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OPTIMIZATION OF DNA EXTRACTION FROM FILAMENTOUS FUNGI *ALTERNARIA SP.* AND *FUSARIUM SP.*

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ABSTRACT

Filamentous fungi have strong cell walls which are lysis resistant and contain high levels of proteins, polysaccharides, and other secondary metabolites. Accurate identification of fungal pathogens using a sequence-based approach required an extraction method that yielded template DNA pure enough for polymerase chain reactions (PCR) or other types of amplification. Therefore, the objective of this study was to develop and standardise a rapid, inexpensive DNA extraction protocol applicable to major fungal phyla, which would yield sufficient template DNA pure enough for PCR and sequencing. In this research, phytopathogenic *Alternaria* and *Fusarium* fungi from contaminated wheat seeds were used. Four methods were tested for genomic DNA isolation: CTAB, an SDS method with modification, the Benjamin Schwessinger method and a Qiagen DNeasy Plant Mini Kit. High qualitative and quantitative characteristics were obtained from the DNeasy Plant Mini Kit and the acid CTAB-buffer (pH ≤ 5). The QIAGEN plant DNeasy and CTAB methods may be applicable to other fungi and effectively implemented in other laboratories.

Keywords: DNA extraction method comparison, filamentous fungi, polymerase chain reaction (PCR) amplification, Fungal DNA extraction techniques, filamentous fungi DNA extraction

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INTRODUCTION

According to FAO, worldwide losses for the wheat were 34% for pests and 12% for diseases [1]. Disease in plants caused by phytopathogenic fungi increased 9 fold in the past 10 year and resulting both in economic deprivation to farmers and to shortfalls in the nutrition provision for local population [2]. These fungi can reproduce both sexually and asexually, while suppressing plant defense [3]. Plants fungal pathogens in both natural and cultivated populations cause of tracheomycosis/vascular wilt, inhibition of growth, root and stem rot [4]. The severity of diseases caused by pathogens depending on the aggressiveness of the pathogen, host resistance [5].

One aim of most plant breeding programs is to increase the resistance of host plants to disease. Accurate identification of fungal phytopathogens is essential for virtually all aspects of plant pathology, from fundamental research on the biology of pathogens to the control of the diseases they cause. Agrotechnical methods will allow monitoring fungal growth, penetration into plants and spreading. Many plant pathogens are difficult to identify using morphological criteria, which can be time consuming and challenging

and requires extensive knowledge in taxonomy [6]. Fungal mycelia from field samples often impossible to detect. In recent years, effective alternative techniques based on polymerase chain reaction (PCR) amplification have been developed to identify micromycetes.

With the advances in polymerase chain reaction (PCR) and DNA sequencing technologies, molecular techniques have now become standard approaches when dealing with fungal identification [7, 8]. The technologies used for genetic identification (amplification, reverse transcription, cloning, sequencing, genotyping) high-quality DNA is required. Ineffective lysis of fungal cells lead to accumulation substances, which are co-extracted with the DNA and inhibit the polymerase chain reaction [9].

Nucleic acid samples can become contaminated by proteins, polyphenolic substances, polysaccharides and secondary metabolites and chemically or mechanically inhibit denaturation and hybridization. The results can be significant distortion. The quality and integrity of the DNA obtained will directly affect the reliability of sequencing and cloning, which used to study population structure [10].

No single DNA extraction methods was optimal for different fungal species. The classical method of fungal DNA preparation is multi-step and includes growing the fungus in liquid or solid medium, disrupting cell walls, removing proteins with phenol and chloroform, and precipitating DNA with ethanol or isopropanol [11].

A fungal cell wall mainly includes 80-90% polysaccharide, proteins, lipids polyphosphates and inorganic ions, which make up the wall-cementing matrix. The general picture of fungal cell walls is that the skeletal microfibrillar wall components, such as β-glucan, chitin, and/or cellulose which makes it difficult to extract DNA. One of the central problems to extract DNA from cell types that possess rigid cell walls and resist lysis techniques [12].

In this regard, isolation of nucleic acids from fungi often requiring the addition lysis steps such as mechanical homogenization, sonication, enzymatic lysis or hazardous chemicals [13].

A number of methods have been developed for DNA extraction from fungal tissues. The efficient DNA extraction protocols which entail the combined use of enzymatic methods (chitinases, glucanases and proteases), physical (glass beads, microwaves, frozen/thaw cycles, grinding in liquid nitrogen) [14]. In addition, polyphenolic and polysaccharide compounds can inhibit the activity of DNA polymerases. This problem can be solved by using a mixture of N-trimethyl ammonium bromid (CTAB), Polyvinyl pyrroledone (PVP), β-mercaptoethanol (βME), bovine serum albumin (BSA) either a spin or vacuum column for efficient removal of inhibitors [15].

