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Research Article

Chromium and Lead Tolerance of Fungi Isolated from Mining Sites in Santo Domingo, Chontales Nicaragua

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Abstract

Few studies have reported the isolation of microorganisms from mining sites in Nicaragua. The objective of this study is to isolate autochthonous fungi from mining sediments of Santo Domingo, Chontales in the central region of Nicaragua and assess them for the tolerance to chromium (Cr) and lead (Pb). For the isolation of fungi, serial dilution and plate seeding on solid cultivation of Potato Dextrose Agar (PDA) was used. The microorganisms were identified by macroscopic observation and microscopy based on the colony colour, shape, hyphae, conidia and spore arrangement. Molecular identification was performed by polymerase chain reaction (PCR) analysis, extracting DNA for amplification of internal transcribed spacer (ITS) regions for ITS1-STS4 for fungi. The PCR product was sequenced and compared with other sequences int the GenBank (NCBI). The fungal genomes *Fusarium oxysporum, Pichia kudriavzevii, Trichoderma harzianum* and *Aspergillus awamori* were identified. The tolerance index (TI) was determined from different concentrations of Cr and Pb, demonstrating that *Fusarium oxysporum, Trichoderma harzianum* and *Aspergillus awamori* are tolerant in the range of 1 to 5 mg L^{-1} for Cr and 52 to 207 mg. L^{-1} for Pb, according to the analysis of variance with the Duncan test. Since the tested species are autochthonous to the contaminated environment in Santo Domingo, they are interesting as a point of departure for soil remediation endeavours in the area.

Keywords

Mycoremediation, Polluted Soil, Tolerance Index, Mining Sites, Nicaragua

1. Introduction

Metalliferous mining constitutes a pillar for the economy in many low and middle income countries (Roe et al., 2016). Mining activities are also associated with severe environmental problems caused by organic and inorganic pollution inorganic pollution [1, 2]. Nicaragua has been mining country since colonial times and the extraction of gold and other precious metals constitute a significant contributor to the economic development of the country. In Nicaragua, both industrial and artisanal mining sites are common which have caused solid and liquid waste discharges affecting the quality of soil, surface water, groundwater, plants and animals [3, 4]. Cadmium, lead, arsenic, and mercury are the elements of

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most concern for human health [5] but other metals, like chromium, copper, and barium also represent considerable risks.

Heavy metals affect the functions of many organisms and ecosystem processes [6] and the interaction between the elemental pollutants and the soil microbiota is crucial for determining the environmental fate of the heavy metals [7]. Within contaminated environments, a great diversity of microorganisms (fungi, yeasts, algae and bacteria) exist and many of them have developed resistance mechanisms, and are well adapted to extreme conditions such as low pH, high temperature, scarce availability of nutrients and high concentrations of organic and inorganic pollutants [8-10]. Therefore, polluted environments are considered promising sites for collection of pollutants tolerant microbiological strains that may be helpful in the development of bioremediation methods, which are commonly regarded as cost effective, ecological, and efficient technologies [11].

Fungi are one of the most diverse microorganisms that are widely distributed in all ecosystems [12, 13]. Thanks to its metabolic capabilities, it can tolerate and/or transform toxic compounds into less harmful forms and accumulate them within their biomass [11]. Fungal species from a number of genera including Aspergillus, Penicillium, Fusarium, Alternaria, Geotrichum, Trichoderma, Coprinellus have been identified to show high tolerance to heavy metal contamination [9, 11, 14, 15].

The identification of metal-tolerant strains of fungi is important for the development of biotechnological strategies that may remediate contaminated soil and water as well as for biomining [16]. Species may be identified based on its phenotypic and morphological characteristics. However, the unique characteristics of fungi makes it difficult to identify only based on morphological classification [17]. The use of Polymerase Chain Reaction (PCR) to amplify the Internal Transcribed Spacer (ITS) region of fungi and subsequent sequencing of the amplified region provides a more reliable basis for identification of fungal species [18]. The objective of this study was to identify fungi isolated from an artisanal gold mining site and assess the tolerance index to Cr and Pb of the identified species.

