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EFFECTS AND MYCO-ACCUMULATION OF LEAD (Pb) IN FIVE *PLEUROTUS* MUSHROOMS

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ABSTRACT

White-rot basidiomycetes execute important functions in bioaccumulation of heavy metals from contaminated nutrient source. This work was conducted to evaluate the effects of lead on the mycelia growth and fruiting body production of five *Pleurotus* mushrooms and their potential to accumulate lead from the lead-infused rice straw-sawdust substrate. The results revealed that the mycelia growth of five *Pleurotus* species was significantly affected by the presence of lead in the culture media in concentration dependent manner. At 100 ppm, *P. salmoneostraminues* showed the highest growth inhibition (47.49 ± 0.20%) while *P. djamour* had the lowest (3.33 ± 0.56%) after 7 days of incubation. Aside from growth inhibition, changes in mycelial morphology were also apparent in *P. salmoneostramineus*, *P. djamour* and *P. florida*. Although statistically insignificant, yields of fruiting bodies of all studied *Pleurotus* were reduced by the presence of lead in the substrate. Their accumulation abilities were varied depending on species type and fructification interval. The maximum accumulation (1.63-2.58 ppm) was noted in the third flush of fructification. *P. florida*, *P. ostreatus* and *P. djamour* recorded the highest maximum accumulation. Therefore, based on

their remarkable tolerance and lead accumulation abilities, these *Pleurotus* mushrooms could play vital character in the application of fungi in environmental protection and conservation.

Keywords: *Pleurotus,* Myco-accumulation, Heavy Metals, Lead (Pb), Bioremediation

INTRODUCTION

Heavy metals are one of the most important environmental pollutants in the entire biosphere owing to their high toxicity to any living forms. As developing country, the heavy metal pollution in the Philippines is enormously increasing due to the rapid industrialization and technological developments. The untreated effluents from the chemical and manufacturing industries, mining sites, and even in agricultural production greatly contribute to the elevation of heavy metal concentrations in the air, soil and water that could pose health threat to mankind. Accumulation of some heavy metals in human and wildlife could raise susceptibility to infectious diseases by targeting the normal functions of the immune system **[1]** and disrupt biological activities of various vital organs such as brain, heart, liver, kidney and bone **[2]**. These toxic metals with no significant role in body functions include mercury, aluminum, cadmium, beryllium and lead.

Lead (Pb) is hazardous and ubiquitous heavy metal pollutant and is from mining drainage, industrial discharges, fertilizers, pesticides, smelting of Pb ore and municipal sewage sludge **[3]**. A number of adverse effects of lead exposure to human have been reported. Lead attacks the central nervous system to cause coma, convulsions and lead poisoning which estimated to 143,000 deaths per year at high levels of exposure, and it could contribute to a variety of body system interruptions resulting to anaemia, hypertension, renal impairment, immunotoxicity, and reproductive organs toxicity when expose at lower concentrations **[4]**.

Considering this vast global concern on the noxious effects of lead, many scientist and researchers are exploring new biotechnology-oriented and environmentally friendly technologies in remediating lead from the contaminated areas. One of the techniques that have been introduced is through bioremediation – exploiting the potentials of living organisms such as plants, fungi and other microbial entities to degrade and accumulate contaminants from the substratum. Fungi are known for their high degrading systems which enable them to accumulate heavy metals via several mechanisms including transformation of metals, efflux, intracellular compartmentalization, biosorption, metabolism dependent accumulation and extracellular precipitation, complexation and crystallization, impermeability and sequestration **[5, 6, 7]**. Singh **[8]** estimated that about 30% of the reported studies on fungal bioremediation are focused to white-rot fungi which could be able to degrade a number of toxic waste materials like polychlorinated biphenyls and dioxins, pesticides, phenols and chlorophenols, effluents from pulp and paper mills, dyestuffs and heavy metals.

Pleurotus species are one of the white-rot basidiomycetes that naturally growing on dead or decaying wooden logs in the tropical forests and on rice straw and sawdust substrate formulation in a rural based mushroom cultivation under Philippines setting. Species of the genus *Pleurotus* and other white-rot fungi are efficient degraders of complex organic compounds present in their substrate due to their active extracellular lignin-modifying enzymes **[9]** such as lignin-peroxidase (LiP), manganese peroxidase (MnP), and various H_2O_2 producing enzymes **[10]**.

