



Ectomycorrhizal Mushrooms as a Natural Bio-Indicator for Assessment of Heavy Metal Pollution

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Abstract: Environmental changes and heavy metal pollution are some of the consequences of anthropogenic activities. Many ecosystems, including edaphic ecosystems, suffer from the effects of pollution. The accurate assessment of soil heavy metal contamination leads to better approaches for remediating soils. The exploration of different ways, including biological methods, to conduct environmental monitoring is still ongoing. Here, we focus on reviewing the potential of ectomycorrhizal fungi as a natural indicator of soil heavy metal pollution. Mycorrhizal fungi fulfill basic criteria required as natural bio-indicators for heavy metal contamination. These fungi use different mechanisms such as avoidance and tolerance to survive in metalliferous soils. Thus, we promote ectomycorrhizal fungi as natural indicators. This review also synthesizes existing research on ectomycorrhizal mushrooms as natural bio-indicators for heavy metal pollution and the elaboration of mechanisms, by which ectomycorrhizal fungi meet the criteria required for a successful bio-indicator.

Keywords: contamination; ectomycorrhiza; heavy metal; homeostasis; mycorrhiza

1. Introduction

Heavy metals (HM) and metalloids are metallic elements having a specific density of more than 5 g cm⁻³, such as mercury (Hg), chromium (Cr), cadmium (Cd), arsenic (As), and lead (Pb) [1]. Heavy metals are adversely affecting living organisms even at low concentrations through bio-accumulation in the food chain [2,3]. Heavy metals are characterized by a long half-life, and they are highly persistent in the environment with the potential for accumulation [4]. The artificial radionuclides or isotopes of different heavy metals cause significant hazards in terms of soil pollution. There are two main sources globally, which are responsible for the presence of different radionuclides in the environment [5]. Nuclear weapon tests and nuclear reactor catastrophes such as Chernobyl in 1986 are the main sources that emitted considerable amounts of α , β , and γ -radionuclides into the atmosphere. For instance, the long-life gamma radioisotope cesium 137Cs is considered an important indicator of radioactive pollution due to its long half-life (30.07 years) [5,6]. Caridi et al. [7] explain that radionuclides are strongly absorbed into the



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Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). sediments and retained for a long time, resulting in genetic mutations, the development of diseases, and soil infertility.

Although cobalt (Co), copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo), nickel (Ni), and zinc (Zn) are also grouped under the HM category, at optimum concentrations, they are beneficial for plant growth and development [8]. Nevertheless, the same metals become toxic when they exceed certain threshold concentrations [8,9]. Even though HM occurs naturally in the crust of the Earth, anthropogenic activities mostly contribute to the HM prevalence in air, soil, and water [10,11]. Since these metals are non-biodegradable, they persist in the environment [12]. Therefore, heavy metal contamination and pollution are major environmental hazards, which, when amplified by inadequate human intervention, could become a serious concern.

Conventional farming practices, increasing industrialization, and the substantial use of fossil fuels have led to high concentrations of heavy metals in the environment [13]. Furthermore, the large quantities of synthetic fertilizers and other agro-chemicals used in farming systems lead to substantially high concentrations of heavy metals in soil and water [1]. In acidic soils, HM becomes highly bioavailable due to competition with H⁺ for binding sites and increased solubility [14,15]. Considering these threats, scientists have focused on finding solutions to minimize the adverse effects caused by heavy metals. In the HM remediation processes in contaminated environments, demarcation of the contamination, identification, and quantification of the HM, specifically in their ionic form, are inevitably important [16–18].

From an ecological perspective, different organisms, including microorganisms, are naturally occurring bioindicators. Bioindicators are used to assess the environmental quality and detect positive or negative changes and their subsequent effects on biotic and abiotic components of the environment [19]. Bioindicator organisms possess the potential to absorb particular pollutants from the surrounding environment and indicate the presence of a particular pollutant within the organism [20]. Although different types of organisms are used as bioindicators in ecological monitoring, ectomycorrhizal mushrooms are well known among them due to their capability of absorbing heavy metals from the surrounding environment [18].

Mycorrhizae are generally considered mutualistic symbioses between plant roots and some fungi [21]. These symbioses are characterized by the bi-directional movement of nutrients where carbon flows to the fungus from the plant and inorganic nutrients move to the plant from the fungus, thereby providing a critical bond between the plant root and soil [22]. In the humid tropics, the following two major types of mycorrhizal associations of trees have been reported: ectomycorrhiza (EM) and arbuscular mycorrhizal fungi (AM) [23]. Both EM and AM are recognized as heavy metal accumulators [24–26].

Nevertheless, the ectomycorrhizal fungi have been identified as a group of organisms that intensely affect ecosystems by facilitating nutrient and water uptake, structuring soil, maintaining food webs, protecting the root systems of trees from pathogenic organisms, extreme environmental conditions, and phytoremediation of contaminated soils [27–29]. Besides that, the vast diversity of EM fungi helps nature to withstand changes in environmental factors due to heavy pollution and global climate change. Moreover, many EM fungi play a major role in bearing commercial value for edible fruiting bodies and in producing metabolites useful in industries [28,30]. Therefore, conservation, broader appreciation, and widening applications of EM fungi are necessary due to their extensive intervention in the successful functioning of ecosystems.

