STUDIES ON *PISOLITHUS* SP.: STRESS RESPONSE, PIGMENT PRODUCTION AND MYCORRHIZATION WITH FORESTRY TREES OF GOA

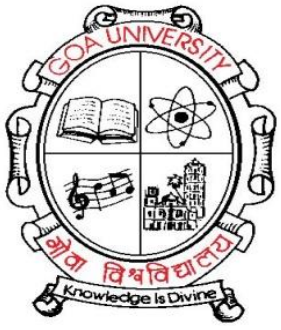
A Thesis submitted to Goa University for the Award of the Degree

DOCTOR OF PHILOSOPHY
in
MICROBIOLOGY

By

LAKSHANGY J. CHARI

Research Guide
DR SANDEEP GARG



Goa University,
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Statement

As required under the University Ordinance O.B.19.8 (VI), I state that the present thesis entitled "**Studies on *Pisolithus* sp.: stress response, pigment production and mycorrhization with forestry trees of Goa**" is my original contribution and the same has not been submitted on any previous occasion. To the best of my knowledge, the present study is the first comprehensive work of its kind from the area mentioned.

The literature related to the problem investigated has been cited. Due acknowledgements have been made whenever facilities and suggestions have been availed of.

Lakshangy J. Chari

Certificate

This is to certify that the thesis entitled "**Studies on *Pisolithus* sp.: stress response, pigment production and mycorrhization with forestry trees of Goa**" submitted by **Lakshangy J. Chari** for the award of the degree of ***Doctor of Philosophy*** in ***Microbiology*** is based on her original studies carried out by her under my supervision. The thesis or any part thereof has not been previously submitted for any other degree or diploma in any University or Institution.

Place: Goa University

Date:

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Certificate from the candidate

As suggested by the External Examiner appropriate corrections are incorporated in the thesis at relevant pages.

Lakshangy J.Chari

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Lakshangy

Dedicated to Almighty

Lord Ganesha

And

to those special people in my life in whose

acts I felt the grace of God

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List of Abbreviations

NH_3	Ammonia	**HSP**	Heat shock protein
NH_4Cl	Ammonium chloride	**H**	Hours
NH_4OH	Ammonium hydroxide	**HCl**	Hydrochloric acid
$(NH_4)_2PO_4$	Ammonium phosphate	**HEPES**	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
BLAST	Basic local alignment search tool	**ITS**	Internal transcribed spacers
β	Beta	**Fe**	Iron
BSA	Bovine serum albumin	**kDa**	Kilo Dalton
H_3BO_3	Boric acid	**Λ**	Lambda
CI	Chloroform-isoamylalcohol	**$MgSO_4$**	Magnesium sulphate
CTAB	Cetyl trimethyl-ammonium bromide	**$MgCl_2$**	Magnesium chloride
$CuSO_4$	Copper sulphate	**Mn**	Manganese
$CaCl_2$	Calcium chloride	**$MnSO_4$**	Manganese sulphate
K_2HPO_4	Dipotassium hydrogen phosphate	**µl**	Microlitre
°C	Degree centigrade	**mM**	Millimolar
DNA	Deoxyribonucleic acid	**µm**	Micrometer
dNTP	Deoxynucleotide triphosphate	**ml**	Milliliter
ECM	Ectomycorrhiza/e/l	**min**	Minutes
EDTA	Ethylene diamine tetra acetic acid	**NCBI**	National Center for Biotechnology information
e.g.	Example	**N**	Normal
$FeCl_3$	Ferric chloride	**ADA**	N-[2-acetamido] iminodiacetic acid
$FeSO_4$	Ferrous sulphate	**ACES**	N-[2-acetamido]-2-aminoethanesulfonic acid
Fig	Figure	**MES**	2-(N-morpholino) ethanesulfonic acid
GMSM	Glucose mineral salt medium	**OD**	Optical density
$g\ L^{-1}$	Grams per litre	**O/N**	Overnight
EC_{50}	Half maximal effective concentration	***p***	para

List of Abbreviations

ppm	Parts per million	**sHSP**	Small heat shock protein
PCI	Phenol-chloroform-isoamylalcohol	**SDS**	Sodium dodecyl sulfate
PMSF	phenylmethylsulfonyl fluoride	**HeLa cells**	Henrietta Lack's 'immortal' cells
PCB	Phosphate citrate buffer	**NaOH**	Sodium hydroxide
Pt	Pisolithus tinctorius	**NaOCl**	Sodium hypochlorite
PAGE	Poly-acrylamide gel electrophoresis	**SPB**	Sodium phosphate buffer
PEG	Polyethylene glycol	**Na_2CO_3**	Sodium carbonate
PCR	Polymerase chain reaction	**SEM**	Scanning electron microscope
PVPP	polyvinyl polypyrrolidone	**Spp**	Species
KCl	Potassium chloride	**H_2SO_4**	Sulphuric acid
PIPES	piperazine-N,N'-bis (2-ethanesulfonic acid)	**S**	Svedberg unit
$K_3Fe(CN)_6$	Potassium ferricyanide	**TEMED**	Tetra methyl ethylene diamine
$K_4Fe(CN)_6$	Potassium ferrocyanide	**Taq**	Thermus aquaticus
KH_2PO_4	Potassium dihydrogen phosphate	**Thiamine HCl**	Thiamine hydrochloride
%	Percentage	**Tris HCl**	Tris hydrochloride
Rf	Resolution factor	**TE**	Tris EDTA
rpm	Revolutions per minute	**UV**	Ultraviolet
rRNA	Ribosomal Ribonucleic acid	**V**	volts
RT	Room temperature	**v/v**	Volume by volume
NaCl	Sodium chloride	**w/v**	Weight by volume
Na_2MoO_4	Sodium molybdate	**$ZnSO_4$**	Zinc sulphate
NaH_2PO_4	Sodium dihydrogen phosphate	**Na_2HPO_4**	Disodium hydrogen phosphate
KOH	Potassium hydroxide	**m**	meter
P	Phosphorus	**ND**	Not determined
ANOVA	Analysis of varience	**PLFAs**	Phospholipid fatty acids
DGGE	Denaturing gradient gel electrophoresis	**RFLP**	Restriction fragment length polymorphism

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CHAPTER I

Introduction and literature survey

INTRODUCTION

Ectomycorrhizae (ECM) also termed as ectotrophic mycorrhizae are the second most predominant type of mycorrhiza found in nature. The distinctive features of a typical ectomycorrhiza are the fungal "mantle" or "sheath" that surrounds the host root, and the fungal hyphae that penetrate between the epidermal or cortical cells to form a network known as the "Hartig net". From the mantle surface, hyphae or bundles of interwoven hyphae "rhizomorphs" radiate out into the surrounding soil. In tropical forests, rhizomorph development can be prolific, sometimes extending for several metres from the tree root. General characteristics of ectomycorrhizal associations are shown in Figure 1.1 (Sylvia *et al.*, 2004).

ECTOMYCORRHIZAL TREE SPECIES

ECM occur on about 10% of the world flora. About 2,000 species of woody plants including gymnosperms and angiosperms are reported as forming ectomycorrhizal associations. Some of the most important plant genera are shown in Table 1.1.

Besides associating with most conifers, including all species of the Pinaceae, ectomycorrhizae are commonly found on most members of the Betulaceae, Dipterocarpaceae, Fagaceae, Juglandaceae, Myrtaceae, and Salicaceae (Marx, 1977; Trappe, 1977; Natarajan *et al.*, 2005).

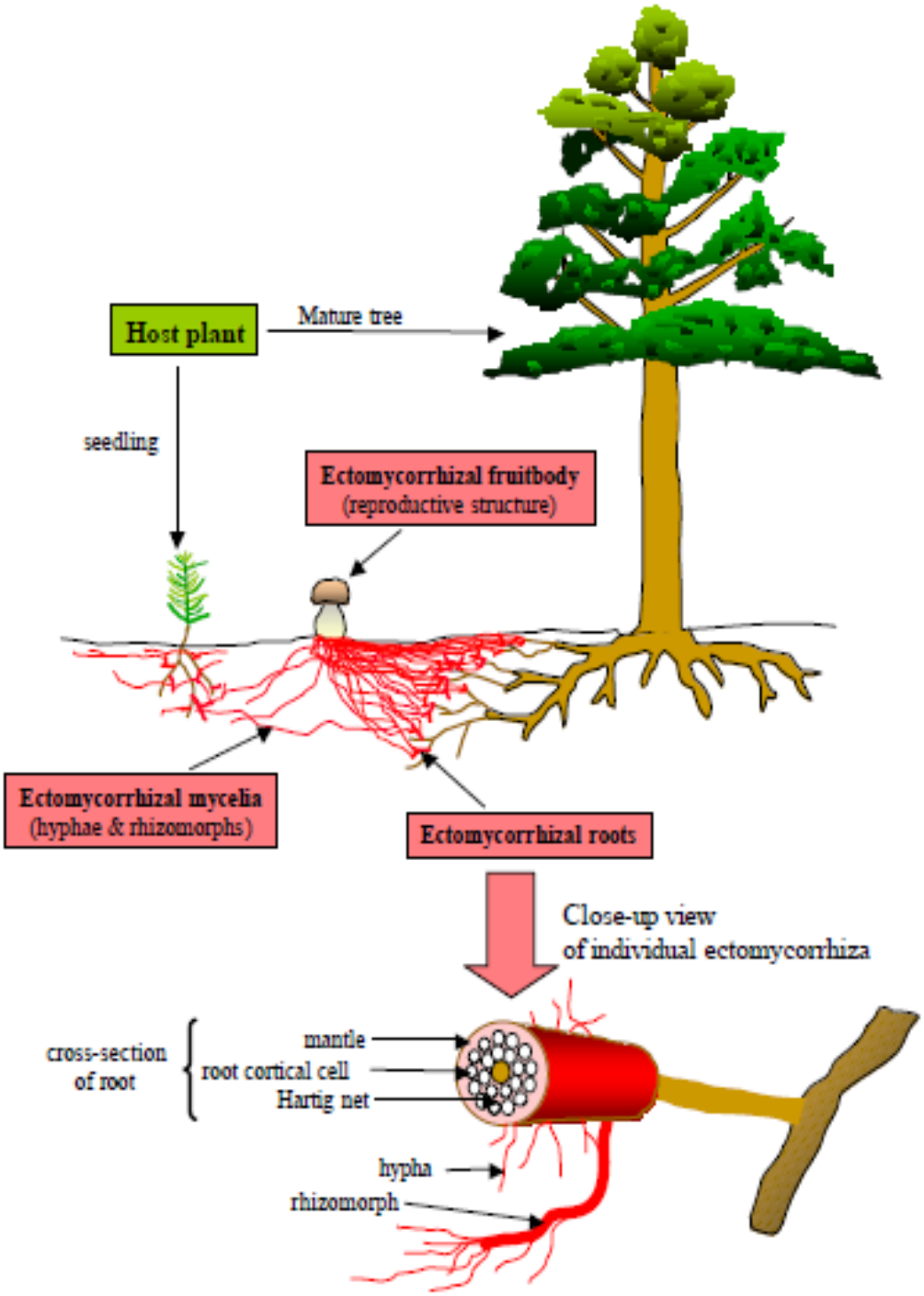


Fig.1.1 General characteristics of Ectomycorrhizal association

(Source: ©Manual of Ectomycorrhizal fungi, Darwin Project, Viet Nam)

Family	Genus
Apocynaceae	*Alstonia, Holasshenia, Wrightia*
Betulaceae	*Alnus, Betula, Carpinus, Corylus, Ostryopsis*
Caesalpiniaceae	*Afzelia, Anthonotha, Bauhini, Berlinia, Brachystegia, Cassia, Erythrophleum, Gilbertiodendron, Intsia, Julbernardia, Monopetalanthus, Paramacrolobium*
Casuarinaceae	*Allocasuarina, Casuarina*
Dipterocarpaceae	*Anisoptera, Balanocarpus, Cotylelobium, Dipterocarpus, Dryobalanops, Hopea, Monotes, Parashorea, Shorea, Vatica*
Euphorbiaceae	*Aldinia, Marcquesia, Uapaca*
Fagaceae	*Castanea, Castanopsis, Fagus, Lithocarpus, Nothofagus, Pasania, Quercus*
Gnetaceae	*Gnetum*
Juglandaceae	*Carya, Juglans*
Mimosaceae	*Acacia*
Myrtaceae	*Compomanesia, Eucalyptus, Eugenia, Leptospermum, Melaleuca*
Nyctaginaceae	*Neea, Pisonia*
Pinaceae	*Abies, Cedrus,Keteleeria, Larix ,Picea, Pinus, Pseodotsuga, Tsuga*
Rosaceae	*Crataegus, Dryas, Malus, Pyrus, Sorbus*
Salicaceae	*Populus, Salix*
Tiliaceae	*Tilia*

Table 1.1 Important families and genera of trees that form ectomycorrhizae (Source: ©Manual of Ectomycorrhizal fungi, Darwin Project, Viet Nam)

ECTOMYCORRHIZAL FUNGI

Over 5,000 species of fungi have been recorded as forming ectomycorrhizae. Most ectomycorrhizal fungi are **Basidiomycetes** and **Ascomycetes**, typically producing large, fleshy fruit-bodies such as mushrooms, toadstools, puffballs, truffles, coral fungi, cup fungi and resupinate fungi. A list of the main families and numbers of ectomycorrhizal genera is given in Table 1.2.

ECM fungi are classified based on their host range and the stage of plant on which they appear. Some fungi have a narrow plant host range, such as *Boletus betulicola* form mycorrhiza only with *Betula* species. Some fungi have broad range such as *Pisolithus tinctorius* (Pt) that forms mycorrhiza with more than 46 tree species belonging to 20 genera (Cairney and Chambers, 1997). Broadly ECM fungi are grouped as early stage and late stage according to their colonization on young roots (plants of age <5 years) and older root system (plants of age >5 years), respectively (Rao *et al.,* 1997) (Table 1.3). Early stage fungi are very important for reclamation and forestry purposes. In older plantations, with the change in canopy and nutritional status of soil the late stage fungi take over. The carbon requirement of late stage fungi is higher than the early stage ECM fungi (Mason *et al.*, 1986).

