In vitro rearing of Pratylenchidae nematodes on carrot discs

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In vitro rearing of Pratylenchidae nematodes on carrot discs.

Abstract — Introduction. This rearing technique is applicable to migratory nematodes (*e.g.*, *Pratylenchus* spp. and *Radopholus similis*) for mass production of nematodes for experimental purposes, conservation of nematodes, and direct studies of nematodes' multiplication or reproduction mechanisms. The principle of the method applied, key advantages, starting plant material and time required are presented. **Materials and methods**. Necessary laboratory materials, and details of the nine steps required for the preparation of carrot discs, preparation of nematodes, inoculation of nematodes and collection of nematodes out of petri dishes are described. Possible troubleshooting is explained. **Results**. Several thousands of nematodes can be extracted out of one carrot disc after 6–8 weeks of culture, depending on nematode species and geographical population.

France (Guadeloupe) / *Musa* sp. / plant nematodes / methods / extraction / laboratory equipment / rearing systems

Élevage *in vitro* de nématodes Pratylenchidae sur disques de carotte.

Résumé — Introduction. Cette technique d'élevage s'applique aux nématodes migrateurs (par exemple, aux espèces de *Pratylenchus* et à *Radopholus similis*) pour une production de masse des nématodes à des fins expérimentales, pour leur conservation, et pour des études de leur multiplication ou de leurs mécanismes de reproduction. Le principe de la méthode appliquée, les principaux avantages, le matériel végétal nécessaire et le temps requis sont présentés. **Matériel et méthodes**. Le matériel de laboratoire nécessaire, ainsi que le détail des neuf étapes nécessaires à la préparation des disques de carotte, la préparation des nématodes, l'inoculation des nématodes et la récupération des nématodes hors des boîtes de Pétri sont décrits. Les problèmes potentiels sont répertoriés. **Résultats**. Plusieurs milliers de nématodes peuvent être extraits à partir d'un disque de carotte après 6-8 semaines de culture, selon l'espèce et la population considérées.

France (Guadeloupe) / *Musa* sp. / nématode des plantes / méthode / extraction / matériel de laboratoire / système d'élevage

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1. Introduction

Application

This technique is applicable to migratory nematodes (*e.g.*, *Pratylenchus* spp. and *Radopholus similis*) for:
– mass production of nematodes for experimental purposes (inoculation on banana plants, biological and genetic diversity studies, etc.),
– conservation of nematodes (collection of isolates – nematotheque),
– direct studies of nematodes' multiplication or reproduction mechanisms.

Principle

Phytoparasitic nematodes can only develop in fresh root tissues. This method allows migratory nematodes to be reared on sterilised carrot discs in petri dishes placed in

an incubator rather than in roots of plants under greenhouse conditions. It has been adapted from the technique described by O' Bannon and Taylor [1].

Fresh carrots are superficially disinfected, then sliced into 0.5-cm discs. They are placed in small petri dishes.

Nematodes are inoculated on the carrot disc. The recommended temperature for rapid multiplication is 27 °C. Under these conditions, populations must be sub-cultured every 6–8 weeks depending on their reproduction rate and on the carrot disc decay. Temperature may be increased up to 30 °C to increase the development rate (for nematode mass production) or decreased down to 20 °C to maintain the populations on the same carrot disc for a longer time (conservation).

After a while (4–8 weeks depending on species, isolates and storing temperature), nematodes will be visible on the petri dish wall. They can be washed out directly with sterile water. For exhaustive collection, nematodes must be extracted from carrot discs. To obtain a viable inoculum, it is recommended to extract the nematodes using an incubation technique [step 9].

Key advantages

This technique allows one to save space since a great number of populations may be maintained in one incubator, and to save time since it allows a rapid mass production of nematodes.

Starting material

The technique requires:
– fresh carrots, medium sized (about 2 cm in diameter), free of any lesion,
– nematodes extracted from root samples, *e.g.,* using an incubation technique for the initiation of culture, or nematodes obtained from former carrot disc culture for a sub-culturing objective.

Time required

About 1 h is necessary to prepare 30–40 petri dishes ready for nematode inoculation; 1 h for nematode sterilisation and preparation (for six different populations); 30 min for inoculation on carrot discs in 30–40 petri dishes.

2. Materials and methods

Laboratory materials

The method requires a laminar flow hood, petri dishes (35 mm in diameter), Parafilm, a Bunsen burner, alcohol (95%), a sterilisable iron punch (15 mm in diameter), dihydrostreptomycin (0.2%), $HgCl_2$ (at 0.01%; *caution*: $HgCl_2$ is a very toxic product, and must be handled according to safety rules), sterile water, a centrifuge with 6 mL × 10 mL tubes, a suction pump, an aerating pump (aquarium type).

3. Protocol

Preparation of carrot discs

• Step 1
Carrot disinfection under the laminar flow hood:
– Take the carrot and plunge it into 95% alcohol for a few seconds.
– Pull it out and flame it rapidly (Bunsen burner).

• Step 2
Prepare carrot discs under the laminar flow hood:
– Cut and discard the two ends of the carrot.
– Chop the middle part into 5-mm-thick slices.
– With a sterilised iron punch, cut the heart of each carrot slice to obtain regular discs of 15 mm in diameter.
– Place one carrot disc into a petri dish (35 mm in diameter).
– Fix the cap of the petri dish with Parafilm to avoid water loss and drying of the carrot disc.

