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Identification of an Indigenous Atrazine Herbicide Tolerant Microbial Consortium in Beans (*Phaseolus vulgaris* L.) as a Potential Soil Bioremediator

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Abstract

The present article reports the isolation and identification of atrazine-tolerant strains of indigenous microorganisms recovered from three representative agricultural sites representing agronomic characteristics of the Tulancingo Valley, Central part of México (disturbed and undisturbed). Biochemical and morphological tests were performed for microorganism's identification and the minimum inhibitory concentration assay was followed to assess atrazine tolerance. Results showed the microorganism populations varied from 10^{-5} to 10^{-6} UFC g^{-1} of soil for bacteria and 10^{4} - 10^{5} conidia g^{-1} of soil for fungi. The bacterial genera isolated and identified were: *Agrobacterium* sp., *Bacillus* sp., *Erwinia* sp., *Micrococcus* sp., *Pediococcus* sp., *Rhizobium* sp., *Serrantia* sp. and *Sphingomonas* sp. Identified fungal genera were: *Alternaria* sp., *Aspergillus* sp., *Mucor* sp., *Cladosporium* sp., *Penicillium* sp., *Fusarium* sp. and *Trichoderma* sp. Tests for herbicide tolerance indicate the isolated microorganisms do not show inhibitory growth at 500 to 2,500 ppm of atrazine concentrations under laboratory conditions. The strains of the fungal genera and *Rhizobium* sp. showed greater tolerance rates to atrazine, based on their growth without inhibition in the presence of 5,000 to 10,000 ppm of the agrochemical. Results suggest the isolated microorganisms may be useful as a viable inoculum for bioremediation purposes in agricultural atrazine-contaminated soils.

Keywords: Agricultural sustainability; Soil recuperation; Agrochemicals

Introduction

Soil is a complex and sensitive biomaterial strongly affected by plant-human management, especially agriculture activities. Agriculture requirements include the use of herbicides which results in negative impact to the environment due to contamination by chemicals. Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine), one of the most used herbicides worldwide, causes disruption to biogeochemical cycles and biodiversity as a result of the permanency of its soil residue [1]. Therefore, it is necessary to apply friendly technologies for remediation and / or recovery of soils. A biological low cost alternative is the in situ bio augmentation of native microorganisms. This technology consists of inoculating microorganisms with physiological, biochemical and molecular ability to absorb, retain, degrade or transform contaminants into less harmful substances. These microorganisms must be recovered from the same soil that is expected to be restored, in order for these microorganisms to be adapted to the agro ecosystem, and to enhance the efficiency of their degradative ability. Atrazine is classified by its biological action as herbicide and is widely used to kill or inhibit the growth of plants considered to be weeds. In 2006, the application of the herbicide atrazine ranged from 29 to 34 million of kilograms of active ingredient per year in agricultural soils [2]; while between 85% and 100% of agricultural soils in industrialized countries are treated with herbicides.

Atrazine is a systemic, selective and residual herbicide, with persistent effects for a period of time. It is transported to environmental processes spheres by volatilization or leaching diffusion. It is considered as one of the pollutants responsible for negatively affecting mammalian reproductive systems, carcinogenicity, teratogenicity and ecotoxicity. Evy and Nilanjana [3] report atrazine effects including changes in aquatic species, plants and mammals. Due to its organizational structure, atrazine can experience natural or induced degradation processes such as: (i) hydrolysing and (ii) oxidative - hydrolytic, two very well studied degradation mechanisms. Reports [4-8] showed that atrazine degradation can be achieved by isolated microbial consortia species in combination with other bioremediation techniques.

The primary limiting factor for atrazine mineralization is the absence of specific microorganisms. However, Sene et al. [6];

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Spaczynski et al. [7]; and Dehghani et al. [8] suggest that rhizosphere bioremediation by native microorganisms has been efficiently achieved by atrazine elimination in soil, when these organisms were isolated from contaminated sites where they grow. And because these native microorganisms grow in contaminated conditions, they may adapt, and develop tolerance, even resistance to such environments, resulting in the generation of metabolic processes that transform the chemical structure of the contaminant into simpler compounds.

This research aims to isolate and identify indigenous microorganisms in the atrazine-contaminated agricultural soils from the Tulancingo Valley, Hidalgo, Mexico and to quantify their herbicide tolerance.

