Cloning and Characterization of Metallothionein genes of *Suillus* Species

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Submitted by

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(July 2016)

DECLARATION

I hereby declare that the work being presented in the M.Tech dissertation entitled "**Cloning and Characterization of Metallothionein genes of *Suillus* Species**" has been carried out by me during the period of July 2015 to July 2016, under the guidance of Dr. M.S Reddy, Professor, Department of Biotechnology, Thapar University, Patiala. Further, I declare that I have not submitted the matter embodied in this dissertation for the award of any other degree or any other qualification of any university or examining body in India/elsewhere.

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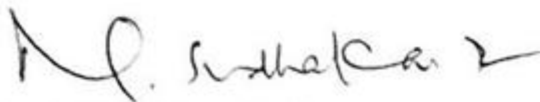
CERTIFICATE

This is to certify that the dissertation entitled "**Cloning and Characterization of Metallothionein genes of *Suillus* Species**" being submitted by **Ms. Tania Kalsotra** in partial fulfillment for the requirement of degree of Master of Technology in Biotechnology in the Department of Biotechnology, Thapar University, Patiala is a bonafide work carried out under the esteemed supervision and conception of **Dr. M.S Reddy**, Professor, Department of biotechnology, Thapar University, Patiala. The report has not been submitted for the award of any other degree or certificate in any other university or institute.



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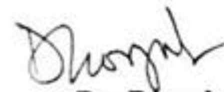

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ABBREVATIONS

Cu	Copper
Cd	Cadmium
$CdSO_4$	Cadmium sulfate
$CuSO_4$	Copper sulfate
cDNA	Complementary DNA
dATP	2-Deoxyadenosine 5'-triphosphate
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
dsDNA	double stranded DNA
ssDNA	single stranded DNA
DNA	Deoxyribonucleic acid
dTTP	2'-Deoxythymidine 5'-triphosphate
ECM	Ectomycorrhizal fungi
EDTA	Ethylenediamine tetra acetic acid
HCl	Hydrochloric acid
$HClO_4$	Perchloric acid
HNO_3	Nitric acid
Kb	Kilo basepair
LB	Luria broth
LA	Luria agar
$MgCl_2$	Magnesium chloride
MT	Metallothionein
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information
Ng	Nanogram
RNase	Ribonuclease
$ZnSO_4$	*Zinc Sulphate*

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ABSTRACT

Metallothioneins are low molecular weight proteins that are rich in cysteine residues and have the ability to bind to various metal ions like (eg: Zn^{2+}, Cu^{2+}). These proteins have been actively involved in the detoxification of heavy metals. They are involved in maintaining the haemostasis of essential traces metals as well as in sequestration of the environmentally toxic metals. The present study is focused on the characterization and evaluation of two MT genes in three *Suillus* species i.e *Suillus sibricus, Suillus himalayensis and Suillus indicus*. These two genes (MT1 and MT2) are found to be rich in the cysteine residues and have C-X-C motifs, which are the characteristic feature of metallothionein genes. The expression level of these genes was found to be increases with the increase in the external copper concentration. Heterologous complementation assays in the metal sensitive yeast strains indicated that both MT1 and MT2 genes encode for a polypeptide that provides tolerance against copper, cadmium and zinc. Higher expression level of MT1 and MT2 were observed in the three *Suillus* species when they were exposed to the different concentration of copper. Moreover there growth was also predicted at different concentration of copper. Thus the data obtained provides the evidence that metallothionien in *Suillus* species protects them from heavy metal stress.

1.1 Mycorrhiza

The term mycorrhiza which literally means fungal root is given to symbiotic relationship of soil fungi with the roots of higher plants. This symbiotic relationship is mutualistic in nature where plants provide nutrition and proper atmosphere to the fungus for its growth and development. The advantages that is provided to the host plant from fungus includes rapid growth, improved uptake of vital nutrients such as phosphate and inorganic nitrogen, enhanced tolerance to biotic and abiotic stress, increased resistance to plant pathogens, synergistic interaction with other advantageous soil microbes and improved soil structure for better aeration and water percolation (Ferrol *et al.,* 2002). Based on the relative location of fungi in roots, mycorrhizae were previously classified as ectomycorrhizae and endomycorrhizae. Now seven types of mycorrhizae are classified which includes: Arbuscular, Arbutoid, Ecto, Ericoid, Ectendo, Monotropoid, and Orchidaceous mycorrhiza (Figure 1). The characterstic feature of different mycorrhizae has been shown in Table 1. The most abundant and widespread mycorrhiza in forest communities are arbuscular mycorrhizal (AM) fungi (Glomeromycota) and ectomycorrhizal (ECM) fungi (Sandeep *et al.,* 2015).

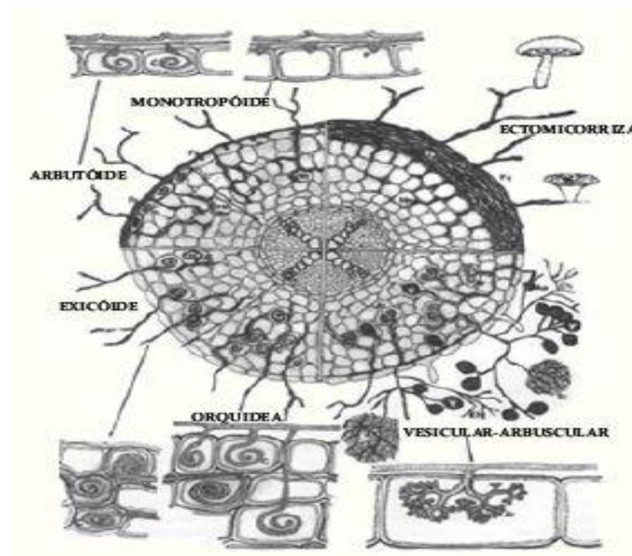

Figure 1: Different Types of Mycorrhiza (Sandeep *et al.,* 2015)

Table 1: Characteristics of different mycorrhiza (Smith and Read, 2010)

Kinds of mycorrhiza	Arbuscular	Ecto	Ectendo	Arbutoid	Monotropoid	Ericoid	Orchid
Fungi septate	-	+	+	+	+	+	+
Fungi aseptate	+	-	-	-	-	-	-
Intracellular colonization	+	-	+	+	+	+	+
Fungal mantle	-	+	+ or -	+ or -	+	-	-
Hartig net	-	+	+	+	+	-	-
Achlorophylly	- (+)	-	-	-	+	-	+
Fungal taxa	Glomero	Basidio/ Asco	Basidio/ Asco	Basidio	Basidio	Asco	Basidio
Plant taxa	Bryo Pterido Gymno Angio	Gymno Angio	Gymno Angio	Ericales	Monotropoideae	Ericales	Orchidales

1.2 Ectomycorrhizal fungi (ECM)

It is an association between fungus (basidiomycete or ascomycete) and root hairs of many plant species in which fungal hyphae forms a dense sheath, called mantle, around the lateral roots of plant. Initially there is very loose formation around the root but later on from the inner zone of mantle, the hyphae penetrate between epidermal cells of the root where it extends between outer cortical cells thereby forming a Hartig net (a network of fungal mycelium formed around the cortical cells due to the intracellular penetration) (Figure2). This Hartig net will act as an interface between both fungus and host plant for the exchange of nutrients, water and other compounds. For nutrients which are unavailable to plant roots, the individual hyphae or rhizomorphs (organized hyphal aggregates) grows out of the mantle and will extend itself into the forest soil and will provide the access of nutrients to the host plant (Luo *et al*., 2014).

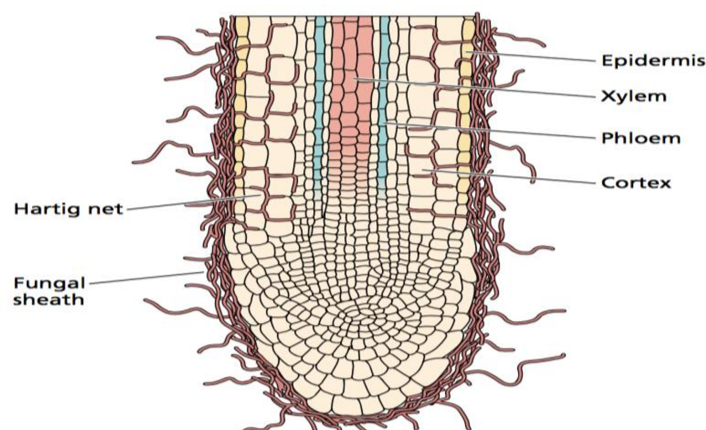

Figure2: Ectomycorrhizal fungi forming a Hartig's net in plant roots.

Almost 10,000 fungal species have been estimated to form ectomycorrhizae with host plant (Finlay, 2008). The plant species that are associated with ectomycorrhizae are mainly from *Betulaceae, Dipterocarpaceae*, *Cistaceae, Ericaceae, Fagaceae, Juglandaceae, Myrtaceae, Mimosaceae*, *Pinacea*e*, Salicaceae, Ulmaceae* families (Sandeep *et al.,* 2015). Various findings have suggested that ectomycorrhizal fungi exhibit the mechanism of metal tolerance and have the ability to detoxify these metals. Studies have shown that such fungus forms symbiotic relationship with the plants and helps their host to survive in the metal contaminated site by reducing the toxic metal stress (Adriaensen *et al.,* 2006; Krznaric *et al.,* 2009). Such type of fungi generally belongs to basidiomycetes or ascomycetes. The basidiomycetes ECM fungus that are found on heavy metal contaminated soils include *Suillus sp., Hebeloma sp., Pisolithus sp., Rhizopogon sp., Scleroderma sp, Lacaria sp. and Amanita muscaria*.

1.3 *Suillus* species

The genus *Suillus* belongs to Suillaceae family (Boletales, Basidiomycota). They are mostly found in the Mediterranean, temperate and boreal region while few of them may also occur in tropical area. Their mycorrhizal association is mainly constricted to the members of Pinaceae family and also with few deciduous species (Verma and Reddy, 2015). It not only provides nutrients to its host plant but also help them in various stress conditions. Various studies have revealed the ability of *Suillus* species to tolerate toxic metals like Zn, Cu and Cd (Adriaensen *et al., 2006;* Muller *et al.,* 2007). Mycorrhizal association of such *Suillus* species provides protection to pine seedlings from metal stress (Krznaric *et al.,* 2010). Such type of *Suillus* species can be used for the regeneration of pine seedlings as well as for large scale

afforestation in the metal contaminated area. Verma and Reddy (2014) obtained eight pure cultures of *Suillus* species (Table2) from basidiocarps collected from the coniferous forest of north western Himalayas in India. The growth and biomass yield was enhanced in *P. wallicharia* by all of these *Suillus* species. However *Suillus sibricus* (SNW06) was observed to be most effective for mycorrhizal association with *P. wallichiana* followed by *S. indicus* (SNW02) while *S. himalayensis (SNW03)* was least effective among all other isolates.

Table2: Eight *Suillus* species collected from coniferous forest of northwestern Himalayas in India (Verma and Reddy, 2014)

S.no	Species	Collection/pun no.	Isolate	MTCC accession no.
1	*Suillus triacicularis*	SHP27/PUN 5538	SNW01	11954
2	*Suillus indicus*	SHP07/PUN 6578	SNW02	11955
3	*Suillus himalayensis*	SHP26/PUN 5537	SNW03	11956
4	*Suillus granulates*	SJK13/PUN 5525	SNW04	11957
5	*Suillus sibiricus*	SJK01/PUN 5520	SNW05	11958
6	*Suillus sibiricus*	SHP05/PUN 6577	SNW06	11959
7	*Suillus sibiricus*	SHP12/PUN 6579	SNW07	11960
8	*Suillus sibiricus*	SUK12/PUN 5532	SNW08	11961

1.4 Heavy metals

Heavy metals are one of the major causes of environmental pollution. Their accumulation in the soil is either due to natural sources or due to human activity (Figure 3). The essential metals like Cu, Zn, Fe, Mo are required in minute concentration for the development of living organisms but can be highly toxic at their elevated concentration while other non-essential metals (like Pb, Ar, Cd) at low concentration can be highly toxic in nature and pose a serious threat for the environment and public health (Luo *et al.,* 2014). Their toxic effects are not limited to the health of living organisms but are also major factor for diminishing of biodiversity as well as productivity thereby causing a great threat to ecosystem by altering its function and structure (Niemeyer *et al.,* 2012; Mayor *et al.,* 2013). These elements are quite stable and cannot be destroyed. So it is essential to detoxify them in order to reduce their harmful effects for all the living organisms present in the environment.

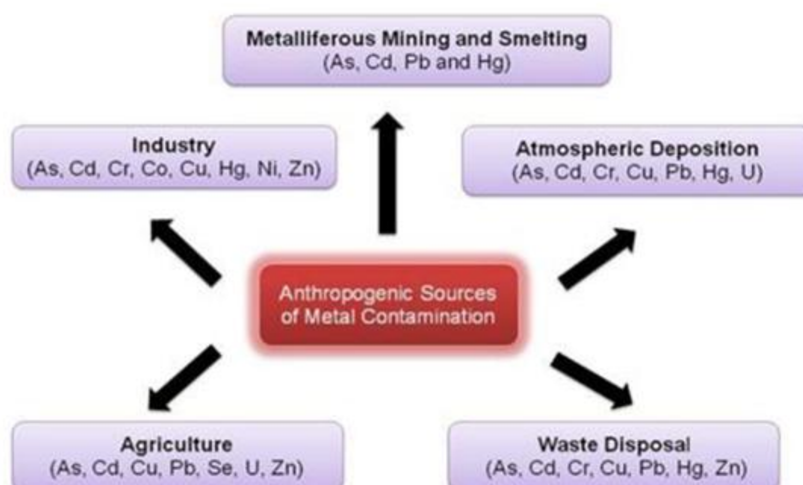

Figure 3: Anthropogenic Sources of heavy metal contamination in environment (Ahemad**,** 2012)

Various approaches (nonbiological and biological) have been proposed to remediate metal contaminated soil. However the conventional approaches like ultrafilteration, reverse osmosis, chemical precipitation, and electro dialysis have been found to be costly and have many drawbacks (Fazli *et al.,* 2015). So bioremediation or phytoremediation techniques are now a days in use in order to detoxify the heavy metals. These techniques involve the use of various microbes/plants in the environment that possess different mechanism for the removal of heavy metals. Such microorganisms or plants do this either by adapting or mutating at high metal concentration in the contaminated site. Among these organisms, the use of mycorrhizal fungi is of great advantage. Various studies have revealed the role of plants associated with ectomycorrhizal fungi or other microbes to detoxify the heavy metal polluted environment (Sabella *et al.*, 2016; Berthelot *et al., 2016*). The ability of different fungi to tolerate the heavy metals like Cu, Zn and to remediate them shows their importance for the bioremediation of heavy metals. This method has its own advantage as it is environmental friendly and cost effective. However the current challenge is to find the hyperaccumulating plants or microorganisms including ectomycorrhizal fungi which can transport these heavy metals into their harvestable parts by absorbing them from soil and detoxify them (Luo *et al.,* 2014). These heavy metals can be remediated by trees in combination with appropriate ectomycorrhizal fungi. The mechanism for the detoxification of metal by ectomycorrhizal fungi includes intracellular chelation or extracellular chelation. Among intracellular

chelators, metallothioneins (MTs) are of key importance that contributes to the detoxification of metal ions into the cell (Clemens, 2001).

