A highly sensitive infection assay method for second-stage juveniles of root-knot nematodes using a one-week-old seedling

Toyoshi Yoshiga

A rapid and highly sensitive assay method to evaluate the infectivity of *Meloidogyne incognita* second-stage juveniles (J2s) is reported. Among the substrates to grow a seedling in a plant culture tube, nursery soils composed of small granular soil particles were the best both in root growth and ease of substrate removal from the root. As a host plant, balsam and cucumber were considered the most appropriate host plants for the assay in the test tube among the 6 plant species. Twenty J2s individuals were inoculated onto a 1-week-old balsam or cucumber seedling in a culture tube containing granular nursery soil, and nematodes in the root system were stained by the acid fuchsin method 1 week after inoculation. The number of infected nematodes increased and then plateaued at seven days after inoculation in both types of seedlings. To demonstrate the efficiency of this method, 1-week-old balsam seedlings were infected with fosthiazate-treated J2s in the presence of fosthizate and dose-dependent infective suppression was observed in the treated J2s. This method enables us to use a small number of nematodes as an inoculum for infection assays and to complete the assay within two weeks from the sowing of seeds. Nematol. Res. 44 (2), 37-42(2014)

Key words: balsam, cucumber, infectivity, *Meloidogyne*

Root-knot nematodes (RKNs, *Meloidogyne* spp.) are endoparasitic nematodes that cause serious damage to agricultural production by not only decreasing crop yields but also reducing crop quality all over the world (Sasser, 1980). Second stage juveniles (J2s) of RKNs enter the elongation zone of roots near the root tips and migrate into the root to establish their feeding site (Wyss et al., 1992). In addition to the direct damage caused by the parasitism of RKNs, the entry of roots by J2s and subsequent plant tissue modification due to nematode infection facilitates the infestation and propagation of other soil pathogens, resulting in severe damage to the plants (Karssen et al., 2013).

One of the most efficient approaches toward the control of nematodes is to disrupt nematode infection. The mechanisms of controlling infection behaviors such as recognition of host root, orientation to the invasion site, stylet probing, and secretion of cell-wall degrading enzymes at the site, are poorly understood. One of the approaches to clarify the mechanisms is pharmaceutical treatment of nematodes, followed by the infection assay, using chemicals such as biogenic amines and their agonists/antagonists. In addition, a reverse genetic approach such as RNA interference (RNAi) (Fire et al., 1998) that targets the knockout of specific genes of nematodes, followed by the infection assay, allow us to understand the mechanism necessary for infection at a molecular level. However, it is often difficult to prepare the large number of treated nematodes needed for these analyses because the amount of chemicals or molecules used for treatment is often limited because of the price and/or availability. In addition, it is critical to use a highly sensitive method for a detailed evaluation of treatment effects on nematode infection. Thus, a small scale and highly sensitive assay method is necessary.

Pot experiments using seedlings such as tomatoes are typically used for infection assays in RKNs, but there are several limitations with this method. It takes several weeks to grow plants even in a small pot to be ready for nematode inoculation. It is necessary to have a designated area to grow plants. A large number of nematodes have to be prepared for infection. Even after preparations for inoculation, the infection rate is usually low. For example, infection rate was less than 13% at day 7 when a tomato seedling in a 220 ml plastic pot with sand was inoculated with 200 J2s (Fujimoto et al., 2011). Using 4-week-old tobacco seedlings, infection rate was approximately 30% at day 7 (Hu et al., 2013). Moreover, it is difficult to collect the whole root system because roots are easily removed and sometimes lost when the soil attached to the roots is washed off, which can result in a low detection rate. A different type of small scale infection assay was performed for the analysis of RNAi-treated RKNs using adzuki plants grown in pouches but the infection
rate was only approximately 30% even 21 days after inoculation (calculated percentage from the results of Shingels et al., 2007). Pluronic gel is used for the in vitro attraction/infection assay for J2s of Meloidogyne spp. (Wang et al., 2009) but the gel has been reported to have adverse effects (Ko and Van Gundy, 1998), thus caution is necessary especially when nematodes and plants are treated with different kinds of chemicals. The aim of this paper was to report a highly sensitive infection assay method for Meloidogyne incognita J2s using a balsam or cucumber seedling in a culture tube with nursery soil.

