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Characterization of copper stress response in *Fusarium tricinctum* M6: A metal-resistant microorganism isolated from an acid mine drainage-affected environment

José Oscar Bonilla[a,b], **Eduardo Alberto Callegari**[c], **María Daniela Paez**[c], **Raúl Andrés Gil**[a,b], **Liliana Beatriz Villegas**[a,b,*]

[a]Instituto de Química San Luis (INQUISAL), CONICET, Chacabuco 917, 5700 San Luis, Argentina

[b]Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis, Chacabuco 917, 5700 San Luis, Argentina

[c]Division of Basic Biomedical Sciences Sanford School of Medicine, University of South Dakota, Vermillion, SD, USA

Abstract

Acid mine drainage-affected environments are interesting microbial niches for the isolation of metal-resistant microorganisms. In this sense, the aim of the present work is to isolate and characterize metal-resistant microorganisms from sediments of an abandoned gold mine located in San Luis (Argentina). For these purposes, the metal removal capacity and the microelemental composition of the biomass exposed to metals were evaluated. Likewise, proteomic techniques were applied to understand the removal and resistance mechanisms. Fusarium tricinctum M6 was isolated and identified as tolerant to Cu(II), Fe(II) and Cr(VI). When faced with 40 μg mL^{-1} Cu (II), the growth was affected by 60% and the removal capacity was 30–35%. Copper was found uniformly distributed in the biomass (5.23% w/w) and variations in the proportion of other biomass constituent elements were detected. When exposed to Cu(II), F. tricinctum M6 showed differential expression of intra and extracellular proteins involved in different metabolic processes. A large number of proteins with metal ion binding sites were detected both at intra and extracellular levels. The results obtained in the present work indicated bioadsorption of the metal on the cell surface and an important readjustment of the protein expression to counteract the stress produced by Cu(II).

*Corresponding author at: Instituto de Química San Luis (INQUISAL), CONICET, Chacabuco 917, 5700 San Luis, Argentina., lbvilleg@hotmail.com, lbvillegas@unsl.edu.ar (L.B. Villegas).

CRediT authorship contribution statement

José Bonilla: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Visualization. **Eduardo Callegari**: Conceptualization, Validation, Formal analysis, Investigation, Data curation, Writing - review & editing. **María Daniela Paez**: Validation, Formal analysis, Investigation, Data curation, Writing - review & editing. **Raúl Gil**: Funding acquisition, Resources, Conceptualization, Supervision, Project administration. **Liliana Villegas**: Methodology, Funding acquisition, Resources, Conceptualization, Supervision, Project administration, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2021.125216.

Keywords

Acid mine drainage; Metal-resistance; *Fusarium*; Intracellular proteomics; Extracellular proteomics

1. Introduction

Despite the benefits that mining activities have for modern human life, throughout history mining has caused significant pollution problems in the sites where operations have been developed. The term Mining Environmental Liabilities (MEL) arose in Latin America and refers to the environmental impacts generated by abandoned mining operations where a regulated and certified mine closure was not carried out (Oblasser and Chaparro Ávila, 2008). One of the most important and documented environmental problems associated with mining activities is the production of Acid Mine Drainage (AMD) (Méndez-García et al., 2015). Acidic water in these environments is produced from oxidative dissolution of sulfide ores that face with oxygen, water, and endogenous microorganisms (Johnson and Hallberg, 2003; Baker and Banfield, 2003). AMD-affected environments present high metal concentrations when compared to other unaffected environments (Fadiran et al., 2014; Bonilla et al., 2018).

Soils and sediments are known as a sink for metals released into the environment from natural or anthropogenic sources (Azarbad et al., 2016). Throughout history, the inherent buffering capacity of soils and sediments when receiving these toxic contaminants has been demonstrated. In these matrices, microorganisms are key biological components for this capacity. Microbial populations of soils and sediments can respond to natural environment changes and cope with the presence of toxic substances, mainly through their genetic malleability (Puglisi et al., 2012).

Long-time exposure to metal contamination leads to the adaptation of microbial communities to survive and persist in contaminated environments. In this sense, microorganisms have developed sophisticated and specific cellular machineries consisting of an extensive network of specialized proteins, transporters and/or involved in gene expression regulation, which respond to both the deficiency and the excess of metals (Ma et al., 2009; Solioz et al., 2010). These cellular mechanisms for maintaining the optimal concentration of metals are known as homeostasis. Microorganisms play an important role in the uptake of metals from the environment by using a multiplicity of homeostatic mechanisms. These mechanisms differ among the different genera and/or microbial species and relatively little is known about them at the molecular level.

The knowledge of the adaptation of different organisms to stress situations, such as the presence of toxic concentrations of metals, is essential to develop strategies for environmental sanitation and treatment of contaminated sites. A very interesting way to study the relation between metals and resistant microorganisms can be Proteomics. Proteome is defined as the total set of proteins expressed by a genome in a cell, tissue or organism at a certain time and condition (Blackstock and Weir, 1999). The proteome is

important not only for analyzing the cell behavior, but also for closing the gap between the organisms' genome and metabolites with a holistic perspective.

Comparison of protein expression profiles obtained under stress conditions caused by the presence of metals, compared to controls without toxic compounds, is useful for the detection of specific changes in the proteome against these conditions (Bonilla et al., 2016). The identification of proteins induced or repressed by the presence of toxic concentrations of a contaminant guide conclusion on the molecular mechanisms involved in the response and removal of metals by resistant microorganisms.

In San Luis (Argentina), there is an abandoned gold mine called "La Esperanza". After its exploitation, the galleries and facilities were abandoned without an adequate closure process, as established by the current legislation, constituting MEL. According to our previous results, the drainage released from this mine possesses AMD characteristics, showing extreme pH values (2.94 ± 0.03) (Bonilla et al., 2018). These results confirm the persistence of the acidic characteristics of the drainage previously reported by Tripole and Corigliano (2005) and Tripole et al. (2006). The water flows into the La Carolina stream, which is used for recreational and tourist activities. The drainage acidifies the stream and increases its sulfate, Zn, Cd and Te concentrations. In the sediments inside the mine, high concentrations of dangerous metals such as Cr, Fe and Cu are detected, some of the most common metals found in environments affected by mining activities, which show biological toxicity when present in high concentrations. Likewise, both the prokaryotic and the eukaryotic community structures in the area are significantly affected. (Bonilla et al., 2018). For these reasons, this work proposes the AMD-affected environment located in San Luis (Argentina) as an interesting microbial niche for the exploration of metal-resistant microorganisms, capable to grow under simple laboratory conditions and with potential application in bioremediation processes, to study their resistance mechanisms at the molecular level.

2. Experimental

2.1. Isolation and selection of metal-resistant microorganisms

For the isolation of metal-resistant microorganisms, a total of 28 sediment samples were collected in sterile flasks from the La Carolina stream bed and from inside the abandoned gold mine, following the scheme specified in our previous studies (Bonilla et al., 2018). Samples were kept at 4 °C before processing. The isolation and initial maintenance of the microorganisms were carried out on the modified Yeast Extract-Glucose Medium (EG*) (g L^{-1}: Glucose 10.0, Yeast Extract 1.0, K_2HPO_4 0.5, KH_2PO_4 0.5, Agar 15.0).

Isolation was carried out through a sequential enrichment method (Perez Silva et al., 2008), using 1% glucose as the unique carbon source. As selection pressure, Fe(II), as $FeSO_4.7H_2O$; Cu(II), as $CuSO_4.5H_2O$; and Cr(VI), as $K_2Cr_2O_7$, were added in increasing concentrations from 1 to 5 μg mL^{-1}. The isolates were kept at 4 °C and were lyophilized for their conservation.