2. Methods and Materials

The study was carried out in the Microbiology Laboratory of the Biotechnology Research Center of the National Autonomous University of Nicaragua, Managua (CIB/UNAN-Managua). Sediment samples were collected from a property used for artisanal gold mining owned by Adonis Gonz áez in Santo Domingo, Chontales, Nicaragua. The gold extraction process in the area is predominantly carried out by amalgamation with mercury and cyanide where water from the Artiguas river and wells are used.

2.1. Study Area

The selected study site was coordinated by the Center for Research in Biotechnology (CIB/UNAN-Managua) and the Ministry of Energy and Mines (MEM) of the municipality of Santo Domingo, department of Chontales, Nicaragua, allowing monitoring to be carried out for the collection of samples on 3rd April 2019, obtaining the following georeferenced 12 °16′16,5″ N, 85 °5′04,5″ W.

2.2. Sampling

The sediment samples were collected with sterile spatulas at four randomly selected sites near the harrows of artisanal mining. The samples were subsequently mixed in order to obtain a representative sample and placed in previously labelled plastic bags, wrapped with aluminium foil and placed in thermos at 4 ℃ to ensure minimal biological activity.

2.3. Isolation of the Fungi

The isolation of the fungi was carried out by plate dilution according to [19]. Approximately 10 g ww of fresh sediment was weighed, followed by the addition of 90 mL of sterile doubled distilled water and stirred for 1 minute. Serial dilutions from 10^{-2} to 10^{-6} were performed, stirring for two minutes. Subsequently, massive surface seeding (0.1 mL) was carried out on the culture medium containing potato dextrose agar (PDA) and chloramphenicol. Each dilution was performed in triplicate; the plates were incubated at 30 ℃ for five days [20]. After the completed incubation the quantification of colony-forming units (CFU)/g of dry sediment was performed. The count was carried out only in plates (dilutions) containing from 30 to 300 colonies [19].

2.4. Morphological Identification of Isolated Colonies

The characteristics of the four isolated colonies were recorded for their morphological structure: shape, size, colour and type of growth formation [21]. During the microscopic observation, a drop of sterile water was placed in the centre of object holder to fix the 2.0 cm transparent adhesive tape containing the sample. The microorganisms were observed on a trinocular microscope OLYMPUS brand, model BX43 with a 40X and 100X magnification. The different characteristics of fungal structures such as hyphae, mycelium and conidia were compared with the contrast reported by Barnett & Hunter [22]. The colony color was observed on both adverse (A) and reverse (B) sides and images were analysed based on the shape of the spore structures visualized under the microscope (C).

2.5. Molecular Identification of Isolated Fungi

The DNA was extracted from solid cultures of each isolated fungus was as described by Almaraz-Sanchez et al. [23]. The molecular identification was performed by PCR, using ITS1-F and ITS4 primers described by Toju et al. [24]. The forward primer used was 5' – CTT GGT CAT TTA GAG GAA GTA A – 3' and reverse primer 5' – TCC TCC GCT TAT TGA TAT GC – 3' according to the Phusion Master Mix protocol (M0531S). 12.5 µL was used for each sample. 4 µL of PCR product was cleaned with 1 µL of ExoSap for a total reaction of 5 µL. The enzymes were activated at 37 ℃ for 30 minutes and subsequently denatured at 85 ℃ for 15 minutes, using an AB 2720 thermal cycler. From the clean product, 1 µL was used with the slightly modified Cycle Sequencing protocol for the Big Dye Terminator v3.1 in a 5 µL reaction. Finally, EDTA at 125 mM was used to precipitate the Cycle Sequencing product according to the M0531S protocol. The pellet was resuspended in 10 µL of formamide and the SeqStudio ABI 3200 sequencer (Sanger sequencing methodology by capillary electrophoresis) was used for reading [18, 25]. The sequences obtained from the molecularly identified species in FASTA format was compared with GenBank NCBI (National Center for Biotechnology Information) database consulted in November 2020.

