

Environment

Potential absorption of mercury-contaminated substrate by *Trichoderma* sp isolated from Brazil Nuts and Amazon Soil

Absorção de mercúrio por *Trichoderma* sp isolado de castanha do Brasil e de solo amazônico

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ABSTRACT

Mercury is an inorganic contaminant with serious harmful consequences to the environment. There has been a continuous rise in its level due to industrialization and other anthropogenic activities, such as the burning of coal and petroleum products, use of mercurial fungicides in agriculture and mercury catalyst in industries, and production of waste by paper industries. Five strains of *Trichoderma* sp., a filamentous fungi, were used in this study to evaluate their resistance to high concentrations of mercury for the purpose of using them for bioremediation. The solid culture medium used was prepared with malt agar 2% with pH 7.0 in which the strains of *Trichoderma* sp. were inoculated. The minimum inhibitory concentration (MIC) of the selected *Trichoderma* sp. isolates was calculated considering the time for growth and concentration of the mercury salt ($\text{Hg}(\text{NO}_3)_2$). At a mercury concentration of 50 mg/mL, maximum growth was first observed in TCH 1 (89.42 ± 0.63 mm) followed by TCH 2 isolate (87.33 ± 0.58 mm). At this concentration, all isolates reached the maximum mycelia growth. When the concentration of 200 mg/L $\text{Hg}(\text{NO}_3)_2$ was used, complete growth inhibition of the isolates was observed. Scanning electron microscopy suggested that differences in sporulation between the control and mercury treatment groups. In conclusion, it can be stated that *Trichoderma* isolates have great potential for bioremediation of sources contaminated with mercury.

Keywords: Bioremediation; Inorganic contaminant, *Trichoderma*; Mercury; Brazil nuts

RESUMO

O mercúrio é um metal pesado com sérias consequências nocivas ao meio ambiente. Cinco linhagens de *Trichoderma* sp. foram isoladas de castanha do Brasil através de cultivo em meio específico. O meio para a cultura de fungos foi preparado com meio malte-ágar 2% com pH 7,0, no qual as cepas de *Trichoderma* sp. foram inoculadas. A concentração inibitória mínima (CIM) dos isolados selecionados de *Trichoderma* foi calculada. O crescimento máximo ocorreu em TCH 1 com mercúrio 50 mg/mL ($89,42 \pm 0,63$ mm) seguido pelo isolado de TCH 2 ($87,33 \pm 0,58$ mm). Nesta concentração, todos os isolados atingiram o máximo de crescimento micelial. Quando a concentração de 200 mg/L de $\text{Hg}(\text{NO}_3)_2$ foi usada, ocorreu inibição completa do crescimento. A microscopia eletrônica de varredura confirmou as diferenças na esporulação entre o controle e o tratamento com mercúrio. O objetivo deste trabalho foi avaliar a resistência do *Trichoderma* a altas concentrações de mercúrio com o objetivo de usá-lo para biorremediação. Concluindo, pode-se afirmar que os isolados de *Trichoderma* têm grande potencial no sentido de serem utilizados na biorremediação de fontes contaminadas pelo mercúrio.

Palavras-chave: Biorremediação; Contaminantes inorgânicos; *Trichoderma*; Mercúrio; Castanha-do-Brasil

1 INTRODUCTION

Mercury is an inorganic contaminant with serious harmful consequences to the environment, especially in the Amazon, where it has been used in gold prospecting (Lima, 1993, Souza et al., 2008). Mercury (Hg) is an extremely toxic metal element and mostly notorious for its long-range mobility in global atmosphere, long term persistence in ecosystem, neurotoxicity and bioaccumulation along food chains (ZHAO et al., 2017; HUANG et al., 2020).

Its level is rising due to industrialization and other anthropogenic activities, such as burning of coal and petroleum products, use of mercurial fungicides in agriculture and mercury catalysts in industries, and production of waste by paper industries and gold prospecting (LIMA, 1993; ZHAO et al., 2017; HUANG et al., 2020).

There is a growing concern about the rationalization of water use, prompting the emergence of ever more stringent laws regarding the disposal of industrial effluents. This has encouraged the improvement of the treatment systems of these effluents (FEISTER, 2013). One of the best alternatives for treating aqueous effluents aiming to reduce the toxicological characteristics of pollutants is through biological degradation, that is, bioremediation. In this sense, the discovery of microorganisms capable of degrading toxic compounds has been sought (BIROLI et al., 2015).

In the Amazon, mining activity is the main route of contaminating the waters and environments. The main inorganic contaminants released during mining is mercury, that is used to capture and retain gold, forming an amalgam, which subsequently needs a mercury

release process through boiling. It is known that for each 1 kg of gold produced, 1.5 kg of mercury (Hg) is used, of which 30% is released to the environment with 20% returning to the rivers through rainfall and 10% released directly into the bodies of water (BONUMÁ, 2006).