The agar diffusion technique was used to pre-select the microorganisms capable to grow in the presence of Fe(II), Cu(II) and Cr(VI) at different concentrations: 100, 250, 500, 750 and 1,000 μg mL^{-1} (Villegas et al., 2004). The plates were incubated at 30 °C for 72 h. Tolerance to the metals was determined by measuring the diameter of the growth inhibition halos around the wells. As control, 50 μL of sterile bidistilled water was used. The assay was carried out in triplicate.

2.2. Minimum inhibitory concentration (MIC)

The Minimum Inhibitory Concentration (MIC) of the metals in liquid medium was performed through a cell viability test modified in our laboratory, using Resazurin dye as indicator of cell viability (Borra et al., 2009). In this work, MIC was defined as the metal concentration interval comprised between the maximum concentration in which cell viability was observed and the minimum concentration in which viability was not detected. For the analysis, concentrations of Fe(II), Cu(II) and Cr(VI) from 15 to 500 μg mL^{-1} were added to 10 mL of culture, containing an initial concentration of 1×10^4 cells mL^{-1} (Rex, 2008). Cultures were incubated during 120 h at 30 °C and 200 rpm.

At the end of the culture time, the cell pellets were washed twice with sterile bidistilled water, resuspended in 1 mL EG* medium and 5 μL of a Resazurin aqueous solution (1 g L^{-1}) sterilized by filtration were added. Finally, pellets were incubated at 30 °C and 200 rpm for 2 h to observe the color variation results. Each metal concentration was evaluated in triplicate.

2.3. Microbial molecular identification

Fusarium tricinctum M6 was identified by PCR amplification and sequencing of different genomic regions. Two pairs of specific eukaryotic primers were used to carry out the amplification: ITS1 – 5.8 S rDNA complete sequence - ITS2 – 28 S rDNA partial sequence (ITS1 F: 5′-TCCGTAGGTGAACCTGCGG-3′; ITS4 R: 5′ TCCTCCGCTTATTGATATGC-3′) and Translation Elongation Factor 1-alpha (EF1 F: 5′-ATGGGTAAGGARGACAAGAC-3′; EF2 R: 5′-GGARGTACCAGTSAT-CATGTT-3′).

The PCR protocol using primers ITS1/ITS4 consisted of a first denaturation cycle at 95 °C for 5 min, followed by 30 cycles of 95 °C for 60 s, 55 °C for 60 s and 72 °C for 90 s. Finally, an elongation cycle was carried out at 72 °C for 7 min. Furthermore, the protocol for amplification using primers EF1/EF2 consisted of one denaturation cycle at 95 °C for 5 min, followed by 35 cycles of 95 °C for 35 s, 54 °C for 30 s, and 72 °C for 90 s. Finally, an elongation cycle was carried out at 72 °C for 7 min

The DNA concentration in the PCR products was determined using Epoch (Biotek) and the integrity of the samples was evaluated through 1% Agarose gel electrophoresis. The PCR products were sent to Macrogen (www.macrogen.com; South Korea) for their purification and sequencing. The sequences were edited with Molecular Evolutionary Genetics Analysis (MEGA v6.0) and were analyzed with BLASTn using NCBI databases (www.ncbi.nlm.nih.gov). Additionally, the Barcode Of Life Data System (Bold Systems v4; www.boldsystems.org) was used for the identification from the ITS sequences.

2.4. Determination of cell growth and removal capability

One hundred mL of liquid EG* medium, supplemented or not with 40 μg mL^{-1} Cu(II), were inoculated with an initial concentration of 1×10^6 cells mL^{-1}. Cultures were incubated at 30 °C and 200 rpm during 120 h. Every 24 h, 3 mL of sample was taken periodically. The samples were centrifuged at 11000 *xg* during 10 min to separate the supernatants from the cell biomass.

The supernatants were used to determine the residual copper concentration through inductively coupled plasma mass spectrometry (ICP-Mass ELAN DRC-e). On the other hand, biomass was used for growth kinetics studies by dry weight techniques. The results were expressed in g L^{-1}. All the assays were carried out in triplicate.

Cultures with Cu(II) added but without the inoculums were included as abiotic controls to verify that the components of the culture medium did not participate in the Cu(II) precipitation.

2.5. Microelemental analysis of microbial biomass

Microelemental analysis and surface mapping of the microbial biomass, in the presence and absence of Cu(II), was carried out by Scanning Electron Microscopy coupled to X-Ray Dispersive Energy Spectrometry (SEM-EDS). The analysis was performed at the UNSL Laboratory for Electron Microscopy and Microanalysis (labmem-UNSL, http://labmem.unsl.edu.ar/).

Cultures obtained in the presence and absence of Cu(II) in the culture medium were centrifuged at 4000 *xg* during 20 min at 4 °C (Centrifuge U-320R) to separate the cell pellet from the supernatant. The biomass was washed three times with sterile bidistilled water and the pellets were processed immediately, without prior preservation.

Samples were mounted on aluminum stubs and dried at room temperature for 7 days. The samples were sputter-coated with carbon (SPI metallizer), and were observed and analyzed under a Zeiss LEO 1450 VP SEM Scanning Electron Microscope, operated at 20 kV, coupled to a Genesis 2000 energy dispersive spectrometer (EDS).

2.6. Collection of intracellular proteins

Cells obtained from culture media in the presence and absence of Cu (II) after 36 h of incubation were harvested by centrifugation at 4000 *xg* during 20 min at 4 °C (Centrifuge U-320R). Pellets were washed twice with phosphate buffered saline (mM: NaCl 124; NaH_2PO_4 10; KH_2PO_4 3) and they were kept at −20 °C. Then, cells were frozen using liquid nitrogen and physically broken using a mortar and pestle. The powder was recovered with Tris-Sucrose buffer (Sucrose 11.29 g dL^{-1}; Tris-HCl 1.5 M pH 8.8 3.33 mL dL^{-1}) and centrifuged at 8500 *xg* during 20 min at 4 °C. Supernatants were used as samples of intracellular proteins and were kept at −80 °C. The assays were performed in biological triplicate.

2.7. Collection of extracellular proteins from cell-free supernatants

For collection of extracellular proteins, the supernatants obtained at 36 h in the presence and absence of Cu(II) were filtered with nitrocellulose membranes (Microclar, 0.2 μm). Then, the supernatants were concentrated 20X using Vivaspin Turbo 15 3000 MWCO ultrafiltration devices with polyethersulfone membrane (PES) (Sartorius). Finally, they were stored at −80 °C. The same procedure was carried out with uninoculated EG* liquid medium, to be used as control in the extracellular proteomic analyses.

The total protein content in both intracellular and extracellular samples was determined by Bradford (1976). Finally, the samples were aliquoted by calculating the necessary volume to obtain 300 μg of intracellular proteins and 25 μg of extracellular proteins. The calculated volumes were lyophilized. The collection of the extracellular proteins was performed in triplicate.

2.8. Shotgun proteomic analysis (nanouhplc-esi-ms/ms)

Lyophilized samples containing intra and extracellular proteins were reconstituted in a solution to achieve a final concentration of 50 mM Tris-HCl pH 8.0 and 1 μg μL^{-1} of protein concentration. Proteins were reduced and alkylated with DTT (Sigma-Aldrich, Saint Louis, MO) and Iodoacetamide (Sigma-Aldrich, Saint Louis, MO), respectively. Insolution digestion was performed with Trypsin (Promega, Madison, WI). Then, the tryptic peptides were concentrated with a centrifugal concentrator (SpeedVac, Thermo Savant). The analysis was carried out by Ultra Performance Liquid Chromatography on a nano scale (nanoUHPLC), coupled to tandem mass spectrometry (nanoUHPLC-ESI-MS / MS) (Supplementary Material 1).