The CTAB extraction method was originally developed by Doyle J.J. & Doyle J.L. (1987) one of the most common methods of DNA isolation [16]. CTAB (N-Cetyl-N, N, N-trimethyl ammonium bromide is a cationic detergent that simultaneously degrades and solubilizes the cell wall and lipid membranes of internal organelles. CTAB is probably the only compound that can separate partial nucleic acids from polyphenols [17] and becomes stable insoluble complexes with nucleic acid in low-salt environment.

MATERIAL AND METHODS

Strains of fungi *Alternaria sp.*, *Fusarium sp.* were isolated from infected plants of spring wheat. Eight isolates were grown in Chapek medium at 25°C for 3-5 days.

Fungal mycelia were collected from culture plates and 50 mg of each mycelium was added into 1.5 Eppendorf tube and centrifuged at 10000 rpm for 5 min. After centrifugation the supernatant was discarded, mycelia was freeze dried (at -20°C).

DNA extraction was done in four different methods:

a) CTAB (Cetyl Trimethyl Ammonium Bromide) method [18];

b) modified SDS (Sodium Dodecyl Sulfate) method [19] ;

c) Benjamin Schwessinger method [20];

d) commercial kits Qiagen DNeasy Plant Mini Kit.

CTAB method. Optimized for the extraction of nucleic acid from fungal samples are lysed and homogenized in CTAB-buffer (2% CTAB, 2M NaCl, 10 mM Na3EDTA, 50 mM HEPES, pH 5,3) and incubated at 65°C for 1 hour. DNA was extracted with chloroform/isoamyl alcohol in a 24:1 ratio at 65°C for 30 min. After centrifugation at 14000 rpm and removal supernatant DNA was precipitated with equal volume of isopropanol. DNA was eluted in 150µl TE-buffer (1 mM ЭДТА, 10mM Tris-HCl, pH 8,0). Current protocol was optimized by using lysis buffer with pH ≤5,3 and chloroform extraction at 65°C.

SDS метод. Samples were homogenized in lysis buffer (50mM Tris pH8, 50 mM EDTA pH8, 3% SDS, 2% 2-Mercaptoethanol) and incubated at 65°C for 1 hour. After centrifugation at 6000 rpm for 5 min the equal volume chloroform/isoamyl alcohol (24:1, v/v) to 600µl of supernatant was added. The equal volume chloroform/isoamyl alcohol (24:1) was added after repeated centrifugation, the tube was centrifuged at 12000 rpm and Na-acetate (3M) was added. DNA was precipitated by adding isopropanol (1 ml), the pellet was washed with 70% ethanol, dried and dissolved in 150µl TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8,0).

Benjamin Schwessinger method. Lysis buffer was made by mixing buffers A+B+C (2.5:2.5:1 + 1%PVP final) A buffer (0.35 M sorbitol, 0.1 M Tris-HCl, pH 9, 5 mM EDTA, pH 8), buffer B (0.2 M Tris-HCl, pH 9, 50 mM EDTA, pH 8, 2 M NaCl 2% CTAB) and buffer C (5% Sarkosyl (N-lauroylsarcosine sodium salt, 0.1%PVP). Lysis buffer also include RNAse. Incubation was carried out for 30 min at 64°C followed by cooling on ice for 5 min. After addition 5M KAc the tube was centrifuged at 12000-14000 rpm. The supernatant transferred to a new tube containing with Chl/IAA (24:1) and centrifuged at 12000-14000 for 30 min and precipitated with isopropanol. DNA was eluted by 150µl TE-buffer (1 mM ЭДТА, 10 mM Tris-HCl, pH 8,0).

DNA extraction with commercial kits (Qiagen DNeasy Plant Mini Kit)

Extraction from fingal pure culture was done according to the manufacturer's recommendations Qiagen DNeasy Plant Mini Kit (Qiagen, Germany).

Quality and quantity determination of DNA samples

The quality and quantity of the DNA obtained were evaluated by measuring the concentration (ng/µl) in a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) [21]. The quality of the DNA yielded by each method was determined by gel electrophoresis in a 1% agarose gel at 90 V for 15-20 min, stained with Ethidium bromide and observed in gel documentation ChemiDoc-It®TS2 Imager (UVP).

