Detection of *Meloidogyne incognita* and *Pochonia chlamydosporia* by fluorogenic molecular probes*

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Fluorescent molecular probes were applied for detection of the plant parasitic nematode *Meloidogyne incognita* and the nematode-egg parasitic fungus *Pochonia chlamydosporia* var. *chlamydosporia*. A region in the *M. incognita* rDNA including ITS2 was selected for amplification and recognition with a real-time PCR assay, based on a combination of three specific motifs, each recognized by a specific fluorescent probe. Similarly, a Scorpion probe was designed for the RT-PCR quantification of *P. c. chlamydosporia*. For this purpose, the ITS-2 rDNA gene of the fungus was sequenced from a number of Italian isolates. A conserved region unique for *P. c. chlamydosporia* found in the ITS-2 rDNA gene was used. The probes allowed recognition of single juveniles of *M. incognita* and of the mycelium- or soil-extracted fungal DNA. The potentialities of the detection procedures are discussed.

Introduction

Molecular probes and diagnostic techniques are increasingly being applied for identification and detection of regulated pests, with significant improvements in speed and reliability of the diagnostic protocols, when compared with traditional visual or morphological methods (Finetti *et al*., 2000; Lévesque, 2001; Boonham *et al*., 2002, 2003; Mishra *et al*., 2003). Molecular detection based on self-fluorescent probes provides, among other features, superior reliability and fidelity after hybridization, coupled to easy laboratory procedures and sensitivity (Tyagi *et al*., 1998; Park *et al*., 2000; Thelwell *et al*., 2000). The fluorescent configurations of the probes remain thermodynamically favoured during successful hybridization. This state is preferred to a quenched self-hybridized configuration, which occurs when target complementary DNA is not recognized (Tyagi *et al*., 1998; Whitcombe *et al*., 1999; Thelwell *et al*., 2000). Due to the reliability and specificity of annealing, molecular probes have a broad application range in field studies, including the detection of pest-associated micro-organisms or of biological antagonists, in epidemiological as well as in host–parasite association studies (Ciancio *et al*., 2000; Schena *et al*., 2002b).

Plant-parasitic nematodes are routinely identified by visual inspection of infected tissues, microscopic examination of roots and determination of morphometric data from temporary or permanent mounts. Recent progress in nematode diagnostics has included serological and other molecular techniques (though self-fluorescent probes have not yet been used). Serological methods have been applied for identification of cyst nematodes and root-knot nematodes (Atkinson *et al*., 1988; Davies & Lander, 1992; Lawler *et al*., 1993; de Boer *et al*., 1996). Methods based on DNA analysis or fingerprinting were applied for identification of *Meloidogyne* spp. and other nematode species (Vrain *et al*., 1992; Harmey & Harmey, 1993; Petersen & Vrain, 1996; Georgi & Abbott, 1998). Methods based on PCR, using ITS2 and other DNA regions, also appeared reliable, allowing identification of cyst and root-knot nematodes (Clapp *et al*., 2000).

Similarly, detection of fungi has rapidly evolved in recent years from visual or culture-based procedures toward molecular and DNA-based identification (Bonde *et al*., 1993; Assigbetse *et al*., 1994; Zervakis *et al*., 2001; Nikolcheva *et al*., 2003). For soil fungi, detection of nematode-parasitic species in the plant rhizosphere has routinely relied on semi-selective media. The method is stringent enough to discriminate among soil species, allowing the culturing and density estimation of the fungus of interest through the counts of the colony-forming units (CFU). In spite of some disadvantages, i.e. the time required by propagules to form a visible growing colony and the need for traditional subculturing and later examination, CFU were extensively used to study plant and rhizosphere colonization by nematode-parasitic fungi like *Pochonia chlamydosporia* var. *chlamydosporia* (Kerry *et al*., 1993; de Leij *et al*., 1993; Bourne *et al*., 1994). In general, CFU counts appear of practical use in ecological study of such fungi, but they show partial selectivity, allowing identification at the species level only through subsequent subculturing of colonies. Molecular applications have improved the specificity of detection and quantitative estimation of *P. c. chlamydosporia* biomass in soil

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(Arora *et al*., 1996; Hirsh *et al*., 2000, 2001). A further step was the use of PCR-based methods using β-tubulin and other gene sequences, allowing in semi-controlled tests the quantitative detection and biomass estimation of *P. c. chlamydosporia* in soil (Mauchline *et al*., 2002).

