

A modified DNA extraction minipreparation protocol for *Fusarium* isolates

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Abstract

We have modified a quick, inexpensive and less prone to contamination protocol by culturing the cryopreserved or fresh mycelium directly in 96-deepwell plate. The method facilitates concomitant assessment of ssDNA fungal diversity by asymmetric PCR (A-PCR) from a single extraction. The DNA yields from *Fusarium* spp. isolates was reasonably high, and a clear DNA band was frequently seen when 10 µl of the PCR product was run in agarose gel. The procedure can be completed in less than 4 hours and 96 samples can be processed at the same time.

Key words: A-PCR, cryopreservation, 96-deepwell plate, *Fusarium*

Introduction

Nucleic acid detection methods such as PCR have become a common tool for fungal identification and diagnosis. Although PCR amplification can be performed directly for various microbial cultures, for filamentous fungi and yeasts prior isolation of DNA is often preferred. As the DNA extraction process eliminates many unknown interfering substances present in the biological material, it plays an important role in ensuring consistent test results. Toward this end, considerable efforts have been made to enable improved DNA preparation from fungi (CENIS 1992, LIU *et al.* 1997). Many of these methods rely on using

a grinder (with or without liquid nitrogen) for initial breaking up of the mycelia. Three widely cited methods (RAEDER and BORDA 1985, LEE and TAYLOR 1990, CENIS 1992) allow the extraction in Eppendorf tubes, simplicity and reducing the scale of the extraction. All methods start from lyophilized or fresh mycelium, ground by hand with a mortar and pestle. However, this protocol is cumbersome, and implies careful sterilization of the material. Protocols for extraction of DNA of fungal cells either are very time-consuming or show poor release of fungal DNA compared to methods of extraction of DNA from human cells (GABAL 1992). We developed a rapid,

low-cost, and reliable DNA extraction procedure for fungi which would not only reduce the workload considerably but also decrease the test turnaround time.

Materials and methods

Culture and harvesting of fungal mycelium

Eppendorf deepwell plate-96, 2.2 ml (Eppendorf) was filled with 550 µl of potato dextrose broth medium (Difco). The isolates were cultured by inoculating some hyphal threads for fresh mycelium or 50 µl skim milk for cryopreservation fungi and were allowed to grow for 2-3 days at 28 °C. The mycelium mats were pelleted by centrifugation for 15 min at 4.000 rpm in a deepwell swing-bucket rotor (microcentrifuge 5804 R; Eppendorf). The mycelium pellet was washed with 600 µl TE buffer and centrifuged again for 5 min at 4.000 rpm. Finally, the TE buffer was decanted.

DNA isolation

Four hundred µl extraction buffer was added. This buffer is the same as that described by Cenis (1992) (200mM Tris HCl pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS). The mycelium was crushed with a conical grinder (Roth), fitting exactly the tube and homogenized by hand or electric potter at 200 rpm for 1 min. Four µl RNase (20 mg/ml) was added to the cell suspension and incubated for 10 min at 65 °C with three times mixing. After that, 130 µl of 3 M sodium acetate, pH 5.2 was added, and

the 96-deepwell plate was incubated at – 02 °C for 10 min. The lysate was centrifuged at 4.000 rpm at 4 °C for 15 min and the supernatant was transferred to a clean 1.2 ml deepwell plate-96. DNA was precipitated by the addition of 650 µl room temperature isopropanol, pelleted at 4.000 rpm for 10 min and washed with ice cold 70% (v/v) ethanol to remove residual contaminants. DNA was resuspended in 100 µl of 1 x Tris-EDTA buffer, and 2 µl of the purified DNA was used in 50 µl of PCR mixture.

Asymmetric PCR

The polymerase chain reaction was performed with minor modifications from the original protocol (WHITE *et al.* 1990). Single-stranded DNA was prepared using primers in a ratio of 50:1 (GYLLENSTEN and ERLICH 1988). Primers used for amplification of the ITS region were ITS1 and ITS4. For each 50-µl reaction, a mixture was made containing 10 ng of genomic DNA, 10mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 0.2 mM of each deoxynucleotide triphosphate (Pharmacia Biotech), 20 pmol of primer ITS1, 0.4 pmol of primer ITS4 and 1 U of Taq DNA polymerase (Promega). Amplification was performed using an automated thermal cycler (Primus 69 HPL, MWG Biotech). The cycling parameters were initial denaturation at 94 °C for 2.5 min, and then 40 cycles of denaturation for 15 s at 94 °C, annealing at 56 °C for 30 s, and extension at 72 °C

for 1.5 min. The amplification was terminated by a final extension for 10 min at 72 °C. Ten µl of PCR product were analysed on a 1.4% agarose gel (80 V for 40min) containing 0.5 µg ml⁻¹ ethidium bromide.

Results and Discussion

The DNA isolation procedure described was found to be effective for all *Fusarium* spp. The average yield of DNA from *Fusarium* mycelium ranged from 4-6 µg. This is enough for at least 50 PCR reactions, as determined by titration of DNA concentration in several reactions. Amplification of ssDNA fragment in *Fusarium* spp. was assayed by asymmetric PCR (A-PCR) (Figure 1), 2 µl of the described fungal miniprep was used as DNA template. The variation of growth rate of different strains is somewhat compensated by the broad range of DNA concentrations allowed by PCR, producing similar amounts of amplified product with different species and strains. Direct addition of mycelium to the extraction buffer, without a separate step for mechanical cell disruption, is favourable when relatively small amounts of DNA are needed for analysis. DE-NIJS *et al.* (1996) found that the DNA extraction buffer was capable of releasing substantial quantities of DNA from the *Fusarium* mycelium (range 0.5-1.6 mg g⁻¹ mycelium), without a separate step for mechanical disruption of cell walls. Other protocols require additional lysis steps like sonification, liquid nitrogen or awkward toxic

chemicals such as phenol-chloroform (HAYNES *et al.* 1996) or guanidine thiocyanate (SANDHU *et al.* 1995). In conclusion, it is likely that this protocol could be applied to the examination of many other fungal species. It provides a rapid reliable and low-cost alternative to the existing DNA purification methods used in molecular plant pathology laboratories.



Fig. 1. PCR fragments produced by asymmetric PCR using ITS1/ITS4 primers. Lanes 1-4, ss DNA amplified from *Fusarium oxysporum* f. sp. *vasinfectum*. Lanes 5-6, *F. oxysporum*, Lanes 7-9, *F. moniliforme*, Lanes 10-12, *F. solani*, Lanes 13-16, *F. semitectum*. The molecular-weight markers represent 100 bp DNA ladder (Promega).

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