Validation of a qPCR method for the quantification of *Pratylenchus penetrans*

Musa Nasamu Bawa

Promoter: Prof.dr. ir. Nicole Viaene
Supervisor: Lieven Waeyenberge

Thesis submitted to obtain the degree of Master of Science in Nematology
# TABLE OF CONTENTS

1. **INTRODUCTION** .......................................................................................................................... 1

2. **MATERIALS AND METHODS** .................................................................................................... 3

   2.1 Specificity of the qPCR primers and probe ................................................................................. 3

   2.1.1 Samples containing *Pratylenchus* spp. from different locations in Belgium ................. 3

   2.1.2 Extraction of nematodes from soil samples and their preparation for molecular processes .......................................................... 5

   2.2 The effect of inhibitors on the performance of the qPCR assay ............................................. 5

   2.2.1 Extraction of soil and plant samples to obtain a nematode suspension mixed with soil and plant soluble components and their preparations for molecular processes 5

   2.2.2 Extraction of plant and soil samples using AZC with/without MgSO₄ and/or kaolin .... 7

   2.3 Dilution experiments .................................................................................................................. 7

   2.3.1 The dilution of plant and soil extracts before addition of nematodes ......................... 7

   2.3.2 The dilution DNA extracted from nematodes in different plant materials extracts ......................... 8

   2.3.3 Dilution of water passed through the Automatic Zonal Centrifuge (AZC) ................. 8

   2.4 Detection of two *Pratylenchus penetrans* in a background of other nematodes .......... 8

   2.5 Practical application of the qPCR assay to detect *Pratylenchus penetrans* in a sample .................................................................................................................................................. 9

   2.6 Reproducibility: influence of qPCR machine .......................................................................... 11

   2.7 Molecular processes used in this study .................................................................................... 11

   2.7.1 DNA Extraction procedure .................................................................................................. 11

   2.7.2 Polymerase Chain Reaction (PCR) and Gel Electrophoresis ......................................... 11

   2.7.3 The quantification test using the qPCR primers and probe ............................................ 12

   2.8 Statistical analysis .................................................................................................................... 13

3. **RESULTS** .................................................................................................................................. 14

   3.1 The Specificity test of the qPCR primers and probe developed on Beta 1-4 endoglucanase gene ................................................................................................................................................. 14
3.2 The effect of inhibitors on the performance of the qPCR assay ........................................ 14
3.2.1 Extraction of plant and soil samples using AZC with/without MgSO4 and/or Kaolin ................................................................. 15
3.3 Dilutions experiments ........................................................................................................ 16
3.3.1 The dilutions of plant and soil extracts prepared from the AZC before addition of nematodes .................................................................................................................. 16
3.3.2 The dilution of plant materials extracts after DNA extraction of nematodes ............ 17
3.3.3 Dilution of the water passed through the AZC ......................................................... 18
3.4 Test to determine what limit could the qPCR assay detect Pratylenchus penetrans in among other nematodes ........................................................................................................ 19
3.5 The application of qPCR assay to detect Pratylenchus penetrans in a sample ............ 19
3.6 Reproducibility: influence of qPCR machine .............................................................. 21
4 DISCUSSION ....................................................................................................................... 22
5 APPRECIATION ................................................................................................................ 26
6 REFERENCES ..................................................................................................................... 27
LIST OF FIGURES

**Figure 1:** Stepwise procedure for preparing nematode suspensions obtained with the Automatic Zonal Centrifuge for molecular processes. ................................................................. 10

**Figure 2:** An agarose gel showing the amplification products obtained from the DNA of 20 individuals of *Pratylenchus penetrans* in the soluble components of 6 plants and 4 soil types. CS-Clay soil, SS-Sandy soil, LS-Loam, OS-Organic soil, MQ-Ultrapure (type 1) water PE- Potato (extracted with antifoam), P-Potato (extracted without antifoam), LK-Leek, Y-Yellow Mustard, C-Carrot, M-Maize, B-Buxus, L-DNA Ladder and (-) - Negative control .................................................................................................................. 15

**Figure 3:** The Ct-values (mean ± standard deviation) of 20 individuals of *Pratylenchus penetrans* (n=2) added to the 1:1, 1:10 and 1:100 dilutions of the suspensions of three plant materials obtained by from the AZC and the water passed through the AZC (AZC-water). ...................................................................................................................... 16

**Figure 4:** The Ct-values (mean ± standard deviation) of three individuals of *Pratylenchus penetrans* (n=2) added to the 1:1, 1:5, 1:10, 1:20 dilutions of the suspensions of four soil types obtained from the AZC with two controls (TW-Tap water and UPW-Ultrapure (type 1) water). ....................................................................................................................... 17

**Figure 5:** The Ct-values (mean ± standard deviation) of 1:1, 1:10 and 1:100 DNA dilutions of five individuals of *Pratylenchus penetrans* (n=2) present in the suspensions of different plant materials obtained from the AZC. M-Maize, C-Carrot, P-Potato, B-Buxus, Y-Yellow mustard, L-Leek, AZC-Tap water passed through the AZC, UPW-Ultrapure (type 1) water, UAZC-Ultrapure (type 1) water passed through the AZC. ................. 18

**Figure 6:** The Ct-values (mean ± standard deviation) of three individuals of *Pratylenchus penetrans* (n=2) added to the 1:1, 1:2, 1:5,1:10,1:50 and 1:100 dilutions of the water passed through the AZC....................................................................................................................... 18

**Figure 7:** The Ct-values (mean ± standard deviation) of three replicates of two individuals of *Pratylenchus penetrans* in a mixture with *Paratylenchus* spp. and *Meloidogyne chitwoodi* at 10 000, 5 000, 1 000, 500, 100 numbers and two controls (the supernatant (free of nematodes (SUP)) and ultrapure (type 1) water (UPW)). ........................................... 19

**Figure 8:** The Ct-values (mean ± standard deviation) of 25 individuals (n=3) of *Pratylenchus penetrans* in the suspensions of a soil sample obtained from the AZC and processed with and without a dilution step. ................................................................................. 20
LIST OF TABLES

Table 1: Associated crop(s) and codes of samples containing Pratylenchus spp. used in this study, together with the Ct values (mean ± standard deviation) of 20 individuals (n=3) of each population obtained in a qPCR reaction. .................................................................3

Table 2: Plants and their source used in this study. .................................................................................6

Table 3: Soils and their origin used in this study. .........................................................................................6

Table 4: The preparation of 100 µl suspensions containing different numbers of nematodes used as a background for a qPCR assay to detect two individuals of Pratylenchus penetrans. .............................................................................................................................9

Table 5: The Ct-values (mean ± standard deviation) of 20 individuals (n=5) of Pratylenchus penetrans (Pp) for each suspension of plant and soil samples except their negative controls and obtained in a qPCR reaction. .................................................................................14

Table 6: The Ct values of three individuals of Pratylenchus penetrans in leek and loam suspensions obtained from the AZC with or without MgSO₄ or Kaolin. .................... 15

Table 7: The Ct-values (mean ± standard deviation) of five individuals each of five populations of Pratylenchus spp. .........................................................................................21
Validation of a qPCR method for the quantification of

