AN IMPROVED METHOD FOR QUANTIFICATION OF *HETERODERA GLYCINES* IN PLANT TISSUES

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ABSTRACT

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An improved maceration method with pretreatment of freezing roots was developed for extraction and quantification of the soybean cyst nematodes (*Heterodera glycines*) in soybean (*Glycine max*) roots. The roots were frozen at −20°C and thawed for two to three cycles to soften and break the tissues before being macerated with a blender, and then centrifuged in sucrose solution. The nematodes in different life stages extracted by this method without staining can be counted under an inverted microscope or a stereomicroscope. The nematode extraction efficiency with this method was approximately 95%. The number of nematodes determined by this new method was highly correlated with that determined by counting nematodes directly from stained roots under stereomicroscope, and the accuracy was also greater. This new method is reliable, efficient, and environmental friendly for quantifying nematodes from plant roots.

Key words: extraction efficiency, freezing, *Heterodera glycines*, nematode extraction, soybean.

RESUMEN

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Se desarrolló un método mejorado de maceración de las raíces mediante la congelación previa de las mismas para la extracción y cuantificación de los quistes del nematodo de la soja (*Heterodera glycines*) en raíces de soja (*Glycine max*). Las raíces se congelaron a −20°C y descongelaron repitiéndose el ciclo dos o tres veces para ablandar y romper los tejidos vegetales antes de macerarlas en una batidora y su posterior centrifugación en una solución de sacarosa. Los diferentes estadios del ciclo de vida del nematodo extraídos mediante este método se pueden contar en un microscopio invertido o bajo una lupa sin necesidad de tinción. La eficiencia de la extracción de los nematodos mediante este método fue de aproximadamente 95%. Se encontró una alta correlación entre el número de nematodos determinados mediante este nuevo método y el conteo directo de las raíces teñidas bajo la lupa y la precisión también fue mayor. Este nuevo método proporciona resultados reproducibles, es eficiente y compatible con el medio ambiente para la cuantificación de nematodos en las raíces de las plantas.

Palabras clave: eficacia de extracción, congelación, *Heterodera glycines*, extracción de nematodos, soja.

INTRODUCTION

Soybean cyst nematode, *Heterodera glycines*, is regarded as the most serious pathogen problem in soybean production worldwide. *Heterodera glycines* infection begins with penetration by second-stage juveniles (J2), followed by development to third-stage juveniles, fourth-stage juveniles, and adult males and adult females in root tissues (Bonner and Schmitt, 1985). Determining the abundance and percentages of the different developmental stages of nematodes in the total population in plant roots is often needed in studies of nematode-host interaction (Sobczak *et al.*, 2005).

Direct counting of nematodes inside root tissues is commonly used to quantify nematode population in roots (Halbrendt *et al.*, 1992; Thies *et al.*, 2002). To improve the visualization of nematodes in plant tissues, several staining procedures have been developed using chemical stains such as lactophenol (McBeth *et al.*, 1941), acid fuchsin (Byrd *et al*., 1983), and cotton blue (Germani and Plenchette, 2004). Thies *et al.* (2002) used natural and non-toxic red food color to stain nematodes in roots, to eliminate the possibility of environmental and health risks.

However, counting nematodes in root tissues is time-consuming. Generally, stained roots are mounted on slides and individual nematodes are counted directly under a microscope. Sometimes the stained roots are placed in a dish and nematodes in the roots can be counted under a stereomicroscope without making slides (Germani and Plenchette, 2004; Pegard *et al*., 2005, Parkunan *et al.,* 2009). When a stereomicroscope is used, a combination of focus adjustment and rolling the root sample over a slide or in a dish is needed. In some cases, nematodes are clustered inside a small area of root tissue making it difficult to count them accurately, and the anatomical details required for the identification of species or developmental stages might be indistinct as a consequence of the staining (Stetina *et al.,* 1997). In addition, it is difficult to soften the hard or lignified tissues of mature root systems for slide specimens. Staining and counting is generally used for the quantification of nematodes in a small number of root samples. Large numbers of root samples may be difficult to be analyzed within a reasonable period of time with this method.

