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A N I N T E R N A T I O N A L J O U R N A L

A simplified DNA Extraction Method for PCR Analysis of *Camarotella* spp.

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ABSTRACT

This work aimed to optimize an efficient and simple protocol for DNA extraction of Camarotella species, an obligate plant pathogen that cause verrucosis or "lixa" on coconut tree and other palms, facilitating the molecular studies of these biotrophic microorganisms. The method proposed enabled a fast, reproducible and reliable DNA extraction from Camarotella species.

Key words: *Camarotella torrendiella, C. acrocomiae*, verrucosis on coconut, DNA, PCR

INTRODUCTION

The use of DNA molecular techniques has been largely applied for species characterization. However, to obtain good results, specific methodologies for certain groups of fungi are usually needed. Several methods of fungal DNA extraction have been already developed (Raeder and Broda, 1985; Fulton et al., 1995; Rogers and Bendich, 1985; Doyle and Doyle, 1987; Doyle and Doyle, 1990; Graham et al., 1994; Walsh et al., 1991). The main disadvantage of these methods is the need to cultivate the fungus to obtain the mycelium, making difficult the DNA extraction for obligate parasites. This work aimed to optimize an efficient and simple protocol for DNA extraction of *Camarotella* species, an obligate plant pathogen that cause verrucosis or "lixa" on coconut tree and other palms, facilitating the molecular studies of these biotrophic microorganisms. A two step PCR analysis was developed through which the product of the first reaction was reamplifyied in a second reaction. In this way, the sensitivity of the assay was improved.

MATERIALS AND METHODS

To confirm that the fungal DNA extraction was adequate for PCR analysis, DNA of eight isolates of *Camarotella torrendiella* (Bat.) Bezerra and Vitória (2008) and five of *Camarotella acrocomiae* (Mont.) K. D. Hyde and P. F. Cannon (1999) were tested.

The genomic DNA of *Camarotella* species was obtained from the hymenial layer of the ascomata. The upper part of the perithecium wall was cut and the ascogenous hymenium was removed with the aid of a fine needle and placed into a PCR tube which was stored at – 80ºC for later use (ranging from 12-24 h).

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A single ascogenous hymenium was transferred to a PCR tube containing 3 µl of the *Cell Lysis Buffer* (0.05M NaOH, 0.25% [w/v] SDS). Then, the solution was mixed in a vortex during 2 min at maximum speed and then incubated at 80ºC for 15 min to obtain the DNA. The sample was stored at -20ºC for later use.

Total PCR reaction volume was 50 µl with the following components and final concentrations: 2.5 mM dNTP (Gibco Life Sciences, Life Technologies Ltd, Paisley, UK), 0.2 pmol of each primer (Amersham Biosciences), 5 U of *Taq* enzyme (from *Thermus aquaticus*) (HT Biotechnology Ltd, Cambridge, UK) and manufacturer's buffer 1x and 1.5 mM of $MgCl_2$. The mix for the PCR reaction was placed directly in the tube containing 3 µl of templates.

Amplification of ITS-1, 5.8S rDNA, and ITS-2 was performed using primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3'). The 5' two-thirds of the 18S rDNA was selectively amplified using the primers NS1 (5'-GTAGTCATATGCTTGTCTC-3'), NS2 (5'-GGCTGCTGGCACCAGACTTGC-3'), NS3 (5'-GCAAGTCTGGTGCCAGCAGCC-3') and NS4 (5'-CTTCCGTCAA TTCCTTTAA-3'), (White et al., 1990). PCR reactions for synthesis of double-stranded DNA were carried out in a Mastercycler gradient Eppendorf with an initial denaturing step at 95°C for 2 min, followed by 40 cycles at 95°C for 30 s, 55°C for 30 s and 72°C for 2 min. The reaction was completed by a final stage at 72°C for 10 min. PCR products were visualized by electrophoresis in 1.2 % (w/v) agarose gel in the presence of ethidium bromide.

The product of the first reaction was reamplifyied in a second reaction. A second PCR reaction (50 µl) was carried out with 12.6 µl of the first amplified PCR product as DNA template, 2.5 mM dNTP (Gibco Life Sciences, Life Technologies Ltd, Paisley, UK), 0.2 pmol of each primer (Amersham Biosciences), 5 U of *Taq* enzyme (from *Thermus aquaticus*) (HT Biotechnology Ltd, Cambridge, UK) and manufacturer's buffer 1x and 1.5 mM of $MgCl_2$,.

RESULTS AND DISCUSSION

The time of storage of the hymenium used for DNA extraction influenced the DNA quality. Consequently, good DNA was obtained only with the hymenium stored for 12-24 h.

