Final Report

EUPHRESCO Program Meloidogyne II for non-competitive research projects

Project Title

Development and validation of innovative diagnostic tools for detection and identification of the quarantine nematode Meloidogyne enterolobii in support of integrated plant protection strategies in the EU member states

Background

Root-knot nematodes (*Meloidogyne* spp.) pose a significant risk to agricultural production systems all over Europe. *Meloidogyne enterolobii* (syn. *M. mayaguensis*) is a polyphagous species. It has been found on many host plants including ornamentals and important agricultural crops. *M. enterolobii* is considered to be a very aggressive root-knot nematode species that is able to reproduce on root-knot nematode-resistant plants such as tomato and pepper cultivars. The ability of *M. enterolobii* to overcome all known sources of resistance to root-knot nematodes makes it extremely difficult to manage this species, particularly in organic farming systems where chemical control is not an option.

Several interceptions of ornamental plants infested with *M. enterolobii* by the Dutch and German authorities in 2008, lead to adding this species to the EPPO (European Plant Protection Organisation) A2 list for quarantine organisms being locally present in the EU in 2010. One of the main questions raised during the pest risk analysis (PRA) for *M. enterolobii* was the possible source(s) and route(s) of introduction into Europe. The presence of *M. enterolobii* in greenhouses in Switzerland and plastic tunnels in France clearly demonstrates that there are pathways for introduction which have to be identified. To ensure that appropriate phytosanitary measures and management strategies are at hand to protect European agriculture against quarantine nematodes, reliable detection and identification tools are needed.

The EPPO diagnostic standard for *M. enterolobii* has recently been published (Bulletin OEPP/EPPO Bulletin 2011 (41): 329-339), but does not include state of the art real-time PCR methods for fast and reliable detection and identification. As most European NPPOs (National Plant Protection Organisations) have no experience with this new nematode species, training and test performance studies (formerly ring test) are required in support of NPPO's.

This project was done to deliver new and validated molecular-based protocols for detection and identification of *M. enterolobii* and consequently improved diagnostic expertise in Europe. It was also done to support the decision making process for eradication or integrated control strategies once the nematode has been detected.

Project benefits

Research conducted to address the following policy and science needs and objectives

- **Provision of new detection and identification tools in support of plant inspection services and extension personnel. These tools were tailored and validated for the suspected roots of entry such as soil and rooted plants (e.g. ornamentals).**
- **Provision of expertise and training in support of NPPOs. This was achieved by test performance studies on nematode extraction protocols and newly developed molecular detection and identification protocols. In addition, a workshop was organized to provide hands on training for Barcoding as new tool for nematode identification.**

The NPPOs have greatly benefitted from the project through enhanced knowledge on detection and identification tools of this new quarantine root-knot nematode species, and thus by supporting their decision making processes once an introduction has occurred. Protocols validated during this project will further be used to timely update EPPO diagnostic standards. The project facilitated sharing of knowledge and expertise in the field of *M. enterolobii* detection and identification to increase awareness of this and other quarantine root-knot nematode species in Europe.

The start date of the project was Jan 2012 and it ended June 2013 just as planned.

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Research topics / work plan

The aim of this project was to coordinate ongoing and future research in the field of *Meloidogyne enterolobii* detection and identification and to validate new methods in a collaborative way. It contained six main topics:

1. Test performance study on nematode extraction methods to detect motile stages (second stage juveniles) of *M. enterolobii* in soil samples.
2. Test performance study on fast and simple DNA extraction from target nematode in complex nematode suspension after extraction from soil samples (Lysis buffer; Holterman et al., 2009). DNA needs to be clean enough (without inhibitors) for further by molecular detection/identification tools. Labs were encouraged but not required to use additional extraction methods. The aim was to test a fast and simple DNA extraction protocol that can be harmonized between NPPO laboratories in the future.
3. Methods development and in-house validation of qPCR methods to detect, identify and potentially quantify *M. enterolobii* in complex nematode suspensions obtained from soil samples (develop protocols for robust, specific and sensitive detection/identification that can be harmonized between NPPO labs, independent of lab equipment and chemistry).
4. Test performance study on protocols developed under topic 3.
5. Final meeting to communicate and discuss results. Optional workshop on Barcoding as new diagnostic tool for fast and reliable nematode identification was offered to interested groups.
6. Publication of results in peer-reviewed research journals and discussion for inclusion of protocols into EPPO diagnostic standard protocol. Identification of future research priorities in the field of *Meloidogyne enterolobii* research.

The results of each research topic are described in detail hereafter. Project partners contributed their knowledge and expertise at all times, made suggestions and participated in the test performance study. Thus it was a small but very successful European project.

Work plan - Topic 1 – Validation of extraction methods for detection of M. enterolobii from soil samples (harmonization of extraction protocols)

Introduction

As demonstrated by a previous test performance study on *M. chitwoodi* and *M. fallax* (Euphresco project *Meloidogyne* I; Den Nijs and van den Berg, 2012), the choice of extraction method can greatly influence the results. The aim of this topic was to test (if possible) one common method for extraction of motile stages of *M. enterolobii* from soil samples and compare the results across participating labs (test performance study). Participants could choose one additional method that is routinely used in their diagnostics lab according to their national requirements for comparison (proficiency test). The recommended standard (Oostenbrink Elutriator) was based on the outcome of the previous TPS on *M. chitwoodi* and *M. fallax*. All partners taking part in this test performance study provided import permits if required by their national authorities.

Material and Methods:

1. Soil samples were collected in November 2012 from the original site of first report (organic farm greenhouse in Switzerland)
2. Soil was sieved (5 mm) and homogenized with a soil mixer
3. 100 ml of soil (10 replicates) was filled in plastic bags and stored at 3°C
4. Seven out of eight EU partners participated in this part of the test performance study. Participating EU partners were: CH-Agroscope (test organizer), BE-ILVO, DE-JKI, FR-LSV, IT-CRA-ABP, NL-NRC, TR-PPRI. UK-FERA did not participate in this part of the study. Soil samples were shipped to participating labs on 03.-04.12.2012 and received on 04.-06.12.2012, except for Italy, who requested the samples in February 2013. Samples were shipped to Italy on 12.02.2013 and received on 13.02.2013. A second set of samples was sent to Germany on 15.02.2013 and received on 16.02.2013.
5. A detailed extraction protocol for the preferred method (Oostenbrink elutriator plus incubation (organic fraction) for 14 days was provided with the soil samples. Additional methods could be used by labs as required by their national regulations and additional samples sets were provided for this purpose when requested.
6. Nematodes were counted in extracted samples and identified to genus level (*Meloidogyne* spp., other plant parasitic nematodes and saprophytic nematodes) based on morphology.
7. Costs for sample preparation and shipment were covered by the test organizer (Agroscope), costs for laboratory equipment and supplies, reagents and personnel were provided by each participating partner, which is in agreement with non-competitive funding mechanisms of Euphresco projects.

