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IMPACT OF METALS EXPOSURE AND RESILIENCY VALUES IN WILD FILAMENTOS FUNGI

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ABSTRACT

Human activities result in environmental stress produced by heavy metals or organic contaminants, changing the community structure and tolerance. Fungal adaptation to metals, cotolerance and resilience or power to recuperate after being stressed with other factor, had been scarcely studied. The aims of this research were to evalute the Cd-adaptation of soil-fungi isolated from contaminated areas, to assess the resistance to cobalt and lead, and to test the resilience ability. *Alternaria alternata, Aspergillus conicus, Cylindrocarpon didymum* and *Gliocladium viride* were selected as they developed in Cd-cultures. The Cd-isolated strains removed twice as much Co and Pb than those of the non-polluted parental; and the metal amount removed were correlated with the Cd-concentration. The resiliency resembled their tolerance abilities. A 10-16 % biomass decrease were observed in *A. alternata* and *A. conicus* during 10-20 days; whereas a 48-56 % decrease were obtained with *C. didymum* and *G. viride*. The Cd-adaptation on the cotolerance development to other metals in *A. alternata* and A. *conicus* were a remarkable data, cotolerance to Co and Pb indicated that the mechanisms conferring resistance was not unique for Cd-ions mainly in *G. viride* and *C. didymum*. In conclusion, tolerant fungi to Cd, Co and Pb, and resistance developed by repeatedly subculturing the strains with increasing HM levels were obtained. Fungi adapted to metals suggesting that tolerance could also be acquired in natural environments; thus the fungal training to metals could be a remarkable technology for the preservation of any habitats and to implement diverse micoremediation strategies.

Keywords: Cotolerance, Heavy Metals, Micoremediation, Resiliency, Soil Filamentous Fungi

INTRODUCTION

Human activities result in environmental stress produced by heavy metals or organic contaminants release that provoke changes in the community structure and could development community tolerance; moreover, the exposure to one type of stress may cause tolerance towards this particular factor but may also develop co-tolerance to another type of disturbance **[1, 2]**. This happens when detoxification of different stresses relies on similar physiological processes; however, when another mechanisms is required, the organism would generate new responses which would demand extra energy **[3, 4]**. Damages reparation increase the costs of maintenance and in non-stressed environments life support represents more than 80% of organisms' energy; therefore, an increase of maintenance energy will cause a decrease of energy available for growth and reproduction **[5, 6]**.

Polluted habitats usually have both toxicants, metals (HM) and organics **[7, 8]**, but fungi showed a significant versatility and advantages than bacteria and algae in contaminated areas, due to their ability to uptake a wide range of substrates, cosubstrates utilization, morphological variations and nutritional habits **[9, 10, 11]**. Fungi comprised largest biomass increasing the adsorption surface, being more effective for metal removal and sequestration by living and non-living cells **[12, 13]**. Evenmore, fungal adaptation to HM had been confirmed in species isolated from stressed environments **[14, 15]**, but cotolerance and resilience had been scarcely studied **[16]**. Thus, the aims of this research were to evalute the adaptation to cadmium of soil filamentous fungi isolated from industrial contaminated areas, to assess the ability to confer its resistance to cobalt and lead, and to test the resilience or power to recovering after being stressed with other factor.

MATERIALS AND METHODS

Sampling Area and Isolation of Cd-Resistant Fungi

The filamentous fungi were isolated from contaminated sediments of the industrial area, La Plata, Argentin; the sediments features of the sampled site, the isolate methodology, the basal medium (BM) and the culture conditions were previously described **[17]**. The filamentous fungi were cultured in BM with 10.0 g glucose (BMG) as carbon source and 100 ppm Cd as $CdSO_4$, pH 5.4, to selected Cd-tolerant species.

Different culture media were used to identify the sporularing isolates. On the basis of micromorphology, colonial features, upper and down agar-plates colour and reproductive forms under diverse

cultures conditions (with or without light, UV exposure, 20-28 ºC, 5.5-7.7 pH), the fungal species were identified by scanning electron microscopy **[18]**. Non sporulating strains were rejected.