To facilitate their quantification, nematodes can be extracted from tissues. Fallis (1943) extracted parasites from tissue using a Waring blender. Taylor and Loegering (1953) introduced the maceration of root tissues with a blender to extract nematodes. Dropkin *et al.* (1960) developed a procedure using enzyme digestion to release adult females of root-knot nematodes from galled roots infected by the root-knot nematodes. Hussey (1971) used both enzymatic and mechanical maceration to release root-knot nematode females and separated the nematodes from debris by centrifugation in a 20% sucrose solution. However, the enzyme digestion method requires constant agitation of plant materials in the enzyme solution for 5 to 36 h (Stetina *et al.*, 1997). Escobar and Rodriguez-Kabana (1980) used sucrose solution floatation to separate *Radopholus similus* from mechanically macerated banana roots. To quantify potato cyst nematode juveniles, Marks and McKenna (1981) liberated the nematodes from lactophenol-treated roots into water using a blender, and separated the nematodes from the root debris by sieving.

McSorley *et al.* (1984) compared several methods for extraction of nematodes from plant roots. In most cases, blender maceration in combination with centrifugation was superior for extracting migratory stages, although other methods may also be effective for certain nematodes and hosts. However, results for the sedentary endoparasite *Meloidogyne incognita* were inconsistent (McSorley *et al.*, 1984). The number of nematodes recovered by mechanical maceration may depend on the nature of the roots, the duration of the maceration time, the carrier solution (water or NaOCl) and other factors (McSorley *et al*., 1984; Stetina *et al*., 1997).

The objectives of the present study were to 1) determine if the root tissues pretreated by freezing is feasible for extraction of *H. glycines* from soybean roots, 2) to evaluate the blending time and quantity of roots for their effects on the extraction efficiency, and 3) to compare the accuracy of the maceration-centrifugation method with direct counting of nematodes in root tissues.

MATERIALS AND METHODS

Experiment 1 – effect of pretreatment of roots on extraction efficiency

This experiment was conducted at the Southern Research and Outreach Center, University of Minnesota, USA. Nematode-infected root samples were prepared from pot culture. Soil was heated in an oven at 63°C for 8 h and then 85 g soil was placed in 100-cm^3 cone-tainers. One soybean 'Sturdy' seed was sowed at about 2 cm depth in each cone-tainer. After 1 week, four 3-cm-deep holes were made adjacent to the plant with a glass stick (0.3 cm diameter) and each plant was inoculated with 2,000 *H. glycines* J2 by pipetting 1 ml suspension of 500 J2 to each hole. The plants were maintained in the growth room at 28°C and a 16 h light photoperiod per day, and watered daily. Forty-eight hours after inoculation, the roots were harvested.

The experiment involved three treatments: 1) direct blending – the fresh roots were directly blended; 2) boiling – the roots were treated with 1.5% NaOCl for 4 min, rinsed thoroughly with tap water, and then heated with tap water to boiling prior to blending; and 3) freezing – the roots were soaked in water and frozen at –20°C for 24 h and then thawed prior to blending. An individual plant was used as a replicate, and four individuals were included for each treatment. After treatment, each sample was blended separately for 60 s in about 50 ml water in a blender (Waring 30–110 ml, Cole-Parmer). After blending, the materials were poured on a 250-µm-aperture sieve nested in a 25-µm-aperture sieve, and rinsed with tap water. The nematodes on the 25-µm-aperture sieve were collected by centrifugation in 63% sucrose solution at 1,110g for 5 min, and the nematodes in the pellicle (the floating pad) were poured on a 25-µm-aperture sieve, rinsed, collected, and counted under an inverted microscope. The nematodes retained in the pellet fraction and in the residue on the 250-µm-aperture sieve were counted under a stereomicroscope following acid fuchsin solution staining (Byrd *et al.*, 1983).

