



Extraction of potato cyst nematodes from soil samples: Cobb's decanting and sieving method vs. Fenwick's method

Extração de nemátodes de quisto de amostras de solo: método de decantação e crivagem de Cobb vs. método de Fenwick

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ABSTRACT

Potato cyst nematodes are a threat to several agricultural crops around the world with some species considered quarantine pests and subjected to strict regulatory measures in many countries. Usually, cysts nematodes co-exist in the soil with other species of plant-parasitic nematodes, so, a time and cost-efficient extraction technique becomes of primary importance. The ideal extraction method should be able to obtain cysts as well as detecting the presence of other motile plant-parasitic nematodes with a potential impact on potato farming (such as *Meloidogyne* sp. and *Pratylenchus* sp.). In recent years, studies have been carried out to test the efficiency of various methods of nematode extraction but few results have been published. Therefore, to test if a method that extracts simultaneously cysts and motile nematodes can be used instead of the reference method that extracts cysts only, the efficiency of Cobb's decanting and sieving technique was compared to Fenwick's technique. As a result, in the 74 samples evaluated, a greater number of cysts were extracted from 24 samples using Fenwick's method and from 11 samples employing Cobb's decanting and sieving technique. The statistics results showed a significance level of 0,05 using Fenwick's can allowing to conclude that this method is much more efficient than Cobb's decanting and sieving technique, and confirming it should not be replaced by alternative methods for cysts extraction.

Keywords: extraction techniques, *Globodera pallida*, *Globodera rostochiensis*, nematodes, PCN.

RESUMO

Os nemátodes de quisto constituem uma ameaça para diversas culturas agrícolas em todo o mundo. Algumas espécies são consideradas organismos nocivos de quarentena e encontram-se sujeitas a rigorosas medidas regulamentares em muitos países. Como os nemátodes de quisto coexistem no solo com outras espécies de nemátodes fitoparasitas, é necessário um método de extração que optimize custos e tempo. Idealmente, o método de extração deveria permitir detetar, para além dos quistos, a presença dos estádios juvenis de outros nemátodes fitoparasitas com potencial impacto agrícola (como *Meloidogyne* sp. e *Pratylenchus* sp.). Nos últimos anos, foram realizados alguns estudos para testar a eficiência de vários métodos de extração de nemátodos, mas poucos resultados foram publicados. Nesse sentido, foi comparada a eficiência do método de decantação e crivagem de Cobb com o método de Fenwick, visando testar se um método que extrai simultaneamente quistos e formas móveis pode ser usado em vez do método de referência para a extração de quistos. Nas 74 amostras avaliadas foi extraído maior número de quistos em 24 amostras pelo método de Fenwick e em 11 amostras pelo método de decantação e crivagem de Cobb. Estes resultados permitem concluir, para um nível de significância de 0,05, que o método de Fenwick foi mais eficiente que o de decantação e crivagem de Cobb na extração de quistos, não devendo ser substituído pelo outro método para este efeito.

Palavras-chave: *Globodera pallida*, *Globodera rostochiensis*, métodos de extração, nemátodes, NQB.

INTRODUCTION

The cyst nematodes comprise approximately 100 species belonging to six different genera. However, the most economically important species belong to the genera *Globodera* and *Heterodera*, since they represent a greater threat to several agricultural crops worldwide (Lilley *et al.*, 2005). Within the genus *Globodera*, two species stand out, the potato cyst nematodes (PCN), *Globodera pallida* (Stone, 1973) and *G. rostochiensis* (Wollenweber, 1923), while within the genus *Heterodera* four species are of great importance, *H. glycines* Ichinohe, 1952 (soybean cyst nematode), *H. avenae* Wollenweber, 1924 (cereal cyst nematode), *H. schachtii* Schmidt, 1871 (beetroot cyst nematode) and *H. zaeae* Koshy, Swarup and Sethi, 1970 (corn cyst nematode).

The species *G. rostochiensis*, *G. pallida* and *H. glycines* are considered to be harmful quarantine organisms and are part of the EPPO List A2 (quarantine organisms already present in the EPPO region, A2/125, A2/124 and A2/167, respectively) (EPPO, 2016). These species are described in the EPPO PM 7/40 (3) and PM 7/89 (1) diagnostic protocols, respectively, and are subject to stringent regulatory measures when detected (EPPO, 2008, 2013a). The success of such protocols depends on the efficient detection and monitoring of cysts in soils and other substrates (Kimpinski *et al.*, 1993).

