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Isolation and Characterization of Mercury Resistant *Trichoderma* Strains from Soil with High Levels of Mercury and Its Effects on *Arabidopsis thaliana* Mercury Uptake

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Abstract

Traditional mining activities are usually correlated with high levels of soil pollution, which is a major environmental concern. Extensive mining activities have taken place in the San Joaquin region in the State of Querétaro, México resulting in high levels of mercury soil pollution (up to 1532 ± 300 mg/kg). We isolated mercury-resistant fungal strains from the San Joaquin region soils and identified them through morphologic characteristics and ITS rDNA region sequence analysis. We determined that fungi isolated belong to the genus *Trichoderma*. All the isolates selected showed the ability to catalyze the volatilization of Hg. For air sampling, an active sampling device was constructed and using acid $KMnO_4$ as an absorbent, the concentration of mercury in solution was determined through the cold vapor atomic absorption method. The results show mercury volatilization from the fungal species assay, with a maximum of 213.04 ± 32.6 μg/m^3 while mycelium accumulation ranged from less than 17.5 ± 2.9 to 20.0 ± 3.4 μg/g. The fungal isolates were also evaluated for their ability to reduce mercury uptake in *Arabidopsis tha-*

liana. These observations suggest the utility of *Trichoderma* for the mobilization of mercury in those contaminated soils.

Keywords

Volatilization, Mercury, *Trichoderma*, Soil Bioremediation

1. Introduction

The San Joaquin region is a unique locality in Querétaro State, which has an extensive history of commercial-scale mercury extraction in México. During the period 1950 to1970, intense mercury mining took place [1]. Today the mines are completely abandoned and economic activity is based in local agriculture. Nevertheless, mercury accumulation from waste produced by the mining operations represents a significant source of pollution in this area [2].

Although diverse investigations have shown the detrimental effects of mercury in various organisms, the ability of several microbial species to detoxify Hg has also been reported [3]. In particular, both Gram negative and Gram positive bacteria are able to detoxify Hg(II) by converting it to the volatile and less toxic form, Hg^0 [4] [5]. Mercury resistance mechanisms have been well studied in several bacterial species. It is mediated by the transcriptional activator, MerR; in the presence of Hg(II) the *mer* operon is expressed. Hg(II) is reduced through the action of mercuric reductase (*merA*), and bacteria can also cleave organomercurials using organomercuriallyase (*merB*) [4] [5]. Previous work from our laboratory showed that microorganisms associated with the rhizosphere of plants that grow in soils contaminated with high metal concentrations may possess the genetic machinery to produce resistance to mercury [2].

In other studies, diverse fungal species such as *Aspergillus*, *Fusarium*, *Mucor*, *Penicillium*, *Rhizopus*, *Clonostachys* and *Trichoderma* have received much attention because of their particular metabolic properties, such as the ability to participate in the degradation of pollutants or to interact with toxic metals. Fungi tolerate and detoxify metals by biosorption, bioaccumulation, resistance mechanisms that include cell membrane metal efflux, intracellular chelation by metallothione in proteins and glutathione-derived-peptides called phytochelatins and by metal compartmentalization in vacuoles and by extracellular precipitation [6] [7] [8].

Members of the genus *Trichoderma* are saprophytic microorganisms ubiquitously distributed, and this genus includes a number of fungal strains that are used as biocontrol agents due to their abilities to antagonize a wide range of phytopathogenic fungi, bacteria and oomycetes [9]. This antagonism involves competition for nutrients and space, the production of antibiotics and the induction of systemic resistance in plants [10]. For the last two decades *Trichoderma* isolates have been used extensively in biological control and their influ-

ence on several microbial populations has been extensively investigated [9]. The wide distribution and ecological plasticity of *Trichoderma* are closely related to their ability to produce a wide range of lysing enzymes, to degrade substrates and to possess high resistance to microbial inhibitors. The influence of several soil parameters on the survival of *Trichoderma* spp. has been investigated, including temperature, pH and the presence of heavy metals and/or toxic compounds such as Ni, Cd, Hg, Pb and Zn [11]. Therefore, these fungi should possess effective systems that efficiently inactivate and/or remove harmful compounds from the soil or from the cell.