Results:

The following methods were used by participating labs for extraction of Meloidogyne spp. from soil samples:

ACW:

1. **Centrifugal flotation**: sample (100 ml soil) washed through 50 and 37 µm sieve; kaolin added and centrifuged at 2000xg for 2 min; discard supernatant; resuspend pellet in Ludox (1.18 g/ml); centrifugation at 2000xg for 2 min, pass over **50 and 37 µm sieve**; debris on Baermann; Counts after 72 h and 14 d
2. **Centrifugal flotation**: sample (100 ml soil) washed through 50 and 37 µm sieve, collect, add kaolin and centrifuge at 2000xg for 2 min; discard supernatant; resuspend pellet in Ludox (1.18g/ml); centrifugation at 2000xg for 2 min, pass over **20 µm sieve**; debris on Baermann; count after 72 h
3. **Oostenbrink dish**: sample (100 ml soil) in dish; count after 72 h

ILVO:

1. **Oostenbrink elutriator (with incubation of organic fraction)**
2. **Automated zonal centrifugation**

JKI:

1. **Centrifugal flotation (incubation of organic fraction)**
2. **Centrifugal flotation with additional sample set 16.02.13 (without incubation)**

LSV:

1. **Oostenbrink elutriator (adapted from Verschoor & De Goede (2000) + Oostenbrink dish (incubation)**
2. **Oostenbrink elutriator (adapted from Verschoor & De Goede (2000) + Centrifugation**

NRC:

1. **Oostenbrink elutriator (with incubation of organic fraction)**
2. **Oostenbrink elutriator (with incubation of organic fraction)**

PPRI:

1. **Oostenbrink elutriator**
2. **Modified Baermann method**

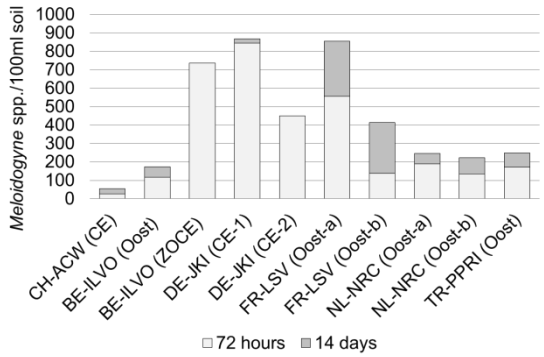
CRA-ABP:

1. **Cotton wool filter method (= modified Baermann method)**

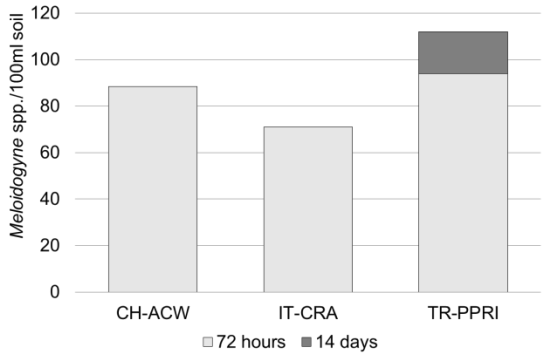
Results:

- Every participant received the soil samples within 1-2 days
- Independent from the extraction method used, each sample from every sample set yielded *Meloidogyne* spp. juveniles
- Storage at 3°C (or other temperatures) did not affect the success of *Meloidogyne* extraction (Nov. 2012-April 2013)
- The chosen method did affect the total number of *Meloidogyne* species detected in the soil samples (Fig. 1 & Fig. 2)
- Nematode counts per 100 ml soil are given below in the two figures.
- Overall variation (54 to 846 Mel./100ml soil) between lab participants was lower when compared to previous TPS on *M. chitwoodi* and *M. fallax* (0 to 6815 Mel./100ml)
- Oostenbrinck dish extraction yield a sufficient number of *Meloidogyne* juveniles with low variation between participating labs.

Effect of the extraction method (**Oostenbrink elutriator/Centrifugation**) on number of recovered *Meloidogyne* spp. per 100ml/soil



Effect of the extraction method (Oostenbrink dish/ modified Baermann) on number of recovered *Meloidogyne* spp. per 100ml/soil



Summary

- The method used had a higher impact on outcome than lab participant (opposite to previous study)
- Numbers of other plant parasitic nematodes (if counted) and saprophytic nematodes did correlate well with *Meloidogyne* numbers
- Total number of *Meloidogyne* spp. depended on method:
    - Baermann funnel/Oostenbrink dish = lower yield
    - Oostenbrink elutriator/centrifugation flotation and automated zonal centrifugation: moderate to high yield

Conclusions

- Depending on the type of sampling (Monitoring, Import, on Farm trials) an extraction method with the highest level of detection should be selected
- Shipping/storage is not a critical factor as long as storage is above 0°C
- Incubation of the organic fraction (for 14 days) can increase yields when densities of the target species are low and time permits

Work plan - Topic 2 – Testing a fast and simple lysis buffer for sufficient and sensitive target DNA extraction from nematode suspensions

Introduction

In order to successfully implement real-time PCR assays for detection and identification of *M. enterolobii*, the DNA extraction step prior to real-time testing is critical and needs to result in sufficient amounts of target DNA (no inhibitors) for successful detection. Furthermore, we have learned from previous test performance studies to keep it simple by comparing only a few methods at a time and providing most of the material needed for a given test. One DNA extraction method was finally chosen using a simple lysis buffer protocol previously developed in the Netherlands, which could easily be prepared by any participating lab (independent of a company). In addition labs were encouraged to use other DNA extraction methods (optional) for comparison with this lysis buffer protocol, which did not require any other DNA purification steps following extraction.

Organization of the test performance study (topics 2 & 4; timeline):

A total of eight European partners participated in the study of which seven participated on the molecular part (topics 2 and 4). 10 sets with 18 samples per set were provided to these seven participating labs. Some labs got two sets upon request for optional additional method testing (e.g. France and Germany got samples 1-8 twice for comparing different DNA extraction methods; the Netherlands got two sample sets for comparison of different qPCR tests. Samples were shipped to the labs on 11.12.2012 and were received one to two days later (12.-13.12.2012).
Analyses of samples: samples containing nematode suspensions were to be analysed directly, if possible, otherwise should be stored frozen or at 4°C until analysis. DNA was to be analysed within 4-6 weeks after dispatch. Most of the results were sent to the TPS organizer (Agroscope) between the end of January 2013 (deadline) and end of February.

Organization of the test performance study (topics 2 & 4; sample codes per set):

Topic 2 included samples 1-8, which were used for detection and identification of *M. enterolobii* in complex nematode suspensions from soil. Nematode suspensions consisted of 500 individuals per 1.5 ml water spiked with target nematode (*M. enterolobii*) juveniles (L2). DNA extraction was to be performed with the lysis buffer (including β-mercaptoethanol and proteinase K) according to the protocol provided with the samples.

Topic 4 included samples 9-18, which were used for linearity and specificity studies on target (*M. enterolobii*) DNA and non-target DNA (other *Meloidogyne* spp. or other nematode genera frequently found in soil samples). For a detailed list on sample codes see Table 1.

Tab. 1 List of sample codes (topics 2 & 4) and tube contents sent to participating labs.

Sample codes	Tube content	
1	Soil A only	Detection
2	Soil A + 2 L2	Detection
3	Soil A + 10 L2	Detection
4	Soil B only	Detection
5	Soil C only	Detection
6	Lysis buffer	Identification
7	L. buffer + 2 L2	Identification
8	L. buffer + 10 L2	Identification
9	Me DNA 100x dil	Linearity
10	Me DNA 1000x	Linearity
11	Me DNA 10000x	Linearity
12	Me DNA 100000x	Linearity
13	*M. hapla* DNA	Specificity
14	*M. graminicola* DNA	Specificity
15	*Nacobus abberrans* DNA	Specificity
16	*Globodera rostochiensis* DNA	Specificity
17	*M. chitwoodi* DNA	Specificity
18	*M. fallax* DNA	Specificity

Material and Methods (topic 2):

1. Samples were shipped to participating labs according to the organization of the TPS mentioned above
2. Seven out of eight EU partners participated in this part of the test performance study. Participating EU partners were: CH-Agroscope (test organizer), BE-ILVO, DE-JKI, FR-LSV, NL-NRC, TR-PPRI, and UK-FERA. IT-CRA-BP did not participate in this part of the study. Samples were shipped to participating labs on 03.-04.12.2012 and received on 04.-06.12.2012
3. A detailed extraction protocols for the preferred method (lysis buffer extraction) was provided with the samples. Additional methods could be used by labs as required by their national regulations and additional samples sets were provided for this purpose.
4. Costs for sample preparation and shipment have been covered by the test organizer (Agroscope), costs for laboratory equipment and supplies, reagents and personnel were provided by each participating partner, which is in agreement with non-competitive funding mechanisms of Euphresco projects.

