ΦYTON
REVISTA INTERNACIONAL DE BOTÁNICA EXPERIMENTAL
INTERNATIONAL JOURNAL OF EXPERIMENTAL BOTANY
FUNDACION ROMULO RAGGIO
Gaspar Campos 861, 1638 Vicente López (BA), Argentina
www.revistaphyton.fund-romuloraggio.org.ar

Fungi degrading metsulfuron methyl in agricultural soils of Argentina

Hongos que degradan metsulfurón metil en suelos agrícolas de Argentina

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Abstract. Strains of *Mucor*, *Penicillium* and *Trichoderma*, able to use metsulfuron-methyl (MM) as a sole carbon and energy source, were isolated from agricultural soils in Argentina. This is the first time this ability is recorded in the *Mucor* or *Trichoderma* species. When the isolated strains were compared in relation to their capacity to use the herbicide, those of *Trichoderma* showed the best results. Because of this, they were selected for further assays. The effect of metsulfuron on growth in liquid media was tested. Also, the ability of conidia to germinate in water agar medium containing the target herbicide was evaluated. All the strains could grow from spores using only metsulfuron-methyl as a carbon and energy source. These strains of *Trichoderma* are promising candidates to be used in the recovery of soils and waters contaminated with this herbicide.

Keywords: Degradation; Herbicides; Soils; Sulfonylurea; *Trichoderma*.

Resumen. Cepas de *Mucor*, *Penicillium* y *Trichoderma* capaces de usar metsulfurón metilo (MM) como única fuente de carbono y energía fueron aisladas de suelos agrícolas de Argentina. Esta es la primera vez que esta habilidad se registra en especies de los géneros *Mucor* y *Trichoderma*. Cuando las cepas aisladas se compararon en cuanto a su capacidad para utilizar el herbicida, las de *Trichoderma* fueron las más eficientes. Debido a esto se las seleccionó para realizar otros ensayos. Se evaluó el efecto del MM en el crecimiento de las cepas en medio líquido y la capacidad de los conidios para germinar en medio de cultivo adicionado con MM. Todas las cepas de *Trichodema* lograron crecer a partir de conidios utilizando solamente metsulfurón metilo como fuente de carbono y energía. Estas cepas son candidatos promisorios para ser utilizados en la recuperación de suelos y aguas contaminadas con este herbicida.

Palabras clave: Degradación; Herbicidas; Suelos; Sulfonilureas; *Trichoderma*.

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Recibido / Received 31.III.2012. Aceptado / Accepted 15.IX.2012.

INTRODUCTION

The herbicide metsulfuron-methyl (MM) [methyl 2-(4-methoxy-6-methyl-1,3,5-triazine-2-ylcarbamoylsulfamoyl) benzoate] is a member of the sulfonylurea group. Sulfonylureas are extensively used in several countries for the control of a wide range of weeds in cereals, pastures and other winter crops. Among these products, MM is widely used because it has high herbicidal activity at low application rates (Ye et al., 2003). Metsulfuron is absorbed by leaves or roots, so it is mainly used as a post-emergency herbicide (Papa & Massaro, 2005). Herbicide residues represent a potential risk as pollutants in soils and waters and they have been reported to cause damage to rotation of substitution crops (Moyer et al., 1994; Ahonsi et al., 2004; Yu et al., 2005). Up to 48% of the applied MM is converted to bound residues in soil (Pons & Barriuso, 1998; Ye et al., 2002). Their phytotoxicity is mainly caused by the MM parent compound that becomes available during plant growth (Ye et al., 2003). Low levels of these residues may have unintentional effects on non-target animals or plants (Li et al., 2005). The residual effect depends on the active ingredient, dose, natural degradation and crop sensibility.

Biological and biochemical mediated processes in soils are very important for ecosystem functioning (Zabaloy et al., 2008). Soil microbes are the driving force behind many soil processes including transformation and degradation of xenobiotics (Yu et al., 2005). Several microorganisms are known to metabolize sulfonylurea herbicides (Berger et al., 1998; He et al., 2007; Filimon et al. 2012). However, little research has been done on the microbial degradation of MM, with most studies focusing on its environmental fate and behavior (He et al., 2007). As pioneering works, Zanardini et al. (2002) isolated a *Pseudomonas* strain able to degrade MM under co-metabolic conditions. Also, Boschin et al. (2003) studied the pathway of biodegradation of MM in rich medium using a common soil fungus, *Aspergillus niger* Tiegh. Using the herbicide as a selective agent, Yu et al. (2005) isolated a strain of *Curvularia* sp. able to use it as sole source of carbon and energy, and studied several features of herbicide degradation in pure culture and soils. He et al. (2006) isolated four bacteria, nine filamentous fungi and twenty actinomycetes capable to use MM, and selected an unknown strain of *Penicillium* sp. (DS11F) as the most effective degrader. Thereafter, He et al. (2007) inoculated wheat rhizosphere with a highly effective degrading *Penicillium* strain, previously isolated from treated soil, and probed that the inoculation enhanced the degradation of MM. Although the mentioned microorganisms do not degrade the total MM present in soils, they can decrease significantly its level, so they would be able to minimize their undesirable effects on soils. The potential of microorganisms as detoxifying agents has led to an increasing research on this subject (He et al., 2007).

