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Tolerance and Stress Response of *Macrolepiota procera* to Nickel

Paula Baptista,*,† Sílvia Ferreira,† Elisa Soares,§ Valentim Coelho,† and Maria de Lourdes Bastos§

†CIMO/Escola Superior Agrária, Instituto Politécnico de Bragança, Campus Santa Apolónia, Apartado 1172, 5301-855 Bragança, Portugal, and §REQUIMTE/Serviço de Toxicologia, Faculdade de Farmácia, Universidade do Porto, R. Aníbal Cunha 164, 4099-030 Porto, Portugal

Nickel (Ni) is an essential element for many organisms; however, it is very toxic at high concentrations and also depending on the species. In macrofungi the mechanisms underlying their Ni tolerance are poorly documented. This study examines, for the first time, the participation of the antioxidative system in *Macrolepiota procera* exposed to different Ni^{2+} concentrations and their relation with Ni tolerance. The effect of the pH on Ni tolerance was also evaluated. The fungus was cultivated on solid medium with different $NiCl_2$ concentrations (0.05, 0.2, 0.8 mM) at pH 4, 6, and 8, and fungi growth and Ni uptake were determined. The antioxidative enzymes catalase (CAT) and superoxide dismutase (SOD) and the production of hydrogen peroxide (H_2O_2) were evaluated on fungal submerged cultures within the first hours of Ni^{2+} exposure. Results showed that *M. procera* growth decreased when Ni^{2+} concentrations increased, reaching a maximum growth inhibition (>80%) up to 0.2 mM of $NiCl_2$. Ni uptake increased proportionally to Ni increase in the medium. Both Ni tolerance and Ni accumulation were affected by medium pH. Microscope observations showed differences in the size of spores produced by fungi at different Ni concentrations. Ni exposure induced oxidative stress, as indicated by the production of H_2O_2, the levels of which seem to be regulated by the antioxidant enzymes SOD and CAT. The time variation pattern of SOD and CAT activities indicated that the former has a greater role in alleviating the stress. The results obtained suggested that tolerance of *M. procera* to Ni^{2+} is associated with the ability of this macrofungus to initiate an efficient antioxidant defense system.

KEYWORDS: *Macrolepiota procera*; nickel; stress; Ni^{2+} tolerance; Ni^{2+} accumulation; hydrogen peroxide; antioxidant enzymes

INTRODUCTION

The Parasol mushroom, *Macrolepiota procera* (Scop.) Singer, is an edible saprotrophic fungus very common in Portugal. It is highly appreciated due to its delicious and delicate texture, good taste, and faint nutty aroma of the cap. From a nutritional point of view *M. procera* is rich in proteins and carbohydrates, contains high amounts of dietary fiber, and has low fat content and energy (*1*, *2*).

A wide variety and abundance of mineral contents are the most characteristic features of this species. These mushrooms are relatively rich in potassium, phosphorus, magnesium, calcium, sodium, copper, zinc, rubidium, iron, selenium, lithium, and manganese (*2*−*10*). *M. procera* also accumulates in its fruit body toxic metals such as mercury, cadmium, chromium, nickel, lead, thallium, barium, cobalt, arsenic, and silver (*2*, *4*−*9*, *11*−*14*). Some of these elements, such as copper, iron, manganese, zinc, nickel, and cobalt, are important as micronutrients for fungal growth and metabolism. In contrast, chromium, cadmium, lead, mercury, and silver have unknown essential biological functions, being recognized as toxic (*15*, *16*). When heavy metal concentrations, both essential and nonessential, are at excessively higher levels than those required for fungi, heavy metal ions form unspecific complex compounds in the cell, which leads to toxic effects (*17*, *18*). Moreover, growth inhibition, morphological and physiological alterations, and changes in fungi reproduction can be observed (for a review see ref *19*).

There is evidence that fungi can tolerate and detoxify heavy metals present in excess concentrations in the growth medium by using several mechanisms. These mechanisms usually include (i) metal valence alteration, (ii) sequestration and precipitation of heavy metals as a result of the extra- and intracellular excretion of metal-chelating substances, (iii) intracellular compartmentalization or volatilization of the metal, and (iv) active uptake (*15*, *16*, *20*−*23*). In contrast, less information is available on the involvement of the antioxidative system in the fungi as protection against heavy metal stress. The few studies carried out, especially in yeast and *Aspergillus* species, reveal that the heavy metal tolerance was correlated with reactive oxygen species (ROS) generation in cells and with antioxidative defense systems efficiency (*24*−*28*). Among the enzymatic defense, superoxide dismutase (SOD) and catalase (CAT) perform an important part of

*Corresponding author (telephone + 351 273303332; fax + 351 273 325405; e-mail pbaptista@ipb.pt).

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a cell's defense against ROS. In contrast, in macrofungi there are only a few reports on stimulation of antioxidative enzymes in response to heavy metals in the growth medium (*29*−*32*), and no evidence has been reported concerning production of ROS. In these studies it was shown that cadmium treatment induced SOD (*31*) and MnSOD (*30*) activity in the ectomycorrhizal fungus *Paxillus involutus* and changes in the CuZnSOD isoenzyme pattern in the ectomycorrhizal fungus *Rhizopogon roseolus* (*29*). Similarly, it was verified that copper, zinc, and manganese treatment induced SOD activity in the entomopathogenic fungi *Cordyceps militaris* and that zinc caused a decrease of membrane lipid peroxide levels in mycelia (*32*).

As far as we know the response of the antioxidative system to heavy metal induced stress has not yet been studied in saprobe macrofungi. Because saprobe fungi can accumulate in its fruit body large concentrations of heavy metals when compared to mycorrhizal fungal species (*33*, *34*), interest in elucidating the mechanisms underlying the resistance of saprobe fungi to metal stress is very high. This ability to accumulate metals can also be an opportunity for fungal mycelia application in selective sorption of individual heavy metal ions from polluted soils.

In this study, we investigated the mechanisms' response to nickel (Ni^{2+}) exposure in the macrofungi *M. procera*, due to its recognized ability to accumulate in its fruit body a large number of toxic metals, including Ni (*33*). The selection of nickel was by the existence of a large area of serpentine soils typically characterized by phytotoxic concentrations of heavy metals such as Ni, Cr, and Co in the Trás-os-Montes region and because it is ubiquitously distributed throughout the world. In a first approach, we evaluated Ni tolerance and accumulation by *M. procera* and the effect of pH in these processes. The induction of macro- and microscopic morphological changes on *M. procera* mycelium by Ni^{2+} was also examined. Finally, we studied the stress induction (H_2O_2 production) and the defense response (ROS scavenging enzymes) on fungal submerged cultures within the first hours of Ni^{2+} exposure (0−72 h).

