Assessment of DNA contents of soil fungi

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Abstract

DNA extractions from microorganisms under soil conditions have been published to a great extent during the last decade. A quantitational approach was, however, rarely attempted so that until today there is hardly a data source available which gives information on DNA contents of microorganisms, i.e. fungi, on a dry weight basis. This is however a very necessary aspect, particularly when an extraction protocol per se should be tested for its efficacy or particularly as well in ecological studies, where it would be important to have an unbiased extraction procedure. That is, the procedure used should be optimal for DNA extraction of both bacteria and fungi to the same extent. Since the majority of extraction protocols used today were designed to extract DNA from bacteria, it was found necessary to examine our own extraction procedure (Blagodatskaya et.al., 2003) for its applicability to extract DNA from soil fungi.

DNA extractions from 25 soil fungal species resulted in a mean dsDNA recovery of 3.3 µg mg^{-1} dry weight with a range of 0.92 µg mg^{-1} to 6.32 µg mg^{-1}. However, a modification of the original extraction procedure by omitting aurintricarboxylic acid (ATA) but adding a Glucanex-lyticase enzyme complex which was tested on ten fungal species resulted in a doubling of dsDNA yields with a mean of 6.45 µg mg^{-1} dry weight (range 4.2 µg mg^{-1} to 8.7 µg mg^{-1} dry weight), whereby eight of the ten species gave DNA yields above 6.0 µg mg^{-1} dry weight, which indicates that in our study the DNA yields were very much comparable between species. No apparent relationship existed between the innate growth rate of fungal species and DNA yields obtained; but yields decreased with the age of the fungal cultures.

As investigated by video-fluorescence microscopy a complete hyphal disintegration did not seem necessary for obtaining a high DNA yield but rather the rapidity of the extraction process together with the miniaturization of the samples promoted a high recovery.

Keywords: soil fungi, dsDNA extraction, lytic enzymes

Zusammenfassung

Bewertung der DNA-Gehalte von Bodenpilzen

DNA Extraktionen von Mikroorganismen unter Bodenbedingungen sind während der letzten zehn Jahre vermehrt publiziert worden. In den meisten Fällen wird keine quantitative Aussage von DNA-Gehalten der Organismen auf Trockengewichtsbasis gegeben. Dieses ist aber ein sehr notwendiger Aspekt, wenn es darum geht das eigene Protokoll auf die Extraktionsgüte zu überprüfen und ebenso ein wichtiger Aspekt in ökologischen Untersuchungen, wo es nicht wünschenswert wäre, wenn das Extraktionsprotokoll bevorzugt nur DNA aus Bakterien oder nur Pilzen extrahieren würde. Da die Mehrheit der heutigen Extraktionsprotokolle zur DNA-Extraktion für Bakterien entwickelt wurde, erschien es notwendig, unser eigenes Extraktionsprotokoll (Blagodatskaya et al., 2003) dahingehend zu überprüfen, inwieweit es sich für die DNA-Extraktion von Pilzen eignet.

DNA-Ausbeuten von 25 Pilzarten lagen bei 3.3 µg mg^{-1} Trockengewicht (TG) in einer Bandbreite von 0.92 µg mg^{-1} bis 6.32 µg mg^{-1}. Dieses ist ein befriedigendes Ergebnis verglichen mit DNA-Gehalten aus Pilzen unter Reinkultur. Eine Modifizierung des Extraktionsprotokolls durch Weglassen von Aurintricarboxylsäure (ATA) und Zufügen von einem Glucanex-Lyticase Komplex ergab eine Verdopplung der Ausbeuten an dsDNA mit einem mittleren Wert, der bei 6.45 µg mg^{-1} TG lag. Dabei ergaben acht von den zehn hier getesteten Arten eine Ausbeute über 6.0 µg mg^{-1}. Dies deutet darauf hin, dass in unseren Untersuchungen die DNA-Werte der Pilze sehr gut vergleichbar sind. Es wurde kein Zusammenhang zwischen der Wachstumsrate der Arten und der potentiellen DNA Ausbeute gefunden; jedoch wurde die Ausbeute mit dem Alter der Organismen geringer.

Über Video-Fluoreszenzmikroskopie konnte gezeigt werden, dass eine komplette Desintegration der Hyphen nicht notwenig war, um hohe DNA-Ausbeuten zu erhalten; eher ist die Schnelligkeit des Extraktionsprozesses zusammen mit der Miniaturisierung der Proben ausschlaggebend für hohe Ausbeuten.

