

**Cloning and Characterization of Metallothionein  
Genes of Ectomycorrhizal Fungi  
*Suillus himalayensis* and *Suillus indicus***

*A Dissertation*

*Submitted in partial fulfillment of the requirements*

*For the award of degree of*

**MASTER OF SCIENCE**

**IN**

**BIOTECHNOLOGY**

Submitted by

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Under the esteemed guidance of

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## DECLARATION

I, the undersigned, hereby declare that the research work presented in the Msc. project entitled "Cloning and characterization of metallothionein genes in ectomycorrhizal fungi *Suillus himalayensis* and *Suillus indicus*." is an authentic record of the work carried out by me under the supervision and guidance of Dr. M.S Reddy, Professor, Department of Biotechnology, Thapar University, Patiala.

Further, I declare that no part of this dissertation has been submitted for a degree or any other qualification of any other university or examining body in India/elsewhere.

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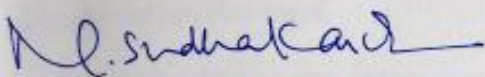
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### CERTIFICATE

This is to certify that dissertation entitled, "Cloning and characterization of metallothionein genes in ectomycorrhizal fungi *Suillus himalayensis* and *Suillus indicus*" submitted by **Ms. Radhika** in partial fulfilment of the requirements for the award of Masters in Science Degree in Biotechnology at Thapar University, Patiala is an authentic work carried out by her under our supervision and guidance.

To the best of our knowledge, the matter embodied in this dissertation has not been submitted to any other university/ institute for award of any Degree or Diploma.



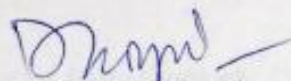
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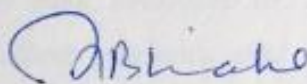
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## **ABSTRACT**

Adverse effects of heavy metals have been known for a long time and exposure to heavy metals continues and is even rapidly increasing. Use of mycorrhizal fungi is one of the best known methods to cope up with heavy metal contamination. Mycorrhizal fungi plays a vital role to ensure plant survival and establishment of vegetation in heavily polluted places as it is in direct contact with plants and soils. In this study we have focused on molecular mechanisms in metal detoxification processes. In this study, metallothioneins were identified from, ectomycorrhizal fungi, *Suillus himalayensis* and *Suillus indicus* to obtain a deeper insight into the detoxification mechanisms of toxic heavy metals by the organism. These two strains were checked for their heavy metal tolerance on copper by growing them on liquid medium supplemented with different concentrations of copper. In result, we found that there was significant decrease in growth by increasing concentration of Cu. Primers were designed by retrieving the EST library from the genome of *Suillus luteus*. Five different genes encoding MTs were identified in the genome of *Suillus luteus*. For MT gene induction, mycelium was exposed to heavy metal stress. RNA was isolated from the mycelium and cDNA was synthesized. cDNA was amplified with gene specific primers to verify cDNA. Four metallothionein genes were recognized and further cloned and characterized. Metallothioneins genes were screened for metal resistance in Eukaryotes by transformation of metal-sensitive *Saccharomyces cerevisiae* mutants. To validate the functionality of the genes, Yeast functional complementation assays were performed. Genes were expressed in one *Saccharomyces cerevisiae* mutant strain, and the transformants were monitored on synthetic media, SD-Ura, with and without metal supplements. The present study identifies metallothionein from *Suillus indicus* and *Suillus himalayensis* as a potential tool against copper.

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## **ABBREVIATIONS**

SNW02	<i>Suillus indicus</i>
SNW03	<i>Suillus himalayensis</i>
RNA	Ribonucleic acid
DNA	Deoxy ribonucleic acid
cDNA	Complementary Deoxy ribonucleic acid
ECM	Ectomycorrhizal
VAM	Vesicular arbuscular mycorrhizas
MT	Metallothionein
PC	Phytochelatin
NAS	Nicotinamine synthase
ROS	Reactive oxygen species
TBE	Tris Boric acid EDTA
dNTP	Deoxy nucleotide tri phosphates
LB	Luria Broth
LA	Luria agar
YPD	Yeast peptone dextrose
ME	Malt extract

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# 1. INTRODUCTION

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Heavy metal contamination is becoming increasingly common all over the world. Prime causes of heavy metal contamination are natural sources (volcanic eruptions, wind dust) and anthropogenic activities. In this study we are more concerned about soil polluted with heavy metals, which has become significant environment problem due to rapid increase in anthropogenic activities such as mining. These polluted soils cause hinderance in the normal growth of plants due to phytotoxicity and altered metabolism. Plants have their own mechanisms to resist negative effects of heavy metals, by combining proteins with heavy metals and using enzymes to detoxify heavy metal pollution. There are various methods to remediate metal polluted soils also, from physical, chemical to biological approach. Bioremediation is an effectual method of treating heavy metal polluted soils. Phytoextraction is another common method of phytoremediation as it ensures the complete removal of the pollutant (Chibuike and Obiora 2014). Microorganisms and plants usually employ different mechanisms for the bioremediation of contaminated soils. Combining both plants and microorganisms is an approach for bioremediation as it ensures a more effective clean-up of heavy metal polluted soils. However, success of this approach majorly depends on the species of organisms involved in the process.

## 1.1 Mycorrhiza

A mycorrhiza is a symbiotic (generally mutualistic, but occasionally weakly pathogenic) relationship between a fungus and the roots of a vascular plant. In this association the fungus colonizes the host plant's roots either intracellularly or extracellularly. According to the early morphological classification (based on the relative location of fungi in roots) mycorrhizae were divided into three types; ectomycorrhizal, endomycorrhizal and ectendomycorrhizal associations (Peyronel et al. 1969). But later it was found that VAM, ericoid and orchid mycorrhizae are unallied types of „endomycorrhizal“ associations with contrasting anatomical features (Brundrett, 2002). Thus, the term „endomycorrhiza“ is not

valid because it includes several phylogenetically and functionally disparate association types. The mycorrhizae have been divided into different types. Ectomycorrhiza is the common form of symbiosis. Other forms are arbuscular, arbutoid ericoid, monotropoid, and orchid mycorrhizae.

## **1.2 Ectomycorrhizas**

The characteristic property of ectomycorrhiza is that the hyphae of ectomycorrhizal fungi do not penetrate individual cells within the roots. Fossil records and molecular clock dating have suggested that ECMs evolved about 200 million years ago (Cairney 2000). Ectomycorrhizae consist of a hyphal sheath covering the root tip and a Hartig net of hyphae helps in surrounding the plant cells within the root cortex. Ectomycorrhizae influence the host plant in numerous ways. It was demonstrated that ectomycorrhizal fungi can improve plant nutrition by enhancing nitrogen, phosphate and potassium uptake (Harley and Smith 1983). Nutrients are taken up by ectomycorrhizal mycelium by active absorption and specific cell membrane transporters following degradation using exoenzymes (Smith and Read 1997). Nutrients exchange between fungus and plant occur using cell-to-cell contact in ectomycorrhizas, especially in the Hartig net. ECM as a transmitting organs between soil and roots, transport carbohydrates for growth of mycelium and fruitbodies from roots and have to satisfy tree's demand for water and nutrients. (Agerer 2006). Increased absorbing surface via fungi mycelia improves water relations of plants also in dry conditions (Morte et al. 2000; Allen et al. 2003). There are many signaling pathways and several novel nutrient transporters are identified between plants and fungi (Bonfante and Genre 2010).

## **1.3 *Suillus himalayensis* and *Suillus indicus* (Taxonomy, Habitat and distribution)**

*Suillus* is a genus of basidiomycete fungi in the family Suillaceae and order Boletales . Species in the genus are associated with trees in the pine family (Pinaceae), and are mostly distributed in temperate locations in the Northern Hemisphere, although some species have been introduced to the Southern hemisphere.

The British botanist Samuel Fredrick Gray first named the genus *Suillus* in the first volume of his 1821 work *A Natural Arrangement of British Plants*. They are commonly known as "slippery jacks" because the cap of the fruit body is sometimes slimy. *Suillus* is derived from the Latin word *sus*, meaning "pig". Species of *Suillus* are found all over the Northern Hemisphere where members of the tree family Pinaceae can be found. Although a few species are also found in tropical regions (usually mountainous areas) most are limited to temperate areas.

*Suillus* isolates exhibit metal tolerance to many toxic metals, and this property of *Suillus* isolates have been shown to protect mycorrhizal pine seedlings from metal stress (Adriaensen et al. 2005)

**Table1:** Different *Suillus* isolates (SNW01–SNW08) obtained from the basidiocarps collected from coniferous forests of the northwestern Himalayas, India

S.no.	Species	Collection/pun no.	Isolate	MTCC accession no.
01	<i>Suillus triacularis</i>	SHP27/PUN5538	SNW01	111954
02	<i>Suillus indicus</i>	SHP07/PUN6578	SNW02	11955
03	<i>Suillus himalayensis</i>	SHP26/PUN5537	SNW03	11956
04	<i>Suillus granulates</i>	SJK13/PUN5525	SNW04	11957
05	<i>Suillus sibricus</i>	SJK01/PUN5520	SNW05	11958
06	<i>Suillus sibricus</i>	SHP05/PUN6577	SNW06	11959
07	<i>Suillus sibricus</i>	SHP12/PUN6579	SNW07	11960
08	<i>Suillus sibricus</i>	SUK12/PUN5532	SUK12/PUN5532	11961

Fresh basidiocarps of *Suillus* species were collected from conifer forests of the northwestern Himalayan region of India during monsoon seasons (Verma and Reddy 2014). Eight pure cultures were obtained from the basidiocarps of a range of *Suillus* species and have been designated from “SNW01–SNW08” (SNW stands for „*Suillus* species from north western Himalayas”) (Verma and Reddy 2014).

Plants possess sophisticated defense strategies to tolerate heavy metal toxicity. Physical barriers such as thick cuticles, trichomes and cell wall are first line of defense against metal (Abolghaseem 2015). Once heavy metals overcome physical barriers, plants initiate cellular defense mechanisms to nullify adverse effects of heavy metals. Biosynthesis of cellular biomolecules is the primary way to tolerate heavy metal toxicity. This includes low-molecular weight proteins such as glutathione, phytochelatins and metallothioneins or various cellular exudates like flavonoids and phenolic compounds, specific amino acid (proline, histidine) and hormones such as salicylic acid and ethylene (Viehweger 2014; Dalvi and Bhalerao 2013; Sharma and Dietz 2006).

ECM fungi play a crucial role in metal detoxification. So, it is important to know how ECM fungi respond to high heavy metal concentrations in the soil solution (Meharg 2003). To use mycorrhizal fungi for bioremediation and soil protection purposes, we need to develop the understanding of the molecular mechanisms that underlie the metal detoxification processes in these fungi. Potential amelioration of metal toxicity of plants by ECM fungi has been proposed by a number of authors (discussed in literature survey). The mechanisms by which they are able to detoxify these metals are numerous and diverse in their action (Gadd 1993). Metal detoxification is a result of different mechanisms like restriction to metal uptake, increased efflux, extracellular and intracellular complexation. Extracellularly, chelation is done by- (organic acids, heavy metal binding in extrametrical mycelium, heavy metal binding of cell wall components and transport mechanisms).

Chelation of metals in the cytosol occurs due to presence of the two classes of peptides, phytochelatins and metallothioneins (Rauser 1999; Clemens 2001).

## 1.4 Metallothioneins

Metallothionein is a low molecular weight, cys-rich protein. It was first isolated from the horse kidney (Margoshes and Vallee 1957). MT proteins comprise of 61 amino acids and an unusual feature to bind both essential and non-essential heavy metals. They bind to heavy metals by the thiol group of its cys residues (Kagi et al. 1974; Hamer 1986). MTs are distinguished by their low molecular weight, lack of aromatic amino acids and presence of 7–12 metal ions per molecule (Hamer 1986; Bremner and Beattie 1990). MTs are found in many living organisms, such as mammals (Sakulsak 2012), fungi (Averbeck et al. 2001; Ramesh and Reddy 2008) and cyanobacteria (Xu et al. 2009). MTs were first classified by (Rauser 1990) into three classes. Class I MTs are first identified in mammals and all show sequence similarity with the equine renal MT. Class I MTs are also found in fungi, like *Neurospora crassa* (Lerch 1980). Class II metallothioneins have been found in yeast (*Saccharomyces cerevisiae*) (Steffens 1990). Class III metallothioneins were first identified in *Schizosaccharomyces pombe* (Murasugi et al. 1984). This class of metallothioneins is found to be in fungi (Kneer et al. 1992). Later the second classification was done by Binz and Kagi in 1999 on the basis of taxonomic parameters and it results in the classification of 15 MT families.

## 1.5 Phytochelatins

Phytochelatins (PCs) are another group of metal binding molecules synthesized by fungi for metal detoxification. Phytochelatins comprise of just three amino acids (Cysteine, Glycine and Glutamic acid), and these amino acids are arranged generally in a specific  $(\text{--GluCys})_n\text{Gly}$  conformation. This conformation allows the identification of the origin of PCs. One potential strategy for the effectual removal of heavy metals is the use of organisms that provide heavy metal resistance and accumulation capacity (Dhankher et al. 2002). Carboxylamide bond in PCs suggests that they are a product of biosynthetic pathway but not a direct result of expression of a metal resistant gene (Murphy et al. 1997). These PCs are found in algae and plants (Grill et al. 1988).

In the present study, metallothionin genes were identified from the ECM fungi, *Suillus himalayensis* and *Suillus indicus*, which have not been reported till date. Metallothionine genes were cloned and characterized.

## OBJECTIVES

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- Identification of metallothionein genes in *Suillus himalayensis* and *Suillus indicus*
- Cloning and Characterization of metallothionein genes
- Yeast functional complementation assays

## 2. REVIEW OF LITERATURE

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### 2.1 Heavy metals – occurrence and their toxicity for plants

The heavy metal refers to a metal that has a relatively high density and is toxic even at low concentration. Heavy metals include silver (Ag), lead (Pb), cadmium (Cd), nickel (Ni), cobalt (Co), iron (Fe), zinc (Zn), chromium (Cr), iron (Fe), arsenic (As), and the platinum group elements. Heavy metals have atomic density greater than  $4 \text{ g/cm}^3$ , or 5 times or more, greater than water (Duruibe et al. 2007). Heavy metal accumulation in soils is of great concern in agriculture due to the adverse effects on food and marketability, growth of crop due to phytotoxicity and environmental health of soil organisms (Gill 2014). Toxic metals can cause harmful effects in many ways, but mainly as result of their strong coordinatingabilities (Ochiali 1987). Heavy metal toxicity in plants depends upon the bioavailability of these metals in soil solution, which is a role of pH, organic matter and cation exchange capacity of the soil (Shah et al. 2011). Rise in conc. of heavy metals in plants results various adverse effects like inhibition of growth, decline in physiological and biochemical activities, structure damage (Cheng 2003). Toxic effects include the blocking of functional groups of biologically important molecules (e.g. enzymes and transport system for essential nutrients and ions) the displacement or substitution of essential metal ions from biomolecules and functional conformational modifications, denaturation and inactivation of enzymes and disruption of cellular and organellar membrane integrity (Ochiali 1987). In addition, heavy metal excess may stimulate the formation of freeradicals and reactive oxygen species, resulting in oxidative stress (Dietz et al. 1999). According to (Berthelin et al. 1995), metal bioavailability is a function not only of their total concentration but also of physico-chemical (e.g. pH, Eh, organic matter, clay content) and biological (e.g. biosorption, bioaccumulation and solubilization) factors.

