Biodegradation **15:** 59–65, 2004.

Bioremediation of BTEX hydrocarbons: Effect of soil inoculation with the toluene-growing fungus *Cladophialophora* sp. strain T1

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Accepted 13 October 2003

Key words: aromatic hydrocarbons, bioremediation, gasoline pollution, microbial interactions, soil fungi

Abstract

The biodegradation of a mixture of benzene, toluene, ethylbenzene, xylene, (BTEX) and methyl-*tert*-butyl ether (MTBE) was studied in soil microcosms. Soil inoculation with the toluene-metabolising fungus *Cladophialophora* sp. strain T1 was evaluated in sterile and non-sterile soil. Induction of biodegradation capacity following BTEX addition was faster in the soil native microflora than in axenic soil cultures of the fungus. Toluene, ethylbenzenes, and the xylenes were metabolized by the fungus but biodegradation of benzene required the activity of the indigenous soil microorganisms. MTBE was not biodegraded under the tested environmental conditions. Biodegradation profiles were also examined under two pH conditions after a long term exposure to BTEX. At neutral conditions the presence of the fungus had little effect on the intrinsic soil biodegradation capacity. At an acidic pH, however, the activity of the indigenous degraders was inhibited and the presence of *Cladophialophora* sp. increased significantly the biodegradation rates of toluene and ethylbenzene. Comparison of the BTEX biodegradation rates measured in soil batches combining presence and absence of indigenous degraders and the fungal inoculum indicated that no severe antagonism occurred between the indigenous bacteria and *Cladophialophora* sp. The presence of the fungal inoculum at the end of the experiments was confirmed by PCR-TGGE analysis of small subunits of 18S rDNA.

Abbreviations: BTEX – benzene, toluene, ethylbenzene, and xylene; MTBE – methyl-*tert*-butylether; TGGE – temperature gradient gel electrophoresis

Introduction

Gasoline leaking from underground storage tanks, distribution facilities, and various industrial operations represents a prime source of soil and aquifer contamination (Swoboda-Colberg 1995). Among the contaminants present in gasoline, benzene, toluene, ethylbenzene, and xylene (BTEX) are classified as priority pollutants because of their high mobility and toxicity (Mehlman 1992). Modern gasoline formulations are commonly supplemented with the additive methyl-tert-butyl ether (MTBE), which allows a reduction on the amount of BTEX (Mays 1989). Although less toxic than BTEX, MTBE appears to be more recalcitrant under natural conditions (Deeb et al. 2001). BTEX and MTBE are the most water-soluble components of gasoline and therefore these compounds predominate in groundwater contaminant plumes from recent accidental gasoline spills.

Bioremediation of hydrocarbon pollution relies on the biodegradation activity of soil microorganisms. Bacteria and fungi capable of degrading BTEX and MTBE have been isolated from soil (Yadav & Reddy 1993; Hardison et al. 1997; Deeb et al. 2001). However, while most of the studies have focused on bacteria, little is known on the contribution of fungi to

bioremediation of BTEX and MTBE. Fungally mediated biodegradation of soil pollutants has mainly been assayed with white-rot fungi (Pointing 2001). These organisms oxidize aromatic hydrocarbons by co-metabolism, and significant mineralization can only be achieved through the synergic interaction of fungi and bacteria (Kotterman et al. 1998; Boonchan et al. 2000). Interestingly, fungi capable of growing on volatile aromatic hydrocarbons as their sole source of carbon and energy have recently been isolated from soil (Cox et al. 1993; Prenafeta Boldú et al. 2001; Woertz et al. 2001). These fungal isolates were found to be tolerant to acidic and dry conditions, characteristics that make them very suitable as biocatalyst in air biofilters treating BTEX vapors (van Groenestijn et al. 2001).

This paper describes the effect of inoculation of the fungus *Cladophialophora* sp. strain T1 on the biodegradation of a mixture of BTEX and MTBE in a soil microcosm. This fungus was previously isolated from a BTEX polluted soil by enriching for toluene assimilation and was found able to degrade the alkylbenzene (TEX) components in pure liquid culture (Prenafeta Boldú et al. 2001, 2002). Here, the result of soil inoculation with conidia of *Cladophialophora* sp. on the BTEX biodegradation profiles was evaluated. The effect of the soil pH was also studied after a long term exposure to pollution.