For molecular identification, the GenBank BLAST (Basic Local Alignment Search Tool) was used to find similar regions between the sequences and the identities of the isolates (determined based on the highest score basis). Multiple alignment was performed between six and ten sequences according to the number of base pairs. Those were downloaded in the FASTA format, which allowed the identification of functional regions; the degree of similarity between them, which were aligned in rows, but parsed in columns. The MEGA software version 10.2.0 was used for the alignment.

The phylogenetic tree was constructed using the Neighbour-Joining (NJ) method and the quality control analysis was performed using the Kimura 2 parameters model and the bootstrap method, which evaluated the reliability with 500 default repetitions of the program [24]. The phylogenetic analysis performed from the nucleotide sequencing identified in the study was grouped independently.

2.6. Metal Tolerance Assessment

The isolated fungi were assessed for their tolerance to Pb and Cr according to Muñoz-Silva et al. [26] with some modification. The solid medium was prepared using 5.85 g PDA and 10 mL chloramphenicol that was added to 150 mL of distilled water. The medium was sterilized in an autoclave at 121 ℃ for 15 minutes, and then removed from the autoclave to cool down to approximately 40 ℃. The solution stock of heavy metal was prepared from salts of K_2Cr2O_7 and Pb $(CH_3COO)_2.3H_2O$ and added in separate mediums of the following concentrations: 1, 3, 5 and 13 mg L^{-1} for Cr and 52, 104, 207 and 622 mg L^{-1} for Pb.

The spiked medium was homogenized and subsequently, poured into Petri dishes and allowed to solidify and then the fungal colonies were added to the centre of each dish. The plates were incubated at 30 ℃ for seven days and the mycelial growth was monitored and recorded at each 24 hours interval. All experiments were conducted in triplicate and a control without metal were used. The Tolerance Index (TI) was calculated from the fungal growth in the presence of metals, divided by the fungal growth in the control (containing no heavy metals) during the same period. The heavy metal tolerance was classified according to Oladipo et al., [20] as: 0.00–0.39 (very low tolerance), 0.40–0.59 (low tolerance), 0.60–0.79 (moderate tolerance), 0.80–0.99 (high tolerance) and 1.00–>1.00 (very high tolerance), the higher the values, the higher the fungal tolerance to the heavy metal.

2.7. Statistical Analysis

Analysis of variance was performed using ANOVA used to test the statistical significance at $p \leq 0.05$ between the spiked substrate and control. The Duncan test was used to compare the means.

3. Results

3.1. Morphological Identification of the Isolated Fungi

The identification based on morphological characteristics and microscopic observations is displayed in (figure 1). The four fungal isolates were identified as belonging to the genera: I). Fusarium, presenting white cottony-looking mycelium that turned violet on the third day; II). Pichia, presenting yeast-like characteristics without cottony appearance, white to cream colour; III). Trichoderma, presenting a spectrum of yellow-greenish to white mycelia and IV). Aspergillus, presenting black and white ellipses.

