Applied Soil Ecology 45 (2010) 218–224

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

Evaluation of the *atz*B gene as a functional marker for the simazine-degrading potential of an agricultural soil

Martínez-Iñigo M. José[b], Gibello Alicia[a], Lobo Carmen[b], Nande Mar[a], Vargas Raquel[a], Garbi Carlos[a], Fajardo Carmen[a], Martín Margarita[a,*]

[a] Complutense University Avenida Puerta de Hierro s/n, 28040 Madrid, Spain
[b] Instituto Madrileño de Investigación y Desarrollo Rural, Agrario y Alimentario (IMIDRA), Finca "El Encín" Km 38, 2 A-II Apdo 127, 28800 Madrid, Spain

ARTICLE INFO

Article history:
Received 6 February 2009
Received in revised form 21 April 2010
Accepted 24 April 2010

Keywords:
Simazine
Urea
Biodegradation
*atz*B gene
FISH

ABSTRACT

The occurrence of natural simazine-degrading bacteria could be an important limiting factor to the use of the herbicide in those agricultural soils with a wide history of herbicide applications. In this work the potential of an agricultural soil to degrade simazine and the effect of the addition of urea was assayed in both fertilised and unfertilised soil microcosms. A culture-independent approach based on the fluorescence *in situ* hybridisation (FISH) technique, using a specific oligonucleotide probe (AtzB1), was applied to detect simazine-degrading bacteria in the soil microcosms. The presence of the *atz*ABC genes in the agricultural soil was confirmed by PCR from soil-extracted DNA. The percentage of AtzB1 probe-target cells in the urea-untreated soil was higher than in the urea-treated one. Moreover, the greatest percentage of AtzB1 probe-target cells in the urea-untreated soil was accompanied by a greater degradation rate, compared to the urea-fertilised one. Our results indicate that the proposed approach was sensitive enough to detect changes in the natural simazine degradation capacity of the soil after fertilisation practices, which typically involve a nitrogen increase.

1. Introduction

Simazine (2-chloro-4,6-bis(ethylamine)-*s*-triazine) is an *s*-triazine-ring herbicide classified as relatively persistent in soil, but intensively used to control, through photosystem II inhibition, pre- and post-emergence of broadleaf weeds among major vegetable and ornamental crops (Fielding et al., 1992). In fact, the presence of simazine in the soil–water system is considered to be an environmental hazard, and because of its estrogenic effect on various cell lines in laboratory experiments, simazine has recently become a compound subjected to control (Sanderson et al., 2001).

The natural occurrence of soil microorganisms capable of mineralising simazine has been reported in agricultural soils where the herbicide had been repeatedly applied (Santiago-Mora et al., 2005). The ring system of the *s*-triazine herbicides is used by soil microorganisms mainly as a source of nitrogen (Bichat and Mulvaney, 1999; Mandelbaum et al., 1995; Radosevich et al., 1995; Struthers et al., 1998; Topp et al., 2000a,b), since only the ring-side chains are available as a source of energy, due to the full oxidation state of the ring carbons after the side chains are removed. However the biodegradation of triazines by adapted microbial populations can be affected by the availability of carbon and nitrogen in the soil (Abdelhafid et al., 2000). Soil nitrogen starvation promotes the biodegradation of N-heterocyclic compounds, including triazines, whereas mineral or organic supplementary sources of nitrogen decrease triazine ring mineralisation (Sims, 2006).

Natural soil attenuation by native microbial populations under simazine selective pressure, seems to be the most suitable strategy for simazine removal from long-term contaminated soil sites, since other procedures, such as bio-augmentation and the use of recombinant simazine-degrading bacteria, have not been considered to be efficient alternatives (Strong et al., 2000; Morán et al., 2006). On the other hand, in fields where triazines are heavily used, the enhanced degradation of triazines by soil-bacteria could result in a loss of herbicide function (Krutz et al., 2008; Shaner and Henry, 2007). Therefore, the study of the soil ecology requires improved monitoring tools to manage microbial populations involved in *s*-triazines degradation.