Experiment 2 – measures to reduce the number of nematodes in the pellet fraction

In experiment 1, a few nematodes were retained in the pellet fraction. Experiment 2 was aimed to find a method to reduce the number of nematodes in the pellet and increase the extraction efficiency. The experiment was conducted at the Southern Research and Outreach Center, University of Minnesota, USA. *Heterodera*

glycines-infected root samples were prepared by inoculating soybean seedlings in sand with nematode cysts. Sixty grams of fine sand was placed in a 100-cm^3 cone-tainer and a 1-week-old soybean seedling grown in vermiculite was placed on the surface of the sand. Fifty cysts of *H. glycines* in 1.5 ml water were placed around the root system and then 65 g sand was added. The inoculated plants were maintained in the greenhouse at 28°C with artificial light for 16 h per day. Two weeks after inoculation, the shoot of each plant was cut at the cotyledonary node. A label was tied to each plant with cotton string, and several plants were stained together. The roots were stained with acid fuchsin solution according to the method described by Byrd *et al*. (1983) without glycerin destaining, and stored at 4°C before being further processed. The roots of each plant were frozen and blended as described in Experiment 1. The mixture of nematodes and root tissues was used for the following treatments.

The experiment comprised three treatments: 1) pour the nematode suspension as much as possible after centrifugation, as a control; 2) clean the lid and the wall of the top part of the tube after centrifugation by scratching with a finger, and wash the materials from the finger to the 25-µm-aperture sieve; 3) after the first centrifugation, centrifuge the bottom fraction again in 63% sucrose solution. The percentage of nematodes in the pellet to the total nematodes (pellet+pellicle) was determined.

Experiment 3 – effect of blending time and root biomass on the efficiency of nematode extraction

Soybean roots infected by *H. glycines* were obtained from pot culture and field plots at the Northeast Institute of Geography and Agricultural Ecology, Chinese Academy of Sciences, Harbin, China. In pot culture, soybean (cv. Hefeng 25) seeds were sown on July 21, 2009 at one seed per pot (height 8 cm, diameter 7 cm, 300 ml). After 1 week, 540 J2 of *H. glycines* were added to each pot. The pots were maintained under outdoor natural conditions and the roots were collected 3 days after inoculation. In the field plots, seeds of soybean cv. Longxuan-5 were sown in naturally infested soil on May 5, 2009 and plants sampled randomly on July 7, 2009.

The root samples from the pot culture were soaked in tap water, frozen at –20°C for at least 24 h, and then thawed at room temperature. This procedure was repeated twice before blending. The field samples were treated following the same procedure, but repeated three times due to the fibrous nature of the roots.

The experiment was a two-way factorial design involving blending time (15 s, 30 s or 1 min) and root biomass (roots of one, two or three plants) with four replicates. After blending in approximately 150 ml water in a blender (Joyyang Company, China), samples were poured on a 380-µm-aperture sieve nested in a 25-µm-aperture sieve and rinsed with tap water. The materials on the 380-µm-aperture sieve were collected, stained with acid fuchsin solution according to the method described by Byrd *et al.* (1983) without glycerin destaining and rinsed with tap water twice, and then the nematodes retained in the root tissues were counted under a stereomicroscope. The materials on the 25-µm-aperture sieve were transferred to a 50-ml tube and centrifuged in 63% sucrose solution at 1,500g for 5 min. The resulting pellicle fraction and pellet fraction were collected separately, and the nematodes in the pellicle and pellet were counted under an inverted microscope and a stereomicroscope, respectively.

Meanwhile, a total of additional 11 soybean plants from the above field plots were blended for 30 s and then nematode mixture was used to determine the damage of the blending to the nematodes, including second-stage juvenile (J2), swollen J2 (SJ2), third-stage juvenile (J3), and fourth-stage juvenile (J4), adult female and male in the nematode mixture.

Experiment 4 – comparison of the accuracy of the maceration-centrifugation method and the direct counting method

This experiment was performed at the Southern Research and Outreach Center, University of Minnesota, USA. The aim was to compare the freezing-blending-centrifugation method with direct counting of nematodes in stained roots. Plants produced in Experiment 2 were used for this experiment. The roots were bleached with 1.5% NaOCl for 4 min, rinsed thoroughly with tap water, stained with acid fuchsin solution according to the method described by Byrd *et al.* (1983) without glycerin destaining, and stored at 4°C before being further processed. For each plant root system, the nematodes in stained root tissues were counted under a stereomicroscope. After that, the root samples were labeled and the nematode population density was determined by the freezing-blending-centrifugation method. These samples were frozen at –20°C, thawed and then blended (Waring Blender 30–110 ml, Cole-Parmer). The samples were poured on an 850-µm-aperture sieve nested in a 25-µm-aperture sieve and rinsed with deionized water. The nematodes in the 25-µm-aperture sieve were collected by centrifugation in 63% sucrose solution and counted with an inverted microscope.