Materials and Methods

Study area

The study area (agricultural land) belongs to the Tulancingo Valley, Hidalgo, Mexico (central), with polygon coordinates: 20° 10' 29" north latitude, 98° 16' 52" west longitude, 19° 57' 20" north latitude and 98° 15' 59" west longitude. Dominated by a humid temperate climate (Cw), the average annual temperature is 14.5°C, with a maximum 30°C from March to July, and a minimum temperature of 3°C from August to February. Precipitation, in the form of rain, is recorded during the months of March through to September, with a minimum of 33.3 mm h^{-1} and a maximum of 190.7 mm h^{-1} with average between 500-553 mm per year [9].

Sampling

Conditions for selection of the sampling site were as follows: (i) Soils with a record of continuous pesticide application and (ii) soils with a record of no pesticide application. Sampling of soils was performed three times during the annual cycle of the legume crop. The sampling depth was 0 cm - 25 cm using a tubular auger (7 cm diameter). Total sample weight was 1 kg.

For sampling collection, the procedures described in the Mexican Official Standard Norm AS-01 method [10] for transport, storage and methods of microbiological, physical and chemical soil analysis were followed. Soil samples for microbiological analysis was transported in labeled sterile plastic bags (Nasco - Whirl - Pak®), to prevent cross contamination. Each of the samples was thoroughly mixed and sieved (2 mm), and then stored at 4°C for physical chemical analysis.

Physical and chemical analytical methods in experimental soils

The physical properties determined in the experimental soils were: available moisture, density, field capacity, moisture, texture and wilting point. Chemical properties determined were: cation exchange capacity, total nitrogen, organic carbon, organic matter and pH. All physical and chemical analytical methods were performed according to the procedures established in the Mexican Official Standard Norm [10].

Diversity of microorganisms in the rhizosphere of legume *Phaseolus vulgaris* L.

In order to determine biodiversity and viability of bacteria and fungi in the representative soils, the following were determined: (i) total score in bacteria (as described in the Mexican Official Standard Norm [11] and (ii) fungi presence, as described by the Waksman method [12]. Six working dilutions (10^{-1} to 10^{-6}) were used in each case. Surface plating with three replications was performed. In order to determine a statistical record of measurement in time for soil conditions (with and without atrazine application), quantification of microorganisms was performed consecutively for three weeks from the soil sampling date.

Selection and identification of microorganism strains

The used selection criteria and identification of native strains of rhizosphere bacteria and fungi were the comparison of the microorganism growth by soil sample. After the incubation period, a count of viable cells was performed, and the time of growth recorded. Macroscopic evidence for selection of isolates on soil extract agar for bacteria included: (i) colony appearance, (ii) elevation, (iii) shape, (iv) turnover margin or (v) color. Microscopic identification was performed by differential Gram stain.

In fungi, the morphological identification of strains included: (i) thalli color, (ii) thalli texture (iii) growth form (length and radius), (iv) appreciation of the hyphae and (v) the appearance, shape and color of conidia.

The fungal strains were examined by microscopy micro culture (Riddell method) [13] after using the staining technique for micro fixed cultivation of lacto phenol blue with the use of photomicrographs taken through a Motic BA300 microscope, objectives contrast bright field and coupled to a docked digital 480 Moticam camera (software Motic Images Plus 2.0).

Gross observation of bacteria was conducted at 24, 48, 72, 92 and 132 hour intervals after isolation; while for fungi, changes along the fungal morphology life cycle were recorded.

The strains of candidate microorganisms (fungi and bacteria) were purified by consecutive plating (spline surface). Bacteria AES were purified in solid medium and fungal strains by a hyphal needling technique in potato dextrose agar (PDA) (Merk Millipore®) and then incubated. This procedure was repeated four times until purification of the isolated strains was obtained; and finally, the inbred strains were stored in inclined test tube for subsequent identification.

