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A Preliminary Investigation on Metal Bioaccumulation by *Perenniporia fraxinea*

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Abstract The lignicolous macrofungus *Perenniporia fraxinea* has drawn increased attention due to its role as a pathogen of ornamentals in urban sites. The present study investigated the bioaccumulation of heavy metals by *P. fraxinea*. Sporophores were collected from urban and suburban areas in Pavia (Northern Italy) and analyzed for metals content (Cd, Hg, Pb, Ni, Cr, Cu, Fe, Mn, Zn) by inductively coupled plasma mass spectrometer and inductively coupled plasma optical emission spectroscopy, after microwave acidic digestion. On the basis of the obtained results the potential bioaccumulation capability of *P. fraxinea* was investigated. The isolates were grown in a culture medium enriched with different concentrations of Cd and Hg, chosen as probes of environmental pollution, and Cu for comparison. As *P. fraxinea* grows in the presence of Cd, Hg and Cu, it seems to be a potential tool in environmental monitoring.

Keywords Bioaccumulation · Heavy metals · Lignicolous macrofungi · *Perenniporia fraxinea* · Environmental monitoring

Several species of macrofungi are well known to accumulate heavy metals (Işildak et al. 2004; Cocchi et al. 2006; García et al. 2009; Cenci et al. 2011; Zhu et al. 2011) in the different stages of the development of the organism, namely mycelium, sporophore and rhyzomorphs, if present (Wargo and Carey 2001). This capability varies greatly depending on the species (Sesli et al. 2008; Melgar et al. 2009) and it is often affected by environmental conditions, particularly in highly industrialized regions (Wang and Hou 2011). Up to now, heavy metal bioaccumulation in lignicolous macrofungi has been poorly investigated for monitoring purposes (Radulescu et al. 2010; Skrbic et al. 2012). Due to their peculiar enzymatic pool, lignicolous macrofungi have an essential ecological role. They are able to degrade lignocelluloses of the plant by saprotrophism, parasitism or both (Maciel et al. 2010), thus compromising physiological and mechanical stability by wood rotting (Schwartze et al. 2000; Rinn 2011). The degradation of lignocelluloses only occurs in the presence of specific heavy metals: peroxidases are flavohemeproteins (Mn peroxidases contain Mn too); Cu is present in catalytic sites of laccases; Fe also participates as a cofactor in cellobiohydrolases (CBH) and cellobiose-dehydrogenases (CDH). Moreover, Fe ions are involved in Fenton reactions, which have an important role in lignocellulose conversion by brown rot fungi; they specifically degrade plant cell wall polysaccharides, leaving lignin residues, hence their name (Arantes et al. 2012; Hatti-Kaul and Ibrahim 2013). Besides, metals like Fe, Zn and Mn are cofactors in superoxide dismutases (SOD) and catalases (CAT), which are needed to contrast oxidative stress (Krumova et al. 2008). In this regard, lignicolous macrofungi should be considered as a sensitive tool for environmental monitoring. Indeed, many *taxa* are able to grow and produce long-lived woody-consistency sporophores even when they are in polluted areas. This ability can be exploited to obtain consistent data on environmental pollution (Čurdová et al. 2004; Skrbic et al. 2012).

Fungi mainly take up metal ions from free water in soil, but wet or dry atmospheric deposition also provides inputs

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(Baldrian and Gabriel 2002; Skrbic et al. 2012). Elements can enter the fungal membrane depending on chemical and physicochemical properties such as membrane electric potential and charge/ionic radius ratio, or they can be complexed by nucleophile sites (carboxylic, hydroxyl and amino groups) or precipitated as crystal (mostly oxalate) or colloid (Zimmermann and Wolf 2002). The presence of metal ions in the environment, depending on their concentration, may favor or interfere with many different fungal physiological processes (Gadd 2010). Indeed, at low concentrations, metals like Fe, Mn or Zn are essential fungal micronutrients, while higher concentrations can lead to toxicity; other metals, such as Cd and Hg, are toxic at any concentration (Baldrian 2003; Karaman and Matavulj 2005; Nies et al. 2015). As the concentration of environmental metals is largely influenced by human activities, its monitoring is a matter of environmental and biological concern.

