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ORIGINAL PAPER

Strains of the soil fungus *Mortierella* show different degradation potentials for the phenylurea herbicide diuron

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Abstract Microbial pesticide degradation studies have until now mainly focused on bacteria, although fungi have also been shown to degrade pesticides. In this study we clarify the background for the ability of the common soil fungus *Mortierella* to degrade the phenylurea herbicide diuron. Diuron degradation potentials of five *Mortierella* strains were compared, and the role of carbon and nitrogen for the degradation process was investigated. Results showed that the ability to degrade diuron varied greatly among the *Mortierella* strains tested, and the strains able to degrade diuron were closely related. Degradation of diuron was fastest in carbon and nitrogen rich media while suboptimal nutrient levels restricted degradation, making it unlikely that *Mortierella* utilize diuron as carbon or nitrogen sources. Degradation kinetics showed that diuron degradation was followed by formation of the metabolites 1-(3,4-dichlorophenyl)-3-methylurea, 1-(3,4-dichlorophenyl)urea and an hitherto unknown metabolite suggested to be 1-(3,4-dichlorophenyl)-3-methylideneurea.

Keywords Fungal biodegradation · Co-metabolism · Pesticide · Fungal genetics · Phylogenetic relationships

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Introduction

Pesticide pollution is one of the major concerns regarding contamination of the environment. Pesticides are mainly used on agricultural land, but also in private gardens, along railways and other public areas. Herbicides account for the largest part of overall pesticide use worldwide (Grube et al. 2011). Among these are the phenylurea herbicides which are frequently found as environmental pollutants (Eriksson et al. 2007; Lapworth and Gooddy 2006; Struger et al. 2011). The phenylurea herbicide diuron [*N*-(3,4-dichlorophenyl)-*N,N*-dimethyl-urea] is on the EU water framework directive's list of priority substances (European Parliament EU 2008) and as it is often found in groundwater at concentrations exceeding the EU limit of 0.1 μg l^{-1} (Lapworth and Gooddy 2006; Torstensson 2001) biodegradation of this compound is a central issue.

The majority of studies on microbial pesticide degradation have focused on bacteria (e.g. El-Bestawy and Albrechtsen 2007; El-Deeb et al. 2000; Simonsen et al. 2006; Sørensen et al. 2008), whereas fewer studies examine the role of fungi (Harms et al. 2011). Several fungi are known to produce enzymes that are able to degrade aromatic herbicides and have therefore been suggested as candidates for bioremediation (Entry et al. 1996).

Fungi can account for up to 75 % of soil microbial biomass (Harms et al. 2011) and their hyphae can grow to a length of 10^2–10^4 m g^{-1} soil (Ritz and Young 2004). Fungi may play an important role in bioremediation of soil as the fungal hyphae can grow into micropores between soil aggregates and through air filled gaps (Wösten et al. 1999) and in this way gain a better access to the compound to be degraded.

Earlier studies have shown that certain fungal species/strains have the ability to degrade diuron (Badawi et al. 2009; Khadrani et al. 1999; Tixier et al. 2000, 2001; Vroumsia et al. 1996). These studies have given insight into formation of different metabolites as well as toxicity of the parent compound and the metabolites. The studies usually include a number of species and consequently compare differences in diuron degradation potential among fungal species. Such differences may likely reflect differences in enzymatic abilities and adaptation to different habitats, but little is known about the variation within fungal genera or species concerning their ability to degrade pesticides.

The genus of *Mortierella* is found within the zygomycetes. Species from this genus are common soil fungi and some of these have been found to be capable of degrading phenylurea herbicides e.g. isoproturon (Rønhede et al. 2005) and diuron (Tixier et al. 2000, 2001; Vroumsia et al. 1996). Nevertheless, it is not known whether this ability is a general characteristic of the genus or the tested isolates were unique in this respect. In the present study we therefore compare *Mortierella* isolates, originating from the same agricultural field, to investigate if the ability to degrade diuron is restricted to specific strains or it is a general feature that they share.

Though many microorganisms have been shown to degrade pesticides, the physiological significance of the degradation remains unclear. For some pesticide degrading bacteria it has been shown that they are able to utilize the compound as a carbon or a nitrogen source (e.g. El-Deeb et al. 2000). Whether the same could be the case for fungal degradation remains unclear. Alternatively, degradation could also be a co-metabolic detoxification process, as some compounds may share characteristics with natural compounds. To answer these questions we investigate if *Mortierella* can utilize diuron as a carbon and a nitrogen source, or whether degradation is caused by co-metabolism.