To identify the genera of the strains, macroscopic and microscopic description of each one were completed after each biochemical tests; these tests were performed using the Analytical Profile Index System (API tests, bioMérieux®). API tests include: motility, catalase enzyme production, oxidase (cytochrome c oxidase production), NO_3 (nitrate reductase and nitrite reductase enzymes production), tryptophan deaminase enzyme prodcution, glucose fermentation (hexose sugar), lactose (lactose fermentation as a carbon source), urease (urease enzyme production), xylose (xylose fermentation as a carbon source), gelatinase enzyme production (which liquefies gelatin), mannose fermentation (hexose sugar), mannitol (mannitol fermentation as a carbon source), maltose (maltose fermentation as a carbon source), citrate (use of citrate as a carbon source) and starch (α-amylase and oligo-1, 6-glucosidase enzymes production).

In fungi, all strains were examined by microscopy microculture with the use of photomicrographs. Images were extrapolated with the results of lacto phenol blue staining, the reported bibliography, as well as morphological criteria [14].

Bioassays of inhibition of growth tolerance

Atrazine herbicide tolerance was determined with the use of the minimum inhibitory concentrations (MIC) assays performed on eight bacterial strains, seven fungal strains and three unidentified fungal genera using the qualitative disc diffusion method [15].

Fungi and bacteria were plated in 9 cm petri dishes containing 20 mL of potato dextrose agar (PDA) solid culture media (Sigma - Aldrich™) while Mannitol agar (MA) media were used for *Rhizobium* sp. Strains of the identified genera were inoculated with the use of the surface extension technique where sterile Whatman paper discs of 0.5 cm diameter were placed on the agar, equidistant and near the border using vernier caliper. Paper discs impregnated with 10 µL of standard solution of atrazine were used. Tested concentrations were: 0, 500, 1000, 1,500, 2,000, 2,500, 5,000, 7,500 and 10,000 mg L^{-1}. A repetition for each treatment was used. The plates were incubated at 25°C for 5 to 7 days until the growth in the control plates reached the edge of the plates; then, after the incubation time, the inhibition growth zone was measured using an electronic micrometer (IP54) and recorded.

The inhibition percentage of fungal and bacterial growth was calculated by using equations 1 and 2:

$$\% \text{ growth} = \left(\frac{\phi atz}{\phi b}\right) \times 100\ \% \quad (1)$$

$$\% \text{ CI} = 100 - \% \text{ growth} \quad (2)$$

Where Øatz represents the diameter (mm) of growth of the microorganism in the treatments exposed to atrazine at each assessment. Øb is the diameter of the negative control microorganism growth in each evaluation and % CI is the percentage of inhibitory growth.

The growth rate of the microorganism inhibition was calculated with the use of equation number 3:

$$V = \frac{\phi C}{t} \quad (3)$$

Where V is the speed of growth, ØC represents the diameter (mm) of the microorganism growth (mm) and t is the incubation time (days).

Statistical analysis

The experimental design used for the physical and chemical characterization of soils was completely random. Each sample had three replicates. Analysis of variance (ANOVA) and subsequently Tukey test $p > 0.05$ were performed in order to assess significant differences.

Result and Discussion

Physical and chemical parameters of the soil samples

The values of the physical and chemical parameters found in the soil samples collected at the three study sites are reported in (Table 1). The pH value for the soil samples was classified as moderately acid for two of the sites (Capulin and Santana) and neutral in the Tepantitla sample. At these intervals, nutrients and contaminants in the soil solution are more available [16]. Also, at these pH values, the rhizosphere microorganisms as bacteria and fungi can grow without a significant reduction of their population.

The values for total nitrogen classified samples at intervals of high (0.15 - 0.25%) and very high (> 0.25), according to Mexican Official Standard Norm [10]. For the organic matter (OM), content of organic carbon (OC), the rule states that values between 4.1% - 6.0% of OM indicate low fertility. According to other reports such as Rodriguez and Rodriguez [17] the amount of organic matter and organic carbon is classified as high and very high (> 3.5%).

Finally, based on the texture (proportion of soil particles: Clay, Silt and Sand) samples are classified as loam and sandy loam [10]. The soil with a clay texture was that of Capulin. The main feature of the sandy loam soils is their low ability to retain nutrients and water, which can be important for soil agrochemical interactions.

Santana and Tepantitla medium texture soils were classified as loam; while Capuline´s as clay texture. Medium-textured or loam soil types are ideal for agricultural production, due to their extensive production capacity and water availability. This soil can be considered versatile for its high cation exchange capacity, which varies in the range of 20 to 45 cmol kg^{-1} (Table 1).

