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ORIGINAL PAPER

Bioremoval of copper by filamentous fungi isolated from contaminated soils of Puchuncaví-Ventanas Central Chile

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Abstract Pollution represents a high risk to plants, animals, and human beings, causing an imbalance and affecting the environment. Soil is considered a universal sink, containing the highest load of environmental pollution. Puchuncaví-Ventanas sector, decreed as a saturated contamination zone in 1993, is considered one of the most affected areas by industrial pollution and belongs to one of the 5 sacrifice zones of Chile. The localities of Puchuncaví and Ventanas have heavy metal pollution levels that exceed up to 99% of the limits allowed by Canadian standards. The objective of this study was to characterize heavy metal tolerance and removal potential of filamentous fungi isolated from polluted soils for their use in decontamination systems and in situ soil improvement. Six fungal strains were selected based on their tolerance and a high capability to accumulate heavy metals, achieving copper bioaccumulation of 84% (*Mortierella* sp. strain LG01), 49% (*Clonostachys* sp. strain CQ23) and 48–77.5% (*Trichoderma* sp. strain LM01A). *Trichoderma* sp. strain LM01A was able to remove 41% of copper from contaminated soil under ex situ conditions. Some fungal strains belong to beneficial fungal genera, which are used as bioproducts in agriculture. The results of this study highlighted the use of *Trichoderma* sp. in soils contaminated, which may be of special interest in agriculture due to the large amounts of copper sulfate still applied as a pesticide in Chile and the world.

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Introduction

Soil pollution by heavy metals presents a great threat to living beings and the ecosystem due to its high toxicity and harmful effect on the food chain (Sandoval, 2006; Wuana & Okieimen, 2011). Some heavy metals are essential for all living organisms; however, in high concentrations, they are toxic (Hassaan et al., 2016; Iztileu et al., 2013; Pérez-Torres et al., 2020; Van der Voet, 2013). Heavy metals have high persistence in soils and the environment and may affect the human body by contact, inhalation, ingestion or through the food chain from absorption by plants. In Chile, there is an area highly contaminated with heavy metals, called the "sacrifice zone", located in Puchuncaví-Ventanas (Central Chile). In this place, there are numerous factories, whose industrial activities can cause problems for the health of people and the environment (Altimira et al., 2012). The main industrial activities correspond to a copper smelting and refinery complex, and a complex of coal-fired thermoelectric plants with three operating units. These two complexes are responsible for 68.1 and 30.7%, respectively, of SO_2 emissions and have contributed to soil pollution by heavy metals, mainly with Cu, Cd, As and Pb (Ministerio-del-Medio-Ambiente, 2014; Parra et al., 2014; Salmani-Ghabeshi et al., 2015). Among the heavy metals, copper (Cu) is one of the most toxic and arises from many sources, such as industry, mining and agriculture. Although most organisms use Cu^{2+} as an essential element for their growth and development (Serra & Guasch, 2009) The presence and persistence of high levels of Cu^{2+} in the environment cause harmful effects on humans and other organisms, mainly due to moderate accumulation over time.

Chile lacks environmental regulations for soils, although there are some sectoral decrees regarding the use of land from some ministries, such as housing, agriculture and mining; these are not intended to set a specific pollutant limit to protect soil quality in the country (Salmani-Ghabeshi et al., 2016).

There are various techniques to decontaminate soils contaminated with heavy metals, which can be physical–chemical (conventional) or biological (Lai et al., 2011; Wuana & Okieimen, 2011). Some of the physicochemical methods have shown some long-term deficiencies and damage to the environment, in addition to a high cost. These methods require the use of various chemical agents that can cause damage to the growth of agricultural crops and care must be taken when applying it on sandy-textured soils, due to the high risk of contaminating groundwater (Beltrán-Pineda & Gómez-Rodríguez, 2016; Lai et al., 2011; Wu et al., 2010).

Numerous studies of biological treatments of contaminated soils have been carried out, to use clean, more efficient and economical technologies (Beltrán-Pineda & Gómez-Rodríguez, 2016). These treatments are mainly focused on the application of phytoremediation (use of plants) or bioremediation (use of microorganisms) to reduce, eliminate, or immobilize organic and inorganic pollutants that have accumulated in soils (Gadd, 2010; Haferburg & Kothe, 2007; Sandoval, 2006). Due to the non-degradability of heavy metal contaminants, the principles of microbial remediation in soils contaminated with heavy metals mainly include biosorption, bioenrichment and bioconversion to less toxic phases (Beltrán-Pineda & Gómez-Rodríguez, 2016; Bravo et al., 2020); they can use the negative charges on the cell surface to immobilize heavy metal ions through electrostatic adsorption or complexation, store the adsorbed toxic metals in different parts of the cells, or bind them in the extracellular matrix (Park & Chon, 2016). Fungi predominate in the soil and have fundamental properties as biotransformers of metals and minerals, contributing to geological phenomena, such as rock weathering, and in the environmental fate of contaminating metals (Gadd, 2010; Gutiérrez et al., 2010). Fungal cells interact in various ways with metals and minerals, from the cell wall to the cell organelles. It has been reported that both yeasts and filamentous fungi can store high amounts of heavy metals (Gutiérrez et al., 2010). Fungi from areas contaminated with heavy metals have high tolerance and accumulation capability (Ayele et al., 2021; Beltrán-Pineda & Gómez-Rodríguez, 2016; Dusengemungu et al., 2020; Oladipo et al., 2018; Vaishaly et al., 2015). This process can be carried out through biomineralization, biotransformation, chemisorption, biosorption and bioaccumulation (Complexation into biopolymers, or enrich heavy metals by interacting with specific

heavy metal-binding macromolecules, including metal-binding proteins), these last two being the most common mechanisms in fungi (Ayele et al., 2021; Beltrán-Pineda & Gómez-Rodríguez, 2016; Oladipo et al., 2018; Shakya et al., 2016). The removal capacity depends on many factors, among them, the growth phase of the microorganism, which is related to the physiological-chemical state, age, and relationship with the medium. The identification of the growth phase allows indirect identification of the probable removal mechanism among other factors involved in this process (Bishnoi, 2005). Heavy metals adhere to the cell wall composed of cellulose, chitin, or both (Ayele et al., 2021; Shakya et al., 2016). The cell wall has many functional groups that favor metal uptake, such as carboxyl, amino, hydroxyl, phosphate and sulfate groups (Ayele et al., 2021; Shakya et al., 2016; Vaishaly et al., 2015). Once the heavy metal is incorporated into the cytoplasm, it is sequestered by proteins called metallothioneins (Beltrán-Pineda & Gómez-Rodríguez, 2016; Shakya et al., 2016).

Liaquat et al. (2021) reported *T. citronoviridae* and *F. solani* to be the best mycoremediation fungi in Pb and Cu contamination, finding minimum inhibitory concentrations between 400 and 1000 mg kg^{-1}. Previous studies carried out by Nongmaithem et al. (2016) found two strains of *Trichoderma* with high levels of tolerance to Ni and Cd, showing an increase in biomass when 40 mg kg^{-1} Ni was evaluated. Kumar and Dwivedi (2021) reported the capability of *T. lixii* CR700 to remove Cu^{2+} from PDB media and from real tannery wastewater via surface sorption and accumulation mechanism. This study evaluates the in vitro and ex situ capability of fungal strains isolated from polluted soils of Puchuncaví-Ventanas sector (Central Chile) to tolerate heavy metals and to remove copper.

