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Native fungi as metal remediators: silver myco-accumulation from metal contaminated waste-rock dumps (Libiola Mine, Italy)

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ABSTRACT

Metal contamination constitutes a major source of pollution globally. Many recent studies emphasized the need to develop cheap and green technologies for the remediation or reclamation of environmental matrices contaminated by heavy

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metals. In this context, fungi are versatile organisms that can be exploited for bioremediation activities.

In our work we tested silver (Ag) bioaccumulation capabilities of three microfungal strains (*Aspergillus alliaceus* Thom & Church, *Trichoderma harzianum* Rifai, *Clonostachys rosea* (Link) Schroers, Samuels, Seifert & W. Gams) isolated from a silver polluted site.

The aim was to select silver tolerant native strains and test their potential silver uptake.

Among the three species tested, *T. harzianum* was the most efficient strain to tolerate and accumulate silver, showing an uptake capability of 153 mg/L taken at the Ag concentration of 330 mg/L.

Our study highlights the potential use of native microfungi spontaneously growing in sulphide-rich waste rock dumps, for silver bioaccumulation and bioremediation.

Keywords: heavy metals, Ag, sulphide mine, mycoremediation, *Trichoderma*, Tolerance Index.

INTRODUCTION

Metal contamination represents a major source of global pollution [1]. Many industrial and mining activities lead to the artificial redistribution of toxic metals in the terrestrial environment, consequently concentrating these metals in soils and surface waters. Thirteen trace metals and metalloids (Ag, As, Be, Cd, Cr, Cu, Hg, Ni, Pb, Sb, Se, Tl, and Zn) are considered priority pollutants [2-3]. Metal ions such as Cu^{2+}, Ni^{2+}, Zn^{2+}, Pb^{2+}, Cd^{2+}, Cr^{6+}, Ag^{+}, and Fe^{2+} can damage cells, proteins, and enzymes, inhibit spore germinations, and limit mycelial and plant growth. Some of these elements (e.g. Zn, Co, Cu, Ni, and Fe) are necessary as trace elements for organismal growth, whereas other metals (e.g. Cd, Ag, Cr, Cs, and Pb) have no physiological role in any biological system [4-3-5]. Many studies show that both categories of elements are toxic to organisms at high concentrations [6-7-8-9]. Among these elements, silver represents an historically and economically important noble metal. It is used in medicine, modern industry, jewelry, photography and in electrical batteries. Ag concentration in soils rarely exceeds 1000 ppb (range of means worldwide = 30 to 8000 ppb; average 50 ppb) and higher levels are generally indicative of pollution by human activity, such as mining [10]. The most common soluble form of silver in the pH range of most soils is Ag^{+} which at low pH has medium mobility and may be sorbed selectively on clay, humus and iron oxides [10]. However, Ag^{+} is a potent biocide and one of the

most toxic metals to many organisms (bacteria, algae, and fish) [11-12]. Among toxic metals Ag is classified with the highest rate of phytotoxicity and mammalian toxicity and has no known biological function [10-13-14]. Chaperon and Sauvé [15] demonstrated that silver can reduce enzyme activities in soil and water. Hence, the removal of silver from wastewaters or mining waste-rock dumps is an important environmental requirement.

Fungi are ubiquitous soil organisms and often constitute the majority of soil biota, especially in acid soil (pH<5.5) [16-17-3]. They are tolerant to metals and possess resistance mechanisms such as metallothionein or phytochelatin proteins, which can bind and deactivate toxic metals, or they may store heavy metals in vacuoles [18-4-7]. Many fungi are able to adapt to the presence of metals in the environment; in fact they are even able to grow and to colonize soils affected by metal exploitation, such as mine soils, waste-rock dumps and tailing deposits, which are characterized by extreme edaphic, physical and chemical conditions [19-20]. Mines are generally harmful to the environment and may be the cause of major pollution [21]. In particular, sulphide ores are stable in dry and anoxic environments, but the exposure to both oxygen and water triggers their spontaneous oxidation [22] resulting in strong acidification of soils and circulating waters (Acid Mine Drainage) as well as ecotoxic metal mobilization [23]. In this context fungi can be considered as pioneer organisms, contributing to remediate, clean and prepare substrates for subsequent plant colonization [24-25]. In particular, the use of wild native microfungi to accumulate heavy metals from derelict mine soils may

represent an innovative, potentially cheap, and sustainable remediation technique to achieve and promote plant recolonization [26].

Silver reaches high concentration values in the Libiola sulphide mine (NW Italy) and it is involved in soil and surface water contamination, affecting plant growth and their soil colonization capability [27].

In this study we compared three previously isolated strains of microfungi for their Ag accumulation capability.