Materials and methods

Fungal strain

Cladophialophora sp. strain T1 (ATCC MYA-2335, CBS 110553) was isolated as previously described (Prenafeta Boldú et al. 2001). During the present investigation, this organism was routinely maintained at 4 °C on mineral medium (Hartmans & Tramper 1991) agar slants supplemented with 2% glucose.

Chemicals

BTEX hydrocarbons were obtained from Acros Organics (Geel, Belgium), Sigma-Aldrich Chemicals (Steinheim, Germany), Lab-Scan Ltd. (Dublin, Ireland) and Merck KGaA (Darmstadt, Germany). All chemicals were of analytical grade.

Soil characteristics

Soil for the experiments was collected from the Agricultural Test Station Kelekamp (Wageningen, The Netherlands), air-dried and stored in the dark at 4 °C prior to use. The physico-chemical properties of the soil are shown in Table 1. This location had no known history of hydrocarbon exposure.

Soil-batch microcosms

Soil samples (approx. 30 g dry-weight) were placed into 250 ml Boston flasks. The soil was subsequently soaked with sterile de-mineralized water and, after removing the excess by gravity, flasks were sealed with teflon-coated valves (Mininert, Phase Separations, Waddinxveen, The Netherlands). Sterile control batches were prepared by autoclaving for 50 min at 120 °C. A mixture of BTEX and MTBE (10 μl), that contained (in volume) 14% of benzene, 43% of toluene, 14% of ethylbenzene, 5% of each xylene isomer, and 14% of MTBE, was spiked into the batches. A spore suspension of *Cladophialophora* sp. (containing approx. 10^4 viable conidia) was used as inoculum. Four different treatments were assayed: untreated soil containing active native microflora (N); autoclaved soil inoculated with the fungus (F); non-autoclaved soil containing both the indigenous microbes and fungal inoculum (N + F); and non-inoculated autoclaved soil (abiotic control). The content of BTEX and MTBE was followed in the gas phase and the equivalent concentration in the water phase was calculated from reported water/air partition coefficients (Mackay & Shiu 1981; Amoore & Hautala 1983). Experiments for each soil treatment were performed in triplicate.

Two additional sets of batches were prepared as described previously, but in these cases the pH was adjusted by soaking soil with either a phosphate buffer (50 mM, pH = 7) or a phosphoric acid solution (pH = 4). These flasks were closed with a cotton plug and placed into two 30 l desiccators. A volume of 1 ml of the previously described BTEX plus MTBE mixture was diluted in 20 ml dibutylphthalate and placed into the desiccators. The water activity was kept to 0.9 by placing a salt solution (140 g NaCl l^{-1}). The desiccators were closed and incubated at 21 °C in the darkness for 60 days. After this time, the desiccators were opened and the batches left overnight for aeration. The cotton plugs were aseptically replaced by teflon-coated valves, and 10 μl of the BTEX plus MTBE solution was added to the batches prior to

Table 1. Physicochemical properties of the soil

Texture	Grain size distribution (%)	Density (kg m^{-3})	f_{oc}[1] (g g^{-1})	pH	Porosity (m^3 m^{-3})	FC[2] (%)
Sandy soil						
Sand	93.3					
Silt	2.8	2650	0.028	6.1	0.5	20
Clay	3.9					

[1] f_{oc}: Organic carbon content.
[2] FC: Water by weight at field capacity.

chromatographic analysis. Statistically significant differences on the biodegradation rates were established by subjecting data to a one-way analysis of variance (ANOVA) test. Significance was considered to be at the $p < 0.05$ probability level. Soil samples were taken at the end of experiments for pH and TGGE analysis.

Analytical methods

Samples of the head space (100 μl) were injected into a HP 6890 Series gas chromatograph (Hewlett Packard, U.S.A.). A CP-Wax 52CB column (Chrompack B.V., Middelburg, The Netherlands) was used as the stationary phase. The carrier gas was nitrogen used at a flow of 1.9 ml min^{-1}. The temperature of the column and the flame ionization detector was 110 and 300 °C respectively. BTEX and MTBE were identified and quantified by using reference compounds.