1.5 Metallothionenins

MTs are low molecular weight proteins (<10KDa) that contains highly conserved cysteine residues and high metal content and has no aromatic amino acids or histidine (Bellion *et al.*, 2007). They are involved in maintaining the haemostasis of essential traces metals (eg: Zn, Cu) as well as in sequestration of the environmentally toxic metals (eg: Cd and Hg). The characteristic feature of MT is the presence of C-X-C, C-X-X-C or C-X-Y-C motifs, where X and Y represent an amino acid other than cysteine (Reddy *et al.,* 2014). The ability of MTs to bind with heavy metals such as Cu, Cd and Zn is due to the presence of sulfhydryl residues i.e cysteine. The direct activation of the protection phenomenon by MTs is achieved by their transcription which is typically regulated by the metal ions that binds to the protein. It has been reported that various cellular processes like detoxification, tolerance against free radical toxicity, differentiation, development and UV response involves the use of MTs (Coyle *et al.,* 2002). MTs are present in various living organisms including plants, mammals, fungi and cyanobacteria.

Metallothioneins are subdivided into three classes based on their heterozygous amino acid sequences (Binz and Kagi, 1999):

- Class I MTs was first identified in mammals and resembles to the eciuine renal MT. They are also found in certain fungi, such as *Neurospora crassa* and *Agaricus bisporus.*
- Class II metallothioneins are present in cynobacteria, yeast (*Saccharomyces cerevisiae*) and nematode *(Caenorhabditis elegans*) and they do not share extensive sequence homology with Class I proteins.
- Class III metallothioneins were first found in *Schizosaccharomyces pombe*. Their structure generally have poly (γ-glutamylcysteinyl)-glycine. They are also named as phytochelatins as they are commonly found in plants. This class of metallothioneins is now known to be widespread in fungi.

Two new classes of MT's have been identified on the basis of their metal binding ability: Cu-thioneins and Zn-thioneins (Palacios *et al.,* 2011).When exposed to copper enriched media,

Cu-thioneins forms homometallic Cu-MT complexes while Zn thionein forms homometallic Zn-MT complexes or Cd-MT complexes when they are exposed to Zn or Cd enriched media.

Till date very limited research has done on the heavy metal tolerance ability of the *Suillus* species in India. The present study is focused on configuring the ability of *Suillus species* for the metal tolerance invitro. For this purpose a full-length cDNA encoding metallothenins (MT1 and MT2) is cloned and characterized from three *Suillus* species i.e. *Suillus sibricus*, *Suillus indicus* and *Suillus himalayensis*, The metals used in the current study are copper, cadmium and zinc. To validate the functional role of metallothionein as a heavy metal binding ligand, these genes are expressed in *S. cerevisiae* metal- sensitive mutant strains.

2.1 Heavy Metals

The term heavy metal refers to the naturally occurring elements with the specific density of more than 5 gm/cm^3 that are found throughout the earth's crust. Their atomic weight ranges between 63.6 and 200.6 (Lakherwal, 2014). They are considered as environmental pollutants due to their toxic effects on human, plants and animals. Various metals like copper, chromium, iron, manganese, nickel, selenium, zinc, and molybdenum are essential micronutrients that are required for the functioning of various biochemical and physiological processes in the living organisms for their biochemical and physiological functions (Tchounwou *et al.,* 2012). However if the concentration of these essential elements increases it may result in variety of fatal diseases. Other metals like arsenic, lead, cadmium, and mercury are nonessential metals and their minute quantity can be highly toxic and may cause a bad impact on the environment and human health (Hall, 2002). The degree of toxicity of such metals not only depend on abiotic factors like metal concentration, humidity or pH value of the soil but also depends on biotic factors like presence of metal liberating microbes (Bellion *et al.,* 2006). Once they enter in the environment it is very difficult to degrade them as they are non-degradable in nature. Due to rapid industrialization there accumulation in the environment is steadily increasing (Clemens *et al.,* 2013). They pose threat not only for the terrestrial organism but also for aquatic organisms. Moreover they have also introduced in the food chain via plants, fishes, water and air.

2.2 Sources of heavy metal

The presence of heavy metal in the environment is either due to natural phenomenon or due to human activities. Natural source includes weathering of minerals, erosion and volcanic eruptions, forest fires, particles released by vegetation. Various anthropogenic activities directly or indirectly contaminate soil by releasing heavy metals in the soil. The activities like mining and smelting operations, production and use of metals or metal containing compounds in industries, and agriculture are involve in contamination of environment with heavy metals. The processing of metals in refineries, burning of coal or petroleum in power plants, use of microelectronics, nuclear power stations, manufacturing of batteries are the major source of heavy metal contamination from industries. The use of insecticides, herbicides, fungicides,

dye-stuffs all are manufactured in industries and are then used for agricultural applications thereby causing accumulation of heavy metals in the soil and water (Su, 2014)

2.3 Heavy metal detoxification

2.3.1 Conventional methods

It includes chemical precipitation, electrodialysis, coagulation, flocculation, ultrafilteration, reverse osmosis and adsorption techniques etc. These methods can be used efficiently where the concentration of metal is high or moderate but are ineffective where the metal ion concentration is low (Lakherwal, 2014). Due to the high reagent requirements and high energy demand this method is costly. Moreover the sludge or other waste products generated by this method is toxic and requires proper disposal (Fazli *et al.,* 2015).

2.3.2 Phytoremediation

This method involves the use of plants to remove, decrease or to decompose pollutants from the environment.

2.3.3 Bioremediation

In this method different microbes are use to remove pollutants from environment. Different microbes use different mechanism for removal of contaminants. They achieve this either by detoxifying, degrading, transforming or by neutralizing the toxic compound.

2.4 Mechanisms of heavy metal detoxification in mycorrhizal fungus

The toxicity cause by heavy metals in both mycorrhizal fungi and their host plants can be due to the molecular dysfuctioning of biomolecules like enzymes and transcriptional factor, which is caused by the displacement of heavy metals. The heavy metal can also inhibit the function of target biomolecules by binding to their thiol groups. Moreover these metals can block the thiol groups which can result in the over production of ROS (Sharma and Dietz, 2009; Schützendübel and Polle, 2002). For the protection against heavy metal toxicity it is necessary to activate the detoxification processes which is achieved by the proper coordination between the mechanism at cellular and molecular level (Figure 4). Various studies have revealed the cellular processes that are involved in the detoxification of excess heavy metals (Bellion *et al.,* 2006; Gallego *et* al., 2012; Seth *et al.,* 2012)

The cellular processes for detoxification of heavy metals mainly include:

a. Heavy metal binding to cell wall
b. Extracellular chelation of metal ions by secreted ligands.
c. Decrease uptake of metal ions or enhaced efflux
d. The uptake of metal ions will decrease or the metal ions will be pumped out of cytosol
e. Intracellular chelation by Metallothioneins (MTs) or phytochelatins (PCs)
f. Compartmentation of metal ions in vacuoles or in other subcellular structures.
g. Repair of metal damaged biomolecules
h. Antioxidative mechanisms that allow fungus to overcome oxidative stress by directly or indirectly reducing the accumulation of ROS species.

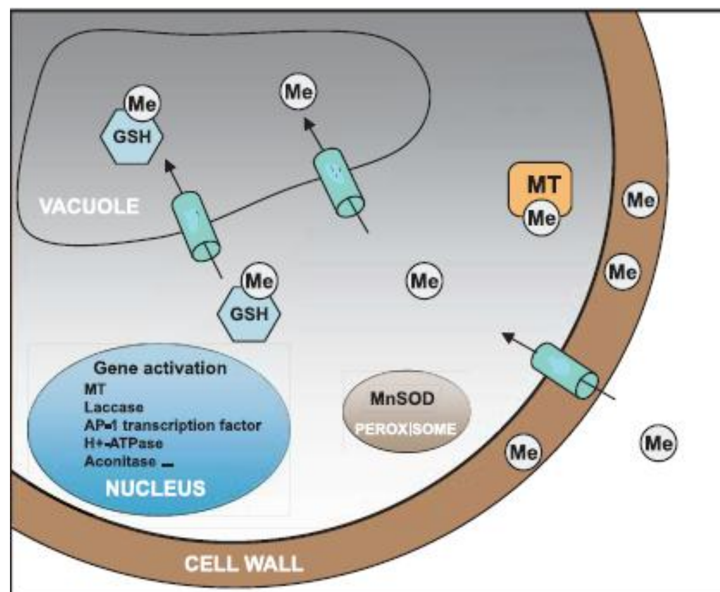

Figure 4: Heavy metal tolerance mechanisms in ectomycorrhizal fungi (Lanfranco, 2007)

The molecular processes in mycorrhizal plants mainly includes

a. Transcriptional regulation of heavy metal ion transporters which generally involves the modification in the activities of transporters for the transport and uptake of metal.
b. Differentially expressed transcriptomes which modulates availability of various molecules that can chelate heavy metals inside or outside cells.
c. Alteration of trancriptomes of host plants by ectomycorrhizae to mediate tolerance against different stresses including heavy metals

Few of these mechanisms are present constitutively while others are activated when there is increase in the concentration of metals (Colpaert *et al.,* 2011).

2.5 Metallothioneins

The ability of metallothioneins to bind the metals varies for different host species in response to different metals. The expression of different metallothionein isoforms in response to different metals is different in ectomycorrhizal fungi.

Hlozkova *et al.,* (2016) reported the presence of three Metallothionein genes (*AsMT1, AsMT2, AsMT3*) in *Amanita strobiliformis*. It was observed that when these genes were transformed in metal sensitive strain of *S. cerevisiae* they confer an increase tolerance against cadmium. *AsMT3* also confers tolerance against Zn. The yeast having *AsMT1* gene shows the best tolerance against copper.

Reddy *et al.,* (2016) studied the role of Metallothionein gene (*PaMT1*) isolated from *Pisolithus albus* for metal detoxification. It was observed that the *Eucalyptus tereticornis* associated with *Pisolithus albus* showed an upregulation of *PaMT1* when they were exposed to Cd or copper as compared to nonmycorrhizzal plants thereby suggesting that when the concentration of copper or cadmium increases the expression of *PaMT1* also enhances. However *PaMT1* expresses more in the presence of copper than cadmium.

Reddy *et al.,* (2014) characterized two MT genes (*LbMT1* and *LbMT2*) from the ectomycorrhizal fungus *Laccaria bicolor* and studied their different expressions to different metal stress. Both genes expressed differently when exposed to different metals. When exposed to copper both the genes express well but the expression of *LbMT1* was higher compare to *LbMT2*. In case of Cd only *LbMT1* was expressed while in the presence of Zn neither of two was expressed.

Sacky *et al.,* (2014) isolated three MT genes from *Hebeloma mesophaeum*, and were characterized. All the three MT isoforms (*HmMT1, HmMT2, HmMT3*) confers tolerance against copper and cadmium however *HmMT1* expresses best among the three in response to copper and cadmium but did not confer tolerance against silver. It was found that zinc and cadmium were the stronger inducers of *HmMT1* while *HmMT3* was induced in the presence of silver.

Blaudez and Chalot (2011) identified a gene (*HcZnT1*) from *Hebeloma cylindrosporum* that confers tolerance to only zinc metal. This gene encodes for ZnT1 transporters that were located in endoplasmic reticulum and plays crucial role in maintaining the concentration of

zinc in Hebeloma cells. ZnT1 transporters provide Zn ions to endoplasmic reticulum for their proper functioning and also help in detoxification of cytosol under zinc stress.

Osobova *et al.,* (2011) reported three isoforms of Metallothionein (*AsMT1a, AsMT1b and AsMT1c*) in *Amanita strobiliformis* and studied their role for the sequestration of intracellular silver ions. It was observed that *AsMT1a* was involved in the sequestration of silver in *A. strobiliformi*.

Ramesh *et al.,* (2009) characterized two MT genes (*HcMT1, HcMT2*) from *Hebeloma cylindrosporum*. Both *HcMT1* and *HcMT2* were capable of providing tolerance against copper. Moreover the presence of Cd induces the transcription of *HcMT1* but it did not affected *HcMT2*. Both the MT genes were not induced in the presence of zinc, lead and nickel.

Bellion *et al.,* (2007) studied the characterization of MT gene (*Pimt1*) from *Paxillus involutus*. It was found that Pimt1 gene encodes for MT that provides tolerance against copper and cadmium. Both copper and cadmium were the strong inducers of Pimt1 in *Paxillus involutus*.

2.6 Metal tolerance in *Suillus* species

Suillus species are important genera of ectomycorrhizal fungi with almost 50 different species described worldwide. Various researches have revealed the ability of *Suillus* species to tolerate heavy metal stress.

Ruytinx *et al.,* (2013) studied the ability of ectomycorrhizal basidiomycete *Suillus bovinus* to tolerate high zinc concentration which is achieved by the removal of zinc out of the cytoplasm into apoplast. This removal of zinc out of the cell wall results in the lower influx of zinc in host plants.

Sousa *et al.,* (2012) studied the effect of Cd exposure on *Pinus pinaster* colonized with two ectomycorrhizal fungi: *Suillus bovinus* and *Rhizopogon roseolus*. It was observed that in the presence of *S bovinus* the shoot growth was enhanced by 30% at contaminated soil while no significant effect was shown on contaminated soil having *R. roseolus*. They hypothesized that the association of ECM fungi with host plant improves the development as well as metal uptake of *P. pinaster* seedlings. They further concluded that such type of fungi should be used at metal contaminated areas for restoration processes by woody species.

Krznaric *et al.,* (2010) studied that *Suillus luteus* isolated from Zn and Cd polluted soil protects the host plant (*Pinus sylvestris*) from high metal stress by reducing the nutritional starvation and transfer of excessive metal.

Krznaric *et al.,* (2009) studied the effect of cadmium on the pine seedlings inoculated with Cd tolerant *Suillus luteus* strain. The results suggested that scot pine seedlings inoculated with Cd tolerant *S. luteus* showed improved plant protection, enhanced fungal biomass and nutrients uptake as compare to the plants inoculated with Cd sensitive isolate. Such *S. luteus* isolates which shows tolerance to cadmium can be used for pine forest establishment on cadmium contaminated soil.

Johansson *et al.,* (2008) observed that when Pb and Cd treatments were given to ectomycorrhizal scot pine seedlings the production of oxalate and low molecular weight organic acids were increased by 15-45%. They compared mycorrhizal seedlings with non mycorrhizal seedlings and found that the presence of mycorrhizal seedlings generally increase exudation of organic acids. The ectomycorrhizal fungi used in their studies were: *Hebeloma velutipes*, *Piloderma byssinum, Paxillus involutus, Suillus bovinus*, *Suillus variegates* and *Rhizopogon roseolus*. They concluded that the performance of mycorrhizal seedlings was improved due to detoxification of metal ions which is achieved by increasing the exudation of organic chelators that chelates Pb and Cd.

