Zinc tolerance and accumulation capability by *Penicillium* strains isolated from Neutral Mine Drainage (NDM) colloidal precipitates

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Abstract

Zinc, at low concentration, is one of the oligo elements essential for life. As well as many others metals, high environmental Zn concentrations are toxic for several organisms and can compromise their lifecycle. Neutral (or alkaline) Mine Drainage (NMD) are metal-rich solutions with near-neutral pH values, often generated by weathering of sulphide minerals (mainly Zn and Pb sulphides), circulating in mining areas. As this phenomenon favours the dispersion of metals and contributes to the rising of concentration of toxic elements in the groundwaters, NMD threatens the surrounding environment. The effectiveness of autochthonous fungal strains in bioremediation process of ecotoxic metal is today well founded. We investigated the Zn tolerance and accumulation capability of four *Penicillium* strains (two *P. janthinellum*, one *P. olsonii*, and one *P. waksmanii*) isolated from an NMD environment. The experimental results showed a positive growth response of all strains at 750 ppm of Zn, while only two strains (*P. olsonii* and *P. waksmanii*) were able to grow at 1500 ppm of Zn. Further tests showed a bioaccumulation capability by *P. olsonii* strains up to 8600 ppm. These data, reporting the bioaccumulation capabilities of fungal species that have not been studied previously, are a further confirmation of the potential usefulness of autochthonous microfungi in bioremediation of heavy metals, and highlight the need of increase the number of tested strains.

Keywords: extreme environments; woodwardite; microfungi; mycoextraction; *Penicillium olsonii*

Riassunto

Accumulo di Zn in quattro ceppi di *Penicillium* (due *P. janthinellum*, un *P. olsonii* e un *P. waksmanii*) isolati da un ambiente soggetto a NMD. I risultati ottenuti hanno mostrato una risposta di crescita positiva di tutti i ceppi in presenza di 750 ppm di Zn, mentre solo due ceppi (*P. olsonii* e *P. waksmanii*) sono stati in grado di crescere a concentrazioni pari a 1500 ppm di Zn. Inoltre, ulteriori prove hanno dimostrato una capacità di bioaccumulo del ceppo di *P. olsonii* fino a 8600 ppm. Questi dati, evidenziando la capacità di bioaccumulo di specie poco studiate in precedenza, sono un'ulteriore conferma della potenziale utilità dei microfunghi autoctoni nel biorisanamento di terreni contenenti metalli pesanti.

**Parole chiave:** ambienti estremi; woodwardite; microfunghi; micoestrazione; *Penicillium olsonii*

1. **Introduction**

The weathering of sulphide minerals can generate solutions with low pH values, and a high amount of dissolved sulphates and metals of environmental concern. The formation of these acid solutions is typical of sulphide ore mining environments, and it is known as Acid Mine Drainage (AMD) (Blowes et al., 2003). When mine waters have near neutral pH, the definition of Neutral (or alkaline) Mine Drainage (NMD) is applied (Scharer et al., 2000). Although high concentrations of dissolved metals, especially Zn and Pb, can be reached in these waters, this phenomenon has received less attention than AMD. Nonetheless, these waters represent a threat to the quality of the surrounding aquatic system (Iribar, 2004; Frau et al., 2015). In NMD from Cu mines one of the most important secondary mineral in the precipitates is woodwardite, \((\text{Cu}_{1-x}\text{Al}_x)(\text{OH})_2(\text{SO}_4)_{x/2}\cdot n\text{H}_2\text{O}\), a member of the hydrotalcites group (Dinelli et al., 1998; Tumiati et al., 2008; Carbone et al., 2013). This mineral forms a complete isomorphic series with zincowoodwardite (Witzke and Raade, 2000), thus Cu can be substituted by Zn, making woodwardite a good sink for this element. The precipitation of colloidal particles generally produces a positive effect on the water quality (Srake et al., 2011), although colloids can be transported for long distances (Schemel et al., 2000), or become a secondary source of contaminants (Consani et al., 2017). For these reasons, mine waters and related precipitates have a major role in increasing the concentrations of ecotoxic elements in surrounding freshwaters and soils, and therefore are a potential risk for the environment and consequently for human health.

