Tolerance and antioxidant response to heavy metals are differentially activated in *Trichoderma asperellum* and *Trichoderma longibrachiatum* (#107254)

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Tolerance and antioxidant response to heavy metals are differentially activated in *Trichoderma asperellum* and *Trichoderma longibrachiatum*

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Heavy metals (HMs) pollution reduces the community of soil microorganisms, including fungi from the genus *Trichoderma*, which are plant growth promotors and biological control agents. Because of potential effects on crop productivity, the toxic effects of HMs in *Trichoderma* are of interest. However, studies on the biochemical and molecular response to oxidation caused by exposure to Cu, Cr, and Pb and whether this antioxidant response is species-specific are scarce. Thus, we compared the tolerance of *Trichoderma asperellum* and *T. longibrachiatum* to Cu, Pb, and Cr and evaluated the expression of genes related to the antioxidant response, including peroxidase (*GPX*), catalase (*CAT*), and cysteine synthase (*CYS*) as well as the activity of peroxidase and catalase. The isolates of *Trichoderma* were selected because we previously reported that they are promotors of plant growth and agents of biological control. Our results revealed that, with exposure to the three HMs, the *Trichoderma* cultures formed aggregates, and the culture color changed according to the metal and the *Trichoderma* species. The tolerance index (TI) indicated that the two *Trichoderma* species were tolerant of HMs (Cu>Cr>Pb). However, the TI and conidia production revealed that *T. longibrachiatum* was more tolerant of HMs than was *T. asperellum*. The three HMs caused oxidative damage in both *Trichoderma* species, but the enzyme activity and gene expression were differentially regulated based on exposure time (72 and 144 h) to the HMs and the *Trichoderma* species. The main changes occurred in *T. asperellum*; the maximum expression of the *GPX* gene occurred at 144 h in response to all three HMs, whereas the *CAT* gene was upregulated at 72 h in response to Cu but downregulated at 144 h in response to all three HMs. The *CYS* gene was upregulated in response to the three metals. The peroxidase activity increased with all three HMs, but the catalase activity increased with Cu and Pb at 72 h and decreased at 144 h with Pb and Cr. In *T. longibrachiatum*, the *GPX* gene was upregulated with all three

HMs at 72 h, the *CAT* gene was upregulated only with Pb at 72 h and was downregulated at 144 h with HMs. Cr and Cu upregulated *CYS* gene expression, but expression did not change with Pb. The peroxidase activity increased with Cu at 144 h and with Cr at 72 h, whereas Pb decreased the enzyme activity. In contrast, catalase activity increased with the addition of all three metals at 144 h. In conclusion, *T. longibrachiatum* was more tolerant of Cu, Cr and Pb than was *T. asperellum*, but exposure to all three HMs caused oxidative damage to both *Trichoderma* species. The antioxidant response varied with *Trichoderma* species and according to the exposure time to each HM; peroxidases and catalases were activated, and the expression of the genes *GPX* and *CYS* was upregulated, whereas the *CAT* gene was downregulated. These findings indicate that the antioxidant response to HMs was genetically modulated in each *Trichoderma* species.

Tolerance and antioxidant response to heavy metals are differentially activated in *Trichoderma asperellum* and *Trichoderma longibrachiatum*

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Abstract

Heavy metals (HMs) pollution reduces the community of soil microorganisms, including fungi from the genus *Trichoderma*, which are plant growth promotors and biological control agents. Because of potential effects on crop productivity, the toxic effects of HMs in *Trichoderma* are of interest. However, studies on the biochemical and molecular response to oxidation caused by exposure to Cu, Cr, and Pb and whether this antioxidant response is species-specific are scarce. Thus, we compared the tolerance of *Trichoderma asperellum* and *T. longibrachiatum* to Cu, Pb, and Cr and evaluated the expression of genes related to the antioxidant response, including peroxidase (*GPX*), catalase (*CAT*), and cysteine synthase (*CYS*) as well as the activity of peroxidase and catalase. The isolates of *Trichoderma* were selected because we previously reported that they are promotors of plant growth and agents of biological control. Our results revealed that, with exposure to the three HMs, the *Trichoderma* cultures formed aggregates, and the culture color changed according to the metal and the *Trichoderma* species. The tolerance index (TI) indicated that the two *Trichoderma* species were tolerant of HMs (Cu>Cr>Pb). However, the TI and conidia production revealed that *T. longibrachiatum* was more tolerant of HMs than was *T. asperellum*. The three HMs caused oxidative damage in both *Trichoderma* species, but the enzyme activity and gene expression were differentially regulated based on exposure time (72 and 144 h) to the HMs and the *Trichoderma* species. The main changes occurred in *T. asperellum*; the maximum expression of the *GPX* gene occurred at 144 h in response to all three HMs, whereas the *CAT* gene was upregulated at 72 h in response to Cu but

downregulated at 144 h in response to all three HMs. The *CYS* gene was upregulated in response to the three metals. The peroxidase activity increased with all three HMs, but the catalase activity increased with Cu and Pb at 72 h and decreased at 144 h with Pb and Cr. In *T. longibrachiatum*, the *GPX* gene was upregulated with all three HMs at 72 h, the *CAT* gene was upregulated only with Pb at 72 h and was downregulated at 144 h with HMs. Cr and Cu upregulated *CYS* gene expression, but expression did not change with Pb. The peroxidase activity increased with Cu at 144 h and with Cr at 72 h, whereas Pb decreased the enzyme activity. In contrast, catalase activity increased with the addition of all three metals at 144 h. In conclusion, *T. longibrachiatum* was more tolerant of Cu, Cr and Pb than was *T. asperellum*, but exposure to all three HMs caused oxidative damage to both *Trichoderma* species. The antioxidant response varied with *Trichoderma* species and according to the exposure time to each HM; peroxidases and catalases were activated, and the expression of the genes *GPX* and *CYS* was upregulated, whereas the *CAT* gene was downregulated. These findings indicate that the antioxidant response to HMs was genetically modulated in each *Trichoderma* species.

Introduction

Heavy metals (HMs) are defined as chemical elements with an atomic number greater than 20 and a density greater than 5 g cm^{-3} that originate from a natural source. According to these criteria, cadmium (Cd), lead (Pb), mercury (Hg), chromium (Cr), nickel (Ni), copper (Cu) and zinc (Zn) are HMs (*Ali and Khan, 2018*). The natural sources of HMs are minerals, volcanic eruptions and rock fragments (*Zhang and Wang, 2020*), but other principal sources of HMs are anthropogenic activities such as mining, the textile industry, paint manufacturing, wastewater irrigation, and the intensive use of agrochemicals such as pesticides and fertilizers (*Alengebawy et al., 2021*). In cultivated soils, the excessive use of Cu- and Pb-based pesticides is one of the main factors that increases the concentrations of these metals in soils (*Facchinelli et al., 2001*). Similarly, the intensive use of fertilizers for long periods increases the accumulation of Cu, Cd, and Zn in agricultural soils, which reduces soil fertility and crop productivity (*Alengebawy et al., 2021*). HMs also reduce the soil microbial community (bacteria and fungi) and the activity of microbial enzymes, which are indicators of soil quality and health (*Keiblinger et al., 2018*; *Raiesi and Sadeghi, 2019*); therefore, HMs are toxic to plants and the soil microbial community. In fungi, Cu and Cr toxicity is due to the production of reactive oxygen species (ROS) that damage cells (*Belozerskaya and Gessler, 2007*; *Viti et al., 2014*). Pb does not directly participate in the reactions that lead to ROS production, but the metal accelerates the oxidation of DNA, proteins, and antioxidant enzymes that counteract oxidative damage (*Gurer and Ercal, 2000*). The damage to the soil microorganism community caused by HMs is ecologically relevant to crop productivity because bacteria and fungi can promote plant growth and are agents of biological control of pathogens (*Saeed et al., 2021*). Fungi of the genus *Trichoderma* possess these functions; in addition, *Trichoderma* fungi induce defense responses in plants under conditions of abiotic and biotic stress (*Macías-Rodríguez et al., 2020*; *Poveda, 2022*). Similar to other microorganisms, *Trichoderma* fungi are exposed to HMs; several *Trichoderma* species

