Comparison of Methods for Isolating Fungal DNA

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Abstract

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In this study methods of fungal DNA isolation were optimised and compared. The aim of the isolation processes was to obtain DNA of sufficient quality and quantity necessary for its amplification, as most detection techniques require DNA amplification before the proper DNA detection itself. For this purpose, classic methods of DNA extraction were compared and optimised while isolations using commercial kits were also done. The methods were evaluated from several perspectives, with focus especially laid on the isolated DNA not contain PCR inhibitors which would prevent DNA amplification, thus inhibiting the detection itself. For optimising the individual methods, collection strains of the genus *Aspergillus* were used. After the evaluation, two most suitable methods were selected and chosen for isolating potentially aflatoxigenic moulds taken from food samples. These methods were the commercially supplied kit for isolating DNA from plant leaves from Sigma and a classic method according Cenis in combination with the cell wall disruption by means of liquid nitrogen.

Keywords: fungal DNA; DNA isolation; PCR; *Aspergillus*

The genus *Aspergillus* is classified among filamentous microscopic fungi, which are among the most frequent food contaminants. From the viewpoint of human health, in particular food is a very suitable and potentially high-risk substrate for the colonisation, growth, and reproduction of toxigenic micromycetes and, consequently, for the production of mycotoxins. Aflatoxins are secondary metabolites produced especially by *A. parasiticus* and *A. flavus* (Bennett & Papa 1988; Mayer *et al.* 2003) strains, and also by *A. nomius* and *A. tamarii* (Goto *et al.* 1996). They can be found in various kinds of food and feedstuffs such as barley, wheat flour, maize, cereals (Villa & Markaki 2009), rice, beans, nuts and nut products, spices, and beer (Yang *et al.* 2004). As the micromycetes' macroscopic and microscopic features are variable and their analysis is time-consuming, molecular biological methods are used for determining toxigenic moulds. The molecular biological methods detect the differences in DNA among the individual types of micromycetes. Polymerase chain reaction (PCR) is a general method used most often. The basic matrix for PCR is template DNA, which is the genetic material of the microorganism.

To acquire DNA, cultures grown especially in liquid medium are used. Cultures grown on agar are scarcely ever used for direct isolation, as the inhibitors from the agar can influence DNA extraction (McCartney *et al.* 2003). The crucial problem is to find a method effective both from the perspective of both speed and sensitivity with

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small amounts of DNA from a limited number of cells. The most difficult steps in the isolation of *Aspergillus* DNA is to disrupt the cell wall without causing damage to genomic DNA (Bir *et al.* 1995). Most methods use mechanical or mechanical-physical techniques for the disruption of the cell wall, such as disruption with glass beads (van Burik *et al.* 1998; Haugland *et al.* 2002; Loeffler *et al.* 2002; Kabir *et al.* 2003; Somashekar *et al.* 2004), grinding in liquid nitrogen (Raeder & Broda, 1985; Shapira *et al.* 1996; Färber *et al.* 1997; Sweeney *et al.* 2000; Mayer *et al.* 2003; de Aguirre *et al.* 2004; Manonmani *et al.* 2005), mill grinding (Cenis 1992), alternating freezing and thawing (Griffin *et al.* 2002), ultrasound in combination with lysis buffer containing sodium dodecyl sulphate (SDS) (Van Burik *et al.* 1998; Zachová *et al.* 2003), and microwave radiation (Tendulkar *et al.* 2003). Enzymes can also be used for digesting the cell wall (Jin *et al.* 2004). Depending on the chemical composition of the cell wall, lysozyme, cellulose, or chitinase are used. The methods for cell wall disruption are often combined (Bir *et al.* 1995; Sandhu *et al.* 1995; Yuan *et al.* 1995; Kerenyi *et al.* 1999; Moody & Tyler 1999; Barros *et al.* 2007; Bakri *et al.* 2009). Protein digestion is mainly performed by proteinase K. SDS is almost a regular component of lysis buffers, and RNase A enzyme is often used for removing RNA (Atkins & Clark 2004). Phenol-chloroform extraction joined with isopropanol precipitation is often used (Shapira *et al.* 1996; Al-Samarrai & Schmid 2000; Jin *et al.* 2004; Manonmani *et al.* 2005). For washing and purifying DNA, isopropanol and ethanol can also be used (Griffin *et al.* 2002). These methods are time-consuming and require a high level of skill. In recent years, commercial kits have been used more frequently. Extra steps are still required initially to lyse the cell wall prior to purification, as fungal cell walls are extremely strong and difficult to lyse by traditional extraction techniques (Fredericks *et al.* 2005; Grubisha & Cotty 2009; Luo *et al.* 2009; Yamamoto *et al.* 2010).

