Simultaneous molecular detection and quantification of two cyst forming nematodes

Mehrdad Madani, Sergei A. Subbotin*, Lieven Waeyenberge and Maurice Moens

Agricultural Research Centre, Department of Crop Protection, Burg.Van Gansberhelaan 96 , 9820 Merelbeke, Belgium
*Present address: Department of Nematology, University of California, Riverside, CA 92521, USA

The potato cyst nematode, *G.pallida,* and the beet cyst nematode, *H.schachtii,* are major nematode pests in potato and sugar beet production, respectively. Their impact on the crop depends largely on pre-plant nematode density. Due to their damaging potential, potato cyst nematodes (*G.pallida* and *G.rostochiensis*) are subject of quarantine regulations in many countries; also *H.schachtii* is on the quarantine list of several non-European countries. Cyst forming nematodes are frequently present in mixed populations. Precise identification and knowledge about the number of nematodes in field soil are necessary to develop effective integrated pest control.

The **real-time PCR technique** is an appropriate technique for obtaining simultaneously information on both the species identity and the number of nematodes present in the sample.

- It measures the nematode number indirectly by assuming that the number of target DNA copies in the sample is proportional to the number of targeted nematodes.
- The quantitative information in a PCR comes from those few cycles in which the amount of DNA grows logarithmically from barely above background to the plateau.
- It continuously monitors the sample using hybridization probes (TaqMan, molecular beacons, or FRET) or double stranded dyes such as SYBR green I (Fig. 1).
- The detection and quantification of target DNA are obtained simultaneously and faster in a single closed tube assay.
- The automated system overcomes the laborious process of estimation of the quantity of the PCR product after gel electrophoresis.

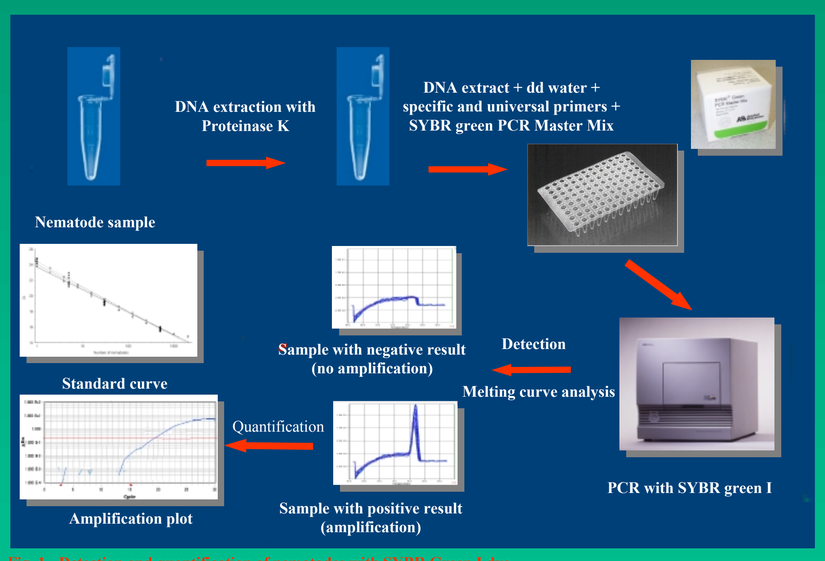

Fig. 1 - Detection and quantification of nematodes with SYBR Green I dye.

SYBR green I fluorescent dye.

Advantages: (1) easy to use, (2) no sequence specificity, (3) detects any PCR product, (4) low cost, and (5) binds to any double-stranded DNA.

Disadvantage: Dye binds also to any non-specific product including primer dimers. Melting curve analysis can overcome this problem.

Melting curve analysis. Non-specific PCR products tend to melt at a much lower temperature than the longer specific products. The length of amplicons can be used to differentiate amplification products separated by less than 2°C in Tm. The presence of only a single peak with melting temperatures of 84.6 ± 0.1°C and 84.1 ± 0.1°C was acquired for *H.schachtii and G.pallida*, respectively (Fig. 2).