In addition to previous applications of molecular probes, we have explored the potential of DNA-based fluorescent molecules (Scorpions and molecular beacons) when tailored for, and targeted towards, phytoparasitic nematodes and their associated antagonistic fungi. The root-knot nematode *Meloidogyne incognita* was selected as the target pest, due to its worldwide distribution, and the egg-parasitic fungus *P. c. chlamydosporia* was selected as target antagonist, due to its biocontrol potentials and widespread occurrence in soils (de Leij *et al*., 1993). We proceeded to the design and testing of species-specific molecular probes for both organisms, in a first attempt to develop methods and protocols suitable for detection, epidemiology and quantitative measurements at the isolate or population levels. First data on the application of these probes are now presented.

Materials and methods

Nematode population and fungal isolates

A population of *M. incognita* from Molfetta (IT) was maintained in pots in a glasshouse, with the original soil planted with cherry tomato. Isolates INEM-VC-13, INEM-VC-18 and INEM-VC-21 of *P. chlamydosporia* came from Italy and were maintained in 15% glycerol at −20 °C.

DNA extraction and sequencing

Total DNA was extracted from *P. chlamydosporia* mycelium according to Arora *et al*. (1996). Cultures of isolates were grown on corn meal agar (CMA, Oxoid) at 24–26 °C for 7 days. Mycelium was scraped from the agar surface and transferred to 1.5 mL microtubes with addition of 600 µL of extraction buffer (50 mM Tris pH 7.2, 50 mM EDTA, 3% SDS, 1% 2-mercaptoethanol). The tubes were then vortexed for 30 s and incubated at 65 °C for 1 h. DNA was purified by addition of 600 µL of phenol/chloroform/isoamyl alchol (25 : 24 : 1) and centrifuged at 12 000 *g* for 15 min. The aqueous phase (300 µL) was then removed, and 30 µL of 3 M Na acetate and 165 µL of isopropanol were added with subsequent mixing. DNA was recovered by centrifugation for 2 min at 12 000 *g*, and the pellet was washed with 70% ethanol, resuspended in 20 µL TE and stored at −20 °C.

DNA amplification and sequencing

Total DNA was extracted from isolates INEM-VC13, INEM-VC18 and INEM-VC 21 of *P. c. chlamydosporia*. An aliquot of 50 ng of genomic DNA was subjected to PCR in 50 µL of a reaction mixture containing 1X PCR buffer (Roche, Mannheim, DE), 3.0 mM $MgCl_2$, 200 µM of each dNTPs, 200 nM of each primer and 1.25 U of Taq DNA polymerase (Roche, Mannheim, DE). Cycling conditions were an initial denaturation step at 94 °C for 5 min, followed by 5 cycles at 94 °C, 45 °C and 72 °C for 30 s and 1 min, respectively, and 30 cycles at 94 °C, 50 °C and 72 °C for 30 s and 1 min, respectively. A final extension was carried out at 72 °C for 7 min.

Primer pairs were: NBRC 9249 U1 (5′-GAGGTGAAATTCTTGGATTTATTG-3′) and NBRC 9249 D (5′-CGCCGAAGCAACGGTTGTAAATGG- 3′); NBRC 9242 U2 (5′-GCT TGGGCTCCAGGGGGAGTATGGT-3′) and NBRC 9242 D; NBR29242 U3 (5′-GCTTGC GTTGATTACGTCCCTGCC-3′) and NBRC 9242 D; NBRC 9242 U4 (5′-GGAAACTCA CCAGGTCCAGACACA-3′) and NBRC 9242 D.

