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Root-lesion nematode (*Pratylenchus* spp.) extraction from maize roots: a comparison of three methods

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Abstract This study examines differences in extraction efficiency of three methods for extracting root-lesion nematodes (*Pratylenchus* spp.) from maize roots. The Baermann funnel, Whitehead & Hemming tray, and centrifugal-sugar flotation methods were evaluated for efficiency and clarity using microscopic observation. Statistical analysis confirmed significant differences among the methods. The centrifugal-sugar flotation method yielded the highest nematode count (1874±76 per 5 g of roots) but the clarity of the observation field under the microscope was lower due to root residues compared to the other two tested methods. The Baermann funnel method yielded 35.9% extraction efficiency (672±46 per 5 g of roots) compared to the centrifugal-sugar flotation method with higher clarity of the observation field than other tested methods. The Whitehead & Hemming tray presented a moderate level of observation field clarity compared to other tested methods with a nematode extraction efficiency of 60.8% (1140±53 per 5 g of roots) compared to the centrifugal-sugar flotation method. The results suggest that the Whitehead & Hemming tray could be a viable choice for nematode extraction, especially when both nematode numbers and microscopic clarity are important considerations. Understanding the restrictions of each methodology enhances the accuracy of nematode quantification leading to improved and updated data for maize producers in New Zealand.

Keywords Endoparasitic nematode, microscopic observation, morphology, centrifugal-sugar flotation, Baermann funnel, Whitehead & Hemming tray, New Zealand

INTRODUCTION

In New Zealand, recent surveys indicated the root-lesion nematode (*Pratylenchus* spp.) to be the dominant genus and most widely distributed in maize and wheat fields (Thiellier & Kularathna 2023; Thiruchchelvan et al. 2023). Root-lesion nematodes are migratory endoparasitic nematodes (Orlando et al. 2020). They complete their life cycle and subsequent colonisation in host roots rather than infesting the soil (Davis & MacGuidwin 2000). Due to their endoparasitic nature, they directly damage host-plant roots and therefore could contribute to large yield losses in host plants (Lopez-Nicora et al. 2023).

Nematode numbers and density are correlated with damage in many cases. Therefore, it is essential to use accurate extraction techniques to ensure management decisions are appropriate. Numerous extraction methods are employed by nematologists, e.g.,

- Baermann funnel (Baermann 1917; Marais et al. 2017; Schumacher & Grabau 2022)
- decanting and sieving (Jenkins 1964; Marais et al. 2017)
- Whitehead & Hemming tray (Hooper & Evans 1993; Prot et al. 1993; Bell & Watson 2001; Marais et al. 2017)
- centrifugation and sugar flotation (Jenkins 1964; De Waele et al. 1987)
- platform shaker (Behn 2012; Batista da Silva 2013) and
- Seinhorst's mystified methods (Behn 2012; Marais et al. 2017)

depending on the type of nematode species, crop, and the resources available (Marais et al. 2017).

However, the efficiency of these methods can vary based on factors like host plant and nematode species extracted (Marais et al. 2017). Disregarding endoparasitic nematode populations in maize roots for diagnostic and advisory purposes can be both inaccurate and could lead to misinterpretation of results.

This study aims to assess the variability of extraction methods to determine the optimal extraction approach for root-lesion nematode from maize roots by utilising three techniques: Baermann funnel; Whitehead & Hemming tray, and centrifugal-sugar flotation. These three methods were chosen because they are commonly employed globally in nematology studies and are simple and time-effective compared with other methods such as the platform shaker and Seinhorst's mystified methods. Using an optimal extraction method will enable more accurate and reliable nematode quantification, aiding in the formulation of precise recommendations for maize producers in all New Zealand's agricultural areas.

