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In planta PCR-based detection of early infection of plant-parasitic nematodes in the roots: a step towards the understanding of infection and plant defence

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Abstract The polyphagous obligate parasites *Meloidogyne spp.* devastate a wide range of crop plants including bananas and plantains. Their infestations impact agriculture worldwide. Therefore, an effective combating regime against this nematode species and an in-depth understanding of plant-nematode interaction are essential. Early detection of infection by visual inspection is not possible. This hampers early control strategy efforts and makes in-depth research of the early infection and plant defence unfeasible. A simple and robust *in planta* PCR-based nematode detection method is described here as the first crucial step. This PCR-based detection assay exploits the existence of the Internal Transcribed Spacer 1 (ITS 1) region of the ribosomal DNA (rDNA) gene family in the nematodes for early detection of nematode penetration into the roots. The results demonstrate that this detection assay is suitable to serve as a molecular screening tool for plant root diagnostic purposes.

Keywords Banana · DNA extraction · *In planta* detection · *Meloidogyne incognita* · Nematode · PCR

Abbreviations

IP	Infected plant root
N	Nematode individual
ND	Nematode DNA
NP	Nematode mixed with plant roots
NPD	Nematode and plant DNA
P	Plant roots
PD	Plant DNA
TR	Transformed tomato roots infected with *Meloidogyne incognita*

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Introduction

Bananas and plantains (*Musa* spp.) are the developing world's fourth most important food commodity in terms of gross value of production. They constitute a

major staple food for millions of people as well as providing a valued source of income through local and international trade (Frison and Sharrock 1998). The production of bananas and plantains is hampered by many diseases and pests (Jones 2000). Nematodes are considered one of the major biotic constraints to *Musa* production (Gowen et al. 2005).

Worldwide, the burrowing nematode *Radopholus similis* is considered as the most important nematode of bananas and plantains. However, in the absence of *R. similis*, other nematode species, such as root-knot nematodes (*Meloidogyne* spp.) may also become important pathogens (De Waele and Davide 1998). *Meloidogyne* spp. are worldwide in distribution attacking many economically important crops. At least five species have been reported on *Musa* in the warm and tropical areas (Gowen et al. 2005). The species most commonly recorded in *Musa* are *M. incognita* and *M. javanica*.

The most obvious symptoms of an infection with *Meloidogyne* species are the swollen, galled primary and secondary roots hence the name root-knot nematodes (RKN). Attributes such as stunted growth, thin pseudostems and small fruit bunches in the *Musa* diploids Pisang Mas and Pisang Nangka resulting from RKN infection have been reported in Malaysia (Razak 1994). Additionally, in the Philippines, RKN caused yield losses ranging from 26% to 57% in the *Musa* triploid Giant Cavendish (Davide and Marasigan 1985).

The predominant developmental stage of *Meloidogyne* species in the soil is the second-stage juvenile (J2; Qiu et al. 2006). J2 individuals accumulate and penetrate at the region of cell elongation just behind the root tip. Once inside the roots, the J2 will move along the vascular cylinder toward a so-called zone of differentiation where they will become sessile, develop into females and induce procambial cells to form giant cells. These cells will function as feeding cells during the rest of the life of the sedentary adult female. As a reaction to colonisation by the nematode, plant cells neighbouring the giant cells will divide and enlarge causing the formation of galls or root-knots (Gheysen and Jones 2006; Williamson and Gleason 2003).

Banana roots—as most plant roots—show no symptoms (galling) during the early stage of J2 infection. Therefore, early detection of infection by visual inspection is not possible. This hampers early control strategy efforts and makes in-depth research of the early infection and plant defence unfeasible. Currently, the *in planta* detection methods used by crop scientists are laborious and time consuming. Typical steps include selective staining of root tissues, isolation of the nematodes, slide preparation and microscopy observations (McCartney et al. 2003; Qiu et al. 2006; Sundelin et al. 2009). For these reasons, the application of molecular techniques is an attractive alternative that offers a more effective, sensitive and rapid screening strategy.

During the past two decades, several molecular tools have been developed for the identification and comparison of nematodes at genera, species and population levels (Powers et al. 2005; Qiu et al. 2006; Subbotin and Moens 2006; Williamson et al. 1997; Zijsltra et al. 2004). Several reports on discriminating nematode species by using taxonomically informative regions such as the mitochondrial DNA (mtDNA), Internal Transcribed Spacer (ITS) regions and the *28S* ribosomal DNA (rDNA) gene have been published (Subbotin and Moens 2006). Additionally, it has also been proven that the Polymerase Chain Reaction (PCR) assay is sensitive enough to allow identification of a single nematode at species level (Qiu et al. 2006).