Results (Assessment of TPS; sample stability)

The assessment of sample stability is related to target samples for detection (nematode suspensions samples 1-5). We found an overall excellent sample stability over a period of 6-8 weeks (investigation period) with only a few variations noticed (data not shown). Direct DNA extraction of nematode suspensions and short storage (< 5 days) at 4°C yielded good DNA quality for qPCR (very similar Ct-values; Ct-value difference only 1-2). Longer storage of nematode suspensions at -18/20°C prior to DNA extraction works also well. This was mostly done by labs, which could not analyze samples directly (close to Christmas break). However, storage of nematode suspensions at 4-6°C for longer periods before DNA extraction is not recommended, since it yielded lower DNA content or a decrease in DNA quality (higher Ct-values >2-3).

Results TPS : (topic 2 only) :

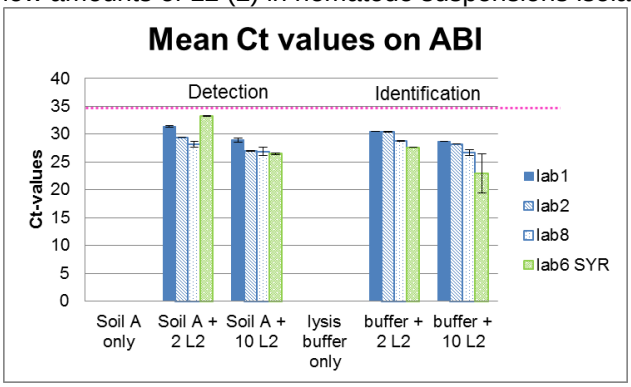
- DNA extraction with the lysis buffer was done by all labs and was followed by COI qPCR according to the protocols for DNA extraction and qPCR provided by the TPS organizer. Performance of DNA extraction can only be evaluated based on qPCR results.
- Results of COI qPCR for detection and identification samples are used for evaluating DNA extraction
- All COI qPCR results were as expected (for detection, identification)!
- No differences between labs or qPCR platforms used were seen by looking at +/- reactions, i.e. all labs provided similar results (positive samples were tested positive and negative samples were tested negative by all labs). Thus results are in 100 % agreement between labs (Table 2).
- DNA extraction by Wizard Magnetic Beads + KingFisher (done by France) was as good as the simple lysis buffer in plus/minus detection (Table 2), however Ct-values were higher (about 2 Ct-values), probably due to loss of sensitivity/DNA, which is known from DNA extraction kits using magnetic beads and thus as expected. One should note here that magnetic beads based DNA extraction kits usually provide very clean and pure DNA, which is sometimes required in downstream experiments. However, our purpose here was to test a simple and fast lysis buffer that can be self-prepared and provides good DNA content for following qPCR research without any further cleaning steps.
- DNA extraction by the ZYMO Research Tissue and Insect MicroPrep-Kit (done by Germany) was less reliable (Table 2). The Ct-values for the detection samples are still okay but identification values failed (all values negative; not identifying two of two positive samples). However, please note, that this was just an additional preliminary test and included only one replication per data set provided by the German participant. So to draw any final conclusions about the performance of this kit additional data should be provided.

Table 2. Results of test performance study (topic 2 & 4) of all participating labs and their equipment used based on plus/minus reactions of all samples in a sample set

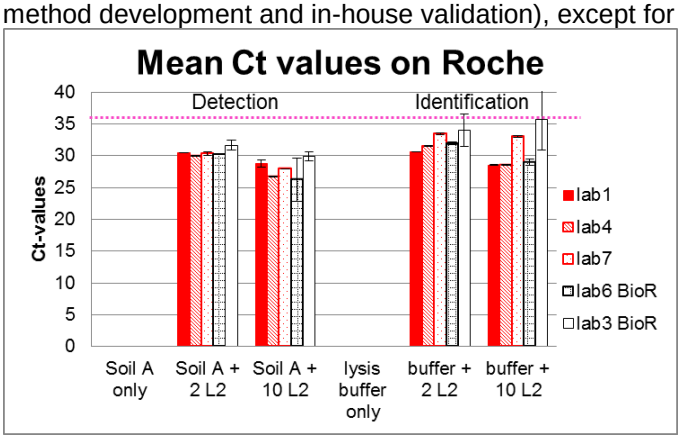
sample code	content			expected results		lab1		lab2		lab3		lab4		lab6		lab7	lab8
			Tigano	ABI	Roche	ABI	Roche	ABI	Wishart	BioRAD	ZYMO	WizardMagnR	Roche	BioRAD	SYRBGreen	Roche	ABI
1	Soil A only	detection	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	Soil A + 2 L2	detection	-	+	+	+	+	+	+ (615bp)	+	+	+	+	+	+	+	+
3	Soil A + 10 L2	detection	+/-	+	+	+	+	+	+/-	+	+	+	+	+	+	+	+
4	Soil B	detection	nt	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	Soil C	detection	nt	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	lysis buffer only	identification	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	buffer + 2 L2	identification	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+
8	buffer + 10 L2	identification	+/-	+	+	+	+	+	+	+	-	+	+	+	+	+	+
9	Me DNA 1/100	linearity	+ (520bp)	+	+	+	+	+	+ (615, 350, 2	+	nt	+	+	+	+	+	+
10	Me DNA 1/1000	linearity	+	+	+	+	+	+	+	+	nt	+	+	+	+	+	+
11	Me DNA 1/10000	linearity	+	+	+	+	+	+	+/-	+	nt	+	+	+	+	+	+
12	Me DNA 1/100000	linearity	-	+/-	+/-	+	+	+	-	+	nt	+	+	+	+	+/-	+
13	M. hapla	specificity	nt	-	-	-	-	-	-	-	nt	-	-	-	-	-	-
14	M. graminicola	specificity	nt	-	-	-	-	-	-	-	nt	-	-	-	-	-	-
15	N. abberrans	specificity	nt	-	-	-	-	-	-	-	nt	-	-	-	-	-	-
16	G. rostoch.	specificity	nt	-	-	-	-	-	-	-	nt	-	-	-	-	-	-
17	M. chitwoodi	specificity	nt	-	-	-	-	-	-	-	nt	-	-	-	-	-	-
18	M. fallax	specificity	nt	-	-	-	-	-	-	-	nt	-	-	-	-	-	-
	ntc	specificity	-	-	-	-	-	-	-	-	nt	-	-	-	-	-	-
											only 1 rep	2 reps					

Results TPS : (topic 2, continued) :

- All labs using ABI platforms provided similar results, despite different Ct-values, for target nematode detection and identification, i.e. lower Ct-values with increasing amounts of target DNA (10 vs 2 *M. enterolobii* L2) (Fig. 3)
- All values were below the cut-off Ct-value of 35 (cut-off Ct-value defined for ABI platforms under topic 3; method development and in-house validation)
- The SYRBGreen assay results (provided by the Netherlands) for detection and identification samples were similar to COI qPCR results, despite different Ct-values, except for detection of low amounts of L2 (2) in nematode suspensions isolated from soil background (Fig.3)



- All labs using Roche platforms provided similar results, despite different Ct-values, for *M. enterolobii* detection and identification samples (lower Ct-values with increasing amounts of target DNA (10 vs 2 L2). Labs using BioRad platforms provided similar results as labs using Roche platforms; even Ct-values were very similar.
- All Ct-values were below the cut-off Ct-value of 36 (defined for Roche platforms under topic 3; method development and in-house validation), except for one BioR platform (Fig.4).