have been isolated from soils and water contaminated by HMs, and their tolerance of HMs has been evaluated (*Tansengco et al., 2018*; *Liaquat et al., 2020*; *Sun et al., 2020*; *Mushtaq et al., 2023*). Other studies have shown that HMs, such as Cr and Cu, cause oxidative damage in *Trichoderma* (*Kumar and Dwivedi, 2019*; *Pérez-Torres et al., 2020*), but studies on antioxidant enzyme activity have produced contradictory results that suggest that this antioxidant response may depend on the particular *Trichoderma* species and the specific HM. Previously, we reported that the activity of catalases and peroxidases in three isolates of *T. asperellum* increased with exposure to Cu-based fungicides, and that the enzyme activity depended on the specific isolate of *T. asperellum* (*Pérez-Torres et al., 2020*). In contrast, in *T. lixii*, catalase activity has been reported to decrease with Cr, and peroxidase and superoxide dismutase activities depended on the Cr concentration (*Kumar and Dwivedi, 2019*). In addition, studies on the expression of detoxification-related genes in response to the exposure of *Trichoderma* to HM are scarce. However, transcriptomic analysis of *T. harz*ianum treated with Cd showed that the gene expression of proteins with oxidoreductase activity increased, suggesting that these proteins are related to protection against oxidation and other toxic effects of Cd (*Oshiquiri et al., 2020*). On the basis of these findings, we evaluated tolerance; oxidative damage; expression of detoxification-related genes such as *GPX*, *CAT*, and *CYS*; and catalase and peroxidase activity in *T. asperellum* and *T. longibrachiatum* exposed to Cu, Cr, and Pb. These *Trichoderma* species were selected because we previously evaluated their potential as promotors of plant growth, agents of biological control and inducers of vegetal resistance (*Ortega-García et al., 2015*; *Zapata-Sarmiento et al., 2020*; *Rodríguez-Hernández et al., 2023*; *Camacho-Luna et al., 2021*; *Camacho-Luna et al., 2023*). Cu and Cr were evaluated because their toxicity is due to the production of ROS (*Belozerskaya and Gessler, 2007*; *Viti et al., 2014*), whereas Pb is a metal that accelerates the oxidation of DNA and proteins, including those of antioxidant enzymes (*Gurer and Ercal, 2000*). The accumulation of these HMs in soils is caused by the intensive use of agrochemicals (*Alengebawy et al., 2021*). This study contributes to the knowledge of the genetic and biochemical mechanisms related to the antioxidant response to Cu, Cr and Pb exposure in two *Trichoderma* species with potential agricultural use.

Materials & Methods

Evaluation of the growth, conidia production and tolerance of *Trichoderma asperellum* and *T. longibrachiatum* exposed to Cu, Cr and Pb

Trichoderma asperellum was previously isolated and identified by *Ortega-García et al. (2015),* and *T. longibrachiatum* was isolated and identified by *Camacho-Luna et al. (2022)*. Fungal strains were grown in a culture medium of potato, dextrose, and agar (PDA, Bioxon™, Becton Dickinson from Mexico) at 25 ± 2 °C with a 12 h light:12 h dark cycle. After 8 d, conidial suspensions (1×10^7 conidia mL^{-1}) were obtained and used to evaluate the growth, tolerance and conidia production of *Trichoderma* exposed to HMs following the methodology of *Pérez-Torres (2020)*. The Erlenmeyer flasks contained a culture medium of potato and dextrose (Difco™, Becton, Dickinson and Company, Maryland, USA) and 100 mg/L of each HM. Copper (Cu) was

added as $CuSO_4 \times 5H_2O$ (Sigma–Aldrich Co., St. Louis, MO), chromium (Cr) was added as $K_2Cr_2O_7$ (J. Baker, Avantor Performance Materials, Mantsonford Road, Radnor, PA) and lead (Pb) was added as $Pb(NO_3)_2$ A.C.S. (Fermont, Productos Químicos Monterrey, S.A. de C.V., Monterrey, N.L.). For each HM and *Trichoderma* species, four Erlenmeyer flasks were prepared. The controls were Erlenmeyer flasks inoculated with each *Trichoderma* species without HMs. After 6 d, the mycelia were recovered and dried, and the dry weight (DW) was obtained. The number of conidia in the culture broth was quantified using a Neubauer hemocytometer. Conidia production was expressed as the number of conidia per gram DW. Images of *Trichoderma* cultures developed in Erlenmeyer flasks were obtained with a Samsung camara (64 megapixels, F1.8). A second experiment was performed under the same experimental conditions, and fresh mycelial biomass was collected at 72 and 144 h and used to evaluate lipid peroxidation, gene expression and peroxidase and catalase activity.

Determination of malondialdehyde (MDA) content

Lipid peroxidation was evaluated by measuring the MDA content and reaction with thiobarbituric acid (TBA) as described by *Pérez-Torres et al. (2020).* The MDA index, which is related to the MDA content of mycelium samples of *Trichoderma* treated with and without metal, was calculated.

RNA isolation and RT–qPCR relative expression analysis

Total RNA was extracted from fresh mycelia of *T. asperellum* and *T. longibrachiatum* with TRIzol® Reagent for small scale isolation (Invitrogen, Carlsbad, CA, USA) after 72 and 144 h of exposure to Cu, Pb and Cr and treated with RNase-free DNase I (Thermo Fisher Scientific Carlsbad, CA, USA). Isolated total RNAs were kept at − 80 °C until use for complementary DNA (cDNA) synthesis. The total RNA concentration was calculated with an Epoch-2 microplate reader (Biotek® Winooski, VT, USA). The integrity of RNA was checked through 1.2 % agarose gel electrophoresis under denaturing conditions. RT–qPCR was performed according to *Rodríguez-Hernández et al. (2014)* with a Step One Real-Time PCR Detection System (Applied Biosystems, Waltham, MA, USA), and StepOne Software v2.1 (Applied Biosystems). For the synthesis and quantification of cDNA, the iTaq Universal SYBR® Green One-Step kit (Bio-Rad,USA) was used. RT-qPCR was performed in 10 µl reaction mixture containing 100 ng of total RNA as template, 5 µl of iTaq universal SYBR Green reaction Power mix (2X), 300 nM of each oligonucleotide, and 0.125 µl of iScript reverse transcriptase. All samples were amplified in triplicate as follows: 10 min at 50 °C (cDNA synthesis) and 1 min at 95 °C (polymerase activation) followed by 40 cycles at 95 °C (DNA denaturation) and 1 min at 60 °C (tubulin), 58 °C (*GPX*, gluthatione peroxidase and *CYS*, cysteine synthase) and 55 °C (*CAT*, catalase). The primers corresponding to the *Tubulin* gene (were: *TUB-F* 5′-GACACTACACTGAGGGTGCT-3′ and *TUB-R* 5′-GTATGACGGGTTGGACAGCT-3′; the primers corresponding to the *Catalase* gene were: *CAT-F* 5´-ACTGCTGAGGGTTGCCCAAT-3' and *CAT-R* 5`-CGAATTCACCATATGCACCAG-3`, the primers corresponding to the

Gluthatione peroxidase gene were: *GPX-F* 5`-ATTCAGCGGACAAATTCAGTGC-3´ and *GPX-R* 5´-CAGCCTTACGGAGCTCCG-3´; and the primers corresponding to the *Cysteine synthase* gene were: *CYS-F* 5´-ATGTTCCGACAAACTGCGCAG-3´ and *CYS-R* 5´-GCAGCCGGTCTCCTCAG-3´. The melting curve was generated with cycles of 5–95 °C for 15 s, with increases of 0.5 °C at each cycle. We used tubulin as a reference for all experiments, and the gene expression levels were evaluated using the $2^{-\Delta\Delta CT}$ method (*Livak and Schmittgen, 2001*). For each sample, three replicates (n = 3) and their respective technical replicates were analyzed.

Determination of enzyme activities

Peroxidase activity was determined by the methods described by *Stasolla and Yeung (2007)* and *Pérez-Torres et al. (2020)*. Briefly, fresh mycelia (300 mg) were extracted with sodium phosphate buffer (50 mM, pH 6.0) containing EDTA (100 mM), DTT (1 mM), and PMSF (1 mM). The extract was centrifuged, and the recovered supernatant was used as the enzyme extract. The protein content was measured by *Bradford's (1976)* method and calculated with a standard curve constructed with bovine serum albumin. The reaction enzyme was prepared with the enzyme extract using guaiacol as the substrate. The tetraguaiacol content (extinction coefficient of 26.6 mM cm^{-1}) was calculated. The enzyme activity was expressed as the tetraguaiacol content per minute per milligram of protein.