MATERIALS AND METHODS

Isolation and Maintenance of *Macrolepiota procera* Mycelium. The mycelium of *M. procera* was isolated from sporocarps collected under *Quercus rotundifolia* stands at Donai-Bragança (northeastern Portugal). Fungal isolation was performed on Melin−Norkans (MMN) agar medium at pH 6.6 [NaCl, 0.025 g/L; $(NH_4)_2HPO_4$, 0.25 g/L; KH_2PO_4, 0.50 g/L; $FeCl_3$, 0.050 g/L; $CaCl_2$, 0.50 g/L; $MgSO_4 \cdot 7H_2O$, 0.15 g/L; thiamin, 0.10 g/L; casamino acids, 1.0 g/L; malt extract, 10 g/L; glucose ,10 g/L; agar, 20 g/L], following the method of Brundrett et al. (*35*). The strain was maintained in the same medium at 25 °C, in the dark, and was subcultured regularly.

Screening for Nickel Tolerance and Effect of pH in This Process. The tolerance of the *M. procera* to Ni^{2+} was tested on MMN agar medium amended with $NiCl_2$ and adjusted to different pH values (4.0, 6.0, and 8.0). The amendment of medium was done by the addition of filter-sterilized solutions of $NiCl_2$ in sterile water to give final concentrations of 0.05, 0.2, and 0.8 mM. The control treatment was performed in the same medium without Ni, by the addition of sterile distilled water. The pH of the medium was adjusted to 4.0, 6.0, and 8.0 with 1 M NaOH or 1 M HCl before autoclaving. Ten milliliter of medium was transferred to 9.0 cm diameter Petri dishes, and autoclaved cellophane membrane was placed aseptically on the surface of the agar. Each Petri dish was centrally inoculated with a single circular 5 mm mycelial plug removed from the edge of an actively growing colony. Dishes were sealed with Parafilm and incubated in the dark at 25 °C for up to 40 days. Seven replicate dishes were prepared for each metal concentration and pH combination. Radial mycelial growth was measured at days 3, 6, 9, and 12, using two cardinal diameters previously drawn on the bottom of the dish. This measure was performed only up to 12 days because at that time control colonies occupied the whole agar surface. Growth inhibition (in percentage) was calculated relative to control, and radial growth rate (mm/day) was also determined.

Macroscopic and Microscopic Characterization of the Colonies. The colonies were macroscopically and microscopically characterized. Morphological alterations of *M. procera* such as colony texture and color, border appearance and color, reverse color, medium coloration, and exudates were registered. At the end of 12 days of *M. procera* incubation the morphology of hyphae was observed. The hyphae were placed on a glass slide, mounted on lactophenol cotton blue, and examined using a light microscope (Leitz Laborlux 12).

Determination of Ni Contents in Mycelium. *Reagents and Materials*. All of the solutions were prepared with doubly deionized water, and the chemicals used were of Suprapur grade (Merck). Nickel standard working solutions were prepared daily from 1000 mg/L solutions (Spectrosol, BDH) in HNO_3 (0.2%). To avoid contamination of the samples, all PTFE materials (Teflon vessels, pipettes, micropipet tips, and autosampler cups) were immersed in freshly prepared 15% v/v pro analysis HNO_3 (Merck) during 24 h and then rinsed thoroughly with doubly deionized water.

Apparatus. Metal quantification was carried out in a Perkin-Elmer HGA-850 furnace installed in a model *A*Analyst 300 spectrometer with deuterium arc background correction, equipped with an AS-800 autosampler and a HP Deskjet 920C. Analyses were performed using Perkin-Elmer HGA tubes with integrated platform. The furnace program was used with an ashing temperature of 1300 °C and an atomization temperature of 2500 °C.

Sample Preparation. Forty days after incubation, the mycelia of *M. procera* were carefully scraped off the cellophane and were dried in an oven at 30 °C, during 7 days. The dried mycelia samples were reduced to powder in an agata mortar, and approximately 0.10−0.20 g was weighed and transferred to a Teflon container, which, after the addition of 2 mL of HNO_3 and 250 μL of H_2O_2, was closed for digestion overnight in a stove thermostatically controlled at 105 ± 1 °C. The digested solution was transferred to a decontaminated tube and diluted to a convenient volume with doubly deionized water.

Method Validation. The accuracy of the method was evaluated by using the Certified Reference Material Lichen CRM 482, the Ni certified value being 2.47 ± 0.07 μg/g and the found value, 2.45 ± 0.05 μg/g. The precision of the method was 9.6 and 9.7% for instrumental and analytical procedures, respectively. The linearity was 0.63−50.0 μg/L. The detection limit was 0.63 μg/L, and the quantification limit was 2.08 μg/L. Considering 0.10 g and 10 mL final volumes we found 0.063 and 0.208 μg/g for the detection and quantification limits, respectively.

Mycelium Elicitation by Ni^{2+}. The induction of oxidative stress response of the *M. procera* to Ni was assessed on mycelium growth on MMN liquid medium amended with $NiCl_2$ at pH 6. Suspension culture of *M. procera* was obtained by transferring eight hyphal plugs (5 mm diameter), collected from colony margins actively growing on MMN agar medium, to 250 mL of liquid medium in a 750 mL culture flask. Fungus cultures were maintained in the dark at 25 °C, without agitation, for 15 days. The assay was performed by transferring 100 mL of this mycelium, previously lightly shaken, into 50 mL of liquid medium. After 7 days of culture, the mycelium was elicited by adding 100 μL per flask of filter-sterilized aqueous solutions of $NiCl_2$ to give final concentrations of 0.05, 0.2, and 0.8 mM. Mock inoculations were done with sterile distilled water (100 μL per flask). After gentle shaking, the flasks were maintained under the same conditions. After 3, 6, 24, 48, and 72 h of contact between *M. procera* fungi and $NiCl_2$, the mycelia were recovered by filtration through filter paper (Whatman no. 3) and were washed three times with sterile water. The mycelia were immediately ground to a fine powder in liquid nitrogen and stored at −80 °C until analysis of H_2O_2 contents and enzyme activities. Three replicate flasks were prepared for each metal concentration and elicitation time.

Quantification of H_2O_2. Hydrogen peroxide content was determined according to the method of Loreto and Velikova (*36*). Briefly, samples (70 mg FW) were homogenized in 2.0 mL of 0.1% (w/v) trichloroacetic acid, and the homogenate was centrifuged at 14000*g* for 15 min at 4 °C. From each supernatant, an aliquot of 0.5 mL was added to 0.5 mL of 10 mM phosphate buffer (pH 7.0) and 1.0 mL of 1 M KI, and the absorbance was measured at 390 nm. H_2O_2 was quantified by taking into

account a calibration curve using solutions with known H_2O_2 concentrations.

Extraction and Quantification of Proteins. Approximately 1.0 g (FW) of ground mycelium was homogenized in 4 mL of 80 mM phosphate buffer (pH 7.0), 1 mM benzamidine, 0.1% (v/v) 2-mercaptoethanol, 1 mM EDTA, 0.1% (v/v) Triton X-100, and 1% (w/v) polyvinylpolypyrrolidone at 4 °C. The homogenates were centrifuged at 14000*g* for 20 min at 4 °C, and the supernatants were recovered. Protein was quantified by the Coomassie Blue microassay using BSA as standard (*37*).

Enzymatic Assays. All enzymatic assays were performed using freshly prepared protein extracts, which were kept on ice until analysis.