Conclusions

- Lessons learned from previous TPS were to keep it simple and test only single parameters at once (not too many methods simultaneously) and provide most of the material needed for the tests to decrease amount of variation between labs. We followed this by testing **only one DNA extraction method** (lysis buffer) and **only one qPCR** method in the presented test performance study.
- Regarding qPCR assays: The previous TPS on *M. chitwoodi* and *M. fallax* detection suggested to provide the range of threshold, cut off Ct-values, and TM values as well as controls at the limit of detection (LOD). We did this by an in-house validation of methods by the TPS Organizer under topic 3. Data were not provided to participating labs because of keeping it as a blind study; but data were available prior to the start of the TPS.
- Improvement of reaction conditions (better efficiency/sensitivity) was reached by all partners by providing most of the solutions needed (buffers, primers) by the TPS organizer. This was also suggested by the previous ring test.
- Data generated were not too many and could therefore still be handled and evaluated (less variance between labs using different equipment).
- Before starting the TPS cut-off Ct-values were defined for different equipment and chemistry used and thus robustness of test under different settings was determined.
- No difficulties were encountered or reported by any of the eight participants during the TPS 2012/2013 or during the final meeting. However, as mentioned before, all participants were encouraged to provide their expertise and knowledge prior to the TPS, and thus a calculation mistake in the qPCR protocol was discovered by the Belgium participant and the protocol was adjusted accordingly.
- The purpose of topic 2 was to test a lysis buffer for DNA extraction. It worked well for all labs and was simple to use and fast to prepare. No complaints or negative comments were encountered. Thus we suggest the inclusion of this lysis buffer protocol for DNA extraction from complex nematode suspensions into the next revision of the EPPO Standard protocol for diagnostics of *M. enterolobii* (PM 7/103).

Introduction

This topic aimed at improving the diagnostic process of *M. enterolobii* currently in use (conventional PCR assays; EPPO Bulletin 41, 329-339) by introducing real time PCR (qPCR) assays on a routine basis. Because real-time PCR is often dedicated for identification, but rarely for detection or even quantification of the pest in complex matrices (soils, roots) in a realistic sample size, this topic aimed at evaluating qPCR tests for specific and sensitive *M. enterolobii* detection and quantification in suspensions obtained by extraction from soil and root samples. The best qPCR protocol that was developed under this topic was then tested in the test performance study (topic 4).

Material and methods:

1. We developed and in-house validated two new qPCR protocols based on previously generated and published sequence data of *M. enterolobii* using parts of the IGS2 and COI gene sequences
2. Primers and probes for qPCR development were selected according to the most suitable gene regions for best sensitivity and specificity. We aimed at a detection level of one individual target nematode in a 100ml soil sample consisting of many different background nematodes.
3. To reach this level of specificity and sensitivity preliminary tests, based on Taqman MGB (Minor Groove Binding) probes, SYBR Green I assays as well as Roche Universal LNA (Locked Nucleic Acid) probes, suggested the use of LNA probes will be best suited for this purpose.
4. The IGS2 and the COI qPCR assays were tested on two platforms (Applied Biosystems and Roche Light Cycler LC480) with different chemistries (Roche, Applied Biosystems, Quiagen, Eurogentec). LNA probes using the Roche Light Cycler Probes Master on Roche platforms and the TaqMan Environmental Master Mix on ABI platforms provided best reliable results and protocols were thus optimized accordingly. Methods and protocols were optimized independent of platform and chemistry used, that can be used be any laboratory capable of molecular diagnostic analyses (independent of buffers or kits of a company; methods harmonization).
5. In-house validation experiments included: Optimization of primer and probe concentrations used in the two assays; finding the right Tm for primers and probes; finding the limit of detection (LOD), i.e. cut-off Ct-values for each qPCR assay and platforms and chemistries used.
6. Analytical sensitivity was validated using standard curves based on dilutions of *M. enterolobii* DNA obtained by bulk extraction of juveniles in lysis buffer. Analytical specificity was validated with 16 *M. enterolobii* populations from world-wide collections, eight non-target *Meloidogyne* species including close relatives and four other genera. Furthermore, repeatability, selectivity, and reproducibility were tested.
7. The optimized protocol for DNA extraction and nematode detection was tested and validated with spiked and non-spiked plant and soil samples. The final optimized protocols were then tested in the test performance study (topics 2 & 4).

Time schedule:

All research (methods development and in-house validation) was done by the TPS organizer at Agroscope Research Station from January – October 2012 prior to the start of the ring test in November/December 2012.

Results:

- Development of new diagnostic qPCR methods using LNA probes. LNA-probes were used due to the following advantages :
    - Dark quenchers (non-fluorescent) at the 3’ end have no background fluorescence such as TAMRA

- They are much shorter than normal TaqMan & even MGB probes (8-9 nt)
- Locked nucleic acids are DNA nucleotide analogues with higher affinity to target DNA (methyl bridge between first O-Atom and fourth C-Atom on ribose ring; DNA backbone)
- Higher specificity and higher detection rate of short target DNA sequences (e.g. frequently used for base pair mismatch discrimination assays or SNP genotyping assays)

- Why choosing COI as gene region for qPCR method development? The QBol project provided promising barcoding sequences for species differentiation of *Meloidogyne* spp. The cytochrome oxidase subunit I gene (COI) is a less variable coding region and demonstrated good discriminatory power between species, with the exception of some tropical *Meloidogyne* spp. (M. *javanica, M. incognita, M. arenaria*). However, little to no variation was observed within species. 46 bases were unique to *M. enterolobii* and 62 diagnostic SNP's could be identified when compared to other *Meloidogyne* species.

- Why choosing IGS2 as gene region? The intergenic spacer region 2 (IGS2) demonstrated a high intra-individual variation (variable non-coding region), thus the large subunit (LSU, 28S) of the ribosomal DNA was selected as barcoding region for nematode identification. However, we looked more closely into this gene region of *M. enterolobii* comparing it with IGS2 gene sequences of closely related *Meloidogyne* spp. and were able to identify a region with SNPs specific enough to distinguish it from other *Meloidogyne* spp.

- In-house validation: Analytical specificity: No cross-reactions with eight non-target *Meloidogyne* spp. including close relatives and four other genera. All *M. enterolobii* populations (16) reacted highly specific with developed assays. No differences were observed between platforms used, assays were highly repeatable and reproducible (Tab. 3).

  Table 3: Results of IGS2- and COI qPCR specificity tests using DNA of *M. enterolobii* and close *Meloidogyne* relatives and other nematode genera frequently found in soil samples.