Thus, our objective in this review is to synthesize work on the potential of EM fungi to act as a natural bio-indicator of heavy metal contamination in soil and how EM mushrooms meet the criteria of successful bio-indicators since there are no studies in the available literature on how EM mushrooms meet the requirements of a successful bio-indicator. Furthermore, we present an overview of the physiology of EM fungi, supportive mechanisms of metal homeostasis, and characteristics of EM mushrooms to be a successful bio-indicator. Furthermore, the analysis of heavy metal concentrations adsorbed to EM mushroom fruiting bodies also assesses the suitability of mushrooms for human consumption when harvested from contaminated substrates or soil.

2. Ectomycorrhizal Fungi

Ectomycorrhizal mushrooms include approximately 10,000 species, mainly belonging to Basidiomycetes, Ascomycetes, and Zygomycetes that form associations with host plants [31,32] and were found to have evolved 130 million years ago [31]. Caesalpiniaceae, Pinaceae, Fagaceae, and Dipterocarpaceae are the major families known to host EM mushrooms in tropical, subtropical, temperate, and boreal forests [33]. Among the different classes of EM fungi, Agaricomycetes are found to be the dominant class in forming mycorrhizal symbiosis [34].

Ectomycorrhizal mushrooms possess a dual lifestyle in soil, being symbionts and facultative saprotrophs [35]. Due to the ecological symbiosis maintained by EM mushrooms with the root systems of trees, the number of interactions maintained with the inhabited environment is higher compared to purely saprotrophic mushrooms [36].

Ectomycorrhizal fungi are capable of enclosing their hyphae, convolving the root tips, and creating a hyphal mantle. Within the mantle, hyphae grow between cortical and epidermal cells where the lumen of roots is not penetrated [37]. This formation results in a structure called the Hartig net (Figure 1). A Hartig net makes an interface for nutrient and water exchange between fungi and roots [35]. Meanwhile, the hyphae extend out of the fungal mantle to the soil and explore the faraway regions of the rhizosphere, and increase the nutrients available for the host [35]. Hence, EM mushrooms act as an integral part of plant nutrition. The plants intern, transferring carbohydrates to the fungus [35]. Apart from sharing nutrients, the host plant also receives benefits, including increased tolerance for salt and drought stresses [38], tolerance to heavy metals [38,39], and resistance to plant pathogens [40,41]. Furthermore, EM mushrooms protect their hosts [42,43], mitigating drought stress and producing vitamins and hormones for plant development [31,44].



Figure 1. (a) Illustration of root colonization structures in ectomycorrhiza (b) Hartig net formation of EM fungi. Outbreak of hyphae of mycelium in intracellular space with no penetration in lumen [37] Mechanisms underlying beneficial plant-fungus interactions in mycorrhizal symbiosis.

Ectomycorrhizal fungi possess crucial symbiotic relationships with plants that grow on heavy metal contaminated sites such as AM and influence plants to alleviate heavy metal toxicity [45,46]. The enhanced Cd tolerance of *Paxillus involutus* by *Populus canescens* [38] and Cu and Cd tolerance of *Eucalyptus tereticornis* by *Pisolithus albus* [47] were some of the evidences that EM helped for host heavy metal tolerance. Furthermore, the alleviated

Cd toxicity of *Pinus pinaster* seedlings was found with the EM fungi *Suillus bovinus* and *Suillus granulatus* [48]. *Inocybe curvipes* enhanced the Pb and Zn tolerance of the Masson pine trees [49]. According to Krznaric et al. [50], pine trees that have an association with *Suillus luteus* possess the ability of heavy metal tolerance.

In contrast to AM, the symbiotic compatibility and stress tolerance of EM are speciesspecific to some extent, and therefore, knowing EM fungal community dynamics can lead to understanding the processes of forest ecosystems and help to facilitate the tools of bioindicators in different environmental stress conditions, including heavy metal toxicity due to contamination of soil [51]. Furthermore, the changes in EM fungal dynamics can therefore be correlated with altered tree responses to stress conditions, including heavy metal toxicity [52]. According to Milenge et al. [53], environmental stress factors could affect photosynthesis and, hence, reduce the sugar availability to EM, which might lead to changes in EM dynamics. Similarly, due to stress factors, the changes in the EM community might alter plant nutrient uptake and photosynthesis and affect plant performance [52]. As a consequence, both EM fungi and the host plant together can act as bioindicators.