2.9. Bioinformatic analysis

Bioinformatics tools were used for the analysis of the mass data by searching against Swiss-Prot (SP) and NCBI databases at MASCOT v2.7.1 (www.matrixscience.com, local license). Customized databases with orthologous microorganisms from SP and NCBI were used due to the lack of specific databases available at the repository sites. The analysis was carried out against a database customized by the combination of four *Fusarium* spp.: *F. graminearum*, *F. oxysporum*, *F. pseudograminearum* and *Fusarium* sp. v20190509, 20190214, 20190510, 20190214, respectively (496,327 sequences, 220,659,562 residues).

The comparative study was carried out using ProteoIQ v2.8 (local license). The lists of unassigned peptides were analyzed with protein-BLAST (https://blast.ncbi.nlm.nih.gov) to recover proteins with a homology percentage between 80% and 100%. Additionally, complementary bioinformatics tools (analysis of Gene Ontology, Pathways, String-db, KEGG, Expasy and The Reactome, for example) were used to organize, visualize and interpret the results and their validation.

The extracellular protein-localizations were analyzed by the sub-CELlular LOcalization predictor (CELLO v2.5, http://cello.life.nctu.edu.tw/) (Yu et al., 2004 and 2006). For intra and extracellular proteomic analyses, biological triplicates and analytical duplicates were performed.

3. Results

3.1. Isolation, characterization and identification of metal-resistant microorganisms

Eight microorganisms were isolated following the methodology with selection pressure and pre-selection based on the qualitative tolerance to Fe(II), Cu(II) and Cr(VI) in solid medium, and the most tolerant microorganism was selected. Cell viability assays were carried out on the selected microorganism in the presence of increasing concentrations of the metals. The isolated was able to grow in the presence of 125 μg mL^{-1} Cr(VI) in the culture medium, and was also capable to grow in the presence of up to 90 μg mL^{-1} Fe(II) and 60 μg mL^{-1} Cu(II). Based on these results, the MIC value (μg mL^{-1}) for Fe was stablished in the range $90 < MIC \leq 125$; in the case of Cr(VI), in the interval $125 < MIC \leq 250$; and for Cu(II), at $60 < MIC \leq 90$.

The molecular identification was carried out through PCR amplification of different genomic regions and sequencing of the PCR products. Primers specific for eukaryotic organisms were selected based on macro and microscopic observations, which indicated that the selected isolate was eukaryotic. Using ITS1/ITS4 primers, a 248 bp sequence was obtained (NCBI Accession Number KY596033). From these data, we determined that the microorganism belonged to the *Fusarium* genus (99.2% identity with different species of the *Fusarium* genus). To identify the microorganism at the species level, EF1/EF2 primers were selected, which are widely used for the identification of species belonging to the *Fusarium* genus (Karlsson et al., 2016). In this analysis, a 625 bp sequence was obtained (NCBI Accession Number MN175470). With this additional information, the microorganism was identified at the genus and species level, and was named as *Fusarium tricinctum* M6.

3.2. Growth and removal assays

Firstly, a removal study was carried out by facing the microorganism with each metal under study. For this analysis, we worked with a metal concentration corresponding to 50% of the maximum concentration in which cell viability was observed. The evaluation consisted of determining the total metal concentration through ICP-MS at initial time and after 120 h of culture. These data showed that the selected microorganism did not show the ability to remove Cr(VI) or Fe(II) under the evaluated conditions. However, variations were observed when exposed to copper (data not shown). For this reason, a more in-depth study of the growth and removal capacity was performed by confronting the microorganism with Cu(II) in the culture medium (Fig. 1). The Cu(II) concentration was selected taking into account the MIC values previously stablished. For these assays, 40 μg mL^{-1} Cu(II) were added in the culture medium.

As shown in Fig. 1–A, the presence of Cu(II) inhibited the growth of *F. tricinctum* M6 in approximately 60% in relation to the reference culture, reaching the stationary phase after 48 h of culture. The cell-free supernatants obtained at different times in the presence of the metal were used to determine the remaining Cu(II) concentration. The maximum Cu(II) removal was obtained after 48 h of culture (34.7%) and it kept constant over time (Fig. 1–B).

3.3. Microelemental analysis of microbial biomass in the presence and absence of Cu(II)

SEM-EDS was applied to evaluate the elemental composition analysis of *F. tricinctum* M6 in the absence or the presence of Cu(II). The biomass was obtained at 36 h of culture in the exponential growth phase. This study aimed to determine the cellular distribution of the metal and the effect of the Cu(II) presence on other constituents' elements of the biomass to establish relations among them.

As can be seen in Fig. 2, the SEM images show the toxic effects of Cu (II) on the microbial biomass morphology. For example, significant variations can be observed in the average width of the hyphae. In the biomass obtained in the absence of Cu(II), hyphae showed 3.368 μm as average width, while the hyphae exposed to the metal were about 15% thinner. EDS analyses showed that C, O, P, S, K and Ca were common elements for both conditions. However, in the presence of the metal, the proportion of P was higher (2.58% w/w), while the peaks of K (1.08% w/w) and Ca (0.38% w/w) appeared in lower intensity. In the presence of Cu(II), the Na and Mg peaks disappeared, while N and Cu peaks appear (6.02%, 5.23% w/w, respectively).

The results of the microelemental composition of *F. tricinctum* M6 indicate that the Cu presence affected the homeostasis of other mono and bivalent elements like Na, K, Mg and Ca, may be causing an imbalance that leads to growth inhibition. Likewise, relations between copper and non-metallic elements such as N and P were significant. Moreover, it is important to highlight that a uniform distribution of copper was observed with no accumulation of the metal in the biomass, suggesting that Cu can be adsorbed on the cell membrane.

3.4. Intracellular proteomic analysis

A shotgun proteomic analysis was carried out to evaluate the differential intracellular proteins expression in *F. tricinctum* M6, both in the presence and absence of Cu(II) in the culture medium. In this study, a total of 181 proteins were identified (Fig. 3). As shown in Fig. 3–A, 63 proteins were detected exclusively in cells obtained in the presence of Cu (II), while 41 were identified in the absence of the metal. Likewise, 77 proteins were detected both in the presence and absence of Cu(II).

A semi-quantitative comparative analysis of the relative abundance was performed on the 77 proteins found in both conditions, using a label-free approach through the normalized spectral counts (SpCs) (Fig. 3B). In this analysis, only 17 proteins showed significant differences in their relative abundances, where 14 were over-expressed and three were down-regulated in the presence of the metal (Table 1).

Proteins over-expressed in the presence of the metal were related to proteins responsible for the protein biosynthesis (Bars 1, 3, 7, 11 and 12), nucleic acid-binding proteins (4), carbohydrate metabolism (5 and 8), redox stress indicator proteins (6, 9, 10 and 13) and calmodulin, a Ca (II)-mediated intracellular signaling protein. In addition to calmodulin, phosphoglycerate mutase also presents bivalent metal ion binding sites, such as Mn(II) (Table 1).

Table 2 shows the proteins identified only in the group exposed to Cu (II), which can be grouped according to their cellular functions obtained from Gene Ontology (GO) in proteins involved in: i) Protein biosynthesis (8 proteins), including translational elongation factors, ribosomal proteins and folding proteins; ii) Oxidation-reduction processes (9); iii) Degradation proteins (11), such as proteases, and proteasome and ubiquitination proteins; iv) Nucleic acid binding proteins (7); v) oxidative stress indicator proteins, such as glutathione reductase; vi) Kinases/Phosphatases (3), which can be important for the enzymatic activation or inactivation; vii) Energy metabolism (4); viii) Carbohydrate metabolism (5), as proteins involved in the glycolytic process and the Krebs cycle; and ix) Proteins responsible for post-translational modifications (2) (Fig. 4).