Sub-division	Order	Family	No of confirmed genera
Basidiomycotina	Agaricales	Amanitaceae	2
		Boletaceae	13
		Cortinariaceae	10
		Entolomataceae	3
		Gomphidiaceae	5
		Hygrophoraceae	5
		Paxillaceae	2
		Strobilomycetaceae	3
		Tricholomataceae	6
	Russulales	Elasmomycetaceae	4
		Russulaceae	5
	Gautieriales	Gautieriaceae	1
	Hymenogastrales	Hydnangiaceae	1
		Hymenogastraceae	1
		Octavianinaceae	4
		Rhizopogonaceae	2
	Phallales	Hysterangiaceae	3
	Lycoperdales	Mesophelliaceae	1
	Melanogastrales	Leucogastraceae	2
		Melanogastraceae	2
	Sclerodermatales	Astraceae	1
		Sclerodermataceae	3
	Aphyllophorales	Cantharellaceae	3
		Clavariaceae	7
		Corticiaceae	4
		Hydnaceae	7
		Thelephoraceae	2
Ascomycotina	Pezizales	Balsamiaceae	3
		Geneaceae	1
		Geoglossaceae	3
		Helvellaceae	2
		Pezizaceae	4
		Pyronemataceae	3
		Terfeziaceae	4
		Tuberaceae	2
	Elaphomycetales	Elaphomycetaceae	1
Zygomycotina	Endogonales	Endogonaceae	2

Table 1.2 Taxonomic distributions of ectomycorrhizal fungi
(Source: ©Manual of Ectomycorrhizal fungi, Darwin Project, Viet Nam)

Early stage fungi	Late stage fungi
Cortinarius globuliformis	*Amanita citrina*
Cortinarius sanguineus	*Amanita muscaria*
Hebeloma crustuliniforme	*Amanita phalloides*
Hebeloma populinum	*Amphinema byssoides*
Hebeloma sacchariolens	*Austrogauteria manjimupana*
Inocybe brevispora	*Boletus edulis*
Inocybe lacera	*Boletus multicolor*
Inocybe lanuginella	*Cartinarius basirubesiens*
Inocybe umbrina	*Castreum camphoratum*
Laccaria bicolor	*Hydnum imbricatum*
Laccaria laccata	*Lactarius glyciosmus*
Laccaria proxima	*Lactarius hepaticus*
Laccaria tortilis	*Lactarius pubescens*
Paxillus involutus	*Leccinium scabrum*
Paxillus muelleri	*Leccinium versipelle*
Pisolithus microcarpus	*Mesophellia trabalis*
Pisolithus tinctorius	*Russula betularum*
Protubera canescens	*Russula emetica*
Ramaria sinapicolor	*Russula grisea*
Rhizopogon luteolus	*Russula lepida*
Sclerderma verrucosum	*Suillus tomentosus*
Sclerderma paradoxum	*Suillus umbonatus*
Suillus bovinus	*Tricholoma flavovirens*
Suillus subluteus	*Tricholoma pessundatum*
Thelephora terrestris	*Xerocomus badius*

Table 1.3 Early stage and Late stage ECM fungi (Source: Garg, 1999)

BENEFITS OF ECM ASSOCIATION

Central to the success of this symbiosis is the exchange of nutrients between the symbionts is bidirectional movement of nutrients. Plant provides carbon and essential vitamins to the fungus and the fungus provides inorganic nutrients to the host, thereby creating a critical linkage between plant root and soil (Sylvia *et al.*, 2004). ECM particularly improves uptake of nutrients present with low mobility in the soil e.g. phosphorus (Bolan, 1991). ECM fungi assist plant through augmenting its hydraulic conductivity, tolerance to drought and resistance to soil-borne pathogens (Marx, 1969; Sylvia *et al.*, 2004). ECM fungi also improve structure and aggregation efficiency of soil (Bogeat-Triboulot *et al.,* 2004). Further benefit comes to host tree by increased tolerances to extreme conditions of soil temperature, pH and high concentrations of heavy metals (Hung and Chien, 1978; Colpaert and Van Assche, 1987; Marx and Cordell, 1989; Jentschke and Godbold, 2000). ECM fungi are also known to produce various growth promoting substances, organic acids, antibiotics and fatty acids (Ho, 1987). Through these different mechanisms, a mycorrhizal plant is benefitted by increased growth, improved fitness and better survival.

Plants live in communities and their roots are connected by common mycelia of mycorrhizal fungi. Fungal hyphae can be connected to more than one plant of the same or different species. Plants can host different mycorrhizal fungi of the same or different species (Carlisle *et al.*, 2001). This

helps the host plant and fungi to share carbon, nitrogen, phosphorus, and other resources within the community.

Besides being beneficial to plants, many ECM fungi are source of food for men and animals e.g. *Agaricus bisporus*, *Boletus edulis*, *Lactarius deliciosus*, *Pleurotus* spp., *Tuber* spp., thus contributing to the economy of many human communities. Edible mushrooms are a high value, non-timber forest product of increasing commercial importance in international markets. Ecologically, ECM fungi play important role in stability of forest ecosystems.

Some mushrooms and polypores are used for dye making e.g. *Pisolithus tinctorius* yields tan to gold dye, several varieties of *Dermocybe* species yield pink, salmon and red dyes, *Gymnopilus spectabilis* yields butter yellow dye, and *Omphalotus olivascens* yields gray, green and purple dyes (Fig 1.2) (Dustin, 2011).

. 	Mushroom dye sample, clockwise from top left: *Pisolithus tinctorius*, *Democybe* spp., *Gymnopilus spectabilis*, *Omphalotus olivascens*
Yarns dyed with the dyer's puffball *Pisolithus tinctorius*	

Fig.1.2 Yarns dyed with mushroom pigments

Important role of ECM fungi in the association

ECM fungi are a special group of soil fungi that are beneficial to plants. These fungi are multi-purpose, accomplishing many essential functions for their plant partner, the most important being their role in the absorption of water and nutrients from the soil. The increased uptake is the result of a significant increase in the plant-soil interface that can be attributed to several factors. Firstly, the hyphae of the ectomycorrhizal fungi grow out beyond the nutrient depletion zone that develops around the root. Secondly, the colonized roots are extensively branched increasing the absorptive surface area. Third factor contributing to the effective absorption of nutrients by mycorrhizae is the narrow diameter of hyphae relative to roots due to which hyphae can penetrate into small soil pores inaccessible to roots or even root hairs. Another factor is that soil solution is less depleted at the surface of a narrow absorbing unit such as hypha (Sylvia *et al.*, 2004).

Availability of phosphorus (P) for plant growth is a limiting factor in soil. Mechanisms suggested for increased uptake of P by ectomycorrhizal plants include exploration of larger soil volume, faster movement of P into mycorrhizal hyphae and solubilisation of soil phosphorus (Bolan, 1991). An ECM plant has better ability to resist drought as the fungi increases the movement of water into the plant. Enhanced osmotic adjustment and leaf hydration of host plant and reduced oxidative damage or improved nutritional status have also been proposed to explain the contribution of mycorrhizal fungi symbiosis to the drought resistance of host plants (Auge, 2001; Porcel

and Ruiz-Lozano, 2004). Some ECM fungi are seen to excrete phytohormone to elevate drought resistance (Han *et al.*, 2006; Zhang *et al.*, 2011). Extrametrical hyphae of ECM fungi forms extensive hyphal network and liberates various chemicals in the soil. This holds the soil particles together and helps to improve the quality of soil (Bogeat-Triboulot *et al.*, 2004).

Ectomycorrhizae can enhance the resistance of plant to soil-borne pathogens. The mechanisms proposed to explain the protective effect include; development of a mechanical barrier (especially the mantle of EM) to infection by pathogens, production of antibiotic compounds that suppress the pathogen, competition of nutrients with the pathogen, including production of siderophores and induction of generalised host defence mechanisms (Sylvia *et al.*, 2004; Zak, 1964, Marx, 1972; Yamaji *et al.*, 2005). Table 1.4 summarises the antagonistic activity of various ECM fungi against soil-borne pathogens.

ECM fungi	Test fungi	Test sample
Laccaria laccata *Lactarius deliciosus* *Leucopaxillus cerealis* *Pisolithus tinctorius* *Suillus luteus*	*Armillaria mellea* *Cylindrocladium scoparium* *Fomes annosus* *Fusarium oxysporum* *Phytophthora* sps. (9) *Polyporus tomentosus* *Poria weirii* *Pythium* sps (24) *Rhizoctonia* sps. (5) *Sclerotium bataticola* *Thanatephorus* sps.(3) Soil Bacteria	Mycelia Disc Culture Filtrate
Amanita citrina *Laccaria bicolour* *Laccaria laccata* *Pisolithus tinctorius* *Suillus brevipes* *Tricholoma flavovirens* *Tricholoma Pessundaium*	**Ascomycetes** *Cylindrocarpon sp.* *Cylindrocladium floridanum* *Cylindrocladium sp.* *Gliocladium roseum* *Hypoxylon mammatum* *Leucostoma kunzeii* *Pyropyxis rubra* *Rhizina undulate* *Truncatella hartigii* *Valsa friesii* **Basidiomycetes** *Armillaria mellea* *Coltrichia tomentosus* *Fomes pini* *Polyporus schweinitzii* *Rhizoctonia praticola* *Rhizoctonia solani* **Imperfecti** *Fusarium oxysporum* **Phycomycete** *Pythium debaryanum* **ECM-Ascomycete** *Sphaerosporella brunnea*	Cell Free Culture Media

Table 1.4 Antagonistic activities of various ECM fungi (continued)

ECM fungi	Test fungi	Test sample
Pisolithus tinctorius	**Phytopathogen** *Phytophora* sp. *Rhizoctonia solani* *Fusarium solani* *Pythium debaryanum* *Pythium ullimum* *Verticillium dahliae* *Pyrenochaeta terrestris* *Cochliobolus sativus* *Septoria musira* *Brunchorstia pinea* **Dermatopathogenic** *Microsporum gypseum* *Trichophyton equinum*	Fraction - Pisolithin A
Pisolithus tinctorius SMF	*Fusarium solani* *Phanerochaete chrysosporium* *Geotrichum candidum* *Verticillium dahliae*	Fungal Colony Culture Filtrate
Paxillus sp.60/92	*Pythium vexans*	Culture Filtrate

Table 1.4 Antagonistic activities of various ECM fungi (Marx, 1969; Kope and Fortin, 1989; Tsantrizos *et al.*, 1991; Suh *et al.*, 1991; Yamaji *et al.*, 2004)

ECM fungi tolerate several metal species. The tolerance of ECM fungi could be either adaptive, constitutive or induced (Meharg, 2003). Metal exclusion mechanisms possibly operating in ectomycorrhizae include sorption of metals in the hyphal sheath, reduced apoplastic mobility as a result of hydrophobicity of the fungal sheath, exudation of chelating substances, or sorption on the extrametrical mycelium (Meharg, 2003; Gadd, 1993; Jentschke and Godbold, 2000). ECM fungi may alter metal sensitivity of their hosts, by either directly affecting metal availability and specification or indirectly modifying plant physiological processes, for e.g., by phytohormone action

(Jentschke and Godbold, 2000). In ECM fungi binding of heavy metals by phosphates (polyphosphate granules) and sulphydryl molecules (metallothioneins, phytochelatins) is also proposed (Galli *et al.*, 1994; Collin-Hansen, 2007).

ECM fungi increase the tolerance of host plant to extremes of soil temperature. Fungi survive extreme temperature by forming vegetative propagules such as spores or sclerotia, synthesizing stress proteins and accumulation of protective organic compounds (Tibett *et al.*, 2002; Ferreira *et al.*, 2005; Ferreira *et al.*, 2007). ECM fungi produce growth promoting substances like auxins isobutanol, isobutyric acid, indole acetic acid, gibberellins, cytokinins, vitamins, volatile organic acids, etc. (Satyanarayana *et al.*, 1996; Ho, 1987). Thus, an ECM plant grows and survives in low fertility or disturbed soils (Harley and Smith, 1983).

Pisolithus

Pisolithus, a member of class Gasteromycete, has wide global distribution. Pt has been recorded in a range of habitats including forest, urban and orchard sites, as well as eroded and mine-site soils (Marx, 1977; Malloch and Kuja, 1979). Carpophores are usually seen in relatively dry sites with little humus or along road side areas. Sites where this fungus exists are commonly characterised by high summer soil temperature, extreme acidity, draughtiness, low fertility and high levels of toxic metals. This combined with ability of *Pisolithus* to form extensive mycorrhizal relations with numerous tree species

makes it quite popular in reclamation efforts. Fungus is an early coloniser and is generally regarded as poorly competitive with other ectomycorrhizal fungi (Marx *et al*., 1984; McAffee and Fortin, 1986). However, because of its adaptability to adverse soil conditions, ease of manipulation and wide geographic distribution and tree host range, it is the most commonly used Bio-Inoculant. It is first of all early stage EM fungi to be made available commercially.

Pisolithus tinctorius (Pt) typically forms bright yellow ECM with a thick fungal sheath and well developed Hartig net. There are inter-isolate differences in the degree of sheath development (Marx *et al.*, 1970). Pt produces an extensive extrametrical mycelia phase which differentiates into rhizomorphs and sclerotia. Rhizomorphs are important in channeling nutrients and water to and from the host and in protecting extrametrical mycelium against diverse environmental conditions. The mycelia of Pt can exceed 30 m in diameter from the host tree in the field (Anderson *et al.*, 1998). Sclerotium acts as a storage organ and helps the fungus to withstand edaphic stresses in a vegetative state. Thus, the formation of sclerotia, along with rhizomorphs, may be important in the reported success of Pt in stressful soil conditions.

