Wild soil fungi able to degrade the herbicide isoproturon

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Hongos de suelo nativos con potencial degradador del herbicida isoproturon

Resumen. El herbicida isoproturon (IPU) es un contaminante fuerte debido a su elevada solubilidad acuosa y bajo potencial para la degradación microbiana. El problema ambiental se debe a su amplio empleo en la agricultura convencional, áreas urbanas, algicida y aditivo de pinturas. Especies fúngicas degradaron el herbicida en cultivos in-vitro, produciendo derivados hidroxilados frecuentes en areas agrícolas. Los objetivos planteados fueron aislar las especies fúngicas representativas de suelos tratados con IPU, evaluar su potencial transformador, e identificar los derivados. La mayoría de los aislamientos produjeron 1-OH-IPU y 2-OH-IPU, indicando que los hongos pueden ser la causa de estos compuestos en las muestras ambientales. De los 35 aislamientos, 10 de ellos fueron especies dominantes y activas degradadoras. *Aspergillus* ochraceus, *Fusarium flocciferum*, *Talaromyces helicus*, *Acremonium strictum*, *Mucor hiemalis*, *Paecilomyces lilacinus* y *Penicillium frequentans* transformaron el herbicida produciendo cantidades significativas de diversos derivados. Esta es la primera mención de dicha actividad para estas especies.
Palabras claves: bioremediación, hongos filamentosos de suelo, potencial degradador, metabolitos hidroxilados

Abstract. The herbicide isoproturon (IPU) is a strong contaminant due to it water solubility and low microbial degradation. Environmental concerns arised from the worldwide use of this herbicide in conventional agriculture, urban areas and algicide in antifouling paints. Several fungi have shown the ability to degrade IPU and its derivatives; however, hydroxylated metabolites were frequently detected in agricultural areas. The aims of this study were to isolate representatives fungi from polluted soils, to study their IPU-degradation potential and to identify the transformation products. *Aspergillus ochraceus*, *Fusarium flocciferum*, *Talaromyces helicus*, *Acremonium strictum*, *Mucor hiemalis*, *Paecilomyces lilacinus* and *Penicillium frequentans* uptook the herbicide and produced significant amounts of diverse derivatives. The fungi produced 1-OH-IPU and 2-OH-IPU, being the source of the hydroxylated IPU-derivatives detected in environment. Therefore, this study demonstrated that selected fungi could be used for the polluted soils bioremediation.
Keywords: bioremediation, filamentous species, biodegradation potential, hydroxylated metabolites

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Introduction

The herbicide isoproturon, 3-(4-isopropylphenyl)-1,1,dimethylurea (IPU) and other phenylurea compounds had been detected as priority pollutants (Environment Agency, 2000; Environment Agency, 2001) due to their high water solubility and low microbial degradation potential that determined the levels exceeding the limit concentrations (Stig *et al*., 2005). Environmental concerns arised from the widespread IPU used being the most extensively pesticide in conventional agriculture (Suhadolc *et al*., 2004). IPU and its derivatives had shown detrimental effects to algae (Pérés *et al*., 1996; Mostafa and Helling, 2001) invertebrates, ciliated protozoan (Perrin-Ganier *et al*., 2001) and microbial activity as 50% methanogenesis reduction (Attaway *et al*., 1982; Remde and Traunspurger, 1994) also genotoxic effects had also been revealed (Behera and Bhunya, 1990; Hoshiya *et al*., 1993). Degradation can involve abiotic and biotic processes, where microbial transformation is especially interesting, since it is the only known pathways to complete mineralisation of aromatic compounds (Alexander, 1981; Mansour *et al*., 1999). Slow natural biodegradation rate, 5-25% within 2-3 months was more frequent than quick and extensive IPU-mineralisation only observed in previously field-treated soils (Sørensen *et al*., 2002).

