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The importance of organic matter when assaying *Meloidogyne chitwoodi* soil populations

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Summary. Methods to detect *Meloidogyne chitwoodi* in soil samples were compared. It was found that the organic soil fraction can constitute a large infection reservoir. Preliminary root maceration had no effect on the yield of juveniles when a cotton wool filter extraction method was used. Conversely, extracting nematodes by centrifugation, root maceration positively influenced the recovery of nematodes. The largest *M. chitwoodi* recovery from the organic soil and mineral soil fractions, and from unfractionated soil, was obtained using zonal centrifugation. Increasing the density of the $MgSO_4$ solution from 1.12 to 1.22 resulted in an increased recovery of old eggs. Small root-knot nematode densities were detected most efficiently with a bioassay and zonal centrifugal extraction.
Key words: conventional centrifugation, extraction method, *Meloidogyne chitwoodi,* soil fraction, root maceration, zonal centrifugation.

Accuracy in quantitative population estimation is required in all fields of applied nematology, and is directly dependent on the sampling and extraction methods used. Therefore, the heterogeneity of the nematode distribution in the target area needs to be considered when developing sampling methods (Webster & Boag, 1992; Been & Schomaker, 1996; Marshall *et al.*, 1998), whilst the extraction method should ideally extract all nematodes present in the soil sample including both the organic fraction (OF) and the mineral fraction (MF) (Coolen & D'Herde, 1972).

Extraction methods for *Meloidogyne* spp. have been reviewed by Barker (1985) and can be split into two types: 1) methods that depend on the nematode's motility (*e.g.* cotton wool filter, Baermann funnel); and 2) those that do not depend on the nematode's motility (*e.g.* centrifugation-flotation). Currently, methods do not provide a means for extracting nematodes from the OF and the MF simultaneously. Thus, to assay the entire soil population multiple methods have to be used. Alternatively, a technique that combines the extraction from both fractions needs to be developed.

Very low infections of *Meloidogyne chitwoodi* Golden, O'Bannon, Santo & Finly, 1980 can seriously damage crops (Santo *et al.*, 1981). Therefore, accurate identification of soil infestations are required for successful implementation of management strategies. Consequently, the choice of extraction method is very important. This study presents details of extraction procedures for *M. chitwoodi* including a comparative analysis of extraction methods and, a bioassay to estimate low *M. chitwoodi* infection levels in soil samples.

MATERIALS AND METHODS

Nematodes. Soil used in this study (80.5% sand, 16.7% silt, and 2.8% clay) was collected from a field cropped in the previous year with black salsify (*Scorzonera hispanica* L.) heavily infested with *M. chitwoodi*. The OF consisted of root fragments and debris remaining in the soil from this crop.

Fractionating the soil. To separate the OF from the MF, soil was suspended in water and the suspension poured onto two sieves: a 2 mm-sieve over a 0.2 mm-sieve. Depending on the experiment, the MF passing through the smallest aperture sieve was discarded or collected. The OF was always caught on the 0.2 mm-sieve, and subsequently was homogenised in a jar filled with water.

Maceration time and nematode recovery. To obtain information on the optimal maceration time for freeing nematodes from the OF, 100 ml aliquots were taken from the homogenised OF-suspension. Each sample, containing *ca* 2 g root fragments, was diluted to 250 ml and macerated in a 500 ml jar of a Waring Blender for 0, 7, 15, 30, 60, or 120 seconds at 20,500 rpm. From this blended suspension, nematodes were separated either through a cotton wool filter (nematodes collected and counted daily during 20 days) or by zonal centrifugation (ZC) (Hendrickx, 1995). For ZC the suspension was diluted to 1000 ml, of which 500 ml were centrifuged. The six blending times were repeated five times for each of the separation techniques.

Soil extraction procedures. The efficiency for determining total *M. chitwoodi* soil populations was compared for three extraction methods: 1) conventional centrifugation (Coolen & D'Herde, 1972); 2) ZC (Hendrickx, 1995); and 3) elutriation (D'Herde & Van Den Brande, 1964). For each of these methods, comparison was made for the unfractionated soil and for each of the two fractions separately.

