

A rapid method for isolation of total DNA from pathogenic filamentous plant fungi

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ABSTRACT. DNA isolation from some fungal organisms of agronomic importance is difficult because they have cell walls or capsules that are relatively unsusceptible to lysis. We have developed a fast DNA isolation protocol for *Fusarium oxysporum*, which causes fusarium wilt disease in more than 100 plant species, and for *Pyrenophaeta terrestris*, which causes pink root in onions. This protocol was based on the sodium dodecyl sulfate/phenol method, without β-mercaptoethanol and without maceration in liquid nitrogen; it uses phenol/chloroform extraction to remove proteins and co-precipitated polysaccharides. The $A_{260/280}$ absorbance ratios of isolated DNA were around 1.9, suggesting that the DNA fraction was pure and may be used for further analysis. Additionally, the $A_{260/230}$ values were higher than 1.8, suggesting negligible contamination by polysaccharides. The DNA isolated by this protocol is of sufficient quality for molecular applications; this technique could be applied to other organisms that have similar substances that hinder DNA extraction.

Key words: Genomic DNA extraction; *Fusarium oxysporum*; *Pyrenophaeta terrestris*; Polymerase chain reaction; Filamentous fungi

INTRODUCTION

In Mexico, onion (*Allium cepa*) holds fourth place in the production of vegetables and is cultivated nearly all over the country; moreover, the country is the principal exporter of this vegetable to the United States (Quintana-Sierra et al., 2005). In Baja California, Mexico, production has increased steadily from approximately 2689 ha in 2005 to 3674 ha in 2007 (SIACON, 2006). However, the presence of diseases caused by pathogenic agents can limit production in Baja California. Pink root disease and fusarium basal rot are two of the important diseases in onion, which are caused by *Pyrenopeziza terrestris* and *Fusarium oxysporum*, respectively. These fungi occur in most parts of the world and survive for many years in the soil (Nasr Esfahani and Ansari Pour, 2008). The isolation of pure DNA is crucial for the study of gene expression in these filamentous fungi, because it is a pre-requisite for several molecular biology techniques, including gene isolation by polymerase chain reaction (PCR), Southern blotting, and the construction of genomic DNA libraries.

However, DNA extraction from these filamentous fungi has been described as being rather complicated, because most of the available protocols include the growth of mycelium in liquid culture, followed by maceration in liquid nitrogen, and usually require additional lysis steps, such as mechanical disruption or sonication, enzymatic digestion or use of toxic chemicals (Al-Samarrai and Schmid, 2000; Alaey et al., 2005). Additionally, although some methods do not involve maceration in liquid nitrogen, they are still time consuming and require special columns (Noor Adila et al., 2007). A number of protocols have been established for fungal DNA. However, many of these protocols are apparently suitable for certain groups or morphological forms of fungi but may not be versatile and efficient for extracting nucleic acids from diverse groups of filamentous fungi (Raeder and Broda, 1985; Bolano et al., 2001). Therefore, the objective of this study was to develop an easy and rapid protocol for the isolation of good quality total DNA from filamentous fungi such as *F. oxysporum* and *P. terrestris* biomass.

MATERIAL AND METHODS

Fungal material

Fusarium oxysporum strain ICA-F1 and *P. terrestris* ICA-P1 were grown on potato dextrose agar (Baker®) on 9-cm diameter Petri dishes at 25°C until mycelium completely covered the agar surface. Mycelia were collected by adding sterile distilled water containing 0.05% (v/v) Tween 80 to the surface of the culture and gently scrubbing with a sterile spatula. The mycelial suspension was transferred to a 1.5-mL microtube and centrifuged at 3000 g, at 4°C for 5 min. The supernatant was discarded and the pellet (100 mg mycelia) was stored at -80°C until further use.

DNA extraction buffer and solutions

The tubes and bottles were treated with 0.1% diethyl pyrocarbonate (DEPC, Sigma, Cat. No. D-5758) solution at 37°C overnight, autoclaved twice at 121°C for 20 min, and then dried at 100°C before use. Tips used for DNA extraction were DNase-free and RNase-free (Axygen®, USA). The extraction buffer was 3% SDS (w/v) containing 0.5 mM EDTA, 1.0 M

NaCl, and 0.1 mM hydroxymethyl-hydrochloride (Tris-HCl, pH 8.0). Additionally, a mixture of chloroform/phenol (1:1, v/v) was also prepared.

DNA extraction procedure

Extraction buffer (0.2 mL) was added to 10 mg of each fungal mycelium and the suspension shaken vigorously for 15 s. Next, 0.2 mL chloroform-phenol mix was slowly added and incubated at 65°C for 5 min. The mixture was cooled to room temperature and centrifuged at 10,000 g, at 4°C for 5 min. The supernatant was transferred to a new microtube, and an equal volume of cold absolute isopropanol or ethanol was added and the contents mixed thoroughly for precipitating total DNA at -20°C for 20 min; the mixture was then centrifuged at 10,000 g for 10 min. The pellet was washed twice with 75% ethanol and centrifuged at 10,000 g, at 4°C for 5 min.

The supernatant was discarded and the pellet was resuspended in 0.03 mL DEPC-treated MiniQuantum (deionized) water, and stored at -80°C until further use. Concentration, yield, and quality control indices based on absorbance readings at 230, 260, and 280 nm ($A_{260/280}$ and $A_{260/230}$ ratios) were carried out with 2 μ L resuspended total DNA. Ten microliters of total DNA solution was loaded onto a 1% agarose gel, and electrophoresed to separate DNA.