Muller *et al.,* (2007) studied the gene expression of *Suillus luteus* exposed to high Zn concentration. Their findings suggested that the uptake and transport of Zn in *S. luteus* is regulated at transcriptional level. The genes that encodes for the metal transporters or for other proteins that are involved in ubiquitin dependent degradation of proteins may be responsibe for defense against heavy metals.

Adriaensen *et al*., (2006) reported that the Zn adapted *S. bovinus* improves the growth of *Pinus sylvestris* seedlings exposed to excessive Zn. To evaluate the effect of Zn in excessive concentration, an exposure of 150 µM Zn was given to mycorrhizal and nonmycorrhizal pine seedlings for 9 months. It was observed that the growth and chlorophyll concentration of nonmycorrhizal plants were inhibited strongly when exposed to excess Zn while the plants colonized with *S. bovinus* were healthier and their growth was also better. Thus they hypothesized that the *Suillus bovinus* provides resistance to the host plant against Zn toxicity.

Adriaensen *et al.,* (2005) discovered an ectomycorrhizal fungus *Suillus luteus* that was associated with pine trees in central Norway. They observed that *S. luteus* has potential to protect pine trees from the toxic effect of Cu. Further they studied the existence of co-tolerance in *S. luteus* to Cu and Zn. The result indicated that the *Suillus* species from Cu mines showed high tolerance ability for Cu, whereas the *Suillus* species that were tolerant to Zn were sensitive to Cu and vice versa.

Blaudez *et al.,* (2000) studied thirty nine ectomycorrhizal fungi for the tolerance against cadmium, copper, nickel, zinc. It was observed that *Suillus luteus*, *Suillus variegates* and *Pisolithus tinctorius* exhibit high tolerance for Cu, Cd and Zn as compared to *Paxillus involutus*, which shows high tolerance to Ni.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Biological materials and culture conditions

A pure culture of *Suillus sibricus, Suillus indicus and Suillus himalayensis* (collected from coniferous forests of the northwestern Himalayan region) were maintained on malt extract medium (appendix) at 25^0C in the dark conditions.

3.1.2 Bacterial strain and culture conditions

E.coli DH5α strain was used for the transformation of metallothionein gene. The strain was maintained at 37^0C on Luria agar medium.

3.1.3 Yeast strains and culture conditions

The *Saccharomyces cerevisae* strains used for complementation assays were *DTY4*, (*MATα*, *trp1-1*, *leu2-3*, *leu2-112*, *gal1*, *his−*, *ura3-50*, *cup1Δ::URA3+*), *DTY3* wild-type strain (*MATα*, *trp1-1*, *leu2-3*, *leu2-112*, *gal1*, *his−*, *ura3-50*, *URA3−*), *ycf1* (*MATa*, *his3D1*, *leu2D0*, *met15D0*, *ura3D0*, *YDR135::kanMX4*), BY4741 strain (*MATa*, *his3D1*, *leu2D0*, *met15D0*, *ura3D0* and *zrc1* (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ZRC1::kanMX4). All the strains were maintained at 30^0C on YPD medium.

3.1.4 Heavy metals

Copper ($CuSO_4$), Cadmium ($CdSO_4$) and Zinc ($ZnSO_4$)

3.2 Tolerance of ECM fungi to different metals

The tolerance of ECM fungi (*Suillus sibricus, Suillus indicus and Suillus himalayensis)* to different concentrations of copper was determined by growing them on malt extract broth. From the freshly grown fungus, 3-4 discs were cut and added in each flask having 50 ml malt extract broth. The flasks were incubated at 25^0C for 5 days in the dark conditions, so as to initiate the growth. After 5 days, $CuSO_4$ was added in different flasks with varying concentrations (0, 100, 200, 300 and 400 µM) and were incubated at 25^0C for 21 days in the dark. After 21 days, the mycelium was harvested and washed with saline water followed by distilled water. The mycelium was then dried in oven at 50-60^0C for 4-8 hours and the dry

weight was noted. Further the mycelium was digested with $HNO_3/HClO_4$ and the metal uptake by mycelium was calculated using atomic absorption spectroscopy.

3.2.1 Nitric acid / Perchloric acid digestion

1. 200-400 mg sample of air dried mycelia was weighed in digestion tubes.
2. 10 ml of Conc. HNO_3 was added in it.
3. In acid proof digestion chamber with fume exhaust system, the digestion tubes were placed on electric heater for 1 hr at 100^0C.
4. The tubes were then cooled and 5 ml $HClO_4$ was added and was heated at 100^0C.
5. The digestion was continued until the contents become colorless and only white fumes appear.
6. The acid content was reduced till white matter was left in the digestion tubes and the tubes were then cooled.
7. The samples were then dissolved in required amount of MQ H_2O and the heavy metal uptake was examined with atomic absorption spectroscopy.

3.3 Molecular methods

3.3.1 RNA isolation

The *Suillus* species (*Suillus sibricus, Suillus indicus and Suillus himalayensis)* were grown on malt extract agar plates overlaid with cellophane sheets for 15 days at 25^0C. After 15 days, the cellophane sheets with mycelium were shifted on to malt extract agar plates having $CuSO_4$ in varying concentrations (0, 100, 200, 300 and 400 µM) and incubated at 25^0C for 48 hours. After 48 hours, the mycelium was scraped off from the cellophane sheets and crushed into fine powder by using liquid nitrogen and was stored at -80^0C. The isolation of total RNA was done from the frozen mycelia powder by using QiAzol lysis reagent (QIAGEN).

1. To approximately 100 mg of powdered mycelia, 1 ml of Qiazol reagent was added and vortexing was done for the appropriate homogenization.
2. The samples were then kept at 15^0C for 10 minutes and then centrifugation was done at 12000g for 10 minutes at 4^0C.
3. The supernatant were collected in fresh tubes and 200 µl of chloroform was added and mixed by inverting the tubes.

4. The sample solutions were then incubated at 15^0C for 2-3 minutes and centrifuged at 12000g for 15 minute at 4^0C.
5. The upper aqueous layer containing RNA was removed from the sample solutions and was then transferred to the fresh tubes.
6. To the aqueous layer, 500 µl of chilled isopropanol was added to precipitate RNA and the tubes were stored at -20^0C for 1 hour.
7. After 1 hour, centrifugation was done at 12000g for 10 minute at 4^0C.
8. The supenatent was discarded and the pellet was washed with 75% of chilled ethanol.
9. Centrifugation was done at 7500g for 10 minute at 4^0C.
10. Supernatent was discarded and pellet were air dried and dissolved in DEPC treated water and stored at -80^0C

3.3.2 Electrophoretic analysis of RNA on agarose gel

1.5% of agrose gel was prepared in 0.5x TBE (Appendix) using a 5x TBE (Appendix). Before pouring, the gel was stained with Ethidium Bromide (0.5 µg/ml) to visualize RNA band. After the solidification of gel, RNA sample was mixed with loading dye and was then loaded into wells. The voltage was supplied for the migration of RNA samples. The band of RNA was then visualized in UV illuminator.

3.3.3 Spectrophotmetric analysis:

The quantification of RNA was evaluated by using nanodrop spectrophotometer. The purity of RNA was evaluated by 260/280 ratio. If the 260/280 ratio is approximately 2 it indicates pure RNA. Lower ratio represents the presence of contaminants that can absorb at or near 280nm.

3.3.4 cDNA synthesis:

Once the purity and quantity of RNA was checked the next step was preparation of cDNA from total RNA. The cDNA was synthesized using revert aid first strand cDNA synthetic kit. For making cDNA, 1 µl oligo dT primer (50 µM), 1 µl dNTP mixture (10mM), 5 µg RNA template and RNase free water were added in a tube to make final volume to 10µl. The reaction mixture was heated at 65^0C for 5 minute and was then cooled immediately on ice. In the reaction mixture, 4 µl of 5X primer script buffer, 0.5 µl RNase inhibitor, and 1 µl Reverese transcriptase were added and the final volume was made to 20 µl by using RNase

free water. Gentle mixing was done and the tubes were kept at 42^0C for 60 minutes and then at 70^0C for 15 minutes. The samples were then stored at -20^0C until further use.

3.3.5 Quantitative reverse transcription PCR (qRT-PCR)

After cDNA synthesis the gene expression analysis for *S. indicus, S. sibricus and S. himalayensis* were performed using Real Master Mix SYBR ROX qPCR kit. The final reaction volume consist of 12.5 µl master mix, 1µl forward primer, 1 µl reverse primer, 0.75 µl cDNA template and 9.75 µl H_2O. The mastercycler® ep realplex real-time PCR system was used to perform qPCR reaction. The cycling program used for qPCR was as follow: 95^0C for 2min (1 cycle), 95^0C for 15 s, 55^0C for 15 s and 68°C for 20 s (45 cycles). As an internal reference for normalization of qPCR data, Sact genes were used. Level of gene expression as compare to control were thus calculated by using the formula

$$R = \frac{(E_{target})^{\Delta CP_{target}(control-sample)}}{(E_{ref})^{\Delta CP_{ref}(control-sample)}}$$

3.3.6 Gene amplification:

Primer designing

Five putative Metallothionein gene sequences of *Suillus luteus* with the accession number GR975901, GR975896, GR975716, GR975715, GR975714 were retrieved from the EST library of NCBI (www.**ncbi**.nlm.nih.gov/). The transcripts of these genes were obtained from ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Further BLASTp was used to obtain the homologous sequences. The five sequences retrieved from EST library were:

GR975901

GTATGCATCTAGATTGATGAGTCCTGAGTAAAACGCCTCTGCTCGACGAACATCCATACA
TCTCCTACTATAATCATAGGCCGACAACATAATACACGACGAGGCCGAGTTCAAGATTCG
ATATTCAACGTTCAATCAACATTTGCACTCTCCAGGCTTGCATTGGCACGAAGTGCCGCA
TGAGCAGCTCGACGAGCCACAGTTGTTGTTAGAAACAAGGACTTCAGTAGCGGTGGACA
TATTGTGATAGATCACTACGCAGT

GR975896

GCGATGCATCTAGATTGACTGCGTAGTGATCTATCACAATATGTCCACCGCTACTGAAGT
CCTTGTTTCTAACAACAACTGTGGCTCGTCGAGCTGCTCATGCGGCACTTCGTGCCAATG
CAAGCCTGGAGGGTGCAAATGTTGATTGAACGTTGAATATCGAATCTTGAACTCGGCCTC

GTCGTGTATTATGTTGTCGGCCTATGATTATAGTAGGGGATGTATGGATGTTCGTCGAGC
AGAGGCGTTTTACTCAGGACT

GR975716
CCTGGTACTCGCGATGCATCTAGATTGACTGCGTAGTGATCTATCACAATATGTCCACCG
CTACAGAAGTCCTTGTTTCTAACAACAACTGTGGCTCGTTGATTAGCACATGCGGCACTT
CGTGCCAATGCAAGACTGGAGAGTGCAATGTTGATTGAACGTTGAATATCGACTCTGGA
ACTCGGCCTCCTCGTGTATTATGTTGTCGGCCTTTG

GR975715
GATGAGTCCTGAGTAATACGCCTCTGCTCGACGAACATCCATACATCTCCTACTATAATC
ATAGGCCGACAACATAAAACACGACGAGGCCGAGTTCAAGATTCGATATTCAACGTTCA
ATCAACATTTGCACTCTCCAGGCTTGCATTGGCACGAAGTGCCGCATGAGCAGCTCGACG
AGCCACAGTTGTTGTTAGAAACAAGGACTTCAGTAGCGGTGGACATATTGTGATAGATC
ACTACGCAGT

GR975714
GCTCGGTACTCGCGAATGCTCTAGATTGACTGCGTAGTGATCTTCTGCGACTGCGTCTGG
TCTACCGCTTCTGGAGCTAAAACCTCTGCCACTACCACTGCATCTTCTGGTACAACTCAG
AAGACCGGCGCTGCCAGTAGCCTTTCTGTCTCTTCGGCAATGGGTGTTGCCGGTGTGATG
AGTCCTGAGTAATACGCCTCTGCTCGACGAACATCCATACATCTCCTACTATAATCATAG
GCCGACAACATAATACACGACGAGGCCGAGTTCAAGATTCGATATTCAACGTTCAATCA
ACATTTGCACTCTCCAGGCTTGCATTGGCACGAAGTGCCGCATGAGCAGCTCGACGAGCC
ACAGTTGTTGTTAGAAACAAGGACTTCAGTAGCGGTGGACATATTGTGATAGATCACTAC
GCAGT

The multiple sequence analysis of these genes was performed (Figure 5) so as to analyze the similarity between them. After multiple sequence alignment the four transcripts were found to be highly similar which indicates the presence of two MT (MT1 and MT2) genes in *Suillus* species. Gene specific primers were designed manually (Table 3) and restriction sites were added to the 5'end of each primer. *Bam*H1 site was added to the 5'end of forward primer and *Eco*R1 at 5'end of reverse primer.

>GR975901 MSTATEVLVSNNNCGSSSCSCGTSCQCKPGECKC-
>GR975896 MSTATEVLVSNNNCGSSSCSCGTSCQCKPGGCKC-
>GR975714 MSTATEVLVSNNNCGSSSCSCGTSCQCKPGECKC-
>GR975715 MSTATEVLVSNNNCGSSSCSCGTSCQCKPGECKC-
>GR975716 MSTATEVLVSNNNCGSLIS-TCGTSCQCKTGECNVD

Figure 5: Multiple sequence alignment of different Metallothionein EST of *Suillus* species

Table 3: PCR primers designed for the amplification of MT (MT1, MT2) genes of *Suillus himalayensis* (*ShMT1* and *ShMT2*), *Suillus sibricus* (*SsMT1* and *SsMT2*) and *Suillus indicus*(*SiMT1* and *SiMT2*)

ShMT1F:	**5'-**CGGGATCCATGTCCACCGCTACTGAAGTC**-3'**
ShMT1R:	5'-CCGGAATTCTCAACATTTGCACTCTCCAGG-3'
ShMT2F:	5'-CGGGATCCATGTCCACCGCTACTGAAGTC-3'
ShMT2R	5'- CCGGAATTCTCAATCAACATTGCACTCTCCAG-3'
SsMT1F:	5'-CGGGATCCATGTCCACCGCTACTGAAGTC-3'
SsMT1R:	5'-CCGGAATTCTCAACATTTGCACTCTCCAGG-3'
SsMT2F:	5'-CGGGATCCATGTCCACCGCTACTGAAGTC-3'
SsMT2R	5'- CCGGAATTCTCAATCAACATTGCACTCTCCAG-3'
SiMT1F:	5'-CGGGATCCATGTCCACCGCTACTGAAGTC-3'
SiMT1R:	5'-CCGGAATTCTCAACATTTGCACTCTCCAGG-3'
SiMT2F:	5'-CGGGATCCATGTCCACCGCTACTGAAGTC-3'
ShMT2R	5'- CCGGAATTCTCAATCAACATTGCACTCTCCAG-3'

Underlined sequences are *Bam*H1and *Eco*R1 sites.

cDNA synthesis was confirmed by amplifying it with metallothionein gene specific primers. The amplification reaction mixture was prepared as given in table 4.