Among lignicolous macrofungi, there is a growing interest in *Perenniporia fraxinea* (Bull.) Ryv., which is a worldwide distributed pathogen of broadleaves. This fungus develops very tenacious sporophores up to several decimeters wide and causes intense white rot, particularly at the base of host trees (Ryvarden and Gilbertson 1994; Bernicchia 2005). This species is present in Asia (mainly Japan), North America and Europe, both in sub-Atlantic and sub-Mediterranean climates (Szczepkowski 2004), and causes problems especially in urban areas. According to a recent report, analysis based on genetic data suggests that *P. fraxinea* spreads through spores instead of root contacts from adjacent infected trees (Sillo et al. 2016).

The present work aims to undertake, for the first time, an explorative study on the capability of *P. fraxinea* to bioaccumulate heavy metals. Sporophores collected from some urban and suburban areas were analyzed for metal content by inductively coupled plasma mass spectrometer (ICP-MS) and inductively coupled plasma optical emission spectroscopy (ICP-OES), after microwave acidic digestion. On the basis of the measured metal ions concentrations, isolates from the investigated sporophores were subsequently grown in cultural medium spiked with proper concentrations of Cd and Hg chosen because of their relevant environmental concern and Cu for comparison. This with the aim to evaluate the possibility to exploit *P. fraxinea* biomass as a possible tool for environmental pollution monitoring.

Materials and Methods

The sporophores of *P. fraxinea* were collected from four areas in Pavia (Northern Italy): two urban sites with moderate traffic (BCS, Borgo Calvenzano Street and VS, Via Scala), an urban park (VP, Vernavola Park) and a suburban area in the municipality of Zerbolò (CV, Cascina Venara). BCS and VS samples were collected from *Platanus* x *acerifolia* (Aiton) Willdenow and *Celtis australis* L., respectively, VP samples on *Robinia pseudoacacia* L. and CV samples on *Populus alba* L.

All fungal samples had grown no more than 10 cm above soil level. Fungal sporophores were manually cleaned, gently brushed using ultrapure water, dried at 37 °C in a ventilated oven and stored at −20°C. Before analysis, samples were unfrozen and homogeneously chopped.

Each sporophore was identified through macro and micro-morphological features by means of stereomicroscope, optical microscope and suitable reagents (KOH, Melzer's reagent-IKI) following taxonomical keys reported in specialized handbooks (Ryvarden and Gilbertson 1994; Breitenbach and Kränzlin 2000; Bernicchia 2005).

To isolate mycelium in pure culture, amounts from each sporophore (up to 10 mm^3) were incubated into Petri plates containing 2% malt extract agar (MEA) (Biokar Diagnostics) in sterile conditions. The isolates were maintained at 25°C.

After macro and micro-morphological checks, molecular analysis was performed on pure cultures to confirm the identification of mycelia. DNA extraction was performed by means of DNeasy® Plant Mini Kit QIagen; internal transcribed spacer—ITS region was amplified using the primer pair ITS1 and ITS4: PCR was performed according to SIGMA protocols for single reagents (Savino et al. 2014). Sequencing was carried out by Macrogen Inc., and the sequences were matched with available databases in Mycobank (http://www.mycobank.org) and BLAST (http://www.ncbi.nlm.nih.gov). All the strains isolated are preserved in the culture collection of fungi belonging to DSTA-University of Pavia (MicUNIPV).

Analytical reagents and methods are described as follows. Ultrapure HNO_3 (65% w/w) and certified multi-standard solution Merck VI for ICP-MS were supplied by Merck (Milan, Italy), H_2O_2 (30% w/w), $Cd(NO_3)_2{\cdot}4H_2O$ and $CuCl_2{\cdot}2H_2O$ were purchased from Carlo Erba Reagents (Milan, Italy). Multi-element standard solutions were prepared in 0.5% ultrapure nitric acid by diluting ICP stock solution. Ultrapure water (resistivity 18.2 MΩ cm^{-1} at 25°C) was produced in the laboratory by a Millipore Milli-Q system. The pH of the solutions was measured by a combined Orion glass electrode 9102 SC, standardized in H^+ activity. The trueness (accuracy and precision) of the overall procedure was verified for a certified reference material (BCR 482, lichen).