Schlüsselwörter: Bodenpilze, dsDNA Extraktion, lytische Enzyme

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1 Introduction

The possibility to extract DNA directly from soil microbial communities has initiated new approaches in microbial ecology for nearly two decades now. Methods such as the polymerase chain reaction are used for the detection and monitoring of microorganisms in soil microbial communities (Holben et al., 1988; Harris, 1994; see also discussion Ogram, 2000), for description of the community composition with respect to the ratio of fungal to bacterial DNA in soil (Harris, 1994) or as an additional sum parameter for estimating microbial biomass or its growth in situ (Marstorp and Witter,1999; Blagodatskaya et al., 2003; Joergensen and Emmerling, 2006). Growth rates are traditionally used in microbial physiology to quantify the response of microbial cells to its environment (Pirt, 1975). It is one of the most sensitive microbial parameters for the detection of detrimental or positive impacts on microbial cells. Principally this approach could be adapted to microbes under soil condition, i.e. agricultural soils, for studying the effects of pesticides, heavy metal toxicity, pollutants, or global change effects on microbial communities (total biomass of bacteria and fungi). This requires optimal extraction of soil DNA. Published extraction procedures vary widely, but for more than a decade now DNA extraction by direct lysis methods (Ogram, 1987; Tien et al., 1999; Ogram, 2000; Robe et al., 2003) are most commonly applied. The cell lysis protocol which we adopted for soil extraction (Blagodatskaya et al., 2003) was based on publications by Sandaa et al. (1997) who introduced the fluorometric detection of dsDNA using PicoGreen® (Molecular Probes, Inc.) as a fluorescent dye and several published sources with respect to lysis procedures (Ogram, 1987; Sayler et al., 1991; Clegg et al., 1997; Marstorp and Witter, 1999). As an alternative to the often used freeze-thawing cycle procedure for physical disruption, we adopted a bead-beating method, a method most commonly used now with reports on superior DNA yields (Moré et al., 1994; Tanaka et al., 1996; van Burik et al., 1998; Müller et al., 1998; Haugland et al., 1999; Reeleder et al., 2003; Fredricks et al., 2005; Griffiths et al., 2006).

The reason for reconsidering the above techniques is that fungi have a more rigid cell wall than bacteria. The possibility that a lysis protocol could favour bacterial DNA extraction existed. Tien et al. (1999) presented evidence that a direct lysis procedure might not extract fungal DNA. A similar observation was mentioned recently by Fierer et al. (2005). Since our final goal is to quantify total microbial biomass growth in soil we found it necessary to check if the lysis protocol used would as well be optimal for DNA extraction from fungi. In total twenty-five fungal species belonging to different taxa were analysed using the direct lysis method (Blagodatskaya et al., 2003).

Here we report on the optimization of the extraction protocol and comparisons of fungal DNA yield with respect to the pertaining literature. In addition, aspects on the physical disruption of cell walls as analyzed by video-fluoreszence microscopy or innate growth rates of fungal species and DNA yields are discussed.

2 Materials and methods

2.1. Fungal species, culture conditions and harvesting procedure

Strains of fungal species used in this study and their sources are given in Table 1. Stock cultures were maintained on Martin`s agar as slant cultures and stored at 4 °C until use. For production of mycelium for DNA extraction, small pieces of fungal slant cultures were transferred to 200 ml fresh sterile modified Czapek-Dox liquid medium with yeast extract in 250 ml Erlenmeyer flasks and adjusted to pH 7.0. Per fungus and experiment three to five replicate cultures were set up. The flasks were kept at room temperature (20 - 25 °C) on an orbital shaker (Infors AG) at 100 rev min^{-1} in daylight. Mycelium of replicate cultures was harvested and combined by vacuum filtration using a Buchner funnel with sterile glass fibre filters GF 6 (Schleicher & Schuell). Mycelia was placed in Falcon tubes under sterile conditions and washed twice with 0.2 % sodium hexametaphosphate by centrifugation (5000 rev. min^{-1} for 10 min) to remove media sticking to the hyphae. The pellet was transferred into a new Falcon tube under sterile conditions containing sterile TE buffer (10 mM Tris/1 mM EDTA, SIGMA) in the ratio mycelia:TE buffer (1:5 w/v). The pellet was macerated with an Ultra-Turrax® (IKA) (~18.000 rev. min^{-1}) for one minute with cooling on ice. Shaft and rotor of the dispersing device had been cleaned beforehand with 70 % ethanol and sterile HPLC grade H_2O. The mycelium was again harvested by centrifugation (5000 rev. min^{-1} for 10 min) and the pellet collected was blotted dry with sterile paper towels.

Mycelium dry weight was assessed by pre-weighted glass dishes plus mycelium and then oven-dried (105 °C, 24 h) and again weighed.