Statistical analysis

Data from Experiment 1 and Experiment 2, and the damage of blending to nematodes in Experiment 3 were subjected to one way analysis of variance using a general linear model procedure, and means were separated using the least significant difference test ($P < 0.05$). Data from Experiment 3 were also analyzed using a general linear model procedure with two-way analysis of variance and means were separated by Fisher's protected least significance difference test at

Table 1. Effect of pretreatment of root tissues on efficiency of extraction of *Heterodera glycines* second-stage juveniles from soybean roots.

Treatment	Number of nematodes recovered from 250-µm-apeture sieve	Pellet	Pellicle	Extraction efficiency[y]
Directly blending	12.8±3.5a[z]	63±7.2	927± 11.3	93.1±1.10
Freezing	0.8±0.5b	45±5.2	882±28.2	94.9±1.43
Boiling	0.3±0.3b	62±4.7	852±4.9	92.5±1.54
$F^{2,9}$	11.54	0.36	0.75	0.81
P	0.003	0.707	0.499	0.474

[y]Extraction efficiency = nematodes in pellicle fraction × 100/total nematodes.
[z]The data are mean ± SE (n = 4). The means followed by the different letters within a column are significantly different according to the least significant difference test at $P < 0.05$.

$P < 0.05$. In Experiment 4, the data were analyzed using a paired-samples T test (two tail test) for the difference between means and Pearson correlation analysis to compare the two methods. All analyses were performed using SPSS 13.0.

RESULTS

Experiment 1 – effect of pretreatment of roots on extraction efficiency

Pretreatment of roots with freezing and pretreatment with boiling reduced the number of nematodes in the materials caught on the 850-µm-aperture sieve compared with the samples without pretreatment ($F_{2,9}$ = 11.539, $P = 0.003$). However, the number of nematodes recovered from the pellet and pellicle fractions, the percentage of nematodes in the pellicle and the extraction efficiency did not differ significantly among the three treatments (Table 1).

Experiment 2 – measures to reduce the number of nematodes in the pellet fraction

Cleaning the lid and tube wall as much as possible following pouring of the upper portion of materials slightly reduced the number of nematodes lost. Compared with the treatment of pouring only, re-centrifuging the pellet significantly reduced the percentage of nematodes in the second pellet ($F_{2,9}$ = 9.217, $P = 0.007$) from 11.1% to 2.4% (Fig. 1).

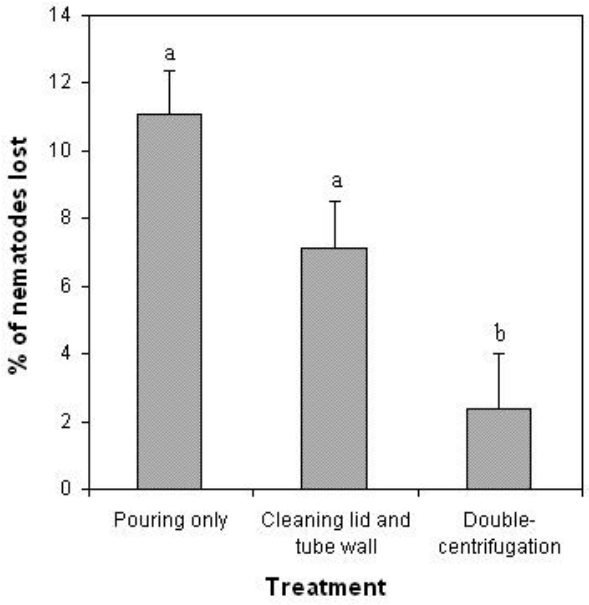

Fig. 1. Impact of different approaches to reduce the percentage of nematodes in the pellet after centrifugation in sucrose solution. The data are means ± SE (n = 4). Different letters above the bars denote significant difference according to least significant difference test at $P < 0.05$.