Primers were designed on the nucleotide sequence of strain NBRC 9249 of *P. c. chlamydosporia* (AB100362), to amplify fragments of 939, 741, 637 and 213 bp, respectively. The primer NBRC 9249 D was selected in the internal transcribed region 1 (ITS1), conserved among different strains of *P. c. chlamydosporia*, whereas primers NBRC 9242 U1, U2, U3 and U4 were designed in the 18S rRNA fragment. Amplified products were analysed by electrophoresis in 1.2% agarose gel and visualized by staining with a 10 mg mL^{-1} solution of ethidium bromide (Sambrook *et al*., 1989).

Amplicons from primers NBRC 9242 D/NBRC 9242 U1 were purified with QIAquick PCR purification Kit (QIAGEN), ligated in pDRIVE vector (Quiagen), following the manufacturer's instructions, and cloned in *Escherichia coli* DH5α (Sambrook *et al*., 1989). Selected plasmids were purified by the boiling method (Sambrook *et al*., 1989) and sequenced by automatic sequencing (MWG, DE). Multiple alignments of nucleotide sequences were performed using the default options of CLUSTAL X 1.8 (Thompson *et al*., 1997), a Windows interface for the CLUSTAL W multiple sequence alignment programme (Thompson *et al*., 1994).

Fungus probe construction

A ribosomal gene fragment amplified and sequenced from extracted DNA of isolate INEM-VC-21 was used for *P. c. chlamydosporia* detection. The fragment sequenced included the 18S gene and ITS-2 rDNA region. For detection, a fragment specific for *P. c. chlamydosporia* in the ITS-2 was selected through BLAST analysis (Altschul *et al*., 1997). Sequences of the 18S and ITS-2 rDNA genes available in GenBank for the fungi closest to *P. c. chlamydosporia* INEM-VC-21 shown by BLAST analysis were used for alignment with the Pileup utility of the program GCG (Fig. 1). Sequences resulting from BLAST query included the following fungi: *Nomurea rileyi* (AB100361), *Verticillium suchlasporium* var. *catenatum* (AB113353), *Paecilomyces carneus* (AB103379) and *P. chlamydosporia* var. *catenulata* (AJ292398 and AJ292399). Other *P. c. chlamydosporia* sequences were also included in the alignment, in order to identify regions specific for *P. chlamydosporia* only, and suitable for the construction of probes and primers. A Scorpion primer for a region specific for *P. c. chlamydosporia* ITS2 was then designed and applied for RT-PCR detection with

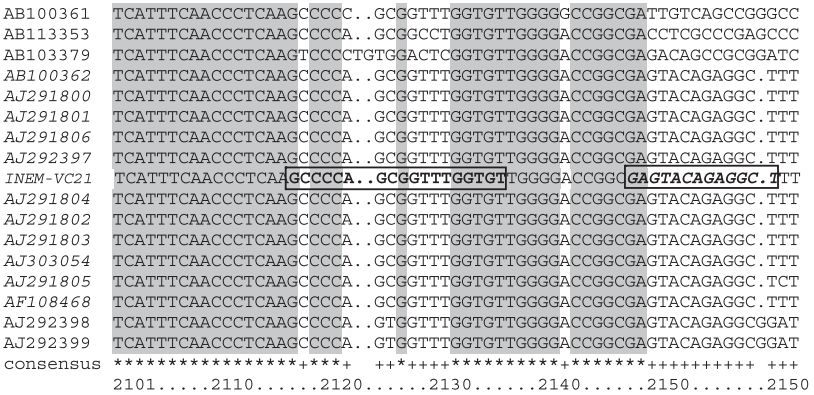

Fig. 1 Alignment of the ITS-2 rDNA sequences deposited in GenBank for the closest species to the sequence obtained from *Pochonia chlamydosporia* var. *chlamydosporia* INEM-VC21 rDNA (italic), as shown by BLAST analysis. Analysis includes sequences from other isolates of *P. c. chlamydosporia* (italic) and from *Nomurea rileyi* (AB100361), *Verticillium suchlasporium* var. *catenatum* (AB113353), *Paecilomyces carneus* (AB103379) and *P. chlamydosporia* var. *catenulata* (AJ292398 and AJ292399). In marked rectangles the primer (bold) and the target (bold, italic) regions used by the Scorpion probe for detection of INEM-VC-21.

