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

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

Short Title: Detoxification of metsulfuron methyl by *Trichoderma* strains.

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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 *T. 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* 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.

Keywords: Bioassay; Detoxification; Metsulfuron methyl; Spectrophotometric quantification; *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 and soil and water resources become contaminated 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 environmentally compatible, but it is well known that residues of MM may cause damage to rotation or substitution crops (Kotoula et al. 1993; Noy and Holloway 2001; Chen et al. 2009) and for that reason it is desirable to remove MM residues efficiently in order 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 metsulfuron methyl 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-Móndejar et al. 2010). *Trichoderma* strains seem to be dominant in their ability to degrade glyphosate (Krzysko-Lupicka and Orlik 1997; Singh and Walker 2006). Vroumsia 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 metsulfuron methyl (TRIMET) (MM) 60 % a.i. was purchased from Tamer Co. (China). Mineral salts medium (MSM) was formulated as in Yu et al. (2005). Metsulfuron methyl solutions (5×10^{-4} , 5×10^{-3} and 0.1 mg 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 (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 ($9.2 \text{ g MM a.i. Ha}^{-1}\text{Yr}^{-1}$). 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°C on MEA or PDA. *Trichoderma* strains, namely T5, T6 and T7, were cultivated on MEA and PDA at 25°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°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) was performed with the primers Ef728M (5'-CATYGAGAAGTTCGAGAAGG-3') and Ef2 (5'-GGARGTACCAGTSATCATGTT-3') (Carbone and Kohn 1999). The PCR reaction for the fungal DNA was performed in 20 µl of total volume 1x Master Mix New England Biolabs, 1.5 mM MgCl₂, 50-100 ng µl⁻¹ genomic DNA. The amplification program consisted first of a denaturation step at 94 °C for 1 min, then 30 cycles at 94 °C for 1 min and 55 °C for 1 min, then 72 °C for 1 min with a final elongation step at 72 °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 v 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 v. 7.0.5.3 (Hall 1999). Heuristic searches were conducted using TNT ver. 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 10000 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 Digrafsky 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 (5×10^{-4} , 5×10^{-3} and 0.1 mg MM l⁻¹). 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

5×10^{-4} and 0.1 mg MM l^{-1} . 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 in order to quantify the MM residues in the mixtures obtained, as explained above (see *Inoculum preparation*). 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* 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 hours 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, ten 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 (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 *Trichoderma 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 $\text{CI}=54$ and a retention index $\text{RI}=78$ from 418 informative characters. The MP strict consensus tree based on tef1 is shown in Figure 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

In order 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 $\text{Abs} = 0.3745 \text{ mg MM l}^{-1} + 1.32028$, with a correlation coefficient of 0.9875. No absorbance at 233nm 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⁻¹, 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 Figure 2a.

When we used the mixtures of MM plus *Trichoderma* inocula, no significant differences were found between the average hypocotyl lengths of seedlings irrigated with 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 $p < 0.05$) (Figure 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, b; Liu et al. 2008). Classical morphological identification of *Trichoderma* species can be rather difficult, mainly due to 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 ITS and tef1 (Druzhinina and Kubicek, 2005; Samuels et al., 2011). 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 in this we confirm the identity of the three strains as *T. harzianum* Rifai *sensu lato* (Barrera 2012).

Metsulfuron methyl 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; He et al. 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; He et al. 2007). He et al. (2006) demonstrated that metsulfuron methyl has different effects on soil microorganisms. Metsulfuron methyl 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 to those obtained by chromatographic methods. Zanini et al. (2009) demonstrated that the UV–Vis spectrum of commercial metsulfuron methyl (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 metsulfuron methyl. 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 ($0.0005 \text{ mg ml}^{-1}$). 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 four thousand 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 ($0.0005 \text{ mg ml}^{-1}$), 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, RAPDs), 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 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⁻¹. 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 enhancement 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 metsulfuron methyl 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.

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Table 1 Spectrophotometric quantification of metsulfuron methyl transformation by *Trichoderma harzianum* strains (T5, T6 and T7). Table shows the percentage of transformation of MM after incubation with *T. harzianum* strains for 7 days.

MM Initial (mg ml ⁻¹)	T5	T6	T7
0.0005*	100%	100%	100%
0.005	36%	22%	100%
0.1	0%	92.6%	100%

(*)Field dose

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Figure captions

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.

Fig. 2 Effect of 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⁻¹. c-d. Plantlets irrigated with a mixture of MM plus T5 strain; c) 5 x 10⁻³ mg MM ml⁻¹; d) 0.1 mg MM ml⁻¹. Arrows point to hypocotyls. Scale bar = 5 mm. **B)** Means and SE of hypocotyl length of the control plantlets and plantlets treated with MM- *T. harzianum* mixtures.

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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.
199x236mm (300 x 300 DPI)

Effect of 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⁻¹. c-d. Plantlets irrigated with a mixture of MM plus T5 strain; c) 5 x 10⁻³ mg MM ml⁻¹; d) 0.1 mg MM ml⁻¹. Arrows point to hypocotyls. Scale bar = 5 mm. b. B) Means and SE of hypocotyl length of the control plantlets and plantlets treated with MM- *T. harzianum* mixtures
169x64mm (300 x 300 DPI)