For both centrifugation methods, the OF was macerated for 60 sec at 20,500 rpm in a 500 ml jar of a Waring Blender containing 250 ml water. Fifty ml of unfractionated soil or soil fractions were used in the conventional centrifugation method; whereas, 100 ml soil samples were used for ZC.

When using the elutriation method, the unmacerated OF from 100 ml soil samples was incubated on a cotton wool filter from which nematodes were collected daily during a period lasting 20 days. Nematodes from the MF, and the unfractionated soil samples, were elutriated (water flow = 1 litre/min). Each of the extraction procedures were repeated ten times.

$MgSO_4$ density and nematode recovery by centrifugation. Extraction methods based on the centrifugation-flotation technique rely on the separating effect of high density solutions. To examine the effect of different $MgSO_4$ densities, *M. chitwoodi* eggs were extracted from fresh or old roots. Infected fresh roots were obtained from tomato plants grown for 40 days in heavily *M. chitwoodi* infested soil. Roots were cut in 0.5 cm pieces and macerated (30 sec; 20,500 rpm) in a 1000 ml jar of a Waring Blender containing 250 ml water. Infected old root fragments were collected by fractionating field infested soil as previously described. As it was more difficult to liberate nematodes from these old roots, fragments were macerated for 2 min at 20,500 rpm in a 1000 ml jar of a Waring Blender containing 250 ml water. The suspensions of macerated roots and nematodes were thoroughly homogenised and 80 ml aliquots were collected. These were extracted by the conventional centrifugal-flotation method (Coolen & D'Herde, 1972) or by the ZC method. For the conventional method suspensions were processed in 100 ml centrifugation tubes with $MgSO_4$ at densities of 1.12, 1.14, 1.16, 1.18, 1.20, or 1.22. In contrast, the ZC extracted nematodes from the total aliquot and $MgSO_4$ at a density of 1.18 were used. All procedures were repeated five times for each of the densities and root ages.

Assaying low infections. *M. chitwoodi* infested soil was thoroughly mixed with a quantity of the same soil, that had previously been sterilised for 4 h at 100 °C, to obtain mixtures of 100, 50, 10, 5, or 1% (w/w) of the original nematode infestation level. From these mixtures the nematode infestation was assayed by: 1) ZC, 2) elutriation, and 3) a bioassay. For the ZC and elutriation methods, 100 ml soil samples were separated into MF and OF and both of these fractions were processed as mentioned previously. For the bioassay, plastic pots were filled with 500 ml unfractionated soil, planted with a 10 day-old tomato plant (cv. Marmande) and kept in the greenhouse (20-25 °C) for 40 days. At the end of this period, each plant was uprooted, the roots were washed free of adhering soil and examined for the presence of nematodes. Each method was repeated ten times.

RESULTS

Maceration. Maceration time affected the recovery of *M. chitwoodi* from the OF depending on the separation method (Fig. 1). ZC extracted no nematodes when the OF had not been macerated. The number of nematode eggs and juveniles liberated from the OF increased with increasing maceration time, reaching a maximum at 60 s maceration time. However, significant differences were present only between the non-macerated control and those of all of the five maceration times. When nematodes were separated on the cotton wool filter, highest recovery was obtained from non-macerated roots. Increasing maceration time did not significantly influence the numbers of nematodes.