With this great impact of white-rot fungi like *Pleurotus* species on environmental protection and rehabilitation through mycoremediation created an interest to investigate the growth response and myco-accumulation abilities of five *Pleurotus* species namely; *P. cystidiosus*, *P. djamour*, *P. florida*, *P. ostreatus*, and *P. salmoneostramineus*, on the substrate contaminated with lead.

MATERIALS AND METHODS

Source of mushroom species

The studied *Pleurotus* species namely, *P. cystidiosus* (MC020-1), *P. djamour* (MC021-0), *P. florida* (MC021-1), *P. ostreatus* (MC024-1) and *P. salmoneostramineus* (MC027-0) were obtained from the culture collection of the Center for Tropical Mushroom Research and Development, Science City of Munoz, Nueva Ecija, Philippines.

Preparation of culture inoculant

From the 7-day old pure cultures of the five mushrooms, an agar block of approximately 10 mm^2 x 3 mm was aseptically inoculated into sterilized potato sucrose gulaman (PSG) plates and incubated at 30°C to allow mycelia growth. After 7 days of incubation, a flame sterile 10 mm diameter cork borer was used to prepare mycelial discs as culture inocula in growth response evaluation.

Preparation of lead contaminated culture media

Potato sucrose gulaman with varying concentrations of lead was used as culture media in the evaluation of the growth response of the five mushrooms. The decoction of 300 g of potato in 1.2 L of distilled water, 24 g of white gulaman, and 12 g of sucrose were combined and boiled until homogenized. Three hundred ml of the medium was prepared for each concentration (1 ppm, 10 ppm, and 100 ppm) of the lead sulfate ($PbSO_4$) and a lead free medium served as the control, 0 ppm. Each prepared medium was dispensed in a flask, cotton plugged and properly labelled. These were sterilized in an autoclave at 121°C, 15 psi for 30 min.

Evaluation of mycelia growth response

The different media were pour plated and aseptically inoculated with mycelia discs of mushrooms. Triplicate was done per treatment per mushroom. The inoculated plates were incubated at 30 °C to allow mycelia growth. The daily mycelia growth was measured and the mycelia density was described as very thin (+), thin (2+), thick (3+), very thick or cottony (4+). The percentage inhibition of mycelia growth was also calculated as follows: Growth inhibition (%) = [(growth diameter of control – growth diameter of treated) / growth diameter of control] x 100.

Preparation of grain spawn

Palay seeds (200 g) were boiled until slit opening of the husk occurred. Seeds were drained, maintained at 65% moisture content, and dispensed into polypropylene plastics. Each bag with 40 g of boiled palay seed was plugged with cotton and wrapped with recycled paper. These were sterilized in an autoclave at 15 psi, 121°C for 30 min and aseptically inoculated with mycelia discs of mushroom. Grain spawn were incubated at 30°C for 10 days which served as the inoculant of the fruiting bags containing substrate infused with lead.

Evaluation of the myco-accumulation ability of the five mushrooms

The ability of the fruiting bodies of five *Pleurotus* species to accumulate lead from the rice straw – sawdust based substrate was also studied. Naturally composted rice straw was chopped into 1 inch long and mixed with composted sawdust following the 7:3 v/v substrate formulations with 65% moisture. Formulated substrate (700 g) was compacted in a polypropylene plastic. Each bag was infused with 2 ml of 1000 ppm of lead sulfate with 5 replicates for each mushroom species. Bags without lead served as the control. These were individually provided with opening using cut pvc pipe, plugged with cotton, covered with recycled paper, and sterilized at 15 psi, 121 °C for 45 minutes. After cooling, each bag was inoculated with grain spawn and subsequently incubated at 30 °C in the incubation room. Once completely colonized with mycelia, fruiting bags were transferred into the growing house with 80-90% RH to allow fruiting body development. The fruiting bodies were harvested, weighed and air-dried up to the last flush. The accumulated amounts of lead of the fruiting bodies from the first up to the third flush were analyzed using atomic absorption spectrophotometer.

Statistical analysis

Data were analyzed using analysis of variance ANOVA in one way classification analysis. LSD was used to determine the significant treatment comparison at 5% level of significance. T-test was also used to compare the effect of lead in the substrate on the yield. The SAS computer program was used for analysis.