MATERIALS AND METHODS

Nematode culture and root sample collection:

Root-lesion nematode-infested soil was collected from a maize field in Dorie, Canterbury, New Zealand (43°53'04.0"S 172°04'33.4"E). Plant debris and stones were manually removed. The soil was thoroughly mixed before being placed into multiple 2-L plastic pots. Subsequently, each pot was planted with a maize hybrid P8500 seed (untreated) obtained from Pioneer Seeds New Zealand. The maize plants were grown at an average temperature of 17±3 °C from May to July 2023 in a glasshouse at Lincoln University. Root samples were collected from maize plants 60 days after planting as this is the maximum period for a two-cycle root-lesion nematode lifecycle and the commonly used period against maize for pot trails (Batista da Silva, 2013; McDonald & van den Berg, 1993). The roots were washed thoroughly with tap water to ensure cleanliness. Excess water was gently removed by blot drying using tissues (Kimwipes, KIMTECH Science). Cleaned roots (~500 g) collected from 20 plants were chopped into 1 cm pieces using scissors and were mixed thoroughly to make a composite sample.

Extraction methods

Baermann funnel

This method was originally developed by Baermann (1917) with later modifications by Marais et al. (2017) and Schumacher and Grabau (2022). A 15 cm length of rubber tubing was fitted at the end of a conical funnel. A clamp was attached near the free end of the tubing. The funnel and tube were filled with water, the clamp was opened, and sufficient water was drained off to remove air bubbles trapped in the tube then a wire mesh was placed on top of the funnel. The funnel was refilled with water to cover the wire mesh. Subsequently, a tissue (Kimwipe) was carefully placed on top of the wire mesh and submerged in water

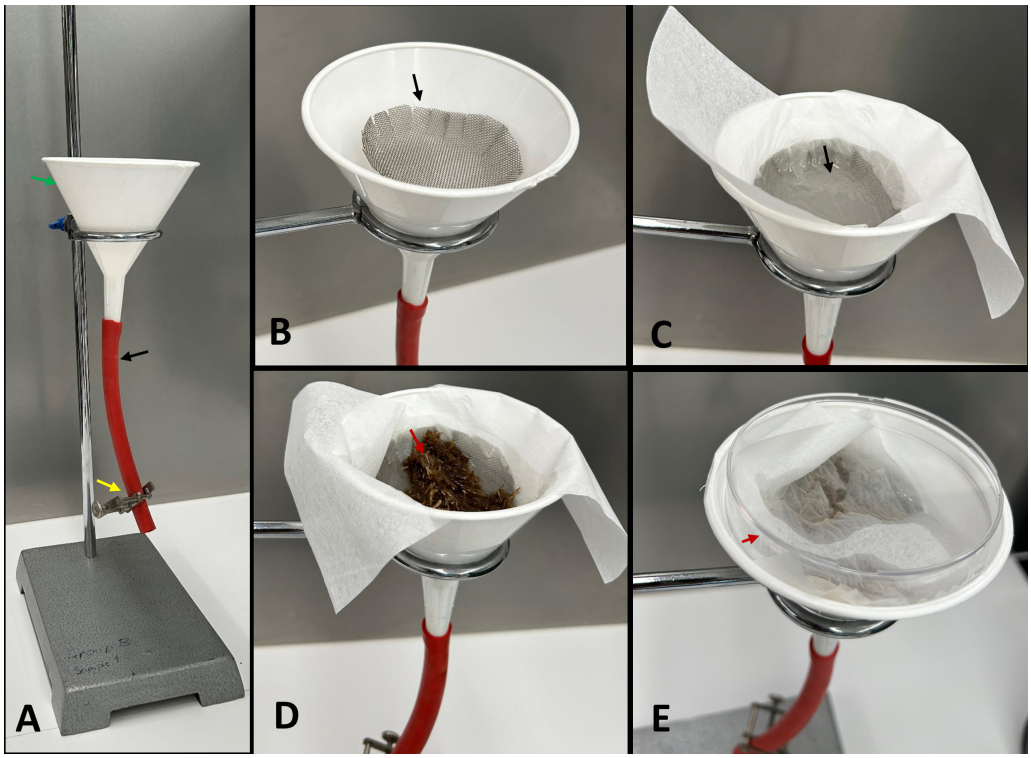

Figure 1 Set up the Baermann funnel for root-lesion nematode extraction from maize roots. A: the set-up funnel (green arrow), rubber tube 15-cm (black arrow), and the clamp (yellow arrow); B: wire mesh (arrow) placed; C: a Kimwipe tissue placed and water submerged the wire mesh (arrow); D: root macerate spread (arrow); E: root macerate covered with a Kimwipe and a Petri plate to avoid desiccation of the sample.