In Argentina, metsulfuron-methyl was introduced in the middle ´80s and it has been widely used, especially in the southern Pampas. Little research has been done in Argentina regarding the microbial degradation of MM. Zabaloy et al. (2008) studied bacterial functional diversity in several Argentinean soils treated with different herbicides, including MM. González Matute et al. (2012) studied the feasibility of the application of degraded mushroom compost of *Agaricus blazei* to degrade metsulfuron methyl. However, up to date, there has been no attempt to study the native soil fungi involved in MM degradation. Our objectives were (1) to isolate fungal strains from agricultural soils of Argentina capable of growing with MM as a sole carbon and energy source, (2) to select those with better performance in media containing the herbicide, and (3) to evaluate their response to different concentrations of the herbicide.

MATERIALS AND METHODS

Sampling, isolation and selection of fungi. Soil sampling was carried out on mid autumn (April, 2007) in the experimental station of INTA Bordenave, Argentina (37° 51´ 55´´ S, 63° 1´ 20´´ W). Soils in the area are classified as Silt Loam (pH: 7.76, carbon content: 4.09%). Average temperatures in the region are 10 °C in winter and 28 °C in summer, and rainfall, mostly distributed in autumn and spring (INTA web) is about 672 mm per year. Samples were taken from plots with different history of tillage and herbicide application (Table 1). In each plot, several soil cores were taken from the upper 20 cm and then mixed to make a composite sample. Samples were stored in polyethylene bags at 5 °C before use.

Table 1. Tillage and herbicide history of each experimental, sampled plot.
Tabla 1. Sistema de labranza e historia de aplicación de herbicida de cada parcela muestreada.

Soil	Tillage system	MM doses (g a.i./Ha/Year)	Time of application (in years)	Date of last application
S2	mechanical tillage	4.2 (regular)	3	30/03/2007
S3	direct seeding	9.2 (heavy)	7	30/06/2006
S4	direct seeding	4.2 (regular)	3	11/05/2006

Before isolation of fungi, an enrichment method was employed. Three sub-samples (10 g each) were taken from each composite soil sample. Each soil sub-sample was added into 120 mL of a mineral salt solution "MMS" (formulated as in Yu, et al. 2005) in 250 mL Erlenmeyer flasks, and incubated 22 days at 25 °C in an orbital shaker. To each flask, metsulfuron methyl "MM" (Riedel-de-Häen, Pestanal 99.3% a.i., Germany) was added three times: 1 mg (day 1), 6 mg (day 8) and 12

mg (day 15). On day 22, 1 mL was taken from each flask and suspended in 9 mL of fresh sterile MMS plus 1 mg of MM. After a week (day 29), this last procedure was repeated adding 6 mg of MM, and on day 34 the same procedure was done adding 12 mg of MM. The highest concentration reached during the enrichment was four thousand times higher than the maximum field dose. After one week, isolation was performed from the last solution and three aliquots (1 mL each) were taken and plated on malt agar (Malt extract 10 g, agar 20 g, distilled water 1 L). Plates were incubated 7 days at 25 °C, and colonies were identified to genus level on the basis of colonial morphology, cultural and microscopic features according to Domsch et al. (1980) and Chaverri & Samuels (2003). For selection, individual colonies were inoculated on plates with metsulfuron methyl enriched media (MMA) that consisted of saline agar (MSS 1 L, agar 20 g) and 1 mg a.i. herbicide/plate (metsulfuron methyl Trimet, origin China, company Tamer). Plates were incubated 7 days at 25 °C, those strains that could grow on MMA were selected, and their growth rate was estimated. For each strain, five MMA plates were centrally inoculated with 6-mm mycelial plugs taken from the margins of active growing colonies. Plates were incubated for a week at 25 °C and colony diameter was measured daily. Faster growing strains (*Trichoderma*) were selected to perform the following assays. All the strains are preserved at the LEBBAH, and the *Trichoderma* strains are also kept at the USDA (United States Departament of Agriculture, BPI collection).