Experiment 3 – effect of blending time and root biomass on the efficiency of nematode extraction

In samples from both pot and field plots, the mean percentage of nematodes in the pellicle fraction was approximately 95%. Increasing blending time from 15 s to 60 s or increasing the root biomass from one to three plants did not affect the percentage of nematodes recovered from the materials on the 380-µm-aperture sieve, the pellet, or the pellicle. The interaction between blending time and root biomass was not significant (Table 2 and 3).

The rate of damaged nematodes differed significantly among the life stages ($F_{4,55}$ = 64.985, $P < 0.001$). Males exhibited the highest breakage rate, followed by J3 and J4, and the lowest rates were in J2+SJ2 and females. In average, 14.8% of total nematodes from the 11 individual plants were damaged (Fig. 2).

Table 2. Effect of blending time and root biomass on efficiency of extraction of second- and third-stage juveniles of *Heterodera glycines* from roots of soybean grown in pot culture.

		% of nematodes recovered from[y]		
Treatment	Level	Tissues on 380-µm-apeture sieve	Pellet	Pellicle
Blending time	15s	0.12±0.07	4.26±1.35	95.0±1.35
	30s	0.05±0.02	5.03±1.83	94.5±1.96
	60s	0.02±0.02	4.15±1.03	95.3±1.09
Number of plants	1	0.10±0.07	4.94±1.61	94.1±1.80
	2	0.09±0.05	4.78±1.52	94.6±1.49
	3	0±0	3.71±1.15	96.1±1.11
Analysis of Variance (F-value)[z]				
Time (T)		1.18	0.10	0.40
Plants (P)		1.27	0.19	0.06
T × P		0.75	0.21	0.19

[y]Data are means of four replicates.
[z]All F-values are not significant at $P > 0.05$.

Table 3. Effect of blending time and root biomass on efficiency of extraction of *Heterodera glycines* juveniles and adults from roots of soybean grown in field plots.

		% of nematodes recovered from[y]		
Treatment	Level	Tissues on 380-µm-apeture sieve	Pellet	Pellicle
Blending time	15s	0.13±0.05	5.36±1.39	90.5±1.64
	30s	0.12±0.05	3.92±1.35	94.3±2.31
	60s	0.12±0.04	4.53±1.05	93.2±1.61
Number of plants	1	0.05±0.03	4.63±1.48	92.8±1.92
	2	0.14±0.03	3.53±0.78	94.7±1.14
	3	0.18±0.06	5.67±1.39	90.5±2.36
Analysis of Variance (F-value)[z]				
Time (T)		1.18	0.10	0.40
Plants (P)		1.27	0.19	0.06
T × P		0.75	0.21	0.19

[y]Data are means of four replicates.
[z]All F-values are not significant at $P > 0.05$.

Experiment 4 – comparison of the accuracy of the maceration-centrifugation method and the direct counting method

The numbers of nematodes recovered with the two methods were highly correlated ($R^2 = 0.931$, $P < 0.001$) (Fig. 3). The number of nematodes counted with the freezing-blending-centrifugation method was 24% greater than that counted directly from stained roots ($P = 0.0024$) (Fig. 4).

DISCUSSION

Most plant tissues become soften after being frozen and thawed because the large ice crystals formed in the tissues during freezing usually cause irreversible damage to plant cell structures after thawing (Reid, 1983; Ando *et al.*, 2009). In the present study, the plant roots were soaked in water before freezing and ice crystals formation at –20°C might have caused more serious damage to the root tissues than if they had not been soaked. The pretreatment with freezing

could improve the separation of nematodes from the root tissue during the blending process. As shown in Table 1, freezing reduced the percentage of nematodes retained in the tissue caught on the 250-µm-aperture sieve compared to the direct blending without freezing pretreatment. As with other maceration methods, freezing-blending centrifugation greatly reduced the time needed to quantify nematode population densities in plant roots compared with visualizing the nematodes in stained root tissues (Marks and McKenna, 1981; McSorley *et al.*, 1984; Hooper, 1986; Stetina *et al.*, 1997; Das *et al.*, 2008). Moreover, the present modified maceration method by means of freezing avoided the potential risks posed by the use of chemicals such as NaOCl, acid fuchsin (Byrd *et al.*, 1983), and cotton blue (Germani and Plenchette, 2004).