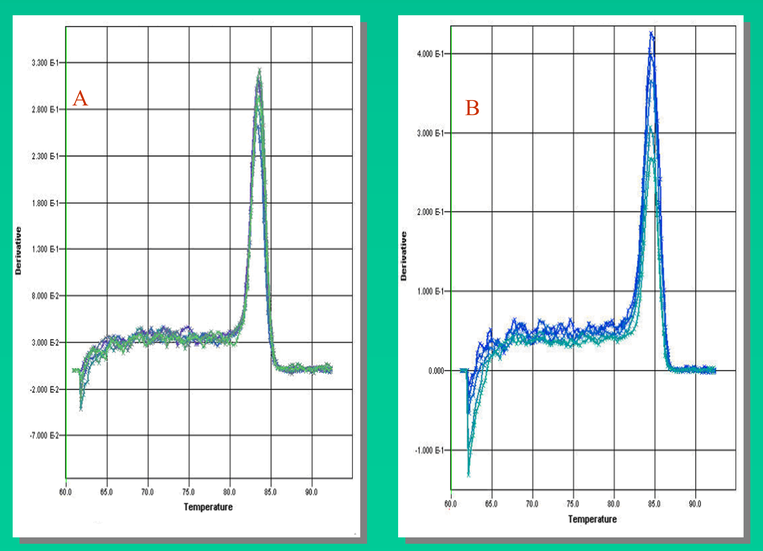

Fig. 2 - Melting curves (fluorescence vs temperature) of specific amplicons for *H.schachtii* (A) and *G.pallida* (B).

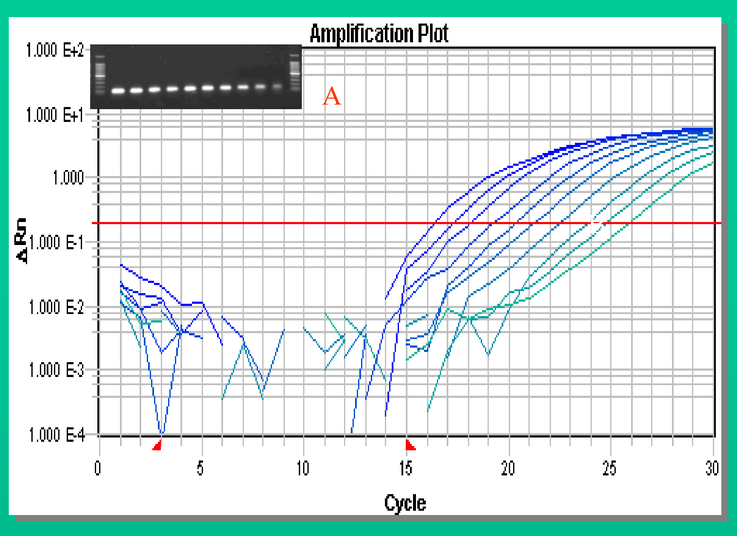

Fig. 3 - Amplification plot with gel analysis of amplicons obtained for *Heterodera schachtii* in serial dilution.

Amplification. rDNA of *Globodera* species was amplified with the species specific primers described by Bulman & Marshall (1997); rDNA of *Heterodera schachtii* was amplified with primers slightly modified from Amiri *et al.* (2002). PCR performed with the specific primer combinations produced for all studied *G.pallida* or *H.schachtii* populations only the single expected fragment of about 115 bp (Fig. 3A).

Standard curve construction. Results from runs of three serial dilutions of DNA samples starting from 2000 juveniles for both species showed a highly significant negative correlation between the Ct value and the nematode number over the range studied (Figs 3B and 4). High reproducibility was observed either within and between replicates of the same or different sample preparations.

Validation test and detection limit. DNA extracted from different precise numbers of juveniles was amplified by Real Time PCR. The numbers of *G. pallida* second stage juveniles present in the sample and the expected numbers detected by real-time PCR matched highly; the match was less perfect for *H.schachtii* (Fig. 4). The technique enables us to detect a single second stage juvenile of *G.pallida H.schachtii* in a sample.