Investigating the capability of some organisms to survive in these inhospitable conditions is an intriguing task, since the same organisms could constitute possible agents able to remediate the environment. Recently, among the organisms that can survive in polluted areas, fungi have shown a huge potential in bioremediation of organic and inorganic pollutants (Gadd, 2010; Aytar et al., 2014; Jakubiak et al., 2014; Zotti et al., 2014; Pozdnyakova et al., 2006). In particular, as concern heavy-metal mycoremediation (bioremediation using fungi), several fungal strains were tested with various metals, such as V (Ceci et al., 2012), Cu (Yap et al., 2011; Wang and Wang, 2013), Ni (Cecchi et al., 2017a), Ag (Cecchi et al., 2017b), Pb (Zucconi et al., 2003), and Zn (Fan et al., 2008). Surely, among fungi, *Penicillium* is one of the main and more common genera. This genus includes more than 300 widespread species, characterised by a wide ecological range. *Penicillium* species have been isolated from various environments and substrata such as soil, freshwater and seawater, food stocks, etc (Domsch et al., 2007; Pitt and Hocking, 2009). Owing to this ecophysiological flexibility, *Penicillium* species have been often exploited for industrial and biotechnological purposes (Adrio and Demain, 2003). For the same reasons, as shown by Leitão (2009), several studies in the last few years have highlighted how *Penicillium* species could be fruitfully exploited for mycoremediation purposes.

In this context, several studies were carried out in order to assess the microbial flora occurring in AMD environments (Colmer and Hinkle, 1947; Das et al., 2009; Bond et al., 2000; Zotti et al., 2014);
conversely, only recently few authors started to study the microbial communities (mainly bacterial) in NMD environments (Lindsay et al., 2009; Majzlan et al., 2011; Dockrey et al., 2014).

Our work investigated the Zn tolerance and accumulation capability of four *Penicillium* strains (belonging to three different species) isolated from a NMD environment. These strains were mostly recurrently isolated during a survey (unpublished data) carried out in the Libiola Cu-Fe sulphide mine located near the Sestri Levante town (Liguria, North-Western Italy). In this precipitate, Zn is one of the major constituents (Carbone et al., 2013), and reaches high concentrations between 0.5% to 2%: for this reason we focused our study on this element.

More specifically, the goal of our work was to verify the *in vitro* tolerance and bioaccumulation capability of native *Penicillium* strains on two different substrata: greenish-blue colloidal precipitate and ZnSO₄·7H₂O enriched media (MUD-MEA and Zn-MEA). The achieved results propel us to deepen our research in order to find the best conditions for exploiting the capability of these strains.

2. Materials and methods

2.1. Study area

The study area was located at the Libiola mine site (lat. 44°17’18” N, long. 9° 26’42” E), located about 8 km NE of Sestri Levante (Eastern Liguria, Italy). The mine area extends over an area of about 4 km² within the basin of the Gromolo torrent (Fig. 1).

The climate of the area is classified as Mediterranean, is characterised by an average temperature during the year of 15 °C (seasonal average 5 °C in Winter, 10.5 °C in Spring, 19 °C in Summer, 13 °C in Autumn) and rainfall during the year range between 1100 and 1600 mm/year. The mineralisation is a Volcanogenic Massive Sulphide (VMS) deposit occurring, as massive lenses and stockwork mineralised veins, within the basalts associated with the Internal Ligurian Ophiolites. Pyrite and chalcopyrite are the dominant sulphide minerals in the site, with minor amounts of sphalerite and pyrrhotite, and gangue minerals are primarily composed by quartz and chlorite with minor amounts of calcite (Zaccarini and Garuti, 2008). The host rocks are mainly represented by basalts, serpentinites, and to a lesser extent by ophiolitic breccias (Ferrario and Garuti, 1980). At one site inside the mining area, the Margherita adit, the precipitation of greenish-blue precipitates takes place (Dinelli et al., 1998; Carbone et al., 2013). Seasonal variations led to pH oscillations from 6.3 to 7.7, and do not affect the
chemical composition of the precipitates, which is always composed mainly by Al, Si, and Cu. The amount of Zn varies from 0.5% to 2% (Carbone et al., 2013).

2.2 Colloidal precipitates sampling

On the whole five samples of colloidal precipitates by NMD were collected at the Margherita mine adit in April 2015. More precisely one sample was used for mineralogical characterization, and the remaining four were processed for microfungal isolation. The sampling was performed with a plastic syringe, then the solid + liquid suspension was stored in polypropylene bottles, filtered through 16 μm filters and air-dried. The pH, Eh and Electrical Conductivity (EC) of related waters were measured in the field using a portable pH-metre “PH330i” (WTW, Germany), equipped with “SenTix41” and “SenTix ORP” electrodes and a “Eutech XS COND6+” conductivity metre.