Table 4: PCR reaction mixture for amplification of gene

Component	Concentration
10X Taq buffer containing $MgCl_2$,	2.5 µl
2 mM DNTPs	2 µl
Forward primer	1 µl
Reverse primer	1 µl
Taq polymerase (5U/ µl Sigma-Aldrich)	0.3 µl
Template	1 µl
MQ H_2O	17.2 µl
Total	25 µl

The PCR was then run in thermocycler for 35 cycles with following PCR program (Table 5) and amplified product was run on 1.5% agarose gel and visualized in gel documentation system.

Table 5: PCR program for gene amplification

Steps	Temperature	Time period
Initial Denaturation	94^0C	3 minutes
Denaturation	94^0C	1 minutes
Annealing	55^0C	1 minutes
Elongation	72^0C	1 minutes
Final Elongation	72^0C	8 minutes

3.3.7 Purification of amplified gene:

The amplified gene (MT1 and MT2) was purified by using Gene jet PCR purification kit. The procedure used for purification was as follow:

1. To the amplified gene mixture binding buffer was added in 1:1 ratio followed by vigorous mixing.
2. The solution was then transferred to the GeneJET purification column and centrifugation was done at 12000rpm for 1 minute.
3. 700 µl of wash buffer was then added in the column and centrifugation was done at 12000 rpm for 1 minute. The flow through was discarded.
4. The empty column was again centrifuged for 1 minute at 12000 rpm to remove any residual wash buffer
5. The column was then tranfered to new microcentrifuge tube
6. 30 µl of MQ H_2O was added at the centre of column to elute DNA and centrifugation was done at 12000 rpm for 1 minute.
7. The DNA was then stored at -20^0C for further use.

3.3.8 Plasmid Isolation (QIAprep Spin Miniprep Kit)

1. In 20 ml of LB+ampicillin the bacterial culture containing pFL61was inoculated and incubated at 37^0C for 16-18 hrs with shaking at 120 rpm.
2. After 16 hrs, the culture was centrifuged at 13000 rpm for 1 minute.

3. Supernatant was discarded and the pellet was resuspended in 250 µl of P1 buffer.
4. Vortexing was done for proper mixing.
5. 250 µl of P2 buffer was then added and mixing was done by inverting the tube 4-6 times.
6. 350 µl of N3 buffer was added and mixing was done by inverting the tube.
7. The solution mixture was then centrifuged at 13000 rpm for 10 minutes.
8. The supernatant was transferred into QIA prep spin column.
9. The column was then centrifuged at 13000 rpm for 1 minute and the flow through was discarded.
10. 500 µl of PB buffer was added in the column and centrifuged at 13000 rpm for 1 minute. The flow through was discarded.
11. The column was washed with 750 µl of PE buffer and centrifuged at 13000 rpm for 1 minute. The flow through was discarded.
12. The empty column was again centrifuged at 13000 rpm for 1 minute
13. The column was then placed on new 1.5 ml microofuge tube.
14. Finally 25 µl of MQ water was added in the column and centrifugation was carried out at 13000 rpm for 1 minute to recover the plasmid pFL61.

3.3.9 Restriction digestion

Both plasmid (pFL61) and amplified MT genes were digested with two different restriction endonucleases (*Eco*R1 and *Bam*H1) for proper orientation of cloned gene and to prevent the self annealing. For double restriction digestion, firstly plasmid (pFL61), MT1 and MT2 genes were digested with *Eco*R1 and then with *Bam*H1 restriction enzyme. The reaction mixture was prepared by adding 4 µl 10X Tango buffer, 1.5 µl *Eco*R1, 3 µl nuclease free water and 10 µl DNA sample (pFL61/ gene). The mixture was then incubated at 37^0C for 2 hours. After 2 hours, *Bam*H1 (1.5 µl) was added in the reaction mixture and the reaction mixture was again incubated at 37^0C for 2 hours. Finally the reaction was inactivated by keeping the reaction mixture at 80^0C for 10 minutes. The digested genes and plasmid were run on 1.5% agarose gel and the required bands were excised using Thermo Scientific GeneJet Gel Extraction kit as per the prescribed protocol. The eluted samples were quantified on nanodrop.

3.3.10 Ligation

For ligation reaction the vector and insert was added into 1:3 ratio using the ligation formula:

$$\frac{ng\ insert = ng\ vector \times kbp}{kbp\ vector}$$

The following reaction mixture (Table 6) was prepared using thermo scientific T4 DNA ligase.

Table 6: Reaction mixture for ligation reaction

Component	Concentration
Plasmid DNA	1 µl
Insert DNA	2-3 µl
10x Tango buffer	3 µl
Thermo scientific T4 Ligase	1 µl
Water, nuclease free	2-3 µl
Total volume	10 µl

The reaction mixture was incubated at 16^0C for 1-2 hours and then at 4^0C for overnight.

3.4 Bacterial transformation

The ligated product was further transformed into *E.coli* DH5α cells and the positive clones were screened.

3.4.1 Preparation of competent cells:

1. A single colony of *E. coli* DH5α was inoculated in 20 ml of freshly prepared luria broth (appendix) and was incubated at 37^0C for 16 hours with shaking at 120 rpm.
2. After 16 hrs, 200 µl of above culture was transferred into fresh LB medium (20 ml) and was incubated at 37 hrs for 2-3 hrs.
3. After 2 hrs, OD was measured at 590 nm to ensure that it is less than 0.5.
4. The above culture was poured in prechilled aukrages and was kept on ice for 10 minutes.
5. Centrifugation was done at 5000 rpm for 10 minutes at 4^0C.
6. The supernatant was discarded and the pellet was re-dissolved in 10 ml of chilled 0.1 M $CaCl_2$ and was incubated on ice for 10-15 minutes.

7. Centrifugation was carried out at 5000 rpm for 10 minutes at 4^0C.
8. The supernatant was discarded and the pellet was re-suspended in 1 ml of chilled 0.1 M $CaCl_2$ and was stored on ice for 12-24 hours.
9. After 24 hrs 200 µl of glycerol was added in 1 ml of competent cells to make glycerol stocks.

3.4.2 Transformation:

1. 100 µl of competent cells were transferred in pre-chilled micro centrifuge tubes and 5 µl of either plasmid or ligated product (~100 ng) was added in it.
2. Gentle mixing was done and the tubes were stored on ice for 30 minutes.
3. After 30 minutes the tubes were kept on 42^0C for 90-120 seconds and were then immediately placed on ice and were allowed to chill for 2-3 minutes.
4. 1 ml of LB was added to the above tubes and was incubated at 37^0C for 1 hour.
5. After 1 hour, centrifugation was done at 11000 rpm for 1 minute.
6. The supernatant was discarded and the pellet was resuspended in 100 µl of LB media and was spreaded on LA+ ampicillin plates.
7. The plates were then incubated at 37^0C for 12- 16 hours.

3.4.3 Bacterial colony PCR:

In order to screen the positive clones, the bacterial colonies were subjected to colony PCR. Using a toothpick the bacterial colony was picked and was dissolved in 5 µl MQ H_2O in PCR tubes followed by incubation at 95^0C for 10 minutes. The tubes were immediately transferred on ice for 3 minutes and centrifugation was done by using minicentrifuge. 3 µl of supernatant was then transferred into the reaction mixture containing 2 µl 10X Taq buffer, 1.5 µl 2 mM dNTPs, 1 µl Forward primer, 1 µl reverse primer, 0.3 µl Taq polymerase and 11.3µl MQ H_2O. The PCR was then run as explained in section 3.3.5.

3.5 Yeast transformation (Lithium acetate method)

The plasmids (pFL61+MT1 and pFL61+MT2) were isolated from positive clones identified by bacterial colony PCR and were further transformed into metal sensitive yeast strains *(DTY4, ycf1 and zrc1)*

1. In 20 ml of YPD broth (appendix), the yeast strain was inoculated and incubated at 30^0C for overnight at 210 rpm.

2. On next day the O.D of the culture was measured at 600 nm and the culture was re-inoculated in such a way that its O.D reaches up to 1.
3. The culture was incubated at 30^0 C for 2-3 hrs at 210 rpm until its O.D reaches to 2.
4. The culture was then transferred to 50 ml Falcon tube and was centrifuged at 3000 rpm for 5 minutes at room temperature.
5. The supernatant was discarded and the pellet was resuspended with 25 ml H_2O and was again centrifuged at 3000 rpm for 5 minutes.
6. The supernatant was discarded and the pellet was dissolved in 1 ml H_2O.
7. The yeast suspension was then transferred in 1.5 ml microfuge tube and was centrifuged for 30 seconds.
8. The supernatant was discarded and the pellet was dissolved in 1 ml H_2O.
9. The aliquots of 100 µl were made and were centrifuged for 30 seconds.
10. The supernatant was discarded and in each tube 240 µl of PEG 3500 (50% w/v), 36 µl LiAc (1M), 10 µl Ss DNA, 1 µl DNA template, 74 µl H_2O was added and was mixed well.
11. The tubes were then incubated at 42^0C for 1 hour and was the centrifuged for 30 seconds.
12. The supernatant was discarded and the pellet was re-suspended with 1 ml YPD broth and was incubated at 30^0C for 1 hr.
13. Again the centrifugation was done for 30 sec and the supernatant was discarded.
14. The pellet was washed with 1ml of H_2O and was centrifuged again for 30 seconds.
15. The supernatant was discarded and the pellet was again dissolved in 1 ml of H_2O.
16. Spreading was done in metal containing SD-Ura selective plates.
17. The plates were then incubated at 30^0C for 2 days.

3.6 Yeast complementation assays:

The functional role of MT1 and MT2 genes in providing heavy metal resistance was analyzed by yeast functional complementation assays including drop test and growth kinetics.

3.6.1 Drop Assay

1. In SD-Ura broth (appendix1), the cultures of *DTY4, ycf1, zrc1* yeast carrying PFL61, *PFL61-ShMT1, PFL61-ShMT2, PFL61-SiMT1, PFL61-SiMT2, PFL61-SsMT1* and *PFL61-SsMT2* was inoculated and incubated at 30^0C and 210 rpm for 48 h.
2. After 48 hrs, the O.D of each culture was measured at 600 nm and was adjusted to 1.

3. The serial dilution of each culture was done upto $10^{-4.}$
4. The drops of 5 µl were spotted on the SD-Ura plates supplemented with and without metals (150 µM $CuSO_4$, 40 µM $CdSO_4$ and 10 mM $ZnSO_4$).
5. The plates were then incubated at 30^0C for 2 days and photographed.

Note: Here BY4741 the wild type strain of *ycf1, zrc1*was used as reference for Cd and Zn and DTY3 is used as reference for copper.

3.6.2 Liquid Assay

1. In SD-Ura broth, the yeast BY4741*, ycf1, zrc1* (carrying *PFL61, PFL61-ShMT1 gene, PFL61 ShMT2 gene, PFL61 SiMT1, PFL61 SiMT2, PFL61 SsMT1, and PFL61 SsMT2*) was inoculated and incubated at 30^0C for 48 hrs at 210 rpm.
2. After 48 hrs the OD of each culture was measured at 600 nm and the mid log precultures of yeast (carrying the insert, empty vector) were again inoculated in such a way that their initial O.D reaches up to 0.02.
3. The cells were then allowed to grow at 30^0C for 6 hrs at 210 rpm.
4. After 6 hrs the metals (150 µM $CuSO_4$, 40 µM $CdSO_4$ and 10 mM $ZnSO_4$) were added in the culture and OD of the culture was taken.
5. The OD of culture was again taken after every 3 hrs and the process was continued till 48 hrs.
6. After 48 hours, the growth pattern of each

4. RESULTS

4.1 Bioinformatics analysis

Five metallothionein ESTs of *Suillus luteus* were retrieved from NCBI (www.ncbi.nlm.nih.gov/). The open reading frame of the sequences obtained was found by NCBI's ORF finder. The transcripts so obtained were analyzed by multiple sequence analysis tool 'MultAlin (multalin.toulouse.inra.fr/multalin/). It was observed that ShMT1, SiMT1and SsMT1 cDNA contains an ORF of 102bp that encodes for polypeptide containing 34 amino acids with molecular weight of approximately 3419.8 Da. *ShMT2, SsMT2 and SiMT2* contains a 105 bp ORF that encodes for polypeptide containing 35 amino acids with molecular mass 3570.97 Da. It was also observed that the MT1 gene of *Suillus* species contains seven residue and three C-X-C motifs while MT2 gene contains five cysteine residues and only one C-X-C motif. Both genes donot contain any aromatic amino acid.

ORF of MT1 and MT2

```
Suillus MT1
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 gagtgcaaatgttga 136
        E  C  K  C  *
```

```
Suillus MT2
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 gagtgcaatgttgattga 158
        E  C  N  V  D  *
```

BLASTp was done to find the homology between the known sequence of *Suillus leutus* and metallothionein transcripts of some other basidomycetes (Figure 6).