In general, molecular-based identification procedures are carried out by using purified DNA of hand-picked nematode individuals isolated from infested soil and infected plant roots (Hubschen et al. 2004; Powers et al. 2005; Qiu et al. 2006). Although reports were published on *in-planta* detection of aerial nematodes in stem and leaf tissues (Zouhar et al. 2007; McCuiston et al. 2007), no comparable detection system targeting nematodes in the root tissues has been published so far. However, there were reports of molecular *in planta* detection of other pathogens such as nematophagous fungi in tomato roots (Hirsch et al. 2000), potato roots (Atkins et al. 2005) and soil-borne fungi in watermelon roots (Lovic et al. 1995).

The objective of our study was to develop and optimise an early PCR-based nematode detection assay *in planta* despite possible interference of inhibitors from plant roots such as polysaccharides, reactive secondary metabolites (Ding et al. 2008) and humic acid from the soil (Wilson 1997; Yeates et al. 1998). To achieve this objective, a pair of PCR primers was designed to amplify the ITS1 region of rDNA of *M. incognita*. Two modified plant DNA

extraction protocols were also compared in our study to assess PCR tolerance for the presence of inhibitory plant and soil components. To the best of our knowledge, the present paper reports for the first time a successful PCR-based early *in planta* nematode detection assay for roots using *M. incognita* J2 and banana roots as an experimental system.

Materials and methods

DNA isolation

DNA was isolated from five different sources: a) nematode individual(s) b) banana roots c) nematode(s) mixed with 50 mg banana roots, d) nematode infected banana roots (50 mg) and e) *Agrobacterium rhizogenes* transformed tomato roots infected with *M. incognita*, denoted as N, P, NP, IP and TR respectively. All DNA isolation procedures were conducted in 2 ml micro-centrifuge tubes. DNA isolation from nematode individual(s) was done according to Madani et al. (2005) (with minor modification). DNA extraction from samples involving plant tissues (P, NP, IP and TR) was carried out by using modified protocols of Dellaporta et al. (1983) and Khayat et al. (2004) as described in detail below. Note that DNA was isolated from 50 mg (fresh weight) root tissues for all experiments involving plant tissues.

Modified from Dellaporta et al. (1983)

One millilitre of DNA extraction buffer [100 mM Tris (pH 8.0), 50 mM EDTA, 500 mM NaCl, 1% (w/v) DTT and 600 μg per ml Proteinase K] was used per 50 mg (fresh weight) root tissue. The sample was incubated overnight at 65°C and Proteinase K was deactivated at 95°C for 15 min. Following the addition of 60 μl of 10% (w/v) SDS, the sample was vortexed for 5 s and incubated at 55°C for 20 min. Subsequently, 330 μl of a 3.0 M potassium acetate solution in 11.5% (v/v) acetic acid was added. After thorough mixing, the sample was incubated on ice for 30 min and centrifuged (13,000 rpm, room temperature) for 10 min. The supernatant was transferred to a new tube and centrifuged similarly for 5 min. One microlitre of RNase A (20 mg per ml) was added to the sample prior to 15 min incubation at 37°C. An equal volume of isopropanol was added. The sample was thoroughly mixed, incubated for 20 min at room temperature and centrifuged (13,000 rpm, room temperature) for 15 min. The resulting supernatant was discarded and the DNA pellet was washed with 500 μl of 70% (v/v) ethanol. After 5 min of centrifugation (13,000 rpm, room temperature), the ethanol was discarded, the pellet was air-dried and finally dissolved in 30 μl dH_2O.

Modified from Khayat et al. (2004)

One millilitre of extraction buffer [4% (w/v) CTAB, 100 mM Tris-HCl (pH 8.0), 1.4 M NaCl, 50 mM Na-EDTA (pH 8.0) and 1% (w/v) DTT] was used per 50 mg (fresh weight) root tissue. The sample was incubated for 30 min at 55°C prior to centrifugation (5,000 rpm, room temperature) for 5 min. The supernatant was transferred into a fresh 2 ml centrifuge tube. RNase A was added at a final concentration of 200 μg per ml prior to 1 h incubation at 37°C. An equal volume of phenol:chloroform:isoamylalcohol [25:24:1] was added, thoroughly mixed by brief vortexing and centrifuged (5,000 rpm, 4°C) for 5 min. The upper aqueous phase was transferred into a fresh 2 ml tube. An equal volume of chloroform: isoamylalcohol [24:1] was added and mixed before centrifugation as mentioned above. Similarly, the upper aqueous phase was transferred into a fresh 2 ml tube. An equal volume of ice-cold isopropanol was added and mixed prior to incubation at −80°C for 1 h. The sample was thawed without agitation at 4°C prior to centrifugation (8,500 rpm, 4°C) for 20 min. The supernatant was discarded while the pellet was carefully washed twice with 1 ml of 70% (v/v) ethanol. After each washing step, the sample was centrifuged (5,000 rpm, room temperature) for 5 min. Finally, the pellet was air-dried and dissolved in 30 μl dH_2O.