From work conducted to date, considerable level of variation displayed within Pt in different aspects of the symbiosis, makes it difficult to extrapolate the physiological capabilities of the fungus.

Cultivation and Physiology of ECM fungi

Unlike endomycorrhizal fungi, ECM fungi are neither intracellular nor require the presence of host for cultivation in laboratory. These features make it possible to use it on a large scale. The requirement is to properly understand the physiology of the fungus so that the cultivation procedures can be manipulated. Detailed study is thus required to optimise the culture parameters for the promising ECM fungi.

Axenic culture of ECM fungi can be established *in vitro* from the spores, sporocarp tissues or mycorrhizae. Usually Hagems (Modess), modified Melin Norkrans (MMN), modified Pridham-Gottlieb, Potato dextrose agar media, KHO and Glucose mineral salt medium (GMSM) (Table 1.4) have been used by researchers to establish *in vitro* culture of these fungi or investigate the physiology (Garg, 1999; Litchfield and Lawhon, 1982; Marx, 1969; Mason, 1980; Modess, 1941). Except GMSM and KHO, all the media contain one or more complex organic component. MMN is the most commonly used media for ECM research. Majority of *in vitro* physiological studies on ECM fungi have been conducted using MMN. However, it is not possible to examine physiological characteristics using complex organic as biochemical details are not revealed completely.

Medium Component	MMN[1]	Hagem[2] (Modess)	MPG[3]	KHO[4]	GMSM[5]
Macro-nutrient (g L^{-1})					
Glucose		5.00	30.00	20.00	20.00
Sucrose	10.00			5.00	
Casein hydrolysate					
Peptone			10.00		
Malt extract	3.00	5.00			
Yeast extract			2.00		
NH_4Cl		0.50		0.50	1.00
$(NH_4)_2HPO_4$	0.25				
$(NH_4)NO_3$			3.00		
$(NH_4)_2C_4H_4O_6$					
KH_2PO_4	0.50	0.50	2.38	0.50	1.00
K_2HPO_4			5.65		
KCl					0.10
$MgCl_2.4H_2O$					0.30
$MgSO_4.7H_2O$	0.15	0.50	1.00	0.50	
$CaCl_2$	0.05				0.132
NaCl	0.025				0.10
Na_2EDTA					0.03752
$FeCl_3$*	1.2mL	1.2mL			
$FeSO_4.7H_2O$			0.0011		0.01053
$Fe_2(SO_4)_3$				0.05	
$FeC_6H_5O_6.3H_2O$					
$Fe(NO_3)_3.9H_2O$					
Micro-nutrient (µg L^{-1})					
$CuSO_4.5H_2O$			6400		97
H_3BO_3					2784
$MnCl_2.4H_2O$			1900		
$MnSO_4.H_2O$					3380
$Na_2MoO_4.2H_2O$					338.46
$ZnSO_4.7H_2O$			1500		201
Acid (µl L^{-1})					
H_2SO_4					240
Vitamins (µg L^{-1})					
Thiamine. HCl	100	50			100
Biotin					40
pH	5.50	4.60-4.80	4.00/6.00	5.50	6.0

Marx, 1969 (1); Modess, 1941 (2); Litchfield and Lawhon, 1982 (3); Cudlin *et al.*, 1992 (4); Garg, 1999 (5); * 1% Ferric chloride solution

Table 1.5 Media used to cultivate ECM fungi

A relatively large number of studies have addressed the existence of inter- and intra-specific variation in the growth response of diverse arrays of ECM fungal species for physiological growth parameters. The effect of temperature range from 20°C to 37°C (Gupta *et al.*, 1997; Daza *et al.*, 2006) and pH range of 2.0 to 11.0 (Hung and Chien, 1978; Hung and Trappe, 1983; Gupta *et al.*, 1997; Yamanaka, 2003) in axenic condition for ECM fungi have been evaluated. The growth response of *Pisolithus* and other ECM fungi under saline condition with upto 500mM NaCl have been assessed (Dixon *et al.*, 1993; Kernaghan *et al.*, 2002; Bois *et al.*, 2006; Matsuda *et al.*, 2006; Madsen and Mulligan, 2007). Heavy metal tolerance in ECM fungi have been studied for metal species such as aluminium (Al), cadmium (Cd), chromium (Cr), copper (Cu), iron (Fe), lead (Pb), manganese (Mn), mercury (Hg), nickel (Ni), and zinc (Zn) (Thompson and Medve, 1984; Tam, 1995; Colpaert *et al.*, 2000). The response of ECM fungi under varying water potential induced using potassium chloride, sodium chloride, sucrose or polyethylene glycol was evaluated *in vitro* (Mexal and Reid, 1973; Coleman *et al.*, 1989; Whiting and Rizzo, 1999; Chen *et al.*, 2003; Dunabeitia *et al.*, 2004; Zhang *et al.*, 2011).

Stress

Mycorrhizal fungi are exposed to all or many of the environmental stresses that other fungi may experience. These include extremes of temperature and pH, anoxia, water stress, physical fragmentation, toxic metals and other pollutants, as well as anthropogenic stresses arising from applications of fertilisers, lime and wood ash. Fungi can respond to these

stresses by altering their morphology, modifying their external environment or adapting their internal metabolism (Finlay *et al.,* 2008). When any stress is applied, the most prominent physiological reactions are the production of a set of novel proteins or an increase in the quantity of certain types of existing proteins. Stress proteins are also known as heat shock proteins since they were first discovered against thermal stress. Heat shock proteins can be classified into three categories according to their molecular size: (I) high-molecular size, with molecular weight between 69 and 120 kDa, (II) medium-molecular size, with molecular weight between 39 and 68 kDa, and (III) low-molecular size, with molecular weight below 38 kDa. The small heat shock proteins (sHSPs) ranging from 12 to 42 KDa are synthesized ubiquitously in eukaryotic and prokaryotic cells in response to heat and other stresses (Ferreira *et al.*, 2005).

Synthesis and accumulation of protective organic compounds, such as sucrose, glycogen and trehalose is one of the tolerance mechanism found in fungi to live in adverse environmental conditions (Fillinger *et al.*, 2001). Trehalose has been reported as part of the physiological adaptation to various abiotic stresses in yeast and fungi (Plesofsky-vig and Brambl, 1993; Fillinger *et al.*, 2001). Ferreira *et al.* (2007) have demonstrated an accumulation of intracellular trehalose and an increase in trehalase activity in the mycelium of *Pisolithus* sp. RV82 in response to heat shock at 42°C.

Pigments/Phenolics in ECM fungi

Fungi are a good source of biological pigments. Characteristic pigments are produced by a wide variety of fungi (Duran *et al.*, 2002). Besides being a textile dye and food colorant the fungal pigments are found to be active against bacteria, yeast, fungi, protozoa and the insects. The cytotoxic activity of naphthoquinones against mouse leukemia and *HeLa* cells has been reported. Along with the antibiotic and toxic activities, naphthoquinones revealed mutagenic and carcinogenic properties (Medentsev and Akimenko, 1998**)**. Recently, the presence of naphthoquinone, a naphthalenoid pulvinic acid (pisoquinone) has been demonstrated in a white skinned variant of the ectomycorrhizal gastromycete *Pisolithus arhizus* (Gill and Kiefel, 2011).

ECM fungi produce various pigmented compound. Pigments in higher fungi are well documented (Velisek and Cejpek, 2011; Zhou and Liu, 2010). The fruiting bodies of some macro-mycetes can give a range of pinks, blues, yellows, reds, and browns (e.g., *Boletus, Cortinarius, Hydnellum, Hygrocybe* spp.). The chromophores of mushroom dyes contain a variety of fascinating organic compounds. Their pigmentation may vary with the age and some undergo distinctive colour changes on bruising. Many of the pigments of higher fungi are quinones or similar conjugated structures that are mostly classified according to the perceived biosynthetic pathways, reflecting their structure, to pigments derived from (*i*) the shikimate (chorismate) pathway, (*ii*) the acetatemalonate (polyketide) pathway, (*iii*) the mevalonate (terpenoid)

pathway and (*iv*) nitrogen containing pigments. Phenolic compounds produced by ECM fungi are antimicrobial and involved in the protection against invasion by pathogens and competitive organisms. Antimicrobial phenolics compounds in ECM are synthesized preferentially in fruiting bodies, soil hyphae and the mantle (Nehls *et al.,* 1999). This probably explains host root protection that has been observed in ECM symbiosis.

Assessment and quantification of ectomycorrhiza

A variety of methods have been used to examine ectomycorrhizal associations. Primarily root systems of target plant species are excavated taking care to ensure that fine roots are well represented in samples and to exclude entangled roots of other species. Once separated from soil, the root samples are cleaned with water over a fine sieve to ensure that finest laterals are not lost. Fresh roots are assessed with dissecting microscope for external features. Unstained ECM roots can usually be distinguished from non-mycorrhizal roots by differences in their colour, thickness, texture and branching patterns (Brundrett *et al.*, 1995). The characteristics of ECM associations are typical and governed by the mycorrhizal fungus and the host plant. *Pisolithus* sp. forms a prominent golden yellowish mycorrhizal association with the host plant which is easy to identify by visual inspection. *Pinus radiata* roots show dichotomously branched root tips with several ECM fungi.

Internal features of the root confirm the presence of ECM association. Sectioning and staining of the ECM roots are necessary to visualise the Hartig net in the root cortex (Bevege, 1968; Brundrett *et al*., 1984). Fresh roots are chopped into 2-4 cm long segments. Root fragments are cleared by heating in 10% KOH and stained with Trypan Blue (0.05%) or Chlorazol Black E (CBE-0.03%) in lactoglycerol. Structural details of the stained specimen are observed using compound microscope. Alternatively, Acid Fuchsin can be used to stain fungal structures in roots and observed in combination with fluorescence microscopy. Diagnostic features of ectomycorrhizal roots are summarised in Table 1.6. Three dimensional details of interactions between fungus and host is revealed using electron microscopy (Brundrett *et al*., 1995).

ECM roots are usually quantified by sampling seedlings, or washing roots from soil cores, taking care to exclude contaminating roots of non-target species. Assuming that roots are young and healthy, each mycorrhizal root tip will contain an active Hartig net zone. These root tips are counted to quantify the intensity of the association, and their numbers should be expressed relative to root length and soil volume. The root length of a sample can be measured with the Gridline Intersect Method (Brundrett *et al*., 1995), while simultaneously measuring the length of mycorrhizal roots or separately counting the total number of mycorrhizal tips. In this method, the cleared and stained root sample is randomly dispersed in dish with gridlines using forceps and dissecting needle. The dish is placed under the dissecting microscope and

the gridlines and roots are focussed. Follow all the horizontal lines and count intersects with roots and mycorrhizas, separately. Procedure needs to be repeated for vertical lines. Total the counts of mycorrhizal roots got for horizontal and vertical lines. Similarly, total the counts for non-mycorrhizal roots. The ratio of the mycorrhizal roots and total roots give the percentage of root length colonised by mycorrhizal fungi. For certain mycorrhizae, the intensity of branching within a mycorrhizal cluster varies considerably and can be quantified with a Branching Density Index. ECM associations with minimal changes to the host roots for e.g., *Eucalypts* can be recognised with practice, but require more careful examination of roots.

ECM extramatrical mycelium play very important role in scavenging nutrients and water from the soil. The extent of extramatrical mycelium provides physiological competitiveness to the ECM. ECM extramatrical mycelial biomass estimates in soils are usually obtained by measuring total hyphal length or by measuring the amount of fungal-specific biomarkers such as ergosterol and phospholipid fatty acids (PLFAs) (Olsson, 1999; Ekblad *et al*., 1998; Wallander *et al*., 1997). Amounts of PLFA 18:2ω6,9 are generally used as indicators of the presence and abundance of fungi in soils and also assumed that each fungal species in a community contributes to the PLFA profile in proportion to its biomass. Ergosterol is a fungus-specific component of membranes and its content is therefore believed to be correlated with the amount of metabolically active fungal biomass. Ergosterol is considered as better indicator of vital fungal biomass than other available measures. These

methods are limited to the estimate of total fungal mass present in the soil and cannot differentiate the species. The estimates of particular EM fungus can be determined in the control conditions, i.e., only in the laboratory (Ek, 1997; Bidartondo *et al*., 2001; Wallander *et al*., 2001).

Landeweert *et al*. (2003) demonstrated the possible use of molecular methods such as denaturing gradient gel electrophoresis (DGGE), restriction fragment length polymorphism (RFLP), competitive polymerase chain reaction (PCR), real-time quantitative PCR and cloning-sequencing to identify and quantify EM mycelia from soil. Such molecular techniques enable the use of genes as biomarkers and facilitate identification of particular fungi directly from a mixed population environment as in soil. The three molecular methods i.e., DGGE, a clone and real-time quantitative PCR, showed consistent results and enabled identification and relative quantification of two ECM fungi, *Suillus bovinus* and *Paxillus involutus*, in mixed-species environment. Increase and decrease in the amounts of DNA of *S. bovinus* and *P. Involutus,* respectively were detected over time in an environment containing a more complex community. These methods clearly revealed the relative changes in fungal biomass of the two ECM species over a period.

Feature	Characteristics
Mycorrhizal development	Root branching patterns and density Root thickening Reduction in root elongation
External hyphae	Presence, abundance, distribution Arrangement - single, strands, rhizomorphs Structure - colour, wall, thickness, clamp connections, crystals Sclerotia
Mantle	Thickness Colour, changes with bruising, or age Hyphal organisation in surface and in deeper layers - loose or compact hyphae or pseudo-parenchyma, prosenchyma Hyphae - thickness, wall structure, clamp connections, septae Cystidia, crystals, exudates Reactions to chemicals (Melzer's reagent, KOH, etc.) Staining reactions Odour Fluorescence
Hartig net	Presence, thickness Hyphal organisation Hyphal structure

Table 1.6 Diagnostic features of ectomycorrhizal roots that may be associated with particular mycorrhizal fungi (Brundrett *et al.*, 1995)

Application in forestry

The biological requirement of many species of forest trees for ECM associations was observed in an attempt of forestation of cutover lands and other treeless areas and the introduction of exotic tree species, where native ECM fungi are deficient or reduced in species diversity (Marx *et al.*, 2002).