Pratylenchus penetrans

Musa B. NASAMU

Department of Nematology, Faculty of Science, University of Ghent

Karel Lodewijk Ledeganckstraat 35, 9000 Gent, Belgium

Summary—Detection and quantification of Pratylenchus penetrans in plants roots and soils are essential for effective management of the root lesion nematode. The purpose of this study was to validate the qPCR assay based on the β-1, 4-endoglucanase gene (Mokrini et al., 2013). This was done by investigating if the qPCR assay could specifically detect only and all populations of P. penetrans. The influence of plant and soil suspensions obtained from the Automatic Zonal Centrifuge (AZC) on the performance of the qPCR was evaluated. In addition, the sensitivity of the assay in detecting two individuals of P. penetrans in a background of 100, 500, 1000, 5000 and 10,000 other nematodes was checked. The reproducibility of the assay was also tested on two different qPCR equipment. A test on how the assay can be applied in routine laboratory was performed. The assay detected the 14 populations of P. penetrans out of the 29 populations of Pratylenchus species tested with an average Ct value of 28.87±0.8. No other Pratylenchus species was detected. The plant and soil suspensions from the AZC inhibited the qPCR assay from detecting P. penetrans except the suspension of maize roots. The assay could detect two individuals of the nematode in 1000 individuals of a mixture of Paratylenchus spp. and Meloidogyne chitwoodi. The results obtained by using this assay can be reproduced on another qPCR equipment. The introduction of a dilution step will be necessary to detect P. penetrans in nematode suspensions extracted with the AZC using a qPCR assay. The qPCR assay can be used successfully for the detection P. penetrans.

Keywords—inhibitor, extraction, suspension, specificity, sensitivity, selectivity, reproducibility.


1 INTRODUCTION

The root-lesion nematode *Pratylenchus penetrans* is a migratory endoparasitic nematode. It is an important nematode pest of potato, carrot, leek among many crops (Castillo & Vovlas, 2007). The nematode has all its developmental stages in the soil, plant roots and tubers. They can penetrate plant roots, tubers and migrate within it causing lesions. Also, they interact with other pathogens and increase the severity of their damage. Strawberry suffers attack from a fungus *Verticillium dahlia* and the incidence increases as *P. penetrans* attack the roots creating extra opening for the fungi to enter (Xu et al., 2015). It is also reported that *Fusarium* wilt is increased in the presence of *P. penetrans* in pea (Oyekan & Mitchell, 1971; Kyndt et al., 2014).

The distribution of *P. penetrans* is reported in many areas especially in the temperate regions. The nematode reduces the yield and/or quality of potato, vegetables, such as carrot, pea, bean, and ornamental plants, including rose, tulip and lily in Europe (Green & Verdejo, 1985; Talavera et al., 2001; de la Pena et al., 2007; Pudasaini et al., 2007; Sogut & Devran, 2011; Esteves et al., 2015). In North Africa, the nematode was recorded in Algeria and Tunisia on several crops (Troccoli et al., 1992) and wheat in Morocco (Mokrini et al., 2012; Mokrini et al., 2016). In Québec, *P. penetrans* was reported as the dominant species in potato fields (Olthof, 1987). They were also detected in high numbers in most of the surveyed fields where crops were grown annually as monocrop in eastern Washington, USA (Kandel et al., 2013).

Distinguishing *P. penetrans* from *P. fallax* and other closely related *Pratylenchus* spp. using traditional microscopic methods is based on subtle morphological differences in lip annule number, tail shape, and vulva position. Seinhorst J. (1968) considered that *P. fallax* differs from *P. penetrans* only in having a crenate tail tip. Gao et al. (1999) described *P. vulnus* from a geographical isolate (Japan) to exhibit characters similar to *P. penetrans* especially the subspherical spermathecal feature. More so, the description of *P. lentis* with three annuli and a large and ovoid spermatheca full of sperm shares similarity to *P. penetrans* (Troccoli et al., 2008). In addition, since there are no distinct differences among the species of *Pratylenchus* and intra-specific variations often occur, morphological traits are often not sufficient to accurately identify *Pratylenchus* spp. (Yu et al., 2012). This process of delimiting species can be time consuming and difficult to count and identify from large samples, especially when other nematodes are present.

Molecular techniques offer an alternative to the time-consuming traditional methods of nematode identification based on morphology. A number of techniques have been used over
the years: analysing isozyme banding patterns (Ibrahim et al., 1995) and total protein patterns after SDS-PAGE with silver staining by Jaumot et al. (1997), dot-blot hybridisation assay by Setterquist et al. (1996), PCR-RFLP analysis (Orui, 1996; Orui & Mizukubo, 1999; Waeyenberge et al., 2000) and species-specific PCR (Al-Banna et al., 2004; Yan et al., 2008; Waeyenberge et al., 2009). Moreover, to quantify Pratylenchus species, qPCR assays were developed by Berry et al. (2008), Sato et al. (2010) and Yan et al. (2013). The PCR-based techniques almost always investigated the ITS-rDNA region. However, the ITS-sequences of *P. penetrans* show extensive polymorphism within the species or an individual (Orui, 1996; Uehara et al., 1999; Waeyenberge et al., 2000). With these limitations it is not suitable to use it as a reliable template for primer design to quantify the species during a qPCR (Mokrini et al., 2013).

Recently, the β-1,4-endoglucanase gene was first used and validated by Mokrini et al. (2013) to detect and quantify *P. penetrans*. In this study, a different and more robust approach to validate this qPCR assay was considered.

The following are the research questions:

i. Specificity: will the qPCR assay detect all populations of *Pratylenchus penetrans* and not any population of other *Pratylenchus* spp. with emphasis on these species being present in Belgium?

ii. Selectivity: is there an influence of the matrix from which nematodes were extracted on the qPCR performance?

iii. Sensitivity: what is the limit to which *P. penetrans* can be detected in a background of other nematode species?

iv. Practical application: can this qPCR assay be used in routine laboratories to detect *P. penetrans* in samples?

v. Reproducibility: is it possible to reproduce the result of this assay on different qPCR machines and on the same day.
2 MATERIALS AND METHODS

2.1 Specificity of the qPCR primers and probe

2.1.1 Samples containing Pratylenchus spp. from different locations in Belgium

The qPCR primers PpenMFor/PpenMRev and a probe PpMPb were developed by (Mokrini et al., 2013) based on the β-1,4 endoglucanase gene. The ability of these primers and probe to detect all populations of *P. penetrans* and not to any other *Pratylenchus* species will be interesting to test.

The Diagnostic Centre for Plants of the Institute for Agricultural Research and Fisheries (ILVO) at Merelbeke received soil samples from different locations in Belgium for identification of nematode composition. The samples containing a single species population and mixed populations of the genus *Pratylenchus* were selected, but not samples that contained *P. penetrans* mixed with other species of *Pratylenchus*. To test the specificity of the qPCR assay, three cultures of *Pratylenchus* species and 26 soil samples were collected and processed (Table 1).

### Table 1: Associated crop(s) and codes of samples containing Pratylenchus spp. used in this study, together with the Ct values (mean ± standard deviation) of 20 individuals (n=3) of each population obtained in a qPCR reaction.

