APPLICATION OF MOLECULAR METHODS FOR DETECTION OF *POCHONIA CHLAMYDOSPORIA* FROM SOIL

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ABSTRACT

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Two extraction and detection procedures were compared for isolation of the nematode parasitic fungus, *Pochonia chlamydosporia*, from soil. The extraction methods compared were a traditional, chloroform-based DNA extraction protocol, and the use of nucleic acids captured by magnetic beads. Sensitivity and specificity tests were developed using DNA extracted from a sandy soil treated with a *P. chlamydosporia* isolate VC21, or from cultures of the same isolate used as a control. The fungus DNA was detected through molecular hybridization (dot-blot) or Real-time PCR. Two probes, of 160 and 197 nucleotides respectively, were synthesized for dot-blot. They recognized an internal region of a serine protease (VCP1) gene, or a fragment of the mitochondrial rRNA gene (NMS). For Real-time PCR a molecular beacon specific to a 23 bp fragment within the VCP1 gene was used. The conventional extraction did not yield DNA pure enough to allow PCR, but permitted its identification through the dot-blot analysis. This protocol allowed the contemporary test of several samples with a detection limit of 500 ng of total DNA from soil. The use of magnetic beads in the DNA extraction from soil simplified the procedure allowing the collection of amounts of high quality DNA, ready for use in Real Time PCR in minutes.

Key words: fungi, nematode, parasitism, *Pochonia chlamydosporia*, probes, rhizosphere, soil.

RESUMEN

Rosso, L. C., Ciancio, A. y Finetti-Sialer, M. 2007. Aplicación de técnicas moleculares para la detección de *Pochonia chlamydosporia* en suelo. Nematropica 37:1-8.

Se compararon dos metodologías de extracción y detección de ADN del hongo *Pochonia chlamydosporia*, parásito de nematodos, a partir de suelo. Dichas metodologías consistieron en un protocolo tradicional de extracción con cloroformo y otro basado en la purificación de ADN por esferas magnéticas. Se desarrollaron ensayos de sensibilidad y especificidad con ADN extraído de un suelo arenoso inoculado con *P. chlamydosporia* o extraído de cultivos del mismo hongo usados como control. La detección del ADN del hongo se llevó a cabo mediante hibridación de los ácidos nucleicos (dot blot) o reacción en cadena de la polimerasa en tiempo real (Real-time PCR). Se utilizaron dos sondas de 160 y 297 nucleótidos para el dot-blot. Dichas sondas reconocieron una región interna del gen de la serín proteasa (VCP1) o un fragmento del ARNr mitocondrial (NMS). En la Real-time PCR se utilizó un indicador (beacon) molecular complementario a un fragmento de 23 pb del gen de la VCP1. Con la extracción convencional no se obtuvo ADN de suficiente pureza para permitir amplificación por PCR, pero permitió su identificación con el dot-blot. Este procedimiento permitió el análisis simultáneo de varias muestras a partir de 500 ng de ADN de suelo. La extracción de ADN del suelo mediante esferas magnéticas, más sencilla y rápida que el método con cloroformo, produjo ADN de pureza suficiente para su amplificación por PCR cuantitativa en tiempo real.

Palabras clave: hongos, nematodos, parasitismo, *Pochonia chlamydosporia*, rizósfera, sondas, suelo.

INTRODUCTION

The fungus *Pochonia chlamydosporia* (Goddard) Zare and Gams is a parasite of nematode eggs, actively studied for application as a biological control agent of important phytoparasitic species. This fungus is a common component of microbial soil communities and may play a key role in nematode regulation in the plant rhizosphere (Kerry *et al.*, 1993; De Leij *et al.*, 1993; Viaene and Abawi, 2000). The potential exploitation of *P. chlamydosporia* requires the elucidation of several ecological factors, including host specificity and trophic preferences (Hirsh *et al.*, 2001; Morton *et al.*, 2003).