Materials and methods

Sampling zone

The Puchuncaví-Ventanas industrial zone is in a district of the Valparaíso Region, Valparaíso Province, Central Chile. The specific soil sampling sites are shown in Fig. 1. La Greda (LG) sampling point (32° 44′ 52.89″ S, 71° 28′ 22.08″ W) is located 1.8 km to the NNW of the main emission sources, while Los Maitenes (LM) (32° 45′ 53.36″ S, 71° 27′ 17.60″ W)

Fig. 1 Geographical location of the industrial complex and the soil sampling site at the Puchuncaví Valley, Chile. The wind rose indicates the prevailing wind direction in the industrial complex. Table inside show details of geographical location and elevation of the sampling points

is located 2.4 km to the W. The Costa Quilén sampling site (CQ) (32º 43′ 07.76″ S, 71° 28′ 47.56″ W) is located 4.9 km N of the industrial pole, Valle Alegre (VA) (32° 48′ 23.87″ S, 71° 26′ 19.55″ W) is 6.3 km SW and Puchuncaví (PU) (32° 43′ 12.83″ S, 71° 24′ 12.78″ W) 8.6 km NW. A Control Site (CS) was selected (32° 51′ 42.91″ S, 71° 27′ 15.98″ W) 11.3 km S of the industrial complex, not affected by industrial activity (Gorena et al., 2020).

Soil sampling and physicochemical analysis

The soil samples were taken and analyzed according to a previously described (Salmani-Ghabeshi et al., 2015). They were taken in each sampling site randomly and composite with a plastic shovel from the first 510 cm of depth previous elimination of stones, leaves, seeds and/or roots, and stored in plastic bags. The soil samples were dried in an oven at 50 °C for 72 h and sieved with a 2 mm polypropylene mesh in agreement with the standard ISO 11464. Soil subsamples were homogenized in an agate planetary mill and sieved through a 0.2 mm mesh using a stainless steel mesh. The samples were then stored in the fridge (4 °C) until the analysis.

For the acid digestion, 400 mg (±0.1 mg) of soil sample or reference material was weighed and placed in glass digestion vessels (Corning, USA) to later add 3.5 mL of HNO_3 and HCl mixture in a 3:1 proportion. The mixture was left to react for some minutes at room temperature. The vessels were then covered with stoppers and taken to a microwave (Ultrawave by Milestone, USA). The samples were finally diluted with pure water to a total volume of 25 ml. Standard Reference Material Soil Contest 3A, from LGC standards UK, was used to assess the accuracy of the experimental results.

The digested samples were assayed on an Agilent Tech 7900 series ICP-MS equipment by standard protocol. The main instrumental parameters were as follows: RF power 1550 W, Ar plasma flow rate 15 L min^{-1}, washing time 60 s, and 3 replicates per sample. Quantification was performed by internal standard calibration using Inycom (Zaragoza, Spain) multi-element ICP-MS calibration standards. Blank samples were assayed, and no significant concentrations of the study elements were found. The concentrations of elements in the soil samples are expressed in dry soil weight terms.

For the identification and quantification of soil elements, while conducting fungal analysis, measurements were carried out using a portable XRF spectrometer S1 TITAN (Bruker, Germany) (Silva et al., 2019). For the measurement, special plastic capsules (Bruker, Germany) filled with dry sieved soil were used.

The analysis of texture, organic matter and cation exchange capacity of the soils was carried out at the Laboquimterra company (Quillota, Chile).

Further analytical details of this chapter can be found in the supplementary material (S.I and S.II).

Characterization of fungal strains

Isolation of fungal strains

Isolation of native fungal strains from soil samples from the study sites was performed as described by Garg et al. (2021). In a 250 mL beaker, 10 g of soil sample was added in 100 mL of sterile distilled water and shaken in an orbital shaker at 150 rpm for 24 h. The supernatant (100 µL) was plated on rose Bengal chloramphenicol agar plates. The isolation plates were incubated at 24 °C and after 7 days the total microbial count expressed as colony-forming unit per gram (CFU/g). To obtain pure strains, successive isolations of small portions of mycelium were made on rose Bengal chloramphenicol agar medium. The same isolation protocol was performed for each soil from each sampling site.

Molecular identification of fungal strains

For molecular identification of selected strains, DNA was extracted from the fungal mycelium of five days of growth as described by Huanca-Mamani et al. (2014) with some modifications. Subsequently, the Polymerase Chain Reaction (PCR) was used to amplify the internal transcribed spacer regions 1 and 2 (ITS1 and ITS2) contained in the ribosomal DNA (rDNA). The PCR mixture (50 µL) was performed with the GoTaq G2 Green Master Mix (Promega) Kit, 0.6 µM of each primer and 200 ng of DNA template. The ITS region was amplified using the following cycling parameters: 3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C and finally 10 min at 72 °C (Oskiera et al., 2015; Skoneczny et al., 2015). The

amplification of the sequences ITS1–ITS2 (650 bp amplicon length) was performed using the primers ITS6 (GAAGGTGAAGTCGTAACAAGG) and IST4 (TCCTCCGCTTATTGATATGC) (Cooke & Duncan, 1997; White et al., 1990). The PCR products were sent to the Pontificia Universidad Católica de Chile (Santiago, Chile) for sequencing, and sequences obtained were analyzed and compared to the NCBI (National Center for Biotechnology Information) gene bank with Blastn.

In vitro evaluation of heavy metal tolerance of fungal strains in a liquid system using leachates

To preliminarily assess the tolerance of all isolates to increasing concentrations of heavy metals, a soil leachate containing heavy metals was used. Liquid culture medium in the absence of heavy metals corresponded to the normal growth control of each fungus. To obtain the soil leachate, 50 g of soil was added in 450 mL of distilled water, acidified with 1 M HCl until obtaining pH 3 and left under stirring for 1 h (Baran et al., 2011) (Fig. SP.III.1). Then, it was filtered to obtain the supernatant without solid particles, and 1 M NaOH was added until obtaining a pH 6 that allows the growth of filamentous fungi. The presence of metals in the leachate was checked with a semi-quantitative total heavy metal strip test (SimplexHealth, UK) during the extraction process (Table SP.III.1). The solution was evaporated to a volume of 225 mL and a concentrated soil leachate was obtained. Once the concentrated leachate was obtained from each soil, a tolerance test was performed using 24-well microplates. 500 µL of leachate from each soil was added to the first column of the plate; four serial dilutions of the leachate were made using liquid culture medium until the dilution of 10^{-4}. The growth control was placed on the last column of the plate, which corresponds to a liquid culture medium without leachate. The test was carried out in duplicate, evaluating two fungal strains for each plate. In each well, a 5 mm agar disk containing mycelium of seven days of previous growth was inoculated. The fungal strains inoculated in each leachate corresponded to the isolates from their respective soils. The fungal strains were incubated on the plate at 24 °C and their ability to grow in the presence of the leachate was analyzed qualitatively after 3 days, time in which it was possible to observe differences in the growth of the strains studied (Awasthi et al., 2017; Zegzouti et al., 2020).