Materials and methods

Chemicals and media

Analytical-grade standards were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany): Diuron [*N*-(3,4-dichlorophenyl)-*N,N*-dimethyl-urea] (CAS no. 330-54-1; 97.5 % purity), DCPMU [1-(3,4-dichlorophenyl)-3-methylurea] (CAS no. 3567-62-2; 97.5 % purity), DCPU [1-(3,4-dichlorophenyl) urea] (CAS no. 2327-02-8; 99 % purity), and 3,4-DCA [3,4-dichloroaniline] (CAS no. 95-76-1; 99 % purity). 3,4-DCAA [3,4-dichloroacetanilide] (CAS no. 2150-93-8) was purchased from Sigma-Aldrich (St. Louis, Missouri). Acetonitrile-D3 was from Eurisotop (99.8 % D).

Potato dextrose agar (PDA) supplied with ampicillin (5 mg l^{-1}) and streptomycin (5 mg l^{-1}) was used for fungal isolation. After isolation all fungi were maintained on PDA plates. Liquid glucose-mineral media used for degradation experiments were made according to Badawi et al. (2009).

Isolation and characterization of fungi

Fungi were isolated from an agricultural field in Græse, Denmark (55°51′51N, 12°5′44E) with a previous history of phenylurea (isoproturon) application. For isolation of fungi PDA medium with ampicillin and streptomycin was used.

To characterize the isolates, DNA was extracted from fungal mycelia using the following protocol: 20 µl Chelex, 40 µl TE buffer and mycelia heated to 95 °C for 2 min followed by centrifugation at 13,000×*g* for 2 min, DNA now in the supernatant was transferred to clean tubes and frozen (−18 °C). PCR amplification of the internal transcribed spacer (ITS) region was performed as described by Gardes and Bruns (1996) with the following modifications: Amplification for 35 cycles consisting of denaturation

for 35 s at 94 °C, annealing for 55 s at 55 °C and extension at 72 °C for 45 s rising with 4 s per cycle. 10× diluted fungal DNA was amplified with primers ITS 1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990). PCR products were sequenced by Macrogen (Seuol, Korea) and the sequences aligned in *MEGA* version 5 (http://www.megasoftware.net/index.php). The most similar sequences were found by BLAST search in Genbank. The phylogenetic relationship between the isolates and related sequences from Genbank was made by Maximum likelihood in *MEGA* version 5 (Tamura et al. 2011), and the robustness of the phylogenetic tree tested by 1,000 bootstraps.

Fungal degradation potential

Five *Mortierella* isolates were chosen for further studies (see Table 1). The experiment was conducted to test the potential of the different fungal strains for degradation of diuron. It was carried out in 100 ml screw-cap flasks with Teflon-lined lids. Initially 25 µl of stock solution diuron (5,000 ppm) dissolved in acetonitrile was added to flasks, acetonitrile was allowed to evaporate, and 25 ml of glucose-mineral medium was added to each flask to give a final concentration of 5 mg diuron l^{-1}. Inoculation for each flask was done with three agar plugs (ø = 4 mm) with mycelium cut from PDA plates. The experiment was carried out in triplicates of each treatment, and with an abiotic treatment to serve as control.

Fungal cultures were incubated on an orbital shaker at 110 rpm in the dark at 8 °C for 43 days. Repeated aliquots sampling from flasks were done with 1 ml sterile syringes, transferring samples to Ultra performance liquid chromatography (UPLC) vials through a syringe filter (0.2 µm PTFE membrane, 15 mm). Samples were frozen (−18 °C) until UPLC analysis.

Diuron degradation by *Mortierella* sp. LEJ701 at different C and N concentrations

One fungal strain, *Mortierella* sp. LEJ 701, was used to investigate the effect of different carbon and nitrogen concentrations on fungal diuron degradation. Experimental setup was done as above with the following changes: for different glucose treatments three different media were used with 5, 0.5 or 0 g glucose l^{-1} (only glucose available to the fungi is from the agar plug $\leq$0.045 g l^{-1}). For each treatments (three glucose levels and with/without diuron) 12 flasks were prepared for destructive sampling of triplicates at four predetermined sampling times. At sampling aliquots were taken for UPLC analysis as described above.

In addition, effects of different nitrogen concentrations were also studied. Again the setup had six treatments (three nitrogen levels and with/without diuron). NH_4Cl in the media was (l^{-1}): 1, 0.04 or 0 g (only N available to the fungi is from the agar plug $\leq$0.001 g l^{-1}). Three additional flasks were used where fungal mycelia were removed after 10 days to test if extracellular enzymes would continue the diuron degradation in the media. In both experiments described above fungal biomasses were collected on filters and dry-weights were measured (80 °C for 20 h).