3. The Role of Ectomycorrhizas in Heavy Metal Stress Tolerance of Host Plants

3.1. Heavy Metal Deposition in EM Fungi

Heavy metals are absorbed by EM fungi from soil solutions. Soil contamination by heavy metals occurs mainly due to natural phenomena and anthropogenic activities. Heavy metal deposition of Basidiomycetes varies depending on temperature, humidity, nature of metal, soil pH, substrate, mushroom species, and also ecosystem processes [54–56]. Due to the sensitivity of EM fungi to heavy metal contaminants, EM mushrooms can be used as an active and passive biomonitoring tool for metal deposition [57,58]. The estimations of biological effects caused by heavy metals are measured by monitoring the population dynamics of EM mushrooms, community variations, and morphological changes [59,60]. Reduced hyphal extension, morphological changes in mycelia, biomass reduction, and increased hyphal branching are major indications of declines of some EM species due to metal stress [61,62].

The mobility of the heavy metal changes due to the nature of metallic compounds since the availability of cations depends mainly on the anions. Ni, Co, Pb, and Cr have less mobility compared to Cd and Hg [9,63,64]. The ectomycorrhizal fungi *Suillus granulatus, Lactarius deliciosus, Tuber melanosporium,* and *Tuber brumale* showed higher Cu biosorption with surplus supplies of potassium (K) and three [65].

The EM mushrooms are capable of withholding heavy metals absorbed by soil solutions and, hence, protecting the host plants. This mechanism of the EM mushrooms relies mainly on the capacity of the fungus to continue proliferation through the substrate, producing new biomass in the presence of high metal concentrations [65–68]. The deposition levels of heavy metals in EM fungi have been extensively studied in different regions and contamination levels [64,69]. *Lactarius deliciosus, Russula delica,* and *Russula albida* from Canakkale, Turkey were found to have deposited the higher concentrations of Cd, Cu, and Pb [24,64,70,71], while Zn was deposited in *Russula delica.* A higher level of Cr was recorded in *Lactarius deliciosus* [64,70–72]. *Russula albida* was found to absorb higher Ni, Cr, Mn, and Zn concentrations, and it was in the range of 1–5 mg kg⁻¹ [72].

Akin et al. [64] carried out two different experiments to compare the absorption of Cu and Zn by *Baorangia bicolor* in Çanakkale, Turkey. *Baorangia bicolor* was compared with *Retiboletus fuscus*, *Russula delica*, and *Russula crustosa* for Cu absorption and found that *R. fuscus* and *R. delica* absorbed more Cu compared to *B. bicolor*. A comparison of *B. bicolor* with *R. crustosa* and *Lactarius representaneus* for Zn absorption resulted in a higher absorption rate of *R. crustosa* and *L. representaneus*. Further, *Imleria badia* showed higher sensitivity to Pb by absorbing a concentration of 0.448 ± 0.03 mg kg⁻¹ of biomass [64,70,71,73].

Crane et al. [15] revealed the response of EM fungi towards Hg concentration and showed that *Amanita muscaria*, *Coccobotrys xylophilus*, *Laccaria laccata*, *Piloderma bicolour*, *Pisolithus arhizus*, and *Suillus decipiens* are influenced by Hg at the immature stage of their

growth, reducing the growth of the fruiting bodies. The changes in morphology depend on the concentrations of Hg and the exposure time. Among hyper-accumulating mycorrhizal mushrooms, such as *Gomphidius glutinosus*, *Craterellus tubaeformis*, and *Laccaria amethystina*, which are all associated with pines, *G. glutinosus* has been seen to absorb the most via the mycelium and concentrate radioactive cesium (Cs) more than 10,000-fold over ambient background levels [74].

Studies carried out in Slovakia, Turkey, and Northern Poland revealed that different EM fungi such as *Boletus edulis* and *Paxillus involutus* hold considerably higher concentrations of Hg, Pb, Cd, and Cu [15,75–77]. Tuzen et al. [78] and Dermirbas et al. [58] revealed that *Tricholoma terreum* is one of the EM fungi studied for retention of different metal ions. Furthermore, Yilmaz et al. [79] explained that *T. terreum* tolerates and holds a higher number of metal ions compared to other EM fungi, and his results explained that the highest contents of HM were represented by Fe (744), Zn (179), and Cu (51) mg kg⁻¹. Durken et al. [80] demonstrated that *Paxillus rubicundulus* can hold Pb (0.69), Cd (0.78), Hg (0.21), Fe (37.0), Cu (51.0), Mn (10.8), Zn (16.8) mg kg⁻¹ in a study conducted in Turkey.

It was reported from Northern Greece and Turkey that Boletus sp. and Hydnum repandum, Russula delica, Tricholoma terreum, Butyriboletus appendiculatus, Leccinum scabrum, Psilocybe coronilla, Tricholoma scalpturatum, and *Suillus granulatus* are a few edible EM fungi that withhold Pb, Cd, Hg, Cu, Mn, Zn, and Fe in higher quantities compared to other edible EM fungi [80–84].