In vitro evaluation of specific tolerance to copper of fungal strains in semisolid medium

From the preliminary test, the fungal strains with the highest tolerance to heavy metals were selected and their tolerance to copper was evaluated. Mycelia growth of the fungal strains was subjected to increasing concentrations of copper ($CuSO_4$ $5H_2O$) incorporated into potato dextrose agar (PDA). Copper concentrations (mg kg^{-1}) were: 64, 127 and 200. These concentrations were chosen based on reports of fungal tolerance, the limits of the copper standards and the copper concentrations present in the soils studied (Maldaner et al., 2020; Văcar et al., 2021). In each plate, four 5 mm diameter disks of PDA with mycelium of each strain (with seven days of growth) were placed at the ends of the plate. Fungi grown on plates with PDA medium without copper sulfate, corresponded to control treatment (Maldaner et al., 2020; Xu et al., 2015). The strains were incubated at 24 °C for 7 days (time in which the control achieves 100% coverage of the Petri dish). Copper tolerance was evaluated by comparing mycelial growth recorded in the control treatment and growth recorded in the presence of copper. Mycelial growth was recorded by measuring the mycelial diameter using Sigma Scan Pro 5 software. Results are expressed as percentage of growth. The assay was performed in triplicate.

In vitro evaluation of copper removal capability of fungal strains

The selected strains were grown in PDA medium plates with 800 mg kg^{-1} of copper sulfate pentahydrate (equivalent 200 mg kg^{-1} Cu) (Kumar & Dwivedi, 2021). A 5 mm diameter agar disk containing the fungus grown 7 days earlier was placed in the center of each plate (Carvajal et al., 2021). Plates were incubated at 24 °C for the necessary time until the maximum mycelial diameter reached by the strain with the lowest growth rate. Subsequently, the mycelium was carefully removed from the agar plate and measurement of metals in the mycelium was performed using a portable S1 TITAN XRF spectrometer

($n=3$). The detection of heavy metals in each fungal strain was performed directly using dry mycelial biomass to evaluate the removal capability. The control corresponded to the growth of each strain on PDA medium without copper. The assay was performed in triplicate. The relative copper content in each mycelium was calculated based on the copper content observed in each treatment divided by the treatment with the highest copper removal, which was finally assigned a relative copper content equal to 1 (Eq. 1).

$$\text{Relative copper content} = \frac{\text{Cu concentration in mycelium}}{\text{maximal Cu concentration in mycelium in trail}}$$

In vitro copper removal kinetics

The strains that showed the best results in the evaluation of in vitro copper removal capacity were inoculated in Petri dishes with PDA medium containing 800 mg kg^{-1} of copper sulfate pentahydrate (Ezzouhri et al., 2009; Kumar & Dwivedi, 2021; Mohammadian et al., 2017; Văcar et al., 2021). A disk of PDA agar with mycelium of 5 mm in diameter was placed in the center of the plate and incubated at 24 °C. Every 24 h, for 5 days, the mycelium of each strain was carefully removed from the agar plate, dried and measurement of metals in the fungus was performed using a portable S1 TITAN XRF spectrometer ($n=3$). The control corresponds to the measurements made using a portable S1 TITAN XRF spectrometer every 24 h on mycelium grown on PDA medium in the absence of copper. The assay was performed in triplicate. To evaluate the possible removal mechanism occurring for each strain, mycelial radius growth was measured. Additionally, the maximum growth rate (μ_{max}) of each strain was calculated to identify the growth phase. The radial growth rate (RGR) was determined by using a linear model, and for the maximum growth rate (μ_{max}), the Gompertz was used, which is a nonlinear model (Baumer et al., 2008; Mas-Diego et al., 2019).

Ex situ removal of heavy metals in contaminated soils under laboratory conditions

The capability to removal heavy metals was evaluated using one of the selected strains. A solid support for fungal growth (based on ground corn) was placed in the lower part of a closed plastic container. On this support, a 5 mm disk of PDA agar with the fungal inoculum was added; contaminated soil (from La Greda), previously autoclaved, was placed on the support inoculated with the fungus. A filter paper (MN 640 m) was placed between the support and the contaminated soil to separate the compartments and facilitate the subsequent extraction of the mycelium. Another filter paper was placed on top of the soil to keep the system isolated. The system was sealed with a lid and a 22 µm syringe filter was inserted to favor

Table 1 Reference soil quality standards

Elements [mg kg^{-1}]	Reference soil quality standards					
	Canada (AEP, 2016)	Netherlands (Target, 2000)	Austria (Gendebien et al., 2001)	France (Gendebien et al., 2001)	Germany (Gendebien et al., 2001)	Australia (ADEC, 2010)
Sulfur (S)	–	–	–	–	–	600
Arsenic (As)	26	55	20	–	20	20
Barium (Ba)	2000	625	–	–	–	300
Calcium (Ca)	–	–	–	–	–	–
Titanium (Ti)	–	–	–	–	–	–
Iron (Fe)	–	–	–	–	–	–
Copper (Cu)	91	190	100	100	60	100
Manganese (Mn)	–	–	–	–	–	500
Potassium (K)	17	–	–	–	–	–
Lead (Pb)	600	530	100	100	100	600
Vanadium (V)	130	–	–	–	–	50
Zinc (Zn)	360	720	300	300	200	200

oxygen exchange (Fig. SP.III.2). Soil sampling of the system was performed after 15 and 30 days (Oyewole et al., 2019). The control corresponded to the same system sealed without fungal inoculum. The determination of metals in the soil was carried out using a portable XRF spectrometer S1 TITAN (Bruker, Germany), in Geochem application and DualSoil method. The soils were measured in plastic capsules with soil previously dried in an oven at 70 °C for 24 h (n = 5).

Statistical analysis

Data of the removal capability of fungal strains were evaluated through a one-way analysis of variance (ANOVA) and the means were compared by Duncan's Multiple Range Test (Statgraphics Centurion XV program v 16.1.15) to identify significant differences among the treatment and control group. Statistical differences were established with a p value ≤ 0.05. All data are presented as mean ± standard deviation (mean ± S.D.).

Result and discussion

Physical–chemical analysis of the soils

Based on the international standards used as a reference (Table 1), La Greda and Los Maitenes have copper and arsenic pollution (ADEC, 2010; AEP, 2016; Gendebien et al., 2001; Target, 2000).

Table 2 Physicochemical characteristics and element concentration by ICP-MS in soil samples collected in the studied area of Puchuncaví-Ventanas

Location	Texture				C.E.C	pH	Organic matter
	Sand (%)	Silt (%)	Clay (%)	Textural class	(meq/100 g)		(%)
La Greda	74.1	5.3	20.6	Sandy loam	7.7	5.1	2.93
Los Maitenes	81.2	6.7	12.1	Loamy sand	0.9	5.3	0.47
Puchuncaví	64.5	9.2	26.3	Sandy loam	2.4	5.0	1.69
Valle Alegre	72.3	5.3	22.4	Sandy loam	23.0	5.3	7.73
Costa Quilén	81.9	2.1	16.0	Loamy sand	3.7	5.1	1.61
Control Site	54.9	22.0	23.1	Sandy clay loam	32.2	5.6	30.5

Element concentration (mg kg^{-1})

Location	S	K	Ca	Ti	V
La Greda	**13,695.27 ± 1634.85**	3,160.76 ± 269.34	7,469.43 ± 614.41	232.21 ± 45.81	**82.14 ± 11.29**
Los Maitenes	**11,760.58 ± 1551.24**	3,241.86 ± 272.52	5,145.21 ± 231.17	288.95 ± 33.61	**69.36 ± 6.04**
Puchuncaví	**13,708.74 ± 1660.31**	2,213.40 ± 251.47	10,082.55 ± 939.56	265.20 ± 61.83	**56.07 ± 11.15**
Valle Alegre	**13,891.42 ± 1688.87**	5,456.50 ± 253.91	15,492.43 ± 1212.76	313.24 ± 44.78	**94.24 ± 6.67**
Costa Quilén	**10,636.36 ± 1925.13**	4,634.84 ± 747.75	19,253.99 ± 2485.95	361.5679.72	**130.72 ± 28.22**
Control site	**12,633.47 ± 1617.59**	2,332.93 ± 186.43	3,247.76 ± 300.63	331.48 ± 48.83	**74.97 ± 5.36**