Comparison tests (Tukey 0.05) of the physical and chemical properties showed significant differences with the soil of Santana, as compared to the other two soil sites analyzed. Taking into account that properties are not only important for the soil fertility and genesis characterization, but also because retention capacity can be strongly affected by them, having an effect on the adsorption and degradation of atrazine rates in the soil [18,19].

Based on a references search [20] and the resulting data, it is estimated that the three sites are classified with good agricultural fertility. The soil sample from the Santana site (coming from an intensive agricultural area, with regular pesticides application) could be vulnerable to deterioration and short-term negative environmental impacts.

Diversity of microorganisms in the rhizosphere of *Phaseolus vulgaris* L.

The results and conditions of the total bacterial count at all sites are shown in (Figure 1). An interval of 1.09×10^5 to 2.0×10^6 CFU g^{-1} soil was obtained. A higher bacterial population was found in the rhizospheric soils, compared to the population found in the non-rhizospheric soils.

The bacterial population in the Santana soil (all three conditions of the site) is in the same range of that found in the undisturbed soils (no atrazine application), and the total bacteria count is similar to that reported by Sangabriel et al. [21]. The soil bacteria population in the rhizosphere of *Phaseolus coccineus* cultured on fuel oil - contaminated and uncontaminated conditions ranged in 1.7×10^6 CFU g^{-1}. Sangabriel et al. [21] also observed a total increase in the bacteria population after a phytoremediation process using *Phaseolus coccineus*.

Other references [22-24] cite locations of rhizospheric soils treated with legume crops for bioremediation processes with a total count of bacteria ranging from 10^5 to 10^6 CFU g^{-1}. These results are similar to the ones found in this experiment, regardless of the condition of the site, hydrology, geographic location or textural class. For fungi, it was observed that the population number (total count) in studied soils is

	Capulín	Tepantitla	Santana	Standard Error
% Moisture	14.89_a	15.86_a	15.20_b	0.2
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	0.0094
% Organic Carbon	2.86_a	3.36_b	4.08_c	0.012
% Organic Matter	4.94_a	5.79_b	4.04_c	0.0086
CEC (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 differences (α = 0.05, Tukey).

Table 1: Comparison of means for physical and chemical properties of the soils studied.

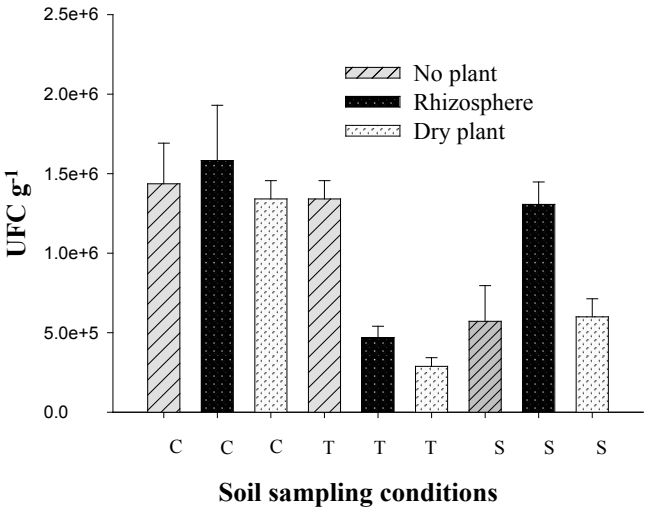

Figure 1: Total count of aerobic bacteria (CFU g^{-1}) of the three agricultural soils of the Tulancingo Valley, Hidalgo, Mexico. C (Capulin soils), T (Tepantitla soils), S (Santana soils). Error bars indicate the standard error of the mean of triplicate replicates.