3.2. Heavy Metal Deposition in Edible EM Mushrooms

The deposition of heavy metals in mushrooms is an important concern for edible mushrooms. Wild edible fungi are belonging to several trophic groups as saprotrophic or termite associated (growing in mutualistic relation with termites) or ectomycorrhizal [84,85]. Some EM genera, i.e., edible *Amanita., Lactarius, Lactifluus,* and *Russula,* are common in some forests. These edible EM fungi are harvested and consumed by people [85]. *Boletus edulis (Bull.), Tricholoma matsutake* (S. Ito and S. Imai), *Lyophyllum shimeji, T. bakamatsutake* Hongo, *T. portentosum* (Fr.), *Rhizopogon roseolus* (Corda) *Th. Fr., Suillus grevillei* (Klotzsch) *Singer, Boletus edulis* Bull., *Amanita caesareoides Lj. N. Vassiljeva, Entoloma sepium (Noulet and Dass.) Richon and Roze,* Cantharellus cibarius *Fr.,* and *Tuber indicum Cooke & Massee, Cantharellus cibarius, and Lactarius hatsudake* are some of the examples of edible mycorrhizal mushrooms [84]. Heavy metal pollution causes detrimental effects on humans, other organisms, and the environment. Hence, it is important to study and investigate the metal content and accumulation in local wild mushrooms since they are a significant nutritional source in many countries [70].

Heavy metal contents of six edible EM mushroom species viz. *Cyanoboletus pulverulentus, Cantharellus cibarius, Lactarius quietus, Russula xerampelina,* and *Suillus grevillea* were estimated by Arvay et al. [86]. The highest mean concentrations of some metal elements were recorded in *S. grevillei* as 107, 104, 81.6, and 434 mg/kg (dried mass basis) for Zn, Cu, Mn, and Fe, respectively. Furthermore, the highest content of Co was found in *L. quietus* at 0.90 mg/kg (dm). Mleczek et al. [81] stated that toxic metals (Al, Cr, Hg, Ni, As, and Pb) amounts are higher in wild mushrooms compared to cultivated mushrooms.

4. Assessment of Heavy Metals in ECM Mushrooms

Despite the habits of the mushroom species, mushrooms are known as potential bioindicators of environmental pollution, including HM contamination in soil. It is an obviously accepted factor that different mushroom species absorb and accumulate HM in varying concentrations depending on their anatomy, physiology, and habitat conditions. Nevertheless, the methods applied to assess the concentration of different HM in mushroom varieties are common. Nowakowski et al. [18] elaborated on different methods of assessing HM in mushrooms in a study carried out in North-East Poland. Dried powdered mushrooms are known to be the most frequently applied form to assess HM

accumulated. Furthermore, for extended and comprehensive illustrations of results, the HM content in both the cap and stipe of the mushrooms can be separately quantified.

According to Nowakowski et al. [18], the Hg content in the mushrooms can be assessed in a single-purpose atomic absorption spectrometer AMA-254, where the dried powdered samples are burnt at 600 °C in an oxygenated atmosphere. The amount of released Hg can be gathered as Hg vapor to measure at different steps. For experiments carried out on a wet basis, the results were obtained on calculations based on the water content in the sample.

Omeljaniuk et al. [87] demonstrated that for the assessment of Pb and Cd in mushrooms, the electrothermal atomic absorption spectrometry analytical technique (ETAAS) is applicable. Determination of Pb and Cd concentrations in mineralized samples can be performed at a particular wavelength depending on the conditions that apply. The element content for the wet basis was calculated based on the water content determination in the sample.

Jablonska et al. [88] and Nowakowski et al. [18] suggest that for the determination of As content, inductively coupled plasma mass spectrometry (ICP-MS) is frequently applied with a kinetic energy discrimination chamber (KED). In this setup configuration, polyatomic interferences can be corrected by collisions and kinetic energy discrimination. ICP-MS conditions are generally varied depending on the samples that are used [18].

All the above-described assessing methods can be applied to both dried and wet forms of mushrooms, where the calculations depend on the water content of the mushroom. Besides that, in the application of the above techniques, the calibration curves should be created from working standard solutions according to the standard solution of the particular HM considered for the test.

In the case of HM in edible mushrooms, the assessment of health risks for humans is another vital concern. On this behalf, different parameters, measures, and limitations are imposed and implemented by the World Health Organization. Those measures which are recurrently applied are relevant to HM, PTWI (provisional tolerable weekly intake) for Hg, PTMI (provisional tolerable monthly intake) for Cd, and BMDL (benchmark dose lower confidence limit) for As and Pb [89]. Thus, depending on the assessed amounts of HM in mushrooms and threshold levels for different measures, one can determine the pollution levels of HM in soils.

5. Mechanisms of Symbiotic Mycorrhizal Tolerance to Heavy Metal

5.1. Anatomical Mechanism of Mycorrhizal Symbiosis

With special structures such as hyphae mantle, Hartig net, external hyphae, and sclerotia, EM fungi and plants together generate a "mycorrhiza" [37]. Those structures have unique characteristics of enrichment and accumulation effects on heavy metals. This phenomenon was well-demonstrated by Leonhardt et al. [90] on the capability of accumulating Zn in five *Russula* species, and the accumulated amount of Zn reached around $326-845 \text{ mg} \cdot \text{kg}^{-1}$ in spore fruit. The study further demonstrated that there was a positive correlation between the number of organic acids secreted by EM fungi and the concentration of heavy metals held by mycelium. This result of Leonhardt's experiment explains that EM fungi bring down the toxicity of absorbed heavy metals in the fruit body by organic acids. In mycorrhizae, the external mycelium of EM fungi is the main point of heavy metals in the soil. It is well evident that under high Mn concentrations, the mycelium of EM fungi is severely damaged and destroyed due to larger amounts of Mn in organelles and the cytoplasm of mycelial cells.

