

# Molecular identification of three isolates of *Trichoderma harzianum* isolated from agricultural soils in Argentina, and their abilities to detoxify in vitro metsulfuron methyl

María Belén Vázquez, Viviana Barrera, and Virginia Bianchinotti

**Abstract:** Metsulfuron methyl (MM) is a sulfonylurea herbicide used worldwide for the control of weeds in cereal crops. In a previous study, three *Trichoderma* strains (T5, T6, and T7) capable of using MM as a sole carbon and energy source were isolated. In this study, the three strains were identified as *Trichoderma harzianum* using genetic markers, and the transformation of MM by the *T. harzianum* strains was quantified using spectrophotometry. Solutions of different phytotoxic doses of MM were incubated with plugs of mycelia of the *Trichoderma* strains and the resulting mixtures were used to assess MM detoxification. The toxicity of the degradation products was tested with a bioassay using pre-germinated seeds of *Lens culinaris* Medik. and mycelia. Strain T7 was more efficient in transforming MM at higher concentrations than the T5 and T6 strains. In the bioassay, T5 showed the best performance at higher MM doses. We conclude that both T5 and T7 strains are promising for further studies regarding treatment or amelioration of MM contaminated soils.

**Key words:** bioassay, detoxification, metsulfuron methyl, spectrophotometric quantification, *Trichoderma*.

**Résumé :** Le metsulfuron méthyle (MM) est une sulfonylurée herbicide utilisée à travers le monde pour contrôler les mauvaises herbes des champs de céréales. Dans une étude précédente, trois souches de *Trichoderma* (T5, T6 et T7) capables d'utiliser le MM comme unique source de carbone et d'énergie avaient été isolées. Dans l'étude actuelle, les trois souches ont attribuées à *Trichoderma harzianum* par des marqueurs génétiques, et la transformation du MM par les souches de *T. harzianum* a été quantifiée par spectrophotométrie. Des solutions comprenant différentes doses phytotoxiques de MM ont été incubées avec des boulettes de mycélium des souches de *Trichoderma* et les mélanges résultants ont été utilisés afin d'évaluer la détoxification du MM. La toxicité des produits de dégradation a été testée par un dosage biologique à l'aide de graines pré-germées de *Lens culinaris* Medik. et du mycélium. La souche T7 était plus efficace que les souches T5 et T6 dans la transformation du MM à des concentrations plus élevées. Lors de l'essai biologique, la souche T5 affichait la meilleure performance à des doses de MM plus élevées. Les auteurs concluent que les souches T5 et T7 sont prometteuses en vue d'études futures dans la perspective de traitement ou d'amélioration de sols contaminés au MM. [Traduit par la Rédaction]

**Mots-clés :** essai biologique, détoxification, metsulfuron méthyle, quantification par spectrophotométrie, *Trichoderma*.

## Introduction

In the last three decades, herbicides have become an essential part of modern agriculture but their large-scale use has led to a global environmental problem. The efficiency of herbicides on weeds is partly influenced by the dose, but, generally, higher quantities of herbicide are employed than is necessary. Herbicides or their residues accumulate, soil and water resources become contami-

nated, and the sustainability of the whole ecosystem is being threatened.

Sulfonylureas are widely utilized as pesticides because they are effective even at low doses (2–75 g of active ingredient per hectare, He et al. 2007). Metsulfuron methyl (MM) is a sulfonylurea employed extensively against weeds in cereals, pastures, and other winter crops (Pons and Barriuso 1998). Sulfonylureas are considered

Received 20 April 2015. Accepted 10 August 2015.

**M.B. Vázquez.\*** Laboratorio de Micología, Centro de Recursos Renovables de la Zona Semiárida, Centro Científico Tecnológico Bahía Blanca, Consejo Nacional de Investigaciones Científicas y Técnicas.

**V. Barrera.** Bioinsumos Fúngicos, Instituto de Microbiología y Zoología Agrícola, Instituto Nacional de Tecnología Agropecuaria, Castelar, Argentina.

**V. Bianchinotti.** Laboratorio de Micología, Centro de Recursos Renovables de la Zona Semiárida, Centro Científico Tecnológico Bahía Blanca, Consejo Nacional de Investigaciones Científicas y Técnicas; Universidad Nacional del Sur, Bahía Blanca, Argentina.

**Corresponding author:** María Belén Vázquez (e-mail: [mbvazquez@criba.edu.ar](mailto:mbvazquez@criba.edu.ar)).

\*Present address: Camino La Carrindanga Km 7, Bahía Blanca, Argentina.

environmentally compatible, but it is well known that residues of MM may cause damage to rotation or substitution crops (Kotoula-Syka et al. 1993; Noy and Holloway 2001; Chen et al. 2009) and, for that reason, it is desirable to remove MM residues efficiently to preserve the soil quality for future crops.

The main mechanisms of degradation of sulfonylurea herbicides in the soil and water are microbial degradation and chemical hydrolysis. In alkaline soils, biodegradation is considered to be predominant over chemical hydrolysis (Yu et al. 2005; He et al. 2007). The role of the microbes in MM degradation is just beginning to be researched. Among soil bacteria, Zanardini et al. (2002) isolated a *Pseudomonas* that degraded MM efficiently in liquid medium, Huang et al. (2007) isolated a *Methylophil* species that could utilize MM as the sole carbon or nitrogen source, and Lu et al. (2011) isolated an *Ancylobacter* with the same ability. Fungi are organisms with a strong capability of degrading complex substances. Boschini et al. (2003) studied the biodegradation of MM in rich medium using *Aspergillus niger*, a common soil fungus. Yu et al. (2005) isolated a *Curvularia* species with the capacity to degrade MM in pure cultures and soil. He et al. (2007) showed that an unidentified *Penicillium* species enhanced the degradation of this herbicide in a simulated wheat rhizosphere. Working with spent mushroom compost enzymes of *Agaricus blazei*, González Matute et al. (2012) demonstrated the ability of this compost to degrade MM to other compounds of lower phytotoxicity than the herbicide.