The main aim of this work was to compare the methods of DNA isolation in the moulds of genus *Aspergillus* with special regard to the amount and purity of the DNA acquired, and to apply these to the potentially aflatoxigenic moulds isolated from various foods samples, in particular tea leaves, spices, and medicinal plants. The acquired DNA was then amplified by PCR and the product was detected electrophoretically.

MATERIAL AND METHODS

Strains used. The following strains were used: *A. parasiticus* CCM F-108 (until 2005 designated as *A. flavus* CCM F-108), *A. parasiticus* var. *globosus* CCM F-550, *A. nidulellus* CCM F-266, *A. niger* CCM 8189, *A. ochraceus* CCM F-803, *A. versicolor* CCM F-585, *Acremonium ochraceum* CCM F-365, *Cladosporium herbarum* CCM F-455, *Fusarium graminearum* CCM F-683, *Fusarium poae* CCM F-169, *Fusarium sporotrichioides* CCM F-164, *Mucor racemosus* CCM 8190 and *Penicillium chrysogenum* CCM 8034 from the Czech Collection of Microorganisms – CCM (Brno, Czech Republic).

The strains *A. flavus* CCF 3171, *A. flavus* CCF 3164, *A. flavus* CCF 3170, *A. flavus* CCF 3196, *A. parasiticus* CCF 141, *A. parasiticus* CCF 3137, *A. tamarii* CCF 3206 and *A. tamarii* CCF 3152 came from the Culture Collection of Fungi – CCF of the Department of Botany of the Faculty of Science of Charles University (Prague, Czech Republic).

Strains *A. flavus* var. *columnaris*, *Eurotium chevalieri*, *Eurotium repens*, *Penicillium diversum*, and *Rhizopus oryzae* were obtained from the collection of the Department of Biological and Biochemical Sciences of the University of Pardubice (Czech Republic). Isolates from real food samples were also used (bio teas, spices, herbs).

Stock cultures were kept inoculated on MEA (Malt Extract Agar) agar slants. After 3–5 days of aerobic cultivation at 30°C the strains were held at refrigerator temperature. The osmophilous moulds *Eurotium chevalieri* and *Eurotium repens* were inoculated on MY40 (Malt Yeast 40) agar slants. After 7–14 days of aerobic cultivation at 25°C, the strains were held at refrigerator temperature. The cultures were re-inoculated once every two months.

Preparation of fungal strains for DNA isolation. Mould strains of the *Aspergillus* genus were inoculated into 500 µl of potato dextrose broth in Eppendorf tubes. The tubes were incubated for 3 days at 30°C. After incubation, mycelium was filtered through a Büchner funnel, washed through twice with distilled water and again filtered dry. The mycelium prepared in this way was weighed into test tubes and DNA isolation followed. The amount of mycelium used for DNA isolation was weighed in the range of 1–100 mg.

Reagents and chemicals. The growth media for the preservation and growth of moulds came from Himedia (India). The primers were acquired from

Generi Biotech (Hradec Králové, Czech Republic), and the chemicals for the preparation of solutions and buffers were acquired from Sigma-Aldrich and Merck (Prague, Czech Republic).

Isolation according to Cenis (1992) – various means of disrupting the mycelium. To the mycelium were added 500 µl of TE buffer of pH 8.0, and after centrifugation (16 500× g for 5 min) and TE buffer removed, 300 µl of extraction solution (200mM Tris HCl at pH 8.5, 250mM NaCl, 25mM EDTA, 0.5% SDS) were added. The mycelium was ground using an Eppendorf micropestle (USA) and 150 µl of sodium acetate (3M, pH 5.2) was added. The test tube was held at –80°C for 10 minutes. After defrosting and centrifuging (16 500× g for 5 min), the supernatant was transferred to another test tube and an equimolar amount of isopropanol was added to it. After 5 min of incubation at room temperature, the supernatant was centrifuged (16 500× g for 15 min) and the resulting DNA was washed with by 300 µl of 70% ethanol. Again after centrifuging (16 500× g for 15 min) and of ethanol removal, DNA was dried at 100°C and dissolved in 50 µl of TE buffer. DNA thusly prepared was used for the PCR.