(Fig. 1A-C). Exactly 5 g of chopped maize roots (< 1 cm pieces) were added to 150 mL of tap water and blended for 30 seconds using the pulse setting of a kitchen stick blender (Living and Co. New Zealand). The mixture was then placed on a 38-µm mesh-sized sieve (Glenammer, UK) to remove excess water. The moist roots remaining on the sieve were transferred to the Baermann funnel and uniformly spread over the tissue (Fig. 1D). Water was gently poured along the inner surface of the funnel to cover the sample (without complete submersion) and the sample was covered with a Kimwipe and a Petri plate to prevent desiccation (Fig. 1E). The sample was then allowed to incubate at 25±3 °C for 48 hours. During this time any nematodes present in the blended root sample were assumed to have migrated into the surrounding water in the funnel. The clamp on the tube was opened and the water containing nematodes (nematode suspension) was drained off and collected in a 100-mL beaker. Excess water was removed using a 38-µm sieve and nematodes were washed off the sieve using ~10-15 mL water into a 50-mL sterilised sample bottle for counting.

Whitehead & Hemming Tray

This method is a modified version of the Baermann funnel method. It is also known as the "Baermann tray" or "maceration-filtration" (Hooper & Evans 1993; Prot et al. 1993; Bell & Watson 2001; Marais et al. 2017). A flat-bottom plastic sieve (2-mm aperture size) was placed on a plastic tray (12 cm in diameter) (Fig. 2A-B), and a layer of Kimwipes was placed on the plastic sieve. As with the Baermann funnel method, exactly 5 g of chopped roots were blended with 150 mL tap water for 30 seconds using a kitchen stick blender on pulse setting to make a homogenised macerate of the roots. The drained root macerate was spread on the sieve, and the outer tray was filled with sufficient water to wet the lower surface of the sample material, without covering the sample with water (Fig. 2C). The sample was covered with a Kimwipe and a Petri plate to prevent desiccation (Fig. 2D). The sample was then allowed to incubate at 25±3 °C for 48 hours. During this time any nematodes present in the blended root sample were assumed to have migrated into the surrounding water in the tray. The nematode suspension was drained off and collected in a 200-mL beaker using water washing. Excess water was removed using a 38-µm mesh sieve and nematodes were washed off the sieve using ~10-15 mL water into a 50-mL sterilised sample bottle for counting.

Centrifugal-sugar flotation

This method was developed by Jenkins (1964) for soil with later modifications for roots by De Waele et al. (1987) and Marais et al. (2017). A comparison of these reported centrifugal-sugar flotation methods and the simplified centrifugal-sugar flotation method in this study is shown in Table 1.

A simplified centrifugal-sugar flotation method for the roots was used in this study, drawing ideas from the original centrifugal-sugar flotation nematode extraction method from soil described by Jenkins (1964). Therefore, the procedure used here involved a reduced number of sieves by omitting the finest sieve (25-µm), eliminated kaolin and adjusted the sugar concentration, and centrifugation parameters aligning it with soil nematode extraction principles. Using a stick blender over a kitchen domestic blender saved time as maceration was efficient with 150 mL water and 5-second breaks. This updated approach ensures effective rapid extraction compared to the reported methods for roots.

