BIOREMEDIATION PERSPECTIVES USING AUTOCHTHONOUS SPECIES OF *Trichoderma* sp. FOR DEGRADATION OF ATRAZINE IN AGRICULTURAL SOIL FROM THE TULANCINGO VALLEY, HIDALGO, MEXICO

[PERSPECTIVAS DE BIORREMEDIACIÓN CON *Trichoderma* sp. NATIVO PARA DEGRADAR ATRAZINA EN SUELO AGRÍCOLA DEL VALLE DE TULANCINGO HIDALGO, MÉXICO]

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SUMMARY

The objective of this study was to show an *in vitro* bioremediation methodology for atrazine-contaminated soils through the use of local strains of native fungi isolated from the *Phaseolus vulgaris* L rhizosphere present in cultivable soils as well as to evaluate its resistance and capacity for atrazine degradation. A *Trichoderma* sp. species was identified in three cultivable soils from the Tulancingo Hidalgo, México region (contaminated with and without atrazine), which resisted atrazine concentrations of 10,000 mg L^{-1}. Tests showed that the strain grows exponentially in atrazine-contaminated soil over a range of 10^5-10^6 CFU g^{-1} in 15 days using atrazine as the only carbon and nitrogen source, while the control and witress showed a decrease of 10^0-10^3 UFC g^{-1} in the same period of time. For the atrazine degradation experiments, a treatment of the application of *Trichoderma* (10^4 - 10^5 CFU mL^{-1}) was applied to sterilized and non-sterilized soil contaminated with 500 mg Kg^{-1} of atrazine, evaluated at four time intervals (5, 10, 20 and 40 days). Statistical differences were found (α=0.050, Tukey) among treatments with the fungi and the test days. The native *Trichoderma* strain degraded 89% of the atrazine in 40 days. It showed that it is viable and cultivable in soil bioremediation.

Keywords: *Trichoderma*; native; biodegradation; herbicide.

RESUMEN

El objetivo del trabajo fue exponer una metodología *in vitro* para biorremediación, de suelos contaminados con atrazina, empleando cepas de géneros fúngicos nativos aislados de la rizósfera del *Phaseolus vulgaris* L en suelos agrícolas, se evaluó su resistencia y capacidad para degradar atrazina. Se identificó el género *Trichoderma* sp. nativo en tres suelos de la región de Tulancingo, Hidalgo, México (contaminados con y sin aplicación de atrazina). *Trichoderma* sp. resistió concentraciones de 10,000 mg L^{-1} del agroquímico. En los ensayos, la cepa creció paulatinamente en suelo contaminado con atrazina, alcanzando intervalos de 10^5-10^6 UFC g^{-1} en 15 días utilizando al herbicida como única fuente de carbono y nitrógeno, mientras que el testigo y control presentaron disminución de 10^0-10^3 UFC g^{-1} en el mismo tiempo. Para la degradación de atrazina se empleó un tratamiento con aplicación de *Trichoderma* (10^4 - 10^5 UFC mL^{-1}) a suelo estéril y no estéril, contaminado con 500 mg Kg^{-1} de atrazina, evaluados a cuatro tiempos (5, 10, 20 y 40 días). Se encontraron diferencias estadísticas (α=0.050, Tukey) entre los tratamientos con el hongo y los días del ensayo. La cepa de *Trichoderma* sp. nativa degradó 89% de atrazina en 40 días. Mostró ser viable y cultivable en remediación de suelos.

Palabras clave: *Trichoderma*; autóctono; biodegradación; herbicida.

INTRODUCTION

Pesticides can be a source of contamination for their use in agricultural practices. Improper handling and the persistence of some of them have caused negative impacts to soil, water, groundwater and even food, which can potentially cause damage to the environment and human health since these substances can bioaccumulate and biomagnify in living organisms or even arrive by diffusion and / or advection at different trophic levels (Madigan *et al.,* 2006; Cooper *et al.,* 2007).

In 2006, the atrazine herbicide was the most widely applied herbicide worldwide (29-34 million kg of active ingredient per year) in agricultural soils (Joo *et al.,* 2010). In Mexico, 1-2 kg of the active ingredient per hectare of this pesticide was applied in 1995, occupying the third place nationally (12.8%) of herbicides, with a clear tendency to increase its use in a rate of 10% annually (INE, 2007). Although in the country there is no official record of atrazine use, it is estimated that in only the central part of the country 376, 561 hectares of maize and sorghum the were treated (Bayer, 2009).

Atrazine (C_8 H_{14} $ClN_{5)}$ is a pre-emergent herbicide with the systemic action of inhibiting photosynthesis considered toxic in the short and medium term, and it is not easily degraded. Some research shows that this product causes serious environmental changes, can act as endocrine breaker (DE), affecting reproductive function in vertebrates, and cause teratogenic effects in humans (Cooper *et al.,* 2007; Raymundo *et al.,* 2009). It has been classified by the Environmental Protection Agency (EPA) as a restricted-use pesticide and classified in group C as a possible carcinogen (EPA, 2006).

For the persistency and negative effects that it may cause, atrazine (ATZ) is on the list of toxic substances as a candidate to be prohibited or have its use minimized worldwide (UNEP, 2001). However, in Mexico, to date no regulations exist that limit or restrict its application. That is why it is necessary to find alternatives to eliminate its effects on the ecosystems. One alternative is bioremediation. This process incorporates biological organisms such as microorganisms (mainly bacteria and fungi) to the contaminated environment to induce and accelerate the biodegradation process (Gopinath and Sims, 2011).

In the case of the use of microorganisms for remediation processes, they must be capable of degrading the herbicide and, moreover, must be harmless to the environment variation. Microorganisms must be isolated from native populations present in the same soil where they are intended to act. In this regard, Robinson (1996), reports that in systems with wild populations of microorganisms, degradation processes are ecologically more adaptable, there is more population diversity, genetic flexibility, metabolic and rhizosphere interactions and horizontal resistance. Thus, specific groups of microorganisms (single strain or consortia) with herbicide degradation are isolated, identified, their potential evaluated, produced and returned to the soil in specific crops so that the bioremediation could represent the best mechanism for the removal of atrazine in soil (Barriuso *et al.,* 2008).