UPLC analysis of diuron and metabolites

Ultra performance liquid chromatography was performed for quantitative analysis of diuron and its metabolites in the culture media. The system used was

Table 1 Fungal strains isolated from soil and tested for diuron degradation potential

Strain	Most related organism[a]	GenBank nr.	Genetic similarity (%)	Diuron degradation (%)[b]
LEJ701	*Mortierella* sp. (strain CBS 118520)	AJ890432.1	99	100.0 (±0.00)
LEJ702	*Mortierella elongata* (isolate RR 171)	AJ878504.1	100	33.6 (±2.29)
LEJ703	*Mortierella alpina* (strain CBS 224.37)	AJ271630.1	99	Nd
LEJ704	*Mortierella* sp. 68	EU877758.1	99	9.2 (± 5.88)
LEJ705	*Mortierella sarnyensis* (CBS 121162)	FJ161927.1	97	Nd

Initial diuron concentration 5 mg l^{-1}

Nd no degradation

[a] BLAST search in GenBank of PCR amplified DNA (ITS) sequence

[b] Diuron degradation experiment conducted in liquid media at 8 °C for 43 days

an Acquity UPLC (Waters, Milford, MA) equipped with a UV detector (200–225 nm absorbance) and an Acquity BEH C_{18} column (1.7 μm particle size, 2.1 mm inner diameter, and 100 mm length) set at a constant flow rate of 0.4 ml min^{-1}, column temperature of 43 °C, and an injection volume of 10 μl. The initial mobile phase was composed of 30 % acetonitrile and 70 % water with a 5 min gradient ending with 35 % acetonitrile and 65 % water. Hereafter conditions returned to the starting values giving a total analysis time of 6 min per sample.

Retentions times for the compounds analyzed were: DCPU 2.45 min, DCPMU 3.33 min, 3,4-DCAA 3.96 min, diuron 4.24 min, and 3,4-DCA 4.72 min. In addition, an unknown metabolite with a retention time of 2.16 min was also detected.

Biosynthesis of the unidentified metabolite

Mortierella sp. LEJ701 was inoculated in flasks containing glucose-mineral medium and diuron (5 mg l^{-1}), as described above, for large scale production of the unknown metabolite for identification by nuclear magnetic resonance (NMR) and mass spectroscopy (MS). Flasks were incubated on an orbital shaker at 110 rpm in dark at 8 °C for 20 days. Sampling was done with 1 ml sterile syringes, transferring samples to 2 ml vials through syringe filters (0.2 μm PTFE membrane, 15 mm). Samples were frozen (−18 °C) until HPLC analysis.

Preparative HPLC

Reverse phase HPLC was used to purify the unknown metabolite, and the identity was determined by NMR spectroscopy and MS. The system used was an ÄKTApurifier (GE Healthcare, Munich, Germany) equipped with a UV detector (210–212 nm absorbance) and a Phenomenex Luna C_{18} column (5 μm, 100 Å; 4.6 × 100 mm). Preparative HPLC was performed using a gradient program with a flow rate of 1.0 ml min^{-1}. The initial mobile phase was composed of 30 % acetonitrile and 70 % water with a 30 min gradient ending with 50 % acetonitrile and 50 % water. The injection volume was 1.8 ml. Fractions of 0.5 ml were collected. The identity and elution positions of the known compounds, diuron, DCPU, DCPMU and 3,4-DCA, were confirmed by mass spectrometry.

NMR spectroscopy

Fractions containing the unknown metabolite were isolated, pooled, lyophilized and dissolved in CD_3CN (99.80 %D) to a volume of 600 μl and transferred to a 545 NMR tube. A parallel sample of diuron was prepared from lyophilized powder and analyzed and a comparative sample of the same volume of HPLC buffer at a matched % of acetonitrile was lyophilized and dissolved in CD_3CN (99.80 % D). For all samples 1D ^1H NMR spectra were recorded on a Varian INOVA 750 MHz NMR spectrometer equipped with a 5 mm triple-resonance probe and Z-field gradient at 298 K utilizing 128 (diuron), 80.000 (solvent) or 80.000 (metabolite) transients. The spectra were transformed and analyzed using MesTreNova (Mestrec Research, Spain) and referenced to internal CD_3CN at 1.94 ppm.

MS analyses

Two μl fractions from the preparative HPLC were diluted with 8 μl 0.5 % formic acid and analyzed by LC–MS. The set-up consisted of an EASY nanoLC (Proxeon, Odense, Denmark) in front of a micrOTOF Q II (Bruker, Bremen, Germany). HPLC conditions were: A 75 × 100 mm 3 μm C18 column (Thermo Scientific, Copenhagen, Denmark) eluted at 300 nl min^{-1} with a 10 min gradient from 10 to 50 % B (A: 0.5 % HCOOH in H_2O, B: 0.5 HCOOH in CH_3CN).