One of the best techniques to evaluate the bioremediation capacity of a microorganism is to determine the amount of substance that causes the inhibition of growth of the microorganism tested, gauging its capacity to resist and absorb the element that is adsorbed in the medium in which it is being placed. This value is known as the Minimum Inhibitory Concentration (MIC) (OSTROSKY, 2008).

Microorganisms naturally resistant to metal ions have the potential to be used for bioremediation. Therefore, such microorganisms must be identified and selected. Compared to other strategies and conventional physico-chemical processes that are used to minimize mercury hazards in the environment, bioremediation is an ecologically adequate, efficient, inexpensive, and highly accepted public alternative (ZINKEVICH et al., 1994, SAYER; GADD, 2001, VIDALLI, 2001, SPROCATI et al., 2006, MELO; AZEVEDO, 2008). Bourdineaud et al. (2020) isolated bacteria of the genus *Pseudomonas* that live on rocks with 2952 mg/kg of mercury. The feasibility of using fungi for bioremediation has also been demonstrated (OLIVEIRA et al., 2018, SU et al., 2020).

The species of the genus *Trichoderma* are fungi composed of imperfect filaments with teleomorphs. They belong to the order Hypocreales and division Ascomycetes (JAKLITSCH et al., 2014). They are commonly isolated from the soil and are known for their potential in the biocontrol of an extensive range of plant pathogenic fungi. When in an endophytic relationship with plants, they induce the defense of plants and enable their growth (YEDIDIA et al., 1999, HARMAN et al., 2004, NONGMAITHEM et al., 2016). It is a species that plays an important ecological role, acting as decomposers of plant residues, as well as the degradation of industrial chemical compounds and the bioaccumulation of both soil and water metals (EZZI; LYNCH, 2005, ANAND et al., 2006). Evidence suggests that when they are in polluted habitats, they have considerable tolerance for metals accumulating in them in high amounts (MOHAMMADIAN et al. 2016). Therefore, they may become dominant organisms in some polluted environments and may play an important role in metal removal technology (TING; CHOONG, 2009). However, the behavior of this species after exposure to metal-containing compounds may differ, depending on the

type of metal and the fungus strain. Although the tolerance to cadmium and nickel has been reported, limited information in this regard is available (NONGMAITHEM et al. 2016).

Here, we conducted bioremediation study on species of strains of the genus *Trichoderma* to evaluate their resistance in high concentrations of mercury. In order to achieve this, we first identified and selected the strains that showed potential for bioremediation.

2 MATERIALS & METHODS

2.1 Methodology of isolation from *Trichoderma* sp.

The five isolates used in the study were obtained from the Brazil nuts (*Bertholletia excelsa*) (*Trichoderma* sp. (TCH) 2, 3 and 4) and Amazon soil (*Trichoderma* sp. (TCH) 1 and 5). To obtain the isolates, malt extract (2%) and antibiotic chloramphenicol were used. Isolates from Brazil nut urchins were obtained by surface scraping the structures of microorganisms. After the isolation procedures, the Petri dishes containing the isolates were transferred to an incubator type; Biochemical Oxygen Demand (B.O.D).

The experiment was adjusted to a photoperiod of 12 hours and temperature of 26 ± 1 °C. After seven days of incubation under the conditions described above, the colonies and morphological structures (conidiophores and conidia) of the isolates were evaluated for identifying the *Trichoderma* isolates at the genus level, based on the morphological keys of the sections and species developed by Gams and Bisset (1998).

The samples were collected from the Brazil nut trees in the city of the Laranjal do Jari (TCH 2, 3 and 4), and the solo in the city of the Macapá (TCH 1 and 5).

2.2 Preparation of the culture medium

The culture medium of the fungi was prepared using malt-agar medium 2% at a pH 7.0. In the Petri plate (90 mm in size), 25 ml of the culture medium was poured where the *Trichoderma* sp. strains were inoculated (experiment made in triplicates).

There are some reasons to use a nitrate salt as the source of Hg in inhibition studies. Inorganic components are hardly found in metallic form, and this is one of the reasons the

heavy metal is totally unsuitable. For this experiment, nitrate was chosen because it is more frequent in nature and more reactive in aquatic environments. Mercury nitrate ($\text{Hg}(\text{NO}_3)_2$) (Sigma Aldrich, analytical grade) was chosen instead of HgSO_4 because of low solubility of HgSO_4 . Sulphate salts are inert to fungi. Therefore, nitrate salts are often used in biosorption studies (ZOTTI et al., 2014; NONGMAITHEM et al., 2016; LI et al., 2017).