Among the proteins identified in cells grown in the presence of Cu (II), a significant amount of metal ion binding proteins was detected. These proteins generally use metals as cofactors to carry out their cellular functions. Some of these proteins are Inosine-5′-monophosphate dehydrogenase, which uses potassium, and degradation proteins with metalloendopeptidase activity, such as Cys-Gly metallodipeptidase dug1, Mitochondrial-processing peptidase, Peptidase alpha, F-box domain-containing protein and Predicted protein NECHADRAFT_94534. Other proteins that also bind bivalent metal ions are Zn(2)-C6 fungal-type domain-containing protein, Fructose-bisphosphate aldolase, Enolase, S-adenosylmethionine synthase, ATP phosphoribosyltransferase and Cysteine desulfurase. These proteins are involved in a wide variety of cellular processes.

3.5. Extracellular proteomic analysis

The differential expression analysis of extracellular proteins was carried out through shotgun proteomics in cell-free supernatants concentrated at 20X. The supernatants were obtained from cultures of *F. tricinctum* M6 both in the presence and absence of Cu(II) in the culture medium.

In this study, 32 proteins were detected in cells grown in absence of Cu and 22 proteins in the metal exposed cells. However, when the location of the identified proteins was analyzed by CELLO v2.5 (http://cello.life.nctu.edu.tw/), only eight proteins in the control group and six in the Cu(II) exposed group showed a significant probability to be secreted into the extracellular space (Table 3). These results showed that the rest of the identified proteins probably come from lysis or cell disruption, a process that naturally occurs in the microbial cultures.

Some of the extracellular proteins detected in the control cells in *F. tricinctum* M6 are mainly involved in carbohydrate metabolism. Also, proteins with functions not characterized were detected. When analyzing the proteins of the cells exposed to the metal, proteins with the capability to sequester bivalent ions were identified, such as PAD domain proteins, which are capable of binding Ca(II). Proteins with functions in carbohydrate metabolism, such as Beta-xylanase, or involved in oxidation-reduction processes were identified.

Additionally, proteins able to sequester metal ions + 2 were detected in supernatants of cells exposed to Cu(II) with no probability to be secreted into the extracellular space, according to CELLO v2.5. These proteins include RBR-type E3 ubiquitin transferase (Uniprot accession

number A0A3M2SJW4), CCR4-NOT transcription complex subunit 4 (A0A0J9UXE0), Zinc finger protein (A0A2H3RXW3), and uncharacterized proteins CEP52_005114 (A0A428TZU7) and FPSE_03992 (K3VLP0). Notably, these proteins were not identified in the control supernatant of *F. tricinctum* M6 (data not shown). Therefore, they could play an important role in the homeostasis mechanisms of Cu(II).

4. Discussion

In the present work, through a sequential isolation method with selection pressure, a microorganism tolerant to Fe(II), Cu(II) and Cr(VI) was isolated and identified as *F. tricinctum* M6. This microorganism showed Cu(II) removal capacity from liquid culture medium. Numerous works reported the ability of *Fusarium* genus members to tolerate and remove metals. For example, Zhang et al. (2012) isolated *F. oxysporum* from a Pb and Zn mining area in China and this microorganism resisted the presence of 0.8 mM Cu, showing a similar resistance to *F. tricinctum* M6. In other work, *F. oxysporum* MUCL 791 showed Cu(II) removal from aqueous solution (Simonescu and Ferdeş 2012). Likewise, *F. oxysporum* was used for the treatment of sewage sludge and showed removal capacity against Cd, Sr and Cu (Moursy et al., 2015). Most of these members have been isolated from sites with a high contamination level caused by anthropogenic activities, demonstrating that the contaminated sites are promising natural niches for the isolation of metal-resistant microorganisms.

4.1. Microelemental composition of microbial biomass exposed to Cu(II)

The microelemental composition of *F. tricinctum* M6 in the presence and absence of Cu(II) was analyzed through SEM-EDS. This study was carried out at the exponential growth phase to analyze the presence, concentration and distribution of copper in the microbial biomass exposed to the metal and, at the same time, to detect variations regarding the control cultures in the absence of the metal. Peaks corresponding to copper were detected when the microorganism was exposed to the metal. Likewise, a uniform distribution of the metal and morphological variations produced by the presence of Cu(II) were observed.

SEM-EDS has been widely used on metal-resistant microorganisms. For example, Lu et al. (2006) confirmed the presence of Pb, Cd and Cu on the surface of *Enterobacter* sp. J1 and observed morphological changes, obtaining consistent results with those obtained in the present work. Microbial morphological changes in the presence of metal were also detected by Villegas et al. (2009) and Damodaran et al. (2013). Recently, Palanivel et al. (2020) observed cell surface alterations and Cu signal in *Pseudomonas stutzeri* LA3. These authors proposed that the metal peak detection using SEM-EDS is related to cellular surface adsorption rather than to intracellular bioaccumulation.

In the present work, Cu(II) influenced other constituent elements of the microbial biomass. In the presence of copper, *F. tricinctum* M6 presented higher proportion of P. The peak of N was also detected, while a lower proportion of K, Ca, and disappearance of Na and Mg were observed, when compared to the control. Some authors claim that ion exchange mechanisms on the cell surface might be involved in the metal removal mechanisms from aqueous solutions. In this sense, Michalak et al. (2011) demonstrated the superficial exchange of

alkali and alkaline earth metals by metal ions when exposed *Enteromorpha* sp. to Cu(II), Mn(II), Zn(II) and Co(II). In the presence of metals, Cu peaks appeared on the surface of the organism, while the Ca and Mg peaks decreased in intensity, and the K and Na peaks disappeared. The importance of the Cu:Na, Cu:Mg, Cu:K and Cu:Ca relation is clearly observed in *F. tricinctum* M6 exposed to Cu(II). Shinde et al. (2012) also proposed that Ni(II) removal by *Yarrowia lipolytica* involves an ion exchange mechanism on the yeast cell surface. The disappearance of the K peaks was also reported by Xu et al. (2012) when studying the biosorption of Cd in *Penicillium chrysogenum*. In a work published by Salvadori et al. (2014), the presence of copper in *Rhodotorula mucilaginosa* biomass was confirmed and variations in the elemental composition were also observed, since in the presence of the metal, N and P peaks appeared, and Na, Ca and K peaks disappeared.

In the same line of research, Sun et al. (2015) used SEM-EDS in *S. cerevisiae* exposed to copper and demonstrated an important relation between Cu:Na and Cu:K, suggesting that copper adsorption can be related to the K release from the cell surface. These authors also related the N peak appearance with the complex formation between Cu(II) and nitrogenous compounds of the culture medium. In *F. tricinctum* M6, an important relation between Cu:K is also observed, as well as the appearance of the N peak, which can be related to what these authors have stated. A similar conclusion was reached by Sheng et al. (2016) who studied Cd accumulation in *Lactococcus lactis*, indicating that electronegative elements can be responsible for the metal biosorption. Finally, Sivaperumal et al. (2018) exposed the adsorption capacity of Cs by *Nocardiopsis* sp. 13 H, detecting a microelemental imbalance with disappearance of Mg and appearance of Na, P and K.

The works previously mentioned indicate that the metal presence causes an important variation of the microelemental composition in resistant microorganisms. In this way, it can be proposed that electronegative elements such as P and N may be involved in the uptake of metal ions, while an ion exchange with other metals can also occur on microbial surfaces.

