean metal basket with a few layers of Kimwipe paper and fit on a glass Petri dish. Allow a 1-cm distance between the bottom of the basket and the dish.
2. Pour the extracted nematode eggs onto the paper in the wire basket. Add enough liquid so that the bottom of the wire basket touches the water surface but is not immersed in water. Cover the top with a plastic lid.
3. Every day check for water evaporation and add some water in the Petri dish so that the bottom of the basket touches the water. This will prevent the eggs from drying out.
4. Every other day, collect the water that contains the J2s from the Petri dishes into a beaker. If not used immediately, aerate the collected inoculum at room temperature using a lab air supply or air generated by an aquarium pump. Use the aerated inoculum within 2 days. J2s may be collected from the hatching set up over a period of 6-8 days.

5. Tomato Root Infection in Pots or Trays and Evaluation of Infection

1. Use three aliquots of the collected nematodes to count the number of J2s in a counting slide or dish, calculate the average and determine the desired volume for inoculation. Typically 3000 J2s are used per plant in pots and 500 J2s for plants grown in seedling trays.
2. Stir the inoculum on a magnetic stirring plate at low speed.
3. Before you inoculate the plants, make sure that the soil is moist but not too wet. For pot inoculation, make three holes of about half-pot depth in the sand around each tomato root system using a pencil (**Figure 2**). Inoculate each plant by delivering the J2s into the three holes using a pipette. Afterwards, cover the holes.
4. For the high-throughput screens in trays, use a modified sealed tip needle with holes on the sides glued to a 5-ml pipette tip along a pipetter (**Figure 3**) to deliver the J2s into the soil. Alternatively, nematodes can be delivered as in step 5.3.
5. Maintain the plants in a greenhouse at 24-27°C for six to eight weeks. Continue to fertilize twice a month with Miracle-Gro.
6. For evaluation of infection, carefully remove the plants from the pots and wash the roots (as described in step 3.3).
7. Stain the egg masses blue by submerging the roots in 1 mg/L erioglaucine, for 15 min.
8. Rinse the roots in water and evaluate by counting the stained egg masses on individual root system. An illuminated desk magnifier can be used to help visualize the egg masses.
9. If the screened plants are needed for further genetic studies, do not cut the tops before washing the roots for evaluation. After staining and counting the egg masses, transplant the seedlings in organic soil, prune the tops heavily to reduce transpiration, and maintain in a greenhouse.

6. Cowpea Root Infection in Pouches and Evaluation of Infection

1. Count the nematode inoculum and stir on a magnetic stirring plate as described earlier.
2. Remove the pouches from the hanging folders and place on a horizontal surface. Inoculate each pouch with 1500 J2s in 5 ml. Lift the plastic cover of the pouch and distribute the nematodes evenly over the surface of the roots.
3. Keep the pouches in a horizontal position for 24 h after inoculation, covered with dark paper to exclude light, then return to the hanging folders in the growth chamber.
4. Water the plants as needed, once or twice daily, with half-strength Hoagland's solution (Hoagland & Arnon, 1950; **Table 1**) until a response to the fertilizer is observed, typically enhanced foliage greening and more vigorous shoot growth. Usually the plants are watered with half-strength Hoagland 3 days in a row. Afterwards, keep the pouches moist with water.
5. Approximately 30 days (range 28-35 days) after inoculation, infuse each pouch with about 10-20 ml of 75 mg/L erioglaucine. Keep the pouches flooded with the dye in a horizontal position overnight.
6. Drain the pouches and evaluate the root systems by counting the egg masses under an illuminated desk magnifier.
7. If the screened plants are needed for further genetic studies or breeding work, carefully pull out the roots from the paper, transplant in organic soil in pots and maintain in a greenhouse.

7. Representative Results

The appropriate stages of tomato and cowpea plants for nematode inoculations for the two systems described are shown in **Figures 1** and **2**. In addition, examples of well-infected roots of tomato and cowpea are shown in **Figures 4** and **5**. As in most disease resistance screens, it is

advisable to use at least 6-10 plants per genotype for nematode inoculation to calculate the average of the infection rate. Variation in nematode infection between plants, of the same genotype, can be reduced by using uniform plant size and accurate amount and delivery of inoculum.

The use of trays and growth pouches enables screening of hundreds to thousands of plants in a small growth space. Growth pouches also allow fast and efficient non-destructive evaluation of root-knot nematode infections with no need for washing roots (**Figure 4**).