The methods of cell wall disruption were also tested using liquid nitrogen and using ultrasound were tested. In the former case, the mycelium was frozen with liquid nitrogen and ground using a mortar and pestle. Then it was weighed into test tubes, and the isolation of DNA according to Cenis (1992) followed by the addition of 300 µl of extraction solution, followed by 150 µl of 3M sodium acetate. The test tube was then stirred and held at –80°C for 10 minutes. The following method was identical with that just described.

In the case of disrupting the mycelium by ultrasound, after adding the extraction solution, the test tube was placed into an ultrasonic bath for 30 minutes. The following steps were identical.

Isolation using commercially available solutions and kits. Fungal DNA was isolated using a commercially available solution of DNAzol®ES (Molecular Research Center, Inc., Cincinnati, USA), a kit designated for isolating DNA from plant leaves (REDExtract-N-Amp™ Plant PCR kit; Sigma-Aldrich, St. Louis, USA), a kit designated for isolating DNA from plant seeds (REDExtract-N-Amp™ Seed PCR kit; Sigma-Aldrich, St. Louis, USA), a kit designated for isolating DNA from plants (Nucleospin® Plant kit; Macherey-Nagel, Düren, Germany), a kit designated for isolating DNA from type-II plants (GenElute Plant Genomic DNA Miniprep Kit; Sigma-Aldrich, St. Louis, USA) and a kit designated for isolating DNA from food (Nucleospin® Food kit; Macherey-Nagel, Düren, Germany). Also the Phire® Plant Direct PCR kit (Finnzymes, USA) was tested. When isolating DNA, the instructions of the manufacturers were followed.

Determining DNA concentration

Spectrophotometric detection of DNA concentration. The acquired DNA was diluted (1 µl of DNA + 99 µl of 1× TE buffer), and the absorbance at the wave lengths of 260 nm and 280 nm was measured with a spectrophotometer. The buffer in which the DNA was dissolved during the isolation was always used as a blank (i.e. most frequently the TE buffer) which might be replaced with distilled water. DNA purity was determined from the absorbance ratio A_{260}/A_{280}.

Fluorometric detection of DNA concentration using PicoGreen solution. DNA concentration was detected fluorometrically using PicoGreen fluorescent stain, which was a component of the Quant-iT™ PicoGreen® Assay kit. For measuring DNA samples, always 100 µl of diluted DNA (1 µl of DNA + 99 µl of 1× TE buffer) was pipetted, 100 µl of PicoGreen (200× diluted in 1× TE buffer) was added and stirred. The microtitration plate was then incubated for 5 min at room temperature in the dark. After incubation, fluorescence was measured with the Tecan Infinite M200 fluorometer with excitation maximum set at 480 nm and emission maximum at 520 nm. DNA concentration was calculated from the calibration curve, with each sample and individual points on the calibration curve being measured five times.

Determining maximum amplifiable dilution of DNA. DNA was diluted 10×, 100×, 1000×, and, when a large initial amount of DNA was acquired, also 10 000×. DNA thus diluted was used for PCR. After PCR, the separation of the PCR product followed, and the lowest concentration of DNA providing a visible band after electrophoresis was determined as the maximum amplifiable dilution of DNA (i.e. such dilution revealing a positive reaction after DNA amplification).

Semi-quantitative determination on agarose gel. The acquired DNA was amplified by PCR, the product was separated in 1% agarose gel stained with ethidium bromide, and the detection was

performed using UV light. DNA concentration was determined by comparing the intensity of the bands with the quantitative markers pBR322 DNA/*Alu*I and ΦX174 DNA/BsuRI (HaeIII).