Although ectomycorrhizal fungi are naturally present in many soils, there is a lot of variation in their ability to colonize and benefit plants. It is therefore advantageous to inoculate the seedling with specific ECM fungus that would be beneficial within the specific environment. This so called 'mycorrhization control' is usually done by planting seedlings that have been previously inoculated in the nursery with the chosen fungal strain. This practice improves not only the survival of the seedlings upon transplanting but also their subsequent growth (Marx and Cordell, 1989; Marx, 1980). The economic benefits of this practice, in terms of increased productivity, have been demonstrated in plantations in the United States of America and in France (Marx and Cordell, 1989; Selosse *et al.*, 2000). However, in order for mycorrhizal inoculation to become a routine practice in nurseries, it is necessary to establish methods for inoculant production at industrial scale.

Efforts have been made to develop various inoculum formulations for application. The results of these studies suggest that vegetative inoculum forms are the most suitable for inoculating plant seedling in nurseries (Trappe, 1977; Marx and Cordell, 1989). However, the availability of vegetative inoculum is limited because of very slow growth rate and fragile nature of the ECM fungi *in vitro*.

Isolates of ectomycorrhizal fungi are generally selected on the basis of their compatibility and efficiency, where compatibility means the ability to colonize roots while efficiency means the ability to promote growth of the plant host. The efficiency is usually tested by evaluating parameters such as the height of the inoculated plant, the diameter of the stem, the overall dry mass of the plant and the nutrient content of the plant, especially phosphorus and nitrogen (Marx *et al.*, 1991). In the nursery, these studies can be performed in periods up to 6 months. However, under field conditions, it is necessary to follow plant development for several years (Selosse *et al.*, 2000).

The ideal ECM inoculant must contain sufficient numbers of infective propagules, remain viable during storage, transport and maintain its infectivity for several months after its production. Furthermore, the formulated inoculant must be easy to apply, free of contamination and production process should be cost efficient (Schwartz *et al.*, 2006). Moreover, the inoculants should

preferably contain a cocktail of different fungal species adapted to different soil properties and be compatible with other ingrediants (i.e., rhizobacteria, biostimulants, surfactants and other organics) that may be part of the product formulation (Marx *et al.*, 2002). Table 1.7 summarises the commercial inoculum of ECM fungi. Three main types of ectomycorrhizal inoculants commonly used include soil, fungal spores and vegetative mycelia. Also methods involving entrapment of fungal materials in natural polysaccharide gels, including immobilization of mycorrhizal root pieces, and spores, in some cases co-entrapped with other plant-beneficial microorganisms are being used.

Commercial product	Type/ process	Species	Company	Reference
BioGrow Blend®	spores	--	Terra Tech, LLC	Mycorrhizal Inoculants
MycoApply®-Ecto	spores	--	Mycorrhizal Applications Inc.	Mycorrhizal Applications
Mycorise Pro Reclaim®	Propagules ecto+endo	--	Symbio Technologies Inc.	Estelle *et al.*, 2003
Myke® Pro LF3	Propagules	--	Premier Tech Biotechnologies	Horticultural Professional
MycorTree®	Spores	--	Plant Health Care, Inc.	Plant Health Care Products
MycoRhiz®	Mycelium/ SSF	*Pisolithus tinctorius*	Abbot Laboratories	Marx *et al.*, 1982
Somycel PV	Mycelium/ SSF	*Hebeloma crustuliniforme* *Laccaria laccata* *Paxillus involutus*	INRA-Somycel S.A.	INRA, 1990
Ectomycorrhizal Spawn	Mycelium/ SSF	*Laccaria laccata* *Pisolithus tinctorius*	Sylvan Spawn Laboratories, Inc.	Marx *et al.* 1989
-	Mycelium/ Submerged	*Hebeloma crustuliniforme*	Rhone Poulene-INRA	INRA, 1990
Mycobead®	Mycelium/ Submerged	Eleven ECM strains	Biosynthetica Pty. Ltd.	Kuek, 1996
Ectovit®	--	--	Symbio-m Ltd.	The Keeler Group & Mycorrhiza

Table 1.7 Commercial availability of ectomycorrhizal fungi inoculants

Soil inoculum

A thin layer of soil obtained from natural forests, old nurseries or established plantations is spread on the top of nursery bed and mixed with the soil or planting substrate (Mikola, 1973). This method is still used in many parts of the world, particularly in developing countries. The use of forest soil inoculums has major disadvantages. Species composition of ECM fungi in the inoculums is usually not known, and the inoculums may also contain harmful microorganisms and noxious weeds (Marx, 1975). Moreover, there is no precise information about the fungal species that are being introduced and their infection potential (Castellano and Molina, 1989). Despite these disadvantages, soil inoculant is recommended if no other type of inoculant is available with the premise that any ECM are better than none.

Spore inoculum

Fungal spores, obtained from fruiting bodies harvested in natural forests, old nurseries or established plantations, have also been used in many parts of the world (Theodorou, 1971)). They are easily obtained and can be easily applied to plants. They can be mixed with sand, clay, or vermiculite carrier before being added to soil, suspended in water and drenched or irrigated, dusted or sprayed, pelleted and broadcast, and encapsulated or coated onto seeds (De la Cruz, 1990; Marx *et al.*, 1984; Theodorou and Bowen, 1973; Castellano and Molina, 1989). This type of inoculant is limited

to those fungal species able to produce large numbers of spores and fruiting bodies. As spores are generally collected from multiple fruiting bodies, they tend to present a higher genetic variability than vegetative inoculant. The availability of spores is erratic during the year, hence the need to collect and store large numbers of fruiting bodies when they are abundant (Marx *et al.*, 1991; Cordell *et al.*, 1988). According to Marx *et al.* (1991), root colonization by this type of inoculant is slower than that presented by vegetative inoculant of the same fungal isolate. Most commercial forms of ECM inoculum are spore based. These formulations contain approximately 10 million spores per unit (Soil Moist – JRM Chemical, Inc.). The amount of inoculum applied to the individual plant depends on the height of the plant at the time of application.

Vegetative inoculum

Addition of mycelia obtained from pure cultures of ectomycorrhizal fungi, also called vegetative inoculant, has proven to be the most suitable method. Pure cultures are generally obtained from fruiting bodies or from mycorrhizas and maintained under laboratory conditions. For commercial production of vegetative inoculant, mycelium has to be grown in solid substrate or in liquid culture.

Production of vegetative ECM inoculants

Microbial inoculants are conventionally produced using solid-state fermentation and submerged liquid fermentation. Both this methods were reviewed by Walter and Paau (1993). Liquid systems are attractive for culture and production of biological products. Liquid substrates are easily mixed, producing more uniform conditions for culture growth than solid substrates. They also allow easier and quicker changes of culture variables such as pH, dissolved oxygen, temperature, stirrer speed and nutrient concentration. In comparison, solid-state fermentation has many disadvantages. Spatial homogeneity within the bed of solid particles is typically poor. It takes long time to achieve sterilisation as heat transfer ability of bed is poor. The risk of failure to sterilize the substrate, increases the cost of the process. Moreover, solid-state fermentation systems are difficult to monitor, either by sampling or with probes. As a result, scale-up of solid-state fermentation bioreactors is more complex. The main advantages of solid-state fermentation are the reduction of bacterial contamination due to the low water activity in solid substrates, the low costs of installation and the simplified design of bioreactors.

Cultivation of ectomycorrhizal fungi in solid substrates

Solid-state fermentation has been the most common technique used to date for production of vegetative inoculants of ectomycorrhizal fungi. The preferred substrate for this process has been a mixture of peat and vermiculite, supplemented with a nutritive solution (Marx *et al.*, 1982). Vermiculite is a low price material and absorbs nutrient solutions well. It also provides a bed structure that enables good aeration of the substrate. The mixture is generally distributed in glass flasks or plastic bags, being inoculated with mycelium plugs or a mycelium suspension, obtained from a previous culture in solid or liquid medium, respectively. It then takes 2–4 months of incubation for the final product to be ready to inoculate seedlings (Alves *et al.*, 2001). This method has been used for the propagation of ectomycorrhizal fungi in the laboratory, and also at industrial level for commercial production (Marx *et al.*, 1982). When applied to the planting substrate, the mycelium remains protected inside the vermiculite particles, where it can survive until receptive roots are produced by the host plant (Marx and Kenney, 1982). Inoculation of plants with vegetative inoculants produced in solid substrates has been done in the USA (Castellano and Molina, 1989; Marx *et al.*, 1982), France (Selosse *et al.* 2000), Mexico (Valdes, 1986), Brazil (Krugner and Filho, 1980), and Liberia (Marx *et al.*, 1985), among other countries, with the inoculants showing high infectivity towards several plant species.

Among the ectomycorrhizal fungi applied as vegetative inoculants, *Pisolithus tinctorius* has been the most frequently applied, due to several factors. The fungus has a wide geographical distribution, a wide host range, tolerates environmental stress well and, is relatively easy to cultivate (Theodorou, 1971; Castellano, 1990). Vegetative inoculant of other ectomycorrhizal fungal species such as *Hebeloma crustuliniforme*, *Laccaria laccata*, *Suillus luteus*, *Cenococcum geophillum* and *Thelephora terrestris*, have also been tested with plant hosts of economical interest (Castellano, 1990).

Submerged cultivation of ectomycorrhizal fungi

Techniques that are currently used for the submerged culture of microorganisms of industrial interest are adapted for the production of ectomycorrhizal inoculants. Compared to solid-state fermentation, liquid fermentation requires less space and time since in liquid medium the contact between phases is maximized and nutrients are more efficiently utilized. After cultivation in bioreactors, mycelium may be immobilized in calcium alginate gel or other types of polymeric matrices. Nursery studies have shown that this type of inoculant is more efficient than those produced by solid-state fermentation, probably due to better survival of the fungus inside the gel when applied to the planting substrate (Le Tacon *et al.*, 1985). Pradella *et al.* (1991)

were the first to study the production of Pt under submerged conditions. Thereafter, intensive research on submerged cultivation of ECM fungi has been carried out to improve the biomass production. Inoculum produced by the submerged aerobic culture of mycelia immobilized within hydrogel beads was found to be of high efficacy (Kuek *et al.*, 1992). Moreover, Kuek (1996) studied the effect of variations in some physicochemical culture parameters on biomass yield of ECM fungi *Laccaria laccata*. Garg (1999) formulated a chemically defined medium suitable for improved biomass production of ECM fungus, and attempted production of *Laccaria laccata* and *Laccaria fraterna* inoculum under submerged condition. Rossi and Oliveira (2011) found a variation of the Pridham-Gottlieb medium efficient for the production of biomass. Rossi *et al.* (2002, 2006, 2007) used airlift bioreactor and attempted to optimise the operational conditions. Despite being a very promising technique submerged fermentation still has limited application. The major drawbacks are the relatively poor growth obtained in most cultivation systems and frequent contamination by saprophytic microorganisms during cultivation, and operational steps overall increasing the production costs (Rossi *et al.*, 2007).

ECM fungi as Bio-inoculants

Over the past few decades a tremendous amount of research has been done on the practical use of mycorrhizal fungi demonstrating their effectiveness in improving survival and health of many plant species on diverse landscapes. Considering this aspect, one cannot overlook the

reforestation success in USA of Ohio Division of Mine Land Reclamation – Abandoned Mined Lands (AML) program that used Pt-inoculated pine and oak seedlings to provide a low cost, low maintenance reclamation method for abandoned mine-lands (Cordell *et al.*, 2000). Another example is the reclamation of Utah Copper Mine Site using Pt and VAM fungi (Marx *et al.*, 2002). Reddy and Natarajan (1997) have demonstrated the promising application of *Laccaria fraterna, L. laccata, Pisolithus tinctorius, Rhizopogon luteolus, Scleroderma citrinum* and *Thelephora terrestris* wth several species of *Pinus*, in reforestation of the Nilgiri and Palni hills of Tamilnadu, South India. Sesa Goa, a mining company has used *Pt-Acacia mangium* combination for reclamation of reject dumps.

Aims and objectives of the research work

Pisolithus, a Gasteromycete, has widespread global distribution and is often found in adverse environments. Sites where this fungus exists are commonly characterised by high soil temperature, extreme acidity, draughtiness, low fertility and high levels of toxic metals. This combined with ability of *Pisolithus* to form extensive mycorrhizal relations with numerous tree species makes it quite popular in reclamation efforts. Moreover, *Pisolithus tinctorius* is reported to have wide spectrum of antibiotic activity. *Pisolithus tinctorius* yields beautiful spectrum of gold to chestnut brown colour dye which are being used to dye the yarns.

Thus, *Pisolithus tinctorius* is a well known economically and ecologically important ECM fungus. Although *Pisolithus* is highly focused ECM species, a great deal of detailed study is needed to tap the fungi and its products for ecological and industrial utilisation.

In view of this, the following objectives were undertaken for study:

1) Stress resistance mechanism of *Pisolithus*:

- Effect of varied stress conditions on growth: water activity, pH, temperature, salinity and metals (Mn and Fe).
- Identification of stress related protein/peptides in whole cell protein.

2) Pigment/s and/or phenolics produced by *Pisolithus*:

- Production of pigment/s and/or phenolics under stationary as well as submerged conditions.
- Purification, characterization and determination of possible biological activity of pigment/s and/or phenolics.
- Parametric optimization for improving production.

3) Mycorrhizal synthesis:

- Selected tree species of forestry importance in Goa will be tested to undergo mycorrhizal synthesis with *Pisolithus*.