Catalase activity was determined by the methods of *Beers and Sizer (1952)* and *Pérez-Torres et al. (2020)*. Fresh mycelium (300 mg) was ground with extraction buffer containing sodium phosphate (100 mM at pH 7.0), EDTA (1 mM), DTT (1 mM), and PMSF (1 mM). The extract was centrifuged and used as the enzyme extract. The enzyme reaction was developed using a H_2O_2 substrate solution. The absorbance at 240 nm was measured and used to calculate the H_2O_2 content using an extinction coefficient for H_2O_2 of 0.04 mM cm^{-1}. The peroxidase activity was expressed as the H_2O_2 content per minute per milligram of protein.

Statistical analysis

The mean and standard deviation (n=4) of the dry biomass, tolerance index, conidia production, MDA index, and enzyme activity data were analyzed with a one-way analysis of variance (ANOVA) and Tukey's test ($p < 0.05$) to determine significant differences between the treatments. Statistical analysis of normalized expression was performed with a one-way ANOVA using GraphPad Prism version 9.5.0 (GraphPad Software, San Diego, California, USA). A Dunnett post hoc test was used for multiple comparisons.

Results

Changes in growth and conidia production and tolerance of *T. asperellum* and *T. longibrachiatum* to Cu, Cr and Pb

T. asperellum: Without HM (control), the color of the *T. asperellum* culture was bright green, and the hyphal growth was disaggregated; however, with Cu, the culture color changed to orange, and with Pb and Cr the intensity of the green color decreased. In the presence of all three

metals, the hyphae grew and formed aggregates (Fig. 1a). With exposure to Cu, the dry biomass of *T. asperellum* was similar to that of the control. However, with exposure to Pb and Cr, the dry biomass decreased by 40% and 18%, respectively, compared with that of the control (Fig. 1b). In the presence of Cu, the conidia production of *T. asperellum* decreased but was at the same order of magnitude as that of the control. With exposure to Pb and Cr, conidia production decreased significantly by two orders of magnitude in comparison with that of the control (Fig. 1c). The tolerance index (TI) values with exposure to Cu, Pb and Cr were 0.92, 0.60 and 0.82, respectively (Fig. 1d).

T. longibrachiatum: Without exposure to HM (control), the *T. longibrachiatum* culture had a bright yellow color, and hyphal growth was disaggregated. With Cu, the culture color was pale yellow, and with Cr and Pb the culture color was lime green; with exposure to all three metals, the hyphae grew and formed aggregates (Fig. 1e). With exposure to Cu, the dry biomass was similar to that of the control, whereas with exposure to Pb, the dry biomass decreased by 18% compared with that of the control. In contrast, the dry biomass increased with Cr compared with that of the control (Fig. 1f). In the presence of the three metals, the conidia production of *T. longibrachiatum* decreased but was at the same order of magnitude as that of the control (Fig. 1g). The TIs with exposure to Cu, Pb and Cr were 1.01, 0.81 and 1.33, respectively (Fig. 1h). These TI values were greater than those observed in *T. asperellum*.

Cu, Cr and Pb cause lipid peroxidation in both *Trichoderma* species.

For both *Trichoderma* species without metals (control), the MDA content changed with the incubation time of the culture (Figs. 2a - b). For this reason, the MDA index was assumed to be an indicator of oxidative stress. In both *Trichoderma* species, the MDA index values were highest at 72 h after exposure to metals. In *T. asperellum*, the increase in the MDA index at 72 h exhibited a rank order of Cu > Pb = Cr, whereas in *T. longibrachiatum,* the increase in the MDA index exhibited a rank order of Pb > Cu > Cr (Figs. 2c - d).

Cu, Pb and Cr cause differential expression of detoxification-related genes in *T. asperellum* and *T. longibrachiatum*

We evaluated the transcriptional profiles of the *GPX*, *CAT*, and *CYS* genes, which encode enzymes involved in the antioxidative response, in both *Trichoderma* strains after exposure to HMs. In *T. asperellum*, expression of the *GPX* gene was upregulated after 72 h of exposure to Pb and Cr, whereas it was downregulated after exposure to Cu (Fig. 3a). The most notable changes with respect to the control were found after 144 h of treatment with the three metals. Expression of the *GPX* gene increased 188.5-fold in response to exposure to Cu, 7.6-fold in response to Pb and 61-fold in response to Cr (Fig. 3d). Expression of the *CAT* gene was upregulated at 72 h of exposure to Cu (4.6-fold), whereas expression of the gene did not change with exposure to Pb or Cr; however, after 144 h, expression of the gene was downregulated with all three metals (Figs. 3b - e). Expression of the *CYS* gene was upregulated after 72 h of treatment with all three metals. With Cu, the expression increased up to 4-fold at 72 h and 144 h; with Pb, expression

increased 13.8- and 3.2-fold at 72 and 144 h, respectively; and with Cr, expression increased 2.8-fold at 72 h and 2.5-fold at 144 h (Figs. 3c - f).
In *T. longibrachiatum*, *GPX* gene expression was upregulated at 72 h of exposure to all three metals: 0.7-fold for Cu, 0.6-fold for Pb and 4.1-fold for Cr. At 144 h of exposure to Cu, expression was downregulated, and with Pb and Cr exposure, expression was upregulated by 5.9-fold and 0.4-fold, respectively (Figs. 4a - d). At 72 h, the *CAT* gene was downregulated with Cu and upregulated with Pb (2.2-fold), but at 144 h, the *CAT* gene was downregulated with exposure to all three metals (Figs. 4b - e). The *CYS* gene was upregulated with exposure to Cr (2.1-fold) at 72 h and with exposure to Cu at 144 h (11.9-fold). Pb did not affect expression of the *CYS* gene (Figs. 4c - e).

Cu, Pb and Cr differentially induce peroxidase and catalase activation in *T. asperellu*m and *T. longibrachiatum*

The peroxidase activity of both *Trichoderma* species also depended on the exposure time and the metal. In both *Trichoderma* species, exposure to Cu for 72 h did not change peroxidase activity, but at 144 h, the enzyme activity increased 0.8-fold compared with that of the controls. Compared with that of the controls, the peroxidase activity of *T. asperellum* increased 3.2-fold after exposure to Pb for 72 h, whereas the enzyme activity decreased in *T. longibrachiatum*. In contrast, in both *Trichoderma* species, exposure to Pb for 144 h did not affect peroxidase activity. Exposure to Cr for 72 h increased peroxidase activity in both *Trichoderma* species, but exposure to Cr for 144 h decreased peroxidase activity in both *Trichoderma* species compared with that in their controls (Fig. 5).
In *T. asperellum* exposed to Cu for 72 and 144 h, catalase activity increased 0.2- and 0.8-fold, respectively, in comparison with that of the controls. In contrast, in *T. longibrachiatum*, exposure to Cu for 72 h decreased catalase activity, but at 144 h, catalase activity was 3.9-fold greater than that of the control. After exposure to Pb for 72 h, catalase activity increased 0.8- to 1.3-fold in the two *Trichoderma* species; however, after exposure to Pb for 144 h, catalase activity depended on the *Trichoderma* species. In *T. asperellum*, enzyme activity decreased, and in *T. longibrachiatum*, catalase activity increased 2.9-fold compared with that of the controls. In *T. asperellum*, exposure to Cr for 72 and 144 h reduced catalase activity, whereas in *T. longibrachiatum*, exposure to Cr decreased catalase activity at 72 h but increased it by 1.9-fold at 144 h (Fig. 6).
Without heavy metals (control), the culture color of *T. asperellum* was green and the hyphal growth was disaggregated, however with heavy metals, the culture color and hyphal growth changed depending on the heavy metal. With Cu, the culture of *T. asperellum* changed to orange color, with Pb the culture conserved a green color and with Cr the cultures decreased the intensity of its color. In the presence of the three metals, the hyphae grew forming aggregates (Fig. 1a). With exposure to Cu, the dry biomass of *T. asperellum* was similar to the control. Whereas the dry biomass of *T. asperellum* cultures exposure to Pb and Cr decreased by 40 and 18% respectively, compared to the control (Fig. 1b). With the presence of Cu, the conidia

production of *T. asperellum* decreased but on the same order of magnitude compared to the control. With exposure to Pb and Cr, the conidia production of *T. asperellum* decreased significantly by two orders of magnitude, in comparison with the control (Fig. 1c). The tolerance index values with the exposure to Cu, Pb and Cr were 0.92, 0.6 and 0.82, respectively (Fig. 1d). The culture of *T. longibrachiatum* growing without the exposure to heavy metals (control), had a bright yellow color and the hyphal growth was disaggregated. With Cu, the culture color of *T. longibrachiatum* was pale-yellow, and with Cr and Pb was lime green; and with the exposure of all metals, the hyphae grew forming aggregates (Fig. 1e). With exposure to Cu, the dry biomass of *T. longibrachiatum* was similar to the control; whereas with exposure to Pb, the dry biomass of *T. longibrachiatum* decreased 18%, related to the control. In contrast, the dry biomass of *T. longibrachiatum* increased with Cr compared to the control (Fig. 1f). With the presence of the three metals, the conidia production of *T. longibrachiatum* decreased, but on the same order of magnitude that than found in the control (Fig. 1g). The tolerance index with the exposure to Cu, Pb and Cr were 1.01, 0.81 and 1.33, respectively (Fig. 1h).