PCR and detection of PCR product by electrophoresis. Three genes were selected for PCR: *apa-2*, *ver-1* and *aflR*, the regulatory gene of aflatoxin B1 biosynthesis. For PCR involving *apa-2* and *ver-1* (which were amplified simultaneously), the method according to Shapira *et al.* (1996) was used as modified by Zachová *et al.* (2003). For the regulatory gene *aflR*, the method according to Sweeney *et al.* (2000) was used. The primers used for DNA amplification are listed in Table 1. The sequences of primers VER 496, VER 1391, APA 450, and APA 1482 were taken from Shapira *et al.* (1996), while those of AFLR 620 and AFLR 1249 from Sweeney *et al.* (2000).

The typical reaction mixture for PCR consisted of 1 µl of template DNA, 2.5 µl of reaction buffer (10× diluted), 2.0 µl of dNTP mix, 1.5 µl of $MgCl_2$ (1.5 mmol/l), 0.25 µl of each of the primers (1µM each), 0.25 µl of Thermo-Start DNA polymerase (1.25 U; ABgene, Epsom, UK) and PCR water to make the total volume of 25 µl. DNA amplification was performed in a thermal cycler (Robocycler 2000 Gradient; Perkin-Elmer, Massachusetts, USA). The initial denaturation was conducted at 95°C for 10 min, followed by 30 reaction cycles (denaturation at 95°C for 1 min, annealing at a temperature depending on the primers used for 2 min, extension at 72°C for 2 min), and final extension at 72°C for 5 minutes. For each PCR, positive and negative controls were included.

Detection of PCR product

PCR product was separated in 1% agarose gel stained with ethidium bromide in the medium of 1× TBE buffer. Electrophoresis was continued for 50 min at 100 V. The detection was performed using UV light at 300 nm and Bio-Capt software. The size of the fragments was determined by comparison with DNA marker 155–970 pb, DNA marker 200–1500 pb, and quantitative markers pBR322 DNA/*Alu*I and ΦX174 DNA/BsuRI (HaeIII).

RESULTS AND DISCUSSION

The main criteria for evaluating the methods used in DNA isolation for potentially aflatoxigenic moulds were the purity and amount of the DNA acquired. The isolation methods should provide a sufficient amount of pure DNA which can be further amplified by PCR while not containing inhibitors at the same time inhibitors which would block PCR (Olexová *et al.* 2004). For this purpose, the classic method of DNA extraction according to Cenis (1992) was optimised and compared with the isolations using commercial kits. A total of six commercially available kits were used for detecting moulds of the genus *Aspergillus*. These kits were designated for DNA extraction from plant leaves, seeds, from types I and II plants, and from food. We based the selection of the kits on the presumption of a similar composition of the (plant) cell wall, and we also took into account the possible matrixes for the occurrence of potentially aflatoxigenic moulds (seeds, food, plants). Also tested was the Phire® Plant Direct PCR kit, which serves for DNA detection from plant leaves and seeds without actual DNA amplification being preceded by its isolation. With all the methods tested, the influence of the initial cell wall disruption (using liquid nitrogen, Eppendorf micropestle and ultrasound) was monitored. The amount of DNA acquired was always related to the original amount of mycelium used for DNA isolation. Each method for DNA isolation was simultaneously used for the various species of the genus *Aspergillus*,

Table 1. List of primers used for DNA amplification

Primer	Sequence (5' → 3')	Length (pb)
VER 496	ATG TCG GAT AAT CAC CGT TTA GAT GGC	895
VER 1391	CGA AAA GCG CCA CCA TCC ACC CCA ATG	
APA 450	TAT CTC CCC CCG GGC ATC TCC CGG	1032
APA 1482	CCG TCA GAC AGC CAC TGG ACA CGG	
AFLR 620	CGC GCT CCC AGT CCC CTT GAT T	630
AFLR 1249	CTT GTT CCC CGA GAT GAC CA	

Table 2. Comparison of methods used for determining DNA concentration (in μm/g) and purity