2.2. Extraction of fungal DNA

2.2.1. Lysis protocol I

25 - 100 mg mycelium (wet weight) was transferred to 2.0 ml sterile polypropylene microfuge tubes (Neo-Lab) which contained in total 600 mg acid-washed glass beads (SIGMA), 200 mg of each size (106 µm, >212 µm, >710 µm). In total, one ml lysis solution TE buffer (50 mM/ 5mM) was added: containing 1 % (10 mg) sodium

Table 1:
DNA recovered from soil fungal mycelium with lysis protocol I

Species	Strain[a] no.	Age of culture (days)	dsDNA µg mg^{-1} (dry weight)	Mean growth rate[b] (cm/day)
Alternaria spec. Nees	F1	6	5.34 ± 0.16	0.73
Aspergillus niger van Tieghem	F7	4	4.51 ± 0.37	0.65
	F7	14	2.83 ± 0.31	
	F7	35	1.12 ± 0.12	
Botrytis allii Munn	F14	19	4.00 ± 0.27	0.53
Cladosporium cucumerinum Ellis & Arth.	F85	26	1.58 ± 0.05	0.29
	F85	34	1.14 ± 0.07	
Cladosporium herbarum (Pers.) Link ex Gray	F86	19	2.41 ± 0.12	0.33
	F86	35	0.79 ± 0.10	
Fusarium culmorum (W.G. Smith) Sacc.	F94	7	3.01 ± 0.04	1.20
	F94	25	1.93 ± 0.09	
Fusarium oxysporum Schlecht.em. Sny. & Hans.	DSMZ/2018	10	3.81 ± 0.12	1.12 [c]
Hormodendrum olivaceum (Corda) Bon.	F52	7	1.77 ± 0.02	0.41
Linderina pennispora Raper & Fennel	Z13	21	0.92 ± 0.04	0.26
Mortierella ramanniana (Möller) Linnem.	Z16	4	5.65 ± 0.50	0.43
	Z16	12	3.59 ± 0.14	
	Z16	35	1.55 ± 0.15	
Mucor flavus Bain.	Z24	12	4.44 ± 0.43	0.33
Mucor mucedo Mich. ex St.-Am.[#]	Z26	3	2.80 ± 0.18	1.42
Oidiodendron rhodogenum Robak	F55	6	4.97 ± 0.11	0.14
Paecilomyces lilacinus (Thom) Samson	F56	12	5.00 ± 0.41	0.51
Penicillium camembertii Thom	F61	14	2.45 ± 0.16	0.45
Penicilium funiculosum Thom	F90	8	5.70 ± 0.40	0.45
	F90	15	3.47 ± 0.28	
Penicillium notatum Westling	F66	8	6.32 ± 0.27	0.20
	F66	21	2.19 ± 0.06	
Penicillium notatum (Fleming) Westling	F65	8	4.92 ± 0.01	0.34
	F65	13	3.56 ± 0.26	
Pythium ultimum Trow	DSMZ/62987	5	4.75 ± 0.02	3.0 [c]
Rhizoctonia solani Kühn	DSMZ/63010	36	2.12 ± 0.04	> 2.8 [c]
Stachybotris chartarum (Ehrenb. ex Link) Hughes	F73	8	3.15 ± 0.10	0.15
Trichoderma harzianum Rifai	DSMZ/63059	8	2.60 ± 0.19	1.80 [c]
	DSMZ/63059	13	5.68 ± 0.07	
Trichoderma (Gliocladium) virens (Miller, Giddens & Foster) Arx[#]	DSMZ/1963	4	3.26 ± 0.06	1.16 [c]
Trichoderma viride Pers. ex Gray	F77	4	3.70 ± 0.21	1.42
	F77	34	2.60 ± 0.01	
Trichothecium roseum (Pers.) Link ex Gray	DSMZ/860	34	3.02 ± 0.08	0.90[c]

[a] Fungal strains marked with F were obtained from the culture collection of the Institute of Microbiology, University of Braunschweig; strains designated with DSMZ were purchased from the German Collection of Microorganisms and Cell Cultures, Braunschweig
[b] Unless otherwise stated cultures were grown on agar plates at 25 °C (incubator)
[c] Growth conditions and growth rates according to Domsch et al., 1980
Species with additional growth studies and DNA monitoring (see Figure 2)
± Standard deviation of mean DNA values obtained from three extractions and two measurements each (> n = 6), see Material and Method section

dodecylsulfate (SDS) (SIGMA), 1 mM (0.42 mg) aurintricarboxylic acid (ATA) (SIGMA) and 0.02 % (0.2 mg) proteinase K (PK) (Roth). The enzyme lyticase (yeast lytic enzyme from *Arthrobacter luteus*, 360953 MP Biomedicals) was added at a final concentration of 300 U, and chitinase (SIGMA) at a final concentration of 0.1 % (1 mg). All ingredients had been solved in TE buffer (50 mM/5 mM). The final pH of the lysis solution was 7.7. All ingredients were used fresh. Tubes were then either vortexed for 25 min (Vortex-Genie2T with platform for reaction tubes at speed 8, Scientific Industries) or placed in a FastPrep® instrument (Q. Biogen, Inc.) for 2 x 45 sec at speed 6.5 and then transferred to a linear shaking water bath (gentle speed) at an incubation temperature of 65 °C and left for 60 - 90 min. Tubes were then centrifuged at 10.000 rev min^{-1} for 5 min. The supernatant containing the DNA extract was processed as described under 2.3.