Five grams of chopped maize roots (< 1 cm pieces) were added to 150 mL of tap water and blended for 3×15 seconds each time followed by a break of 5 seconds macerated roots were passed through 250-µm and 38-µm mesh sieves. The

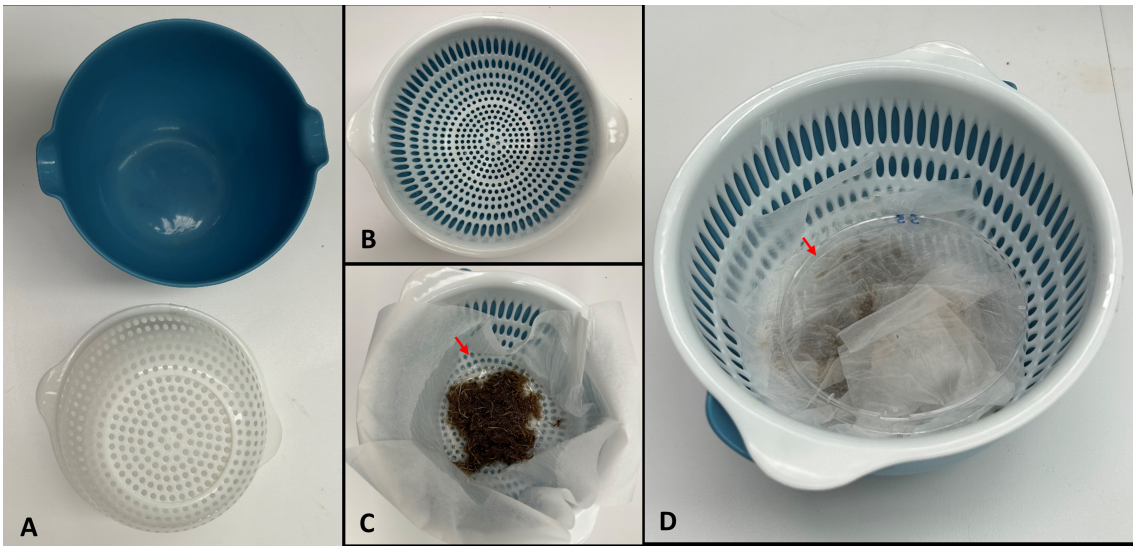

Figure 2 Set up the Whitehead & Hemming tray for the root-lesion nematode extraction from the maize roots. A: plastic tray (blue) and plastic sieve (white); B: plastic sieve placed inside the plastic tray; C: root macerate spread over the Kimwipe and the water level touching the root samples; D: root macerate covered with Kimwipe and a Petri plate to avoid desiccation of the sample.

Table 1 Comparison of the centrifugal-sugar flotation nematode extraction methods for soil and roots.

Steps	Study			
	Soil (original)	Roots (modified)		
	Jenkins 1964	De Waele et al. 1987	Marias et al. 2017	This study (simplified)
Weight of roots or soil (g)	100 g soil	1.25, 2.5, 5 and 10 g	Not < 5 g	5 g
Length of roots (mm)	N/A	5-10	2-5	< 10
Blender type	N/A	Domestic	Kitchen	Stick (Living & Co. NZ)
The volume of tab water used to blend/mix (mL)	1000	250	250	150
Blender regime	3 × Manually mixing and stirring, allowed to settle for 30 seconds	High speed for 2 minutes	Medium speed for 30 – 45 seconds	3 ×15 seconds followed by a break of 5 seconds, Pulse setting
Sieve sizes (μm)	53 and 45	750 and 45	1000, 150, 45, 38 and 25	250 and 38
Centrifugation with tap water	1750 rpm for 5 minutes (~576 × g or RCF)	1750 × g for 5 minutes	3484 × g for 7 minutes	Not done
Adding Kaolin	Not done	2 mL	5 mL	Not done
Sugar concentration (% w/v)	45.4	62.4	62.4	45.4
Centrifugation with sugar	1750 rpm for 1 minute	1750 × g for 1 minute	3484 × g for 3 minutes	1750 rpm for 1 minute (576 × g or RCF)

N/A - not applicable, RCF - relative centrifugal force

retentate approximately 15 mL in the 38-μm mesh sieve was transferred into a 50-mL centrifuge tube. Subsequently, 35 mL of sucrose solution (45.4% w/v) was added and mixed thoroughly before the tube was centrifuged (Thermo Scientific-Multifuge X1R, Germany) for 1 minute at 576 × g RCF. After centrifugation, the supernatant was passed through a 38-μm-aperture sieve and the retentate in the sieve was washed carefully using water dispensed from a wash bottle to collect extracted nematodes into a 50-mL sterile specimen bottle with a lid.

