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The use of real-time PCR and species-specific primers for the identification and monitoring of *Paecilomyces lilacinus*

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Abstract

The *Paecilomyces lilacinus* is the most widely tested fungus for the control of root-knot and cyst nematodes. The fungus has also been implicated in a number of human and animal infections, difficulties in diagnosis often result in misdiagnosis or delays in identification leading to a delay in treatment. Here, we report the development of species-specific primers for the identification of *P. lilacinus* based on sequence information from the ITS gene, and their use in identifying *P. lilacinus* isolates, including clinical isolates of the fungus. The primer set generated a single PCR fragment of 130 bp in length that was specific to *P. lilacinus* and was also used to detect the presence of *P. lilacinus* from soil, roots and nematode eggs. Real-time PCR primers and a TaqMan® probe were also developed and provided quantitative data on the population size of the fungus in two field sites. PCR, bait and culture methods were combined to investigate the presence and abundance of the fungus from two field sites in the United Kingdom where potato cyst nematode populations were naturally declining, and results demonstrated the importance of using a combination of methods to investigate population size and activity of fungi.

Keywords: *Paecilomyces lilacinus*; PCR diagnostics; Species-specific primers; Potato cyst nematodes; Real-time PCR

1. Introduction

Paecilomyces lilacinus is the most widely tested fungus for the control of root-knot and cyst nematodes. Many authors have investigated its use in the field to control nematode populations [1–4] and it has been routinely isolated from infected nematode eggs in soils suppressive to plant parasitic nematodes [5–9]. At present, it is the only commercially available fungal formulation to control nematode pests, and the commercial strain 251 is registered for sale in a number of countries [[1]; www.biocontrol.co.za/pl; www.prophyta.com].

The fungus has also been isolated from clinical samples [10–14], not only from patients who are immuno-compromised and, therefore, at greater risk from opportunistic pathogens, but also from otherwise healthy patients. The diverse incidences and epidemiology of the infections by the fungus are varied and were reviewed by Okhravi et al. [1]. Morphological characteristics of the fungus are often overlooked by histopathologists leading to a misidentification and a delay in the correct form of treatment [13].

PCR has provided a rapid and powerful tool to amplify specific regions of DNA to identify organisms. Specific primers exist for a number of fungal species

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including those under investigation as potential biological control agents of plant parasitic nematodes [15–17]. Relatively simple and rapid kit-based DNA extraction methods have allowed DNA sufficiently pure for PCR amplifications to be extracted from a range of soil, plant and clinical samples, making the process both routine and consistent. A selective medium has also been developed for the isolation and quantification of *P. lilacinus* from soil and plant samples [18], requiring the correct identification of conidia and are less sensitive than other methods. The use of culturable methods for identification and estimates of fungal abundance also requires a level of expertise, and between 3 and 5 days incubation before colonies can be identified and are prone to contamination. DNA based PCR methods, although not providing information on the viability of the fungus do provide a rapid, accurate and sensitive method of detection.

Conventional PCR provides a direct method for identification of the fungus. When it is combined with other methods such as serial dilution plating on a selective medium and/or baiting soil with a host such as nematode eggs the technique can provide a diagnostic method for the abundance and biological control capability of the fungus [17]. Conventional PCR is unable to provide quantitative data on fungal populations, but the development of competitive and real-time PCR has enabled this important advance in molecular diagnostics. Although competitive PCR [19] is quantitative, it requires the construction of a competitor molecule in the reaction, optimisation, and serial dilution of each sample to derive quantification and has been superseded by real-time PCR. TaqMan® based real-time PCR relies on the combination of a primer set and an additional dual-labelled fluorogenic probe to allow continuous monitoring of amplicon synthesis during thermocycling and requires no post-PCR handling for target quantification [20]. It has been used to successfully determine the abundance of a number of fungal species [17,20–25] and to investigate their ecology in soil [17,23,25].

To determine the efficacy of a biological control agent it is important to develop tools to monitor and identify the specific isolate released into the environment and to re-isolate it from soil and its pest target [26]. Several authors [27–29] have investigated the use of arbitrary primers to develop DNA profiles for specific isolates, and to monitor cultures following application in the field. Here, the development and testing of specific PCR primers and a real-time PCR probe and primer set based on the ITS region of the fungal genome are reported to identify, and detect *P. lilacinus* from nematode, root and soil samples, to investigate the incidence of the fungus in two test soils from the UK, and to determine if the primers can detect both these and medical isolates of the fungus.

2. Materials and methods

2.1. Isolates and media

Fungal cultures were taken from the Rothamsted culture collection or from other sources stated in Tables 1 and 2. Isolates were stored at 4 °C on corn meal agar (CMA-Oxoid, Basingstoke, UK) plates until needed, then sub-cultured onto fresh corn meal agar and incubated at 28 °C. In the pot trials the *P. lilacinus* isolate used was determined by the nematode host. Where potato cyst nematodes (PCN-*Globodera pallida*) were added, isolate RES Pl1 was used, whereas where root knot nematodes (*Meloidogyne incognita*) were added, isolate 251 was used. For clinical isolates, DNA was extracted from pure cultures by Dr. Chris Linton (Mycology Reference Laboratory, Bristol, UK) and supplied for testing.

2.2. Optimisation of species-specific primers

Primer set (PaeF: 5′ CTC AGT TGC CTC GGC GGG AA 3′; PaeR: 5′ GTG CAA CTC AGA GAA GAA ATT CCG 3′) was designed from an ITS sequence submitted to the GenBank/EMBL database (Accession Number AF368804). Primers were compared to other sequences in the database through BLAST and FASTA searches to confirm specificity, and their design was optimised using the NetPrimer software (Biosoft International, www.premierbiosoft.com/netprimer.html). PCR reactions of 20 μl contained 10 μl 2 × RedTaq mega mix (Sigma, UK), 2 pmol of each primer and 20 ng template DNA. Optimised PCR conditions were as follows: 95 °C followed by 35 cycles of 94 °C for 1 min, 61 °C for 1 min, 72 °C for 1 min, with a final incubation at 72 °C for 5 min. PCR products were separated on 2% agarose gels.

2.3. Confirmation of P. lilacinus primer specificity using fungal isolates

DNA was extracted from the fungal cultures listed (Tables 1 and 2) using the method described by Klimyuk et al. [30]. DNA from cultures was added to the PCR reagents and the conditions followed as described above.

2.4. Detection of P. lilacinus in soil

DNA was extracted from two soils (Ely, Cambridgeshire and Spalding, Lincolnshire, UK) where it was reported that potato cyst nematode populations were naturally declining [31]. Triplicate DNA samples were extracted from 10 soil cores from each site using a soil DNA extraction kit (Mo-Bio Laboratories Inc, California, USA). All soil DNA extractions were diluted 1:10 before using in PCR reactions. DNA (20 ng) was added to a PCR as above with minor modifications, namely