2.2.2. Lysis protocol II

50 mg mycelium (wet weight) was transferred to sterile microfuge tubes containing glass beads as described under 2.2.1. Lysis solution was identical to lysis protocol I except that aurintricarboxylic acid (ATA) was not added. As an alternative to chitinase, the enzyme complex Glucanex® (from *Trichoderma harzianum* L1412 Sigma) was used and added at a final concentration of 4 % (40 mg/ml per microfuge tube). Lyticase was applied in the same concentration as under 2.2.1.Tubes were placed in a FastPrep® instrument (Q. Biogen, Inc.) for 2 x 45 sec at speed 6.5 and then transferred to a rotating (450 rev min^{-1}) thermomixer (Eppendorf) at an incubation temperature of 65 °C as an alternative to a water bath and left for 90 min.

Proof of the lysing efficiency was determined exemplarily by plating the contents of the mycelial lysis solution of *Mucor mucedo* and *Fusarium culmorum* after the bead beating step (FastPrep®) on petri dishes containing Martin`s agar. Plates were placed in an incubator at 25 °C for 14 days. During this period no growth occurred.

2.3. Determination of extracted DNA

The quantity of DNA obtained in the extracts was determined by making a 100-fold dilution of the extract (50 µl aliquot in 4.95 ml TE (10 mM Tris/1 mM EDTA buffer)). From this, aliquots of 0.5 ml were then transferred to plastic disposable cuvettes (Sarstedt, No.67.742). A 200-fold dilution of the dsDNA stain PicoGreen® (Molecular Probes) (Sandaa et al., 1998) was prepared according to the product's information sheet of the manufacturer and 0.5 ml of the dye was added to the cuvettes (making a final 200-fold dilution of the extract) and left to react at room temperature in the dark for 2 min. Fluorescence intensity was measured with an SFM 25 fluorescence photometer (Kontron Instruments AG). Samples were exited at 480 nm and fluorescence emission intensity was measured at 525 nm. The fluorescence photometer was calibrated using 1000 ng bacteriophage lambda DNA (SIGMA, No D3779) as standard. Lysis solution or lysis solution containing lytic enzymes together with PicoGreen® was used as a blank. The efficiency of the fluorescence photometer was checked beforehand by generating a DNA standard curve according to the experimental protocol of the PicoGreen® manufacturer.

DNA yield was either determined immediately after extraction or extracts were stored in the refrigerator (4 °C) for maximal 48 h. DNA yield was expressed as µg DNA mg^{-1} dry weight.

2.4. Microscopy

The fluorescence dye acridine orange (AO) (Clark, 1981) was diluted 1:6000 fold in tap water. 0.5 ml of the mycelia:TE buffer (1:5 w/v) as described under 2.1. and 0.5 ml of the AO solution were mixed and the fungal material stained for 10 min in the dark. After staining, the hyphal material was washed twice in Eppendorf cups containing Hepes buffer with 2 % glucose by centrifugation at 10.000 rev min^{-1} for 5 min. The pellet was transferred to 1 ml Hepes buffer and 20 µl were placed on a glass slide with a cover slip for examination under the microscope.

Fluorescent images were obtained with an epi-fluorescent microscope (Zeiss-Axioplan), a triple band filter set (Zeiss Nr. 25; exitation: 400 - 570 nm, emission: 460 - 610 nm). Pictures were captured via a cooled CCD camera (DIE-470T, Optronics Engeneering). For image processing a software Lucia G, vers. 3.52a (NIKON) was used. Objectives used were either a Zeiss Plan-Neofluar 10x/0.35 or 20x/0.50.

2.5. Determination of cell carbon

Fungal mycelium was obtained and dried as described under 2.1. Hyphae were homogenized using a ball mill MM301 (Retsch GmbH, Germany). 100 mg dry weight material was taken for carbon analysis using a TruSpec CN Determinator (Leco Corporation). Four replicates per fungus were analysed.

2.6. Statistical analysis

Pair-wise comparison between groups of treatments were done using one-way ANOVA (Holm-Sidak method: overall significance level = 0.05) with Sigma Stat for Windows-Vers.3.0 (SPSS Science).

3 Results and discussion

3.1. Observations and pre-steps taken on DNA extraction for lysis protocol I

To assess whether our DNA extraction procedure used for soils (Blagodatskaya et al., 2003) would be applicable as well for fungal DNA extractions, some comparative analyses were done which preceded protocol I and observations made there will be described in short. This extraction procedure for total soil DNA is based mainly on the one described by Marstorp and Witter (1999) with the exception that the step of freeze-drying and grinding in liquid nitrogen was left out and as an alternative the bead beating procedure was employed (van Burik et al., 1998; Müller et al., 1998; Haugland et al., 1999; Griffin et al., 2002; Yamada et al., 2002; Griffiths et al., 2006) and by Sandaa et al., (1998) with respect to lytic ingredients and the fluorometric quantification of DNA using PicoGreen® as a specific dsDNA marker. At the end the bead beating procedure allowed a miniaturization and a great reduction of time in the whole process.

