Hydroxylation of the Herbicide Isoproturon by Fungi Isolated from Agricultural Soil

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Several asco-, basidio-, and zygomycetes isolated from an agricultural field were shown to be able to hydroxylate the phenylurea herbicide isoproturon [N-(4-isopropylphenyl)-N',N'-dimethylurea] to N-(4-(2-hydroxy-1-methylethyl)phenyl)-N',N'-dimethylurea and N-(4-(1-hydroxy-1-methylethyl)phenyl)-N',N'-dimethylurea. Bacterial metabolism of isoproturon has previously been shown to proceed by an initial demethylation to N-(4-isopropylphenyl)-N'-methylurea. In soils, however, hydroxylated metabolites have also been detected. In this study we identified fungi as organisms that potentially play a major role in the formation of these hydroxylated metabolites in soils treated with isoproturon. Isolates of Mortierella sp. strain Gr4, Phoma cf. eupryena Gr61, and Alternaria sp. strain Gr141 hydroxylated isoproturon at the first position of the isopropyl side chain, yielding N-(4-(2-hydroxy-1-methylethyl)phenyl)-N',N'-dimethylurea, while Mucor sp. strain Gr22 hydroxylated the molecule at the second position, yielding N-(4-(1-hydroxy-1-methylethyl)phenyl)-N',N'-dimethylurea. Hydroxylation was the dominant mode of isoproturon transformation in these fungi, although some cultures also produced traces of the N-demethylated metabolite N-(4-isopropylphenyl)-N'-methylurea. A basidiomycete isolate produced a mixture of the two hydroxylated and N-demethylated metabolites at low concentrations. Clonostachys sp. strain Gr141 and putative Tetracladium sp. strain Gr57 did not hydroxylate isoproturon but N demethylated the compound to a minor extent. Mortierella sp. strain Gr4 also produced N-(4-(2-hydroxy-1-methylethyl)phenyl)-N'-methylurea, which is the product resulting from combined N demethylation and hydroxylation.

Isoproturon [N-(4-isopropylphenyl)-N',N'-dimethylurea] (Fig. 1) is a phenylurea herbicide that is used in Europe mainly for the control of annual grasses and broad-leaf weeds in winter cereals. Environmental concerns have arisen from the frequent finding of isoproturon in surface water and groundwater at concentrations exceeding the European Union limit for drinking water (0.1 μg liter−1) (12, 31, 32). Due to the widespread use and high water solubility of isoproturon, it is on the list of 33 priority substances that seriously threaten surface and groundwater set up by the European Union in the Water Framework Directive.