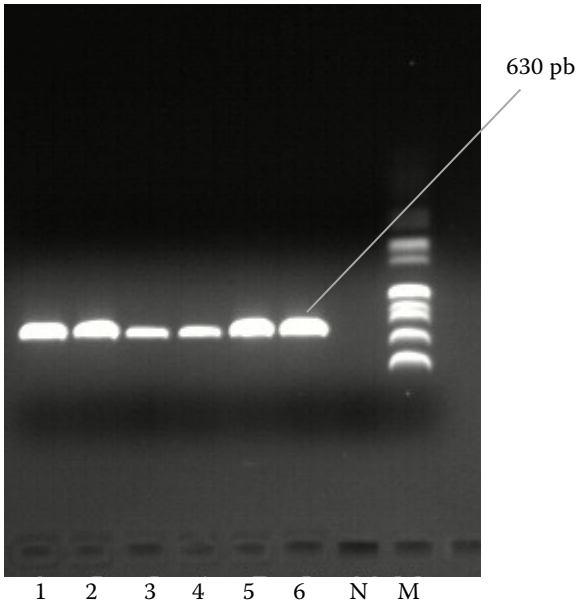
		1a		1b		1c		2		3		4		5		6	
		c	A_{260}/A_{280}	*c*	A_{260}/A_{280}	*c*	A_{260}/A_{280}	*c*	A_{260}/A_{280}	*c*	A_{260}/A_{280}	*c*	A_{260}/A_{280}	*c*	A_{260}/A_{280}	*c*	A_{260}/A_{280}
A. flavus CCF 3171	DNA spectrophotometrically	1057 ± 25	1.98 ± 0.05	1251 ± 17	1.85 ± 0.05	423 ± 16	1.84 ± 0.03	1068 ± 29	1.92 ± 0.05	948 ± 31	1.75 ± 0.02	827 ± 21	1.42 ± 0.04	561 ± 44	1.49 ± 0.02	367 ± 23	1.52 ± 0.02
	DNA fluorometrically	853 ± 22	–	961 ± 22	–	289 ± 25	–	1021 ± 27	–	926 ± 25	–	652 ± 19	–	495 ± 31	–	418 ± 31	–
	maximum amplifiable dilution	10^2	–	10^3	–	10^1	–	10^4	–	10^4	–	10^3	–	10^2	–	10^2	–
A. parasiticus CCF 3137	DNA spectrophotometrically	993 ± 25	1.72 ± 0.03	1033 ± 25	1.80 ± 0.03	515 ± 15	1.89 ± 0.02	923 ± 21	1.75 ± 0.05	1011 ± 19	1.91 ± 0.03	1005 ± 25	1.58 ± 0.05	778 ± 38	2.06 ± 0.02	554 ± 20	1.60 ± 0.01
	DNA fluorometrically	795 ± 20	–	897 ± 28	–	345 ± 21	–	855 ± 27	–	853 ± 26	–	811 ± 22	–	648 ± 11	–	711 ± 17	–
	maximum amplifiable dilution	10^2	–	10^3	–	10^2	–	10^3	–	10^3	–	10^3	–	10^3	–	10^3	–
A. flavus CCF 3170	DNA spectrophotometrically	942 ± 15	1.98 ± 0.02	977 ± 20	1.94 ± 0.04	306 ± 15	1.78 ± 0.04	1004 ± 19	1.81 ± 0.05	615 ± 25	1.86 ± 0.03	610 ± 19	1.29 ± 0.03	496 ± 36	1.69 ± 0.04	748 ± 33	1.71 ± 0.03
	DNA fluorometrically	867 ± 22	–	957 ± 19	–	338 ± 20	–	969 ± 26	–	584 ± 18	–	495 ± 25	–	523 ± 21	–	659 ± 19	–
	maximum amplifiable dilution	10^3	–	10^4	–	10^1	–	10^3	–	10^2	–	10^2	–	10^3	–	10^3	–
A. parasiticus CCM F-108	DNA spectrophotometrically	1057 ± 18	2.01 ± 0.02	1078 ± 11	1.77 ± 0.05	572 ± 32	1.95 ± 0.03	1118 ± 33	1.59 ± 0.05	719 ± 22	1.66 ± 0.02	744 ± 35	1.30 ± 0.02	868 ± 19	1.77 ± 0.05	557 ± 21	1.37 ± 0.03
	DNA fluorometrically	851 ± 29	–	869 ± 24	–	419 ± 33	–	890 ± 20	–	851 ± 31	–	654 ± 24	–	712 ± 35	–	482 ± 28	–
	maximum amplifiable dilution	10^2	–	10^3	–	10^2	–	10^3	–	10^3	–	10^3	–	10^3	–	10^2	–
A. parasiticus var. *globosus* CCM F-550	DNA spectrophotometrically	1021 ± 32	1.88 ± 0.04	1043 ± 22	1.72 ± 0.04	584 ± 27	1.68 ± 0.02	932 ± 12	1.73 ± 0.05	757 ± 40	1.81 ± 0.04	693 ± 25	1.61 ± 0.03	512 ± 15	1.64 ± 0.02	770 ± 14	1.44 ± 0.04
	DNA fluorometrically	903 ± 19	–	1025 ± 29	–	474 ± 16	–	841 ± 27	–	589 ± 10	–	560 ± 21	–	569 ± 27	–	645 ± 26	–
	maximum amplifiable dilution	10^3	–	10^4	–	10^2	–	10^2	–	10^3	–	10^2	–	10^3	–	10^3	–