MATERIALS AND METHODS

Study Area

The Libiola mine (NW Italy) is a derelict Fe-Cu sulphide mine, exploited between 1864 and 1962. During this period, over 1 million tons (Mt) of sulphides were produced [28]. Five major waste-rock dumps are present in the mining area; they were built piling up heterogeneous sterile rocks (or sulphide mineralizations with metal concentration below the economic cut-off grade) derived from the exploitation of two major open-pits and over 30 km of underground galleries [29]. In the whole area Acid Mine Drainage (AMD) processes occur and persist over time [30-27]. Surface and underground waters are generally characterized by pH values as low as 3, very high sulfate contents (from 10^3 to 10^4 mg/L), and excessive concentrations of potentially toxic metals [31]. The mine soils and, in

particular, the waste-rock dumps are characterized by extreme edaphic conditions [27-32]: high metal concentrations, low pH values and low availability of essential macronutrients. The most critical metals occurring in the waste-rock dumps and in the surrounding soils [29-27-32] are related to sulphide mineralizations (Cu ≤ 13347 ppm; Zn ≤ 1126 ppm; Co ≤ 408 ppm) and host rocks, i.e. basalts and serpentinites (Cr ≤ 2587 ppm; Ni ≤ 3579 ppm). Moreover, anomalously high Ag concentrations are common and scattered over the mining area. Previous studies reported concentrations of silver as high as 9780 ppb in soils [32], 11000 ppb in waste-rocks [30], and 3200 ppb in stream sediments precipitating from mine-waters [31]. The diffuse presence of silver in the materials derived from mining operations agrees well with the high silver concentration reported for the strata bound sulphide deposits of Libiola (up to 10600 ppb), where they occur within pyrite (Ag-bearing pyrite) or as accessory minerals (native silver, acanthite, argentite and electrum) in the pyrite-chalcopyrite mineralizations [33-34-35].

Silver Nitrate ($AgNO_3$) Bioaccumulation Test

Silver bioaccumulation tests were conducted with three microfungal strains (*Aspergillus alliaceus* (ML 35-12), *Trichoderma harzianum* (ML 8-12), *Clonostachys rosea* (ML 38-12)). These microfungi were isolated at the Libiola mine from bare dump soil samples collected between 5 and 20 cm depth below ground surface after removing the fraction > 2 mm; the isolation method was described in Zotti et al.[29]. This method allowed obtaining culturable fungal

strains, which were later identified by macro-micromorphological characteristics and molecular analysis (ß-tubulin locus DNA sequence), and preserved in the culture collection of the Mycological Laboratory of DISTAV (University of Genoa, Italy).

The three microfungal strains were compared for their silver tolerance. All experiments were conducted in triplicate. The medium was prepared by adding 330 mg/L $AgNO_3$ to the Malt Extract Agar (MEA). In addition a control medium (MEA) was prepared. The media were autoclaved at 121 °C for 20 min. Ten Petri dishes (12 cm Ø) for species (5 for each medium) were centrally inoculated with fungal solutions obtained by diluting fungal conidia in a semisolid suspension of Tween 80 (polysorbitan 80). The conidia were counted using a Burker chamber to quantify the inocula (8 X 10^5 conidia ml^{-1}). Then, Petri dishes were kept at 24 °C for 14 days in the dark to verify the growth capability of each strain. The growth was monitored by measuring the fungal colony diameters of all Petri dishes for two weeks. In order to evaluate the silver effect on fungal growth the Tolerance Index based on diameters (TId) was calculated according to Anahid et al. [7] (equation 1):

(diameter of mycelia grown in the presence of silver/diameter of mycelia grown in the control) x 100 (1)

The strain with the highest TId was subsequently selected to grow on 330 mg/L $AgNO_3$ enriched, solid MEA covered with an autoclaved cellophane microporous

membrane (BioRad). 20 Petri dishes were inoculated with the selected microfungal suspension, kept at 24 °C in the dark, and harvested after 14 days by removing the mycelium from the cellophane with a sterile plastic spatula. In addition the strain was grown on 20 control medium (MEA) plates.

Mycelia were dried at 60 °C for 48 h to determine the dry weight (DW). The Tolerance Index based on dry weight (TIdw) was calculated to quantify the fungal silver tolerance following Fomina et al. [36] and Crane et al. [37] (equation 2):

(treated fungal dry weight/control fungal dry weight) x 100 (2)

The TIdw allowed to evaluate the inhibition of biomass production on the Ag-enriched media compared to the Ag 0 mg/L controls. It ranged between 1 and 100, where the lower the TIdw, the greater the Ag toxicity.

Analytical methods

The dried mycelium samples were sent to the ALS Analytical Laboratory (Sweden). Ag concentration of the samples was assessed by acid digestion followed by inductively coupled plasma mass spectrometry (ICP-MS) analysis. 20 Petri dishes represent the necessary number of dishes to obtain the adequate fungal amount (≥ 1 g) for the ICP-MS analysis. This technique was capable of detecting metals and several non-metals at concentrations as low as one part in 10^{12} (part per trillion). This was achieved by ionizing the sample with inductively coupled plasma and then using a mass spectrometer to separate and quantify those ions [38]. The element concentration was measured in triplicate for quality

