[Research Note]**Protocol for root-knot nematode culture by a hydroponic system and nematode inoculation to *Arabidopsis***

Hidetaka Nishiyama¹, Bui Thi Ngan¹,
Satoru Nakagami¹, Chica Ejima¹, Takashi Ishida¹
and Shinichiro Sawa^{1,*}

Root-knot nematodes (*Meloidogyne incognita*) are one of the most economically damaging nematodes; thus it is important to know the molecular mechanisms involved in the phases of nematode infection. To use this nematode as a material for research, we must constantly prepare sufficient numbers of nematodes, preferably sterilized. Here we have developed alternative methods for culture, sterilization, and inoculation of root-knot nematodes that are especially suitable for the use of *Arabidopsis thaliana* Heynh as host plants. Nematol. Res. 45(1), 45-49 (2015).

Key words: hydroponic culture system, *Meloidogyne incognita*, protocol, root-knot nematode

INTRODUCTION

The root-knot nematode, *Meloidogyne incognita* (Kofoed and White), is one of the most common and economically important destructive nematode pathogens on many crops (Mai, 1985; Sasser and Freckman, 1987; Moens *et al.*, 2009). High numbers of nematode juveniles of uniform age are utilized for many research applications, including studies of molecular mechanisms involved in the nematode infection process, breeding for host resistance, nematicide screening, biochemical analyses, and biological control experiments (Lambert *et al.*, 1992; Wuyts *et al.*, 2006; Goto *et al.*, 2011; Ejima *et al.*, 2011; Kiyohara and Sawa, 2012).

Alternatives to nematode cultures have been developed. Gnotobiotic root-culture systems were useful for host-parasite interaction analysis in soil (Zuckerman and Brzeski, 1966; Koenig and Barker, 1985) and on nutrient agar medium (Huettel and Rebois, 1985; Verdejo *et al.*, 1988). These were not suitable, however, for routine

inoculum production because the infective juvenile yields were low, and maintenance of these cultures was costly and time-consuming (Lambert *et al.*, 1992). Another culture method (Dropkin *et al.*, 1969) consists of root-knot nematode-infected plants grown in gravel contained in crocks and fertilized with a circulating nutrient solution. Juveniles were collected by draining fluid from the crocks. Although this method produced inoculum of this pathogen for an extended period, the juveniles collected were of mixed age, and the yield was low. A hydroponic nematode culture system (Lambert *et al.*, 1992) enabled the ready production and retrieval of freshly hatched, infective root-knot nematode juveniles. This system, however, requires around 50-60 days from the start of setting up to collecting large numbers of second-stage juveniles (approximately 320,000 second-stage juveniles).

Furthermore, a sterilized nematode infection system enabled us to examine various aspects of molecular mechanisms without concern for the effects of various contaminating organisms. A model plant is useful for analyzing a nematode infection system, because we can utilize many genetic mutants, marker lines, or other tools. In this context, it is necessary to establish simple and effective systems for sterilizing nematodes and inoculating them into *Arabidopsis*.

This paper presents the protocol for a modified hydroponic nematode culture system to collect large numbers of infective root-knot nematode juveniles (approximately 500,000 second-stage juveniles every 2-3 days just after the system is set up), nematode sterilization, and inoculation to *Arabidopsis*.

PROTOCOL**1. Root-Knot Nematode hydroponic culture.****1.1. Tomato growth and nematode inoculation to tomato.**

1. Tomato seeds, *Solanum lycopersicum* (Dunal) "Pritz (Kaneko Seeds Co., Ltd., Japan)", are soaked in water for 1 h. A jiffy-pot (42 mm in diameter, Sakata Seed Corporation, Japan; Fig. 1A) is soaked in water until it is completely swollen. Tomato seeds are then sown into the jiffy-pot. The jiffy-pot is placed onto a 13-cm-diameter plate and wrapped (Fig. 1B) with to trap the heat and humidity needed for germination, and incubated in a growth chamber at 27°C with a 24 h light period.

2. Six day-old seedlings (Fig. 1C) are transplanted into a 19-cm-diameter pot filled with autoclaved soil. The pots are placed in a growth chamber at 27°C with a 24 h light period and are watered daily

¹ Graduate School of Science and Technology, Kumamoto University, 2-39-1, Chuo-ku, Kumamoto 860-8555, Japan

*Corresponding author, e-mail: sawa@sci.kumamoto-u.ac.jp

with sterile tap water.