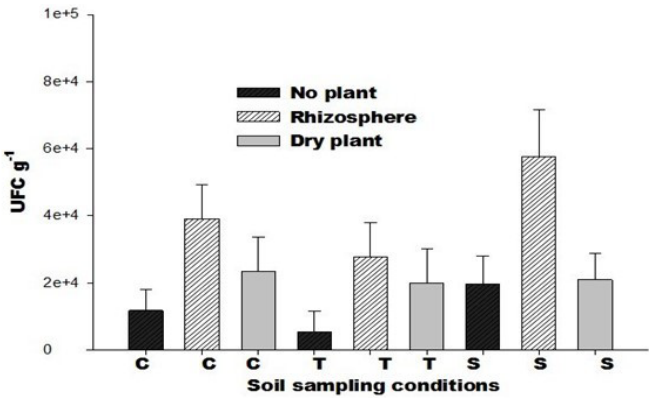

Figure 2: Fungi total account (CFU g^{-1}) of the three agricultural soils from Tulancingo Valley, Hidalgo, Mexico. Where C corresponds to the sample of the Capulin soil, T corresponds to Tepantitla soil and S correspond to Santana soil. Error bars indicate the standard error of the mean of triplicate replicates.

also higher in the rhizosphere area at rates rhizosphere / soil (R / S) of 2:1, in all of the three sites (Figure 2): Capulín (C), Tepantitla (T) and Santana (S).

The proportion of conidia per gram of soil (CFU g^{-1}) reported here was found within the estimated range considered by Maier and Pepper [24]. They reported CFU g^{-1} ranging from 10^5 to 10^6 in the rhizhosphere. Also, Calvo et al. [22] and Córdova et al. [25] reported ranges of 10^3 CFU g^{-1} soil, in non- rhizospheric soils in the study area.

Selection and identification of bacterial and fungal strains

The selected fungi strains and bacteria were isolated specifically from the rhizosphere of Santana soil (disturbed) in order to consider the issues addressed by Robinson [26], who relates the genetic resistance of microorganisms in wild systems to their ability to self-regulate and adapt to contaminated (disturbed) environment. Tables 2 and 3 present a summary of the identified organisms to genus level.

As for the identification of *Rhizobium* sp., isolated from nodules of the bean plant, phenological characteristic of the bean plant observed where the nodes extracted to isolate the bacterium *Rhizobium* sp. Results showed an average of 3.2 knots, 3.8 inflorescences and 11.47 cm root length in all the selected plants, the average was 23 peripheral root nodules, pink appearance, different sizes, average diameter of 0.34 cm and a volume of 0.10 mL (in 1 mL conical vial).

The microscopic observation of fresh bean nodules revealed the existence of living organisms in a bacillus form, beige color, translucent and with a mucilaginous consistency. Average Bacillus length was 109.85 µm and Gram negative under a fresh staining. Comparison of morphological and biochemical tests proved that the isolated bean nodule is the bacterial genus *Rhizobium* sp. (Table 3). Similar results were reported by Acosta and Martinez [27] and Richardson et al. [28]. In these reports, Rhizobium strains were isolated from nodules of legumes growing in pesticide contaminated soils. Also, *Agrobacterium* sp., *Bacillus* sp., *Rhizobium* sp., *Sphingomonas* sp., *Micrococcus* sp. and *Serrantia* sp. have been reported for their degradative capacity of organic pollutants, including the herbicide atrazine [4,29,30].

Based in the macro, microscopic and biochemical evidence, eight bacteria genera were identified in the soils, as follows: *Agrobacterium* sp., *Bacillus* sp., *Erwinia* sp., *Micrococcus* sp., *Pediococcus* sp., *Rhizobium*

Genera				
	Bacillus sp.	*Micrococcus* sp.	*Serrantia* sp.	*Sphingomonas* sp.
Macroscopic description				
	Colonia aspect viscous. Circular convex elevation. Entire margin. Color: reddish-brown, slimy	Circular aspect of the colony. Flat. Yellow color. Creamy. Viscose.	Circular form of the colony. Convex elevation. Brown to reddish color. Viscose.	Circular convex colony. Circular orange. Flat. Entire margin. Yellow
				Colonies circular in entire dome margin. Creamy. Opaque beige
Microscopic description				
	Gram negative Bacillus	Gram positive Cocus	Gram negative Bacillus	Gram negative Bacillus
Biochemical tests				
Catalase	+	+	+	+
Citrate	-	-	NA	Negative
Gelatine	+	+	NA	Positive
Glucose	NA	Positivo	+	Negative
Glucose OF	NA	NA	F	Oxidative
Lactose	-	+	-	NA
Maltose	+	+	NA	Negative
Mannitol	NA	+	+	Negative
Manose	+	+	NA	Negative
Mobility	+	++	+	+
NO_3	NA	NA*	+	Negative
Oxidase	NA	-	-	+
Starch	-	NA	NA	NA
Tryptophan	NA	NA		Negative
Urea	+	-	NA	Negative
Xilose	+	+	NA	NA

Note + = positive; - = negative
• NA = Not Applicable.