Generally, the nematode extraction methods take into account different aspects: size, shape, mobility and the specific density of nematodes. These items lead to a variety of nematode extraction methods, described in the general EPPO diagnostic protocol PM 7/119 (1) where various methods are explained including Fenwick's technique (EPPO, 2013b). Despite Cobb's decanting and sieving method (Coob, 1918) is commonly used to extract motile forms from the soil, it can also be used for cyst extraction as has been described on previous works (Mota & Eisenback, 1993a, b; Van Bezooijen, 2006; Berger, 2007; Coyne *et al.*, 2007; Skantar *et al.*, 2007, 2011).

In recent years, some studies have been carried out to test the efficiency of various methods of nematode extraction, but few results have been published (eg. Bellvert *et al.*, 2008; Den Nijs & van den Berg, 2012; Kumar *et al.*, 2012). An ideal extraction method would allow all nematodes in a sample to be recovered at

all stages of their life cycle, regardless of temperature and soil type (composition, organic matter content and texture) and at low cost (work, equipment, water). However, none of the existing methods meets this ideal and, therefore, the nematology laboratories have to choose the most appropriate method for each situation (Van Bezooijen, 2006).

Cysts extraction methods are based on the fact that dry cysts contain air, causing them to float on the water surface. For an efficient recovery of cysts, the soil must be dried and passed through a 4 mm mesh sieve to remove the coarser material. To obtain a representative sample, a dry soil amount ranging from 100 to 500 g is used (Marks & Brodie, 1998; Van Bezooijen, 2006). Samples can be dried at room temperature or in a kiln, being this process essential for the accuracy of the diagnosis because if it is not well performed it can lead to false negative results. Drying of the soil at very high or non-gradual temperatures may impair the viability of the cysts contents, thus soil samples should not be dried at a temperature above 30 °C and with at least 40% air moisture (EPPO, 2013b).

Extraction of cysts nematodes using Fenwick's can

The Fenwick can is a metal can tapering at the top with a sloped base. The can has a sloping collar below the rim. Dry soil is placed in a receptor with a <2 mm screen over the can and washed through into the can. Heavy soil particles fall to the bottom of the can, whereas dried cysts and light soil debris float to the surface and are siphoned over the rim to a collecting sieve. The Fenwick can has been adapted by different manufacturers and laboratories and can vary in dimensions. It has the advantage of allowing the processing of a great number of samples and recovering a large number of cysts, but the soil samples must be previously dried and a large amount of water is expended (Fenwick, 1940; Oostenbrink, 1950; Van Bezooijen, 2006; EPPO 2013b).

Extraction of cysts nematodes using Cobb's decanting and sieving method

This method makes use of the differences in size, shape, nematode mobility, and sedimentation

rate of nematodes and soil particles. The sample is gently shaken in a container filled with water, separating the nematodes from the soil particles. The heavy particles sediment and the nematode suspension is decanted and passed through a set of sieves, where they are retained. This sieving method is performed with a series of sieves decreasing in size (1000-500 μm , 375-350 μm , 175 μm , 100 μm , 45 μm) so that nematodes of different sizes are collected separately (Mota, 1989; Van Bezooijen, 2006; Coyne *et al.*, 2007; EPPO, 2013b).

The advantage of using Cobb's decanting and sieving method is that in one procedure it is possible to extract cyst and motile forms of the nematodes, thus saving time and water. However, Marks & Brodie (1998) argue that only about 70% of the cysts can be recovered by the decanting and sieving method, and should not be used in routine analyses.

The present research was undertaken at the Laboratory of Nematology, which is part of the reference laboratory for Plant Health of the National Institute for Agrarian and Veterinary Research, I.P. (INIAV), in Oeiras, Lisbon, and responsible for the analyses of potato cyst nematodes in samples from the national survey programme. The main objective of this study was to compare the efficiency of the two cysts nematode extraction techniques, where Fenwick is the method used by the EPPO diagnostic protocols and Cobb's decanting and sieving technique a cost and time efficient alternative for cysts extraction. For the purposes of this study, Cobb's method was chosen due to its ability to extract nematodes of different sizes. So that, the laboratory would have an alternative cyst extraction method and could give fast and efficient responses to the high number of samples received.

