The Root-Knot Nematode Culture and Nematode Inoculation to *Arabidopsis*

1. The Root-Knot Nematode Culture.

1.1. Tomato growth and nematode inoculation to tomato.

1. Tomato seeds, *Solanum lycopersicum* "Pritz" are soaked in sterile water for 1 hour. The jiffy-pot is soaked in water to completely swell. 8 tomato seeds are sown in a jiffy-pot (Fig. 1). The jiffy-pot was wrapped to trap the heat and humidity for germination and incubated in chamber at 27^{o}C with a 24h light period .

Fig. 1. Tomato seeds are sown in a jiffy-pot and grown in the 19-cm-diameter pots

2. After germination, each tomato plant is soon replanted to a 19-cm-diameter pot which is filled with autoclaved soil. Pots are placed in a growth chamber at 27^{o}C with a 24h light period and are watered daily with water (once a week with hyponex solution) .
3. 4- to 5-week-old plant is first inoculated on the surface of the soil with approximately 20,000 *Meloidogyne incognita* second-stage juveniles (J2s) produced in hydroponic culture.
4. Inoculations are repeated at 3-day intervals, then inoculation will be finished after 6 times of inoculation (with approximately 120,000 J2s have been applied to a tomato plant) (Fig. 2).

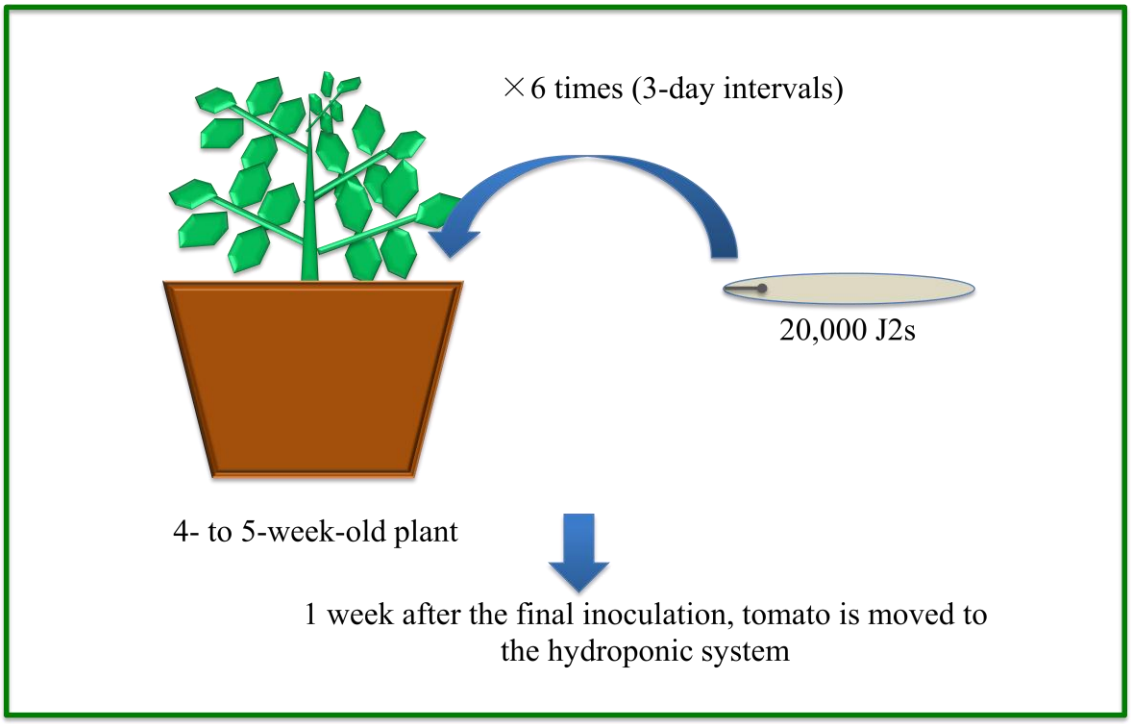

Fig. 2. Nematode inoculation to tomato

1.2. Hydroponic system construction

1. The chamber of the hydroponic system (Fig. 3) is a modified 3-L polypropylene Buchner Funnel (Lambert, K. N. et al. 1992).
2. The plastic grid covering the bottom of the funnel top is removed with a hot scalpel.
3. The funnel top and bottom are then welded together with plastic glue.
4. Silicone tubing and a T- connector are attached to the bottom of the funnel and produced an air inlet and drain.
5. The tubing connection to the funnel is secured with hose clamps, which prevented the tubing from slipping.
6. Air flow is generated by an air pump (non-noise W300). The air line from the air pump is attached to the silicone tubing of the air inlet by a plastic reducer.
7. A pinchcock is attached to the silicon tubing to be close for hydroponic system working and open for solution draining.
8. The chamber lid is a 23-cm-diameter grey acrylic disk.
9. 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.
10. A 7.0-cm-high of 4.0-cm-diameter acrylic tubing is welded onto each

4.0-cm-diameter hole with acrylic solvent-glue created a plant support tube. A 5.0-cm-high of 2.0-cm-diameter acrylic tubing is welded onto 2.0-cm-diameter hole with acrylic solvent-glue functioned as a vent.