The DNA concentration and quality ($A_{260/280}$ and $A_{260/230}$) were determined using a NanoDrop® ND-1000 spectrophotometer (Nanodrop Products, Wilmington, DE, USA).

Primer design and PCR amplification

The primer pair MiITS1F 5′ATTGTTGTG WAACGGCT3′/MiITS1R 5′TAGTCCTAACATGT CACC3′ was designed to target the ITS1 region of rDNA of *M. incognita* (Fig. 1). The 91 bp target

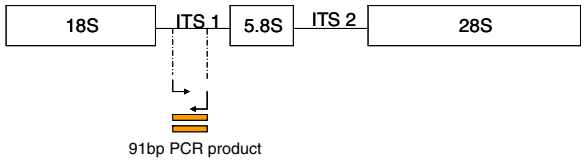

Fig. 1 Schematic diagram showing the primer annealing sites at the ITS1 spacer region of ribosomal DNA yielding a 91 bp PCR product in *Meloidogyne incognita*

sequence is located in a highly conserved region as determined by alignment of the ITS1-5.8S-ITS2 fragments of 21 ribosomal DNA clones of *M. incognita* using ClustalX (version 1.81; Thompson et al. 1997). The clone variants are available in the GenBank database (Acc. No. FJ534516 and FJ534515).

PCR was performed in a 25 µl or 50 µl reaction mixture containing 1X PCR Buffer (New England Biolabs or NEB, Hertfordshire, UK), 1.5 mM $MgCl_2$ (NEB, UK), 80 µM of each dNTP, 1 µM of each primer, 0.04 U per µl *Taq* Polymerase (NEB, UK) and sterile dH_2O in 0.2 ml microcentrifuge tube. All PCR reactions were run on the Thermocycler Mastercycler Gradient (Eppendorf, Hamburg, Germany). The PCR program consisted of an initial denaturation step of 3 min at 94°C followed by 38 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C with a final elongation step of 72°C for 5 min as described by Fallas et al. (1996).

Agarose gel electrophoresis

A 10 µl sample of each PCR reaction was separated on a 1.5% (w/v) agarose gel in 1X sodium borate buffer [10 mM NaOH; pH adjusted to 8.5 using H_3BO_3 (Brody and Kern 2004)] prior to running at 250 V for 28 min. Following electrophoresis, the gel was stained with 0.5 µg per ml ethidium bromide for 20 min followed by destaining in distilled water for 20 min. The gels were viewed on a UV (302 nm wavelength) light trans-illuminator and images were captured with a digital camera (Camedia-C3030 Zoom, Olympus).

Primer specificity test

The primer pair was tested for its specificity on readily available DNA templates of a) J2 nematode individual(s) of *M. incognita* (b) pooled individuals of *M. javanica*, *R. similis, P. coffeae* and *P. penetrans*, c) banana root (50 ng DNA) mixed with nematode and d) banana root (50 ng DNA) denoted as ND, NPD and PD respectively. Samples ND and NPD contained 5 µl DNA extracts from a varying number of J2 individuals (1–100 J2 individuals). Sample PD served as negative control. Samples without visible signal were subjected to a second PCR round for confirmation with 3 µl of a 10× diluted primary PCR product as template.

PCR products (91 bp) of ND and NPD samples were sent for direct sequencing (VIB Genetic Service Facility, University of Antwerp, Belgium) to verify that the signal obtained on agarose gel originated from the nematode template and not the plant template.

Primer sensitivity test

All DNA templates used from this experiment onwards were freshly isolated from the tissue samples. J2 individuals were hand-picked and placed into a 2 ml microcentrifuge tube containing 20 µl dH_2O. The numbers of individuals tested in this experiment were 1, 10, 50 and 100. Banana roots free from nematode infections were isolated and thoroughly washed. After air drying, the roots were weighed. Fifty milligrams of banana roots (fresh weight) were grounded into fine powder with mortar and pestle in liquid nitrogen. This fine powder was transferred into the tube containing J2 individual(s) in 1 ml extraction buffer (NP). These NP samples were subjected to DNA isolation as described above. Samples P (50 mg) and TR (50 mg) were included as negative and positive controls, respectively. The PCR reactions were conducted in two reaction volumes (*i.e.* 25 µl and 50 µl) for both DNA extraction protocols with 25 ng template DNA used in each reaction.

Early detection of targeted nematode infection of single primary root

This experiment emulated a field inoculation procedure of single primary root described by De Schutter et al. (2001).