There are 90 naturally occurring elements out of which 50 are heavy metals but only few are of biological importance (Weast 1984). Based on solubility under physiological conditions 17 heavy metals may be available for living cells and of importance for organism and ecosystem. Among these Fe, Mo, Mn, Co, Cu, Ni and Zn are essential as micronutrients

(Reeves and Baker 2000). But many other heavy metals such as Ag, Au, Cd, Pb and Hg have no biological role, studied by (Bruins et al. 2000). These metals are considered as non-essential and toxic to microorganisms even at minute concentration.

Plants make use of different mechanisms to exploit different essential heavy metals for their activity and to lessen exposure to non-essential heavy metals.

According to (Manara 2012) mechanisms to detoxify heavy metals are:

- Reduction of uptake of metal ions into root cells by restricting metal ions to the apoplast.
- Binding them to the cell wall or to cellular exudates.
- Metal transport, chelation, trafficking, and sequestration into the vacuole.
- Activation of oxidative stress defense mechanisms and the synthesis of stress related proteins and signaling molecules like heat-shock proteins, hormones and reactive oxygen species (ROS).

## **2.2 Sources of heavy metal contamination**

Prime sources of heavy metals in the environment are Natural sources and Anthropogenic sources.

### Natural Sources of heavy metals

- Geologic parent material or rock outcroppings as composition and concentration of heavy metals depends on the rock type and environmental conditions
- Volcanoes have been reported to emit high level of Al, Zn, Mn, Pb, Ni, Cu and Hg .
- Wind dusts and volcanic eruptions.

### Agricultural sources of heavy metals

- Inorganic and organic fertilizers.
- Cadmium enhancement also occurs due to the application of sewage sludge, manure and limes ( Yanqun et al. 2004).

## Industrial Sources of Heavy Metals

- Mining
- Refinement (soil heaps and tailing, transport of ores, smelting).
- For example, coalmines are sources of As, Cd, etc., which enrich the soil around the coalfield directly or indirectly. The utilization of Hg in gold mining and the mobilization of significantly high amounts of Hg from old mines have become a significant source of this pollutant to the environment.

## Domestic effluents

- Untreated waste water.
- Waste substances which have passed over sewage outfalls and discharged to receiving water bodies often end up into the sea from coastal residential areas.

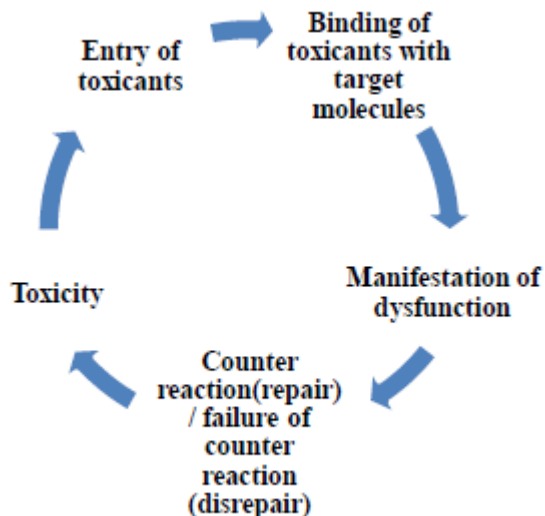
## **2.3 Mode of Action and Toxicity of Heavy metals**

Trace elements enter into plant system due to their chemical properties such as reduction and oxidation reactions in physiological conditions. These heavy metal ions which are obligatory for life are the considerable cause of their toxicity when present in surplus amount. The strength of toxicity depends upon Absorption, Concentration and perseverance of the toxicant at its location (Shanker AK 2008).

The final toxicants behind these toxic effects are:

- Metal species that binds with the endogenous target molecule for example receptors, enzymes, protein, DNA or lipid that critically alters the biological environment and results in toxic damage.
- Metabolite of the parent compound or ROS or RNS generated during in vivo transformations of the toxicant.
- Endogenous molecule or compound synthesized in response to primary toxicant exposure.

These toxicants work through various biological processes such as absorption, distribution of toxicants to the site of action, reabsorption and metabolic activation (Langman and Kapur 2006).



**Fig1:** Mode of action of toxic elements (Shanker AK 2008)

The heavy metals are directly involved in the redox reaction that results in the formation of  $O_2^-$  and subsequently in  $H_2O_2$  and  $OH^-$  production via the Haber-Weiss and Fenton reactions (Dietz et al 1999). Exposure of plants to redox inactive heavy metals result in oxidative stress through indirect mechanisms like interaction with antioxidant defense system, disturbance in the electron transport chain or induction of lipid peroxidation. Another important mechanism of heavy metal toxicity is its ability to bind strongly to oxygen, nitrogen and sulfur atoms (Nieboer and Richardson 1980). Because of these features, heavy metals can inactivate enzyme by binding to cys residues. For example, Cadmium binding to SH groups of structural protein and enzyme leads to misfolding and inhibition of their activity (Dalcorso et al. 2008; Hall 2002).

Based on the aforementioned, it is concluded that heavy metal toxicity is attributed to three significant reasons:

1. Stimulation of ROS by auto-oxidation and the Fenton reaction or by modification of the antioxidant defense system (Schutzendubel A and Polle A 2002).
2. Direct interaction with proteins due to their affinities with thiol, histidyl and carboxyl groups, causing the heavy metal to target structural, catalytic and transport sites of the cell (Hall 2002).
3. Displacement of necessary metal ions from specific binding sites, causing function to collapse (Sharma and Dietz 2009).

## **2.4 Heavy metal detoxification by fungi**

### **Mycorrhizas**

Mycorrhizal fungi help in phytostabilization of toxic heavy metals. Plants with mycorrhizal association accumulate metallic toxicants in vesicles as well as in fungal hyphae, hence these metallic toxicants are immobilized and do not restrain the growth and uptake of micronutrients. Many mycorrhizal fungi have shown to overcome the stress of heavy metals (Hildebrandt et al. 2007). Tolerance to heavy metals varies among different fungal groups. Fungal colonization can be decrease if less tolerant strains are in association with mycorrhizae. There is a report on inhibition of mycorrhizal colonization in the presence of Cu and Cd in soil (Weissenhorn et al. 1993; Kaldorf et al. 1999; Griffioen 1989). This has explained the blocking of some physiological and biochemical processes in mycorrhizal fungi.

Arbuscular mycorrhizae have been reported to develop strategies which can alleviate heavy metal stress (Hall 2002). For example, AM can bind to heavy metals by releasing an insoluble glycoprotein known as glomalin (Gohre and Paszkowski 2006).

Ectomycorrhizal fungi have also been studied for metal tolerance. Ectomycorrhizal symbioses can play a crucial role in protecting plants from toxic metals. The response of ectomycorrhizal fungi to toxic metals is significant since these organisms participate in crucial symbiotic relationships with trees that grow at polluted sites, and alleviate metal toxicity in the host plants (Courbot et al. 2004).

Potential amelioration of metal toxicity to plants by ectomycorrhizal (ECM) fungi has been proposed by a number of authors (Silva et al. 2013; Colpaert 2011; Blaudez et al. 2000; Gadd 2004; Bellion 2006; VanTichelen et al.2001; Tam 1995).

Along with the role of ectomycorrhizal fungi in metal tolerance by the host plant there are mechanisms that have been proposed involve different exclusion processes that restrict movement of metal to the host roots. These processes have been extensively reviewed and assessed (Jentschke and Godbold 2000).

### ***Suillus* species**

**Table2:** Metal tolerant *Suillus* isolates that have been shown to protect mycorrhizal pine seedlings from metal stress.

<b>Sr.no</b>	<b><i>Suillus sp.</i></b>	<b>Metal tolerant</b>	<b>Author</b>	<b>Year</b>
<b>1.</b>	<i>Suillus luteus</i>	Copper	Adriaensen K et al.	2005
<b>2.</b>	<i>Suillus bovinus</i>	Zinc	Adriaensen, Vangronsveld , Colpaert	2006
<b>3.</b>	<i>Suillus luteus</i>	Zinc	Muller et al.	2004
<b>4.</b>	<i>Suillus luteus</i>	Cadmium	Krznaric et al.	2010

Number of investigations has been carried out using ectomycorrhizal tree seedlings on metal detoxification in host plants

**Silva et al. (2013)** have explored the copper resistance of ectomycorrhizal fungi isolates *Pisolithus microcarpus* , *Pisolithus sp.*, *Suillus sp.* and *Scleroderma sp.* at different copper concentration in solid and liquid media. The results were like - In the solid culture medium the isolates *Pisolithus microcarpus* , *Pisolithus sp.* and *scleroderma sp.* have shown higher average diameter of mycelium and on the other hand isolate *Suillus* exhibited significant reduction in the diameter of the mycelium in the different copper concentration. In the liquid culture medium,

the four considered isolates showed significant reduction in the mycelium diameter, dry mass of the fungal mycelium and in the pigment production. These results indicate that the liquid medium show higher negative effect of Cu on the growth of fungi than solid medium, even with the application of smaller doses of copper.

**Huanq et al. (2006)** focused on the resistance of ectomycorrhizal fungi (*Gomphidius viscidus*, *Boletus edulis*) to copper and cadmium. The results indicated that there was reduction in biomasses of the two species. *Gomphidius viscidus* has shown higher tolerance to Cu but less to Cd than that of *Boletus edulis*. It is also reported that with the increasing concentrations of Cu or Cd, the Cation exchange capacity of *Gomphidius viscidus* increased, but the Cation exchange capacity of *Boletus edulis* dropped.

**Jourand et al. (2010)** have proved that how mycorrhizas contribute significantly to the adaptation of the host plant in metalliferous soils. In this study, it was explained that *Pisolithus albus* has a very high tolerance to nickel, at around 1600 micromolar, or 400 times the concentration tolerated by other strains. Attempts at mycorrhizal colonisation were conducted with this strain on *Eucalyptus globulus*, which is naturally sensitive to nickel. It is proved to be ten times more nickel tolerant than those colonised by a sensitive mycorrhizal fungi. They also present opportunities for using mycorrhizal symbiosis in the adaptation of plants to their environment.

**Adriaensen et al. (2005)** investigated an ectomycorrhizal fungus *Suillus luteus* that determine its heavy metal tolerance in Norway. In this study they hypothesized that this population had developed adaptive Cu tolerance and was able to protect pine trees against Cu toxicity. They also tested for the existence of cotolerance to Cu and Zn in *S. luteus*. The results indicated that copper mine isolates exhibited high copper tolerance but the zinc tolerant isolates were shown to be copper sensitive. This indicates the evolution of metal-specific tolerance mechanisms is strongly triggered by the pollution in the local environment. A dose-response experiment was also conducted by them in which the copper sensitivity of nonmycorrhizal *P.sylvestris* seedlings were compared to the sensitivity of mycorrhizal seedlings colonized either by a Cu sensitive or Cu tolerant *Suillus. luteus* isolate.

**Courbot et al. (2004)** explored the main mechanisms involved in metal detoxification. They appeared to involve the chelation of metal ions with thiol containing compounds, such as metallothioneins, phytochelatins, and glutathiones. In this study they used high-performance liquid chromatography (HPLC) for the simultaneous measurement of thiol containing compounds from cysteine residues and its derivatives (glutamylcysteine, glutathione) to high molecular mass compounds (phytochelatins). In the result, it is found that glutathione and glutamylcysteine compounds increased when the ectomycorrhizal fungus *Paxillus involutus* was exposed to cadmium and an additional compound metallothionein was also increased drastically in mycelia when exposed to cadmium.

**Blaudez et al. (2000)** determined in vitro tolerance to heavy metals of thirty nine ectomycorrhizal fungi (*Paxillus involutus*, *Pisolithus tinctorius*, *Suillus bovinus*, *Suillus luteus*, *Suillus variegatus*) on cadmium, copper, nickel zinc and measured inhibition of biomass production. In the results, it was observed that *Suillus luteus*, *S. variegatus* and *P. tinctorius* show more tolerance to Cu, Cd and Zn as compared to *P. involutus*, whereas the reverse was true for Ni.

**Van tichelen et al. (2001)** studied the copper toxicity in Scots pine colonized with *Suillus bovinus* and *Thelephora terrestris*. It was concluded that mycorrhizal infection protects pine from copper toxicity and there was greater reduction in root biomass in non infected plants and enhanced copper uptake in the shoots.

**Coalpaert et al. (2011)** studied the effects of toxic concentrations of heavy metals on ectomycorrhizal populations and communities. In this study, Selection and adaptations within *Suillus* species that colonise the host plants in harsh environments is discussed. It is concluded that the evolutionary adaptation of *Suillus* species in metalliferous soil is beneficial in the survival of host trees.

**Coalpaert et al. (2008)** screened twenty-one isolates of *Suillus luteus* for their tolerance to the heavy metals (Zn, Cd, Cu, Ni) and measured inhibition of radial growth and biomass production. The Zn and Cd tolerance of the *S. luteus* isolates from the polluted soil were considerably higher than the tolerances measured in the isolates from the nonpolluted site. It is observed that the

elevated soil metal concentrations were responsible for the evolution of adaptive zinc and Cadmium tolerance.

**Krznaric et al. (2010)** revealed the tolerance of ectomycorrhizal fungus *Suillus luteus*. In this study fungus is inoculated in pine seedlings and exposed to high zinc and cadmium doses. In the result it is found that, there was severe reduction of nutrient uptake in non-mycorrhizal pines and the seedlings inoculated with mycorrhizal fungus were more resistant to Zn and Cd even at high metal exposure.

**Tam (1995)** studied five ectomycorrhizal fungus with nine different heavy metals and observed considerable variation among the ectomycorrhizal fungus and concluded that the fungus *Pisolithus tinctorius* provides tolerance to zinc and copper.

## **2.5 Mechanism of heavy metal detoxification and tolerance**

Many essential heavy metals are required for normal growth and development of plant but elevated doses of both essential and non-essential heavy metals can lead to toxicity. Plants have evolved tolerant races to cope up with heavy metal toxicity and survive on metalliferous soils. Decontamination or detoxification processes mediated by plants is referred to as phytoremediation. It is the most commonly used and rapidly expanding process to detoxify heavy metals from root level. Decontamination of heavy metals is a challenging task as heavy metals cannot be degraded and hence stay in the soil (Ma et al. 2011a; Rajkumar et al. 2010).

All plant species are capable to resist heavy metals to some extent. On the basis of accumulation of metals, plant kingdom is divided into two groups: non-accumulator plants and hyper accumulator plants (Katrin 2014).

