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ORIGINAL ARTICLE

Rapid and reliable DNA extraction and PCR fingerprinting methods to discriminate multiple biotypes of the nematophagous fungus *Pochonia chlamydosporia* isolated from plant rhizospheres

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Abstract

Aims: To develop a simple, rapid, reliable protocol producing consistent polymerase chain reaction (PCR) fingerprints of *Pochonia chlamydosporia* var. *chlamydosporia* biotypes for analysing different fungal isolates during co-infection of plants and nematodes.

Methods and Results: DNA extracted from different *P. chlamydosporia* biotypes was fingerprinted using enterobacterial repetitive intragenic consensus (ERIC)-PCR. Four extraction methods (rapid alkaline lysis; microLYSIS®-PLUS; DNeasy®; FTA® cards) gave consistent results within each protocol but these varied between protocols. Reproducible fingerprints were obtained only if DNA was extracted from fresh fungal cultures that were free of agar. Some DNA degradation occurred during storage, except with the FTA® cards, used with this fungus for the first time, which provide a method for long-term archiving. Rapid alkaline lysis and ERIC-PCR identified fungal isolates from root and nematode egg surfaces when plants were treated with different combinations of fungal biotypes; the dominant biotype isolated from the rhizosphere was not always the most abundant in eggs.

Conclusions: ERIC-PCR fingerprinting can reliably detect and identify different *P. chlamydosporia* biotypes. It is important to use fresh mycelium and the same DNA isolation method throughout each study.

Significance and Impact of the Study: This evaluation of methods to assess genetic diversity and identify specific *P. chlamydosporia* biotypes is relevant to other mycelial fungi.

Introduction

The fungus *Pochonia chlamydosporia* (syn. *Verticillium chlamydosporium*) (Goddard) Gams and Zare (2001) is a facultative parasite of eggs of sedentary endoparasitic nematodes such as cyst (*Heterodera* spp. and *Globodera* spp.), root-knot (*Meloidogyne* spp.) and false root-knot nematodes (*Nacobbus* spp.). A range of laboratory and field trials has confirmed its potential as a biological control agent (Atkins *et al.* 2003; Verdejo-Lucas *et al.* 2003). Analysis of genetic variation in the fungus at the subspecies level demonstrates that it is related to host nematode preference (Morton *et al.* 2003; Mauchline *et al.* 2004). These studies relied on single-step polymerase chain reaction (PCR) fingerprinting of DNA extracted from cultured isolates. Many approaches have been used successfully to provide PCR fingerprints of fungi using primers that recognize universal consensus, repeated or arbitrary sequences, including in fungi with biological control potential such as *Trichoderma* spp. (Arisan-Atac *et al.* 1995). The most appropriate primers depend on the fungal species under investigation (Bridge *et al.* 1998). Primers designed originally to amplify the enterobacterial repetitive intragenic consensus (ERIC) sequence

(Versalovic *et al.* 1991) have subsequently been shown to perform as arbitrary primers suitable for fingerprinting a range of bacteria and fungi despite not necessarily amplifying genuine ERIC sequences (Gillings and Holley 1997). Fungi where ERIC-PCR fingerprinting has proved useful for discriminating different isolates include *P. chlamydosporia* (Arora *et al.* 1996; Morton *et al.* 2003), *Leptosphaeria maculans* (Jedryczka *et al.* 1999) and *Pandora neoaphidis* (Tymon and Pell 2004). However, problems related to PCR inhibitors (e.g. agar and humic materials from soil), DNA extraction method, DNA degradation and PCR conditions can affect the resultant fingerprints, limiting their use, especially for large scale experiments involving the analysis of genetic variation in the fungus (Morton *et al.* 2003; Mauchline *et al.* 2004).

Rapid methods for PCR identification to the species level often use ribosomal gene targets and can detect very small amounts of fungal DNA (Griffin *et al.* 2002) which may have undergone some degradation. The ERIC-PCR amplicons in *P. chlamydosporia* that provide diagnostic fingerprints range from 100–1000 bp and rely on sequences homologous to the ERIC primers spread throughout the genome, and if the average fragment size in the genomic DNA substrate is too small, the number of bands will be reduced. Also, degraded DNA can give misleading results, for example through generation of chimeric PCR products that confuse profiles based on amplicon size (Pääbo *et al.* 1990). A reliable fungal DNA extraction protocol suitable for diagnostic PCR assays to identify fungi to species, subspecies or individual biotype level is essential for studying pathological and ecological interactions. There is also a need for relatively simple and quick methods of DNA extraction that do not rely on expensive kits or specialized equipment, such as physical disruption of cells followed by alkaline lysis to release genomic DNA (Klimyuk *et al.* 1993).