The aim in this work was the isolation of mercury-resistant fungal strains that grow in soils which have a long history of involvement in mercury extraction in central México. Identification through morphological and molecular approaches revealed three strains, capable to grow in high mercury concentrations, belonging to the species *Trichoderma virens*. The use of an active sampling device and the Cold Vapor AAS method revealed that *Trichoderma* has the ability to catalyze the conversion of Hg^{+2} to Hg^{0} and the volatilization of Hg^{0} and finally, fungi-plant interaction assays demonstrated that *Trichoderma* isolates reduced mercury accumulation in *Arabidopsis thaliana* tissues.

2. Materials and Methods

2.1. Isolation of Mercury-Resistant Fungi

The soil samples were collected from San Joaquin, Querétaro, México. Soil pH and organic matter content were analyzed using standard methods [12]. Using aseptic conditions, soil samples were serially diluted in potato dextrose broth (PDB; Difco; Detroit, MI, USA) and immediately thereafter, 0.1 ml was spread over potato dextrose agar medium (PDA; Difco; Detroit, MI, USA), amended with 50 $mg{\cdot}L^{-1}$ of $HgCl_2$. The plates were incubated at 28˚C during 4 days for isolation of fungal strains. Hg-resistant colonies were picked and purified on the same medium. Purified isolates were screened on the basis of their tolerance to $HgCl_2$ [13]. From each isolate, mycelium was inoculated on PDA plates supplemented individually with 0, 25, 50, 75, 100, 150, 200 and 250 $mg{\cdot}L^{-1}$ of $HgCl_2$. The inoculated plates were incubated at 28˚C for at least 11 days and radial growth was recorded. We selected three isolates for further study based on their ability to grow in the presence of higher concentrations of $HgCl_2$ (100 and 200 $mg{\cdot}L^{-1}$) and for their clear ability to catalyze the volatilization of Hg as accessed via the non-radioactive X-ray method [14].

The effect of the mercury on the radial growth of the isolates tested was estimated by measuring the radius of the colony extension (mm) against the control grown in medium without mercury. Three replicates of each concentration and controls without metal were used.

2.2. Identification of Fungal Strains

Fungal Isolated were characterized to the genus level on the basis of macroscopic

characteristics (colony morphology, color, appearance and shape) and microscopic characteristics (septation of mycelium, shape, diameter and texture of conidia) [15] [16].

Isolates were further identified by analyzing ITS 18S ribosomal deoxyribonucleic acid (rDNA) sequences. For molecular characterization, fungal strains were grown for 4 days at 28˚C in PDB medium supplemented with 50 $mg{\cdot}L^{-1}$ of $HgCl_2$, harvested and processed for DNA extraction using standard procedures [17]. Amplification of the fungal ribosomal 18S rDNA gene and the internal transcribed spacer region (ITS) was performed using the conserved primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [18]. The reactions were performed in 30 µl volume with PCR Master Mix (Fermentas, Lithuania). The amplification conditions using a C1000 Thermal Cycler (Bio-Rad Laboratories, Inc.; Berkeley, CA, USA) were as follows: 95˚C for 5 min, 30 cycles of 94˚C for 30 s, 60˚C for 45 s, and 72˚C for 1.5 min, with a final 8 min elongation step at 72˚C. The amplification products were purified using the DNA Clean & Concentrator-5 kit (Zymo Research; Irvine, CA, USA) and cloned into the pJET1.2/blunt cloning vector (Thermo Scientific; Waltham, MA, USA) according to the specifications of the manufacturer. Sequencing reactions were performed by the LANBAMA-IPICYT laboratory (San Luis Potosi, México). The 18S rDNA ITS sequences obtained were compared against nucleotide sequences from GenBank [19].