Element concentration (mg kg^{-1})

Location	Mn	Fe	Cu	Zn	As	Ba	Pb
La Greda	**591.21 ± 146.36**	39,406.86 ± 4044.30	**815.87 ± 355.28**	160.55 ± 26.69	**53.80 ± 24.36**	78.99 ± 11.96	**82.24 ± 36.51**
Los Maitenes	**617.71 ± 41.95**	36,454.41 ± 15,517.98	**293.20 ± 365.56**	93.06 ± 60.68	**24.76 ± 22.82**	49.08 ± 4.71	38.80 ± 46.55
Puchuncaví	**800.74 ± 156.53**	29,853.73 ± 6348.26	**81.88 ± 19.47**	62.26 ± 10.52	9.83 ± 1.87	92.23 ± 9.57	23.83 ± 3.97
Valle Alegre	**909.61 ± 83.30**	41,921.12 ± 3571.85	**82.63 ± 16.68**	75.46 ± 8.46	6.60 ± 0.77	86.51 ± 6.37	18.70 ± 5.93
Costa Quilén	**746.05 ± 95.63**	58,912.02 ± 10,933.45	39.59 ± 6.59	113.07 ± 14.28	12.51 ± 1.26	71.78 ± 4.94	29.32 ± 2.34
Control site	**864.67 ± 213.34**	29,875.28 ± 1914.84	42.46 ± 2.68	32.29 ± 3.00	< 6	48.44 ± 11.54	7.46 ± 0.92

The results marked in bold correspond to the values that exceed the norms

The physical–chemical and elemental concentration analyses of the different soil samples showed that the soil from La Greda, the locality closest to the industrial complex in the area (1.8 km), had a higher concentration of heavy metals, both copper, arsenic and lead. It was observed that as the locality becomes more distant from the industrial zone, the concentration of copper, arsenic and lead decrease (Table 2).

According to the Australian standard, all soils showed manganese and vanadium contamination (values exceeding the limits of the standard), while only Costa Quilén showed vanadium contamination according to the Canadian standard. None of the soils under study showed lead, barium, or zinc contamination.

The soils sampled were characterized by being mainly sandy loam. Unlike clay soils, sandy soils lack binding capacity and can lead to pollution at the phreatic level (Tahir & Marschner, 2016). It was observed that the soils sampled have low organic matter content, except for the control site with high values. Generally, a low cation load and a low percentage of organic matter were observed, except at the control site, where both values were increased.

Characterization of fungal strains

Hundred and thirteen filamentous fungi strains were isolated from the different soil samples, of which 22 corresponded to native isolates from La Greda soil (LG), 21 from Valle Alegre soil (VA), 20 from Puchuncaví soil (PU), 15 from Los Maitenes soil (LM), 22 from Costa Quilén soil (CQ) and 13 from Sitio Control (CS).

The growth of each strain in the presence of a heavy metal concentration curve carried out in 24-well plates (liquid system), allowed to preselect those that showed high tolerance to soil leaching (Fig. SP.III.3). The in vitro copper tolerance test (0, 64, 127 and 200 mg kg^{-1} Cu) on PDA medium performed with these fungal strains showed a decrease in mycelial growth rate and coloration in some mycelia as the copper concentration increased (Fig. 2). However, all strains had tolerance to high copper concentrations since in most of the cases, fungal growth was not affected at 64 mg kg^{-1} (1 mM) (Ezzouhri et al., 2009; Văcar et al., 2021), being considered potential metal accumulators. All the growth percentages recorded after 5 days of incubation in PDA medium (200 mg kg^{-1} Cu) were higher than 40%. The mycelial growth of strains LM01A, LM10 and CQ23 stood out with 100% growth (Fig. 3).

Twelve isolates were identified based on morphological characterization and by partial sequencing of ITS1–ITS2 region, showing between 76 and 100% similarities to NCBI database entries. The analysis showed an affiliation of sequences to *Mortierella*, *Fusarium*, *Clonostachys*, *Trichoderma, Trichocladium and Microphaeropsis* species; all of them with agronomic importance (Table 3).

From the heavy metal tolerance tests (Figs. 2 and 3), it was possible to select putative strains of *Mortierella elongata* (LG01), *Trichoderma harzianum* (LM01A), *Trichoderma asperellum* (LM10), *Clonostachys solani* (CS12A), *Clonostachys rosea* (CQ23) and *Fusarium solani* (VA12B), due to their high tolerance to copper and other metals present in soil leachates.

In vitro *evaluation of copper removal capability of fungal strains*

From the first isolation of 113 fungal strains from the soils under study, 12 strains showed high tolerance to heavy metals. Tolerance to heavy metals is defined as the capacity of an organism to grow in the presence of metals and survive their toxicity (Anahid et al., 2011). Based on the above, several of the strains isolated from the Puchuncaví-Ventanas area are tolerant. In the case of copper, most of the organisms use Cu^{2+} as an essential element for their growth and development. However, this is toxic to most microorganisms, even at small concentrations, promoting strains to develop mechanisms to survive in that environment (Binsadiq, 2015).

In this study, the tolerance of each fungal strain was evaluated in the presence of increasing concentrations of copper on PDA medium plates. From the twelve preselected strains, six strains of different fungal genera showed better growth in increasing concentrations of heavy metals and copper, which were identified as putative *Mortierella elongata*, *Trichoderma harzianum*, *Trichoderma asperellum*, *Clonostachys solani*, *Clonostachys solani* and *Fusarium solani*. The isolation of various fungal species from soils contaminated with heavy metals has been reported (Liaquat et al., 2020; Mohammadian et al., 2017; Oladipo et al., 2018). Fungi such as *Penicillium*, *Aspergillus niger*, *Trichoderma harzianum*, *Komagataella phaffi (Pichia pastoris)* and *Phanerochaete* have been reported for their ability to efficiently remove heavy