Description	Max score	Total score	Query cover	E value	Ident	Accession
metallothionein [Paxillus involutus]	47.8	47.8	100%	9e-07	71%	AAS19463.1
metallothionein [Pisolithus albus]	45.4	45.4	100%	8e-06	63%	AJO67962.1
metallothionein [Piriformospora indica]	44.7	44.7	82%	1e-05	75%	ACT83730.1
metallothionein [Russula atropurpurea]	44.7	44.7	100%	2e-05	62%	AHA31882.1
hypothetical protein CC1G_05129 [Coprinopsis cinerea okayama7#130]	44.7	44.7	97%	2e-05	67%	XP_001833429.
hypothetical protein MPER_09911 [Moniliophthora perniciosa FA553]	43.9	43.9	91%	3e-05	68%	EEB91695.1
metallothionein 2 [Amanita strobiliformis]	43.9	43.9	100%	3e-05	62%	AGO04615.1
Metallothionein [uncultured eukaryote]	42.0	42.0	100%	2e-04	59%	CCG34103.1
metallothionein [Ganoderma lucidum]	41.6	41.6	100%	3e-04	65%	ABP02008.1
hypothetical protein JAAARDRAFT_29377 [Jaapia argillacea MUCL 33604]	40.0	40.0	91%	0.002	65%	KDQ63354.1
hypothetical protein TRAVEDRAFT_25481 [Trametes versicolor FP-101664 SS1]	40.0	40.0	70%	0.002	79%	XP_008031815.
metallothionein 1 [Laccaria bicolor]	38.1	38.1	100%	0.007	65%	AHI43933.1
hypothetical protein RSAG8_02323 [Rhizoctonia solani AG-8 WAC10335]	38.5	38.5	64%	0.008	82%	KDN48970.1
hypothetical protein HYDPIDRAFT_117423 [Hydnomerulius pinastri MD-312]	37.4	37.4	100%	0.021	76%	KIJ60168.1
hypothetical protein RSAG8_02324 [Rhizoctonia solani AG-8 WAC10335]	37.0	37.0	61%	0.021	81%	KDN48971.1
metallothionein [Taiwanofungus camphoratus]	37.0	37.0	100%	0.025	59%	ABF69031.1

Figure 6: BLASTp analysis shows homologous sequences of metallothioneins from different fungi

The sequences so obtained were then aligned together by using a multiple alignment tool "Multalin" (Figure 7).

```
>Suillus MT1                  -----------------MSTATEVLVSNNNCGSSSCSCGTSCQCKPGECKC----------
>Suillus MT2                  -----------------MSTATEVLVSNNNCGSLISTCGTSCQCKTGECNVD---------
>Paxillus involutus           -----------------MNTITSVPVNFNNCGSNSCGCGSSCACKPGECKC----------
>Coprinopsis cinerea          ----MVQLSAHLLFVPHADLALQNPVNYNNCGSSSCGCGASCACKPGECKC----------
>Russula atropurpurea         -----------------MSPVIQNPVNEHHCGNSSCTCGDSCQCKPGECKC----------
>Amanita strobiliformis       -----------------MQSESQSLVSFANCGSNSCNCGASCACKPGDCKC----------
>Hebeloma mesophaeum          MSRTRTPDCTCGTCECAPTCTCAAPVNQSGCGSSSCTCTS-CACKPGECKC----------
>Hydnomerulius pinastri       MQKYLTQPTRFSIRPIIMNTVTSVPVSQNNCGSSSCGCGSTCQCKPGECKC----------
>Agaricus bisporus            MSKCGEACACANNCQCCSNN--EVPKNQH-CGMSSCGCGDSCGCKPDECKC----------
>Physcomitrella  patens       ---------------------------MSGCGNSACCCGSDCQCSPGNCRCRTMDAPNFGD
>Taiwanofungus camphorates    -----------------MFSATTVPVN-NACGSGDCKCTSTCACKPGDCKCTSTCACKPGD
>Ganoderma lucidum            -----------------MYSTTDVVKN-AACGSSSCNCGATCACKPGECKC----------
>Piriformospora indica        -----------------MISETIVPVN-QNCGNSSCSCGDSCQCKPGECKC----------
```

Figure 7: Multiple sequence alignment of different homologous metallothionein sequences showing C-X-C motifs.

It was observed that all the sequences contain seven cysteine residues at same location. These cys residues are the characterstic feature of ectomycorrhizal MT genes. With these aligned sequences a phylogenetic tree was constructed using maximum parsimony method (Figure 8)

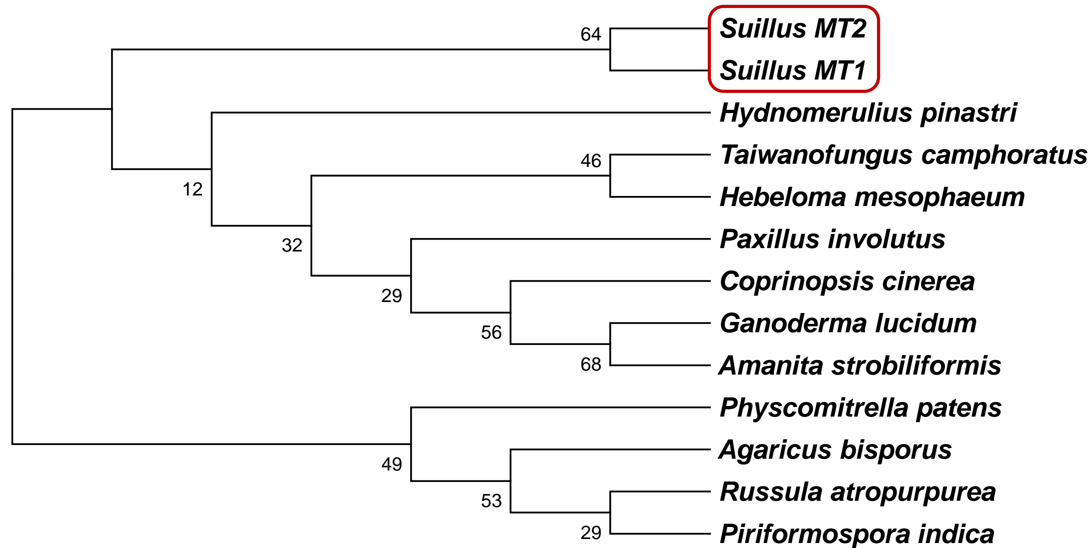

Figure 8: Phylogenetic analysis of metallothionein gene using maximum parsimony method. The bootstrap values for 1000 replicates are indicated on branch nodes.

4.2 Effect of copper concentration on the growth of *Suillus* species:

The *Suillus* species (*Suillus sibricus, Suillus indicus, Suillus himalayensis*) were grown in malt extract broth for 21 days at different concentration of copper to analyse the affect of copper metal on the biomass. The dry weight so obtained was observed and calculated. It was observed that the growth of *Suillus sibricus* was increased initially in the presence of copper when compared to control. The maximum growth was seen at concentration of 100 µM after which there was decrease in the growth of *S. sibricus* when the concentration of copper was further increased. In *Suillus himalayensis* and *S. indicus* there was decrease in the growth with the increase in the concentration of copper (Figure 8). Table 7 shows the different variation in overall growth of mycelium when exposed to different concentration of copper metal.

Table 7: Dry weight of *Suillus* species at different concentration of copper

Copper concentration (µM)	Mycelium dry weight (mg/50ml)		
	S. himalayensis	***S. indicus***	***S. sibricus***
0	45.925±3.45	16.04±3.38	49.15±3.75
100	34.525±3.61	11.88±3.17	94.6±4.37
200	27.025±3.62	6.56±0.81	77.87±3.46
300	25.025±2.80	4.04±0.38	63.05±3.66
400	14.575±1.79	3.36±1.40	44.225±4.25

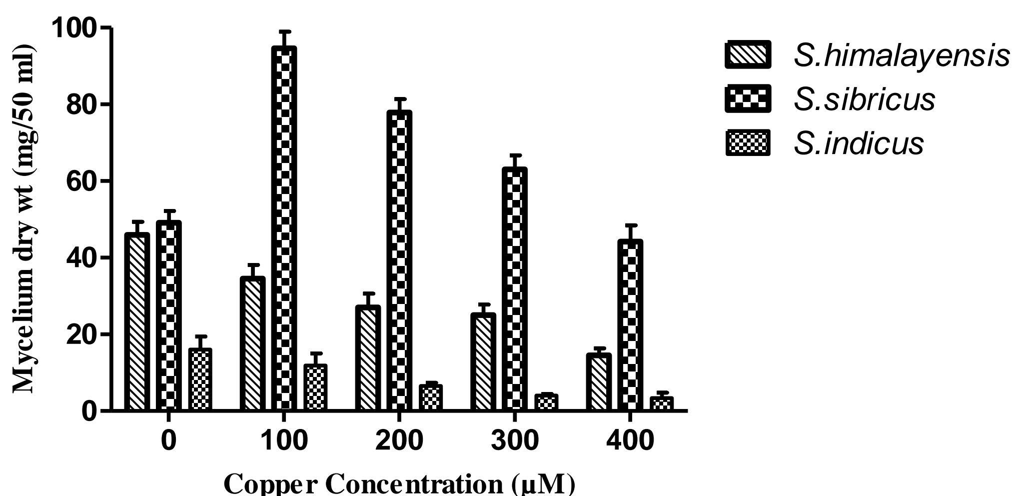

Figure 9: Effect of different copper concentrations (0, 100, 200, 300 and 400 µM) on the growth of *S. sibricus, S. indicus, S. himalayensis*.

4.3 Heavy metal uptake

The accumulation of heavy metals under different stress concentrations was found to be increase with the increase in the concentration of copper for all the three *Suillus* species. Maximum accumulation was observed at 400 µM concentration of copper in all the three species. However among the three strains *S. himalayensis* has shown higher uptake of copper than *S. sibricus* and *S. indicus* when compared with control (Figure 10).

Table 8: Accumulation of copper in three *Suillus* species at different concentration of copper

Copper concentration (µM)	Heavy metal uptake (µg/mg)		
	S. himalayensis	*S. indicus*	*S. sibricus*
0	0.0108±0.0012	0.086±0.003	0.0046±0.006
100	0.742±0.052	0.43±0.11	0.58±0.0059
200	1.54±0.023	0.981±0.076	0.951±0.056
300	1.72±0.053	1.112±0.038	1.62±0.064
400	2.42±0.034	1.210±0.031	1.845±0.12

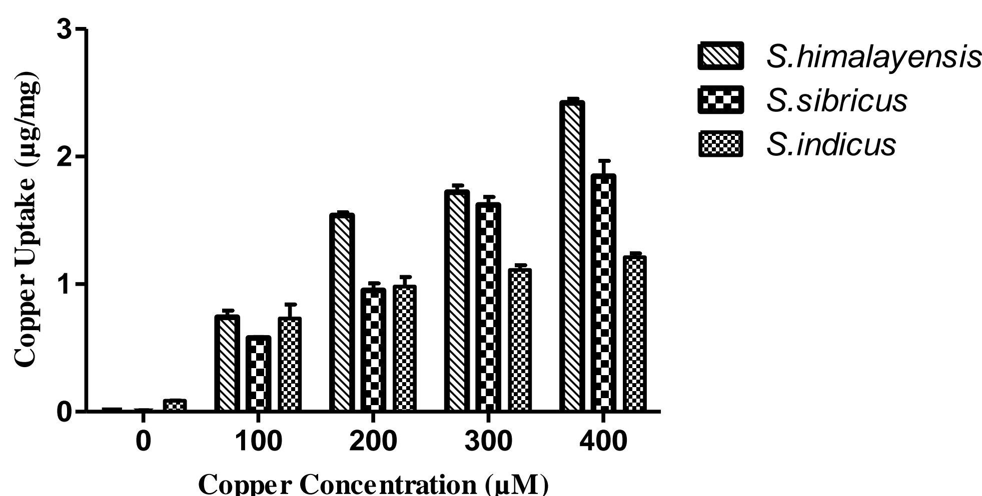

Figure 10: Copper uptake by *S. himalayensis, S. sibricus and S. indicus* at different concentration of copper

4.4 Quantification of gene expression through Real-time PCR

4.4.1 RNA Isolation

The total RNA was isolated from all the three *Suillus* strains (*S. sibricus, S. indicus, S. himalayensis*) grown at different copper concentrations (Figure11). The concentration of total RNA was quantified using nanodrop (Table 9).The quality of rRNA was checked by running it on agrose gel (Figure 11 A). It was observed that the bands of RNA are intact which indicates that the RNA is of good quality and can be further used for cDNA synthesis. To verify the synthesis of cDNA, PCR amplification was done (Figure 11 B).

Table 9: RNA isolation from all the three *Suillus* species at different copper concentration

Copper concentration (µM)	RNA concentration (ng/µl)		
	S. himalayensis	***S. indicus***	***S. sibricus***
0	2824	4124	2561
100	2553	3567	2359
200	3358	2278	2966
300	3606	3648	2275
400	2609	3598	2775

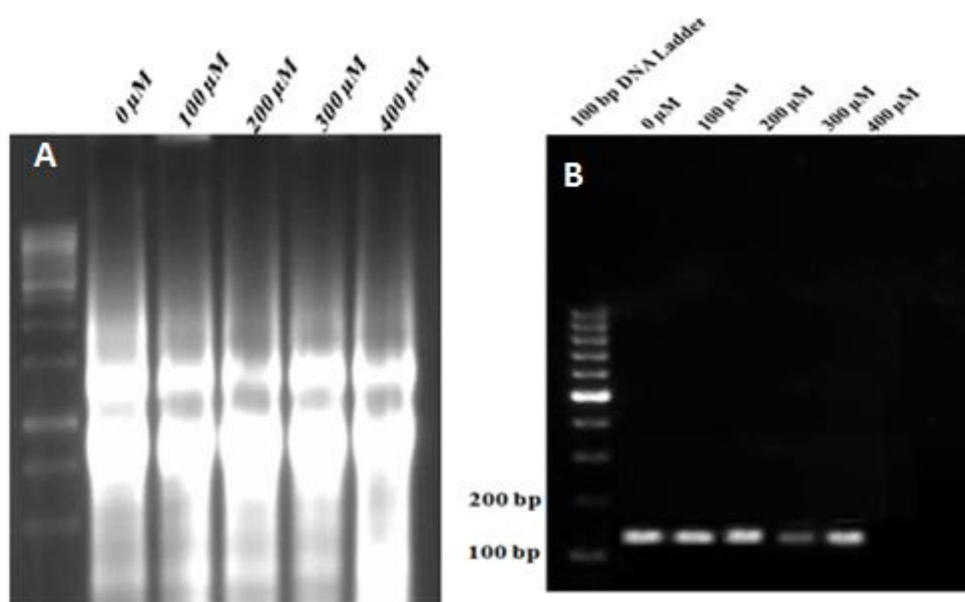

Figure 11: (A.) Total RNA isolation from *Suillus* treated at different concentration of copper (0, 100, 200, 300 and 400 µM). (**B.**) PCR amplification to verify cDNA synthesis.

4.4.2 qPCR

qPCR analysis was done to check the expression of gene at different copper concentration. It was observed that the expression of gene was highest at 400 µM. MT2 gene expresses much better than MT1 gene in presence of copper. It was observed that the MT1 gene and MT2 gene in all the three species shows a huge variation at different copper concentration (Table 10, Table 11).

Table 10: Variation in the expression level of MT1 gene in three *Suillus* species that were incubated for 48 h in the malt extract medium supplemented with the different copper concentration

Copper concentration (µM)	Fold Expression MT1 gene		
	S. himalayensis	***S. sibricus***	***S. indicus***
100	4.89±0.32	1.48±0.13	1.647±0.21
200	12.29±0.23	2.56±0.26	2.313±0.13
300	15.032±0.44	6.020±0.06	4.16±0.36
400	21.11±0.098	9.713±0.38	6.320±0.21

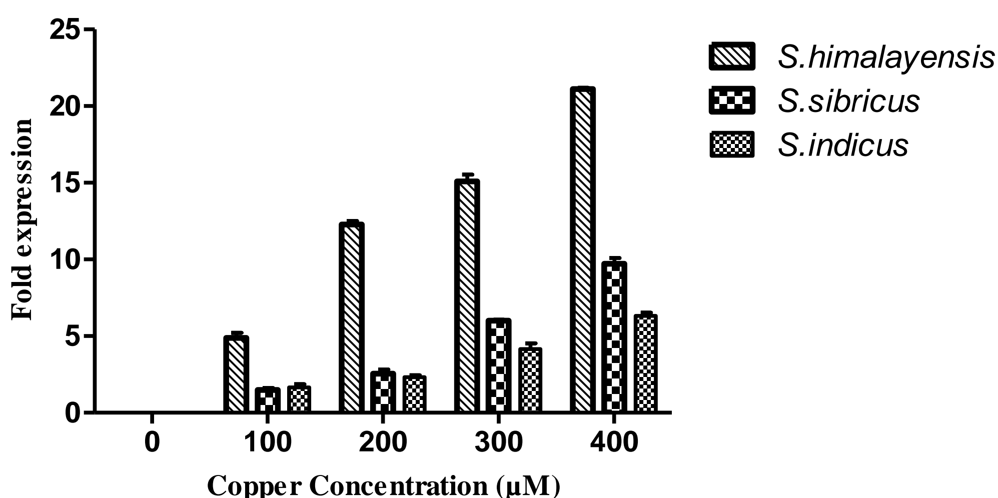

Figure 12: Expression level of MT1 gene in S. *himalayensis, S. sibricus and S. indicus* after 48 hours of incubation in the medium supplemented with an increasing copper concentration.