2.3 Toxicological prediction

In order to identify some undesirable properties of compounds with mercury, the server was used to predict toxicity (<http://tox.charite.de/tox/>). To achieve this goal, identifying the properties was based on the similarity of functional groups with the molecules in question both in vitro and in vivo and also in toxicological properties such as Toxicological Class, generation of toxic fragments, and LD_{50} values.

2.4 Cultivation of *Trichoderma* sp. in solid medium and Minimum Inhibitory Concentration (MIC)

To analyze the radial growth of the selected strains of *Trichoderma* that were inoculated in the malt agar medium (2%), incubated for 72 hours at room temperature (27 ± 1 °C) in Petri plates (90 mm) and supplemented with different concentrations of the $\text{Hg}(\text{NO}_3)_2$ (0, 100, 150, 175 and 200 ppm).

Thereafter, the halo ring size after growth was measured using a digital caliper. Inhibition of growth was calculated using the following formula: $\text{MIC} = (X - Y)/X \times 100$, where MIC is the Minimum Inhibitory Concentration, X is the median of radial growth of the control group free of inorganic contaminant ($\text{Hg}(\text{NO}_3)_2$; 0.0 ppm) and Y is the median of radial growth obtained by culturing the fungus in medium with the inorganic contaminant.

The Minimum Inhibitory Concentration (MIC) of the metal causing 50% inhibition of growth (MIC_{50}) of the selected microorganism *Trichoderma* sp. was calculated from inhibition of growth peculiar to mercury ($\text{Hg}(\text{NO}_3)_2$).

Growth was measured using the halo method with Digimes (Brazil) digital caliper. The first 24 hours were left free of measurement to enable the growth of the fungi. Then the fungi

growth measures were checked every 12 hours until the total growth in the 90 mm plate was completed, in 72 hours in the control experiment.

The control group was characterized as a culture medium free of inorganic contaminant. Meanwhile the mercury was inserted into the culture medium at the concentrations of 0, 100, 150, 175 and 200 mg/L of mercury nitrate ($\text{Hg}(\text{NO}_3)_2$).

2.5 *Trichoderma* sp. biomass and quantification of mercury

To obtain biomass, the selected species of *Trichoderma* was inoculated in malt medium (2%) incubated at room temperature (27 ± 1 °C) using shaker 75 rpm. Small portion (0.5 mm) of the fungus was transferred to Erlenmeyer (250 ml) containing 100 ml of the malt medium (2%) supplemented with different concentrations of the target metal (0, 100, 150, 175 and 200 mg/L) and incubated in triplicates at a temperature of 27 ± 1 °C for 120 hours. After this period, the culture medium with biomass was filtered with Whatman N^o.1 filter paper which was being washed with deionized water in order to remove the culture medium. The collected mycelium was dehydrated in an oven at 60 °C for 48 hours and the dry weight was measured using a digital scale with precision of 0.1 mg.

After filtering, the quantification of mercury in the supernatant was performed using an atomic absorption spectrophotometer (AAS Shimadzu model 6300).

After a period of 120 hours, the biomass of each isolate grown in the modified medium with different concentrations of $\text{Hg}(\text{NO}_3)_2$ (0, 100, 150, 175 and 200 mg/L) was measured and the concentration and growth were calculated.

2.6 Evaluation by Scanning Electron Microscopy (SEM)

The mycelium of the *Trichoderma* sp. strains was analyzed with Scanning Electron Microscopy (SEM). We attempted to analyze the possible differences between the control group and those that were inoculated in the medium with different concentrations of mercury nitrate $\text{Hg}(\text{NO}_3)_2$.

2.7 Statistical analysis

The experiments were conducted using a completely randomized factorial design with three replicates, considering the isolates as factor A and metal concentrations as factor B. For all parameters using Program R (2015), one-way analysis of variance was used. The comparison of the mean values of the two samples was done by the paired test with a significance level of $p < 0.05$. The identical letters in the results indicate non-significant differences between treatments within each isolate.

3 RESULTS

3.1 Effect of mercury on growth

The effects of different concentrations of mercury on the mycelia growth of *Trichoderma* sp. isolated from “nuts of the Brazil” were tested and summarized in Table 1. TCH 2 strain reached the maximum radial growth. Notwithstanding the exception of the TCH 1 strain, the growths of all were similar with a non-significant difference (Fig 1). Maximal growth of the strain in 50 mg/L mercury occurred in TCH 1(89.42±0.63 mm) followed by the isolate TCH 2(87.33±0.58 mm). In this concentration, all isolates reached the maximum of mycelia growth (around 90 mg/L) in comparison with other concentrations of mercury. When mercury concentration of 200 mg/L was used, complete inhibition of mycelia growth occurred. ANOVA showed that there were significant differences in inhibition between *Trichoderma* sp. and inhibition by mercury concentrations. This fact occurred when using any of the isolates that did not show significant differences between them. There was a difference between the control group and the group with mercury medium of 175 mg/L dosage (Table 1).