The advent of molecular techniques, such as functional gene-targeted fluorescence *in situ* hybridisation (FISH) and PCR amplification, has improved our ability to analyse the native microbial composition of soil populations without the need for cultivation (Jen et al., 2007; Martín et al., 2008). In studies on the natural attenuation capacity of soils contaminated with *s*-triazine herbicides, the monitoring of functional genes encoding one of the key enzymes associated with this process is considered to be an attractive approach to determine the soil remediation potential. We focused on the *atz*B which serves as the point of intersection of

* Corresponding author. Tel.: +34 913943911; fax: +34 913943883.
E-mail address: margamar@vet.ucm.es (M. Margarita).

0929-1393/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.apsoil.2010.04.008

multiple *s*-triazine biodegradation pathways and that is completely essential for microbial growth on *s*-triazine herbicides. Unlike *atz*A and *trz*N, which are largely distributed between Gram-negative and Gram-positive bacteria, respectively, *atz*B has been found in phylogenetically diverse bacteria (Seffernick et al., 2007). Moreover, *atz*B nucleotide specific sequences have previously been used as probes for FISH detection of bacterial strains that are capable of degrading simazine (Martín et al., 2008).

Thus, the objective of this study is to evaluate the utility of the *atz*B gene as a functional biomarker to detect variations in the natural simazine attenuation capacity of a soil due to common fertilisation practices, which typically involve an increase in nitrogen content.

2. Materials and methods

2.1. Soil sampling and physicochemical analysis

Bulk soil samples were collected from the surface layer A (0–10 cm depth) of a Calcic Haploxeralf loam (USDA) located in Meco (Central Spain). These samples had been exposed to repeated applications of simazine in earlier cultivation practices. The soil samples were air-dried and sieved (<2 mm) before analyses. The soil texture was analysed according to the methods of Day (1965), while the organic matter and total nitrogen content were determined using the Walkley–Black (Nelson and Sommers, 1996) and Kjeldahl methods (Ministerio de Agricultura, 1992), respectively. The soil pH was measured in a 1:2.5 soil-to-water ratio. The level of carbonates was determined according to the methods of Loeppert and Suarez (1996). Urease activity was determined using methods described by Nannipieri et al. (1978).

2.2. Simazine-degrading bacterial community in the soil

2.2.1. Cultivation and isolation of bacteria

An enrichment of the soil sample (1 g) was performed in 9 ml of Luria Bertani (LB) medium (Sambrook et al., 1989) at 28 °C for 1 h. After enrichment, the suspension was serially diluted 10-fold in 0.9% saline buffer. Bacterial enumeration was performed in triplicate by plating 0.1 ml of each appropriate dilution on LB agar and a modified minimal R medium agar plates (Selifonova et al., 1993) containing 100 mg of simazine per liter (R-S) as the sole nitrogen source and 300 $\mu g\,ml^{-1}$ cycloheximide in order to prevent fungal growth; plates were incubated for 5 days at 28 °C.

2.2.2. Identification of bacterial isolates

The pure bacterial cultures, isolated from the soil samples previously plated on LB and R-S media, were characterised by a conventional microbiological analysis (morphological characterisation, KOH test, Gram staining, and oxidase and catalase tests) and a large fragment of 1500 bp of the 16S rRNA gene of each isolate was amplified using the universal primers: pA (5′-GAGAGTTTGATCCTGGCTCAGGA; positions 7–27 *Escherichia coli* numbering) and pH* (5′-AAGGAGGTGATCCAGCCGCA; positions 1541–1522), as described previously (Aranaz et al., 2008). PCR products were purified with a QIAquick PCR purification kit (Qiagen) and sequenced using the ABI Prism 377 DNA sequencer (Applied Biosystems) from Secugen facilities (Centro de Investigaciones Biológicas, CSIC, Spain). Sequence data were analysed by the BLASTn (http://www.ncbi.nlm.nih.gov) program.