Superoxide dismutase (SOD; EC 1.15.1.1) activity, determined according to the Beyer and Fridovich method (*38*), was based on the ability of SOD to inhibit the reduction of NBT by the superoxide radicals generated photochemically. The reaction mixture consisted of 100 mM phosphate buffer (pH 7.8), 0.2 mM EDTA, 19.8 mM L-methionine, 0.05% (v/v) Triton X-100, 57 μM NBT, 0.9 μM riboflavin, and protein extract (50−250 μL). After 6 min of incubation at 30 °C under continuous light, absorbance was read at 560 nm. One unit (U) of SOD activity is defined as the amount of enzyme required to cause 50% inhibition of NBT reduction under the above assay conditions.

Catalase (CAT; EC 1.11.1.6) activity was determined by following the decomposition of H_2O_2 (*39*). Reactions were performed in 80 mM phosphate buffer (pH 7.0) containing 1 mM H_2O_2 and initiated by adding 20−50 μL of protein extract. The decrease in absorbance was evaluated at 240 nm and 25 °C. One unit (U) of CAT activity is defined as the amount of enzyme necessary to decompose 1 μmol min^{-1} H_2O_2 under the above assay conditions.

Data Analysis. Data from enzyme activities, quantification of H_2O_2, and radial growth of mycelium are presented as the mean of three or seven independent experiments displaying the respective SE bars. Differences among means were done by analysis of variance (ANOVA), using SAS v. 9.1.3, and averages were compared using the Tukey test ($p < 0.05$).

Correlations between *M. procera* mycelium growth and Ni accumulation with Ni medium concentration were tested by regression analysis using the same software package.

RESULTS

Screening for Nickel Tolerance and pH Effect. The tolerance of *M. procera* to nickel was screened by measuring the radial growth of fungi cultured on MMN agar amended with $NiCl_2$ compared to control (without $NiCl_2$) at pH 4.0, 6.0, and 8.0. The obtained results showed that Ni affects *M. procera* growth and that the pH of the medium influences this process (**Figure 1**). At pH 4.0 and 6.0, all Ni concentrations significantly suppressed *M. procera* growth (**Figure 1A**,**B**). This inhibition reached a peak after 9−12 days of fungus medium inoculation from 33.4 to 89.2% at pH 4.0 and from 42.5 to 83.3% at pH 6.0. In contrast, at pH 8.0, only in the first 3 days was a significant reduction of fungus growth observed (**Figure 1C**). Afterward, growth was only significantly inhibited at 0.2 and 0.8 mM $NiCl_2$, whereas at 0.05 mM $NiCl_2$ the growth of *M. procera* was stimulated.

The analysis of growth rates at day 12 (**Figure 2**) showed no significant differences between *M. procera* grown at 0.2 and 0.8 mM $NiCl_2$. However, those rates were significantly lower than those observed at 0.05 mM $NiCl_2$.

For all of the tested pH values a significantly negative linear regression between *M. procera* growth and concentration of $NiCl_2$ amended on agar medium was found (**Table 1**).

Macroscopic and Microscopic Characterization of the Colonies. During 12 days, macroscopic and microscopic observations of *M. procera* colonies grown on Ni^{2+}-amended and nonamended media, at different pH values, were made to evaluate morphological alterations. The results obtained showed that neither macroscopic nor microscopic alterations in mycelial morphological characteristics were registered at different Ni concentrations and pH media (**Figures 3** and **4**). In all treatments *M. procera* mycelium was white, with few aerial growths, without exudates or rifts. The hyphae were septate, hyaline, and anastomosing. The spores were elliptical with a dimension of 10−15 × 4−6 μm.

However, a Ni influence on the spore size produced by fungi was observed. The spores from fungi cultured in media with

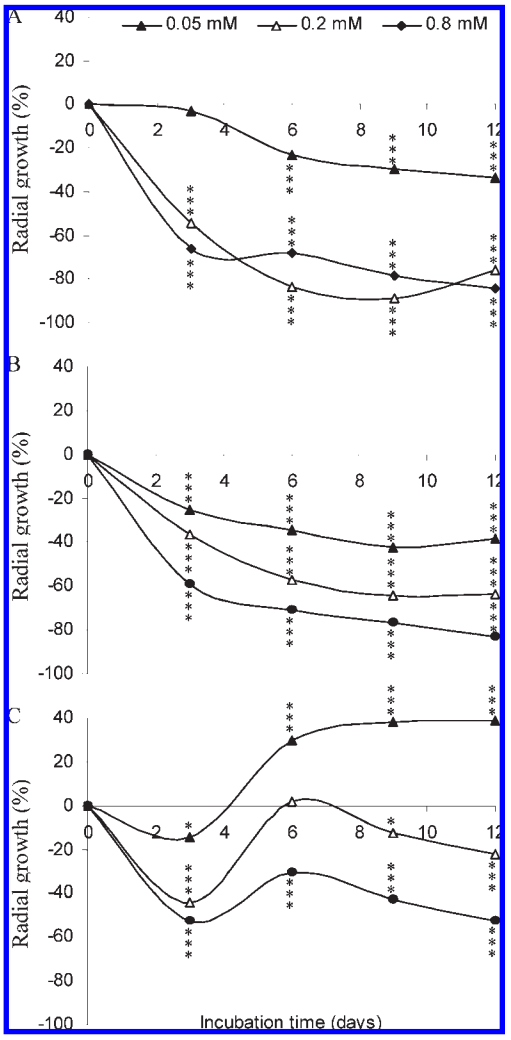

Figure 1. Differences of radial growth (%) of *Macrolepiota procera* cultured on MMN agar unamended (0 mM) and amended with $NiCl_2$ (0.05, 0.2, and 0.8 mM) at pH 4 (**A**), 6 (**B**), and 8 (**C**), for 12 days. Statistical significance: ∗, $p < 0.05$; ∗∗, $p < 0.01$; ∗∗∗, $p < 0.001$.

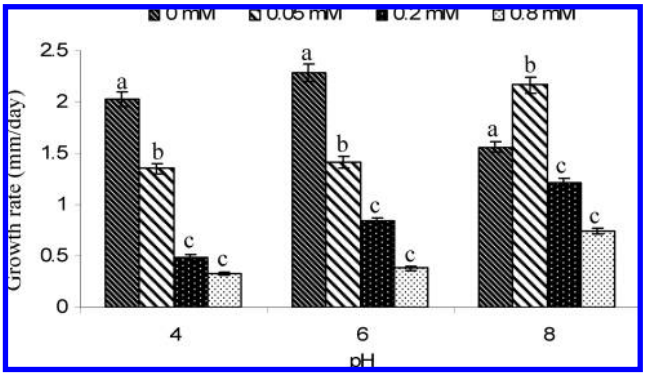

Figure 2. Growth rates (mean ± SE, $n = 7$) of *Macrolepiota procera* grown on MMN agar unamended (0 mM) or amended with $NiCl_2$ (0.05, 0.2, and 0.8 mM) at pH 4, 6, and 8, at day 12. Bars with different letters indicate values with significant differences at $p < 0.05$, within each pH value.

0.2 and 0.8 mM $NiCl_2$ had higher dimension (12−25 × 5−11 μm) than spores from control medium and medium with 0.05 mM $NiCl_2$. For 0.8 mM $NiCl_2$ at pH 8, the presence of crystalline structures with a dimension ranging from 15 to 20 μm was also observed (single arrow in **Figure 4**).