Majority of plants are non-accumulator plants. However, all have to cope with heavy metals for growing in metalliferous soils. Hence, all plants possess potential mechanisms to survive with even toxic heavy metals (Hall 2002; Clemens 2006). The simplest strategy exercised by plants is to evade metal uptake from soil or preventing metal movement into shoots. Additionally, plants employ various elements for the acquirement and sequestration of essential metals. But this can

cause induction of many toxic symptoms (Fodor et al. 2002) such as impairments of chlorophyll synthesis that result in changed ratios of chlorophyll a and b (Viehweger and Geipel 2010) and photosynthetic activity (Kupper et al. 2007), dwarfism of plants.

Metal tolerance is associated with hypertolerant plants, which are able to exclude metals to minimize metal accumulation. This is the key difference to hyperaccumulating plants.

Nevertheless, metal hypertolerance is linked to hyperaccumulating plants.

Metal hyperaccumulating plants involve three mechanisms of metal accumulation (Katrin 2014):

- Overexpression of transport systems for superior sequestration,
- Tissue specific expression of proteins
- High metal chelating concentrations.

**Becher et al. (2004)** found that metal hyperaccumulation in *Arabidopsis halleri* is higher than nonaccumulator *A. thaliana*.

**Pence et al. (2000)** revealed that the Zn<sup>2+</sup> transporter ZnT1 (Zn<sup>2+</sup> transporter) expression is higher in the case of hyperaccumulator *Noccaeacaerulescens* than the non-accumulator *N. arvensis*.

Knowledge of metal hyperaccumulation has a broad relevance in inhibiting the accumulation of toxic metals, biofortification and phytoremediation.

## **2.6 Microbes associated with plants in heavy metal detoxification**

Plants growing in metalliferous soils consist of diverse group of microorganisms (Idris et al. 2004), which have tendency to tolerate high doses of metal. Among the microorganisms involved in heavy metal detoxification, the mycorrhizal fungi have been recurrently reported for having heavy metal tolerance. This indicates that they play important role in phytoremediation of metal polluted sites (Miransari 2011; Orłowska et al. 2011; Colpaert et al. 2011). Rhizosphere bacteria also deserve special attention for improving the phytoremediation process by releasing

chelators (e.g., organic acids, siderophores), oxidation and reduction reactions (Khan et al. 2009; Kidd et al. 2009; Ma et al. 2011a; Rajkumar et al. 2010; Uroz et al. 2009). Similarly the metal tolerant mycorrhizal fungi have also been frequently reported in hyperaccumulators growing in metal polluted soils indicating that these fungi have evolved a heavy metal-tolerance and that they may play important role in the phytoremediation of the site ( Miransari 2011; Orłowska et al. 2011).

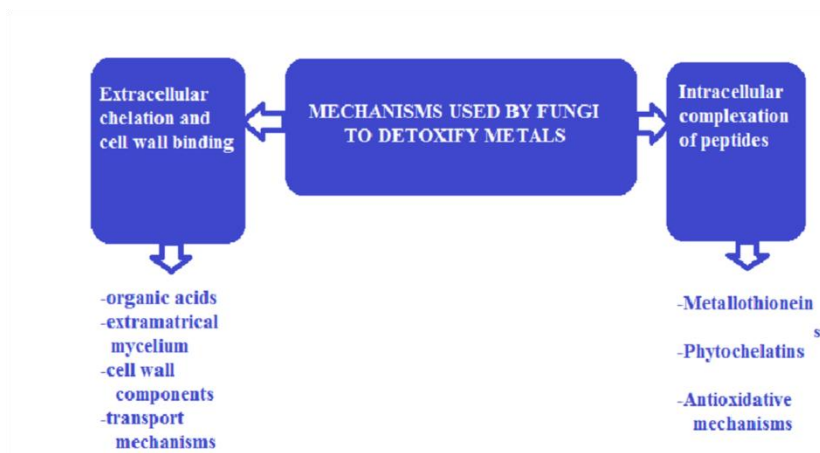
**(Verbruggen et al. 2009)** concluded that plants have developed two key strategies to resist heavy metal exposure:

- Excluder strategy (in which plants try to resist entrance of heavy metals in the roots, by restricting soil metal bioavailability.
- Tolerance strategy (which helps in confinement and detoxification of heavy metals in a controlled way.

Plant system is capable of alleviating adverse effects of toxic metals to some extent. Plants defense system is ineffective at high doses of heavy metals. In this case, mycorrhizal fungi helps in phytostabilization of toxic heavy metals.

According to Belion et al. (2006) there are two protective strategies by which fungi can resist heavy metal toxicity at cellular and molecular levels:

- Extracellular chelation
- Intracellular chelation



**Fig 2:** Mechanisms involved in metal detoxification

Biosynthesis of cellular biomolecules is the prime way to neutralize metal toxicity. This involves the induction of low molecular weight proteins or chelators such as nicotianamine, organic acids, metallothioneins glutathione and phytochelatins or cellular exudates such as specific amino acids (proline and histidine), phenolic compounds, protons, and hormones (salicylic acid, ethylene) (Viehweger 2014).

Some of the defense mechanisms are discussed below:

**Nicotinamine (NA)** - Nicotianamine is a nonproteinogenic amino acid that has ability to bind a range of heavy metals, such as Cu, Ni, Zn, etc. NA is synthesized from the enzyme nicotianamine synthase (NAS) by combining the three molecules of S-adenosyl-methionine (Higuchi 1999). Zn-NA complexes have been detected in *Schizosaccharomyces pombe* (Trampczynska 2010). For regulating metal homeostasis in plants, metal-NA complex is transported over cellular membranes by YSL protein (Curie2009). The expression of NAS genes is associated with NA concentrations and thus contribute to NA mediated metal homeostasis.

**Organic acids** - Organic acids are low molecular weight compounds, produced by plant associated microbes. They have proposed role in heavy metal solubility and in the mobilization of mineral nutrients. Organic acids are involved in complexation reaction by binding to metal ions. However, the stability of the complexes is dependent on various factors such as number of carboxylic groups in the organic acids, binding form of heavy metals as well as the pH of soil

solution (Ryan et al. 2001). (Ramachandran et al. 2006; Sauer et al. 2008) described organic acid biosynthesis and excretion mechanisms in bacteria and fungi. Organic acids released by plant-associated microbes play an important role in the complexation of toxic and essential ions and increase their mobility for plant uptake. (Mihalik et al. 2012) investigated that citrate facilitates translocation of uranium from root to shoot interfering Fe and Zn transport. This explains the importance of organic acids in sequestration of metal ions in plant tissues and cellular compartments (Ma et al. 2011a).

**Phytochelatin** - are short chain thiol-rich, low-molecular weight peptides synthesized from glutathione by the enzyme phytochelatin synthase. Its general structure is ( $\gamma$ -glutamyl-cysteinyl)  $n$ -glycine ( $n= 2$  to 11). Phytochelatin has a high affinity to bind heavy metals when they are present in high concentration (Gupta et al. 2013). Phytochelatin has been found in many living organisms from fungi to many different animal species (Bundy et al. 2014). In plants, PCs are identified to be part of the self-protective act not only against metal stress but also against other stress such as excess amount of heat, salt concentration, UV-B and herbicide (Zagorchev et al. 2013). PCs are reported as biomarkers for the detection of heavy metal stress in plants (Saba et al. 2013). PCs are first manufactured and actively shipped in the cytosol in the form of metalphytochelatin complexes (Song et al. 2014). PCs can attach to some heavy metal cations and anions such as Cu, Cd, Zn via sulfhydryl and carboxyl groups (Gupta et al. 2013). Nevertheless,  $Cd^{2+}$  ions are found to be the most effectual stimulator of PCs synthesis as it can induce PCs upto 6-fold stronger than  $Cu^{2+}$  and  $Zn^{2+}$  in *Rauwolfia serpentina* (Kotrba 1999) and *Picea rubens* Sarg (Thangavel et al. 2007). PCs can be produced or accumulated in roots and aerial organs (Heiss et al. 2003) investigated that prolonged exposure of Cd to *Brassica juncea* have resulted in 3-fold higher PCs accumulation in leaves than roots (Szalai et al. 2013) found that treatment of Cd on maize plants for a longer duration led to higher level of phytochelatin synthase in leaves and minimized PCs action in roots. PCs are found to be not effective among the Cd responsive thiols in *Paxillus involutus* (Courbot et al. 2004) and *Suillus bovinus* (Colpaert et al. personal communication) and this confirms the lack PCs in fungi, except in *Candida glabrata* (Zhou and Goldsbrough 1995).

**Metallothionein** - Metallothioneins (MTs) are intracellular metal-binding proteins, present in nearly all living organisms. MT was first isolated from horse kidney by Margoshes and Vallee (1957). MT has remarkable features that confer a degree of specificity and stability to predict the properties of metallic ingredients. These unique biomolecules have low molecular weight around (<7000 Da), highly conserved 18–23 cys residues and no aromatic amino acids. Expression of MT gene and its protein can be synthesized by variety of metals such as Cu, Cd, Zn, cytotoxic agents and stress-producing conditions (Andrews 2000). MTs also work against oxidative stress in which they act as a hydroxyl radical scavenger (Viarengo et al. 2000). MTs are synthesized after mRNA translation, contrary to PCs which are the enzymatically synthesized peptides. MTs are found in wide variety eukaryotes including fungi, invertebrates, mammals and plants as well as in some prokaryotes (Du et al. 2012; Cai and Ma 2003). MTs show different characteristics and functions based on their occurrence in different organisms. MTs are involved in nullifying the adverse effects of heavy metals and apart from this they are also known to be active agents in cellular events including ROS scavenger (Wong et al. 2004), repair of plasma membrane and damaged DNA (Grennan 2011).

Metallothioneins have been reported in variety of eukaryotes like fungi, mammals, plants as well as in prokaryotes.

**Guo et al. (2013)** reported a novel metallothionein gene in sugarcane, designated as ScMT2-1-3. It was found that under  $\text{Cu}^{2+}$  stress expression of MT was upregulated but under  $\text{Cd}^{2+}$  stress expression was downregulated. In this study, it is concluded that ScMT2-1-3 is considerably involved in Cu detoxification but in case of Cd more testification is needed.

**Grennan (2011)** reported that MT isoforms from types 1a, 2a, 2b and 3, in *Arabidopsis*, are involved in Cu chelation, while type 4a and 4b act as a Zn binder.

**Suhy et al. (1999)** studied Zn metabolism using transgenic mice and suggested that mammalian MT1 and MT2 function as chaperons for the synthesis of metalloproteins.

**Jing et al. (2009)** characterized metallothionein gene, SmtA, of cyanobacteria and transgenic plants are synthesized by cloning SmtA gene in *Arabidopsis*. In the results, it was observed that

in high zinc stress, the transgenic plants were over-expressing SmtA gene and showing higher survival rate.

**Lanfranco et al.(2002)** identified a metallothionein gene named as *GmarMT1*, in arbuscular mycorrhizal fungus, *Gigasporamargarita*. After complementation assays, it was revealed that *GmarMT1* gene was successful in conferring tolerance against Cd and Cu toxicity.

**Gonzalez-Guerrero et al. (2007)** identified a metallothionein gene (*GintMT1*) in *Glomus intraradices* and studied its tolerance in case of Cu, Cd and oxidative stress. In gene expression analyses, it was concluded that *GintMT1* gene was unregulated in response to Cu and oxidative stress but not to Cd. In the functional analysis, it was observed *GintMT1* encodes a functional metallothionein.

**Courbot et al. (2004)** found that there was drastic increase in expression of metallothionein gene (*PiMT1*) when exposed to Cd in fungus *Paxillus involutus*. Additional compounds are found, glutamylcysteine, glutathione and phytochelatins, in ectomycorrhizal fungus *Paxillus involutus* in response to cadmium by using high-performance liquid chromatography method.

**Bellion et al. (2007)** characterized the metallothionein gene (*PiMT1*) gene and observed that the gene was over-expressed in case of Cu and Cd but not in Zn. Yeast complementation studies also showed that *PiMT1* was capable to complement the hypersensitivity of mutant strains against Cu and Cd.

**Hryniewicz et al. (2011)** investigated the effects of single and joint inoculations of ECM fungus (*Hebeloma mesophaeum*) and bacterial strain (*B. cereus*) on growth and metal extraction of willows. In this study, expression of MT1 was also observed in mycorrhizal and nonmycorrhizal willows and their consequences for the phytoextraction.

**Ramesh (2009)** identified two metallothionein genes in *Hebeloma cylindrosporum*. In this study, expression of metallothionein genes were checked in the presence of different heavy metal doses using competitive RT-PCR. Then both MT genes were functionally characterized using complementation assays in metal sensitive yeast mutants.

**Table3:** Fungi in which metallothionein has been reported.

S.No.	Fungus	Type of Fungus	Metallothionein genes	Stress	Reference
1	<i>Hebeloma mesophaeum</i>	Ectomycorrhizal fungus	HmMT1, HmMT2 & HmMT3	Zn, Cd & Ag	Sacky et al. 2014
2	<i>Heliscus lugdunensis</i>	Aquatic fungus	Nec 1U-MT1	Cd	Loebus et al. 2013
3	<i>Laccaria bicolor</i>	Ectomycorrhizal fungus	LbMT1 & LbMT2	Cu, Cd, Zn, H <sub>2</sub> O <sub>2</sub>	Reddy et al. 2014
4	<i>Amanita strobiliformis</i>	Ectomycorrhizal fungus	A MT1a, 1b & 1c encode isomorphic AMT1s	Ag	Osobavar et al. 2011
5	<i>Hebeloma cylindrosporum</i>	Ectomycorrhizal fungus	MT1 & MT2	Cu & Cd	Ramesh et al. 2008
6	<i>Paxillus involutus</i>	Ectomycorrhizal fungus	PiMT1	Cu, Cd but not Zn	Bellion et al. 2007
7	<i>Yarrowia lipolytica</i>	Yeast	CRF1	Cu & Cd	Gracia et al. 2002
8	<i>Candida glabrata</i>	Yeast	MT1, MT2	Cu	Mehra et al. 1989
9	<i>Saccromyces cerevisiae</i>	Yeast	CRS5	Cu	Culotta et al. 1994
10	<i>Saccromyces cerevisiae</i>	Yeast	Cu-MT	Cu	Butt et al. 1994
11	<i>Gigaspora margarita</i>	Arbuscular mycorrhizal fungus	GmarMT1	Limited carbon supply	Bergero et al. 2007

## 3. MATERIALS AND METHODS

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### 3.1 Materials

#### 3.1.1 Biological materials

##### Ectomycorrhizal fungal isolates and culture conditions

Fresh basidiocarps of *Suillus* species were collected from conifer forests of the northwestern Himalayan region of India during monsoon seasons (Verma and Reddy 2014). Eight pure cultures were obtained from the basidiocarps of a range of *Suillus* species and have been designated from “SNW01–SNW08” (SNW stands for „*Suillus* species from north western Himalayas”) (Verma and Reddy 2014). In the present study, *Suillus himalayensis* and *Suillus indicus* were used. Both the strains were maintained on malt extract (2%) medium at temperature 25°C.