2.3. Measure of Mercury Emission by Fungal Isolates under Laboratory Conditions

For the determination of mercury emission from the fungal isolates under controlled environmental conditions, an active sampling device was used. This device was constructed utilizing an air pump with a 1.5 $L{\cdot}min^{-1}$ air flow, an Erlenmeyer flask of 1000 mL with a rubber cap and flow valves (connected to the pump) and an output connected to a 250 mL Erlenmeyer flask containing 20 mL of an acidic solution of 10% $KMnO_4$ as an absorbent for the volatile Hg° released through active sampling [20] [21] [22]. This solution captures Hg° according to the equation:

$$2KMnO_4 + 3Hg^{\circ} + 4H_2SO_4 \rightarrow 2MnO_2 + 3HgSO_4 + K_2SO_4 + 4H_2O$$

For these experiments, 10^4 conidia of fungal species, determined microscopically, were germinated and grown in 200 ml of PDB medium at 28˚C during seven days using the active sampling device. Thereafter, a concentration of 100 $mg{\cdot}L^{-1}$ of $HgCl_2$ was added to culture medium. The sampling was initiated by opening the chamber connection valves and pumping air for 5 minutes at a flow rate of 1.5 $L{\cdot}min^{-1}$ through the air chamber after 1, 3 and 6 h of growth in the presence of $HgCl_2$. The pumped air flowed through a hose into the potassium permanganate collector container. Once the sampling was finished, three portions of 5 ml of potassium permanganate were taken from the collector container for determination of mercury concentration. After this incubation time (6

h), the mycelial masses were harvested in order to determine their dry weights. Metal uptake was estimated as the amount of metal (μg) per unit of mycelium dry weight (g). The mercury concentrations of the air samples and mycelium obtained after 6 h of treatment were analyzed using the cold vapor atomic absorption (CVAAS) technique, using a GBC HG 3000 device.

2.4. Determination of the Hg° Volatilized to the Air by the Fungi

Determination of mercury concentration was performed using the formula described by [23].

$$k = \frac{m}{c * 10^{-3} * t}$$

where: c = the Hg concentration in the air ($\mu g/m^3$), m = Hg mass collected in the active sampler (μg), k= sampling rate ($L \cdot min^{-1}$), equal to the airflow rate through the apparatus, and t = sampling time (min).

2.5. *Arabidopsis thaliana* mercury Uptake Assay

To determine if isolated fungi affect mercury uptake in *Arabidopsis thaliana* plants under laboratory conditions, *A. thaliana* wild type accession Col-0 seeds were surface sterilized and sown on Murashige and Skoog (MS) [24] agar-solidified medium supplemented with 0.3% sucrose, pH 5.7 and placed in a growth chamber (26˚C, 16 h: 8 h, light: dark) for 7 d. For mercury uptake bioassays, *Arabidopsis* seedlings grown on plates for 7 d were inoculated with 10^6 conidia of fungal species and transferred to plastic pots (top diameter, 66 mm, bottom diameter 53 mm; height 54 mm), filled with 50 g of air-dried Sunshine Professional Growing Mix (Sun Gro, Horticulture; Agawam, MA, USA). *Arabidopsis* plants were incubated in a growth chamber as above under a 12-h light/dark photoperiod. The pots were irrigated with half-strength Hoagland solution [25] during 14 d for plant-fungal interaction, after that, 10 $mg \cdot L^{-1}$ of $HgCl_2$ was added to the Hoagland solution and irrigated during 15 days and mercury concentration in plant tissues was determined. Twenty five plants were used per treatment.

2.6. Statistical Analysis

Data from soil content and volatilization experiments were analyzed by completely random two-factorial design. Analysis of variance (ANOVA) was done, followed by a Tukey's multiple comparison test ($\alpha = 0.05$). Tukey's tests are used in conjunction with ANOVA to compare the means. The statistical analysis was done using the Statistical Analysis System (SAS Institute, Carry, NC, USA).