MATERIAL AND METHODS

Soil samples

The soil samples analysed were collected in Portuguese potato fields during 2015 and 2016 and were provided by the Laboratory of Nematology of INIAV, in Oeiras. In no case was the origin of these samples known.

Cysts nematode extractions

Extractions were carried out on 74 samples of previously dry soil according to the EPPO PM/40 (3) protocol. A fraction of 300 g of each soil sample was extracted using Fenwick's can and an equal amount was used for Cobb's decanting and sieving method. Subsequently the cysts extracted by each technique were counted.

Comparison of the efficiency of the methods for extracting cysts from soil samples

The comparison of efficiency of the two methods was achieved through a comparison test of means for paired samples using the software R (<https://www.r-project.org/>). For this comparison were only used samples where one or more cysts were detected in at least one of the methods. A difference variable (D) was created and defined as $D_i = \text{Fenwick } i - \text{Cobb } i$ where "Fenwick" and "Cobb" are the number of cysts detected by the Fenwick and Cobb methods in the i^{th} sample, respectively.

The hypothesis tests were performed with a significance level $\alpha=0.05$. The assumption of normality of this variable was analysed by the Shapiro-Wilk test, with the same level of significance. The statistic used in the hypothesis tests: difference of means was $Z = \bar{D} / (sD / \sqrt{n})$, where \bar{D} is the mean of the variable D, sD is the sample's standard deviation and n is the number of observations. Subsequently, the χ^2 (chi-square) test was used, with the same level of significance, to allow identification of samples where there were major differences between the two methods.

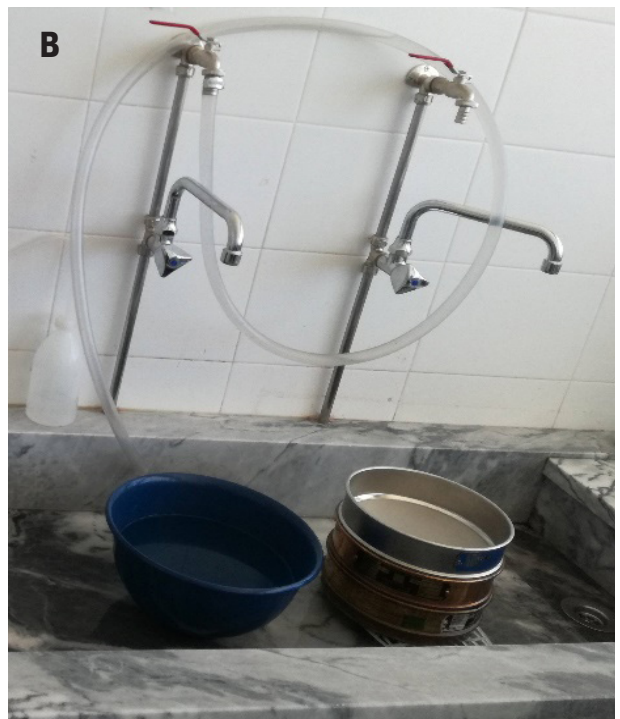


Figure 1 - Extraction methods. A: Fenwick can; B: Cobb's decanting and sieving technique; C: cysts collected.

RESULTS AND DISCUSSION

From the 74 samples analysed, no cysts were collected in 35 samples by any of the methods. In the remaining 39 samples a greater number of cysts were extracted from 24 samples using Fenwick's can and a greater number of cysts were gathered in 11 samples using Cobb's decanting and sieving method. In four samples the same number of cysts was collected.

It was sought to determine whether the efficiency of the two methods was different with a significance level of 0.05 and it was verified that this assumption could not be assumed, so, the hypothesis test that involves the Normal distribution (to the detriment of Student's *t*) was chosen, assuming a high number of samples. Under these conditions, statistics revealed that the performance of the two extraction methods is significantly different, with a *p*-value of 0.02539, less than 0,05, which allowed excluding the hypothesis of equality.

The next proposed hypothesis was that the Fenwick method was more efficient than the decanting and sieving method. The obtained p value of less than 0.05 (p-value = 0.012698), allowed supporting the hypothesis, with a significance level of 0.05, confirming that the Fenwick method is more efficient than decanting and sieving, meaning that it recovers a larger number of cysts from soil.

