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

**Mohamed Al Nagi[1], Kamel A. Abd-Elsalam[2, 3], Ali H. Bahkali[1]
and Asran-Amal Abdel Mongy[2, 3]**

[1]King Saud University, College of Science, Botany and Microbiology Department, Riyadh, Saudi Arabia

[2]Plant Pathology Research Institute, Agricultural Research Center (ARC), Giza, Egypt

[3]Unit of Excellence in Nano-Molecular Plant Pathology Research (ARC), Giza
Corresponding author. E-mail: abd-elsalam.k.a@post.com

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 polyvinylpolypyrro-lidone (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. 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 deoxynucleiotide (dATP, dCTP, dGTP and dTTP), 2.5 mM MgCl 2, 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, Techne, 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–TCCTC CGCT TATTG ATATGC–3') and ITS5 (5'–GGAAG TAAA AGTC GTAAC AAGG–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).
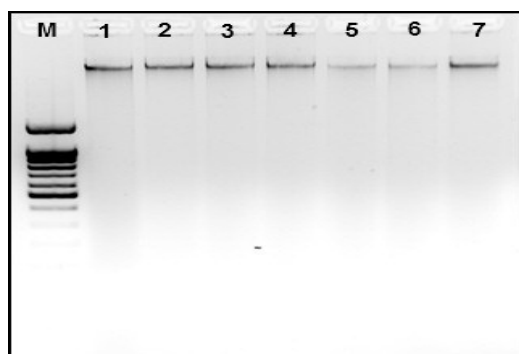

Figure 1. Electrophoresis of DNA extracted from the seven soil samples. 5% of eluted DNA was loaded on 1% agarose gel, and electrophoresis was done for 40 minutes. DNA was extracted from three distinct soil types including (Lane 1, *R. solani*-infested sand soil; Lane 2, *Fusarium*-infested sand soil; Lane 3, *M. phaseolina*-infested loam soil; Lane 4, *Rhizoctonia*-infested loam soil; Lane 5, *Fusarium*-infested clay soil; Lane 6, *M. phaseolina*-infested clay soil; Lane 7, three types of soil were infested with three fungal genera), M = 100-bp DNA molecular weight ladder (Bioron)

The suitability of extracted DNA for downstream molecular processes was further verified by restriction endonuclease digestion, RAPD-PCR and ITS amplification. The isolated DNA was amenable to restriction digestion using CfoI (Figure 2).
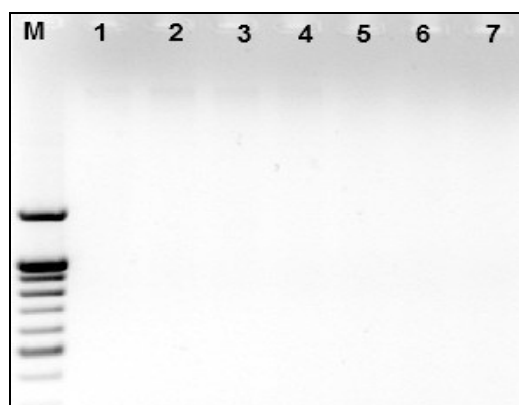

Figure 2. Agarose gel electrophoresis of the DNA restricted with CfoI. DNA was extracted from three distinct soil types including (Lane 1, R. solani-infested sand soil; Lane 2, *Fusarium*-infested sand soil; Lane 3, *M. phaseolina*-infested loam soil; Lane 4, *Rhizoctonia*-infested loam soil; Lane 5, *Fusarium*-infested clay soil; Lane 6, *M. phaseolina*-infested clay soil; Lane 7, three types of soil were infested with three fungal genera), M = 100-bp DNA molecular weight ladder (Bioron)

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).
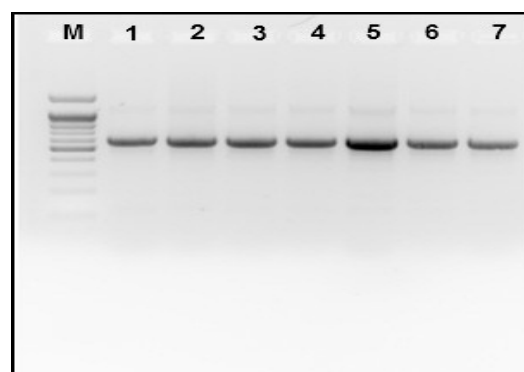

Figure 3. Agarose gel (1.5%) electrophoresis of the amplified internal transcribed spacer (ITS) products obtained with the ITS4/ITS5 primer pair. DNA was extracted from three distinct soil types including (Lane 1, *R. solani*-infested sand soil; Lane 2, *Fusarium*-infested sand soil; Lane 3, *M. phaseolina*-infested loam soil; Lane 4, *Rhizoctonia*-infested loam soil; Lane 5, *Fusarium*-infested clay soil; Lane 6, *M. phaseolina*-infested clay soil; Lane 7, three types of soil were infested with three fungal genera), M = 100-bp DNA molecular weight ladder (Bioron)