Lysozyme, a necessary enzyme in soil DNA extraction (bacteria), did not increase fungal DNA yields and was omitted. On the contrary, proteinase K, an enzyme which inactivates DNases but degrades as well the histones that package the DNA into chromosomes, was very essential. If this step was excluded, yields decreased (see also Tsai and Calza, 1992). DNA yields increased with time of incubation (65 °C , water bath) with proteinase K. A 90 minute incubation time was chosen, since a more prolonged incubation time did not lead to significantly higher yields. Pre-incubation with lytic enzymes such as lyticase or chitinase is the common procedure for obtaining protoplasts (see below under 3.2.1). In contrast to the finding by Karakousis et al., (2006) enzymatic pre-incubation per se significantly decreased DNA yields in all cases, the longer the pre-incubation time lasted, the lower the DNA yield. For that reason the lytic procedure was designed in such a way that all lytic steps would take place in the microfuge tube.

There was a trend in increases of DNA yield when the initial weight of hyphal material was reduced from 100 mg to 50 mg or 25 mg. This occurrence was, however, not studied in detail.

Previous to the FastPrep® beater for physical cell disruption continuous vortexing was applied for 25 min (2.2.1). With the exception that the FastPrep® beater shortens the whole procedure very much, there were no significant differences with respect to DNA yield between the two instruments.

Storage of samples after DNA extraction under freezing conditions is detrimental to DNA estimations. Best results were obtained when the extracts were analyzed immediately or were stored in the refrigerator (4 °C) for a maximum of 48 h.

A recent paper by Karakousis et al. (2006) on cell wall disruption and lysis methods assessed the degree of disintegration of hyphal material of a number of fungal strains by various treatments. Their assumption was the greater the effect on cell wall disintegration, the greater the potential yield of DNA. Unfortunately, a quantitative assessment in terms of DNA yield on a dry weight basis and its allocation to the different disruption procedures was not done. In contrast to this report, our experience with epifluorescence microscopy using the vital dye acridine orange as shown in Figure 1 or FUN-1 for viability testing (Millard et al., 1997) (not shown) revealed hyphae which were not disintegrated, however showed a diminished fluorescence in comparison to controls, a sign that the cells were not metabolically active or "dead". The fact that no growth occurred after the bead beating procedure (see 2.2.2) is a sign that the organisms were killed. Apparently, total cell wall desintegration does not seem necessary for obtaining high yields of fungal DNA. However, the lytic enzymes seem to weaken the cell wall or support porous conditions so that the lysis solution can react more easily.

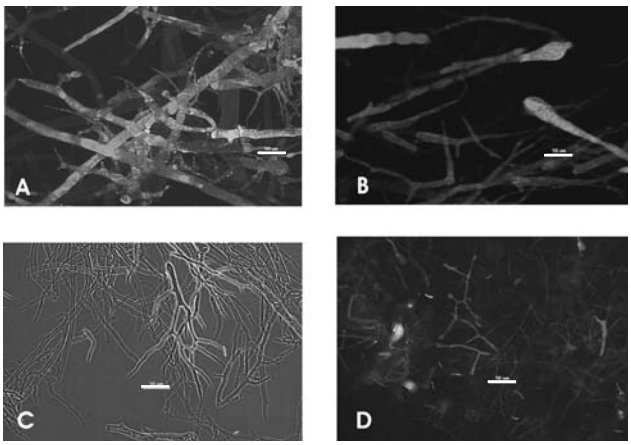

Figure 1:

Hyphae of *Mucor mucedo* after different disintegrating treatments and examined by fluorescence video-microscopy using acridine orange as a vital dye. (A) *M. mucedo*, no treatment. (B) Image taken after 3 min in lyticase. (C) Image taken after 6 h in Glucanex and viewed under phase 3. Cell wall structures are clearly visible but cells seem hyaline (empty). (D) Image taken after bead beating. To summarize: disintegrating treatments left cell walls intact; however the fluorescence diminished as seen between images (A) and (B + D). White bar measures 50 µm. Magnifications were 200x (A - C) and 100x (D), respectively. *M. mucedo* stands exemplarily for a number of fungi examined in this way.

3.2. DNA yield and age of fungal culture

Twenty-five strains of common soil fungal species were analyzed for their DNA yield using lysis protocol I. Total

dsDNA values are shown in Table 1. The mean DNA yield amounted to 3.3 µg mg^{-1} dry weight (range 0.92 to 6.32). This seemed low compared to bacterial DNA, which takes 3 % - 13 % and more of the cells dry weight (Stouthamer, 1973; Brock and Madigan, Neidthard, 1987; Christensen et al., 1995; Makino et al., 2003) depending on cell size and growth (Simon and Azam, 1989; Christensen et al., 1995). There was a trend that young mycelium had a higher DNA content (Table 1). Other authors reported similar observations (Gottlieb and van Etten, 1964; Dorn and Rivera, 1966; Karakousis et al., 2006). No obvious relationship between potential DNA yield and the natural growth rate of species was detected (Table 1). This is demonstrated exemplarily in Figure 2. The fungi *Mucor mucedo* and *Trichoderma* (*Gliocladium*) *virens*, both of which have fast growth rates, show different patterns of DNA yield over time. While the DNA yield of *M. mucedo* decreased with time, that of *T. virens* stayed constant at the beginning and then increased.