183 measurement assurance and the percentage coefficients of relative standard
184 deviation were below 10%, reaching maximum values of about 25% only for
185 those concentrations close to the detection limit of the element (i.e. 2 ppb Ag).
186
187 RESULTS
188
189 After two weeks *Trichoderma harzianum* resulted the most $AgNO_3$ tolerant (TId
190 100) and fastest growing strain (Petri dishes completely covered). *Clonostachys*
191 *rosea* grew more slowly (TId 40 after two weeks) and covered Petri dishes in 28
192 days, whereas *Aspergillus alliaceus* showed a difficult germination capability (TId
193 0). The *C. rosea* and *T. harzianum* weekly growth pattern was reported in Figure
194 1.
195 ICP-MS analysis of *Trichoderma harzianum* dried mycelium revealed a Ag
196 accumulation capability of 153 mg/L. This meant that 46.364% of the silver was
197 stored up. The *T. harzianum* TIdw value measured was 83,6.
198
199
200 DISCUSSION
201
202 Previous studies showed that the waste-rock dumps from the Libiola mine are
203 characterized by an extremely poor flora and by a peculiar mycobiota [32-29]. This
204 peculiar biota is strongly conditioned by the severe edaphic conditions occurring
205 within the waste-rock dump and in the adjoining soils, which do not allow a

206 homogeneous colonization by the Mediterranean vegetation, that grows in the
207 unpolluted areas bordering the mine. Soil microorganisms, in particular fungi,
208 may take on an important role in the reactivation of biogeochemical cycles and in
209 soil metal remediation. In this context, we tested silver tolerance capability of
210 three native fungal strains known to uptake ecotoxic metals. Fomina et al. [37] and
211 Ceci et al. [9] showed the high copper, cadmium, and vanadium accumulation by
212 *Clonostachys rosea*, whereas Joseph et al. [39] evaluated the *Aspergillus alliaceus*
213 metal accumulation (Cu, Zn and Sn) and corrosion capacity. Furthermore,
214 Harman et al. [40] evidenced that *Trichoderma* species are highly resistant to a
215 variety of toxins and xenobiotic compounds, including antibiotics, fungicides and
216 heavy metals. Some studies showed the high metal uptake and tolerance capability
217 of *Trichoderma harzianum*, in particular for the group IIb metals (Cd, Zn, Hg) and
218 lanthanides [41-42-43-44]. Among the species used in this study only *Clonostachys*
219 *rosea* had been previously tested for silver uptake [45]. Our results prove the great
220 capability of *Trichoderma harzianum* to grow on silver contaminated substrata,
221 and suggest the use of this specie in a silver remediation protocol for the
222 contaminated soils and wastewaters of the Libiola mine. In fact, the TIdw value
223 (83,6) shows a high fungal silver tolerance, which increased over time [7]. Only
224 few previous studies investigated fungal - and bacterial - silver accumulation
225 capabilities [46-45-47-48-49-50], likely because Ag toxicity represents a strong limiting
226 factor for the selection of tolerant and efficient accumulator microorganisms.
227 Many studies used relatively low silver concentrations to evaluate fungal and
228 bacterial toxic metals accumulation or adsorption capabilities [45-47]. For example,

Kisielowska et al. [50] tested *Aspergillus niger* Tiegh. silver bioleaching, by evaluating Ag fungal content. They achieved fungal silver accumulation from 7.024% to 41.495% taken at initial concentration of 13.76 mg/L. Our work highlighted that the selected *Trichoderma harzianum* strain resulted much efficient to tolerate and accumulate silver, showing an uptake capability of 46.364% taken at initial concentration of 330 mg/L.

Our research suggest a potential use of fungal silver bioaccumulation as a cheap and sustainable remediation technique, especially if silver recovery from the mycelium can be achieved as well. Ag is a noble metal with high economical importance, and its recovery possibility would represent a great resource. Many studies on precious metal biosorption show that their recovery is possible and not too expensive [48-51-52-50-53]. Jacobsen [54] even considered this method as the most efficient alternative to the traditional techniques such as pyrometallurgical and hydrometallurgical processes widely used to recover precious metals from wastewater. Silver could be recovered by fungal mycelium high-acid digestion or incineration [55-56], but further studies need to be conducted. Using native silver accumulating fungal strains for soil and surface water decontamination would constitute a more sustainable way to mitigate environmental damage. More research is needed into the potential of silver recovery after fungal bioaccumulation, including its economic feasibility.

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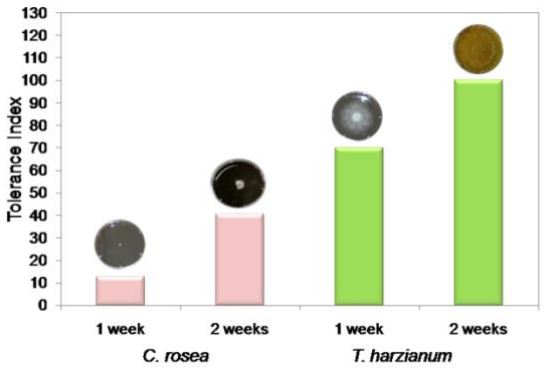
FIGURE CAPTIONS

Figure 1. *Clonostachys rosea* and *Trichoderma harzianum* growth pattern.



426 Fig. 1