RESULTS AND DISCUSSION

Mycelial growth, density and growth inhibition

Lead is not considered as essential element for the growth and development of any living organisms. It causes various diseases and severe lethal effects to animals including human being and solely lethal in plants **[11]**. In fungi particularly mushrooms, however, lead at certain concentration inhibits mycelial growth and reduces fruiting body production **[12]**. The effect of lead on mushroom could directly recognize based on the mycelial growth responses on contaminated media. In the present study, the mycelial growth responses of the five *Pleurotus* species on the media contaminated with varying levels of lead was evaluated. **Table 1** presents the mycelial growth diameter, growth inhibition and mycelia density of the five *Pleurotus* species on lead contaminated potato sucrose gulaman after 7 days of incubation. Apparently, in all studied *Pleurotus* species, lead treated media significantly recorded lower mycelial growth diameter compared to lead free media. The highest concentration, 100 ppm, significantly had the lowest mycelial growth diameter, indicating that the effect of lead is concentration dependent, as lead concentration increased the mycelial growth diameter decreased. However, among the five *Pleurotus* species grown at 100 ppm lead concentration, *P. djamour* showed the highest mycelial growth diameter (87.00 ± 0.50) while *P. salmoneostraminues* had the lowest (47.17 ± 0.28) after 7 days of incubation. This hindered growth could probably explained by the inactivation of some enzymes and proteins responsible in the growth and development of mycelia due to the toxic effects of lead ions. Bruins *et al.* **[13]** reported that lead ions could inhibit enzyme activity, disrupt membrane function and oxidative phosphorylation, and alter nucleic acids and proteins and even osmotic balance of the microorganisms. Furthermore, extensive damage to the cell membrane due to irreversible K^+ efflux and inhibition of energy-dependent H^+ efflux from cells is strong indication of heavy metal toxicity **[14]**.

The morphology of the mycelia like density and color can also be affected by heavy metals **[15, 16]**. In terms of mycelial density, *P djamour* and *P. florida* only showed thick mycelia on lead treated media as compared to the very thick mycelia on lead free media. In contrast, *P. salmoneostramineus* which produced thick mycelia on lead free media had very thick mycelia when cultured on the lead contaminated media. Likewise, more dense mycelia of *L. edodes* were observed in culture media supplemented with cadmium and mercury **[17]**. This could probably be attributed to changes in branching of the hypha, in which branching is prevalently active in the lateral hyphae than in the hyphal tips. However, no variation in mycelial density was noted between lead free and lead treated media in both *P. cystidiosus* and *P. ostreatus*.

Growth inhibition is one of the most important responses of fungi to heavy metal stress. Similarly, growth inhibition was apparently observed in lead treated media and was found increasing as the lead concentration increased. The lowest percentage growth inhibition was noted in *P. djamour*, followed by *P. florida* and *P. ostreatus*, strongly suggest their high lead tolerance. On the other hand, the highest percentage growth inhibition was found in *P. salmoneostramineus*, followed by *P. cystidiosus*, indicating their quite sensitivity to lead concentrations. This obviously means that the level of tolerance and inhibition among *Pleurotus* species is varied. In the previous study, *P. sajor-caju* showed more than 85% growth inhibition when cultured on media with 15 μg/ml of lead **[18]**. Moreover, lead at 0.2 mM in liquid culture strongly inhibited the growth of other basidiomycetes such as *P. chrysosporium*, *P. sanguineus,* and *T. versicolor* **[19]** and the very aggressive *C. comatus* also responded sensitivity on 100 ppm lead-contaminated medium **[12]**. Apart from lead, growth inhibitions of *Pleurotus* species were also apparent to other highly toxic metals like mercury, cadmium, copper, cadmium, and zinc **[16, 20, 21]**.

Despite of these reported fungal sensitivity and inhibition caused by lead and other toxic metals, it is still reasonable to assume that mushrooms have wide variety of tolerance mechanisms against toxic pollutants. In some fungi like *Aureobasidium pullulans*, lead toxicity is prevented due to the existence of the excreted extracellular polymeric substances (EPS) in which more than 90% of lead could bind to it, impeding the penetration of lead in the inner cellular part **[22, 23]**. The tolerance and degrading system of *P. ostreatus* against Benzo[a]pyrene is mediated by enzymes like synthetic laccase mediator, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate), natural mediator vanillin and metal ions such as copper, zinc and manganese **[24]**. These important abilities of fungi, especially mushroom, directed towards a number of studies on their bioaccumulation potential of various toxic pollutants.