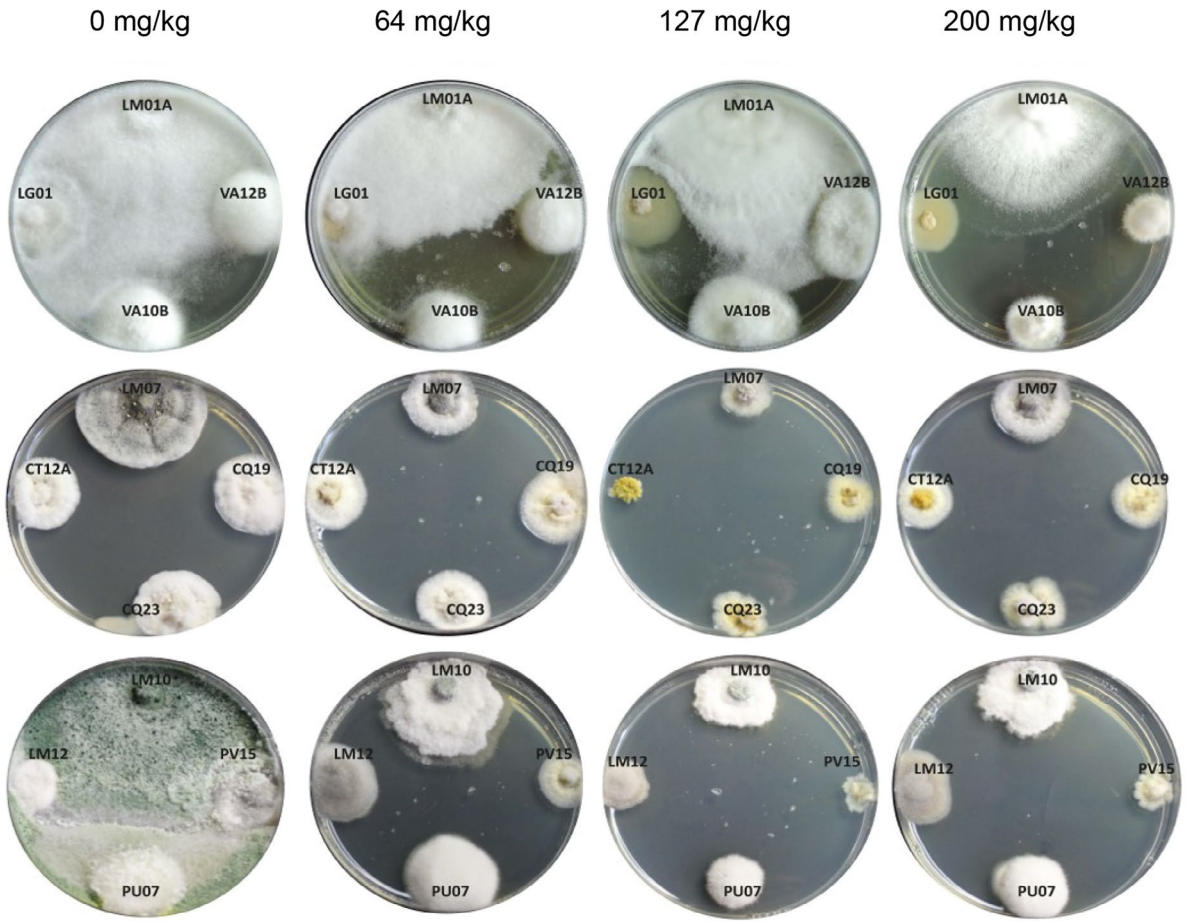

Fig. 2 Growth of preselected cells at increasing copper concentration. The first column corresponds to the control (growth in PDA without copper sulfate). Columns to the right correspond to increasing copper concentrations (64, 127 and 200 mg kg^{-1}). In each row, four different strains are visualized on each plate. The test was performed in triplicate

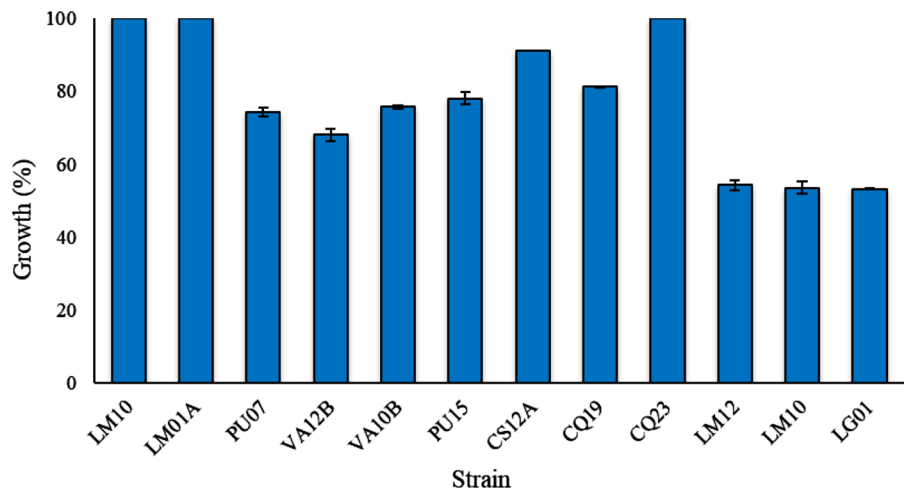

Fig. 3 In vitro tolerance of fungal strains in the presence of 800 mg kg^{-1}copper sulfate (equivalent to 200 mg kg^{-1} Cu) (strain selection screening). Growth (%) measured at 5 days of incubation, was calculated in relation to the control without copper for each strain. All values represent mean value of triplicate measurements ± SD

Table 3 Identification of fungal strains isolated from soil by ITS1–ITS2 sequence analysis

Soil sample	Strain	Hit blast	Identity (coverage) %	GenBank accession
La Greda	LG01	*Mortierella elongata*	99 (100)	MH047197.1
Los Maitenes	LM01A	*Trichoderma harzianum*	100 (100)	MT584872.1
	LM07	*Trichocladium asperum*	97 (100)	AY706336.1
	LM10	*Trichoderma asperellum*	100 (100)	MT529846.1
	LM12	*Microsphaeropsis* sp.	99 (91)	MN153956.1
Puchuncaví	PU07	*Fusarium* sp.	81 (93)	AF289654.1
	PU15	*Clonostachys byssicola*	100 (91)	LT220545.1
Valle Alegre	VA10B	–	–	ND
	VA12B	*Fusarium solani*	100 (100)	MT560338.1
Costa Quilén	CQ19	*Clonostachys rosea*	76 (91)	MT588112.1
	CQ23	*Clonostachys rosea*	99 (93)	MH047188.1
Control site	CS12A	*Clonostachys solani*	100 (100)	MH855181.1

ND Not determined

metals (Iram et al., 2013; Liaquat et al., 2020). Fungi have become predominant organisms in heavy metal contaminated environments and have revealed their tolerance to heavy metals. The presence of melanins (a dark pigments) in fungi located in the fungal cell wall can decrease the noxious effect of heavy metals (Liaquat et al., 2020). In this study, not only the growth retardation of some fungal strains analyzed was evidenced, but also the coloration of the mycelium was observed when they were in the presence of copper. This could account for the production of this compound, present in most fungi, as a response to exposure to harmful elements. Further analysis of the presence of melanin in these strains will be required in future work.

In this study, fungal strains isolated from heavy metal contaminated soils could remove copper from the medium on plates ranging from 84% (*M. elongata*) to 24% (*T. asperellum*), when copper removal was evaluated in vitro with 200 mg kg^{-1} copper (maximum concentration tested). This difference may be due to the mechanisms of toxicity and resistance to heavy metals are variable among genera and species (Binsadiq, 2015). Relative copper content in mycelium for each selected fungal strain is shown in Fig. 4. The highest removal percentages were obtained with *M. elongata* (LG01; 84%), followed by *C. rosea* (CQ23; 49%), *C. solani* (CT12A; 40%), *F. solani* (VA12B; 39%), and the lowest removal with *T. asperellum* (LM10; 24%) (Fig. 4). Based on these results, two strains were selected for copper removal kinetics: *M. elongata* strain LG01, since it presented the best copper removal results and *T. harzianum* strain LM01A, which presented high removal capability together with *C. rosea* (CQ23) but its growth in PDA medium with copper sulfate was the fastest.