Lipid peroxidation in the two *Trichoderma* species exposed to heavy metals

In the two *Trichoderma* species and without metals (control), the MDA content changed with the incubation time of the culture (Figs. 2 a - b). For this reason, it was calculated the MDA index as an indicative of oxidative stress. In the two *Trichoderma* species, the MDA index values highest were found at 72 h with the exposure to metals. In *T. asperellum*, the increase of MDA index at 72 h was in the next order: Cu > Pb > Cr; whereas in *T. longibrachiatum* the increase of MDA index was in the next order: Pb > Cu > Cr (Figs. 2 c - d).

Cu, Pb and Cr causes differential expression of detoxification-related genes in *T. asperellu*m and *T. longibrachiatum*

In the tests with Cu, Pb and Cr in liquid medium, the mycelium of the two strains *T. asperellum* and *T. longibrachiatum* was collected. Twice, after 72 and 144 h, total RNA was extracted to determine the expression of the genes *GPX* (gluthatione peroxidase), *CAT* (catalase) and *CYS* (cysteine synthase), which are involved in the response to oxidative stress caused by treatment with Cu, Pb and Cr. In *T. asperellum*, the relative *GPX* gene expression was strongly induced after 72 h under Pb at 30856-fold compared to the control, while a high expression level was detected after 144 h under Cu exposure at 35568-fold compared to the control (Figs. 3 a - d). The relative expression level of *CAT* gene showed the highest induction after 72 h of Cu exposure with 0.00180633-fold compared to the control, but after 144 h, the expression of *CAT* gene was suppressed when exposed to Cu, Pb and Cr (Figs. 3 b - e). The relative expression level of the *CYS* gene showed a high induction by Pb exposure by 1009-fold after 72 h compared to the control and by 409-fold after 144 h for Cu, Pb and Cr exposure compared to the control (Figs. 3 c - f).

In *T. longibrachiatum*, gene expression was analyzed after 72 and 144 h (Fig. 4). The *GPX* gene showed a high induction under Cr exposure after 72 h by 209351-fold compared to the control,

while a high expression level was detected after 144 h under Pb exposure at compared to the control (Figs. 4 a - d). The *CAT* gene under Pb exposure after 72 h by 0.011353-fold compared to the control. As in *T. asperellum*, after 144 h, the *CAT* gene expression in *T. longibrachiatum* was suppressed upon exposure to Cu, Pb, and Cr (Figs. 4 b - e). Finally, *CYS* gene expression was increased 1697-fold upon exposure to Cr after 72 h and under Cu exposure at 144 h gene expression was increased 9100-fold compared to the control (Figs. 4 c - f). These results showed that the early activation of the expression of three genes related to detoxification in *T. asperellum* but in *T. longibrachiatum* indicates a different mechanism by exposure to heavy metals.

Peroxidase and catalase activity in *Trichoderma* exposed to heavy metals

In the two *Trichodema* species without exposure to metals (control), the highest peroxidase activity was found at 144 h (Fig. 5). The peroxidase activity in both *Trichoderma* species also depended on the exposure time and the metal. In the two *Trichoderma* species the exposure to Cu for 72 h did not change the peroxidase activity, but at 144 h increased in 0.8 times the peroxidase activity, compared with their controls. The exposure to Pb for 72 h increased 3.2 times the peroxidase activity in *T. asperellum*, while the enzyme activity decreased in *T. longibrachiatum*, related with their controls. In contrast, in the two *Trichoderma* species at 144 h of exposure to Pb not changed the peroxidase activity. In relation with Cr, the exposure to the metal for 72 h increased the peroxidase activity in both *Trichoderma* species, but the exposure to Cr for 144 h decreased the peroxidase activity in the two *Trichoderma* species, related with their controls (Fig. 5).

In the absence of metals (control), the catalase activity decreased at 144 h in the two *Trichoderma* species (Fig. 6). In *T. asperellum* with the exposure to Cu for 72 and 144 h, the catalase activity increase in comparison with their controls. Whereas in *T. longibrachiatum*, the exposure to Cu for 72 h decreased the catalase activity, but at 144 h increased 3.9 times the catalase activity, in relation with the control. With the exposure to Pb for 72 h, the catalase activity increased in the two *Trichoderma* species; but with the exposure to Pb for 144 h, the catalase activity depended on the *Trichoderma* specie. In *T. asperellum* decreased the enzyme activity and in *T. longibrachiatum* increased the catalase activity, compared with their controls. In *T. asperellum*, the exposure with Cr for 72 and 144 h reduced the catalase activity; while in *T. longibrachiatum*, the catalase activity decreased at 72 h but increased at 144 h of exposure with Cr (Fig. 6).

Discussion

Mycelial growth, conidia production and tolerance to Cu, Cr and Pb depend on the *Trichoderma* species

Exposure of the two *Trichoderma* species to Cu, Cr, and Pb induced changes in mycelium pigmentation and culture morphology. The nature of these changes depended on the *Trichoderma* species and the HM. In general, the mycelium pigmentation and morphology of *T. longibrachiatum* were the less affected by exposure to the three HMs compared with those of *T.*

asperellum, suggesting that *T. longibrachiatum* was more tolerant of Cu, Pb, and Cr than was *T. asperellum*. Changes in the mycelium pigmentation and culture morphology of *Trichoderma* caused by exposure to HMs have been reported in other *Trichoderma* species. The mycelium of *T. virens* exposed to Cu turned from green to a darker color (*Siddiquee et al., 2013*). In *T. harzianum* exposed to Cd, Cr, and Pb, the fungus grew and formed white aggregates with orange pigmentation on the edges (Liaquat et al., 2020). Mycelial pigmentation is a characteristic that could be related to the tolerance of metals (*Martino et al., 2000*), and we suggest that pigment production could be related to the tolerance of *T. asperellum* and *T. longibrachiatum* to Cu, Cr, and Pb. The absence of yellow pigments did not affect the mycelial growth of *T. reesei* or improve conidiation or tolerance to oxidative stress (*Zhang et al., 2021a*). In *T. longibrachiatum*, hexaketide metabolites such as sorbicillinoids have been identified (*Meng et al., 2016*), and in *T. asperellum*, polyketides have been identified (*Wu et al., 2017*). However, the role of polyketides in tolerance to HMs in these two *Trichoderma* species has not been studied and could be the subject of future studies.

The TI values for the three HMs were greater in *T. longibrachiatum* than in *T. asperellum*; in addition, pigmentation and sporulation under exposure to HMs were less affected in *T. longibrachiatum* than in *T. asperellum*, which indicates that *T. longibrachiatum* is more tolerant of Cu, Pb and Cr than is *T. asperellum*. It has been previously reported that the tolerance to Cu, Pb and Cr depends on the *Trichoderma* species: this was observed for Cu with *T. atroviridae*, *T. harzianum* and *Trichoderma* spp. (*Yazdani et al., 2009*; *Mohammadian et al., 2017*; *Maldaner et al., 2020*); for Pb with *T. harzianum*, *T. virens* and *T. viride* (*Liaquat et al., 2020*, *Tansengco et al., 2018*; *Joshi et al., 2011*); and for Cr with *T. harzianum* and *T. gamsii* (*Liaquat et al., 2020*, *Tansengco et al., 2018*).

Exposure to heavy metals causes lipid peroxidation in both *Trichoderma* species.

In both *Trichoderma* species without exposure to HMs (controls), the MDA content increased with culture time, suggesting that ROS could be related to the mycelial growth of *Trichoderma*. ROS are involved in several development processes (mycelial growth and conidia and sclerotia formation) and pathogenicity in fungi. ROS are produced via the enzyme NADPH oxidase (*Nox*), which consists of three catalytic subunits, *NoxA*, *NoxB*, and *NoxC*, and the regulatory subunit *NoxR* (*Zhang et al., 2020*; *Zhang et al., 2021b*). In *T. atroviride*, the *NoxR* and *Nox1* genes have been shown to participate in the development and differentiation of the fungus (*Cruz-Magalhães et al., 2019*).