Detection of microbial antagonists in the environment is an important step in the development of biological control agents suitable for practical or commercial applications. In the plant rhizosphere, nematode parasitic fungi may be detected and studied through traditional as well as molecular methods (Atkins *et al.*, 2005; Hirsh *et al.*, 2000; Mauchline *et al.*, 2002). Some protocols, already available to detect and quantify plant pathogens through the use of molecular techniques, may also be applied to nematode biocontrol agents (Gao *et al.*, 2004; Shurko *et al.*, 2004; Zhang *et al.*, 2006). Molecular methods have proven to be sensitive enough for identification and quantification of *P. chlamydosporia* and other nematode endoparasitic fungi at the species level (Atkins *et al.*, 2005; Zhang *et al.*, 2006), and include Real-time PCR (RT-PCR) or other similar protocols based on specific probes, mainly applied to DNA from plant roots or pure fungal cultures (Hirsh *et al.*, 2000; Ciancio *et al.*, 2005; Peteira *et al.*, 2005; Zhu *et al.*, 2006). Microbial estimation by DNA detection methods in soil is a complex task. This is due to the abundance of compounds from soil or of plant origin which affect PCR amplification as they precipitate together with nucleic acids, or inhibit the enzymatic reaction (Barthelet *et al.*, 1996; Cheryl *et al.*, 1998; Niemi *et al.*, 2001; Filion *et al.*, 2003). The high sensitivity displayed by some detection protocols when using DNA with low amounts of inhibitors (i.e., extracted from pure cultures), was not observed when using DNA extracted from roots or plant rhizosphere field soil (Ciancio *et al.*, 2005). PCR-based protocols, however, were successfully applied to quantify *P. chlamydosporia* and other nematode endoparasites in soil (Mauchline *et al.*, 2002; Ciancio *et al.*, 2005; Zhang *et al.*, 2006). Hence, for practical exploitation of PCR-based protocols in the detection of nematode antagonists like *P. chlamydosporia* in soil, it is useful to simplify the detection procedures with special attention to the nucleic acid extraction and purification steps.

Among DNA hybridization methods, dot-blot analysis is a fast and simple procedure often applied in clinical diagnostics or for detection of plant pathogens, i.e. viruses, fungi or nematodes (Astruc *et al.*, 1996; Schurko *et al.*, 2004; Uehara *et al.*, 1999). Dot–blot analysis consists of the serial deposition of droplets of sample solutions with the DNA of interest on a nylon membrane. The single strand DNA is anchored to the membrane and then hybridized with single-strand DNA probes labelled with digoxygenine. The heteroduplex is revealed by autoradiography of the reaction signal produced by an enzyme and linked through an anti-digoxygenin antibody to the DNA probe and its own substrate. This simple protocol allows the analysis of a large number of samples and may provide a first indication of the amounts of DNA present in the samples, based on the spot's intensity and protocol sensitivity.

In this paper we describe results concerning the detection of *P. chlamydosporia* in soil. We assayed two methods for DNA extraction from soil, a traditional chloroform-based and

an innovative procedure based on the use of DNA-binding magnetic beads. The DNA extracted was then used in two different molecular detection procedures, dot-blot hybridization and Real time PCR.

MATERIALS AND METHODS

Sample Preparation

Sterilized sandy soil originating from Ragusa, Italy and stored in 2 liter pots, was inoculated adding 1 g of commercial product corresponding to 5×10^6 spores/g of *P. chlamydosporia* var. *chlamydosporia* isolate INEM VC-021 originating from soil, collected at Metaponto, Italy. This amount corresponds to the dose used in *P. chlamydosporia* treatments, which exceeded the natural density of fungus. The same soil left untreated was used as a control. Mycelium and spores of the same isolate growing in Petri dishes were used for DNA extraction from pure cultures. Prior to nucleic acid extraction, 50 g of fungus inoculated and non-inoculated soil samples were suspended in 100 ml of sterile distilled water (DW) and kept under constant agitation for 1 h, to break soil aggregates. The soil suspension was first passed through a 150 µm aperture sieve and then through a 5 µm sieve. The remaining material on the latter sieve was transferred onto filter paper and dried at room temperature for 2 days and used immediately.