A symptom of heavy metal toxicity is mycelial growth inhibition, along with changes in mycelial morphology. Some filamentous fungi, such as *Trichoderma* spp., have shown unusual hyphal morphologies at concentrations of 203 mg kg^{-1} copper (De Padua, 2021). In this study, the 6 selected strains showed a decrease in growth rate and less sporulation in culture medium with 800 mg kg^{-1} copper sulfate (equivalent 200 mg kg^{-1} Cu). However, in seven days, *T. asperellum* strain LM10, *T. harzianum* strain LM01A and *C. rosea* strain CQ23 showed 100% growth with respect to the control (PDA medium without copper), while the strain most affected by copper sulfate toxicity was *M. elongata*, reaching a percentage of 56% growth. Kumar and Dwivedi (2021) reported that fungus *T. lixii* CR700 also removed Cu^{2+} (100 mg kg^{-1}) by intracellular sequestration/accumulation mechanism in addition to extracellular surface sorption mechanism. Organisms accumulate the metal from outside to inside the cell via active (metabolic dependent) or passive mechanisms. Metal can enter the cell through ion exchange, as a passive mechanism, while in the case of active transport, it is driven by many types of transporting agents which are called a transporter. In fungi, many types of transporters have been reported for the transport of the heavy metals from outside to inside of the cell or from periplasm to cytoplasm or from cytoplasm to vacuoles or from inside to outside of the

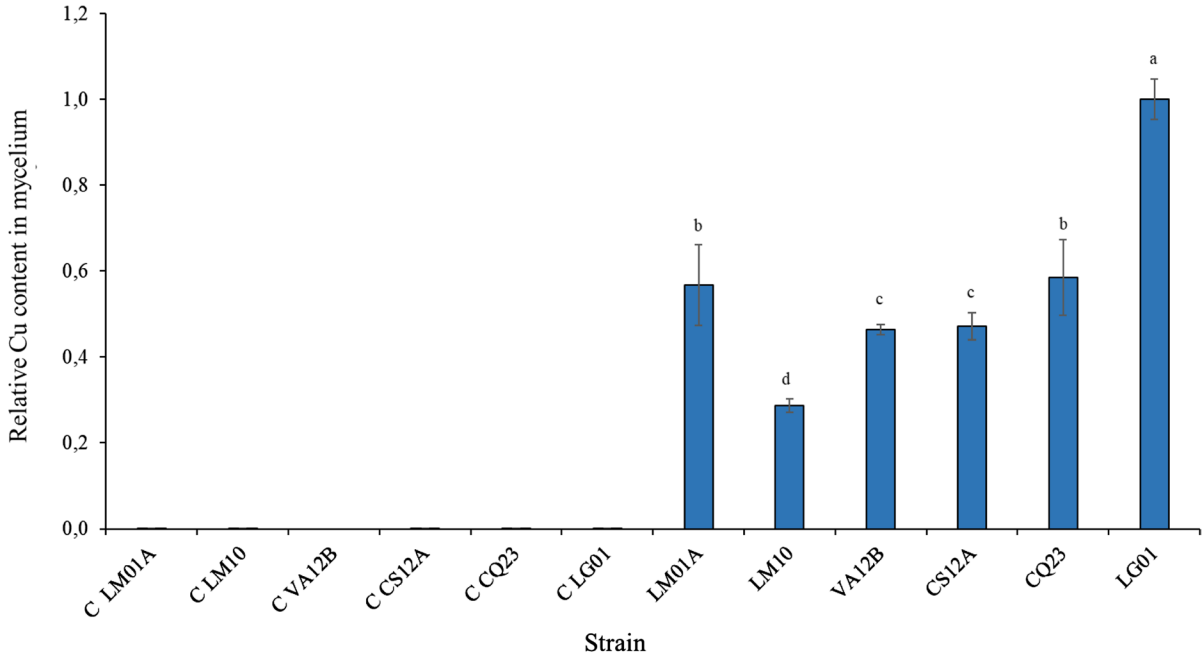

Fig. 4 In vitro copper removal. Prefix C corresponds to the control of each strain. LM01A: *T. harzianum*, LM10: *T. asperellum*, VA12B: *F. solani*, CS12A: *C. solani*, CQ23: *C. rosea* and LG01: *M. elongata.* Plates were incubated at 24 °C for the necessary time until the maximum mycelial diameter reached by the strain with the lowest growth rate. All values represent mean value of triplicate measurements ± SD

cell (in case of efflux pump) (Kumar & Dwivedi, 2021). Copper transporters responsible for transferring copper from the outside to the inside of cells have been described (Puig & Thiele, 2002), where they can subsequently bind to metal chelating proteins (e.g., phytochelatin and metallothionein). Once inside bioaccumulation can occur, the metal ions are precipitated in different cellular organelles

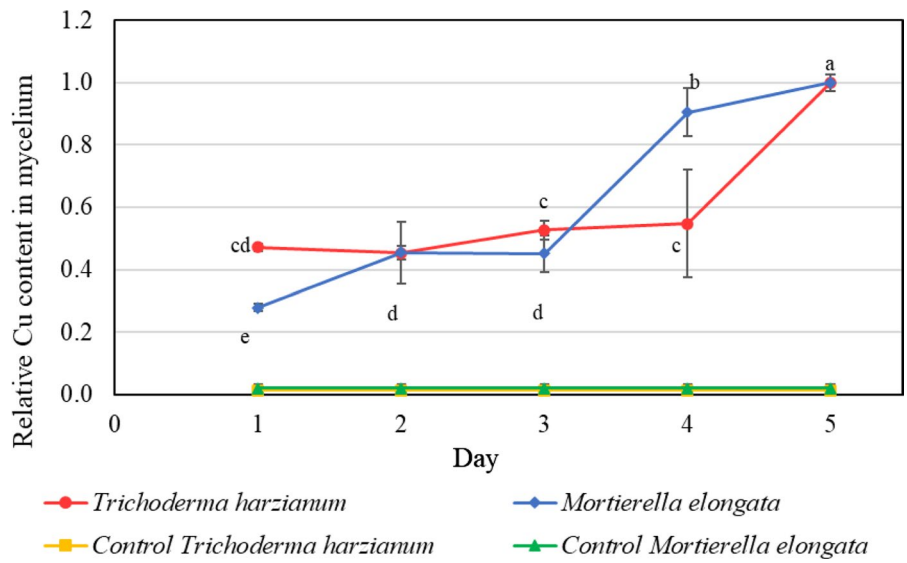

Fig. 5 In vitro copper removal kinetics of *T. harzianum* strain LM01A and *M. elongata* strain LG01 fungi in presence of copper sulfate (800 mg kg^{-1}, equivalent 200 mg kg^{-1} Cu). Control corresponds to the fungus without copper. Lowercase letters indicate significant differences between the different days of copper removal. All values represent mean value of triplicate measurements ± SD

(compartmentalization), thereby generating the non-toxic form of metal ions. Compared to biosorption, bioaccumulation is feasible only in live cells and is thus a metabolism dependent process (Feng et al., 2017; Rhee et al., 2016; Priyadarshini et al., 2021). Overexpression of many types of proteins, including heat shock protein, transporter protein, etc., was reported, which may aid in Cu^{2+} detoxification and accumulation inside the cell of Penicillium janthinellum strain GXCR (Feng et al., 2017).

In vitro *copper removal kinetics*

Figure 5 shows the kinetics of copper removal by *T. harzianum* strain LM01A and *M. elongata* strain LG01. A higher initial removal rate was observed for *T. harzianum* strain LM01A. *M. elongata* strain LG01 tended to increase the removal of copper at longer incubation times. The highest copper removal was observed on the fifth day, with values close to 155 mg kg^{-1} copper measured in the mycelium (77.5% removal).

The radial growth rate (RGR) was determined using a linear model obtaining Control_LM01A (1.1 mm/h), Control_LG01 (0.6 mm/h), Copper_LM01A (0.6 mm/h) and Copper_LG01 (0.3 mm/h). Using the Gompertz model, the maximum specific growth rate (μ_{max}) of Control_LM01A (0.24 h^{-1}), Control_LG01 (0.07 h^{-1}), Copper_LM01A (0.10 h^{-1}) and Copper_LG01 (0.03 h^{-1}) were determined.