Tissue cultured plantlets of the banana cultivar ‘Grand Naine’ were transplanted into 200 ml plastic pots containing sand:peat (2:1) soil mixture. The plants were left to acclimatise under greenhouse

conditions for 8 weeks with fertiliser being applied twice a week starting from the second week after transplantation. The plants were then transferred into 1 l pots with one of the primary roots selected for inoculation. The selected root of each plant was placed across a 3.5-cm diameter plastic Petri dish (Fig. 2) and left in the soil for 3 days prior to inoculation. Slits to place the root were first made by melting the Petri dish's wall with heated forceps.

M. incognita J2 individuals were obtained as described above. Five hundred *M. incognita* J2 individuals were inoculated at each inoculation site with two inoculation sites per target root (Fig. 2). The treatment was done in two replicates with duplicates of non-inoculated roots serving as negative controls (denoted as NIPC). Plants were daily harvested from 2 days after infection (dai) onwards till 5 dai.

Upon harvest, the target site of inoculation was excised from the root system and the root fragment was thoroughly washed to ensure that a positive PCR signal originates from penetrated nematodes. The root fragment was then air-dried and weighed. The weighed root fragment (denoted as IP) was snap-frozen in liquid nitrogen prior to DNA isolation by the protocol modified from Khayat et al. (2004) as described above. Alternatively, this snap-frozen IP (or NIPC) sample can be stored in −80°C till further use. PCR was conducted in 50 µl reaction mixture using 25 ng template DNA per reaction. P and TR samples were included as negative and positive controls, respectively.

Results

Primer specificity test

The nematode-specific signal was detected in nematode DNA (denoted as ND) (Fig. 3a) and the mixture of nematode and plant DNA (denoted as NPD) (Fig. 3b) samples. The signal was detectable in the ND and NPD samples containing as low as one J2 *M. incognita* individual. Banana plant DNA samples (PD, lanes 10 & 11 in Fig. 3a) were negative. Sequencing of PCR products yielded single peaks (data not shown) and matched (100% identity) with the *M. incognita* ITS1 region sequences in the GenBank (Acc. No. FJ534516 and FJ534515). A species specific test was additionally performed on *M. javanica*, *R. similis, P. coffeae* and *P. penetrans*. A positive signal was only detected in *M. javanica* (results not shown).

DNA isolation from samples involving plant tissues and primer sensitivity test

Low DNA purity with average A_{260}/A_{280} and A_{260}/A_{230} ratios of 1.39 (±0.22, n=23) and 0.29 (±0.69,

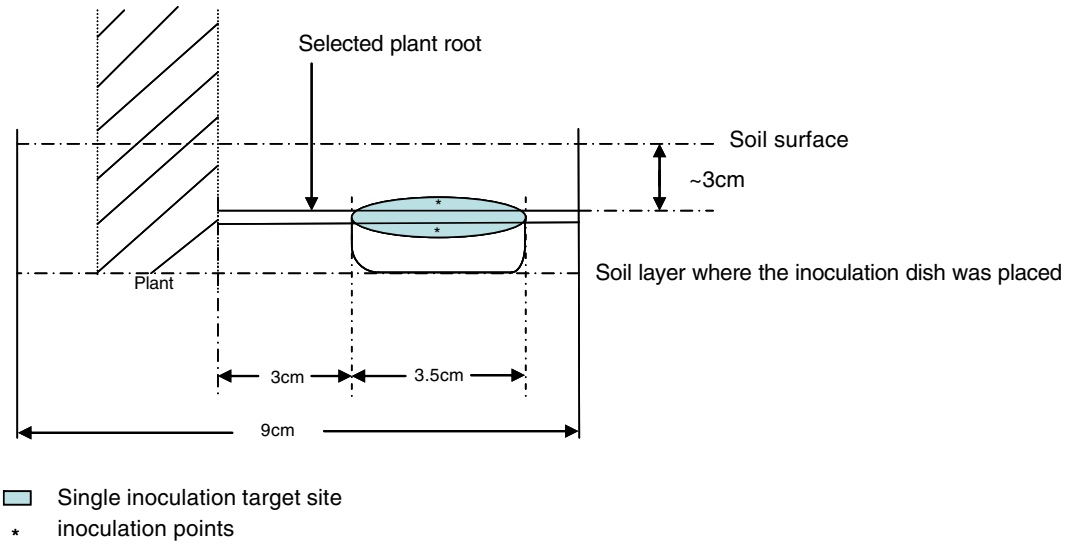

Fig. 2 Schematic diagram depicting the single inoculation target site and two inoculation points on a single primary root of an 8 months old greenhouse-grown banana plant in a 1 l pot