***Trichoderma* assays.** Biomass estimation was done in malt broth (Malt extract 20 g, distilled water 1 L) and metsulfuron methyl broth (160 mg MM Trimet, MMS 1 L). The experiment was repeated three times. Mycelial plugs of each strain were taken from the margin of active growing colonies. Plugs were inoculated in bottles with 120 mL of each media and they were incubated in an orbital shaker at 25 °C. After a week, the mycelium was collected by filtration, and the pellets were dried to constant weight.

Tolerance and conidial germination were evaluated on solid media with three concentrations (5 x 10^{-4}, 5 x 10^{-3} and 0.1 mg/L) of metsulfuron methyl against a control with no herbicide. Conidia from 2-week-old malt agar cultures were harvested with 10 mL of 0.1% Tween 80. From the resulting conidial suspension, 0.1 mL were poured onto solid media (MSS 1 L, agar 20 g) enriched with the herbicide. The whole experiment was done in triplicate. Plates were incubated at 25 °C for a week; growth and conidial germination were qualitatively assessed under a stereoscopic microscope.

RESULTS

Thirty strains belonging to seven fungal genera were recovered on malt agar from agricultural soils in Argentina after applying an enrichment method with metsulfuron methyl (Table 2). All the strains were tested on their ability to grow on a solid media with the herbicide as a sole carbon and energy source (MMA). Only two strains of *Penicillium*, three of *Trichoderma* 3 and two of *Mucor* could grow on that medium (Table 2). When growth rate on MMA was compared among

Table 2. Genera and number of strains of fungi recovered on malt agar using an enrichment method with MM. The number of strains able to grow in MMA is also shown.
Tabla 2. Géneros y número de cepas de hongos recuperados en agar malta utilizando un método de enriquecimiento con MM. También se presenta el número de cepas capaces de crecer en MMA.

Soil	Genera	Number of strains recovered in malt agar	Number of strains able to grow in MMA
2	*Alternaria*	1	-
	Trichoderma	3	-
3	*Alternaria*	1	-
	Penicillium	8	2
	Rhizopus	1	-
	Trichoderma	6	3
4	*Cladosporium*	2	-
	Fusarium	2	-
	Mucor	6	2

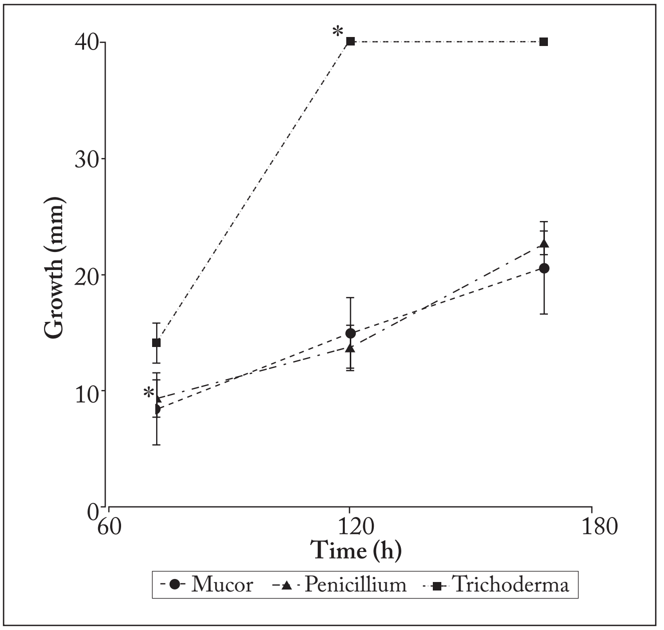

Fig. 1. Average growth of *Mucor*, *Penicillium* and *Trichoderma* strains on MMA (1 mg MM per plate) at 25 °C. Asterisks indicate beginning of sporulation and bars indicate the standard error.
Fig. 1. Crecimiento promedio de las cepas de *Mucor*, *Penicillium* y *Trichoderma* en MMA (1 mg MM por placa) a 25 °C. Los asteriscos indican el inicio de la esporulación y las barras indican el error estándar.

them, those of *Trichoderma* had an average growth of 6.35 ± 0.41 mm/d versus 3.06 ± 0.20 mm/d and 2.91 ± 0.41 mm/d registered for *Penicillium* and *Mucor* strains, respectively (Fig. 1). *Penicillium* and *Trichoderma* strains sporulated on MMA, being the sporulation much more abundant in the second ones (visual evaluation).