CHAPTER II

Growth response of Pisolithus sp. isolate

INTRODUCTION

In vitro growth response of ECM fungi to various growth factors show variation between and within species (Hung and Trappe, 1983; Matsuda *et al.*, 2006). Earlier studies report ECM fungi to tolerate pH 2-8 & 6-11 (Hung and Trappe, 1983), temperature of 20-40°C (Gupta *et al.*, 1997), 0-3% sodium chloride (Matsuda *et al.*, 2006), water potential of 0 to -3MPa (Coleman *et al.*, 1989), 0-500 ppm manganese (Thompson and Medve, 1984) and 0-400 ppm iron (Tam, 1995). The wide tolerance relates the wider ecological distribution of ECM fungi. ECM fungi promote plant growth and productivity even in adverse soil conditions or stressed environment (Harley and Smith, 1983). Various physical and biochemical mechanisms have been suggested by which the ectomycorrhizal fungi confer stress resistance to host plants. Recently stress proteins are being investigated in ECM fungi. An increase in the content of glutathione and γ-glutamyl cysteine was observed when *Paxillus involutus* was exposed to cadmium. An additional compound with a 3 KDa molecular mass, probably related to a metallothionein, increased drastically in mycelia exposed to cadmium (Courbot *et al.*, 2004). *Pisolithus* isolate RV82 exposed to heat shock for 30 min showed the synthesis of 70, and 28, 26 and 15-18 KDa sHSPs (Ferreira *et al.*, 2005). Liang *et al.* (2007) found 14 upregulated and 8 downregulated proteins in *Boletus edulis* under salt shock (4% NaCl for 6 h). All these studies have been carried out in complex organic medium that is modified Melin-Norkran's medium (MMN), widely used media for cultivation of ECM fungi. Demarcation of stress zones in an ECM fungus for

various edaphic factors, necessitate a detailed physiological study in axenic culture conditions in chemically defined medium.

The ease with which the fungus can be grown in vitro has facilitated extensive study of its physiology and so also the simplicity of mycorrhizal synthesis under controlled conditions with a range of host plants.

2.1 MATERIALS AND METHOD

2.1.1 Fungal culture

Pisolithus sp. isolate obtained from iron ore mine at Codli, Goa was used for the study.

2.1.2 Culture isolation

Basidiocarps that were in mycorrhizal association with *Acacia mangium* were collected from iron ore mine at Codli, Goa. Sporocarps were washed with sterile water. Sporocarps were surface sterilised with sodium hypochlorite solution containing 2% (v/v) of active chlorine (Appendix B) and cut opened aseptically using sterile scalpel. Inner tissue of mushroom was picked using sterile forceps and placed onto the modified Melin Norkran's agar medium (MMN, pH 6.5) (Marx, 1969) (Appendix A) and plates were incubated at room temperature (RT).

2.1.3 Culture maintenance

Pure fungal mycelia were maintained by periodic transfer on MMN and Glucose Mineral Salt Medium (GMSM, pH 6.0) (Garg, 1999) (Appendix A). The plates were maintained at RT and sub-cultured after every 45 days.

2.1.4 Culture identification

2.1.4.1A Morphological identification

Morphological characteristics of mushrooms such as their size, colour, presence or absence of volva, stipe, ring, scales, reticulum, zonation, striation, warts, cap, areolae and gills were noted. Transverse sections of the sporocarps were prepared. Mycelial growth and morphology of dried spores were examined microscopically. Fungal hyphae were stained with Lactophenol Cotton Blue solution (Appendix B) (HiMedia) and observed under Olympus BX51 compound microscope. Images were captured with Olympus DP71 digital camera attached with the microscope. Basidiospores samples were prepared for scanning electron microscopy using method described by De Melo and Faull (2004) with slight modifications (Appendix C) and examined with a JEOL, 5800LV, Japan, Scanning Electron Microscope (SEM).

2.1.4.1B Molecular Identification

Molecular identification of the isolated mycelia was performed using sequence data of the ITS region of the nuclear ribosomal DNA. Total genomic DNA was extracted from 100mg fresh mycelium according to CTAB method as described by Gardes and Bruns (1993) and Graham *et al.* (1994) (Appendix C). The ITS region of rDNA was amplified using the primer pair ITS 1 and ITS 4 (White *et al.*, 1990). Amplification was carried out using BIOERXP thermal cycler in a 50 µl reaction mixture (Appendix C). The sequencing of amplified rDNA was done by Bangalore Genei, India. The sequences of variable region (ITS) and complete 5.8S rRNA were compared with the sequences available in the public nucleotide databases at the National Center for Biotechnology information (NCBI) using its world wide web site (http://www.ncbi.nlm.nih.gov/entrez), and the BLAST (Basic local alignment search tool) algorithm. Sequence alignment and comparison was done using the multiple sequence alignment software ClustalX2 and data was converted into PHYLIP format. Minor modifications were done manually on the basis of conserved domains wherein columns containing more than 50% gaps were excised. A Neighbour-joining (NJ) tree was obtained with 500 seeds and 10,000 bootstraps. The final tree obtained was rooted and drawn using MEGA4. The sequence obtained has been deposited in GenBank.

2.1.5 Growth response of *Pisolithus* sp.

2.1.5.1 Effect of various growth factors

The *in vitro* growth response of *Pisolithus* sp. was checked using GMSM with varying particular medium component or growth condition.

An inoculum disc (0.8mmX0.5mmX0.5mm) was cut from the actively growing edge of 20-days-old colonies on MMN were used to inoculate solid media in petri plate and 20 ml liquid medium in 100ml conical flask. Inoculated media were incubated at RT for a period of 30 days.

2.1.5.1A Effect of growth media

The isolate of *Pisolithus* sp. was grown on complex organic MMN and chemically defined GMSM agar medium.

2.1.5.1B Effect of buffering systems

Pisolithus sp. was grown on GMSM agar amended with various buffer systems, HEPES buffer (0.02 M), Phosphate-citrate buffer (PCB) (0.02 M), and Sodium phosphate buffer (SPB) (0.02 M) (Appendix B). GMSM without any buffer was used as control.

2.1.5.1C Effect of phosphate

Effect of varying concentration of phosphate was checked from 0.007 to 0.107 M. The molarity of phosphate in the medium (pH 6.8) was adjusted using 0.1 M SPB solution (Appendix B).

2.1.5.1D Effect of Phosphate buffers

The growth of *Pisolithus* sp. was checked on GMSM (pH 6.8) with 0.02 M and 0.04 M SPB and PCB (Appendix B). The amount of buffer was in addition to 0.007 M of phosphate present in basal medium.

2.1.5.1E Effect of pH

The effect of pH was determined by adjusting GMSM medium to pH 2.2 to 8.0 using 0.04 M PCB in addition to the phosphate present in basal medium (Appendix B).

2.1.5.1F Effect of Temperature

GMSM solid and liquid medium inoculated with *Pisolithus* sp. were incubated at different temperature from 10 to 50ºC.

2.1.5.1G Effect of Sodium chloride (NaCl)

Pisolithus sp. was grown using GMSM agar and broth with varying concentration of NaCl from 0 to 4%.

2.1.5.1H Effect of water potential

Pisolithus sp. was grown in GMSM broth adjusted to different water potential by varying amount of polyethylene glycol (PEG 6000) from 0 to 35%.

2.1.5.1I Effect of Manganese

Growth response of *Pisolithus* sp. to manganese (Mn) was determined on GMSM. Basal GMSM medium was prepared containing micronutrient solution without $MnSO_4$. Different amount of Mn from 0 to 10,000 ppm was prepared by incorporating suitable amount of $MnSO_4$ salt solution (50,000 ppm Mn) (Appendix A).

2.1.5.1J Effect of ionic form of iron

Fungal isolate was grown on GMSM amended with 2.121, 40 and 50 ppm Fe using $FeSO_4.7H_2O$ or $FeCl_3$ salts as a source of iron (Appendix A). Basal GMSM medium was prepared without Fe. Iron stock solution was added to basal medium after autoclaving, before pouring the plates.

2.1.5.1K Effect of iron

Growth response of *Pisolithus* sp. to iron (Fe) was determined from 0 to 100 ppm by incorporating suitable amount of 1000 ppm Fe stock solution of $FeCl_3$ salt (Appendix A).

2.1.5.3 Analytical procedures

2.1.5.3A Determination of growth

2.1.5.3Aa Colony Diameter

Fungal growth was recorded by measuring colony diameter at 30 days of incubation. The diameter of each colony was measured thrice by rotating the plate every-time at 60^o to the previous. To account for non-symmetry the values obtained were averaged and reported along with standard errors.

2.1.5.3Ab Biomass quantification

Growth in liquid medium was determined as dry biomass. Fungal masses were collected and washed with sterile distilled water. Mycelium was kept in pre-weighed aluminium cups at 80ºC. Dry weight of fungal biomass was recorded after every 2 h till constant weights of cups were obtained.

The pH of agar medium and culture broth were determined using pH-paper and pH-analyser (LABINDIA), respectively.

2.1.5.3B Statistical Analysis

Four replicates were maintained for each experimental condition. Impact of parameters on growth of *Pisolithus* sp. and significant differences between treatments were assessed by analysis of variance (ANOVA) at $P<0.001$ and treatment means were compared by least significant difference ($P<0.05$) using Student-Newman-Keuls Method.

2.1.6 Stress response of *Pisolithus* sp.

2.1.6.1 Biomass for protein analysis

Pisolithus sp. was grown in GMSM broth at optimum and stress conditions (Table 2.1). Stress was defined as the conditions of physical parameter that cause reduction in growth with less than 60% of maximum biomass obtained. Effect of pH, temperature, sodium chloride, PEG and manganese were studied. After incubation of 30 days fungal masses were collected and washed with sterile deionized water. Wet weight of the mycelium was taken and protein extraction was carried out (Chen and Chen, 2004; Osherov and May, 1998; Burgess *et al.*, 1996) (Appendix C). Protein precipitate was dissolved in 100mM Tris HCl, pH 8.0. Protein content was quantified by using Folin-Lowry's method (Lowry *et al.*, 1951) (Appendix D). The protein sample was electrophoresed on 12% SDS-Polyacrylamide Gel

(Sambrook *et al.*, 1989) (Appendix C). Protein bands were visualised using silver staining method (Blum *et al.*, 1987) (Appendix C).

2.2 RESULTS AND DISCUSSION

2.2.1 Culture identification

Sporocarps of *Pisolithus* sp. were of 1.0 to11.5 cm in diameter, rounded or club-shaped, yellowish smooth shiny surface, with deeply rooted fibrous base (Fig.2.1A). A Transverse Section reveals yellowish brown spore sacs (peridioles) developing in a black gelatinous matrix (Fig.2.1B). Mature dry spores were of 7.5 to 8.5 µm in diameter, cinnamon brown, globose and bearing triangular shaped flattened curved spines (platelet spines) were seen under Scanning Electron Microscope (Fig.2.1C). These characteristics match the results reported by Kope and Fortin (1990). Vegetative growth of *Pisolithus* sp. PT1 isolate consists of conspicuous aerial mycelium emerging from a golden yellowish cottony growth with a dull yellow appearance on the leading edge (Fig.2.1D). After the isolate grew maximally, the colony changed to a tan colour. Mycelia exuded brown pigment that coloured the medium from yellowish (GMSM) to brownish (MMN). Moreover, PT1 colonies seldom exuded brown pigmented droplets from the aerial hyphae (Fig.2.1F). The hyphae grew interlaced and numerous clamp connections occurred throughout the 20 days old culture (Fig.2.1E). These features are similar to that observed by Hile and Hennek (1969).

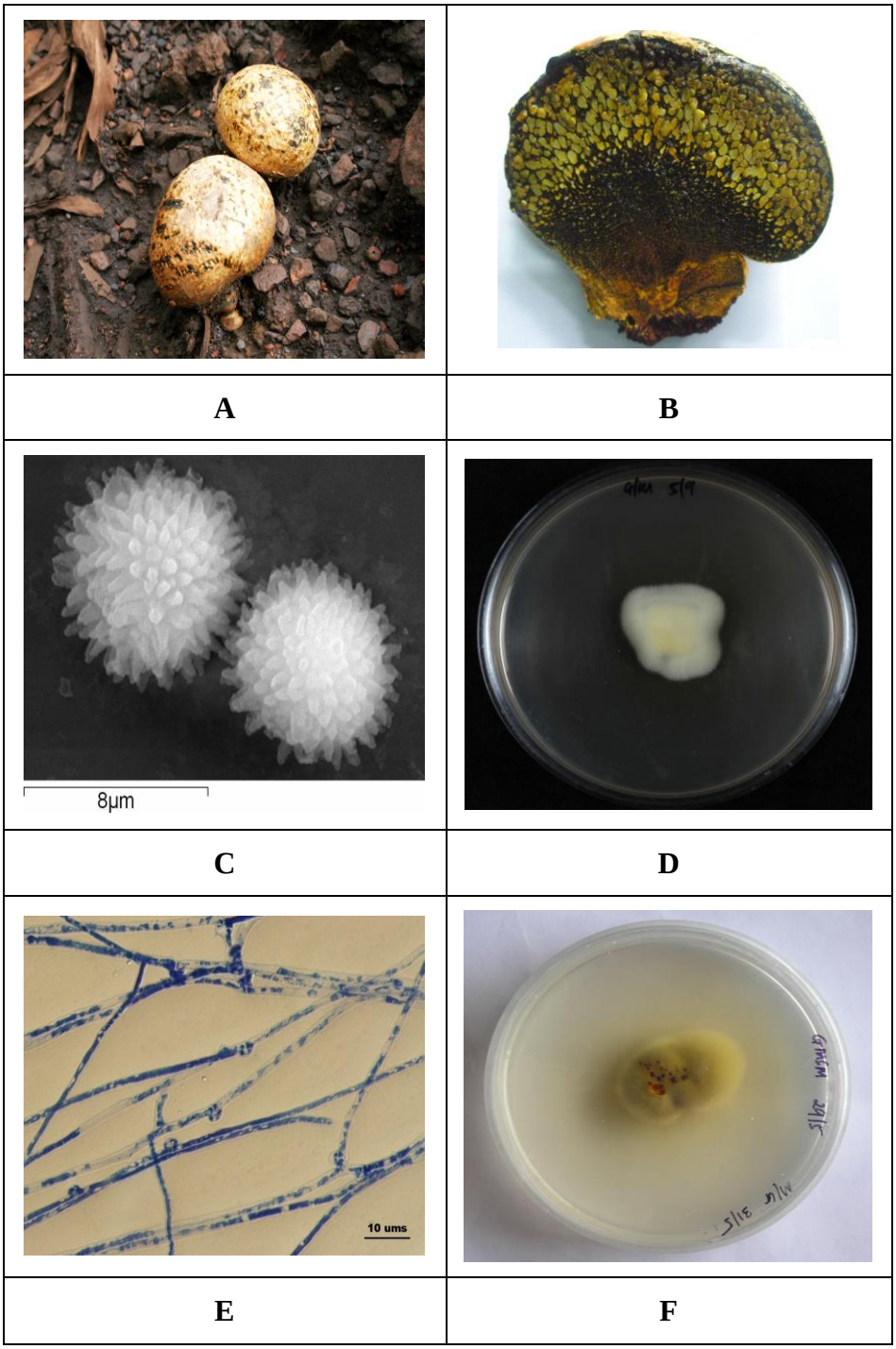
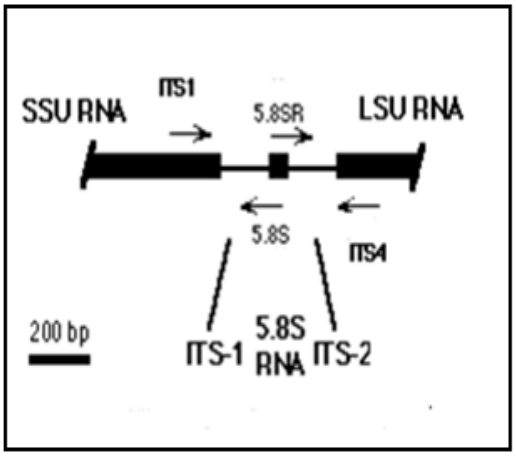