5.2. The Molecular Biological Mechanism of Plants Regulated by EM Fungi

Molecular mechanisms are one of the major mechanisms that regulate interactions between symbiotic plants and heavy metals. Studies by Majorel et al. [91] explained the molecular mechanism of Ni efflux by using two *Pisolithus albus* strains as a Ni-tolerant strains and a Ni-sensitive strains. It was described that three genes of C3578 (p-type ATPase), C5339 (ATP binding cassette), and C17235 (major facilitator superfamily) were expressed only in Ni-tolerant strains, whilst no expression was found in the Ni-sensitive strains.

Besides, EM fungi help to reduce the toxicity of heavy metals by inducing the genetic expression of different metallothioneins under metal stress. This phenomenon uses two *Hebeloma mesophaeum* strains collected from contaminated and clean areas [92]. With investigations performed to assess transcription levels of HmMTs in a *Hebeloma* strain exposed to Cd showed that the level of HmMT3 transcription was three times higher in isolates taken from contaminated areas compared to isolates from the uncontaminated site. Those findings concluded that metallothionein provides a considerable protective effect on *Hebeloma* isolates from contaminated areas.

EM fungi are capable of altering the transcription of gene expression of symbiotic plants. This controls the levels of defense enzymes generated by plants against heavy metals [34]. With similar results, Benes et al. [93] explained that overexpression of a metal tolerance protein gene (CsMTP8) boosts the Mn tolerance in tea plants. Furthermore, they revealed that even at excessive levels of Mn, the accumulation of Mn can be reduced with the overexpression of genes.

The molecular regulation of gene expression on the production of proteins that control heavy metal absorption, transport, and chelation plays a vital role in strengthening heavy metal tolerance and toxicity resistance. However, further studies are necessary on the gene regulation on HM tolerance to make the maximum use of EM fungi for mycoremediation.

5.3. Extracellular and Cellular Mechanisms to Sustain Metal Tolerance in Ectomycorrhizal Fungi5.3.1. Metal Homeostasis in Ectomycorrhizal Fungi

EM mushrooms prevent metal toxicity in plant species [46,94]. For maintenance of a successful mycorrhizal association that protects host vegetation in metalliferous soil, EM mushrooms hold significant traits such as survival in toxic soil or substrate, effective and efficient transfer of nutrients to the hosts, and poor transfer of metal toxins to host vegetation [95]. EM mushrooms possess a higher capacity for tolerating the stress of heavy metals. They exercise the following two types of interacting mechanisms with heavy metal homeostasis: 1. Mechanism of Avoidance and 2. Mechanism of Tolerance.

5.3.2. Mechanism of Avoidance

In the avoidance mechanism, the organism inhibits the metal entry. This is performed by extracellular precipitation by excreting di and tricarboxylic acids, and oxalic acids through biosorption to cell walls with chitin and glucosamine, either reducing uptake or increasing efflux. However, even with the action of avoidance, 20–30% of metal is found in the vacuoles and cytosol of cells.

During studies conducted in Europe, it was demonstrated that *Pinus* species are major hosts for *Suillus* species that are common in mining sites and near smelters [68,95–97]. Wilkinson and Dickinson [98] demonstrated that EM symbiosis exists on highly metal-contaminated sites that are colonized by mycotrophic *Pinus* trees and that resistance of pine vegetation to metal toxicity is achieved through association with well-adapted EM fungi. EM mushrooms are capable of binding large amounts of metals, though it is unlikely that this affinity is an effective way of avoiding metal insertion into cells. The hyphae present in most EM mushrooms are covered with a layer of water-repellent proteins and hydrophobins that control metal uptake from soil [99]. This process somehow controls the entrance of metal pollutants to avoid the excess absorption of heavy metals into EM mushrooms, but it does not totally avoid the entrance of metal contaminants.

Binding heavy metals to the fungal cell wall is another mechanism that has been extensively discussed under the avoidance mechanism. The cell wall of the fungi is the prime site of interaction with metal substances and the first barrier against excess metals. EM fungi bear a larger number of potential binding sites on cell walls due to the presence of free amino, phosphate, hydroxyl, carboxyl, and mercapto groups, which help to hold a substantial fraction of metals. The existence of melanin among cell walls enhances both

metal biosorption capacity and strength, being an effective strategy for avoiding metal entering cells [100]. However, with the saturation of hyphal walls with metals at higher metal concentrations, the protective potential of cell walls narrows down. This occurs as a result of equilibrium between the cytosol of a fungal cell and soil solution, which finally causes the entrance of some quantities of metals into the cytosol. This phenomenon was described by Blaudez et al. [101], Ahoenen-Jonnarth et al. [102] and Bellion et al. [99] on the increased oxalate exudation of EM fungi in *Paxillus involutus* during exposure to Cd. They described that uptake of Cd is reduced by more than 85% in the presence of oxalate and suggested that increased exudation levels of oxalate were an effective way to avoid Cd entry into living cells of EM fungi. Besides the above evidence, Colpaert et al. [94] demonstrated a similar mechanism of tolerance with relevance to Zn in *Suillus bovines*. Nevertheless, with the currently available information for the date, there is no doubt that extracellular complexation mechanisms are greatly beneficial for EM fungi.