Most of the studies used enrichment-culture techniques to isolate phenylurea-degrading microorganisms (Stangroom *et al*., 1998), but some isolates from agricultural soils failed to extensively degrade the herbicide (Bending *et al*., 2001). The addition of IPU-metabolite, 3-(4-isopropylphenyl)-1-methylurea (MDIPU) as carbon and energy source yielded a mixed culture able to mineralized MDIPU and 4-isopropyl-aniline (4IA), with enhanced herbicide degradation (Sørensen and Aamand., 2001), evenmore, others studies indicated that co-operative metabolic activities may be involved in the IPU-transformation (Sebai *et al*., 2004). In soils, however, hydroxylated metabolites have frequently been observed; therefore, the aims of this study were: 1) to identify wild filamentous soil fungi as organisms that potentially play a role in the formation of these hydroxylated intermediates in soils treated with isoproturon, 2) to confirm their IPU-potential uptake and 3) to identify the transformation products.

Materials and methods

Soil samples. Samples were taken from the upper 10 cm of an agricultural area near La Plata, Argentina. Soil borne fungi were obtained from organic soil particles by sieving through a 2 mm sieve, and 5.0 g soil was added to a L-liter sterile screw-cap bottle with 500 mL of 0.1% (wt/vol) $Na_4O_7P_2 \cdot 10H_2O$ to disperse clumps and colloids, and shaken horizontally for 1 h at 4°C (Thorn *et al*., 1996).

The suspension was poured through a stack of two sieves with grids of 0.5 mm and 63 µm. After a brief rinse with cold tap water, the 0.5 mm sieve was removed, and the contents of the 63 µm sieve were washed for 2 min under cold tap water. When the mineral fraction settled and most of the water run out, 1 mL was collected from the dense suspension of organic particles and used for isolation of fungi.

Fungal isolation. Filamentous fungi were isolated by plating 100 µL of a 1x 10^2 dilution of the soil organic particles on mineral medium (MM) (Romero *et al*., 2005) with 5.0 g IPU /Liter and 2% agar. The isoproturon was allowed to dissolve overnight before use. pH was adjusted to 6.5 with 1 M HCl, after autoclaving and cooling to 50°C, the IPU-MM was supplemented with 40 mg/L tetracycline, 20 mg penicillin/Liter and 20 mg streptomycin/Liter. The dishes were incubated in the dark at 27 °C and screened for 3 weeks. Hyphae growing from particles were transferred to other IPU-MM plates to confirm the biodegradation potential.

Fungal isolates were characterized on the basis of colony morphology, spore structures microscopy and nucleotide sequences of the internal transcribed spacers (ITS) of rRNA genes. Mycelium from cultures was extracted in 40 µL Tris-EDTA buffer (pH 8.0) with 10 µL 20% Chelex. A 2 µL aliquot of the extract was used as the template for a PCR using primers ITS1F and ITS4 (White *et al*., 1990). Amplification was performed with a thermocycler (PCT-200; MJ Research Inc., Massachusetts) using the PCR conditions described by Gardes and Bruns (1993). PCR products were purified (StrataPrep PCR purification kit; catalog no. 211189-1; Stratagene) and sequenced by MWG-Biotech AG.

Degradation analysis. Three filamentous fungi were examined for IPU-degradation. The isolates were inoculated as 1 mL portions of spore suspensions of each culture, prepared by vortexing sporulating mycelium in 3 mL distilled water containing 1 g/L Tween 80 and 8.5 g/L NaCl. Each fungus was inoculated into 100 mL bottles with IPU-MM, and incubated in the dark, at 27°C, 150 rpm for 30 days, by triplicate. Three different control flasks were incubated in the same conditions, one without IPU, another non-inoculated bottle and a third one inoculated and sterilized flask, in order to allow differentiation between fungal exudates, to assess IPU-uptake and its metabolites production in the chromatographic analysis. One mL sample was collected every fifth day with a syringe and passed through a polytetraflouroethylene filter (diam 17 mm; Titan 2 HPLC filter; 0.20 µm membrane; no. 42213-PC) into glass vials for the chromatographic analysis. An HPLC system (1050 HP; Hewlett-Packard) with a UV/VIS detector was used with a Hypersil 5 µm C18 column (250 by 2 mm; Phenomenex).

The biomass of the fungi was determined at the end of the experiment, 30^{th} day, by filtering the mycelia onto filter paper disks; then samples were air dried to a constant weight in a fume hood. The assays were done by triplicate, and the results are reported as arithmetic means with 5 % standard deviation.