There are fungi and bacteria that exhibit the ability to transform the atrazine molecule through alkylation and chlorination reactions, leading to various degradation products such as Hydroxyatrazine (HA), Desethylatrazine (DEA) and Desisopropylatrazine DIA (Govantes *et al*., 2009). However, microorganisms have also been isolated with the ability to completely mineralize this molecule (Lima *et al.,* 2009).

Atrazine degradation may be carried out by isolated species or microbial consortia (Gopinath and Sims, 2011; Ma *et al.,* 2011; Sene *et al.,* 2010; Govantes *et al.,* 2009). For this, the microorganisms are used in processes such as: bioaugmentation (inoculation of microorganisms), phytoremediation (using plants) and rizodegradation (remediation plant-microbe association at root level). It has been reported that atrazine can be degraded by various genera of bacteria such as *Pseudomonas* sp. (Monard *et al.,* 2008), *Bacillus sp.* (Getenga *et al.,* 2009), *Serrantia sp.* (So and Kyung, 2009), *Rhizobium* sp. (Chaudhary *et al.,* 2011; Abou-Shanab *et al.,* 2006), *Flavobacterium* and *Variovorax oryzihabitans paradoxus* (Belimov *et al.,* 2005), as well as fungal genera, among which are: *Aspergillus* (Gopi *et al.* 2012; Bajwa *et al.,* 2010), *Rhizopus* (Gopi *et al.,* 2012), *Fusarium moniliforme* (Bajwa *et al.,* 2010), *Penicillium* (Gopi *et al.,* 2012; Bajwa *et al.,* 2010) and *Trichoderma* (Smith *et al.,* 2005; Davidchik *et al.,* 2008; Quinto *et al.,* 2008; Sene *et al.,* 2010).

Trichoderma is an antagonist genera that can be found in the rhizosphere, where it competes with other microorganisms for nutrients and space. Some species of this genus produce enzymes that break down toxic substances and allow the cleaning of xenobiotic contaminants in soil (Perez *et al.,* 2009; Sene *et al.,* 2010), such as pesticides, organochlorine and organophosphorus compounds (Argumedo *et al*., 2009). Organochlorine pesticides such as DDT, dieldrin and endosulfan and classified as highly toxic persistent, have been degraded by *Trichoderma*

species (Llado *et al.,* 2009). This ability suggests that this as fungal genera could be used as a potential biorremediador of soils contaminated with agrochemicals.

Studies reported by Harman *et al.* (2004), mention that the *Trichoderma* spp strain T22, with genetic modification, was able to withstand concentrations up to 2,000 mg of cyanide per gram of soil exceeding the limits set by the EPA. This tolerance is quite remarkable and its activity is associated with the cellular system of intoxication based on the production of the enzyme permeases. Another study by Tang *et al.* (2009), showed that throught molecular breeding of the *Trichoderma atroviride* strain T23 in liquid medium at laboratory level, they achieved degradation levels between 81% and 96% of 600 g ml $^{-1}$ of pesticides dichlorides, compared with 72% achieved by the original strain without improvement.

There are few reports on pesticide bioremediation involving the genus *Trichoderma* sp., as a native rhizosphere strain (regularly they are exogenous GM strains) and even less research on their behavior and effects in terrestrial environments as an atrazine biodegrader. However, this genus has shown its biological potential for bioremediation activity in soil with a high level of development in agricultural applications (King *et al.,* 2004; Shmoll and Schuster, 2010). On the other hand, alternatives of bioaugmentation with genetically modified organisms are viable, but expensive and inaccessible for most farmers in the country and, in some cases, being exogenous strains, could limit or modify the native microenvironment which is to be improved.

The aim of this study was to evaluate the ability of a native rhizosphere strain of *Trichoderma* sp., of *Phaseolus vulgaris* L. to degrade atrazine under *in vitro* conditions for bioremediation purposes.

MATERIALS AND METHODS

Area of Study

To estimate the native and rhizospheric conditions of the study area, a selection criteria was set: a minimum of five continuous cycles of planting beans *(Phaseolus vulgaris),* soils with different water regimes (rained and irrigated) and soils with and without herbicides applications. It was determined that the landrace bean known as "moro" is a common crop in the region and meets the criteria for this experiment.

Three agricultural sites in the Valley of Tulancingo, Hidalgo, were selected. Two were agricultural soils of rained regime with no record of pesticide application, (El Capulin, located in the town of Cuautepec de Hinojosa, latitude: 20° 0' 23.27" N, longitude: 98° 14' 59.09" W, and the second in the town of Tepantitla: latitude: 20° 3' 2.90" N, longitude: 98° 19' 16.54" W). The third, representative of agricultural area with continued use of agrochemicals, was Santana Hueytlalpan, in the municipality of Tulancingo, Hidalgo: latitude 20° 10' 29.82" N, longitude: 98° 16' 52.72" W.

Three soil samplings were extracted during the annual cycle of the crop: soil without plant inflorescence, plant soil (rhizosphere) and dry soil. Sampling was performed according to the procedures described in the Official Mexican Norm (NOM-021-RECNAT-2000) for transport, storage and methods of microbiological, physical and chemical soil analysis.

Sampling depth was 0-25 cm using a tubular auger (7 cm diameter), and because no variability in the soil composition was observed it was not necessary a compound soil sampling, due to the uniformity of selected soils.

In the valley of Tulancingo (polygon: 20° 10' 29" N, 98° 16' 52" W, 19° 57' 20" N and 98° 15' 59" W) a subhumid temperate climate predominates (Cw), with an average annual temperature of 14.5 ° C, with maximum temperatures of 30 ° C in the months of March to July, and from August to February reaching the minimum of 3 ° C. The rainfall regime may occur from March to September with a minimum of 33.3 mm h $^{-1}$ and a maximum of 190.7 mm h $^{-1,}$ with an average of between 500-553 mm per year (National Meteorological System, 2011; University Weather Station, 2009).