Because the MDA content increased with culture time, the MDA index was calculated. The greatest increase in the MDA index occurred at 72 h in both *Trichoderma* species exposed to the three HMs, which indicates that the principal oxidative process occurred at 72 h. Similarly, in *Trichoderma* and other fungi, the increase in MDA content caused by exposure to HMs was indicative of oxidative stress; this was observed for *T. harzianum* with exposure to Cu (*Tavsan and Ayar Kayali, 2013*), for *T. lixii* with exposure to Cr (*Kumar and Dwivedi, 2019*), for *Pleurotus ostreatus* and *P. opuntia* with exposure to Pb and Cr (*Zhang et al., 2016*; *Li et al.,*

2017; *Yadav et al., 2023*), and for *Phanerochaete chrysosporium* with exposure to Pb (*Huang et al., 2017*).

Cu, Pb and Cr induced differential expression of detoxification-related genes in *T. asperellu*m and *T. longibrachiatum*

Analysis of the expression of the *GPX*, *CAT*, and *CYS* genes in *T. asperellum* and *T. longibrachiatum* under exposure to Cu, Pb and Cr revealed differential behavior in the two *Trichoderma* species based on metal and exposure time, which indicates that gene activation related to the antioxidant response to the oxidative damage caused by the HMs could indicate a genetic mechanism that is differentially regulated in the two species. The *GPX* gene was differentially expressed between the two *Trichoderma* species. Studies of gene expression related to the antioxidant response in *Trichoderma* are scarce. However, our data are consistent with the results obtained from a transcriptome analysis of *T. harzianum* exposed to Cd, which revealed upregulated expression of proteins with oxidoreductase activity, such as glutathione S transferases (*Oshiquiri et al., 2020*), suggesting that the *GPX* gene participates in the antioxidant response to HM exposure in *Trichoderma*.

With respect to the expression profile of the *CAT* gene, our results revealed early expression under exposure to Cu in *T. asperellum* and under exposure to Pb in *T. longibrachiatum*. At 144 h, the *CAT* gene was downregulated in both *Trichoderma* species following exposure to HMs. The expression profile of the *CAT* gene in fungi has been poorly studied. However, plants treated with Cd and Pb show an abundance of *CAT* gene transcripts, suggesting that the *CAT* gene participates in the antioxidant response to exposure to HMs (*Aydin et al., 2016*). These data suggest that the *CAT* gene could be involved in the early response to the oxidative damage caused by exposure to HMs in *Trichoderma*.

The amino acid cysteine is the precursor of metallothioneins, glutathione and phytochelatins (*Clemens, 2006*). To determine whether the cysteine synthase (*CYS*) gene is involved in the antioxidant response of both *Trichoderma* species under exposure to Cu, Pb, and Cr, we evaluated its expression level. Notably, in *T. asperellum,* the *CYS* gene was upregulated after 72 h of exposure to all three HMs, whereas in *T. longibrachiatum*, the *CYS* gene was upregulated only after exposure to Cu and Cr but not Pb, which suggests that the *CYS* gene is not involved in the response to Pb. Similarly, expression of the *OASTL* gene encoding an enzyme involved in cysteine synthesis in *T. harzianum* was induced with exposure to Cd, Pb, Hg and Zn (*Raspanti et al., 2009*). Therefore, we suggest that the *CYS* gene participates in the antioxidant response in *Trichoderma*. This is the first report of *CYS* gene expression in the genus *Trichoderma*, specifically in *T. asperellum* and *T. longibrachiatum,* under exposure to Cu, Pb, and Cr.

4.4 Cu, Pb and Cr caused differential peroxidase and catalase activities in *T. asperellu*m and *T. longibrachiatum*

Antioxidant enzymes are a cellular mechanism that reduces the toxic effects of excess ROS, and this antioxidant mechanism is essential for maintaining the cellular redox balance (*Belozerskaya*

and Gessler, 2007). In *Trichoderma*, the antioxidant enzyme activity of some *Trichoderma* species exposed to HMs has been evaluated. However, the results are contradictory and suggest that the antioxidant response may depend on the particular *Trichoderma* species, the *Trichoderma* isolate and the HM. In three isolates of *T. asperellum* exposed to Cu-based fungicides, the activity of catalases and peroxidases depended on the isolate of *T. asperellum* (*Pérez-Torres et al., 2020*). In contrast, in *T. lixii*, catalase activity has been reported to decrease with Cr, and peroxidase and superoxide dismutase activities depended on the Cr concentration (*Kumar and Dwivedi, 2019*). On the basis of these results, this study evaluated the peroxidase and catalase activities of two *Trichoderma* species exposed to Cu, Pb and Cr. Our results revealed that, in both *Trichoderma* species exposed to Cu, the activity of peroxidases and catalases increased principally at 144 h. This result confirms that catalases and peroxidases respond to the oxidative stress induced by Cu-based fungicides in three isolates of *T. asperellum* (*Pérez-Torres et al., 2020*). In other fungi, such as *Aspergillus niger* (*Cavalcanti Luna et al., 2015*), *Pleurotus* sp. (*Mohamadhasani and Rahimi, 2022*) and *Alternaria alternata* (*Shoaib et al., 2015*), the activity of catalase or peroxidase also increased with exposure to Cu, suggesting that the increase in antioxidant enzyme activity is a mechanism to reduce the toxic effects of Cu via the degradation of hydrogen peroxide to water.

Our results also revealed that in both *Trichoderma* species exposed to Pb and Cr, peroxidase and catalase activities were dependent on the exposure time and HM. Studies on antioxidant enzyme activity in response to the oxidative stress caused by Pb and Cr in *Trichoderma* are scarce. In *T. lixii* exposed to Cr, catalase activity decreased with increasing Cr concentration, whereas peroxidase activity depended on the Cr concentration (*Kumar and Dwivedi, 2019*). In *Phanerochaete chrysosporium*, changes in catalase and peroxidase activities depended on the exposure time to Cd and Pb, and it has been suggested that changes in enzyme activity are a cellular response to protect against the oxidative damage caused by ROS (*Zhang et al., 2015*; *Huang et al., 2017*). Thus, our results show that the catalase and peroxidase activities in response to oxidative stress induced by HMs depends on the HM and exposure time to the metal. The antioxidant response to HMs is genetically modulated in each *Trichoderma* species.

Conclusions

The isolate of *T. longibrachiatum* was more tolerant to Cu, Cr, and Pb than was the isolate of *T. asperellum*. However, the three HMs induced oxidative damage in both *Trichoderma* species, and in response to oxidative damage, the activities of peroxidase and catalase and the expression of detoxification-related genes (*GPX*, *CAT*, and *CYS*) were induced differentially in *T. asperellum* and *T. longibrachiatum* depending on the exposure time and the HM (Cu, Pb, or Cr). These findings indicate that the antioxidant response to HMs was genetically modulated in each *Trichoderma* species. To our knowledge, this is the first report of *CYS* gene expression in the genus *Trichoderma* and its role in the antioxidant response to Cu, Pb, and Cr. This study contributes to the understanding of the genetic and biochemical mechanisms of the antioxidant response of *Trichoderma* fungi, which have potential use in food crops.

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Figure 1

Growth, sporulation and tolerance of *T. asperellum* and *T. longibrachiatum* exposed to Cu, Pb and Cr for 144 h.

Image of liquid cultures (a and e) showing the changes in mycelial growth and culture pigmentation. Dry biomass (b and f), conidia production (c and g), and tolerance index (d and h). The control cultures were grown without metals. The mean ± standard deviation (n=4) was calculated and analyzed using a one-way analysis of variance (ANOVA), and significant differences in dry biomass and conidia production between treatments were determined using Tukey´s test ($P<0.050$).