Nematode	Number of populations/Isolates	Source/Reference collection	IGS2	COI
M. enterolobii	16	CH, F, NL, USA	+	+
M. incognita	6	CH, D, NL	-	-
M. hapla	2	CH, NL	-	-
M. fallax	1	CH	-	-
M. arenaria	2	CH, NL	-	-
M. javanica	2	NL	-	-
M. ethiopica	1	SI	-	-
M. chitwoodi	1	D	-	-
M. graminicola	1	D	-	-
Bursaphelenchus xylophilus	1		-	-
Bursaphelenchus mucronatus	1		-	-
Nacobus abberrans	1	F	-	-
Globodera rostochiensis	1	CH	-	-

- In-house validation (analytical sensitivity): Both qPCR assays were highly sensitive in detecting one individual target nematode juvenile (L2) in a nematode suspension obtained from 100 ml of soil at a Ct-value of 30. The limit of detection (LOD) cut-off values for the COI qPCR assay was defined at a Ct-value of 35.2 on Applied Biosystems (ABI) platforms and was set at a Ct-value of 36.0 on Roche Light Cycler (LC480) platforms. This assay was finally

selected for the TPS (topics 2 & 4).

- In-house validation (selectivity I). Selectivity as defined in PM 7/98 (EPPO Bulletin 40, 5-22; 2010) is not relevant for nematodes identification as they are previously isolated from the matrix. If test is used as a detection test, insensitivity of the test to variation of matrix, in our study different amount of background nematodes, should be determined. When we compared results of the COI qPCR assay on two platforms using standard curves of pure *M. enterolobii* DNA with spiked standard curves, to which 100 or 1000 background nematodes in 200 µl lysis buffer had been added, we noticed first of all no inhibition of qPCR reactions. Spiking pure samples with background nematodes did shift Ct-values slightly (about 1-2 Ct-values), resulting in a slight loss of sensitivity, but it did not affect the slope of the regression, i.e. validity of COI qPCR assay.

- In-house validation (selectivity II): In this experiment matrix variation consisted of a background of 1000 background nematodes which very spiked with different amounts of target nematodes (L2 juveniles of *M. enterolobii*). *M. enterolobii* was correctly detected in nematode suspensions containing DNA of 1000 other nematodes. One individual L2 was detected at a Ct-value of 30-32 in samples with background nematodes vs 29-31 in samples without background. Ct-values increase with higher amounts of target nematodes (Me L2) present.

- Sensitivity of the COI qPCR in finding *M. enterolobii* target DNA in complex nematode suspensions on different real-time PCR platforms using different chemistry. One *M. enterolobii* juvenile could be detected in a suspension of 1000 background nematodes. Non-target DNA presence reduces sensitivity of assays only slightly.

- In-house validation (selectivity III): Detection of *M. enterolobii* in spiked/non-spiked Bonsai soil and root samples was tested by the two different qPCR assays (COI qPCR; IGS2 qPCR) and two different real-time platforms and chemistries used (ABI 7500 vs. Roche LC 480). Samples contained low to moderate numbers (0 to >40) of other *Meloidogyne* species *(M. incognita, M. javanica*) in the background. Spiked samples meant one *M. enterolobii* L2 juvenile was added. All non-spiked Bonsai samples (soil and roots) were negative for Me, since average Ct-values were above Ct cut off values of 35-36±1. Spiked Bonsai samples (soils and roots) were positive for Me. The detection of one *M. enterolobii* L2 was possible at an average Ct-value of 29-31. The tests are non-selective, i.e. insensitive to matrix variation (soil, roots). Numbers in table are average Ct-values (n = 6).

Summary:

- We developed two new innovative species specific qPCR methods for the detection/quantification and identification of the root-knot nematode Me from soil or plant roots.

- The assays could distinguish *M. enterolobii* from other tropical root-knot nematodes (*M. incognita, M. ethiopica, M. arenaria, M. javanica, M. hapla, M. fallax*).

- The assays were highly sensitive and detected one juvenile in a suspension of nematodes from 100 ml soil at an average Ct-value of 30 or one target nematode juvenile in a background of 1000 other nematodes. By using standard curves of pure target nematode DNA it is possible to quantify the amount of target in an unknown sample.

- The assays were highly repeatable with a high level of agreement between replications of samples tested under the same conditions (very small Standard deviations)

- The assays were highly reproducible and provided consistent results even when tested under

different conditions (time, equipment).

- The assays were non-selective, i.e. insensitive to matrix variation (as defined in three independent selectivity experiments).

Conclusions

- These assays will speed up routine diagnostics, especially when needed for many samples, and result in faster implementation of phytosanitary measures for this Q-Organism (help in decision making)
- Surveys/monitoring studies for the distribution, spread, survival of *M. enterolobii* or its introduction into EU countries is possible. Import controls at port of entry are now also possible and can stop invasion into non-contaminated areas (import/export control)
- The developed and in-house validated COI qPCR assay has also been validated in the TPS study (topics 2 & 4) by seven participating EU partners.
- Assays are simple to use and can be done by any laboratory capable of molecular biological studies. Assays do not depend on knowledge of nematode morphology.
- The new real-time PCR assay will be included in the revised version on the diagnostic standard for *M. enterolobii.*

- ***Work plan - Topic 4 – Test Performance Studies for the specific detection and identification of M. enterolobii in nematode suspensions obtained from root and soil samples***

Introduction

Real-time PCR assays have been used successfully for identification of other quarantine root-knot nematode species such as *M. chitwoodi* and *M. fallax*. These assays are mostly dedicated to high throughput identification as many samples can be analyzed in parallel. However, assays able to detect the target nematode in a complex matrix or from a nematode suspension obtained after extraction from soil or roots is also desirable.

This test performance study aimed at testing the robustness of a lysis buffer for DNA extraction from target nematodes (topic 2) followed by molecular detection or even quantification and identification (topic 4). Testing the robustness of the optimized DNA extraction and qPCR protocols under different laboratory constellations and by different laboratory personnel is critical for successful out-house validation. The newly developed and in-house validated COI qPCR protocol for *M. enterolobii* detection and identification (topic 3) was therefore tested under different laboratory constellations (equipment, chemistry and personnel) using the same samples provided by the TPS organizer. This allows an assessment of performance of laboratories in using qPCR tools. The outcome of this topic can further help NPPOs in their decision making process by providing a fast and reliable diagnosis of Q-pathogens followed by fast implementation of phytosanitary measures such as control or management strategies, and assessment of efficiency of eradication or assessment of treatment assays after an introduction has occurred. In addition, molecular based detection and identification methods do not require tedious and time consuming nematode identification to species level by morphometrical characters using microscopic examination which can be only correctly performed by experienced taxonomists.

Organization of the test performance study (topics 2 & 4; timeline):

A total of eight European partners participated in the study of which seven participated on the molecular part (topics 2 and 4). 10 samples with 18 samples per set were provided to these seven participating labs. Some labs received additional sets upon request for optional additional method testing (e.g. France and Germany got samples 1-8 twice for comparing different DNA extraction methods; the Netherlands got two sample sets for comparison of different qPCR tests. Samples were sent to the labs on 11.12.2012 and were received one to two days later (12.-13.12.2012).
Analyses of samples: samples containing nematode suspensions were to be analysed directly, if possible, otherwise should be stored frozen or at 4°C until analysis. DNA was to be analysed within 4-6 weeks after dispatch. Most labs followed this guideline and results of the sample stability study (assessment of TPS) can be seen on page 10. Analyses results were sent to the TPS organizer (Agroscope) between the end of January 2013 (deadline) and end of February.