Based on the high growth rate and the beneficial characteristics of *Trichoderma* spp. in agriculture, *T. harzianum* strain LM01A was selected to perform the ex situ treatment of contaminated soil. The strain LM01A showed the highest growth in culture medium in presence of copper sulfate (800 mg kg^{-1}, equivalent 200 mg kg^{-1} Cu) and the highest copper removal after 5 days. *Trichoderma* is a good candidate to conduct bioremediation processes of heavy metals, due to its adaptability, its high growth rate in different conditions and the benefits it brings to soils. The high growth rate of strain LM01A, under these conditions, would facilitate its establishment in the soil with a higher mycelial biomass production and a higher bioremediation capability.

The removal of heavy metals by cells involves a first stage of biosorption (Tejada-Tovar et al., 2015), which is a fast process that occurs up to 4 h (Bishnoi, 2005). In a second step, the contaminants are transported into the cell by a membrane transport system (Tejada-Tovar et al., 2015). During the lag period or early stages of cell growth, the biosorption of metal ions increases and then decreases when the cultures reach the stationary phase (Bishnoi, 2005). Several filamentous fungi have been isolated and evaluated for their heavy metal biosorption capability for their potential application in Cu waste bioremediation. *Trichoderma*, *Penicillium* and *Aspergillus* strains have Cu biosorption capability compared to other fungal genera such as *Geotrichum*, *Monilia* and *Fusarium* (Dusengemungu et al., 2020).

In this study, *T. harzianum* strain LM01A and *M. elongata* strain LG01 were selected due to their high copper removal capability and their copper removal kinetics were analyzed. *T. harzianum* strain LM01A showed a relatively constant removal during the first four days of growth in the presence of copper sulfate, increasing considerably on the fifth day. In contrast, *M. elongata* increased its removal daily that correlates with fungal growth. A removal of close to 155 mg kg^{-1} of copper in five days (77.5%) by both strains was observed. The removal kinetics differs between the two strains, which could indicate that they have different mechanisms for interacting with the copper present in the culture medium.

Biosorption is a rapid process dependent on the age of the cells, increasing during the early stages of growth (Bishnoi, 2005). Bioaccumulation is an active, slow and more energy-consuming process. Bioaccumulation occurs when soluble metal ions are transported between the cell membrane and the cell (metabolism-dependent intracellular uptake), resulting in solid particle accumulation or precipitation in vacuoles (Dusengemungu et al., 2021; Tejada-Tovar et al., 2015). The rigid cell wall of fungi, as well as their branched filamentous growth type, is features that convert fungi into efficient decomposers and attractive candidates for heavy metal biosorption (Dusengemungu et al., 2020). One of the advantageous features of the fungal cell wall is the presence of chitin, chitosan and its deacetylated derivative, N-acetyl D-glucosamine polymer, which improves metal uptake and binding (Araújo et al., 2020; Azmana et al., 2021; Wang et al., 2020). Through mechanisms mediated by wall components, filamentous fungi accumulate metal ions in their mycelium (biomass) and spores (intracellular/biosorption and extracellular/adsorption processes) (Dusengemungu

et al., 2020). These processes are important for fungal life and performance, energy uptake and redox reactions (Igiri et al., 2018; Naveena et al., 2012; Siddiquee et al., 2015).

In this study, *T. harzianum* strain LM01A showed a high growth rate, tending to reach the stationary phase after covering the entire surface of the PDA plate (Itoh et al., 2017; Pinasthika et al., 2018). Here, copper removal occurs by biosorption, reaching equilibrium between sorbate and biosorbent in the first period (Sandoval, 2006). On the fifth day, an increase in removal was observed, which could be explained by bioaccumulation. *M. elongata* showed slower mycelial growth in the presence of copper, with an extended lag phase, allowing a gradual increase in copper removal by biosorption. The radial growth rates allowed to establish the relationship between the different fungal strains exposed to copper and their controls. *T. harzianum* strain LM01A has a high growth rate, both in the control condition and in the condition exposed to copper. Despite the above, the capability of biosorption and bioaccumulation in its initial phase of growth is lower than that presented by *M. elongata* strain LG01. *M. elongata* has a low growth rate but its biosorption and bioaccumulation capability is higher. During the latency phase, both strains undergo a process of adaptation to the medium by the presence of copper. According to the kinetics of copper removal from the medium, *M. elongata* strain LG01 increases copper removal after the third day and *T. harzianum* strain LM01A shows an increase in removal from the fourth day, both reaching very similar values on the fifth day. These results coincide with previous investigations, where the maximum removal capability is observed at the end of the exponential phase and the beginning of the stationary phase (Dusengemungu et al., 2020; Jayaraman & Arumugam, 2014; Maldaner et al., 2020).

Ex situ removal of heavy metals in contaminated soils under laboratory conditions

The physicochemical analysis of the soil showed that the La Greda soil is the only one that exceeds by far the copper concentration limits allowed in the international standards considered in Table 1. It was interesting to perform the ex situ test in the presence of the most contaminated soil to demonstrate the capability of this strain to remove copper. The test was carried out with soil extracted from the locality, with which the ex situ isolated system described in the methodology was elaborated in the presence of the selected fungal strain (putative *T. harzianum* strain LM01A). In this study, the heavy metal removal capability of the *T. harzianum* strain was evaluated 15 and 30 days after its application in the contaminated soil of La Greda (ex situ system). *T. harzianum* strain was used for this assay because of its high growth rate in the presence of copper (200 mg kg^{-1}) and its high in vitro removal capability.

A significant decrease in Ca, Cu, S, Zn and K was observed with respect to the control when the soil

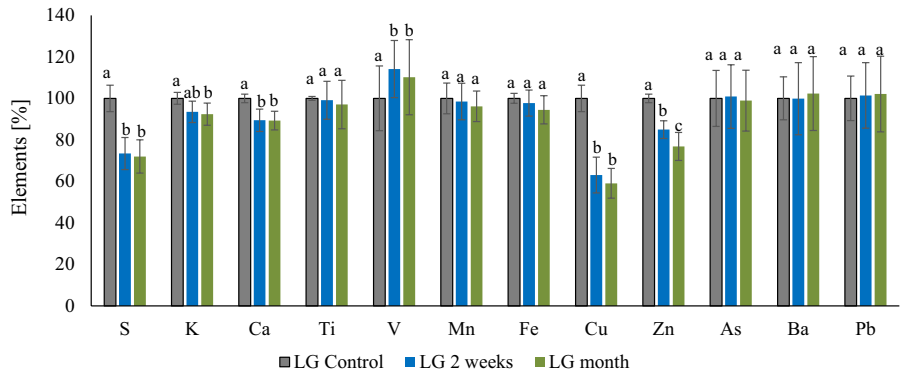

Fig. 6 Element content (%) in La Greda soil treated ex situ with *T. harzianum* strain LM01A. LG Control: corresponds to soil without treatment with the fungus; LG2 weeks: soil treatment with the fungus after two weeks; LG month: soil treatment with the fungus after one month. Lower case letters indicate significant differences between the different time of the treatment for each element

was subjected to the fungus (Fig. 6), showing that the LM01A strain can grow at very high concentrations of these different metals, and it can present different mechanisms of resistance and/or adaptation to toxic/non-toxic metals.