Isolate INEM VC-021 was cultured in Petri dishes on corn meal agar (CMA) for two weeks at 24°C. The mycelium was then collected from the surface by scraping with a sterile spatula and frozen at -20°C in vials until processed.

Nucleic Acid Extraction

For DNA chloroform extraction, 500 mg of the dried soil filtrate were used. Cell disruption was performed by adding glass beads (456 µm diam, Sigma-Aldrich) and keeping the vials on a vortex for 5 min in the presence of 1 ml of the extraction buffer (Na_2HPO_4, 0.12 M; NaCl, 1.5 M; CTAB, 2%). Vials were then centrifuged at 4°C for 20 min at 12.10^3 rpm (13.4 rcf) to separate the debris. Nucleic acids were purified from the supernatant with a volume of chloroform, centrifuged at 12.10^3 rpm (13.4 rcf) for 10 min and then precipitated with isopropanol in the presence of 1:10 sodium acetate (3 M) for one h at 4°C. The pellet, collected after a 15 min centrifugation at 13.10^3 rpm (15.7 rcf), was washed with 70% ethanol, dried and then suspended in nuclease free DW. The same protocol was applied for DNA extraction from 0.1 mg of *P. chlamydosporia* mycelia.

DNA extraction with magnetic beads (E.Z.N.A Mag-Bind Kit Omega Bio-tek, GA, USA), was carried out following the manufacturers' instructions. Cells were disrupted as previously described, using glass beads in 400 µl of extraction buffer (SP1, Omega Bio-tek) with 2 µl (10 mg/ml) RNAse. The vials were then incubated at 65°C for 10 min with subsequent addition of extraction buffer (SP2, Omega Bio-tek), and then centrifuged 10 min at 12.10^3 rpm (13.4 rcf). An amount of 10 µl of a magnetic bead suspension in presence of binding buffer (MGB) was then added to the supernatant for each vial. After 5 min incubation at room temperature, the vials were placed on the magnet separator device for 20 min. After discarding the cleared supernatant, the vials with the magnetic beads were removed from the support, resuspending the beads in 500 µl of washing buffer (SPM, Omega Bio-tek). This operation was repeated twice. The DNA was then released from the magnetic beads by adding 50 µl of elution buffer and stored at 4°C until used.

The DNA amounts obtained with the different procedures were measured through absorbance readings at 260 nm, using a

spectrophotometer (DU 800 Beckman Coulter, USA). The purity index was calculated following the relation of absorbance at 260/280 and 260/230 nm, indicative of the presence of proteins and polyphenols, respectively (Manchester, 1995).

Molecular Detection of P. chlamydosporia

For dot-blot hybridization, chemiluminescent probes produced by PCR using the PCR Dig probe synthesis kit (Roche) were used. Two probes, a 197 nt region of the mitochondrial gene coding for an RNA of the small ribosomal subunit (NMS, GenBank accession number AY556047), and a 160 nt region of an alcaline serinprotease coding gene (VCP1, GenBank AJ427460), were used. For probes amplification and synthesis, the DNA from the cultured fungus was used, using the following primers: NMSf, 5'-cagtgaggaatcttggtcacg; NMSr, 5'-cttacgtattaccgcgactgct; For0, 5'-ctcgaggctgcccaac; Rev0, 5'-tgcatgcactaggctcgg.

Subsamples of 1.0, 0.5 and 0.1 µg of DNA extracted from soil, and of 1.0, 0.5, 0.3, 0.2 and 0.1 µg of DNA from pure cultures were used. Before hybridization each subsample was treated with a denaturing solution (NaOH, 0.5 N; NaCl, 1.5 M; 1:1 v/v). Five µl droplets of each subsample were then placed on a positively charged nylon membrane. DNA was permanently fixed to the membrane through UV exposure for 3 min. The pre-hybridization step was performed with 5 ml of Dig Easy Hyb (Boehringer Mannheim) at 50°C in a hybridization oven for 60 min. The digoxygenin labelled probe was denatured by boiling for 5 min and then placed on ice immediately. After removal of the pre-hybridization solution, 5 ml of Dig Easy Hyb (containing 1 µl of probe/ml of solution) were added and incubated overnight at 50°C. After incubation, the nylon membrane was washed twice at room temperature, immersed for 15 min in agitation with the ×2 SSC (NaCl, 3M; Sodium citrate 0.3M; pH 7) solution; 0.1% (w/v) Sodium dodecylsulfate (SDS), and later washed twice for 15 min in ×1 SSC solution, 0.1% (w/v) SDS at 65°C.