Nickel Uptake by *M. procera* Mycelium and pH Effect. Nickel uptake by *M. procera* was assessed by determination of Ni concentration in mycelia grown on both Ni-enriched and control media at pH 4.0, 6.0, and 8.0, after 40 days of incubation. The results obtained showed that Ni accumulation was affected by the $NiCl_2$ concentrations added to the medium and by the pH (**Table 2**). *M. procera* Ni concentration is proportional to the concentration of $NiCl_2$ added to the culture medium. The highest mean levels of Ni were found in mycelia grown in 0.8 mM $NiCl_2$, whereas the lowest mean values were found in the control mycelia. In general, mycelia from colonies grown at pH 4.0 and 6.0 presented higher Ni values than those grown at pH 8.0. This was particularly evident in colonies grown in the highest $NiCl_2$ concentrations (0.8 mM). For all of the pH values tested a highly significant positive linear regression between mycelia Ni accumulation and concentration of $NiCl_2$ added to the medium culture was found (**Table 1**).

Table 1. Correlation between the Evaluated Parameters (Mycelium Growth and Ni Mycelium Accumulation) and Concentration of $NiCl_2$ Amended to MMN Agar Medium at pH 4.0, 6.0, and 8.0

pH	equation curve	r^2	p^a
	Mycelium Growth		
4	$y = -0.2663x + 0.5973$	0.532	***
6	$y = -0.2624x + 0.6494$	0.578	***
8	$y = -0.2231x + 0.5778$	0.384	***
	Ni Mycelium Accumulation		
4	$y = 0.0018x - 0.0604$	0.858	***
6	$y = 0.0016x - 0.0396$	0.766	***
8	$y = 0.0022x - 0.0193$	0.609	***

[a] ***, $p < 0.001$ (extremely significant correlation).

Reactive Oxygen Species Formation. To ascertain the involvement of ROS during the first hours of *M. procera* exposure to Ni^{2+}, the quantification of hydrogen peroxide was performed on mycelia cultivated in liquid medium elicited with $NiCl_2$ at different concentrations (0.05, 0.2, 0.8 mM). As depicted in **Figure 5A**, the addition of Ni^{2+} to the growth medium caused changes in mycelia H_2O_2 production when compared to controls. In cultures elicited with 0.05 and 0.2 mM $NiCl_2$ the patterns of H_2O_2 production were similar between them within the first 24 h of exposure. In both treatments the first peak of H_2O_2 production was observed at 3 h after Ni^{2+} exposure with a significant increase (up to 1.5-fold) when compared to the control. Afterward, an additional peak of H_2O_2 production (1.3-fold increase) was observed in mycelium elicited with 0.2 mM $NiCl_2$ at 48 h after exposure. Treatment with 0.8 mM $NiCl_2$ led to a stronger variation in the production of H_2O_2. At this Ni concentration two peaks of H_2O_2 production were observed. The first peak occurred 3 h after $NiCl_2$ addition, exhibiting a significant increase (up to 4.8-fold) when compared with the control. After a

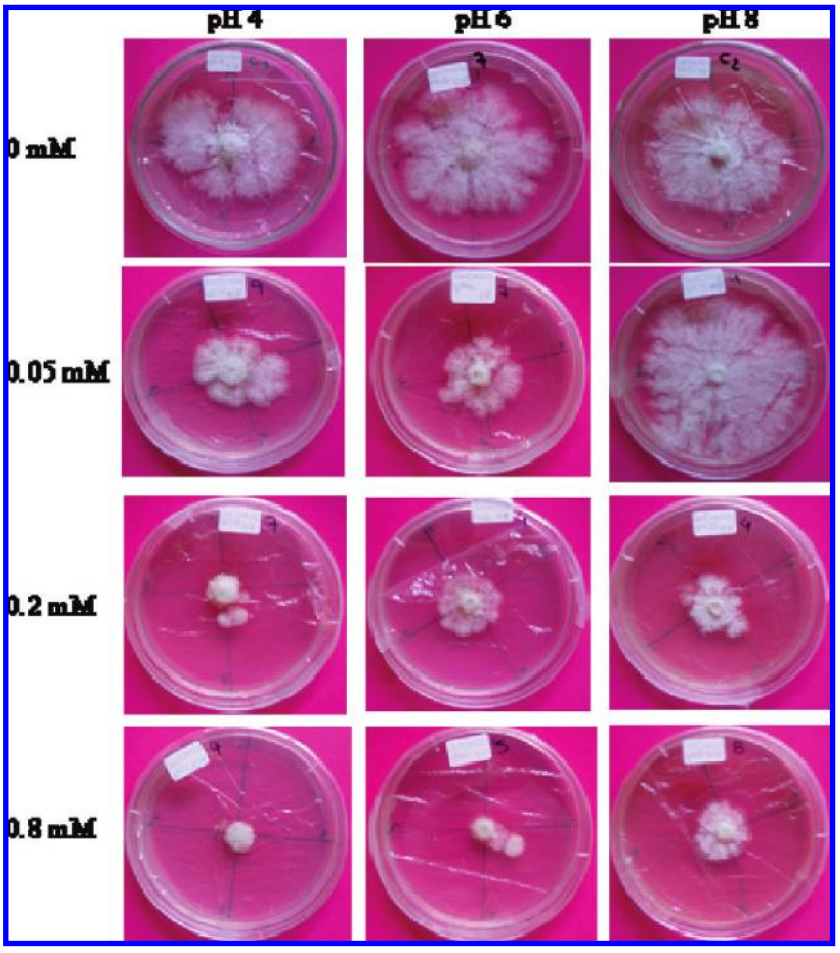

Figure 3. Macroscopic mycelial of *Macrolepiota procera* grown on MMN agar unamended (0 mM) or amended with $NiCl_2$ (0.05, 0.2, and 0.8 mM) at pH 4, 6, and 8, at day 12.