Physical and chemical analytical methods in soil.

Soil samples were dried at room temperature (22-25 ° C), cracked and sieved with a 32 mesh sieve of 0.5 mm aperture (AS-01 method of NOM-021-RECNAT-2000). In order to characterize these soils, the following physical properties were determined: texture (Bouyoucos, 1962); real density (Buckman and Brady, 1966), and moisture by the gravimetric method (AS-05-021-RECNAT NOM-2000).

The chemical characterization covered cation exchange capacity (Mehlich, 1948), pH: measured in water (AS-02-021-NOM RECNAT-2000); organic carbon and organic material by Walkley and Black (1934), and total nitrogen (Kjendahl, 1983).

Isolation and characterization of rhizospheric *Trichoderma* spp.

Three samples of the sites under experiment were taken during the annual crop cycle (landrace beans "Moro") and the total count of fungi was determined by the Waksma method (1922), in 6 working dilutions (10^{-1} - $10^{-6)}$, surface planting on Potato Dextrose Agar (PDA) without acidification. The plates were incubated at room temperature in the dark for seven days. At the end of the incubation time, and for the condition of the soil (with and without chemicals) a viable cell count was performed, recording the morphologic similarities and macroscopic characteristics (shape, texture, growth, structure, hyphae, conidia, etc.). The colonies of fungi with similar characteristics among the three sites were purified for their isolation by the hyphal puncture technique and stored in PDA in a slant tube for later identification.

The fungal identification at genus level, was related to the macro and microscopic description of each isolate. Subsequently, the strains were examined by microscopy micro culture (Riddell, 1950) using photomicrographs obtained with a Motic BA300 microscope, objectives brightfield, phase contrast that are coupled to a digital camera Moticam 480 (Motic Images Plus 2.0 software). Images obtained with lactophenol blue staining were compared, and morphological criteria (Barnett and Hunter, 2006) identified *Trichoderma* as the main genera.

Resistance trials

The fungal strains, resistance to atrazine was determined by applying Minimum Inhibitory Concentration (MIC) tests to 23 different strains of fungi isolated by the qualitative disk diffusion method (Bauer *et al.,* 1966), with three repetitions. PDA dishes were inoculated with the different fungus genera identified by the surface extension. Sterile filter paper discs (0.5 cm in diameter) impregnated with 10 µL of atrazine solutions at concentrations of 0, 500, 1000, 1500, 2000, 2500, 5000, 7500 and 10,000 mg L^{-1} were used. The inhibition zone (no growth area) present on each disk, in relation to the mycelial growth, was determined.

***Trichoderma* sp. biomass production**

Massive biomass production of *Trichoderma* sp. was carried out in petri dishes containing PDA (propagated by the hyphal tip technique) at room temperature and with five days in darkness. Mycelium was removed by scraping the dish with three consecutive washes of 10 mL sterile water (Guigon *et al.,* 2010). The suspension of conidia obtained from each dish was kept in a 1 L sterile glass jar with screw cap.

***Trichoderma* sp. growth kinetics**

Samples of 100 g of sterile soil, saturated at field capacity with a concentration of 100 ppm of atrazine (Gesaprim 50FW ®), were placed in 250 mL jars with screw cap. The samples were inoculated with a suspension of strains of *Trichoderma* sp. (Concentration of about 10^4 - 10^5 CFU g^{-1} of mycelium). The jars were stored at room temperature for 15 days. The total count of the fungus (Waksma, 1922) was monitored at the first day of storage (time 0) and at 5, 10 and 15 days as described above. A Blank (sterile soil) and a Control (non-sterile soil), under the same conditions was set.

Atrazine degradation in soil

The soil for this experiment came from the Tempantitla experimental site without pesticide application. Samples were air-dried and sieved with a No. 16 mesh 1 mm opening. A homogeneous mixture 80/20 v/v Agrolita ® - perlite was prepared. 50 g of soil mixture were placed in jars and sterilized at 110 ° C for 45 minutes for three consecutive days. The jars were saturated at field capacity (Rodriguez and Rodriguez, 2002) with a solution of 500 ppm of technical grade atrazine (Gesaprim 50FW ®).

Two soil conditions were used: sterile and non-sterile. Flasks were inoculated with 10 mL of the suspension of *Trichoderma* (10^4 – 10^5 CFU * mL $^{-1)}$ and stored for 40 days under greenhouse conditions. To evaluate the treatments, the soil was analyzed at 5, 10, 20 and 40 days after inoculation. A blank (sterile soil inoculated with (*Trichoderma*) and a control (non-sterile soil inoculated with *Trichoderma*) was set. In each time period, the soil was air dried and stored in plastic bags for herbicide removal.

Atrazine extraction and quantification

5 g of dry soil were placed in 50 mL conical tubes and homogenized with 10 mL of methanol and stirred orbitally at 300 revolutions per minute (rpm) for 24 hours in darkness. The tubes were centrifuged 10 min at 5000 rpm and 14 ° C. The supernatant was filtered and collected in 10 mL glass jars. Methanol was evaporated to dryness at 25 ° C, the residue was re-suspended in 5 mL of Liquid Chromatography High Resolution (HPLC) grade acetonitrile. Finally, the samples were filtered with a 0.45 microns Millipore Nylon® membrane and the liquid was collected in jars for HPLC analysis (Fragoeiro and Magan, 2005).

The liquid filtrate was analyzed with a HPLC Agilent 1200®, Infinity Series, diode array detector with a Poroshell® column 120 EC-C18 4.6 x 50mm 2.7 micron. The elution in the chromatography mobile phase began in 70/30 water/acetonitrile (v/ v) at a flow rate of 0.5 mL min $^{-1}$ and sample injection of 10 microliters. The retention time was 2.84 minutes for atrazine with a detection limit of 1 mL hg $^{-1}$. The working solutions for the calibration curve of atrazine (0, 0.2, 0.4, 0.6, 0.8 mg mL $^{-1)}$ were prepared from the stock solution of 1 mg mL $^{-1}$ (atrazine 98% analytical reagent) by dilution with HPLC grade acetonitrile.