4.2. Proteomic analysis

Using gel-free proteomic approach, the present study carried out the differential expression analysis of intracellular and extracellular proteins of *F. tricinctum* M6 in the presence and absence of Cu(II). The proteins were obtained at the exponential growth phase, where the highest metabolic activity is observed. It is important to highlight the methodological challenge represented by the unavailability of the sequenced genome of the isolated microorganism, as well as proteomes or transcriptomes specific databases. One of the alternatives to overcome this difficulty is to use available databases of orthologous microorganisms (Bonilla et al., 2020), as done in the present work. The importance of the integration and the comparison of strengths and limitations of proteomic databases is still debated in different fields of study (Subba et al., 2019).

4.2.1. Differential expression of intracellular proteins in the presence of Cu(II)—

When exposed to high concentrations of copper, *F. tricinctum* M6 showed differential expression of intracellular protein profiles. Similarly, proteins related to protein biosynthesis and carbohydrate metabolism have been found in *Cyanothece* sp. CCY 0110

exposed to Cu(II) (Mota et al., 2015). Chiapello et al. (2015) observed an increased production of chaperones, proteins involved in protein biosynthesis, energy production, carbohydrate metabolism and stress redox in *Oidiodendron maius* against Cd and Zn. All these data coincide with the results obtained in the present work when confronting the microorganism with Cu(II).

In the presence of copper, stress proteins and proteins for misfolded proteins response were detected in *Streptococcus pneumoniae* (Guo et al., 2015). In parallel, Chen et al. (2015) detected proteins related to oxidative stress and detoxification in plants exposed to Cu(II). These works agree with the observed in the present study. Likewise, consistent with the presence of oxidative stress, the metal-resistant yeast *R. mucilaginosa* showed higher expression of proteins related to energy synthesis, protein synthesis and degradation (Ilyas et al., 2016). Proteins related to oxidative stress, such as oxidoreductases, have also been reported in *Meyerozyma guilliermondii*, a manganese-resistant yeast isolated from acid mine drainage-affected environments (Ruas et al., 2019), and in *Acinetobacter calcoaceticus* against Cu (Kang et al., 2020).

Over-expression of calmodulin was detected in *F. tricinctum* M6 exposed to Cu(II). Calmodulin is a protein with important roles in the response to different stresses, including metal stress (Zhang et al., 2016). Notably, this protein can also be activated by the presence of other bivalent metal ions (Mills and Johnson, 1985). The signaling mechanisms activated by metals also affect the expression of kinases and phosphatases (Tiwari and Lata, 2018). In *F. tricinctum* M6 exposed to Cu(II), E3 ubiquitin ligase was detected, which has been related to abiotic stress in plants in a study carried out by Wu et al. (2016).

The energy metabolism was affected in *F. tricinctum* M6 exposed to Cu(II). Proteins related to energy metabolism have been reported as important in the response to metals by Zou et al. (2015). In agreement, authors as Feng et al. (2017) state that the tolerance to Cu(II) by *Penicillium janthinellum* GXCR depends on the energy generation, which is necessary to carry out carbohydrate metabolism. Izrael-Živković et al. (2018) detected proteins involved in energy metabolism, protein biosynthesis and post-translational modifications in *P. aeruginosa* exposed to Cd(II). Recently, the importance of the energy metabolism to face metals was also observed when exposing *Lactobacillus plantarum* against lead (Liu et al., 2019) and *Streptomyces* sp. MC1 to Cr(VI) (Bonilla et al., 2020).

Proteins related to carbohydrate metabolism, and involved in replication, transcription and translation have been reported recently in *R. mucilaginosa* AN5 (Kan et al., 2019). In a recent study, *Streptomyces* sp. MC1 exposed to Cr(VI) also showed higher expression of proteins related to protein biosynthesis, proteins involved in oxidation-reduction processes and chaperones with a key role for misfolded protein repair (Bonilla et al., 2020). These works are in concordance with the results obtained with *F. tricinctum* M6 against Cu(II), where the same protein groups are reported as necessary proteins to face the stress produced by the presence of copper and to maintain the metabolic balance.

Metals are known to be inhibitors of the folding protein process and, at the same time, inhibit the denatured proteins refolding, either naturally or chaperones assisted (Chiapello et

al., 2015). This problem enhances the misfolded or defective proteins degradation and causes an increased expression of proteasome and ubiquitination proteins. Likewise, the presence of metals promotes a greater chaperones expression, as observed in this study, which are key components for protein folding and for stress response (Steurer et al., 2018; Kan et al., 2019). To achieve the normal cellular function, cells exposed to Cu(II) can be forced to synthesize more proteins, which explain the increased expression for proteins involved in the protein biosynthesis, and consequently, for proteins involved in nucleic acid modeling and metabolism. The synthesis of new proteins may demand higher energy expenditure by the cells, as observed in *F. tricinctum* M6. To counteract the oxidative stress caused by the Cu(II) presence, the microorganism shows higher expression of stress response proteins and oxidoreductases. Likewise, an increase expression for kinases and phosphatases is observed, which fulfill functions in the enzymatic activation or inactivation (Tiwari and Lata, 2018).

As mentioned in the Results Section, in the presence of Cu(II) *F. tricinctum* M6 expressed numerous proteins that possess metal ion binding sites, involved in a wide variety of cellular processes. Metalloproteomics studies the expression of metalloproteins and their changes in the biological time and space. Proteins that control metal homeostasis and proteins regulated by metals bind transiently to the metal ions (Maret, 2010). Therefore, metalloproteomes appear as interesting dynamic structures for metal-resistant organisms (Lancaster et al., 2014).

Chiapello et al. (2015) detected metalloenzymes in *O. maius* against Cd and Zn, proposing that these proteins may be important for metal tolerance, not only through their enzymatic activity, but also due to their ability to sequester bivalent metal ions. Metal ion binding proteins have also been detected in *P. aeruginosa* exposed to Cd(II) (Izrael-Živković et al., 2018). In this work, the authors proposed that Cd (II) has the ability to replace the metals used by these proteins as cofactors. Taking into account our results, Cu(II) may also replace the natural cofactors of many proteins at the metal binding sites. In this sense, Farcasanu and Ruta (2018) and Fein et al. (2019) affirm that proteins with metal ion-binding sites present high affinity for a wide variety of metal ions and their promiscuity and increased expression may be key for the metal tolerance in resistant microorganisms.

4.2.2. Differential expression of extracellular proteins in the presence of Cu(II)—

The proteins identified in the extracellular space of *F. tricinctum* M6 exposed to Cu(II) were mainly related to carbohydrate metabolism and oxidation-reduction processes. Likewise, proteins able to chelate metal ions were also identified, such as PAD domain protein, which binds Ca(II).

What happens in the extracellular environment when cells are exposed to metals is still unknown and, to the best of our knowledge, there are only few studies related to this area (Giner-Lamia et al., 2016). For example, the one carried out by Kim et al. (1995), who detected extracellular copper response proteins (CRX) in *Methanobacterium bryantii* BKYH exposed to Cu(II). Protein secretion was also stimulated by Mg(II) in *Vibrio parahaemolyticus* (Bhattacharya et al., 2000). Martino et al. (2002) demonstrated that Zn induced the protein secretion in *O. maius*. In the present work, Beta xylanase was identified

in supernatants of *F. tricinctum* M6 exposed to Cu(II). This enzyme is involved in the carbohydrate metabolism and has previously been associated to the early response to metals in basidiomycetes (Zhao et al., 2015).