The electrophoretic mobility of DNA compared with known molecular weight to determine the size of fragments GeneRuler DNA Ladder Mix (100-10,000 bp) (#SM0332, Thermo Fisher Scientific).

Polymerase chain reaction (PCR)

DNA quality of the samples obtained with different protocol was assessed by PCR amplification of ITS region (ITS1 5′GGAAGTAAAAGTCGTAACAAGG 3′; ITS4 5′TCCTCCGCTTATTGATATGC3′). The ITS region was amplified in a 25 µl reaction using 25 ng DNA, 1x DreamTaq buffer (1.5 mM MgCl2), 0.3 µM pimer, 200 µM dNTP, 0.2 U Taq DNA Polymerase. The PCR conditions were as follows: 95°C for 2 min, 30 cycles of 95°C for 10 s, 50°C for 30 s, and 72°C for 1 min, and a final cycle at 72°C for 3 min.

PCR products were electrophoresed on a 1,2%(w/v) agarose gel in 1x THE (20 mM Tris-HEPES, pH 8.06) at 90 V for 1 hour.

DNA sequencing and sequence analisys

The DNA sequencing was performed with the genetic capillary electrophoresis analyzer ABI3700 (Applied Biosystems Thermo Fisher Scientific), Sanger's method, (BigDye® Terminator chemistry).

The sequences were visualized and edited using Ugene (Bowtie, UGENE Genome Aligner) and compared to the NCBI (http://www.ncbi.nlm.nih.gov).database using BLAST [22] to confirm the identity of the species.

RESULTS AND DISCUSSION

Comparison of effectiveness protocols for isolation DNA

Molecular methods like PCR, sequencing plays an important role in the identification and study of microorganisms. DNA extraction is a critical step used in molecular genetic approaches for the study of microorganisms. There are no standardized protocol for nucleic acid extraction and common protocols needs optimization.

In the present study, we compared four methods of fungal DNA extraction using different lysis buffer (table 1).

Table 1. Comparison of Methods for DNA extraction

	CTAB method	SDS method	Modified method from Schwessinger B.	Qiagen DNeasy Plant Mini Kit
The main components of the lysis buffer	2% CTAB	3% SDS, 2%mercaptoethanol	2% CTAB 5% Sarkosyl 0.1% PVP	200 mM NaOH, 1% SDS
Principle of the method	Lysis buffer contains detergents and chaotropic salts, DNA was deprotonated in chloroform and further extracted using organic solvent.			Spin columns use a silica membrane for selective adsorption of DNA
Lysis conditions	1 hour 65°C		30 min 64°C	10 min 65°C
Precipitation	Isopraponol	Isopraponol	Isopraponol	Mixture of substances with guanidinium chloride
Extraction time, h	1 h 30 min	1 h 5 min	2 h	35 min

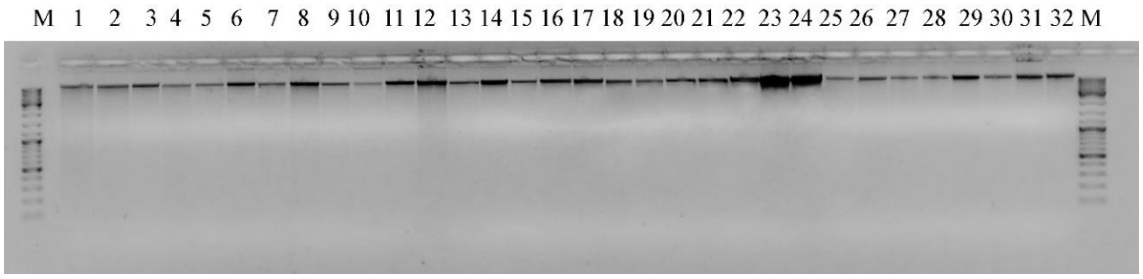
The main differences between the extraction methods is composition of lysis buffer. Subsequent purification and precipitation steps almost identically, and after lysis samples mixed with chloroform, forming a biphasic mixture. DNA precipitated from aqueous phase with isopropanol and dissolved in TE buffer. The exception is commercial kit Qiagen DNeasy Plant Mini Kit, in which all the main stages are patented.

Optimized protocol uses lysis buffer containing CTAB to obtain DNA is relatively less time-consuming and takes 1.5 hour. The detergent forms an insoluble complex with the nucleic acids, which separated from proteins, polysaccharides and other molecules by chloroform purification. DNA extraction using toxic chloroform making this method less attractive and require additional costs of utilization. In addition, the method require homogenization the samples and sterilization instruments to prevent contamination. The advantages of SDS and Benjamin Schwessinger methods, involves thermal lysis, it is not require a preliminary homogenization. However, using of mercaptoethanol poses a danger to researchers.