PCR and TGGE parameters

DNA was extracted from the soil batches and from pure cultures of *Cladophialophora* sp. by using a FastDNA Kit (Bio 101, Vista, CA) and purified by using a Wizard Kit (Promega, Madison, WI). DNA samples were amplified in 50 μl PCR mixtures containing the following final concentrations or total amounts: 1–10 ng of DNA, 50 mM Tris (pH 8.3), 2 mM $MgCl_2$, each deosynucleoside triphosphate (dNTP) at a concentration of 250 μM, 400 nM of forward and reverse primer, and 0.5 U of *Taq* DNA polymerase. All reagents were combined and heated at 94 °C for 4 min, followed by 35 cycles of PCR: 94 °C for 35 s, 55 °C s for 50 s followed by 72 °C for 2 min. The fungal-specific primers nu-SSU-0817-5′ (TTA GCA TGG AAT AAT RRA ATA GGA) and nu-SSU-1196-3′ (TCT GGA CCT GGT GAG TTT CC) were used, which generated 422 bp amplicons of the fungal 18S rDNA gene (Borneman & Hartin 2000). A rich GC-clamp was added to the forward primers for TGGE separation. PCR amplification was confirmed in agarose gels (12 g l^{-1}) containing 0.5 mg l^{-1} of ethidium bromide. The gels were run in 1 × TAE buffer at 80 V for 45 min and visualized using a UV transilluminator.

Each PCR-amplified sample (4 μl) was mixed to 1 μl of loading buffer and loaded onto a temperature gradient gel electrophoresis (TGGE) equipment (Biometra Göttingen, Germany). An 8% polyacrylamide gel (8 × 8 × 1 mm^3) containing 6 M urea, 1.25 × TAE, 0.1% TEMED, 1 g l^{-1} ammonium persulfate, and 5 ml of a solution of 400 g l^{-1} acrylamide/bis was used. TGGE was performed at 130 V for 2 h 30 min, the temperature gradient from 36 to 42 °C being run parallel to the migration. A silver staining protocol was applied to the gels. Bands were cut out and eluted in 20 μl of PCR buffer for 2 h with intervals of shaking. The eluted fraction (15 μl) was used for re-amplification and, after purification, the products were sent for sequencing (MWG Biotech AG, Ebersberg, Germany). Obtained sequences were deposited GenBank and homology searches were performed against other available sequences from this database.

Results

Degradative activity experiments

Biodegradation activity was induced in indigenous microorganisms from an agricultural, non-polluted soil within 3 days after addition of a mixture of BTEX and MTBE (Figure 1). MTBE was not degraded but the BTEX were depleted after 8 days, at a maximum rate of 3.8, 7.8, 2.2, and 1.9 μmol kg^{-1} h^{-1} ($n = 6$, $r^2 > 0.96$) respectively for benzene, toluene, ethylbenzene, and all three xylene isomers. Biodegradation was also observed in sterile soil inoculated with conidia of *Cladophialophora* sp. strain T1 but only the alkylbenzene components (TEX) were consumed. In this case,