3. Results

3.1. Identification and Properties of Isolates

The soil samples used to obtain fungal strains in this study were slightly acidic (pH 6.5), presented 2.3% of organic matter and showed high levels of mercury,

1532 ± 300 mg/kg. Twenty fungal isolates were selected based on their ability to grow consistently in PDA medium containing 50 $mg \cdot L^{-1}$ of $HgCl_2$ without loss of viability after several passages in fresh medium. From twenty fungal strains that were isolated, three were selected for further study based on their ability to grow in the presence of higher concentrations of $HgCl_2$ (100 - 200 $mg \cdot L^{-1}$) and were designated as strains 11, 20-D and 20-F. Maximal level of mercury resistance (200 $mg \cdot L^{-1}$) was observed for the strain 20-F, whereas strain 11 and 20-D showed lower resistance (100 $mg \cdot L^{-1}$); the remaining 17 fungal strains obtained in this study tolerated mercury concentrations between 25 - 50 $mg \cdot L^{-1}$. Radial growth in solid medium in the presence of two concentrations of $HgCl_2$ (100 and 200 $mg \cdot L^{-1}$) was measured daily for 11 days (**Figure 1**) for strains 11, 20-D, and 20-F. Mercury concentration at 100 $mg \cdot L^{-1}$ did not substantially affect the radial growth rate of any of the fungal strains assayed, for instance, the average radial growth rate was reduced only 5% and 12% of the growth rate of the non-mercury control. In contrast, strain 20-F was able to grow slowly on $HgCl_2$ at 200 $mg \cdot L^{-1}$, reaching up to 50% of non-mercury control growth while growth of strains 20-D and 11 were severely affected at 200 $mg \cdot L^{-1}$ (**Figure 1**). From these data, we can conclude that strain 20-F show a higher level of tolerance to $HgCl_2$ than strains 20-D and 11.

Although not a quantitative method, the volatilization of Hg by mercury tolerant isolates was determined by the X-ray film method [14] after 12 h of incubation at room temperature and measurement of dark spots on the film (data not shown). Hg volatilization was observed for all selected fungal isolates. From these data we conclude that fungal strains presented volatilization responses at different mercury concentrations, and mechanisms involving detoxification or bioaccumulation could play important roles in this process.

Fungal identification and validation were obtained by DNA sequence analysis of ~600 bp fragments of ITS 18S rDNA prepared by PCR as described in Materials and Methods. Comparison of those sequences using the BLAST algorithm indicated that rDNA sequences belong to genus *Trichoderma virens* (**Figure 2**). The GenBank accession number for the nucleotide sequences are: strain 11:KU990872, strain 20-D:KU990873, strain 20-F:KU990874.

3.2. Mercury Volatilization Determination

Considering the qualitative mercury volatilization results described above and the fact that fungal strains play an important role in colonization and decontamination of metal polluted ecosystems, we decided to grow the fungal isolates in PDB medium at 28˚C during seven days in an active sampling device to investigate their capacity to respond to the presence of mercury and to evaluate the potential impact of mercury volatilization by the isolated fungal strains under controlled environmental conditions. The results in **Table 1** show *T. virens* strain 20-D is the strain with higher ability to volatilize mercury under our assays conditions. Mercury volatilization was observed for the strains during the first

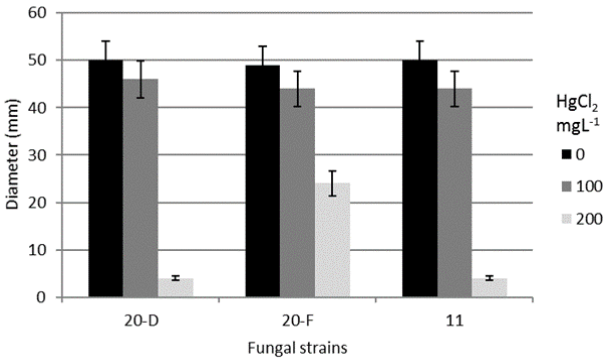

Figure 1. Growth of isolates 11, 20-D and 20-F during 11 days in PDA medium alone or in the presence of mercury.