Organization of the test performance study (topics 2 & 4; sample codes per set):

Topic 2 included samples 1-8, which were used for detection and identification of *M. enterolobii* in complex nematode suspensions from soil. Nematode suspensions consisted of 500 individuals per 1.5 ml water spiked with target nematode (*M. enterolobii*) juveniles (L2). DNA extraction was to be performed with simple lysis buffer (including β-mercaptoethanol and proteinase K) according to the protocol provided with the samples.
Topic 4 included samples 9-18, which were used for linearity and specificity studies on target (*M. enterolobii*) DNA and non-target DNA (other *Meloidogyne* spp. or other nematode genera frequently found in soil samples). For a detailed list on sample codes see Table 1.

Tab. 1 List of sample codes (topics 2 & 4) and tube contents sent to participating labs.

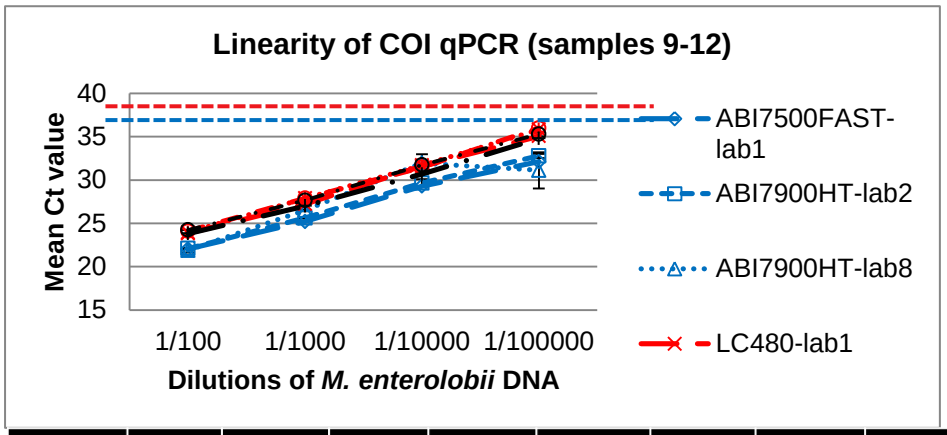
Sample codes	Tube content	
1	Soil A only	Detection
2	Soil A + 2 L2	Detection
3	Soil A + 10 L2	Detection
4	Soil B only	Detection
5	Soil C only	Detection
6	Lysis buffer	Identification
7	L. buffer + 2 L2	Identification
8	L. buffer + 10 L2	Identification
9	Me DNA 100x dil	Linearity
10	Me DNA 1000x	Linearity
11	Me DNA 10000x	Linearity
12	Me DNA 100000x	Linearity
13	*M. hapla* DNA	Specificity
14	*M. graminicola* DNA	Specificity
15	*Nacobus abberrans* DNA	Specificity
16	*Globodera rostochiensis* DNA	Specificity
17	*M. chitwoodi* DNA	Specificity
18	*M. fallax* DNA	Specificity

Material and Methods (topic 4):

1. During the start-up period (until Oktober 2012), participating partner laboratories were asked to provide an inventory list of equipment and methods available and used in their lab for detection and identification of *M. enterolobii*. This list also included a letter of commitment with information on what they are willing to perform and the number of sample sets required.
2. All partners taking part in this test performance study provided import permits if required by their national authorities.
3. Seven out of eight EU partners participated in this part of the test performance study. Participating EU partners were: CH-Agroscope (test organizer), BE-ILVO, DE-JKI, FR-LSV, NL-NRC, TR-PPRI, and UK-FERA. IT-CRA-BP did not participate in this part of the study. Samples were sent to participating labs on 11.12.2012 and received on 12.-13.12.2012
4. Costs for sample preparation and shipment have been covered by the test organizer (Agroscope), costs for laboratory equipment and supplies, reagents and personnel were provided by each participating partner, which is in agreement with non-competitive funding mechanisms in Euphresco projects.
5. Nematode suspensions were obtained from 100 ml soil samples collected on Swiss field sites (A-C) in November 2012. Nematode suspensions contained 500 individual nematodes per 1.5 ml tap water and were spiked or non-spiked with different amounts of target nematodes (0, 2, or 10 *M. enterolobii* L2 juveniles).
6. 18 samples per set (10 sets) were sent by express mail to each of the seven participating labs.
7. Sample sets included pure and mixed suspensions of nematodes as well as template target and non-target DNA samples (Tabl.1).
8. A detailed COI qPCR protocol, based on the results of topic 3, for labs using Applied Biosystem or Roche Light Cycler Real-time platforms and appropriate chemistry was provided simultaneously with the samples. Additional methods could be used by labs as required by their national regulations and additional samples sets were provided for this purpose.
9. Primers were also provided by the test organizer and sent along with the samples, due to their unpublished sequences but LNA probes as well as appropriate master mixes depending on the equipment used were ordered by each lab separately prior to the test performance studies.
10. Except for samples provided as dried DNA (samples 9-18), all samples (1-8) had to be extracted by the DNA-extraction method suggested under topic 2.
11. Each sample was tested at least twice (replicate) within the same run for each test. At least one run was performed.
12. Results were reported to the organizer for analyse and evaluation between the end of January 2013 (deadline) and end of February 2013.
13. Results were reported to all TPS participants (by oral presentations and hand-outs) and discussed at the final meeting held at the Agroscope Research Station in Wädenswil, Switzerland on 11.-12.04.2013.

Results (Assessment of TPS; linearity, different equipment)

The assessment of sample linearity is related to target DNA dilutions (samples 9-12) and included the analysis for data of all sample sets (eight). No problems with sample labelling or confusion of tube tags were reported by participants. We found an overall excellent sample linearity (Adjusted R^2 per sample set 0.991-1.0, Fig. 1) of DNA dilutions sent to participating labs with only one exception (R^2 = 0.781). Keeping the Cut-off Ct-values for the different equipment used in different laboratories in mind (see results of topic 3), BioRAD machines were comparable to Roche Light Cycler machines in that they provided similar Ct-values in the range of 24 to 36, while ABI machines resulted in lower Ct-values (22-33). In any case DNA was detected in all samples depending on target DNA concentration, with the lowest DNA concentrations being close to the Cut-off Ct lines (35 for ABI machines and 36 for the Roche Light Cycler and BioRAD machines, respectively).



Adj R^2/ sample set	Lab 1 (ABI)	Lab 2 (ABI)	Lab 8 (ABI)	Lab 1 (Roche)	Lab 4 (Roche)	Lab 7 (Roche)	Lab 3 (BioR)	Lab 6 (BioR)
	0.991	0.995	0.781	0.991	1.0	0.991	0.998	0.992

Fig. 1

Results TPS : (topic 4 only) :

- DNA extraction with simple lysis buffer was done by all labs on samples 1-8 and was followed by COI qPCR according to the protocols for DNA extraction and qPCR provided by the TPS organizer.
- All COI qPCR results were as expected (for detection, identification, linearity and specificity; Tab.2).
- No differences between labs or qPCR platforms used were seen by looking at +/- reactions, i.e. all labs provided similar results (positive samples were tested positive and negative samples were tested negative by all labs). Thus results are in 100 % agreement between labs (Table 2).
- Conventional PCRs done by CH-ACW (Tigano et al., 2010) and BE-ILVO (Wishart et al., 2002) provided also good results, but were less sensitive in detection and identification of small amounts of target nematodes compared to the COI qPCR, which is expected due to higher sensitivity of qPCR methods. Furthermore, the conventional PCR by Wishart et al., 2002 was more sensitive in detection and identification of target nematodes than the conventional PCR using the Tigano et al., 2010 SCAR primers.
- SYRB Green assay results provided by NL-NRC were comparable to COI qPCR results

