Development of a direct quantitative detection method for *Meloidogyne incognita* and *M. hapla* in andosol and analysis of relationship between the initial population of *Meloidogyne* spp. and yield of eggplant in an andosol

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A real-time PCR-based detection method was developed for the root-knot nematodes (RKNs) *Meloidogyne incognita* and *M. hapla* in andosol. Different numbers of second-stage juveniles (J2) were artificially added into 20 g of soil not containing *M. hapla* and *M. incognita* and then DNA was extracted from the soils. There were significant correlations ($r^2 = 0.8857$, $P < 0.05$ in *M. incognita* and $r^2 = 0.9978$, $P < 0.01$ in *M. hapla*) between the threshold cycle (Ct) values and the number of nematodes added. Next, soils were collected at transplanting time from different sites (12 plots) in a field naturally infested with *M. incognita* and *M. hapla* to measure the initial population densities. RKNs were distributed heterogeneously in the field: the initial population ranged from 0 to 24 J2/20 g soil with the Baermann method, while that of *M. incognita* and *M. hapla* from 0.6 to 713 J2 equivalent (J2eq)/20 g soil and from 0.0 to 115 J2eq/20 g soil, respectively, with the real-time PCR method. The yield was determined by the sum of commercial sized eggplants harvested for 3 months of the cultivation period. The yield decreased in the plots with an initial population of RKNs more than 2 J2/20 g soil with the Baermann method. In real-time PCR, the yields were low in the plots with the sum of initial *M. incognita* and *M. hapla* more than 128 J2eq/20 g soil. The present study established a quantification method with real-time PCR for *M. incognita* and *M. hapla* in andosol and evaluated the relationship between the initial population of *Meloidogyne* spp. and the yield of eggplant. Nematol. Res. 43(2), 21-29 (2013)

Key word: Ct value, gall index, real-time PCR, root-knot nematode

INTRODUCTION

Root-knot nematodes (RKNs), *Meloidogyne* spp., cause significant losses of yield and quality in many crops all over the world, and the annual global losses have been estimated as US\$100 billion worldwide (Oka *et al.*, 2000). There are four common species of RKN in the tropical and temperate areas: *M. incognita*, *M. hapla*, *M. arenaria* and *M. javanica* (Moens *et al.*, 2009). Among them, *M. incognita* and *M. hapla* are distributed widely in Japan, and cause damage to cash crops, fruit and root vegetables, such as cucumber, tomato, eggplant and sweet potato in Japan (Ichinohe, 1992).

The species of RKNs are separated from each other on the basis of the morphology of perineal pattern, stylet knob

shape, and the length of stylet and dorsal gland orifice (Moens *et al.*, 2009). However, it is difficult to identify species of RKNs with traditional techniques, and the identification of RKNs needs mature skills and a long time even for specialists. Thus, an alternative identification method is required. New identification methods of RKNs have been developed with some enzymes, such as esterase and malate dehydrogenase, and with DNA-based methods (Blok *et al.*, 2009). The rapid development of DNA technology has been a breakthrough for overcoming the weak points of traditional identification methods (Atkins *et al.*, 2005; Berry *et al.*, 2007). Since Madani *et al.* (2005) reported real-time PCR specific primers for quantitative purposes of the potato cyst nematode *Globodera pallida* and the sugarbeet cyst nematode *Heterodera schachtii*, many specific primers have been designed for different plant-parasitic nematodes such as *Bursaphelenchus xylophilus* (Leal *et al.*, 2007), *Pratylenchus penetrans* (Sato *et al.*, 2007), *M. incognita* and *G. rostochien-sis* (Toyota *et al.*, 2008), *M. javanica*, *P. zae* and *Xiphinema elongatum* (Berry *et al.*, 2008), *H. glycines* (Goto *et al.*, 2009) and *P. thornei* (Yan *et al.*, 2012). However, no specific primers have been developed for *M. hapla* until now.