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>Species</th>
<th>Crop(s)</th>
<th>Ct</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture Gottem</td>
<td><em>P. crenatus</em></td>
<td>Carrot</td>
<td>Undet.</td>
</tr>
<tr>
<td>DCP/2016/000054</td>
<td><em>P. crenatus</em> + <em>P. fallax</em></td>
<td>Maize</td>
<td>Undet.</td>
</tr>
<tr>
<td>DCP/2016/000055</td>
<td><em>P. crenatus</em> + <em>P. fallax</em></td>
<td>Potato</td>
<td>Undet.</td>
</tr>
<tr>
<td>DCP/2016/000330</td>
<td><em>P. crenatus</em> + <em>P. neglectus</em> + <em>P. thornei</em></td>
<td>Potato</td>
<td>Undet.</td>
</tr>
<tr>
<td>Ledegem/1</td>
<td><em>P. crenatus</em></td>
<td>Pasture field</td>
<td>Undet.</td>
</tr>
<tr>
<td>DCP/2016/000025</td>
<td><em>P. neglectus</em></td>
<td>Vegetables</td>
<td>Undet.</td>
</tr>
<tr>
<td>DCP/2016/000333</td>
<td><em>P. neglectus</em></td>
<td>Potato</td>
<td>Undet.</td>
</tr>
<tr>
<td>DCP/2016/000344</td>
<td><em>P. neglectus</em></td>
<td>Vegetables</td>
<td>Undet.</td>
</tr>
<tr>
<td>DCP/2016/000345</td>
<td><em>P. neglectus</em></td>
<td>Vegetables</td>
<td>Undet.</td>
</tr>
</tbody>
</table>

Under.-Undetermined
Table 1: (continued)

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>Species</th>
<th>Crop(s)</th>
<th>Ct</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCP/2016/000347</td>
<td><em>P. neglectus</em></td>
<td>Vegetables</td>
<td>Undet.</td>
</tr>
<tr>
<td>Culture Turkey</td>
<td><em>P. thornei</em></td>
<td>Carrot</td>
<td>Undet.</td>
</tr>
<tr>
<td>DCP/2016/000332</td>
<td><em>P. thornei + P. neglectus</em></td>
<td>Potato</td>
<td>Undet.</td>
</tr>
<tr>
<td>DCP/2016/000434</td>
<td><em>P. thornei</em></td>
<td>Buxus</td>
<td>Undet.</td>
</tr>
<tr>
<td>DCP/2015/005159</td>
<td><em>P. vulnus</em></td>
<td>Buxus</td>
<td>Undet.</td>
</tr>
<tr>
<td>DCP/2016/000435</td>
<td><em>P. vulnus</em></td>
<td>Apple</td>
<td>Undet.</td>
</tr>
<tr>
<td>Culture Kerkom</td>
<td><em>P. penetrans</em></td>
<td>Carrot</td>
<td>30.1±3.4</td>
</tr>
<tr>
<td>DCP/2015/004529</td>
<td><em>P. penetrans</em></td>
<td>Lettuce</td>
<td>28.5±1.2</td>
</tr>
<tr>
<td>DCP/2015/005158</td>
<td><em>P. penetrans</em></td>
<td>Lettuce</td>
<td>28.4±0.4</td>
</tr>
<tr>
<td>DCP/2016/000022</td>
<td><em>P. penetrans</em></td>
<td>Vegetables</td>
<td>29.9±1.7</td>
</tr>
<tr>
<td>DCP/2016/000169</td>
<td><em>P. penetrans</em></td>
<td>Pear</td>
<td>29.2±2.1</td>
</tr>
<tr>
<td>DCP/2016/000349</td>
<td><em>P. penetrans</em></td>
<td>Vegetables</td>
<td>28.8±2.0</td>
</tr>
<tr>
<td>DCP/2016/000350</td>
<td><em>P. penetrans</em></td>
<td>Vegetables</td>
<td>28.4±1.5</td>
</tr>
<tr>
<td>DCP/2016/000351</td>
<td><em>P. penetrans</em></td>
<td>Vegetables</td>
<td>28.8±1.0</td>
</tr>
<tr>
<td>DCP/2016/000525</td>
<td><em>P. penetrans</em></td>
<td>Peony</td>
<td>28.1±1.0</td>
</tr>
<tr>
<td>DCP/2016/000686</td>
<td><em>P. penetrans</em></td>
<td>Yellow mustard</td>
<td>28.9±0.6</td>
</tr>
<tr>
<td>DCP/2016/000863</td>
<td><em>P. penetrans</em></td>
<td>Lettuce</td>
<td>27.9±0.1</td>
</tr>
<tr>
<td>Ledegem/2</td>
<td><em>P. penetrans</em></td>
<td>Lettuce</td>
<td>27.6±1.3</td>
</tr>
<tr>
<td>Ledegem/3</td>
<td><em>P. penetrans</em></td>
<td>Vegetables</td>
<td>29.1±0.2</td>
</tr>
<tr>
<td>Zwalm/1</td>
<td><em>P. penetrans</em></td>
<td>Vegetables</td>
<td>30.5±0.2</td>
</tr>
</tbody>
</table>

Undet. - Undetermined
2.1.2 Extraction of nematodes from soil samples and their preparation for molecular processes

Two hundred ml of soil was collected from each of the 26 soil samples (Table 1) and washed into a 1 000 ml beaker through an 850 µm sieve. Where the plant roots were obtained on the sieve, they were collected and blended at high speed for one minute using the Waring Commercial \textsuperscript{©} Laboratory Blender. The blended roots were afterwards washed into the same beaker through another 850 µm sieve.

The 1 000 ml beakers containing the soil and blended roots were placed on the Automatic Zonal Centrifuge (AZC) for nematode extraction (Hendrickx, 1995). The extract collected in 150 ml glass beakers were kept undisturbed for over 3 h to let the nematodes settle to the bottom of the beakers. The supernatants were sucked off using a vacuum pump BioChem-Vacuum Center BVC 21 NT VARIO until a volume of 40 ml was left in the beakers.

The nematode suspension was poured into a counting dish and observed with a dissecting microscope. Individuals belonging to the genus \textit{Pratylenchus} from each sample were picked using a pig hair needle and placed into a staining block. The \textit{Pratylenchus} species were further identified based on their morphological features.

Eppendorf tubes of 0.2 ml were labelled appropriately and 25 µl of ultrapure (type 1) water was pipetted into each. The identified \textit{Pratylenchus} species were transferred into the Eppendorf tubes so that three tubes each contained one nematode and three other tubes contained five nematodes. This was done for each sample (Table 1). The nematodes in the tubes were stored at -20°C and afterwards DNA extraction was performed (section 2.6.1) and further molecular processes as described below (sections 2.6.2 and 2.6.3).

2.2 The effect of inhibitors on the performance of the qPCR assay

The nematode \textit{Pratylenchus} is a migratory endoparasite and can be found in plant materials and soils. These habitats can contain chemicals (inhibitors) that could have an influence on the amplification process of the qPCR.

2.2.1 Extraction of soil and plant samples to obtain a nematode suspension mixed with soil and plant soluble components and their preparations for molecular processes

Some plants (Table 2) and soil types (Table 3) were selected for this test as they have been reported as host or suitable habitat of \textit{Pratylenchus penetrans} (Kleyburg & Oostenbrink, 1959; Townshend, 1973; Brodie, 1999).
Table 2: Plants and their source used in this study.