Degradation of isoproturon in soil takes place primarily through microbial processes, although photodegradation of isoproturon has been demonstrated (17). Microbial degradation can lead to complete mineralization of isoproturon, which can mitigate the release of the herbicide to the environment, but it may also result in metabolites with possible detrimental properties. The degradation pathways have recently been reviewed by Sørensen and coworkers (29) and proceed either by consecutive N demethylation to N-(4-isopropylphenyl)-N'-methylurea (trivial name, monodemethyl-isoproturon [MDIPU]) and N-(4-isopropylphenyl)urea (trivial name, didemethyl-isoproturon [DDIPU]), followed by hydrolysis to 4-isopropylaniline, or directly from isoproturon to 4-isopropylaniline (Fig. 1). The microbial pathways have been studied mainly in bacterial cultures, and four strains able to mineralize isoproturon have been isolated by enrichment from soils previously treated with isoproturon (1, 5, 27, 30, 36). A Sphingomonas sp. isolated from a British soil seems to degrade isoproturon by initial N demethylation via MDIPU to DDIPU, followed by hydrolysis to 4-isopropylaniline before mineralization of the ring structure (30), while an Arthrobacter sp. isolated from the same field mineralizes the urea side chain directly and accumulates 4-isopropylaniline (5, 36). In soils treated with isoproturon, however, several hydroxylated metabolites have been detected. Based on these findings, a parallel pathway has been proposed (21, 26). This pathway is initiated by hydroxylation of the isopropyl side chain of isoproturon to N-(4-(1-hydroxy-1-methylethyl)phenyl)-N',N'-dimethylurea (2-OH-IPU), which is subsequently N demethylated to 2-OH-DDIPU and 2-OH-DDIPU, and hydroxylated 4-isopropylaniline is the final known end product. To our knowledge, no bacteria able to perform this pathway have been isolated from soils, although products hydroxylated at the first position of the isopropyl side chain were produced in modest amounts by bacterial enrichments from soil [N-(4-(2-hydroxy-1-methylethyl)phenyl)-N',N'-dimethylurea (1-OH-IPU)] (16) and by two species of soil algae [N-(4-(2-hydroxy-1-methylethyl)phenyl)-N'-methylurea (1-OH-DDIPU)] (20).
claving and cooling to 55°C, the medium was supplemented with 4 mg of Benlate 50 (50% benomyl; Dupont) suspended in 2 ml of 1:1 acetone–70% ethanol, 1.0 g lignin (catalog no. 370959; Aldrich) dissolved in 5 ml of 1 M KOH, 0.4 ml guaiacol (catalog no. G5502; Sigma), 40 mg of tetracycline (catalog no. T3838; Sigma), 20 mg penicillin G (catalog no. 194537; ICN Biomedicals), and 20 mg of streptomycin (catalog no. 194541; ICN Biomedicals) dissolved in sterile distilled water.

Malt extract agar (MEA) contained (per liter) 10 g malt extract broth and 20 g agar; the pH was adjusted to 6 with 1 M NaOH. To prepare malt extract agar with antibiotics (MEA+a), MEA was autoclaved and cooled to 50°C, and then tetracycline, penicillin G, and streptomycin were added as described above for lignin-guaiacol-benomyl medium.

Malt extract broth contained (per liter) 10 g malt extract broth, pH 6, Spez- ieller Nährstoffer agar contained (per liter) 0.2 g sucrose, 0.2 g glucose, 1.0 g K2HPO4, 0.5 g NaCl, 0.066 g MgSO4 ·7 H2O, 0.04 mg CuSO4 ·5 H2O, 0.021 mg ZnCl2, 0.041 mg CoCl2 ·6 H2O, and 0.025 mg Na2MoO4 ·2 H2O; the pH was adjusted to 6.5 with 1 M HCl. Glucose medium was mineral medium with 5.0 g/liter glucose. Lignin-guaiacol-benomyl medium (35) contained (per liter) 0.5 g Ca(NO3)2 ·4 H2O, 1.3 g KH2PO4, 0.5 g NaCl, 0.066 g MgSO4 ·7 H2O, 0.04 mg CuSO4 ·5 H2O, 0.021 mg ZnCl2, 0.041 mg CoCl2 ·6 H2O, and 0.025 mg Na2MoO4 ·2 H2O; the pH was adjusted to 6.5 with 1 M HCl. Glucose medium was mineral medium with 5.0 g/liter glucose. Lignin-guaiacol-benomyl medium contained (per liter) 0.1 g NH4NO3, 0.1 g KCl, 0.02 g FeSO4 ·7 H2O, 0.05 g Ca(NO3)2 ·4 H2O, 2.0 g malt extract broth (Lab M, International Diagnostic Group, Lancashire, United Kingdom), and 15 g agar (Difco). After auto-

Fungi seem to have a widespread ability to transform isoproturon since many fungi are able to remove isoproturon from the medium when they are grown in liquid culture (3, 9, 14, 37). The products have not been characterized or quantified in detail, however, and most of the fungi studied thus far have not originated from agricultural soils where the herbicide was used. The objectives of this study were to isolate representatives of the fungal community from an agricultural soil, to study their potential for isoproturon transformation, and to identify the transformation products. Among the fungi that we isolated, asco-, basidio-, and zygomycetes were found to transform isoproturon mainly to 1-OH-IPU and 2-OH-IPU, thus indicating that soil fungi could be the source of the hydroxylated metabolites of isoproturon detected in environmental samples.