Table 2: Results of macroscopic, microscopic and biochemical tests of isolated bacterial genera (part I).

Genera				
	Agrobacterium sp.	*Erwinia sp.*	*Pediococcus* sp.	*Rhizobium* sp.
Macroscopic description				
	Circular form of the colony. High grow. White to pale brown color. Viscous consistency.	Sharply colony form. Convex margin. Yellow color.	Circular form of the colony. Lisa. Viscous consistency. Bright yellow color.	Circular colony form. Convex. White to pale brown color. Translucent. Mucilaginous aspect.
Microscopic description				
	Gram negative Bacillus	Gram negative Bacillus	Cocus gram positive	Gram negative Bacillus
Biochemical tests				
Catalase	+	-	-	+
Citrate	NA	NA	+	NA
Gelatine	NA	-	+	NA
Glucosa OF	F	NA	NA	Oxidative
Glucose	Positive	-	NA	-
Hugyleftson test	-	NA	NA	Oxidative
Lactose	NA	+	+	+
Maltose	NA	NA	+	+
Manitol	NA	NA	+	+
Manose	A	NA	+	NA
Mobility	+	+	+	+
NO_3	-	NA	NA	NA
Oxidase	+	NA*	NA	+
Starch	NA	NA	-	NA
Trypthophan		NA	NA	+
Urea	+	NA	+	NA
Xilose	+	NA	+	NA
YMA media (bromo timol blue)	-	NA	NA	+

Note + = positive; - = negative
* NA = Not Applicable

Table 3: Results of macroscopic observations, microscopic and biochemical evidence of isolated bacterial genera (part II).

sp., *Serrantia* sp. and *Sphingomonas* sp. For fungi, seven genera and 23 candidate species were identified and recorded as follows: *Alternaria* (one), *Aspergillus* (one), *Cladosporium* (one), *Fusarium* (three), *Mucor* (two), *Penicillium* (seven) and *Trichoderma* (five). Three unidentified fungal strains were also observed (Table 4).

The frequency of the microorganisms identified by soil condition, highlight the *Micrococcus* sp. and *Bacillus* sp., presence. It can be inferred then, that the microorganism are native and present under different conditions and able to persist over the agricultural cycle of the crop. *Rhizobium* sp. is present in a lower frequency; it was identified only in rhizospheric soil and was isolated from de nodules of the bean plant. Therefore, the condition of the soil (soils without plants) seems to be a limiting factor and responsible for the lower frequency of this bacterium.

Results in terms of frequency and types of fungal genera identified in this study are similar to studies from Ibiene et al., Gopi et al., Martinez et al., and Maldonado et al. [30-33], who report the genus Aspergillus, Fusarium, Mucor, Penicillium and Trichoderma to be present in metals, pesticides and hydrocarbure contaminated soils.

Growth inhibition bioassays

Exposure to contaminants can cause the reduction and / or modification of the microbial soil population. However, some microorganisms persist and increase their population. This may indicate the ability of the population to develop and adapt to such conditions. The results of the minimum inhibitory concentration trials (MIC) to atrazine concentrations are summarized in Table 5. The identified genera grew without inhibition halo at concentrations of 0-2,500 mg L^{-1} of atrazine.

Bacillus sp., *Erwinia* sp., *Micrococcus* sp., *Pediococcus* sp., *Sphingomonas* sp., and *Serrantia* sp. exhibit inhibitory growth at rates up to 5,000 mg L^{-1}, increasing to 7,500 mg L^{-1} and is completely inhibited at 10,000 mg L^{-1} of atrazine. It should be noted that Agrobacterium