We isolated several fungal species from alkaline agricultural soils in Argentina that could degrade MM, amongst which the *Trichoderma* strains showed the best ability (Vázquez and Bianchinotti 2013). *Trichoderma* species are easily isolated from soils worldwide (Howell 2003) and they are used successfully as biocontrol and bioremediation agents (López-Mondéjar et al. 2010). *Trichoderma* strains seem to be dominant in their ability to degrade glyphosate (Krzyśko-Lupicka and Orlik 1997; Singh and Walker 2006). Vrousia et al. (1996) reported that fungal species belonging to *Trichoderma* are good degraders of diuron. Previously, we demonstrated that the *Trichoderma* strains isolated from agricultural soils in Argentina were able to grow with MM as a sole carbon and energy source (Vázquez and Bianchinotti 2013). In this study, we identified these strains to species using molecular methods and we evaluated their efficacy in removing MM from liquid media using spectrophotometry. The toxicity of the degradation products was tested with a bioassay.

## Materials and methods

### Chemicals and media

Commercial MM (TRIMET) 60% active ingredient was purchased from Tamer Co. (China). Mineral salts medium (MSM) was formulated as in Yu et al. (2005). MM solutions ( $5 \times$

$10^{-4}$ ,  $5 \times 10^{-3}$ , and  $0.1 \text{ mg} \cdot \text{L}^{-1}$ ) were prepared in MSM, pH 7. Malt extract agar (MEA), potato dextrose agar (PDA), and potato dextrose broth (PDB) were from Britania (Argentina).

### Fungal strains cultivation and identification

The strains used in this study belong to the collection of the Laboratorio de Micología (Centro de Recursos Renovables de la Zona Semiárida, Consejo Nacional de Investigaciones Científicas y Técnicas (CERZOS CONICET)). They were isolated from soil samples from an experimental field in Argentina ( $37^{\circ}51'55''\text{S}$ ,  $63^{\circ}1'20''\text{W}$ ), with a history of direct seeding and 7 years of MM application (9.2 g MM active ingredient per hectare per year). Soils in the area are classified as silt loam (pH 7.76, carbon content 4.09%). Before isolation of fungi, an enrichment method with MM was employed (Vázquez and Bianchinotti 2013). Stock cultures were maintained at  $4^{\circ}\text{C}$  on MEA or PDA. *Trichoderma* strains, namely T5, T6, and T7, were cultivated on MEA and PDA at  $25^{\circ}\text{C}$  in darkness for 7 days. Cultural and microscopic features were studied following Domsch et al. (1980) and Chaverri and Samuels (2003).

### Phylogenetic analysis and DNA extraction

Colonies were incubated in PDB at  $25^{\circ}\text{C}$  for 48–72 h, under 12 h white fluorescent light photoperiod. Two hundred and fifty milligrams of mycelium was frozen in liquid nitrogen and ground into a fine powder in a mortar. DNA was extracted using a DNeasy Plant Mini Kit plant and fungi DNA purification kit (QIAGEN). The DNA obtained was quantified by electrophoresis with 1% agarose gels at 120 mV for 15–20 min and the bands were compared with Precision Molecular Mass Standard (BIO-RAD) and visualized by fluorescence with ethidium bromide. The amplification of the translation elongation factor 1 alpha (tef1-alpha) was performed with the primers Ef728M (5'-CATYGAGAAGTTCGAGAAGG-3') and Ef2 (5'-GGARGTACCAGTSATCATGTT-3') (Carbone and Kohn 1999). The polymerase chain reaction (PCR) for the fungal DNA was performed in 20  $\mu\text{L}$  of total volume 1x Master Mix New England Biolabs,  $1.5 \text{ mmol} \cdot \text{L}^{-1} \text{ MgCl}_2$ , 50–100  $\text{ng} \cdot \mu\text{L}^{-1}$  genomic DNA. The amplification program consisted first of a denaturation step at  $94^{\circ}\text{C}$  for 1 min, then 30 cycles at  $94^{\circ}\text{C}$  for 1 min and  $55^{\circ}\text{C}$  for 1 min, then  $72^{\circ}\text{C}$  for 1 min with a final elongation step at  $72^{\circ}\text{C}$  for 3 min. The resulting products were purified with Wizard SV Gel and PCR Clean-Up System (Promega). Sequencing was conducted using a BigDye™ Terminator version 3.1 (Applied Biosystems) based on Sanger's method. The resulting products were purified using ethanol precipitation and run with a Genetic Analyzer 3130xl at the Laboratory of Mycology and Microbiology USDA (Beltsville, USA).

The alignments of tef1-alpha sequences were done using Clustal W algorithm analysis in Bioedit version 7.0.5.3 (Hall 1999). Heuristic searches were conducted using TNT version 1.1 (Goloboff et al. 2008). During the

search, equal weights and no additive characters were used, and gaps were treated as missing data. Before searches, all uninformative characters were deactivated. The searches were done using Multiple TBR + TBR with 10 000 hold and 1000 replicates. Bootstrap values were calculated from 1000 replicates. All the characters were considered with the same weight.

