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Original Article

A simple, rapid, safe and low-cost method to extract DNA from phytopathogenic fungi

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Abstract

The aim of this study was to develop an easy, fast, non-hazardous and inexpensive technique for extraction of genomic DNA from multiple plant fungal pathogens. Samples of pure fungal growth of *Fusarium equesti* , *Neoscytalidium dimidiatum* , *Fusarium proliferatum* and *Alternaria alternata* isolated from diseased wheat, grapevine, potato and lily plants respectively were ground with sterilized sand and NaOH (2N), followed by a centrifuging process to separate the sand grains and cellular components of fungi from the DNA. Subsequently, the DNA was mixed with Tris buffer (1 M) pH 8. The ITS region of rDNA was successfully amplified, sequenced and analyzed from the extracted DNA of the four pathogenic fungi. This new approach provides a simple, rapid, safe and low cost way to obtain DNA samples of sufficient quantity and quality for use in molecular assays for the identification of plant fungi.

Keywords: DNA extraction, Fungi, PCR, Sequence, Phylogeny analysis

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Introduction

There are an enormous number of phytopathogens including viroids, viruses, phytoplasmas, fungi, bacteria, nematodes that interact with different crop, vegetable, fruit and ornamental plants and cause various diseases. The impact of these pathogens can be vary from minor symptoms to devastations that can destroy large areas of food crops which lead to inadequate food supplies for approximately 10% of the global population (Agrios,2005; Strange and Scott, 2005). With a view to reduce the losses caused by these pathogens, the rapid and accurate identification of the causative organism is essential to reveal mechanisms of its virulence and accurately predict the disease severity in order to determine an effective and feasible control method (Strange and Scott, 2005).

Considerable progress has been achieved in understanding various aspects of plant-pathogen interactions through the application of several molecular techniques. For instance, molecular diagnostic techniques are widely used to identify plant pathogens, even those are in very low concentration or mixed with others pathogens (Agrios, 2005; Punja et al., 2007). These molecular diagnosis methods mainly depend on polymerase chain reaction (PCR) by employing molecular markers. This approach has succeeded in the diagnosis of phytopathogenes including fungi using conserved molecular markers such as internal transcribed spacer (ITS) region. This region locates within ribosomal DNA and consists of three parts ITS1-S5.8-ITS2 that are distinguished

among fungal species of different or even the same genus. Therefore, it is widely utilized to characterize, classify and determine the phylogenetic relationship among fungal populations (White et al., 1990; Tapia-Tussell et al., 2006).

A sufficient quantity of DNA must be extracted in order to implement the PCR test successfully. Any method of DNA extraction includes four main steps: the first comprises breaking down the cell walls of the organism via several techniques and substances such as liquid nitrogen, glass beads (Lee et al.,1988; Wu et al., 2001) dry ice (Griffin et al.,2002) magnetic or glass beads (Faggi et al.,2005), enzymatic digestion (Li et al., 2002), benzyl chloride (Xue et al., 2006), or a combination of these (Zhang et al.,2008). The second step involves separation of the DNA from cellular components through a centrifugation process and use of buffers such as cetyl trimethyl ammonium bromide (CTAB) (Doyle and Doyle, 1987). Then, a purification process is conducted to separate DNA from proteins using phenol and chloroform, followed by precipitation using ethanol or isopropanol alcohol (Ashktorab and Cohen, 1992). Many DNA extraction methods are available (Tapia-Tussell et al., 2006; Lee et al.,1988; Cenis, 1992; Min et al.,1995; Löffler et al., 1997; Al-Samarrai and Schmid 2000). Additionally, there are many commercial kits produced by special companies such as Qiagen/Germany and Promega/USA to achieve the same purpose. Despite the fact that the above methods provide sufficient quantities and qualities of DNA, they usually demand longer time to be processed as a result of comprising many steps and buffers to remove contamination proteins, polysaccharides and RNA. These extra steps might cause inadvertent mistakes. In addition, they may include some materials such as liquid nitrogen which is required extra attention to deal with and extra cost to purchase and store. In addition, most commercial kits for DNA extraction are expensive. Thus, it has been necessary to develop an easy, fast, safe and low-cost DNA extraction method and this was the object of this study.

Material and Methods

Fungal isolations

In previous studies, *Fusarium equesti* was isolated from rotted seeds and seedlings of wheat *Triticum estivum*, *Neoscytalidium dimidiatum* isolated from grapevine *Vitis vinifera* showed dieback symptoms, *Fusarium proliferatum* isolated from rotted root and crown of potato *solanum tuberosum* and *Alternaria alternate* from leaves of lily *Lilium candidum* displayed irregular brown spots symptoms. These fungi were sub-cultured and incubated at 25 °C in darkness for 7 days following previous procedures (Narayanasamy, 2011; Al-Saadoon et al., 2014; Martin and Dombrowski, 2015).