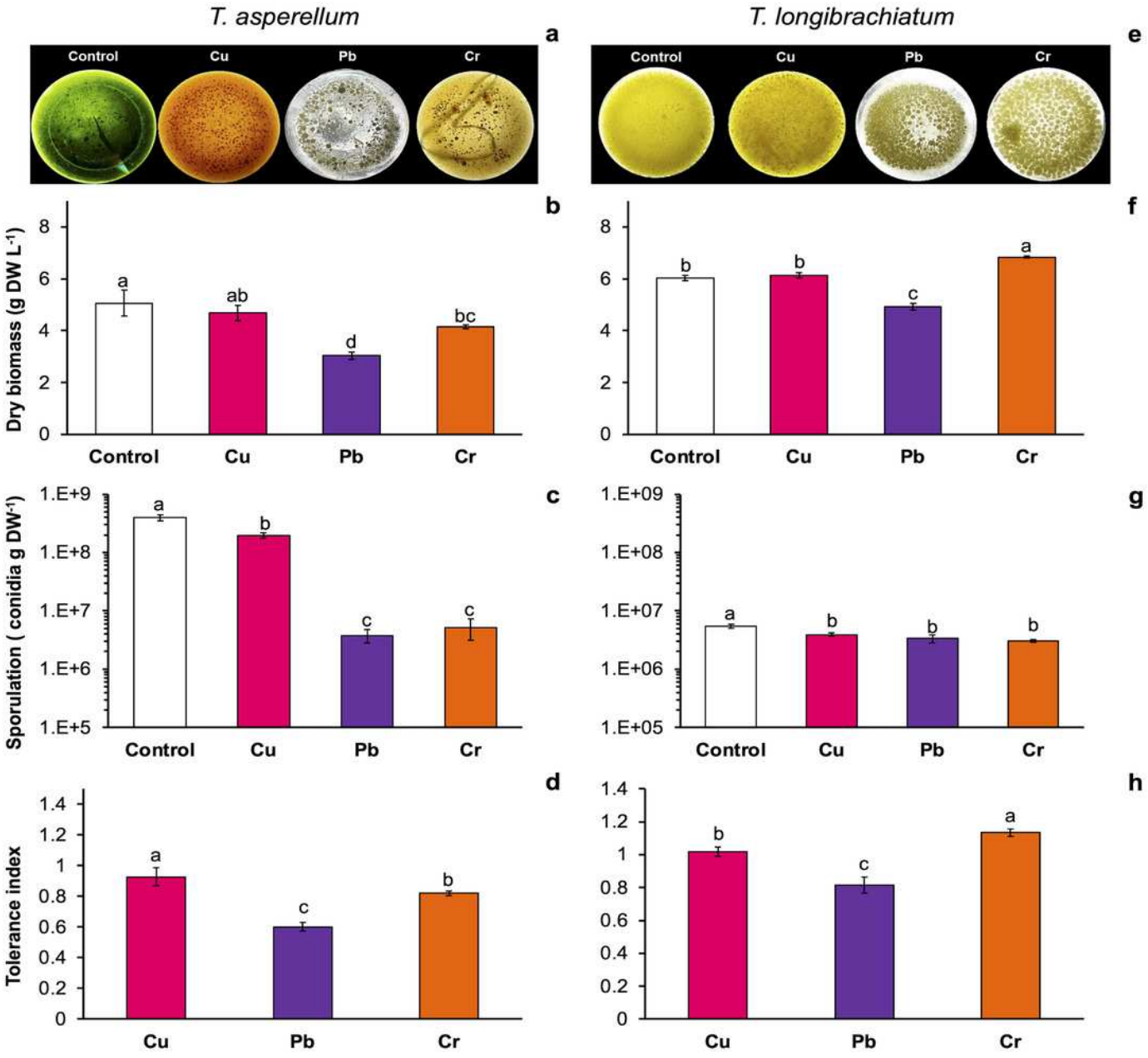

T. asperellum
T. longibrachiatum
a
e
Control
Cu
Pb
Cr
b
f
Dry biomass (g DW L-1)
c
g
Sporulation (conidia g DW-1)
d
h
Tolerance index

Figure 2

Malondialdehyde (MDA) content (a and b) and the MDA index (c and d) of *T. asperellum* (a and c) and *T. longibrachiatum* (b and d) after exposure to Cu, Pb, and Cr for 72 and 144 h.

The control cultures were grown without metals. The mean ± standard deviation (n=4) was calculated and analyzed using a one-way analysis of variance (ANOVA), and significant differences in the MDA content between treatments were determined using Tukey´s test (P<0.050).

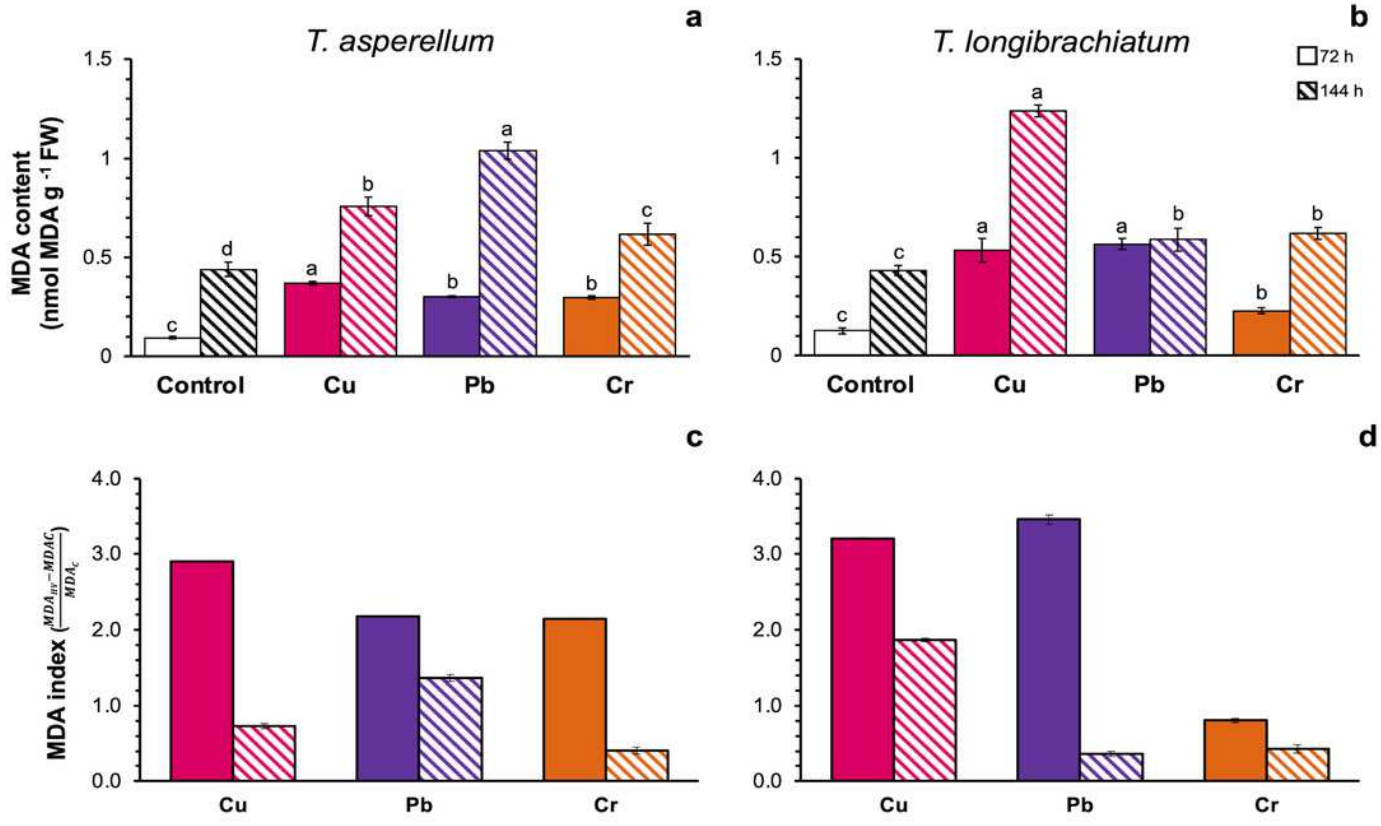


Figure 3

Normalized expression levels of the *CAT*, *GPX* and *CYS* genes in *T. asperellum* exposed to Cu, Pb and Cr for 72 and 144 h.

The control cultures were grown without metals. The relative expression of *CAT*, *GPX*, and *CYS* was normalized against that of the *Trichoderma TUB* (*tubulin*) gene. The experiments were repeated in triplicate. A one-way ANOVA with a Dunnett post hoc test was used for multiple comparisons, and asterisks indicate significant differences with (*) $P<0.0332$, (**) $P<0.0021$, (***) $P<0.0002$ and *(****)* $P<0.0001$.