1.3. Nutrient solution

To prepare sterile nutrient solution for hydroponic culture, the following six stock solutions (Table 1) are individually syringe filter-sterilized (0.45-μm filter).

Table 1. Six stock solutions for hydroponic culture

Stock Solution	Reagent	Amount	Stock Conc^n	Working Conc^n	Maker
Stock Solution 1 (500 ml)	KNO_3	92.0 g	1.820 M	2.730 mM	Wako
Stock Solution 2 (500 ml)	$Ca(NO_3)_2.4H_2O$	326.5 g	2.767 M	1.384 mM	Wako
Stock Solution 3 (500 ml)	$MgSO_4.7H_2O$	199.5 g	1.619 M	0.810 mM	Wako
Stock Solution 4 (500 ml)	$NH_4H_2PO_4$	54.0 g	0.939 M	0.470 mM	Wako
Stock Solution 5 (500 ml)	FeNa.EDTA	6.6 g	0.036 M	0.018 mM	Sigma
Stock Solution 6 (1000 ml)	$MnCl_2.4H_2O$	1810.0 mg	9.100 mM	4.550 μM	Wako
	$CuSO_4.5H_2O$	100.0 mg	0.400 mM	0.200 μM	Wako
	$ZnSO_4.7H_2O$	251.6 mg	0.875 mM	0.437 μM	Wako
	H_3BO_3	2860.0 mg	46.278 mM	23.139 μM	Wako
	$Na_2MoO_4.2H_2O$	26.9 mg	0.111 mM	0.055 μM	Wako

To make 3 L of working solution (for one hydroponic system), the stock solutions are added to the 3 L of sterile water:

4.5 ml Solution 1
1.5 ml Solution 2
1.5 ml Solution 3
1.5 ml Solution 4
1.5 ml Solution 5
1.5 ml Solution 6

Fig. 3. The Hydroponic culture system

1.4. Hydroponic nematode culture

1. Hydroponic system is placed in a growth chamber at 25^{o}C with a 24-h light period.
2. 1 week after the final inoculation, tomato plants are gently taken out of from the pots.
3. Tomato roots are gently washed and soil is gently and thoroughly removed to avoid plugging the tubing in the hydroponic system.
4. Roots are cleaned by washing with ddH_2O for two times and with sterile water for one time.
5. 7.0 cm from the stock of the stem is wrapped around in a sponge and wedged into the plant support tube to prevent light and contaminants from entering the chamber and to support the plant. Roots are wrapped in an aluminum foil sheet to compress and protect them and inserted them through the support tube. Then the plant is inserted through the lid, the aluminum foil sheet is removed, and the lid with the 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 sheet to prevent contaminants from entering the chamber of the system.
7. The pinchcock is completely locked.
8. The chamber of the system (3L polypropylene Buchner funnel) 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 filling the funnel.
11. Plants are pruned, and flowers are removed as needed.

2. Nematode Collection

After the hydroponic system working, at 2- to 3- day intervals, second-stage juveniles (J2s) are collected.

1. To collect J2s, stop the air flow working, open the pinchcock, the solution is drained into a 3L glass beaker, and the funnel is cleaned and refilled by the sterile nutrient solution. The drained solution is passed through a sterile 45-μm-pore sieve that removed large root pieces.
2. Then solution is passed through a sterile filter system with 10-μm-filter membrane to concentrate J2s on 10-μm-filter membrane.
3. Transfer the filter membrane to a sterile glass plate. This will yield a small volume of water filled with J2s.
4. Transfer this solution to a sterile Kimwipe filter (made with 8 layers of Kimwipe tissue) placed on the top of 200-ml glass beaker filled with sterile water. Leave it for 4 hrs for the active J2s move through the tissue into the beaker and remove small root pieces and nematode eggs.
5. After 4 hours, tissue is gently removed, and collect the J2s.

3. Nematode Sterilization

1. Collect nematodes, J2s from hydroponic culture system.

All steps from here onwards should be conducted in the clean bench!

2. Transfer the nematodes to the sterile filter system (use the 10-μm filter membrane) and wash thoroughly with ～800 ml sterile water.
3. Transfer the filter membrane to a sterile glass plate. This will yield an enough volume of solution containing J2s for the 6^{th} step (Fig. 4).

