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Distribution of *Meloidogyne chitwoodi* in potato tubers and comparison of extraction methods

Nicole VIAENE *, Tina MAHIEU and Eduardo DE LA PEÑA

Institute for Agricultural and Fisheries Research (ILVO), Plant Unit, Crop Protection, Burg. Van Gansberghelaan 96, 9820 Merelbeke, Belgium

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Summary – *Meloidogyne chitwoodi* and *M. fallax* are quarantine organisms in Europe. One measure to restrict the spread of these nematodes is careful inspection of potato tubers. The distribution of *M. chitwoodi* in heavily infected tubers was studied and several methods for extraction of these nematodes from tubers were compared. The majority of the nematodes (96%) were found in the first 5.25 mm of the tuber, corresponding to the depth of the vascular ring. About half of them were found between 1.75 and 3.50 mm deep. Incubation of small pieces of tuber on Baermann funnels in the misting chamber during 36 days yielded about 12 times fewer juveniles than mixing potato tissues and extracting them using zonal centrifugation, a process that took about 1 h. Enzymatic maceration of potato tissues for 24 or 48 h did not liberate more nematodes than 2 min of blending the tissues at high speed. More nematodes, with 95% consisting of eggs, were extracted by zonal centrifuging than by pouring the macerated suspension over a set of 500 μm, 250 μm and 20 μm sieves.

Keywords – Columbia root-knot nematode, detection, enzymatic extraction, quarantine.

The root-knot nematodes *Meloidogyne chitwoodi* and *M. fallax* can cause severe reductions in quality of potatoes and vegetables, especially carrot and black salsify (Santo *et al*., 1988; Van der Beek *et al*., 1998). In potatoes, typical symptoms are galling of roots and of the surface of infected tubers. Brown necrotic spots develop around the females, which are located just below the skin (Finley, 1981).

The spread of *M. chitwoodi* and *M. fallax* in Europe is mostly restricted to the south-east of The Netherlands and the adjacent north-east of Belgium (Waeyenberge & Moens, 2001; Karssen, 2002; den Nijs *et al*., 2004). However, they have also been reported in France, Germany and Switzerland (Daher *et al.*, 1996; Anon., 2004). Outside Europe, *M. chitwoodi* is widespread in the Pacific Northwest of the USA (Ingham, 2000) and also occurs in South Africa (Fourie *et al*., 2001). *Meloidogyne fallax* has been found in New Zealand (Marshall *et al*., 2001), South Africa (Fourie *et al*., 2001) and Australia (Nobbs *et al.*, 2001). As both species have many hosts, allowing their rapid build-up and distribution, it is of utmost importance to restrict their spread and to develop techniques that allow quick and reliable detection of the species.

Meloidogyne chitwoodi and *M. fallax* are listed as quarantine organisms in the European Union and special requirements exist for planting and movement of seed potatoes (Anon., 2000). Current regulations suggest visual inspection of plants and tubers of seed potatoes, after an appropriate induction method, as the procedure to detect infection by *M. chitwoodi* or *M. fallax*. The induction involves incubation of the tubers, which allows for accumulation of degree-days resulting in the development of both the nematodes and symptoms (Griffin, 1985; Pinkerton *et al.*, 1991; Van Riel, 1993). However, the development of external symptoms varies with variety, infection level and, to a lesser extent, with incubation conditions (Van Riel, 1993). It has been observed that tubers without symptoms can harbour *M. chitwoodi* (L. Molendijk, pers. comm.). Obviously, inspection of seed potato tubers by extracting nematodes from the tubers, instead of visually checking for symptoms after incubation, will improve detection and allow faster processing of samples. Detection of any given stage of the nematode should be performed with the most appropriate extraction technique available and by using the most appropriate parts of the tuber, so that the chances of detection are as high as possible.

* Corresponding author, e-mail: nicole.viaene@ilvo.vlaanderen.be

In view of this, the main objective of the work presented here was to evaluate different methods of extraction of root-knot nematodes from potato tubers. We also investigated the spatial distribution of *M. chitwoodi* in the potato tubers to clarify which part of a tuber should be analysed to increase the chance of detecting the nematode.