Table 11: Variation in the expression level of MT2 gene in three *Suillus* species that were incubated for 48 h in the medium supplemented with the different copper concentration

Copper concentration (µM)	Fold Expression MT2 gene		
	S. himalayensis	***S. sibricus***	***S. indicus***
100	9.84±0.98	3.68±0.45	2.78±0.54
200	12.04±0.51	7.46±0.53	2.86±0.35
300	20.11±0.68	8.33±0.87	7.06±0.64
400	29.44±0.73	13.36±0.43	11.15±0.46

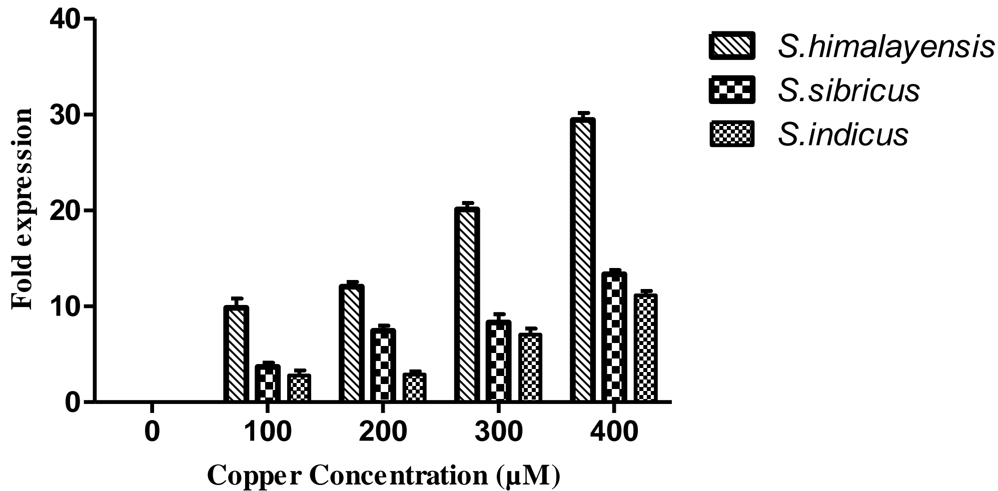

Figure 13: Expression level of MT2 gene in S. *himalayensis, S. sibricus and S. indicus* after 48 hours of incubation in the medium supplemented with an increasing copper concentration.

4.5 Yeast complementation assay

To validate the functional role of *SsMT1, SsMT2, ShMT1, ShMT2, SiMT1 and SiMT2* protein products as a heavy metal binding ligand, these genes were expressed in the metal sensitive mutant strains of *S. cerevisiae*. The growth of the resulting transformant was monitored by drop test on media supplemented with metals and without metals.

4.5.1 Drop Assay

Copper stress

The growth of *DTY4* (PFL61) was inhibited at 150 µM $CuSO_4$ whereas the same cells *expressing pFL61-SsMT1, pFL61-SsMT2, pFL61-ShMT1, pFL61-ShM2, pFL61-SiMT1and pFL61-SiMT2* were able to grow in $CuSO_4$ 150 µM medium. This clearly shows the role of MT1 and MT2 genes in providing copper tolerance (Figure 14).

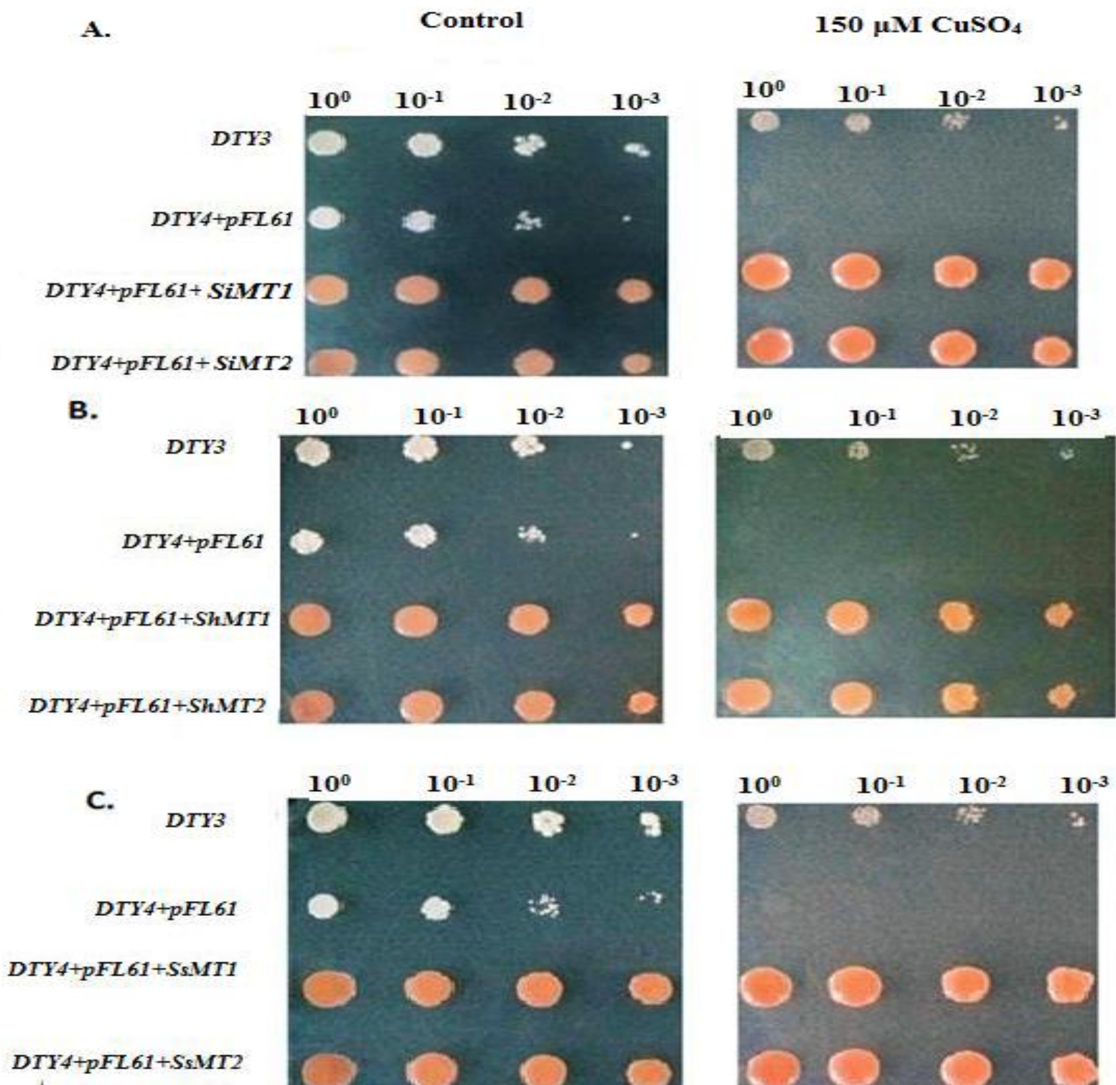

Figure 14: Functional complementation of Cu sensitive yeast strain on selective media **A**. Cu sensitive strain DTY4 was transformed with empty vector *pFL61* or *pFL61-SiMT1* and *pFL61-SiMT2*. **B.** Mutant strain with *pFL61-ShMT1* and *pFL61-ShMT2*. **C.** Mutant strain with *pFL61-SsMT1* and *pFL61-SsMT2*.

Cadmium Stress

The *pFL61-ShMT1*, *pFL61-ShM2*, *pFL61-SiMT1and pFL61-SiMT2* was transformed in the *ycf1* mutant strain that lacks the ability to grow on cadmium. It was observed that when the MT genes were transformed in the *ycf1* strain, the growth of cadmium sensitive (*ycf1)* transformed with empty vector was inhibited at 40 µM $CdSO_4$, whereas the *ycf1* cells carrying the expressing, *pFL61-ShMT1*, *pFL61-ShMT2*, *pFL61-SiMT1and pFL61-SiMT2* expression plasmid were able to grow on media supplemented with cadmium at a similar rate to parental wild type strain BY4741 (Figure 15).

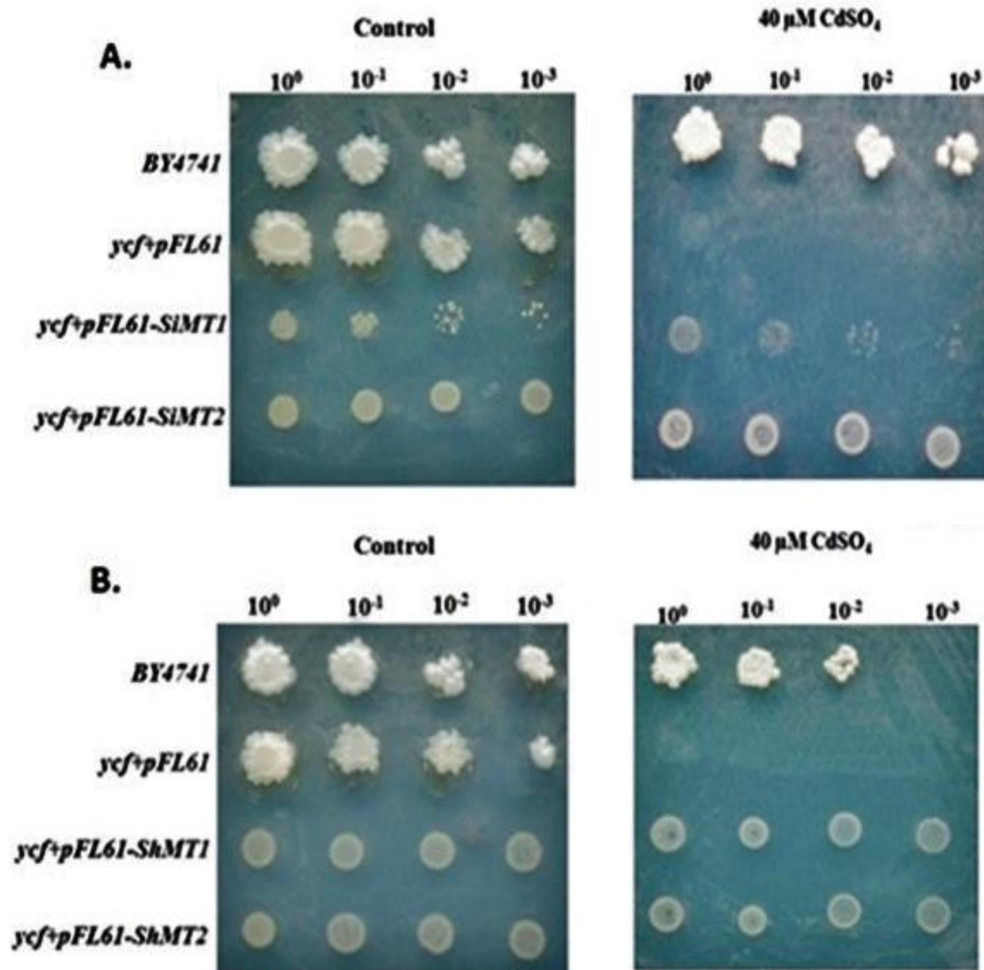

Figure 15: Functional complementation of Cd sensitive yeast strain on selective media **A.** Cd sensitive strain was transformed with empty vector *pFL61 or pFL61-SiMT1 and SiMT2.* **B.** Mutant strain with *pFL61-ShMT1* and *pFL61-ShMT2*

Zinc stress

The *pFL61-ShMT1*, *pFL61-ShMT2*, *pFL61-SiMT1and pFL61-SiMT2* were also transformed *in zrc1,* a mutant strain for zinc. It was observed that zinc sensitive (*zrc1*) yeast strain transformed with *pFL61-ShMT1, pFL61-ShM2, pFL61-SiMT1and pFL61-SiMT2* plasmid was able to grow at 10 mM $ZnSO_4$ whereas the *zrc1* with empty vector was unable to grow at same condition (Figure 16).

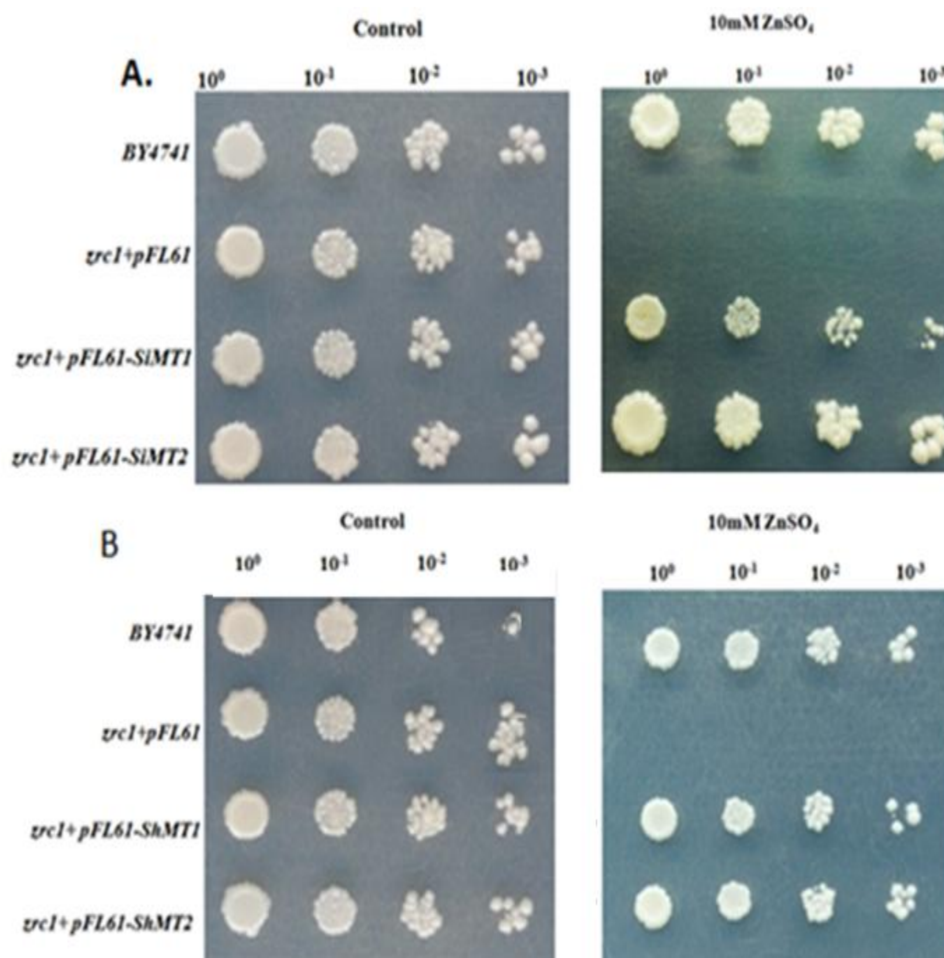

Figure 16: Functional complementation of Zn sensitive yeast strain on selective media. **A.** Zn sensitive strain was transformed with empty vector *pFL61 or pFL61-SiMT1* and *pFL61-SiMT2*. **B.** Mutant strain with *pFL61-ShMT1* and *pFL61-ShMT2*.