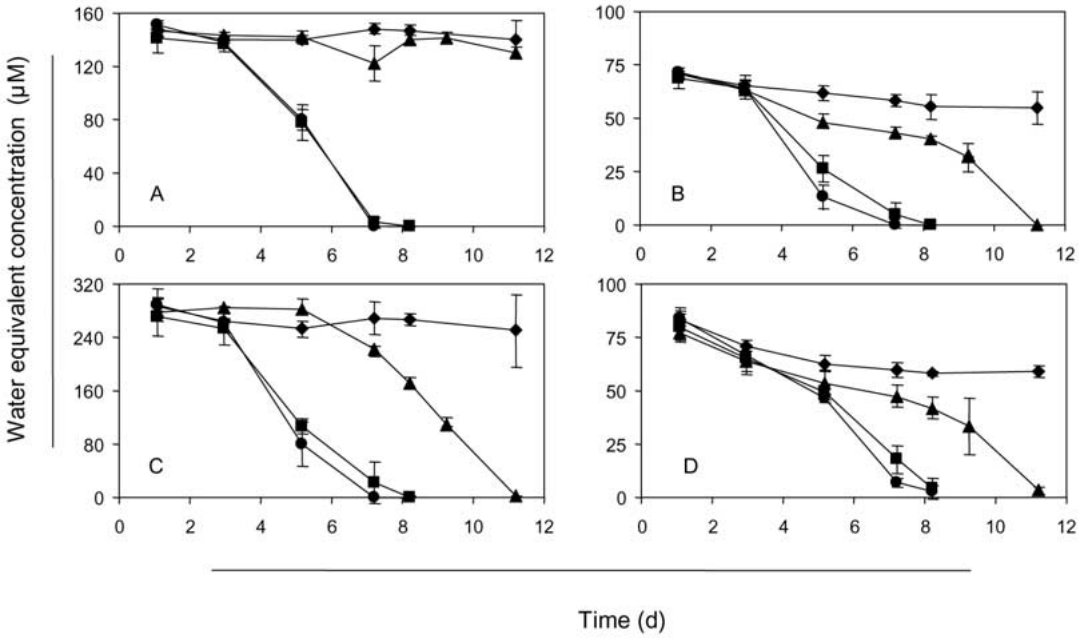

Figure 1. Degradation patterns of a mixture of benzene (A), toluene (B), ethylbenzene (C), and the total xylene isomers (D) in soil microcosms. Substrates were added at time 0 and incubation was performed at 21 °C. Soil containing the active indigenous microflora (■); the previous soil inoculated with *Cladophialophora* sp. strain T1 (●); autoclaved soil containing *Cladophialophora* sp. (▲); and abiotic control consisting of autoclaved soil (♦). Error bars correspond to the standard deviation of three different experiments.

the maximal rates were similar to those attained by the indigenous soil microflora, except that a lag phase of about 7 days occurred. No differences were observed on the biodegradation patterns in non-sterile soil with or without the fungal inoculum.

As mentioned above, the biodegradative activity was also measured in a second set of soil batches after a 60 day exposure to an atmosphere containing BTEX and MTBE. Two pH conditions, neutral and acidic, were tested (Table 2). Prolonged incubation with the volatiles resulted in a general increase of the BTEX biodegradation capacity of the indigenous soil microorganisms and of the inoculated *Cladophialophora* sp; no lag phase was detected in either case. Differences in the rates of biodegradation measured in the native soil microbes and in axenic soil cultures of fungus were not significant for toluene, ethylbenzene, or *o*- and *m*-xylene, while degradation of *p*-xylene was slower with *Cladophialophora* sp. and benzene was not degraded at all by this fungus. Inoculation of neutral non-sterile soil with *Cladophialophora* sp. had little effect on the overall BTEX levels and only the increase in ethylbenzene degradation was found to be significant. Under acidic conditions, the biodegradation activity of the native degraders was considerably lower than the levels obtained at neutral pH. Inhibition of BTEX breakdown due to acidity was less severe in axenic soil containing *Cladophialophora* sp. than in non-sterile soil, being the biodegradation rates of toluene and ethylbenzene higher than these obtained in natural soil (Table 2). Fungal inoculation of acidic non-sterile soil also resulted in a substantial increase on the biodegradation of toluene and ethylbenzene. Biodegradation of benzene always required the activity of the indigenous soil microflora and the presence of *Cladophialophora* sp. did not have any effect on the benzene degradation rate. In contrast to the BTEX, MTBE was not significantly biodegraded in any of the treatments.

TGGE microbial profiles

Amplicons of the expected length were generated from the total soil DNA using the universal fungal primers. Significant PCR product yields were only obtained from microcosms that had previously been inoculated with *Cladophialophora* sp. Unexpectedly, despite several attempts, DNA amplification from neutral non-sterile soil containing the fungal inoculum was always low. The TGGE pattern from the inoculated soils showed bands identical to those obtained with

Table 2. Maximal degradation rates (in μmol kg^{-1} h^{-1}, at 25 °C) of BTEX and MTBE measured in soil microcosms[1] after 60 days of exposure. The values correspond to the average and standard deviation of three independent experiments