MATERIALS AND METHODS

Media and chemicals. Mineral medium contained (per liter) 1.0 g (NH4)2SO4, 1.3 g K2HPO4, 0.5 g NaCl, 0.066 g MgSO4 ·7 H2O, 0.04 mg CuSO4 ·5 H2O, 0.021 mg ZnCl2, 0.041 mg CoCl2 ·6 H2O, and 0.025 mg Na2MoO4 ·2 H2O; the pH was adjusted to 6.5 with 1 M HCl. Glucose medium was mineral medium with 5.0 g/liter glucose. Lignin-guaiacol-benomyl medium (35) contained (per liter) 0.5 g KH2PO4, 0.2 g MgSO4 ·7 H2O, 0.1 g NH4NO3, 0.1 g KCl, 0.02 g FeSO4 ·7 H2O, 0.05 g Ca(NO3)2 ·4 H2O, 2.0 g malt extract broth (Lab M, International Diagnostic Group, Lancashire, United Kingdom), and 15 g agar (Difco).
TABLE 1. Fungal isolates and most closely related fungi from a BLAST search of the GenBank database

<table>
<thead>
<tr>
<th>Isolate*</th>
<th>Results of BLAST search (% identity)</th>
<th>N demethylation</th>
<th>Hydroxylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mortierella sp. strain Gr4 (zygo)</td>
<td>Mortierella cf. hyalina (91)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fusarium sp. strain Gr6 (asco)</td>
<td>Fusarium spp. (100)</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Mucor sp. strain Gr22 (zygo)</td>
<td>Mucor hiemalis (100)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gr57 (asco)</td>
<td>Tetracladium maxilliforme (99)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Phoma cf. eupyrena Gr61 (asco)</td>
<td>Phoma spp., Podascus spp., and Didymella planata (98)c</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Yeast Gr86 (bas)</td>
<td>Cryptococcus sp. (100)</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Acremonium sp. strain Gr141 (asco)</td>
<td>Clonostachys sp., Nectria gliocladioides, and Bionectria ochroleuca (100)c</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Alternaria sp. strain Gr174 (asco)</td>
<td>Alternaria spp. (100)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Basidiomycete strain Gr177</td>
<td>Coprinus lagopus (93)</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

As, ascomycete; zygo, zygomycete; bas, basidiomycete.

More than 160 isolates were obtained from the Græse soil using the three isolation strategies. From these isolates, 10 fungi were selected to represent the zygo-, asco-, and basidiomycetes. The ITS rRNA genes of the 10 selected fungi were sequenced, and the fungi were identified to the genus level when possible (Table 1). A BLAST search of the ITS sequences confirmed the morphological identification except for the fungus morphologically identified as Acremonium sp. strain Gr161, for which no close matches were detected. Strain Gr177 could be identified as a basidiomycete by its clamp connections, and its sequence most closely resembled sequences of isolates of Coprinacea. Gr57 did not sporulate, but the closest matching sequence was the sequence from Tetrad cladium maxilliforme, an aquatic hyphomycete which is also often isolated from soils (7).