Table 2. Results of test performance study (topic 2 & 4) of all participating labs and their equipment used based on plus/minus reactions of all samples in a sample set

sample code	content			expected results		lab1		lab2		lab3		lab4		lab6		lab7	lab8
			Tigano	ABI	Roche	ABI	Roche	ABI	Wishart	BioRAD	ZYMO	WizardMagn	Roche	BioRAD	SYRBGreen	Roche	ABI
1	Soil A only	detection	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	Soil A + 2 L2	detection	-	+	+	+	+	+	+ (615bp)	+	+	+	+	+	+	+	+
3	Soil A + 10 L2	detection	+/-	+	+	+	+	+	+/-	+	+	+	+	+	+	+	+
4	Soil B	detection	nt	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	Soil C	detection	nt	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	lysis buffer only	identification	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	buffer + 2 L2	identification	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+
8	buffer + 10 L2	identification	+/-	+	+	+	+	+	+	+	-	+	+	+	+	+	+
9	Me DNA 1/100	linearity	+ (520bp)	+	+	+	+	+	+ (615, 350, 2	+	nt	+	+	+	+	+	+
10	Me DNA 1/1000	linearity	+	+	+	+	+	+	+	+	nt	+	+	+	+	+	+
11	Me DNA 1/10000	linearity	+	+	+	+	+	+	+/-	+	nt	+	+	+	+	+	+
12	Me DNA 1/100000	linearity	-	+/-	+/-	+	+	+	-	+	nt	+	+	+	+	+/-	+
13	M. hapla	specificity	nt	-	-	-	-	-	-	-	nt	-	-	-	-	-	-
14	M. graminicola	specificity	nt	-	-	-	-	-	-	-	nt	-	-	-	-	-	-
15	N. abberrans	specificity	nt	-	-	-	-	-	-	-	nt	-	-	-	-	-	-
16	G. rostoch.	specificity	nt	-	-	-	-	-	-	-	nt	-	-	-	-	-	-
17	M. chitwoodi	specificity	nt	-	-	-	-	-	-	-	nt	-	-	-	-	-	-
18	M. fallax	specificity	nt	-	-	-	-	-	-	-	nt	-	-	-	-	-	-
	ntc	specificity	-	-	-	-	-	-	-	-	nt	-	-	-	-	-	-
											only 1 rep	2 reps					

Evaluation of the COI qPCR assay (out-house validation): definition and calculation of performance criteria (according to PM 7/98, EPPO Bull. 40: 5-22) (Tab.3)

Table 3.

Laboratory/Reference	Accepted reference positive value	Accepted reference negative value
Positive samples set result	PA = positive agreement	PD = positive deviation
Negative samples set result	ND = negative deviation	NA = negative agreement
Undefined samples set result	ND = negative deviation	NA = negative agreement

Performance criteria	Definition	Calculation
Accuracy	Closeness of agreement between the test result and the accepted reference value (ISO 5725-1) *Comments: the mode of calculation used to determine the accuracy neans that a balanced evalutation can be performed even if the sizes of N^+ and N^- are not equal*	= [(sum PA / N^+) + (sum NA / N^-)]/2
Sensitivity (SE)	A test's capacity to obtain a positive result for the samples for which the accepted reference value is positive	= sum PA / N^+
Specificity (SP)	A test's capacity to obtain a negative result for the samples for which the accepted reference value is negative	= sum NA / N^-

Key: N^+ = number of samples for which the accepted reference value is positive = sum PA + sum ND
N^- = number of samples for which the accepted reference value is negative = sum NA + sum PD

Tab.4: Evaluation of the COI qPCR assay: calculating the performance criteria on results of COI qPCR sent by all labs (8 sample sets were analyzed containing 18 samples each)

1 Soil A only	detection
2 Soil A + 2 L2	detection
3 Soil A + 10 L2	detection
4 Soil B	detection
5 Soil C	detection

9 Me DNA 1/100	linearity
10 Me DNA 1/1000	linearity
11 Me DNA 1/10000	linearity
12 Me DNA 1/100000	linearity
13 M. hapla	specificity
14 M. graminicola	specificity
15 N. abberrans	specificity
16 G. rostoch.	specificity
17 M. chitwoodi	specificity
18 M. fallax	specificity
ntc	specificity

6 lysis buffer only	identification
7 buffer + 2 L2	identification
8 buffer + 10 L2	identification

	Detection	Specificity	Identification	
sensitivity	100	100	100	
specificity	100	100	100	
accuracy	100	100	100	
e.g. PA 2 (detection)	PD 0	x8 tests	16	0
ND 0	NA 3		0	24
ND 0	NA 0		0	0
N^+ 16+0= 16	SE= 16/16 = 1 x 100 = 100%			
N- 24+0= 24	SP= 24/24 = 1 x 100 = 100%			
	A = [1+1]/2 = 1 x 100 = 100%			
e.g. PA 4 (linearity)	PD 0	x8 tests	32	0
ND 0	NA 7(specificity)		0	56
ND 0	NA 0		0	0
N^+ 32+0= 32	SE= 32/32 = 1 x 100 = 100%			
N- 56+0 = 56	SP= 56/56 = 1 x 100 = 100%			
	A = [1+1]/2 = 1 x 100 = 100%			
e.g. PA 2 (identification)	PD 0	x8 tests	16	0
ND 0	NA 1		0	8
ND 0	NA 0		0	0
N^+ 16+0= 16	SE= 16/16 = 1 x 100 = 100%			
N- 8+0= 8	SP= 8/8 = 1 x 100 = 100%			
	A = [1+1]/2 = 1 x 100 = 100%			

Tab 5.

		Detection	Specificity	Identification
M. enterolobii	Sensitivity	100	100	100
	Specificity	100	100	100
	Accuracy	100	100	100

- All positive and negative results for detection, identification and specificity are 100 % in agreement between different laboratories and equipment using the COI qPCR assay
- The assay is very sensitive, specific and accurate for detection and identification of *M. enterolobii*.
- Inter-laboratory comparison confirms intra-laboratory results!

Results of TPS (topic 2 & 4; detection and identification according to equipment)

- All labs using ABI platforms provided similar results, despite different Ct-values, for target nematode detection and identification, i.e. lower Ct-values with increasing amounts of target DNA (10 vs 2 *M. enterolobii* L2) (Fig. 2)
- All values were below the cut-off Ct-value of 35 (cut-off Ct-value defined for ABI platforms under topic 3; method development and in-house validation)
- The SYRBGreen assay results (provided by the Netherlands) for detection and identification samples were similar to COI qPCR results, despite different Ct-values, except for detection of low amounts of L2 (2) in nematode suspensions isolated from soil background (Fig.2)

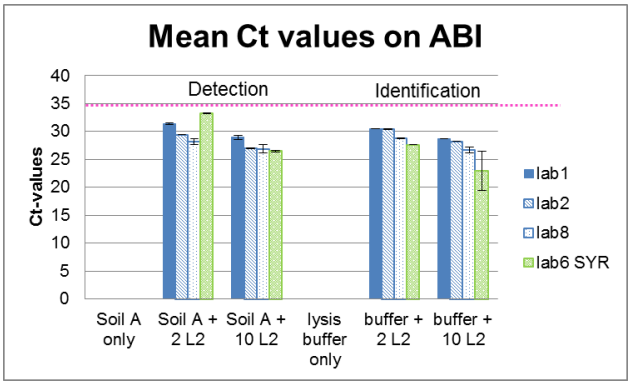


Fig.2

- All labs using Roche platforms provided similar results, despite different Ct-values, for *M. enterolobii* detection and identification samples (lower Ct-values with increasing amounts of target DNA (10 vs 2 L2). Labs using BioRad platforms provided similar results as labs using Roche platforms; even Ct-values were very similar.
- All Ct-values were below the cut-off Ct-value of 36 (defined for Roche platforms under topic 3; method development and in-house validation), except for one BioR platform (Fig.3).