##### Yeast fungal isolates and culture conditions

The *Saccharomyces cerevisiae* strains used for complementation assays were: one Cu sensitive strain (DTY4; *MAT $\alpha$* , *trp1-1*, *leu2-3*, *leu2-112*, *gal1*, *his3 $\Delta$ 1*, *ura3-50*, *cup1 $\Delta$*  : : *URA3<sup>+</sup>*) referred as *cup1 $\Delta$*  (Lerch 1980) and one wild strain BY4741 (*MAT $\alpha$* , *his3D1*, *leu2D0*, *met15D0*, *ura3D0*). Both the strains were maintained at 30°C on YPD medium.

##### Bacterial strain and culture conditions

The *E.coli* DH5 $\alpha$  strain was used for its transformation with metallothionein genes. This strain was maintained at 37°C on Luria-agar medium.

#### 3.1.2 Heavy Metal

In the present study, copper (as CuSO<sub>4</sub>) was used as heavy metal stress.

## 3.2 Methodology

### 3.2.1 Heavy metal tolerance of *Suillus himalayensis* and *Suillus indicus*

The potential to tolerate the heavy metal stress in *Suillus himalayensis* and *Suillus indicus* was tested by growing pure mycelial culture in liquid malt extract medium supplemented with different concentrations of copper. For this, the liquid malt extract medium (pH-5.5) was prepared and divided 50 ml in 250 ml flasks. Each of the 50 ml broth was inoculated with three mycelial discs, cut from freshly growing culture and incubated at 25°C in dark conditions for 4-5 days. Both the strains were allowed to initiate the growth and after 5 days of incubation supplemented with different concentration of Cu (0, 100, 200, 300, 400 µM) in the form of CuSO<sub>4</sub>. The flasks were kept uninterrupted at 25°C and after 21 days of incubation, the mycelium was harvested, washed with distilled water and dried. The dry weight of the mycelium at different metal stresses was recorded for both strains *Suillus himalayensis* and *Suillus indicus*.

### 3.2.2 Bioinformatics analysis

The putative metallothionein gene sequences for *Suillus* strain were searched from the EST library at NCBI database ([www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)). 5 putative genes were retrieved with the accession numbers GR975901, GR975896, GR975716, GR975715, GR975714. Out of these five genes, two were reverse complemented. The transcripts of these genes were obtained from the ORF finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>), which were then compared using multiple alignment tool. From these putative sequences primers were designed. The putative gene sequences were verified using the blastp (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) analysis. All the five transcripts were compared using the multiple sequence alignment tool MULTALIN (<http://multalin.toulouse.inra.fr/multalin/>). The transcripts obtained were characterized by the phylogenetic analysis using MEGA6 software. Two out of the five ESTs were selected as the putative metallothionein genes for the *Suillus* strain. Gene specific primers were designed for the amplification of these genes from the *Suillus* cDNA by reverse transcriptase PCR method. Specific restriction sites were added to the 5' end of these primers.

### 3.2.3 MOLECULAR METHODS

#### 3.2.3.1 Isolation of Total RNA

Both the strains (*Suillus indicus* and *Suillus himalayensis*) were grown on malt extract agar medium for 21-24 days at 25°C. Mycelium was then subcultured on processed cellophane sheets for 15 days at 25°C. After 15 days of growth period, mycelium (along with cellophane sheets) was transferred to Cu (200Mm) amended ME plates and incubated for 48 hrs and then scraped out of the plates and crushed into fine powder with the help of liquid nitrogen and stored at -80°C. Total RNA isolation was performed from the frozen mycelia powder using the TRIzol reagent (Invitrogen, Life Technologies, USA) method.

Procedure:

1. One ml of TRIzol Reagent was added to the approx. 100 mg of mycelium and homogenized vigorously.
2. Homogenized samples were incubated at 15°C for 5 minutes to allow the complete dissociation of nucleoprotein complexes and then centrifuged at 12000g for 10 minutes at 4°C.
3. 200µl of chloroform was added to the sample solution and vigorously shaken for 30 seconds, incubated for 2-3 minutes at 15°C and then centrifuged at 12,000 gfor 15 minutes at 4°C.
4. The upper aqueous phase was removed from the sample into a fresh tube and then the RNA was precipitated from the aqueous layer by adding 500µl of isopropanol and storing it at -20°C for 20-30 minutes.
5. Precipitated RNA was centrifuged at 12000g for 10 minutes at 4°C.
6. The supernatant was discarded and the pellet washed with 75% ethanol and centrifuged at 7500g for 5 minutes at 4°C.
7. At the end, RNA pellet was dried and RNA dissolved in DEPC treated water and stored at -80°C.

RNA sample was loaded on agarose gels (1.5%) prepared in 0.5X TBE buffer (pH 8.0) (Appendix) using a 5X TBE buffer (Appendix II). Prior to pouring, ethidium bromide (EtBr) (0.5 µg/ml) was added to visualize RNA bands, as it intercalates between base pairs. The RNA sample was then migrated on electrophoretic apparatus and visualized on Gel Documentation system to check the integrity of the samples.

### **3.2.3.2 Spectrophotometric quantification**

The concentration and purity of RNA sample was checked on NANODROP Spectrophotometer (Thermo-Scientific). The purity of sample was evaluated by the ratios, O.D 260/230 nm and O.D 260/280 nm respectively. Sample purity was indicated by a value higher than or closer to 2.1 for RNA.

**3.2.3.3 cDNA Synthesis by reverse transcription PCR (RT-PCR)** cDNA was synthesised from total RNA by RT- PCR method (The Reverse AIDTM First Strand cDNA Synthesis Kit, Fermentas Life Sciences, USA). 5 µg of total RNA and 1 µl of oligo dT was denatured at 65°C for 5 minutes and immediately cooled on ice. The first strand cDNA was synthesized with the help of 2µl of 10mM dNTP mix, 1µl of Ribolock RNase inhibitor (20U/µl), 1µl of Rev. Aid Reverse Transcriptase(200U/µl) and 4µl of 5x Reaction Buffer. The reaction was carried out for at 42°C for 60 minute and then at 70°C for 5 minutes. After the completion of cycle, cDNA sample was stored at -20°C.

**3.2.3.4 Gene amplification** cDNA was verified by amplifying the metallothionein gene with primers. PCR reactions were carried out with M.Q water 17.2µl, Taq Buffer 2.5µl (10x PCR reaction buffer containing MgCl<sub>2</sub>), dNTPs 2µl (2mM dNTP mix), Forward primer 1µl (100µM MTF), Reverse Primer 1µl (100µM MT1R for MT1 gene); Reverse primer 1µl (100µM MT2R for MT2 gene), Taq polymerase 0.3µl (5U/µl Sigma-Aldrich) and template (5µg) 1µl. Total reaction volume was 25µl.

The tubes were kept in a thermocycler for 35 cycles of denaturation, annealing and elongation, with the following PCR program:

**Table 4:** PCR program for gene amplification

Steps	Temperature	Time period
<b>Initial Denaturation</b>	94°C	3minutes
<b>Denaturation</b>	94°C	1 minutes
<b>Annealing</b>	55°C	1 minutes
<b>Elongation</b>	72°C	1 minutes
<b>Final Elongation</b>	72°C	8 minutes

The reaction was carried out for 35 cycles and amplified product was run on a 1.5% agarose gel and visualized on Gel Documentation system.

PCR primers used for the amplification of metallothionein genes :

**For *Suillus himalayensis***

<i>ShMT1F</i>	5- CGGGATCCATGTCCACCGCTACTGAAGTC-3
<i>ShMT1R</i>	5- CCGGAATTCTCAACATTTGCACTCTCCAGG- 3
<i>ShMT2F</i>	5- CGGGATCCATGTCCACCGCTACTGAAGTC-3
<i>ShMT2R</i>	5-CCGGAATTCTCAATCAACATTGCACTCTCCAG-3

**For *Suillus indicus***

<i>SiMT1F</i>	5- CGGGATCCATGTCCACCGCTACTGAAGTC-3
<i>SiMT1R</i>	5- CCGGAATTCTCAACATTTGCACTCTCCAGG- 3
<i>SiMT2F</i>	5- CGGGATCCATGTCCACCGCTACTGAAGTC-3
<i>SiMT2R</i>	5-CCGGAATTCTCAATCAACATTGCACTCTCCAG-3

### 3.2.3.5 Cloning of Metallothionein gene products

#### Double restriction digestion

In this double restriction digestion, DNA samples (plasmid (pFL61), MT1 and MT2 genes) were digested with EcoR1(10U/L) and BamH1(10U/L) (Thermo Scientific Restriction & Modifying Enzymes).

Then following reaction components were added in the sequence for each reaction:

- In the first step, plamid(425 ng/μl), MT1 gene(450.2 ng/μl) and MT2 gene(432.1 ng/μl) were digested with EcoR1 and incubated at 37°C for 2 hours.
- After 2 hours of incubation, samples were digested with BamH1 and incubated for 2 hours at 37°C.

**Table 5:** Restriction Digestion of plasmid pFL61 and MT genes

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Sample	Volume
Enzyme(EcoR1+BamH1)	2μl+2μl
DNA sample	10μl
Buffer	4μl
M.Q Water	2μl
Total	20μl

- ❖ 2X Tango Buffer was used for this restriction digestion as it was recommended by double digest calculator-Thermo scientific because BamH1 show less star activity with this buffer.
- ❖ After the completion of digestion protocol, enzymes were inactivated at 80°C for 20 minutes.
- ❖ Same procedure was followed for another strain.

## **Purification of digested DNA**

After restriction digestion of DNA, samples were purified using Thermo Scientific GeneJET Gel Extraction kit as it ensures us to have no traces of undigested vector that can interfere during transformation. After restriction digestion samples were migrated on the 1% gel at 70V.

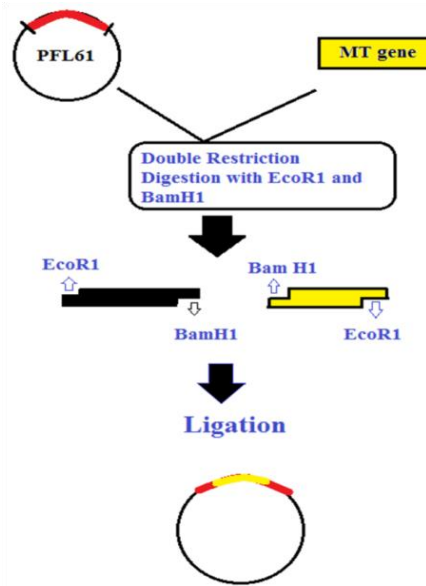
- Gel slice containing DNA fragment was excised with the help of clean scalpel and weight of the gel slice was recorded.
- Binding buffer was added to the gel slice in 1:1 volume and incubated at 50°-60°C for 10 minutes until the gel slice was completely dissolved.
- In case of metallothionein genes, 1:2 volume of 100% isopropanol was added in the gel solution and mixed thoroughly.
- 800µl of gel solution was transferred to the purification column and centrifuged for 1 minute. Flow-through was discarded and column was placed back into the same tube.
- 700µl of wash buffer was added to the column and flow through was discarded.
- The empty column was centrifuged for an additional step to remove residual wash buffer.
- The column was transferred into a clean 1.5ml microcentrifuge tube and eluted with 30 µl of lukewarm M.Q water and stored at -20°C. After elution procedure concentration of eluted sample was recorded on nanodrop.

## **Ligation**

Plasmid and insert were ligated in the ratio 1:3. Reaction mixture was incubated overnight at 4°C

(**Table 6:** Reaction mixture for ligation)

Plasmid DNA	<b>20-100 ng</b>
Insert DNA	3:1 ratio of plasmid
10x T4 DNA Ligase buffer	2µl
Thermo Scientific T4 DNA Ligase (Cat #EL0016)	1U
Water, nuclease-free	To 20µl
Total volume	20µl



**Fig: 3** Restriction digestion and ligation of vector and MT genes

### 3.2.3.6 Transformation of the ligate (Plasmid+Gene) in *E.coli* DH5 $\alpha$

#### Preparation of competent cells

1. A single colony of *E.coli* DH5 $\alpha$  was picked from a freshly grown plate and transferred into 20ml Luria broth (LB). The culture was incubated for 16-17 hours at 37°C with vigorous shaking.
2. After the 16-17 hours of incubation, 500 $\mu$ l of above saturated culture was transferred to 50 ml of LB broth and incubated at 37°C for 3 hours. Growth was monitored by determining O.D<sub>600</sub> after every one hour.
3. The above culture was poured in pre-chilled 50ml oak ridge tubes and stored at 0°C for 10 minutes.
4. The cells were recovered by centrifugation at 5000 rpm for 10 minutes at 4°C. The supernatant was poured-out from the cell pellet.
5. The pellet was resuspended in 10ml of chilled 0.1M CaCl<sub>2</sub> and stored on ice for 10 minutes.

6. The cells were recovered by centrifugation at 5000 rpm for 10 minutes at 4°C.
7. The fluid was decanted and cells were resuspended in 1ml of ice-cold 0.1M CaCl<sub>2</sub>.
8. Stored on ice for 12-24 hours. After 24 hours glycerol stock was prepared by adding glycerol 200µl in 1ml of competent cells.

### Transformation

1. 100µl of competent cells were transferred to three pre-chilled 1.5ml microfuge tube. In the first tube 5µl plasmid was added and in another two tubes ligated product was added.
2. All the contents were mixed gently and stored on ice for 30 minutes.
3. After 30 minutes, all three tubes were kept in pre-heated waterbath at 42°C for two minutes and then tubes were rapidly transferred on ice for two minutes.
4. Then 1ml LB broth was added to each tube and incubated at 37°C for 60 minutes.
5. After 60 minutes of incubation, centrifuged at 8000 rpm for 5 minutes. 500 µl of supernatant was discarded and the remaining solution was mixed with with the pellet.
6. 100µl of the above solution was spreaded on LB+Amp plates. And the plates were incubated overnight at 37°C.

### **3.2.3.7 Bacterial colony PCR**

1. 5µl M.Q water was taken in two PCR tubes tubes and a pinhead colony from each sample patch was mixed thoroughly in water.
2. The tubes were incubated at 95°C for 10 minutes and after 10 minutes tubes were rapidly transferred on ice for 3 minutes and used as a template.

PCR reaction was carried out with M.Q water 11.2µl, Taq Buffer 2µl (10x PCR reaction buffer containing MgCl<sub>2</sub>), dNTPs 1.5µl (2mM dNTP mix), Forward primer 1µl (100µM MTF), Reverse Primer 1µl (100µM MT1R for MT1 gene); Reverse primer 1µl (100µM MT2R for MT2 gene), Taq polymerase 0.3µl (5U/µl Sigma-Aldrich) and template 3µl. Total reaction volume was 20µl.

**Table7:** PCR program for running the colony PCR

Steps	Temperature	Time period
<b>Initial Denaturation</b>	94°C	3minutes
<b>Denaturation</b>	94°C	1 minutes
<b>Annealing</b>	55°C	1 minutes
<b>Elongation</b>	72°C	1 minutes
<b>Final Elongation</b>	72°C	8 minutes

The reaction was carried out for 35 cycles and amplified product was run on a 1.5% agarose gel and visualized on Gel Documentation system.