DNA extraction methods

The commercial kit method

The DNeasy Plant Mini kit (Qiagen, Hilden, Germany) was used to extract the total DNA from pure 7 days old of four fungi with a modification in the first step of extraction process. This modification was by using glass beads size 150-600 mm (Sigma-Aldrich, USA). These glass beads were autoclaved for 20 minutes at 121°C under 15psi. A sample of 100 mg each fungal growth was scraped from each pure 7 days colony utilizing sterilized scalpel blade and mixed thoroughly with approximately 5-10 mg of the glass beads using a stick homogenizer rather than using liquid nitrogen. After that all other extraction steps were operated following the manufacturer`s instructions. The DNA concentration and purity was then measured using spectrophotometer (APEL Co., Japan) and stored at -20°C (Narayanasamy, 2011).

The developed DNA extraction method

In the new method 100-200 mg of pure 7 days mycelia of each fungal pathogenic isolate was collected using a sterile scalpel blade and mixed with 5-10 mg of washed sand granules size 100-200 µm. A 100 microliters of NaOH (2N) was added to the mixture and the fungus growth was then ground with the assistance of a stick homogenizer, followed by centrifuging at 14000 rpm for 5 minutes. This was for the purpose of separating the sand grains and fungal cellular components from the DNA. Subsequently, 15 microliters of the supernatant containing DNA was transferred and mixed with 35 microliters of Tris solution buffer concentration 1 M and pH 8 using Vortex [Never vortex the DNA because it will shear the DNA leading to degraded integrity of the DNA. Increased concentration is not always the sign of intact DNA]. The purity and concentration of the DNA was evaluated by the same above spectrophotometer. All DNA samples were then kept at -20°C. To verify this extraction method, genomic DNA of 10 replicates of each fungal isolate were extracted and evaluated using polymerase chain reaction test.

Polymerase Chain Reaction (PCR) test

The PCR test was applied for all replicates using pairs of universal primers ITS1 (5`-TCCGTAGGTGAACCTGCGG-3`) and ITS4 (5`-TCCTCCGCTTATTGATATG ATGC-3`) that target the ITS region of fungi (White et al., 1990). The Ready-To-Go PCR Beads kit (GE Healthcare, UK) was used to perform the PCR test by following the manufacturer`s guidelines. Two microliters of total DNA isolated from the pathogenic fungi using both methods were added to each reaction mentioned above separately.

The PCR amplification programme started with initial denaturation for 5 min at 95°C, then 40 cycles consisting of three steps: denaturation at 95°C for 40 seconds, primer annealing at 55°C for 40 seconds and extension at 72°C for 1 min. A final extension step for 5 min at 72°C was included. PCR products were electrophoresis analyzed and visualized on 1.5 % agarose gels stained with ethidium bromide using a UV transilluminator (Edvotek Inc, USA) (White et al.,1990; Gardes and Bruns,1993; Jima, 2012).

DNA sequencing and bioinformatics analysis

All samples of DNA (PCR products) were sequenced using the Macrogen, Inc sequencing service Applied Biosystems (ABI 3730xI automated Sequencer, Seoul, South Korea; http:// www.macrogen.com).The concentration of PCR product was between 50-150 ng/µl. Sequencing was performed using the ITS1 primer. Analysis of chromatograms and nucleotide sequences were conducted using Chromas (version2.6.4; http://technelysium.com.au /wp/chromas/) software. Basic Local Alignment Search Tool (BLAST) was used to compare fungal obtained sequences with published sequences from the National Center for Biotechnology Information (NCBI) website (http://www.ncbi. nlm.nih.gov /BLAST). A plasmid Editor (ApE version1.17; http://biolog ylabs. utah.edu/ jorgensen/ wayned /ape/) software was also used to compare the DNA sequences of both DNA extraction methods. Subsequently, Molecular evolutionary genetics analysis (MEGA6 version 6.0; https://www.megasoftware.net/) software developed by Tamura et al. (2013) was operated for phylogenetic analysis of all tested fungi.

Results and Discussion

In order to investigate whether the new approach could be applied successfully to extract the genomic DNA from many different fungi, it was utilized for four pathogenic fungal strains belong to genera infecting crop, vegetable, fruit and ornamental plants. DNA extracted by both methods was left undiluted and utilized directly for PCR amplification of the rDNA ITS region using ITS1 and ITS4 primers. PCR products (Figure 1) were obtained from all fungal DNA samples. This indicated that the quality of DNA extracted by the two methods was sufficient and comparable. The PCR products of the amplified rDNA ITS region of each fungal pathogenic strains were sequenced successfully. The sequence data showed that the four fungal genomic DNA samples extracted with this new method were almost identical to those extracted via the DNeasy Plant Mini kit. The BLAST analysis of these fungal sequences showed them to share more than 95% nucleotide sequence identity with many of those strain sequences available in NCBI database. Additionally, phylogenetic analysis of their sequences by MEGA6, applying a neighbour-joining technique clustered them together with several fungi from the same species. The fungal identification was confirmed based on sequence comparison and phylogenetic analysis.