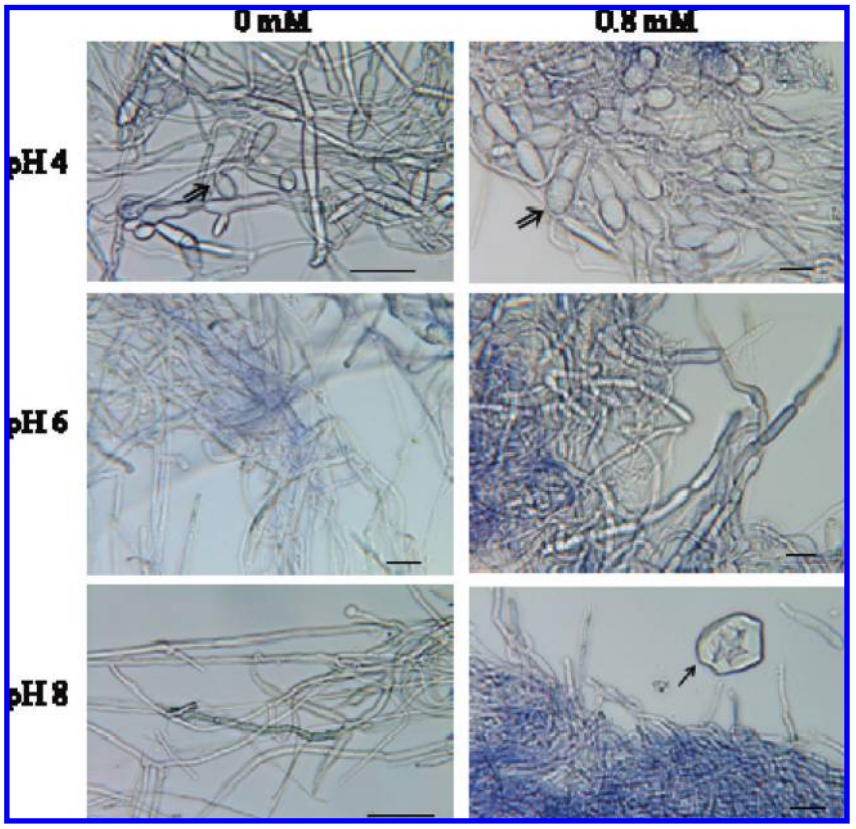

Figure 4. Microscopic mycelia of *Macrolepiota procera* grown on MMN agar unamended (0 mM) or amended with $NiCl_2$ (0.8 mM) at pH 4, 6, and 8, at day 12. The spores are indicated by a double arrow and the presence of crystalline structures by a single arrow. Bars = 15 μm.

Table 2. Amounts of Nickel in Mycelia of *Macrolepiota procera* Grown on MMN Agar at Different $NiCl_2$ Concentrations (0, 0.05, 0.2, and 0.8 mM) and pH (4.0, 6.0, and 8.0) (Mean ± SD; $n = 3$), at Day 40[a]

	Ni (μg/g of dry wt) in mycelia at			
pH	0 mM $NiCl_2$	0.05 mM $NiCl_2$	0.2 mM $NiCl_2$	0.8 mM $NiCl_2$
4	0.55 ± 0.05 a	56.00 ± 24.45 b	251.67 ± 19.04 c	415.33 ± 34.12 d
6	0.46 ± 0.14 a	45.10 ± 15.53 b	300.33 ± 17.90 c	421.33 ± 64.07 d
8	0.49 ± 0.12 a	33.13 ± 1.31 b	231.33 ± 32.47 c	251.33 ± 14.01 c

[a] In each row different letters indicate significant differences at $p < 0.05$.

significant decrease in H_2O_2 levels at 24 h after elicitation, the second statistically significant increase (up to 2.7-fold) relative to control was observed at 48 h after $NiCl_2$ addition. Afterward, H_2O_2 levels decreased until 72 h after $NiCl_2$ addition.

Activity of ROS-Scavenging Enzymes. SOD and CAT activities were determined in protein extracts from *M. procera* mycelium during the time course of elicitation by $NiCl_2$ at pH 6. The results obtained showed that treatment with 0.05 and 0.2 mM $NiCl_2$ did not cause statistically significant changes on CAT activity in comparison with control (**Figure 5B**), although a significant peak induction (up to 1.9-fold when compared to control) was observed on mycelium elicited with 0.8 mM $NiCl_2$ after 6 h of exposure.

In contrast, SOD activity was strongly changed when compared to control for all of the $NiCl_2$ concentrations studied (**Figure 5C**). In mycelium elicited with 0.05 and 0.2 mM $NiCl_2$ the pattern of SOD activity was similar within the first 24 h of exposure. For these concentrations, two significant peak inductions were observed. The first significant increase was detected after 3 h of fungus exposure, reaching the highest levels of SOD activity for these Ni concentrations (18.0 and 25.4 U/mg of protein, respectively, at 0.05 and 0.2 mM $NiCl_2$). The second significant peak induction was observed after 24 h of fungus exposure to Ni. Afterward, SOD activity was slightly increased, but this increase was not significantly higher than those observed for control. In mycelium elicited with 0.8 mM $NiCl_2$ only one significant increase of SOD activity was observed: this induction occurred 3 h after fungus exposure, reaching the highest levels of SOD activity in the experiment (30.3 U/mg of protein). Afterward, SOD activity decreased and practically remained constant until the end of the experimental period, showing only values significantly higher than the control after 6 h of elicitation.

DISCUSSION

Many fungi can survive and grow in high concentrations of toxic metals (*15*). This is achieved by physiological and morphological strategies employed to combat metal stress and by numerous and varied mechanisms of metal resistance and tolerance (*23*). The involvement of the antioxidative system of the fungi in protection against heavy metal stress should therefore be considered in connection with the metal tolerance of these organisms. However, this aspect has been neglected, and the effect of heavy metal stress on the antioxidative system of fungi is practically unknown, especially on saprotrophic macrofungi. The main goal of the present work was to evaluate this relationship in the saprobe macrofungi *M. procera* when exposed to different Ni^{2+} concentrations.

The results obtained showed that Ni^{2+} inhibited *M. procera* growth. However, this inhibition was > 50% (when compared to control) only at the higher Ni^{2+} tested concentrations (0.2 and 0.8 mM). The inhibition of macrofungi growth by toxic metals was previously demonstrated in several ectomycorrhizal fungi, namely, aluminum, zinc, copper, chromium, cadmium, and lead for *Laccaria laccata* (*40*−*42*); cadmium, copper, lead, zinc, and chromium for *Suillus bovinus* (*41*,*42*); cadmium, copper, lead, and

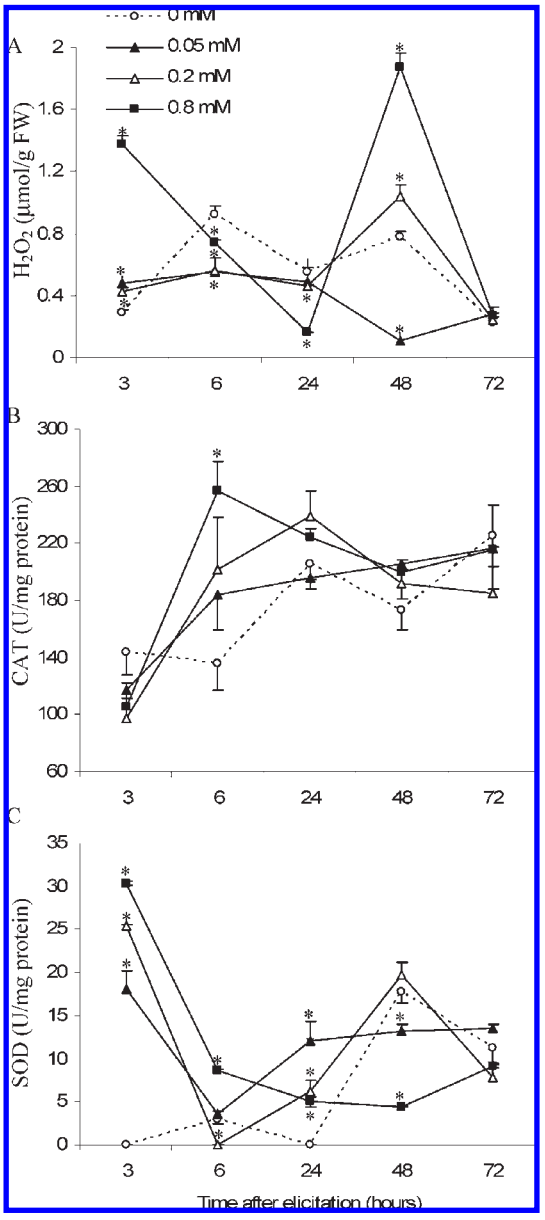

Figure 5. Quantification of H_2O_2 levels (**A**) and determination of CAT (**B**) and SOD (**C**) activities in *Macrolepiota procera* mycelium elicited with $NiCl_2$ (0.05, 0.2, and 0.8 mM) for 72 h. Mock-elicited mycelium (0 mM) was used as control. Mean ± SE ($n = 3$) is shown. Asterisk indicates values that differ significantly from controls at $p < 0.05$.