Microwave-assisted acid digestion of sporophores, mycelia and BCR was performed by a MarsXpress microwave system supplied by CEM (CEM s.r.l., Cologno al Serio, Italy), equipped with a 16 PFA PTFE (Xpress, 55 mL) vessel carousel and internal temperature control.

0.3 g of mycelium or sporophore samples and 0.1 g of BCR were weighted into the PFA-PTFE vessels. 5 mL of HNO_3 and 2 mL of H_2O_2 were added. Microwave heating was performed at 800 W for 15 min at 180°C. After cooling, the contents were evaporated to a small volume (about 0.5 mL) by an XpressVap™ accessory, diluted to 10 mL in calibrated polypropylene tubes and analyzed by ICP-MS and ICP-OES. Reagent blanks were prepared following the same procedure applied to samples. Measurements were performed by ICP-MS (quadrupole Elan DRC-e, PerkinElmer, Shelton, CT, USA) equipped with a standard ICP torch, cross flow nebulizer, nickel sampler and skimmer cones and dynamic reaction cell™ (DRC), and by an ICP-OES PerkinElmer Optima 3300 DV, according to the operating conditions suggested by the manufacturer.

Mycelium growth tests in culture medium spiked with Cd, Hg and Cu were only performed on the VS strain. This was grown in Petri dishes with 2% MEA (Malt Extract Agar) for a week at room temperature. Three flasks containing 1.5 L 2% ME (Malt Extract) were sterilized by autoclave (20 min, 120°C), cooled and spiked with Cd (1–10 mg L^{-1}), Hg (0.1–5 mg L^{-1}) and Cu (10–50 mg L^{-1}) solutions. The native pH of the spiked solutions was about 4.7. Each flask was inoculated with three agar plugs (10 mm per side) from the above-mentioned strain and stored in static growth at room temperature (25°C) for 4 weeks. After this time, the cultured fungal biomass was separated from the culture liquid by filtration, washed with ultrapure water and dried at 37°C until constant weight was achieved. Then it was submitted to microwave acidic digestion before ICP-MS and ICP-OES analyses. A control test was performed on a blank 2% ME solution inoculated with the same *P. fraxinea* strain under the same conditions. All experiments were performed in triplicate (n = 3).

Results and Discussion

This paper investigated the potential capability of *P. fraxinea* to bioaccumulate heavy metals. Cu, Fe, Mn and Zn were selected as nutrients for animal, plant and fungi life, while Cd, Hg, Ni, Cr and Pb were considered because they are usually monitored in environmentally polluted areas due to their toxicity for living organisms (Čurdová et al. 2004; Skrbic et al. 2012).

Heavy metal concentrations were determined after acidic digestion (see "Materials and Method" section) in n sub-samples of *P. fraxinea* sporophores collected in three urban sites (BCS, VS and VP) and in a suburban area (CV). The results are reported in Table 1.

Copper concentrations ranged from 6.0 to 35 mg Kg^{-1}, iron from 189 to 310 mg Kg^{-1}, manganese from 17 to 34 mg Kg^{-1}, and zinc from 18 to 57 mg Kg^{-1}. Cu, Fe, Mn and Zn were expected to reach higher values than the other elements due to their essential role and this is consistent with most studies dealing with such an issue (Karaman and Matavulj 2005; Kalač 2010; Giannaccini et al. 2012). As regards the other metals, their concentrations ranged from tenths of a unit up to 1.7 mg Kg^{-1}. Particularly Cd and Hg reached higher levels in VS than those found in the other sampling sites. These results may be justified by taking into account that VS sporophores were collected near a busy road. Although average concentrations of heavy metals in fungal sporophores vary among the species, the concentrations found in the samples studied are in line with those reported in recent literature (Čurdová et al. 2004; Skrbic et al. 2012). The trueness of the procedure was confirmed on BCR 482 reference material obtaining satisfactory accuracy and precision (93%–95%, RSDs not exceeding 10%, n = 3).