A literature search on fungal DNA contents is shown in Table 2. Although extraction protocols have changed considerably over the last 40 years, DNA yields are pretty much comparable with respect to order of magnitude with the exceptions of a few low values. Considered in this search were papers with DNA yields expressed quantitatively in µg mg^{-1} dry weight (or wet weight). There is a large amount of information on DNA yield where no quantitation was attempted (mainly those on PCR work) or where the information was too vague, or the values were related to unusual units not suitable for comparative purposes.

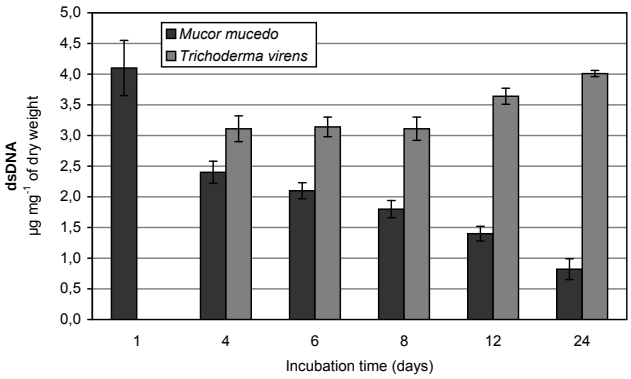

Figure 2:

Comparison of two soil fungi *M. mucedo* and *T. virens* with similar fast growth rates but with differing dsDNA yields during their growth period. Error bars indicate standard deviation of DNA yield of triplicate extractions with two DNA determinations each from one experimental set up.

Table 2:

Examples of studies on fungal DNA extraction and DNA yield (arranged by year of publication)

		DNA	
Species	**Age of culture (days)**	**µg mg^{-1}(mycelial dry weight)**	**Reference**
Penicillium atrovenetum	2 - 4	4.1 - 6.0	Gottlieb & van Etten, 1964
Aspergillus nidulans	1	2.0	Dorn & Rivera, 1966
Paracoccidioides brasiliensis	2 - 8	6.0 - 9.0	Ramirez-Martinez, 1970
Aspergillus oryzae	3	2.0 - 4.0	Bajracharya & Mudgett, 1980
Schizophyllum commune	?	0.05 - 0.1[a]	Specht, et al., 1982 [55]
Aspergillus nidulans	2?	1.5	Raeder & Broda, 1985
Coprinus cinereus	2	1.2	"
Phanerochaete chrysopsorium	1	1.2	"
Aspergillus sp.	15 h	1.6	Bainbridge et al., 1990
Fusarium graminearum	10	1.9 - 5.2[a]	de Nijs et al., 1996
Fusarium culmorum	10	5.4 - 7.7[a]	"
Fusarium poa	10	5.6	"
Aspergillus fumigatus	7	0.74[a]	van Burik et al., 1998
Apergillus flavus	10	0.34	Al-Samarrai & Schmid, 2000
Apergillus niger	10	0.39	"
Rhizopus nigricans	10	0.45	"
Penicillium citrinum	10	0.87	"
Fusarium graminearum	10	0.38	"
Phaeocryptopus gaeumannii	> 90	~0.4	Winton et al., 2002
Aspergillus niger	n.d.	0.4 - 0.5[a]	Punekar et al., 2003
Aspergillus nidulans	"	"	"
Aspergillus fumigatus	1	2.5 - 3.5[a]	Jin et al., 2004
Six fungal species	14 - 21	0.1 - 1.0[a]	Karakousis et al. 2006

a) Data were cited on a fresh weight basis

Table 3:
Amount of DNA extracted from fungal species with lysis protocol I plus lytic enzyme amendments

Species	Taxon (Class)	dsDNA (µg mg^{-1} mycelial dry weight)		
		control[a]	lytic enzymes	
			lyticase	chitinase
Pythium ultimum[b]	Oomycetes	4.06 ± 0.37	5.02 ± 0.63**	4.85 ± 0.57
Mucor mucedo	Zygomycetes	3.03 ± 0.35	3.05 ± 0.3	3.60 ± 0.12***
Penicillium camembertii	Ascomycetes (Fungi imperfecti)	4.49 ± 0.47	4.33 ± 0.10	4.70 ± 0.10*
Fusarium culmorum	Ascomycetes (Fungi imperfecti)	2.82 ± 0.19	3.41 ± 0.49	3.62 ± 0.74**
Rhizoctonia solani	Basidiomycetes (Hyphomycetes)	2.92 ± 0.32	3.29 ± 0.11**	3.54 ± 0.24***

[a] Protocol I without lytic enzyme amendments.
[b] Age of culture in days: P. ult.-6; M. muc.-4; Pen. cam.-6; Rh. sol.-6; Fus. cul.-7.
± Standard error of mean (SEM). DNA values obtained from at least three repeated experiments with triplicate extractions and duplicate DNA determinations each.
Significances are expressed as *=P<0.05, **=P<0.01, ***=P<0.001 with respect to control values.