Substrate	N		F		N + F	
	pH = 7.1	pH = 4.6	pH = 6.5	pH = 3.2	pH = 6.8	pH = 3.7
MTBE	ND[2]	ND	ND	ND	ND	ND
Benzene	12.0 ± 0.7	6.6 ± 1.8	ND	ND	13.2 ± 2.1	7.1 ± 0.2
Toluene	65.7 ± 4.9	11.8 ± 1.1	51.8 ± 14.0	33.3 ± 6.6	71.7 ± 6.0	46.3 ± 16.9
Ethylbenzene	9.7 ± 2.4	4.9 ± 1.4	13.3 ± 0.9	7.9 ± 1.3	24.2 ± 7.0	9.2 ± 1.6
o-Xylene	5.2 ± 1.4	3.0 ± 0.5	4.5 ± 1.7	3.3 ± 0.7	5.8 ± 2.4	3.9 ± 0.5
m-Xylene	5.2 ± 0.5	5.0 ± 0.9	4.9 ± 1.8	v3.3 ± 0.5	9.1 ± 1.5	3.4 ± 0.5
p-Xylene	4.7 ± 0.4	4.9 ± 0.7	1.6 ± 0.2	0.3 ± 0.1	6.7 ± 1.1	4.1 ± 1.0

[1]N: soil containing the native microflora; F: soil containing the fungus *Cladophialophora* sp. strain T1. The pH was measured at the end of the experiments.
[2]Not depleted (the gas phase content after 14 days was similar to that of abiotic controls).

a pure culture of the fungus (Figure 2). Purity of the original culture was subsequently confirmed by performing a single spore isolation. The new culture showed again the same TGGE pattern. Three individual bands of the gel were excised, re-amplified, and successfully sequenced. Bands *a* and *b* showed an identical sequence that was deposited in GenBank under the accession number AY150798; band *c* was also sequenced and deposited as GenBank AY150799. Alignment searches with other sequences in this database showed the highest homology levels (100% for bands *a* and *b*, and 98% for band *c*) were obtained for 18S rDNA genes from other strains in *Cladophialophora*, *Phialophora*, *Ramichloridium*, and *Rhinocladiella*, anamorph genera that are related to the teleomorph *Capronia* (Untereiner 2000).

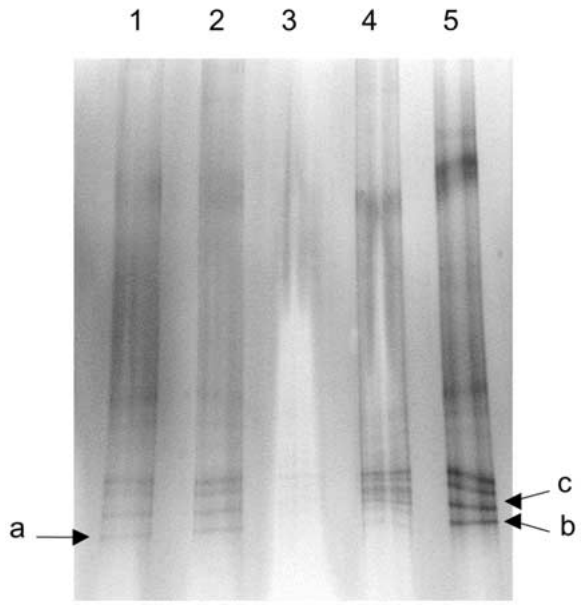

Figure 2. TGGE profiles for fungal 18S rDNA fragments obtained from the soil samples inoculated with *Cladophialophora* sp. strain T1: acidified autoclaved soil (Lane 1); acidified and non-autoclaved soil (Lane 2); non-acidified non-autoclaved soil (Lane 3); non-acidified and autoclaved soil (Lane 4); pure culture of fungal strain T1 (Lane 5). Arrowed bands *a*, *b*, and *c* were sequenced; results are given in the text.

Discussion

This study shows that *Cladophialophora* sp. strain T1 was able to breakdown alkylbenzene hydrocarbons from a mixture of BTEX in a soil microcosm. So far, biodegradation of BTEX hydrocarbons has mainly been characterized using submerged bacterial cultures growing under optimal or near-optimal laboratory conditions (Alvarez & Vogel 1991; Chang et al. 1993; Oh et al. 1994; Duetz et al. 1998; Goudar & Strevett 1998; Deeb & Alvarez-Cohen 1999; Deeb et al. 2001). In this situation bacteria usually multiply faster than fungi and, hence, they are the predominant organisms that have been more often selected and comprehensively studied (Prenafeta Boldú et al. 2001). The relatively low fungal growth rates might be the reason for the extended lag phase seen here in sterile soil inoculated with *Cladophialophora* sp. Despite the long lag phase, maximal biodegradation rates for toluene, ethylbenzene, and xylenes were generally similar for both native soil microorganisms and for *Cladophialophora* sp. The substrate specificity of *Cladophialophora* sp., as observed in soil cultures,

was consistent with that seen previously in liquid cultures. Toluene and ethylbenzene both served as growth substrates, the xylenes were cometabolized (the *p*-xylene isomer at a relatively low rate), and benzene was not degraded (Prenafeta Boldú et al. 2002).