• Step 3
Check carrot contamination:
– Store the prepared petri dishes in an incubator maintained at 27 °C. Leave them there

for at least 1 week to detect any fungal or bacterial contamination.

Note: take care to prepare at least 1.5 times more petri dishes than those expected to be inoculated.

Preparation of nematodes

• Step 4

Prepare the suspension of nematodes:

– Pour the suspension of nematodes extracted from banana roots or obtained from former carrot disc culture into a test tube.

– Let it decant for at least 1 h.

– Reduce the volume of water to 5 mL by placing the tube of the suction pump at the surface of the water and progressively following the lowering of the water's surface. *Caution:* operate very carefully to avoid any agitation of the water and of the decanted nematodes.

– Agitate the remaining 5 mL volume of water to put the decanted nematodes in suspension and homogenise their distribution.

– Collect it with a pipette and pour it into a 10-mL centrifuge tube.

– Centrifuge at 250 *g* for 3 min.

• Step 5

Under the laminar flow hood, disinfect the suspension of nematodes:

– Discard the supernatant and pour in 5 mL of the $HgCl_2$ (0.01%) solution, and agitate.

– Centrifuge at 250 *g* for 3 min.

– Discard the supernatant and pour in 5 mL of sterile water; agitate.

– Centrifuge at 250 *g* for 3 min.

– Discard the supernatant and pour in 5 mL of the dihydrostreptomycin (0.2%) solution; agitate.

– Centrifuge at 250 *g* for 3 min.

– Discard the supernatant and pour in 5 mL of sterile water; the suspension is ready for inoculation.

Inoculation of nematodes

• Step 6

Check the petri dishes which were stored in the incubator the week before (see step 3); discard all petri dishes with any trace of contamination on the carrot disc.

• Step 7

Under the laminar flow hood, remove the Parafilm, open the petri dishes and inoculate with nematodes:

– Collect 0.5 mL or less of sterile nematode suspension with a pipette and spread it on the carrot disc.

– Place the cap on the petri dish and fix it with Parafilm (see step 3).

Note 1: except for specific experimental purposes (*e.g.*, study of reproduction rate), the nematode inoculum does not need to be standardised precisely. However, it is recommended to inoculate at least 10 nematodes on one carrot disc and not to inoculate more than 50 of them.

Note 2: for a given population, it is recommended to inoculate at least three (ideally five) replicates (= petri dishes). For mass multiplication, it is better to multiply the number of replicates than to increase the number of inoculated nematodes on one carrot disc (see note 1).

Nematodes collected out of petri dishes

• Step 8

Simply wash out the nematodes swarming outside the carrot disc in the petri dish with sterile water.

• Step 9

For a more complete collection, extract the nematodes from the carrot disc (or plant roots for culture initiation):

– Dilacerate the carrot disc (or plant roots) into small pieces.

– Place the carrot fragments on a cellulose or nylon gauze (*e.g.*, cellulose handkerchief).

– Place the gauze on a small sieve with large mesh aperture (1–2 mm).

– Place the sieve in a beaker and fill with water until carrot discs are completely immersed (*figure 1*).

– Let the nematodes migrate freely through the cellulose or nylon gauze and the sieve meshes, for 24–48 h.

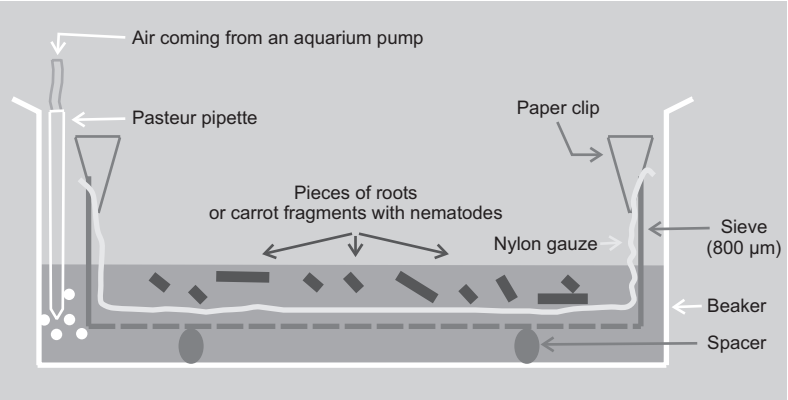

Figure 1.
Device allowing the collection of nematodes from carrot fragments after their inoculation in petri dishes.

– Remove the sieve out of the beaker and pour the water containing the nematodes in a tube or a beaker.

Note: water must be permanently aerated using an aerating pump (*e.g.*, aquarium pump) to keep the nematodes alive and highly mobile.

Troubleshooting

Two main problems can occur:

(a) There is a rapid decay of carrot discs, which can be because carrots were stored too long, there was an internal contamination of carrots, or nematodes were not disinfected enough.

Solution: use fresh carrots and proceed carefully according to the preparation steps described.

(b) No or limited development of nematodes are observed. Three reasons can be advanced:

– Inoculation was achieved with wounded or dead nematodes.

Solutions:

• avoid forced and traumatic extraction of nematodes such as centrifugal flotation [2],

• check regular water aeration during the extraction process (step 9),

• check nematode vitality before inoculation, by observing a control sample under a binocular microscope.

– Carrots were stored too long.

Solution: use fresh carrots.

– Carrots used were dried.

Solution: make sure the Parafilm tightly covers the connection between the petri dish and its cap.

4. Typical results obtained

Several thousands of nematodes can be extracted out of one carrot disc after 6–8 weeks of culture, depending on nematode species and geographical population.

References

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