Table 1 – Growth rate of *Trichoderma* sp. isolated in mercury in medium malt with culture time of 72 hours

Train	0 mg/L		50 mg/L		100 mg/L		150 mg/L		175 mg/L		ANOVA
	Diam(mm)	Diam.(mm)	%ic	Diam.(mm)	%ci	Diam.(mm)	%ci	Diam.(mm)	%ci	F	
TCH-1	90	89.42±0.63	1	76.17±1.26	17	73.67±0.58	8	58.16±1.40	37	587.4**	
TCH-2	90	87.33±0.58	3	81.67±4.93	9	82.13±1.03	9	81.5±4.80	9	4.712*	
TCH-3	90	85.67±1.15	5	82.60±0.53	8	81±0.29	0	80.53±0.50	10	78.7**	
TCH-4	90	86.67±0.28	4	86.67±1.53	4	86.40±0.53	4	76±1.73	16	73.17**	
TCH-5	90	82.42±0.80	8	79.17±2.52	2	80±1.00	1	75.33±0.57	11	62.52**	

*significant at 5%. **significant at 1%.

3.2 Toxicological prediction

The LC₅₀ (lethal concentration) of Mercury Nitrate for mammals is 25 mg/kg and of toxicological class 2 (Table 2). It is not carcinogenic but toxic if swallowed and in contact with skin. The Polar Molecular Surface (MPSA) is a very useful parameter when it comes to predicting drug transport properties. Defined as the sum of the surface of polar atoms (usually oxygen, nitrogen, and hydrogen) bound to a molecule, this parameter is related to intestinal absorption capacity and also to brain penetration barrier. The toxicity prediction using the server <http://tox.charite.de/tox/> shows that the ability to support the presence of mercury by humans is restricted to 25 mg/L (Table 2). *Trichoderma* sp. was resistant up to 200 mg/L of mercury, a dosage 8 times greater than that which a human can tolerate.

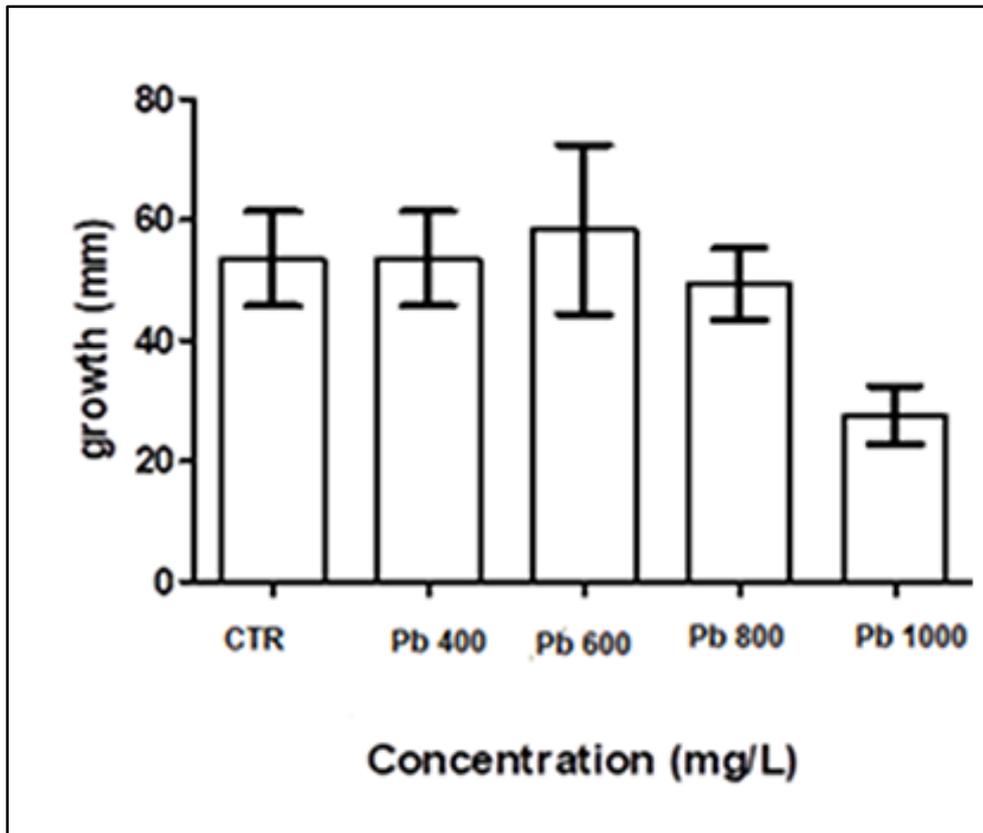
Table 2 – Toxicological Prediction of Mercury Nitrate Hg (NO₃)₂