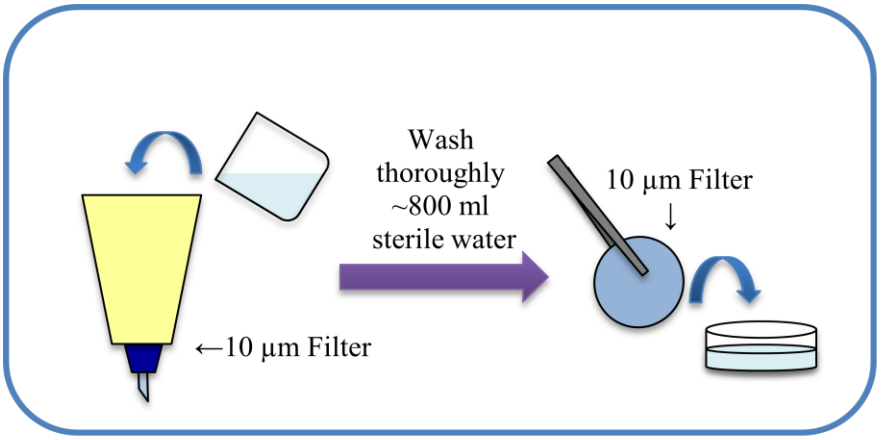

Fig. 4. Nematodes are washed by using sterile filter system (Step 2~3)

4. Insert 4 ml of 10% sucrose into a sterile 10ml glass tube.
5. Add 3 ml of 50% sucrose slowly from the bottom. Make sure not to mix the gradient.
6. Tilt the sucrose gradient to its side and gently top up with 3 ml nematode solution that we got from 3rd step.
7. Spin the sucrose gradient at 3500 rpm for 15 minutes. Make sure it is balanced inside the centrifuge.
8. After spinning, there will be 6 layers, with 2 prominent white layers (fig. 5). Proceed to the rinsing step as fast as possible.

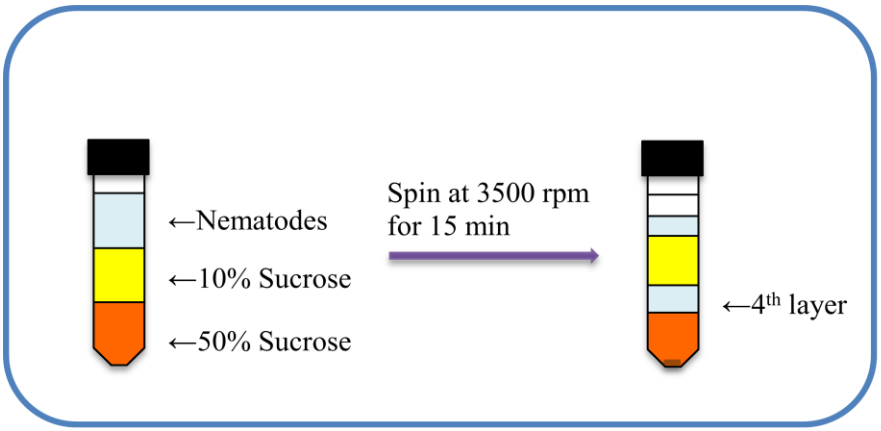

Fig. 5. After spinning, there will be 6 layers, nematodes are 4th layer (Step 4~8)

9. Discard the top three layers and transfer the 4th layer (2nd white layer from the top) to a sterile plate.
10. Prepare the sterile filter system and use the 10-μm filter membrane.
11. Rinse the filter system with sterile water first, to prevent nematodes from sticking to the filter membrane during the rinsing step.
12. Transfer the nematodes to the filter and wash thoroughly with sterile water (～800 ml).
13. Transfer the 10-μm filter membrane to a sterile plate. This will yield a small volume of water filled with J2s.
14. Transfer this solution to a sterile Kimwipe filter (made with 6 layers of Kimwipe tissue) placed on the top of 100-ml glass beaker filled with sterile water and leave it for 2 hrs for the J2s to pass through.
15. After 2 hours, Kimwipe tissue is gently removed, collect the J2s and wash it once again with sterile water (～800 ml) using the 10-μm filter membrane.
16. Transfer the 10-μm filter to sterile plate and pass the J2s through another sterile Kimwipe filter. Leave it for 2 hrs.
17. Collect the J2s that pass through the Kimwipe filter and wash it with sterile water (～800 ml) using the 10-μm filter membrane.
18. Transfer the 10-μm filter membrane to a sterile glass plate. This will yield a small volume of water filled with J2s (Fig. 6).
19. Re-suspend J2s in sterile water and use for inoculation.

