IMPROVED METHOD FOR DNA ISOLATION FROM DIFFERENT TYPES OF SOIL INFESTED WITH THREE FUNGAL GENERA

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ABSTRACT

In the current study, we describe a DNA isolation method that is based on an easy, quick polyvinylpolypyrrolidone-precipitation to release phytofungi from the soil, combined with lysozyme- RNase and -SDS lysis of the fungal population. DNA extracts were subjected to different techniques, including gel electrophoresis, restriction enzyme digestion, RAPD and ITS-PCR amplification. The proposed method yielded high-quality DNA, which was transparent, non-viscous and lacked visible contamination of RNA. Isolated DNA was efficiently digested with restriction enzymes. DNA extracted from soil was pure enough to be utilized at high concentrations for PCR amplifications. The extracted DNA was of high quality and allowed direct detection of specific genes by the polymerase chain reaction (PCR). The amplicon length of the fragment ITS4/ITS5, ranged in size from 550 to 680 bp. A polymerase chain reaction method used to detect soil-borne plant pathogens such as Fusarium spp., Rhizoctonia solani and Macrophomina phaseolina in the soil was developed and used with a range of soil textures. A direct method for the extraction of DNA from soil samples, which can be used for PCR-mediated diagnostics without a need for further DNA purification, was developed. The developed protocol seemed adequate to the range of soil textures that were artificially infested by a variety of soil-borne pathogens.

Key words: humic acid, soil-borne fungi, SDS-based method, soil DNA extraction.

INTRODUCTION

DNA extraction from fungi in soil often fails because of humic substances that are co-extracted with the DNA and subsequently inhibit polymerase chain reaction (PCR) analyses (Damm and Fourie, 2005). Two problems in isolating DNA from soil are: efficiently breaking cells and eliminating humic acids. Factors affecting cell rupture in soil have been evaluated by combining physical and chemical treatments (Frostegard et al., 1999). Many methods have been developed to remove humic substances from soil DNA, including polyvinylpolypyrrolidone (PVPP) (Frostegard et al., 1999; Zhou et al., 1995).

Generally, physical methods have shown efficiency for disruption of vegetative forms, small cells and spores (More et al., 1994). However, the majority utilize additional physical methods for cell disruption, such as freezing in liquid nitrogen followed by grinding (Volossiouk et al., 1995). Many of these methods are based on using a grinder (with or without liquid nitrogen) for initial breaking up of the mycelia.

The most popular detergent treatment includes SDS at 1% and salt concentrations of 1 M or, more often, coupled with heating and shaking (Edgcomb et al., 1999; Melo et al., 2006). Some researchers have used PVPP powder in batch or in spin columns to bind humic materials and purify DNA extracts (Cullen and Hirsch, 1998).

Few common protocols for a variety of soils and pathogens are found in the literature (Kageyama et al., 2003). In the current study, chemical (SDS-lysis) and enzymatic (lysozyme) were used to recover DNA from soil infested with soil-borne fungi. The DNA extraction protocol and PCR quantification
assay can be performed in less than 2 h and is adaptable to detect and quantify genomic DNA from several soil-borne fungi.

**MATERIAL AND METHODS**

**DNA Extraction from soil**

Typical field soils, including sand, loam, and clay, artificially infested with three soil-borne fungi, was taken from six diverse regions in Riyadh city in Saudi Arabia. One disk of fungal inoculum taken from one-week-old culture on potato dextrose agar (PDA) was aseptically introduced into sterilized Petri dishes (4 cm) and allowed to colonize 10 gram soil for two weeks. In the basic procedure, 100 mg of soil sample and five hundred milligram of PVPP were ground with liquid nitrogen by using a mortar and pestle for about 5 min or until a fine powder remained. Four hundred micro litre of sterile extraction buffer (200 mM Tris-HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) was added to homogenized soil for two weeks. In the basic procedure, 100 dishes (4 cm) and allowed to colonize 10 gram of soil sample and five hundred milligram of soil for two weeks. In the basic procedure, 100 mg of soil sample and five hundred milligram of PVPP were ground with liquid nitrogen by using a mortar and pestle for about 5 min or until a fine powder remained. Four hundred micro litre of sterile extraction buffer (200 mM Tris-HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) was added to homogenized soil. Then, 10 μl of 20 mg ml⁻¹ RNase A (20 mg ml⁻¹ final concentration) were added and mixed well. Samples were incubated at 65°C for 25 min. The aqueous DNA-containing top layer was transferred to new microtube and 160 μl 3M sodium acetate (pH 5.2) was added and incubated at 20°C for 10 min. Tubes were centrifuged in a microfuge at 13,000 rpm and 0.3 ml of the supernatant transferred to another tube. An equal volume of isopropanol was added and incubated at room temperature for 15 min. The aqueous phase was precipitated by centrifugation at 13,000 rpm for 1 min. DNA pellets were washed two times in 70 % (v/v) ethanol, vacuum dried for some minutes and re-suspended in 150 μl Tris EDTA buffer.

**DNA fragmentation and quantification**

DNA was electrophoresed through 1% agarose gels (Agarose MP, Roche Diagnostics Nederland BV) containing 1 μg ethidium bromide per ml of 1× Tris-acetate-EDTA (TAE) buffer. The Gel Doc 1000 system (Gel Documentation and Analysis Systems, Uvitec, Cambridge, UK) was used for image capturing under UV illumination and the graphic files were exported as 8-bit TIFF images.

**Restriction endonuclease digestion**

Restriction endonuclease CfoI (Roche-Germany) was used to test digestibility of DNA obtained by the proposed method. The digestion was done under conditions specified by producer. The restriction fragments were run in 1.5% agarose gels and stained with ethidium bromide (0.1 mg/mL) for 10-15 min. Fragment size was estimated by comparison to electrophoretic mobility of the 100 bp DNA ladder.