<table>
<thead>
<tr>
<th>Plants</th>
<th>Plant part used</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato (<em>Solanum tuberosum</em>)</td>
<td>Tuber peels</td>
<td>Bintje DCP B15-1491*</td>
</tr>
<tr>
<td>Leek (<em>Allium ampeloprasum</em>)</td>
<td>Roots</td>
<td>Shop</td>
</tr>
<tr>
<td>Carrot (<em>Daucus carota</em>)</td>
<td>Root peels</td>
<td>Shop</td>
</tr>
<tr>
<td>Buxus (<em>Buxus sempervirens</em>)</td>
<td>Roots</td>
<td>Green house at ILVO</td>
</tr>
<tr>
<td>Yellow mustard (<em>Sinapis alba</em>)</td>
<td>Roots</td>
<td>ILVO research field</td>
</tr>
<tr>
<td>Maize (<em>Zea mays</em>)</td>
<td>Roots</td>
<td>LG3220**</td>
</tr>
</tbody>
</table>

*ILVO Diagnostic centre, **ILVO Entomology

Five grams (5g) of the plant materials (roots or tuber peel) were blended at high speed and washed through an 850 µm sieve into a 1 000 ml beaker and placed on the AZC. This was repeated for five more sub-samples (6 in total). For the extraction of potato peels, 10 ml antifoam (Extran AP33 Merck, Germany) was added.

Table 3: Soils and their origin used in this study.

<table>
<thead>
<tr>
<th>Soils</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clay</td>
<td>Ledegem</td>
</tr>
<tr>
<td>Sandy</td>
<td>DCP/2016/000574</td>
</tr>
<tr>
<td>Loamy</td>
<td>Zwalm</td>
</tr>
<tr>
<td>Organic</td>
<td>Agrofino, universele potgrond</td>
</tr>
</tbody>
</table>

Two hundred ml of each soil sample was washed through an 850 µm sieve into a 1 000 ml beaker and placed on the AZC. This was repeated for five more sub-samples of each sample (6 in total).

The two controls used for this experiment were tap water passed through the AZC (AZC water) and ultrapure (type 1) water.

The plant and soil extracts of 120 ml each were stirred and 1 ml of each was pipetted into a staining glass. Using the dissecting microscope, the pipetted sample was checked for the presence of *Pratylenchus* species. When the absence of *Pratylenchus* was confirmed, 25 µl extract was pipetted into a 0.2 ml Eppendorf tube. This was repeated for the 6 replicates of each soil type and plant material. To five tubes of each extract, 20 *P. penetrans* (source: culture Kerkom, ILVO) were added while the last tube was used as a negative control. The remainder of the various extracts of the soil and root were collected into 50 ml tubes, sealed with parafilm and stored at -20°C.

The process of DNA extraction was performed as described in section 2.6.1. Hereafter, the samples were subjected to PCR and qPCR processes as described below (sections 2.6.2 and 2.6.3).
In order to confirm some of the results, selected tubes containing extracts of the plant materials and soils were defrosted by placing them at room temperature for a day. The defrosted tubes were shaken to allow proper mixture and immediately 25µl were pipetted into an Eppendorf tube of 0.2µl. To these tubes, three individuals of *P. penetrans* (source: culture Kerkom, ILVO) were added. Ultrapure (type 1) water, defrosted and fresh water passed through the AZC were included in the test. Each sample was replicated twice.

### 2.2.2 Extraction of plant and soil samples using AZC with/without MgSO₄ and/or kaolin

Tests using the AZC with and without MgSO₄ and kaolin were performed to assess the influence of these chemicals on the qPCR assay. Materials free of nematodes (loam soil and roots of leek plant) and tap water were passed through the AZC. For each matrix, the normal nematode extraction protocol and extraction without MgSO₄ were performed. The extraction without kaolin and extraction without both MgSO₄ and kaolin were also performed for the matrices except loam soil. Ultrapure (type 1) water and tap water (not passed through the AZC) were included as controls. Three individuals of *P. penetrans* (source: culture Kerkom, ILVO) were added to 0.2 ml Eppendorf tubes containing 25 µl of each suspension obtained from the AZC and each control. The process of DNA extraction was performed as described in section 2.6.1 and followed by qPCR process (section 2.6.3).

### 2.3 Dilution experiments

#### 2.3.1 The dilution of plant and soil extracts before addition of nematodes

Dilutions of the extracts of some plant materials and soils were tested to check the performance of the qPCR assay at their lower concentrations since their undiluted forms did not allow the detection of *P. penetrans* (section 3.2). Carrot, maize, potato (extracted with antifoam) and all soil types (Table 3) were selected. The dilutions for the plant materials were 1:1, 1:5, 1:10, 1:100 and for the soils 1:1, 1:10 and 1:20. The dilutions were prepared and 25 µl was pipetted into Eppendorf tubes of 0.2 µl. The AZC water and ultrapure (type 1) water were also included independently to each test of plant materials and soils. The samples were replicated twice. Twenty and three *P. penetrans* (source: culture Kerkom, ILVO) were added to the plants and soils extracts and their corresponding AZC and ultrapure (type 1) waters respectively. The DNA extraction and qPCR processes were performed (sections 2.6.1 and 2.6.3) respectively.
2.3.2 The dilution DNA extracted from nematodes in different plant materials extracts.

The DNA extracted from the nematodes in some plant extracts from section 2.2.1 were 1:10 and 1:100 diluted to check the effect diluting will have on the performance of the qPCR assay. Buxus, carrot, leek, maize, yellow mustard and potato (extracted with antifoam) were used for the test. The DNA extracts of the nematodes in the controls (AZC and ultrapure (type 1) waters) were also diluted. Their undiluted samples were also included in the test. Each sample was replicated three times and qPCR process was performed (section 2.6.3).

2.3.3 Dilution of water passed through the Automatic Zonal Centrifuge (AZC)

The suspension obtained from tap water passed through the AZC (AZC water) was diluted to 1:1, 1:2, 1:5, 1:10, 1:50 and 1:100. Undiluted ultrapure (type 1), AZC and tap waters were included to the test. To 25 µl of each, three *P. penetrans* (source: culture Kerkom, ILVO) were added, then DNA extraction was performed as described below (section 2.6.1) and qPCR (section 2.6.3) was performed on the samples.

2.4 Detection of two *Pratylenchus penetrans* in a background of other nematodes.

To determine the sensitivity of the qPCR assay in detecting *P. penetrans* in a background of other nematodes. Two individuals of this species (source: culture Kerkom, ILVO) were placed in a series of 100, 500, 1 000, 5 000 and 10 000 other nematodes (mixture of 98% *Paratylenchus* spp. and *Meloidogyne chitwoodi* and 2% free living nematodes). *Paratylenchus* spp. and the free living nematodes were obtained from a sandy soil after an extraction using the AZC. *Meloidogyne chitwoodi* was obtained from a culture on potato after an extraction using the Baermann funnel. The absence of *Pratylenchus* spp. was confirmed by observing the extracts under a dissecting microscope.

To prepare the numbers above, the nematodes extracts were put together to a volume of 700 ml of suspension. The content was transferred into three 250 ml measuring cylinders to allow the nematodes to settle for over 3 h. Afterwards, the supernatant of each was removed to a volume of 30 ml. The three volumes were all transferred into another 250 ml cylinder to settle for 3 h. Again supernatant was taken out to leave 30 ml.

The 30 ml of suspension was transferred into a 500 ml cylinder and 470 ml of water was added to the suspension to dilute possible inhibitors (MgSO\(_4\) and others). The mixture was allowed to settle for 3 h and supernatant was taken out to leave 100 ml. This 100 ml of suspension was
then transferred into a 100 ml cylinder. After 3 h, the supernatant was taken out to leave 10 ml which contain the nematodes. This suspension contained approximately 10 000 nematodes in 1 ml when counted using a dissecting microscope, therefore, 500 µl, 100 µl, 50 µl and 10 µl had 5 000, 1 000, 500 and 100 nematode individuals, respectively (Table 4).