#### Inoculum preparation and MM detoxification assay

The strains were incubated on MEA for 2 weeks at 25 °C. Fungal mycelium was harvested with a Digralsky loop using 10 mL of distilled water for each dish. In that way, a 120 mL suspension was obtained for each strain. From each mycelial suspension, 10 mL aliquots were taken and inoculated in bottles containing MSM and different concentrations of MM ( $5 \times 10^{-4}$ ,  $5 \times 10^{-3}$ , and 0.1 mg MM·L<sup>-1</sup>). The bottles were incubated in an orbital shaker at 25 °C for 7 days.

#### Fungal detoxification of MM

The efficiency of the strains in degrading MM was evaluated using a spectrophotometer. Quantification was performed with UV-Vis spectroscopy using a Shimadzu UV-Vis (UV-1601PC) spectrophotometer equipped with a glass cell with an optical path length of 1 cm. The absorbance of MM was measured at 233 nm according to Zanini et al. (2009). A calibration curve was constructed using standard solutions of MM with concentrations ranging between  $5 \times 10^{-4}$  and 0.1 mg MM·L<sup>-1</sup>. A linear regression method was used to draw the calibration curve. The growing mycelium was removed by filtration and the absorbance was measured in the remaining solutions to quantify the MM residues in the mixtures obtained, as explained above (see Inoculum preparation and MM detoxification assay). The amount of residual herbicide in each MM fungal solution was estimated using the calibration curve. The absorbance of liquid cultures by the three fungal strains incubated under the same conditions as the mixtures, but with no herbicide, was also measured.

#### Toxicity of MM detoxification products

The toxicity of the degradation products after fungal transformation was tested using a lentil seed bioassay (Paul et al. 2009). Pre-germinated seeds of *Lens culinaris* Medik. were sown in plastic well trays containing 500 g of uniformly distributed sterile perlite as the supporting medium. Thirty seedlings were used per treatment. Trays were kept at 25 °C and exposed to 16 h of light per day. The lentils were irrigated with the previously obtained mixtures (10 mL per seedling). Growth response curves at three different concentrations of MM in MSM with no fungi were also developed, using the same methodology, to confirm that the hypocotyl length is an adequate indicator of the concentration of the herbicide present in the medium. After 3 and 5 days of incubation, 10 seedlings per treatment were randomly removed and the length of the hypocotyl was measured with a digital

gauge. The mean lengths of the hypocotyls were compared using analysis of variance (ANOVA) and Fisher tests (least significant difference (LSD)) at a significance level of 5%. Statistical analysis was performed using Info Stat Statistical software (Di Rienzo et al. 2012).

## Results

### Phylogenetic analysis

The three *Trichoderma* strains studied here (T5, T6, and T7) displayed morphological characteristics that corresponded with the description of *T. harzianum* provided by Chaverri et al. (2003). The sequences of T5-TEF, T6-TEF1, and T7-TEF1 were grouped in the same clade by maximum parsimony (MP) tef1 analysis with *T. harzianum* reference sequences AF348100 and AF348101. The tef1 data set included 34 taxa and 660 characters. The tef1 MP analysis yielded 26 optimal trees of 1424 steps with a consistency index CI = 54 and a retention index RI = 78 from 418 informative characters. The MP strict consensus tree based on tef1 is shown in Fig. 1 with the accession numbers of the 30 reference sequences and *Sphaerostilbella aureonitens* which was used as an outgroup. The GeneBank accession numbers of the isolates T5-TEF, T6-TEF1, and T7-TEF1 are KT275197, KT275198, and KT275199, respectively.

### Fungal detoxification of MM

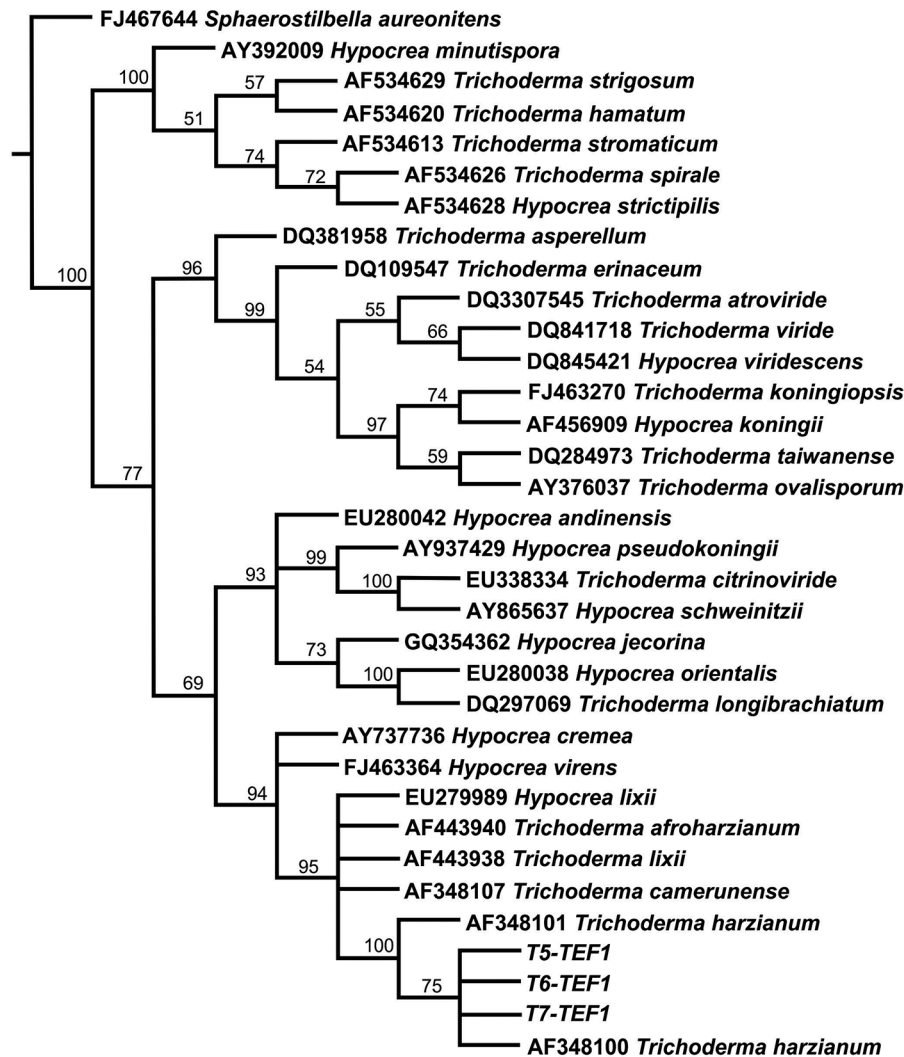
To quantify the amount of MM transformed in the detoxification assay, we produced a calibration curve for MM at 233 nm using a UV-Vis spectrophotometer. The linear equation obtained was absorbance =  $0.3745 \text{ mg MM} \cdot \text{L}^{-1} + 1.32028$ , with a correlation coefficient of 0.9875. No absorbance at 233 nm was detected for the strains grown without MM. The three *Trichoderma* strains showed different levels of efficiency in their ability to transform metsulfuron methyl. The most efficient was T7; no herbicide was detected at any of the tested concentrations after 7 days of incubation. The T5 and T6 strains showed less ability to transform high doses of MM than T7 (Table 1). The coefficient of variation of the spectrophotometric quantifications was less than 1% in all cases.