Toxicological Class	2
LD ₅₀	25 mg/kg
Molecular Weight	324.6
Number of Hydrogen Acceptors	6
Number of atoms	9
Number of connections	6
Polar Molecular Surface (MPSA)	137,76

3.4 Minimal Inhibitory Concentration (MIC)

As noted in the Table 1, the inhibition of growth was very low in all treatments up to 175 mg/L dose. The strain TCH 1 was the most sensitive to 175 mg/L at approximately 37% (58.16 ± 1.40 mm in relation to 90 mm of the control). The results of *Trichoderma* growth at 200 mg/L are not stated in this table because at this concentration, the total growth inhibition occurred. Although TCH 4 presented spores in the sporulation stage, it showed inhibition around 18% of its growth at 72 hours (growth of 80.53 mm, while the control was 90 mm in diameter). No differences were observed between concentrations of 100 to 175 mg/L of the mercury (Table 1 and Fig 2). The total inhibition of growth occurred at 200 mg/L.

Fig 1 – Growth of *Trichoderma* in culture medium of agar-malt (2%) with mercury (Hg) 175 mg/L where CTR is the negative control (without mercury) and Tch are the different isolates of *Trichoderma*



The effects of mercury on the radial growth of selected strains of *Trichoderma* sp. from “nuts of the Brazil,” were observed as shown in Fig 2. The growth of *Trichoderma* sp. was significantly influenced by inorganic contaminants; notably strong inhibition of growth occurred in strain TCH 1 (mean of the control was 53 mm while growth mean in the presence of the mercury was 28.93 mm for TCH 1). The Minimum Inhibitory Concentration also occurred in other strains but less accentuated at this mercury (Hg) concentration and more or less equivalent, the growth mean of the control was 53 mm while growth media in the presence of the mercury was around 40 mm for TCH 2, 3, 4, and 5. The parametric test showed that there were significant growth differences when using mercury nitrate in relation to the control (mercury nitrate free). The difference between the tests of 100, 150, and 175 mg/L was significant in relation to the control, but not significant among them. Fig 2 shows how mercury affected sporulation.

Fig 2 – Photo showing the cultivation of *Trichoderma* sp. in malt-agar medium; A) TCH 2 photo cultured in medium with Hg of 175 mm where it is noticed that there was a delay in the sporulation process evidenced by whitish color. B) TCH 2 photo show fungi cultured in medium with 0 (Hg(NO₃)₂) where the complete maturation process with sporulation evidenced by the dark green color is visualized

A)



B)



3.5 Effects of alkaline pH of the medium on the growth of *Trichoderma sp*

The results of the effects on the alkaline pH are shown in Table 3 where the results of inhibition at pH 9 were visualized. The *Trichoderma* isolates which were more resistant to this pH and showed constant growth with inhibition around 21% were TCH 3 and 4 which. In the control group, growth inhibition due to the pH of the medium was also perceptible.

Table 3 – Growth of *Trichoderma sp.* in malt- agar medium 2% with pH 9.0 and 175 mg/L Hg(NO₃)₂ concentration

Trat	TCH 1	TCH 2	TCH 3	TCH4	TCH 6	
HHS	Ctr	Ctr	Ctr	Ctr	Ctr	
1	224	-----	-----	-----	-----	
2	336	-----	-----	-----	-----	
3	448	-----	12.83 ± 1.26	14.33 ± 0.29	-----	
4	660	26.83 ± 1.89	41.67 ± 2.08	43.83 ± 3.06	38.17 ± 3.21	42.33 ± 2.75
5	772	47.67 ± 2.08	62.17 ± 1.04	65.50 ± 0.50	56.67 ± 0.76	46.17 ± 0.29
		Hg	Hg	Hg	Hg	Hg
1	224	0	0	0	0	0
2	336	0	0	0	0	0
3	448	0	0	4.50 ± 2.18	0	0
4	660	0	0	12.17 ± 2.08	12.00 ± 2.50	-----
5	772	0	0	36.00 ± 4.77	23.00 ± 0.50	21.33 ± 1.26

The presence of mercury (Hg) bound to the pH factor caused strong inhibition. Only THC 3, 4 and 5 after 48 and 60 hours were able to show growth in medium with mercury at pH of 9, yet with perceptible inhibition. The Minimum Inhibitory Concentration was 50% for THC 3, 59% for THC 4 and 51% for TCH 5.

3.6 Effects of acid pH of the medium on the growth of *Trichoderma sp.*

The effects of low pH on the growth of *Trichoderma sp.* are shown in Table 4. Thus, we observed 16% TCH 1 inhibition, 4% TCH 2 and TCH 3 inhibition, 5% TCH 4 inhibition, and 8% TCH 5 inhibition. The Minimum Inhibitory Concentration was 50% for THC 3, 59% for THC 4 and 51% for THC 5 (Table 4).