Eppendorf tubes of 1.5 ml containing the different nematodes numbers were spun at high speed (16 100 rpm) for 10 min and supernatant was taken away and left with 100 µl for tubes containing 10 000 and 5 000 nematodes while to the other tubes, the supernatant free of nematode was added (Table 4) to make up to 100 µl i.e. 50 µl and 90 µl for the tubes containing 500 and 100 nematodes respectively. The tube containing 1 000 nematodes already contained 100 µl. To each tubes two individuals of *P. penetrans* (source: culture Kerkom, ILVO) were added.

**Table 4**: The preparation of 100 µl suspensions containing different numbers of nematodes used as a background for a qPCR assay to detect two individuals of *Pratylenchus penetrans*.

<table>
<thead>
<tr>
<th>Number of nematodes in initial volume</th>
<th>Volume of supernatant added/subtracted</th>
<th>Number of nematodes /100 µl</th>
<th>Number of nematodes /µl</th>
<th>Number of P. penetrans added</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 000 in 1000 µl</td>
<td>-900 µl</td>
<td>10 000</td>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td>5 000 in 500 µl</td>
<td>-400 µl</td>
<td>5 000</td>
<td>50</td>
<td>2</td>
</tr>
<tr>
<td>1 000 in 100 µl</td>
<td>0 µl</td>
<td>1 000</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>500 in 50 µl</td>
<td>+50 µl</td>
<td>500</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>100 in 10 µl</td>
<td>+90 µl</td>
<td>100</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

The controls used in this experiment were the supernatant from the nematode suspension and ultrapure (type 1) water, 100 µl of each was used. To each of the 1.5 ml Eppendorf tubes, 100 µl of WLB⁺ (section 2.6.1) was added for DNA extraction and qPCR process was performed (section 2.6.3). The suspensions containing the mixture with 10 000, 5 000, 1 000, 500 and 100 nematodes and controls were replicated three times.

### 2.5 Practical application of the qPCR assay to detect *Pratylenchus penetrans* in a sample

This test aimed at developing a protocol for quantification of *P. penetrans* after nematode extraction using the AZC, taking care of the inhibition of the qPCR assay (section 3.2). A soil sample (DCP/2016001794) was mixed well and 200 ml of the soil was extracted.
nematodes in the suspension from the AZC were allowed to settle for 3 h and the supernatant was taken off, leaving 35 ml in a beaker (Figure 1). Eight replicate sub samples (200 ml each) were extracted. The number of *P. penetrans* were counted in the suspensions of two of the sub samples, while six other subsamples were quantified using the qPCR assay. Of these, three suspensions were dilated with tap water to 1:10 and three were left undiluted. The content of each beaker was poured into a 50 ml Eppendorf tube, this was allowed to settle down for another 3 h. Then, the supernatant was taken off and 5 ml was left in the three tubes selected for the dilution of the suspension. To the 5 ml left in these 50 ml tubes, 45 ml of tap water was added. The undiluted samples were gently shaken and together with the diluted samples, they were allowed to settle for another 3 h.

After 3 h, 3 ml of each samples was pipetted from the bottom of the tube and divided over two 1.5 ml Eppendorf tubes. The tubes were centrifuged at 12 000 rpm for 10 min. The supernatant was removed to leave 500 µl in each tube. For DNA extraction (section 2.6.1), 500 µl of WLB+ was added to each. Then, 950 µl from each tube was pipetted and transferred to a separate 2 ml Eppendorf tube and followed by the qPCR process (section 2.6.3).

![Figure 1: Stepwise procedure for preparing nematode suspensions obtained with the Automatic Zonal Centrifuge for molecular processes.](image)

Figure 1: *Stepwise procedure for preparing nematode suspensions obtained with the Automatic Zonal Centrifuge for molecular processes.*
2.6 Reproducibility: influence of qPCR machine

The protocol for quantifying Pratylenchus penetrans was tested on two different qPCR machines. The two machines Bio Rad qPCR machine CFX96™ and AB Applied-7900HT Fast Real-Time PCR System (ILVO). Six samples contained the DNA of P. penetrans while two samples contained the DNA from P. vulvus and two other from P. neglectus. Each sample contained with 5 individuals. The test was performed on the same day.

2.7 Molecular processes used in this study

2.7.1 DNA Extraction procedure

DNA extraction aims to release the DNA of the nematode(s) for downstream molecular processes. Proteinase K stock solution (20mg/ml), Lysis Buffer Minus (WLB−) and β-mercaptoethanol were the stock solutions used to prepare Lysis Buffer Plus (WLB+). The Lysis Buffer Minus (WLB−) breaks open the cells of the nematode to allow β-mercaptoethanol reduce the disulphide bonds present in proteins while Proteinase K destroys endogenous nucleases. To prepare 1 000 µl of WLB+, the three stock solutions were mixed under a fuming hood at 950 µl of WLB−, 40 µl of Proteinase K and 10 µl of β-mercaptoethanol and afterwards stored at -20°C. The maximum time the WLB+ was stored was one month.

The volume of WLB+ (Holterman et al., 2006) used for a sample was equivalent to that of the sample. For instance, 25 µl of the WLB+ was pipette into each of the tubes containing Pratylenchus spp. in a volume of 25 µl of ultrapure (type 1) water. The 0.2 ml tubes containing the nematode lysate mix was incubated at 65°C for 1.5 h to allow activity of the Proteinase K. This was immediately followed with a step to inactivate the Proteinase K at 99°C for five min. The DNA extraction reaction ran in a Thermocycler (Bio Rad T100™ Thermal Cycler).

Alternatively, the 1.5 ml tubes were incubated at 65°C for 1.5 h in a Thermomixer (Eppendorf: Thermomixer for 1.5 ml) followed by 5 min at 99°C on a heat block.

Both DNA extraction methods release unpurified DNA from the nematodes.

2.7.2 Polymerase Chain Reaction (PCR) and Gel Electrophoresis

In molecular biology, the technique used to amplify a piece of DNA to generate many of copies of a particular DNA sequence is the Polymerase Chain Reaction (PCR). The products of the PCR (amplicon) are separated according to their molecular sizes by an electrical field through a gel. This technique is described as gel electrophoresis.
The successes of the DNA extraction step were tested by performing PCR and gel electrophoresis. In each PCR experiment, a negative control lacking DNA template and a positive control with DNA from *P. penetrans* in ultrapure (type 1) water were included. The PCR amplification temperature/time conditions were a denaturation step at 95°C for two min, 35 cycles of DNA template denaturation for 30 s at 95°C, annealing for 30 s at 55°C and polymerisation for 1 min at 72°C. A 5 min final polymerisation step at 72°C followed the last cycle.

Five µl of each PCR product and 2.5 µl O’ GeneRuler™ DNA ladder of 100 bp were used for electrophoresis in 1X TAE buffer (Sambrook *et al.*, 1989) in a 1.5% agarose gel (SeaKem®LE Agarose) for 30 min at 100 V. The gel was stained with 0.003% ethidium bromide (0.02 g/ml) for 15 min and rinsed before viewed on a UV transilluminator and photographed.

Universal primers (Waeyenberge, unpublished) were used to amplify a part a segment of the 28S rRNA gene. The PCR was performed in a 0.2 µl Eppendorf tube with final volume of 50 µl of reaction mixture. The mixture contained 10 µl of Green GoTaq® Flexi Buffer (Promega), 6 µl of MgCl₂ solution (Promega) and 1µl of PCR Nucleotide Mix (Promega); in addition, 0.3 µl each of forward and reverse primers, 0.25 µl of Go Taq® G2 Hot Start Polymerase (Promega), 1 µl of template DNA/ultrapure (type 1) water and 31.15 µl of ultrapure (type 1) water.