zinc for *Hebeloma crustuliniforme*, *Suillus luteus*, and *Lactarius turpis* (*42*); aluminum, cadmium, copper, lead, and zinc for *Paxillus involutus* (*42*, *43*); aluminum, arsenic, cadmium, chromium, nickel, and lead for *Laccaria fraterna* (*44*); and aluminum, arsenic, cadmium, chromium, nickel, lead, iron, copper, and zinc for *Pisolithus tinctorius* (*44*−*46*). Among all of the heavy metals tested, Ni was indicated to be the most toxic as expressed by the growth decrease and ectomycorrhiza formation (*46*). When solid medium was added with 200 μmol L^{-1} Ni^{2+}, a reduction of the growth rates of *P. tinctorius* by 70−72% or even a nongrowth was verified (*46*). In our work, a consistent growth of *M. procera* in medium supplemented with similar Ni^{2+} concentrations suggested that this isolate is tolerant to Ni.

We have also verified that *M. procera* Ni accumulation was positively correlated with Ni^{2+} concentrations added to the medium. *M. procera* growing in 0.8 mM Ni^{2+} accumulated up to 900-fold Ni when compared to control fungi. This means that *M. procera* is able to remove a significant amount of Ni from solution. A similar observation has been made for Ni and other heavy metals in several macrofungi (*19*), namely, *Pleurotus* spp. (*47*, *48*), *Paxillus involutus* (*49*), and *Trametes versicolor* (*48*). The mechanisms by which fungi remove metals from medium are diverse. However, fungi can do it essentially by (i) biosorption to biomass (cell walls, pigments, and extracellular polysaccharides), (ii) intracellular accumulation and sequestration, and (iii) precipitation of metal compounds onto or around hyphae (*23*).

It was noted that both Ni accumulation by *M. procera* and Ni-induced toxicity decreased as the pH of the medium increased. At pH 8.0 a significant stimulation of *M. procera* growth at 0.05 mM Ni was even observed, in comparison to control. This is probably because this metal is an essential element for several organisms, being a constituent of many enzyme systems (*50*). Both growth and the accumulation of toxic metals by fungi were previously reported as being affected by the pH status of the growth media (*15*, *23*). This could be due to the effects of the pH on metal speciation and mobility. In acidic pH, metals exist as free ions, whereas at alkaline pH the ions precipitate as insoluble hydroxides or oxides (*15*). Therefore, the decreased Ni toxicity with the pH increasing, observed in the present study, might result from the formation of hydroxylated species as previously reported by Gadd (*15*). In fact, on colonies grown on 0.8 mM Ni at pH 8.0 the presence of crystalline structures that possessed morphology and dimensions similar to nickel hydroxide (*51*) was observed. This suggests that one of the mechanisms of metal tolerance of *M. procera* is linked to nickel hydroxide production, which presumably precipitates Ni as an insoluble form that is not taken up by fungi. The low bioaccumulation capacity of *M. procera* at pH 8.0 could also be due to this precipitation because the formation of hydroxide precipitate reduces the amount of free nickel ions. The production of hydroxylated species by fungi is a common phenomenon that provides a means of immobilizing soluble metal ions, thus decreasing bioavailability and increasing tolerance to these metals (*23*). External pH may also affect metal biosorption to fungal cell wall (*15*, *52*). Decreasing pH to a certain value may increase the concentration of free metal ions in solution that are more available to metal binding sites of the fungal cells. However, at very low pH values, the production of H^+ may compete with metal ions for sorption sites (*15*). Thus, this may be the reason for the slightly higher Ni accumulation by *M. procera* observed at pH 6.0 when compared to *M. procera* grown at pH 4.0.

Toxic metal exposure was reported to cause morphological changes on mycelium (*19*, *23*, *53*). However, in our study the only difference noted was in the size of spores produced by *M. procera*, which was influenced by Ni^{2+} concentration. The increase in spore size observed on colonies grown at 0.2 and 0.8 mM Ni when compared to control could be an adaptive response to stress elicited by the high concentrations of this metal. It could be also derived from the binding of Ni^{2+} to the cell walls and/or Ni accumulation in vacuoles. This can be inferred from the results of several studies on ultrastructural localization of heavy metals in fungal spores, which showed its deposition on the surface of the cell wall and its accumulation in the spore vacuoles (*54*, *55*). However, the explanations must be viewed with caution and need further confirmation.

In our work, evidence was given for the capacity of Ni^{2+} to induce ROS production. *M. procera* exposure to high Ni^{2+} concentrations (0.2 and 0.8 mM) displayed two production peaks of H_2O_2. As far as we know this is the first time there is direct evidence for ROS production by macrofungi in response to heavy metals. Similar findings have been observed only in the yeast *Candida intermedia* when exposed to copper, zinc, and selenium (*26*).

The efficient decomposition of ROS requires the synchronized action of several antioxidant enzymes. SOD dismutates superoxide to H_2O_2, whereas CAT converts H_2O_2 to nonreactive oxygen species. In the present work, a rapid and significant increase in SOD activity was coincident with the first ROS peak, occurring 3 h after Ni^{2+} exposure. These data suggest that early SOD activity could contribute to the high levels of H_2O_2 observed, as a consequence of the dismutation of formed superoxide radical. Enhanced synthesis of SOD has also been observed in the macrofungi *P. involutus* (*30*, *31*), *C. militaris* (*32*), and *Rhizopogon roseolus* (*29*), after heavy metal exposure. However, this temporal correlation established between SOD activity and H_2O_2 production was not observed until the end of the present experiment. In fact, the second burst observed 48 h after Ni^{2+} exposure was not coincident with an increase in SOD activity. These data suggest that an alternative pathway for H_2O_2 production might be present at longer Ni^{2+} exposure times.