Table 4 – Growth of *Trichoderma* sp. in malt-agar medium 2% with pH 4.0 and 175 mg/L Hg(NO₃)₂ concentration

Tch 1 hs	Ctr	Hg	ic	Test t P value
24				
36				
48	52.83±2.57	43.50±1.50	17	
60	65.33±1.53	44.67±0.29	31	0.01
72	90±0	74.50±0.50	16	
Tch 2	Hg	Ci		Test t P value
24				
36				
48	50.83±1.26	36.00 ±1.26		0.04
60	55.67±1.26	54.50±1.15		
72	90±0	86.00±1.00		
Thc 3 Hs	Ctr	Hg	ci	Test t P value
24	11.67±0.76	7.67±0.76	34	
36	28.67±0.58	18±1.00	37	
48	53.00±3.12	40,83±1.53	23	0.02
60	60.50±1.32	57.67±3.25	5	
72	90±0	86.57±0.58	4	
Thc 4 Hs	Ctr	Hg	ci	Test t P value
24	11.50±0.87	7.87±0.29	33	p-value = 0.00002542
36	25.00±2.0	16.33±0.58	35	
48	46.17±1.04	32.33±0.58	30	
60	55.33±1.61	43.50±0	21	
72	90±0	85.50±1.00	5	
Thc 5 hs	Ctr	Hg	ci	Test t P value
24				
36				p-value = 0.0002385
48				
60	37.67±2.89	37.17±1.26	1	
72	84.00±0.87	77.00±0.87	8	

3.7 Culture of *Trichoderma* sp. in liquid medium

Table 5 shows the behavior of *Trichoderma* when cultured in liquid medium in the presence of 175 mg/L Hg. Interestingly, the pH of the TCH 2, 3 and 5 mercury isolates remained at 7.5 and 7.7 while the pH of the isolates TCH 1 and 4 were around 4.4 and 5.5. The control showed more uniformity. With the control although there is variation this one was not so accentuated. The high pH and only mildly low pH depict different strategies to cope with the stress caused by the high concentration of Hg.

Table 5 – Growth of the biomass of THC using malt-agar medium with 175 mg/L mercury

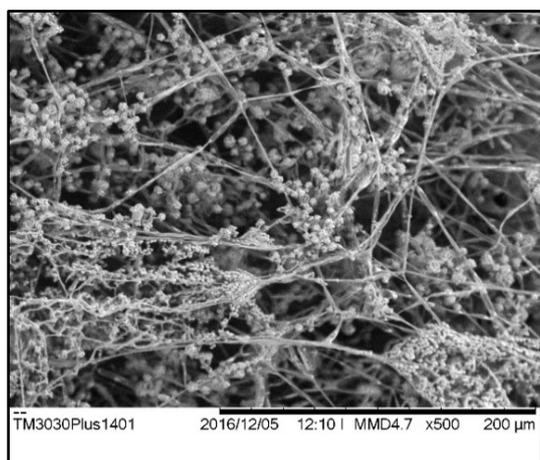
trat		Biomass	pH		biomass	pH	Ci %
1	Tch 1	196 ± 20.22	3.31	TCH 1	133.33 ± 22.50	4.04	32
	CTR			Hg 175			
2	Tch 2	163.33 ± 15.7	5.95	TCH 2	152.33 ± 7.23	7.79	7
	CTR			Hg 175			
3	Tch 3	145 ± 4.36	5.91	TCH 3	144.67 ± 3.51	7.74	0
	CTR			Hg 175			
4	Tch 4	464 ± 33.65	4.85	TCH 4	461.67 ± 8.08	5.52	0.01
	CTR			Hg 175			
5	Tch 5	459.67 ± 33.98	4.38	TCH 5	417.33 ± 2.52	7.56	0.09
	CTR			Hg 175			

3.8 Analysis of the sporulation of *Trichoderma sp.* by Scanning Electron Microscopy (SEM)

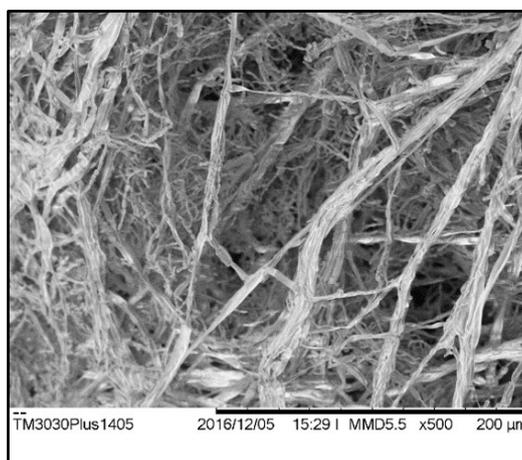
Effect of increasing the concentration of mercury in the culture medium of *Trichoderma* was perceptible. A delayed process of sporulation was observed in *Trichoderma* grown in media containing mercury compared to *Trichoderma* grown in mercury-free medium, (control) (Fig 3). This effect was also visible both in the color and distribution profile of spores. The figure 3 Fig 3 shows the results of the analysis by scanning electron microscopy.