### **3.2.3.8 Bacterial Plasmid isolation (QIAprep Spin Miniprep Kit)**

1. 20 ml LB+Amp was inoculated with bacterial culture and kept at 37°C for 16-17 hours.
2. The LB inoculated with culture was centrifuged at 12000 rpm for 1 minute.
3. The bacterial pellet was resuspended in 250µl of buffer P1.
4. 250µl of buffer P2 was added and mixed thoroughly by inverting the tube 4-6 times (until the solution becomes clear and viscous).
5. The 350µl of N3 buffer was added and mixed immediately and thoroughly by inverting the tube.
6. The above solution was centrifuged at 13000 rpm for 10minutes in a table-top microcentrifuge.
7. The supernatant from above step was taken in QIAprep spin column with the help of a pipette.
8. The column was centrifuged for 1 minute at 13000rpm and flow-through was discarded.
9. QIAprep spin column was washed by adding 500µl of PB buffer and centrifuged for 1 minute at 13000rpm. The flow-through was discarded.
10. Then, column was washed with 750µl of PE buffer and centrifuged for 1 minute at 13000rpm. The flow-through was discarded.
11. To residual wash buffer, column was centrifuged again for 1 minute at 13000 rpm.

12. QIAprep spin column was placed in 1.5ml microfuge tube.
13. In last, 25µl of M.Q water was added to the center of the column and centrifuged for 1 minute at 13000 rpm.

### 3.2.3.9 Yeast Transformation (Lithium acetate method)

Copper sensitive strain DTY4 referred to as *cup1* and mutant of *S. cerevisiae* were used for transformation of (pFL61, PFL61-*SHMT1* and *SHMT2*)- First set, (pFL61, PFL61-*SIMT1* and *SIMT2*)- *Second set*. The transformed cells were selected on complete SD -ura and SD -ura with metal.

1. 20ml of YPD medium was prepared and inoculated with a single yeast colony and kept at 30°C overnight with shaking at 250 rpm (primary culture).
2. Next day, first OD<sub>600</sub> was taken and according to first OD<sub>600</sub> inoculation of 40ml YPD media was done to achieve the OD<sub>600</sub> 1 and the final volume 50ml.
3. The culture was further incubated at 30°C for 2 hours with shaking (230 rpm) and checked the OD<sub>600</sub>.
4. The culture was transferred into 50ml falcon tube and centrifuged at 3000rpm for 5 minutes. The supernatant was discarded.
5. The pellet was resuspended in 25ml of autoclaved distilled water and centrifuged at 3000rpm for 5 minutes. The supernatant was discarded.
6. The pellet was suspended in 1ml of distilled water.
7. The cell suspension was taken into 1.5ml eppendorf and centrifuged for 30 sec. The supernatant was discarded and pellet was resuspended in distilled water to make up the final volume 1 ml.
8. In an autoclaved 1.5ml tube, 0.1 µg of sample DNA (pFL61, pFL61-MT1 and pFL61-MT2) and 3 µl of salmon sperm carrier DNA was added and mixed gently.
9. 100µl of yeast competent cells were added and mixed well by vortexing. Then 240µl of PEG and 36µl of 1M LiAc was added and mixed thoroughly with rapid pipetting.
10. Tubes containing reaction mixture were incubated at 30°C for 30 minutes with shaking at 250 rpm.
11. Then tubes were kept at 42°C for 1 hour in pre-heated waterbath.

12. After 1 hour the cells were centrifuged for 30 second and supernatant was discarded.
13. Transformed cells were plated on SD without Ura medium and SD- ura supplemented with copper.
14. The plates were incubated at 30°C for 2-4 days and observed the results.

#### **3.2.3.10 Yeast functional complementation assays**

For functional complementation assays, cultures of *DTY4* yeast cells carrying sample DNA (pFL61, pFL61-MT1 and pFL61- MT2) were grown in each respective medium at 30°C with shaking at 250 rpm. Yeast cultures were adjusted to same OD<sub>600</sub> and 5 µl serial dilutions were dotted on SD without Ura plates and on SD without Ura supplemented with 150µM CuSO<sub>4</sub>. Then plates were incubated for 3days at 30°C and results were observed.

Simultaneously in other experiment, falcon jars containing 20 ml of fresh SD-Ura medium were inoculated with mid-log preculture of *DTY4* cells containing (pFL61, PFL61-*SHMT1* and *SHMT2*)- First set, (pFL61, PFL61-*SIMT1* and *SIMT2*)- *Second set* to attain a starting optical density of 0.02 at 600nm. Cells were allowed to grow at 30°C at 220 rpm and CuSO<sub>4</sub> (150µM) were added after 5 hours of inoculation. The OD<sub>600</sub> of the cultures were measured at 3 hours interval for 42 hours. All the analysis was performed by using Graph Pad Prism 5.1 software.

## 4. RESULTS

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### 4.1 Bioinformatic analysis

The 5 metallothionein ESTs of *Suillus luteus* were retrieved from the EST library at the NCBI website ([www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)), with the accession numbers GR975901, GR975896, GR975716, GR975715, GR975714 .

#### > GR975901

```
GTATGCATCTAGATTGATGAGTCCTGAGTAAAACGCCTCTGCTCGACGAACATCCATACATCTCCTACTATAATCATAG
GCCGACAACATAATACACGACGAGGCCGAGTTCAAGATTTCGATATTCAACGTTCAATCAACATTTGCACTCTCCAGGCT
TGCATTGGCACGAAGTGCCGCATGAGCAGCTCGACGAGCCACAGTTGTTGTTAGAAACAAGGACTTCAGTAGCGGTGGA
CATATTGTGATAGATCACTACGCAGT
```

#### > GR975896

```
GCGATGCATCTAGATTGACTGCGTAGTGATCTATCACAATATGTCACCCTACTGAAGTCCTTGTTCCTAACAACAAC
TGTGGCTCGTCGAGCTGCTCATGCGGCACCTTCGTGCCAATGCAAGCCTGGAGGGTGCAAATGTGATTGAACGTTGAAT
ATCGAATCTTGAACCTCGGCCTCGTCGTGATTATGTTGTCGGCCTATGATTATAGTAGGGGATGTATGGATGTTTCGTCC
AGCAGAGGCGTTTTACTCAGGACT
```

#### > GR975716

```
CCTGGTACTCGCGATGCATCTAGATTGACTGCGTAGTGATCTATCACAATATGTCACCCTACAGAAGTCCTTGTTC
TAACAACAACCTGTGGCTCGTTGATTAGCACATGCGGCACCTTCGTGCCAATGCAAGACTGGAGAGTGCAATGTTGATTGA
ACGTTGAATATCGACTCTGGAACCTCGGCCTCCTCGTGTATTATGTTGTCGGCCTTTG
```

#### > GR975715

```
GATGAGTCCTGAGTAATACGCCTCTGCTCGACGAACATCCATACATCTCCTACTATAATCATAGGCCGACAACATAAAA
CACGACGAGGCCGAGTTCAAGATTTCGATATTCAACGTTCAATCAACATTTGCACTCTCCAGGCTTGCATTGGCACAAGT
GCCGCATGAGCAGCTCGACGAGCCACAGTTGTTGTTAGAAACAAGGACTTCAGTAGCGGTGGACATATTGTGATAGATC
ACTACGCAGT
```

#### >GR975714

```
GCTCGGTACTCGCGAATGCTCTAGATTGACTGCGTAGTGATCTTCTGCGACTGCGTCTGGTCTACCGCTTCTGGAGCTA
AAACCTCTGCCACTACCACTGCATCTTCTGGTACAACCTCAGAAGACCGGCGCTGCCAGTAGCCTTTCTGTCTCTTCGGC
AATGGGTGTTGCCGGTGTGATGAGTCCTGAGTAATACGCCTCTGCTCGACGAACATCCATACATCTCCTACTATAATCA
TAGGCCGACAACATAATACACGACGAGGCCGAGTTCAAGATTTCGATATTCAACGTTCAATCAACATTTGCACTCTCCAG
GCTTGCATTGGCACGAAGTGCCGCATGAGCAGCTCGACGAGCCACAGTTGTTGTTAGAAACAAGGACTTCAGTAGCGGT
GGACATATTGTGATAGATCACTACGCAGT
```

The ORF of the sequence were found using the ORF finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>)

### GR975715

401 atgtccaccgctactgaagtccttgtttctaacaacaactgtggc

M S T A T E V L V S N N N C G

356 tcgtcgagctgctcatgcggcacttcgtgccaatgcaagcctgga

S S S C S C G T S C Q C K P G

311 gagtgcaaatgtga 297

E C K C \*

### GR975901

240 atgtccaccgctactgaagtccttgtttctaacaacaactgtggc

M S T A T E V L V S N N N C G

195 tcgtcgagctgctcatgcggcacttcgtgccaatgcaagcctgga

S S S C S C G T S C Q C K P G

150 gagtgcaaatgtga 136

E C K C \*

### GR975896

41 atgtccaccgctactgaagtccttgtttctaacaacaactgtggc

M S T A T E V L V S N N N C G

86 tcgtcgagctgctcatgcggcacttcgtgccaatgcaagcctgga

S S S C S C G T S C Q C K P G

131 ggggtgcaaatgtga 145

G C K C \*

## GR975716

```
51 atgtccaccgctacagaagtccttgtttctaacaacaactgtggc
M S T A T E V L V S N N N C G
96 tcgttgattagcacatgcggcacttcgtgccaatgcaagactgga
S L I S T C G T S C Q C K T G
141 gagtgcaatggtgattga 158
E C N V D *
```

## GR975715

```
225 atgtccaccgctactgaagtccttgtttctaacaacaactgtggc
M S T A T E V L V S N N N C G
180 tcgtcgagctgctcatgcggcacttcgtgccaatgcaagcctgga
S S S C S C G T S C Q C K P G
135 gagtgcaaattgga 121
E C K C *
```

```
> GR975901 MSTATEVLVSNNNCGSSSCSCGTSCQCKPGECKC-
> GR975896 MSTATEVLVSNNNCGSSSCSCGTSCQCKPGGCKC-
> GR975715 MSTATEVLVSNNNCGSSSCSCGTSCQCKPGECKC-
> GR975714 MSTATEVLVSNNNCGSSSCSCGTSCQCKPGECKC-
> GR975716 MSTATEVLVSNNNCGSLISTCGTSCQCKTGE CNVD
```

**Fig 4:** The transcripts analysed using the multiple sequence alignment tool MULTALIN(<http://multalin.toulouse.inra.fr/multalin/>)

After multiple sequence alignment, we found that there are two type of sequences and from those sequences primers were designed. We have analysed that the encoded proteins contained between six and two mostly conserved Cystein residues, all of them are arranged as C-x-C doublets. *ShMT1* and *SiMT1* cDNA contains ORF around 100 bp encoding 34 aa with a predicted molecular mass of 3419.8 Da and isoelectric point of 5.88. *ShMT2* and *SiMT2* cDNA has an ORF around 100 bp encoding a polypeptide containing 35 aa with calculated a molecular mass of 3570.97 Da and isoelectric point of 4.14. Other characteristics of *ShMT1* and *SiMT1* are the presence of seven cystein residues with no aromatic amino acid (representing about 20.5% of the total amino acid content). There are three C-x-C residues. *ShMT2* and *SiMT2* sequence contains five cystein residues (representing about 14.2% of the total amino acid content) with no aromatic amino acid and only one C-x-C motif.

The selected MT sequences were characterized by the BLASTp analysis.

### For *ShMT1*

<a href="#">metallothionein [Paxillus involutus]</a>	44.7	44.7	100%	8e-05	68%	<a href="#">AAS19463.1</a>
<a href="#">metallothionein 2 [Amanita strobiliformis]</a>	42.7	42.7	100%	4e-04	62%	<a href="#">AGO04615.1</a>
<a href="#">metallothionein [Pisolithus albus]</a>	42.4	42.4	100%	6e-04	60%	<a href="#">AJO67962.1</a>
<a href="#">metallothionein [Piriformospora indica]</a>	42.0	42.0	82%	7e-04	71%	<a href="#">ACT83730.1</a>
<a href="#">metallothionein [Russula atropurpurea]</a>	42.0	42.0	100%	7e-04	59%	<a href="#">AHA31882.1</a>
<a href="#">hypothetical protein MPER_09911 [Moniliophthora perniciosa FA553]</a>	41.2	41.2	91%	0.001	65%	<a href="#">EEB91695.1</a>
<a href="#">hypothetical protein CC1G_05129 [Coprinopsis cinerea okavama7#f130]</a>	41.2	41.2	97%	0.001	64%	<a href="#">XP_001833429.2</a>
<a href="#">Metallothionein [uncultured eukaryote]</a>	38.9	38.9	100%	0.010	56%	<a href="#">CCG34103.1</a>
<a href="#">metallothionein [Ganoderma lucidum]</a>	38.5	38.5	100%	0.014	62%	<a href="#">ABP02008.1</a>
<a href="#">GI16332 [Drosophila mojavensis]</a>	41.2	41.2	61%	0.019	56%	<a href="#">XP_002011059.1</a>
<a href="#">hypothetical protein TRAVEDRAFT_25481 [Trametes versicolor FP-101664.SS1]</a>	37.0	37.0	70%	0.051	75%	<a href="#">XP_008031815.1</a>
<a href="#">hypothetical protein JAAARDRAFT_29377 [Jaapia argillacea MUCL_33604]</a>	36.6	36.6	91%	0.077	61%	<a href="#">KDDQ63354.1</a>
<a href="#">hypothetical protein RSAG8_02323 [Rhizoctonia solani AG-8 WAC10335]</a>	35.8	35.8	64%	0.17	77%	<a href="#">KDN48970.1</a>
<a href="#">metallothionein 1 [Laccaria bicolor]</a>	35.4	35.4	100%	0.20	62%	<a href="#">AHI43933.1</a>
<a href="#">metallothionein [Taiwanofungus camphoratus]</a>	35.4	35.4	100%	0.22	59%	<a href="#">ABF69031.1</a>
<a href="#">hypothetical protein HYDPIDRAFT_117423 [Hydnomerulius pinastri MD-312]</a>	34.7	34.7	100%	0.33	74%	<a href="#">KJL60168.1</a>

**Fig5a:** Homologous sequences found in BLASTp analysis for *ShMT1*

## For *ShMT2*

Description	Max score	Total score	Query cover	E value	Ident	Accession
<input checked="" type="checkbox"/> <a href="#">metallothionein [Paxillus involutus]</a>	35.4	35.4	91%	0.20	59%	<a href="#">AAS19463.1</a>
<input checked="" type="checkbox"/> <a href="#">metallothionein [Pisolithus albus]</a>	34.7	34.7	91%	0.35	55%	<a href="#">AJO67962.1</a>
<input checked="" type="checkbox"/> <a href="#">metallothionein [Russula atropurpurea]</a>	33.5	33.5	91%	0.97	50%	<a href="#">AHA31882.1</a>
<input checked="" type="checkbox"/> <a href="#">metallothionein 2 [Amanita strobiliformis]</a>	32.0	32.0	91%	3.7	50%	<a href="#">AGO04615.1</a>
<input type="checkbox"/> <a href="#">hypothetical protein MPER_09911 [Moniliophthora perniciosa FA553]</a>	31.6	31.6	82%	4.1	55%	<a href="#">EEB91695.1</a>
<input type="checkbox"/> <a href="#">Metallothionein [uncultured eukaryote]</a>	31.2	31.2	94%	5.3	48%	<a href="#">CCG34103.1</a>
<input type="checkbox"/> <a href="#">hypothetical protein RSAG8_02323 [Rhizoctonia solani AG-8 WAC10335]</a>	31.2	31.2	71%	5.9	56%	<a href="#">KDN48970.1</a>
<input checked="" type="checkbox"/> <a href="#">metallothionein [Piriformospora indica]</a>	31.2	31.2	74%	6.2	54%	<a href="#">ACT83730.1</a>
<input type="checkbox"/> <a href="#">hypothetical protein CC1G_05129 [Coprinopsis cinerea okavama7#130]</a>	31.2	31.2	82%	7.1	55%	<a href="#">XP_001833429.2</a>
<input type="checkbox"/> <a href="#">hypothetical protein JAAARDRAFT_29377 [Jaapia arqillacea MUCL 33604]</a>	30.8	30.8	85%	7.7	50%	<a href="#">KDG63354.1</a>