5.3.3. Mechanism of Tolerance

Even with the presence of cell-wall binding capacities and extracellular chelation of EM fungi, larger quantities of metals enter the cells. These metal contents then undergo intracellular detoxification mechanisms to minimize the metal burden in the cytosol through the tolerance mechanism. Generally, this process is performed in the following two different ways: bonding with non-protein thiols and transport to intracellular compartments.

During the process of bonding with non-protein thiols, the metals entering cells are intracellular chelated. Cells synthesize chelators enriched with thiols such as mettalothiones and glutathiones within vacuoles. These proteins bind heavy metals in the cytosol, forming metal peptide complexes that are actively transported to vacuoles.

Metal tolerance based on the availability of metallothionein such as peptides in ectomycorrhizal fungi was elaborated by Morselt et al. [103] using *Pisolithus tinctorius*. The role of metallothioneins is very significant in the maintenance of metal tolerance. Research shows isolated metallothionein genes from primitive organisms and also those that are well developed. The potential of the metal-binding capability of metallothioneins depends on the metal type and host species. Metallothioneins exist in different isoforms where they express themselves variably with different responses depending on the species. The presence of genes for three metallothionein isoforms in *Amanita strobiliformis* is a good example of this phenomenon [104]. The genes for three isoforms are up-regulated in the presence of Cu and Ag (Silver), Cd and Zn.

Reddy et al. [104] described two genes, LbMT1 and LbMT2, as metallothionein genes present in *Laccaria bicolor* and elaborated on their differential response to Cu, Cd, and Zn. The responses of both genes, LbMT1 and LbMT2, increased proportionately with Cu concentration, whilst only LbMT2 responded to increasing Cd levels. In the same experiment, Reddy et al. [104] explained clearly that LbMT1 and LbMT2 genes enhance Cd tolerance in EM fungi such as *L. bicolor*. In the case of *Hebeloma mesophaeum*, isoforms such as HmMT1, HmMT2, and HmMT3 have been characterized, where HmMT1 is induced by Cd and Zn, whilst HmMT2 is induced by silver (Ag).

A similar study of *Hebeloma cylindrosporum* found two metallothionein genes, HcMT1 and HcMT2. Among the two genes, HcMT1 becomes expressive only for Cu, while HcMT2 becomes expressive in the presence of Cd and Cu. An RT-PCR analysis was conducted to find the expression level of both genes with relevance to varying concentrations of Cd and Cu [49]. In the same study, it was found that different isoforms of MT genes respond differently toward different metals in EM mushrooms. Some studies have revealed that *Pisolithus albus* and *Paxillus involutus* where a single metallothionein gene has been made out that is induced by Cd and Cu [49].

Glutathione is a polypeptide that is produced as a response to heavy metal stress. Ramesh et al. [105] and Courbot et al. [106] explained that increased levels of glutathione in EM fungi such as *Paxillus involutus* and *Laccaria laccata* occur in response to Cd [107]. Production of glutathione in EM fungi is induced by Cd, As, Cr, Pb, and Cu where glutathione has been seen as a Cd chelator [108]. Putative genes have been identified for encoding glutathione synthetase in *Hebeloma cylindrosporum*, *Laccaria bicolor*, and *Paxillus involutus* [99]. Besides the abovementioned, Howe et al. [109] demonstrated the Cu-binding metallothioneins in different EM fungi, such as *Laccaria laccata* and *Paxillus involutus*.

The environment with high levels of heavy metals alters some species of EM mushrooms. In some cases, higher metal concentrations reduce the diversity, composition, and richness due to selection pressure on mushroom populations. Scientists have suggested adaptive metal tolerance against Al, Ni, Zn, Cd, or Cu for ECM mushroom species such as *Pisolithus arhizus, Pisolithus albus, Suillus* spp., and *Leucoagaricus* spp. that have adapted. This shows a similar situation to plant species, though the evolution of metal-tolerant EM species is small in number.

Among EM mushroom species, *Suillus luteus* shows a distinct behavior with genotypes adapted to different soil types and soil conditions. Ecotypes of *S. luteus* are available with specific tolerance against higher concentrations of Zn, Cu, and Cd [110], while *S. bovinus* shows adaptive tolerance against high Zn concentrations [111]. The tolerance level of elevated metal concentrations is described as a specific phenomenon present in every live cell for metal homeostasis. Though metals are essential contributors to micronutrients, they become toxic when the free concentration of ions exceeds particular thresholds in the cytoplasm.