Routine HPLC analysis of IPU, MDIPU (3-(4-isopropylphenyl)-1-methylurea), DDIPU (3-(4-isopropylphenyl)-urea) and 4-isopropyl-aniline (4IA) was performed using the isocratic method (Juhler *et al*., 2001) and identified by comparison with authenthic standards and literature data. Hydroxylated intermediates, 1-OH-IPU, 2-OH-IPU and 1-OH-MDIPU, were detected with a gradient method using acetonitrile and water as eluents at 0.3 mL/min flow rate. For the first 8 min 15% (by volume) acetonitrile was used, then it was raised linearly to 45% between 8-12 min., and between 23-25 min the acetonitrile level decreased to 15%. Equilibration time before the next injection was 7 min, with 4 µL injection volume and the 45°C column temperature. Phenylureas were detected and quantified at 245 nm, and 4IA was detected and quantified at 200 nm. The retention times were: 7.0 min for 1-OH-MDIPU; 9.8 min for 2-OH-IPU; 11.1 min for 1-OH-IPU; 17.8 min for DDIPU; 18.7 min for MDIPU; 20.0 min for IPU; and 22.0 min for 4IA (Del Pilar Castillo *et al*., 2001; Gerecke *et al*., 2001). The reported IPU-derivatives are the arithmetic means of three separated experiments with replicated batch cultures; standard deviation was no more than 5 %. The analytical standards were purchased from Ehrenstorfer GmbH, Augsberg, Germany: isoproturon (CAS no. 34123-59-6), MDIPU (CAS no. 34123-57-4), DDIPU (CAS no. 56046-17-4) and 4IA (CAS no. 99-88-7).

Results and discussion

About 35 isolates were obtained from the IPU-polluted soils using the isolation strategies, then, the 10 dominants fungi, that represented 70 % of the isolates, were selected and identified to the genus level when possible. *Acremonium strictum, Alternaria alternata, Aspergillus ochraceus, Fusarium flocciferum, Mortierella* spp.*, Mucor hiemalis,*

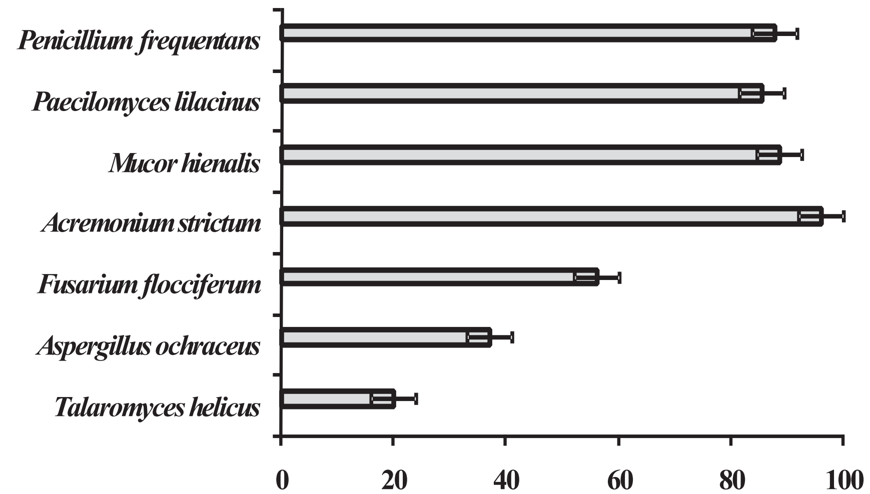

Figure 1. IPU residual levels at the 30th day incubation time (expressed as IPU-percentage in relation to the control flasks).

Phoma spp., *Paecilomyces lilacinus, Penicillium frequentans, Talaromyces wortmannii* and *Trametes versicolor* were identified as IPU-degrading isolates. *A. ochraceus, A. strictum, F. flocciferum, M. hiemalis, P. lilacinus, P. frequentans* and *T. helicus* were used in further IPU-bioassays to isolate and identify the derivatives as they showed the higher metabolic activity againt the herbicide.