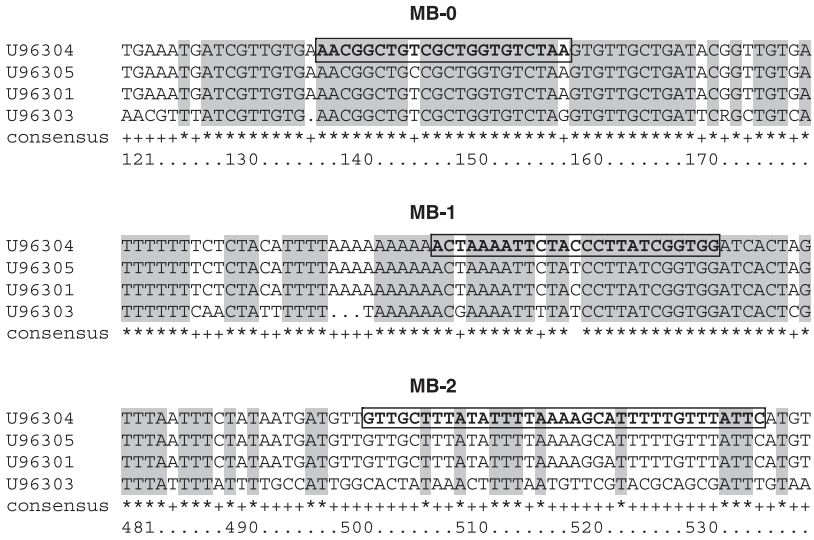

Fig. 2 Regions (bold) recognized in the rDNA of the nematode *Meloidogyne incognita* by the molecular beacons MB-0, MB-1 and MB-2 used for detection. The probes recognize three regions (in rectangles), whose combination is specific for *M. incognita*. They are characterized by nucleotide polymorphism in the alignment of the ITS-1, 5.8S and ITS-2 rDNA of four nematode species. The GenBank sequences used are: U96304 for *M. incognita*, U96301 for *M. arenaria*, U96305 for *M. javanica* and U96303 for *M. hapla*. Shaded nucleotides show conserved regions (asterisks); symbol ' +' shows nucleotide similarity.

an iCycler. The Scorpion molecule included a fluorescein group (FAM) attached to its 5′ end, followed by the stem-loop probe sequence, the methyl red (MR) quencher and the hexylene glycol (HEG) stopper, separated by a short spacer sequence from the primer.

Probe: 5′ FAM-ccgcggAGCCTCTGTACTCccgcgg-MR-HEG-nnnnnnnnnn-GCCCCAGCGGTTTGGTGT-3′.

Reverse primer: 5′-CTGATGCGAGGTTGTGCT-3′.

For probe position see Fig. 2.

Nematode probe construction

A set of three molecular beacons (MB-0, MB-1 and MB-2) was designed for the identification of single juveniles proceeding from the *M. incognita* population. The probes, whose combination was specific for *M. incognita*, were constructed on three different regions found in the alignment of the ITS-2 rDNA regions of sequences available in GenBank (Fig. 2). The alignment was constructed as previously described, using four sequences obtained from *M. incognita* (U96304), *M. javanica* (U96305), *M. arenaria* (U96301) and *M. hapla* (U96303). For each probe, forward and reverse primers were also selected, in order to amplify regions no longer that 200 bp:

Probe MB-0: forward 5′-GTGATGTTCAAATTTGAATTCG-3′; reverse 5′-GAGTCCTAACATGTCACCACATA-3′.

Probe MB-1: forward 5′-GAGCCTCTTAAGTGAGGC-3′; reverse: 5′-TGCTGCGTTCTTCATC-3′.

Probe MB-2: forward 5′-AACGTCTGGTTCAGGGTC-3′; reverse 5′-TCGACTGAGTTCAGGTCAA-3′.

Each combination of primers and probe differentiated *M. incognita* from one or more *Meloidogyne* species, with highest stringency with all probes. The nucleotidic positions of the molecular beacons in U96304 are: MB-0 = 138–159; MB-1 = 297–321; MB-2 = 502–536 (Fig. 2).