The HPLC values obtained were fitted to Equation 1, of the standard curve:

$Y = 8694X + 684.7$ [1], $R^2 = 0.987$ (Equation 1)

This equation gives the retained mass of atrazine (X). The overall mass balance for atrazine in the test was:
M-input = M + M-retained-downgraded (Equation 2)
Where:
M = mass of atrazine,
From equation (2) the degraded atrazine milligrams were obtained:
M-degraded = M-M-input-hold (Equation 3)

Experimental design

For the characterization of physical and chemical soils a simple variance of means statistical analysis with a completely randomized design (Tukey: $\alpha = 0.05$) model was used.

The biodegradation of atrazine was a statistical model of the analysis of the variance of one factor in a completely randomized design with fixed effects under repeated measurements over time. The Tukey test ($\alpha = 0.05$) was applied for means comparison between treatments.

In both cases, the data obtained were analyzed with the statistical program Number Cruncher Statistical System (NCSS), version 2007.

RESULTS AND DISCUSSION

Physical and chemical parameters of the soil samples

The values of the physical and chemicals parameters found in the soil samples collected at the three study sites are given in Table 1. The Capulín and Santana sites showed a moderately acid pH levels, and Tepantitla (NOM-021-RENACT-2000) was neutral. In these intervals, nutrients are more available, as are contaminants in the soil solution (Rinnan *et al.,* 2007).

The total soil nitrogen results were variable for each study site and within the Official Mexican Norm (NOM-021-RENACT-2000) they are classified in intervals of high (0.15 to 0.25%) and very high (> 0.25). Regarding the content of organic matter (OM) and organic carbon (OC), the norm states that values between 4.1-6.0% MO, indicate a low fertility content. On the contrary for Rodriguez and Rodriguez (2002), the amount of organic matter and organic carbon in agricultural classification is high and very high (> 3.5%). Finally, regarding texture, soils are classified as sandy loam and loam (NOM-021-RENACT-2000).

Table 1. Mean comparison of the chemical and physical characteristics of the soils studied.

	Agricultural soils of the Tulancingo Valley, Hidalgo, Mexico.			
	Capulín	Tepantitla	Santana	Standard error
% Humidity	14.89_a	15.86_a	15.20_b	0.20
pH (1:2 in water)	6.35_a	6.69_a	5.78_b	0.12
% Total Nitrogen	0.18_a	0.24_b	0.30_c	$9.48*10^{-3}$
% Organic carbon	2.86_a	3.36_b	4.08_c	0.012
% Organic material	4.94_a	5.79_b	4.04_c	$8.60*10^{-3}$
CIC (Cmol(+)kg^{-1})	29.34_a	59.63_b	25.46_c	3.63
Density (g cm^{-3})	2.38_a	2.43_a	2.29_a	0.11
Texture	Sandy loam	loam	loam	

For each line different letters indicate significant difference (with $\alpha = 0.05$, Tukey).

The physical and chemical results showed that there are significant differences in the Santana soil (pesticide application site) compared to the soil without pesticide application. Furthermore, one must consider that these properties are not only important in the genesis and fertility of the soil, but also affect retention capacity, adsorption and atrazine degradation in soil (Bridges *et al.,* 2008; Raymundo *et al.,* 2009). Comparing the results with bibliographical observations and references, it was found that the three sites are at intervals of agricultural fertility, but the soil of Santana, because of its record of intensive agricultural use of pesticides, could be vulnerable to deterioration and negative environmental impacts in the short term.

Isolation and characterization of the rhizospheric *Trichoderma* sp.

It was observed that there is a higher total count of fungi in the rhizosphere (rhizospheric soil) in a ratio rhizosphere / soil (R / S) of 2:1, at the three sites Capulín (C), Tepantitla (T) and Santana (S). (Figure 1).

The proportion of Colony Forming Units per gram of soil (CFU g^{-1}) is within the range estimated by Maier and Pepper (2009), of 10^5 to 10^6 CFU g^{-1} in rhizospheric soils. Mainly, because two of the agricultural soils (C and T) are temporary regime without fertilization. These results indicate that the diversity and number of fungal genera present are in an appropriate ranges.

From macro and microscopic laboratory descriptive tests and experimental data, it was possible to identify 7 kinds of fungi (23 species posibles), as follows: *Alternaria* (one), *Mucor* (two), *Clandosporium* (one), *Fusarium* (three), *Aspergillus* (one), *Penicillium* (seven) of *Trichoderma* (five) and three unidentified fungal strains.

The growth of *Trichoderma* sp. in culture media began with the observation of colonies with small green specks inconidium cushions, and with visible airborne spores. The microculture microscopic observations showed hyaline conidiophores, branched, not verticillated, in groups of phialides producing conidia (fialosphoros) hyaline in ovoid shape and in terminal clusters (Barnett and Hunter, 2006; Sources and Ferrera, 2007).

Resistance tests and biomass production of *Trichoderma* sp.

The identified 23 strains of fungi showed no growth inhibition halos of mycelium for atrazine concentrations of 0 to 10.000 mg $L^{-1,}$ by the CMI method. This behavior shows that the genera isolated resist high concentrations of agrochemical (Gopi *et al.,* 2012; Gopinath and Sims, 2011; Sene *et al.,* 2010).