Fungal Growth

Four fungi able to grow on subsequent plating with increasing Cd levels (150 ppm, 200 ppm and 250 ppm) in BMG, pH 5.5, were chosen for further assays. The growth rate was measured as the incorporation of radioactively labelled acetate into ergosterol as this substance is a fungus specific ones **[19]**. Fungal liquid cultures (100 ml) inoculated with 5 ml with 250 ppm Cd-culture were incubated overnight with 0.50 ml acetate solution and 0.10 ml of ^{14}-C acetate solution ([1.2.-^{14}C] acetic acid, sodium salt, 2.07 GBq mmol -1, Ammersham, UK). One ml of 5 % formalin was added to stop the incorporation at 1, 10 and 20 days incubation times, so three sets of each fungi and HM were incubated. The samples were centrifuged, and the supernatants were discarded. Ergosterol was extracted and analyzed by HPLC being a sensitive method for determining fungal biomass **[20, 21]**. A conversion factor of 4.2 mg ergosterol / mg dry weight mycelium was obtained from ergosterol levels determined for the freeze-dried fungal biomass developed in the *in-vitro* Cd-assays; with 85 % ergosterol counting efficiency **[22]**.

The inoculum were determinated by filtration with nitrocellulose disc filters (MSI Micron Sep; Ø 47 mm, pore 0.45 μm) and filters were dried at 90ºC for 4 days, to constant weight. Three control cultures of each fungi were implemented, one without Cd, a 2nd one inoculated with each fungi and sterilized to check the metal sorption to the non-living biomass, and a non-inoculated 3rd flask. The experiments and the controls were done in triplicate, incubated at 27ºC, 130 rpm, in the dark

Resiliency Experiment

The changes over time in culture mediun without Cd or resilience were determined at different sampled times, 1, 10 and 20 days, by transferring 1ml of the culture with the higher metal concentration to a flask with BMG without Cd. The dry weight (average values) of these assays were compared with the control biomass as measure of resilience or power to recovering after being stressed **[23]**.

Cotolerance Assays

The fungi that grew with 250 ppm Cd were transferred to BMG amended with cobalt or lead. The isolates were trained by serial culture, every 7 days, to BMG with 10, 50, 100 and 200 ppm Co (as $CoCl_2$. $6H_2O$), or 50, 200, 400 and 600 ppm Pb (as $Pb(NO_3)_2$). The flasks were incubated at 27ºC, 130 rpm, in the dark, by triplicate. Not exposed strains were grown and maintained on the

same medium without HM.

Resistance (R) values, mean values of the dry weight, were normalised by the controls:

R (resistance) = % change = [(x treated – x control)/ x control] • 100 % , where x was the fungal biomass of each culture types. Three replicates were done for each fungi and metal concentration, and the experiments were performed twice.

Statistical Analysis

The data are expressed as the arithmetic mean ± standard error. Analysis results were not normally distributed, so nonparametric test Spearman rank correlation was used to correlate metal levels with fungi growth. For other analyses we used ANOVA with Cd, Co and Pb and time as fixed factors, with a significant level set at P = 0.01. In the statistical analyses the average change of all three sampling time points (1, 10 and 20 days) was taken as the measure of resistance or resiliency.

RESULTS AND DISCUSSION

Alternaria alternata, *Aspergillus conicus*, *Cylindrocarpon didymum* and *Gliocladium viride* were selected as they developed in Cd-cultures. A non-significant biomass decrease were observed in nontraining and Cd-training fungi with 100 ppm Cd. In the experiments with higher Cd levels, *A. alternata* and *A. conicus* tolerated the metal concentration; however *C. didymum* and *G. viride* showed 45-55 %, 78-82 % and 80-90 % biomass decrease with 150, 200 and 250 ppm Cd, respectively **(Figure 1)**.

Further experiments evaluated the sensitivity of the isolated fungi to cobalt and lead. *A. alternata* and *A. conicus* tolerated similar Co concentrations, producing a 30-31 % and 45-50 % diminution of its respective mycelium. By other hand, in the *C. didymum* and *G. viride* assays a higher significant drop were observed, 70-75 % biomass decrease were obtained with 50 ppm Co, and 85-90 % with 100 and 200 ppm Co, respectively **(Figure 2)**.