A significant uptake was observed in *T. helicus* cultures, approximatly 80% IPU disappeared within 15 days, considering as 100% the initial IPU-level (Figure 1). Demethylation to MDIPU and DDIPU, and two hydroxylation position, at the first and second points on the isopropyl side chain, yielding 1-OH-UPI, 2-OH-IPU and 1-OH-MDIPU metabolites with this fungi (Figure 2). The integrated area of the detected compounds mirrored the area of the residual IPU-peak, and the total integrated area was almost constant in the cultures.

A significant IPU-uptake was also detected in the other fungi cultures, as 63 and 54% of the herbicide had disappeared at the end of the 30th incubation day in *A. ochraceus* and *F. flocciferum* assays, respectively. *A. ochraceus* produced the same derivatives as *T. helicus* but in minor amounts and within 25th days; while in the *F. flocciferum* cultures brief 1-OH-MDIPU and non DDIPU levels were observed. *A. strictum, M. hiemalis, P. lilacinus* and *P. frequentans* cultures exhibited a transient IPU-transformation, as a significant residual IPU-levels were detected at 30th day. Moreover, demethylation was dominant for *A. strictum,* and *M. hiemalis* produced more hydroxylated intermediates at the first position; while *P. lilacinus* and *P. frequentans* produced similar amounts of 1-OH-IPU, 2-OH-IPU and low MDIPU concentrations.

In the remaining fungal cultures, the herbicide initial levels were quite similar to the controls, suggesting that these species are not active degradors of the studied pesticide.

IPU was not degrade in the three abiotic controls, a decreae of only 0.65% was observed in the aninoculated bottles, 0.48% in the control without IPU as substrate, but a slight higher value (0.95%) was observed in the inoculated and sterilized flasks. The first values could be to IPU-absortion to the soil particles, the last one could be explained by the present of diverse substances produced during the sterilization processes.

Other researches had pointed out that MDIPU was the main metabolite produced by pure cultures of soil fungi (Sandermann *et al*., 1998; Sørensen and Aamand., 2001) and 1-OH-IPU had only been measured in cultures derived from soils (Elkhattabi *et al*, 2004; Spliid and Køppen, 1998). In the