As in the intracellular space, proteins with metal binding sites were detected in the *F. tricinctum* M6 supernatants exposed to copper. These proteins did not show a significant probability to be secreted into the extracellular space, according to CELLO v2.5. Interestingly, Desvaux et al. (2009) have defined Exoproteomics as the study of all the proteins present in the extracellular space, whether secreted or not by the cells. In this way, the authors recognize the importance of all the proteins identified in the supernatants for metal-resistance. It is known that exoproteins found in the presence of metals include many functional categories, the composition of the exoproteome varies under different growth conditions and many of the detected proteins appear as proteins with unknown functions (Giner-Lamia et al., 2016). This clearly provides an interesting framework for studies of the extracellular space.

In the exoprotein content of *F. tricinctum* M6 in the presence of Cu(II), many of the identified proteins possess metal ion binding sites. These proteins may play a fundamental role in the Cu(II) sequestration and transport, mitigating the toxic effects for the cell. Most of the detected exoproteins show intracellular functions. However, their functions in the extracellular space remain unknown. These results indicate that, when faced with metal stress, the microorganism responds not only by adjusting the intracellular protein profiles, but also by adjusting the protein expression in the extracellular space.

5. Conclusions

The acid mine drainage-affected environment located in La Carolina (San Luis, Argentina) constitutes an interest microbial niche for the isolation of metal-tolerant microorganisms. This fact is an indication of the relation between the environmental conditions and the adaptive capacity of the endogenous microbiota.

The microelemental composition of the biomass exposed to Cu(II) indicates that the surface electronegative elements and the ionic exchange mechanisms play an important role in the copper ions adsorption. Proteomic studies showed that protein expression by *F. tricinctum* M6 changed to cope with metal toxicity. The differential expression allows the microorganism to counteract the metabolic imbalances that the metal toxicity produces. Likewise, proteins identified in the extracellular space may be crucial for the metal sequestration and transport.

In *F. tricinctum* M6 exposed to copper, a large number of proteins with bivalent metal ion binding sites at intra and extracellular levels were detected. The evidence regarding the high affinity of these proteins for a wide variety of metal ions would indicate that Cu(II) ions have the ability to replace the metals used by these proteins as cofactors, either activating or inactivating the original protein activity. The results obtained in this work show that there is not only one resistance mechanism in *F. tricinctum* M6, but a combination of different strategies, including proteomic changes, sequestration of the metal ions and surface

adsorption. The study of metal-tolerant organisms is important not only for our mechanistic understanding of selective incorporation and/or metal immobilization, but also for the efforts to harness these skills in bioremediation processes.

5.1. Limitation of the study and future perspective

Future research is needed to in depth into the robustness of the isolated microorganism when faced with the actual environmental conditions of the affected area (low pH values, presence of co-pollutants, temperature, among other). Likewise, the microorganism should be analyzed not only for its application in AMD-affected environments, but also for its application in environments affected by industrial and domestic effluents. The microorganism can also be faced with other bivalent metal ions to confirm the importance of the metal ion-binding proteins for the resistance showed by *F. tricinctum* M6 against Cu(II). Finally, the microorganism can also be evaluated against metals in a resting-cell approach instead of a growing-cell method.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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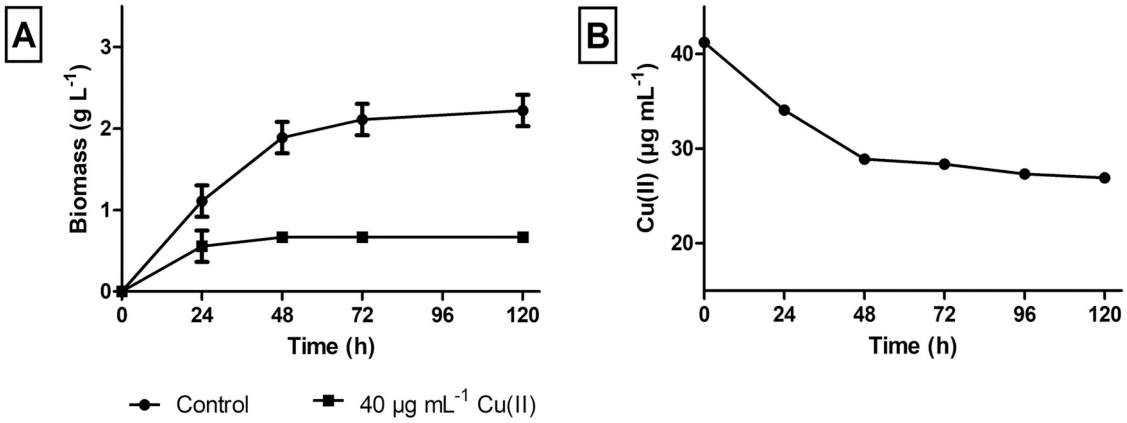

Fig. 1.

Growth evaluation of *F. tricinctum* M6 in the presence and absence of 40 μg mL^{-1} Cu(II) in the culture medium (**A**). Copper remaining concentration in the supernatants of *F. tricinctum* M6 exposed to the metal (**B**).