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Andosol is the major soil type in agricultural land in Japan and the most serious damage is caused by RKNs in this soil type (Takakura, 1984). Chemical control with fumigant or non-fumigant types of nematicides is frequently used, as an insurance against nematode damage, without a survey of plant-parasitic nematodes in fields. In almost all eggplant-producing areas of the country, nematicides are routinely used for the prevention of yield losses caused by RKNs (Hagiya, 1992). To avoid such an insurance-like application of nematicides, information about the economic threshold level is essential. Economic threshold levels have been developed using the Baermann method for *Meloidogyne* spp. in carrot (Sano, 1988), burdock (Yamada, 1992), spinach (Di Vito *et al.*, 2004), potato (Vovlas *et al.*, 2005), carrot (Gugino *et al.*, 2006), eggplant (Schomaker and Been, 2006), celery (Vovlas *et al.*, 2008), and cucumber and tomato (Plant protection station, 2011). However, these economic threshold levels may be underestimated and variable. The extraction efficiency of nematodes from soil using the Baermann method is estimated at about 50%, and the method fails to extract eggs and dormant forms of nematodes (Ingham, 1994). Indeed, the Baermann method extracted only 30% of plant-parasitic nematodes recovered by the double-layer centrifugation method which extracts passive nematodes in soil as well as active nematodes (McSorley and Frederick, 2004). In addition, Den Nijs and Van Den Berg (2013) revealed that the variance of *Meloidogyne* counts was the highest for the Baermann method, and concluded that the Baermann method is not advisable for survey purposes. An alternative quantification method with real-time PCR has been developed that enables quantifying all forms of nematodes (Goto *et al.*, 2009; Min *et al.*, 2011). In fact, *M. incognita* was detected with the real-time PCR method in soils which *M. incognita* could not be detected with the Baermann method (Min *et al.*, 2012), suggesting economic threshold levels should be evaluated based on the nematode populations determined with real-time PCR. According to our previous reports, calibration curves for estimating the density of a target nematode in soil differed depending on the type of soils used (Sato *et al.*, 2010), but were similar within a single soil type, andosols (Goto *et al.*, 2010). Min *et al.* (2011) used sandy soils for quantifying *M. incognita*, but no calibration curve was developed for andosols, the major soil type in Japan, at present.

The objectives of this study were to develop a rapid quantification method using real-time PCR for *M. incognita* and *M. hapla* in an andosol and to evaluate the relationship between the initial population of *Meloidogyne* spp. and the yield of eggplant in an andosol.

MATERIALS AND METHODS

Soil and nematode:

Andosol without *Meloidogyne* spp. was collected from fields in Tokyo Metropolis, Japan, and used to make a calibration curve. The absence of *Meloidogyne* spp. was confirmed by the Baermann method and the real-time PCR method, as described below. Nematodes were purchased from the NIAS (National Institute of Agrobiological Sciences) Genebank in Japan.

Primers:

Specific primers for *M. hapla* [Mh-f (5'-ATGTTGGTACGCAGCGATTGTGTA-3') - Mh-r (5'-CAGCGGGTGATCTCGACTGA-3')] were designed based on the ITS1 sequences of *M. hapla* (AY268108), *M. incognita* (AB053484) and *M. arenaria* (AF077086) (Table 1). Specific primers for *M. incognita* used in this study were RKNf (5'-GCTGGTGTCTAAGTGTGCTGATAC-3') - RKNr [5'-GAGCCTAGTGATCCACCGATAAG-3'] reported by Toyota *et al.* (2008) (Table 1).

Primer specificity was evaluated using *M. hapla* and *M. incognita* with real-time PCR. DNA was extracted from individual J2s, according to the method of Iwahori *et al.* (2000) with slight modifications. A nematode was put into a drop of water on a glass slide, air-dried and cut with a sterile needle under a microscope. Then, 10 µl of lysis buffer (10 mM tris(hydroxymethyl)aminomethane-HCl buffer (Tris-HCl), pH 8.0, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% IGEPAL CA-630, 100 µg/ml proteinase K) was added to the nematode. The buffer with the nematode was transferred into a 200 µl tube, frozen at -85 °C for 15 min. After thawing, the sample was incubated at 65 °C for 1 h to degrade the nematode's body and then at 98 °C for 10 min to inactivate proteinase K. This solution was used as a DNA template in real-time PCR described below.

Preparation of calibration curves:

Different numbers (5, 20, 80, and 500) of *M. incognita* or *M. hapla* J2 were added to 20 g of the reference soil. Each infestation level was prepared in triplicate and DNA was extracted in duplicate using the method reported by Min *et al.* (2012). Soil specimens were air-dried at 60 °C for one night, and each of the 20 g air-dried soil samples were pulverized in duplicate with a ball mill (Mixer Mill MM400, Retsch Co., Ltd., Haan, Germany) for 2 min. Soil (0.5 g) was put into a 2-ml tube with 0.75 g of zirconia beads (0.1 mm in diameter) and 0.25 g of glass beads (0.5 mm in diameter), and 1,000 µl of lysis buffer (0.5% sodium dodecyl sulfate (SDS), 10 mM Tris-HCl, 50 mM EDTA, pH 8.0) and 100 mg

Table 1. Comparison of sequences in the positions of the primers used in this study.