MATERIALS AND METHODS

Nematodes:

M. incognita (Nishigoshi strain) (Sano et al., 2002) was maintained with Solanum lycopersicum (Pritz: Kaneko Seeds Co., Ltd.) (mini-tomato) that was grown in a growth chamber at 27°C (16 hr light/8 hr dark). The J2s hatched out from egg masses within 48 hr and were collected on a 5-µm sieve, washed with deionized water three times on the sieve and used for the assays. Substrates for plant seedling:

River sand, glass beads (GMB-80, Nippon Rikagaku Kikai, Japan), and nursery soils (‘Kenbyō’, Yae Nogei Co., Ltd., Japan; Golden Ryujyou baiyoudo, Iris Ohyama, Japan; Jiffy mix, Sakata Seed Corporation, Japan) were compared as a potential substrate for the infection assay using a balsam or cucumber seedling. A seed of balsam (Impatiens balsamina, Tohoku Seed, Co., Ltd., Japan) or cucumber (Haya midori, Tohoku Seed, Co., Ltd., Japan) was placed onto the bottom of a plant culture tube (25 mm diameter, 100 mm height; Iwaki, AGC Techno Glass Co. Ltd.). To remove any M. incognita J2s was inoculated onto the surface of the nursery soil particles of a 1-week-old seedling using a glass disposable Pasteur pipette (5 3/4 inches length; Iwaki, AGC Techno Glass Co. Ltd.). To remove any J2s still attached to the inside of the pipette after the initial inoculation, approximately 100 µl of deionized water was drawn up by the same pipette and inoculated onto the same seedling. This last step was repeated, resulting in the inoculation of a total of 300 µl aliquot of the J2 suspension. A new Pasteur pipette was used for each seedling. The inoculated seedlings were

| Name     | Species             | Variety                  | Company                      |
|----------|---------------------|--------------------------|------------------------------|
| Balsam   | Impatiens balsamina | Tsubakisaki yae tokusen kongou | Utane Seed Co., Ltd., Japan |
| Carrot   | Daucus carota       | Kuroda gosun ninjin      | Atariya Noen Co., Ltd., Japan |
| Cucumber | Cucumis sativus     | Haya midori              | Tohoku Seed, Co., Ltd., Japan |
| Cucumber | Cucumis sativus     | Tokiwa kazemidori        | Tohoku Seed, Co., Ltd., Japan |
| Cucumber | Cucumis sativus     | Yume midori              | Tohoku Seed, Co., Ltd., Japan |
| Egg plant| Solanum melongena   | Seiguro nakanaga nasu Shiki | Tohoku Seed, Co., Ltd., Japan |
| Okura    | Abelmoschus esculentus | Gokaku okura          | Tohoku Seed, Co., Ltd., Japan |
| Tomato   | Solanum lycopersicum | Kyouryoku beijyu        | Takii & Co., Ltd., Japan     |
| Tomato   | Solanum lycopersicum | Pritz                   | Kaneko Seeds Co., Ltd., Japan |
incubated in a growth chamber.

Before staining, the roots were carefully washed with tap water in a small plastic container to remove all soil particles without losing any roots, and each entire root system was processed with acid fuchsin stain (Byrd Jr. et al., 1983). Nematodes were observed and counted under a stereomicroscope.

Fosthiazate treatment:
The sensitivity of the assay method was demonstrated by evaluating the effects of the organophosphate fosthiazate on the nematode. Fosthiazate standard (066-03381, Wako Pure Chemical Industries, Ltd., Osaka, Japan) was dissolved first in a small amount of methanol and then in Milli-Q water to make a 100 μg/ml solution from which serial dilutions were prepared. Fosthiazate solution was mixed with an equal volume of nematode suspension in a 3- or 6-cm Petri dish to a final concentration of 0, 0.1, or 1 μg/ml and the suspension was then placed in 25 °C dark conditions for 24 hr. Concurrently, 2 ml of fosthiazate solution of 0, 0.1, or 1 μg/ml was added to a tube containing a 1-week-old balsam seedling, completely soaking the roots of the seedling in the solution. After 24 hr of soaking, as much of the solution as possible was removed using a Pasteur pipette. The plant growth did not appear to be harmed by soaking in the fosthiazate solution (unpublished observation). Twenty J2s that were treated with fosthiazate for 24 hr were inoculated as an approximately 50 μl aliquot to an unwashed fosthiazate-treated seedling. The same concentration of plain fosthiazate solution (not containing nematodes) was used to rinse the nematodes from the pipette making the total inoculation volume of nematode suspension 150 μl per seedling. The