	Soils with no plants			Rhizosphere soils			Soils with dry plants		
Genera	**T**	**C**	**S**	**T**	**C**	**S**	**T**	**C**	**S**
Agrobacterium sp.	+	-	+	+	+	+	+	+	+
Alternaria	+	+	+	+	+	+	+	+	+
Aspergillus sp.	+	+	+	+	+	+	+	+	+
Bacillus sp.	+	+	+	+	+	+	+	+	+
Cladosporium	+	+	+	+	+	+	+	+	+
Erwinia sp.	+	-	+	+	+	+	+	+	+
Fusarium sp. 1	+	+	+	+	+	+	+	+	+
Fusarium sp. 2	+	+	+	+	+	+	+	+	+
Fusarium sp. 3	+	+	+	+	+	+	+	+	+
Micrococcus sp.	+	+	+	+	+	+	+	+	+
Mucor sp. 1	+	+	+	+	+	+	+	+	+
Mucor sp. 2	+	+	+	+	+	+	+	+	+
Pediococcus sp	-	+	+	+	+	+	+	+	+
Penicillium sp. 1	+	+	+	+	+	+	+	+	+
Penicillium sp. 2	+	+	+	+	+	+	+	+	+
Penicillium sp. 3	+	+	+	+	+	+	+	+	+
Penicillium sp. 4	+	+	+	+	+	+	+	+	+
Penicillium sp. 5	+	+	+	+	+	+	+	+	+
Penicillium sp. 6	+	+	+	+	+	+	+	+	+
Penicillium sp. 7	+	+	+	+	+	+	+	+	+
Rhizobium sp.	-	-	-	+	+	+	+	+	+
Serrantia sp.	+	-	+	+	+	+	+	-	+
Sphingomonas sp.	+	-	-	+	+	+	+	+	+
Trichoderma 1	+	+	+	+	+	+	+	+	+
Trichoderma 2	+	+	+	+	+	+	+	+	+
Trichoderma 3	+	+	+	+	+	+	+	+	+
Trichoderma 4	+	+	+	+	+	+	+	+	+
Trichoderma 5	+	+	+	+	+	+	+	+	+
Unidentified 1	+	+	+	+	+	+	+	+	+
Unidentified 2	+	+	+	+	+	+	+	+	+
Unidentified 3	+	+	+	+	+	+	+	+	+
Trichoderma 1	+	+	+	+	+	+	+	+	+
Trichoderma 2	+	+	+	+	+	+	+	+	+
Trichoderma 3	+	+	+	+	+	+	+	+	+
Trichoderma 4	+	+	+	+	+	+	+	+	+
Trichoderma 5	+	+	+	+	+	+	+	+	+
Unidentified 1	+	+	+	+	+	+	+	+	+
Unidentified 2	+	+	+	+	+	+	+	+	+
Unidentified 3	+	+	+	+	+	+	+	+	+

T: Tempantitla, C: Capulín, S: Santana

Table 4: Diversity of microorganisms isolated and identified by soil condition.

Minimum Inhibitory Growth Concentration (mg L^{-1})						
Genera	0-2500	5000	7500	10000	Rise time (days)	Growth rate (mm day^{-1})
	% CI*	% CI	% CI	% CI		
Agrobacterium	0	0	6.25	13.75	6	13.33
Alternaria	0	0	0	0	7	11.42
Aspergillus sp.	0	0	0	0	6	13.33
Bacillus sp.	0	13.75	18.75	100	5	16
Cladosporium	0	0	0	0	5	16
Erwinia sp.	0	12.5	20	100	3	26.66
Fusarium sp. 1	0	0	0	0	5	16
Fusarium sp. 2	0	0	0	0	5	16
Fusarium sp. 3	0	0	0	0	5	16
Micrococcus sp.	0	5	13.75	100	3	26.66
Mucor sp. 1	0	0	0	0	7	11.42
Mucor sp. 2	0	0	0	0	7	11.42
Pediococcus sp.	0	16.25	18.75	100	5	16
Penicillium 1	0	0	0	0	7	11.42
Penicillium 2	0	0	0	0	7	11.42
Penicillium 3	0	0	0	0	7	11.42
Penicillium 4	0	0	0	0	7	11.42
Penicillium sp. 5	0	0	0	0	7	11.42
Penicillium sp. 6	0	0	0	0	7	11.42
Rhizobium sp.	0	0	0	2.5	4	11.42
Serrantia sp	0	6.25	16.25	100	4	20
Sphingomonas	0	8.75	15	100	4	20
Trichoderma 1	0	0	0	0	5	16
Trichoderma 2	0	0	0	0	5	16
Trichoderma 3	0	0	0	0	5	16
Trichoderma 4	0	0	0	0	5	16
Trichoderma 5	0	0	0	0	5	16
Unidentified 1	0	0	0	0	6	13.33
Unidentified 2	0	0	0	0	6	13.33
Unidentified 3	0	0	0	0	6	13.33

