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Mineralization of hydroxylated isoproturon metabolites produced by fungi

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Abstract

When exposed to the herbicide isoproturon, some soil fungi in pure culture metabolize the substance to hydroxylated metabolites. Hydroxylated metabolites of isoproturon have also been detected in soil studies. In an agricultural soil not previously exposed to isoproturon we found that the hydroxylated isoproturon metabolite *N*-(4-(2-hydroxy-1-methylethyl)phenyl)-*N*′,*N*′-dimethylurea mineralized faster than both isoproturon and its *N*-demethylated metabolite *N*-(4-isopropylphenyl)-*N*′-methylurea (MDIPU), thus indicating that mineralization of isoproturon is stimulated by fungal hydroxylation in this soil. In soils previously treated with isoproturon, in contrast, isoproturon and both its hydroxylated and demethylated metabolites mineralized at almost the same rate with up to 52% of the ^{14}C-ring-carbon being degraded to $^{14}CO_2$ within 63 days. Thus hydroxylated metabolites of isoproturon do not seem to be more persistent than isoproturon, and hence may degrade before they can leach from topsoil and contaminate the aquatic environment. While an isoproturon-mineralizing bacterium *Sphingomonas* sp. SRS2 and a MDIPU-mineralizing mixed bacterial culture were able to deplete the medium of hydroxylated metabolites, little or no mineralization took place. This indicates that other bacteria must be present in the soil that are able to benefit from isoproturon being made available to mineralization by fungal hydroxylation.

Keywords: Phenylurea herbicide; Degradation; Bioconversion; Hydroxylation; *Sphingomonas* sp. SRS2; *Phoma eupyrena*

1. Introduction

Cereal farmers in many parts of the world use the herbicide isoproturon (Fig. 1). In Europe, isoproturon has since the sixties been one of the most heavily used herbicides in winter wheat and barley, where it is used as a pre-emergence herbicide in autumn (Sørensen et al., 2003). Isoproturon is relatively mobile in soil, and its extensive use has resulted in the frequent contamination of isoproturon in surface water and groundwater in Europe. The European Union has accordingly placed isoproturon on a list of 33 priority substances that present a significant risk to or via the aquatic environment (European Council, 2001). Microbial degradation of isoproturon in the topsoil plays an important role in the environmental fate of isoproturon. In some soils bacteria use isoproturon for growth, and completely mineralize the compound to CO_2 and biomass. In other soils, in contrast, isoproturon is transformed into metabolites that may be more mobile than isoproturon itself and therefore a threat to surface water and groundwater (Sørensen et al., 2003).

Bacterial strains that completely mineralize the isoproturon ring carbon into CO_2 and biomass have been isolated from soils that have been exposed to isoproturon for long periods (Sørensen et al., 2001; Bending et al., 2003; Sebai et al., 2004). *Sphingomonas* sp. strain SRS2, a bacterium isolated from a British soil, degrades isoproturon by initial *N*-demethylation to MDIPU (MonoDesmethyl-IsoProtUron) and DDIPU (DiDesmethyl-IsoProtUron) (Fig. 1). The DDIPU is then hydrolyzed to 4-isopropyl-aniline, after which the ring carbon is mineralized (Sørensen et al., 2001). An *Arthrobacter* sp. has been shown to hydrolyze the carbonyl group of the urea side chain of isoproturon, mineralize the side chain and accumulate 4-isopropyl-aniline in the medium (Cullington and Walker, 1999;

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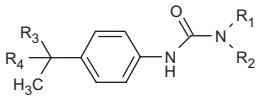
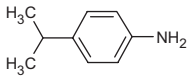


General structure



4-isopropyl-aniline

	R1	R2	R3	R4	IUPAC name
Isoproturon	CH_3	CH_3	CH_3	H	*N*-(4-isopropylphenyl)-*N′*,*N′*-dimethylurea
1-OH-IPU	CH_3	CH_3	CH_2OH	H	*N*-(4-(2-hydroxy-1-methylethyl)phenyl)-*N′*,*N′*-dimethylurea
2-OH-IPU	CH_3	CH_3	CH_3	OH	*N*-(4-(1-hydroxy-1-methylethyl)phenyl)-*N′*,*N′*-dimethylurea
MDIPU	H	CH_3	CH_3	H	*N*-(4-isopropylphenyl)-*N′*-methylurea
1-OH-MDIPU	H	CH_3	CH_2OH	H	*N*-(4-(2-hydroxy-1-methylethyl)phenyl)-*N′*-methylurea
2-OH-MDIPU	H	CH_3	CH_3	OH	*N*-(4-(1-hydroxy-1-methylethyl)phenyl)-*N′*-methylurea
DDIPU	H	H	CH_3	H	*N*-(4-isopropylphenyl)urea
1-OH-DDIPU	H	H	CH_2OH	H	*N*-(4-(2-hydroxy-1-methylethyl)phenyl)urea
2-OH-DDIPU	H	H	CH_3	OH	*N*-(4-(1-hydroxy-1-methylethyl)phenyl)urea

Fig. 1. Chemical structure and names of the herbicide isoproturon and the metabolites included in this study.