### Toxicity of MM transformation products

The results of the bioassay showed that the hypocotyl length was affected by the herbicide concentration present in the medium. Significant differences were found in the hypocotyl length of *Lens culinaris* between the different herbicide concentrations (ANOVA,  $F_{[2,13]} = 15.65$ ,  $p < 0.0003$ ; means: 4.03 cm, 1.14 cm, and 0.32 cm for 0.0005, 0.005, and 0.1 mg MM·mL<sup>-1</sup>, respectively, LSD Fisher,  $p < 0.05$ ). Differences in the hypocotyl length of the control plantlets (irrigated with water) and those treated with the higher herbicide doses are shown in Fig. 2A.

When we used the mixtures of MM plus *Trichoderma* inocula, no significant differences were found between the mean hypocotyl lengths of seedlings irrigated with

**Fig. 1.** Strict consensus phylogenetic cladogram constructed with maximum parsimony analysis with *tef1* sequences. The *tef1* sequences from *Trichoderma harzianum* strains isolated in this study are named T5-TEF1, T6-TEF1, and T7-TEF1. Bootstrap >50% are shown above branches. Terminal nodes are composed of GenBank accession numbers and *Sphaerostilbella aureonitens* was used as an outgroup.



**Table 1.** Spectrophotometric quantification of metsulfuron methyl (MM) transformation by *Trichoderma harzianum* strains (T5, T6, and T7).

Initial MM (mg·mL <sup>-1</sup> )	T5	T6	T7
0.0005*	100%	100%	100%
0.005	36%	22%	100%
0.1	0%	92.6%	100%

**Note:** Table shows the percentage of transformation of MM after incubation with *T. harzianum* strains for 7 days.

\*Field dose.

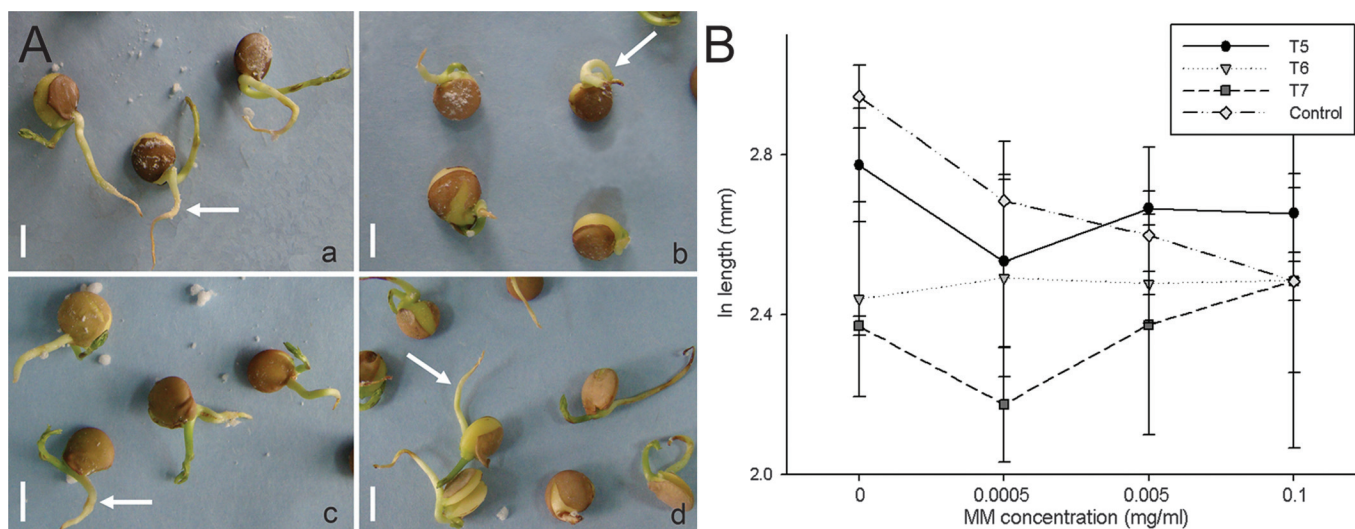
any of the solutions (ANOVA,  $F_{[3,4]} = 6.59$ ,  $p > 0.3$ ). The mean comparison test showed that the hypocotyls of the plantlets irrigated with any MM-T5 solution were longer than those of the control irrigated with the higher herbicide dose (LSD Fisher  $p < 0.05$ ). The plantlets irrigated