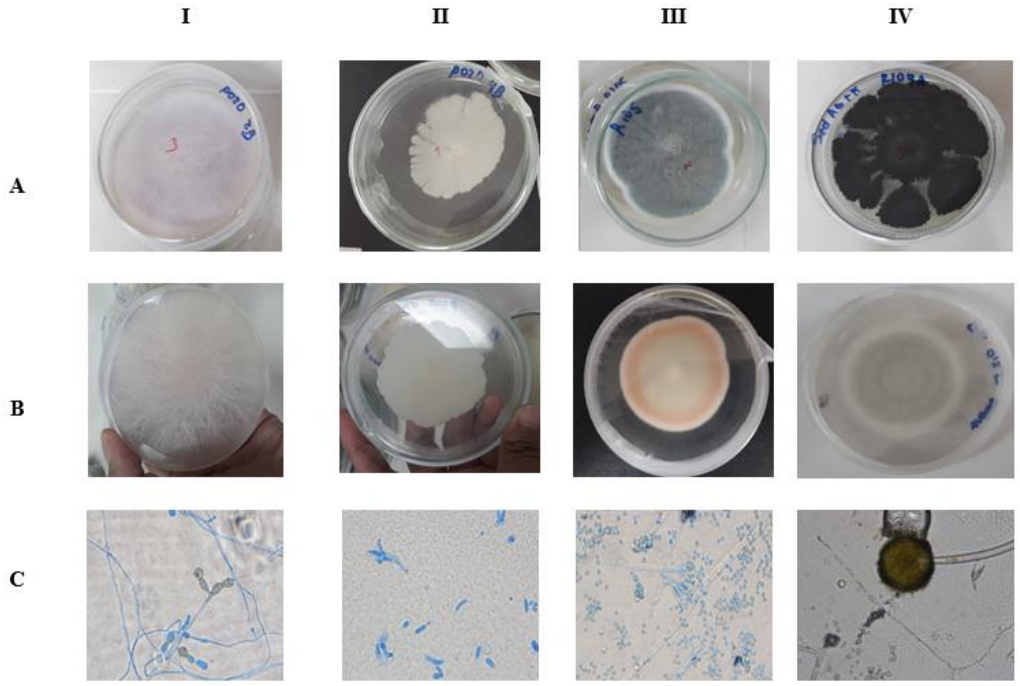

Figure 1. *Macroscopic and microscopic characteristic of fungi isolated from sediments mining (I, II, III y IV). Colonies show front (A) and reverse (B) side of different isolated fungi on PDA medium after five day of incubation and show in the optical microscopic (C) 40 X and 100 X.*

3.2. Molecular Identification as Sequence Analysis

The molecular identification coincided with the morphological observation. Amplification of the ITS regions fungal strains generated fragments of approximately 650 base pairs (pb) in the length. The results obtained of amplified PCR products was estimated to be approximately 568 pb for Fusarium and Pichia, 643 pb for Trichoderma and 619 pb for Aspergillus.

The Phylogenetic analysis showed that the sequences of the species identified as *Fusarium oxysporum* had a 99% (4 nucleotides base differences) similarity to the accession JN232163. The species identified as *Pichia kudriavzevii* had 99% (1 nucleotides base differences) similarity to the MT731410 accession. The species identified as *Trichoderma harzianum* showed 99% (3 nucleotides base differences) similarity to the MF078649 accession and the species *Aspergillus awamori* showed 100% (similarity with the MH856950 accession. Table 1 shows the percentage of similarity of the identified species based on the coincidence of the sequencing of the aligned nucleotides. Figures 2-5 show the sequence and phylogenetic trees of the identified species.

Table 1. *The percentage of sequence similarity of the identified species.*

Sequence code	Taxonomy species	Closet	Identity	Max Score	Percentage Similarity (%)
E10-H1	*Fusarium oxysporum*	Fusarium oxysporum281 (JN232163.1)	510/515	920	99
G10-H2	*Pichia kudriavzevii*	Pichia kudriavzevii L5983 (MT731410.0)	486/491	881	99
A8-3	*Trichoderma harzianum*	Trichoderma harzianum Th43-14 (MF078649.1)	581/585	1055	99
B8-4	*Aspergillus awamori*	Aspergillus awamori CBS 115.52 (MH856950.1)	550/550	1016	100