<i>M. hapla</i> specific primers	Sequence in the position of forward primer (5'-3')	Sequence in the position of reverse primer (5'-3')
<i>M. hapla</i> (AY268108) ¹	ATGTTGGTA-CGCAGCGATTG---T-----AA	CAG---CGGGTGATCTCGA---CTGA
<i>M. incognita</i> (AB053484)	•AAG---A•T-TTTGTTT••CA-----GT ^{2,3}	••••••••G••••••••••
<i>M. arenaria</i> (AF077086)	•AAG---A•T-TTTGTTT••CA-----GT	••••••••G••••••••••
<i>M. javanica</i> (GQ395513)	••••AATC•-----•A-----T•	••GCA••A•C•A•CA••TATCT•TT
<i>M. camelliae</i> (JX912885)	T•••••CGT••TT•GA•••A---GGCCGG••	••••••••C••A••••••••
<i>M. graminis</i> (JN241896)	•C•••-----T•••••G•-----••	••••••••••••••••••••
<i>M. graminicola</i> (JN241866)	•AAA••T•T-TATT•GTT••T-----•C	••••••••••••••••••••
<i>M. incognita</i> specific primers	Sequence in the position of forward primer (5'-3')	Sequence in the position of reverse primer (5'-3')
<i>M. incognita</i> (AB053484)	GCT---GGTGTCT-----AAGTGT-TGCTGATAC	GAGCC---TAGTGATCCA-----CCGATAAG
<i>M. hapla</i> (AY268108)	••••••••••••••••••••G•••••••R••T•	••••••••G•••••••••••••••••••••
<i>M. arenaria</i> (AF077086)	••••••••••••••••••••G••••••••••••	••••••••G••••••••••~••••••••••
<i>M. javanica</i> (GQ395513)	••TTTC•A••TC-----GC-••••C••G•GA•T	••••AATT••••••••GCGAGAG••••••••
<i>M. camelliae</i> (JX912885)	••---A•••G••TGAACCGGGC••AA••C---•A•C•A	••••••••G••••••••~••••••••CG••
<i>M. graminis</i> (JN241896)	••••••••~••••••••••T••••••••••T•	••••••~G••••••••~••••••••G••••
<i>M. graminicola</i> (JN241866)	A••••~••••••••••G••••••••••T•	••A••~G••••••••~••••••••G••••

The *M. incognita* primers were reported by Toyota *et al.* (2008).

¹ Numbers in parentheses after the species names indicates the accession number.

² Dots indicate the same base as above.

³ Hyphens indicate deletion of the corresponding base.

of skim milk were added. The soil was bead-beaten at 5,000 rpm for 1 min two times, followed by centrifugation (13,000 × *g* for 5 min). Then, 600 µl of the supernatant was transferred to a new 2-ml tube, and 377 µl of 5 M NaCl and 270 µl of 10% hexadecyltrimethylammonium bromide (CTAB) were added to the tube. After 10 min incubation at 60 °C, 500 µl of chloroform was added, and the tube was centrifuged at 15,000 × *g* for 20 min. The supernatant (1.1 ml) was transferred to a new 2-ml tube, 500 µl of chloroform was added, and the tube was centrifuged at 15,000 × *g* for 20 min. The supernatant (1.0 ml) was transferred to a new 2-ml tube, then mixed with 600 µl of 20% polyethylene glycol 8,000 (PEG) solution (20% PEG, 1.6 mM NaCl) and centrifuged at 15,000 rpm for 20 min at 4 °C to collect DNA as a pellet. The DNA pellet was washed with 1.0 ml of 70% ethanol and centrifuged at 15,000 rpm for 5 min at 4 °C then dried using VC-15Sp (TAITEC Co., Ltd., Koshigaya, Japan) for 20 min, and suspended in 100 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The DNA was diluted 10-fold and used as a template in real-time PCR.