### 2.7.3 The quantification test using the qPCR primers and probe

The qPCR is a technique in molecular biology that allows the monitoring of a DNA amplification process (PCR) in real time. The qPCR machines measure the accumulation of fluorescent signals produced by SYBR-green or TaqMan probe. In this experiment, the TaqMan probe-based qPCR was used.

In each PCR experiment, two negative controls lacking DNA template (NTC) were included. The qPCR amplification program consisted of the denaturation step 95°C for two min, 40 cycles of DNA template denaturation for 10 s at 95°C, annealing for 30 s at 63°C. The sequences developed for the forward primer, reverse primer and probe were PpenMFor 3′-CCA ACC TCT GCT ACA CTA-5’, PpenMRev 3′-CAG TGC GTT CAT TTA-5’ and PpMPb 3′-CAC TA T TA T GCC GC-5’, respectively (Mokrini *et al.*, 2013). The MGB-probe was labelled with 6-FAM and a non-fluorescent quencher (NFQ).

The qPCR was conducted following the protocol described by (Mokrini *et al.*, 2013). The reactions of 20 µl contained 3 µl of template DNA/ultrapure (type 1) water, 10 µl of Master
mix (SensiMIX II Probe Hi-ROX), 0.8 µl of each primer, 0.4 µl of probe and 5 µl of ultrapure (type 1) water. However, some experiments had the volumes of template DNA and ultrapure (type 1) water modified to 6 µl and 2 µl of ultrapure (type 1) water. The multi-well plates containing these mixtures were spun at high speed for few min then ran on a BioRad qPCR machine CFX96™ or AB Applied 7900HT Fast Real-Time PCR System.

All the Ct values reported were obtained from the AB Applied 7900HT Fast Real-Time PCR System. However, the BioRad qPCR machine CFX96™ was used in the test for reproducibility of the qPCR assay.

2.8 Statistical analysis

One-way analyses of variance (ANOVA) using the statistics software RStudio Desktop 0.99.902 was used. Homogeneity of variances was tested using Levene's test and normal distribution of residuals was assessed with Shapiro Wilk's W test. Suitable transformations (log, square root) were used when data did not meet assumptions. Kruskal–Wallis test was performed when assumptions were still not met after transformation. Post-hoc multiple comparisons tests were carried out with Tukey Kramer HSD. The critical statistics was $p<0.05$. 
3 RESULTS

3.1 The Specificity test of the qPCR primers and probe developed on Beta 1-4 endoglucanase gene.

The primer pair PpenMFor/PpenMRev along with the probe PpMPb detected all 14 populations of Pratylenchus penetrans using the qPCR assay. The mean Ct-values of three repetitions of 5 individuals of a sample varied from 27.6±1.3 to 30.5±0.2 (Table 1). Nonspecific amplifications were not produced when this assay was tested on DNA from other Pratylenchus spp. In addition, DNA was not detected (undetermined) in any of the controls that contained water without DNA.

3.2 The effect of inhibitors on the performance of the qPCR assay

To examine the presence of inhibitors that might interfere with the qPCR assay, six different plants and four soil types were used. The assay was performed on 20 individuals of P. penetrans (Pp) of which DNA was extracted in the presence of the soluble components of the plant and soil suspensions derived from the AZC. The tests were compared with the DNA extracted from 20 Pp in ultra-pure (type 1) water (positive control). The Ct value obtained were undetermined for all the samples tested except for maize where 20 individuals of P. penetrans resulted in a Ct of 27.1±1.7 (Table 5). However, two of the five replicates of the suspension of potato (extracted with antifoam) had Ct values of 25.7 and 25.0. The negative controls were always undetermined while the positive control (ultrapure (type 1) water) had Ct value of 29.5±2.1. However, the tap water passed through the AZC, the second positive control, had an undetermined Ct value.

Table 5: The Ct-values (mean ± standard deviation) of 20 individuals (n=5) of Pratylenchus penetrans (Pp) for each suspension of plant and soil samples except their negative controls and obtained in a qPCR reaction.

<table>
<thead>
<tr>
<th>Plant suspensions</th>
<th>Soil suspensions</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With Pp</td>
<td>Without Pp</td>
</tr>
<tr>
<td>Maize</td>
<td>27.1±1.7</td>
<td>Undet.</td>
</tr>
</tbody>
</table>

Undet.-Undetermined, NTC-No Template Control, UPW-Ultrapure (type 1) water
The amplicons visualised after electrophoresis (Fig. 2) indicated that the *P. penetrans*’ DNA extraction in the soluble components of maize, potato, and yellow mustard were successful. The thickness of their bands were similar to the ultrapure (type 1) water (positive control). However, the other crop and soil suspensions showed weak bands for the DNA of the nematodes whereas the negative control lacking DNA template had no band.

### 3.2.1 Extraction of plant and soil samples using AZC with/without MgSO₄ and/or Kaolin

The extracts of loamy soil, leek and tap water obtained from the AZC gave Ct values whenever MgSO₄ was exempted in the extraction step. However, for all extractions that included MgSO₄ and the negative controls the Ct values were always undetermined (Table 6).

#### Table 6: The Ct values of three individuals of Pratylenchus penetrans in leek and loam suspensions obtained from the AZC with or without MgSO₄ or Kaolin.

<table>
<thead>
<tr>
<th>Extraction using AZC</th>
<th>Without MgSO₄</th>
<th>Without Kaolin</th>
<th>Without MgSO₄ and Kaolin</th>
<th>With MgSO₄ and Kaolin</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZC water</td>
<td>32.9</td>
<td>Undet.</td>
<td>31.2</td>
<td>Undet.</td>
</tr>
<tr>
<td>Leek suspension</td>
<td>32.7</td>
<td>Undet.</td>
<td>31.8</td>
<td>Undet.</td>
</tr>
<tr>
<td>Loam suspension</td>
<td>31.2</td>
<td></td>
<td></td>
<td>33.9</td>
</tr>
<tr>
<td>Ultrapure (type 1) water</td>
<td></td>
<td></td>
<td></td>
<td>30.5</td>
</tr>
<tr>
<td>Tap water</td>
<td></td>
<td></td>
<td></td>
<td>31.5</td>
</tr>
<tr>
<td>NTC</td>
<td></td>
<td></td>
<td></td>
<td>Undet.</td>
</tr>
</tbody>
</table>

Undet.-Undetermined, NTC-No Template Control
3.3 Dilutions experiments

3.3.1 The dilutions of plant and soil extracts prepared from the AZC before addition of nematodes

The undiluted extracts of the crops and the control (AZC water) showed no amplification for the 3 individuals of *P. penetrans* except the undiluted maize extract with Ct value of 28.38±0.1. There was no amplification for the three individuals of *P. penetrans* added to undiluted extracts of carrot and potato, nor in the control (AZC water). However, a Ct value of 28.38±0.1 was produced from 3 individuals added to undiluted maize extract.

The 1:10 and 1:100 dilutions of the three crops extracts allowed the detection of the nematodes. In addition, there were no differences between the two dilutions (Figure 3).