Table 1 Mean metal concentrations (mg Kg^{-1}) in *P. fraxinea* sporophores, standard deviations in parentheses

Analyte	Sampling site			
	VS	BCS	VP	CV
	n = 8	n = 5	n = 5	n = 8
Cu	33 (8)	6.0 (0.3)	11 (5)	35 (6)
Fe	203 (16)	189 (6)	200 (39)	310 (26)
Mn	26 (8)	17 (2)	25 (11)	34 (5)
Zn	41 (5)	20 (1)	18 (6)	57 (7)
Cd	0.6 (0.1)	0.15 (0.05)	0.13 (0.03)	0.12 (0.02)
Hg	1.7 (0.5)	0.51 (0.04)	0.15 (0.03)	0.7 (0.5)
Pb	0.4 (0.1)	0.22 (0.03)	0.35 (0.02)	0.13 (0.04)
Cr	0.7 (0.1)	0.19 (0.04)	0.42 (0.01)	0.18 (0.07)
Ni	0.4 (0.1)	0.38 (0.03)	0.30 (0.04)	0.33 (0.09)

On the basis of the obtained findings, further experiments were carried out on VS mycelia in order to test the potential bioaccumulation capability of *P. fraxinea*. The results of mycelia grown in 2% ME medium spiked with Cd and Hg, the two metals chosen as probes of environmental pollution (Čurdová et al. 2004; Skrbic et al. 2012), are shown in Table 2. For comparison, the same tests were also performed in the presence of Cu because of its biological role (Ludwig et al. 2013) and on a blank ME 2% solution as a control test. The ranges of concentrations were obtained from preliminary growth tests (data not shown) set on literature data (Čurdová et al. 2004), although actually higher than those found in polluted areas. The growth was shown to be influenced by the metal ion concentrations considered. More specifically, *P. fraxinea* can tolerate Cu up to a concentration of 50 mg L^{-1}, Hg up to 5 mg L^{-1}, while 5 mg L^{-1} of Cd was inhibitory to the growth of the organism. Especially in the case of Hg and Cu the accumulation capability was

Table 2 Percentage of growth and metal content (mg Kg^{-1}) in mycelia biomass

Medium	Growth (%)	Metal content (mg Kg^{-1})
Blank	100	<MDL[a]
Cu 10 mg L^{-1}	32	136
Cu 50 mg L^{-1}	3	16,440
Cd 1 mg L^{-1}	23	70
Cd 5 mg L^{-1}	–	–
Hg 0.5 mg L^{-1}	100	490
Hg 1 mg L^{-1}	64	1000
Hg 5 mg L^{-1}	4	36,000

[a]0.005–0.07 ug L^{-1} ICP-MS and 0.005–0.05 mg L^{-1} ICP-OES

enhanced by increasing the metal ion concentrations in the growth medium.

These results indicate that *P. fraxinea* can accumulate amounts of heavy metals. The evidence of an intrinsic high tolerance to Cd, Hg and Cu, even in the early stages of growth, makes this macrofungus a potential tool for monitoring pollution.

This is the first report on *P. fraxinea* capability to bioaccumulate heavy metals. These initial results indicate that it can grow in the presence of heavy metal ions and it is able to accumulate them.

Submerged cultivation of this fungal species in liquid media spiked with three metal ions, chosen as probes, confirmed that these elements were concentrated in fungal biomass. Bioaccumulation has been shown to increase as Cd, Hg and Cu concentration increases, up to values beyond which they become too toxic. Further investigations will be extended to a wider range of metal concentrations in order to obtain a deeper evaluation about the bioaccumulation potential of this fungus.

Since this species is widespread on different broadleaf substrata in Europe, Asia and North America, future studies should be extended to other chemical elements on a larger series of samples collected in areas with varying amounts of pollution to evaluate the potential role of *P. fraxinea* as an environmental pollution probe.

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