2.3 Isolation of native fungal strains

Mycological analysis was performed in August 2013. Four samples were collected sucking 50 ml of precipitates with a sterile syringe, the samples were put in falcon tubes and stored in a thermal bag at 6°C until the arrival to the laboratory; then the samples were stored in a refrigerator at 6°C. In order to select vial strains, the mycological analysis was performed following a dilution plate method modified from Gams (1984), as follows, within 2 days from the sampling. The isolation of the strains was performed plating 1 ml of the precipitates both pure and diluted (1:10, 1:10⁻², 1:10⁻³) on two different media (Rose Bengal Sigma-Aldrich® and Malt Extract Agar added with chloramphenicol Sigma-Aldrich®). A total of 16 Petri dishes (9 cm Ø) were inoculated for each sample. The dishes were incubated at 24°C for 7 days. Given the high level of toxicity of the matrix, the dishes were monitored daily for three more weeks, in order to isolate also the strains whose growth was slowed down by the high heavy metal concentrations.

The strains isolated were identified at genus level by conventional mycological methods by observing their micro- and macromorphological features and considering the different trophic and physiological requirements. In order to identify species belonging to genus Penicillium, molecular analyses ß-tubulin gene was amplified using primers Bt2a and Bt2b (Glass & Donaldson, 1995). The isolated strains were conserved in the culture collection of the Mycological Laboratory of DISTAV (University of Genoa, Italy).

2.4 Tolerance and bioaccumulation tests

Firstly, the ability of the strains to survive and germinate at high Zn concentration was tested on 750 and 1500 ppm Zn enriched Malt Extract Agar (Zn-MEA750 and Zn-MEA1500). The media were prepared by adding 3.706 and 7.412 g of ZnSO₄·7H₂O, respectively, to a standard MEA (1 L of distilled water, 20 g glucose, 20 g malt extract, 1 g peptone, 20 g agar), and autoclaving for 10 minutes at 120°C. In order to separate mycelial biomass (essential for subsequent analysis) from the Zn-MEA, a 9 cm diam. Cellophane Membrane Backing Biorad® disc was placed in each Petri dish. A conidial suspension (5x10⁸ conidia/mL) was obtained by adding 20 ml of deionised water + 10 µL of tween 80 to 14-d-old cultures and by scraping the surface. The conidial suspension was then inoculated on Zn-MEA 9 cm diam. Petri dishes, and incubated at 24°C for 21 days. The colonies were then separated from the media, and analysed with a Tescan Vega scanning electron microscope equipped with an EDS (Energy Dispersive Spectrometry) probe (EDAX manufacturer; see paragraph 2.5) to obtain a first evaluation of the presence and distribution of Zn in the mycelial biomass.

The bioaccumulation capability of the strain that showed the higher Zn signal during the EDS analysis was tested in vitro. The bioaccumulation test was performed on Zn-MEA750 (see above) and MUD-
MEA (1 L greenish-blue precipitates and water suspension, 20 g glucose, 20 g malt extract, 1 g peptone 20 agar). As done during the tolerance test, a 9 cm Cellophane Membrane Backing Biorad disc was placed in each Petri dish. A 5x10^8 per ml conidia suspension was prepared as described above. The conidial suspension was inoculated on MEA (control), MEA enriched with ZnSO_4·7H_2O and MEA-Mud dishes. The mycelium of 14-days culture was analysed by Tescan Vega scanning electron microscope equipped with an EDS (Energy Dispersive Spectrometry) probe in order to verify the heavy metal uptake capacity of the mycelium.

2.5 Instrumental analyses

The mineralogical characterisation of the natural colloidal precipitate samples was determined by X-Ray Diffraction (XRD) with Co Kα radiation (current 20 mA, voltage 40 kV). The sample of colloidal precipitates was ground with an agate mortar and pestle, and mounted on zero-background silicon plates. The analysis was performed scanning the sample between 5 and 80° 2θ at a scan rate of 5°/min.

The major element composition was investigated with Inductively Coupled Plasma-Emission Spectrometry ICP-ES on 1 g of sample digested in hot Aqua Regia, while minor and trace elements were determined by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) analysis of a 0.5 g samples after modified Aqua Regia digestion at Bureau Veritas Mineral Laboratories (Canada).

3. Results and discussion

3.1 Mineralogical characterisation

The XRD results (Fig. 2) show that the greenish-blue precipitates consist mainly of allophane [(Al2O3)(SiO2)1.3-2·2.5-3H2O], an amorphous hydrated Si-Al aluminosilicate, and woodwardite [(Cu1-xAlx)(OH)2(SO4)x/2·nH2O] or hydrowodwardite, which differs from woodwardite only for its higher water content (Witzke, 1999).

![Fig. 2. XRD diagram of the sample (h-wd= hydrowoodwardite; All= allophane).](image)

Fig. 2. Diagramma XRD del campione (h-wd= hydrowoodwardite; All= allofane).

From the bulk chemistry of the sample (Fig. 3) it is evident that the major constituents of the precipitates are Al and Si (11.84% and 9.57%, respectively), followed by Cu (8.14%), S (1.37%), and Fe (1.16%). The concentration of Zn in the sample is 5.729 mg kg\(^{-1}\).
The colloidal precipitate composition shows a high concentration of several toxic metals (see Fig. 3), which are detrimental to living organisms; therefore, this colloidal precipitate can be considered an extreme environment.