Real-time PCR protocol:

Real-time PCR was performed in a StepOne Real time PCR System (Life Technologies Co., Carlsbad, CA) with a final volume of 10 µl containing 2 µl of 10 times diluted template DNA, 0.4 µl of 10 µM either primers for *M. hapla* or primers for *M. incognita* and 5 µl of Fast SYBR® Green Master Mix (Life Technologies) under the manufacturer's

recommended conditions (95 °C for 10 s, (95 °C for 5 s and 60 °C for 20 s, at increasing and decreasing rates of 0.2 °C/s) for 45 cycles). A negative control was also included using distilled water instead of a template DNA. Real-time PCR was done once per each DNA extract, since replicate samples showed almost identical values in real-time PCR.

The Baermann method:

Each soil sample was mixed well, and 20 g of the sub-sample was put in triplicate on two layers of tissue paper (Kimwiper®S-200, NIPPON PAPER CRECIA Co., LTD., Tokyo, Japan) supported on a 1 mm-mesh window screen (Baermann funnel). The Baermann funnels were incubated at 25 °C for 72 h, after being filled with water to the top of the funnel. The extracted nematodes were counted using a microscope.

Field experiments:

Field experiments were conducted in 2011 in a field of Tsukuba Research Institute of Katakura Chikkarin Co., Ltd., Ibaraki, Japan, which was naturally infested with *M. hapla* and *M. incognita*. No fumigants or contact nematicides had been applied in the field since 2009. The properties of the soil were: andosol, 30% sand, 40% silt, 30% clay; LiC, pH (H₂O) 5.8, total C = 24.0 g/kg soil, total N = 3.3 g/kg soil. The field was divided into 12 plots (each 3 × 3 m). Nematode densities at transplanting were assessed using a composite soil sample collected from six sites of each plot.

The initial populations of RKNs were measured by the Baermann method and the real-time PCR method as described above. After basal fertilization (N-P₂O₅-K₂O = 20-20-20 kg/10 a), the plot soil was mixed well with a shovel. The plots were mulched with a protective plastic cover to reduce evaporation, soil erosion and weeds. Eight 1-month-old eggplants, *Solanum melongena* L. 'senryou 2', were transplanted into each plot (total number of plants = 96) and cultivated in 2011 for about 3 months from May 31 to August 24 without irrigation and pesticide application. The yield was determined by weighing all the eggplant's commercial size fruits. After cultivation, eggplant roots were carefully dug out from soil with a shovel, and carefully washed to remove the attaching soil completely. The gall index of roots was assessed per plant using Zeck's scale of 0 to 10 (0: no knots on roots, 10: all roots severely knotted or no root system) (n = 96) (Zeck, 1971). However, this scale is based on the galls caused by only *M. incognita*, *M. arenaria*, or *M. javanica*, but not by *M. hapla*.

Statistical analysis:

Statistical analysis was performed using the software Excel Statistics 2002 (Social Survey Research Information Co., Ltd., Tokyo, Japan).

RESULTS

Primer specificity:

The sequences in the position of Mh forward primer are different between *M. incognita* and *M. hapla*, while the sequences of *M. incognita* in the position of RKN primers are similar to those of *M. hapla* (Table 1). Thus, the newly

designed primers Mh for *M. hapla* did not amplify DNA from *M. incognita*, while primers RKN for *M. incognita* amplified DNA from *M. hapla* at an efficiency of as low as 15% (date not shown).

Preparation of the calibration curve:

When the andosol in Tokyo was inoculated with RKN J2 at densities of 5 to 500 individuals/20 g of soil, highly significant correlations ($r^2 = 0.8857$, $P < 0.05$ in *M. incognita* and $r^2 = 0.9978$, $P < 0.01$ in *M. hapla*) were observed between the Ct values and the number of RKN J2 inoculated into the soils (Fig. 1).

Analysis of relationship between the initial population of *Meloidogyne* spp. and yield of eggplant:

In the Baermann method, both *M. hapla* and *M. incognita* were counted as RKNs since it was impossible to distinguish the species with microscopic observation. The number of RKNs ranged from 0 to 24 J2/20 g soil. In the real-time PCR method, the numbers of *M. incognita* and *M. hapla* were separately measured using the calibration curves obtained in Fig. 1 and ranged from 0.6 to 713 J2 equivalent (J2eq)/20 g soil and from 0 to 115 J2eq/20 g soil, respectively. There was a significant correlation in the number of RKNs between the Baermann and the real-time PCR method ($P < 0.001$), although their absolute values were 26 times different (Fig. 2). There was one site in which RKNs were not detected in the Baermann method, but present at densities of 56 *M. incognita* J2eq/20 g soil and 17 *M. hapla* J2eq/20 g soil with the real-time PCR method.