RT-PCR detection

For PCR from pure fungal cultures or nematodes, a simple method was used for cell disruption, based on vortexing the sample with glass beads in presence of TE (10 mM Tris HCl, pH 8.0; 1 mM EDTA). For real-time detection of *P. c. chlamydosporia*, Scorpion amplification and fluorescence

readings were performed in 96-well microtiter plates, monitoring fluorescence in a spectrofluorometric thermal cycler (iCycler, Bio-Rad Thermal Cycler) for real-time data collection during the annealing/extension steps. Template DNA was extracted from mycelium, in amounts from 2 ng to 0.2 pg. For detection of the fungus, the reaction consisted of an initial denaturing step at 94 °C for 3 min followed by 40 cycles, each consisting of 30 s at 94 °C and 30 s at 50 °C. As controls, H_2O or 1 µL of crude mycelium and chlamydospore extracts released after disruption with glass beads were used.

For molecular-beacon detection of *M. incognita* juveniles, 1–15 specimens were hand-picked from the suspension and double-washed in sterile distilled water. The nematodes were disrupted with glass beads in 10 mM TRIS HCl (pH 8), and a 5-µL template was used for RT-PCR. Controls were H_2O or DNA extracted from the nematode *Xiphinema index* (data not shown). PCR was performed as previously described. The reaction consisted of an initial denaturing step at 94 °C for 3 min followed by 40 cycles, each consisting of 30 s at 94 °C and 30 s at 48–58 °C. Molecular-beacon reactions were run in the presence of the fluorescent probes and 4 mM $MgCl_2$. Monitoring of the probes and display of data were performed through the device-dedicated software.

PCR from artificially inoculated soil

Sandy soil, previously found free from the fungus, was artificially inoculated with *P. c. chlamydosporia*, and 1 g was suspended in 2 mL of sterile distilled water, mixed and allowed to stand for 15 s. The supernatant was then collected and centrifuged at 14 000 rev min^{-1} for 30 min at 4 °C. The pellet containing the chlamydospores was treated with 200 µL of extraction buffer (Na_2HPO_4 0.12 M; NaCl 1.5 M and 2% CTAB), adding 200 mg of glass beads, homogenized for 5 min and then centrifuged for 2 min at 3000 rev min^{-1}. The DNA containing supernatant was phenol-chloroform extracted, vortexed for 3 min and centrifuged for 5 min at maximum speed. Nucleic acids were precipitated adding 1/10 vol of 3 M sodium acetate (pH 5.2) and 0.7 volume of isopropanol, mixed and left to stand for 1 h on ice. After incubation, the DNA from the suspension was collected by centrifugation for 15 min at 14 000 rev min^{-1}. The DNA-containing pellet was washed with 500 µL of 70% ethanol, dried and suspended in 50 µL of sterile distilled water. The DNA obtained was then purified through a chromatographic column (sepharose) and used for amplification and Scorpion detection as previously described. DNA extracted from *P. c. chlamydosporia* was used at different concentrations as positive control. For the pure fungal culture, 1 µL of chlamydospore suspension (corresponding to a total number of 49 chlamydospores) was used with 10^{-2} serial dilutions. Total DNA recovered from soil (2.5 ng μL^{-1}) was used to the same dilution.

Results and discussion

Real-time Scorpion PCR showed reliable amplification and fluorescence readings from the *P. c. chlamydosporia* DNA extracted from the mycelium (Fig. 3). Detection was possible even with a 2 pg initial template from crude mycelium or chlamydospore extract released after disruption with glass beads. No signal was observed from control sterile distilled water. Fluorescence readings were also observed when DNA