- When the plants have grown for 5 to 6 weeks, approximately 20,000 *M. incognita* J2 (Fig. 1D) are inoculated for the first time onto the surface of the soil. The *M. incognita* strain used here was isolated from Koshi (Kumamoto, Japan) and maintained in the NARO Kyushu Okinawa Agricultural Research Center.
- Such inoculations are repeated at 3-day intervals, for a total of 6 times (approximately 120,000 J2 are thus applied to each tomato plant).

1.2. Hydroponic system construction

To construct the hydroponic system (Fig. 2A), the chamber is made by modifying a 3 L polypropylene Buchner funnel (160 mm in diameter, Tokyo Glass Kikai, Japan), as described below.

- The plastic grid covering the bottom of the funnel top is removed with a hot scalpel.
- The funnel top and bottom are then welded together with plastic glue.
- Silicone tubes (18 × 24 mm; 12 × 18 mm; 6 × 10 mm in diameter, ARAM, Japan) and tube connectors (VL12186; E-M; T-2, SANSYO, Japan) are assembled. The connectors are attached to the bottom of the funnel for producing an air inlet and for draining (Fig. 2B).
- The silicone tube connected to the funnel is secured with hose clamps, which prevent the tube from slipping.
- A pinchcock is attached to the silicone tube to be

closed when the hydroponic system is working and opened for draining the solution.

- An air pump (non-noise W300, Japan Pet Design) is connected with a silicone tube as the air line for generating air flow.
- The chamber lid of the hydroponic system is formed from a 23-cm-diameter grey acrylic disk.
- Two 4.0-cm-diameter holes near the center and one 2.0-cm-diameter hole 7.0 cm from the edge were drilled into the disk.
- An acrylic tube (40 × 34 mm in diameter, SANPLATEC, Japan) 7.0 cm long is welded onto each 4.0-cm-diameter hole with acrylic solvent-glue to create plant support tubes. An acrylic tube (20 × 16 mm in diameter, SANPLATEC, Japan) is welded onto the 2.0-cm-diameter hole with acrylic solvent-glue to function as a vent.

1.3. Nutrient solution

To prepare the sterile nutrient solution for hydroponic cultures, six stock solutions, which are described in Table 1, are individually prepared and filter-sterilized (0.45-μm filter).

To make 3 L of working solution for one hydroponic system, the stock solutions are added to 3 L of sterile water as follows: 4.5 mL of Solution 1, 1.5 mL of Solution 2, 1.5 mL of Solution 3, 1.5 mL of Solution 4, 1.5 mL of Solution 5, and 1.5 mL of Solution 6.

Fig. 1. Tomato seedling.

Jiffy-pot, before soaking in water to completely swell (A). After sowing tomato seeds, the jiffy-pot is wrapped to trap the heat and humidity for germination (B). Six day-old seedlings (C). After germination, tomato seedlings are transplanted and grown in 19-cm-diameter pots (D).

Fig. 2. Hydroponic culture system.

The chamber of the hydroponic system is constructed from a modified 3 L polypropylene Buchner funnel (A). Silicone tubes and tube connectors are assembled to attach to the bottom of the funnel for producing an air inlet and a liquid drain (B).

1.4. Hydroponic nematode culture in the hydroponic system

1. The hydroponic system is placed in a growth chamber at 25°C with a 24 h light period.
2. Two weeks after the final inoculation of *M. incognita* J2, the tomato plants are gently taken out of from the pots.
3. The tomato roots are gently washed and all soil is gently and thoroughly removed to avoid plugging the tubing in the hydroponic system.
4. Roots are cleaned by washing twice with distilled water and once with sterile water.
5. To prepare each plant for insertion into the plant support tube, 7 centimeters from the base of the stem is wrapped around with a sponge at a thickness that can be wedged into the plant support tube to prevent light and contaminants from entering the chamber and to support the plant. The roots are then wrapped in an aluminum foil sheet to compress and protect them as the plant is inserted through the support tube. After both plants have been inserted through the lid, the aluminum foil sheets are removed, and the lid with two inserted plants is placed onto the funnel.
6. The vent on the lid and the last silicone tubing part (under the pinchcock) are covered with aluminum foil sheets to prevent contaminants from entering the chamber of the hydroponic system (the modified 3 L polypropylene Buchner funnel).
7. The pinchcock is then completely locked.
8. The system chamber is filled with sterile working

nutrient solution.