Commercial kits for isolation DNA Qiagen Plant DNeasy Kit, (Qiagen, Germany) includes microcentrifuge tube for lysis and spin-columns for removal RNA, proteins

and polysaccharides, also solutions does not involve the use toxic reagents phenol and chloroform. The DNA is adsorbed by silica in the presence of chaotropic salt with high ionic strength. This allows positively charged ions to form a salt bridge between the negatively charged silica and the negatively charged DNA. The DNA washed with high-salt and ethanol, and eluted with low salt. A rapid and less toxic option for DNA extraction is the use of commercial kits, but the price of the extraction can become a limitation when there is a demand to extract DNA from a large number of samples.

The comparative analysis of quality extracted DNA from 8 fungal isolates belonging to the genus *Alternatia* and *Fusarium* (figure 1).



1-8 – DNeasy Plant Mini Kit (Qiagen); 9-16 – B. Schwessinger method; 17-24 – CTAB method; 25-32 – SDS method

Fig. 1. Genomic DNA profile of fungi, extracted using 4 different methods on 1.0% agarose gel

The effectiveness of the DNA extraction method was evaluated by such criteria as the average DNA quantity (μg/μl) per sample, the DNA qualitative indices (A_{260}/A_{280} and A_{260}/A_{230} ratios).

When the quality of the DNA was evaluated, the A260/A280 ratio was greater than 1.8 for all the samples, indicating that the DNA was almost free of proteins [23]. With regard to the A260/A230 ratio, were approx. 2, indicating that the samples do not have impurities of carbohydrates, peptides, phenols, salts, and aromatic compounds A comparative analysis of the methods used is given in table 2.

Table 2. Genomic DNA yield from different fungal pathogens using different extraction methods

Extraction method	Fungal isolates	T.A. (μg)	$D_{260/280}$	$D_{260/230}$
Method by Schwessinger B.	*Alternaria sp.*	39-167 **80,7**	1,9	2,4
	Fusarium sp.	40-157 **86.0**	2,0	2,1
SDS method	*Alternaria sp.*	17-65 **33.1**	1,8	1,3
	Fusarium sp.	15-58 **32.0**	1,6	1,3
CTAB method	*Alternaria sp.*	195-221 **215,7**	1,9	2,0
	Fusarium sp.	34-111 **78**	1,9	1,9
DNeasy Plant Mini Kit (Qiagen)	*Alternaria sp.*	15,2-20,5 **17,1**	1,8	1,9
	Fusarium sp.	32,5-55 **42,5**	1,8	1,9
T.A. = total amount of DNA in each sample diluted in 100 μl of TE Buffer. The upper number indicates range of concentration, lower (highlighted with bold text) note the mean value.				

In our study, concentration of DNA largely depend on method rather than species of filamentous fungi. The average value were the same when using a specific method,

with the only exception of *Alternaria sp.* strains that were extracted by optimized CTAB method. Perhaps, this is due characteristic for some mycelial fungi. In addition, using a slightly acidic pH (5,3) lysis buffer allow to inhibit oxidative processes. The activity of enzyme and chemical reactions almost completely stop, as a result covalent bond is not formed between DNA and cell components. Extraction with chloroform by heating is promote the removal amount of impurities (polysaccharides, polyphenols and pigments).

The SDS method produced the lowest yields per sample – 32-33,1 μg, that's likely that no homogenization in a thermal lysis. The worst quality of the DNA (A260/A280 and A260/A230 ratios) obtained from this protocol, indicating that the samples have inhibitors or proteins and RNA. In contrast, the quality and quantity characteristics from the samples obtained with the commercial kits were high.

This research showed that lysis buffer composition does not significantly affect for DNA quality and quantity of *Alternaria sp.*and *Fusarium sp.* However, commercial kit DNeasy Plant Mini Kit (Qiagen) and acid CTAB-buffer (pH≤5) produced the highest quality of DNA, also both methods employ mechanical disruption of mycelium.

The costs associated with the DNeasy Plant Mini Kit not suitable for large quantities, while the CTAB-method reagents are common to labs that routinely conduct molecular studies.

Analysis of the quality DNA by PCR

DNA quality of the extracted samples was assessed by PCR amplification of the ITS region (Internal Transcribed Spacer) using universal primers such as ITS 1 and ITS 4 (figure 2).