AGGWATAAGR TWRGATTTTG TCKCGTCKCG TCCTWACCRY GWCAGKKYAR GRGKGWGACC
GCGGAGGATC ATTACTGTAG TTTACAACTC CCAAACCCCT GTGAACATAC CACTTGTTGC CTCGGCGGAT
CAGCCCGCTC CCGGTAAAAC GGGACGGCCC GCCAGAGGAC CCCTAAACTC TGTTTCTATA
TGTAACTTCT GAGTAAAACC ATAAATAAAT CAAAACTTTC AACAACGGAT CTCTTGGTTC TGGCATCGAT
GAAGAACGCA GCAAAATGCG ATAAGTAATG TGAATTGCAG AATTCAGTGA ATCATCGAAT CTTTGAACGC
ACATTGCGCC CGCCAGTATT CTGGCGGGCA TGCCTGTTCG AGCGTCATTT CAACCCTCAA
GCACAGCTTG GTGTTGGGAC TCGCGTTAAT TCGCGTTCCC CAAATTGATT GGCGGTCACG
TCGAGCTTCC ATAGCGTAGT AGTAAAACCC TCGTTACTGG TAATCGTCGC GGCCACGCCG
TTAAACCCCA ACTTCTGAAT GTTGACCTCG GATCAGGTAG GAATACCCGC TGAACTTAAG CATATCAWWA
AAACSSSGRR ARRRAGAAAA AAGAACC

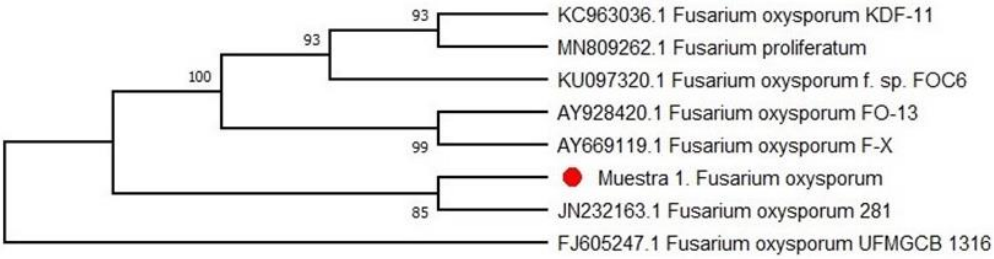

Figure 2. *Sequence and phylogenetic trees of Fusarium oxysporum.*

CMWCRCWAAY AKGMKKMWSC GRKRAGTGAA CCTGCGGAAG GATCATTACT GGRATWRKAC
TACTACACTG CGTGAGCGGA ACGAAAACAA CAACACCTAA AATGTGGAAT ATAGCATATA GTCGACAAGA
GAAATCTACG AAAAAACAAA CAAAACTTTC AACAACGGAT CTCTTGGTTC TCGCATCGAT GAAGAGCGCA
GCGAAATGCG ATACCTAGTG TGAATTGCAG CCATCGTGAA TCATCGAGTT CTTGAACGCA CATTGCGCCC
CTCGGCATTC CGGGGGGCAT GCCTGTTTGA GCGTCGTTTC CATCTTGCGC GTGCGCAGAG
TTGGGGGAGC GGAGCGGACG ACGTGTAAAG AGCGTCGGAG CTGCGACTCG CCTGAAAGGG
AGCGAAGCTG GCCGAGCGAA CTAGACTTTT TTTCAGGGAC GCTTGGCGGC CGAGAGCGAG
TGTTGCGAGA CAACAAAAAG CTCGACCTCA AATCAGGTAG GAATACCCGC TGAACTTAAG CATATCTTAA
AAACSGSCGR RGARRAAAAG AA