![Fig. 1](image_url)

A. Seedlings in a culture tube seven days after sowing. A plant culture tube (25 mm diameter, 100 mm height) contains 1 g of sieved nursery soil for sowing (Kenbyo, Yae Nogei Co., Ltd., Japan) and 600 μl of distilled water, and the tube was capped with a plastic lid. The tube containing a seed was incubated in a growth chamber at 27 °C (16 hr light/8 hr dark) for seven days. a, c: balsam seedling; b, d: cucumber seedling; a, b: side view of a culture tube; c, d: bottom view of a culture tube. B. Acid fuchsin staining of roots of seedlings and nematodes 1 week after nematode inoculation. Whole root systems of a balsam (e) or cucumber (f) seedling after acid fuchsin staining were mounted on a glass slide and covered with a glass coverslip (24 × 50 mm). An enlargement of a part of a balsam root with accumulated nematodes (arrow head) is shown (g).
inoculated seedlings were placed in the growth chamber for 1 week and the nematodes in the roots were stained as previously described. Experiments were repeated twice.

**RESULTS AND DISCUSSION**

Comparison among substrates:

River sand and glass beads were easily removed from the roots but root growth was poor in these substrates. Although root growth was good when nursery soils contained organic substances such as vermiculite and peat moss ('Golden Ryujyou baiyoudo', Jiffy mix), it was difficult to remove these organic substances from the roots without tissue loss. Nursery soils composed of small granular soil particles, such as 'Kenbyo', were among the best both in root growth and ease of substrate removal from the root. Thus, 'Kenbyo' was selected for further experiments as a substrate.

Comparison among seedlings:

Root growth of carrot, eggplant, tomato seedlings was slow and poor in one week. Okura grew too fast and the plant culture tube was too small for its seedling. Among the possible host plants tested for this assay, balsam and cucumber were considered the most appropriate; roots of these seedlings spread relatively well at the bottom of the tube within 1 week of sowing (Fig. 1A) and these seedlings can readily be maintained in the culture bottle for 2 weeks. There was no difference among cucumber varieties. Therefore, these seedlings were chosen as the host plant for the study.

Infection assay using balsam and cucumber seedlings:

When 1-week-old balsam seedlings were inoculated with 20 J2s, a mean of 4.8 nematodes (approximately 20% of the inoculated J2s) was detected in the root.

**Fig. 2** Change in the number of infected nematodes in seedling roots after nematode inoculation. Twenty J2s were inoculated onto a 1-week-old balsam (A) or cucumber (B) seedling and infected nematodes were counted on different days after inoculation. The numbers in parentheses indicate the total number of replicates. Bars represent standard deviation.

**Fig. 3** Infectivity of fosthiazate-treated nematodes. The J2s were soaked in 0, 0.1, or 1 µg/ml fosthiazate solution for 24 hr and 20 J2s were inoculated onto a 1-week-old balsam seedling in the presence of fosthiazate. The numbers in parentheses indicate the total number of replicates. Bars represent standard deviation. Experiments were repeated twice and the results were separately represented as Experiment 1 and Experiment 2.
system 3 days after nematode inoculation, and it increased to 10.4 (approximately 52% of the inoculated J2s) per seedling by day 7 (Fig. 2A). When 1-week-old cucumber seedlings were used for the assay, a mean of 8.3 inoculated nematodes (approximately 41% of the inoculated J2s) was detected 3 days after nematode inoculation, which increased to 14.6 (approximately 72% of the inoculated J2s) by day 7 (Fig. 2B). The number of infected nematodes did not significantly change after day 7 on either type of seedling. The infection rate at day 7 in this method appears to be much higher than that of conventional methods, which is less than 30% (Fujimoto et al., 2011; Shingels et al., 2007). A 7-day-incubation period appeared to be sufficient to evaluate nematode infectivity when these types of seedlings were used. Infection rates were slightly higher when cucumber seedlings were used, but the roots of balsam seedlings were thicker compared with those of cucumber (Fig. 1B); thus, making balsam easier to handle. Nematodes tended to accumulate in the same area of the balsam seedling roots (Fig. 1g). There was no difference in efficiency between the seedlings during acid fuchsin staining and subsequent nematode observation, which suggest that both seedlings are equally appropriate for this assay method.