PCR amplification with tubeQ-08 primer was performed using suggested DNA isolation protocol (Figure 4).
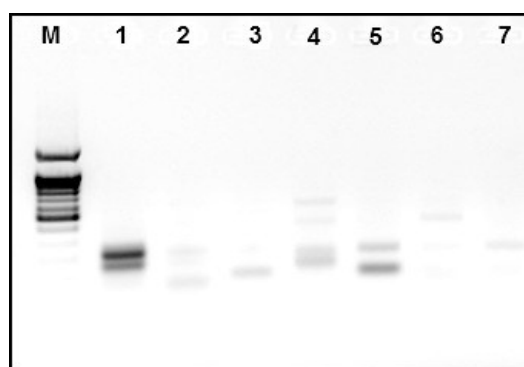

Figure 4. Agarose gel electrophoresis of RAPD-amplified products. DNA was extracted from three distinct soil types including (Lane 1, *R. solani*-infested sand soil; Lane 2, *Fusarium*-infested sand soil; Lane 3, *M. phaseolina*-infested loam soil; Lane 4, *Rhizoctonia*-infested loam soil; Lane 5, *Fusarium*-infested clay soil; Lane 6, *M. phaseolina*-infested clay soil; Lane 7, three types of soil were infested with three fungal genera), M = 100-bp DNA molecular weight ladder (Bioron)

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.

REFERENCES

Abd-Elsalam, K.A., Asran-Amal A., El-Samawaty, A.M.A., 2007. *Isolation of high quality DNA from cotton and its fungal pathogens*. J. Plant Dis. Prot., 114:113-116.

Abd-Elsalam, K.A., Bahkali, A.H., Al-Khedhairy, A.A., Moslem, M.A., 2011. *Freeze- and thaw-based procedures for extraction DNA from activated sludge*. Polish. J. Environ. Studies, 20: 643-648.

Cullen, D.W., Hirsch, P.R., 1998. *Simple and rapid method for direct extraction of microbial DNA from soil for PCR*. Soil Biol. Biochem., 30: 983-993.

333

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

Curlevski, N.J.A., Xu, Z.H., Anderson, I.C., Cairney J.W.G., 2010. *Diversity of soil and rhizosphere fungi under Araucaria bidwillii (Bunya pine) at an Australian tropical montane rainforest site.* Fungal Divers, 40:1-11.

Damm, U., Fourie P., 2005. *A cost-effective protocol for molecular detection of fungal pathogens in soil.* South Afric. J. Sci., 101: 135-139.

Edgcomb, V.P., McDonald, J.H., Devereux, R., and Smith, D.W., 1999. *Estimation of bacterial cell numbers in humic acid-rich salt marsh sediments with probes directed to 16S ribosomal DNA.* Appl. Environ. Microbiol., 65: 1516-1523.

Frostegard A., Courtois, S., Ramisse, V., Clerc, S., Bernillon, D., Le Gall, F., Jeannin, P., Nesme, X., and Simonet, P., 1999. *Quantification of bias related to the extraction of DNA directly from soils.* Appl. Environ. Microbiol., 65: 5409-5420.

Hyde, K.D., Abd-Elsalam, K.A., Cai, L., 2011. *Morphology: still essential in a molecular world.* Mycotaxon, 144: 439-451.

Kageyama, K., Komatsu, T., Suga, H., 2003. *Refined PCR protocol for detection of plant pathogens in soil.* J. Gen. Plant Pathol., 69: 153-160.

Melo, S.C.O., Pungartnik, C., Cascardo, J.C.M., Brendel, M., 2006. *Rapid and efficient protocol for DNA extraction and molecular identification of the basidiomycete Crinipellis perniciosa.* Genet. Mol. Res., 5: 851-855.

More, M.I., Herrick, J.B., Silva, M.C., Ghiorse, W.C., Madsen, E.L., 1994. *Quantitative cell lysis of indigenous microorganisms and rapid extraction of microbial DNA from sediment,* Appl. Environ. Microbiol., 60: 1572-580.

Poussier, S., Cheron, J.J., Couteau, A., Luisetti, J., 2002. *Evaluation of procedures for reliable PCR detection of Ralstonia solanacearum in common natural substrates.* J. Microbiol. Methods, 51: 349-359.

Robe, P., Nalin, R., Capellano, C., Vogel, M.T., Simonet, P., 2003. *Extraction of DNA from soil.* Eur. J. Soil Biol., 18: 190.

Volossiouk, T., Robb, E.J., Nazar, R.N., 1995. *Direct DNA extraction for PCR-mediated assays of soil organisms.* Appl. Environ. Microbiol., 61: 3972-3976.

White, T.J., Bruns, T.D., Lee, S., Taylor, J., 1990. *Analysis of phylogenetic relationships by amplification and direct sequencing of ribosomal RNA genes.* In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, New York: 315-322.

Whitehousea, C.A., Hottel, H.E., 2007. *Comparison of five commercial DNA extraction kits for the recovery of Francisella tularensis DNA from spiked soil samples.* Molecul. Cell Prob., 21, 92.

Yeates, C., Gillings, M.R., Davison, A.D., Altavilla, N., Veal, D.A., 1997. *PCR amplification of crude microbial DNA extracted from soil.* Lett. Appl. Microbiol., 25: 303-307.

Zhou, J., Bruins, M.A., Tiedje, J.M., 1995. *DNA recovery from soils of diverse composition.* Appl. Environ. Microbiol., 62: 316-322.