2.2.3. PCR amplification of the atzABC genes from soil DNA

DNA from the soil sample was extracted with the Ultra Clean Soil DNA Kit (MoBio Laboratories, Inc., Solana Beach, CA, USA). DNA extracted from a pure culture of *Pseudomonas* sp. strain ADP (Realpure Spin Kit Real, Durviz S.L.) was used as a template for the positive control of the PCR. PCR was carried out using specific primers designed from conserved regions of the catabolic genes, *atz*A, *atz*B, and *atz*C, as previously reported (de Souza et al., 1998; Devers et al., 2004). The reaction conditions were: 1 min at 95 °C, 40 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C, plus an additional 10-min step at 72 °C. The PCR products were separated on a 1.5% agarose gel, and the resolved DNA bands were compared to amplicons from the positive control. To check the specificity of the primers, the amplicons obtained from the *Pseudomonas* ADP DNA were sequenced and compared to the target gene sequences available in the GenBank/EMBL databases using the http://www.ncbi.nlm.nih.gov software.

2.3. Microcosm experiments

The experiment was a completely randomized design with three replications and the following treatments: (a) soil supplemented with simazine (Si) and (b) soil supplemented with simazine and urea (SiU).

2.3.1. Degradation studies

The degradation of simazine was studied in two sets of triplicate soil microcosms with 200 g of soil in 1 L Erlenmeyer flasks, where the moisture content was adjusted to 22% (w/w). One set of microcosms (Si) was prepared with soil that was supplemented with simazine (99% purity, Sigma–Aldrich, St. Louis, MO) at the agricultural dose of 4.5 $mg\,kg^{-1}$ of soil. To study the effect of urea on simazine degradation, a second set of soil microcosms (SiU) was prepared with soil that was supplemented with simazine (4.5 $mg\,kg^{-1}$ soil) and with urea at an agricultural dose of 500 $mg\,kg^{-1}$ of soil. The sterile soil samples, treated only with sterile water, were used as controls. All of the microcosms were incubated for 30 days in a climatic chamber at 21 ± 0.5 °C in the dark.

Sub-samples of urea-treated and -untreated microcosms were removed periodically, after 0, 10, 17, 24 and 30 days, in order to monitor the chlorinated herbicide content. Simazine was extracted from the soil microcosm samples with methanol, and the concentrations of the herbicide in the extracts were measured by HPLC, as previously reported (Sánchez et al., 2005; Martín et al., 2008). The analyses were performed with a Waters model 616PDA996 photodiode array detector, equipped with the Millennium 20/10 software for data analysis. Simazine separation was performed on a Novapack C-18 (3.9 mm × 150 mm) column with an injection volume of 10 μl, using a mobile phase consisting of 40% acetonitrile in water at a flow rate of 0.5 $ml\,min^{-1}$. Simazine was monitored at 214 nm and was identified by coelution with a standard in HPLC analysis.

2.3.2. Mineralisation studies

Mineralisation assays of [U-ring ^{14}C] simazine (Sigma–Aldrich, St. Louis, MO) were also performed in two sets of microcosms. One-gram samples of soil of the Si microcosms and the SiU microcosms, supplemented with urea (500 $mg\,kg^{-1}$ soil), were placed in 50 ml biometer flasks sealed with Teflon stoppers, containing 10 ml of sterile PJC medium (Hareland et al., 1975) and 2 μCi of [U-ring ^{14}C] simazine (Sánchez et al., 2005; Martín et al., 2008). In all cases, a non-radioactive simazine solution was added to obtain a final concentration of 5 $mg\,l^{-1}$. The incubations were performed in triplicate, and controls were prepared with sterile soil samples.

The resulting $^{14}CO_2$ from the mineralisation was trapped in a vial with 1 ml of 1N NaOH solution. The final radioactivity was measured by scintillation counting with a Hewlett-Packard model 2500 TR scintillation spectrometer. The sampling was performed after 0, 10, 17, 24 and 30 days.