Fig.2.1 Morphological and colonial characteristics of *Pisolithus tinctorius* PT1.
(A) Fruiting bodies, (B) Transverse section of sporocarp, (C) Scanning electron micrograph of basidiospores (7500X; JEOL 5800LV SEM), (D) Vegetative mycelium growing on MMN medium, (E) Hyphae with clamp connections (F) Pigment exuded from aerial hyphae on GMSM

The genus *Pisolithus* are widely regarded as conspecific (Cairney, 2002; Anderson *et al.*, 1998). Recent molecular analysis have revealed considerable genetic variation within the species suggesting that *P. tinctorius* group worldwide comprises a complex of several species (Martin *et al.*, 1998, Anderson *et al.*, 2001, Martin *et al.*, 2002, Moyersoen *et al.*, 2003, Reddy *et al.*, 2005), which cannot be separated by morphological studies. Thus, in *Pisolithus* spp., even though the fruit bodies used to obtain pure cultures are unambiguously identified based on their morphological characters, the identity of the isolated mycelia should also be confirmed by molecular methods. The internal transcribed spacer (ITS) region containing two variable non-coding regions that are nested within the rDNA repeat between the highly conserved small subunit, 5.8S, and large subunit rRNA genes is a convenient target region for molecular identification of fungi (Gardes and Bruns, 1993).

Molecular identification of *Pisolithus* sp. PT1 isolate was carried out based on the ITS region of the nuclear rDNA. The rDNA ITS region of the *Pisolithus* isolate (PT1) was amplified with conserved fungal primers ITS1 and ITS4. The size of the ITS fragment was approximately 396 bp, which includes ITS1, 5.8S and ITS2 regions (Fig.2.2). The ITS region was sequenced and the sequence data was submitted to GenBank nucleotide database.

Accession number provided by GenBank was **JF810704**. A BLAST (NCBI) search using the ITS region of isolate PT1 showed 95% and above homology to known taxa of Pisolithaceae with maximum homology to *Pisolithus tinctorius* and *Pisolithus albus*. The evolutionary relationship of the isolate with selected species of the Boletales was analyzed using the Neighbor-joining (NJ) method. Phylogenetic analyses clearly revealed its evolutionary relatedness with *Pisolithus tinctorius* (Fig.2.3). From the phylogenetic tree it was evident that isolate PT1 forms a separate group with the closest relatives being *Pisolithus tinctorius*, *Pisolithus albus*, and *Pisolithus arhizus*. Within this group, it was seen that the sequence of the isolate aligned to *Pisolithus tinctorius*.



Accession Number JF810704 396 bp DNA linear

source	1..396	
rRNA	<1..8	/product="18S ribosomal RNA"
misc_RNA	9..175	/product="internal transcribed spacer 1"
rRNA	176..340	/product="5.8S ribosomal RNA"
misc_RNA	341..>396	/product="internal transcribed spacer 2"

ORIGIN

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        1  gatcattatc gaaactcgaa aggcgtggag gggcaccttc gctgggtcct tcgaagccct
       61  ttttactttg tccacacctc tgtgcacccc gttcgcgcga ggttcttcgg aacctgcgcg
      121  atgctatctc gaactcgtat gtctacagaa tgtaacctag cgtcggaaaa gaaaatacaa
      181  ctttcagcaa cggatctctt ggctctcgca tcgatgaagg acgcagcgaa tcgcgataag
      241  taatgtgaat tgcagatttt ccgtgaatca tcgaatcttt gaacgcacct tgcgctcctt
      301  ggtattccga ggagcatgcc tgtttgagtg tcatcgaaaa tctcaagcca agctgttttg
      361  actttggtcg aaaaagcctg gatttttgga gtgttg
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Fig.2.2 The rDNA ITS region of the *Pisolithus* isolate PT1, amplified with conserved fungal primers ITS1 and ITS4. The size of the ITS fragment was approximately 396 bp, which includes partial 18S (8 bp, red), ITS1 (167 bp, green), 5.8S (165 bp, blue) and partial ITS2 (56 bp, yellow) regions.

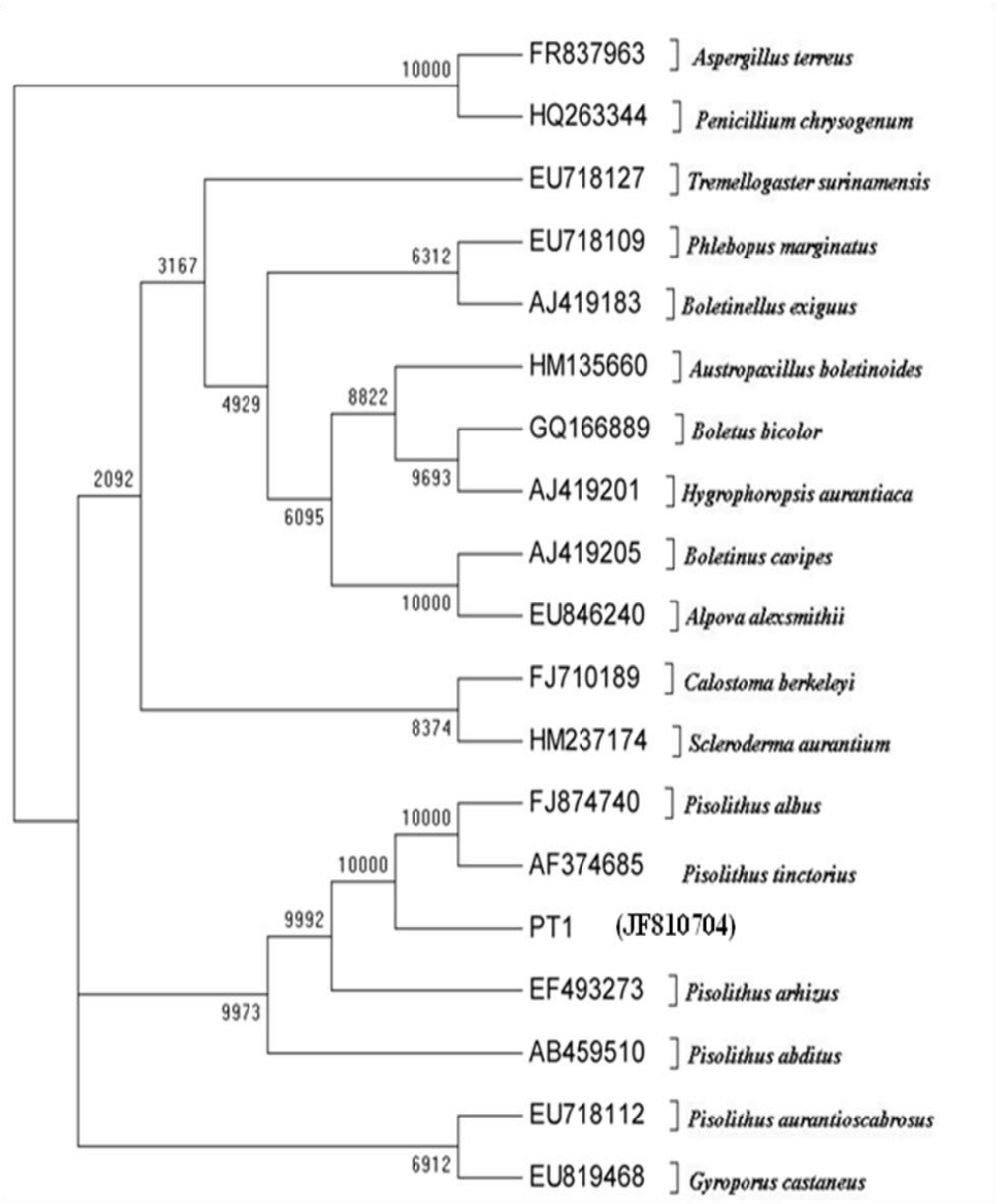


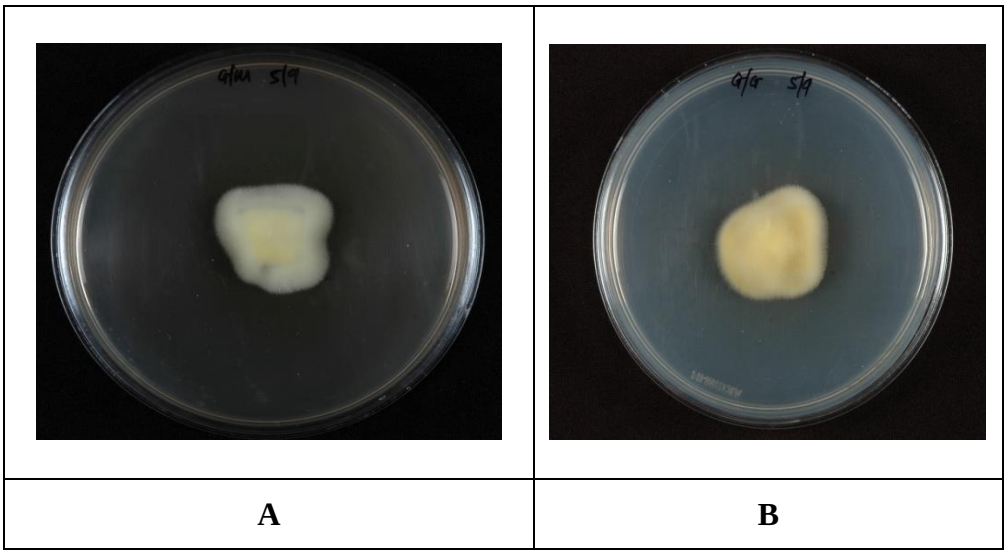
Fig.2.3 Phylogenetic position of *Pisolithus* sp. PT1 isolate. Tree inferred by maximum likelihood analysis based on rDNA sequences, including ITS1, 5.8S and ITS2 regions. *Penicillium chrysogenum* and *Aspergillus terreus* were used as outgroups. The numbers at the nodes indicate the bootstrap value at which a given branch was supported in 10000 replications

2.2.2 Growth response of *Pisolithus tinctorius* PT1

Pisolithus being ubiquitous organism is subjected to different environmental conditions during growth and hence believed to have wider tolerance range. In the past, research work on ECM fungi and their response to various growth parameters have been conducted and noted differential response between species and within the species (Matsuda *et al.*, 2006; Cairney, 1999; Hung and Trappe, 1983). The growth of *Pisolithus tinctorius* PT1 on GMSM and MMN agar medium (Fig.2.4) and liquid medium (Fig.2.5) revealed that the isolate grew better on GMSM. Isolate PT1 began to grow faster on GMSM as the medium contained simple easily assimilable nutrients. The isolate showed dense compact growth on GMSM as compared to conventional MMN. The isolate when grown in MMN and GMSM resulted into drastic lowering of pH of growth media to 2.0-3.0. Earlier studies have revealed that ECM fungi produce organic acids such as oxalic and citric acids that aid in phosphate availability (Bolan, 1991; Lapeyrie *et al.*, 1991). Elaboration of such organic acids by ECM fungi in growth medium could be responsible for lowering of pH of the medium. The available phosphate in the medium was not able to maintain the pH during the growth of PT1. It is inevitable to have controlled pH of growth medium in order to clearly identify the effect of pH on the growth of ECM fungi (Giltrap and Lewis, 1981; Yamanaka, 2003). However, there is no attempt made earlier to understand the effect of maintained pH on the growth of *Pisolithus tinctorius*, although very important fungus both economically and ecologically. There are sporadic studies where initial pH of complex MMN medium was adjusted before

inoculation (Hung and Trappe, 1983; Gupta *et al.*, 1997; Sundari and Adholeya, 2003). It is difficult to assess the effect of pH on the fungal growth on conventional complex culture media as they have low buffering capacities (Child *et al.*, 1973). Attempts were made to incorporate the inert buffer in the medium to control pH during the growth of Pt and other ECM fungi, but observed that buffers such as N-[2-acetamido] iminodiacetic acid (ADA), N-[2-acetamido]-2-aminoethanesulfonic acid (ACES), 2-(N-morpholino) ethanesulfonic acid (MES) and piperazine-N,N'-bis (2-ethanesulfonic acid) (PIPES) interfere the metabolism of ECM fungi by either stimulating or inhibiting the growth (Giltrap and Lewis, 1981; Hilger *et al.*, 1986).