9. Air flow is adjusted to a gentle bubbling.
10. At 2- to 3-day intervals, the nutrient solution is changed by draining the solution and refilling the chamber (see below).
11. Plants are pruned, and flowers are removed as needed.

2. Nematode collection

After the hydroponic system is working, the J2 nematodes are collected at 2- to 3-day intervals.

1. To collect J2s, the solution is drained into a 3 L glass beaker. The funnel is cleaned and refilled with the sterile nutrient solution. The drained solution is passed through a sterile 45-µm-pore sieve to remove large root pieces.
2. The solution is then passed through a sterile filter system with a 10-µm-filter membrane to concentrate J2s onto the 10-µm-filter membrane (Millipore, TCTP04700).
3. The filter membrane is transferred to a sterile glass plate containing 10 mL of sterile water. This will yield a small volume of water filled with J2s.
4. The solution is transferred to a sterile Kimwipe filter (made with 8 layers of Kimwipe tissues) placed on the top of a 200-mL glass beaker filled with sterile water and left for 4 h to allow the active J2s to move through the tissue into the beaker and remove small root pieces and nematode eggs.
5. After 4 h, the nematode solution is collected.

Table 1. Six stock solutions for hydroponic culture

Stock Solution	Reagent	Amount	Stock Conc.	Working Conc.
Stock Solution 1 (500 mL)	KNO ₃	92.0 g	1.820 M	2.730 mM
Stock Solution 2 (500 mL)	Ca(NO ₃) ₂ ·4H ₂ O	326.5 g	2.767 M	1.384 mM
Stock Solution 3 (500 mL)	MgSO ₄ ·7H ₂ O	199.5 g	1.619 M	0.810 mM
Stock Solution 4 (500 mL)	NH ₄ H ₂ PO ₄	54.0 g	0.939 M	0.470 mM
Stock Solution 5 (500 mL)	FeNa-EDTA	6.6 g	0.036 M	0.018 mM
Stock Solution 6 (1000 mL)	MnCl ₂ ·4H ₂ O	1810.0 mg	9.100 mM	4.550 µM
	CuSO ₄ ·5H ₂ O	100.0 mg	0.400 mM	0.200 µM
	ZnSO ₄ ·7H ₂ O	251.6 mg	0.875 mM	0.437 µM
	H ₃ BO ₃	2860.0 mg	46.278 mM	23.139 µM
	Na ₂ MoO ₄ ·2H ₂ O	26.9 mg	0.111 mM	0.055 µM

3. Nematode sterilization

1. The nematode solution is transferred to sterile 10 mL glass tubes (approximately 50,000 J2s per glass tube).
2. The glass tubes are spun at 1,000 rpm for 1 min.
3. The supernatant is discarded, keeping the nematodes at the bottom of the tube.
4. Five mL of a solution of 0.002% mercuric chloride, 0.002% sodium azide and 0.001% Triton X-100 in sterile water is added, and let stand for 10 min.
5. The nematode solution is spun at 1,000 rpm for 1 min. The supernatant is discarded and the J2s are kept at the bottom of the tube. Five mL of sterile water is added.
6. Repeat the 5th step 3 times.
7. Five mL of sterile water containing 5 µL of 1,000 ppm streptomycin is then added, and the tube is incubated at 12°C for 1 night.
8. The 5th step is again repeated 3 more times.
9. This solution is transferred to a sterile Kimwipe filter (made with 6 layers of Kimwipe tissues) placed on the top of a 100-mL glass beaker filled with sterile water and left for 2 h for the J2s to pass through the tissue.
10. A sterile filter system is prepared for use with a 10-µm-filter membrane.
11. After 2 h, the Kimwipe filter is gently removed, the J2 solution is collected and passed through a sterile filter system with a 10-µm-filter membrane to concentrate J2s onto the membrane.
12. The filter membrane is transferred to a sterile glass plate containing a small volume of water. This will yield a small volume of sterile water filled with J2s.
13. These J2s are re-suspended in sterile water and used for inoculation.

4. Nematode inoculation to *Arabidopsis*

1. *Arabidopsis* seeds are sterilized by incubation in sterile water containing 10% NaOCl and 1% Triton X-10 for 10 min, followed by 3 washes in sterile water.
2. *Arabidopsis* seeds are sown in vertical square plates (D210-16, Simport, USA) containing 30 mL of plant growth media for culturing nematodes (Hutangura *et al.*, 1998). Six *Arabidopsis* seeds are sown in each plate.
3. The plates are then incubated at 4°C for 2 days to promote even germination. Then the vertical plates are placed in a growth chamber at 23°C with a 24 h

light period.