The most rapid degradation of isoproturon was seen with cultures of Mortierella sp. strain Gr4 and Phoma cf. eupyrena Gr61. In these cultures, about 80% of the isoproturon disappeared within 20 days (Fig. 2). This could not be explained by production of MDIPU, DDIPU, or 4-isopropylaniline since only a transient accumulation of MDIPU was observed with Mortierella sp. strain Gr4. However, gradient HPLC analysis revealed a peak at 11.1 min that accumulated with both fungi and a peak at 7.0 min with Mortierella sp. strain Gr4. There was less rapid disappearance of isoproturon with three other fungi. Mucor sp. strain Gr22 produced a peak at 9.8 min, while Alternaria sp. strain Gr174 produced a peak at 11.1 min and basidiomycete strain Gr177 produced a mixture of MDIPU and low concentrations of the compounds with peaks at 9.8 and 11.1 min. The integrated area for the unknown compounds at 7.0, 9.8, and 11.1 min mirrored the area of the disappearing isoproturon peak since the total integrated area was almost constant in the five cultures (Fig. 2). These compounds were not fungal exudates, since they were never observed in cultures.
to which isoproturon was not added. Isoproturon did not degrade in the abiotic control (Fig. 2). None of the remaining cultures exhibited degradation of isoproturon, and the average concentrations of isoproturon at day 25 ranged from 45.6 to 55.2 µmol/liter (coefficient of variation, 5.2%; n = 3). Gr57 and Clonostachys sp. strain Gr141 produced traces of MDIPU, however. With Gr57 the average concentration was 1.0 µmol/liter at day 25, while with Gr141 the average concentration ranged from 1.5 to 2.3 µmol/liter from day 10 to day 25 (coefficient of variation, <74%; n = 3).

Preparative HPLC was performed with media containing 50 mg/liter isoproturon incubated for 14 days with mycelia of Mucor sp. strain Gr22 or Phoma cf. eupyrena Gr61 and media with 50 mg/liter MDIPU incubated for 4 days with Mortierella sp. strain Gr4. By comparison with known NMR assignments we easily identified the HPLC peak at 9.8 min as 2-OH-IPU and the peak at 11.1 min as 1-OH-IPU (11). The peak at 7.0 min did not match any reported NMR assignments for isoproturon metabolites. Together with the typical shifts from the remaining protons of the molecule, the presence of methylene shifts at 3.50 ppm and 3.56 ppm showing vicinal coupling and two separate NH shifts at 7.03 ppm (N3H) and 5.00 (N1H), respectively, confirmed the identification of the HPLC peak at 7.0 min as 1-OH-MDIPU (Table 2).

Growth was observed for all fungi, and the biomasses ranged from 0.14 ± 0.04 to 2.0 ± 0.1 mg/ml (mean ± standard deviation; n = 3), so the lack of isoproturon degradation with some of the fungi was not due to an inability to grow in the medium.

**DISCUSSION**

The 10 fungi tested in the present study represent asco-, basidi-, and zygomycetes and are related to genera consid-
tered to be common soil saprophytes that are often isolated from agricultural soils (7, 19). Basidiomycetes, such as isolate Gr177, are rarely isolated from agricultural soils, but this may be because they are overlooked due to their low growth rates on agar (35).

Several of the fungal cultures rapidly transformed isoproturon with hydroxylated metabolites as the dominant products. To our knowledge, this is the first detailed demonstration of the hydroxylation of isoproturon by soil fungi. N demethylation has previously been reported to be the dominant pathway for fungal degradation of isoproturon, although it has been mentioned that unidentified products might be the result of hydroxylation (2, 3) and hydroxylated isoproturon has previously been suggested to be a fungal degradation product (4). The lignin and manganese peroxidases of the white rot fungus Phanerochaete chrysosporium have also been shown to produce unidentified products that have been suggested to be hydroxylated compounds (6).

In the present study, three isoproturon transformation processes were identified for the soil fungi: N demethylation to MDIPU and two types of hydroxylation, at the first and second positions on the isopropyl side chain, yielding 1-OH- and 2-OH-MDIPU, respectively. Mortierella sp. strain Gr4 hydroxylated only at the first position. This process was dominant for Phoma cf. eupyrena Gr61 and Alternaria sp. strain Gr174, but these organisms also produced traces of 2-OH-MDIPU. Mucor sp. strain Gr22 hydroxylated mainly at the second position, while basidiomycete strain Gr177 produced equal amounts of 1-OH- and 2-OH-MDIPU, although it produced both at low concentrations. The only hydroxylating isolate that did not also N demethylate was Phoma cf. eupyrena Gr61 (Fig. 2). 1-OH-MDIPU is the product of combined N demethylation and hydroxylation and was produced only by Mortierella sp. strain Gr4. Isoproturon did not serve as an energy or nutrient source for the fungi, and the purpose of these fungal transformations is unknown. They may be elicited by a detoxification system, however.