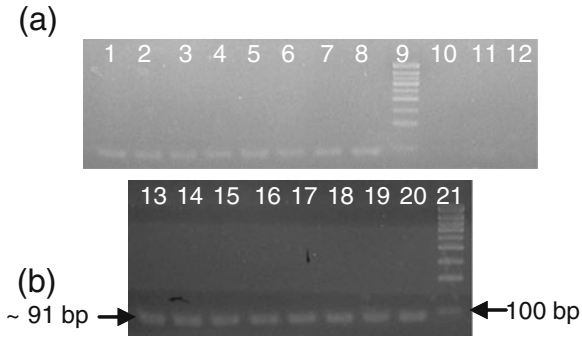

Fig. 3 Primer specificity test on **a** DNA isolated from varying number of J2 individuals (ND) and **b** 50 ng banana plant DNA mixed with nematode DNA extracted from varying number of J2 individuals (NPD). *Lane 1*: ND1, *Lane 2*: ND10, *Lane 3*: ND20, *Lane 4*: ND30, *Lane 5*: ND40, *Lane 6*: ND50, *Lane 7*: ND60, *Lane 8*: ND100, *Lane 9*: 100 bp ladder, *Lane 10 & 11*: PD (banana plant DNA, negative control), *Lane 12*: dH_2O (distilled water, negative control), *Lane 13*: NPD1, *Lane 14*: NPD10, *Lane 15*: NPD20, *Lane 16*: NPD30, *Lane 17*: NPD40, *Lane 18*: NPD50, *Lane 19*: NPD60, *Lane 20*: NPD100, *Lane 21*: 100 bp ladder

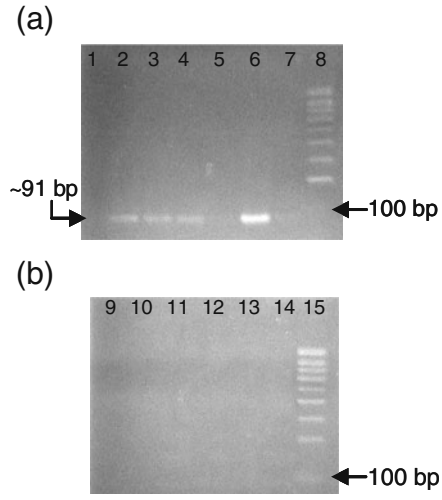

Fig. 4 Primer sensitivity test on **a** DNA isolated from a mixture of 50 mg fresh weight banana root tissue and varying number of J2 individuals (NP) and **b** replicate of 25 ng plant DNA samples (P). DNA isolated from a nematode culture maintained in transformed tomato roots (TR) served as positive control PCR reaction. *Lane 1*: NP1, *Lane 2*: NP10, *Lane 3*: NP50, *Lane 4*: NP100, *Lane 5*: empty, *Lane 6*: TR (positive control), *Lane 7*: dH_2O (distilled water, negative control), *Lane 8*: 100 bp ladder, *Lane 9*: P6, *Lane 10*: P7, *Lane 11*: P8, *Lane 12*: P9, *Lane 13*: P10, *Lane 14*: P11, *Lane 15*: 100 bp ladder

n=23), respectively, were obtained from samples containing banana root tissue (NP and P) using the modified Dellaporta et al. (1983) protocol. By contrast, the modified Khayat et al. (2004) protocol yielded higher DNA quality with average A_{260}/A_{280} and A_{260}/A_{230} ratios of 1.90 (±0.22, n=23) and 1.22 (±0.38, n=23), respectively.

PCR failed to produce the nematode signal in both reaction volumes tested (*i.e.* 25 µl and 50 µl) with NP DNA extracted using the modified Dellaporta et al. (1983) protocol, whereas the nematode signal was successfully amplified in a 50 µl PCR reaction volume from NP DNA extracted using the modified Khayat et al. (2004) protocol (data not shown). Hence, the latter DNA extraction protocol and 50 µl PCR reaction volume were used in subsequent assays.

The lower detection limit of the PCR assay was 10 J2 *M. incognita* individuals (NP10; Fig. 4a), which was tenfold higher than that of the mixed DNA samples (NPD) as mentioned above. No nematode signal was obtained in samples containing DNA extracted from banana plant roots (P samples) or the negative control sample (Fig. 4b). The signal obtained from positive control DNA of nematode infected tomato roots (TR, lane 6, Fig. 4a) demonstrated that *M. incognita* could be detected by the established PCR assay in infected tomato roots.