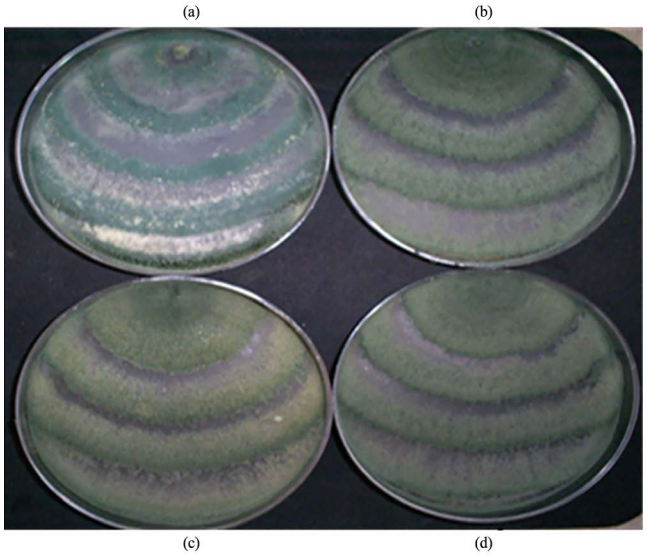

Figure 2. Fungal strains identification. Panel shows fungal strain grow in PDA medium: (a) *Trichoderma atroviride* (control); (b) Isolate 11; (c) Isolate 20-D; (d) Isolate 20-F identified as *Trichoderma virens*.

Table 1. Volatilization of Hg° by active sampling device and mycelium-mercury accumulation.

Strain	Hg total (μg/m³)*			Hg total in mycelium (μg/g)
	1 h	3 h	6 h	
Trichoderma virens 20-D	48.61 ± 9.6[a]	129.65 ± 14.7[b]	213.04 ± 32.6[b]	18.5 ± 4.8[a]
Trichoderma virens 20-F	<1.28	3.9 ± 2.2[c]	<1.28	17.5 ± 2.9[a]
Trichoderma virens 11	4.61 ± 2.4[b]	<1.28	<1.28	20.0 ± 3.4[a]
Control	<1.28	<1.28	<1.28	<1.28

*Determination of mercury emission from the fungal samples under controlled environmental conditions was performed as indicated in Materials and Methods. The fungal strains were grown in the presence or absence (control) of $HgCl_2$ using the concentration indicated in Materials and Methods. Numbers in columns followed by the same superscript letter were not significantly different between treatments according to the statistical test ($\alpha < 0.05$).

hour sampled with 48.61 ± 9.6 μg/m^3 for *T. virens* 20-D. *T. virens* 11 showed a much lower level of mercury volatilization, 4.61 ± 2.4 μg/m^3. *T. virens* 20-F was the less effective isolate for mercury volatilization during the first hour. We observed an increasing efficiency of mercury volatilization for the isolate 20-D at the three and six hour sampling times, for example, at six hours we observed 213.04 ± 32.6 μg/m^3 for 20-D. We can conclude from these data that strain 20-D display the higher level of response to $HgCl_2$ than isolates 20-F and 11.

On the other hand, total mycelial mercury content at the end of the volatilization experiment did not reflect enhanced accumulation in fungal strains with lesser abilities to volatilize mercury. For instance, there was no statistically significant difference in the mercury content of mycelium from the strain 20-D (18.5 ± 4.8 μg/g) as compared with *T. virens* 20-F (17.5 ± 2.9 μg/g) although these strains differed considerably in their volatilization abilities (Table 1). We can conclude from these results that mercury detoxification through volatilization and bioaccumulation play significant roles in the mercury resistance strategies utilized by these microorganisms. More study is required to understand the biochemical and genetic processes involved in these detoxification strategies.