In this paper, three commercial kits are compared with alkaline lysis for their suitability to extract DNA from *P. chlamydosporia* isolates for ERIC-PCR fingerprinting. One of the commercial methods, using Whatman FTA® cards, claims to provide convenient long-term storage of DNA samples without significant degradation. The methods were validated in a study where competition between pairs of *P. chlamydosporia* isolates to infect root-knot nematode eggs on the roots of tomato plants was assessed. Four fungal isolates from a range of geographical locations with different host nematode preferences were tested in pairwise combinations to determine their competitiveness to colonize the rhizosphere and to infect nematode eggs. Understanding host nematode behaviour *in vivo* is an important factor when considering the biological control applications of *P. chlamydosporia*.

Materials and methods

Fungal culture

Isolates representing different biotypes of *P. chlamydosporia* var. *chlamydosporia* were selected from the Rothamsted Collection to represent a range of geographical origins and nematode host preferences [i.e. root knot nematode (RKN) or potato cyst nematode (PCN)]. These were isolates 10 (Brazil, RKN); 190 (Kenya, RKN); 280 (Jersey, PCN); 309 (Zimbabwe, RKN); 400 (Bulgaria, RKN); PcAm1 (Portugal, PCN, kindly provided by Clara Santos, University of Coimbra, Portugal) and *P. chlamydosporia* var. *catenulata* isolate 392 (Cuba, RKN). The fungus was routinely subcultured on Czapek Dox agar (Oxoid, Basingstoke, UK); all fungal colonies to be screened were transferred to corn meal agar (CMA, Oxoid) and incubated for 7 days at 28°C before transferring a small sample of mycelium to 5 ml Czapek Dox Broth (CDB, Oxoid) in a sterile plastic 30 ml capacity universal tube (Sterilin) using a sterile wire hook (a modified bacteriological loop). Samples were incubated with shaking (50 rev min^{-1}) at 28°C for up to 8 days, until the total yield of mycelium was approximately 100 mg (wet wt), when it was transferred into a 1·5 ml capacity microfuge tube. The amount of mycelium varied according to the growth rate of the biotype but this method produced sufficient material for DNA extraction from all isolates tested. The microfuge tubes were centrifuged at 2000 ***g*** at 4°C for 2–3 min before discarding the supernatant and washing the pelleted mycelium by re-suspending and centrifuging twice with 100 μl of sterile distilled water (sdH_2O). Routinely, the washed mycelium was kept on ice before extracting DNA using the methods described below but, to assess quality following storage some samples were freeze-dried overnight and stored for up to 2 months before DNA extraction or subsequent inoculation of CDB.

DNA extraction methods

All DNA extraction procedures were performed using aseptic techniques and subsequent PCRs were prepared in a separate laboratory to minimize the likelihood of cross-contamination. Three of the methods described below extracted DNA from 5 ml CDB cultures as described above. The fourth method, DNeasy®, required larger amounts of fungal material (see below).

Alkaline lysis

This method is based on a plant DNA extraction protocol described by Klimyuk *et al.* (1993). Fresh NaOH (40 μl

0·25 mol l^{-1}) was added to the fresh or freeze-dried samples of washed fungal mycelium from 5 ml CDB cultures, prepared as described above and homogenized (about 30 s) using a sterile plastic micro-pestle attached to an electric drill (Bosch PSR 7,2 VE; max speed 400 rev min^{-1}). Alternatively, for higher throughput, samples could be pulverized using acid washed sterilized glass beads (2–3 mm diameter; Jencons-PLS, East Grinstead, Sussex, UK) in a FastPrep® FP120 cell disruptor (Qbiogene, Cambridge, UK) for 20 s at 5 m s^{-1} and transferred to ice to cool the sample before repeating the step. Samples were immediately heated in a boiling water bath for 30 s, transferred to ice then neutralized and subjected to further lysis by the addition of 40 μl 0·25 mol l^{-1} HCl and 20 μl 10% Nonidet (Sigma, Poole, UK). Samples were boiled for a further 2 min, cooled on ice then divided into aliquots that were stored at either –20°C or –80°C.