**Fig5b:** Homologous sequences found in BLASTp analysis for *ShMT2*

## For *SiMT1*

<input type="checkbox"/> <a href="#">metallothionein [Paxillus involutus]</a>	44.7	44.7	100%	8e-05	68%	<a href="#">AAS19463.1</a>
<input type="checkbox"/> <a href="#">metallothionein 2 [Amanita strobiliformis]</a>	42.7	42.7	100%	4e-04	62%	<a href="#">AGO04615.1</a>
<input type="checkbox"/> <a href="#">metallothionein [Pisolithus albus]</a>	42.4	42.4	100%	6e-04	60%	<a href="#">AJO67962.1</a>
<input type="checkbox"/> <a href="#">metallothionein [Piriformospora indica]</a>	42.0	42.0	82%	7e-04	71%	<a href="#">ACT83730.1</a>
<input type="checkbox"/> <a href="#">metallothionein [Russula atropurpurea]</a>	42.0	42.0	100%	7e-04	59%	<a href="#">AHA31882.1</a>
<input type="checkbox"/> <a href="#">hypothetical protein MPER_09911 [Moniliophthora perniciosa FA553]</a>	41.2	41.2	91%	0.001	65%	<a href="#">EEB91695.1</a>
<input type="checkbox"/> <a href="#">hypothetical protein CC1G_05129 [Coprinopsis cinerea okavama7#130]</a>	41.2	41.2	97%	0.001	64%	<a href="#">XP_001833429.2</a>
<input type="checkbox"/> <a href="#">Metallothionein [uncultured eukaryote]</a>	38.9	38.9	100%	0.010	56%	<a href="#">CCG34103.1</a>
<input type="checkbox"/> <a href="#">metallothionein [Ganoderma lucidum]</a>	38.5	38.5	100%	0.014	62%	<a href="#">ABP02008.1</a>
<input type="checkbox"/> <a href="#">GI16332 [Drosophila mojavensis]</a>	41.2	41.2	61%	0.019	56%	<a href="#">XP_002011059.1</a>
<input type="checkbox"/> <a href="#">hypothetical protein TRAVEDRAFT_25481 [Trametes versicolor FP-101664 SS1]</a>	37.0	37.0	70%	0.051	75%	<a href="#">XP_008031815.1</a>
<input type="checkbox"/> <a href="#">hypothetical protein JAAARDRAFT_29377 [Jaapia arqillacea MUCL 33604]</a>	36.6	36.6	91%	0.077	61%	<a href="#">KDG63354.1</a>
<input type="checkbox"/> <a href="#">hypothetical protein RSAG8_02323 [Rhizoctonia solani AG-8 WAC10335]</a>	35.8	35.8	64%	0.17	77%	<a href="#">KDN48970.1</a>
<input type="checkbox"/> <a href="#">metallothionein 1 [Laccaria bicolor]</a>	35.4	35.4	100%	0.20	62%	<a href="#">AHI43933.1</a>
<input type="checkbox"/> <a href="#">metallothionein [Taiwanofungus camphoratus]</a>	35.4	35.4	100%	0.22	59%	<a href="#">ABF69031.1</a>
<input type="checkbox"/> <a href="#">hypothetical protein HYDPIIDRAFT_117423 [Hydnomerulius pinastri MD-312]</a>	34.7	34.7	100%	0.33	74%	<a href="#">KJ60168.1</a>

**Fig5c:** Homologous sequences found in BLASTp analysis for *SiMT1*

**For *SiMT2***

Description	Max score	Total score	Query cover	E value	Ident	Accession
<input checked="" type="checkbox"/> <a href="#">metallothionein [Paxillus involutus]</a>	35.4	35.4	91%	0.20	59%	<a href="#">AAS19463.1</a>
<input checked="" type="checkbox"/> <a href="#">metallothionein [Pisolithus albus]</a>	34.7	34.7	91%	0.35	55%	<a href="#">AJO67962.1</a>
<input checked="" type="checkbox"/> <a href="#">metallothionein [Russula atropurpurea]</a>	33.5	33.5	91%	0.97	50%	<a href="#">AHA31882.1</a>
<input checked="" type="checkbox"/> <a href="#">metallothionein 2 [Amanita strobiliformis]</a>	32.0	32.0	91%	3.7	50%	<a href="#">AGO04615.1</a>
<input type="checkbox"/> <a href="#">hypothetical protein MPER_09911 [Moniliophthora perniciosa FA553]</a>	31.6	31.6	82%	4.1	55%	<a href="#">EEB91695.1</a>
<input type="checkbox"/> <a href="#">Metallothionein [uncultured eukarvoté]</a>	31.2	31.2	94%	5.3	48%	<a href="#">CCG34103.1</a>
<input type="checkbox"/> <a href="#">hypothetical protein RSAG8_02323 [Rhizoctonia solani AG-8 WAC10335]</a>	31.2	31.2	71%	5.9	56%	<a href="#">KDN48970.1</a>
<input checked="" type="checkbox"/> <a href="#">metallothionein [Piriformospora indica]</a>	31.2	31.2	74%	6.2	54%	<a href="#">ACT83730.1</a>
<input type="checkbox"/> <a href="#">hypothetical protein CC1G_05129 [Coprinopsis cinerea okavama7#130]</a>	31.2	31.2	82%	7.1	55%	<a href="#">XP_001833429.2</a>
<input type="checkbox"/> <a href="#">hypothetical protein JAAARDRAFT_29377 [Jaapia arqillacea MUCL 33604]</a>	30.8	30.8	85%	7.7	50%	<a href="#">KQQ63354.1</a>

**Fig5d:** Homologous sequences found in BLASTp analysis for *SiMT2*

The homologous sequences from different species were retrieved in the FASTA format and were analysed for the conserved regions by the multiple sequence alignment using MULTALIN