A mass balance for the transformation of isoproturon could not be obtained because no authentic standards of the hydroxylated compounds were available. In all cultures, however, the total integrated area of the isoproturon and metabolite peaks remained close to constant (Fig. 2). Isoproturon, MDIPU, and DDIPU have similar responses at 245 nm for similar concentrations, and their UV spectra in the range from 200 to 300 nm are comparable to those of the hydroxylated metabolites. It is therefore reasonable to assume that the peak area of hydroxylated phenylureas explains the removal of isoproturon observed; i.e., hydroxylation was the major degradation process in this study. Sorption and mineralization are other possible means of isoproturon removal from the medium. P. chrysosporium mineralized 14C-labeled isoproturon and 3,4-dichloroaniline, the aniline metabolite of the phenylurea herbicide diuron, but only at a high temperature (39°C) and in a pure oxygen atmosphere (18, 24). No mineralization of isoproturon has been observed with soil fungi incubated under atmospheric air with 14C-labeled isoproturon (3). Only bacteria enriched from soil with a history of isoproturon application have otherwise been able to mineralize isoproturon (27, 29). Berger (3) reported that the mycelium sorbed about one-third of the isoproturon in liquid cultures of Phoma eupyrena and Cladosporium herbarum, while six other fungi did not sorb isoproturon. Sorption must have been a minor mechanism in our study, since almost all the disappearance of isoproturon can be explained by hydroxylation.

Isoproturon can be hydroxylated at two different positions on the isopropyl side chain, yielding 1-OH-IPU and 2-OH-IPU (Fig. 1). Only 2-OH metabolites have previously been detected in soil samples, however (8, 16, 22, 23, 25, 26, 34). In the studies in which 1-OH metabolites were analyzed, they were not detected (26, 33). In our study, in contrast, the soil fungi were able to produce both 1-OH and 2-OH metabolites. It is possible that the two types of metabolites are both produced in soils and that they have markedly different half-lives.

The initial transformation of phenylurea herbicides has previously been shown to be crucial for the rate of mineralization. A mixed bacterial culture from the Grese field mineralized MDIPU readily but isoproturon poorly, thus indicating that the mixed culture depends on the N demethylation being carried out by other organisms (28). In a study with Acinetobacter calcoaceticus, mineralization of the phenylurea chlortoluron was dependent on a transformation catalyzed by a cytochrome P450 monooxygenase. The bacterium was genetically engineered to express cytochrome P450 CYP105D1 and gained the ability to N demethylate and hydroxylate chlortoluron. The transformed strain was then able to mineralize and grow on chlortoluron (15). This raises the possibility of interactions between different microorganisms since it seems possible that the hydroxylated and N-demethylated metabolites of a phenylurea herbicide could serve as substrates for mineralizing microorganisms. Future research should clarify the potential of soils and bacterial isolates to mineralize fungal metabolites of isoproturon. This could determine the persistence of the metabolites and may provide new insight into interactions between fungi and bacteria in the degradation of the herbicide.

A large proportion of the fungi examined in this study transformed isoproturon to hydroxylated metabolites that are also found in environmental samples (25, 26). This suggests that soil fungi could be responsible for production of the hydroxylated metabolites detected in environmental samples. Any conclusion should be drawn cautiously, however, as the activity of the isolated fungi in soil is unknown, and the involvement of other soil organisms cannot be ruled out. The hydroxylated...
metabolites produced by fungi may be important from an environmental point of view as field data suggest that 2-OH-IPU has a greater tendency to leach from the soil than MDIPU (26). The higher mobility of hydroxylated metabolites underlines the need to evaluate the production of these compounds, their ecotoxicity, and their fate in soils.

ACKNOWLEDGMENTS

A. H. Johnsen and A. Kastrup provided valuable assistance in the preliminary identification of metabolites. S. Rønhede was funded by a grant from the Danish Technical Research Council.

REFERENCES