In the presence of a higher metal burden in the cytosol, metal transport proteins assist with metal tolerance through extruding toxic metals from the cytosol or metal sequestration into sub-cellular compartments [99,112]. The compartmentation of metals in other sub-cellular structures was described by Blaudez et al. [101] for the content of Cd in both the cytosol and vacuole of *Paxillus involutes* and concluded that 20% and 30% of Cd were present, respectively, which well displayed the potential for effective detoxification of Cd in stressed cells. Nevertheless, the molecular mechanisms that pave the base for metal transfer in subcellular compartments are still understudied in EM fungi.

6. ECM Mushrooms vs. Saprobic Mushrooms as Bio-Indicators

Previous studies carried out on metal accumulation in different mushrooms have suggested that metal accumulation depends on species and that it differs depending on the ecotype of fungi species involved. It has also been suggested that different metal concentrations are found in higher concentrations in fungi growing in soil's organic layer compared to fungi growing on wood. The relationship between the metal concentration of mycorrhizal mushrooms and the metal concentration in the soil still needs to be uncovered, which will then help to illuminate the role of mycorrhizae in the sequestration and movement of metals in ecosystems, especially in forests and polluted areas.

Forming the relationship between metal concentration in EM fungi and the metal concentration in the surrounding soil is an essential prerequisite to understanding the roles that EM fungi perform in metal sequestration and movement in forest ecosystems. Nevertheless, data from preliminary studies explain that EM fungi can prove very useful as bioindicators of heavy metal pollution [113–115].

The heavy metal stress alleviation by EM fungi was evident in the root cells of the mycorrhizal plants and they had better performance with EM fungi than without, where plant fitness was also shown to be improved by EM fungi [46,94]. In order to protect host plants, an increased capability to survive in the toxic substrate while maintaining efficient and optimum nutrient transfer to the hosts are paramount characteristics of EM fungi compared to saprobic fungi [116]. This phenomenon has been demonstrated by Jentschke and Godbold [46], explaining the improved nutrient uptake of EM fungi from soils with poor nutrient quantities, which enhances the better and healthier growth of host plants. This has been shown by comparing mycorrhizal plant species with non-mycorrhizal plant species [46]. However, the metal absorption of EM fungi over saprobes is yet to be studied in detail to figure out whether EM fungi perform over saprobic fungi. Additionally, the effects of metal contamination on fruiting patterns of EM fungi and saprobic fungi, though,

have not been adequately described, though EM fungi have been described as natural bio-indicators. Previous studies have revealed that EM fungal communities belowground are highly diverse in both structure and composition, which undergo the influence of different land-use practices [117,118]. Nevertheless, despite the ecotype of fungi, saprobic, or EM, some fungal species become better bioindicators compared to others, meeting the major characteristics of a successful bio-indicator. For successful bio-indicators, factors such as relevance, sensitivity, and measurability of the indicator are significant. These indicators are varied and can include different species. For instance, EM mushrooms have been found to be highly sensitive to ecological parameters such as soil quality, wind, and air pollutants since EM mushrooms absorb water and nutrients from surrounding environments [116,119]. EM mushrooms' species composition, diversity, and morphological variation are powerful tools to acquire information about changes in soil quality and other biological processes [38,120]. Habitat degradation, overexploitation, and habitat fragmentation that affect the diversity and integrity of ecosystems are generally applied to EM fungi. EM fungi are also measurable. Due to the unique features of EM mushrooms, they are used as relevant indicators for biodiversity and productivity of ecosystems. The use of EM species for biological monitoring as a bio-indicator is considered an effective tool for early warnings to detect and monitor heavy metal contaminants in the environment.

The basic characteristic of a natural bio-indicator is the ability to respond towards changes in its environment. These responses should be measurable, and they are explained as the sensitivity to stress, but they should not undergo mortality. Heavy metals and metallic compounds are one of the major factors that those EM mushrooms respond to. This can be explained as the capability of EM mushrooms to perform as bio-indicators with their capability of metal detection in soil. Generally, alterations and changes occur in habitats, enabling the formation of two different types of reactions in EM mushrooms. Those are recognized as reactions involving insensibility and those involving intolerance. Hence, the process of bio-indication arises based on the above two factors.

The density of local populations of EM fungi is another concern when using EM fungi as an indicator. The distribution of EM fungi within an area has to be relatively stable despite ordinary climatic, meteorological, and environmental divergence. Indicator species should be abundant and common enough for proper monitoring, determinations, and assessment of pollutant levels for accurate forecasting and decision-making depending on observations.

To become a successful indicator of biological systems, the ecology and life history of the particular organism should be understood and clarified well, as it is easy to make out the changes of organisms when the ecosystem or environment undergoes stress conditions or pollution. At the organism level, scientists examine how individuals interact with biotic and abiotic factors. These factors constitute the ecology of an organism where they affect the behavioral and physical changes of organisms, which we consider bio-indicators.