with any T6 and T7 solutions, with or without MM, were smaller than the control plantlets (LSD Fisher  $p < 0.05$ ) (Fig. 2B). When the efficacy of the three *T. harzianum* strains was compared, plantlets irrigated with MM-T5 grew better than those irrigated with MM-T6 or MM-T7 solutions (LSD Fisher  $p < 0.05$ ). The hypocotyls of plantlets irrigated with MM-T5 showed similar development to the control plantlets irrigated with water (ANOVA,  $F_{[3,56]} = 2.77$ ,  $p > 0.3$ , LSD Fisher  $p < 0.05$ ).

### Discussion

*Trichoderma* is one of the most common fungi recovered from soils. Studies on *Trichoderma* species are important for agricultural purposes because these fungi show innate resistance to a wide range of pesticides; several strains are used as biocontrol agents and some as plant growth promoters (Harman et al. 2004a, 2004b; Liu et al. 2008). Classical morphological identification of *Trichoderma* species can be rather difficult, mainly due to

**Fig. 2.** Effect of metsulfuron methyl (MM) on *Lens culinaris* hypocotyl development. (A) Seedlings of *Lens culinaris* after being treated with water and metsulfuron solutions. (a) Control plantlets irrigated with water. (b) Plantlets irrigated with 0.1 mg MM·mL<sup>-1</sup>. (c–d) Plantlets irrigated with a mixture of MM plus T5 strain; (c) 5 × 10<sup>-3</sup> mg MM·mL<sup>-1</sup>; (d) 0.1 mg MM·mL<sup>-1</sup>. Arrows point to hypocotyls. Scale bars = 5 mm. (B) Means and SE of hypocotyl length of the control plantlets and plantlets treated with MM-*T. harzianum* mixtures.



the overlapping of characters in closely related species (Chaverri et al. 2003; Chaverri and Samuels 2003; Samuels 2006; Jaklitsch 2009). The three *Trichoderma* strains studied here displayed morphological characteristics that corresponded fairly well with the *T. harzianum* description provided by Chaverri et al. (2003). The primers most used for the identification of *Trichoderma* species are internal transcribed spacer (ITS) and *tef1* (Druzhinina and Kubicek 2005; Samuels et al. 2012). There was no evidence of any genetic differences between the three *T. harzianum* strains (T5, T6, and T7) and, as a group, the three strains associate in the *T. harzianum* clade. Based on this, we confirm the identity of the three strains as *T. harzianum* Rifai sensu lato (Barrera 2012).

MM is one of the sulfonylurea herbicides most used worldwide, but several reports mention that it could have undesirable effects on the environment (Noy and Hollaway 2001; He et al. 2006, 2007; Wang et al. 2010). Indigenous soil microorganisms play a significant role in the degradation of xenobiotics. In particular, the importance of biodegradation in soils has been proven for MM (Yu et al. 2005; He et al. 2006, 2007). He et al. (2006) demonstrated that MM has different effects on soil microorganisms. MM application inhibits actinomycetes and heterotrophic bacteria, whereas fungi appear to react in the opposite way and become the dominant microorganisms. MM tolerant fungi are strongly stimulated by the application of this herbicide (He et al. 2006). The degradation or removal of toxic compounds is one of the remarkable capabilities of *Trichoderma* species (Harman et al. 2004a; Pinedo-Rivilla et al. 2009). Our findings supported the hypothesis that these fungi could have a role to play as MM biodegraders.

Spectrophotometric methods have been successfully used to quantify herbicide residues in environmental samples (Jan et al. 2009). Ghosh and Philip (2004) used this method to quantify the degradation of atrazine by microbial consortia. Heinfling et al. (1997) used spectrophotometry to test fungal degradation of several dyes. Brigante et al. (2013) demonstrated that the UV-Vis spectroscopic method gives results that are comparable with those obtained by chromatographic methods. Zanini et al. (2009) demonstrated that the UV-Vis spectrum of commercial MM (TRIMET) exactly matched that of the pure analytical grade herbicide. They found that it is possible to perform spectrophotometric quantification of commercial MM because it has no UV-Vis absorbing impurities and we used UV-Vis spectrophotometry to quantify the detoxification efficacy of the three *T. harzianum* strains (T5, T6, and T7). As in Kundu et al. (2005), we used a linear calibration curve to interpolate the unknown concentration of the herbicide in the MM-fungal solutions. No absorbance was detected for the strains grown without MM. The *Trichoderma* strains showed different levels of efficiency in their ability to degrade MM. Based on the spectrophotometric results, it was found that the three *T. harzianum* strains could transform 100% of the initial dose of MM at recommended field dose (0.0005 mg·mL<sup>-1</sup>). The most efficient was the T7 strain, as this strain transformed 100% of the herbicide in the medium after 7 days of incubation at any of the tested doses. In a previous study, we observed that the T7 strain was also highly tolerant to the herbicide as it could withstand MM levels 4000 times higher than maximum field doses (Vázquez and Bianchinotti 2013). This strain (T7) is promising as it can be used in bioremediation of strongly contaminated