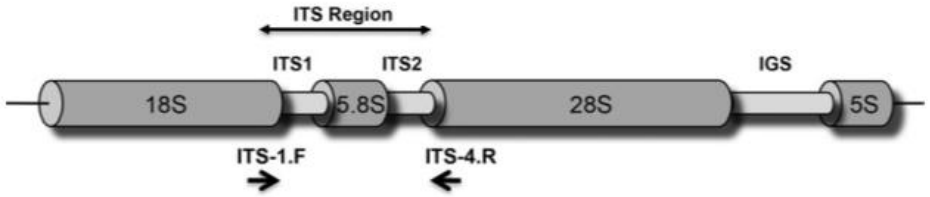


Fig. 2. Polymerase chain reaction (PCR) and sequencing primer sites.
Ribosomal DNA target locus of the ITS [24]

The ITS-primers most commonly used for PCR based on fragments encoding rRNA which are containing conserved and variable spacer sequences. Conserved regions allow for the design of primers of the ITS region or individual parts for the amplification. The rDNA-ITS region most commonly used for to study fungal identification by pyrosequencing and other methods. The correct identification is possible due from variation in PCR with ITS primers [24]. A band of approx. 550 bp corresponding to the ITS region was obtained with the amplification of the primers ITS1/ITS4.

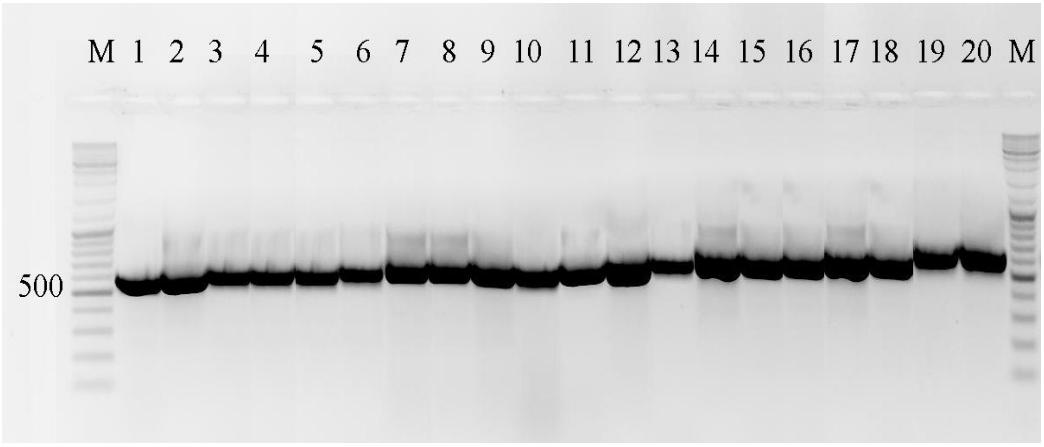


Fig. 3. PCR products amplified using ITS primers

ITS region were successfully amplified from all DNA samples isolated with four different protocols, that the quantity and quality of DNA was sufficient for PCR. PCR products of approximately 500 kb amplified with the ITS primer set using freshly prepared DNA on the DNA extraction using 4 different methods: 1-5 – SDS method, 6-10 – DNeasy Plant Mini Kit (Qiagen), 11-15 – Benjamin Schwessinger method, 16-20 – CTAB method.

Sequencing data were analyzed using Unipro UGene (Bowtie, UGENE Genome Aligner). A threshold of 100% similarity between the sequences from the isolates in this study and sequences from strains which were previously deposited in Genbank. The quality of DNA obtained with different methods was successfully generate amplification products.

ITS region can be used for sequencing and different molecular study. This studies shown that routine methods of extracting DNA from mycelial fungi are also effective as expensive kits. Cost per sample ranging from $2.56/sample to $5.93/sample [25].

The disadvantages of routine methods are a large number of purification steps during extraction with chloroform, but with these methods it is possible to obtain high yields of matrix DNA. But high yields are less importance, because only small amounts of DNA are needed for PCR. In this study we optimized the DNA extraction protocol suitable for PCR. The extraction takes one day (initial lysis steps to sequence analysis).

CONCLUSION

The results have shown that for extraction of genomic DNA from filamentous fungi *Alternaria sp*. and *Fusarium sp.* the most efficient, rapid and cost effective protocol is using acid CTAB with use of hot chloroform, this method capable of obtaining high-quantity and quality DNA from large numbers of samples, which suitable for PCR and other molecular biological experiments.

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