1a – method according to Cenis + Eppendorf micropestle; 1b – method according to Cenis + liquid nitrogen; 1c – method according to Cenis + ultrasound; 2 – DNA isolation using the compound DNAzol® ES (liquid nitrogen); *c* (μg/g) – amount of acquired DNA per g of mycelium; 3 – REDExtract-N-Amp™ Plant PCR kit (Sigma-Aldrich); 4 – Nucleospin® Plant kit (Macherey-Nagel); 5 – GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich); 6 – Nucleospin® Food kit (Macherey-Nagel); *c* (μg/g): amount of acquired DNA per g of mycelium

for reasons of the varying extractability of their DNA. The diverse results in extraction of DNA among the individual strains and species of moulds were pointed out by Guo *et al.* (2005).

In our study, four methods were used to evaluate DNA concentration: spectrophotometric detection, fluorometric detection using PicoGreen compound (Ahn *et al.* 1996; Haque *et al.* 2003; Nicklas & Buel 2003), determination of the maximum amplifiable dilution of DNA (Olexová *et al.* 2004), and semi-quantitative determination of the amount of DNA by staining with ethidium bromide and evaluation on agarose gel.

DNA that could be to amplified was acquired using almost all the methods for isolating DNA (except for REDExtract-N-Amp^{TM} Seed PCR kit, the results of which are not included in Table 2). Phire® Plant Direct PCR kit could not be evaluated from the viewpoint of concentration and purity, as the mycelium was part of the PCR reaction mixture. A problem with false negative reaction occurred only in the case of using a large initial amount of material for DNA isolation with the method according to Cenis (1992). The mycelium of the *Aspergillus* moulds was disrupted using Eppendorf micropestle, ultrasound, and liquid nitrogen. In Figure 1, the positive reaction for the amplification of the *aflR* gene (intensive bands of the product caused by multiplication of the segment limited by a pair of primers) can be seen, specifically for the mould species *Aspergillus parasiticus* CCM F-108. Due to the equal amounts of the initial material for DNA isolation, it can be stated merely from the sharpness and visibility of the zones after electrophoretic separation that the cell wall disruption by liquid nitrogen and Eppendorf micropestle was much more efficient than the cell wall disruption by ultrasound. This fact is also documented by Table 2, which presents the values of DNA concentration for spectrophotometric measurement, fluorometric measurement, and for maximum amplifiable dilution. The capacity for DNA amplification was verified by PCR with primers complementary to the DNA sequence sought. After electrophoresis, the lowest concentration of DNA providing a positive reaction was determined. As stated by Olexová *et al.* (2004), if DNA is isolated for the following use in PCR, the quality of DNA used is best characterised by the concentration of the amplifiable amount of DNA. The amount of DNA acquired by ultrasound homogenisation was not large, and there is a danger of the occurrence of false negative findings. One of the possibilities is the absence of DNA in the PCR reaction mixture due to the insufficient cell wall disruption. Another possible cause is DNA loss during the purification steps, if the amount of DNA is small. Moreover, when using ultrasound, the isolation time is increased by at least 30 minutes. A low extraction of DNA when using ultrasound was also described by van Burik et al. (1998). In contrast, liquid nitrogen allows thorough cell wall disruption of moulds in a much shorter time. However, such thorough cell wall disruption using liquid nitrogen and Eppendorf micropestle can, in some cases, also lead to false negative results. The reason for this is imperfect removal of mycelium during the extraction steps. The remaining mycelium can then act as an inhibitor of DNA amplification. This suspicion was confirmed when processing a large amount of the initial material (100 mg of mycelium), as the acquired DNA lysate needs to be diluted to achieve positive results. The acquired amount of DNA varied according to the method used for the initial cell wall disruption and decreased in