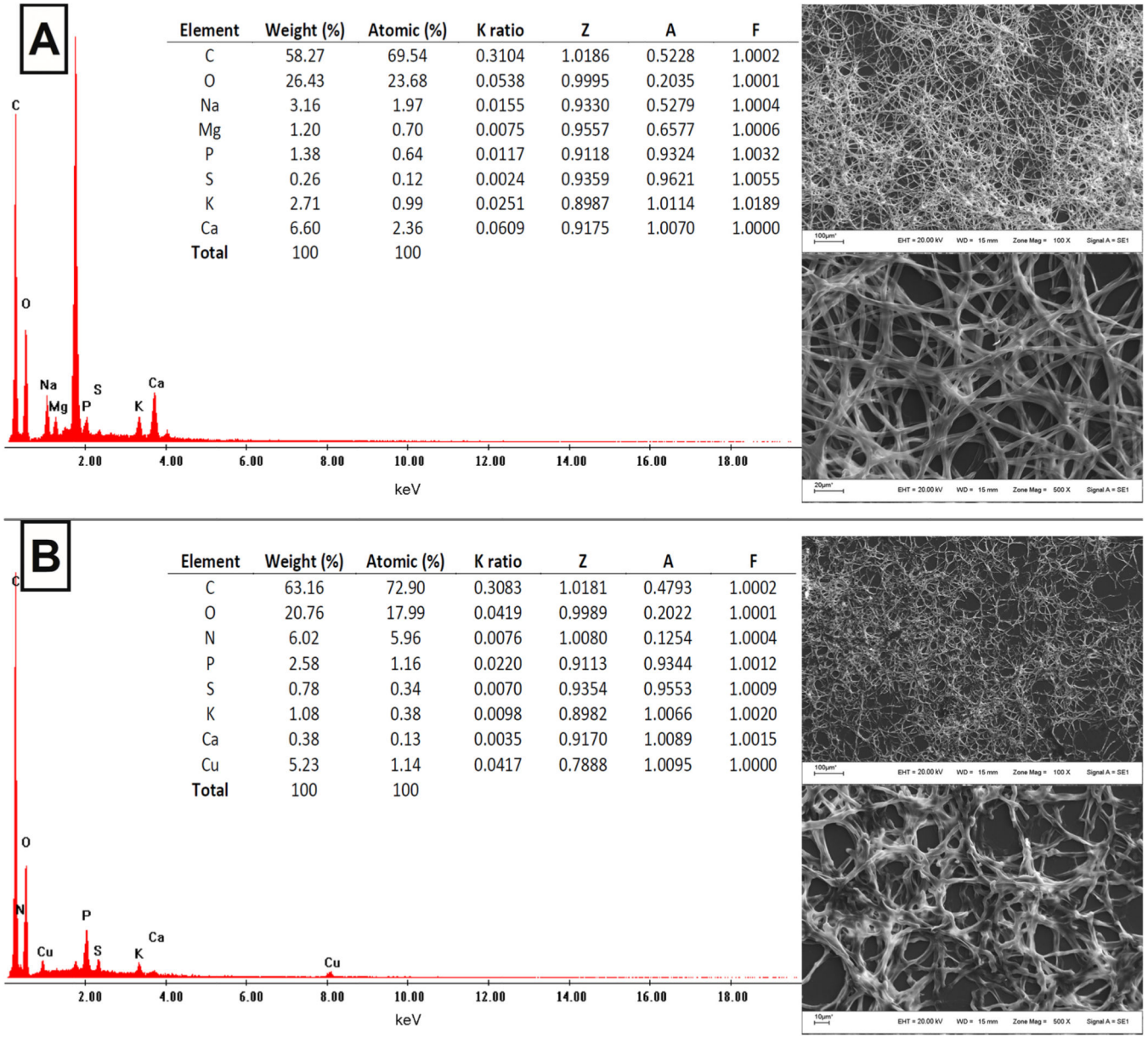

Fig. 2.

Microelemental analysis of *F. tricinctum* M6 in the absence (A) and in the presence (B) of Cu(II) in the culture medium, through SEM-EDS operated at 20 kV.

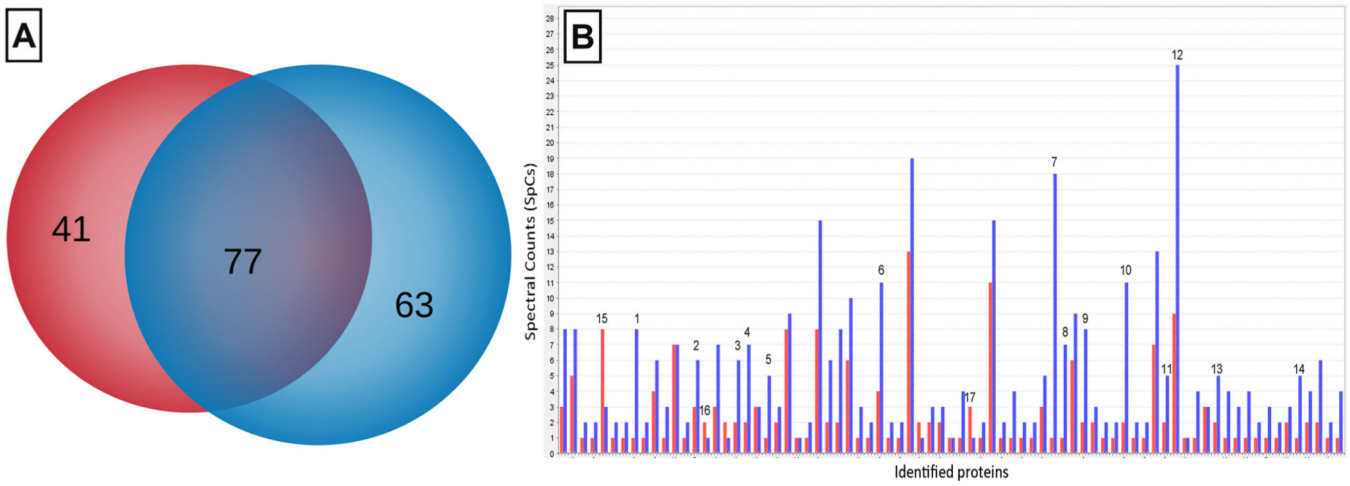

Fig. 3.

Intracellular proteomic analysis of *F. tricinctum* M6. Venn diagram of proteins identified in the presence (blue) and in absence (red) of Cu(II) in the culture medium; the group of proteins shared by both conditions is shown in violet (A). Bar diagram of the semi-quantitative expression of intracellular proteins detected in the presence (blue) and in absence (red) of Cu(II) in the culture medium (B).

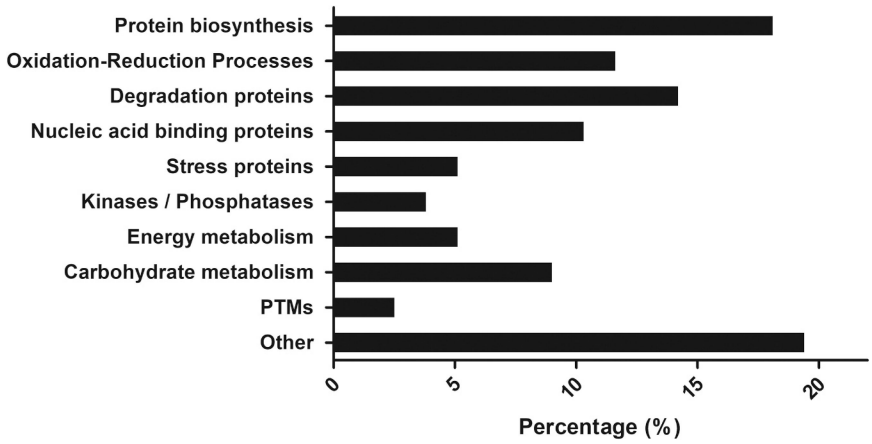

Fig. 4.

Proteins identified in *F. tricinctum* M6 exposed to Cu(II), grouped according to their cellular function.

Table 1

Intracellular proteins of *F. tricinctum* M6 identified both in the presence and absence of Cu(II), over-expressed or down-regulated in the presence of the metal.

Bar Number	Sequence ID[a]	Sequence name[b]	Protein length (AA)[c]	Protein weight (kDa)[d]	Log_2 scale[e]
Over-expressed					
1	A0A0D2Y596	Elongation factor 3	1055	117.03	**1.95**
2	F6KJZ2	Calmodulin (Fragment)	125	14.16	**1.09**
3	A0A0D2XAN5	40 S ribosomal protein S6	239	27.22	**1.58**
4	A0A0D2XC03	Nucleosome assembly protein 1-like 1	404	45.43	**1.79**
5	A0A1C3YN26	Phosphoglycerate kinase	534	57.94	**1.29**
6	A0A2H3H8F9	Heat shock 70 kDa protein 4	778	85.86	**1.66**
7	A0A0D2XZN4	40 S ribosomal protein S3	260	28.52	**3.16**
8	N4TYJ1	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	525	57.45	**1.79**
9	A0A2H3G5U3	Heat shock protein 60	735	77.84	**1.95**
10	A0A2H3GUK9	Peroxiredoxin 1	212	23.80	**1.45**
11	A0A2H3FXB5	60 S ribosomal protein L9-B	250	28.14	**1.29**
12	A0A0P0RS90	Elongation factor 1-alpha	460	49.82	**1.48**
13	A0A0D2YH41	DnaJ like subfamily C member 2	444	50.37	**1.29**
14	Q4I1N3	Sulfate adenylyltransferase	574	64.23	**1.29**
Down-regulated					
15	N1RH59	Ran-specific GTPase-activating protein 1	237	26.45	**−1.50**
16	A0A0D2XM79	Isocitrate dehydrogenase [NADP]	462	51.72	**−1.08**
17	W9IHZ4	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	276	30.46	**−1.58**

[a] Accession numbers from UniProtKB/TrEMBL (https://www.uniprot.org).

[b,c,d] Function annotations were retrieved from NCBInr (http://www.ncbi.nlm.nih.gov) and UniProt (https://www.uniprot.org/).

[e] Heat map information obtained from ProteoIQ. $Log_2 > 1$ indicates over-expression, while $Log_2 < -1$ indicates down-regulation.