Statistics

Statistical analyses were done in SigmaPlot version 11.0 (Systat Software Inc). Results from different treatments were tested for significance with ANOVA including Tukey Test for pairwise multiple comparison procedures. Linear regression was performed to test the correlation between amount of fungal biomass and diuron degradation. Differences are found statistically significant when $p \leq 0.05$. Data are presented as mean ± standard error (SE) unless stated otherwise.

Strain deposition and nucleotide sequence accession numbers

The five *Mortierella* strains LEJ701-LEJ705 are deposited at the Centraalbureau voor Schimmelcultures under the accession numbers CBS 133175-CBS 133179 in the CBS collection of fungi. In addition, the

sequences determined in this study have been deposited in the Genbank database under accession numbers JX206802-JX206806.

Results

Phylogeny of fungal pesticide degraders

The five strains showed very different potential for degradation of diuron (Table 1). The most efficient diuron degrader *Mortierella* sp. LEJ701 was able to degrade all diuron in the medium and the two closely related *Mortierella* sp. isolates LEJ702 and LEJ704 showed some potential for diuron degradation removing 33.6 and 9.2 % of the diuron, respectively. The two strains that did not degrade diuron were found to be only distantly related to the diuron degraders (Fig. 1). The most efficient diuron degrader *Mortierella* sp. LEJ701 had a sequence similarity of 97 and 96 % with the two other degraders *Mortierella* sp. LEJ702 and LEJ 704, respectively. Whereas *Mortierella* sp. LEJ701 only had a sequence similarity of 92 and 91 % with the non-degraders *Mortierella* sp. LEJ703 and LEJ705, respectively.

Substrate effects on diuron degradation

Mortierella clearly responded to the level of glucose in the medium. Diuron degradation was faster and more complete in the medium with a high glucose concentration compared to the degradation in media with intermediate and low glucose concentrations (Fig. 2). The diuron degradation rate in the low glucose medium was very slow, whereas the degradation rates were significantly higher for intermediate and high glucose concentration media ($p < 0.001$) (see Table 3). No significant difference between degradation rates was found for intermediate and high glucose media ($p = 0.154$).

Fungal biomass differed significantly between the three glucose treatments at experimental termination (Table 2, ANOVA Tukey test; all $p < 0.001$). Diuron degradation was positively correlated with fungal biomass ($p < 0.001$, $r^2 = 0.683$; Fig. 3), and the degradation rates per unit of biomass differed significantly for the three treatments (i.e. carbon concentration) (Table 3).

The results showed no effect of diuron on fungal biomass quantity at experimental termination ($p = 0.607$; Table 2), although an alteration in pellet structure was observed. The fungal pellets in treatments with diuron had long hyphal outgrowths from the otherwise spherical pellets.

The effect of nitrogen on diuron degradation was very similar to the effects observed with glucose concentrations. Diuron degradation was slow in the treatment with low nitrogen concentration and significantly higher in the treatments with intermediate nitrogen concentration ($p < 0.001$). Finally the treatment with high nitrogen concentration showed the fastest degradation rate (Table 3; $p < 0.001$).

There was no degradation in the abiotic controls. Furthermore no further degradation was seen by possible exudates after removal of fungal biomass (data not shown).

Accumulation and identification of diuron metabolites

Diuron degradation and accumulation of the two metabolites DCPMU and DCPU corresponded with the patterns shown in previous studies (Badawi et al. 2009). The first metabolite DCPMU accumulated as the diuron concentration decreased and after approximately 10 days the second metabolite DCPU appeared (Fig. 2a). In addition, to these compounds, our results showed an unknown metabolite with a retention time of 2.16 min observed by UPLC (Fig. 4). When this metabolite was included we achieved complete recovery of the diuron initially added to the system (Fig. 2a, b).