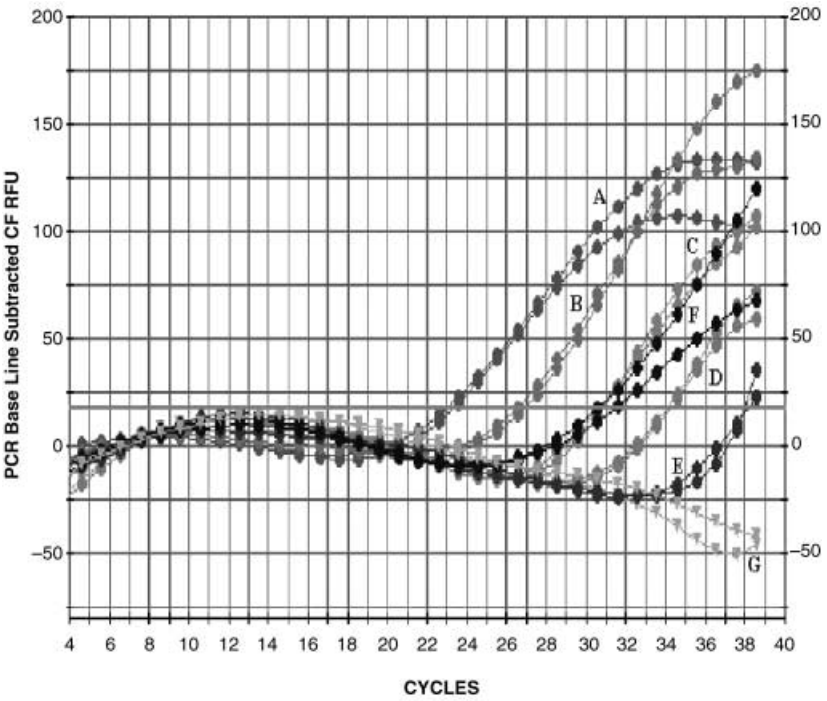

Fig. 3 Real-time Scorpion PCR amplification and fluorescence readings of DNA extracted from mycelium of *Pochonia chlamydosporia* var. *chlamydosporia*. Sample DNA templates in the vials were: A = 2 ng; B = 200 pg; C = 20 pg; D = 2 pg; E = 0.2 pg; F = 1 µL from crude mycelium and chlamydospores extracts released after disruption with glass beads; G = control H_2O.

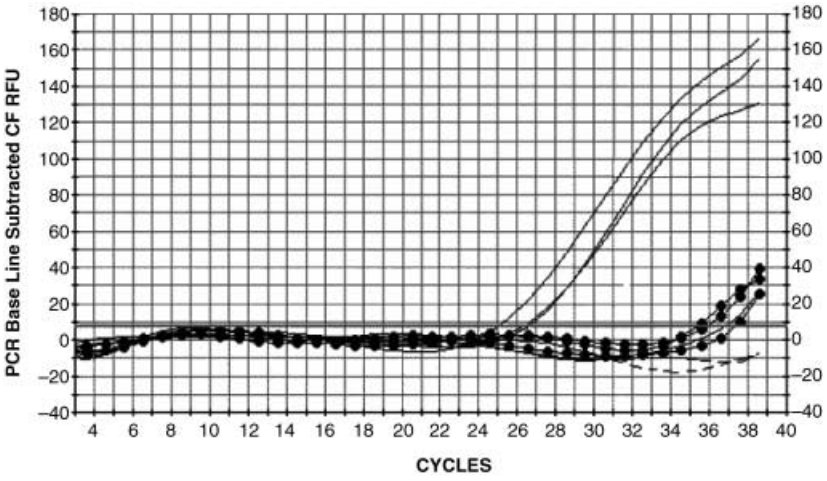

Fig. 4 Detection of *Pochonia chlamydosporia* var. *chlamydosporia* from artificially inoculated soil as shown by the Scorpion florescence readings scored during amplification, beginning at cycle 35 (squared lines). Positive controls were *P. c. chlamydosporia* mycelium extracted DNA (continuous lines); negative controls were soil without added chlamydospores (dotted lines).

was extracted from soil inoculated with chlamydospores. However, an increase in the threshold cycle was observed for the amplification and detection from soil (Fig. 4), indicating either a lower amount of DNA in the template or a reduced efficiency or higher inhibition in the amplification and detection steps (Fig. 4).

Detection from *M. incognita* juveniles showed that the molecular beacons recognized all the three corresponding rDNA regions specific for this species. The intensities scored for the fluorescent emissions appeared inversely related to the length of the region of annealing (Fig. 5). No signal was recorded from sterile distilled water or from *Xiphinema index* DNA (data not shown).