Fig 3 – Scanning electron microscopy (SEM) image showing the sporulation difference in *Trichoderma* isolates grown in medium (A) without mercury and (B) with 175 mg/L Hg(NO₃)₂

A)



B)



4 DISCUSSION

Mercury (Hg) is one of the trace elements that is toxic to the environment and has high accumulation potential. Therefore, it has serious effects on environmental quality and public health (BUCH et al. 2017). Different species of *Trichoderma* have been tested as an alternative in bioremediation with copper (ZOTTI et al. 2017), silver (CECCHI et al. 2011), arsenic (TRIPATHI et al. 2017), and cadmium (TENG et al. 2015, NONGMAITHEM et al. 2016). Nongmaithem et al. (2016) emphasized the importance of *Trichoderma* sp. as having biosorption potential. For this purpose we used five *Trichoderma* isolates which were explored as potential bioremediation agents.

Growth of *Trichoderma* sp. was strongly influenced by mercury, causing considerable inhibition of growth in strain TCH 1 (mean of the control was 53 mm while growth media in the presence of the mercury was 28.93 mm for TCH 1).

Inhibition occurred at minimum inhibitory concentration also occurred in other strains but less pronounced at this mercury (Hg) concentration and more or less equivalent in size (mean for control was 53 mm and around 40 mm for TCH 2, 3, 4 and 5). The paired parametric t test showed no significant growth differences between the treatment group TCH 2, 3, 4 and 5, but showed a significant difference between the growth of TCH 1 and the other strains (TCH 2, 3, 4 and 5).

The effects of different concentrations of mercury on the mycelia growth of *Trichoderma* sp. isolates from Brazil nuts were tested as explicit in Table 1. The maximum radial growth was reached by *Trichoderma* strain 2. Notwithstanding the exception of the TCH 1 strain, the growth of all strains was similar with a non-significant difference seen (see Fig 1). At 50 mg/mL mercury concentration, maximum growth was observed in TCH 1 (89.42 ± 0.63 mm) followed by TCH 2 isolate (87.33 ± 0.58 mm).

At this concentration (50 mg/L), all isolates reached the maximum mycelia growth which is around 90 mm, where there was no inhibition of growth. The effects of increased mercury concentration were measured on the sporulation which was delayed as the mercury concentration increased (Fig. 1).

It should be noted that the growth of *Trichoderma* sp. was influenced by the doses of mercury from 100 mg/L upwards. But the most noticeable effect of mercury was on sporulation, having delayed sporulation when compared with the control (Fig. 1). The stages of sporulation

first occurred with the formation of spores of whitish color which then became greenish and finally dark green color characterizing the final phase of this process. Briefly, it was observed that TCH 1 was more susceptible to growth and more whitish staining, showing intense delay of the spore maturation process.

Among the studied isolates, TCH 3 was the most suitable to survive in environments overloaded with this inorganic contaminant, although all showed tolerance to high doses of mercury. ANOVA showed that there is no significant difference between the species studied in relation to supporting the presence of mercury. However, the Tukey test confirmed that the TCH 3 strain is slightly more resistant. In humans, the fetal mercury poisoning demand occurs mainly in the first month of gestation (MANSOUR et al., 1974). In only 24 hours after ingestion, mercury can be detected in the placenta (GALE; HANLON, 1976).

In female fishes (*Poecilia reticulata*), the rates of mercury accumulation occur in the form of mercury nitrate or mercury chloride and in methyl form. In deionized water of 0.1-20 ng Hg/mL in the presence of complex agents, the release of mercury nitrate from fish occurs in 2 stages with half-life of 4.2 and 67.7 days respectively. Mercury released from mercury nitrate increased with exposure time and becomes methyl mercury in the fish (KRAEMER; NEIDHART, 1974). The order of accumulation of inorganic contaminants in gills was performed as in a succession of cadmium after lead, then mercury, nickel and chromium.

In the case where sulfate reducing bacteria were isolated and their physiology tested to measure their behavior against mercury chloride (HgCl_2), cadmium sulfate CdSO_4 and mercury nitrate $\text{Hg}(\text{NO}_3)_2$, the order of toxicity for the growth of the salts of these metals was first seen in cadmium, followed by lead and finally, mercury. The inhibitory concentration for mercury was 100 mg/kg (LOKA BHARATHI; SATHE, 2017).