Figure 2 shows the daily recovery of *M. chitwoodi* from root fragments after 0, 15, 60, or 120

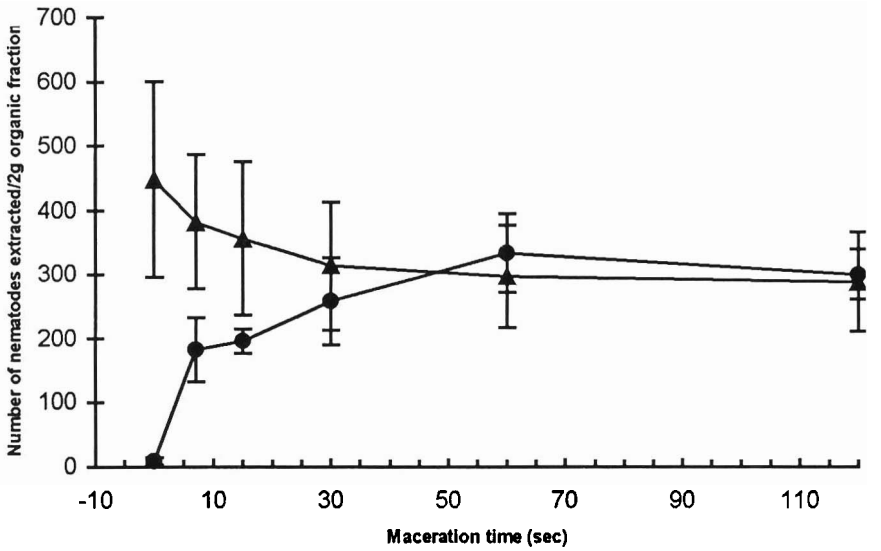

Fig. 1. Total number of *M. chitwoodi* individuals extracted from the organic fraction of a soil by zonal centrifugation (●) and a cotton wool filter (▲) after different maceration times. Bars represent standard errors.

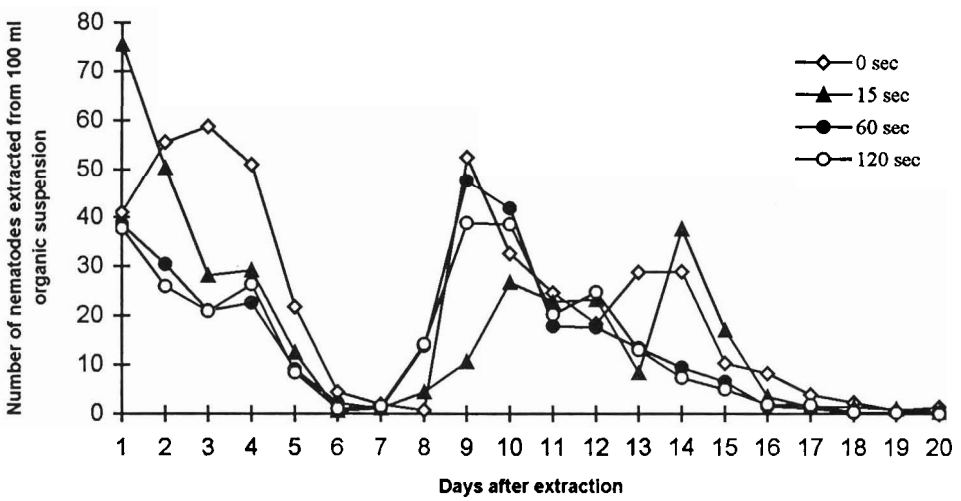

Fig. 2. Influence of three different maceration times of root fragments on the subsequent recovery of *M. chitwoodi* through a cotton wool filter.

seconds of maceration (other maceration times not shown to keep readability of the graphic). Nematodes were recovered from the first day after all maceration times. Nematode recovery from macerated roots was cyclical with peaks occurring at day 1, 9 (60-120 s) and 14 (15 s). By day 16, nematode recovery had nearly stopped for all treatments. A similar cyclical pattern was observed for non macerated roots with peaks at days 3, 9 and a lower peak at day 14.

Soil extraction. The relative efficiencies of methods for extracting *M. chitwoodi* from soil, OF, and MF are shown in Table 1. With each of the fractions, and the unfractionated soil, significant differences were found between the number of nematodes obtained by the three methods. When extracting nematodes from the unfractionated soil, or from the MF, the ZC flotation method yielded the largest number of nematodes with the conventional centrifugation method giving an in

Table 1. Comparison of methods for extracting *Meloidogyne chitwoodi* from soil and its constituent fractions.