The Scorpion probe allowed direct recognition of *P. c. chlamydosporia*, thanks to the specificity of the nucleotide reaction and the unique occurrence of the targeted sequence in the fungus genome. Similarly, due to the specificity of the probe reaction and the unique combination of single nucleotide variations targeted, our method allowed *M. incognita* to be discriminated from most other common *Meloidogyne* spp., through the combined application of all the three molecular beacons.

The data suggests that all the probes tested may be useful, alone or in combination, in soil and plant rhizosphere detection of their corresponding targets. When considering the complex of nematode microbial antagonists and their interactions occurring in soil, density measurements of biological control agents in studies on epidemiology or population dynamics are often biased by the indigenous populations of the soil. In general, there is a need for molecular tools which target micro-organisms in the environment after their intentional or accidental release. Molecular detection has the potential to discriminate between resident microbial populations and cells or propagules derived from micro-organisms intentionally released for biocontrol purposes. The possibility of fungus detection at the population or isolate level largely depends on the information stored in the genome and, in particular, on the occurrence of nucleotide polymorphisms suitable for discrimination. For this purpose, a sufficient amount of DNA data must be made available at the species or isolate levels.

The method for DNA extraction from soil herein applied is suitable for small-volume samplings, allowing the possibility to process several replicates in order to infer data from a larger volume of the plant rhizosphere. In order to increase sensitivity and to reduce the reaction C_t, the assay must be coupled with a nested PCR. Additional work is required to determine the lowest detection levels under natural conditions, as well as the role of soil inhibitors and the flexibility of the combined use of probes, when detecting all organisms interacting in the soil microcosm.

Finally, this is the first application of molecular beacons in the direct recognition of a plant-parasitic nematode. Although the extent of such specific recognition needs further testing, considering the number of *Meloidogyne* species and their evolutionary distances, the diagnostic procedure proved reliable even when recognition was attempted from a single juvenile specimen. The method may hence represent the basis for the development of further molecular tools, useful in nematode diagnostics as well as in the study of plant parasitism and pest management.

Acknowledgements

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Détection de *Meloidogyne incognita* et *Pochonia chlamydosporia* par sondes moléculaires fluorogéniques

Des sondes moléculaires fluorescentes ont été utilisées pour la détection du nématode parasite des plantes *Meloidogyne incognita* et du champignon parasite des œufs de nématodes *Pochonia chlamydosporia* var. *chlamydosporia*. Une région de