The amplified PCR products were not visible, probably due to insufficient DNA template. The preamplified product (12.6 µl) was then used in a second PCR amplification. This allowed the obtaining of a sufficient number of copies for visualization on agarose gel. In this case, the PCR amplification gave satisfactory reproducible fingerprinting (Figs. 1-2).

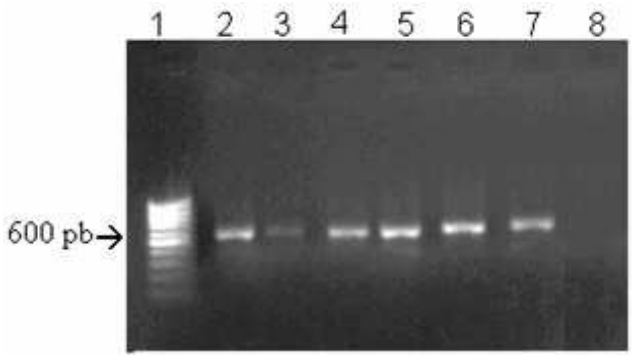

Figure 1 - *Camarotella* spp. isolates reamplified by PCR, using the primers pairs ITS5/ITS4. Line 1, ladder DNA 100 pb; line 2, *Camarotella*. *torrendiella* from *Syagrus botryophora*; line 3, *Camarotella torrendiella* from *Bactris* sp.; lines 4-5, *Camarotella torrendiella* from coconut; line 6, *Camarotella acrocomiae* from coconut; line 7, *Camarotella* cf. *torrendiella* from *Bactris ferruginea*; line 8, negative control.

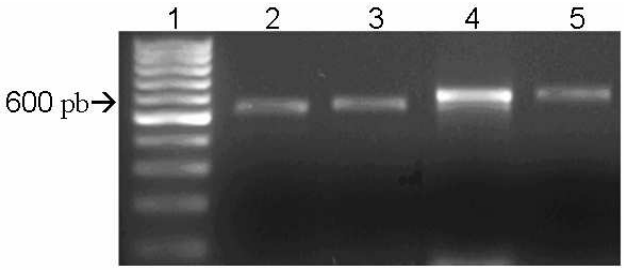

Figure 2 - *Camarotella* spp. isolates reamplified by PCR, using primers NS1/NS2 (Lines 2-3) and NS3/NS4 (Lines 4-5). Line 1, ladder DNA 100 pb; line 2, *Camarotella torrendiella* from coconut; line 3, *Camarotella acrocomiae* from coconut; line 4, *Camarotella torrendiella* from coconut; line 5, *Camarotella acrocomiae* from coconut.

The obtained DNA was appropriate for PCR analysis. The employed method eliminated the necessity of grinding with liquid nitrogen, centrifugation with chloroform, phenol:chloroform and Chelex, using only one *Cell Lysis Buffer* in order to break the wall of the asci and ascospores.

The DNA from cleistothecia of Erysiphales obtained from stromata grounded directly in the extraction buffer, containing phenol: chloroform, and 5% (w/v) Chelex has been reported as a suitable method for DNA extraction from fungal ascomata *in vivo* (Glenn et al., 1998; Saenz et al., 1999). However, this protocol was not appropriate for *Camarotella* spp. Recently, DNA extraction of *Sphaerodothi*s from hymenium withdrawn from perithecial stromata using chloroform has been reported (Silva et al., 2003).These two extractions protocols showed that they were labour intense, time-consuming (more than a day), and needed too much fruiting bodies, about 40-50 for each template, to obtain good quality DNA. Also, DNA quality, analyzed by measuring the 260:280 UV absorbance ratio, did not was reach quality standard (between 1.8 and 2.1). In the present protocol, few samples with extremely low DNA contents presented 260:280 UV absorbance ratio lower than 1.8 (data not shown).

The method proposed in this work enabled a fast and reliable DNA extraction from all *Camarotella* species studied. The present success in obtaining DNA from *Camarotella* spp. might allow the molecular studies which would greatly contribute to improve the taxonomy of the genus.

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RESUMO

A extração e amplificação de DNA são etapas fundamentais para a aplicação de métodos moleculares e para tal, a origem do material é relevante. As espécies do gênero *Camarotella* que causam as lixas do coqueiro e outras palmeiras são biotróficas e o crescimento em meio artificial é controverso, embora Oliveira et. al, (2004) tenham registrado seu cultivo *in vitro*, utilizando meio líquido completo. No entanto, não ficou provado que o micélio formado correspondia ao micélio de *Camarotella.* Em conseqüência das dificuldades com o cultivo dos agentes etiológicos das lixas, foi otimizado um protocolo para extração de DNA genômico a partir do himênio ascógeno *in natura* e um protocolo de PCR para sua amplificação. Nossos resultados são importantes para o estudo da família Phyllachoraceae, pois possibilitará a análise molecular que, nessa família, é limitada pela dificuldade de obtenção de DNA.

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