The effect of different buffer systems, added to maintain the pH of GMSM, on growth of *Pisolithus tinctorius* PT1 is shown in Fig.2.6. The isolate showed more growth on buffers containing higher amount of phosphate as compared to HEPES and plain GMSM (control). The two different phosphate buffers viz. SPB and PCB showed similar growth of the isolate (P>0.05). It was noted that, after 30 days of incubation the final pH of the medium containing SPB and PCB were 3.5 and 4.5, respectively. However, the final pH of the growth media containing HEPES buffer and no-buffer was 3.0. HEPES, an inert buffer was neither able to support maximum growth nor resist the drop in pH, probably because of sub-optimal concentration of buffer (0.02 M) used in the medium. On the contrary, similar molarity of PCB could regulate the pH to 4.5 with stimulating the growth. The selection of sub-optimal concentration of buffer was due to the earlier reported inhibition of higher concentration of inert buffers on the growth of Pt (Hilger *et al.*, 1986).



A	B

Fig.2.4 Thirteen days old growth of *Pisolithus tinctorius* PT1 on solidified MMN (A) and GMSM (B)

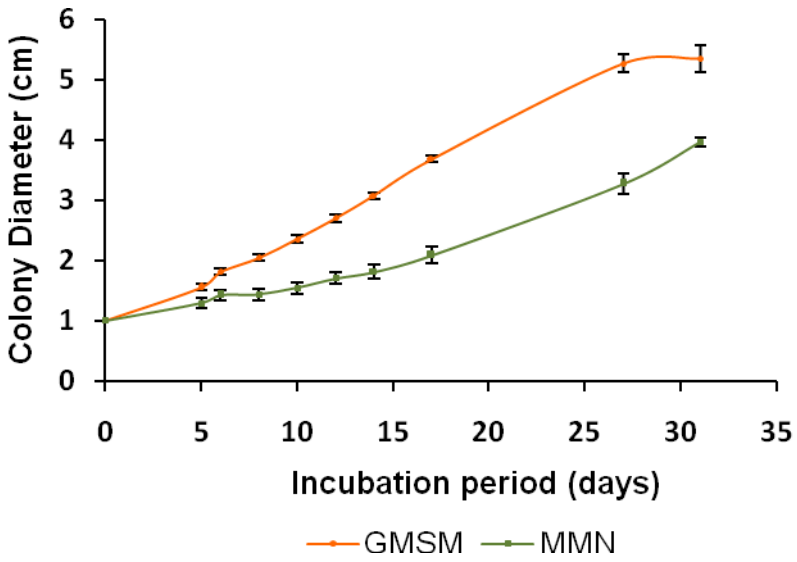


Fig.2.5 Radial growth curve of *Pisolithus tinctorius* PT1 on GMSM and MMN. Bars indicate standard errors

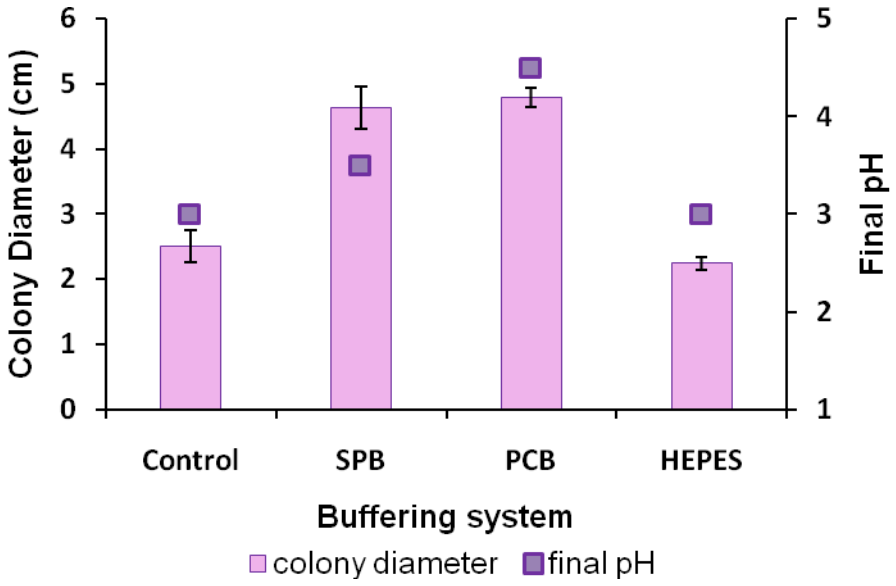


Fig.2.6 Effect of buffering systems on radial growth of *Pisolithus tinctorius* PT1 and final pH of media. SPB - Sodium phosphate buffer; PCB - phosphate citrate buffer; HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Control - GMSM medium without buffer

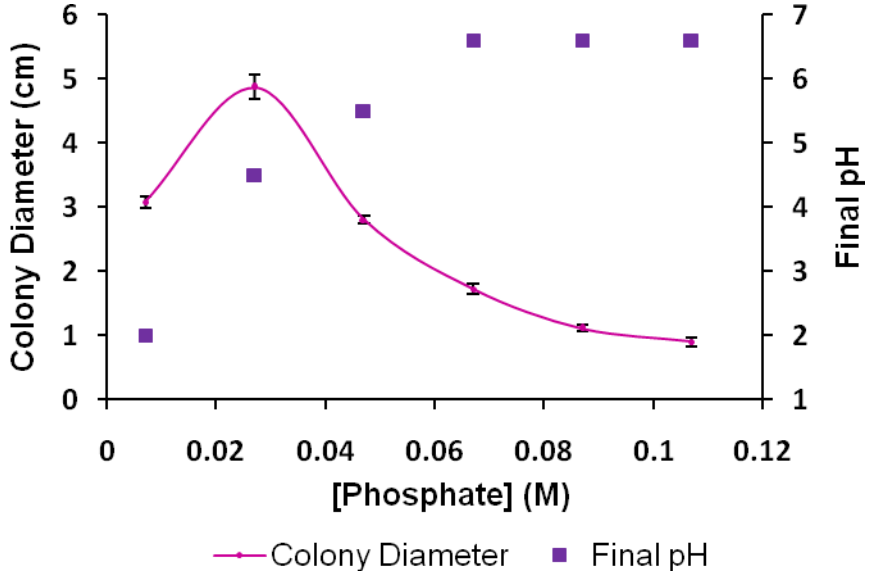


Fig.2.7 Effect of phosphate on radial growth of *Pisolithus tinctorius* PT1 and final pH of GMSM

The growth response of *Pisolithus tinctorius* PT1 to varying phosphate concentration is shown in Fig.2.7. Isolate showed increase in growth with increasing phosphate amount followed by a decrease in growth with further increase in phosphate. Growth was significantly higher with 0.027 M phosphate ($P<0.001$) than any other concentrations. Growth of PT1 isolate on 0.007 M and 0.047 M phosphate were similar ($P>0.05$).The final pH of the growth medium was found in acidic region at lower phosphate concentration where significant biomass was accumulated. The phosphate concentration of 0.067 M could regulate pH to initial pH 6.6. However, there was quite reduction in growth of the isolate. The growth on 0.087 M phosphate concentration was seen only after 20 days and was as feeble mycelia on inoculum disc. Phosphate concentration of 0.107 M completely inhibited the growth of *Pisolithus tinctorius* PT1.

The effect of phosphate concentrations of two phosphate buffers on growth of PT1 and their efficiency in maintaining the pH of growth medium is displayed in Fig.2.8. There was no significant difference in growth obtained in the two buffers with same phosphate concentrations. However, as the molarity of phosphate in the buffers increased the growth of the isolate decreased to similar extent in both the buffers. This response of PT1 could be due to combined effect of pH and increasing phosphate molarity. The final pH with PCB was higher than the corresponding phosphate molarity of SPB. This is the first report where PCB (0.04 M) was found suitable for growth without causing any inhibition and maintaining the pH as set initially, throughout the

growth of fungus. Hence, 0.04 M PCB was used to adjust the different pH of media in pH experiment.

Hung and Trappe (1983) and Gupta *et al.* (1997) have suggested that the best growth of Pt on complex medium spanned three pH units. In present study, *P. ticntorius* PT1 was monitored for effect of pH on growth using PCB (0.04 M) added to already existing 0.007 M phosphate in basal GMSM. The growth of PT1 with varying pH was typically of bell shaped (Fig.2.9). Growth of the isolate increased with increasing pH from 2.0 to 4.0 followed by decrease with increasing pH further from 4.0 to 8.0. Total inhibition on the growth of the isolate was seen at pH 2.2 and pH 8.0. The growth was very meagre at pH 3.0 and started only after 15 days of incubation. However, it was not better than at pH 2.2 and 8.0 ($P>0.05$). Significantly, best colony diameter was observed at pH 4.0 ($P<0.05$), indicating pH 4.0-4.2 as optimum pH for growth of PT1. This report conclusively showed the optimum pH for PT1 was 4.0-4.2 and the pH range for growth was 3.0-7.0. This isolate although acidophilic in nature could tolerate and survive in the soils with acidic to neutral pH and low phosphorus conditions such as mining soil rejects. Srinivasan *et al.* (2000) found no effect on growth of *Pisolithus tinctorius* due to change in pH from 5.5 to 6.5 under a RSM experiment using MMN medium.

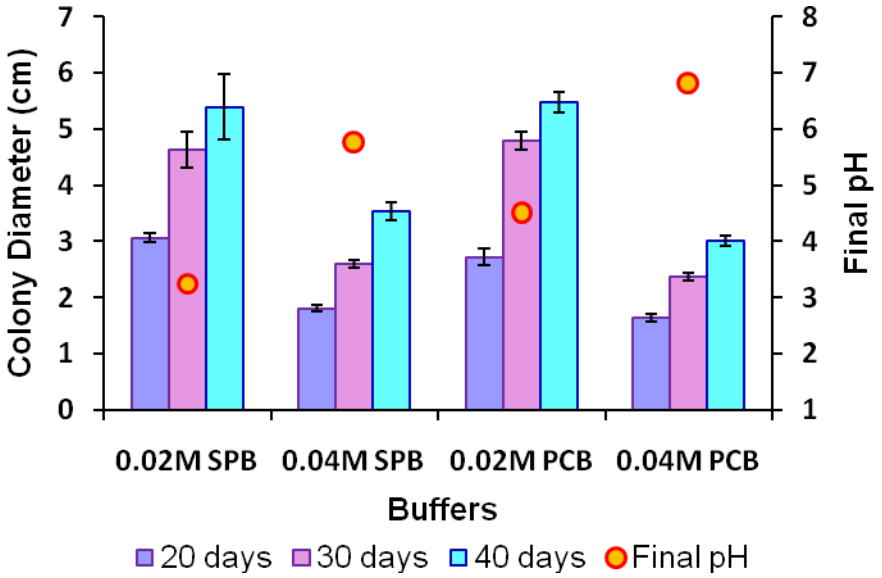


Fig.2.8 Effect of varying strength and different phosphate buffers on radial growth of *Pisolithus tinctorius* PT1 and final pH of the media. SPB - Sodium phosphate buffer; PCB - Phosphate citrate buffer

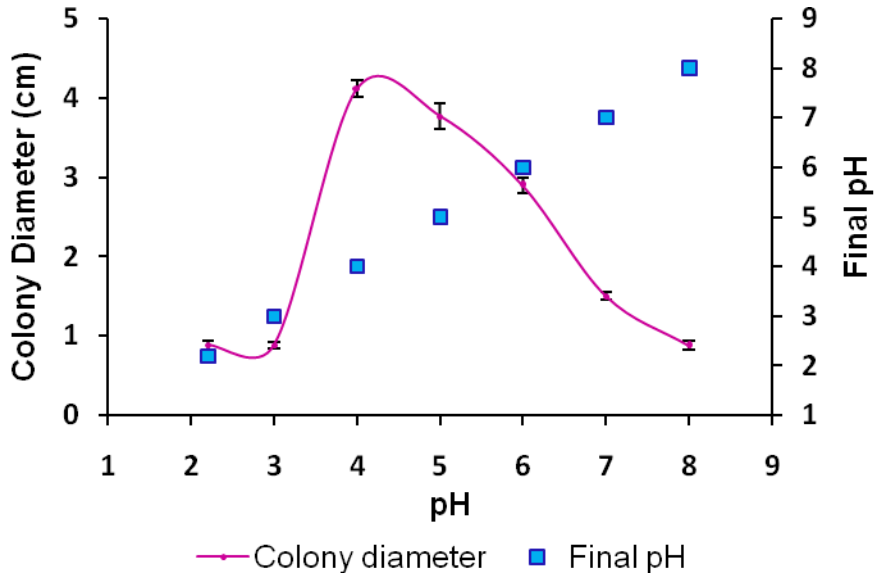


Fig.2.9 Effect of varying pH on growth of *Pisolithus ticntorius* PT1. The various pH of the GMSM was maintained using 0.04 M phosphate citrate buffer (PCB)

Mycorrhizal development is strongly temperature dependent (Mosse *et al.* 1981) and the tolerance in Pt of high temperatures may account for its predominance on mine spoils (Marx, 1975). Darkly pigmented ECM fungi like *Pisolithus* and *Cenococcum* have been found to be more tolerant to high temperatures (Hung and Chien, 1978; Cline *et al.*, 1987). Gupta *et al.* (1997) found 12 isolates among the 60 ECM fungi to grow in the temperature range between 25 to 37°C and have reported Pt as the most tolerant fungus. Growth of *P. tinctorius* PT1 was markedly affected by incubation temperature ($P<0.001$) (Fig.2.10) representing a typical bell shaped curves. There was good correlation of colony diameter and biomass produced. Optimum temperature for maximum growth of the isolate was 28-30°C. Interestingly, there was no significant difference in the biomass obtained at 25 to 30°C ($P>0.05$). PT1 showed growth between 25-37°C. These findings are in accordance with that reported by Gupta *et al.* (1997). Growth at 37 and 42°C was significantly lower than at 25 to 30°C ($P<0.05$). PT1 tolerated and survived at the temperature of 15 and 42°C. The culture was viable at 15and 42°C as the isolate responded when plates were shifted to RT (Fig.2.11). Growth was completely inhibited at 10 and 45°C. This isolate can probably tolerate soil temperature higher than 42°C once in association with the host. Similar behaviour in *Pisolithus* sp. was observed by Ferreira *et al.* (2005). Mycelium stopped growing at the temperatures of 42, 44 and 46°C. Interestingly, isolate recovered its growth after 24-48 h of incubation at 28°C.