Haplotype hypothesis. The variation in detection and quantification of these two species might be due to differences in amount of the target rDNA. The specific primer designed for *H.schachtii* amplifies only one of the ITS-rDNA haplotypes of the species, whereas the primers for *G.pallida* amplify all haplotypes. Although we concluded that the haplotype amplified by the *H.schachtii* primer was present in all studied populations, it is not clear whether it is distributed in the same ratio among all *H.schachtii* populations. The standard curve should be constructed for certain nematode populations.

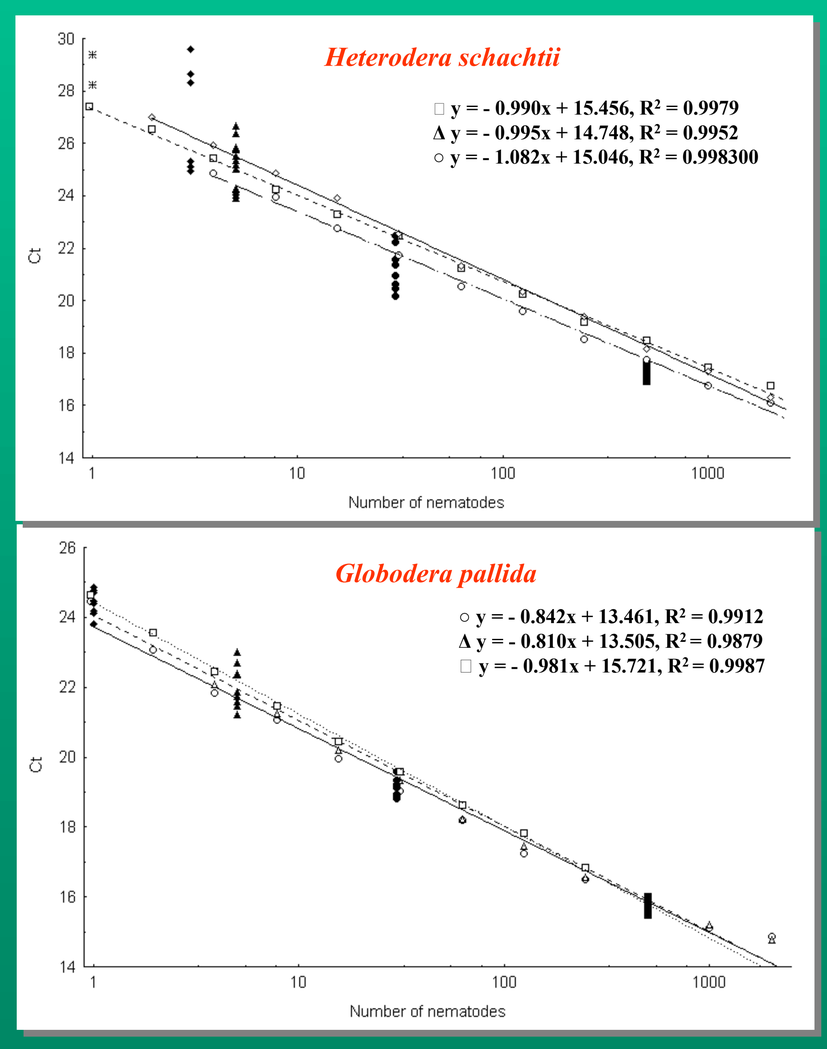

Fig. 4 - Sensitivity, detection range, and specificity of the real-time PCR assay for *Heterodera schachtii* and *Globodera pallida*. The reproducibility of the assay was determined by testing a dilution series of three independent DNA extractions. Bold figures represent the results of the validation test with known numbers of second stage juveniles (1, 3 5, 30, or 500).

REFERENCES

Amiri, S., Subbotin, S.A. & Moens, M. 2002. Identification of the beet cyst nematode *Heterodera schachtii* by PCR. *European Journal of Plant Pathology* 108:497-506.

Bulman, S.R. & Marshall, J.W. 1997. Differentiation of Australian potato cyst nematode (PCN) populations using the polymerase chain reaction (PCR). *New Zealand Journal of Crop and Horticultural Science* 25: 123-129.