For the immunodetection of the hybridization reaction, the membrane was incubated for 5 min under gentle shaking, in a washing solution (maleic acid, 0.1 M; NaCl, 0.15 M; pH 7.5 and 0.3% (v/v) Tween 20). It was then incubated 1 h in a blocking solution (Boehringer Mannheim) with gentle shaking at room temperature. Anti-digoxygenin antibody (1:20000) was then added to the membrane, coupled with alcaline phosphatase in blocking solution and incubated for1 h at room temperature under gentle shaking. Finally, the membrane was washed twice with the washing solution for 15 min and for 3 min in a neutralizing solution (Tris-HCl, 0.1 M; NaCl, 0.1M; pH 9.5).

To reveal the reaction, the membrane was maintained in contact with a radiographic film, which was then developed after exposure for either 3 or 16 h. The intensity of the hybridization signal was registered and quantified using a Gel Doc (Bio-Rad Laboratories, CA, USA) with dedicated Quantity One software.

Nested Real-time PCR

The sensitivity of the nested real-time assay was determined by extracting DNA from 200 mg of the dried soil filtrate with a known concentration of spores of *P. chlamydosporia* (5000, 1000, 100 and 10 spores/g, added before filtration) as previously described. The DNA from uninoculated soil was used as a negative control. Oligonucleotide primer pairs For1/Rev1 and For0/Rev0 were used in the first- and second-round PCR amplification reactions, respectively. Reactions were performed in

a Mx3000 (Stratagene, USA) in a final volume of 25 µl. Cycling conditions included a first step at 95°C for 4 min and a series of 40 cycles at 94°C for 30 sec, 54°C for 30 sec and 72°C for 20 sec. The first-round PCR mix contained 200 µM, dNTPs; 1× PCR buffer; primers 0.5 µM and 1 u of Taq polymerase, Roche and 2µl soil DNA. In the second-round real-time PCRs the probe MB-VCP1, a molecular beacon previously used for *P. chlamydosporia* detection (Ciancio *et al.*, 2005) was used to allow fluorescence readings during amplification The reaction mix contained 200 µM, dNTPs; 1× PCR buffer; primers, 0.5 µM; $MgCl_2$ 4 mM; 1 u of Taq polymerase, Roche; 0.3 µM probe and 1 µl of first round PCR.

RESULTS AND DISCUSSION

The extraction protocols described showed differences in DNA yield and purity (Table 1). Both methods produced DNA of low purity, as shown by the ratios of the 260/280 and 260/230 nm readings, which never reached 1.8, considered the optimal value for nucleic acids (Manchester, 1995). Chloroform extraction yielded more DNA than magnetic beads, with higher 260/280 and lower 260/230 ratios, indicative of a higher incidence of polyphenolic compounds and humic acids present in solution (Manchester, 1995). Treated samples extracted with magnetic beads yielded lower amounts of DNA than controls which, however, allowed PCR amplification, whereas the DNA obtained from the chloroform extraction could not be amplified. The low 260/280 nm ratio observed for the DNA obtained with magnetic beads was indicative of the presence of proteins, which did not affect the amplification protocol.

The probes NMS and VCP1 synthesized for the dot-blot detection of *P. chlamydosporia* showed different levels of sensitivity (Fig. 1). Probe NMS was able to detect up to 100 ng of DNA extracted from the fungus culture, after 3 hrs of membrane exposure, whereas the hybridization signal of VCP1 was observed only after 16 hrs (Fig. 1). The hybridization specificity assays detected *P. chlamydosporia* starting from 500 ng of soil DNA extracted by the chloroform protocol (Fig. 2). The occurrence of very low intensity dark spots in the untreated samples could indicate a possible residue of fungal DNA from dead indigenous hyphae.