soils. Based on our spectrophotometric assays, the strains T5 and T6 were effective in transforming MM at recommended field dose (0.0005 mg·mL<sup>-1</sup>), but at higher doses they were less effective than the T7 strain. *Trichoderma* strains are quite diverse, and this could be the basis for the differences that we found in MM transformation. There is much evidence in favor of the potential of different *T. harzianum* strains based on their intraspecific variability (Harman et al. 2004a). Since Rifai (1969), nine aggregate species were established in *Trichoderma*, based on the overlapping of morphological characters. The combined study of molecular markers (ITS, random amplified polymorphic DNAs), physiology, and morphology of collections of *T. harzianum* from different regions showed that it was composed of many different groups (Kuhls et al. 1996; Samuels 1996; Lieckfeldt and Seifert 2000). *Trichoderma harzianum* was recognized as a species complex, based on the high genetic variability observed when more molecular markers were applied (Druzhinina and Kubicek 2005).

Unless biotic conversion of a xenobiotic leads to a complete mineralization, the products of biological degradation could be less, equal, or even more toxic than the original compound (Fetzner 2002). For this reason, assessment of the toxicity of the resulting products following biological transformation is as important as the study of the ability of an organism to degrade the herbicide. Several methods have been employed to evaluate the toxicity of MM residues. In agreement with Paul et al. (2009), we demonstrated that the hypocotyl length was affected by the concentration of MM present in the medium. Significant differences were found in the hypocotyl lengths of *Lens culinaris* between the different herbicide concentrations. Paul et al. (2009) found that a bioassay using lentils was a useful technique, as it is even more sensitive than high performance liquid chromatography (HPLC) for detecting residues of MM up to 30 days after application. Over time, metsulfuron residues became more tightly bound to the soil and methods that involve solvent extraction, like HPLC, became inefficient at quantifying them. In the bioassay, the bound residues were released from the soil matrix during plant growth and they inhibited the development of plantlets (Paul et al. 2009).

For each MM concentration, we compared the control plantlets with those inoculated with T5 and we found that inoculation with T5 enhanced the development of the plantlets for MM concentrations greater than 0.0005 mg MM·mL<sup>-1</sup>. Harman et al. (2004b) mentioned that *Trichoderma* species associated with roots offer multiple benefits to plants, including protection against disease and enhanced plant growth and development. Our bioassay showed that there might be an interaction between the plant and T5. In controlled systems like ours, where no other microorganism takes part in the relationship, we can reasonably infer that the growth en-

hancement is directly related to the activity of the fungal strain. According to Harman et al. (2004b) this kind of co-metabolic system produces some factors that are only synthesized during the interaction and they could be responsible for the observed results.

## Conclusions

There is no evidence of genetic differences between the *Trichoderma harzianum* strains studied based on the phylogenetic analysis with the *tef1* molecular marker. However, the three strains transformed MM in liquid media at different rates. The T7 strain was the most effective at high MM concentrations and could be considered as a highly promising new tool for the treatment or amelioration of soils strongly contaminated with this herbicide. The T5 strain transformed MM at field doses, and also enhanced hypocotyl development at higher MM doses. *Trichoderma harzianum* showed intraspecific variability which might be the basis for the differences that we found in the MM transformation.

## Acknowledgements

This research was partially supported by a Proyecto de investigación científica y tecnológica del Fondo Nacional para la investigación científica y tecnológica, Argentina (PICT-FONCYT) grant (PICT-07/00380). María Belén Vázquez holds a fellowship from CONICET. Virginia Bianchinotti is a researcher from CONICET.

## References

- Barrera, V.A. 2012. El género *Hypocrea* Fr. (Hypocreales, Ascomycota) en la Argentina. Estudio de la variabilidad molecular de su estado anamórfico *Trichoderma*. Thesis. [www.digital.bl.fcen.uba.ar](http://www.digital.bl.fcen.uba.ar).
- Boschin, G., D'Agostina, A., Arnoldi, A., Marotta, E., Zanardini, E., and Negri, M. 2003. Biodegradation of chlorsulfuron and metsulfuron-methyl by *Aspergillus niger* in laboratory conditions. *J. Environ. Sci. Health Part B*, **38**: 737–746. PMID:14649705.
- Brigante, M., Zanini, G., and Avena, M. 2013. Effects of montmorillonite on the chemical degradation kinetics of metsulfuron methyl in aqueous media. *Appl. Clay Sci.* **80–81**: 211–218. doi:10.1016/j.clay.2013.03.018.
- Carbone, I., and Kohn, L.M. 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia*, **91**(3): 553–556. doi:10.2307/3761358.
- Chaverri, P., and Samuels, G.J. 2003. *Hypocrea/Trichoderma* (Ascomycota, Hypocreales, Hypocreaceae): species with green ascospores. *Stud. Mycol.* **48**: 1–116. ISBN 907035151X.
- Chaverri, P., Castlebury, L.A., Samuels, G.J., and Geiser, D.M. 2003. Multilocus phylogenetic structure within the *Trichoderma harzianum/Hypocrea lixii* complex. *Mol. Phylogenet. Evol.* **27**: 302–313. doi:10.1016/S1055-7903(02)00400-1. PMID:12695093.
- Chen, B., Huang, X., Zheng, J., Li, S., and He, J. 2009. *Candida mengyuniiae* sp. nov., a metsulfuron methyl-resistant yeast. *Int. J. Syst. Evol. Microbiol.* **59**: 1237–1241. doi:10.1099/ijs.0.004614-0.
- Di Rienzo, J.A., Casanoves, F., Balzarini, M.G., Gonzalez, L., Tablada, M., and Robledo, C.W. 2012. Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina. <http://www.infostat.com.ar>.
- Domsch, K.H., Gams, W., and Anderson, T.H. 1980. Compendium of soil fungi. Vol. 1. Academic Press, London.

