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Identification of sulfonylurea biodegradation pathways enabled by a novel nicosulfuron-transforming strain *Pseudomonas fluorescens* SG-1: Toxicity assessment and effect of formulation

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HIGHLIGHTS

- A nicosulfuron-transforming strain *P. fluorescens* has been isolated and characterized.
- Two major metabolites of nicosulfuron were identified (ADMP and ASDM).
- ADMP is 20-fold more toxic toward *A. fischeri* than nicosulfuron.
- Formulated-nicosulfuron biotransformation is lower than that of the active ingredient.
- Other sulfonylureas are biotransformed by this strain through various pathways.

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ABSTRACT

Nicosulfuron is a selective herbicide belonging to the sulfonylurea family, commonly used on maize culture. A bacterial strain SG-1 was isolated from an agricultural soil previously treated with nicosulfuron. This strain was identified as *Pseudomonas fluorescens* and is able to quantitatively dissipate 77.5% of nicosulfuron (1 mM) at 28 °C in the presence of glucose within the first day of incubation. Four metabolites were identified among which ASDM (2-(aminosulfonyl)-N,N-dimethyl-3-pyridinecarboxamide) and ADMP (2-amino-4,6-dimethoxypyrimidine) in substantial proportions, corresponding to the hydrolytic sulfonylurea cleavage. Two-phase dissipation kinetics of nicosulfuron by SG-1 were observed at the highest concentrations tested (0.5 and 1 mM) due to biosorption. The extend and rate of formulated nicosulfuron transformation were considerably reduced compared to those with the pure active ingredient (appearance of a lag phase, 30% dissipation after 10 days of incubation instead of 100% with the pure herbicide) but the same metabolites were observed. The toxicity of metabolites (standardized Microtox® test) showed a 20-fold higher toxicity of ADMP than nicosulfuron. *P. fluorescens* strain SG-1 was also able to biotransform two other sulfonylureas (metsulfuron-methyl and tribenuron-methyl) with various novel pathways. These results provide new tools for a comprehensive picture of the sulfonylurea environmental fate and toxicity of nicosulfuron in the environment.

1. Introduction

Sulfonylureas are a large family of herbicides, widely used for the control of broad leaf weeds in various crops and vegetables, with sales multiplied by 100 during the last 30 years in Europe and North America [1]. Since their discovery in the mid-70s, several active ingredients (AI) were developed and about 20 are now marketed worldwide. They can be classified in 3 sub-groups according to the substituents R_1 and R_2 on the sulfonylurea bridge: (i) R_1 = pyridinic ring and R_2 = pyrimidinic ring (*e.g.* nicosulfuron, rimsulfuron); (ii)

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R_1 = aromatic ring and R_2 = triazinic ring (*e.g.* tribenuron-methyl, metsulfuron-methyl); (iii) others. When applied at low doses varying from 2 to 75 g of AI per hectare, sulfonylureas possess high herbicidal activity, broad spectrum of action, good selectivity and low mammalian toxicity [2,3]. They act as inhibitors of the plant acetolactate synthase (ALS), a key enzyme for branched-chain amino acid synthesis, which stop cell division and plant growth [4]. Nevertheless, because of their high phytotoxicity towards sensitive crops [5,6] and the likelihood of their transport in surface runoff and/or transfer to groundwater [7], their fate and their potential impact on aquatic ecosystems is of concern.

Nicosulfuron is often considered as the representative of the family since it is one of the most-sold sulfonylurea herbicides [8]. This compound is a post-emergence herbicide commonly used on corn cultures at 60 g AI/ha. Its solubility (7.5 g/L) and sorption coefficient (0.14 to 2.15 L/kg according to the soil characteristics) make this compound relatively mobile and bioavailable in soil and water [9,10]. This compound is non-volatile (vapour pressure < 8.10^{-10} Pa) and its photolysis is not expected to be a major route of transformation in soil and water [11,12]. However, nicosulfuron can be transformed *via* microbial activities and chemical hydrolysis [13]. Some bacterial and fungal strains able to biotransform nicosulfuron have been isolated but only with an additional source of carbon (glucose or rich medium) (co-metabolism) [14–18]. The major metabolites identified, ADMP (2-amino-4,6-dimethoxypyrimidine) and ASDM (2-(aminosulfonyl)-N,N-dimethyl-3-pyridinecarboxamide) [11] were also detected in soils, surface water and crops. Previous studies showed toxic effects of nicosulfuron on various aquatic plants, such as macrophytes, phytoplankton, diatoms and freshwater microalgae [11,19–21] but also shifts in the microbial community structure of soil treated, leading to the selection of nicosulfuron-tolerant bacteria [22,23]. Nevertheless, the metabolites have not yet been considered in these toxicity studies. Another key parameter is that the farmers do not spray the herbicide as a pure AI but as a formulation (mixture of AI and co-formulants (surfactants, solvents, synergists, . . .)) [24]. The presence of these chemicals can greatly modify the fate of AI [25] and is not often taken into account.

The aim of our study was to compare the biotransformation kinetics and pathways of pure and formulated nicosulfuron by a newly-isolated bacterial strain from an agricultural soil. The toxicity of nicosulfuron and its metabolites, alone or in mixtures, was also assessed with the normalized Microtox® test. The potential of this strain to biotransform other sulfonylureas was tested and metabolic pathways were proposed.