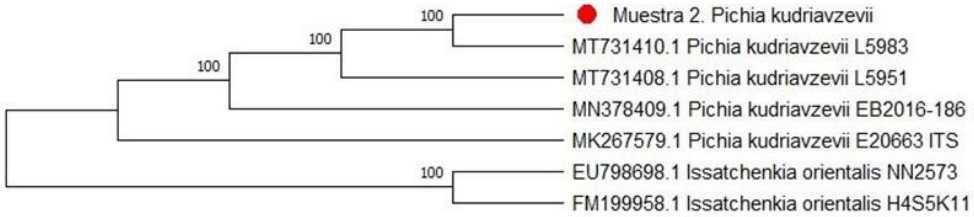

Figure 3. *Sequence and phylogenetic trees of Pichia kudriavzevii.*

AGCGTAAATG GSCTACGCGT GTYGAYCAGC GGTAGGGATC ATTACCGAGA ATTACAACTC
CCAAACCCAA TGTGAACGTT ACCAAACTGT TGCCTCGGCG GGATCTCTGC CCCGGGTGCG
TCGCAGCCCC GGACCAAGGC GCCCGCCGGA GGACCAACCA AAACTCTTAT TGTATACCCC
CTCGCGGGTT TTTTTATAAT CTGAGCCTTC TCGGCGCCTC TCGTAGGCGT TTCGAAAATG AATCAAAACT
TTCAACAACG GATCTCTTGG TTCTGGCATC GATGAAGAAC GCAGCGAAAT GCGATAAGTA ATGTGAATTG
CAGAATTCAG TGAATCATCG AATCTTTGAA CGCACATTGC GCCCGCCAGT ATTCTGGCGG GCATGCCTGT
CCGAGCGTCA TTTCAACCCT CGAACCCCTC CGGGGGGTCG GCGTTGGGGA TCGGCCCTCC
CTTAGCGGGT GGCCGTCTCC GAAATACAGT GGCGGTCTCG CCGCAGCCTC TCCTGCGCAG
TAGTTTGCAC ACTCGCATCG GGAGCGCGGC GCGTCCACAG CCGTTAAACA CCCAACTTCT
GAAATGTTGA CCTCGGATCA GTAGGAATAC CCGCTGAACT TACTCTTTTA AAGAGGGGGG
GGRRRRRRRR RAA

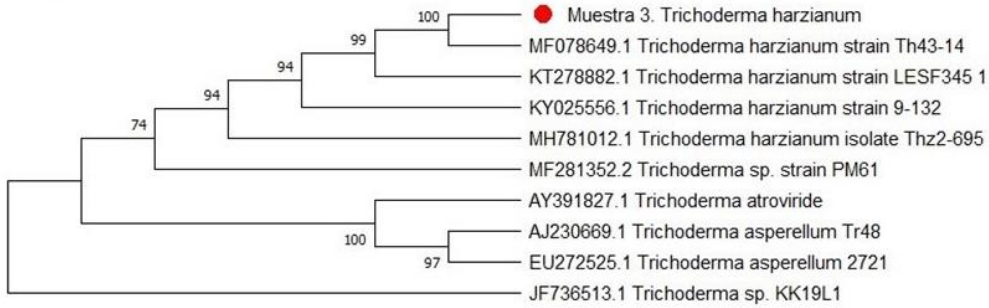

Figure 4. *Sequence and phylogenetic trees of Trichoderma harzianum.*

GGGATACAGA ACTTGGATGY ATYAAYYYKS GGTAGAATAA GCGGAGGAAA GGGTCCTTTG
GGCCCAACCT CCCATCCGTG TCTATTATAC CCTGTTGCTT CGGCGGGCCC GCCGCTTGTC
GGCCGCCGGG GGGGCGCCTT TGCCCCCCGG GCCCGTGCCC GCCGGAGACC CCAACACGAA
CACTGTCTGA AAGCGTGCAG TCTGAGTTGA TTGAATGCAA TCAGTTAAAA CTTTCAACAA TGGATCTCTT
GGTTCCGGCA TCGATGAAGA ACGCAGCGAA ATGCGATAAC TAATGTGAAT TGCAGAATTC AGTGAATCAT
CGAGTCTTTG AACGCACATT GCGCCCCCTG GTATTCCGGG GGGCATGCCT GTCCGAGCGT
CATTGCTGCC CTCAAGCCCG GCTTGTGTGT TGGGTCGCCG TCCCCCTCTC CGGGGGGACG
GGCCCGAAAG GCAGCGGCGG CACCGCGTCC GATCCTCGAG CGTATGGGGC TTTGTCACAT
GCTCTGTAGG ATTGGCCGGC GCCTGCCGAC GTTTTCCAAC CATTTTTCC AGGTTGACCT
CGGATCAGGT AGGGATACCC GCTGAACTTA AGCATATCAA WWAAGCGGGA GGAAAGA