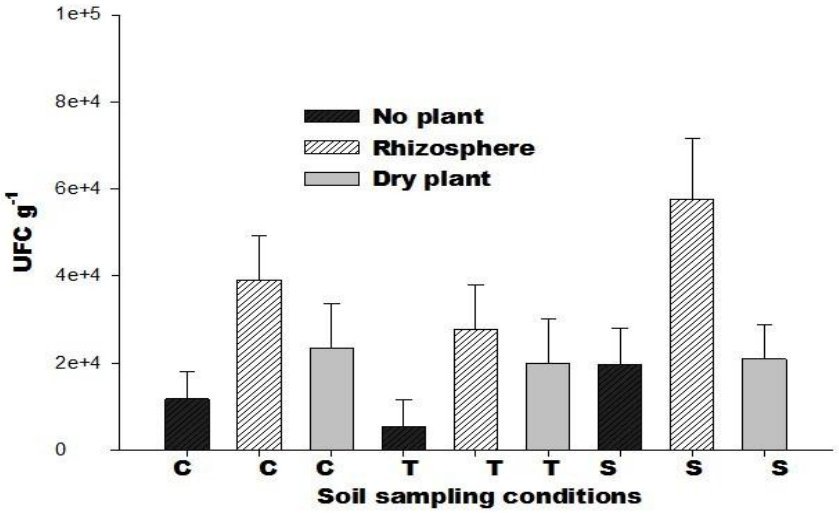

Figure1. Total count of fungi (CFU g^{-1}) of the three agricultural soils form Tulancingo Valley, Hidalgo, Mexico. C (Capulín) and T (Tepantitla) are temporary regime (no herbicide application) and S (Santana) is with irrigation and atrazine application. The soil sampling conditions were: no plant and dry plant rhizosphere**.**

The biomass production test was performed with a strain identified as *Trichoderma genus,* since there are references that point out that this genera represents major advantages in relation to other identified genera. This fungus is characterized as a symbiotic rhizospheric in plants and is used in the bioremediation of soils for extraction and the biodegradation of contaminants (Harman *et al.,* 2004; Argumedo *et al.,* 2009; Ghopita *et al.,* 2011).

Of the five strains of *Trichoderma* sp. (T_1, T_2, T_3, T_4 and T_5), it was identified as T_5 by its uniform growth, pH ranges (4.5 to 7.5) and because it was the only strain that developed mycelium both on agar and on liquid media. 0.12 g of dry mycelium biomass of *Trichoderma* sp. grown on 78.53 / cm^2 of each solid PDA plate was obtained.

Trichoderma sp. growth kinetics

Experimental data indicate that *Trichoderma* sp. (Strain T_5) grew steadily for 15 days at room temperature. On the other hand, the control (non-sterile soil) increased its growth to a lesser extent and the control showed a progressive decrease in CFU g^{-1} soil (Figure 2).

The concentration of *Trichoderma* sp. increased from $1x10^4$ to $1.8x10^6$ CFU g^{-1} soil in 15 days at room temperature using atrazine as the sole carbon and nitrogen source. It can be estimated that bioaugmentation with the fungus was favorable, compared to the control (unidentified microbial consortium).

Atrazine degradation in soil

Significant differences ($\alpha = 0.05$) between treatments from five to 20 days were found. At the end of the experiment (40 days), the amount of atrazine biodegraded by the *Trichoderma* sp. (T) was higher compared to that achieved by the Blank (B) and the Control (Ct) at the same time (Table 2).

Another point to emphasize is the behavior of the treatment with *Trichoderma* sp. in relation to time, where the treatment biodegraded 447.1 mg L^{-1} from an initial concentration of 500 mg kg^{-1} of atrazine (8.9 mg atrazine / 50 g soil), representing 89.4% of the herbicide. It is observed that from day 20 there is a difference in the fungal genera (T) respecting the Control and Blank (Table 2 and Figure 3).

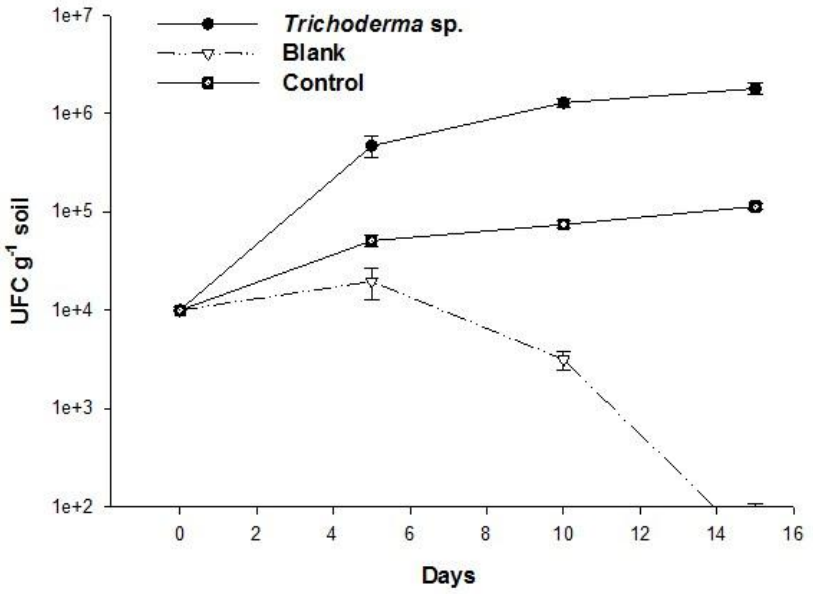

Figure 2. Growth kinetics in CFU g^{-1} of *Trichoderma* sp. in sterile soil in relation to Blank and Control at 0, 5, 10 and 15 days of incubation with an initial 100 ppm atrazine concentration.

Table 2. Biodegradation of atrazine to different treatments.