The unknown metabolite was biosynthesized, purified and analyzed by NMR spectroscopy and MS analyses. From the analytical MS analyses it was directly apparent that the unknown metabolite-fraction from the preparative fractionation contained two different species of molecular mass (m.m.) 204.004 and 215.996 Da, (monoisotopic values), corresponding to DCPU (theoretical m.m. = 203.986 Da) and the unknown metabolite as well as trace amounts of intact diuron (m.m. = 232.019 Da compared to the theoretical 232.017 Da). Hence any quantitative NMR analysis would be meaningless. Despite the very low concentration, and hence low signal-to-noise in the NMR spectrum, it was however immediately apparent that compared to the NMR spectrum of diuron, several sets of peaks originating from the aromatic ring suggested this to be intact also in the metabolite with

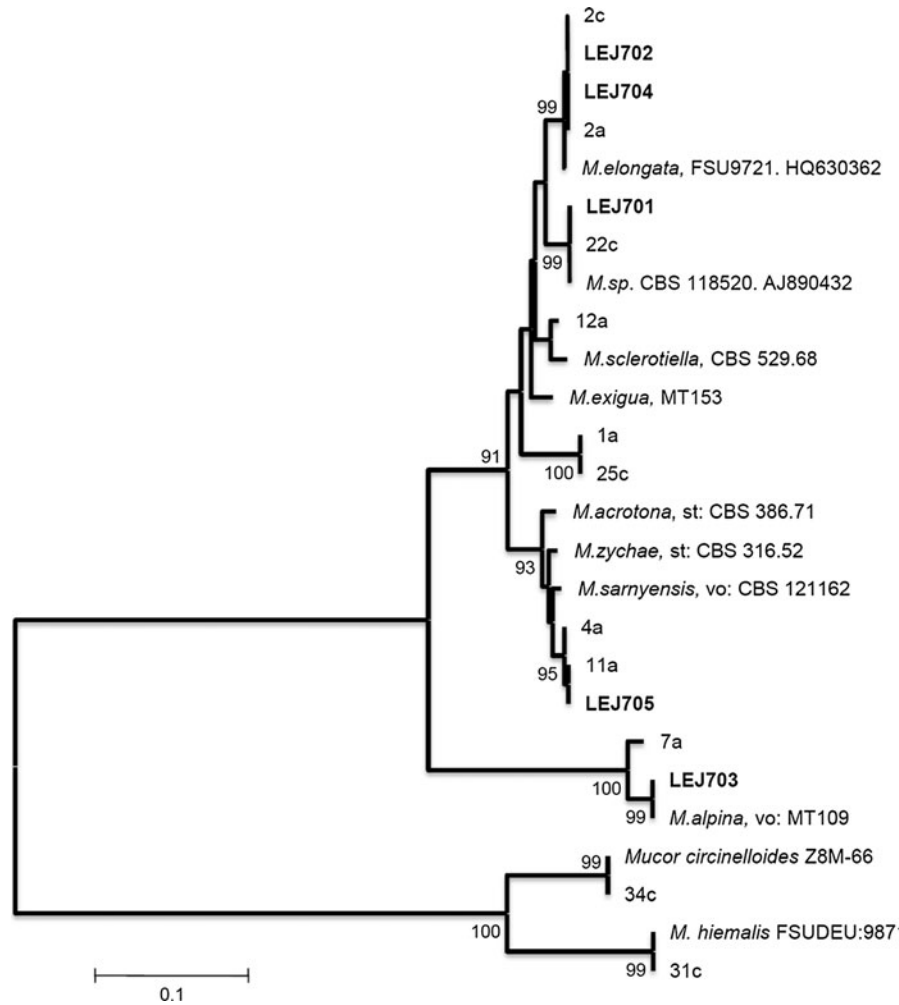

Fig. 1 Maximum likelihood phylogenetic tree showing the five *Mortirella* isolates and chosen isolates from Genbank. The weight of the branches corresponds to their bootstrap values. Bootstrap values lower than 90 are not presented

both chloride atoms as well as the amide proton present. The two chloride atoms were corroborated from the ^{35}Cl–^{37}Cl isotope"signature" in the MS spectra. In addition, from the molecular mass and isotopic pattern using the software application SmartFormula (Bruker Daltonics) the composition of the unknown metabolite was suggested to be $C_8H_6ON_2$ (theoretical m.m. = 215.986 Da) with high confidence. None of the methyl groups were present in the NMR spectrum as concluded from lack of these signals in the aliphatic region, suggesting that the fraction of diuron in the NMR sample was below detection. A small but significant change in the chemical shifts for all the aromatic protons ortho-positioned relative to the amide group suggested a change in the chemistry of this side group compared to diuron, although the structure was not elucidated by NMR due to the sparse amount of sample. The shorter retention time on the UPLC excluded the metabolite as either DCPMU or DCPU, so based on the NMR data, the molecular mass, the suggested atomic composition of the metabolite, as well as the lack of methyl groups, we suggest the metabolite to be 1-(3,4-dichlorophenyl)-3-methylideneurea (DCPMDU). Figure 5 depicts the proposed degradation pathway of diuron, including the most likely position of 1-(3,4-dichlorophenyl)-3-methylideneurea based the degradation kinetics (Fig. 2). The molecular masses were calculated as *m/z* values—1.0078 (H^+).