3.3. Accumulation of Mercury by *Arabidopsis thaliana* Plants

Based on the mercury volatilization of fungal strains examined, it was important to determine whether the addition of these strains affect the uptake of mercury by *A. thaliana* plants. Table 2 shows the result of three independent experiments in which the total mercury concentration in root and rosette leaf tissue was measured. Interestingly, *Trichoderma* strains produced significant reduction ($\alpha < 0.05$) in the total mercury contents of the *Arabidopsis* tissues. For example, the presence of the fungal strain *T. virens* 20-D and *T. virens* 11 reduce mercury concentrations to 14.27 ± 5.2 and 16.18 ± 4.5 μg/g in root tissue, while *T. virens* 20-F reduce mercury concentration to 25.69 ± 6.9 μg/g in root tissue, compared with a level of 48.88 ± 7.9 μg/g in the uninoculated control. By contrast, there were no significant differences in mercury concentration in rosette leaf tissue ($\alpha < 0.05$) when *T. virens* 20-D and *T. virens* 11 strains were used. Only, mercury concentration reduction was observed for *T. virens* 20-F to 22.99 ± 6.9

Table 2. Mercury concentration in rosette leaf and root tissue of *Arabidopsis* plants inoculated with *Trichoderma* strains.

Strain	Hg total (μg/g)	
	rosette leaf tissue	root tissue
Uninoculated control	50.97 ± 10.9^a	48.88 ± 7.9^a
Trichoderma virens 20-D	41.42 ± 5.3^a	14.27 ± 5.2^b
Trichoderma virens 20-F	22.99 ± 6.9^b	25.69 ± 6.9^b
Trichoderma virens 11	55.70 ± 11.4^a	16.18 ± 4.5^b

Numbers in columns followed by the same superscript letter were not significantly different between treatments according to the statistical test ($\alpha < 0.05$).

μg/g of rosette leaf tissue compared with a level of 50.97 ± 10.9 μg/g in the uninoculated control. These results represent up to 75% reduction in the mercury accumulation in root and 50% of reduction in rosette leaf tissue compared with uninoculated controls. Altogether these results shown that the inoculation *Arabidopsis* plants with the *Trichoderma* isolates assayed, reduced significantly the mercury accumulation in the plant tissues.

4. Discussion

Mercury toxicity represents a concern around the world, and several studies of mercury pollution in soil have been reported [26]-[33]. Our results show that the soil examined in the present study presented elevated mercury concentrations significantly exceeding the Mexican Standards for agriculture and residential areas (23 mg/kg, NOM-147-SEMARNAT-SSA1-2004). Although our measurements indicate a high concentration of mercury in this soil, the high concentration of organic matter and the soil pH would explain the fact that diverse plants and microorganisms can still grow in this soil. Several reports confirm that mercury is predominantly found in its divalent Hg^{2+} form associated with organic matter and soluble minerals in soil, and could be less mobile and thus less likely to be accumulated by plants and microorganisms [34] [35].

Several *Trichoderma* species have been used as biological control agents of phytopathogenic fungi and oomycetes of agronomical importance, due to their antagonistic abilities to produce antibiotics and siderophores, and because of their mycophagous properties [36] [37] [38]. The colonization of plant roots by *Trichoderma* confers beneficial effects on plant growth and development by the production of phytohormone-like compounds and the induction of systemic resistance [10] [37]. In addition, *Trichoderma* species are highly resistant to a variety of toxins and xenobiotic compounds, including antibiotics, fungicides and, of primary relevance to the present study, heavy metals [39] [40] [41].