Materials and methods

Different potato tubers cv. Asterix, from a single lot harvested from a field infested with *M. chitwoodi*, were used for each test. Tubers were stored in a cold chamber at 4°C for 4 months prior to the experiments and had an approximate size of 8 cm in length and 5 cm in width. Tubers used in the experiments were carefully washed using a brush to remove soil from the potato surface. More than 30% of the tubers had an uneven surface and about 20% were deformed. When removing a thin (1 mm) slice of the tuber, it was easy to observe females and egg masses.

In the first experiment, the nematode distribution in the tubers was studied by examining different layers of infected potato tubers for the presence of the nematode. Mechanical maceration followed by zonal centrifugation was used to extract the nematodes from the potato tissue. In the second experiment, different layers were examined using the Baermann funnel technique. In the third experiment, zonal centrifugation and sieving were compared as extraction methods for nematodes from potato suspensions obtained by enzymatic maceration. Finally, by combining Experiments 1 and 3, a comparison between mechanical and enzymatic maceration was made.

Nematode distribution in potato tubers

Different layers were carefully removed from five tubers using a sharp knife. Layers were removed until no egg masses or females were visible in the tuber; then an extra layer was removed. For every potato, the thickness of each layer was measured at five places, resulting in 25 measurements per layer. Each layer obtained from five tubers was cut into pieces of about 1 × 1 cm. From these pieces, a 10 g sample was taken and macerated in a Waring blender for 2 min at high speed. The resulting mixture was poured over a 2 mm sieve to remove the remains of potato skin and peel that had not been macerated. The mixture and the rinsing water from both the blender and the sieve were collected in a beaker, and water was added to obtain a total volume of 1 l. Nematodes were extracted from the mixture using an automated zonal centrifuge (Hendrickx, 1995). This machine separates nematodes from soil or macerated plant tissues following the principles of conventional extraction by centrifugal flotation, but the process is completely automated. The nematode suspension with the macerated potato tissue is sub-sampled (500 ml was taken) and automatically transferred together with water and $MgSO_4$ solution (density = 1.2 g ml^{-1}) into a rotor. In this rotor the nematodes are separated from the other components and retained on the interface between the water and the $MgSO_4$ solution. After this zonal centrifugation, nematodes are eluted in a mixture of $MgSO_4$, water and kaolin and collected in a 10 ml vial through the hollow shaft of the rotor. In the extracted suspension all nematode stages, corresponding to 5 g of potato layer, were counted using a dissecting microscope.

Extraction on Baermann funnels in the mistifier chamber

The first five layers of five potatoes were cut in pieces of about 10 × 5 mm. A 10 g sample of each layer was put on a cotton wool filter placed in a Baermann funnel and left under continuous misting for 36 days at a temperature varying between 15 and 18°C. The 25 funnels (one for each layer and tuber) were arranged in a completely randomised block design. The number of hatched second-stage juveniles (J2) was counted every 2-3 days by tapping off about 20 ml of water from each funnel. After the observation period, every funnel was completely emptied into a beaker and the supporting sieve, containing slimy residues, was rinsed into the same beaker. The decomposed potato layer left on the cotton wool filter was also rinsed into the beaker. The content of each beaker was blended for 1 min as in the previous experiment and then nematodes were extracted using zonal centrifugation and counted following the procedure described above.

The experiment was repeated with the first three layers of six potatoes, but the water in the funnels together with rinsing water from the supporting sieve was completely removed 8, 12 and 18 days after the initiation of the experiment. This was done because it was thought that discarding the slimy residues with the water would improve release of J2. The water that was removed was checked for nematodes using the methodology (blending, centrifuging, counting) described above.