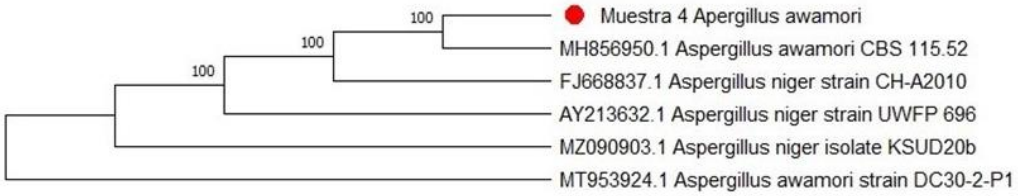

Figure 5. *Sequence and phylogenetic trees of Aspergillus awamori.*

3.3. Tolerance Index

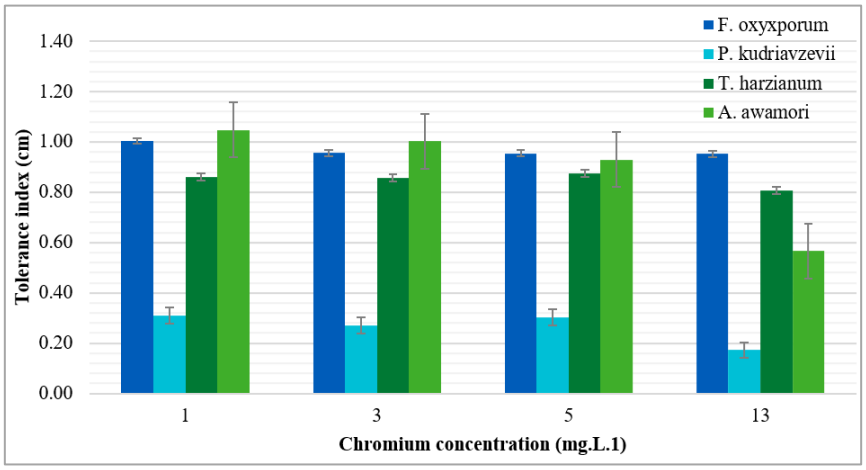

Figure 6. *Chromium tolerance index of the isolated fungi.*

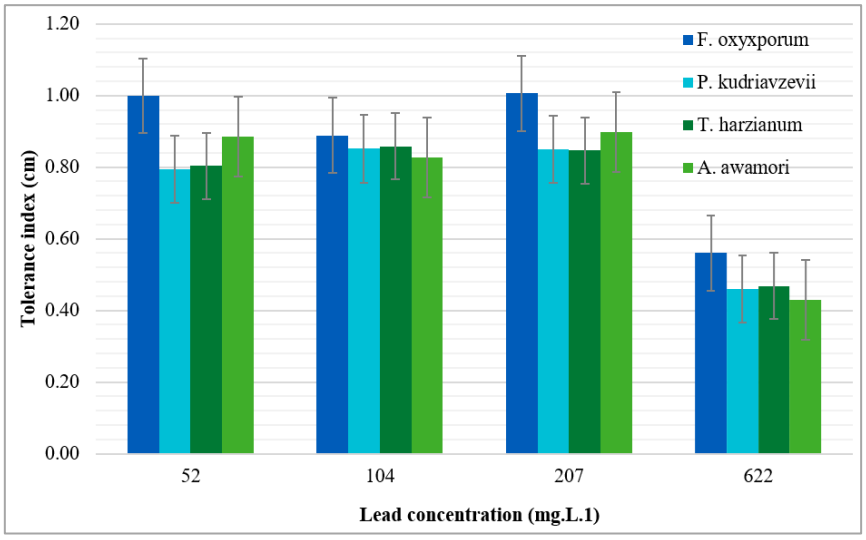

Figure 7. *Lead tolerance index of the isolated fungi.*

The Cr and Pb TI of the species of *F. oxysporum, P. kudriavzevii, T. harzuanun and A. awamori* is shown in figure 6 and figure 7. *F. oxysporum* presented a high TI (0.95-1.00) in the concentration 1-13 mg L^{-1} Cr and the Pb and high and very high T1 (1.01-0.88) in concentration of 52-207 mg L^{-1}. At 622 mg L^{-1} Pb however the TI was low (0.56). *T. harzianum and A. awamori* presented high and very high T1 (0.81- 1.05) in the 1-13 mg L^{-1} Cr and 52-207 mg L^{-1} Pb respectively. However, *A. awamori* indicated low TI (0.57) at 13 mg L^{-1} Cr and for *T. harzianum* and *A. awamori* the TI was low (0.43-0.47) at 622 mg L^{-1} Pb.