In the present work, we selected three *Trichoderma* isolates from mercury contaminated soils, based on their ability to tolerate and volatilize mercury. We found that the strain *T. virens* 20-D showed better efficiency of mercury volatilization during the time evaluated (**Table 1**). Differences in mercury tolerance and volatilization for fungal strains (strain 20D, 20F and 11) isolated from contaminated soil may reflect different adaptation strategies or mechanisms involving permeability barriers, intra- and extracellular sequestration, efflux pumps, enzymatic detoxification, and metal speciation. Also, the results show that all selected *Trichoderma* strains were highly effective to reduce the concentration of mercury in root tissue. With regard to this, differences in mercury accumulation by roots and shoots have been previously reported in tobacco plants [42], *Triticum durum* [43] *Oryza sativa* [44], *Poa annua* [45], and other terrestrial plant species [46]. Previous studies have shown that certain species in the genus *Trichoderma*, isolated from contaminated soils, possess the ability to tolerate diverse metal concentrations through the various mechanisms mentioned above

and we cannot exclude any of these at this point [6]. Further study will be required to determine the mechanisms by which the species identified in our study tolerate mercury.

While little information is available about the mechanisms of mercury tolerance and volatilization in *Trichoderma*, studies of metal tolerance in other fungi, suggest that ABC transporters are critical for resistance to environmental pollutants [47] and that the activity of O-acetylserine (thiol) lyase, which is involved in cysteine biosynthesis, may be involved in the resistance mechanism [48]. Previous works have examined the accumulation of mercury in fungal species [49], but despite those analyses the mechanisms of mercury resistance and volatilization in *Trichoderma* remain elusive. It is clear then that plant-microorganism interactions are affected by mercury mobilization in contaminated soils. More specifically, the expression of genetic systems that lead to the reduction of Hg (II) by *Trichoderma* species make this organism a prominent player in the mobilization of mercury in those contaminated soils.

In bacteria, a group of genes organized in the *mer* operon are involved in transport and reduction of mercury and the demethylation of organomercurials. Resistance to mercury in these organisms is classified as narrow or broad spectrum. For instance, the narrow spectrum system confers resistance only to inorganic compounds, whereas the broad spectrum mechanism confers resistance to inorganic and organic compounds [50] [51] [52]. Recently, two genes, *hgcA* and *hgcB*, were identified which encode a corrinoid protein with a strictly conserved cysteine proposed to be the ligand for cobalt in the corrinoid cofactor, and a ferredoxin-like protein thought to be an electron donor to HgcA [53]. The lack of either gene abolishes mercury methylation by *Desulfovibrio desulfuricans* [54]. In addition to mercury volatilization in bacterial systems, inorganic mercury transformation has been reported in *Saccharomyces cerevisiae* and *Candida albicans* yeasts [55] and via non-enzymatic transformation under abiotic conditions with methylcobalamin as the methyl donor [56]. Examining available *Trichoderma* genomes revealed genes encoding a putative protein homologous to MerP (involved in mercury transport), as well as three paralogs of MerA (bacterial mercuric reductase), and a gene with similarity to *hgcB*; its partner *hgcA* was not found. We hypothesize that the functions of *hgcA* and some genes of the bacterial *mer* operon could be replaced by other *Trichoderma* gene products to complement the functions of the existing homologs and confer high tolerance to mercury. The presence of these genes and the accumulation of mercury in the mycelium in these fungi could explain in part the mercury resistance phenotypes.

5. Conclusion

In conclusion, in the present study the selected representative strains presented a wide range of resistance to $HgCl_2$, from high (200 $mg{\cdot}L^{-1}$), to moderate (100 $mg{\cdot}L^{-1}$), and low tolerance (25 - 50 $mg{\cdot}L^{-1}$). The broad mercury resistance range

could be explained by genetic and physiological differences among the isolated fungi. For instance, we observed that the various strains tested were able to accumulate and volatilize Hg° at different levels. Further work is needed to determine the genetic and molecular mechanisms of mercury resistance and volatilization in these *Trichoderma* isolates and this work may demonstrate their utility in bioremediation systems in the soil of Nuevo San Joaquin, Querétaro State, México.

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