In our study it was verified that *M. procera* exposure to low Ni^{2+} concentrations (0.05 and 0.2 mM) did not cause changes in CAT activity when compared to control. These results are in accordance with those obtained by cadmium elicitation of *P. involutus* that showed nonsignificant variation in CAT activity at low Cd concentration (*31*). The same authors showed that *P. involutus* is protected by activation of other components of the antioxidative system, namely, glutathione reductase. This observation suggested that an alternative mechanism of protection against H_2O_2 could be present in *M. procera* when exposed to low Ni^{2+} concentration. However, in the presence of the highest Ni concentrations the enzyme CAT seems to be involved in H_2O_2 detoxification. In fact, coincidently with the increase in CAT activity observed 6 h after 0.8 mM Ni exposure, the observed decline in H_2O_2 levels could reflect the ability of CAT to scavenge H_2O_2. In contrast, in the macrofungus *P. involutus* an inhibition of CAT activity in response to high Cd concentration was reported (*31*). Therefore, CAT and especially SOD were found to be essential antioxidant enzymes in *M. procera* required for protection against H_2O_2 in response to high Ni^{2+} concentrations in the growth medium.

The results obtained indicated that the ability of *M. procera* to survive in high Ni^{2+} concentrations was correlated with the heavy metal uptake, the ROS generation, and the efficiency of the antioxidative defense system. These findings may contribute to a better understanding of the response mechanisms of saprotrophic macrofungi to metal stress and can offer useful information for programs of bioremediation using fungi.

LITERATURE CITED

(1) Barros, L.; Baptista, P.; Correia, D. M.; Sá Morais, J.; Ferreira, I. C. F. R. Effects of conservation treatment and cooking on the chemical composition and antioxidant activity of Portuguese wild edible mushrooms. *J. Agric. Food Chem.* **2007**, *55*, 4781–4788.

(2) Ouzouni, P. K.; Riganakos, K. A. Nutritional value and metal content profile of Greek wild edible fungi. *Acta Aliment.* **2007**, *36*, 99–110.

(3) Vetter, J. Lithium content of some common edible wild-growing mushrooms. *Food Chem.* **2005**, *90*, 31–37.

(4) Tuzen, M.; Sesli, E.; Soylak, M. Trace element levels of mushroom species from East Black Sea region of Turkey. *Food Control* **2007**, *18*, 806–810.

(5) Falandysz, J.; Szymczyk, K.; Ichihashi, H.; Bielawski, L.; Gucia, M.; Frankowska, A.; Yamasaki, S.-I. ICP/MS and ICP/AES elemental analysis (38 elements) of edible wild mushrooms growing in Poland. *Food Addit. Contam.* **2001**, *18*, 503–513.

(6) Falandysz, J.; Gucia, M.; Mazur, A. Some mineral elements and their bioconcentration factors in parasol mushroom (*Macrolepiota procera*) from the region of Poniatowa in the lubelskie voievodeship. *Bromatol. Chem. Toksykol.* **2007**, *40*, 249–255.

(7) Falandysz, J.; Kunito, R.; Gucia, M.; Mazur, A.; Falandysz, J. J.; Tanabe, S. Some mineral constituents of Parasol mushroom (*Macrolepiota procera*). *J. Environ. Sci. Health B* **2008**, *43*, 187–192.

(8) Yakiz, D.; Konuk, M.; Afyon, A.; Kok, S. M. Minor element and heavy metal content of edible wild mushrooms native to Bolu, North-West Turkey. *Fresenius' Environ. Bull.* **2008**, *7*, 249–252.

(9) Svoboda, L.; Chrastny, V. Levels of eight trace elements in edible mushrooms from a rural area. *Food Addit. Contam.* **2008**, *25*, 51–58.

(10) Falandysz, J. Selenium in edible mushrooms. *J. Environ. Sci. Health C* **2008**, *26*, 256–299.

(11) Vetter, J.; Berta, E. Mercury content of some edible mushrooms. *Z. Lebensm. Unters. Forsch.* **1997**, *205*, 316–320.

(12) Alonso, J.; Salgado, M. J.; Garcia, M. A.; Melgar, M. J. Accumulation of mercury in edible macrofungi: infuence of some factors. *Arch. Environ. Contam. Toxicol.* **2000**, *38*, 158–162.

(13) Figueiredo, E.; Soares, M. E.; Baptista, P.; Castro, M.; Bastos, M. L. Validation of an electrothermal atomization atomic absorption spectrometry method for quantification of total chromium and chromium(VI) in wild mushrooms and underlying Soils. *J. Agric. Food Chem.* **2007**, *55*, 7192–7198.

(14) Falandysz, J.; Gucia, M. Bioconcentration factors of mercury by Parasol mushroom (*Macrolepiota procera*). *Environ. Geochem. Health* **2008**, *30*, 121–125.

(15) Gadd, G. M. Interactions of fungi with toxic metals. *New Phytol.* **1993**, *124*, 25–60.

(16) Gadd, G. M.; Sayer, J. A. Influence of fungi on the environmental mobility of metals and metalloids. In *Environmental Microbe−Metal Interactions*; Derek, R. L., Ed.; ASM Press: Washington, DC, 2000; pp 237−256.

(17) Hughes, M. N.; Poole, R. K. Metal speciation and microbial growth—the hard (and soft) facts. *J. Gen. Microbiol.* **1991**, *137*, 725–34.

(18) Spain, A. Implication of microbial heavy metal tolerance in the environment. *Rev. Undr. Grad. Res.* **2003**, *2*, 1–6.

(19) Baldrian, P. Interactions of heavy metals with white-rot fungi. *Enzym. Microb. Technol.* **2003**, *32*, 78–91.

(20) Tomsett, A. B. Genetics and molecular biology of metal tolerance in fungi. In *Stress Tolerance in Fungi*; Jennings, D. H., Ed.; Dekker, New York, 1993; pp 69−95.

(21) Volesky, B.; May, H.; Holan, Z. R. Cadmium biosorption by *Saccharomyces cerevisiae*. *Biotechnol. Bioeng.* **1993**, *41*, 826–829.

(22) Joho, M.; Inohue, M.; Tohoyama, H.; Murayama, T. Nickel resistance mechanisms in yeasts and other fungi. *J. Ind. Microbiol.* **1995**, *14*, 164–168.

(23) Fomina, M. A.; Burford, E. P.; Gadd, G. M. Toxic metals and fungal communities. In *The Fungal Community. Its Organization and Role in the Ecosystem*, 3rd ed.; Dighton, J., White, J. F., Oudemans, P., Eds.; Taylor and Francis, CRC Press: Boca Raton, FL, 2005; Vol. *23*, pp 733−758.

(24) Jamieson, D. J. Oxidative stress response of the yeast *Saccharomyces cerevisiae*. *Yeast* **1998**, *14*, 1511–1527.

(25) Vido, K.; Spector, D.; Lagniel, G.; Lopez, S.; Toledano, M. B.; Labarre, J. A proteome analysis of the cadmium response in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **2001**, *276*, 8469–8474.

(26) Fujs, S.; Gazdag, Z.; Poljsak, B.; Stibilj, V.; Milačič, R.; Pesti, M.; Raspor, P.; Batič, M. The oxidative stress response of the yeast *Candida intermedia* to copper, zinc, and selenium exposure. *J. Basic Microbiol.* **2005**, *45*, 125–135.

(27) Todorova, D.; Nedeva, D.; Abrashev, R.; Tsekova, K. Cd(II) stress response during the growth of *Aspergillus niger* B 77. *J. Appl. Microbiol.* **2008**, *104*, 178–184.