4.5.2 Liquid assay

The restoration of Cu, Cd and Zn tolerance by all the three *Suillus* species were further confirmed by growing metal sensitive yeast mutants in liquid culture supplemented with and without $CuSO_4$, $CdSO_4$ and $ZnSO_4$.

Growth in presence of Copper ($CuSO_4$)

It was observed that the Cu sensitive yeast strain (DTY4) transformed with MT genes of *S. sibricus, S. himalayensis and S. indicus* grows very efficiently under copper stress. No growth was observed in the metal sensitive mutant strain carrying empty pFL61 vector when exposed to the copper which signifies that the mutant strain carrying the *Suillus* MT genes have attain the tolerance against copper (Table 12,13,14; Figure 17,18,19)

Table 12: Growth variations in the copper sensitive yeast strain (*DTY4*) carrying *SiMT1*, *SiMT2* genes and empty plasmid under copper stress

Time (hours)	**BY4741+Cu**	***DTY4*+ pFL61**	***DTY4*+ pFL61+Cu**	***DTY4*+ *SiMT1*+Cu**	***DTY4*+ *SiMT2*+Cu**
6	0.046±0.006	0.069±0.010	0.072±0.004	0.138 ± 0.008	0.186 ± 0.004
9	0.090±0.005	0.091±0.004	0.109±0.011	0.383 ± 0.009	0.491 ± 0.006
12	0.493±0.002	0.367±0.014	0.118±0.003	0.673 ± 0.004	0.766 ± 0.004
18	1.351±0.052	0.721±0.020	0.115±0.020	0.992 ± 0.004	1.183 ± 0.002
21	1.501±0.010	0.810±0.080	0.137±0.006	1.235 ± 0.035	1.374 ± 0.015
24	1.521±0.040	0.921±0.022	0.137±0.002	1.536 ± 0.036	1.684 ± 0.007
27	1.556±0.002	0.958±0.030	0.143±0.004	1.93 ± 0.067	1.891 ± 0.001
30	1.582±0.033	1.092±0.002	0.147±0.001	2.066 ± 0.041	2.013 ± 0.004
36	1.689±0.017	1.177±0.033	0.156±0.011	2.145 ± 0.062	2.097 ± 0.007
42	1.700±0.006	1.214±0.012	0.160±0.010	2.176 ± 0.062	2.117 ± 0.006
48	1.72±0.012	1.223±0.018	0.161±0.010	2.251 ± 0.043	2.203 ± 0.23

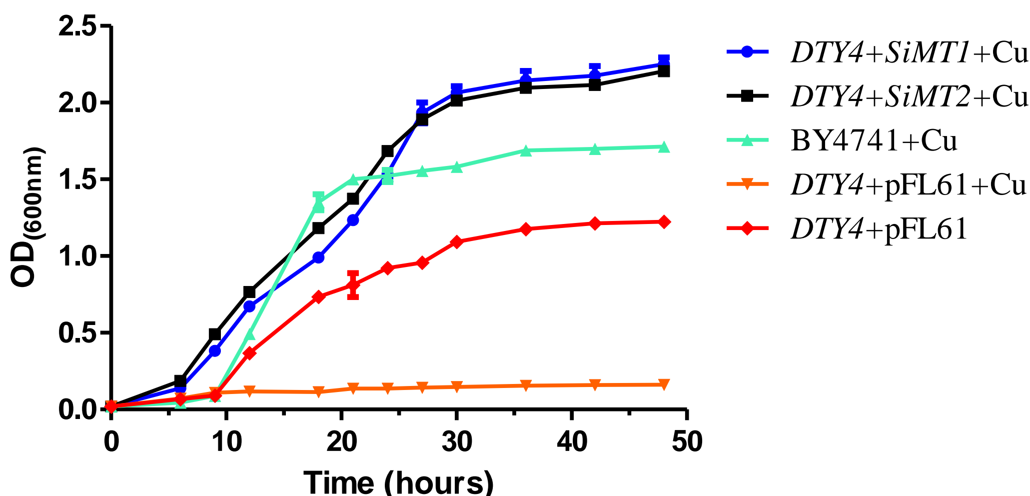

Figure 17: Growth curve of copper sensitive yeast strain expressing *SiMT1 and SiMT2* or empty vector in SD-Ura liquid medium with or without metal supplementation

Table 13: Growth variations in the copper sensitive yeast strain (*DTY4*) carrying *ShMT1*, *ShMT2* genes and empty plasmid under copper stress

Time (hours)	BY4741+ Cu	*DTY4*+pFL61	*DTY4*+pFL61+ Cu	*DTY4*+ *ShMT1*+Cu	*DTY4*+ *ShMT2*+Cu
6	0.046±0.006	0.069±0.010	0.072±0.004	0.116±0.006	0.148±0.004
9	0.090±0.005	0.091±0.004	0.109±0.011	0.372±0.002	0.404±0.001
12	0.493±0.002	0.367±0.014	0.118±0.003	0.736±0.007	0.748±0.003
18	1.351±0.052	0.721±0.020	0.115±0.020	1.031±0.007	1.058±0.001
21	1.501±0.010	0.810±0.080	0.137±0.006	1.422±0.020	1.476±0.020
24	1.521±0.040	0.921±0.022	0.137±0.002	1.708±0.018	1.797±0.001
27	1.556±0.002	0.958±0.030	0.143±0.004	1.915±0.036	1.825±0.005
30	1.582±0.033	1.092±0.002	0.147±0.001	2.003±0.048	1.973±0.001
36	1.689±0.017	1.177±0.033	0.156±0.011	2.087±0.040	2.023±0.033
42	1.700±0.006	1.214±0.012	0.160±0.010	2.105±0.024	2.088±0.014
48	1.72±0.012	1.223±0.018	0.161±0.010	2.193±0.035	2.137±0.011

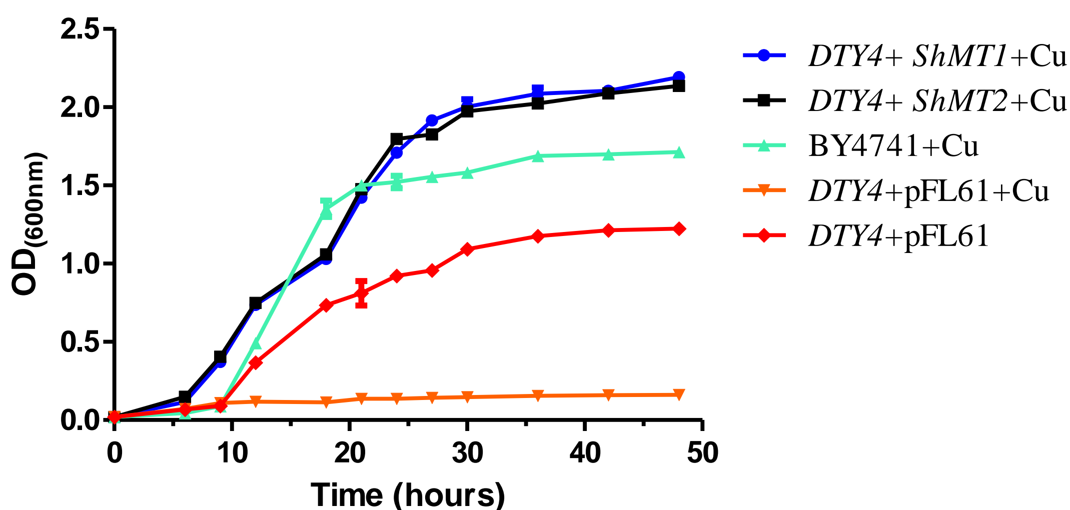

Figure 18: Growth curve of copper sensitive yeast strain expressing *ShMT1 and ShMT2* or empty vector in SD-Ura liquid medium with or without metal supplementation

Table 14: Growth variations in the copper sensitive yeast strain (*DTY4*) carrying *SsMT1*, *SsMT2* genes and empty plasmid under copper stress

Time (hours)	BY4741+ Cu	*DTY4+* pFL61	*DTY4+* pFL61+Cu	*DTY4+* *SsMT1*+Cu	*DTY4+* *SsMT2*+Cu
6	0.046±0.006	0.069±0.010	0.072±0.004	0.123±0.003	0.163±0.011
9	0.090±0.005	0.091±0.004	0.109±0.011	0.427 ± 0.016	0.443±0.022
12	0.493±0.002	0.367±0.014	0.118±0.003	0.723±0.010	0.813±0.018
18	1.351±0.052	0.721±0.020	0.115±0.020	1.012±0.023	1.116±0.038
21	1.501±0.010	0.810±0.080	0.137±0.006	1.458±0.038	1.458±0.054
24	1.521±0.040	0.921±0.022	0.137±0.002	1.758±0.038	1.808±0.016
27	1.556±0.002	0.958±0.030	0.143±0.004	1.804±0.025	1.9385±0.070
30	1.582±0.033	1.092±0.002	0.147±0.001	1.871± 0.049	2.0115±0.037
36	1.689±0.017	1.177±0.033	0.156±0.011	1.988±0.010	2.065±0.012
42	1.700±0.006	1.214±0.012	0.160±0.010	1.996±0.002	2.118±0.009
48	1.72±0.012	1.223±0.018	0.161±0.010	2.051±0.008	2.200±0.019

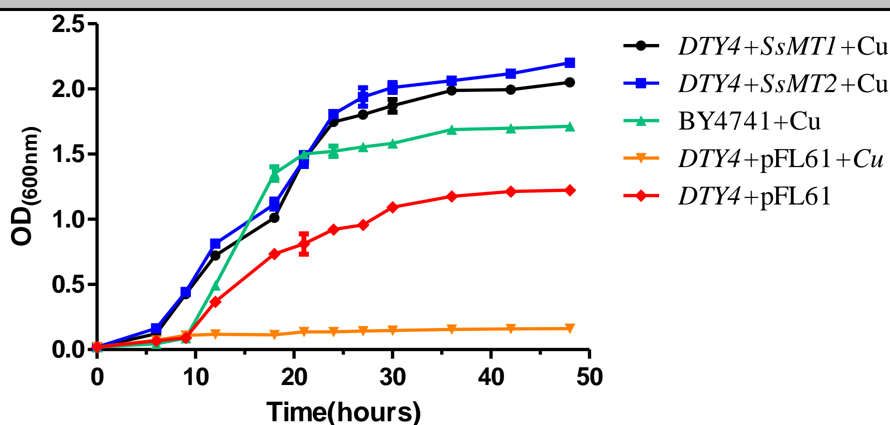

Figure 19: Growth curve of copper sensitive yeast strain expressing *SsMT1 and SsMT2* or empty vector in SD-Ura liquid medium with or without Cu supplementation

Growth in presence of $CdSO_4$

It was observed that the metal sensitive *ycf1* yeast strain transformed with *SiMT1, SiMT2, ShMT1* and *ShMT2* gene grows under cadmium stress. However growth in both the strains was less. No growth was observed in the mutant strain carrying empty pFL61 vector when

exposed to the plates supplemented with 40 μM $CdSO_4$. This indicates the involvement of both MT genes in conferring the ability for tolerance against cadmium (Table 15 and 16; Figure20, 21)

Table 15: Growth variations in cadmium sensitve yeast mutant strain carrying MT gene of *Suillus indicus* under Cd stress

Time (hours)	BY4741+ Cd	*ycf1*+pFL61	*ycf1*+pFL61+ Cd	*ycf1*+*SiMT1* +Cd	*ycf1*+*SiMT2* +Cd
6	0.060±0.001	0.0435±0.003	0.041±0.001	0.039± 0.003	0.068± 0.006
9	0.08±0.005	0.096±0.002	0.0645±0.005	0.090± 0.002	0.100± 0.001
18	0.174±0.007	0.372±0.012	0.064±0.006	0.195± 0.004	0.214± 0.012
21	0.213±0.014	0.697±0.001	0.068±0.001	0.202± 0.003	0.245±0.008
27	0.401±0.046	1.047±0.015	0.067±0.001	0.257± 0.007	0.3004±0.011
30	0.429±0.021	1.278±0.007	0.068±0.001	0.288± 0.010	0.3164±0.007
36	0.437±0.017	1.337±0.023	0.071±0.001	0.310± 0.011	0.3404±0.002
42	0.419±0.006	1.463±0.032	0.071±0.003	0.327± 0.005	0.3574±0.003
48	0.427±0.001	1.527±0.003	0.074±0.002	0.349± 0.009	0.3794±0.010

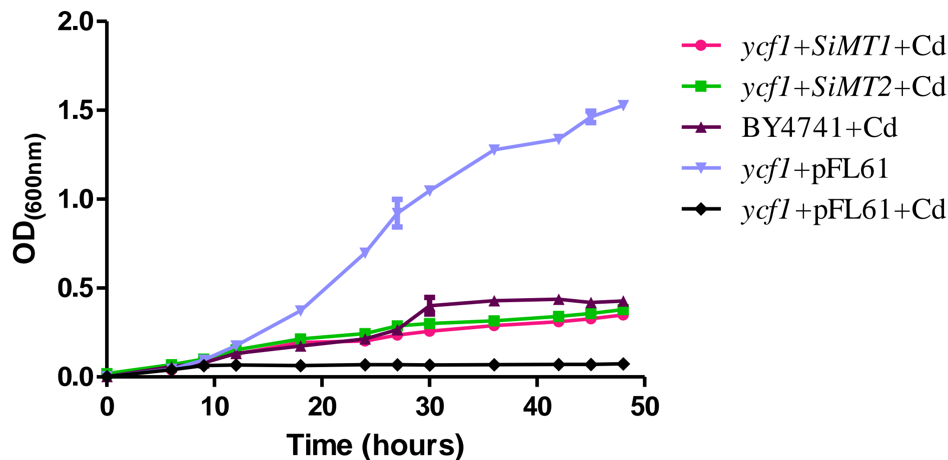

Figure 20: Growth curve of cadmium sensitive yeast mutant strain expressing *SiMT1 and SiMT2* or empty vector in SD-Ura liquid medium with or without Cd supplementation

Table 16: Growth variations in Cd sensitive yeast strain carrying both MT genes of *Suillus himalayensis* under Cd stress