ENZYMATIC MACERATION FOLLOWED BY SIEVING OR ZONAL CENTRIFUGATION

The first layer, 1.75 mm thick on average, of three potatoes was cut up into 1 cm pieces and bulked. Eight 3 g samples were put in a 200 ml plastic cup, and 50 ml enzymatic solution consisting of 10 ml Pectinex (26 000 polygalacturonase units/ml), 10 ml Celluclast (700 endo-glucanase units/ml; Realco, Louvain-La-Neuve, Belgium) and 30 ml phosphate buffer was added. The solution also contained antibiotics at a rate of 50 mg tetracyclin and 50 mg streptomycin per l and had a final pH between 4.5 and 5. Cups were kept shaken at 35°C at 150 rpm. To investigate the influence of shaking time on the liberation of nematodes from the tissues, four mixtures were shaken for 24 h and four were shaken for 48 h. Nematodes were extracted from the potato suspensions using zonal centrifugation or by pouring the suspensions over a stack of sieves with apertures of 20, 250 and 500 μm. Two cups submitted to each shaking time were used for each extraction method. All nematodes retrieved from the 250 μm and 500 μm sieves were counted, whilst for the 20 μm sieve all nematodes were collected in a volume of 100 ml and three 2 ml subsamples were counted. The whole nematode suspension obtained after zonal centrifugation (10 ml) was counted. To measure loss of nematodes through the 20 μm sieve, the solution passing through the sieve was collected in beakers and nematodes were extracted with the zonal centrifuge.

The experiment was thus set up as a two-factor test with shaking time and extraction method as factors, each at two levels and with two replications of each combination. Data were analysed using analysis of variance (ANOVA) and means were separated by the LSD (Least Significant Difference) test. When the requirements for ANOVA were not met, logarithmic transformation of the data was performed.

Results

NEMATODE DISTRIBUTION IN THE TUBER

For each potato tuber, seven layers were cut with an average diam. of 1.75 $\pm$ 0.05 mm. Over 75% of the nematodes were extracted from the first two layers: 29.5% from the first layer and 48.5% from the second layer (Fig. 1). An average of 174 $\pm$ 66 juveniles and 1322 $\pm$ 408 eggs were retrieved per tuber after mechanical maceration followed by zonal centrifuging (Fig. 2). No other stages were found. The majority of the nematodes (96%) resided in the first three layers (from 0 to 5.25 mm in depth), but nematodes were retrieved up to the seventh layer, at 12.25 mm deep (Fig. 2).

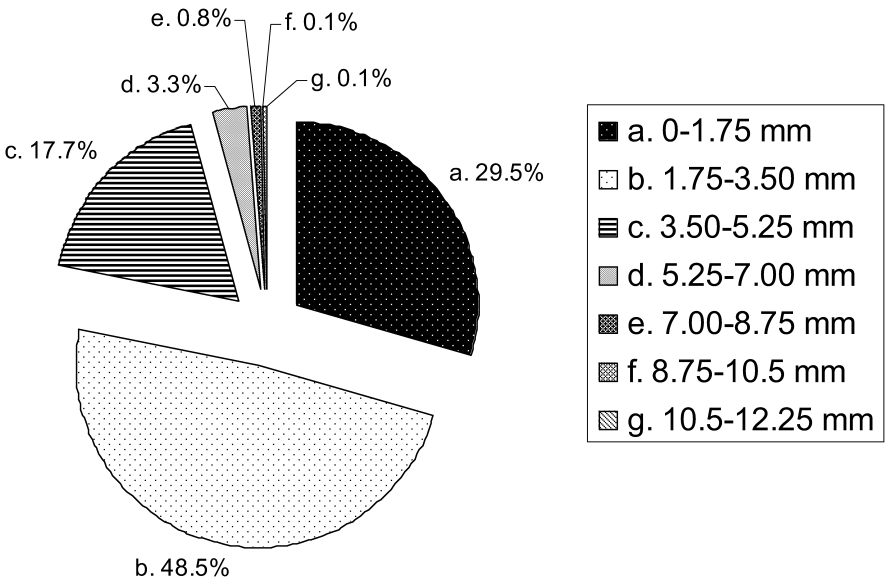

Fig. 1. *Distribution of* Meloidogyne chitwoodi *(eggs and second-stage juveniles) in different layers in a potato tuber. Data are based on five replications representing five tubers.*

Table 1. *Efficiency of the Baermann funnel technique for the extraction of* Meloidogyne chitwoodi *from five potato tubers.*

Tuber[1]	J2 in funnel[2]	J2 in filter[3]	Eggs in filter[3]	Total[4]	Efficiency (%)[5]
1	1	304	6896	7201	0.01
2	101	112	944	1157	8.73
3	582	48	336	966	60.25
4	2	128	1776	1906	0.10
5	0	32	1376	1408	0.00

[1] The first layer (1.75 mm) of five tubers was used.