This statistically significant difference was mainly observed during the first two weeks of treatment. After one month of treatment with *T. harzianum* (LM01A), vanadium increased its concentration in the soil by 10%; calcium, sulfur, zinc and potassium decreased by 11, 28, 23 and 4%, respectively, while copper was the most reduced metal in the soil with a decrease of 41%.

Trichoderma spp. actively interact in soil and plant roots, playing an important role in the environment by facilitating the decomposition of organic wastes, the biodegradation of industrial chemicals and the bioremoval/bioaccumulation of several heavy metals from wastewater and soil; this is due to the unique characteristics of their cell wall, composed mainly of glucan and chitin polymers (Akhtar & Mannan, 2020; Nongmaithem et al., 2016).

Between two weeks and one month of soil treatment, no significant differences in these elements were observed. This could be due to the observed rapid growth of the *T. harzianum* strain on the solid support, which promotes maximum accumulation in fungal biomass within two weeks.

In this study, the ability of *T. harzianum* strain LM01A to remove 41% of copper from contaminated soil under ex situ conditions in two weeks was determined. Kumar and Dwivedi (2021) showed that fungus *T. lixii* CR700 also removed Cu^{2+} by intracellular sequestration/accumulation mechanism in addition to extracellular surface sorption mechanism in five days (in vitro liquid culture in PBD medium). *T. lixii* CR700 showed efficient removal ability for Cu^{2+} and almost half of the Cu^{2+} removal is contributed by surface sorption mechanism. Kumar and Dwivedi (2021) reported accumulated values for *T. lixii* CR700 of 0.51, 1.71 and 1.72 mg/g of dry biomass at the concentrations of 10, 50 and 100 mg/L, respectively. Long et al. (2017) reported 2.8 mg/g dry biomass Cu accumulation in *Aspergillus oryzae* G15, respectively, at 200 mg/L Cu^{2+} concentration. Cu can also be transported or diffused through copper carriers (Peña et al., 2000; Puig & Thiele, 2002), being able to bind to metal-chelating proteins before being compartmentalized in a vacuole or precipitated by the presence of intracellular organic acids (Feng et al., 2017; Rhee et al., 2016; Sutherland & Stillman, 2014). Feng et al. (2017) reported on different types of proteins that can collaborate during the detoxification and accumulation process of copper within the cells of *Penicillium janthinellum*. strain GXCR.

In the case of Zn, there was a gradual decrease in soil concentration, relative to the untreated control, between two weeks and one month of treatment. The bioaccumulation of this element in *T. harzianum* is notoriously slower than the others and did not exceed the reduction observed with copper (Gadd, 2010). El Sayed and El-Sayed (2020) reported that the bioaccumulation of this element in *T. harzianum* is notoriously slower than that of the abovementioned elements and did not exceed the reduction observed with copper. El Sayed and El-Sayed (2020) reported the bioaccumulation process of *F. solani* in response to Zn(II), indicating that pH influences biomass and binding site activities. Biomass is considered a natural ion-exchange material having positively and negatively charged groups. It is also found that increasing pH above 4 limits the competitive effects of hydronium ions and favors proton exchange with Zn(II). Hartikainen et al. (2012) studied the tolerance of saprotrophic fungi (ascomycetes, zygomycetes or basidiomycetes) to copper and zinc and indicated that soil microbial functioning is more vulnerable to Cu than to Zn in soil, establishing a relationship in the bioremediation process of these two elements together. Genes involved in Cu resistance in yeasts include the metallothionein protein CUP2, which is a Cu-binding factor, and CUP1, which binds both Cu and Zn. Hartikainen et al. (2012) reported that Cu is more toxic than Zn to ascomycete and basidiomycete fungi. Therefore, Cu may have a greater effect than Zn on competition between fungal species and on the structure of fungal communities in contaminated soil.

In relation to vanadium, an increase in this element in the soil of 10% was observed after one month of treatment. Vanadium is a metal widely distributed in nature, being an essential part of biological systems (Crans & Kostenkova, 2020; Imtiaz et al., 2015), since there are enzymes, whose active center is composed of this element (Contreras-Cadena et al., 2014). Xu et al. (2019) reported fungal accumulation of V in a medium amended with V oxides, demonstrating the ability of fungi to solubilize V compounds in solid phase, thus introducing previously immobile V into

the biogeochemical cycle and food chain. Furthermore, in the cell, vanadium has affinity for phosphate, carboxyl, and amino groups (Del Carpio et al., 2018; Rehder, 2015), so the fungus can use mechanisms of expulsion of this metal to allow these functional groups to bind to the other elements by biosorption.

Numerous reports have demonstrated that in heavy metal polluted environments, microbial populations develop the ability to adapt to the high contamination levels (Coelho et al., 2020; Narendrula-Kotha & Nkongolo, 2017). In this study, it was determined that filamentous fungi isolated from areas contaminated with heavy metals could remove mainly copper in culture media. *Trichoderma harzianum* strain LM01A isolated from the Puchuncaví-Ventanas industrial zone can remove metals from contaminated soil. Based on the calculated growth rates and RGR, it is possible to propose that the removal is due to biosorption and bioaccumulation mechanisms. As other strains isolated from the industrial zone were also able to remove copper (*Mortierella elongata* and *Clonostachys rosea*). Future trials will be necessary to corroborate the mechanisms that are occurring with the strains analyzed here.

Conclusions

In this study, the filamentous fungi isolated from the Puchuncaví-Ventanas area showed high tolerance to heavy metals and can accumulate them.

A great diversity of fungal strains was observed in the Puchuncaví-Ventanas soils. In this study, we isolated and characterized fungal strains belonging to diverse genera such as *Trichoderma*, *Clonostachys*, *Mortierella* and *Fusarium*. Several native strains showed tolerance to high concentrations of heavy metals, including copper.

Six selected fungal strains showed in vitro copper removal between 84.3 and 24.2%. According to the mycelial radial growth rates and growth velocities obtained for each strain, it is possible to propose that the mechanisms of tolerance and elimination of heavy metals (biosorption and bioaccumulation) are variable among them. In the in vitro removal kinetics assay, a removal of copper (155 mg kg^{-1}) was achieved on the fifth day (77.5% removal) with *T. harzianum* strain LM01A and *M. elongata* strain LG01. After treatment of the contaminated soil of La Greda with *T. harzianum* strain LM01A, removal of Cu, S, Zn, K and Ca was observed, whereas vanadium increased its concentration. The results obtained show that the strains of filamentous fungi studied here have the capability to remove elements and may be used for improving contaminated soil. Due to their vegetative features, filamentous fungi are among the most biofriendly and low-cost biosorbents.

The application of filamentous fungi for bioremediation is an environmentally friendly, effective, and low-cost alternative due to its low technological requirements. Given that some of the selected strains belong to beneficial fungal genera used in agriculture, such as *Trichoderma*, *Clonostachys* and *Mortierella,* they could be applied as an improver treatment for contaminated soils by industrial activities and/or agricultural practices that contribute with high concentrations of metals to the soils, e.g., smelter and refineries, phosphorus fertilizers, copper sulfate or other pesticides. Copper accumulation in these beneficial fungi shows their potential to remove Cu, reducing its impacts on environmental and human health.

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Author contributions All authors contributed to this work. MC, XF and FC conceived the ideas, designed the research and Funding acquisition; PJ, AVer, FD, MO, DM, FJ, XF, MC and AVel performed the experiments and analyzed the data; and XF, MC, PJ, MS and EL wrote the manuscript with feedback from all co-authors.

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Data availability The data in the article are available.

Declarations

Conflict of interest All the authors declare that they have no conflict of interest.

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