Turnbull et al., 2001). In contrast to the above-mentioned soil bacteria, soil fungi metabolize isoproturon to hydroxylated metabolites. Thus 5 of 10 isolates from agricultural soil, including ascomycetes, basidiomycetes and zygomycetes, were found to hydroxylate isoproturon at the isopropyl side chain to generate 2-OH-IPU, 1-OH-IPU and 1-OH-MDIPU (Fig. 1) (Rønhede et al., 2005).

Hydroxylated metabolites of isoproturon have also been detected in soil (e.g. Mudd et al., 1983; Schroll and Kühn, 2004; Elkhattabi et al., 2004; Alletto et al., 2006), and 2-OH-IPU and 2-OH-MDIPU (Fig. 1) have been detected in run-off and in lysimeter leachate after heavy rainfall (Schuelein et al., 1996; Dörfler et al., 2006). The fate of hydroxylated isoproturon metabolites in soil has not been described, and whether or not they can be further degraded and mineralized by bacteria and other soil organisms or instead comprise dead-end metabolites of environmental concern is unknown.

The aim of the present study is therefore to determine whether agricultural soils are able to mineralize hydroxylated isoproturon metabolites produced by fungi and whether previously isolated isoproturon-mineralizing and MDIPU-mineralizing bacteria are also able to degrade and mineralize hydroxylated metabolites of isoproturon.

2. Materials and methods

2.1. Chemicals and media

The analytical standards (purity >98%) for isoproturon (CAS no. 34123-59-6), MDIPU (CAS no. 34123-57-4), DDIPU (CAS no. 56046-17-4) and 4-isopropyl-aniline (CAS no. 99-88-7) were obtained from Dr. Ehrenstorfer GmbH (Augsberg, Germany). The [^{14}C-U-phenyl]-labeled isoproturon (2.15 MBq mg^{-1}) and MDIPU (4.42 MBq mg^{-1}) were purchased from Izotop (Budapest, Hungary). As evaluated by thin-layer chromatography (TLC), the radiochemical purity of our stocks of ^{14}C-isoproturon and ^{14}C-MDIPU were 99% and 90%, respectively.

Buffer 1 (per liter): 1.33 g $Na_2HPO_2 \cdot 2H_2O$, 1.38 g KH_2PO_4 and 9.0 g NaCl. The pH was 6.6.

Medium A (per liter): 2.14 g $Na_2HPO_2 \cdot 2H_2O$, 0.572 g K_2HPO_4, 0.179 g $(NH_4)_2SO_4$, 66.5 mg KNO_3, 66.5 mg $MgSO_4 \cdot 7H_2O$, 13.3 mg $CaCl_2$, 3.80 mg H_3BO_4, 2.05 mg $MnSO_4 \cdot H_2O$, 50 μg $CuSO_4 \cdot 5H_2O$, 27 μg $ZnCl_2$, 54 μg $CoCl_2 \cdot 6H_2O$, and 33 μg $Na_2MoO_4 \cdot 2H_2O$. After autoclaving, 1.33 ml of a sterile filtered solution of $FeCl_3 \cdot 6H_2O$ was added to yield a final concentration of 6.85 mg l^{-1}. Since *Sphingomonas* sp. SRS2 depends on an external source of certain amino acids (Sørensen et al., 2002), $(NH_4)_2SO_4$ and KNO_3 were replaced by 0.133 g casamino acids l^{-1} (Difco) in the experiments with this bacterium.

Malt extract (ME) broth (per liter): 10 g ME broth adjusted to pH 6.0.

Potato dextrose agar (PDA) and R2A were purchased from Difco. R2B was prepared as described by Reasoner and Geldreich (1985). Specieller Nährstoffarmer Agar (SNA) was prepared as described previously (Rønhede et al., 2005).

2.2. Soils

Four soils were used in the study. The soil designated "Jyndevad" was sampled from an agricultural field located near the village of Jyndevad in southern Jutland, Denmark. The field is used for organic farming, and no pesticides