MicroLYSIS®-PLUS

Fresh or freeze-dried samples of washed mycelium from 5 ml CDB cultures, prepared as described above, were transferred into a 2 ml capacity microfuge tube containing acid washed sterilized glass beads (2–3 mm dia, Jencons-PLS) then 40 μl of microLYSIS®-PLUS (Microzone Limited, Haywards Heath, UK) was added. Each sample was pulverized in a FastPrep® cell disruptor as above. Tubes were then centrifuged at low speed (2 min at 6500 ***g*** at 4°C) to precipitate the beads, cell debris and intact mycelium. The supernatant was transferred to PCR tubes (0·2 ml eight strip tubes with attached caps; Starlab (UK) Ltd, Milton Keynes, UK) and subjected to the manufacturer's recommended protocol in a thermal cycler (65°C 15 min, 96°C 2 min, 65°C 4 min, 96°C 1 min, 65°C 1 min, 96°C 30 s, 20°C hold) then stored as above.

FTA® Cards

FTA® cards are a cotton-based cellulose membrane containing lyophilized chemicals that lyse many types of cells and viruses, releasing DNA that binds to, and is protected by the matrix (Moscoso *et al.* 2005). Washed fresh mycelium from 5 ml CDB, prepared as described above, was spread over 25 mm dia circles on FTA® Classic cards (Whatman International Ltd, Maidstone, UK), crushed with a sterile pestle and prepared according to manufacturer's instructions. Samples were left to dry protected from direct light and stored for 2 and 6 months at room temperature, when a disk of 2 mm was removed from each circle and used in an ERIC-PCR.

DNeasy®

Fresh, washed samples of mycelium (1 g wet wt, harvested from 50 ml CDB cultures incubated 4 days at 28°C in 250 ml flasks) were transferred to a sterilized mortar and ground with a sterilized pestle in liquid nitrogen. DNA was extracted from the ground mycelium using DNeasy® Plant Maxi Kit spin columns (Qiagen Ltd, Crawley, W. Sussex, UK) according to the manufacturer's protocol.

PCR protocols

The alkaline lysis, microLYSIS®-PLUS and FTA methods did not involve a DNA purification step so it was not possible to assess quantity and quality of DNA yields, other than by performing PCR and comparing results. DNA concentrations for DNeasy® extraction were obtained by measuring absorbance at 260 nm with a spectrophotometer (NanoDrop ND-1000, Thermo-Fisher Scientific, Waltham, MA) and purity estimated from the A_{260}/A_{280} ratio. The absence of inhibitors at levels sufficient to interfere with PCR in samples was verified and the identity of the fungus confirmed using a species-specific β-tubulin gene PCR assay for *P. chlamydosporia* var. *chamydosporia* (Hirsch *et al.* 2000). Conditions for ERIC-PCR were adapted from Arora *et al.* (1996): reactions made up to 25 μl with sdH_2O contained 12·5 μl Red Taq (Sigma), 2·5 mmol l^{-1} $MgCl_2$ final concentration 1 μl each primer (50 pm μl^{-1} stock) and 3 μl DNA extracted by the alkaline lysis and microLYSIS®-PLUS methods or 1 μl DNA extracted using DNeasy® or a 2 mm disk from FTA® paper. The annealing temperature was reduced from 46°C to 42°C. PCR products were run on a 2% agarose gel (Helena Biosciences, Sunderland, UK) at 100 V for 90 min to give sufficient separation of bands to allow the discrimination of different profiles.

Detection of differences in the relative abundance of paired biotypes of *P. chlamydosporia* in the rhizosphere and in nematode eggs

Isolates 190, 280, 309 and 400 from the Rothamsted collection, representing four different biotypes, were added to peat-based potting compost at a rate of 5000 chlamydospores g^{-1} soil each, in all pairwise combinations, in 12·5 cm dia pots (four replicates for each treatment). A 14 days tomato seedling (cv. Tiny Tim) was planted in each pot and grown for a further 14 days before adding 2000 second stage juveniles of *Meloidogyne incognita* R2: 1135 (originally from North America) around the roots, as described previously (Mauchline *et al.* 2004). Eight weeks after addition of the infective nematodes, plants were harvested and 1 cm root segments and nematode egg masses were sampled for viable fungal propagules by plating dilutions of the root or egg washes on *Pochonia*-selective agar (four plates per treatment) as described previously (Mauchline *et al.* 2004). All colonies were transferred to CMA prior to inoculation of CDB for ERIC-PCR screening, using the alkaline lysis

method described above. The isolates had been chosen partly on the basis of their distinctive ERIC profiles, and each colony that was screened could be assigned to one of the two isolates used for the co-infection by visual comparison of bands.