1, 2 – cell wall disruption by Eppendorf micropestle; 3, 4 – cell wall disruption by ultrasound; 5, 6 – cell wall disruption by liquid nitrogen; N – negative control; M – DNA marker (155–970 pb)

Figure 1. Electrophoresis output for PCR products for DNA of the collection culture Aspergillus parasiticus CCM F-108 (isolation according to Cenis with various types of mycelium disruption; amplification of the gene aflR)

the following order: liquid nitrogen > Eppendorf micropestle > ultrasound.

Very good results concerning DNA quality and quantity were provided by the isolation procedure using the commercially available compound DNAzol®ES (Table 2). DNAzol®ES was tested according to the publication by Guo *et al.* (2005). Those authors have used DNAzol®ES for isolating DNA from 25 species of moulds and compared the method with the DNA isolation method according to Cenis (1992). With almost all species of moulds (23 out of 25 species), the use of DNAzol®ES yielded DNA of higher purity and in 17 cases also the acquired amount of DNA was higher compared to the method according to Cenis (1992). The isolation using this commercially available compound is based on the presence of guanidine in the lysing solution, followed by chloroform extraction of DNA precipitated by ethanol. The results we obtained are in accordance with the results published by Guo *et al.* (2005), i.e. better results were achieved in isolating DNA from the potentially aflatoxigenic mould of the genus *Aspergillus* than in isolating DNA with the use of the classic method according to Cenis (1992). As true with the Cenis method, the acquired amount of DNA varied according to the method used for the initial cell wall disruption and decreased in the same order as in that method. It is apparent from the results that the method of cell wall disruption influenced the resulting amount of DNA acquired also when a commercially available compound was used. The use of DNAzol®ES is among the fastest methods, enabling to acquire DNA from the sample within 90 minutes.

Among the fastest methods was using the kit designated for DNA isolation from plant leaves (REDExtract-N-Amp™ Plant PCR kit). DNA was acquired from the sample within 15 minutes. The same company kit designated for isolating DNA from plant seeds was also tested (REDExtract-N-Amp™ Seed PCR kit). Although these kits for DNA isolation were very similar, DNA isolation using the kit for seeds was unsuccessful. The cause is apparently the excessive effectiveness of the extraction and solution preparation. Seeds have stronger cell walls than moulds, and the highly effective solutions probably disrupted the DNA that was released.

Mycelium disruption using the kit designated for DNA isolation from type-I plants (Nucleospin® Plant kit) is based on the use of extraction buffer containing chaotropic salts (CTAB) and mixtures of detergents. Contaminants such as polysaccharides, plant fibres and other components are removed by centrifugation of the mixture through columns. The isolated DNA is bound to a silicate membrane and gradually washed by purification solutions. After washing, the elution of DNA into a clear microcentrifuge tube follows. After DNA extraction, the kit uses two lysing solutions, C0 and C1. Solution C0 uses a mixture of detergents and CTAB for cell wall disruption, while solution C1 uses only a solution of CTAB. CTAB buffer for isolating mould DNA (the so-called CTAB method) was also used in the work by van Burik *et al.* (1998). CTAB is a cation-active surfactant assisting in both cell wall disruption and polysaccharides removal, the latter ones being PCR inhibitors. DNA isolation was successful when using all of the aforementioned options for moulds cell wall disruption, and also when using both tested buffers. The total isolation time was approximately 60 minutes. In using the Nucleospin® Plant kit, the isolated DNA was insufficiently pure (Table 2), although even this level of purity was sufficient for the amplification using PCR. Another kit from Macherey-Nagel recommended for isolating DNA from food is also based on the effect of chaotropic salts, detergents and proteinase K present in the extraction buffer with the following binding of the isolated DNA on silica columns. The isolation through silica columns is also used by GenElute Plant Genomic DNA Miniprep Kit.