(<http://multalin.toulouse.inra.fr/multalin/>).

```

ShMT1  MSTATEVLVS  NN.NCGSSSC  SCGTSCQCKP  GGCKC.
ShMT2  MSTATEVLVS  NN.NCGSLIS  TCGTSCQCKT  GECNVD
Pisolithus  MOSVNAVLVN  NNGNCGSAAC  ACGSNACKP  GECKC.
Paxillus  MNTITSVPVN  FN.NCGSNCS  GCGSSACKP  GECKC.
Amanita  MOSESQSLVS  FA.NCGSNCS  NCGASCACKP  GDCKC.
Russula  MSPVIQNPVN  EH.HCGNSSC  TCGDSCQCKP  GECKC.
Piriformospora  .MISSETIVP  VNQNCGNSSC  SCGDSCQCKP  GECKC.
Laccaria  .MISTINVP  VSQTCGSSSC  NCGESCACKP  GECKC.
Ganoderma  .MYSTTDVV  KNAACGSSSC  NCGATCACKP  GECKC.

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**Fig6a:** Multiple sequence alignment of MT proteins of basidiomycetous fungi sequences (*Suillus himalayensis*).

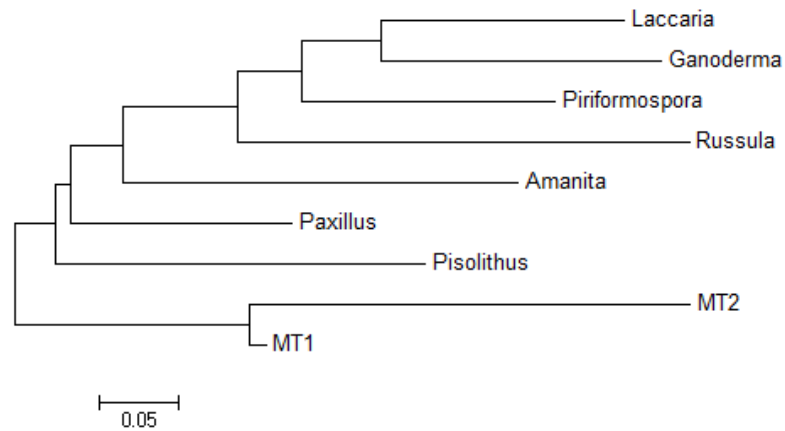
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SiMT1 MSTATEVLVS NN.NCGSSSSC SCGTSCQCKP GECKC.
SiMT2 MSTATEVLVS NN.NCGSLIS TCGTSCQCKT GECNVD
Paxillus MNTITSVPVN FN.NCGSNSC GCGSSCACKP GECKC.
Amanita MQSESQSLVS FA.NCGSNSC NCGASCACKP GDCKC.
Pisolithus MQSVNAVLVN NNGNCGSAAC ACGSNACKP GECKC.
Russula MSPVIQNPVN EH.HCGNSSC TCGDSCQCKP GECKC.
Piriformospora ..MISSETIVP VNQNCGNSSC SCGDSCQCKP GECKC.
Laccaria ..MISTINVP VSQTCGSSSSC NCGESCACKP GECKC.
Ganoderma ..MYSTTDVV KNAACGSSSSC NCGATCACKP GECKC.

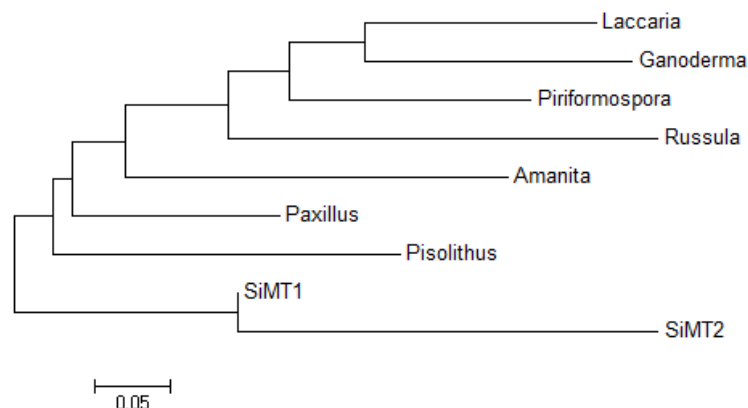
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**Fig6b:** Multiple sequence alignment of MT proteins of basidiomycetous fungi sequences (*Suillus indicus*).

Using MULTALIN software, MT protein sequences were compared with homologous sequences. It was found that MT1 sequence of both the strains has 100% homology with other MT (as they contain three c-x-c doublets). MT2 sequence was different as it contains one c-x-c doublet.



**Fig7a :** Phylogenetic analysis of MTs retrieved from the NCBI database (For *S.himalayensis*)



**Fig 7b:** Phylogenetic analysis of MTs retrieved from the NCBI database (For *S. indicus*)

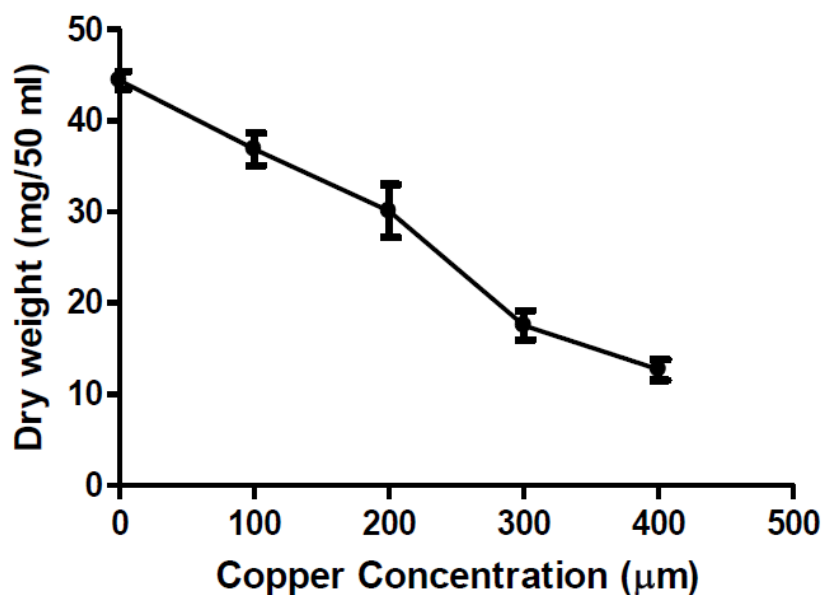
#### 4.2 Screening of *Suillus himalayensis* and *Suillus indicus* for their tolerance to different heavy metals

*Suillus himalayensis* and *Suillus indicus* were tested for their tolerance in the presence of Cu. This experiment was conducted to evaluate the possible interaction between heavy metals and tolerant fungi in regard to the physiological alterations. To evaluate the response of both the strains on different concentrations of Cu, pure mycelial culture was grown in liquid malt extract media and after 21 days of incubation at 25°C mycelium was harvested and dry weight was recorded.

**Table8:** Effect of different Cu concentrations on *Suillus himalayensis*.

Copper Concentration ( $\mu\text{M}$ )	Dry weight (mg/50ml) ( $\pm\text{SE}$ )
0	44.4 $\pm$ 0.987a
100	37.93 $\pm$ 1.785b
200	30.06 $\pm$ 2.894c
300	17.53 $\pm$ 1.605d
400	12.66 $\pm$ 1.146d

The values followed by same letter are non-significant at  $P < 0.5$ . The mean values were compared using Duncan's multiple range test



**Fig.8:** Effect of copper concentrations in the malt extract (2%) medium on the mycelial growth (mg/50ml) of *Suillus himalayensis*

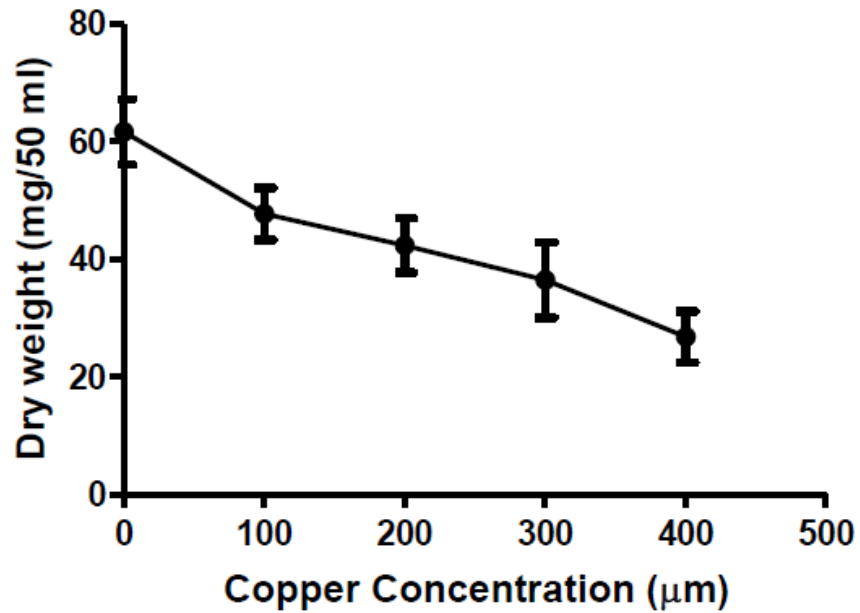
**Table9:** Effect of different Cu concentrations on *Suillus indicus*.

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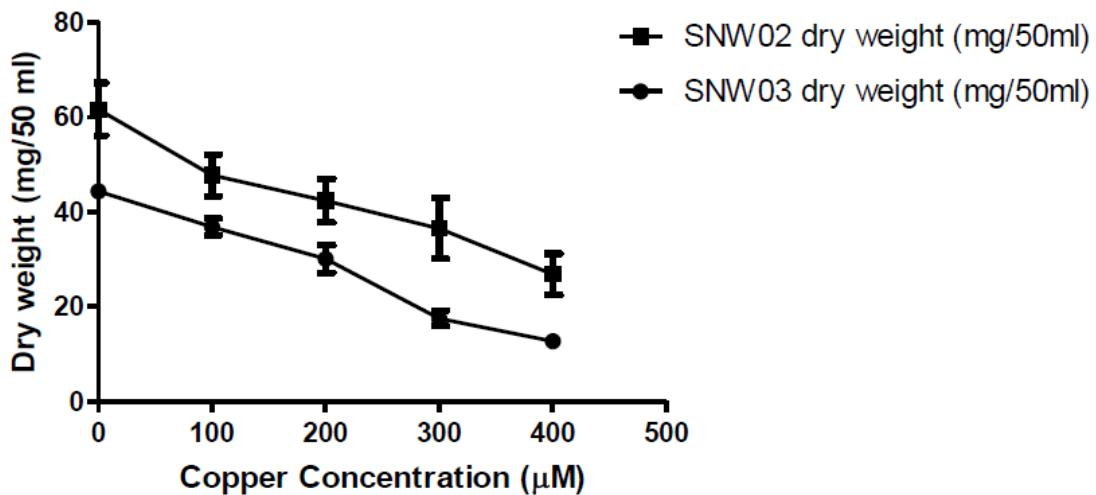
Copper Concentration ( $\mu\text{M}$ )	Dry weight (mg/50ml)
0	61.66a $\pm$ 5.56a
100	47.73 $\pm$ 4.45ab
200	42.3c $\pm$ 4.61bc
300	36.53 $\pm$ 6.40bc
400	26.83 $\pm$ 4.35c

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The values followed by same letter are non-significant at  $P < 0.5$ . The mean values were compared using Duncan's multiple range test.



**Fig. 9:** Effect of copper concentrations in the malt extract (2%) medium on the mycelia growth (mg/50ml) of *Suillus indicus*

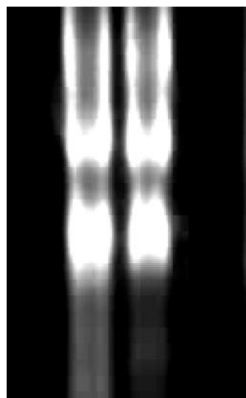


**Fig10:** Effect of copper concentrations in the malt extract (2%) medium on the mycelia growth (mg/50ml) of *Suillus indicus* and *Suillus himalayensis*

From the results it was found that the growth of *Suillus himalayensis* and *Suillus indicus* were adversely affected with high Cu concentration significantly. *S.himalayensis* showed 50% tolerance between (200-300)  $\mu$ M and *S.indicus* showed 50% tolerance between (300-400) $\mu$ M copper concentration and thereafter growth was decreased. So, this signifies that *Suillus indicus* more tolerant to Cu stress than *Suillus himalayensis*.

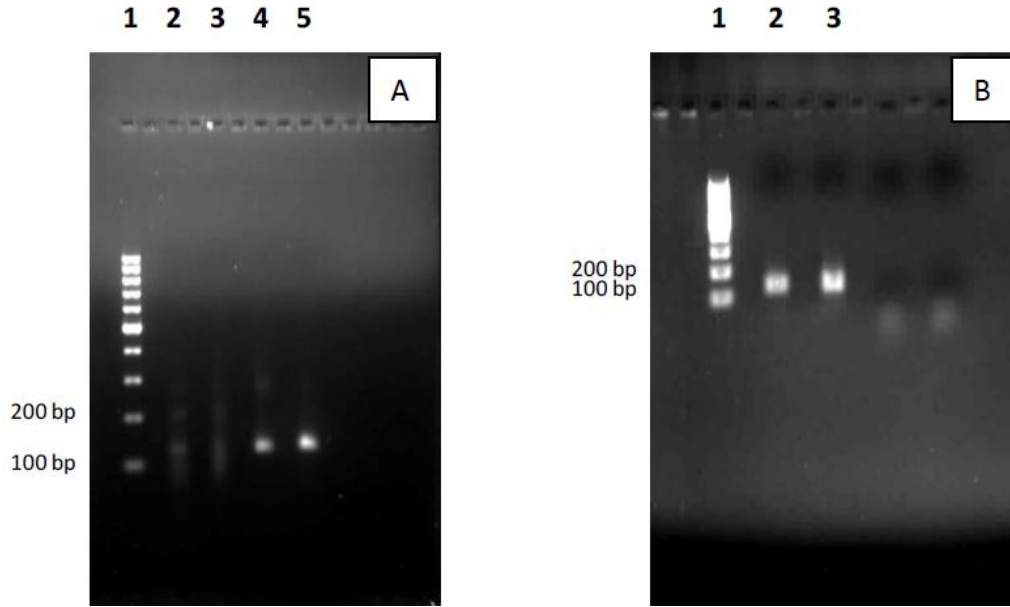
#### **4.4 RNA isolation, cDNA synthesis by RT-PCR and Gene specific PCR**

For the induction of metallothionein genes, *Suillus himalayensis* and *Suillus indicus* were grown on malt extract (ME) agar medium plate overlaid with processed cellophane for 15 days. Then mycelial cultures (along with cellophane) from both the strains were transferred to ME medium plates supplemented with 200 $\mu$ M Cu and incubated for 48 hours. After crushing the sample, total RNA was isolated using TRIzol method (fig:11). cDNA was synthesized with the 5 $\mu$ g total RNA sample by Reverse Transcriptase PCR.



**Fig11:** RNA isolation from *Suillus himalayensis* and *Suillus indicus*

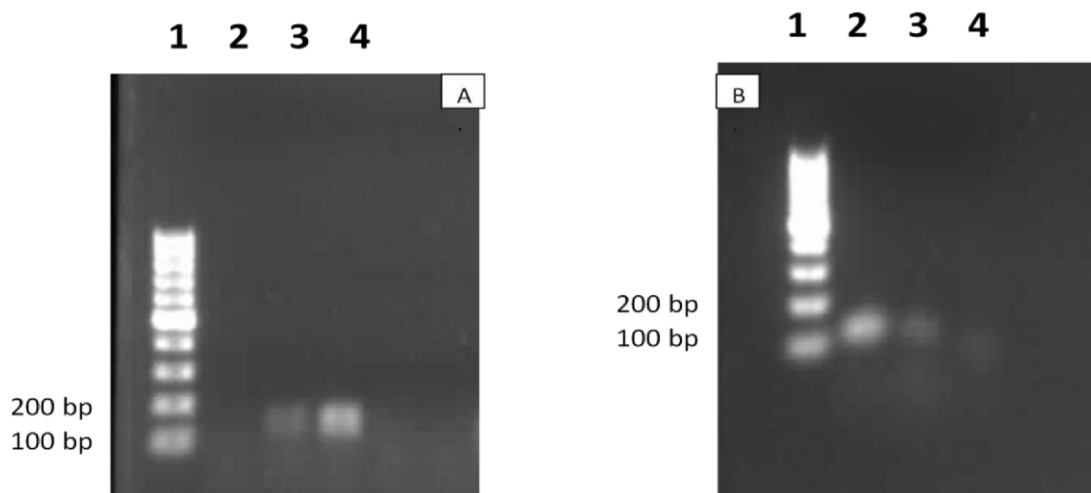
From the cDNA, metallothionein genes were amplified. After amplification, we got an amplicon around 100bp with MT primers.



**Fig12:** A) *ShMT1* and *ShMT2* gene amplification: 1- 100 bp, ladder. 2- *ShMT1* and 3- *ShMT2* B) *SiMT1* and *SiMT2* gene amplification: 1- 100 bp ladder, 2- *SiMT1*, 3 – *SiMT2*

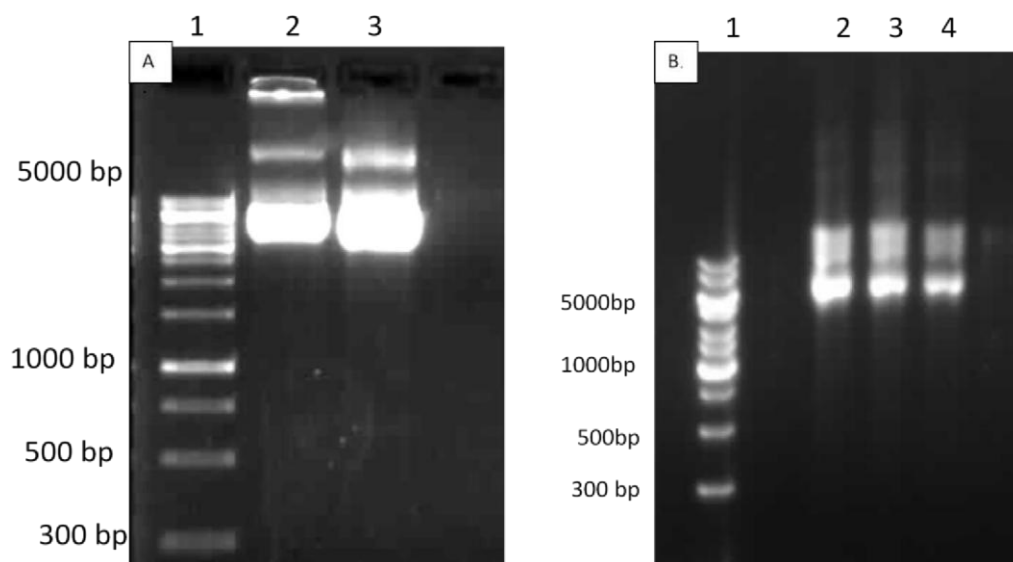
#### 4.4 Cloning of metallothionein genes

The amplified gene products from cDNA and PFL61 (shuttle vector) were digested with EcoR1 and BamH1 for 4 hours. After 4 hours of incubation, gene products and vector were purified using a Qiagen gel extraction kit (Qiagen, Valencia, CA, USA). The purified gene products were ligated with vector and the ligated products were transformed into *E.coli* DH5 $\alpha$  cells. The positive clones were screened by colony PCR (Fig: 13A,B), Plasmid isolation using QIAprep Spin Miniprep Kit (fig:14A,B) and amplification of plasmid using metallothionein gene primers (fig:15).



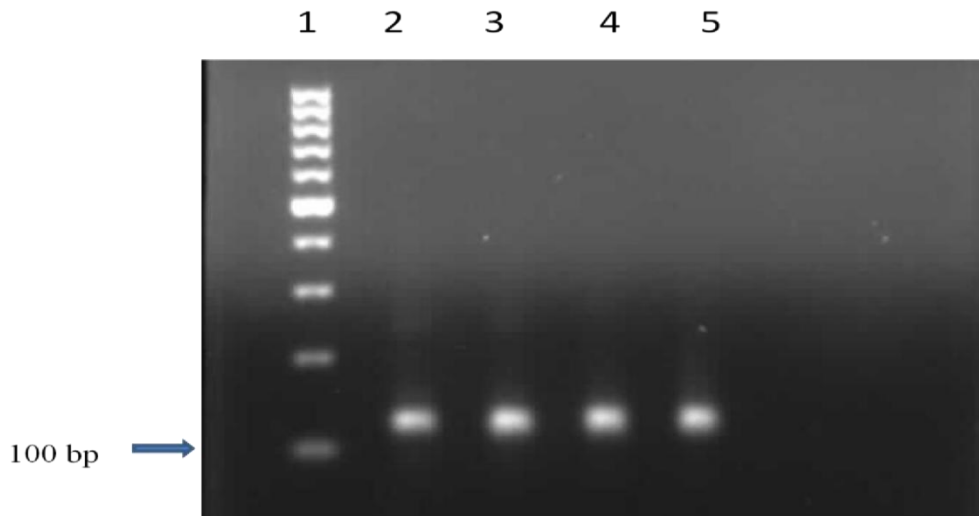
**Fig13:** A) The colony PCR of *ShMT1* and *ShMT2* genes 1- 100 bp ladder, 3-SHMT1, 4-SHMT2. B) The colony PCR of *SIMT1* and *SIMT2* genes 1- 100 bp ladder, 2-SIMT1, 3-SIMT2

From the colonies appeared after bacterial transformation, bacterial colony PCR was performed for the confirmation of positive colonies. In results, we got positive colonies because bands appeared at the size of genes of interest. This was the first method to confirm positive transformants.



**Fig14:** A) Plasmid isolation from cells transformed with *ShMT1*: Lane 1- 1Kb DNA Ladder, Lane2- *ShMT1* plasmid, Lane3- *ShMT2* plasmid; Fig B) Plasmid isolation from cells transformed with *ShMT1*: Lane 1- 1Kb DNA Ladder, Lane2- *SiMT1* plasmid, Lane3- *SiMT2* plasmid

This was the second method to confirm positive transformants. Plasmid was isolated from the colonies appeared after transformation. In the results, bands were appeared at the size of vector used in transformation.



**Fig15:** Plasmid Amplification: Lane1- 100bp ladder, Lane2- *ShMT1* gene amplified from plasmid, Lane3- *ShMT2* gene amplified from plasmid, Lane4- *SiMT1* gene amplified from plasmid, Lane5- *SiMT2* gene amplified from plasmid

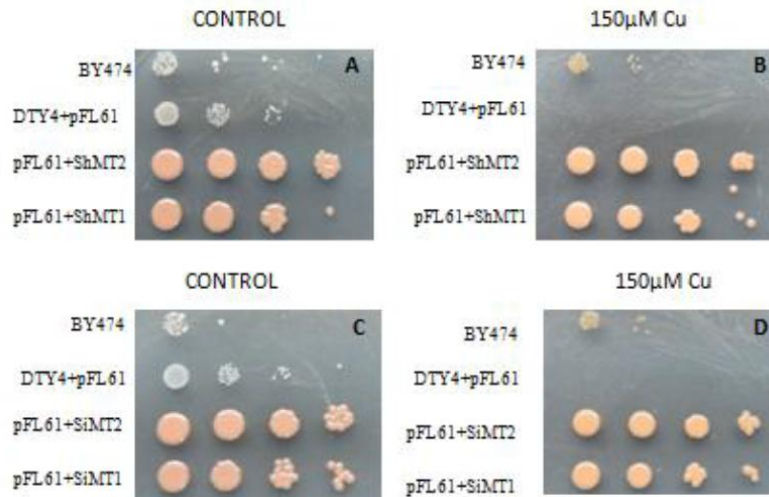
This was the final method to confirm positive transformants. The plasmid isolated from the colonies was further amplified using gene specific primers. In results, bands were appeared at the size of gene of interest.

#### 4.5 Yeast complementation studies

For testing the tolerance ability of metallothionein genes in high copper concentration, metal – sensitive strain, DTY4 was transformed with *ShMT1*, *ShMT2*, *SiMT1* and *SiMT2* genes using Lithium Acetate method. Positive transformants were selected by patching yeast colony on SDUra plates supplemented with copper.

DTY4 carrying PFL61 was grown in SD broth at 30°C with shaking at 230 rpm. Similarly, positive transformants and BY4741 (wild type strain) were grown on SD-Ura broth at 30°C with shaking at 230 rpm. After two days of incubation, cultures were serially diluted upto 0.001 and

5µl sample was spotted on SD-Ura plates and SD-Ura plates with the addition of 150 µM CuSO<sub>4</sub> and incubated at 30°C for 3 days.



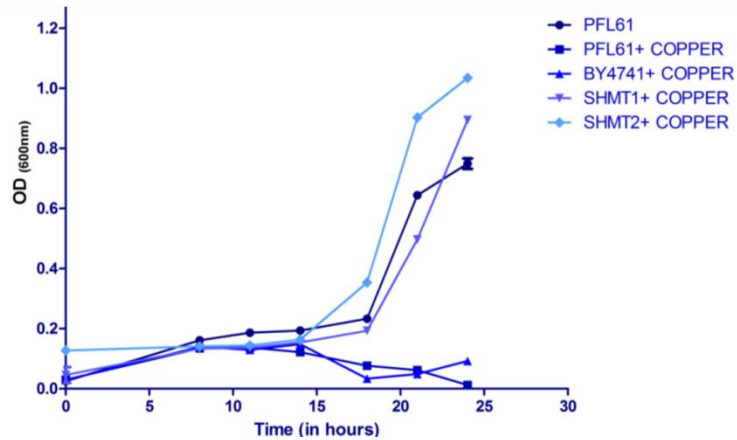
**Fig16:** Functional complementation and growth of Cu-sensitive yeast mutants on selective media.

(A,B) yeast mutant strains were transformed with PFL61 or PFL61- *ShMT1* and *ShMT2*. (C,D) yeast mutant strains transformed with PFL61 or PFL61- *SiMT1* and *SiMT2*. Diluted transformant cultures were spotted on SD-Ura medium with or without metal supplementation.

In the results, it was observed that at 150µM concentration of CuSO<sub>4</sub>, growth of DTY4+ pFL61 was completely inhibited but on SD-Ura, DTY4+ pFL61 has shown significant growth. Transformants carrying metallothionein genes have shown better expression than BY4741 (wild strain). It was also observed that *ShMT2* and *SiMT2* were more tolerant to Cu than *ShMT1* and *SiMT1*. Among the two strains, genes (*SiMT1* and *SiMT2*) isolated from *Suillus indicus* were more tolerant to Cu stress than *Suillus himalayensis*. To check resistance power of transformants, DTY4 carrying PFL61, all the transformants (pFL61+*ShMT1*, pFL61+*ShMT2*, pFL61+*SiMT1*, pFL61+*SiMT2*) and wild type strain BY4741 were grown in liquid medium.