![Fig. 3. Main chemical compounds of the sample.](image)

### 3.2 Tolerant/bioaccumulant strains

As previously reported, we opted to use the native strains to test the Zn tolerance and accumulation capability, since these strains are more likely to survive to the high metal concentrations. In previous works we displayed that autochthonous microfungi evolved adaptation strategies against limiting factors and/or pollutants (Zotti et al., 2014; Cecchi et al., 2017 a,b), and for this reasons are the best candidate to show a positive growth response on contaminated media in laboratory test. However, in our case, as shown in Table 1, the growth response test (G) performed using the 4 most frequent strains present in this NMD area highlight different threshold for different species. Observing the Table 1, it is clear that all strains are able to germinate at 750 ppm of Zn, suggesting that the propagules of these two species have surely adapted to this environment without completely losing the germination capability. However, only two strains (P. olsonii and P. waksmanii) are able to germinate with Zn concentration of 1500 ppm.

<table>
<thead>
<tr>
<th>Strain Closest match (accession)</th>
<th>Identities</th>
<th>G 750</th>
<th>G 1500</th>
</tr>
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<tbody>
<tr>
<td>1 Penicillium janthinellum Biourge (KJ608359.1)</td>
<td>99%</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2 Penicillium janthinellum Biourge (DQ486649.1)</td>
<td>99%</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3 Penicillium olsonii Bainier &amp; Sartory (AY674443.1)</td>
<td>99%</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4 Penicillium waksmanii K.M. Zalessky (JQ965078.1)</td>
<td>99%</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

In spite of the fact that 4 tolerant strains were positive at the 750 ppm G test, and 2 at the 1500 ppm G test, the following accumulation screening performed by SEM and EDS probe on the fungal biomass samples shows that only P. olsonii biomass grown on Zn-MEA750 is able to accumulate Zn (see Fig. 4).
The SEM image in figure 4 shows a well developed conidiophores of the P. olsonii strain on Zn-MEA750; while the results of EDS analysis on the mycelium of the same strain are reported in the figure 5 showing a peak of Zn in the right part of the graphic. We must take into account that this EDS analysis is a semi quantitative measure, therefore it confirms the presence of at least 1% of Zn in the fungal biomass, but cannot provide the exact amount.

For this reason the following bioaccumulation tests with a P. olsonii strain on Zn-MEA750 and MUD-MEA were carried out to quantify the exact amount of Zn bioabsorbed in the biomass. After the incubation period, the ICP-MS analysis on mycelial biomass performed at the ASL Scandinavia AB Lab (Luleå, Sweden) confirms, as shown in table 2, the Zn uptake capability of this P. olsonii strain, with a concentration of 1520 ppm (from MEA-Mud) and 8630 ppm (from Zn-MEA750).

<table>
<thead>
<tr>
<th>Medium Sample</th>
<th>Zn (mg/kg)</th>
</tr>
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<tbody>
<tr>
<td>MEA</td>
<td>b.d.l.</td>
</tr>
<tr>
<td>P. olsonii biomass</td>
<td>8630</td>
</tr>
<tr>
<td>P. olsonii biomass</td>
<td>1520</td>
</tr>
</tbody>
</table>

Tab. 2. ICP-MS analysis on P. olsonii biomass and related medium on MEA, MEA+ZnSO₄·7H₂O, and MEA+Mud
Tab. 2. Analisi ICP-MS del contenuto in Zn nella biomass di P. olsonii cresciuto sui differenti terreni (MEA, MEA+ZnSO₄·7H₂O, e MEA+Mud).
*Penicillium olsonii* is well known as agent of food spoilage, and in the past it has been tested as a potential antagonist of the plant pathogen *Rhizoctonia solani* (Demirci et al., 2011), but till now has not been reported in literature to accumulate heavy metals. Our results represent the first report of *P. olsonii* metal tolerance and bioaccumulation capability, and demonstrate that harsh environmental conditions make a selection which can favour the evolution of strain-specific strategies in fungi. These results invite us to deepen this investigation, in order to increase i) the number of heavy metal tolerant strains ii) the knowledge of the heavy metals uptake patterns of native filamentous fungi.

**Acknowledgement**

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**References**


Witzke T. and Raade G. (2000) Zincowoodwardite, \([\text{Zn}_{1-x}\text{Al}_x\text{(OH)}_2]\text{[\((\text{SO}_4)\frac{x}{2}\text{(H}_2\text{O})_x]\), a new mineral of the hydrotalcite group. Neues Jahrbuch für Mineralogie – Monatshefte, 10: 455-465.}