The isolation of DNA using kits required from 1 h to 2 h (except for REDExtract-N-Amp™ Plant PCR kit). Using classic methods, DNA was also obtained after 2 hours. Also confirmed was the presumption that the extractability of DNA would vary for the individual species of potentially aflatoxigenic moulds of the genus *Aspergillus* (Table 2). With regard to the cost, the use of commercially provided sets is today almost comparable with the expenses for the classic methods. In contrast to the classic methods, however, all the stated steps necessary for acquiring DNA have been optimised, and thus the risk of losing DNA during the purification steps is eliminated. The only risk which could arise in using the kits is the occurrence of PCR inhibitors when isolating DNA from some matrixes, as is suggested in the work of Fleischmann and Heubl (2009). This problem in using commercially available kits did not, however, occur during our study on DNA isolation from potentially aflatoxigenic moulds.

Table 3. PCR results for genes *ver-1*, *apa-2* and *aflR*

Cultures and isolates of genus *Aspergillus*	*ver-1*	*apa-2*	*aflR*
A. parasiticus CCM F-108	+	+	+
A. parasiticus var. *globosus* CCM F-550	+	+	+
A. nidulellus CCM F-266	–	–	–
A. niger CCM 8189	–	–	–
A. ochraceus CCM F-803	–	–	–
A. versicolor CCM F-585	–	–	+
Cladosporium herbarum CCM F-455	–	–	–
Fusarium graminearum CCM F-683	–	–	–
Fusarium poae CCM F-169	–	–	–
Fusarium sporotrichioides CCM F-164	–	–	–
Mucor racemosus CCM 8190	–	–	–
Penicillium chrysogenum CCM 8034	–	–	–
A. flavus CCF 3171	+	+	+
A. flavus CCF 3164	+	+	+
A. flavus CCF 3170	+	+	+
A. flavus CCF 3196	–	–	–
A. parasiticus CCF 141	+	+	+
A. parasiticus CCF 3137	+	+	+
A. tamarii CCF 3206	–	–	–
A. tamarii CCF 3152	–	–	–
A. flavus var. *columnaris*	+	+	+
Eurotium chevalieri	–	–	–
Eurotium repens	–	–	–
Penicillium diversum	–	–	–
Rhizopus oryzae	–	–	–

+ positive result; – negative result

After DNA isolation, the amplification of DNA by PCR followed. In total, three genes were amplified: *apa-2* and *ver-1* simultaneously, and the regulatory gene *aflR* separately.

For the regulatory gene *aflR*, the method according to Sweeney et al. (2000) was used, as optimised by Brožková (2007). The optimisation consisted in finding the optimal annealing temperature (59°C) and concentration of magnesium ions in the reaction (2.5mM). After performing electrophoresis, the product corresponding in the size to 630 pb was recorded. Positive reaction for the presence of the *aflR* gene was also obtained for *Aspergillus versicolor* CCM F-585. It produces the metabolites sterigmatocystin and versicolorin, which are intermediate products in the biosynthesis of aflatoxin B1. For this reason, *Aspergillus versicolor* is classified among potentially toxigenic moulds (Engelhart *et al.* 2002; Ehrlich *et al.* 2005). The results of PCR specificity for all three aforementioned genes are shown in Table 3.

CONCLUSION

In this study, the methods for isolating DNA of potentially aflatoxigenic moulds of the genus *Aspergillus* were compared from the following perspectives: the purity of DNA acquired, amount of DNA, speed of the isolation method, equipment and cost demands. A total of eight methods for isolating DNA were introduced and optimised. These included especially commercially available kits, but also the classic method of DNA isolation was compared using various kinds of cell wall disruption. Eppendorf micropestle, liquid nitrogen and ultrasound were used for cell wall disruption. It was demonstrated that the method of cell wall

disruption considerably influences the effectiveness of DNA isolation, the effectiveness of the cell wall disruption decreasing in the following order: liquid nitrogen > Eppendorf micropestle > ultrasound.

On the basis of the results obtained, two most suitable methods for isolating DNA of *Aspergillus* moulds were selected. From the commercially available sets, it was the kit for isolating DNA from plant leaves from Sigma. Using this kit, DNA was isolated without proper homogenisation of the cell wall. The use of that kit was also the fastest method, as DNA was extracted within 15 minutes. The other successful method was the classic method according to Cenis in combination with cell wall disruption by means of liquid nitrogen.

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