3.2.1. Effect of enzyme amendments of Lyticase and Chitinase to the lysis protocol I

Fungi have a very rigid cell wall with either a chitin-chitosan (Zygomycetes), chitin-glucan (Ascomycetes, Basidiomycetes) complex as the major cell wall component except for Oomycetes which have mainly cellulose instead. In studies for obtaining protoplasts or for extracting DNA from spores, the use of lytic enzymes is very common (Hamlyn et al., 1981; Deshpande, 1986; Tsai and Calza, 1992; Birch and Denning, 1998; Watts et al., 1998; Jung et al., 2000; Balasubramanian et al., 2003; Fari a et al., 2004). To investigate if lytic enzyme amendments would improve our DNA yield lyticase or chitinase was chosen. Five fungal species (*Pythium ultimum*, *Mucor mucedo*, *Penicillium camembertii*, *Fusarium culmorum*, *Rhizoctonia solani*) belonging to different classes were selected. Results are shown in Table 3. The moderate increases in DNA yield were in the range of 5 % - 28 % which were at least significant to the control at $p = < 0.05$. Lyticase was more active on the cellulose-containing fungus *Pythium* while chitinase was mainly active on chitin-containing fungi. A combination of lyticase+chitinase in the lysis solution always reduced the DNA yield.

3.3. DNA yields with lysis protocol II

In the following approach it was attempted to further optimize and simplify the lysis protocol by testing commercially available lytic enzyme complexes. Ten fungi were chosen: the Oomycete *Pythium ultimum*, two Zygomycetes *Mortierella ramanniana* and *Mucor mucedo*, the Hyphomycete *Trichoderma* viride (class Ascomycetes) which has a teleomorphic stage, the Hyphomycete *Rhizoctonia solani* (class Basidiomycete) which has a teleomorphic stage and five Fungi imperfecti (class Ascomycetes) *Botrytis allii*, *Fusarium culmorum*, *Paecilomyces lilacinus*, *Penicillium camembertii*, *Stachybotrys chartarum* with no known sexual stage. During these investigations it became obvious that the addition of aurintricarboxylic acid (ATA), which was thought to be a specific inhibitor of nucleases during DNA extraction (Hallick et al., 1977; Marstorp and Witter, 1999), depressed the potential DNA yield drastically by 44 % (range 16 % - 95 %) in our system (Figure 3a) when data from lysis protocol I (containing ATA) and lysis protocol II (without ATA) were compared. It could be that ATA is not that specific. This observation was made by Bina-Stein and Tritton (1976), noting that ATA will inhibit most enzymes regardless of their specific catalytic functions.

The lytic enzyme complex should have chitinolytic, glucanolytic and cellulolytic properties should the lysis protocol be applied for soil DNA extractions. A vast body of literature reports on the successful use of the enzyme complex Novozym 234 (Novo Nordisk, Denmark) which was commercially produced from the fungus *Trichoderma harzianum*. The substance is today no longer available from the former manufacturer, and as an alternative Glucanex® was chosen, which has the same combination of ß-glucanase, cellulase, protease and chitinase activities and is as well a product of *Trichoderma harzianum*. With Glucanex® as an enzyme amendment, seven of the ten fungi showed a positive response with small increases in DNA yields in the range of 6 % - 14 % when compared to lysis protocol II without Glucanex®. The increases were significant for the majority of fungi ($p = < 0.02 - 0.001$). Glucanex® combined with lyticase increased the DNA yield of eight fungal species even more in the range from 6 % to 24 % ($p = 0.01 - 0.001$) (Figure 3a). A summary of the DNA yield of all ten fungi is given in Figure 3b. The protocol II in combination with a Glucanex-lyticase complex gave the highest DNA yields with a mean of 6.45 µg mg^{-1} dry weight (range 4.2 to 8.7). *Fusarium culmorum* is the only species which did not react to these lytic enzyme amendments, however when Glucanex® was combined with chitinase,

there was an increase in DNA yield by > 11 %. All other fungi did not react to the Glucanex-chitinase combination, the values were either comparable to the lysis solution without lytic enzymes or similar to the Glucanex-lyticase complex or values were even decreased (not shown). It is worthy to point out that in this investigation the DNA yield did not differ very much between fungal species tested. With the exception of *Fusarium culmorum* and *Rhizoctonia solani* with DNA yields > 4.0 µg mg^{-1} dry weight and *Mucor mucedo* with > 5.0 µg mg^{-1} dry weight, all other species gave DNA yields above 6.0 µg mg^{-1} dry weight. This may be due to the fact that the mycelium used was young and sporulation was not pronounced yet.