As *Trichoderma* strains (hereafter named as T5, T6 and T7) showed the better performance on MMA, they were considered the most promissory as herbicide degraders so they were selected to be thoroughly studied. *Trichoderma* growth was estimated in liquid media. The three strains produced about ten times lower biomass in metsulfuron methyl broth than in malt broth; T5 and T7 produced 0.53 g/L, while T6 produced 0.5 g/L. In malt broth the estimated values of biomass were 4.83 g/L for T5, 5.5 g/L for T6 and 4.19 g/L for T7.

Growth and conidial germination were evaluated on solid media with different concentrations of metsulfuron methyl. Germination was not inhibited by any of tested concentrations, but T5 showed a tendency to decrease both mycelium production and sporulation with herbicide increment, while T6 and T7 produced more hyphae and conidia with the increasing MM concentration.

DISCUSSION

In this paper, we report on the recovery of representatives of seven genera of fungi from soils treated with metsulfuron methyl, a sulfonylurea herbicide. All these genera have been previously registered by Cabello (1985). They are considered common inhabitants of soils (Watanabe, 2002) and several contain species that have been reported as tolerant or good degraders of xenobiotic substances. Fungi are well-known for their capacity to use complex substances but fungal biotransformation of herbicides has been less explored than bacterial degradation although they are considered the most effective in herbicide degradation among soil microorganisms (Filimon et al., 2011). From the thirty recovered strains, just a few of *Mucor*, *Penicillium* and *Trichoderma* could grow with the herbicide as the sole nutritive and energy source.

To our knowledge, this is the first time the capacity to degrade MM is described in a *Mucor* species. *Mucor* species are known to degrade 2,4-D and 2,4-DCP (Vroumsia et al., 2005; Joshi & Gupta, 2008) and several species have been mentioned as degraders of phenylurea herbicides such as chlortoluron, diuron and isoproturon (Vroumsia et al., 1996); among them a strain of *M. racemosus* could degrade up to 60% of chlortoluron added to the media. Our results support the idea that the ability to degrade xenobiotic compounds is widespread among *Mucor* species.

The recovery of *Penicillium* strains able to degrade MM is not surprising. Several species in the genus have been reported as degraders of herbicides like 2,4-D and phenylurea, including MM (Vroumsia et al., 1996; Silva et al., 2007; Joshi & Gupta, 2008). All *Penicillium* strains isolated here could grow in MMA, but they did it significantly slower than *Trichoderma* ones. He et al. (2006) also noticed that on a *Penicillium* species isolated from MM treated soils in China, the initial concentration of MM affected its degradation by the fungus, causing toxicity and reducing the vitality of the organism.

The ability of *Trichoderma* to degrade MM is reported here for the first time. Fungal species belonging to the genus *Trichoderma* are easily isolated from the soil worldwide (Howell, 2003). They are successfully used as biocontrol and biorremediation agents (Vroumsia et al., 1996; López-Mondéjar et al., 2010). Howell (2003) considers that the most salient feature of *Trichoderma* species is their ability to parasitize other fungi; several species are mycoparasites of important phytopathogenic fungi and so are used as biocontrol agents but their ability to use xenobiotics has been less explored. Our results indicate that *Trichoderma* strains were the most efficient to grow with high concentration of MM. This is consistent with the early report of Vroumsia et al. (1996) on diuron and would indicate they are good degraders of phenylurea herbicides.

Penicillium and *Trichoderma* strains were effective degraders of metsulfuron methyl and showed better performance on media with the herbicide than *Mucor* strains. The former were recovered from a soil which had been exposed to elevated doses of MM during 7 years while *Mucor* strains come from a soil which has received smaller doses of herbicide during a shorter period. In long-term applications, pesticides would act as strong selection agents favoring the development of an active microbial population with the ability to degrade them (Hernández García et al., 2008; Diez, 2010). We interpreted that *Mucor* strains were less adapted to the herbicide and it could alter them in some way.

In conclusion, we isolated several fungi from agricultural soils treated with MM, and we made the first report of MM degrading capacity on fungi belonging to the genera *Mucor* and *Trichoderma*. From the isolated strains, the *Trichoderma* ones showed better growth in MM media. Our next step will be to focus on the maximum concentrations of MM these fungi could tolerate without altering their degrading capacity. Our results are promissory on the feasibility of using the isolated *Trichoderma* strains to remove and detoxify metsulfuron methyl from water and soils, but this needs more detailed investigations in the future.

ACKNOWLEDGEMENTS

This research was partially supported by a PICT-FONCYT grant (PICT-07/00380). M.B. Vázquez holds a fellowship from CONICET. We thank N. Curvetto for the analytical grade herbicide supply and Mario Vigna (EEA-INTA Bordenave) for collecting the soil samples.

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