Table 5: Minimum inhibitory growth concentrations MIC (%) of atrazine for genera identified.

showed the lowest growth percentage at 7,500 mg L^{-1}, while *Rhizobium* sp. showed the lowest zone of inhibition in the presence of 10, 000 mg L^{-1} of atrazine.

The seven fungal genera (23 possible species) identified showed no growth inhibition halos of mycelium at 10,000 mgL^{-1} atrazine concentrations. These results evidence those genera isolates to be tolerant to high concentrations of the chemical. Assays relating minimum inhibitory concentration and type of microorganisms yield good results in terms of the number of species and tolerance to the herbicide. This last is compared to other studies [6,20,34,35]. Bacteria identified are inhibited to a greater extent from 5,000 mg L^{-1} of atrazine, except *Rhizobium* sp. Moreover, the fungal genera show no growth inhibition at any atrazine concentrations. Of the 15 organisms identified, 74.18% presented no growth inhibition at 10,000 mg L^{-1} of atrazine concentration. Only 3.22% of bacteria (*Agrobacterium sp.* and *Rhizobium sp.)* grew at this concentration and 22.58% (*Bacillus sp., Erwinia sp., Micrococcus sp., Pediococcus sp., Serrantia sp., Sphingomonas sp.*) showed growth inhibition (Table 5). To the best of our knowledge, there are only a few references describing indigenous microorganisms with high tolerance to atrazine. Regularly, tolerance and / or resistance are induced by genetic modification [3,36].

There are reports of the use of effective bioremediation methods of atrazine removal. Moreover, the genetic pathways for atrazine detoxification have been well characterized in bacteria, fungi and plants. Along with this, transgenic microbes and plants expressing atrazine degrading enzymes have also been used. A combination of transgenic plants and microbes have been proposed as a combined synergistic method for atrazine bioremediation [37]. However, the gap between bioremediation laboratory conditions, green house bioremediation trials and an effective real soil cleanliness (or large scale soil decontamination) is still under discus [38]. In order to keep research study cost low, no molecular biology techniques for microorganism identification were performed. Further studies will benefit for the use of such techniques.

Conclusion

Organochlorine pesticides, including atrazine, are the most common used herbicide / pesticide in agriculture and forestry for disease and insect control. These compounds have been linked to a various human health hazards such as cancer, reproductive harm and even birth defects. Because of their residual effects, the molecules can remain in the soil and cause water contamination. Many methods for soil bioremediation have been proposed, but some of them seem not to be cost efficient or tend to be difficult to achieve since it is strongly influenced by natural conditions. Then, it is necessary to find ways to reduce variability of results, which can be achieved by adapting the technology to the local native environment. Intensive research needs to be done to discover the biological activity for natural local resources, including microorganisms.

Therefore, this study has resulted in the isolation and identification of indigenous microorganisms with high tolerance to atrazine concentrations, distributed as follows: (i) eight bacterial genera (*Agrobacterium* sp., *Bacillus* sp., *Erwinia* sp., *Micrococcus* sp. *Pediococcus* sp. *Rhizobium* sp., *Sphingomonas* sp., and *Serrantia* sp.), (ii) seven fungal genera (Alternaria, Aspergillus, Cladosporium, Fusarium, Mucor, Penicillium and Trichoderma) and (iii) three unidentified fungal strains. The microorganisms are native, rhizospheric and specific to the bean plant. The consortium identified genera did not show growth inhibition to 2,500 mg L^{-1} of atrazine concentration assays. In particular, *Rhizobium* sp. and fungal strains showed no growth inhibition to 10,000 mg L^{-1} atrazine concentration. These microorganisms may be suitable to be used in atrazine agricultural contaminated soils bioremediation. Further studies may include molecular biology techniques for microbe identification and atrazine biodegradation mechanisms.

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