There were warnings that lytic enzymes could be contaminated with DNA (Loeffler et al., 1999; Rimek et al., 1999; Borst et al., 2004). In this study this was not the case since DNA contamination of any ingredient would be detected in our system by a high blank.

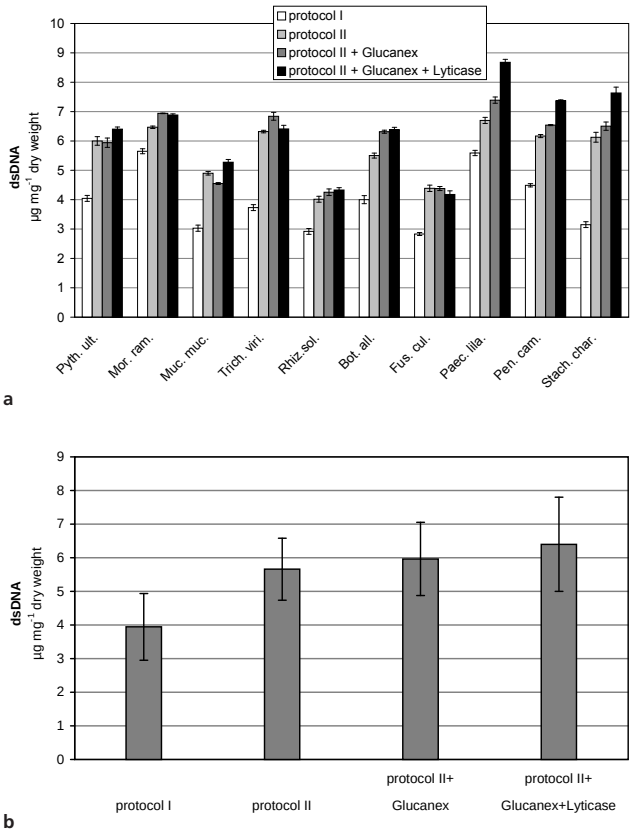

Figure 3a,b:

Contrasting dsDNA yields of ten soil fungi with respect to the lysis protocol applied

a) Yield of individual species. Fungi were grouped according to their taxonomical status (see 3.3). Error bars = standard error. Mean DNA yields were based on at least three separate experiments with triplicate extractions and duplicate DNA determinations each. For complete species names see Table 1.

b) Summary of DNA yields of ten fungal species as given under a). Error bars = standard deviation; significant differences in DNA yield among treatment groups were $^{***}p < 0.001$ or $^{**}p < 0.003$.

3.4. Carbon:DNA ratio

An additional useful parameter besides DNA alone is the C:DNA ratio of organisms as an index for following the nutritional status or growth of cells. This index is more commonly applied in marine science (Jones et al., 1995). Exemplarily three fungal species were analysed for their carbon content. When expressed as a percentage of the total cellular dry weight, the carbon contents for *Pythium ultimum*, *Mucor mucedo* and *Fusarium culmorum* were 44.65 % ± 0.12, 42.59 % ± 0.36 and 41.25 % ± 0.84, respectively. With respect to the DNA yield obtained with extraction protocol II, this resulted in a C:DNA ratio of 70, 80 and 98 for *P. ultimum*, *M. mucedo* and *F. culmorum*, respectively. These C:DNA ratios are very similar to those found for marine microalgae (Jones et al., 1995).

4 Conclusions

The extraction protocol II is suitable for a high yield of DNA recovery from fungal mycelia. The yields obtained belong to the set of high DNA recoveries reported in Table 3. The procedure is relatively simple, quick (~2 h) and with the exception of SDS no poisonous ingredients are used. In recent years a number of miniaturized rapid methods were announced (Liu et al., 2000; Millar et al., 2000; Manian et al., 2001; Knoll et al., 2002; Płaza et al., 2004) for fungal DNA extractions. Unfortunately, none of those gave information on the DNA yield obtained in quantitative terms and a comparative evaluation could not be done.

The DNA yield from young fungal mycelium was in this study very much comparable between species. It amounted to less than one percent of mycelial dry weight and is nearly 10- fold lower than that seen for bacteria. Since a relationship exists between cell size and DNA contents on a dry weight basis (Christensen et al., 1995), whereby with decreasing cell size the DNA content increases (Simon and Azam1989; Christensen et al., 1995), fungal DNA yield must be lower than that for bacteria. In this context it may be of interest to note that the range of DNA per unit biomass dry weight or Carbon:DNA ratio reported here were similar to published values for marine algae (Holm-Hansen et al., 1968; Jones et al., 1995).

Should this method be applied to DNA extraction from soil microorganisms, our original lysis protocol must be modified, omitting ATA. Cell-wall degrading enzymes such as Glucanex® in combination with lyticase should be included since they can improve DNA yields.

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