The present study demonstrated that increasing the blending time from 15 s to 60 s or increasing the root biomass from one to three plants did not affect the nematode extraction efficiency from roots pretreated with freezing. This contrasts to the results of a previous study in which 10 s of blending was most efficient for extracting root-knot nematodes and egg masses (Stetina *et al.*, 1997). Considering the fibrous texture of root samples and the potential increase in breakage rate with increasing blending time, we suggest using 30 s of blending time.

After centrifugation, a small number of nematodes ranging from 3.7% to 5.0% retained in the pellet fraction (Table 2). Two major reasons could account for this. First, some nematodes may have become stuck to the wall of the tube as a result of the high viscosity of the sucrose solution. Second, floating root tissue residue in the sucrose solution could impede the movement of nematodes during centrifugation. After re-centrifugation, the average nematode percentage in the pellet was significantly reduced, to approximately 2% compared to the 11% observed in pellets that had been centrifuged only once (Fig. 1). Similar results with double centrifugation have been reported in previous studies (Greco and D'Addabbo, 1990; McSorley and Parrado, 1987). When a study requires greater accuracy, re-centrifugation of pellets may be necessary.

Damage to nematodes during the mechanical blending process might be a concern for using mechanical maceration methods. In the present study, individual nematodes were observed carefully. The damage rate of *H. glycines* nematodes by blending the soybean roots pretreated with freezing was approximately 14.8%, with the males most vulnerable to the blending due to the longer body length than other stages. For quantification of nematode population, the head portion of a broken nematode can be counted as an individual.

About 95% of the nematodes were recovered in the pellicle. The total number of nematodes counted from the same root samples were 24% greater using the maceration-centrifugation method than when counted directly from stained roots. The results of this

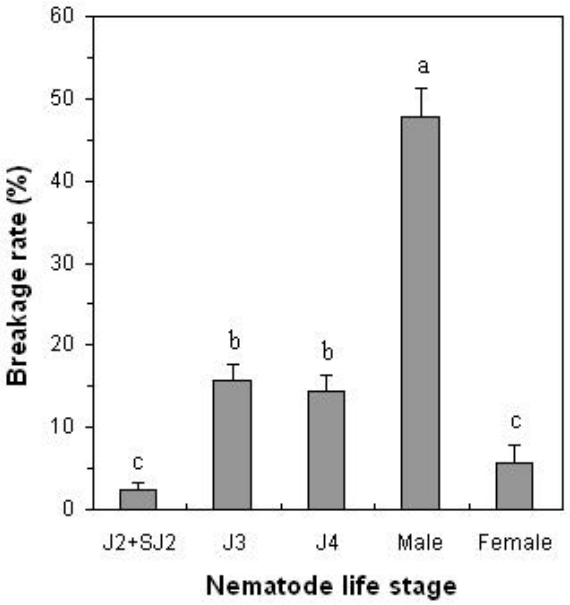

Fig. 2. Breakage rate of nematodes at different stages of *Heterodera glycines* as a result of blending. J2, SJ2, J3 and J4 are second-, swollen second-, third- and fourth-stage juveniles, respectively. The data are means ± SE (n = 11). Different letters above the bars denote significant difference according to least significant difference test at $P < 0.05$.

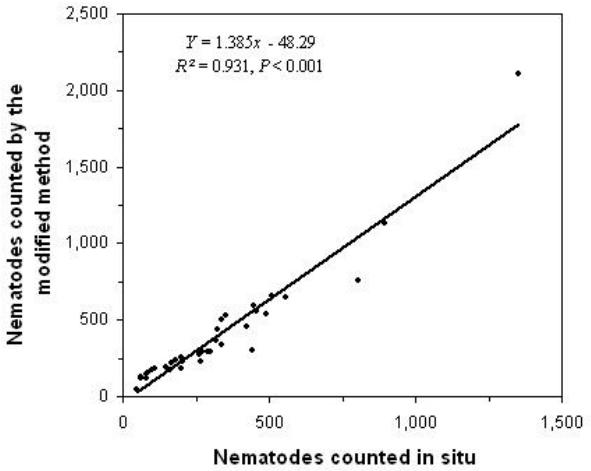

Fig. 3. Linear relationship between the number of nematodes counted directly in situ from stained roots and the number determined by freezing-blending-centrifugation method (modified method).