	Atrazine (mg L^{-1})			
Treatment	5 days	10 days	20 days	40 days
Trichoderma sp. (T)	114.9452_a	168.5607_a	442.3395_b	447.1820_b
Blank (B)	29.6909_a	67.0079_a	59.9570_a	207.0662_b
Control (Ct)	119.8873_a	174.3655_a	112.2000_a	147.4810_a
Standard error	±13.5272	±13.5272	±13.5272	±13.5272

Column means with different letters differ statistically (α = 0.050, Tukey)

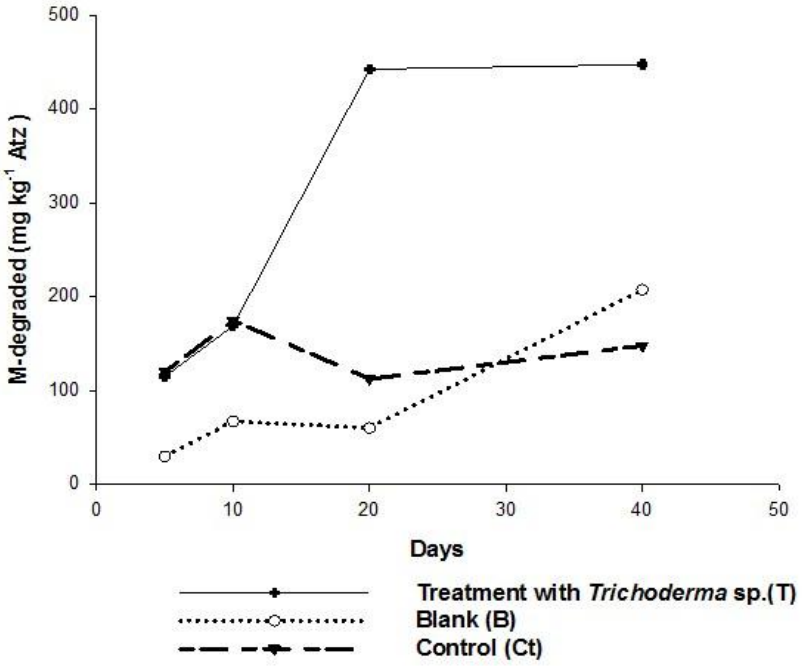

Figure 3. Atrazine mass (mg kg^{-1}) degraded with *Trichoderma* sp. (T) inoculation compared to Blank (B) and Control (Ct), recorded at 5, 10, 20 and 40 days after inoculation.

This behavior is different from that reported by Przybulewska and Sienicka (2008), where the same genera mineralized (up to NH_3 and CO_2) 2.5 mg/50 g of soil in 112 days (10^6 -10^7 CFU g^{-1}), indicating that the results are 3.5 times higher than those reported, 2.8 times less in time, and with the same initial amount of inoculum.

CONCLUSION

The genus *Trichoderma* sp. was isolated, identified and characterized as a native strain of the rhizosphere of *Phaseolus vulgaris* L., which belongs to the agro ecosystem of the valley of Tulancingo, Hidalgo, Mexico, and showed resistance to atrazine herbicide up to 10,000 mg $L^{-1.}$ It is able to degrade up to 89% of the atrazine in 500 mg kg^{-1} of soil under laboratory conditions.

This study also shows that it is feasible to cultivate the native *Trichoderma* sp strain under laboratory conditions with degradation results for this herbicide superior to those already reported.

The next step in this research should include trials with the native rhizospheric strain of *Trichoderma* sp at greenhouse and field level to evaluate is effectiveness for atrazine bioremediation in cultivated soils contaminated with atrazine.

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REFERENCES

Abou-Shanab, R.A., Angle, J.S., Chaney, R.L. 2006. Bacterial inoculants affecting nickel uptake by Alyssum murale from low, moderate and high Ni soils. Soil Biology & Biochemistry. 38: 2882-2889.

Adebusoye, S.A., Picardal, F.W., Ilori, M.O., Amund, O.O., Fuqua, C. 2008. Characterization of Multiple Novel Aerobic Polychlorinated Biphenyl (PCB)-Utilizing Bacterial Strains Indigenous to Contaminated Tropical African Soils. Biodegradation. 19:145-159. Agencia de Protección Ambiental. 2012. http://www.epa.gov/oppsrrd1/reregistration/atrazine.

Argumedo, D.R., Alarcón, A., Ferrera, C.R., Peña, C.J. 2009. El género fúngico *Trichoderma* y su relación con contaminantes orgánicos e inorgánicos. Revista Internacional de Contaminación Ambiental. 25:257-269.

Bajwa, R., Khokhar, I., Mukhtar, I., Mushtaq, S. 2010.Isolation and Identification of Filamentous Fungi from Different Industrial Effluents. Plant Product Research Journal. 14: 32–35.

Barnet, H.L., Hunter, B.B. 2006. Illustratrated general of imperfect fungi, Fourth edition, Apss Press, Minnesota, USA.

Barriuso, J., Ramos, S.B., Lucas, A.L., Probanza, L.A. 2008. Ecology, Genetic Diversity and Screening Strategies of Plant Growth Promoting Rhizobacteria (PGPR). Ahmad, I, Pichtel, J. and Hayat, S.(Eds), Plant-Bacteria Interactions. Strategies and Techniques to Promote Plant Growth, (pag. 1.13), WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim, USA.

Bastos, A.C., Magan, N. 2009. *Trametes versicolor* : Potential for atrazine bioremediation in calcareous clay soil, under low water availability condition. International Biodeterioration and Biodegradation. 63:389–394.

Bauer, A.W., Kirby, W.M.M., Sherris, J.C., Turck, M.1966. Antibiotic susceptibility testing by a standardized single disk method. American Journal of Clinical Pathology. 45:493-496.

Bayer Cropscience: http://www.annualreport2009.bayer.com/en/homepage.aspx [Última consulta 13 Julio 2012].

Belimov, A.A., Hontzeas, V.I., Safronova, S.V., Demchinskaya, G., Piluzza, S., Glick, B.R. 2005. Cadmium-tolerant plant growth-promoting bacteria associated with the roots of Indian mustard (*Brassica juncea* L. Czern.). Soil Biology & Biochemistry. 37: 241-250.

Bridges, M., Brien, H.W., Shaner, D.L., Khosla, R., Westra, P., Reich, R. 2008. Spatial Variability of Atrazine and Metolachlor Dissipation on Dryland No-tillage Crop Fields in Colorado. Journal of Environmental Quality. 37:2212–2220.

Bouyoucos, G.J. 1962. Hydrometer method improved for making particle size analysis of soils. Agronomy Journal. 54:464-465.

Buckman, H. and Brady, N.C. 1966. The Nature and Properties of Soils. The Macmillan Company. United Kingdom.