Fig. 1. Relationship between the Ct value and the number of *Meloidogyne incognita* J2s (A) ($P < 0.05$) or *M. hapla* J2s (B) inoculated in andosol ($P < 0.01$).

Fig. 2. Relationship between the number of root-knot nematodes (*Meloidogyne incognita* and *M. hapla*) in andosol with the Baermann method and the sum of *M. incognita* and *M. hapla* with the real-time PCR method ($P < 0.001$).

Fig. 3. Relationship between the yields of eggplant and the initial population of root-knot nematodes (*Meloidogyne incognita* and *M. hapla*) in andosol with the Baermann method.

Fig. 4. Relationship between the yields of eggplant and the initial population of sum of *Meloidogyne incognita* and *M. hapla* (A), *M. incognita* (B), and *M. hapla* (C) in andosol with the real-time PCR method.

There was a significant correlation between the initial population of RKNs with the Baermann method at transplanting and the yield ($P = 0.007$) (Fig. 3). The yield decreased in the plots with an initial population of RKNs more than 2 J2/20 g soil.

In the case of real-time PCR, yield decreased in plots with the sum of initial *M. incognita* and *M. hapla* more than 128 J2eq/20 g soil (Fig. 4A). Based on the initial populations of *M. incognita*, yield losses were observed in plots containing over 115 J2eq/20 g soil (Fig. 4B). In contrast, there was no significant correlation between the yield and the initial population of *M. hapla* ($P = 0.2$) (Fig. 4C).

In the present study, galls were observed on all the eggplant roots in all the plots and the gall index ranged 1 to 7. It tended to increase with the initial populations of RKNs with the Baermann method, the sum of *M. incognita* and *M. hapla* and of *M. incognita* with the real-time PCR method,

Fig. 5. Relationship between the gall indices and the initial population of root-knot nematodes (*Meloidogyne incognita* and *M. hapla*) in andosol with the Baermann method.

Fig. 6. Relationship between the gall indices and the initial population of sum of *Meloidogyne incognita* and *M. hapla* (A), *M. incognita* (B), and *M. hapla* (C) in andosol with the real-time PCR method.

Fig. 7. Effect of the initial density of root-knot nematodes (RKNs) with the Baermann method on cumulative yields of eggplants in the plots.

: 0 to 2/20 g soil of the initial density of RKNs, : more than 2/20 g soil of the initial density of RKNs. Asterisks indicate a significant difference between the two treatments by Student's t-test ($P < 0.05$).

although there were no significant correlations between them ($P = 0.09$ in Fig. 5, $P = 0.42$ in Fig. 6A, $P = 0.40$ in Fig. 6B). In contrast, there were no such relations between the gall index and the initial populations of *M. hapla* ($P = 0.6$) (Fig. 6C).

Periodical change in eggplant yield:

Twelve plots were separated into two groups based on the initial densities of RKNs (0-2 and >2 J2/20 g soil) and periodical changes in the yields were calculated. There were no marked differences in the yield pattern in the initial period between the two groups, but significant differences became obvious 50 days after transplanting ($P < 0.05$) (Fig. 7).

DISCUSSION

Knowledge of the relationship between pre-plant plant-parasitic nematode population densities in soil and plant growth is essential for the prediction of yield losses caused by nematodes and for choosing the best management practices. This study developed the real-time PCR primers Mh and RKN which sensitively quantified *M. hapla* and *M. incognita*, respectively, in andosol (Fig. 1). This method is advantageous in determining nematode densities, as our previous study already reported (Min *et al.*, 2012). In the field test, RKNs were detected with real-time PCR in the soil which RKNs were not detected with the Baermann method (Fig. 2). This kind of result was in agreement with the previous study (Min *et al.*, 2012). These results further support the merits of the real-time PCR method, especially in enumerating the densities of nematodes not easily detected with the Baermann method, such as dormant or quiescent nematode forms. However, there could be some technical errors in the density estimated with the real-time PCR method, because the andosol used for preparing the calibration curves was different from the andosol used for the field study. Goto *et al.* (2010) reported that the Ct values of soils with the same density of the target nematode were not the same among three kinds of andosols, although the difference among the single soil type, andosol, was much smaller than that between two different soil types (andosol and sandy soil). In addition, the density of *M. incognita* might be overestimated. The primer set RKN amplifies DNA even from *M. hapla* at an efficiency of 15%, as this defect was already reported in a previous paper (Toyota *et al.*, 2008), indicating that 100 individuals of *M. hapla* are detected as