Extraction method	Unfractionated soil (1)	Mineral fraction (2)	Organic fraction (2)
Elutriation	82.4a(3)	37.8a	880.2b
Conventional centrifugation	223b	228.8b	545.8a
Zonal centrifugation	492c	597c	1613.6c

(1) Unfractionated soil: 100 ml soil containing a non-macerated organic fraction and a mineral fraction.
(2) Mineral fraction or macerated organic fraction separated from 100 ml soil.
(3) Data means of ten replicates. Numbers in columns followed by a different letter are significantly different according to Duncan's Multiple Comparison Test at $P<0.05$.

Table 2. Effect of six $MgSO_4$ densities on the extraction by centrifugal methods of *Meloidogyne chitwoodi* eggs from fresh tomato and old roots of black salsify.

Extraction method	$MgSO_4$ density	Number of eggs extracted from 2 g fresh roots	Number of eggs extracted from 2 g old roots
Conventional centrifugation	1.12	189.0a (*)	11.2a
	1.14	284.2b	44.0b
	1.16	305.2b	50.6bc
	1.18	363.6b	65.8cd
	1.20	338.0b	64.0d
	1.22	356.0b	70.2d
Zonal centrifugation	1.18	762.4c	98.0e

(*) Data means of five replicates. Numbers in columns followed by a different letter are significantly different according to Duncan's Multiple Comparison Test at $P<0.05$.

termediate recovery and the elutriation method the poorest recovery. Nematode extraction from the OF was optimal when using the ZC method and the conventional centrifugation method yielded the lowest number of nematodes. With each of the three methods, the number of *M. chitwoodi* extracted from the OF was much higher than obtained from the MF. The number of nematodes extracted from the MF and from the unfractionated soil were similar.

The effect of $MgSO_4$ densities. The effect of $MgSO_4$ densities on the extraction of eggs from fresh or old roots is shown in Table 2. For the two root ages, the largest number of eggs was obtained using ZC extraction ($MgSO_4$ density = 1.18). This number of eggs obtained by ZC differed significantly from that obtained using the conventional centrifugation method with various $MgSO_4$ densities. With this latter method, the number of eggs extracted from old roots increased with increasing $MgSO_4$ densities. With fresh roots maximum egg recovery was obtained with a density of 1.18, and with old roots with a density of 1.22.

Detection of low infections. When comparing methods to assay small *M. chitwoodi* soil densities, all methods detected the nematode presence in all ten replicates of the 10%-, 50%-, and 100%-mixtures. Complete recovery was achieved for the 5%-mixtures, when using ZC or the bioassay; the elutriation method detected the infection in 9 out of 10 replicates. In the 1%-mixtures, *M. chitwoodi* was detected in 3, 6 or 9 out 10 of the elutriated, centrifuged or bioassayed samples.

DISCUSSION

Second stage juveniles of root-knot nematodes occur free-living in the soil or embedded in organic material. Eggs are present as egg masses in the soil, or are attached to, or partially or totally embedded in, organic material. Results from the experiments reveal that the nematode population present in OF is important when estimating nematode numbers of the endoparasitic nematode *M. chitwoodi* in soil samples. Depending on the extraction method used, is was found that the proportion of nematodes occurring in the OF of a

field soil varied between 70 and 96%.

Dickson and Struble (1965) developed a sieving and staining technique to extract egg masses of *M. incognita* from soil. Byrd *et al.* (1972) used this technique with limited success and developed an elutriation method with upright water flow to float root-fragments and egg masses and then used NaOCl to dissolve the gelatinous matrix that binds egg masses. In our experiments most eggs and juveniles from the OF would be lost if the OF was not first separated from the MF when elutriating the sample. Higher yields may have been obtained if the flow rate had been increased, but our samples contained a substantial amount of fine sediment and higher flow rates would have yielded even more sediment. The sediment already present in the samples made counting difficult.