[2] Total number of second-stage juveniles (J2) extracted during 35 days of incubation on a Baermann funnel in the mistifier chamber.

[3] Number of J2 or eggs in decomposed first potato layer on filter of the Baermann funnel, extracted using maceration-centrifugation at the end of the experiment.

[4] Sum of J2 and eggs retrieved from the Baermann funnel and the cotton wool filter.

[5] Expressed as the percentage of the total number of *M. chitwoodi* collected as hatched J2 from the funnel.

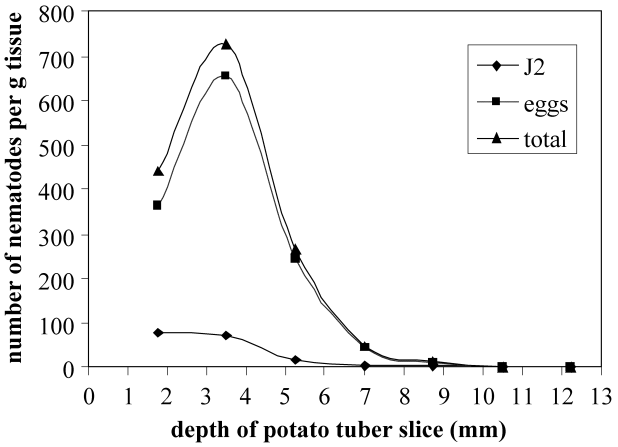

Fig. 2. *Number of second-stage juveniles (J2), eggs and total nematodes (eggs + J2) of* Meloidogyne chitwoodi *in consecutive layers of an infected potato tuber. Each data point is the mean of five replications representing five tubers.*

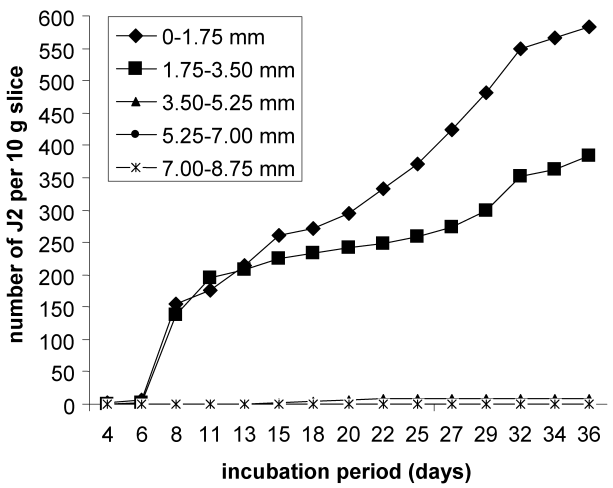

Fig. 3. *Extraction of hatched juveniles of* Meloidogyne chitwoodi *over time from different layers of a potato tuber put on Baermann funnels in the mistifier chamber.*

BAERMANN FUNNELS

The number of juveniles collected from the Baermann funnel over time varied greatly between the tubers (Table 1). A few hatched juveniles, 1-16 per funnel, were collected from all tubers on the first observation day (4 days of incubation). For one tuber, J2 hatched continuously over time during the whole incubation period and a total of 977 J2 per 10 g tissue were found after 36 days (Fig. 3). By contrast, very few J2 were retrieved from two out of the five potatoes (only two and four J2 per tuber), despite clear symptoms of infection with *M. chitwoodi*. Intermediate observations were made for the remaining two tubers; hatch stopped after 32 days, but a total of 33 and 112 J2 per 10 g tissue was counted (unpubl.).