15 individual of *M. incognita*. For example, in a field containing 100 individuals of *M. hapla* and 15 individual of *M. incognita*, the density of *M. incognita* is estimated as 30 individuals of *M. incognita*, two times higher than the actual density. Therefore, caution is needed when quantifying *M. incognita* in fields infested with a large population of *M. hapla*. However, the density of *M. incognita* was much higher than that of *M. hapla* in the field used in this study and therefore, the false detection of *M. hapla* as a small population of *M. incognita* should not be a serious problem, since *M. incognita* would be estimated as 101.5 in a field containing 100 individuals of *M. incognita* and 10 individuals of *M. hapla*. In fields with mixed populations of *M. incognita* and *M. hapla*, the quantification of *M. hapla* using the primer set Mh is essential, since the primer set does not amplify DNA from *M. incognita* at all, enabling the quantification of only *M. hapla*.

Yield losses were observed in plots with an initial population of RKNs more than 2 J2/20 g soil measured with the Baermann method. This level is quite similar to that in a previous study in which the economic threshold level for eggplant was estimated at 1 J2/20 g soil of *M. incognita* (Schomaker and Been, 2006). In contrast, the yield in carrot decreased in fields with 20 to 30 J2/20 g soil of *M. incognita* at seeding time (Sano, 1988). In US, the damage threshold density in carrot estimated in a two-year study on *M. hapla* was 3.0 to 14 eggs/20 ml in organic soil and 12 to 38 eggs/20 ml in mineral soil (Gugino *et al.*, 2006). The yield loss in spinach was observed in soils with more than 40 eggs and J2s of *M. incognita*/20 ml soil (Di Vito *et al.*, 2004). Also, the economic threshold level in burdock, cucumber and tomato was 1.6 J2/20 g soil (Plant protection station, 2011; Yamada, 1992). Vovlas *et al.* (2005 and 2008) estimated the tolerance limits to *M. javanica* and *M. incognita* in potato and celery as 10 to 13 and 3 for eggs and J2s/20 ml soil, respectively. Collectively, the yield loss level of this study in eggplant was comparable to that in burdock, celery, cucumber and tomato, and markedly lower than that in carrot and spinach. While carrot and spinach were cultivated for two months in the previous studies, eggplant and the other vegetables were cultivated for over four months. The length of the cultivation period might cause different yield loss levels. Indeed, there were no differences in the yields in the initial period between the plots having different initial populations of RKNs, but significant differences became obvious 50 days after transplanting ($P < 0.05$) (Fig. 7). This result suggests that crops with shorter cultivation periods might show higher tolerance levels to RKNs.

In the case of real-time PCR, the yield decreased in the plots with more than 128 J2eq/20 g soil of *M. incognita* and

M. hapla or 115 J2eq/20 g soil of *M. incognita* (Fig. 4A, B). These yield loss levels were much higher than the level obtained with the Baermann method. A similar result is reported in a combination of radish and *P. penetrans*, the economic threshold level was less than 1 individual/20 g soil with the Baermann method, while 5 J2eq/20 g soil with the real-time PCR method (Sato *et al.*, 2010). This may be due to the real-time PCR method detecting eggs and dormant forms of nematodes that are not detected with the Baermann method.

The gall index tended to increase with the initial population of *M. incognita*, when the gall index was estimated with the Zeck's scale which measures galls caused by *M. incognita*, *M. arenaria*, and *M. javanica*. The gall size is different between *M. incognita* and *M. hapla*, and the former makes relatively larger galls on lateral roots (Hunt and Handoo, 2009). Thus, relatively larger galls formed by *M. incognita* might have made it difficult to see smaller galls of *M. hapla*, and therefore galls caused by *M. hapla* might be underestimated in this study.

In conclusion, we developed a rapid quantification method using real-time PCR for *M. incognita* and *M. hapla* in an andosol and evaluated the relationship between the initial population of *Meloidogyne* spp. and the yield of eggplant in an andosol.

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