**PCR amplification of template DNA**

PCR reaction mixtures of 20 μl contained 20 ng template DNA, PCR buffer (JenaBioscience, Germany), 0.2 mM of each deoxynucleotide (dATP, dCTP, dGTP and dTTP), 2.5 mM MgCl₂, 1.0 U Taq polymerase (JenaBioscience, Germany), 20 pmol of TubeQ-08 a single 10-mer primer (5’ CTCCAGCGGA-3’) purchased from MWG Biotech, Germany. Amplification was performed in DNA Thermal cycler (Techne TC-312, Technne, Stone, UK). The PCR program parameters consisted of 30 cycles of denaturation at 94°C for 1 min, annealing at 3°C for 1 min and extension at 72°C for 2 min. The final cycle had a 3-min extension step at 72°C. The ITS fragment between 18S and 28S (including ITS1 + ITS2 + 5.8S) of soil fungi was amplified using the PCR primers ITS4: (5’–TCCTCGCTATGGATATGC-3’) and ITS5 (5’–GGAAGTAAAGTCGTAACAGG-3’; White et al., 1990). PCR was carried out, according to the method we reported previously (Abd-Elsalam et al., 2011). The PCR product (5 μl) was electrophoresed through 1.5% (w/v) agarose gel in TAE buffer and stained with ethidium bromide.

**RESULTS**

**DNA yield and fragmentation**

A total of 100 mg of soil samples was homogenized with a mortar and pestle in liquid nitrogen. However, the differences in the yield may be related to the method used for the quantification of the DNA, since absorbance at 260 nm can easily overestimate the DNA concentration, due to its inability to distinguish between DNA and RNA. The proposed method
could achieve a high yield of about 500 ng (total DNA)/100 mg of initial sample, the size of the DNA fragment was about 12 kb. Only one major band was visualized on the agarose gel, no smearing DNA appeared on the gel, though the marker fragment of 100 bp was clearly detectable (Figure 1).

The genomic DNA of soil samples infested with fungal genera were highly amplifiable by PCR, as indicated by the amplification products resolved on 1.5% agarose gel. On amplification using ITS primers the isolated DNA showed high intensity bands.

The PCR products obtained from the rDNA amplification were of approximately 550-680 bp in different types of soil (Figure 3).
DISCUSSION

Simple, rapid, and efficient procedures for DNA extraction from soil infested with fungal genera were developed to yield DNA of purity and quality suitable for PCR assays. Soil grinding in liquid nitrogen was combined with a simple Sodium dodecyl sulphate (SDS) buffer extraction method to provide extracts, which could be directly subjected to PCR amplification. Pulverizing increased the extracellular DNA yield, compared with the yield obtained without any lysis treatment, but none of the subsequent treatments clearly increased the DNA yield (Abd-Elsalam et al., 2007). Also, the use of liquid nitrogen allowed cell disruption under temperature conditions which minimized nucleic acid degradation. During the SDS lyses phase, proteins and polysaccharides become trapped in large complexes that are coated with dodecyl sulphate. The quantity and quality of the DNA obtained by this method were suitable for PCR amplification and other molecular assays. The present method eliminates the need to use phenol or chloroform to obtain high quality DNA from different types of soil.

Probably the most common chemical is the detergent sodium dodecyl sulphate (SDS), which dissolves the hydrophobic material of cell membranes. Additionally, amplification facilitators (such as PVPP, and skim milk) may also be used in cell lysis buffer to reduce effects of humic acids from soil samples on inhibiting PCR. Poussier et al. (2002) reported that the addition of 2% PVP or PVPP to the DNA extraction buffer increased significantly the PCR detection of phytopathogens in tomato, egg-plant, and pepper, but the combination of the addition of 5% PVPP to DNA extraction buffer and 500 ng or 5 mg of (BSA) to the PCR mixture gave the best amplification. Additions of PVPP, sodium ascorbate, and hexadecyl tri-methyl ammoniumbromide (CTAB) in cell lysis buffer can partially remove humic compounds derived from soil samples (Robe et al., 2003). The addition of PVP during homogenization significantly reduced the co-purification of PCR inhibitors with minimal loss of DNA yield. This co-purification is problematic in DNA quantification because humic acids exhibit considerable absorbance at the wavelength used to quantify DNA (260 nm). However, humic acids also exhibit absorbance at 230 nm, and these absorbance patterns have been used to determine the level of contamination of DNA isolated from soil (Yeates et al., 1998). Fungal communities are identified through analysis of environmental DNA (Curlevski et al., 2010, Hyde et al., 2011). DNA internal transcribed spacer (ITS) regions were used to detect the three fungal pathogens tested in this study. Total DNA directly extracted from soil represents many soil inhabitants each having a characteristic ITS region, which is present in fungi, as well as in plants. However, each pathogen was specifically detected in naturally and artificially infested field soils. Purification of the DNA extracted from soil was essential for assaying three phytopathogenic fungi (Fusarium spp., Rhizoctonia solani, and Macrophomina phaseolina) in soil. The procedure is safe, inexpensive, and does not require specialized equipment or generate hazardous wastes. The DNA extraction protocol, including PCR quantification assay can be performed in less than 2 h, and is adequate to detect and quantify genomic DNA from other soil-borne fungi, too.

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REFERENCES

MOHAMED AL NAGI ET AL.: IMPROVED METHOD FOR DNA ISOLATION FROM DIFFERENT TYPES OF SOIL INFESTED WITH THREE FUNGAL GENERA