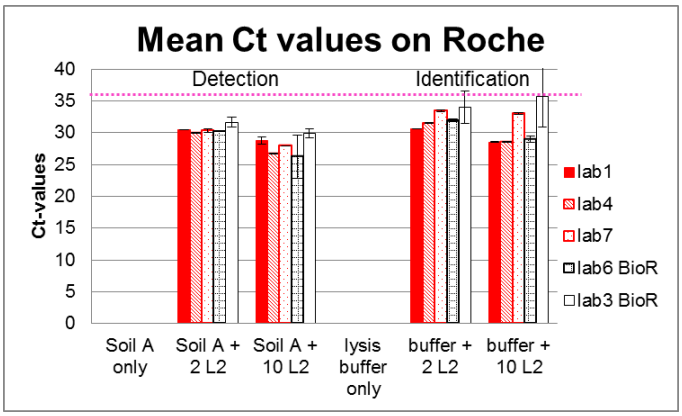


Fig.3

Results of TPS (summary results of COI qPCR test validation (topic 4) according to PM 7/98)

Performance criteria	A	B	C	D	Documentation (where)
Analytical sensitivity	Yes	Yes	Not available, new test	Not available, new test	Lab books; publication; Final report
Analytical specificity	Yes	Yes	Not available, new test	Not available, new test	Lab books; publication; Final report
Selectivity	Yes	Yes	Not available, new test	Not available, new test	Lab books; publication; Final report
Repeatability	Yes	Yes	Not available, new test	Not available, new test	Lab books; publication; Final report
Reproducibility	Yes	Yes	Not available, new test	Not available, new test	Lab books; publication; Final report

A = Data from own laboratory experiments
B = Data from proficiency tests or ringtests
C = Data from manufacturers
D = External information (literature, etc.)

- **Analytical sensitivity (= limit of detection):** in-house validation data could be confirmed by the TPS; The smallest amount of target that can be detected reliably was: 1 L2
- **Diagnostic sensitivity**: not tested; can be done now by comparing COI qPCR with gold standard. But what is the gold standard? Morphological determination, counting? Needs to be defined and agreed by all EU participants
- **Analytical specificity**: in-house validation confirmed by TPS on one population of *M. enterolobii* and six non-target nematode populations: 100 %.
- **Diagnostic specificity**: not tested; can be done now!
- **Reproducibility**: Calculated % agreement for a given level is 100 % (for 1 L2)
- **Repeatability**: Calculated % of agreement for a given level is 100 % (for 1 L2)

Conclusions

- Lessons learned from previous TPS were to keep it simple and test only single parameters at once (not too many methods simultaneously). Also provide most of the materials needed for the tests to decrease amount of variation between labs. We followed this by testing only one DNA extraction method (simple lysis buffer) and only one (COI qPCR) of the two developed qPCR methods (topic 3) in the presented test performance study.
- Regarding qPCR assays: The previous TPS on *M. chitwoodi* and *M. fallax* detection suggested to provide the range of threshold, cut off Ct-values, and TM values as well as controls at the limit of detection (LOD). We did this by an in-house validation of methods by the TPS Organizer under topic 3. Data were not provided to participating labs because we wanted to keep it as a blind study; but suggested data were available prior to the start of the TPS.
- Improvement of reaction conditions (better efficiency/sensitivity of qPCR) were reached by all partners by providing most of the solutions needed (buffers, primers) by the TPS organizer. This was also suggested by the previous ring test.
- Data generated were not too many and could therefore still be handled and evaluated (less variance between labs using different equipment).
- Before starting the TPS cut-off Ct-values were defined for different equipment and chemistry used and thus robustness of test under different settings was determined.
- No difficulties were encountered or reported by any of the seven/eight participants during the TPS 2012/2013 or during the final meeting. However, as mentioned before, all participants were encouraged to provide their expertise and knowledge prior to the TPS, and thus a calculation mistake in the qPCR protocol was discovered by the Belgium participant and the protocol was adjusted accordingly.
- The purpose of topic 4 was to test the robustness and reliability of COI qPCR and confirm in-house validation by an interlaboratory comparison (out-house validation). The excellence of the COI qPCR in detection and identification of *M. enterolobii* was thus confirmed by this TPS. As a result we suggest the inclusion of the COI qPCR protocol into the next revision of the EPPO Standard protocol for diagnostics of *M. enterolobii* (PM 7/103).

Workplan Topic 5 – Workshop: Test performance study on detection and identification of Meloidogyne enterolobii and barcoding as nematode identification tool

Aim:

- Results of the test performance study on best protocol for *M. enterolobii* extraction and detection and identification were presented at the final meeting.
- Interested participants were given the opportunity to familiarize with QBOL and Q-bank techniques as tools for diagnostics of *M. enterolobii* and closely related species.

Methods

Presentations were given covering the following topics:

- Extraction and detection of *M. enterolobii* from soil samples: Results of the test performance study (topic 1)
- Development and in-house validation of two qPCR methods for the detection and identification of *M. enterolobii* (topic 3)
- Detection and identification of *M. enterolobii* using a simple DNA extraction protocol and the COI qPCR: results from the test performance study (topic 2 & 4)
- The use of Q-bol/Q-bank data to identify unknown plant quarantine nematodes (optional for interested users)

Demonstrations/hands-on modules:

- Was done upon request by final meeting participants

Place and Time

The final meeting took place at the Agroscope Research Station ACW in Wädenswil, Switzerland from 11.-12.04.2013. The barcoding-workshop followed the final meeting and was offered on 12.04.2013 from 9-12 am).

Presentations, demonstrations and exchange of information and ideas lasted for 1-2 days. Registration to the meeting was required. Participating costs for the workshop, including hand-outs of presentations, coffee breaks, sandwich lunches and a dinner were estimated roughly at 100 € per Person, but were covered by ACW and the BLW (Bundesministerium für Landwirtschaft der Schweiz). Accommodation and travel costs were covered by everybody's own budget.

We would like to thank all sponsors for the financial support of this successful project and all participating EU-partners for taking part in this important test performance study and for providing results for its evaluation.

Workplan Topic 6 – Communication and Publication of results in peer-reviewed research journals and the EPPO Bulletin on diagnostic standard protocols for detection of Q-organisms

Aims:

- Results of all topics will be included into a final report send to all participants for comments or suggestions in approx. August 2013.
- Results of developed methods for the detection and identification of *M. enterolobii* using more specific and more sensitive new molecular based tools (qPCR; topic 3) will be published in scientific peer-reviewed research journals (in preparation).
- As discussed and agreed upon by all final meeting participants, results of validated methods (in-house and out-house) of the test performance study are to be incorporated into a newly revised EPPO diagnostic standard for *M. enterolobii*. Furthermore, results of the TPS shall be published in a peer-reviewed research journal if possible and in an EPPO Bulletin! This can be done at a later time.
- Future research goals in diagnostic, epidemiology, and integrated management of *M. enterolobii* and other quarantine nematodes in Europe were discussed to minimize dissemination of these organisms in the future.

All available data that have been published can be disseminated by the various parties involved and aid in fundraising for research to fill the knowledge gaps. The reports will also increase awareness of this aspect of spread of quarantine organisms.