Although more sensitive, the NMS probe appeared less specific. The values of hybridization intensity obtained for this probe showed that it cannot discriminate the fungus in soil, since controls/treated intensity ratios were not significantly different from those of the untreated soil. Probe VCP1 was more specific, with significant differences in the intensity readings between inoculated and untreated soil (r^2 = 0.96). Sensitivity, however, became evident at 0.5 µg of initial DNA amounts (Fig. 3).

Table 1. Purity index and DNA obtained for each extraction protocol and soil sample.

DNA Extraction	Soil sample	260/280 nm ratio	260/230 nm ratio	DNA ($ng \cdot g^{-1}$)
Chloroformic	treated	1.22	1.34	875.8
	control	1.54	1.04	871.6
Magnetic beads	treated	1.08	1.38	358.3
	control	1.09	1.73	413.3

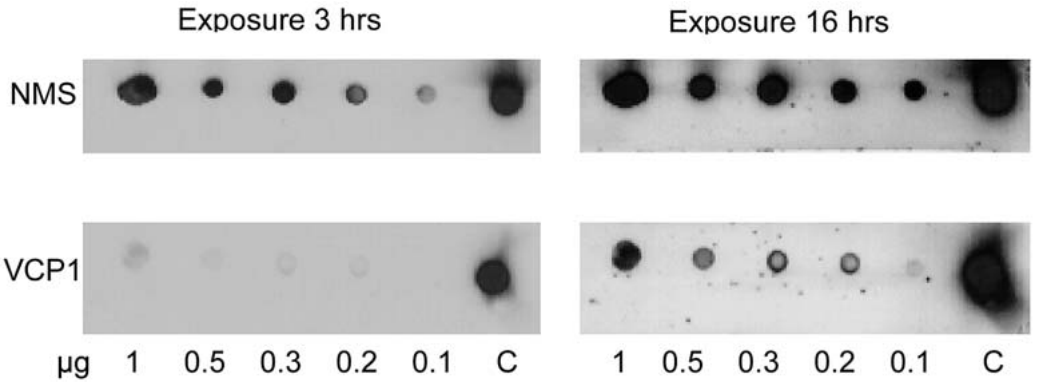

Fig. 1. Sensitivity of probes NMS and VCP1 in dot-blot detection, at two different exposure times, of increasing DNA amounts chloroform-extracted from cultures of *P. chlamydosporia* (isolate INEM-VC21). C: internal control with 1:1000 diluted probe.

In the assays performed with nested RT-PCR, detection of *P. chlamydosporia* DNA extracted with magnetic beads was performed efficiently, with positive fluorescence readings appearing at cycles 11 for 5000 spores/g, 13 for 1000spores/g, 14 for 100 spores/g and 16 for 10 spores/g. Differences in the threshold cycles corresponding to the initial DNA amounts were, however, minimal (Fig. 4). DNA extracted from the untreated soil did not yield significant fluorescent signals. The sensitivity of the real-time PCR assay was equivalent to 10 spores/g of soil of *P. chlamydosporia*.

Magnetic beads appeared useful in the extraction and clearing of DNA from soil, yielding a template suitable for amplification, whereas the chloroform extraction procedure was unsuitable for soil DNA amplification, probably because of insufficient clearing of soil compounds inhibiting the PCR reaction. The purity of the nucleic acids extracted from soil is essential for microbial detection and quantification when

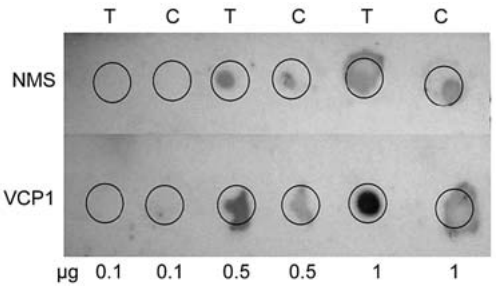

Fig. 2. Specificity of probes NMS and VCP1 in the dot-blot detection of *P. chlamydosporia* using increasing amounts of DNA extracted with chloroform, from treated (T) and untreated control soil (C).