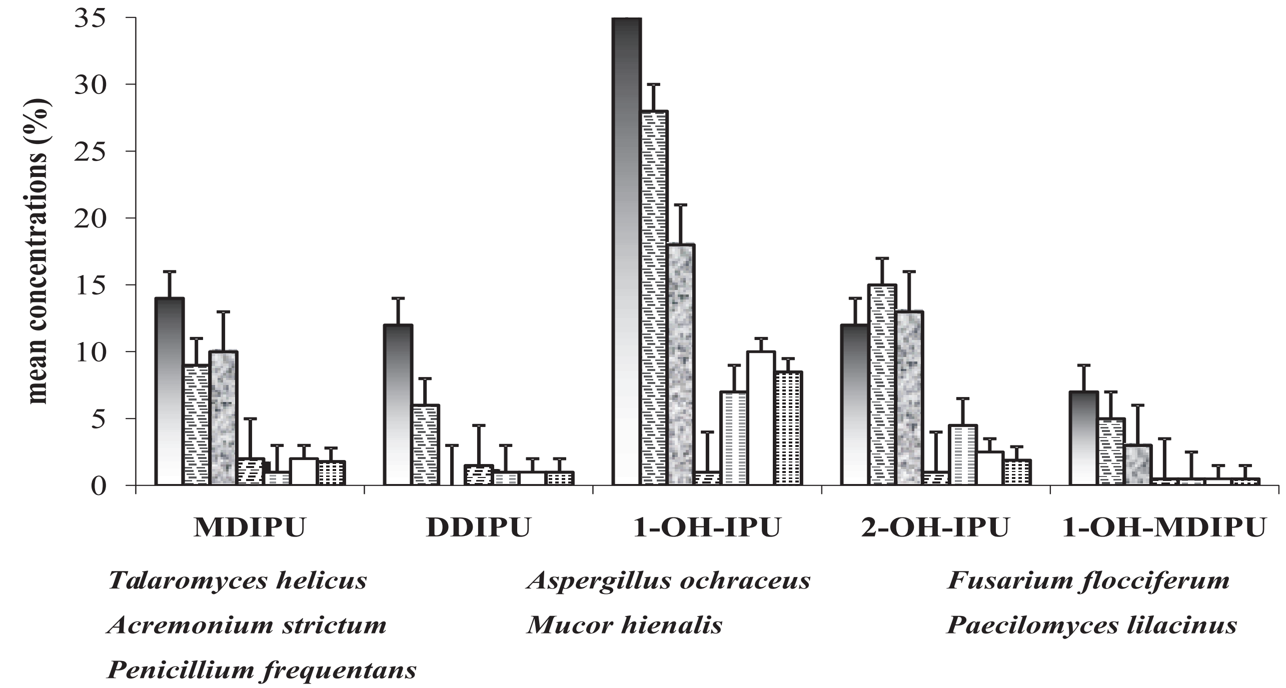

Figure 2. IPU-derivatives produced by each fungal species (mean concentrations at 30 th day incubation time; MDIPU: 3-(4-isopropylphenyl)-1-methylurea; DDIPU: 3-(4-isopropylphenyl)-urea; 1-OH-IPU, 2-OH-IPU and 1-OH-MDIPU: hydroxylate metabolites).

present study, demethylation and two hydroxylation position of IPU were detected in the fungal assays, with a highly significant IPU transformation in the *T. helicus* and *A ochraceus* experiments, as only 20 and 37% residual IPU-levels were detected at the final incubation time.

The fungal hydroxylated metabolites may be important from an environmental point of view as field data suggest that 2-OH-IPU had a greater tendency than MDIPU to leach from the soil (Glässgen *et al*., 1999). The higher mobility of the hydroxylated intermediates underlined the need to evaluate the production of these compounds, their ecotoxicity and their fate in soils (Walker *et al*., 2002; Bolte, 2004).

Growth was observed in *A. ochraceus, F. flocciferum, T. helicus,* as these fungal species were able to uptake the herbicide and produced significant and diverse intermediates; also in the *A. strictum, M. hiemalis, P. lilacinus* and *P. frequentans* experiments, a slight growth were obtained. The biomasses ranged from 2.0 0.1 to 0.14 0.04 mg/mL for the first and second fungi group, respectively. Therefore, the fungal species were able to grow in this medium using IPU as carbon and energy sources, and without a second substrate.

The fungi able to partialy degrade phenylurea herbicides cover a broad range of different species including *Cunninghamella elegans*, *Mortierella isabellina* (Tixier *et al*., 2000), *Talaromyces wortmanii* (Vroumsia *et al*., 1996), *Rhizopus japonicus*, *Rhizoctonia solani* and *Aspergillus niger* (Vroumsia *et al*., 1996). Among other authors results, *Mortierella* spp., *Phoma* spp. and *Alternaria* spp. hydroxylated isoproturon at the first position of the isopropyl side chain 3 (Sandermann *et al*., 1998). This study is the first mention as IPU-degraders for the following filamentous soil fungi *A. ochraceus, F. flocciferum, T. helicus, A. strictum, M.*

hiemalis, P. lilacinus and *P. frequentans*, they uptook the herbicide and produced significant amounts and diverse derivatives. Therefore, enhancing the possibility to detoxify polluted areas by natural bioremediation strategies, as the mentionated micoflora are frequent and active species in polluted sediments. These fungi could be used to accelerate the detoxification of polluted areas (Schuelein *et al*., 1996; Bending *et al*., 2003).

In conclusion, thirty-five isolates were obtained from IPU-polluted soils and 10 dominat of them were active to degradate the herbicide. We suggested that soil filamentous fungi were responsible for the hydroxylated metabolites detected in the environmental polluted samples. The advantage of the fungal activity could be explained by easy metabolized intermediates produced by fungi, and the subsequent degradation by other microorganisms (Sørensen *et al*., 2003). This could determine the hydroxylated derivatives persistence and may provide new insight into interactions between fungi and bacteria in the IPU-degradation (Lehr *et al*., 1996; Sørensen *et al*., 2001).

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