![Figure 3](image)

**Figure 3:** The Ct-values (mean ± standard deviation) of 20 individuals of *Pratylenchus penetrans* (*n=2*) added to the 1:1, 1:10 and 1:100 dilutions of the suspensions of three plant materials obtained by from the AZC and the water passed through the AZC (AZC-water).

The undiluted extracts of four soil types showed no amplification of the three individuals of *P. penetrans*. The dilutions of 1:5, 1:10, 1:20 and 1:100 of all the soil extracts allowed the detection of all the nematodes together with the controls of tap and ultrapure (type 1) water (Figure 4). However, there were no differences between the dilutions of each soil type but the clay dilution of 1:5 was statistically different (p-value 0.04841) from ultrapure (type 1) water.
(positive control) and the 1:10 dilutions of loam and organic soils. In addition, the clay dilution of 1:5 differ with the 1:20 dilutions of loam and sandy soils (Figure 4).

3.3.2 The dilution of plant materials extracts after DNA extraction of nematodes.

The dilutions of maize and ultrapure (type 1) water (positive control) with their undiluted extracts allowed the detection of the *Pratylenchus penetrans*’ DNA in them (Figure 5). The nematodes could be detected in the 1:10 dilutions of carrot, buxus and the ultrapure (type 1) water passed through the AZC but not their undiluted nor 1:100 dilutions. In addition, no amplification was noticed for the undiluted, 1:10 and 1:100 dilutions of the extracts of potato, yellow mustard and the tap water passed through the AZC.

![Figure 4](image)

**Figure 4:** The Ct-values (mean ± standard deviation) of three individuals of *Pratylenchus penetrans* (n=2) added to the 1:1, 1:5, 1:10, 1:20 dilutions of the suspensions of four soil types obtained from the AZC with two controls (TW-Tap water and UPW-Ultrapure (type 1) water).
The inhibitory effect of the tap water passed through the AZC was examined by diluting the extract to 1:100, 1:50, 1:10, 1:5 and 1:2. The qPCR assay detected the three individuals of P. penetrans for the dilutions of 1:5, 1:10, 1:50, 1:100 and the control (ultrapure (type 1) water). The 1:5 dilution of the water had the highest Ct value (35.9 ± 0.5) which was statistically different (p-value < 0.05) from the other dilutions (Figure 6).
3.4 Test to determine what limit could the qPCR assay detect *Pratylenchus penetrans* in among other nematodes

In a background of other nematodes’ DNA, the qPCR assay could detect two *P. penetrans* in mixtures with 100, 500 and 1000 *Paratylenchus* spp and *Meloidogyne chitwoodi*. The Ct values of *P. penetrans* did not differ statistically (p-value=0.078) between mixtures of 100, 500 and 1000 *Paratylenchus* spp and *Meloidogyne chitwoodi*. However, when the nematode was mixed with 5000 or 10000 nematodes (*Paratylenchus* spp and *Meloidogyne chitwoodi*), the two *P. penetrans* could not be detected by the qPCR assay (Figure 7).

![Figure 7: The Ct-values (mean ± standard deviation) of three replicates of two individuals of *Pratylenchus penetrans* in a mixture with *Paratylenchus* spp. and *Meloidogyne chitwoodi* at 10 000, 5 000, 1 000, 500, 100 numbers and two controls (the supernatant (free of nematodes (SUP)) and ultrapure (type 1) water (UPW)).](image)

3.5 The application of qPCR assay to detect *Pratylenchus penetrans* in a sample

The qPCR assay performed on a sample containing 25 individuals of *Pratylenchus penetrans* in 2000 µl of their DNA extract showed mean Ct values of 35.97±0.03 for the extract of the sample when diluted with water. The positive control containing five individuals of *P.
Pratylenchus penetrans in 50 µl of DNA extract of ultrapure (type 1) water showed a Ct value of 31.1±0.2 (Figure 8).

**Figure 8:** The Ct-values (mean ± standard deviation) of 25 individuals (n=3) of Pratylenchus penetrans in the suspensions of a soil sample obtained from the AZC and processed with and without a dilution step.
3.6 Reproducibility: influence of qPCR machine

The results of the qPCR assay performed on two qPCR machines (Bio Rad qPCR machine CFX96™ and AB Applied-7900HT Fast Real-Time PCR System) showed Ct values of three different populations of *Pratylenchus penetrans* (Table 8). There was no amplification for *P. vulnus* and *P. neglectus*. The controls which contained water in place of DNA had undetermined Ct values. The Ct values for the three populations of *P. penetrans* were not different (p value=0.425) when ran on the two different qPCR machines.

**Table 7**: The Ct-values (mean ± standard deviation) of five individuals each of five populations of *Pratylenchus spp.*

<table>
<thead>
<tr>
<th>Species</th>
<th>AB-Applied</th>
<th>Bio-Rad</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pratylenchus vulnus</em></td>
<td>Undet.</td>
<td>Undet.</td>
</tr>
<tr>
<td><em>Pratylenchus neglectus</em></td>
<td>Undet.</td>
<td>Undet.</td>
</tr>
<tr>
<td><em>Pratylenchus penetrans</em></td>
<td>32.53±2.1</td>
<td>33.70±0.9</td>
</tr>
<tr>
<td><em>Pratylenchus penetrans</em></td>
<td>30.84±2.2</td>
<td>31.35±1.8</td>
</tr>
<tr>
<td><em>Pratylenchus penetrans</em></td>
<td>30.75±1.1</td>
<td>30.39±0.1</td>
</tr>
<tr>
<td>NTC</td>
<td>Undet.</td>
<td>Undet.</td>
</tr>
</tbody>
</table>

Undet.- Undetermined, NTC-No Template Control
4 DISCUSSION

The plant parasitic root-lesion nematode *Pratylenchus penetrans* could be detected using the qPCR assay developed by Mokrini *et al.* (2013) on the DNA of 14 populations of *P. penetrans* from Belgium used in this experiment. This confirms the specificity of this assay which was shown to detect the different populations of *P. penetrans* from North Africa and Europe (Mokrini *et al*., 2013). There was no amplification for the other five species of *Pratylenchus*. It has been reported that *P. fallax* has a close relationship to *P. penetrans* morphologically and phylogenetically based on the ITS region (Carta *et al*., 2001; Handoo *et al*., 2001; Subbotin *et al*., 2008). However, the qPCR assay did not detect the two populations of *P. fallax* tested in this research.

The selectivity test showed that inhibitors can have a huge impact on the performance of a qPCR assay. The soluble components of the crops (except maize) and of the soils including the tap water passed through the AZC contained substance(s) that inhibited the detection of three and 20 individuals of *P. penetrans*. This inhibitory effect could be linked to the MgSO$_4$ supplied by the AZC because suspensions obtained from the AZC without the supply of MgSO$_4$ always allow the detection of *P. penetrans*. Moreover, there could be additional inhibitory effect supplied by the soluble components of the crop and soil (Yan *et al*., 2013). However, the soluble components of maize obtained from the AZC allowed the detection of three and 20 individuals of *P. penetrans*. This is comparable to the assay performed with *P. penetrans* extracted from roots of wheat using the AZC (Mokrini *et al*., unpublished). The two crops (cereals) may contain particular substances that counter the effect of the inhibitor (MgSO$_4$) supplied by the AZC. The activity of the inhibitors always resulted in no amplification signals as Ct values were undetermined. This is not in line with the report of Yan *et al.* (2013) that soil inhibitors could influence a qPCR assay by providing higher estimations of amplification. This is the first report of the failure of a qPCR assay performed with the suspension obtained from an AZC.