Yield and myco-accumulation of lead from the substrate

With the established effects of lead on the mycelial growth of the five *Pleurotus* species, the yield and lead accumulation potential of fruiting bodies of these basidiomycetes on lead contaminated substrates was also considered in the present work. Although statistically insignificant, yields of all studied *Pleurotus* species were apparently reduced by the presence of lead in the substrate **(Table 2)**. The results obtained affirmed with the previous finding that the biological efficiency of sporocarp production of *P. sajor-caju* was reduced by lead **[18]**. This yield reduction could be accounted to the morphological changes on stipe length, stipe diameter, and cap diameter of the fruiting bodies as evident effects of lead in *P. tuber-regium* **[20]**. Among the five species grown on contaminated substrate, *P. florida* produced the highest yield of 245.66 ± 7.77 g, followed by *P. ostreatus* and *P. djamour*. Aside from the very low mycelial growth inhibitions as response on the contaminated media, these high yields of fruiting bodies of three *Pleurotus* species strongly dictate their high tolerance and resistance against lead. Hence, they are promising candidates in mycoremediation.

In view of the wealth information on heavy metal contents and/or the ability of various wild edible mushrooms to accumulate heavy metals **[25, 26, 27, 28, 29, 30, 31]**, it was hypothesized that the five *Pleurotus* species exhibit the same bio-potentiality. Myco-accumulation is the sequestration of heavy metals or toxic pollutants by fungi. Mushrooms degrade and accumulate whatever molecules present in the nutritional sources by their aggressive mycelia which ultimately develop into fruiting body. As compared with other agricultural crop plants, mushrooms have very effective mechanisms to uptake heavy metals as shown by their higher metal content **[25]**. In the present study, it is noteworthy that the levels of lead accumulation in five mushrooms were varied depending on the type of species and interval of fructification or flushing **(Table 2)**. The maximum accumulated lead contents in all species were found in the third flush of fruiting bodies with a range of 1.63-2.58 ppm. *P. florida* and *P. ostreatus* recorded the highest while *P. salmoneostramineus* had the lowest maximum accumulation. Surprisingly, it was also apparently observed an increasing level of lead accumulation from the first thru the third flush of fructification suggesting that the mycelia required time-course to accumulate at the maximum level, which can be considered in the future study. This result clearly indicates that the 2 ml of 1000 mg/L lead infused at the bottom part of the fruiting bags were accumulated and translocated in the mycelia and concentrated in the fruiting bodies.

Previously, the mechanism of accumulation of lead by *S. cerevisiae* was studied **[32]**. Lead adsorbed on the surface of the cell, then passed through the cell wall and traversed the cell membrane to penetrate into the cytoplasm. In the cytoplasm, lead is conjugated with glutathione, forming the lead–glutathione complex and is transported to the vacuole **[33]**. However, in mushrooms like *P. ostreatus*, the concentrations of nine metals (Al, Bi, Cd, Cr, Cu, Fe, Mn, Ni and Pb) at cellular level were found higher in cytosol fraction than in cell wall and mixed membrane fractions **[34]**. Also, lead within the fruiting bodies of mushrooms is unevenly distributed. The pileus contained higher amount of lead than the corresponding stipe of *P. tuber-regium* **[35]**.

With this considerable ability, these five *Pleurotus* species could play a tremendous impact in controlling or neutralizing the toxic effect of heavy metal pollution. This will serve as groundwork of many strategies which could be effectively used in environmental biotechnology. For instance, creation of protoplast self-fusion mutant endophytic fungus, *Mucor* sp., to improve stress tolerance and phytoremediation of *Brassica napus* in Cd- and Pb-contaminated soil **[36]**, and development of genetically encoded protein sensors for Pb in fungi **[37]**. Nevertheless, this ability could also pose risks to human health once the high amount of lead is consumed or enters the food chain. As mentioned in the study conducted by García *et al*. **[38]**, the statutory valid limits for lead content in wild-growing edible mushrooms and cultivated mushrooms were 10 mg/kg and 3 mg/kg dry weight, respectively. Although the lead content of mushrooms obtained in the present study were found lower than the prescribed acceptable limits and thus, do not represents immediate toxicological effects, it is still unsafe to assume due to biomagnification issues, because there are some evidence that lead can be biomagnified through the food webs **[39]**.