4. Seven days after sowing of seedlings, approximately 80 sterile J2s are inoculated per plant (approximately 480 sterile J2s per plate with 6 plants) and incubated at 27°C under ambient light conditions.
5. Galls or egg-masses are produced at 2 weeks or 6 weeks post inoculation, respectively.

ACKNOWLEDGEMENTS

We would like to thank Dr. Hiroaki Iwahori (NARO/KARC) for his support in providing us with nematodes for our experiments. S. Sawa, was supported by grants from KAKENHI (23119517, 24114001, and 24114009) and the Fuji protein Foundation.

LITERATURE CITED

- Dropkin, V. H., Helgeson, J. P. and Upper, C. D. (1969) The hypersensitive reaction of tomatoes resistant to *Meloidogyne incognita*. Reversal by cytokinins. *Journal of Nematology* 1, 55-61.
- Ejima, C., Uwatoko, T., Ngan, B. T., Honda, H., Shimizu, N., Kiyohara, S., Hamasaki, R. and Sawa, S. (2011) SNPs of CLAVATA receptors in tomato, in the context of root-knot nematode infection. *Nematological Research* 41, 35-40.
- Goto, D. B., Fosu-Nyarko, J., Sakuma, F., Sadler, J., Flottman-Reid, M., Uehara, T., Kondo, N., Yamaguchi, J. and Jones, M. G. K. (2010) *In Planta* observation of live fluorescent plant endoparasitic nematodes during early stages of infection. *Nematological Research* 40, 15-19.
- Huettel, R. N. and Rebois, R. V. (1985) Culturing plant parasitic nematodes using root explants. In: *Plant Nematology Laboratory Manual* (Zuckerman, B. M., Mai, W. F. and Harrison, M. B., eds.). University of Massachusetts Agricultural Experiment Station, Amherst, MA, 155-158.
- Hutangura, P., Jones, M. G. K. and Heinrich, T. (1998) Optimisation of culture conditions for *in vitro* infection of tomato with root-knot nematode *Meloidogyne javanica*. *Australasian Plant Pathology* 27, 84-89.
- Kiyohara, S. and Sawa, S. (2012) CLE signaling systems during plant development and nematode infection. *Japanese Society of Plant Cell Physiologists* 53, 1989-1999.
- Koenig, S. R. and Barker, K. R. (1985) Gnotobiotic techniques for plant-parasitic nematodes. In: *An Advanced Treatise on Meloidogyne*. Vol. 2 (Barker,

- K. R., Carter, C. C. and Sasser, J. N., eds.), North Carolina State University Graphics, Raleigh, NC, 49-66.
- Lambert, K. N., Tedford, E. C., Caswell, E. P. and Williamson, V. M. (1992) A system for continuous production of root-knot nematode juveniles in hydroponic culture. *Phytopathology* 82, 512-515.
- Mai, W. F. (1985) Plant-parasitic nematodes: Their threat to agriculture. In: *An Advanced Treatise on Meloidogyne*. Vol. 1 (Sasser, J. N. and Carter, C. C., eds.), North Carolina State University Graphics, Raleigh, 11-17.
- Moens, M., Perry, R. N. and Starr, J. L. (2009) *Meloidogyne* species - a diverse group of novel and important plant parasites. In: *Root-knot nematodes* (Perry, R. N., Moens M. and Starr, J. L., eds.). CABI Publishing, Wallingford, 1-17.
- Sasser, J. N. and Freckman, D. W. (1987) A world perspective on nematology: The role of the society. In: *Vistas on nematology* (Veech, J. A. and Dickson, D. W., eds.), Society of Nematologists, Hyattsville, 7-14.
- Verdejo, S., Jaffee, B. A. and Mankau, R. (1988) Reproduction of *Meloidogyne javanica* on plant roots genetically transformed by *Agrobacterium rhizogenes*. *Journal of Nematology* 20, 599-604.
- Wuyts, N., Lognay, G., Swennen, R. and De Waele, D. (2006) Nematode infection and reproduction in transgenic and mutant *Arabidopsis* and tobacco with an altered phenylpropanoid metabolism. *Journal of Experimental Botany* 57, 2825-2835.
- Zuckerman, B. M. and Brzeski, M. W. (1966) Methods for the study of plant parasitic nematodes in gnotobiotic root culture. *Nematologica* 11, 453-466.

Received: July 15, 2014