When focused on ecology and taxonomy of EM mushroom species that are considered as indicator species of metal pollution, *Lactarius deliciosus* [121,122], *Cyanoboletus pulverulentus* [123,124], *Cantharellus cibarius* [125], *Lactarius quietus* [126,127], *Macrolepiota procera* [51], *Amanita muscaria* [128,129], *Pisolithus arhizus* [130,131], *Termitomyces spp.* [132,133], *Gomphidius glutinosus* [134], *Craterellus tubaeformis* [135,136], *Laccaria amethystina* [137], *Imleria badia* [81,138], *Leccinellum griseum* [139], *Russula delica* [140,141], *Baorangia bicolour* [142] are well-known and well-studied species for indication of environmental heavy metals.

EM mushrooms are recognized as commercially important for many reasons. Among the studied mushroom species worldwide, there are approximately 2500 species of edible mushrooms, of which the majority of the most expensive ones belong to the mycorrhizal category. *Tuber melanosporum* Vitt. (Périgord black truffle), *Tuber magnatum* Pico and Vitt. (Italian white truffle), *Tricholoma matsutake* (Ito and Imai) Sing. (matsutake), *Boletus edulis* Bull: Fr. sensu lato (porcini) *Cantharellus cibarius* Fr.: Fr. (chanterelle), and *Amanita caesarea* (Scop.: Fr.) Pers: Schw. (Caesar's mushroom) are well-known for being marketed. Wegiel et al. [143] found that *Imleria badia* Pers. is used to isolate antimitotic polysaccharides.

Hemileccinum impolitum Fr., *Entoloma sinuatum* (Bull.) P. Kumm., *Rhizopogon ochraceorubens* Krombh., *Tricholoma terreum* (Schaeff.) P. Kumm, are considered the best species for the production of alkaloids and Necatorin.

7. Benefits of Using ECM as a Natural Bio-Indicator of Metal Pollution

Studies conducted on assessing the role of EM mushrooms in different environments reveal the benefits of using EM species as a bio-indicator. EM mushrooms have the capacity to perform in different forms of indicators (Figure 2) depending on different applications. The determination of biological impacts on different organisms living in ecosystems, assessing antagonistic and synergetic effects of metal pollutants on organisms, early diagnosis of harmful influences of metal toxins on plants, humans, and other organisms, the capability of having easy counts and calculations, and being an economically feasible option compared to other monitoring systems, as EM mushrooms are naturally available, are major benefits that lead to the application of EM mushrooms as a successful natural bio-indicator of metal pollution.



Figure 2. Different bio-indicator functions, which are performed by EM mushrooms.

EM fungi are capable of detecting pollutants in soils. This could be performed by quantifying the amounts of different pollutants (e.g., heavy metals, organic compounds, inorganic ions, and salts) absorbed into EM fungi to assess whether they exceed the normal threshold concentration of soil. For example, the maximum threshold levels for Pb, Zn, Cu, and Fe are 1–7, 5–10, 0.6–6, and 15 mg/100 g of dry soil [144]. At exceeded levels of threshold concentrations, EM communities could reduce their diversity and species richness. Other than indicating the presence of different pollutants, environmental changes are also indicated by EM fungi. Soil pH, soil moisture, temperature, and humidity are the most influential growth factors for EM fungi. The morphology and distributional patterns of EM fungi change depending on soil chemical attributes, temperature, and humidity. When optimum environmental conditions are not reached, the distributional patterns and morphology of EM mushrooms change. For example, during seasons of higher temperatures, most of the EM species decline, but some *Pisolithus* spp. can survive [130].

As EM fungi associate with host tree species, when the diversity of that vegetation is changed, the EM species that fruit with affinity to host trees also change. Especially for EM species, which are host-specific. *Suillus, Laccaria, Inocybe, Russula,* and *Scleroderma* species are examples of fungi that grow with unique hosts where they affiliate with *Pinus muricata, Pinus radiate, Shorea robustus, Dipterocarpus turbinatus,* and *Hopea odorata* [143]. This phenomenon can be particularly seen with EM species linked to unique hosts, where EM species richness and abundance are affected separately by host species. Atmospheric changes are another influential factor in the diversity of EM fungi. Toxic gases and dust particles disturb the distribution of ectomycorrhizal species. Increased N concentrations cause the reduction and sometimes complete removal of EM species from ecosystems. The purity of both air and the environment can be assessed by EM species.

8. Conclusions

Based on the previous literature, we conclude that EM mushrooms use metal homeostasis mechanisms to sustain themselves in soils polluted with heavy metals. Since EM mushrooms can thrive in highly polluted soils with heavy metals, they have a higher potential to indicate changes in the surrounding environment when the acceptable threshold levels of pollutants are exceeded. EM mushrooms fulfill the bio-indicator criteria (being indicative, abundant, economically important, and well-studied). Hence, EM mushrooms are successful and an appropriate natural bio-indicator with developed mechanisms of metal homeostasis, metal tolerance, and adaptations to survive in heavy metal-polluted soils.

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