study agree with those in a previous study (Marks and McKenna, 1981), which reported greater nematode recovery rate with maceration-centrifugation as compared to direct counting of lactophenol-treated roots under a stereomicroscope. Similarly, enzymatic digestion recovered a larger number of nematodes than the pressed glass plate method of counting nematodes inside root tissue (Araya and Caswell-Chen, 1993). In addition, root samples from field soil may be infected

by more than one nematode species. Using the modified method to release nematodes, it will be easier to identify species and exclude individual nematodes that are not of interest.

After blending, the materials were poured on a sieve nested in another sieve. The top sieve was of 850-µm, 380-µm or 250-µm-aperture; which sieve should be used in a study depends on the nematode stage required. For research on J2 alone, a 250-µm-aperture sieve could be used, considering its body diameter; this sieve catches more residues, thereby reducing the amount of residue in the pellet and improving the efficiency of nematode extraction and counting. For studies involving all stages including mature females and males, an 850-µm-aperture sieve should be used because mature females might not be able to pass through the smaller sieves. For the bottom sieve, a 25-µm-aperture was used in the present study. Again, the sieve aperture size used depends on the objectives of a study. A fine sieve of 20-µm-aperture has been used to extract *Meloidogyne chitwoodi* from potato tubers (Viaene *et al.*, 2007). Taking these findings together, we preferred to use a 850-µm-apertur sieve nested in a 25-µm-aperture sieve to separate *H. glycines* after blending, to collect as many nematodes as possible. For the centrifugation stage, 38% sucrose solution should be used for J2, whereas 63% sucrose solution should be used when other developmental stages are required.

In summary, an improved method for quantifying *H. glycines* nematodes in roots was developed in the present study. It comprises the following steps: 1) Place the roots in a freezer for at least 24 h at –20°C; 2) let the frozen roots be thawed at room temperature; 3) repeat steps 1 and 2 one or two times according to the extent of fibrous texture of the roots; 4) place the root samples in a blender, add a volume of tap water depending on the root biomass and the volume of the blender, and blend the samples for 30 s; 5) pour the mixture into an 850-µm-aperture sieve nested in a 25-µm-aperture sieve, wash the root tissues thoroughly, discard the tissues remaining on the 850-µm-aperture sieve, and collect the mixture of nematodes and root tissue residue on the 25-µm-aperture sieve to a 50-ml tube with 38% sucrose solution for J2 and 63% sucrose for J3, J4 and adults; 6) centrifuge the tube at 1,100-1,500 g for 5 min; 7) collect the pellicle from step 6; 8) centrifuge the pellet in sucrose solution again if needed; 8) collect the pellicle from step 8; 9) combine the pellicles from step 7 and step 9; and 10) count the nematodes in the combined pellicles under an inverted microscope.

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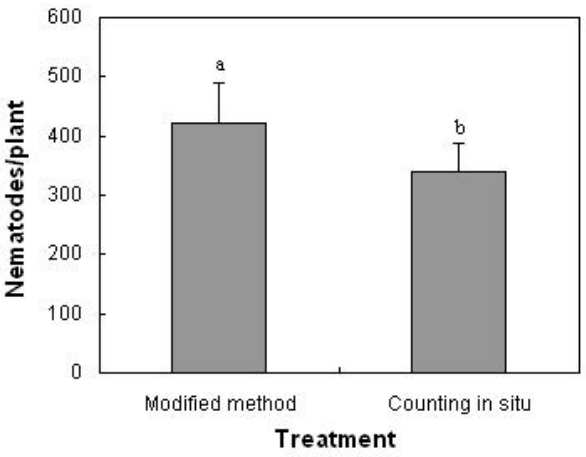

Fig. 4. Average nematode numbers counted directly from stained roots (counting in situ) and determined by the freezing-blending-centrifugation method (modified method). Data are mean ± SE (n=36). Different letters above bars denote significant difference according to least significant difference test at $P < 0.05$.

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