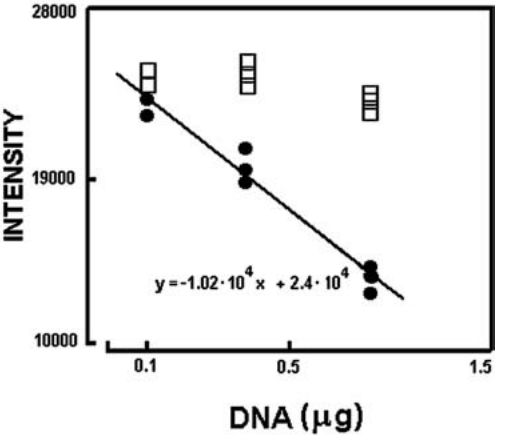

Fig. 3. Intensity of the DNA hybridization signal measured with Gel Doc instrument (Bio-Rad) for the VCP1 probe, in dot-blot detection, of treated (●) and untreated control soil (□).

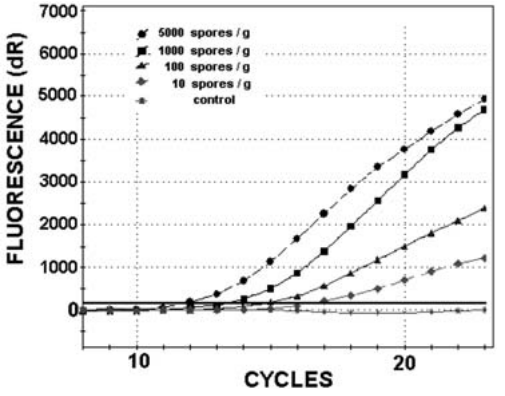

Fig. 4. Detection of *P. chlamydosporia* by nested RT-PCR of DNA extracted with magnetic beads. Fluorescence shown corresponds to different amounts of DNA from treated soil (5000, 1000, 100, 10 spores/g). DNA from untreated soil used as control shows no signal.

DNA amplification methods are applied (Jacobsen, 1995; Barthelet *et al.*, 1996; Cheryl *et al.*, 1998). In the presence of PCR inhibitors, the reaction does not proceed or could yield negligible amounts of undetectable amplification products, giving a false negative. The type of soil and its chemical composition appear as key parameters for soil PCR, and would provide information about the nature of possible inhibitors and lead to more appropriate methods for DNA extraction (Jacobsen, 1995).

The chloroform based extraction remains, however, a suitable method when less sensitive detection procedures are applied, as shown by the higher concentration of DNA yielded per sample and its use in dot-blot hybridization and detection. DNA extraction with magnetic beads from soil appeared as a simple and fast method allowing the production of nucleic acids of acceptable quality in a few minutes (Jacobsen, 1995). The lower DNA amounts obtained with this protocol may be related to the quantity of the DNA binding beads used in the assay, and not to the efficiency of the extraction method itself.

The large number of microbial species present in soil is still an obstacle when applying molecular procedures for quantitative detection. The low specificity of probe NMS suggests that, in spite of a constant upgrade of the genetic data available, a large fraction of the genetic information and diversity present in soil microbial communities is still unknown. The faint signal observed with probe VCP1 in the untreated soil (no fungus added), may be considered as background noise or aspecific binding. This signal should be subtracted when the method is applied for a rough quantification of the fungus density. This suggests the samples in which the DNA was previously eliminated (i.e., through enzymatic treatments or by γ irradiation) should be used as a control in field tests (Mauchline *et al.*, 2002).

Nested RT-PCR data confirm the specificity and suitability of molecular beacons for *P. chlamydosporia* detection in soil, but this method requires a good quality template DNA, as that produced by the magnetic beads. The dot-blot method, although technically demanding and less specific than nested RT-PCR, is also suitable for the contemporary analysis of multiple samples in a batch. The synthesis of non-radioactive probes from DNA produced through PCR is an alternative, low cost tool for *P. chlamydosporia* detection.

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