Table 1: Mycelial growth diameter, percentage growth inhibition and mycelia density of the five *Pleurotus* species on potato sucrose gulaman at varying concentrations of lead after 7 days of incubation				
***Pleurotus* species**	**Lead concentration (ppm)**	**Mycelial growth (mm)**	**Growth inhibition (%)**	**Mycelial density**
P. cystidiosus	**0**	**78.90 ± 0.05[a]**	**0.00 ± 0.00[d]**	**3+**
	1	**77.93 ± 0.19[b]**	**1.22 ± 0.30[c]**	**3+**
	10	**65.53 ± 0.18[c]**	**16.94 ± 0.20[b]**	**3+**
	100	**52.27 ± 0.28[d]**	**33.74 ± 0.32[a]**	**3+**
P. djamour	**0**	**90.00 ± 0.00[a]**	**0.00 ± 0.00[c]**	**4+**
	1	**89.50 ± 0.50[a]**	**0.56 ± 0.56[c]**	**3+**
	10	**88.00 ± 0.25[b]**	**2.22 ± 0.28[b]**	**3+**
	100	**87.00 ± 0.50[c]**	**3.33 ± 0.56[a]**	**3+**
P. florida	**0**	**89.67 ± 0.29[a]**	**0.00 ± 0.00[d]**	**4+**
	1	**88.35 ± 0.29[b]**	**1.47 ± 0.02[c]**	**4+**
	10	**86.54 ± 0.10[c]**	**3.49 ± 0.40[b]**	**3+**
	100	**83.39 ± 0.08[d]**	**7.00 ± 0.39[a]**	**3+**
P. ostreatus	**0**	**84.75 ± 0.05[a]**	**0.00 ± 0.00[d]**	**4+**
	1	**79.00 ± 0.43[b]**	**6.78 ± 0.46[c]**	**4+**
	10	**77.92 ± 0.44[c]**	**8.06 ± 0.57[b]**	**4+**
	100	**72.25 ± 0.25[d]**	**14.75 ± 0.28[a]**	**4+**
P. salmoneostramineus	**0**	**89.82 ± 0.20[a]**	**0.00 ± 0.00[d]**	**3+**
	1	**60.68 ± 0.48[b]**	**32.44 ± 0.67[c]**	**4+**
	10	**57.36 ± 0.12[c]**	**36.13 ± 0.24[b]**	**4+**
	100	**47.17 ± 0.28[d]**	**47.49 ± 0.20[a]**	**4+**
Values are mean ± SD. Treatment means in each species with the same letter of superscript are not significantly different from each other at 5% level of significance using LSD. In mycelial density column, very thin (+), thin (2+), thick (3+), very thick or cottony (4+)				

Table 2: Total yield and accumulated amount of lead in fruiting bodies of the five *Pleurotus* species grown on lead contaminated rice straw-sawdust based substrate					
***Pleurotus* species**	**Lead (ppm)**	**Total yield (g)**	**Accumulated amount of lead (ppm)**		
			1st flush	**2nd flush**	**3rd flush**
P. cystidiosus	**0**	**106.24 ± 5.85[a]**	**ND**	**ND**	**ND**
	1000	**98.28 ± 5.85[a]**	**0.00**	**1.75**	**1.81**
P. djamour	**0**	**179.80 ± 6.77[a]**	**ND**	**ND**	**ND**
	1000	**168.40 ± 16.9[a]**	**0.46**	**1.93**	**2.35**
P. florida	**0**	**246.74 ± 6.98[a]**	**ND**	**ND**	**ND**
	1000	**245.66 ± 7.77[a]**	**0.28**	**1.89**	**2.58**
P. ostreatus	**0**	**202.88 ± 12.5[a]**	**ND**	**ND**	**ND**
	1000	**197.28 ± 14.4[a]**	**0.94**	**2.14**	**2.57**
P. salmoneostramineus	**0**	**123.80 ± 8.50[a]**	**ND**	**ND**	**ND**
	1000	**115.00 ± 7.71[a]**	**0.37**	**0.84**	**1.63**
Values are mean ± SD. Treatment means in each species with the same letter of superscript are not significantly different from each other at 5% level of significance using T-test. ND, not detected.					

CONCLUSION

In conclusion, although mycelial growth and fruiting body production of the five *Pleurotus* were affected by lead at certain concentration, they still possessed remarkable abilities to tolerate and accumulate lead from the contaminated substrate. Findings of the present study affirmed with previous reports on heavy metal accumulation abilities of *P. ostreatus*, *P. florida*, *P. eryngii*, *P. sajor-caju* and *P. citrinopileatus* **[40, 41, 42, 43, 44]**. These clearly prove the applicability *Pleurotus* mushrooms in bioremediation strategies in protecting the Mother Nature against the toxic effects of not only lead but also of the wide variety of hazardous pollutants. However, their mechanisms of accumulation must be considered in future studies.

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