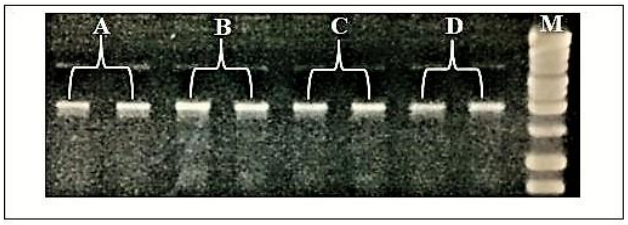

Figure 1: **Analysis of PCR amplicons amplified with genomic DNA samples extracted with the newly developed method and the DNeasy Plant Mini kit.**

The PCR bands appearing on agarose gel resulting of amplification the rDNA ITS region for all investigated fungal strains: (A) *Fusarium equesti* of wheat plant, (B) Neoscytalidium dimidiatum of grape trees, (C) Fusarium proliferatum of potato plant and (D) Alternaria alternate of lily plant.

The number (1) refers to the amplified DNA extracted with the new method while number (2) indicates to the amplified DNA extracted with the DNeasy Plant Mini kit. (M) is the marker.

The simple, fast, safe and low cost approach of DNA extraction developed in this study has significant advantages compared to the DNeasy Plant Mini kit and other different approaches: Time required for the DNA extraction process is decreased significantly (Table 1) due to no liquid nitrogen being required for breaking of the fungal cell walls and no DNA purification process by means of phenol/chloroform that are considered toxic substances. Therefore, it is not hazardous for operators and does not require disposal of toxic wastes (Zhang et al., 2010). This reduction in time was paired with significant decrease in cost. The price of the DNeasy Plant Mini Kit for 50 samples is 221.00 US dollar (https://www.qiagen.com). However, the cost of the new approach for the same number of samples is roughly less than 1 US dollar. This cost was estimated based on price of chemical substances (NaOH and Tris) that are available at the Sigma-Aldrich company (https://www.sigmaaldrich.com) while sand is free. In addition, only a small amount (100-200 mg) of fungal filaments is required. Hence no long period is necessary for cultivation of the fungal strains. This is a valuable feature particularly for slow growing fungi. Additionally, the possibility of DNA contamination, which can lead to a failed PCR test (Kwok and Higuchi, 1989; Manzanilla-Lopez et al., 2008) is reduced because of omitting several steps involving surface contact between the DNA and spatula, mortar or pestle etc. and special tools or instruments such as a low temperature centrifuge are not required. However, since no purification process is included, the new method has the limitation that the quality and quantity of DNA extracted was lower compared with the commercial method (Table 2). Although this limitation did not affect the PCR amplification process that resulted in very clear amplicon bands (Figure 1) that were sequenced and used successfully in the phylogenetic analysis (Figures 2,3,4,5). This new method can be simply learned by normal researchers and it can be feasible for investigation of a large number of fungal samples in shorter time during a study of screening, epidemiology genetic diversity of plant fungi.

Table 1: Comparison of time required for DNA extraction between the new method and the DNeasy Plant Mini kit method

Methods	No. of samples	Time of steps (minutes)				
		Break	Incubation	Purification	Precipitation	Total
New method	1	5	-	5	-	10
DNeasy Plant Mini kit	1	5	15	15	5	40

Legend: The developed method was compared to the DNeasy Plant Mini kit in dividing the DNA extraction process into four steps: breaking of the fungal cell wall, incubation in order to release the DNA into buffer, purification (for the DNeasy Plant Mini kit using several different stages with various buffers and solutions) and purification. Dash (-) sign indicates to the absence of step.

Table 2: Comparison of quality and quantity of DNA extracted by the new method and the DNeasy Plant Mini kit

Fungi Parameters	A		B		C		D	
	1	2	1	2	1	2	1	2
Quality (260/280)	0.6	1.2	0.8	1.5	0.5	1.3	0.6	1.1
Quantity (µg/ml)	7.8	15.2	13.4	18.4	5.8	12.8	11.7	18.1

Legend: Analysis of genomic DNA samples extracted with the newly developed method and the DNeasy Plant Mini kit. Absorbance measurements were made using a spectrophotometer to determine the quality and quantity for all examined fungal strains: (A) *Fusarium equesti* of wheat plant, (B) *Neoscytalidium dimidiatum* of grape trees, (C) *Fusarium proliferatum* of potato plant and (D) *Alternaria alternate* of lily plant. The number (1) denotes to the genomic DNA extracted with the new method while number (2) indicates to the genomic DNA extracted with the DNeasy Plant Mini kit.