For all three methods used in this study (Table 2), extracted nematode samples were kept at 4 °C until counted using an inverted compound light microscope (Olympus CKX53, Japan) at ×40 magnification.

Statistical analyses

Each of the three methods was replicated six times as a set at a time, and each set was repeated three times within a week. Data of root-lesion nematode numbers from all three sets were combined as one data set, representing 18 replicates for each method. Statistical analysis was performed using a one-way ANOVA, and a post hoc Bonferroni test at 95% confidence intervals using GenStat® (23rd Edition A VSNi) statistical software. Data were checked for normality using the w-test in GenStat®. To obtain normality, data were square-root transformed before statistical analysis.

The extraction efficiencies were calculated based on the highest number of nematodes extracted by any method.

RESULTS and DISCUSSION

Number of nematodes

The centrifugal-sugar-flotation method demonstrated the highest extraction efficiency compared to the other tested methods for root-lesion nematode extraction. Therefore, it was assumed that the number of extracted root-lesion nematodes (1874±76 per 5 g of roots) comprised 100% recovery from the roots so was used as the baseline method to compare the extraction efficiency of the other two methods. The Whitehead & Hemming tray method had a relative extraction efficiency of 60.8% (1140±53 per 5 g of roots), and the Baermann funnel method exhibited the lowest efficiency at 35.9% (672±46 per 5 g of roots) compared to centrifugal-sugar-flotation method (Fig. 3). Further, the pairwise contrast statistical test within the ANOVA showed a highly significant difference between all the extraction methods (p<0.001) (Table 3).

Sample clarity

Extracts produced using the Baermann funnel method (Fig. 4A) were clearer under the microscope than those produced using the Whitehead & Hemming tray method (Fig. 4B). Extracts produced using the centrifugal-sugar flotation method had the lowest sample clarity with more root residues observed (Fig. 4C) at ×40 magnification. However, the extracts from the centrifugal-sugar flotation were clearer at ×100 compared to at ×40 magnification (Fig. 5).

Table 2 Comparison of nematode extraction methods used in this study.

Steps	Method		
	Baermann funnel	**Whitehead & Hemming tray**	**Centrifugal-sugar flotation**
Weight of maize roots (g)	5	5	5
Length of roots (mm)	< 10	< 10	< 10
The volume of water used to blend the roots (mL)	150	150	150
Blender regime	30 seconds on pulse setting	30 seconds on pulse setting	3 × 15 seconds each time followed by a break of 5 seconds, Pulse setting
Sieve sizes (µm)	38	38	150 and 38
Incubation conditions	25±3 °C for 48 hours	25±3 °C for 48 hours	N/A
Centrifugation conditions	N/A	N/A	1750 rpm for 1 minute (576 × g or RCF)
Time to complete the procedure	Minimum 48 hours irrespective of the number of samples	Minimum 48 hours irrespective of the number of samples	40 samples within 3 hours

N/A-not applicable

Overall suitability

This study showed that the Baermann funnel method was less effective at extracting root-lesion nematodes from maize roots compared to the Whitehead & Hemming tray. Similar results were reported in rice root systems (Prot et al. 1993). However, the Baermann funnel method could be a good option for both morphological and molecular identification purposes rather than population determination because nematodes were extracted with no deformation of their anatomy compared to other methods tested (Marais et al. 2017). The centrifugal-sugar flotation method yielded higher root-lesion nematodes compared to the other tested methods. This could be due to the ease of nematode separation from root tissue by maceration, sugar-flotation, and centrifugation as mentioned by McSorley et al. (1984) as well as separating immobile nematodes from roots.