Early detection experiment

The presence of J2 *M. incognita* was detected in infected banana root as early as 2 days after inoculation (dai) and the same result was obtained at 3 dai (lanes 1 to 4, Fig. 5). The experiment was conducted up to the 5th dai with all IP samples positive for nematode presence (data not shown). Control, non-inoculated banana roots (NIPC samples)

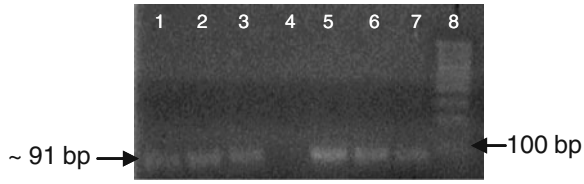

Fig. 5 PCR assay on DNA isolated from infected plant roots 2 and 3 days after infection (IP2 and IP3, respectively) with DNA samples TR (nematode culture maintained in transformed tomato roots) and ND100 (100 individual J2 nematodes) serving as positive controls. *Lane 1 & 2*: IP2, *Lane 3 & 4*: IP3, *Lane 5*: TR3, *Lane 6*: TR4, *Lane 7*: ND100, *Lane 8*: 100 bp ladder

lacked the nematode signal (data not shown), whereas it was again clearly detectable in the positive control TR tomato samples (lanes 5 & 6, Fig. 5).

Discussion

Current infection detection methods used are laborious and time consuming. Often, *M. incognita* infestation is detectable at the later stage of infection, making effective control measures ineffective. Therefore, we developed a diagnostic assay by using a molecular approach as an alternative to current screening assays. A pair of PCR primers was specifically designed to amplify the *M. incognita* ITS1 region. Successful amplification of this region serves as nematode-specific signal that signifies the presence of nematodes in the asymptomatic root system, provided that spurious amplification from other sources can be confidently ruled out. Following optimisation, primer efficiency tests revealed high specificity towards nematode DNA, and this quality was not compromised in the abundance of plant DNA. Moreover, the signal was detectable in all ND and NPD samples containing as low as one J2 *M. incognita* individual. No signal was obtained from banana plant DNA samples (PD) and negative samples that were subjected to a second round of PCR (data not shown), indicating the specificity of the reaction and ruling out false negative results. The specificity of the primer pair for the nematode template was further confirmed with the BLASTn result of the sequenced PCR products that matched (100% identity) with *M. incognita* ITS1 region in the GenBank (data not shown). A preliminary species specificity test conducted on *M. javanica*, *R. similis, P. coffeae* and *P. penetrans* indicates that our primer pair seems to have affinity only for *Meloidogyne* species tested (results not shown).

The efficiency of banana root DNA extraction was also tested by comparing two modified plant DNA extraction protocols published by Dellaporta et al. (1983) and Khayat et al. (2004). Protein and polysaccharide contamination was more abundant for DNA samples extracted using the former protocol. The main difference between the two protocols is the presence of cetyl trimethylammonium bromide (CTAB) in the extraction buffer and a phenol extraction for protein removal in the latter protocol. CTAB is a surfactant that binds the polysaccharides present in the solution under high salt condition (Cold Spring Harbour Protocols 2009) and resulted in higher A_{260}/A_{230} ratios of the NP samples. The CTAB-based extraction method proved to be more efficient for DNA extracted from tissues containing high level of PCR inhibitors including banana roots (Rowhani et al. 1993; Thomson and Dietzgen 1995).

PCR reaction volume also played a crucial role in the detection assay. This was evident from the results obtained for NP DNA samples extracted using the modified Khayat *et al*. protocol, in which successful amplification was observed only in a 50 µl PCR reaction volume. This result also supported the notion that the modified Dellaporta protocol had not produced DNA of sufficient purity, as amplification failed for both 25 µl and 50 µl PCR reaction volumes.

Although the A_{260}/A_{280} ratios obtained in all samples extracted using the modified Khayat et al. (2004) protocol were within the range of 1.8 to 2.0, the A_{260}/A_{230} ratios for all samples were still largely below 2.0. The presence of humic acid was hypothesized to contribute to the low A_{260}/A_{230} ratios acquired from soil samples (Wilson 1997; Yeates et al. 1998). Therefore, the PCR mixture was diluted twice to circumvent PCR inhibition. This step exploited the PCR sensitivity by reducing the concentration of inhibitors relative to target DNA (Wilson 1997).

A tenfold lower detection limit was obtained for NP samples compared to NPD samples. This reduced sensitivity indicated the presence of PCR inhibitors in banana root tissue. Additionally, the signal obtained from positive control DNA of nematode infected tomato roots (TR) demonstrated that *M. incognita* could also be detected by the established PCR assay in infected tomato roots.

Finally, we tested the applicability and the efficiency of this assay by designing a targeted inoculation experiment emulating a field inoculation procedure of a single primary root published by De Schutter et al. (2001). By using this assay, we were able to detect nematode presence in the inoculated root fragments (IP samples) from as early as 2 dai. This was indeed well in advance of the first visual indications of infection (root galling). As such, the outcome of this experiment proved that the detection assay could serve as a molecular tool to diagnose early nematode infection in the root system at stages where there is absolutely no visual indication of infection. Moreover, the nematode signal was also detected in additional,

independent TR tomato samples demonstrating the reliability of this *in planta* PCR-based nematode detection assay (data not shown).