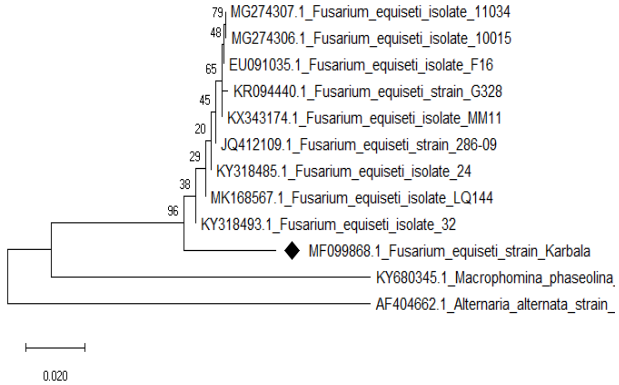

Figure 2: Phylogenetic tree created by analyses of the ITS fragment sequence of rDNA extracted by the developed method.

It showed the relationships among *F. equesti* isolated of diseased wheat plants (indicated with black prism) and other *F. equesti* strains stored at genbank database. The two outgroup fungal strains were *Macrophomina phaseolina* and *Alternaria alternata*.

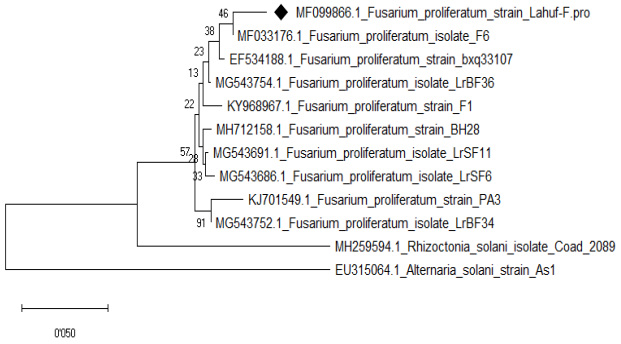

Figure 3: Phylogenetic tree of a maximum-likelihood constructed based on the ITS region sequence of rDNA extracted by the developed procedure with NCBI publicly available sequences of *F. proliferatum*.

It confirmed the relationship among the *F. proliferatum* strain Lahuf-F.pro isolated in this study (signified with a black prism). The two outgroup fungal strains were *Rhizoctonia solani* and *Alternaria solani*

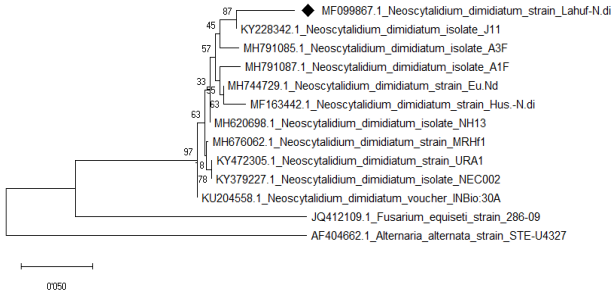

Figure 4: Phylogenetic tree of *Neoscytalidium dimidiatum* fungi based on the ITS sequence of rDNA, extracted by the developed technique.

It showed the close genetic relationship among *N. dimidiatum* strain Lahuf-N.di isolated in this study (indicated with black prism) and those worldwide deposited in genbank database. The outgroup fungi were *F. equesti* and *A. alternata*.

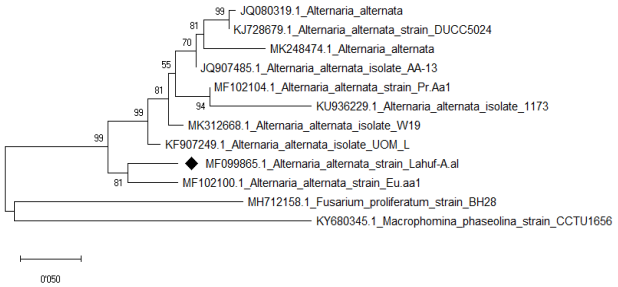

Figure 5: Phylogenetic tree created from the ITS sequences of rDNA extracted by the developed method showing the phylogenetic relationships among *Alternaria alternata* strains.

A. alternata strain Lahuf-A.al obtained in this study (denoted with a black prism) was genetically more similar to those strains of *A. alternata* stored at genbank than *Fusarium proliferatum* and *Macrophomina phaseolina* that were the outgroup strains.

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Contribution of Authors

Lahuf AA: Conceived Idea, Designed Research Methodology, Data Analysis, Manuscript Writing**.** Manuscript final reading and approval
Jaafar OH: Data Collection, Statistical Analysis, Manuscript Writing
Hameed ZL: Literature Review, Literature Search, Data Interpretation

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