Nevertheless, diluting the suspensions from the AZC allowed the detection of the *P. penetrans*. The introduction of dilution lowers the concentration of inhibitor (MgSO$_4$) and therefore the impact on the qPCR assay was less. It was observed that 1:10 and 1:100 dilutions of all the suspensions obtained from the AZC allowed the detection of the three individuals of *P. penetrans*. This is similar to the report by Yan *et al.* (2013) who observed that when a soil has been autoclaved, the *P. neglectus* added afterwards can be quantified more accurately therefore
minimizing the effect of inhibitors. In this report, the suspension dilution that allowed the qPCR assay to detect the nematode in all the samples can be limited to 1:10 (suspension : ultrapure (type 1) water). The higher dilutions of 1:20, 1:50 and 1:100 gave no significant improvement as Ct values were similar. Whereas the lower dilution of 1:5 was not favourable for the assay as it produced a Ct value higher than the previous dilutions. The higher Ct value indicates that a high concentration of MgSO₄ was present in the 1:5 suspension dilution and therefore minimizing the performance of the qPCR assay. A much higher concentration of MgSO₄ in the 1:2 dilution is responsible for total failure of the qPCR assay.

A different approach to reduce the influence the inhibitors in the suspensions obtained from the AZC have on the qPCR assay, was diluting the DNA of *P. penetrans* instead of diluting the suspension. The dilutions were ten (1:10) and one hundred (1:100) times. The 1:10 and 1:100 DNA dilutions of ultrapure (type 1) water and maize suspension gave amplification signals as it detected the DNA of *P. penetrans*. This shows that the qPCR assay is very sensitive as it could detect the DNA of *P. penetrans* when diluted. This report is similar to the findings that a qPCR assay can detect the 1:10 and 1:100 DNA dilutions of *P. penetrans* (Sato et al., 2007; Sato et al., 2011). However, failure of the qPCR assay in detecting the 1:10 and 1:100 DNA dilutions of *P. penetrans* was observed for other crop suspensions. The 1:10 DNA dilutions in the suspensions of carrot, buxus and the ultrapure (type 1) water passed through the AZC were detected but not the 1:100 DNA dilutions. Moreover, the 1:10 and 1:100 DNA dilutions of *P. penetrans* in the suspensions of potato, yellow mustard and tap water passed through the AZC were not detected. These failure of the qPCR assay in detecting the DNA of *P. penetrans* could be linked to the low amounts of the DNA in the diluted suspensions mixture and the presence of inhibitors. Berry et al. (2008) also attributed the failure of a qPCR process in detecting the DNA of *Xiphinema elongatum* to low amounts of DNA in the dilutions. In contrast, Sato et al. (2007) and Yan et al. (2012) could detect the DNA of nematodes in dilutions higher than 1:100; this could be possible as they used purified DNA of the nematode for their tests. Mokrini et al. (2013) could detect the DNA of *P. penetrans* to 1:80 dilution in nuclease free water (1nematode/µl). This dilution is lower than the 1:100 dilution in the suspension that contain the soluble components of plant or soil.

This research reports failure of the qPCR in detecting the 1:10 and 1:100 DNA dilutions of *P. penetrans* in the suspensions of several crops and therefore diluting DNA of *P. penetrans* in the suspensions of crops is not be recommended for use. However, from the former approach of dilution, it is recommended to dilute the suspensions obtained from the AZC before DNA
extraction and qPCR. This can be an effective way to minimize the effect of inhibitors present in the nematode suspensions obtained from the AZC.

This qPCR assay is also considered to be highly sensitive as it could detect an individual of *P. penetrans* (data not shown). Moreover, it could detect two individuals of *P. penetrans* among *Paratylencyclus* spp and *Meloidogyne chitwoodii* to 1000 nematodes. The Ct value obtained from detecting two individuals of *P. penetrans* in 1000 nematodes is similar to those obtained when *P. penetrans* was mixed with 500 and 100 nematodes. Using the traditional method of counting with a stereomicroscope, there will be a great possibility that the two individuals of *P. penetrans* among 1000 *Paratylencyclus* spp and *Meloidogyne chitwoodii* will be not be counted. The sensitivity of the assay is comparable to previous assays. Mokrini *et al.* (2013) reported that the assay detected the DNA of a single individual of *P. penetrans* when mixed with DNA from 80 individuals of *P. thornei* (80/50 µl=1.6nematode/µl). Toyota *et al.* (2008) detected one single second-stage juvenile of *Globodera rostochiensis* in mixed nematode communities of 1000 free living individuals. A single *P. penetrans* could be detected in the presence of 800 individuals of free living nematodes (Sato *et al.*, 2007). The 1000 nematodes may not represent the absolute sensitivity of this assay in a mixture of other nematodes. It is likely that the limit will fall between 1000 and 5000 nematodes.

The reproducibility of this test was demonstrated by the similar range of Ct values obtained from the two different qPCR machines used to perform the test of five individuals of three populations of *P. penetrans* and a population each of *P. vulnus* and *P. neglectus*. The *P. penetrans* were all detected whereas *P. vulnus* and *P. neglectus* were not. Braun-Kiewnick *et al.* (2016) tested samples on two different qPCR machines and had the limit of detection to be different. This difference can be linked to the difference in operations of the machines as manufactured. In as much as similar Ct values were obtained from the different qPCR machines used in this research, results should be treated independently.

This experiment also demonstrated that the qPCR assay can detect DNA of *P. penetrans* from both soil and plant samples. Mokrini (unpublished) also detected *P. penetrans* extracted from wheat. Sapkota *et al.* (2016) also detected *Meloidogyne hapla* from carrot and clay and sandy soils. Sato *et al.* (2011) estimated the number of *P. penetrans* in the samples from different radish fields using a qPCR assay. Interestingly, there was no difference in the Ct values for the DNA of *P. penetrans* in the soluble components of the different soil types used in this study. This is in contrast to the report of Goto *et al.* (2009) that different soil types have different Ct
values. The volumes of nematode suspension used for qPCR and that used for the traditional method of counting to find nematodes numbers in samples differs as given in some reports. Sapkota et al. (2016) used 250 mg of soil for qPCR assay out of 100 g of soil used for the traditional method. Berry et al. (2008) also used 5 ml of nematode suspension for the traditional method of quantification by counting and 1 ml of the same suspension for qPCR thereby the numbers of nematodes could differ and provide false estimation. However in this research, the volume of nematode suspension used for the traditional method of quantification was reduced in a stepwise procedure and therefore the number of nematode in the initial volume remained same with that used for the qPCR and provide better estimation.

The unpurified DNA obtained by the use of WLB+ for allowed the detection of the nematodes (Mokrini et al., 2013; Braun-Kiewnick et al., 2016). However, this method of DNA extraction has low efficiency in the extraction of nematodes’ DNA (Madani et al., 2005). It will be interesting to test the results of this test using alternative DNA extraction methods on the nematode suspensions from the AZC. In addition, a standard Ct for each crop and soil types to estimate nematode numbers can be developed. It would be interesting to further follow up on these hypotheses.

More than two root lesion nematodes species can concomitantly be in the same roots of the host plant. Therefore correct identification is important for successful management. In conclusion, the results of this experiment show that the assay can be used in assessing P. penetrans infestations levels in soils and plants even in the midst of other nematodes. In addition, nematode suspensions obtained from the AZC should be diluted to allow the detection of the nematodes in a qPCR reaction.
5 APPRECIATION

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