In conclusion, we have developed and optimised an *in planta* PCR-based assay for early detection of *Meloidogyne incognita* in the root system. We first showed that the designed primers were functional and amplified the targeted ITS1 region. The assay proved to be sensitive as the nematode specific PCR signal was visible from 10 J2 individuals onwards mixed with 50 mg fresh weight banana root tissue. Additionally, nematode infestation in the roots could be detected as early as 2 days after J2 inoculation. This assay is currently being employed in our lab as an early detection tool for our studies on nematode-plant root interactions. Here, we established a detection assay that could serve as the basis of development of future nematode detection techniques such as quantitative nematode detection by real-time PCR and field nematode survey.

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References

Atkins, S. D., Clark, I. M., Penny, S.-P., Hirsch, R., & Kerry, B. R. (2005). The use of real-time PCR and species-specific primers for the identification and monitoring of *Paecilomyces lilacinus*. *FEMS Microbiology Ecology, 51*, 257–264.

Brody, J. R., & Kern, S. E. (2004). History and principles of conductive media for standard DNA electrophoresis. *Analytical Biochemistry, 333*, 1–13.

Cold Spring Harbour Protocols. (2009). Cetyltrimethyl Ammonium Bromide (CTAB) DNA Miniprep for Plant DNA Isolation. doi:10.1101/pdb.prot5177. Retrieved from http://cshprotocols.cshlp.org/cgi/content/abstract/2009/3/pdb.prot5177.

Davide, R. G., & Marasigan, L. Q. (1985). Yield loss assessment and evaluation of resistance of banana cultivars to the nematodes *Radopholus similis* Thorne and *Meloidogyne incognita* Chitwood. *Philippine Agriculturist, 68*, 335–349.

De Schutter, B., Speijer, P. R., Dochez, C., Tenkouano, A., & De Waele, D. (2001). Evaluating host plant reaction of *Musa* germplasm to *Radopholus similis* by inoculation of single primary roots. *Nematropica, 31*, 295–299.

De Waele, D., & Davide, R. G. (1998). The root-knot nematodes of banana: *M. incognita* and *M. javanica*. INIBAP *Musa* Pest Fact sheet No. 3.

Dellaporta, S. L., Wood, J., & Hicks, J. B. (1983). A plant DNA minipreparation: Version II. *Plant Molecular Biology Reporter, 4*, 19–21.

Ding, L.-W., Sun, Q.-Y., Wang, Z.-Y., Sun, Y.-B., & Xu, Z.-F. (2008). Using silica particles to isolate total RNA from plant tissues recalcitrant to extraction in guanidine thiocyanate. *Analytical Biochemistry, 374*, 426–428.

Fallas, G. A., Hahn, M. L., Fargette, M., Burrows, P. R., & Sarah, J. L. (1996). Molecular and biochemical diversity among isolates of *Radopholus* spp. from different areas of the world. *Journal of Nematology, 28*, 422–430.

Frison, E. A., & Sharrock, S. (1998). The economic, social and nutritional importance of banana in the world. In C. Picq, E. Fouré, & E. A. Frison (Eds.), *Bananas and food security Proceedings of an international symposium held in Douala* (pp. 21–35). INIBAP: Cameroon. France.

Gheysen, G., & Jones, J. T. (2006). Molecular aspects of plant-nematode interactions. In R. N. Perry & M. Moens (Eds.), *Plant Nematology* (pp. 234–254). Wallingford: CABI Publishing.

Gowen, S. R., Quénéhervé, P., & Fogain, R. (2005). Nematode parasites of bananas and plantains. In M. Luc, R. A. Sikora, & J. Bridge (Eds.), *In Plant parasitic nematodes in subtropical and tropical agriculture* (pp. 611–643). Wallingford: CABI Publishing.

Hirsch, P. R., Mauchline, T. H., Mendum, T. A., & Kerry, B. R. (2000). Detection of the nematophagous fungus *Verticillium chlamydosporium* in nematode-infested plant roots using PCR. *Mycological Research, 104*, 435–439.

Hubschen, J., Kling, L., Ipach, U., Zinkernagel, V., Brown, D., & Neilson, R. (2004). Development and validation of species-specific primers that provide a molecular diagnostic for virus-vector longidorid nematodes and related species in German viticulture. *European Journal of Plant Pathology, 110*, 883–891.

Jones, D. R. (2000). *Diseases of banana, Abacá and Ensete*. Wallingford: CABI Publishing.