Table 2

Intracellular proteins of *F. tricinctum* M6 identified in the presence of Cu(II) in the culture medium.

Sequence ID[a]	Sequence name[b]	Protein length (AA)[c]	Protein weight (kDa)[d]
Protein biosynthesis			
A0A2H3HCL8	Elongation factor 1-gamma 1	425	47.78
A0A0D2XB67	40 S ribosomal protein S21	87	9.63
A0A0D2XA48	GrpE protein homolog	244	27.43
A0A0D2XWU0	T-complex protein 1 subunit alpha	565	61.32
A0A0D2Y4M4	40 S ribosomal protein S2	257	27.97
A0A2H3HCC0	40 S ribosomal protein S22	130	14.79
A0A0D2Y2F8	40 S ribosomal protein S19	150	16.58
A0A0D2XJG4	60 S ribosomal protein L27a	149	16.79
Oxidation-Reduction Processes			
N1RL11	NADH-ubiquinone oxidoreductase 29.9 kDa subunit, mitochondrial	235	26.92
N1RDJ1	L-aminoadipate-semialdehyde dehydrogenase large subunit	1163	129.01
A0A0D2XAA8	Inosine-5′-monophosphate dehydrogenase	532	56.83
A0A0D2YFU9	DAO domain-containing protein	533	57.75
A0A0D2XC02	D-3-phosphoglycerate dehydrogenase 2	473	51.62
S0EP01	Probable LEU2-beta-isopropyl- malate dehydrogenase	1084	118.61
I1S2Z0	PKS_ER domain-containing protein	368	39.83
A0A0D2Y544	UDPglucose 6-dehydrogenase	605	66.18
A0A0J9VP92	Uncharacterized protein FOXG_12068	501	54.50
Degradation proteins			
A0A2H3HD18	Vacuolar protease A (Fragment)	429	46.42
A0A0D2Y1V9	26 S protease regulatory subunit 6 A	459	50.92
A0A0D2XML3	E3 ubiquitin-protein ligase	810	91.38
A0A0J9UJ20	26 S proteasome regulatory subunit N7	392	42.69
N1RE30	Cys-Gly metallodipeptidase dug1	460	50.55
W7MDN2	Mitochondrial-processing peptidase subunit alpha	532	57.97
A0A0D2×9D5	26 S protease regulatory subunit 7	440	49.07
I1RFS5	Peptidase alpha subunit	565	61.44
A0A0J9ULK9	F-box domain-containing protein	529	60.77
C7Z9Q6	Predicted protein NECHADRAFT_94534	577	62.78
I1RQ19	Uncharacterized protein FG06152.1	838	91.93
Nucleic acid binding proteins			
A0A2H3THI2	Related to TATA-binding protein associated factor 2 N	348	38.23
A0A0J9WA35	BZIP domain-containing protein	212	24.35
Q4HTT2	Histone H2B	137	14.75
K3VN47	Zn(2)-C6 fungal-type domain- containing protein	1024	116.72
C7YIM9	Uncharacterized protein BRD2103	675	74.94
C7YNQ5	Uncharacterized protein NECHADRAFT_60425	604	65.40
A0A0J9V1M3	Uncharacterized protein FOXG_07428	841	94.08

Sequence ID[a]	Sequence name[b]	Protein length (AA)[c]	Protein weight (kDa)[d]
Stress proteins			
A0A0C4DHU2	Glutathione reductase	506	54.79
Kinases/Phosphatases			
A0A0D2XQI2	3′(2′),5′-bisphosphate nucleotidase	408	43.65
A0A0D2Y8P5	Adenylate kinase	256	28.26
A0A0J9WPA8	Protein phosphatase 2 (Formerly 2 A), regulatory subunit A	514	57.03
Energy metabolism			
A0A0D2XEG1	ATP synthase subunit delta, mitochondrial	165	17.70
A0A0D2XAN8	V-type proton ATPase subunit B	511	56.65
A0A0D2XHL0	V-type proton ATPase subunit E	229	25.83
A0A0J9UCJ3	V-type proton ATPase catalytic subunit A	788	85.90
Carbohydrate metabolism			
A0A0D2Y1Y2	Fructose-bisphosphate aldolase, class II	360	39.61
A0A0D2XEA1	Triosephosphate isomerase	247	27.06
A0A0D2XGP1	Fumarate hydratase, mitochondrial	529	56.80
A0A0D2×829	Enolase	438	47.28
A0A0D2Y4K0	Glucose-6-phosphate 1-epimerase	313	33.58
Post-translational modifications (PTMs)			
A0A0D2YKT2	1,3-beta-glucanosyltransferase	375	41.05
K3VMJ8	Uncharacterized protein FPSE_04909	513	58.50
Other			
N1RHH1	Tropomyosin-2	161	18.79
A0A0D2XNF4	Myo-inositol-1-phosphate synthase	541	59.44
A0A0D2XHT9	Phospho-2-dehydro-3- deoxyheptonate aldolase	317	34.11
N1REE8	Rho GDP-dissociation inhibitor	198	22.16
A0A2H3GY22	Reduced viability upon starvation protein 167	434	49.42
A0A0D2×9R5	S-adenosylmethionine synthase	403	44.27
A0A2H3G4L5	ATP phosphoribosyltransferase	325	35.33
A0A0D2XX43	pHdomain-containing protein	834	91.54
A0A0J9WG59	Abhydrolase_3 domain-containing protein	259	28.70
A0A0D2XWU1	Prolyl-tRNA synthetase	553	62.36
S0DLW9	Related to putative sterigmatocystin biosynthesis lipase/esterase STCI	364	40.68
A0A0J9WKR9	Cysteine desulfurase	401	43.75
C7Z6×3	Predicted protein NECHADRAFT_33672	2106	231.18

[a]Accession numbers from UniProtKB/TrEMBL (https://www.uniprot.org) and NCBI (http://www.ncbi.nlm.nih.gov).

[b,c,d]Function annotations were retrieved from NCBInr (http://www.ncbi.nlm.nih.gov) and UniProt (https://www.uniprot.org/).

Table 3

Extracellular proteins identified in *F. tricinctum* M6 cell-free supernatants obtained in the presence and absence of Cu(II) in the culture medium. Predictions obtained from CELLO v2.5.

Sequence ID[a]	Sequence name[b]	Protein length (AA)[c]	Protein weight (kDa)[d]
Control			
A0A395N2×2	Het-domain-containing protein	538	62.66
A0A090MDG5	Efflux pump FUS6	126	14.32
K3VU44	Beta-glucosidase	832	89.51
C7YSF2	Endo-chitosanase	293	30.69
W7LIK3	Uncharacterized protein FVEG_01594	795	83.27
S0E6U9	Uncharacterized protein FFUJ_06603	787	81.86
A0A0D2XFR8	Uncharacterized protein FOXG_02757	787	81.98
C7YTA7	Predicted protein NECHADRAFT_69214	294	31.91
Exposed to Cu(II)			
A0A428U1G5	PAD domain-containing protein	609	67.34
A0A098DCZ3	Beta-xylanase	563	60.66
C7YUT8	FAD-binding PCMH-type domain-containing protein	653	70.99
W7LGU8	Uncharacterized protein FVEG_14799	124	13.95
C7YKT3	Predicted protein NECHADRAFT_105961	135	14.56
C7YX48	Secreted protein NECHADRAFT_95070	533	59.24

[a] Accession numbers from UniProtKB/TrEMBL (https://www.uniprot.org) and NCBI (http://www.ncbi.nlm.nih.gov).

[b,c,d] Function annotations were retrieved from NCBInr (http://www.ncbi.nlm.nih.gov) and UniProt (https://www.uniprot.org/).