(28) Yasokawa, D.; Murata, S.; Kitagawa, E.; Iwahashi, Y.; Nakagawa, R.; Hashido, T.; Iwahashi, H. Mechanisms of copper toxicity in *Saccharomyces cerevisiae* determined by microarray analysis. *Environ. Toxicol.* **2008**, *23*, 599–606.

(29) Miszalski, Z.; Botton, B.; Turnau, K. New SOD isoform in *Rhizopogon roseolus* (Corda in Sturm) in the presence of cadmium. *Acta Physiol. Plant.* **1996**, *18*, 129–134.

(30) Jacob, C.; Courbot, M.; Brun, A.; Steinman, H. M.; Jacquot, J.-P.; Botton, B.; Chalot, M. Molecular cloning, characterization and

regulation by cadmium of a superoxide dismutase from the ectomycorrhizal fungus *Paxillus involutus*. *Eur. J. Biochem.* **2001**, *268*, 3223–3232.

(31) Ott, T.; Fritz, E.; Polle, A.; Schützendübel, A. Characterisation of antioxidative systems in the ectomycorrhiza-building basidiomycete *Paxillus involutus* (Bartsch) Fr. and its reaction to cadmium. *FEMS Microbiol. Ecol.* **2002**, *42*, 359–366.

(32) Wang, Z.-S.; Gu, Yu-X.; Yuan, Qin-S. Effect of nutrition factors on the synthesis of superoxide dismutase, catalase, and membrane lipid peroxide levels in *Cordyceps militaris* mycelium. *Curr. Microbiol.* **2006**, *52*, 74–79.

(33) Alonso, J.; Garcia, M. A.; Pérez-López, M.; Melgar, M. J. Acumulación de metales pesados en macromicetos comestibles y factores que influyen en su captación. *Rev. Toxicol.* **2004**, *21*, 11–15.

(34) Alonso, J. Metais pesados e outros contaminantes en cogumelos. *Tarrelos* **2006**, *8*, 15–25.

(35) Brundrett, M.; Bougher, N.; Dell, B.; Grove, T.; Malajczuk, N. *Working with Mycorrhizas in Forestry and Agriculture*; ACIAR Monograph 32; CSIRO Publishing: Canberra (Australia), 1996.

(36) Loreto, F.; Velikova, V. Isoprene produced by leaves protects the photosynthetic apparatus against ozone damage, quenches ozone products, and reduces lipid peroxidation of cellular membranes. *Plant Physiol.* **2001**, *127*, 1781–1787.

(37) Sedmak, J. J.; Grossberg, S. E. A rapid, sensitive, and versatile assay for protein using Coomassie Brilliant Blue G250. *Anal. Biochem.* **1977**, *79*, 544–552.

(38) Beyer, W. F.; Fridovich, I. Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. *Anal. Biochem.* **1987**, *161*, 559–566.

(39) Aebi, H. Catalase. In *Enzymes 1: Oxidoreductases, Transferases*; Bergmeyer, H. U., Ed.; Verlag Chemie: New York, 1983; pp 273−286.

(40) Jones, D.; Meuhlchen, A. Effects of the potentially toxic metals, aluminium, zinc and copper on ectomycorrhizal fungi. *J. Environ. Sci. Health A* **1994**, *29*, 949–966.

(41) Raman, N.; Srinivasan, V.; Ravi, M. Effect of chromium on the axenic growth and phosphatise activity of ectomycorrhizal fungi, *Laccaria laccata* and *Suillus bovinus*. *Bull. Environ. Contam. Toxicol.* **2002**, *68*, 569–575.

(42) Fomina, M. A.; Alexander, I. J.; Colpaert, J. V.; Gadd, G. M. Solubilization of toxic metal minerals and metal tolerance of mycorrhizal fungi. *Soil Biol. Biochem.* **2005**, *37*, 851–866.

(43) Rudawska, M.; Leski, T. Aluminium tolerance of different *Paxillus involutus* Fr. strains originating from polluted and non-polluted sites. *Acta Soc. Bot. Pol.* **1998**, *67*, 115–122.

(44) Ray, P.; Tiwari, R.; Gangi, U. R.; Adholeya, A. Detecting the heavy metal tolerance level in ectomycorrhizal fungi *in vitro*. *World J. Microbiol. Biotechnol.* **1995**, *21*, 309–315.

(45) Tam, P. C. F. Heavy metal tolerance by ectomycorrhizal fungi and metal amelioration by *Pisolithus tinctorius*. *Mycorrhiza* **1995**, *5*, 181–187.

(46) Aggangan, N. S.; Dell, B.; Malajczuk, N. Effects of chromium and nickel on growth of the ectomycorrhizal fungus *Pisolithus* and formation of ectomycorrhizas on *Eucalyptus urophylla* S.T. Blake. *Geoderma* **1998**, *84*, 15–27.

(47) Sanglimsuwan, S.; Yoshida, N.; Morinaga, T.; Murooka, Y. Resistance to and uptake of heavy metals in mushrooms. *J. Ferment. Bioeng.* **1993**, *75*, 112–114.

(48) Arica, M. Y.; Arpa, Ç.; Kaya, B.; Bektas, S.; Denizli, A.; Genç, O. Comparative biosorption of mercuric ions from aquatic systems by immobilized live and heat-inactivated *Trametes versicolor* and *Pleurotus sajur-caju*. *Bioresour. Technol.* **2003**, *89*, 145–154.

(49) Blaudez, D.; Botton, B.; Chalot, M. Cadmium uptake and subcellular compartmentation in the ectomycorrhizal fungus *Paxillus involutus*. *Microbiology* **2000**, *146*, 1109–1117.

(50) Hausinger, R. P. Metallocenter assembly in nickel-containing enzymes. *J. Biol. Inorg. Chem.* **1997**, *2*, 279–286.

(51) Xiao-yan, G.; Jian-cheng, D. Preparation and electrochemical performance of nano-scale nickel hydroxide with different shapes. *Mater. Lett.* **2007**, *61*, 621–625.

(52) Schiewer, S.; Wong, M. H. Ionic strength effects the biosorption of metals by marine algae. *Chemosphere* **2000**, *41*, 271–282.

(53) Tuszynska, S. Ni^{2+} induces changes in the morphology of vacuoles, mitochondria and microtubules in *Paxillus involutus* cells. *New Phytol.* **2006**, *169*, 819–828.

(54) Turnau, K.; Kottke, I. Fungal activity as determined by microscale methods with special emphasis on interactions with heavy metals. In *The Fungal Community. Its Organization and Role in the Ecosystem*, 3rd ed.; Dighton, J., White, J. F., Oudemans, P., Eds.; Taylor and Francis, CRC Press: Boca Raton, FL, 2005; Vol. *23*, pp 287−305.

(55) González-Guerrero, M.; Melville, L. H.; Ferrol, N.; Lott, J. N. A.; Azcón-Aguilar, C.; Peterson, R. L. Ultrastructural localization of heavy metals in the extraradical mycelium and spores of the arbuscular mycorrhizal fungus *Glomus intraradices*. *Can. J. Microbiol.* **2008**, *54*, 103–110.

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