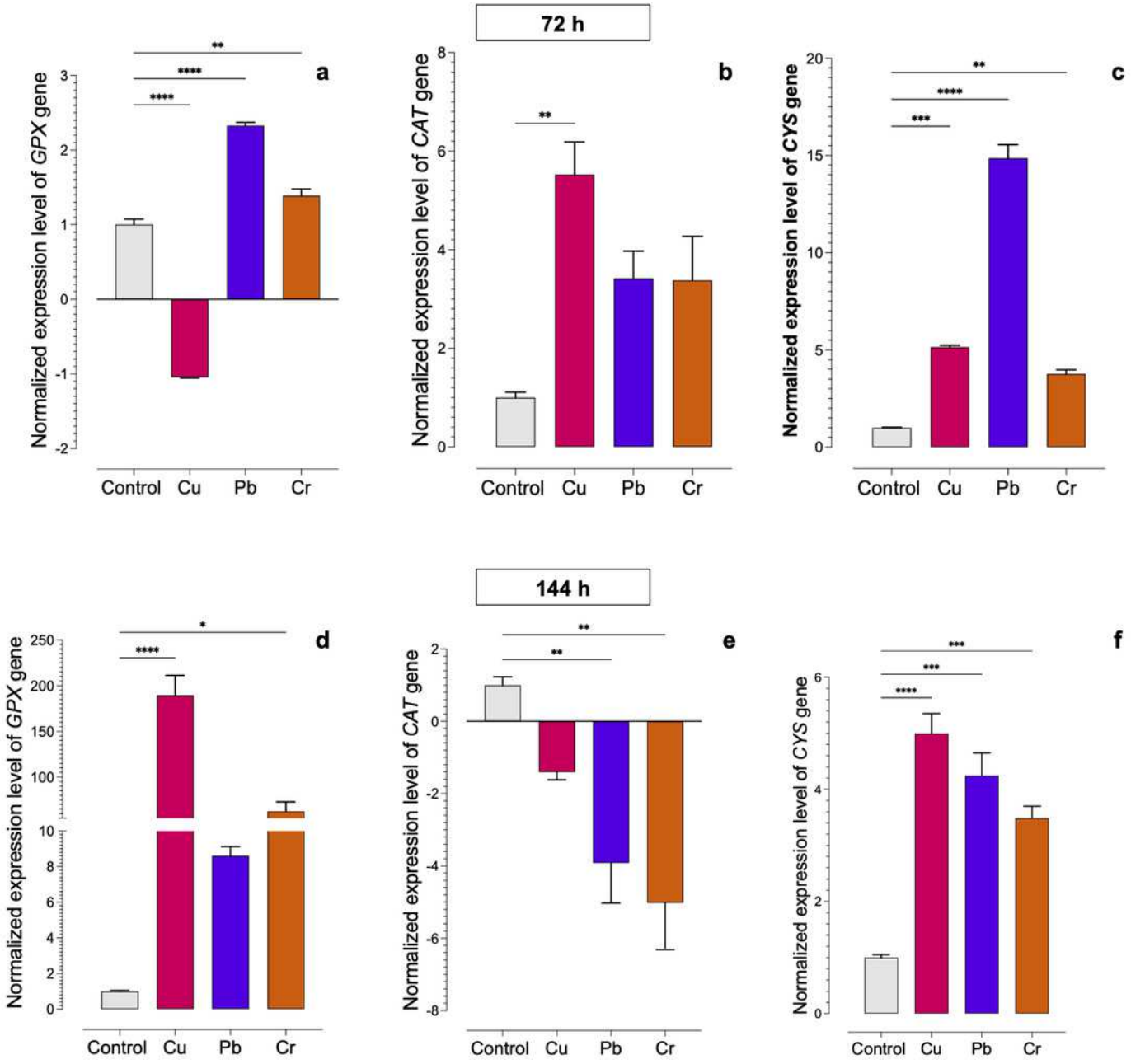

72 h
144 h
a
b
c
d
e
f
Normalized expression level of GPX gene
Normalized expression level of CAT gene
Normalized expression level of CYS gene
Control
Cu
Pb
Cr

Figure 4

Normalized expression levels of the *CAT*, *GPX* and *CYS* genes in *T. longibrachiatum* exposed to Cu, Pb and Cr for 72 and 144 h.

The control cultures were grown without metals. The relative expression of *CAT*, *GPX*, and *CYS* was normalized against that of the *Trichoderma TUB* (*tubulin*) gene. The experiments were repeated in triplicate. A one-way ANOVA with a Dunnett post hoc test was used for multiple comparisons, and asterisks indicate significant differences with (*) $P<0.0332$, (**) $P<0.0021$, (***) $P<0.0002$ and *(****)* $P<0.0001$.

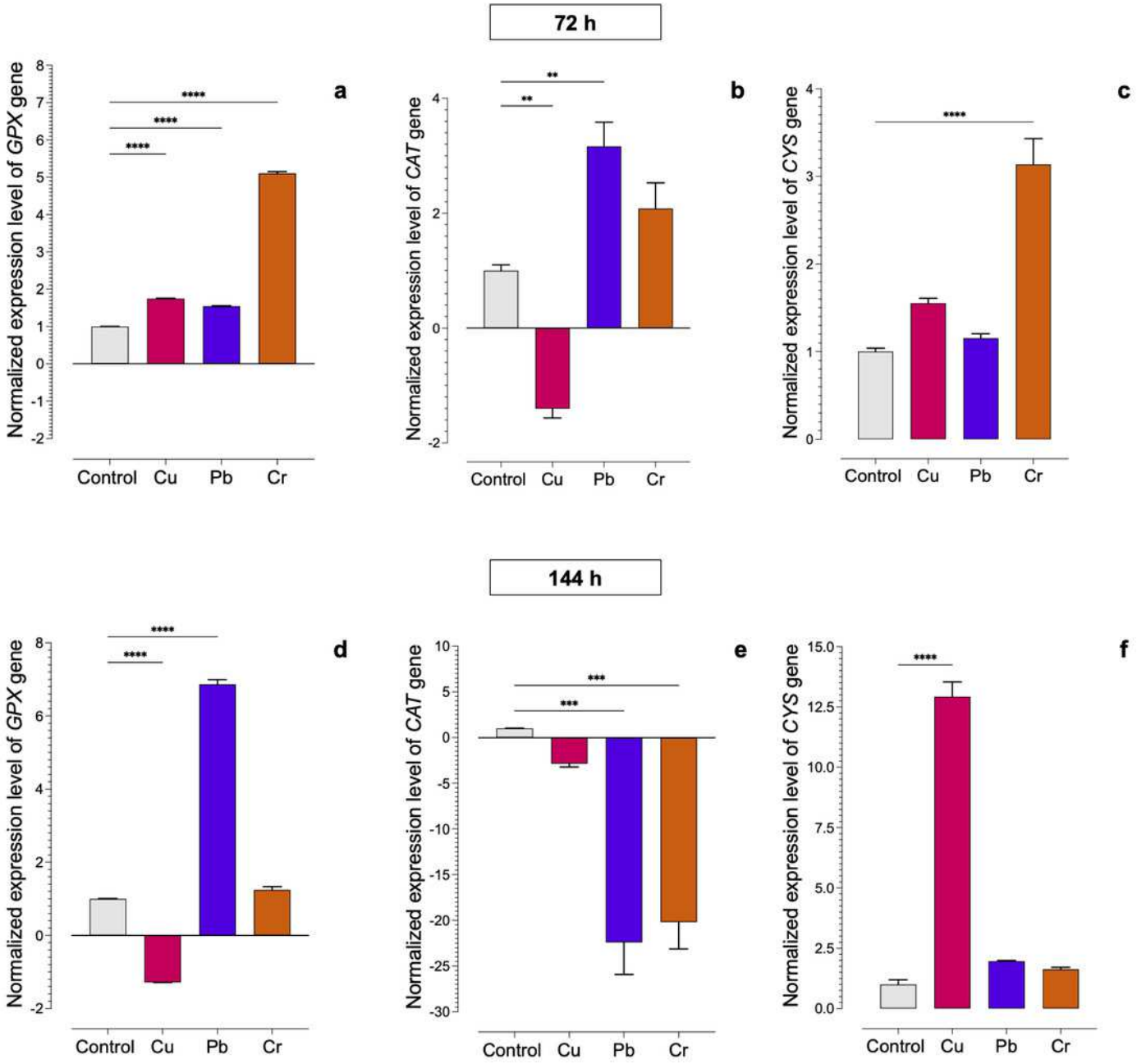

72 h
a
b
c
Normalized expression level of GPX gene
Normalized expression level of CAT gene
Normalized expression level of CYS gene
Control
Cu
Pb
Cr
144 h
d
e
f

Figure 5

Peroxidase activity in *T. asperellum* and *T. longibrachiatum* exposed to Cu, Pb and Cr for 72 (a and b) and 144 h (c and d).

The control cultures were grown without metals. The mean ± standard deviation (n=4) was calculated and analyzed using a one-way analysis of variance (ANOVA). Different lowercase letters indicate significant differences in peroxidase activity between treatments according to a Tukey´s test ($P<0.05$).