Results

All methods extracted sufficient DNA from fresh mycelium for ERIC-PCR amplification (Fig. 1). Freeze-dried mycelium produced insufficient DNA for reproducible DNA fingerprinting profiles, but lyophilized isolates could be cultured by inoculating CDB. All DNA extraction methods from fresh mycelium gave the predicted PCR product of 270 bp with the β-tubulin primers and ERIC profiles with consistent band patterns that allowed identification of individual isolates from the competition experiment. However, the profiles were not always similar when the four methods were compared; the DNeasy® and alkaline lysis methods generated more and larger ERIC-PCR amplicons than the other methods tested. DNA stored on the FTA® cards, in contrast with the other methods, did not generate PCR amplicons larger than 1000 bp, indicating that DNA is preserved as relatively short fragments. DNA degradation occurred over time in samples kept at –20°C, especially after using alkaline lysis to extract the DNA. Nevertheless, all methods provided reproducible profiles from DNA stored for up to 6 months at –80°C and DNeasy® samples were reliable after one year. Samples from the FTA® cards, stored at ambient room temperature, produced more consistent ERIC profiles over time when compared to samples stored at –80°C obtained by using any of the other three methods of DNA extraction, where profiles were observed to lose bands. Although the longest storage period tested here was 6 m, the manufacturer claims that genomic DNA remains stable for >14 years on FTA® cards.

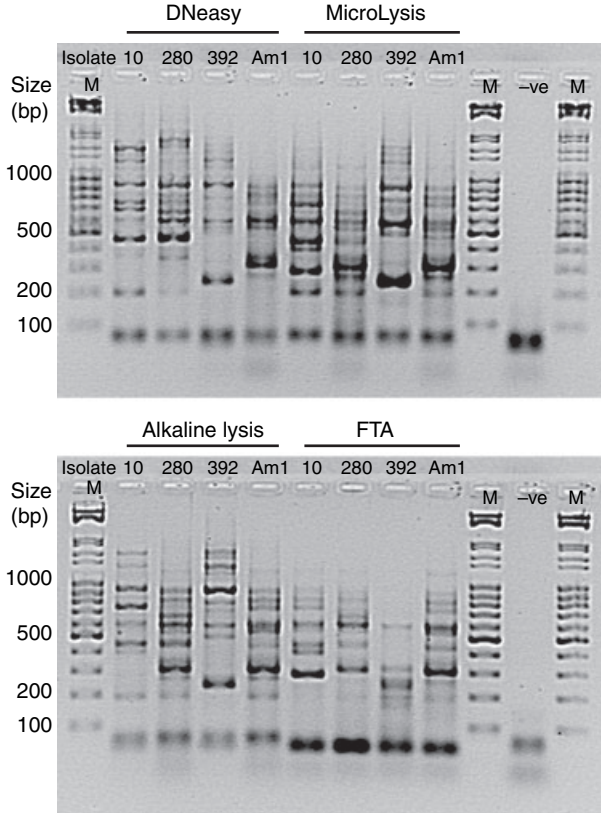

Figure 1 ERIC-PCR products amplified from *Pochonia chlamydosporia* var. *chlamydosporia* genomic DNA. M – size marker; Am1 – PcAm1; -ve – negative control.

The DNeasy® and alkaline lysis methods gave similar PCR profiles when screening fungal colonies from the pot experiment but alkaline lysis enabled many more to be screened (Fig. 2) and around 400 colonies in total were identified. Analysis of results (Fig. 3) indicates, for example, that isolate 309 was out-competed by 280 but dominated 190 on both roots and eggs whereas isolate 400 dominated 190 on roots but was out-competed on eggs. Isolate 280 out-competed all other isolates on roots but both 400 and 190 were found in some eggs.