Discussion

Our results show that the ability to degrade diuron varied among the *Mortierella* strains tested. The most efficient degrader *Mortierella* sp. LEJ701 and the two

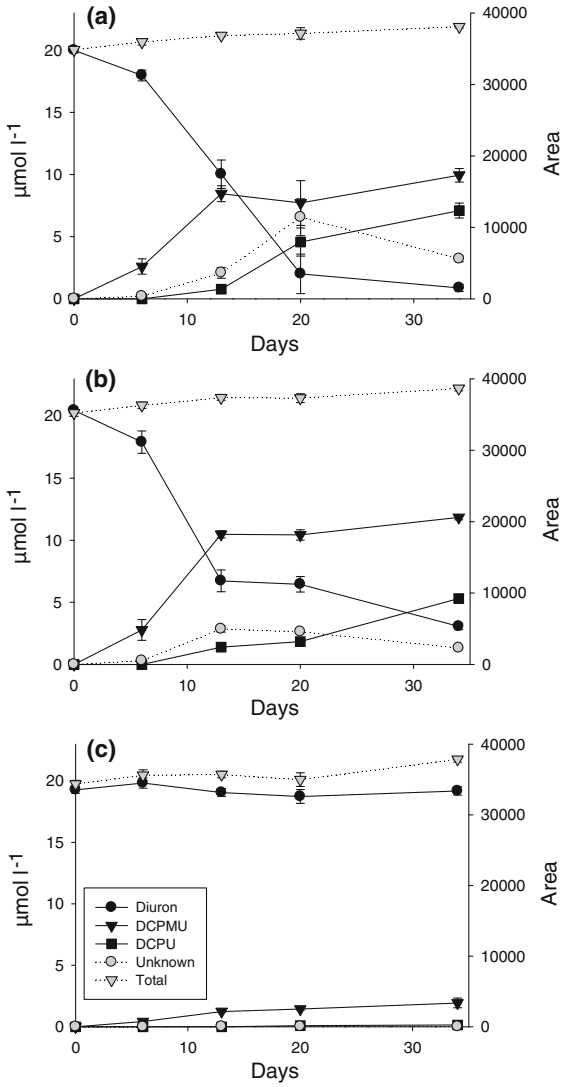

Fig. 2 Diuron degradation kinetics by *Mortierella* sp. LEJ 701 at **a** high, **b** intermediate and **c** low glucose concentrations. Diuron: [*N*-(3,4-dichlorophenyl)-*N,N*-dimethyl-urea], DCPMU: [1-(3,4-dichlorophenyl)-3-methylurea], DCPU: [1-(3,4-dichlorophenyl) urea], and unknown metabolite (RT 2.16 min). *Solid lines* with *black* symbols can be read on both ordinates. *Dashed lines* with *gray* symbols can only be read on the right-hand ordinate (area)

Table 2 *Mortierella* sp. LEJ 701 biomass at different carbon and nitrogen concentrations

Treatment	Diuron biomass (mg)	Control biomass (mg)
Low C	$22.7 \pm 1.1^{A,a}$	$22.4 \pm 3.3^{A,a}$
Intermediate C	$31.1 \pm 1.3^{A,b}$	$32.5 \pm 0.8^{A,a}$
High C	$47.6 \pm 0.1^{A,c}$	$43.8 \pm 2.4^{A,b}$
Low N	$23.5 \pm 1.3^{A,a}$	$26.6 \pm 2.9^{A,a}$
Intermediate N	$38.2 \pm 2.9^{A,b}$	$39.9 \pm 1.5^{A,a}$
High N	$53.8 \pm 3.9^{A,c}$	$58.1 \pm 12.9^{A,a}$

Biomasses at termination of experiments (day 33–34) with diuron (5 mg l^{-1}) or without diuron (control)

Low C ≤0.045 g glucose l^{-1}, *Intermediate C* 0.5 g glucose l^{-1}, *High C* 5 g glucose l^{-1}, *Low N* ≤0.001 g NH_4Cl l^{-1}, *Intermediate N* 0.04 g NH_4Cl l^{-1}, *High N* 1 g NH_4Cl l^{-1}

Numbers are mean ± SE. Different letters show significant difference between treatments—comparing diuron and control treatments (capital letters) and low, intermediate and high C or N, respectively (lower case letters)