The ZC method was shown to yield more *M. chitwoodi* than either the elutriation or the conventional methods. Second stage juveniles of *Meloidogyne* can pass through a 5 µm sieve and at the end of the elutriation the nematode suspension was poured onto a bank of sieves (50 µm and 45 µm). Consequently, the smaller yield of nematodes obtained with the elutriator is not surprising, but finer sieves would quickly become blocked.

Elutriation followed by cotton wool filter extraction provides a slow recovery of nematodes due to the obligatory hatching of the eggs. In our experiments nematode juveniles were collected from a cotton wool filter during a period of 20 days, after which time hatching had ceased. The effect of maceration was strongly influenced by time and the highest immediate nematode recovery was obtained after short maceration times of 7 and 15 secs. Longer maceration had the effect of reducing nematode numbers, probably as a result of nematode mortality. The majority of juveniles collected before days 6 to 7 had been liberated mechanically and thus were readily available. Those obtained later, had hatched naturally from liberated eggs. In non-macerated roots, maximum juvenile hatch was observed only after three days.

Although the ZC method and the conventional centrifugation method have the same working principle, their yield differed. With conventional centrifugation the nematodes move in a direction opposite to that of the heavy fraction (soil or root fragments). Initially, the majority of heavy particles sediment quickly including a number of nematodes. With ZC, the soil suspension is added gradually into the water layer and the nematodes move in the same direction as the heavy soil particles. Thus, the nematodes are not hindered in their movement. With the conventional centrifugation, the nematodes are exposed for a long time to osmotic pressure caused by the $MgSO_4$, whereas with ZC the nematodes are not subject to osmotic stress. This because the density at the water/$MgSO_4$ interface, where the nematodes are collected, is lower than that of the $MgSO_4$ solution. Moreover, the time spent by the nematodes in the heavy solution is much shorter in the ZC than in the conventional method.

Prot and Netscher (1978) used four-week old *Meloidogyne* susceptible tomato plants to bioassay root-knot infestation and stained the roots at day eight after transplantation. Their results showed that a bioassay is the best method to detect *Meloidogyne* at low densities. The results of our bioassay experiment confirmed this earlier study. Unfortunately, this type of bioassay is time consuming and does not address the possibility of reduced, or even complete inhibition of nematode hatching. However, root galls always need to be examined for the presence of *Meloidogyne*. Moreover, a bioassay can only provide an approximation of the number of nematodes present. A more accurate estimation can only be obtained after a quantitative extraction of the nematodes present in the sample.

The variability of sedimentation rate of individuals increases as the heterogeneity of a nematode population increases. The sedimentation rate of *Criconemella xenoplax* varies with the age of the nematode population and the host plant (Viglierchio & Schmitt, 1983). The results from our $MgSO_4$ experiment also show differences in density between newly produced eggs and older eggs. The density of new eggs is lower than that of older ones, indicating that appropriate densities of $MgSO_4$ should be used when extracting nematodes from older roots.

This study has revealed that the organic fraction in soil samples is extremely important, thus a sample should separated into the mineral and organic fractions. The organic fraction should be macerated to liberate the nematodes embedded in the root (fragments) and the suspension obtained should be added to the mineral fraction before further extraction.

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Chen S., Hendrickx G., Moens M. Роль органического вещества при оценке популяций *Meloidogyne chitwoodi* в почве.

Резюме. Проведено сравнение методов для выявления *Meloidogyne chitwoodi* в почвенных пробах. Показано, что органическая фракция может заключать значительную часть инвазионного материала. Первичная мацерация корней не оказывает существенного влияния на выход личинок при использовании экстракции на ватных фильтрах. Напротив, при экстракции центрифугированием мацерация корней позитивно сказывается на выходе личинок. Наивысший выход *M. chitwoodi* из органических и минеральных фракций почвы и нефракционированной почвы был получен с применением зонального центрифугирования. Увеличение плотности раствора $MgSO_4$ с 1.12 до 1.22 привело к повышению выхода старых яиц. Низкие плотности галлообразующих нематод наиболее эффективно обнаруживались методом биопроб и зональным центрифугированием.