Discussion

The alkaline lysis method was relatively quick, easy and cheap and provided good results if samples did not require long-term storage. The DNeasy® method gave higher quality DNA but the requirement of grinding mycelium in liquid nitrogen and costs of kits limited the number of colonies that could be screened. Although FTA® cards were easy and quick to use, and DNA survived well at room temperature, it appears that genomic DNA fragments are relatively short with this method, and may not generate sufficient amplicons for isolate identification by PCR fingerprinting. However, they could prove very useful in archiving fungal DNA samples to be screened using other PCR applications, for example to amplify single target sequences. If fungal DNA is needed for analyses many months or years after an experiment, another option is to revive freeze-dried mycelium by culturing in CDB prior to DNA extraction.

The observation that the four DNA extraction protocols generate different ERIC-PCR profiles is of concern. When screening fungal isolates, whether from a culture collection or co-infection experiment, care should be taken when comparing results obtained with different DNA extraction methods.

Results from the co-infection experiment provide a preliminary indication of the complexity of interactions

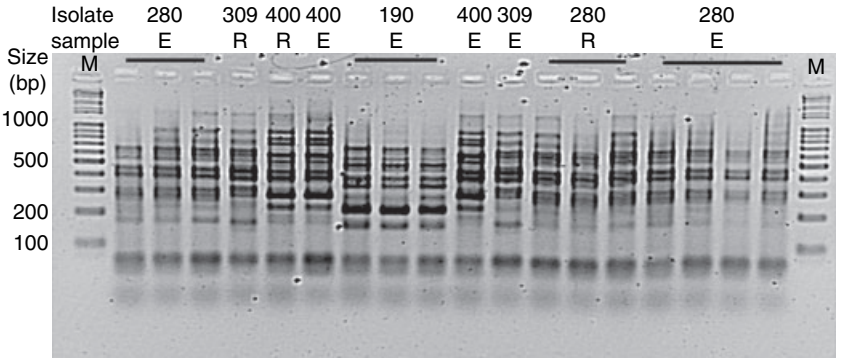

Figure 2 Example of ERIC-PCR screening of fungal colonies from competition experiment, DNA extracted by alkaline lysis: M, size marker ladder; fungal cultures from nematode eggs (E) or tomato roots (R) identified according to distinctive ERIC profiles. Arrows indicate diagnostic bands.

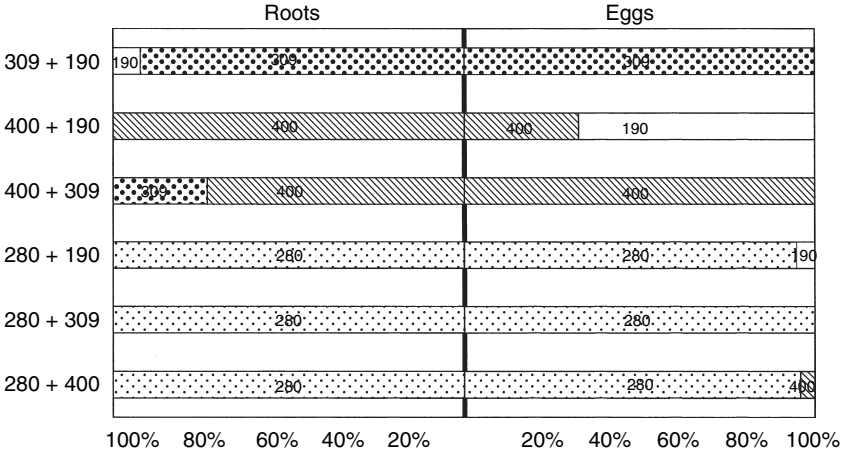

Figure 3 Analysis of tomato plant rhizospheres and nematode egg occupancy by fungal isolates inoculated in pairwise combinations, identified using ERIC-PCR.

between con-specific fungi and indicate that roots and eggs exert different degrees of selective pressure. Further research is needed on the interactions between different biotypes and isolates in the rhizosphere, for which the alkaline lysis method has been shown to provide a cost-effective, rapid and reliable method for screening large numbers of fungal colonies using ERIC-PCR. The results described are relevant to similar studies with other fungi, and using other PCR screening methods and emphasize the importance of using fresh mycelium and employing the same DNA extraction method throughout a study.

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