The effects of Pb on fungal growth patterns reversed that of the other HM, a significant minor decrease was obtained with all the Pb levels. *A. alternata* biomass dropped 20 % respect to the control flasks, and 30-35-40% decrease were observed with the other three fungi. Lead exhibited a significant lower toxicity for filamentous fungi isolated from polluted areas; being the four species tolerant to higher Pb levels **(Figure 3)**.

Even more, the mycelia of Cd-isolated strains removed approximately twice as much Co and Pb than those of the non-polluted parental, cultivated with 0 ppm Cd; and the HM amount removed by both cultures types were correlated with the Cd concentration in the plates. That is, fungi strains isolated in medium amended with Cd, were able to fixed higher HM levels in

further experiments. In the liquid cultures, the mycelia formed a mat, in the control and at 100 ppm Cd an extended wide growth were observed, whereas at the higher Cd-level the colonies were smaller in size and dense.

We found that soils that differ in pollution level had different stability to the applied of a second disturbance; our results supported both concepts of stability: (1) non-stressed systems were more stable because they possessed larger energetic resources, and (2) stressed systems were more stable since they gained abilities, like adaptation and physiological changes, to cope with additional stress. The fungal responses to stress, their nature and size, depended on the kinds of stress factors, especially whether a subsequent stress was applied.

The resilience or power to recovering obtained when fungi were transfered from 250 to 0 ppm Cd, resembled the tolerance ability of the wild fungi. *A. alternata* and *A. conicus* dropped 10 and 16 % of it dry-weight when they were exposed to the higher Cd-levels 10 and 20 days, respectively; whereas a higher decrease (48 and 56 %) were observed in *C. didymum* and *G. viride* the flasks after 10 and 20 days Cd-treatments **(Figure 4)**. The lenght of the exposure time to an stress factor affected the morphology and physiology of the fungi; *A. alternata* and A. *conicus* showed a quick adaptation to HM, on the contrary *C. didymum* and *G. viride* were more sensitive to HM pollution.

The Cd-adaptation on the cotolerance development to other metals in *A. alternata* and A. *conicus* is a remarkable data, and similar results with Cd but not cotolerance had been reported **[24, 25]**. The cotolerance observed with Cd, Co and Pb during training with only one ion, indicated that the cellular mechanisms conferring resistance was not unique for this metal mainly in *G. viride* and *C. didymum.*

The comparative toxicity of the metals to Cd-trained and non-trained (control assays) fungi followed the sequence: Co > Cd > Pb. The ion sizes and its penetration through fungal membranes pores were not responsible for their toxicity, as their respective nonhydrated ionic radii are 0.069, 0.072 and 0.072, respectively. Cell wall composition, secreted organic molecules and vacuoles had been mentionated as mechanism of metal tolerance in fungi **[26, 27]**.

It has been argued that fungi were more resistant to environmental stresses than bacteria **[28, 29]**, and it had been confirmed by fungal growth. Fungal response to multiple stressors had been scarsely studied **[30]** and usually in arbuscular mycorrhizae **[31, 32]**, microbial communities **[12, 33]** and white-rot-fungi **[34-36]**. The “stress on

stress" experiments, like isolating in a BM with Cd and then culturing in Co and Pb, demonstrate the higher resistance of fungi. Mechanisms like lower diffusion of contaminants into cells, lower surface to volume ratio, detecting diverse toxicants at different points of their surface, growth towards desirable sites or away from harmful conditions to avoid contact with pollutants and repair of damaged hyphae were some of the advance responses of fungi **[37]**.