- Druzhinina, I., and Kubicek, C.P. 2005. Species concepts and biodiversity in *Trichoderma* and *Hypocrea*: from aggregate species to species clusters? *J. Zhejiang Univ. Sci. B*, **6**(2): 100–112. PMID:15633245.
- Fetzner, S. 2002. Biodegradation of xenobiotics. In *Encyclopedia of Life Support Systems (EOLSS) Publishers, developed under the Auspices of the UNESCO. Biotechnology, Edited by Doelle and Da Silva. EOLSS Oxford, U.K.* 32 pp.
- Ghosh, P.K., and Philip, L. 2004. Atrazine degradation in anaerobic environment by a mixed microbial consortium. *Water Res.* **38**: 2277–2284. doi:10.1016/j.watres.2003.10.059.
- Goloboff, P.A., Farris, J.S., and Nixon, K.C. 2008. TNT, a free program for phylogenetic analysis. *Cladistics*, **24**: 774–786. doi:10.1111/j.1096-0031.2008.00217.x.
- González Matute, R., Figlás, D., Mockel, G., and Curvetto, N. 2012. Degradation of metsulfuron methyl by *Agaricus blazei* Murrill spent compost enzymes. *Bioremed. J.* **16**: 31–37. doi:10.1080/10889868.2011.628353.
- Hall, T.A. 1999. *Biol. Edit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser.* pp. 95–98.
- Harman, G.E., Howell, C.R., Viterbo, A., Chet, I., and Lorito, M. 2004a. *Trichoderma* species: opportunistic, avirulent, plant symbionts. *Nat. Rev. Microbiol.* **2**: 43–56. doi:10.1038/nrmicro797. PMID:15035008.
- Harman, G.E., Lorito, M., and Lynch, J.M. 2004b. Uses of *Trichoderma* spp. to alleviate or remediate soil and water pollution. *Adv. Appl. Microbiol.* **56**: 313–330. PMID:15566984.
- He, Y.H., Shen, D.S., Fang, C.R., and Zhu, Y.M. 2006. Rapid biodegradation of metsulfuron-methyl by a soil fungus in pure cultures and soil. *World J. Microb. Biotechnol.* **22**: 1095–1104. doi:10.1007/s11274-006-9148-y.
- He, Y.H., Shen, D.S., Hu, L.F., and Zhu, Y.M. 2007. Study on metsulfuron-methyl degradation in simulated wheat (*Triticum aestivum* L.) rhizospheric soil with *Penicillium* sp. inoculation. *Water Air Soil Pollut.* **179**: 297–307. doi:10.1007/s11270-006-9233-8.
- Heinfling, A., Bergbauer, M., and Szewzyk, U. 1997. Biodegradation of azo and phthalocyanine dyes by *Trametes versicolor* and *Bjerkandera adusta*. *Appl. Microbiol. Biotechnol.* **48**: 261–266. doi:10.1007/s002530051048.
- Howell, C.R. 2003. Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: the history and evolution of current concepts. *Plant Dis.* **87**: 4–10. doi:10.1094/PDIS.2003.87.1.4.
- Huang, X., He, J., Sun, J., Pan, J., Sun, X., and Li, S. 2007. Isolation and characterization of a metsulfuron-methyl degrading bacterium *Methylopila* sp. S113. *Int. Biodeter. Biodegrad.* **60**: 152–158. doi:10.1016/j.ibiod.2007.02.005.
- Jaklitsch, W.M. 2009. European species of *Hypocrea* part I. The green-spored species. *Stud. Mycol.* **63**: 1–91. doi:10.3114/sim.2009.63.01. PMID:19826500.
- Jan, M.R., Shah, J., Muhammad, M., and Ara, B. 2009. Glyphosate herbicide residue determination in samples of environmental importance using spectrophotometric method. *J. Hazard. Mater.* **169**: 742–745. doi:10.1016/j.jhazmat.2009.04.003. PMID:19411135.
- Kotoula-Syka, E., Eleftherohorinos, I.G., Gagianas, A.A., and Sficas, A.G. 1993. Phytotoxicity and persistence of chlorsulfuron, metsulfuron-methyl, triasulfuron, and tribenuron-methyl in three soils. *Weed Res.* **33**: 355–367. doi:10.1111/j.1365-3180.1993.tb01951.x.
- Krzyško-Lupicka, T., and Orlik, A. 1997. The use of glyphosate as the sole source of phosphorus or carbon for the selection of soil-borne fungal strains capable to degrade this herbicide. *Chemosphere*, **34**: 2601–2605. doi:10.1016/S0045-6535(97)00103-3.
- Kuhls, K., Lieckfeldt, E., Samuels, G., Kovacs, W., Meyer, W., Petrini, O., Gams, W., Börner, T., and Kubicek, C. 1996. Molecular evidence that the asexual industrial fungus *Trichoderma reesei* is a clonal derivative of the ascomycete *Hypocrea jecorina*. *Proc. Natl. Acad. Sci. USA*, **93**: 7755–7760.
- Kundu, S., Pal, A., and Dikshit, A. 2005. UV induced degradation of herbicide 2,4-D: kinetics, mechanism and effect of various conditions on the degradation. *Sep. Sci. Technol.* **44**: 121–129. doi:10.1016/j.seppur.2004.12.008.