Additionally, centrifugal-sugar flotation is a rapid technique that enables the processing of many samples within a relatively short time (Marais et al., 2017). The other two methods tested take a much longer time to extract nematodes from samples as they need at least 24 to 48 hours irrespective of the sample size to allow migration of the nematodes into the water. In the centrifugal-sugar flotation method, nematodes are passively extracted and therefore around 30 to 40 samples could be processed within 2.5 to 3 hours including the samples preparation and post-cleaning steps. Due to its passive nature of extracting nematodes, this method allows the researcher to extract almost all the nematodes from the samples, irrespective of the mobility of nematodes making this method the best for nematode surveys. However, precautions must be taken when preparing the samples to enhance sample clarity when

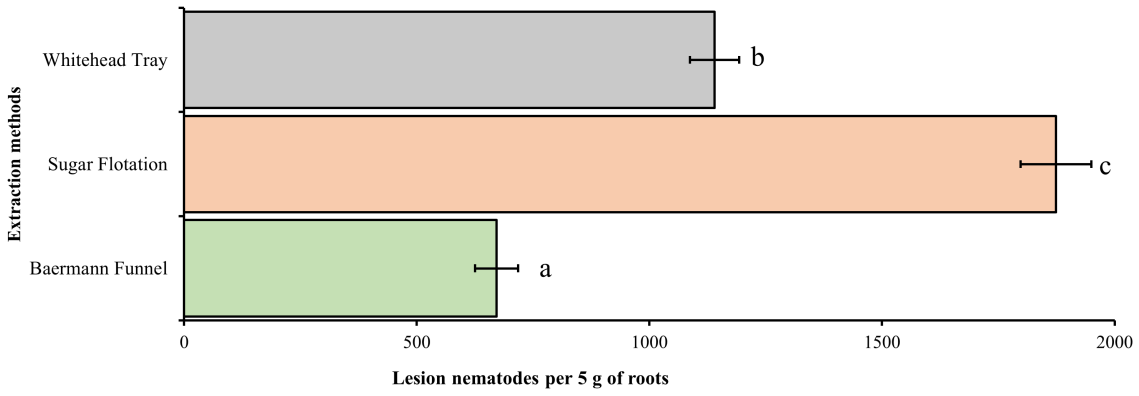

Figure 3 Mean number of root-lesion nematodes extracted from maize roots using each of the three extraction methods. Bars (indicate mean values) with the same letter are not significantly different according to the Bonferroni post hoc test at 95% conference interval. Error bars are the standard error of the mean (SEM).

Table 3 Pairwise contrast of the recovered root-lesion nematode numbers between tested extraction methods.

Source of variation	Statistic		
	Degrees of freedom	Variance ratio (F-value)	F-statistic P-value
Extraction methods	2	99.28	<.001
Baermann funnel and Centrifugal-sugar flotation	1	198.04	<.001
Baermann funnel and Whitehead & Hemming tray	1	41.01	<.001
Centrifugal-sugar flotation, and Whitehead & Hemming tray	1	58.81	<.001
Residual	51		
Total	53		

using this method. Although the Whitehead & Hemming tray method yielded fewer nematodes than the centrifugal-sugar flotation method, a clearer sample was obtained, making observations much easier with less debris contamination. The results suggest that the Whitehead & Hemming tray could be a better option than the Baermann funnel and centrifugal-sugar flotation methods for root-lesion nematode extraction, especially when both nematode numbers and microscopic clarity are important considerations, even though it limits extracting most of the nematodes. This research suggests that each extraction method has advantages and disadvantages. Therefore, the extraction method needs to be selected based on the type of research questions that researchers need to answer. Findings from this current study have provided a valuable understanding of the field of nematology, guiding researchers in choosing the most appropriate method based on their specific research goals and objectives. Future research could explore modifications to the centrifugal-sugar flotation method to enhance clarity during microscopic observation without compromising nematode yield. For instance, manually cutting root fragments into smaller pieces rather than using a blender would result in cleaner samples. However, manual cutting takes longer than blending and would not expose nematodes within deep root tissues in the root fragments, giving a false representation of the nematode numbers. Assessing the effect of changes on nematode morphological and morphometric characteristics by each method needs to be considered in future studies. This could provide insights into the accuracy and reliability of each method for taxonomic purposes where the morphological approach is used for species-level identification.