The results obtained are in line with the conclusions drawn by Marks and Brodie (1998) and Coyne *et al.* (2007), who argue that Cobb's decanting and sieving method should not be used in routine analyses.

Table 1 - Number of cysts extracted from the soil samples by Fenwick's and Cobb's decanting and sieving methods

Samples	Nº of cysts from Fenwick's method	Nº of cysts from Cobb's method	χ^2	p*
H1_2015	0	1		-
H2_2015	2	5	1,25	n.s.
H3_2015	8	2	3,6	n.s.
H4_2015	0	0		-
H5_2015	4	1		-
H6_2015	2	2		-
H7_2015	0	0		-
H8_2015	0	0		-
H9_2015	0	0		-
H10_2015	0	0		-
H11_2015	0	0		-
H12_2015	0	0		-
H13_2015	0	0		-
H14_2015	0	0		-
H15_2015	0	0		-
H16_2015	0	0		-
H17_2015	0	0		-
H18_2015	0	0		-
H19_2015	0	0		-
H20_2015	0	0		-
H21_2015	0	0		-
GB1_2015	2	0		-
GB1A_2015	1	0		-
GB2_2015	2	1		-
GB2A_2015	0	0		-
GB3_2015	13	2	8,067	< 0,01
GB3A_2015	15	3	8,000	< 0,01
GB4_2015	19	2	13,182	< 0,001
GB4A_2015	2	2		-
GB5_2015	0	0		-
GB5A_2015	0	0		-
GB6_2015	6	8	0,286	n.s
GB6A_2015	2	2		-
GB7_2015	0	1		-
GB7A_2015	0	0		-
GB8_2015	0	0		-
GB8A_2015	2	1		-

Samples	Nº of cysts from Fenwick's method	Nº of cysts from Cobb's method	χ^2	p*
GB8AII_2015	0	0		-
GB9_2015	0	0		-
GB9A_2015	0	1		-
GB10_2015	8	4	1,333	n.s.
GB10A_2015	7	7		-
GB11_2015	13	0	12,143	< 0,001
GB11A_2015	28	1	24,333	< 0,001
GB12_2015	0	0		-
GB12A_2015	0	0		-
GB13_2015	0	0		-
GB13A_2015	1	5	2,667	n.s
GB14_2015	28	0	28,000	< 0,001
GB14A_2015	0	14	14,000	< 0,001
GB15_2015	16	3	8,500	< 0,01
GB15A_2015	0	1		-
GB16_2015	0	0		-
GB16A_2015	5	39	26,272	< 0,001
GB17_2015	57	29	9,117	< 0,01
GB17A_2015	76	30	19,962	< 0,001
GB18_2015	93	67	4,225	< 0,05
GB18II_2015	2	0		-
GB18A_2015	1	0		-
GB19_2015	3	18	10,272	< 0,01
GB19A_2015	0	3		n.s
GB1_2016	60	17	23,718	< 0,001
GB2_2016	27	0	26,071	< 0,001
GB3_2016	204	47	97,817	< 0,001
GB4_2016	7	0	6,250	< 0,05
GB5_2016	1	0		-
GB6_2016	0	0		-
GB7_2016	0	0		-
GB8_2016	0	0		-
GB9_2016	0	0		-
GB10_2016	0	0		-
GB11_2016	0	0		-
GB12_2016	0	0		-
GB13_2016	0	0		-

* χ^2 (chi-square) test with a level of significance lower than 0.05, 0.01 or 0.001 (p < 0.05, p < 0.01 or p < 0.001).

CONCLUSIONS

Based on studies previously performed and in this study, it can be concluded that the extraction step is of the utmost importance for the detection of cysts in an area. Despite Cobb's decanting and sieving technique being able to recover cysts from soil samples, Fenwick's technique can recover considerable higher numbers of cysts from a soil core and it must be the method used in routine analyses. However, in other studies, Cobb's decanting and sieving method may be appropriate as a first approach to determine the presence of nematodes (cysts and motile forms) in a field, allowing to give faster answer and simultaneously saving time and

water during the extraction process. Nevertheless, it is necessary to consider that it can lead to false negatives. On the other hand, it would be interesting to have several subsamples and try to evaluate/compare the rate of cysts extraction by both methods using the same number of cysts homogeneously distributed in each sample and to establish a detection limit for the least efficient method.

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