The majority of J2 (96%) were retrieved from the first and second potato layers (data not shown), although in one tuber only a single J2 was retrieved from the first layer (Table 1). The extraction of nematodes from decomposed (first) potato layers on the cotton wool filter, at the end of the experiment, resulted in the discovery of hundreds of individuals of *M. chitwoodi* per 10 g potato layer, of which 95% were eggs (Table 1). The filter of the funnel with the tuber from which most J2 were extracted (Fig. 3), contained the fewest eggs and juveniles; here the Baermann technique showed a 60% efficiency (Table 1).

The repeat test with six tubers showed that removing all the water from the funnels and rinsing the supporting sieves resulted in a two-fold increase in numbers of retrieved J2; this was true at each occasion (8, 12 and 18 days). For example, after 18 days 52, 45 and four J2 were retrieved from 10 g of the first, second and third layer, respectively, when J2 obtained from the rinse were not included; however, 82, 97 and eight J2 per 10 g were obtained from the respective layers when rinsed J2 were included. In both tests, the water in the funnels was cloudy due to large amounts of starch released during the first week of incubation. Starch decomposition resulted in abundant growth of microorganisms during the whole extraction period. All eggs extracted from the rinsing water were black.

Enzymatic maceration followed by sieving or zonal centrifugation

Enzymatic maceration released different stages of *M. chitwoodi* from the tuber tissues: egg masses, eggs, juveniles and mature females. The amount of separate eggs was striking and depended on the extraction method; when centrifuging 95.3% of the total number of nematode stages were eggs, whereas only 47.5% of the stages consisted of eggs when sieving (Fig. 4). More eggs were retrieved by centrifuging than by sieving ($P < 0.01$), where eggs were only found on the 20 μm sieve. Zonal centrifugation of the water passing the 20-μm sieve indicated that there was a 3.5% loss of eggs and juveniles through that sieve (data unpubl.). Averaged over both extraction methods, more eggs were found after 48 h of maceration than after 24 h ($P < 0.01$). There was a significant interaction between time and extraction method ($P < 0.05$) for the numbers of eggs.

No egg masses and significantly fewer females ($P < 0.01$) were found after centrifuging than after sieving (Fig. 4). Egg masses and females were mainly found on the 250 μm sieve, in a few cases still attached to each other. Only third- and fourth-stage juveniles were extracted from the tubers, and this predominantly on the 20 μm sieve in case of sieving. There was no difference in numbers of juveniles extracted by sieving or by centrifuging ($P > 0.1$) (Fig. 4).

When considering the total number of nematode stages, where an egg as well as an egg mass are considered as one unit (as they represents one occasion for detecting *M. chitwoodi*), more nematodes were found when using zonal centrifuging than when using sieves ($P < 0.05$), when both incubation periods were considered together. Incubation for 48 h yielded more nematode stages than incubation for 24 h ($P < 0.05$), when numbers were averaged over both methods. Comparison of the four combinations showed that the sieving method following 24 h of enzymatic maceration yielded significantly fewer nematodes than centrifuging after 48 h of maceration ($P < 0.05$) (Fig. 4). The two other time-method combinations gave intermediate results (Fig. 4).

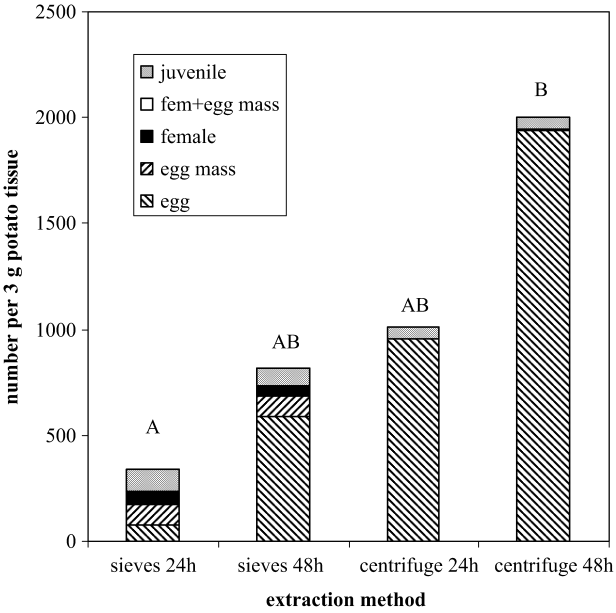

Fig. 4. *Number of different stages of* Meloidogyne chitwoodi *extracted by sieving and zonal centrifuging after 24 and 48 h incubation in an enzymatic solution. Different letters above bars indicate significant differences according to the Least Significant Difference Test (*P *< 0.05).*