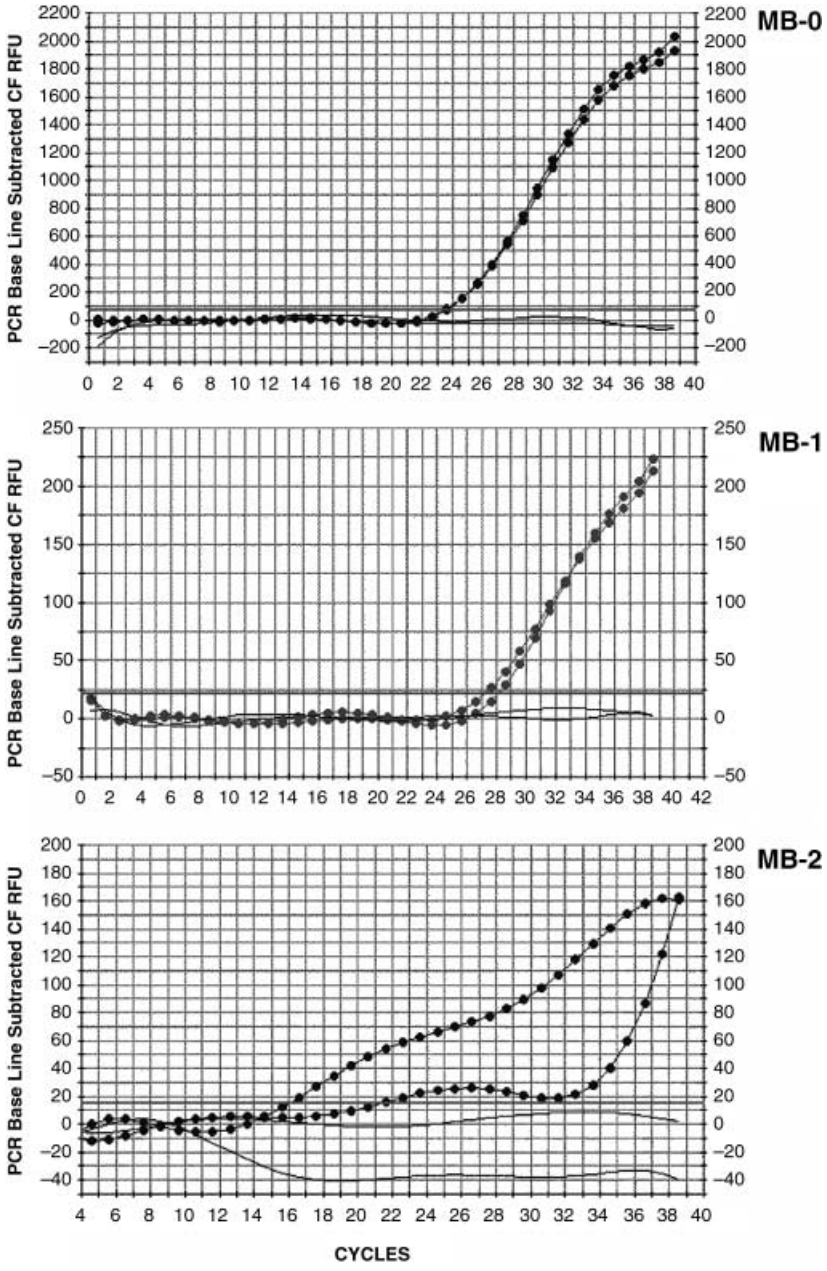

Fig. 5 Molecular-beacon detection from juveniles of *M. incognita*. Controls were H_2O (continuous lines) or DNA from the nematode *Xiphinema index* (data not shown). Fluorescent signals recorded during amplification from all probes show that molecular beacons reacted with all complementary targets.

l'ADNr de *M. incognita* comprenant un ITS2 a été sélectionnée pour être amplifiée et reconnue par une analyse PCR en temps réel basée sur une combinaison de 3 motifs spécifiques, chacun étant reconnu par une sonde fluorescente spécifique. De même, une seconde sonde Scorpion a été élaborée pour la quantification en temps réel par RT-PCR de *P. c. chlamydosporia*. Dans ce but, le gène ITS-2 de l'ADNr du champignon a été séquencé pour un certain nombre d'isolats italiens. Une région unique conservée pour *P. c. chlamydosporia* trouvée dans le gène ITS-2 de l'ADNr a été utilisée. Les sondes permettent la reconnaissance de juvéniles isolés de *M. incognita* et de l'ADN du champignon extrait du mycélium ou du sol. Le potentiel de ces procédures de détection est discuté.

Выявление *Meloidogyne incognita* и *Pochonia chlamydosporia* с помощью флюорогенных молекулярных зондов

Флюоресцентные молекулярные зонды применялись для выявления паразитирующей на растениях нематоды *Meloidogyne incognita* и паразитирующем на яйцах нематод грибе *Pochonia chlamydosporia* var. *chlamydosporia*. Участок рДНК *M. incognita*, включающий ITS2, был отобран для амплификации и распознавания с помощью ПЦР в реальном времени, основанном на сочетании трех специфических рисунков, каждый из которых

распознавался специфическим флюоресцентным зондом. Аналогичным образом был разработан зонд Scorpion для определения количества *P. c. chlamydosporia* с помощью ПЦР в реальном времени. С этой целью ген ITS-2 рДНК гриба был секвенсирован из нескольких итальянских изолятов. Был использован сохраненный участок, уникальный для *P. c. chlamydosporia*, обнаруженный в гене ITS-2 рДНК. Зонды давали распознавание единичных ювенильных особей *M. incognita* и мицелия или извлеченной из почвы грибной ДНК. В статье рассматриваются потенциальные возможности процедур выявления.

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