In the environment, fungal populations produce low molecular weight organic acids to mineralize the nutrients and then absorb them (as if it were an external digestion). The same phenomenon occurs in the biosorption of metals. Some micronutrients are absorbed for their metabolic needs (KAVANAGH, 2005), while toxic trace metals are conditioned inside vacuoles to minimize their toxicity after absorption (GADD, 2010; GADD et al., 2014; BALDRIAN, 2003).

The minimum inhibitory concentration obtained with *Trichoderma* varies according to the inorganic contaminant concentration. For cadmium, *Trichoderma* resisted up to 300 mg/L

(NONGMAITHEM et al. 2016) and 200 mg/L for nickel (NONGMAITHEM et al. 2016). For mercury, inhibition occurred with 200 mg/L of mercury. The behavior of the isolates *Trichoderma* sp. in liquid medium showed that the mercury tolerance increased in liquid medium compared to the solid medium.

Regarding the pH of the culture medium with mercury, it oscillated around 7.5 (the pH of the medium with TCH 1 was 7.04, TCH 2 was 7.79, TCH 3 was 7.74 and TCH 5 was 7.56). In liquid medium, the strategy of the microorganism was perceived as resulted to an increase in pH. The *Trichoderma* isolates in mercury medium oscillated around 7.5 pH (the pH of the medium with TCH 1 was 4.04, TCH 2 was 7.79, TCH 3 was 7.74 and the TCH 5 was 7.56). In liquid medium this strategy of the microorganism was perceived that resulted in an increase of pH. The activity of fungi *Trichoderma asperellum* was not affected by alkaline pH as it exhibited the production of enzyme even in alkaline pH of the culture medium (SRIDEVI et al. 2017).

There were some reasons for studying the effects of pH 4.0 and 9.0 on the organism. The experiment was made with initial pH of 7.0 and after analysis; it was repeated in the acid pH of 4.0 and alkaline pH 9.0. It has been observed that pH is very important in the activation of enzymes to protect against aggressive factors of the environment notably the inorganic contaminants. In some organisms, a higher inhibition occurs at low pH while for some microorganisms at a high pH; there is a greater ability to deal with aggressiveness. Therefore, the behavior of the fungus at different pH was analyzed in acid and alkaline media.

When mercury is added to malt agar medium, what occurs is a decline in pH, but in liquid medium, there is an increase in pH, which suggests a defense mechanism of the microorganism whose biochemical reactions have not yet been fully delineated. It is known that fungi grow over a relatively wide pH range and adapt to extracellular pH through a genetic regulatory system mediated by a key component whose name is PacC, which is a pH transcription regulator gene (HE et al. 2014).

With respect to the effects of alkaline pH on the radial growth of *Trichoderma* isolates in mercury culture medium in the first 48 hours (TCH 2 and 3) or 60 hours (TCH 1,4 and 5), growth was inhibited; sometimes seen only on one spot preventing the spread of *Trichoderma*, but most of the time not even this was noticeable. However at pH 9.0, total inhibition of growth occurred in the first few hours. This pH effect was clear because it also reflected on the control.

With respect to mercury at 200 mg/L there was total inhibition of fungus growth for TCH 1 and 2 whereas for TCH 3, 4 and 5 the growth started only after 60 hours and with noticeable effect of mercury retarding both growth and sporulation. Also, Juntunen et al. (2015) showed that at optimum temperature, Fe protease showed high activity in the pH range of 6-10, with a sharp decline in activity at pH above 9.0.

While at high pH strong inhibition occurred, at lower pH there was only mild retardation of growth and sporulation. This is probably due to the fact that pH 4.0 is very close to the normal pH in which the microorganism survives. For example when cultivated starting as the culture medium at pH 7.0 occurs a decline of the pH therefore to pH 4.6 (Table 1). As shown in Fig. 3 obtained through scanning electron microscopy, the sporulation alteration is detected in medium with and without mercury. Therefore, it is observed that the presence of mercury leads to sporulation retardation (Fig 1) and influences the number of spores produced. This is in accordance with the study of Repaint et al. (2009) in which mercury affected fungal growth and sporulation at the concentrations used.

5 CONCLUSIONS

Trichoderma isolates were able to withstand environments contaminated with mercury and caused growth retardation and sporulation. Therefore, they have the potential to be used for bioremediation of mercury. However, their resistance is eight-fold greater than that of human beings. Microscopic analysis by scanning electron microscopy showed the effects of increased mercury on concentration and in the sporulation. Cultivation in liquid medium showed a high resistance capacity in the aquatic environment with the dosage of mercury, which shows a promising use in bioremediation since the major problem of mercury contamination occurs predominantly in aquatic environment.

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CONFLICT OF INTERESTS

The authors declare that there is not conflict of interests regarding the publication of this paper.

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