- Lieckfeldt, E., and Seifert, K.A. 2000. An evaluation of the use of ITS sequences in the taxonomy of the Hypocreales. *Studies in Mycology*, **45**: 35–44.
- Liu, B., Glenn, D., and Buckley, K. 2008. *Trichoderma* communities in soils from organic, sustainable, and conventional farms, and their relation with Southern blight of tomato. *Soil Biol. Biochem.* **40**: 1124–1136. doi:10.1016/j.soilbio.2007.12.005.
- López-Mondéjar, R., Antón, A., Raidl, S., Ros, M., and Pascual, J.A. 2010. Quantification of the biocontrol agent *Trichoderma harzianum* with real-time TaqMan PCR and its potential extrapolation to the hyphal biomass. *Bioresour. Technol.* **101**: 2888–2891. doi:10.1016/j.biortech.2009.10.019. PMID:19897358.
- Lu, P., Jin, L., Liang, B., Zhang, J., Li, S., Feng, Z., and Huang, X. 2011. Study of biochemical pathway and enzyme involved in metsulfuron-methyl degradation by *Ancylobacter* sp. XJ-412-1 isolated from soil. *Curr. Microbiol.* **62**: 1718–1725. doi:10.1007/s00284-011-9919-z. PMID:21431836.
- Noy, D., and Holloway, K. 2001. Metsulfuron-methyl residues and potential recropping damage in Victorian cropping soils. In *Proceedings of the Australian Weed Conference, Melbourne, Australia*.
- Paul, R., Sharma, R., Kulshrestha, G., and Singh, S.B. 2009. Analysis of metsulfuron-methyl residues in wheat field soil: a comparison of HPLC and bioassay techniques. *Pestic. Manage. Sci.* **65**: 963–968. doi:10.1002/ps.1780.
- Pinedo-Rivilla, C., Aleu, J., and Collado, G. 2009. Pollutants biodegradation by fungi. *Curr. Org. Chem.* **13**: 1–21. doi:10.2174/138527209788921774.
- Pons, N., and Barriuso, E. 1998. Fate of metsulfuron-methyl in soils in relation to pedo-climatic conditions. *Pestic. Sci.* **53**: 311–323. doi:10.1002/(SICI)1096-9063(199808)53:4<311::AID-PS775>3.0.CO;2-C.
- Rifai, M.A. 1969. A revision of the genus *Trichoderma*. *Mycological Papers*, **116**: 1–56.
- Samuels, G.J. 1996. *Trichoderma*: a review of biology and systematics of the genus. *Mycological research*, **100**: 923–935. doi:10.1016/S0953-7562(96)80043-8.
- Samuels, G.J. 2006. *Trichoderma*: systematics, the sexual state and ecology. *Phytopathology*, **96**: 195–206. doi:10.1094/PHYTO-96-0195. PMID:18943925.
- Samuels, G.J., Ismaiel, A., de Souza, J., and Chaverri, P. 2012. *Trichoderma stromaticum* and its overseas relatives. *Mycol. Progr.* **11**: 215–254. doi:10.1007/s11557-011-0743-4.
- Singh, B.K., and Walker, A. 2006. Microbial degradation of organophosphorus compounds. *FEMS Microbiol. Rev.* **30**: 428–471. doi:10.1111/j.1574-6976.2006.00018.x. PMID:16594965.
- Vázquez, M.B., and Bianchinotti, M.V. 2013. Isolation of metsulfuron methyl degrading fungi from agricultural soils in Argentina. *Phyton*, **82**: 113–118. <http://www.revistaphyton.fund-romuloraggio.org.ar/vol82/VAZQUEZ.pdf>.
- Vroumsia, T., Steiman, R., Seigle-Murandi, F., Benoit-Guyod, J.-L., and Khadrani, A. 1996. Biodegradation of three substituted phenylurea herbicides (chlortoluron, diuron, and isoproturon) by soil fungi. A comparative study. *Chemosphere*, **33**: 2045–2056. doi:10.1016/0045-6535(96)00318-9.
- Wang, H., Xu, J., Yates, S.R., Zhang, J., Gan, J., Ma, J., et al. 2010. Mineralization of metsulfuron-methyl in Chinese paddy

- soils. *Chemosphere*, **78**: 335–341. doi:10.1016/j.chemosphere.2009.10.023. PMID:19906402.
- Yu, Y.L., Wang, X., Luo, Y.M., Yang, J.F., Yu, J.Q., and Fan, D.F. 2005. Fungal degradation of metsulfuron-methyl in pure cultures and soil. *Chemosphere*, **60**: 460–466. doi:10.1016/j.chemosphere.2005.01.015. PMID:15950038.
- Zanardini, E., Arnoldi, A., Boschini, G., D'Agostina, A., Negri, M., and Sorlini, C. 2002. Degradation pathways of chlorsulfuron and metsulfuron-methyl by a *Pseudomonas fluorescens* strain. *Ann. Microbiol.* **52**: 25–37.
- Zanini, G.P., Maneiro, C., Waiman, C., Galantini, J.A., and Rosell, R.A. 2009. Adsorption of metsulfuron-methyl on soils under no-till system in semiarid Pampean Region, Argentina. *Geoderma*, **149**: 110–115. doi:10.1016/j.geoderma.2008.11.025.