Polymerase chain reaction

Specific DNA of each fungus was amplified by PCR with Taq DNA polymerase according to manufacturer instructions (Invitrogen, CA, USA). PCR analysis was performed according to the method described by González-Mendoza et al. (2008), and the fungal DNAs (2 μ L) were used as PCR template. To assess the suitability of isolated DNA, PCR was performed with equal amounts of DNA, using B-actin primers (5-TGTTCACCAACCACA GCAGAGCG-3 and 5-CACTGTCCGTCGGGTAACCTCG-3). PCR was carried out using the following protocol: 93°C for 3 min (1 cycle) and 50°C for 1 min and 72°C for 1 min (30 cycles). The PCR products were electrophoresed using a 1.5% (w/v) agarose gel, which was stained with EtBr and visualized under UV light.

RESULTS AND DISCUSSION

Since the currently available DNA extraction protocols are rather costly and time consuming (Sambrook and Russel, 2001), we adapted a rapid DNA isolation method from plants (González-Mendoza et al., 2008) combining chemical reagent digestion without mechanical shearing for lysing the hyphae of either *F. oxysporum* strain ICA-F1 or *P. terrestris* followed by DNA isolation.

The isopropanol and ethanol step allowed effective precipitation of DNA, rendering it more stable. In this study, we also found that grinding the biomass in liquid nitrogen produced poor results (data not shown), and therefore, this step was eliminated. Furthermore, high salt concentration (1.0 M NaCl) in the extraction buffer was introduced to avoid preventing or diminishing the dissolution of polysaccharides during the extraction step. In all cases, we obtained good yields of high-quality genomic DNA (Figure 1). In addition, the absorbance ratios $A_{260/280}$ and $A_{260/230}$ were determined to evaluate quantity, quality, and integrity of isolated DNA. The $A_{260/280}$ was 1.9, suggesting that the DNA fraction was pure and may be used for further analysis. In all samples, the $A_{260/230}$ values were higher than 1.8, suggesting negligible contamination by polysaccharides (Table 1).

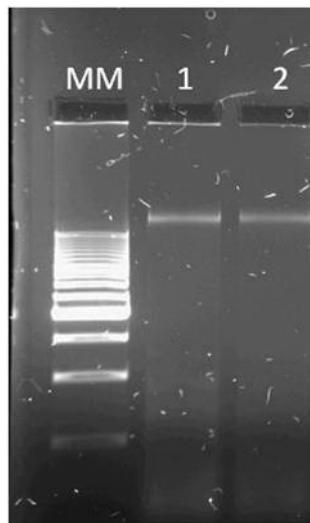


Figure 1. Gel electrophoresis of the total DNA extracted from filamentous fungus. MM = DNA marker (1 kb), lane 1 = *Fusarium oxysporum*, and lane 2 = *Pyrenopeziza terrestris*.

Table 1. Absorbance ratios and yield of total DNA isolated from fungus.

Fungus	A _{260/280}	A _{260/230}	Yield (μg/mg fresh weight)
<i>Fusarium oxysporum</i>	1.83 ± 0.06	1.96 ± 0.08	9.3 ± 0.07
<i>Pyrenopeziza terrestris</i>	1.87 ± 0.09	1.90 ± 0.5	8.6 ± 0.06

Data are reported as means ± SD (N = 3).

To assess the DNA's purity and its use for basic molecular analysis, PCR amplification of a fragment of the *Actin* gene present in both fungi was carried out. Amplification produced a single band of approximately 200 bp specific for the *Actin* gene (Figure 2).

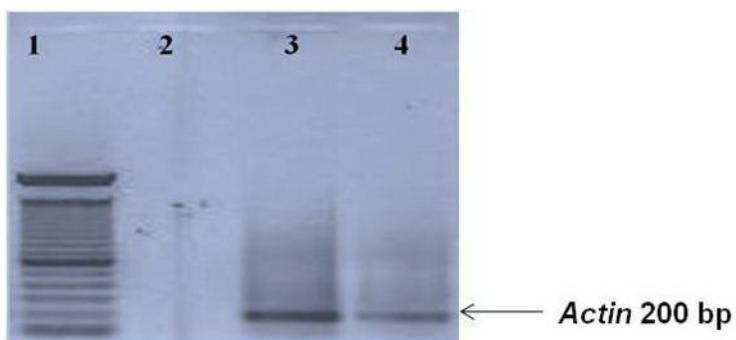


Figure 2. Agarose electrophoresis of the polymerase chain reaction products. Lane 1 = DNA marker; lane 2 = negative control; lanes 3 and 4 = *Actin* gene, 200 bp on *Fusarium oxysporum* and *Pyrenopeziza terrestris*, respectively.

In addition, one of the advantages of this procedure is that the omission of maceration reduces sample handling, minimizing the risk of contamination between samples. This is particularly important in work involving amplification by PCR. Additionally, this protocol provides a rapid, reliable, and low-cost alternative to the existing DNA purification protocols used in research and clinical laboratories (Liu et al., 2000; Pfaza et al., 2004). Therefore, the proposed protocol is an efficient (<2 h) and inexpensive procedure for the isolation of good-quality DNA from *F. oxysporum* strain ICA-F1 and *P. terrestris* strain ICA-P1 for molecular assays.

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