Time (hours)	BY4741+ Cd	*Ycf1*+pFL61	*ycf1*+pFL61+ Cd	*ycf1*+*ShMT1*+ Cd	*ycf1*+*ShMT2*+ Cd
6	0.060±0.001	0.0435±0.003	0.041±0.001	0.049±0.003	0.051±0.001
9	0.08±0.005	0.096±0.002	0.0645±0.005	0.082±0.006	0.106±0.007
12	0.132±0.003	0.176±0.002	0.067±0.0010	0.117±0.010	0.178±0.010
18	0.174±0.007	0.372±0.012	0.064±0.006	0.214±0.017	0.219±0.005
21	0.213±0.014	0.697±0.001	0.068±0.001	0.217±0.036	0.280±0.010
24	0.265±0.009	0.921±0.077	0.068±0.001	0.247±0.026	0.310±0.006
27	0.401±0.046	1.047±0.015	0.067±0.001	0.281±0.012	0.347±0.007
30	0.429±0.021	1.278±0.007	0.068±0.001	0.339±0.020	0.3922±0.005
36	0.437±0.017	1.337±0.023	0.071±0.001	0.352±0.010	0.434±0.006
42	0.419±0.006	1.463±0.032	0.071±0.003	0.383±0.001	0.448±0.005
48	0.427±0.001	1.527±0.003	0.074±0.002	0.394±0.006	0.458±0.008

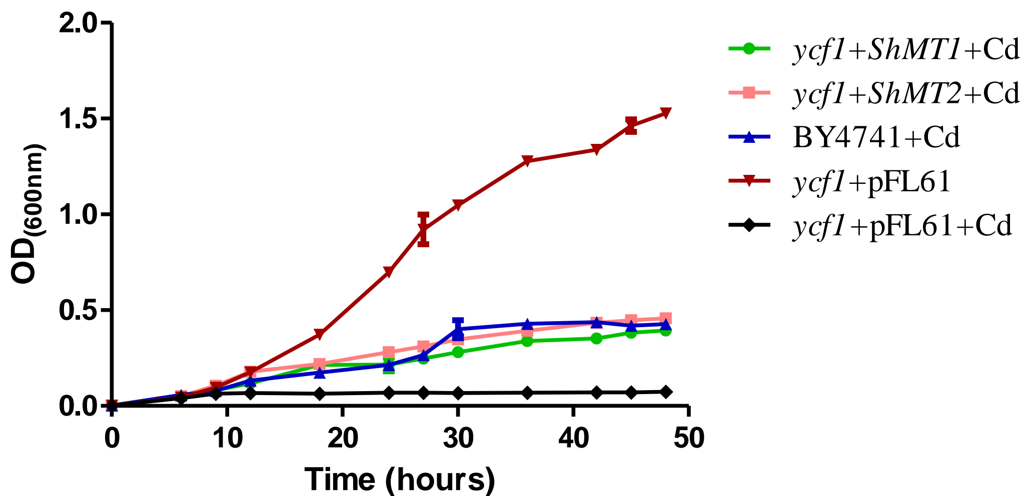

Figure 21: Growth curve of mutant strain expressing *ShMT1 and ShMT2* or empty vector in SD-Ura liquid medium with or without Cd supplementation

Growth curve in presence of $ZnSO_4$

It was observed that metal sensitive *zrc1* strain transformed with *SiMT1, SiMT2, ShMT1 and ShMT2* gene grows under zinc stress. However there growth was found to be less when compared to the wild type strain BY4741. No growth was observed in the mutant strain carrying empty pFL61 vector when exposed to the zinc which signifies that the mutant strain carrying the ShMT and SiMT genes have attain the tolerance mechanism against zinc (Table 17, 18; Figure 22, and 23).

Table 17: Growth variation in zinc sensitive yeast strain carrying MT gene of *Suillus himalayensis* under Zn stress

Time (hours)	BY4741+ Zn	*zrc1*+pFL61	*zrc1*+pFL61+ Zn	*zrc1+ShMT1*+ Zn	*zrc1+ShMT2*+ Zn
6	0.048±0.003	0.065±0.006	0.05±0.007	0.059±0.003	0.055±0.004
9	0.135±0.003	0.141±0.005	0.071±0.004	0.076±0.008	0.112±0.002
12	0.258±0.003	0.540±0.076	0.060±0.010	0.103±0.001	0.168±0.015
18	0.312±0.014	0.674±0.079	0.067±0.004	0.156±0.024	0.180±0.007
21	0.565±0.026	1.007±0.086	0.084±0.002	0.181±0.008	0.285±0.008
24	0.681±0.021	1.129±0.063	0.079±0.001	0.211±0.026	0.349±0.012
27	0.691±0.004	1.150±0.064	0.079±0.009	0.265±0.048	0.447±0.001
30	0.708±0.014	1.232±0.038	0.079±0.001	0.356±0.015	0.482±0.005
36	0.729±0.004	1.290±0.050	0.080±0.001	0.365±0.033	0.536±0.028
42	0.763±0.055	1.285±0.023	0.080±0.001	0.417±0.007	0.627±0.035
48	0.772±0.001	1.152±0.024	0.083±0.001	0.427±0.021	0.671±0.004

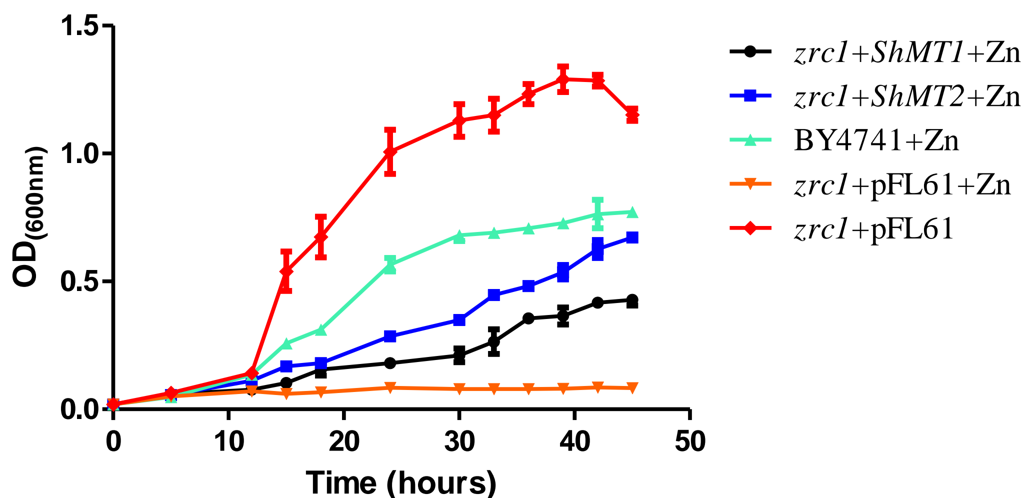

Figure 22: Growth curve of mutant strain expressing *ShMT1 and ShMT2* or empty vector in SD-Ura liquid medium with or without Zn supplementation.

Table 18 Growth variations in zinc sensitive yeast strain carrying MT gene of *Suillus indicus* under Zn stress

Time (hours)	BY4741+ Zn	*Zrc1*+pFL61	*zrc1*+pFL61+ Zn	*zrc1+SiMT1*+ Zn	*zrc1+SiMT2*+ Zn
6	0.048±0.003	0.065±0.006	0.05±0.007	0.048±0.005	0.063±0.001
9	0.135±0.003	0.141±0.005	0.071±0.004	0.077±0.007	0.182±0.001
12	0.258±0.003	0.540±0.076	0.060±0.010	0.108±0.009	0.440±0.044
18	0.312±0.014	0.674±0.079	0.067±0.004	0.143±0.012	0.706±0.035
21	0.565±0.026	1.007±0.086	0.084±0.002	0.266±0.016	0.791±0.070
24	0.681±0.021	1.129±0.063	0.079±0.001	0.344±0.033	0.962±0.005
27	0.691±0.004	1.150±0.064	0.079±0.009	0.572±0.007	1.059±0.042
30	0.708±0.014	1.232±0.038	0.079±0.001	0.733±0.049	1.0535±0.092
36	0.729±0.004	1.290±0.050	0.080±0.001	0.985±0.018	1.087±0.043
42	0.763±0.055	1.285±0.023	0.080±0.001	1.054±0.031	1.090±0.002
48	0.772±0.001	1.152±0.024	0.083±0.001	0.977±0.014	1.082±0.005

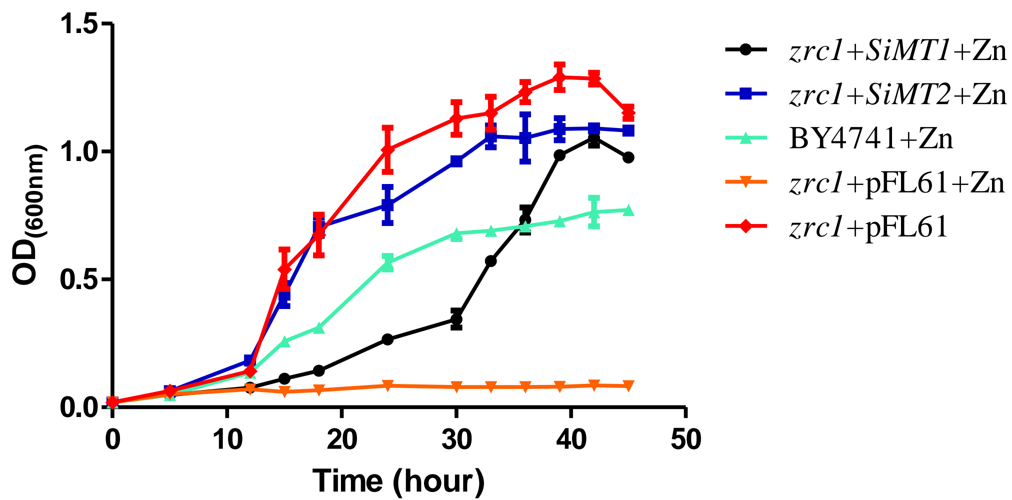

Figure 23: Growth curve of mutant strain expressing *SiMT1 and SiMT2* or empty vector in SD-Ura liquid medium with or without Zn.

5. Summary

The present work is focused on the characterization and evaluation of metallothioneins for their ability to tolerate heavy metal. The two MT (MT1 and MT2) genes from each *Suillus* species (*Suillus indicus, Suillus sibricus and Suillus himalayensis*) are used for this study. Various reports have suggested the role of metallothioneins in the tolerance of heavy metal (Hlozkova *et al.,* 2016**;** Reddy *et al.,* 2016). Five putative Metallothionein sequences of *Suillus luteus* were retrieved from EST library. From ORF finder the transcripts of these genes were obtained and the multiple sequence alignment was done. It was observed that MT1 genes contain an ORF of 102 bp and encodes for the polypeptide that contains 34 amino acids. MT2 gene encodes for the polypeptide that consists of 35 amino acids and has an ORF of 105 bp. When the multiple sequence alignment of their transcript was performed it was noticed that there are seven cysteine residues and three C-X-C motifs in the MT1 gene while MT2 gene consist of five cysteine residues and one C-X-C motif. The presence of cysteine residue and C-X-C motifs are the characterstic feature of metallothionein and indicates that they are closely related to the MTs of several other basidomycetes. The mRNA transcript accumulation pattern of MT1 and MT2 gene in *S. indicus, S. sibricus and S. himalayensis* were also quantified by RT PCR method. It was noticed that MT2 gene expresses more efficiently under copper stress when compared to MT1 gene. The potential of *Suillus sibricus, Suillus indicus* and *Suillus himalayensis* to tolerate heavy metal were also studied by growing the pure mycelium in the malt extract medium supplemented with the copper at different concentrations. The growth of *S. sibricus, S. indicus and S. himalayensis* were adversely affected with the increasing concentration of copper. Among the three *Suillus* species, *S. sibricus* showed the higher tolerance to the copper. The biomass of *Suillus sibricus* was found to be increased initially upto 100 µM concentration of copper after which there was decline in the biomass concentration. It was determined that that *S. himalayensis, S. sibricus and S. indicus* shows different degree of tolerance towards copper. The LD_{50} value of copper stress for *S. sibricus* and *S. himalayensis* was 370 µM and 325 µM while in case of *S. indicus* it was 180 µM. This decrease in the growth of all the *Suillus* species may be because of the accumulation of heavy metals. It was observed that the uptake of heavy metal in all the three *Suillus* species increase with the increase of copper concentration. To validate the functional role of these genes, yeast complementation assay was performed. The complementation studies indicated that the DTY4 strain transformed with *pFL61-SiMT1, pFL61-SiMT2, pFL61-ShMT1, pFL61-ShMT2, pFL61-ShMT1,* and *pFL61-ShMT2* were able

to grow on the medium supplemented with 150 µM $CuSO_4$. While the growth of DTY4 transformed with empty pFL61 was restricted. The MT genes from *Suillus himalayensis,* and *Suillus indicus* were also expressed in Cd sensitive yeast strain and Zn sensitive yeast strain for configuring their ability to induce in the presence of Zn and Cd. The growth of both metal sensitive strains (*ycf1 and zrc1*) transformed with MT1 and MT2 genes of *S. himalayensis and S. indicus* in the presence of Cd and Zn shows that the metallothionein not only provides tolerance against copper but also plays a vital role in the tolerance of Cd and Zn in the *Suillus* species.

From the present study it was concluded that all the three ectomycorrhizal fungus *Suillus himalayensis, Suillus sibricus and Suillus indicus* encodes two MTs (MT1 and MT2) that are highly induced by copper and may be involved in the specific detoxification of these metals. The functional roles of these genes were characterized by the yeast functional complementation assay.

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APPENDIX

Preparation of malt extract media

Add 20 g of malt extract medium in 1 L of distilled water. Adjust the pH to 5.5 by using NaOH. Autoclave it at 121°C for 15 mins. For malt extract plates add agar (15 g) before autoclaving.

Preparation of malt extract media

Add 20 g of malt extract medium in 1 L of distilled water. Adjust the pH to 5.5 by using NaOH. Autoclave it at 121°C for 15mins at 15lbs. For malt extract plates add agar (15 g) before autoclaving.

Preparation of LB

Components	Concentration
Bacto-tryptone	10 g/L
Bacto-yeast extract	5 g/L
NaCl	5 g/L

Adjust pH to7 with NaOH and autoclave at 121^0C for 20 minutes

LB Agar+ Ampicillin

Prepare LB media as above and add agar (15g/L). Autoclave the media. Before pouring on plates, add ampicillin (50 µg/ml) in it.

Composition of YPD for 1L

Components	Concentration
Peptone	20 g/L
Yeast extract	10 g/L
Agar (for plates only)	20 g/L

aComposition of SD-Ura media (pH5.9) for 1 L

Components	Concentration
Yeast Nitrogen Base	6.7
Glucose	20 g
1% Adenine	1 ml
1% Tyrosine	5 ml
Amino acid mixture	0.77 g

Preparation of Agarose Gel Dye (6X)

Components	Concentration
Bromo phenol Blue	0.25 %
Xylene cyanol	0.25 %
Glycerol	30 %

TBE Buffer (5x)

Components	Concentration
Tris HCl	0.045 M (pH 8)
Boric acid	0.45 M
EDTA	0.01M (pH 8)

0.5X TBE buffer

Add 100 ml of 5x TBE buffer in 900 ml of distilled H_2O and mix well.