Chaudhary, K.S., Inouhe, M., Rai, N.M.U., Mishra, K., Gupta, K.D. 2011. Inoculation of *Rhizobium* (VR-1 and VA-1) induces an increasing growth and metal accumulation potential in *Vigna radiata* and *Vigna angularis* L . growing under fly - ash. Ecological Engineering. 37:8:1254-1257.

Cheyns, K., Martin, L.F., Bru, D., Aamand, J., Vanhaecke, L., Diels, J., Merckx, R., Smolders, E., Springael, D. 2011. Long-term dynamics of the atrazine mineralization potential in surface and subsurface soil in an agricultural field as a response to atrazine applications. Chemosphere. 10:450-465.

Cooper, R.L., Laws, S.C., Goldman, J.M., Narotsky, M.G. 2007. Atrazine and reproductive function: mode and mechanism of action studies. Birth Defects Research. 80:98-112.

Davidchik, V.N., Kulikova, N.A., Golubeva, L.I., Stepanova, E.V., Koroleva, O.V. 2008. *Coriolus hirsutus* Laccase Effect on Atrazine Adsorption and Desorption by Different

Types of Soil. Applied Biochemistry and Microbiology. 44:440-445.

Fragoeiro, S., Magan, N. 2005. Enzymatic activity, osmotic stress and degradation of pesticide mixtures in soil extract liquid broth inoculated with *Phanerochaete chrysosporium* and *Trametes versicolor*. Environmental Microbiology 7:348–355.

Fuentes, D.G., Ferrera, C.R. (Eds.). 2007. Ecología de la raíz (segunda edición). Cd. Obregón, Sonora, México.: Sociedad Mexicana de Fitopatología, A.C.

GESAPRIM 50 FW®. Syngenta 2009. Hoja de Seguridad Resolución 350/99. http://www.jedys.com.ar/data/HojaDeSeguridad_359.pdf . [Última consulta 13 Julio 2012].

Getenga, Z., Dörfler, U., Iwobi, A., Schmid, M., Schroll, R. 2009. Atrazine and terbuthylazine mineralization by an Arthrobacter sp. isolated from a sugarcane-cultivated soil in Kenya. Chemosphere. 77:534–539.

Gopi, V., Upgade, A., Soundararajan, N. 2012. Bioremediation potential of individual and consortium Non-adapted fungal strains on *Azo dye* containing textile effluent.Advances in Applied Science Research. 3:303-311

Gopinath, K.R. and Sims, K.G. 2011. Biostimulation for the Enhanced Degradation of Herbicides in Soil. Applied and Environmental Soil Science [Versión electronica] Disponible en: http://www.hindawi.com/journals/aess/2011/843450 .[Última consulta 13 Julio 2012].

Govantes, F., Porrúa, O., García, G.V., Santero, E. 2009. Atrazine biodegradation in the lab and in the field: enzymatic activities and gene regulation Microbial Biotechnology. 2:2. 178–185.

Guigón, L.C., Guerrero, P.V., Vargas, A.F., Carvajal, M.E., Ávila, Q.G.D., Bravo, L.L., Ruocco, M., Lanzuise, S., Woo, S., Lorito, M. 2010. Identificación Molecular de Cepas Nativas de *Trichoderma* spp. su Tasa de Crecimiento *in vitro* y Antagonismo contra Hongos Fitopatogenos. Revista Mexicana de Fitopatología. 28:87-96.

Harman, G.E., Lorito, M., Lynch, J.M. 2004. Uses of *Trichoderma* spp. to Alleviate or Remediate Soil and Water Pollution. Advances in Applied Microbiology. 56:330-331.

Instituto Nacional de Ecología 2007. Estudio: precisión del Inventario de plaguicidas obsoletos y sitios contaminados con éstos http://siscop.ine.gob.mx/descargas/gt/informe_final/informe_final_plaguicidas.pdf.

Joo, H., Choi, K., Hodgson, H. 2010. Human metabolism of atrazine. Pesticide. Biochemistry and Physiology. 98:73–79.

Kjeldahl, J. 1983. Sur une nouvelle méthode de dosage de l'azote dans les substances organiques, Résumé du compte-rendu des travaux du Laboratoire de Carlsberg, Copenhague, en commission chez H. Hagerup, Imprimerie de Thiele.

Kolombet, V.L., Zhigletsova, K.S., Kosareva, I.N., Bystrova V.E., Derbyshev, V.V., Krasnova, P.S., Schisler, D. 2008. Development of an extended shelf-life, liquid formulation of the biofungicide Trichoderma asperellum. World Journal of Microbiology and Biotechnology. 24:123–131.

Llado, S., Jiménez, N., Viñas, M., Solanas A.M. 2009. Microbial populations related to PAH biodegradation in an aged biostimulated creosote-contaminated soil. Biodegradation. 20:593–601.

Lima, P.D., Viana, P. André S., Chelinho, S., Costa, C., Ribeiro, R., Sousa, J.P., Fialho, A.M., Viegas, C.A. 2009. Evaluating a bioremediation tool for atrazine contaminated soils in open soil microcosms: The effectiveness of bioaugmentation and biostimulation Approaches. Chemosphere. 74:187–192.

Liu, X., Parales, E.R. 2009. Bacterial Chemotaxis to Atrazine and Related *s* -Triazines. Applied and Environmental Microbiology. 75:5481-5482.

Ma, Y., Prasad, M.N., Raikumar, M., Freitas, H. 2011. Plant growth promoting rhizobacteria and endophytes accelerate phytoremediation of metalliferous soils. Biotechnology Advances. 29:248-258.

Madigan, M.T., Martinko, J.M., Parker, J. 2006. Brock Biología de los Microorganismos. Décima Edición, Pearson Prentice Hall, Madrid, España.

Mehlich, A. 1948. Determination of catión and anion-exchange properties of soils. Soil Science. 66: 429-445.

Monard, C., Martin, L.F., Vecchiato, C., Francez, A.J., Vandenkoornhuyse, P., Binet, F. 2008. Combined effect of bioaugmentation and bioturbation on atrazine degradation in soil. Soil Biology Biochemistry. 40:2253–2259.