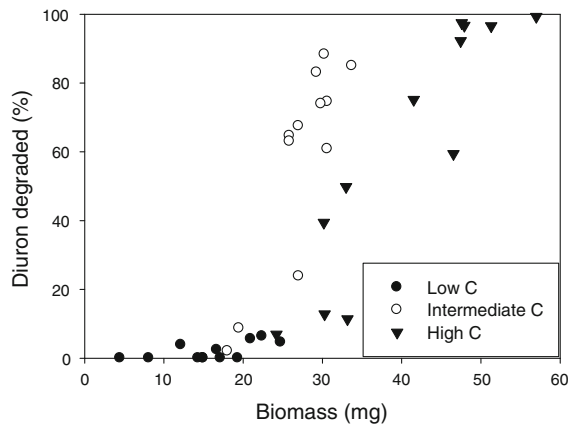

Fig. 3 Multiple scatter plot for all data points between days 6–34. Diuron degradation shown as percent decrease of initial concentration. Low C: ≤0.045 g glucose l^{-1}, Intermediate C: 0.5 g glucose l^{-1}, and High C: 5 g glucose l^{-1}

other degraders *Mortierella* sp. LEJ702 and LEJ704 showed 96–97 % genetic similarity, suggesting that though they are closely related they may belong to separate species. Nevertheless these closely related strains share a particular trait enabling them to degrade diuron by demethylation. Vroumsia et al. (1996) found, among the 90 fungal strains they screened for degradation potential, that four *Mortierella* isolates had degraded 19–54 % diuron after 7 days incubation at 24 °C. These values are within the same range as in the present study, though we achieved this at a much lower temperature—one comparable to average yearly temperature in a temperate climate—with *Mortierella* sp. LEJ701 degrading approximately 50 % diuron within 10 days. Furthermore, their work was not supported by molecular data on the fungal phylogeny, and it is therefore not possible to evaluate the phylogenetic relationship between the isolates. For bacteria, on the other hand, a phylogenetic analysis by

Table 3 Diuron degradation rates for *Mortierella* sp. LEJ 701 at different carbon and nitrogen concentrations

	Degradation rate ($\mu M\ day^{-1}$)	Degradation rate per unit biomass ($\mu M\ day^{-1}\ g^{-1}$)
Low C[A]	0.02 ± 0.02^{a}	1.16 ± 1.10^{a}
Intermediate C[A]	0.53 ± 0.04^{b}	17.17 ± 1.76^{c}
High C[A]	0.61 ± 0.01^{b}	12.82 ± 0.14^{b}
Low N[B]	0.07 ± 0.05^{a}	3.10 ± 2.18^{a}
Intermediate N[B]	0.40 ± 0.01^{b}	10.54 ± 1.05^{b}
High N[B]	0.78 ± 0.03^{c}	14.68 ± 0.75^{b}

Low C $\leq$0.045 g glucose l^{-1}, *Intermediate C* 0.5 g glucose l^{-1}, *High C* 5 g glucose l^{-1}, *Low N* $\leq$0.001 g NH_4Cl l^{-1}, *Intermediate N* 0.04 g NH_4Cl l^{-1}, *High N* 1 g NH_4Cl l^{-1}

Numbers given are mean $\pm$ SE. Initial diuron concentration 5 mg l^{-1}. Different letters show significant difference between treatments—comparing low, intermediate and high C or N, respectively

[A] Degradation rate calculated days 6–34

[B] Degradation rate calculated days 4–33

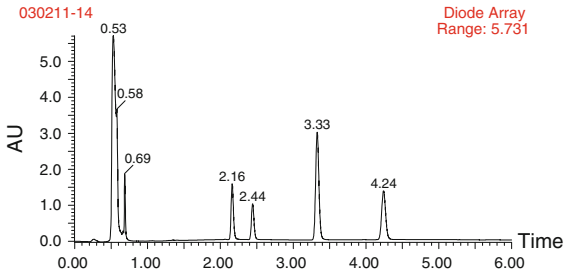

Fig. 4 UPLC chromatogram showing an unknown metabolite. Retention time (RT): Unknown 2.16 min, DCPU 2.44 min, DCPMU 3.33 min, Diuron 4.24

Hussain et al. (2009) demonstrated that isoproturon degrader strains were closely related.

The intra- and inter-specific differences in ability to degrade pesticides clearly demonstrate the need for further studies to clarify the genetic background for this characteristic. Future functional genetic studies should clarify these differences and may facilitate more focused isolation strategies for potential pesticide degraders. For biotechnological purposes this will be important as it may be possible to isolate fungal strains with even higher potential for bioremediation under natural conditions.