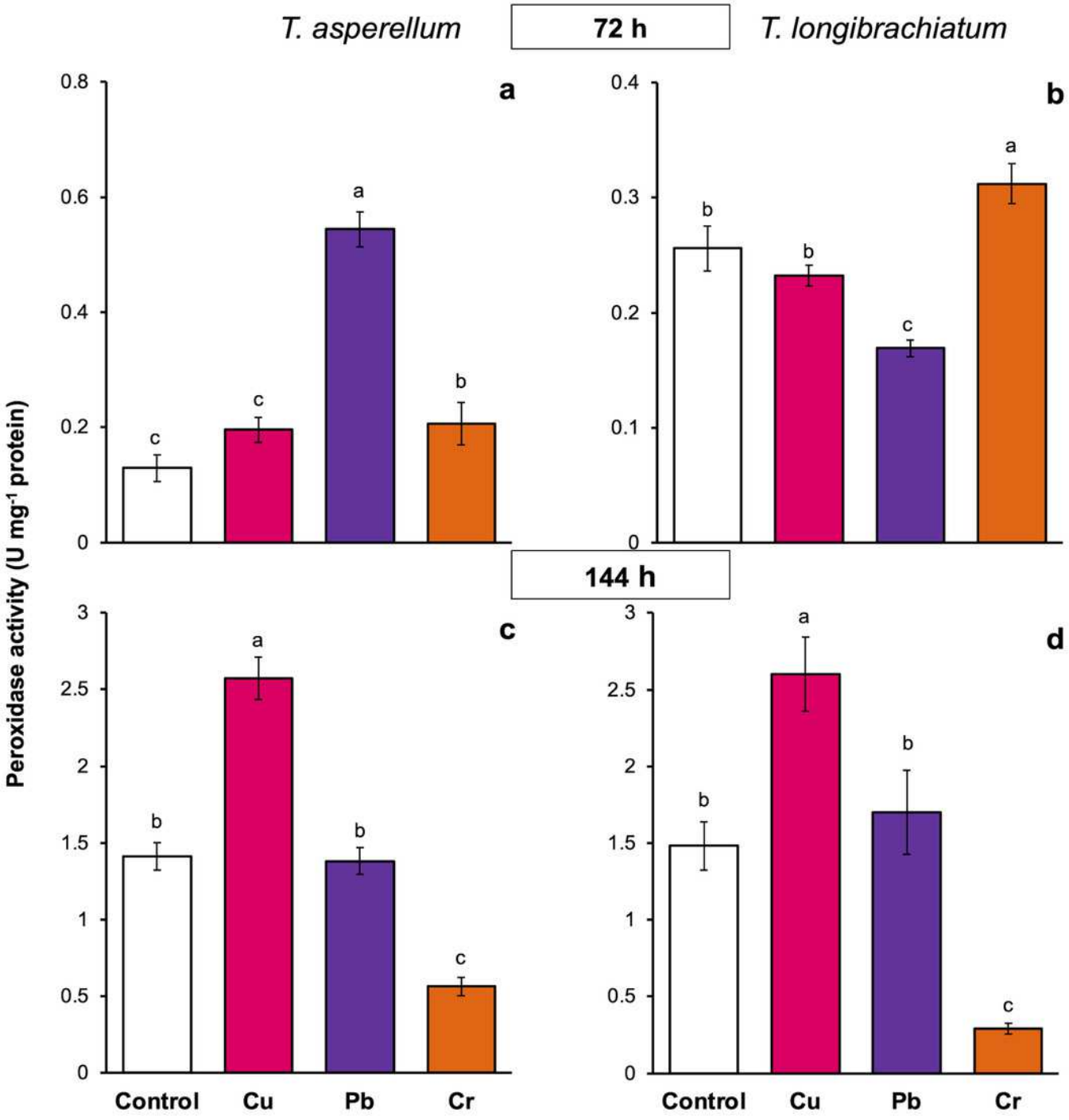
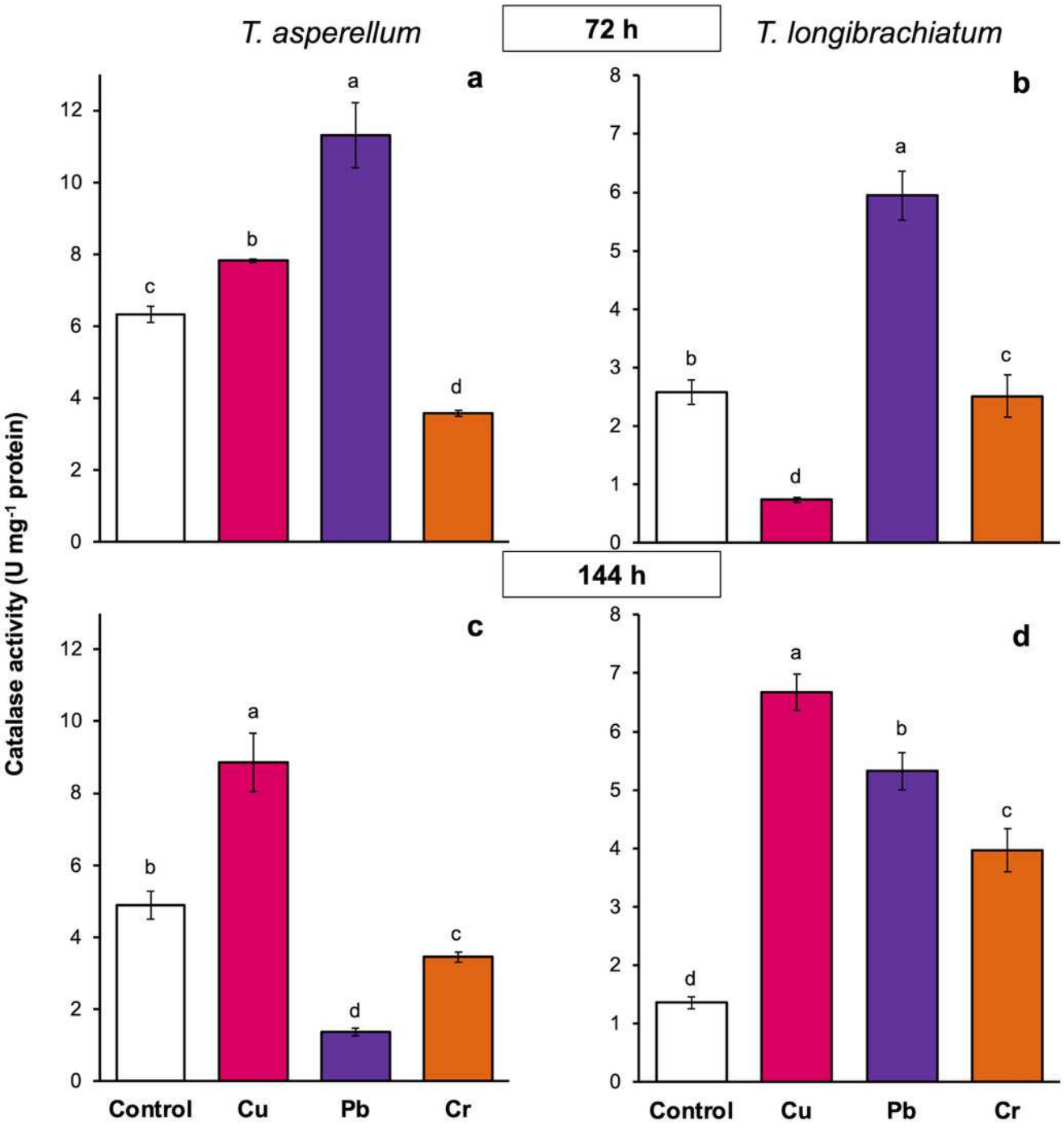

T. asperellum
72 h
T. longibrachiatum
a
b
c
d
144 h
Peroxidase activity (U mg-1 protein)
Control
Cu
Pb
Cr

Figure 6

Catalase activity in *T. asperellum* and *T. longibrachiatum* exposed to Cu, Pb and Cr for 72 (a and b) and 144 h (c and d).

The control cultures were grown without metals. The mean ± standard deviation (n=4) was calculated and analyzed using a one-way analysis of variance (ANOVA). Different lowercase letters indicate significant differences in catalase activity between treatments according to a Tukey´s test ($P<0.05$).


T. asperellum
72 h
T. longibrachiatum
a
b
c
d
144 h
Catalase activity (U mg-1 protein)
Control
Cu
Pb
Cr