Comparison of enzymatic and mechanical maceration

Mechanical maceration (Fig. 2) can be compared with enzymatic maceration (Fig. 4), as tubers from the same batch were used and nematodes were extracted from the macerated tissues from the first potato layer by zonal centrifugation in both experiments. An average of 442 nematodes/g tissue was found after mechanical maceration, of which 82% were eggs. Enzymatic maceration resulted in 502 nematodes/g tissue (averaged over 24 h and 48 h shaking), of which 95% were eggs. A t-test did not detect significant differences ($P < 0.05$) between numbers of nematodes retrieved with both maceration techniques.

Discussion

Distribution of *M. chitwoodi* was predominantly confined to the first 5.25 mm under the potato skin and most nematodes were found in the second layer (between 1.75 and 3.50 mm deep). This confirms the observations of Finley (1981) that lesions of *M. chitwoodi* are spaced around the vascular cylinder of the tuber (about 5 mm under the skin), generally in the cortex, but occasionally within the pith. Therefore, it is recommended that a 5 mm thick layer (as deep as the vascular cylinder) of a potato tuber should be examined for detection of *M. chitwoodi*. However, it is possible that the depth suggested, based on our results using cv. Asterix, could vary among varieties of potatoes, as there is also a variability in the appearance of external symptoms among varieties (Van Riel, 1993).

The Baermann funnel technique applied in a misting chamber was an inefficient method for extraction of juveniles from potato tissue. Not only were the total numbers of nematodes retrieved with this method very low compared to the numbers retrieved with maceration followed by sieving or zonal centrifugation, but it also took much longer to obtain results and there was large variability in numbers of collected juveniles between tubers. Mechanical maceration of the tissues in a blender, instead of cutting the slices into small pieces, might have improved nematode release to some extent (McSorley *et al*., 1999). However, the Baermann funnel technique requires hatching and movement of juveniles, processes hampered by the decomposition of tuber tissues. Disintegration of starch and pectin resulted in slimy residues which clogged the cotton wool filter and the supporting sieve and gave rise to an increase in microorganisms. The latter presumably created an environment detrimental to eggs, hatching and movement of juveniles, due to low oxygen levels and the release of toxins. Rinsing the funnels and examination of the water and the filters showed that the majority of the eggs were dead and many juveniles were stuck in the filter. This method might have been improved by providing aeration to the funnel content or by adding antibiotics to the water in the funnel to decrease bacterial growth and, thus, increase hatching to some extent, but use of these chemicals is not encouraged. In addition, hatching depends on the maturity of the eggs, which varies with age and storage temperature of the tubers before examination (Inserra *et al.*, 1983; Griffin, 1985; Pinkerton *et al.*, 1991).

Zonal centrifuging yielded about three times more nematodes than sieving from potato tissues macerated with enzymes. As the majority of the nematode stages were made up of eggs, these results reflect the conclusions obtained for the number of eggs. When using sieves, other stages (females, egg masses and juveniles) contributed to the total number of nematodes, with most of them found on the 250 μm sieve. The 500 μm sieve only caught an occasional large egg mass. However, in a preliminary test 78 egg masses were caught on this sieve (unpubl.). Either the smaller size of the egg masses or the rinsing with a stronger jet stream might have caused egg masses to pass through the 500 μm sieve in the experiment reported here. The low number of females and absence of egg masses extracted with the zonal centrifuge are a result of the size of these nematode stages; most are too large to be retrieved by the zonal centrifuge. The inferiority of the sieving method compared to centrifuging for retrieval of eggs could be caused by two factors: the aperture of 20 μm was too large or eggs were lost during handling. As only a 3.5% loss of eggs and juveniles was recorded, even if this is perhaps slightly underestimated due to loss in the zonal centrifuge, it is possible that eggs and juveniles were lost with splashing water when rinsing. A finer sieve, *e.g*., with 5 μm aperture, might catch more eggs and juveniles, but as there was little loss through the 20 μm, this option was not taken. Also, a finer sieve might easily become clogged.