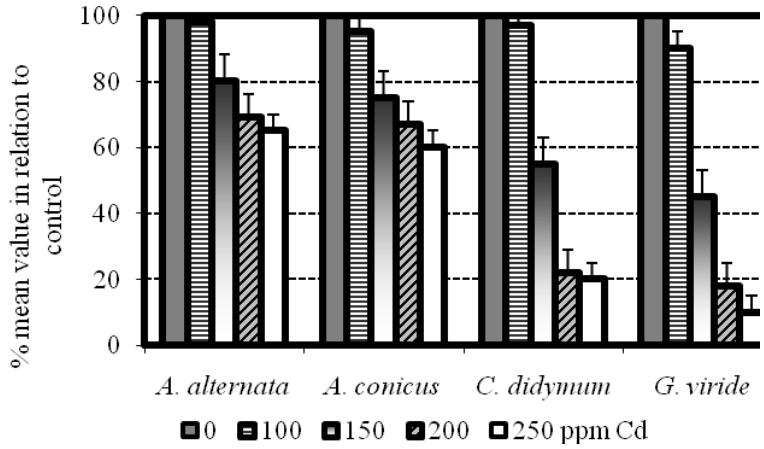

Figure 1: Fungal Biomass Obtained in Cd Cultures

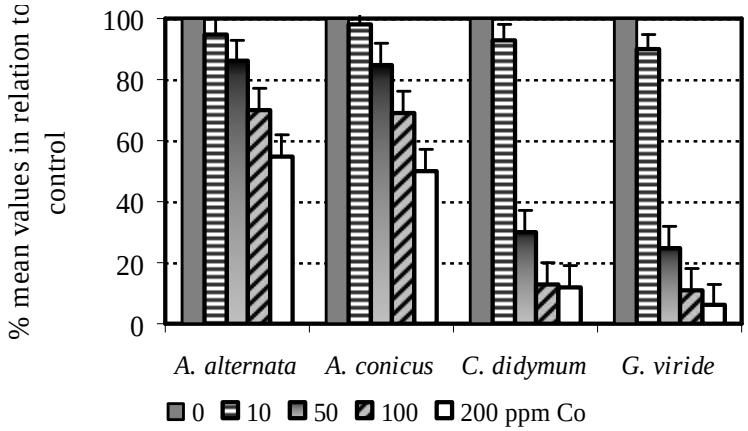

Figure 2: Biomass Obtained in CoAssays

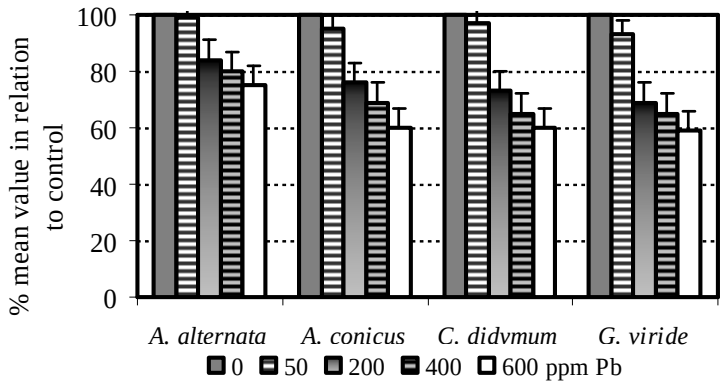

Figure 3: Biomass Obtained Decrease in Pb Experiments

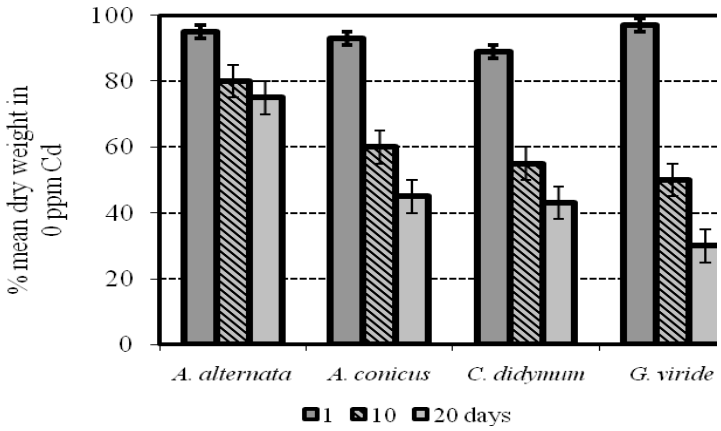

Figure 4: Fungal Biomass Values in Cultures Without Cd, After 1, 10 and 20 Days Exposure

CONCLUSION

Tolerant fungi to Cd, Co and Pb, and resistance developed by repeatedly subculturing the strains with increasing HM levels were obtained *in-vitro*, therefore, physiological adaptation was the prevailed mechanisms rather than gene mutation. The ability of fungi to adapt, even partially, to metals suggested that such tolerance and cotolerance could also be acquired in natural environments; thus the training of fungi to metals could be a remarkable technology for the preservation of any habitats and to implement diverse micoremediation strategies.

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