CONCLUSIONS

The choice of nematode extraction method depends on the specific objectives of a particular study. The centrifugal-sugar flotation method excels in nematode yield but compromises clarity during microscopic analysis. The Baermann funnel method, despite yielding fewer nematodes, offers a clearer observation field, making it suitable for detailed morphological and morphometric studies. The Whitehead & Hemming tray method balances nematode abundance and observation clarity, positioning it as a practical choice for overall efficacy for root-lesion nematode extraction from maize roots.

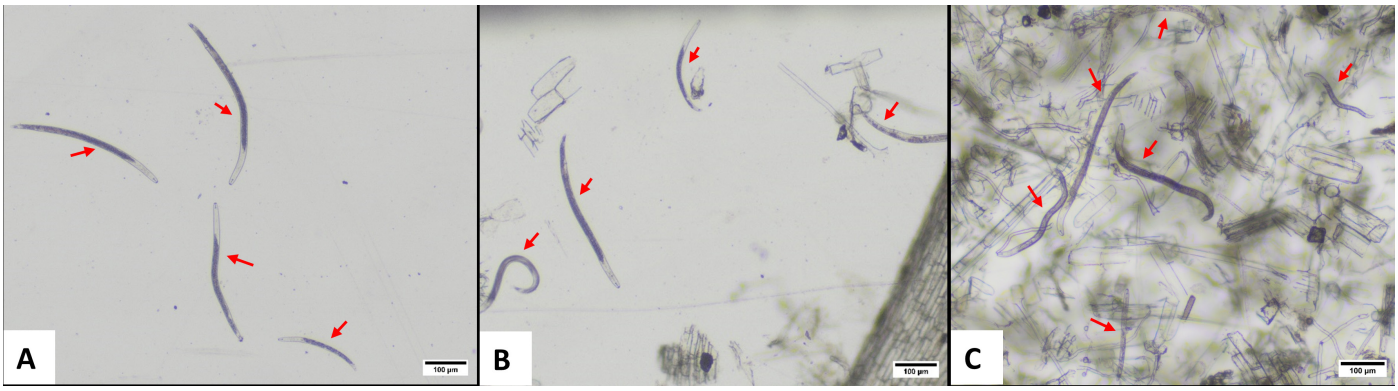

Figure 4 Microscopic view of the extracted root-lesion nematodes in counting disc. A: Baermann funnel; B: Whitehead & Hemming tray; and C: centrifugal-sugar flotation method at ×40 magnification (red arrows indicating the root-lesion nematodes).

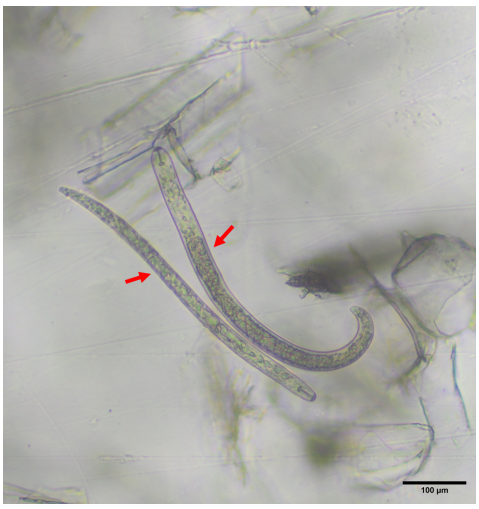

Figure 5 Microscopic view of the extracted root-lesion nematodes in counting disc from centrifugal-sugar flotation method at ×100 magnification (red arrows indicating the root-lesion nematodes).

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