NOM-021-RECNAT-2000. Norma Oficial Mexicana, que establece las especificaciones de fertilidad, salinidad y clasificación de los suelos. Estudios, muestreo y análisis. México. http://www.profepa.gob.mx/innovaportal/file/3335/1/nom-021-semarnat-2000.pdf [Última consulta 13 Julio 2012].

NOM-112-SSA1-1994. Norma Oficial Mexicana, que establece bienes y servicios.Determinación de bacterias coliformes. Técnica del número más probable. México. http://bibliotecas.salud.gob.mx/gsdl/collect/nomssa/index/assoc/hash8ceb.dir/doc.pdf [Última consulta 13 Julio 2012].

Pérez, B.Y., Cantillo, P.T., Ramos, R.E., González, G.M., López, M.M. 2009. Prospección de hongos de suelo con potencialidades para el control biológico en suelos de agroecosistemas cubanos. Fitosanidad. 13:3-5.

Pochon, J., Tardieux, Y. 1962. Techniques d' Analyse en Microbiologie du sol. Editurial de Tourelle, París, France.

Prasad, M.N.V. and Katiyar, S.C. 2010. Drill cuttings and fluids of fossil fuel exploration in North-Eastern India: environmental concern and mitigation options. Current Science. 98:1566-156.

Programa de las Naciones Unidas para el Medio Ambiente (PNUD). 2001. Convenio de Estocolmo sobre contaminantes orgánicos persistentes. http://redalyc.uaemex.mx/pdf/539/53906901.pdf.

Przybulewska, K. and Sienicka, K. 2008. Decomposition of atrazine by microorganism isolated from long-term herbicide experimet soil. Ecological Chemistry and Endengineerings. 15: 501-511.

Quinto, C., Cárdenas, L. 2007.Diálogo para ganar: interacción simbiótica entre una bacteria del suelo y el frijol. Biotecnología. 14:273-280.

Raymundo, E., Nikolskii, I., Duwig, C., Prado, B., Hidalgo, C., Gavi, F. 2009. Transporte de atrazina en un andosol y un versitol de México, Interciencia. 34:330-337.

Razak, A.A., Bachman, G., and Farrag, R. 1999. Activites of microflora in soils of upper and lower Egypt. The African Journal of Mycology and Biotechnology. 98:2557-2551.

Reporte meteorológico universitario de la estación meteorológica del Instituto de Ciencias Agropecuarias de la Universidad Autónoma del Estado de Hidalgo. 2009. http://www.uaeh.edu.mx/principal/clima/index.html . [Última consulta 13 julio 2012].

Rey, M., Llobell, A., Monte, E., Scala, F., Lorito, M. 2004. Genomics of *Trichoderma.* Applied Mycology and Biotechology . 4: 225-248.

Riddell, R.W. 1950. Permanent strained mycologycal preparation obtained by slide cultures. Micología. 82: 265-270.

Rinnan, R., Michelsen, A., Bååth, E., Jonasson, S. 2007. Mineralization and carbon turnover in subarctic heath soil as affected by warming and additional litter. Soil Biology and Biochemistry. 39:3014-3023.

Robinson, R.A. 1996. Return to Resistance: Breeding Crops to Reduce Pesticide Dependence. Davis, CA, agAccess. United States of America.

Rodríguez, H., Rodríguez, J. 2002. Métodos de análisis de suelos y plantas, Criterios de interpretación, Trillas, DF México.

Scott, C., Lewis, E.S., Milla, R., Taylor, M.C., Rodgers, J.W.A., Dumsday, G., Brodie, E.J., Oakeshott G.J., Russell, J.R. 2010. A free-enzyme catalyst for the bioremediation of environmental atrazine contamination. Journal of Environmental Management. 91:2075-2078.

Sene, L. Converti, A., Ribeiro, G.A.S., Cássia, R. 2010. New Aspects on Atrazine Biodegradation. Brazilian Archives of Biology and Technology an International Journal. 53:487-496.

Schuster, A., Schmoll, M. 2010. Biology and biotechnology of Trichoderma. Applied Microbiology & Biotechnology. 87:3. 787.

Sistema Meteorológico Nacional. 2011. http://smn.cna.gob.mx/index.php?option=com_content&view=article&id=103&Itemid=68.

Smith, D., Alvey, S., Crowley, E.D. 2005. Cooperative catabolic pathways within an atrazine-degrading enrichment culture isolated from soil. Microbiology Ecology. 53:265–273.

So, Y.K., and Kyung, S.C. 2009. Isolation and Characterization of a Plant Growth-Promoting Rhizobacterium, Serratia sp. SY5. Journal Microbiology. Biotechnology. 19:1431-1438.

Tang, J.,Liu, L., Hu, S., Chen, Y., Chen, J. 2009. Improved degradation of organophosphate dichoruos by Trichoderma atroviride transformats generated by restriction enzyme- medianted interation (REMI). Bioresurce Technology. 100:480-483.

Tappin, I.A., Loughnanc, J.P., McCarthy, J.A., Fitzsimons, F.M. 2012. Removal of atrazine from river waters by indigenous micoorganismos. Environmental Chemistry Letters. 10: 89-96.

Waksman, S.A. 1922. A method of counting the number of fungi in soil. Journal of Bacteriology. 7(3):341-399.

Walkley, A., and Black, I.A. 1934. An examination of the Degtjareff method for determining organic carbon in soils: Effect of variations in digestion conditions and of inorganic soil constituents. Soil Science. 63:251-263.

Yang, C., Yang, L., Kun, Z., Wang, X., Cuiqing, M., Hongzhi, T., Ping, X. 2010. Atrazine Degradatión by a simple consortium of *Klebsiella* sp. A1 and *Comaonas* sp. A2 in nitrogen enriched medium. Biodegradation. 21:97-105.

Zafar, S., Aqil, F., Ahmad, I. 2007. Metal tolerance and biosurption potential of filamentous fungi isolated from metal contaminated agricultural Soil. Bioresource Technology. 98 (13):2557-2561.

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