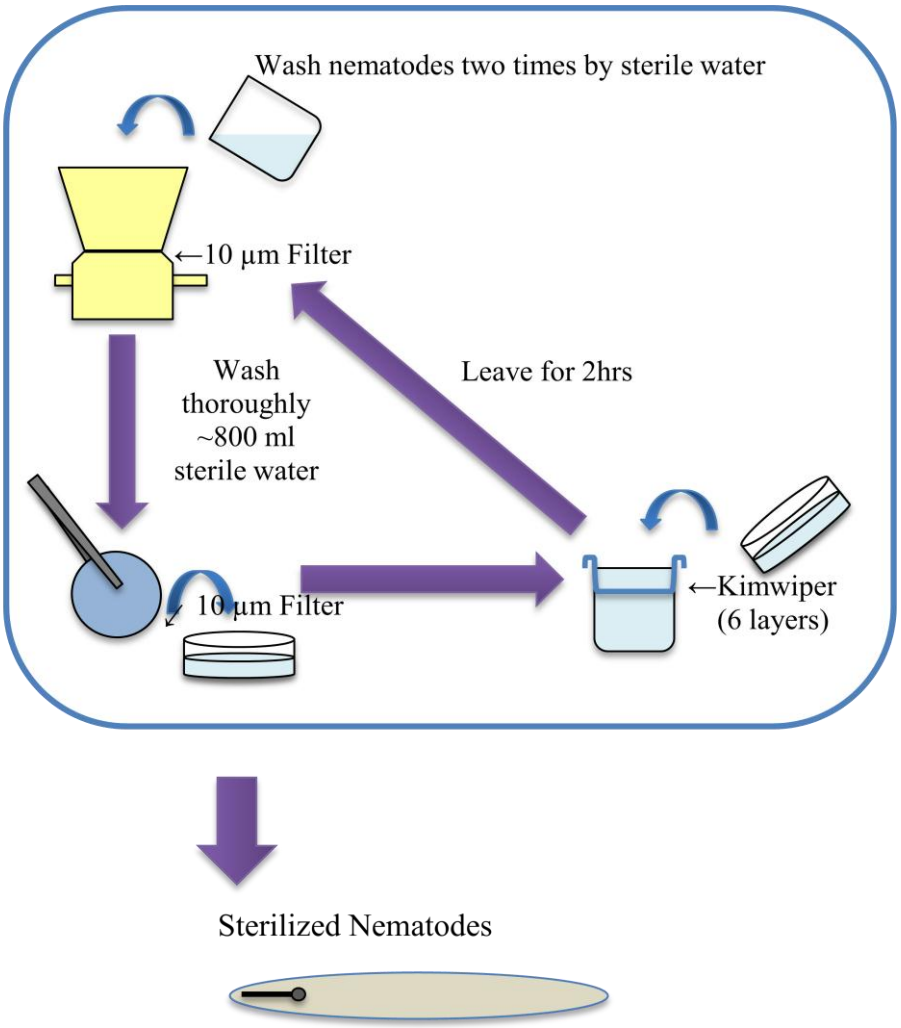

Fig. 6. Nematodes are washed by using sterilize filter system and passed through the Kimwipe filter for 2 times (Step 10~18)

4. Nematode Inoculation to *Arabidopsis*

1. *Arabidopsis* seeds are sterilized by using a 1.5-ml tube containing 1ml solution (NaClO, 100 μL; 10×Triton-X, 10 μL; Sterilize water, 890 μL) for 10 minutes. Then *Arabidopsis* seeds are washed by sterilize water for 3 times.
2. Sterilized *Arabidopsis* seeds is sown in vertical square plate containing 30 ml of plant growth media for culturing nematodes (Hutangura, P. et al. 1998) (Table 2). Six *Arabidopsis* seeds are sown per plate.

Table 2. The plant growth media (500 ml) for culturing nematodes

Reagent	Amount (g)	Final Conc^n	Maker
MS powder	0.55	1/4X	Sigma
Sucrose	2.5	0.5%	Wako
Adjust to pH6.4 with 0.1N KOH			
Phytagel	3.0	0.6%	Sigma
Adjust to 500 ml with ddH_2O and autoclave			

(*Note for making the media: Phytagel clumps very easily, so add slowly to rapidly stirring medium*).

3. After sowing *Arabidopsis* seeds, the plates are incubated for 2 days at 4^{o}C to promote even germination. Then the vertical plates are placed in a growth chamber at 22^{o}C with a 24h light period.
4. At 7 days after sowing (das), seedlings are inoculated with ～120 sterile J2s per plant (～720 J2s per plate with 6 plants) and incubated at room temperature (27^{o}C) under ambient light conditions.
5. Roots are observed at 2 weeks and 6 weeks post inoculation.

References

1. Hutangura, P., Jones, M.G.K., 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.
2. 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.