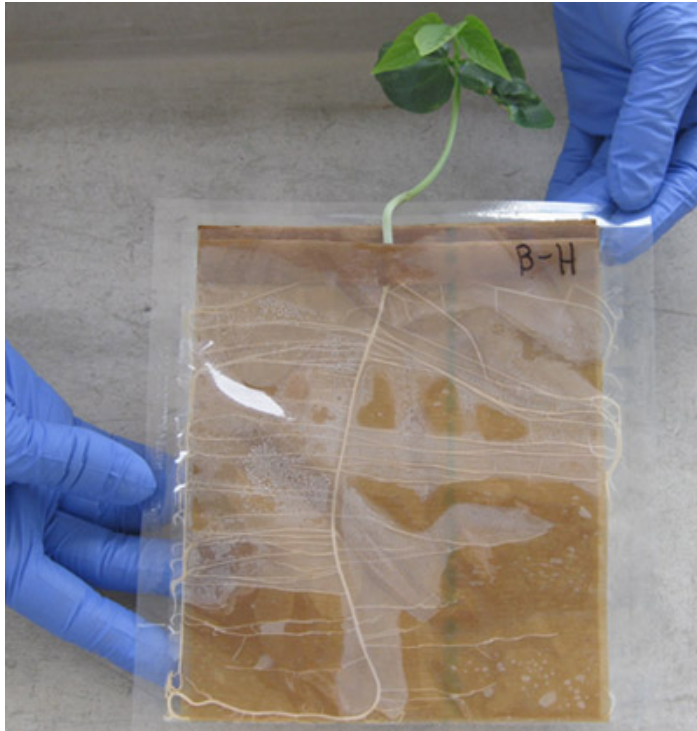


Figure 1. A two week-old cowpea plant grown in a pouch and ready for root-knot nematode inoculation.



Figure 2. A three week-old tomato plant ready for root-knot nematode inoculation.

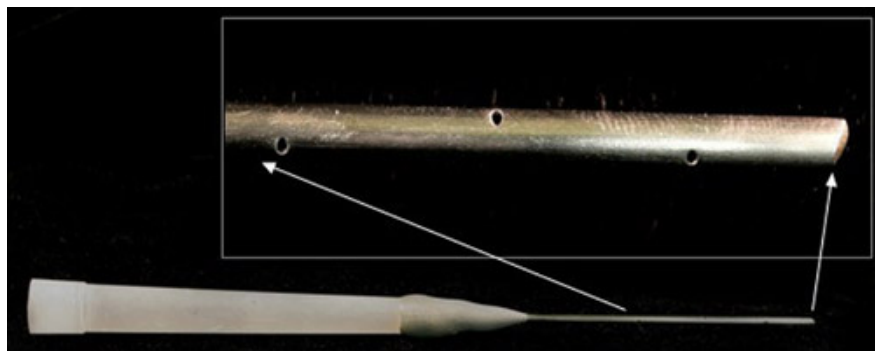


Figure 3. A modified needle and pipette tip used for high-throughput inoculation of nematodes. The bottom of a needle was sealed and three sets of holes were drilled in the needle using a laser beam. Then, the modified needle was glued to a 5-ml pipette tip.



Figure 4. A tomato root system infected with root-knot nematodes.



Figure 5. A cowpea root system with egg masses stained with erioglaucine 30 days post-inoculation grown at 28 °C.

Discussion

There are two critical steps for a successful nematode screen: preparation of a highly infective inoculum and using plants at the proper developmental stage. Hatching rate of root-knot nematode eggs is highly variable and ranges between 5- 50%. Therefore to obtain optimum levels of hatch and highly infective J2s, close attention must be paid to the egg extraction and hatching procedures. Eggs should be exposed to bleach for a minimum time period and bleach should be rinsed out well from the root mixture. When hatching the eggs, the slurry containing the eggs should not be submerged in water. In addition, avoid spilling the eggs into the underlying Petri dish where the hatched juveniles are collected. One way to avoid spilling the eggs during the hatching process is not to add water directly to the Kimwipe as the paper might break. Instead add the water directly to the Petri dish.

For best results, use freshly hatched J2s. If the hatch rate is low and more inoculum is needed, J2s can be stored at 15°C for a longer period. Warm the stored inoculum to room temperature to revive the nematodes prior to inoculation. However, do not store the J2s for prolonged periods even at 15°C as starved J2s will not infect efficiently.

Young seedlings are the best developmental stage for root-knot nematode inoculation. However, this needs to be balanced with the formation of an adequate root system providing sufficient numbers of root tips as points of entry for the J2s. Maintaining optimal plant growth condition is also critical for the screens. Avoid over-watering plants specially those grown in pouches. Over-watering the pouches, as indicated by standing water in the bottom of the pouch, can promote fungal growth and diminish root health.

With both assays further evaluation of nematode infection and calculation of the number of eggs/root system and eggs/gram of fresh root can be performed. For the tomato assays, individual roots are weighed and eggs extracted as described for inoculum preparation (section 3). When processing large number of plant samples, individual root systems could be macerated using a blender for egg extraction. The number of eggs should be counted in at least three aliquots and the eggs/gram of fresh root system calculated. For the pouch assays, the root system is pulled off the paper insert, weighed, and eggs extracted.

Disclosures

We have nothing to disclose.

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