Zonal centrifugation yielded two to three times more eggs and juveniles of *M. chitwoodi* than elutriation and conventional centrifugation (Chen *et al.*, 2000). When using the latter two techniques for extraction of *M. chitwoodi* from macerated tissue, there most probably will not be any difference with the sieving method tested in the present study, which yielded also about a third of the number of nematodes compared to the zonal centrifuge.

Incubation of potato slices in an enzymatic solution on a shaking platform resulted in release of egg masses and their disintegration into eggs, especially when incubation lasted 48 h. About the same number of egg masses (100) were found on the sieves after 24 h as after 48 h of incubation, indicating that more egg masses were released from the potato tissues during the extra 24 h of shaking, replacing the ones that were disintegrated into eggs. It is assumed that the release of egg masses and eggs are a result of the cellulase and pectinase activity. However, according to Finley (1981), egg masses inside tuber tissues are protected by a layer of lignified cortical cells, the so-called protective basket, which cannot be dissolved by pectinases or cellulases. This means that only mechanical disruption of the tissues, caused by the continuous shaking of the suspension, could have set the

egg masses free from the surrounding tissues. Shaking for 48 h resulted in more release of egg masses and eggs than shaking for 24 h, although the benefit of shaking for an extra 24 h was lost when an inferior extraction method (sieving) was used, as shown by the lack of significant differences between numbers of nematodes retrieved by sieving after 48 h and numbers extracted by zonal centrifuging after 24 h. In addition, it was shown that mechanical maceration, using a blender for 2 min, resulted in the release of as many nematodes as enzymatic maceration. Therefore, mechanical maceration, which is much faster and cheaper to perform than enzymatic maceration, is recommended as the technique to release *M. chitwoodi* from the potato tissues.

When dealing with tubers that are only slightly infected, however, all efforts should be made to break down the few egg masses that are present into eggs, thus enhancing the chances of finding one unit of *M. chitwoodi*. A method to disperse eggs from the gelatinous matrix should be applied. This could be performed by blending the tuber tissue in a 5% sodium hypochlorite solution instead of water and incubating them for 40 min (Kanwar *et al*., 1991). However, sodium hypochlorite reacts with magnesium sulphate used in zonal centrifugation, resulting in a deposit in the extracted nematode suspension. This makes microscopic observation of the nematodes impossible. Hence, sodium hypochlorite can only be used in combination with sieving. Cellulase and pectinease, used in the enzymatic solution, probably do not contribute to the breakdown of the gelatinous matrix as it is composed of glycoproteins and produces cellulase by itself (Orion, 1995).

Based on the results described above, and keeping in mind that the goal of the extraction is to have the greatest chance of detecting at least one unit of *M. chitwoodi*, regardless of the nematode stage, the following method is recommended for extraction of *M. chitwoodi* from infected potato tuber tissues. A 5 mm thick layer should be cut from the washed tuber, cut into 1 cm pieces and macerated with water for 2 min at high speed in a Waring Blender. Nematodes are then extracted from the suspension by zonal centrifugation. If the latter extraction method is not available, sieving of the solution containing the macerated tissues, through a stack of sieves with apertures of 500, 250, 20 and 5 μm, taking care to reduce losses as much as possible by careful handling, can be performed. Residues on all sieves should be collected and checked for *M. chitwoodi*.

Although not tested, we can assume that the same method could be applied for extraction of *M. fallax* from potato tubers. However, more research is needed on the distribution of this species in the potato tuber, as it has been observed that *M. fallax* occurs deeper in the tuber than *M. chitwoodi*. Following the detection, proper identification methods (morphological or molecular) should be applied to determine the nematode species.

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