Infection assay using fosthiazate:

Fosthiazate-soaked J2s were inoculated onto 1-week-old balsam seedlings in the presence of fosthiazate to evaluate the sensitivity of this infection assay method. When J2s were soaked in 1 µg/ml fosthiazate solution for 24 hr, only 1.3% of J2s showed undulatory movement and infection was strongly inhibited in the presence of fosthiazate: complete inhibition was observed in Experiment 1 by 1 µg/ml treatment but a mean number of 0.6 nematode/seedling was detected (one or two nematodes in 3 tubes out of 7) in Experiment 2 (Fig. 3). In contrast, although more than 95% of J2s soaked in 0.1 µg/ml for 24 hr showed undulatory movement in the solution, only mean numbers of 1.2 and 2.2 of inoculated J2s caused infection in Experiment 1 and Experiment 2, respectively (Fig. 3). These results suggest that nematode undulatory movement does not always correlate with nematode infectivity. Fosthiazate is an acetylcholinesterase-inhibitor. Low doses of acetylcholinesterase-inhibiting carbamate nematicides disrupt chemoreception in plant-parasitic nematodes, which is a result of the general mechanism of action of these nematicides at low doses (Winter et al., 2002). Low infectivity of J2s soaked in 0.1 µg/ml in this study could have been caused by a similar mechanism.

Conclusions: The new infection assay method presented in this study has many advantages. The assay can be completed within two weeks after sowing. Using small culture tubes enables increased replications under identical conditions in limited space such as a growth chamber. The infection rate is high because they have been inoculated onto roots of a seedling in a small culture tube. Therefore, the infection assay method described in this study is useful for evaluating the infectivity of M. incognita J2s after various treatments.

ACKNOWLEDGEMENTS

I thank Dr. H. Iwahori at NARO Kyushu Okinawa Agricultural Research Center, Kumamoto, Japan, for providing the M. incognita Nishigoshi strain.

LITERATURE CITED

Byrd Jr., D. W., Kirkpatrick, J. T. and Barker, K. R. (1983) An improved technique for clearing and staining plant tissues for detection of nematodes. Journal of Nematology 15, 142-143.

Fire, A. Xu, S-Q, Montgomery, M. K., Kostas, S. A., Driver, S. E. and Mello, C. C. (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391, 806-811.

Fujimoto, T., Tomitaka, Y., Abe, H., Tsuda, S., Futai, K. and Mizukubo, T. (2011) Expression profile of jasmonic acid-induced genes and the induced resistance against the root-knot nematode (Meloidogyne incognita) in tomato plants. Journal of Plant Physiology 168, 1084-1097.

Hu, L., Cui, R., Sun, L., Lin, B., Zhuo, K. and Liao, J. (2013) Molecular and biochemical characterization of the β-1,4-endoglucanase gene Mj-eng-3 in the root-knot nematode Meloidogyne javanica. Experimental Parasitology 135, 15-23.

Karssen, G., Wesemaal, W. and Moens, M. (2013) Root-knot nematodes. In: Plant Nematology, 2nd Edition (Perry, R. and Moens, M. eds.), CABI, Wallingford, 74-108.

Ko, M. P. and Van Gundy, S. D. (1988) An alternative gelling agent for culture and studies of nematodes, bacteria, fungi, and plant tissues. Journal of Nematology 20, 478-485.

Sano, Z., Iwahori, H., Tateishi, Y. and Kai, Y. (2002) Differences in the resistance of sweet potato...
cultivars and lines to *Meloidogyne incognita* populations. Japanese Journal of Nematology 32, 77-86.

Sasser, J. N. (1980) Root-knot nematodes: a global menace to crop production. Plant Disease 64, 36-41.

Shingles, J., Lilley, C. J., Atkinson, H. J. and Urwin, P. E. (2007) *Meloidogyne incognita*: molecular and biochemical characterisation of a cathepsin L cysteine proteinase and the effect on parasitism following RNAi. Experimental Parasitology 115, 114-20.

Wang, C., Lower, S. and Williamson, V. M. (2009) Application of Pluronic gel to the study of root-knot nematode behavior. Nematology 11, 453-464.

Winter, M. D., McPherson M. J., and Atkinson, H. J. (2002) Neuronal uptake of pesticides disrupts chemosensory cells of nematodes. Parasitology 125, 561-565.

Wyss, U., Grundler, F. M. W. and Munch, A. (1992) The parasitic behaviour of second stage juveniles of *Meloidogyne incognita* in roots of *Arabidopsis thaliana*. Nematologica 38, 98-111.

Received: May 3, 2014