The increase of the soil biodegradation capacity and the absence of a lag phase seen when indigenous degraders were exposed over a long term to BTEX atmosphere indicates that BTEX-degrading organisms had become enriched. However, this intrinsic bioremediation was strongly inhibited when the soil pH was acidic. Under this condition, inoculation with *Cladophialophora* sp. resulted in a substantial rise in the biodegradation levels of toluene and ethylbenzene, these are the only BTEX compounds that can effectively be assimilated by the fungus (Prenafeta Boldú et al. 2002). The maximal degradation rates for toluene and ethylbenzene were measured when both the native adapted microflora and the fungus were present. Benzene was only degraded by the indigenous microbes at rate that was independent from the presence or absence of the fungus. Altogether, these results indicate that at an acidic soil pH *Cladophialophora* sp. can successfully compete with the natural soil microflora. Also, it is clear that no strong antagonism occurred between the indigenous BTEX degraders and the introduced *Cladophialophora* sp. At a pH close to neutrality the role of the fungus in biodegradation appears to be of reduced prominence and, except in regard to ethylbenzene, fungal inoculation did not appreciably change the level of indigenous biodegradation. Moreover, less fungal DNA was amplified from the neutral soil sample than was amplified from the others (Figure 2), suggesting that a relatively small fungal biomass had developed under these conditions. A low soil pH has previously been recognized as an important factor favoring the development of fungi over bacteria (Bååth 1998), and acidic soils contaminated with oil hydrocarbons have been found to contain large fungal populations (Bossert & Bartha 1984). Our results showing the fungal involvement in aromatic pollutant biodegradation in acidic soils are consistent with the picture generated by other authors (Middelhoven et al. 1992; Stapleton et al. 1998). Our results also confirm that MTBE is more recalcitrant than BTEX (Deeb et al. 2001).

Monitoring the fate of microbial degraders is important in the evaluation of the effectiveness of soil bioremediation. Analysis of small subunits of the 16S rDNA gene has been used for the study of bacterial BTEX degraders in natural environments (Pedersen & Arvin 1999; Ralebitso et al. 2000). Here, the presence of *Cladophialophora* sp. in soil was assessed by PCR-TGGE analysis of SSU from 18S rDNA. As mentioned previously, sequence analysis of excised bands showed maximal homology with other sequences from the genus *Capronia*, which is the holomorph of *Cladophialophora*. This taxon appears to have experienced a very high degree of evolutive diversification recently and for this reason the 18S rDNA gene is unlikely to contain enough sequence variability to resolve species identification (Untereiner 2000). Interestingly, enrichment of fungi capable of growth on volatile aromatic hydrocarbons has often yielded members of *Cladophialophora* and the closely related genus *Exophiala* (Cox et al. 1993; Prenafeta Boldú et al. 2001; Woertz et al. 2001). Virulent agents of systemic disease are also present among these genera (de Hoog & Guarro 1995) and precise taxonomic identification is therefore needed to prevent the creation of biohazardous conditions during bioremediation.

At present, decontamination of BTEX-polluted soil is commonly carried out through *in situ* soil bioventing (Bowlen & Kosson 1995). With this technique hydrocarbons are removed by a combination of volatilization and by stimulated aerobic biodegradation (Malina et al. 1998). Increasing the biodegradation capacity of soil would either reduce the volatilization of BTEX to the atmosphere or reduce the need to treat this polluted air stream. Fungi growing on volatile aromatic hydrocarbons have already been used successfully for the biofiltration of air containing volatile hydrocarbons (van Groenestijn et al. 2001). This preliminary study indicates that fungal inoculation of soil might be a viable technique to improve biodegradation of BTEX pollutants during bioventing, especially when a low pH limits the activity of the indigenous bacteria. Further studies are still needed to assess the viability and the associated biohazard risk of introducing fungal cultures into soil on a large scale.

Acknowledgement

The authors are indebted to R. Summerbell of the Centraalbureau voor Schimmelcultures for the critical reading of this manuscript.

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