Our results show that it is unlikely that the fungus utilizes diuron as a carbon and/or nitrogen source,

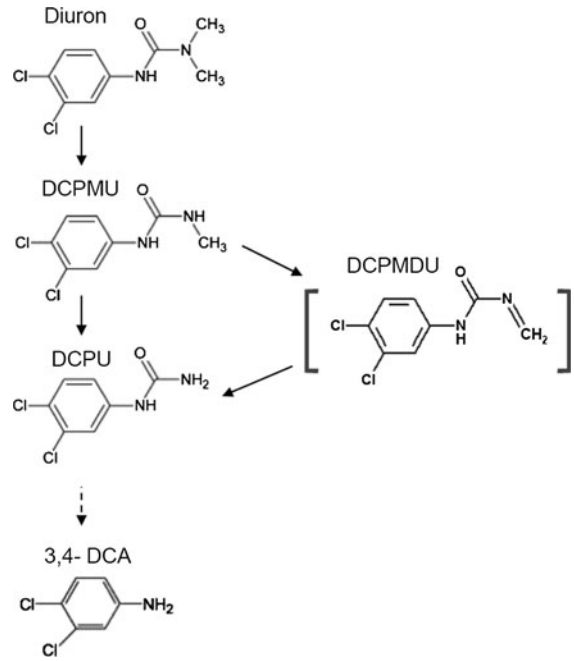

Fig. 5 Proposed degradation pathway of diuron. Diuron: [*N*-(3,4-dichlorophenyl)-*N,N*-dimethyl-urea], DCPMU: [1-(3,4-dichlorophenyl)-3-methylurea], DCPU: [1-(3,4-dichlorophenyl) urea], 3,4-DCA: [3,4-dichloroaniline] and DCPMDU: [1-(3,4-dichlorophenyl)-3-methylideneurea]. 3,4- DCA was only found as trace amounts by MS. The structure and placement of DCPMDU, in *brackets*, are suggested based on the composition $C_8H_6ON_2$ and the degradation kinetics

since growth was not stimulated by diuron addition even at the lowest levels of glucose and NH_4Cl where diuron C and N would otherwise contribute significantly to biomass quantity. On the contrary our results showed that *Mortierella* sp. LEJ701 required an additional carbon and nitrogen source to be able to degrade diuron. Likewise, Rønhede et al. (2005) found that isoproturon did not serve as an energy or nutrient source for the fungi used in their experiment. In contrast, Kulshrestha and Kumari (2011) found that an *Acremonium* strain could utilize the organophosphate chlorpyrifos as a source of both carbon and nitrogen. Since the *Mortierella* strain cannot utilize diuron as a N or C source it seems plausible, that the degradation process is co-metabolic mediated by enzymes excreted by the fungus during growth leading to successive demethylation through the metabolite DCPMDU.

The degradation rate was fastest in the medium with high glucose and NH_4Cl with a C/N ratio of 7.6:1. In comparison, Teng et al. (2010) found that

bioremediation of PAH contaminated soil was most efficient at a C/N ratio of 10:1. Since soil organic matter has a C/N ratio of 10:1 (Robertson and Groffman 2007) bioremediation of diuron is possible in soils without addition of further C and N sources. In addition, we found that diuron degradation occurred even at C/N ratios of 0.8:1 and 190:1 in the intermediate C and N medium, respectively.

We did not detect a decrease in fungal biomass quantity in treatments with diuron (Table 2). This is in contrast to Vroumsia et al. (1996) who reported a minor growth inhibition of *Rhizoctonia solani* exposed to a four times higher diuron concentration. However, we did observe an altered morphology of the fungal pellets during the experiment. The growth response characterized by long hyphal outgrowths from the pellet is often a response to stress e.g. toxic or lack of nutrients (Fomina et al. 2003). Toxicity of diuron or its metabolites towards the fungus can therefore not be excluded, and the co-metabolic degradation process could be a detoxification mechanism.

Ecotoxicity studies by Tixier et al. (2000) have shown that the metabolites DCPU and DCPMU are three times more toxic than diuron. It is consequently of concern that the degradation of diuron by common soil fungi leads to accumulation of these compounds (see Fig. 2). We have no information on the toxicity of the new metabolite since only small amounts could be synthesized, but seen in a regulatory context all these metabolites should be included in monitoring programs as well as in approval of pesticides on the basis of their toxicity and potential accumulation in the environment.

In conclusion, we have shed light on the diversity of diuron degradation potential between *Mortierella* strains. Also we have shown that diuron degradation by *Mortierella* is a co-metabolic process resulting in accumulation of its metabolites. Further research should include degradation of these metabolites. We suggest a possible way of achieving fast diuron degradation beyond these intermediate metabolites could be by developing consortia incorporating both fungal and bacterial strains.

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