Khayat, E., Duvdevani, A., Lahav, E., & Balesteros, B. A. (2004). Somaclonal variation in banana (*Musa acuminata* cv. Grande Naine): genetic mechanism, frequency and application as a tool for clonal selection. In S. M. Jain & R. Swennen (Eds.), *Banana improvement: cellular, molecular biology and induced mutation* (pp. 97–109). Science Publishers Inc: Plymouth.

Lovic, B. R., Valadez, V. A., Martyn, R. D., & Miller, M. E. (1995). Detection and identification of *Monosporascus* spp with genus-specific PCR primers and nonradioactive hybridization probes. *Plant Disease, 79*, 1169–1175.

Madani, M., Subbotin, S. A., & Moens, M. (2005). Quantitative detection of the potato cyst nematode, *Globodera pallida*, and the beet cyst nematode, *Heterodera schachtii*, using Real-Time PCR with SYBR green I dye. *Molecular and Cellular Probes, 19*, 81–86.

McCartney, H. A., Foster, S. J., Fraaije, B. A., & Ward, E. (2003). Molecular diagnostics for fungal plant pathogens. *Pest Management Science, 59*, 129–124.

McCuiston, J. L., Hudson, L. C., Subbotin, S. A., Davis, E. L., & Warfield, Y. C. (2007). Conventional and PCR detection of *Aphelenchoides fragariae* in diverse ornamental host plant species. *Journal of Nematology, 39*, 343–355.

Powers, T. O., Mullin, P. G., Harris, T. S., Sutton, L. A., & Higgins, R. S. (2005). Incorporating molecular identification of *Meloidogyne* spp. into a large-scale regional nematode survey. *Journal of Nematology, 37*, 226–235.

Qiu, J. J., Westerdahl, B. B., Anderson, C., & Williamson, V. M. (2006). Sensitive PCR detection of *Meloidogyne arenaria,* M. incognita and M. javanica extracted from soil. *Journal of Nematology, 38*, 434–441.

Razak, A. R. (1994). Plant parasitic nematodes, a potential threat to commercial cultivation of banana in Malaysia. In R. V. Valmayor, R. G. Davide, J. M. Stanton, N. L. Treverrow, & V. N. Roa (Eds.), *Banana nematodes and weevil borers in Asia and the Pacific. Proceedings of a conference-workshop on nematodes and weevil borers affecting bananas in Asia and the Pacific, Serdang, Selangor, Malaysia* (pp. 34–45). Los Baños: INIBAP/ASPNET.

Rowhani, A., Chay, C., Golino, D. A., & Falk, B. (1993). Development of a polymerase chain reaction technique for the detection of grapevine fanleaf virus in grapevine tissue. *Phytophatology, 83*, 749–753.

Subbotin, S. A., & Moens, M. (2006). Molecular taxonomy and phylogeny. In R. N. Perry & M. Moens (Eds.), *Plant nematology* (pp. 33–58). Wallingford: CABI.

Sundelin, T., Collinge, D. B., & Lubeck, M. (2009). A cultivation independent, PCR-based protocol for the direct identification of plant pathogens in infected plant material. *European Journal of Plant Pathology, 123*, 473–476.

Thomson, D., & Dietzgen, R. G. (1995). Detection of DNA and RNA plant viruses by PCR and RT-PCR using a rapid virus release protocol without tissue homogenisation. *Journal of Virological Methods, 54*, 85–95.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research, 25*, 4876–4882.

Williamson, V. M., & Gleason, C. A. (2003). Plant–nematode interactions. *Current Opinion in Plant Biology, 6*, 327–333.

Williamson, V., Caswell-Chen, E., Westerdahl, B., Wu, F., & Caryl, G. (1997). A PCR assay to identify and distinguish single juveniles of *Meloidogyne hapla* and *M. chitwoodi*. *Journal of Nematology, 29*, 9–15.

Wilson, I. G. (1997). Miniriview: Inhibition and facilitation of nucleic acid amplification. *Applied and Environmental Microbiology, 63*, 3741–3751.

Yeates, C., Gillings, M. R., Davidson, A. D., Altavilla, N., & Veal, D. A. (1998). Methods for microbial DNA extraction from soil for PCR amplification. *Biological Procedures Online, 1*, 40–47.

Zijsltra, C., van Hoof, R., & Donkers-Venne, D. (2004). A PCR test to detect the cereal root-knot nematode *Meloidogyne naasi*. *European Journal of Plant Pathology, 110*, 855–860.

Zouhar, M., Marek, M., Douda, O., Mazáková, J., & Ryšánek, P. (2007). Conversion of sequence-characterized amplified region (SCAR) bands into high throughput DNA markers based on RAPD technique for detection of the stem nematode *Ditylenchus dipsaci* in crucial plant hosts. *Plant Soil and Environment, 53*, 97–104.