P. kudriavzevii, presented very low TI (0.17-0.31) in 1-13 m.L^{-1} Cr and moderate and high (0.79-0.85) in 52-207 mg L^{-1} Pb, and low (0.46) at 622 mg L^{-1} Pb.

4. Discussion

The fungal isolates collected at the artisanal mining site in Santo Domingo were of four genera (*Fusarium, Pichia, Trichoderma* and *Aspergillus*) and all of them presented good growth on culture medium. The results were consistent with a number of studies where these genera have been found in and isolated from polluted soils and sediments [26-32]. The morphological observations coincided with the phylogenetic analysis that had a similarity >99% which is acceptable for fungal identification [33].

All species, except *P. kudriavzevii* presented maximum tolerance to Cr at the first three concentrations (1, 3 and 5 mg L-1) with a notable TI decrease of *A. awamori* at the highest concentration (13 mg L^{-1}). These results suggest that *F. oxysporum, T. harzianun* and *A. awamori* are interesting candidates for biotechnological applications in moderately to highly polluted substrates. The Pb tolerance was more evenly distributed between the species with *F. oxysporum* being the most tolerant in all concentrations. The species TI in substrates with mixed contamination is yet to be determined but previous studies confirm the tested species tolerance to Cr and Pb and indicate high tolerance to other metals such as Ni and Cu [12, 29, 34-36]. The fact that the tested species are autochthonous to the contaminated environment in Santo Domingo makes them an interesting point of departure for soil remediation endeavours in the area. Metal tolerance mechanisms in fungi are complex and depend not only on the isolate tested but also on the site of its isolation [37]. The identification of autochthonous metal tolerant strains of fungi is also important for the development of biotechnological strategies that may remediate contaminated soil and water as well as for biomining [16]. Appropriate fungal remediation techniques may include fungal biomass used as biosorbents for removal of metals from aqueous solutions or mycoextraction from soils and sediments [38, 39].

5. Conclusions

The fungal isolates collected at the artisanal mining site in Santo Domingo were of four genera (Fusarium, Pichia, Trichoderma and Aspergillus) which all presented good growth in laboratory conditions. All species but *P. kudriavzevii* presented high or very high tolerance to Cr making them interesting candidates for biotechnological applications in moderately to highly polluted substrates. The Pb tolerance was acceptable in all tested specied, *F. oxysporum* being the most tolerant. Since the tested species are autochthonous to the contaminated environment in Santo Domingo, they are interesting as a point of departure for soil remediation endeavours in the area.

Abbreviations

CIB/UNAN	Microbiology Laboratory of the Biotechnology Research Center of the National Autonomous University of Nicaragua
BLAST	Basic Local Alignment Search Tool
CFU	Colony-Forming Unit
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
FASTA	Software Package for DNA and Protein Sequence Alignment
ITS	Internal Transcribed Spacer
MEM	Ministry of Energy and Mines
NCBI	National Center for Biotechnology Information
NJ	Neighbour-Joining
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
TI	Tolerance Index

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Author Contributions

Martha Jarquín Pascua: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft

María Teresa Plata Oviedo: Data curation, Investigation, Methodology, Supervision, Validation, Writing – review & editing

Martha Lacayo Romero: Conceptualization, Investigation, Methodology, Project administration, Supervision, Validation, Writing – review & editing

Henrik Haller: Data curation, Investigation

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Conflicts of Interest

The authors declare no conflicts of interest.

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