**Table10:** Growth variation (mean±SE) in metal sensitive yeast with 150µM CuSO<sub>4</sub> (*ShMT1* and *ShMT2*)

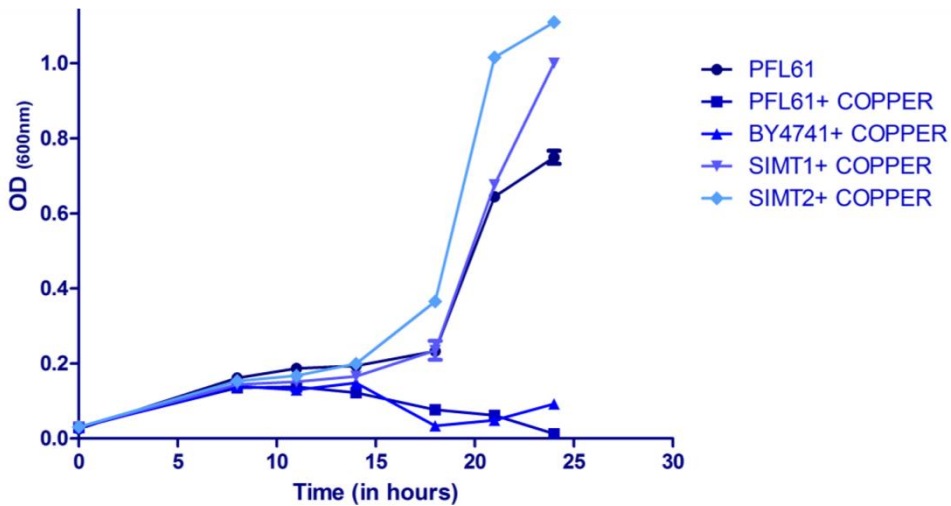
TIME(hours)	PFL61	PFL61+Cu	BY4741+Cu	<i>ShMT1</i> +Cu	<i>ShMT2</i> +Cu
0	0.025±0.005	0.030±0.0005	0.029±0.004	0.046±0.026	0.127±0.007
8	0.162±0.004	0.135±0.0050	0.139±0.004	0.134±0.001	0.141±0.002
11	0.187±0.002	0.138±0.0025	0.130±0.001	0.137±0.000	0.144±0.001
14	0.194±0.001	0.122±0.0030	0.148±0.001	0.155±0.004	0.164±0.002
18	0.233±0.001	0.077±0.0040	0.034±0.006	0.194±0.001	0.354±0.011
21	0.645±0.009	0.062±0.00300	0.049±0.001	0.498±0.003	0.903±0.007
24	0.750±0.018	0.012±0.0025	0.093±0.002	0.895±0.005	1.035±0.004



**Fig17:** Growth curve of Yeast mutant strains transformed with *ShMT1* and *ShMT2* genes in SD-Ura liquid medium with or without metal supplementation

**Table11:** Growth variation (mean±SE) in metal sensitive yeast with 150µM CuSO<sub>4</sub> (*SiMT1* and *SiMT2*)

TIME(hours)	PFL61	PFL61+Cu	BY4741+Cu	<i>SiMT1</i> +Cu	<i>SiMT2</i> +Cu
0	0.025±0.005	0.030±0.0005	0.029±0.004	0.030±0.0005	0.030±0.0005
8	0.162±0.004	0.135±0.0050	0.139±0.004	0.144±0.0010	0.154±0.0035
11	0.187±0.002	0.138±0.0025	0.130±0.001	0.152±0.0015	0.167±0.0005
14	0.194±0.001	0.122±0.0030	0.148±0.001	0.166±0.0040	0.199±0.0005
18	0.233±0.001	0.077±0.0040	0.034±0.006	0.235±0.0250	0.366±0.0085
21	0.645±0.009	0.062±0.00300	0.049±0.001	0.676±0.0015	1.016±0.0020
24	0.750±0.018	0.012±0.0025	0.093±0.002	1.000±0.0090	1.109±0.0015



**Fig18:** Growth curve of Yeast mutant strains transformed with *SiMT1* and *SiMT2* genes in SD-Ura liquid medium with or without metal supplementation.

From the liquid medium growth assay (Table: , Fig: ), it was conferred that the metallothionine gene (pFL61+*ShMT1*, pFL61+*ShMT2*, pFL61+*SiMT1*, pFL61+*SiMT2*) were expressed more significantly than BY4741 (wild strain), pFL61+Cu and pFL61 on 150 $\mu$ M copper concentration.

It was also observed that expression level of *ShMT2* and *SiMT2* was higher than *ShMT1* and *SiMT1*. Among the two strains, genes isolated from *Suillus indicus* were more tolerant to copper than *Suillus himalayensis*.

With increasing time of incubation pFL61-*ShMT1*, pFL61-*ShMT2*, pFL61-*SiMT1*, pFL61-*SiMT2* transformed DTY4 cells growth was increasing on SD-Ura supplemented with 150 $\mu$ M.

## DISCUSSION

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In the present study, metallothioneins of ectomycorrhizal fungus were characterized and evaluated for their metal tolerance ability. Two MT genes from *Suillus himalayensis* and two genes from *Suillus indicus* were used for this study. Various *Suillus* strains have been reported for metal tolerance (Table 2) but metallothionein has not been reported in any *Suillus* strain till date. The potential to tolerate heavy metal stress was tested by growing mycelial culture in liquid malt extract medium at different copper concentrations. From the results, it was observed that tolerance level of both the strains was different. *Suillus indicus* have shown better tolerance than *Suillus himalayensis*. In *Suillus indicus*, 50% growth was inhibited in between (300-400) $\mu$ M while in *Suillus himalayensis*, 50% growth was inhibited in between (200-300) $\mu$ M. Five putative metallothionein gene sequences were found in the EST library of *Suillus luteus*. Transcripts of these genes were obtained from the ORF finder and from the ORF sequences primers were designed. Genes of interest were cloned in *E.coli* to increase copy no. of the vector plasmid and then genes were transformed into metal-sensitive yeast strain. For functional verification Drop assay and Liquid assay was performed. In results, it was observed that *ShMT2* and *SiMT2* expressed more significantly. Several possible detoxification mechanisms have been investigated for explaining the variation in tolerance ability to copper among species. PiMT1 gene in *Paxillus involutus* have been shown to express under Cu stress (Bellion et al. 2007). Ramesh et al. (2008) identified two metallothionein genes (HcMT1 and HcMT2) in *Hebeloma cylindrosporum* and validate its functionality by Yeast complementation assay. Garacia et al. (2002) isolated crf1 gene that codes for transcription factor and confers resistance to copper, with MTs as target genes. Reddy et al. (2014) demonstrated metallothionein genes (*LBMT1* and *LBMT2*) of *Laccaria bicolor* in metal detoxification. Espey et al. (2003) investigated that well characterized metallothioneins of lower eukaryotes (*Neurospora crassa*) are synthesized mainly in response to toxic concentrations of copper but not by other stresses. Some agents which are capable of stimulating formation of free radicals are also known to be responsible for the induction of MT gene transcription (Bauman et al. 1991). In this study, we have improved our understanding of the copper tolerance mechanisms in *Suillus himalayensis* and *Suillus indicus* by demonstrating the key role of a MT in copper tolerance. Knowledge about metal detoxification mechanisms in ECM fungi will lead to powerful applications in Biotechnology.

## CONCLUSION

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This study focused on the identification of metallothionein genes and validation of its function in eukaryotes. Various molecular mechanisms were used in this study. *Suillus himalayensis* and *Suillus indicus* were tested for their resistance power against different concentrations of copper and from the results it was cleared that in the presence of 400 $\mu$ M CuSO<sub>4</sub> both the strains show least growth. The results indicated that both the strains showed different degree of tolerance against Cu. From the present data, it was conferred that *Suillus indicus* was little more tolerant to Cu than *Suillus himalayensis*. For the identification and characterization of metallothionein genes, primers were designed from the EST library of *Suillus luteus*. Five different genes encoding MTs were found. For the MT gene induction, Cu metal stress (200 $\mu$ M) was given to mycelium and RNA was isolated from the mycelium of the strains. cDNA was synthesized by Reverse transcription PCR and then cDNA was amplified for the presence metallothionein gene, by using gene specific primers. Four metallothionein genes were successfully identified from both the strains. To increase the copy number of plasmid inserted with gene of interest, Ligated products were transformed into *E.coli* DH5 $\alpha$  cells. For the functional verification of metallothionein genes, metal sensitive mutant yeast strain were transformed with gene of interest. For confirming yeast transformation and expression level of metallothionein gene, Yeast functional complementation studies were performed. MT genes were expressed in *Saccharomyces cerevisiae* metal-sensitive mutant strain and growth of the transformants was monitored on synthetic media (SD-Ura) with and without metal supplements (150 $\mu$ M Cu). From the results, it was conferred that among two metallothionein genes in each strain, *ShMT2* and *SiMT2* were more tolerant to Cu than *ShMT1* and *SiMT1* and among all the genes, *SiMT1* and *SiMT2* were more tolerant to copper than *ShMT1* and *ShMT2*.

So, this study has proved that these metal tolerant genes can be an efficient tool in phytoremediation.

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## Appendix

MEDIA	COMPOSITION
<b>Malt Extract media</b>	<ul style="list-style-type: none"> <li>• Malt Extract 30g/L</li> <li>• Mycological peptone 5g/L</li> <li>• Agar 15g/L</li> </ul>
<b>LB broth</b>	<ul style="list-style-type: none"> <li>• Bacto-tryptone 10 g/L</li> <li>• Bacto-yeast extract 5 g/L</li> <li>• NaCl 5 g/L</li> </ul>
<b>LB/amp<sub>+</sub> agar plates</b>	<ul style="list-style-type: none"> <li>• Prepare LB broth <input type="checkbox"/> Add agar(1.5g/ml)</li> <li>• Autoclave the media.</li> <li>• Cool it</li> <li>• Add ampicillin( 50μg/ml)</li> <li>• Pour media in the plates</li> </ul>
<b>SD-Ura Medium</b>	<ul style="list-style-type: none"> <li>• Add YNB 0.67g</li> <li>• Add Glucose 2g</li> <li>• Add 1% Adenine (1g)</li> <li>• Add 1% Tyrosine (1g)</li> <li>• Add 200 mg</li> <li>• Add amino acid mixture without Uracil 0.072 mg.</li> <li>• Add agar (1.5-2)g.</li> <li>• Autoclave</li> </ul>
<b>YPD Medium</b>	<ul style="list-style-type: none"> <li><input type="checkbox"/> Peptone 20 g/L</li> <li><input type="checkbox"/> Yeast extract 10 g/L</li> <li><input type="checkbox"/> Agar (for plates only) 20 g/L</li> </ul>

	<input type="checkbox"/>	
<b>Polyethylene Glycol (PEG)</b>	<input type="checkbox"/>	PEG 4000 40%
	<input type="checkbox"/>	TE buffer 1X
	<input type="checkbox"/>	LiAc 1X
<b>TBE Buffer (10x)</b>	<input type="checkbox"/>	Tris-HCl 0.09 M (pH 8)
	<input type="checkbox"/>	Boric acid 0.9 M
	<input type="checkbox"/>	EDTA 0.02 M (pH 8)
<b>Agarose gel loading dye (6X)</b>	<input type="checkbox"/>	Bromophenol Blue 0.25%
	<input type="checkbox"/>	Xylene Cyanol FF 0.25%
	<input type="checkbox"/>	Glycerol In Water 30.0%