2. Experimental

2.1. Chemicals and media

Nicosulfuron (purity 99.6%), metsulfuron-methyl (purity 99.2%), tribenuron-methyl (purity 99.1%), rimsulfuron (purity 99.9%) and ADMP (2-amino-4,6-dimethoxypyrimidine, purity 98.0%) were purchased from Sigma Aldrich (France) and ASDM (2-(aminosulfonyl)-N,N-dimethyl-3-pyridinecarboxamide, purity 98%) from J and K Scientific (Germany). Mineral salt medium (MSM), pH 6.7, was composed of (/L): 1 g $(NH_4)_2HPO_4$, 1 g KH_2PO_4, 1 g KNO_3, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.1 g NaCl, 0.02 g $CaCl_2$, 0.005 g $FeSO_4{\cdot}7H_2O$, 1 mL each of a salt and vitamin stock solutions. The salt stock solution contained (/L) 20 g boric acid, 18 g $MnSO_4{\cdot}H_2O$, 2 g $ZnSO_4$, 1 g $CuSO_4$, 2.5 g Na_2MoO_4, 0.01 g $Co(NO_3)_2$. The vitamin stock solution contained (/L): 2 mg biotin, 5 mg thiamine-HCl. The glucose-mineral salt medium (GSM) was composed of 5 g/L glucose in MSM. The MSM and GSM agar plates were prepared by adding 1.5% (w/v) agar into the liquid media. Tryptic soy broth (TS) medium (Sigma Aldrich, France) is composed of (/L): 17 g casein peptone, 3 g soya peptone, 5 g NaCl, 2.5 g K_2HPO_4, 2.5 g glucose (pH 7.3).

2.2. Enrichment culture and isolation of nicosulfuron-transforming bacteria

Nicosulfuron herbicide-contaminated soil was collected from the surface layer (0–5 cm) of an agricultural field in Limagne (Puy-de-Dôme, France) planted with maize and treated with a nicosulfuron/mesotrione/S-metolachlor cocktail used at the recommended agronomic dose (60 g/150 g/1920 g AI/ha, respectively). 60 g of air dried, sieved (Ø = 2 mm), homogenised soil was added to 500 mL (10% dry soil w/v) of MSM or GSM in 2L flasks. The final nicosulfuron concentration in the enrichment media was initially 0.05 mM. Two additional enrichment steps were realized every 3 weeks by subculturing 10 mL of the culture into 490 mL of fresh medium containing 0.1 and then 0.5 mM of nicosulfuron. The flasks were incubated at 28 °C on an orbital shaker at 150 rpm in darkness. The cultures were sampled weekly for bacterial isolation on MSM or GSM agar plates containing nicosulfuron (final concentration corresponding to that of the enrichment step). Pure colonies were tested for their capacity to transform nicosulfuron in liquid MSM or GSM. Agar plates and liquid culture were incubated at 28 °C in the dark for at least eight days. The liquid cultures were daily sampled for nicosulfuron quantification by HPLC (cf. Section 2.5.1). Among the isolates coming from the final step of the enrichment, a pure strain transforming completely the nicosulfuron in GSM medium was selected and named SG-1.

2.3. Identification of the nicosulfuron-transforming strain SG-1

The identification of the isolated strain SG-1 was carried out by Vitek® test (Biomérieux, France) and 16S rDNA gene sequencing. Bacterial genomic DNA of strain SG-1 was extracted *via* the QIAamp kit (Qiagen) following the manufacturer's recommendations and used as template for 16S rDNA gene PCR amplification using universal primers 27f (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492r (5′-GGT TAC CTT GTT ACG ACT T-3′) as described by Batisson et al. [26]. The PCR products were sequenced by Eurofins using the primers 27f, 338f (5′-ACT CCT ACG GGA GGC AGC AG-3′), 518r (5′-ATT ACC GCG GCT GCT GG-3′), 968f (5′-AAC GCG AAG AAC CTT AC-3′) and 1492r. The sequence was deposited in GenBank under the accession number KU291443.

The SG-1 16S rDNA sequence was compared to the closest sequences retrieved in GenBank database using BLAST (http://www.ncbi.nlm.nih.gov/BLAST). The phylogenetic analysis was carried out by multiple alignments (Clustal Ω) followed by construction of a phylogenetic tree (MEGA 6.0 software).

2.4. Biotransformation of nicosulfuron and other sulfonylurea herbicides

The biotransformation of nicosulfuron (0.1, 0.5 or 1 mM) by the SG-1 strain was determined in GSM. SG-1 was pre-cultured in TS medium, washed in NaCl 0.8% and used to inoculate 150 mL of GSM in 500 mL flasks (10^5 cfu.mL^{-1} final). Uninoculated media served as abiotic controls. Each experiment was carried out in triplicate. The cultures were incubated in the dark at 28 °C under agitation at 150 rpm on a rotary shaker and sampled (2 × 1 mL) periodically for growth (Optical Density $OD_{\lambda = 600nm}$) and determination of nicosulfuron residual concentration (HPLC) measurements. The metabolite analysis (HPLC, ^{1}H NMR and LC/ESI–MS) and toxicological bioassays (Microtox® test) were carried out at an initial 1 mM nicosulfuron concentration.

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