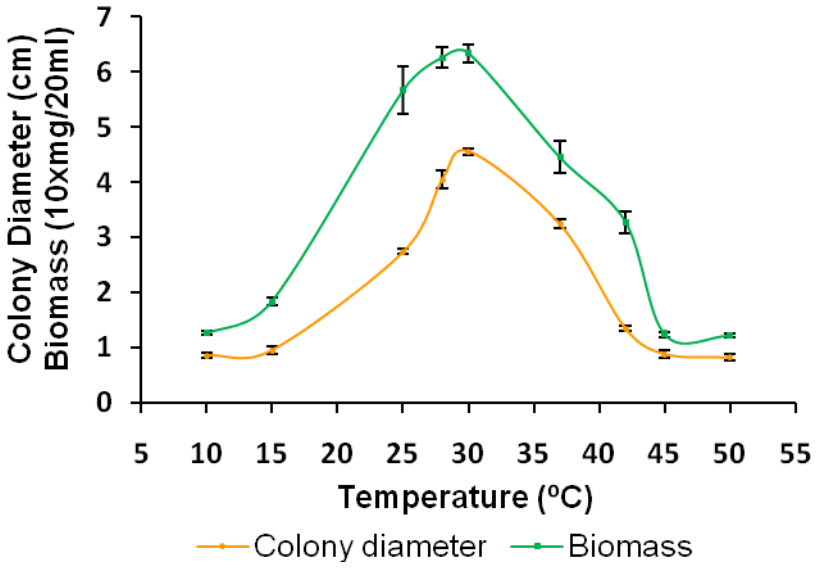


Fig.2.10 Effect of incubation temperature on growth of *Pisolithus tinctorius* PT1

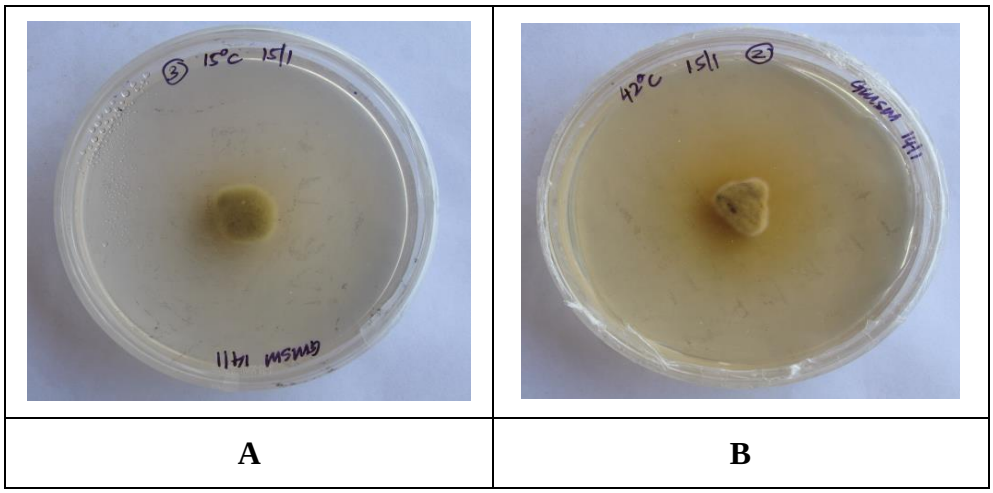


Fig.2.11 Recuperation of growth of *Pisolithus tinctorius* PT1 incubated at (A) 15°C and (B) 42°C after re-incubation at room temperature (RT)

The major difference in the experimental set up was that in their study the isolate was subjected to heat shock of 2 h whereas in present study the isolate was incubated at 42°C for a month. Survival of fungus in soils having temperature higher than 50°C in mining sites could be due to spores as mycelium of PT1 was found dead at this temperature.

The class Basidiomycetes was reported to be the least tolerant with over half the species unable to withstand more than 2% NaCl (Tresner and Hayes, 1971). The growth response of *P. tinctorius* PT1 was found at all the tested amount of NaCl except 4% (Fig.2.12). Growth of PT1 at 3% NaCl indicates its capacity to tolerate high salt concentration. However, the growth was significantly lower than the other concentration ($P<0.05$). Growth of the isolate was better with increasing concentration of NaCl from 0.01 to 1% in GMSM agar. Maximum colony diameter was recorded at 1% NaCl. There was very good correlation between colony diameter on GMSM agar and biomass obtained in GMSM broth with varying amount of NaCl. Unlike growth on GMSM agar, maximum biomass was found at 0.01% NaCl. There was no significant difference in the amount of biomass accumulated in the broth having NaCl from 0 to 0.5% ($P>0.05$). Similarly colony diameter at 0.05 to

1% was not significantly different (P>0.05) indicating optimum NaCl concentration for growth could lie between 0.01-1%. EC_{50} value of the isolate was found to be approximately 2.3%. These findings are in consistent with that reported for ECM fungi. Pt is seen to tolerate more amount of NaCl than other ECM fungi such as *C. graniforme*, *T. terrestris*, *Laccaria bicolor* and *S. luteus* (Saleh-Rastin, 1976; Dixon *et al.*, 1993; Bois *et al.*, 2006; Matsuda *et al.*, 2006). Gupta *et al.* (1997) reported Pt to tolerate upto 3% NaCl concentration. Interestingly, a coastal strain of Pt was found to be inhibited during *in vitro* growth with high concentration setting of sodium ions (Nagarajan and Natarajan, 1999). *Pisolithus* species could grow above 1.2% NaCl concentration and suggested EC_{50} value for Pt could be well in excess of 1.2% NaCl (Chen *et al.*, 2001, Matsuda *et al.*, 2006). Recently, Madsen and Mulligan (2007) found Pt to be tolerant upto 1.7% NaCl. The wide distribution of present isolate along the west coast of India including mining sites and rain-forest could be due to its ability to tolerate the variable salt concentration found in such diverse ecosystems.

ECM fungi show intra and interspecific variable response to PEG induced water stress (Coleman *et al.*, 1989, Dunabeitia *et al.*, 2004, Zhang *et al.*, 2011). PEG 6000 is inert, non-ionic and virtually impermeable chains used to induce water stress and maintain uniform water potential throughout the experimental period. As PEG reduces solidification of agar, fungal isolate was grown in liquid medium. *In vitro* growth of *Pisolithus* sp. to water stress

is not yet carried out, although response to water stress of *Pinus pinaster* inoculated with dikaryotic strains of *Pisolithus* sp. has been investigated (Lamhamedi *et al.*, 1991) and reported that seedlings colonized with certain dikaryotic were more sensitive to water stress than seedlings colonized with other dikaryons. In present investigation *P. tinctorius* PT1 showed growth in mesic zone i.e., water potential ≤ -1.0 MPa created in the medium using PEG 6000 (Fig.2.13). Further, it has tolerated upto 10% of PEG in the medium (P<0.001).

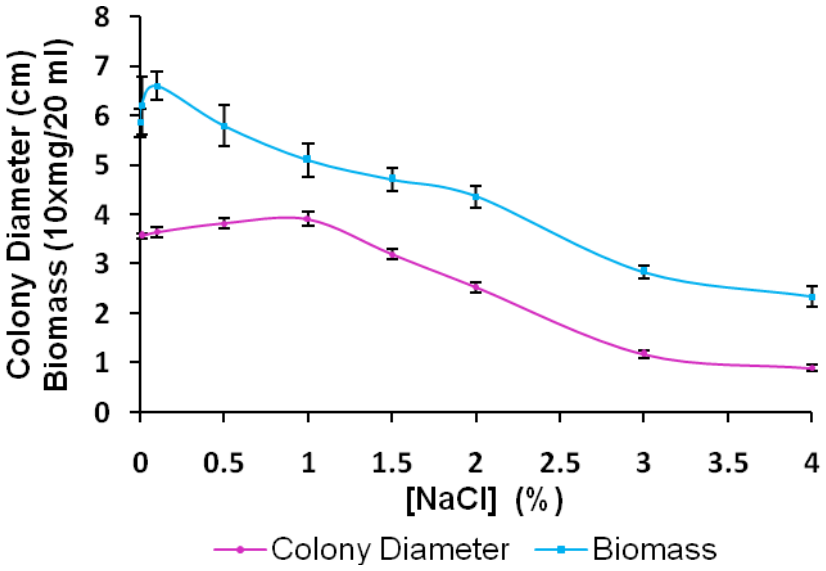


Fig.2.12 Growth response of *Pisolithus tinctorius* PT1 to varying amount of NaCl in GMSM

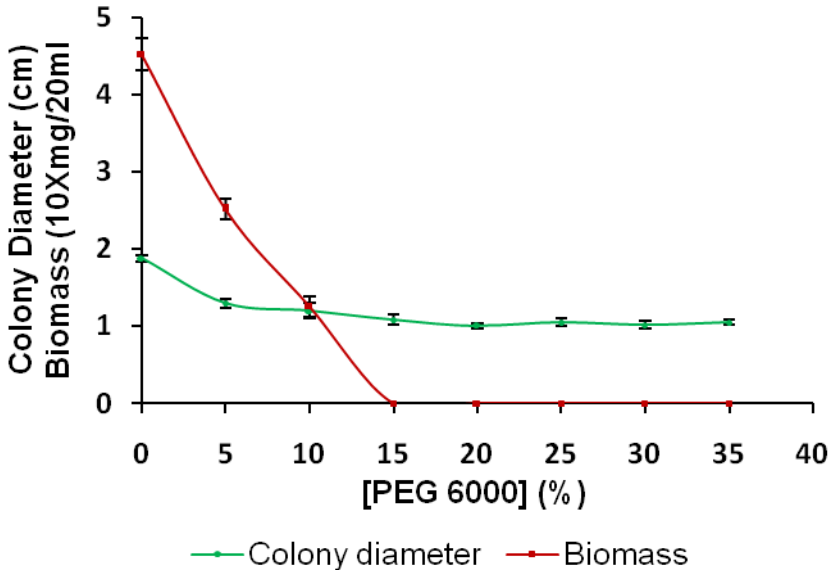


Fig.2.13 Effect of varying amount of PEG 6000 in the medium on growth of *Pisolithus tinctorius* PT1

The growth of the fungus was completely inhibited by PEG at 15% and above. Maximum growth of the isolate was observed when no PEG was added in the medium ($P<0.05$). Coleman *et al.* (1989) reported that drought tolerance depends more on fungal species than on annual precipitation at the site of collection.

Pisolithus tinctorius has been demonstrated to help loblolly and shortleaf pine seedlings establishment in acid coal spoils having high contents of Fe and Mn (Marx and Artman, 1979). Isolate investigated in the present study, showed ectomycorrhizal synthesis with *Acacia mangium* growing extensively on iron ore mining rejects rich in Fe and Mn.

Thompson and Medve (1984) tested *C. graniforme, S. luteus, T. terrestris* and Pt for manganese tolerance and reported *C. graniforme* as the most tolerant and *T. terrestris* as least tolerant. They found that the growth of Pt was not affected significantly even at the highest tested concentration of Mn i.e., 500 ppm. Further, there was no impact of type of salt used as source of Mn on Pt.

Pisolithus tinctorius PT1 actively responded to Mn concentration *in vitro* (Fig.2.14) and appeared to be Mn-tolerant. Isolate showed increase in colony diameter with increasing [Mn] from 0-1000 ppm. There was no significant difference in growth of PT1 with 500 to 2500 ppm Mn ($P>0.05$),

suggesting 500 to 2500 ppm Mn as optimum for maximum growth of the isolate. Increasing concentration beyond 2500 ppm drastically affected the growth. However, there was no inhibition of growth of isolate upto 10,000 ppm. The growth was similar at 8,000 and 10,000 ppm (P>0.05). Interestingly the isolate survived, tolerated and grew at 10,000 ppm. This isolate possibly can tolerate even higher Mn concentration as its growth was not inhibited.

The ionic form of the iron in the medium significantly affected the growth of *Pisolithus tinctorius* PT1 (P<0.001) (Fig.2.15). The isolate preferred ferric iron more than ferrous. Ferrous iron was inhibitory at 50 ppm in contrast to ferric where inhibitory concentration was found 60 ppm. Further investigation on this may highlight the preference of ionic form of iron by ectomycorrhizal fungi. The growth pattern of the isolate in present study, under the influence of ferric iron is irregular (Fig.2.16) and similar to that observed by Tam (1995). However, the tolerance range of iron for Pt reported by him was much higher than the experimental concentrations tested in current investigation. The isolate showed an initial decrease in growth as the iron amount was slightly increased, followed by a gradual increase upto 45 ppm [Fe] and then sudden decrease at 50 ppm (P<0.05). Medium supplemented with 60 to 100 ppm [Fe] completely inhibited the growth of the isolate (P<0.05). Large numbers of fruiting bodies of *P. tinctorius* were seen during onset of monsoons on iron ore mining sites of western India. The predominance of this fungus could be explained because of its tolerance to high manganese and ferric iron content.

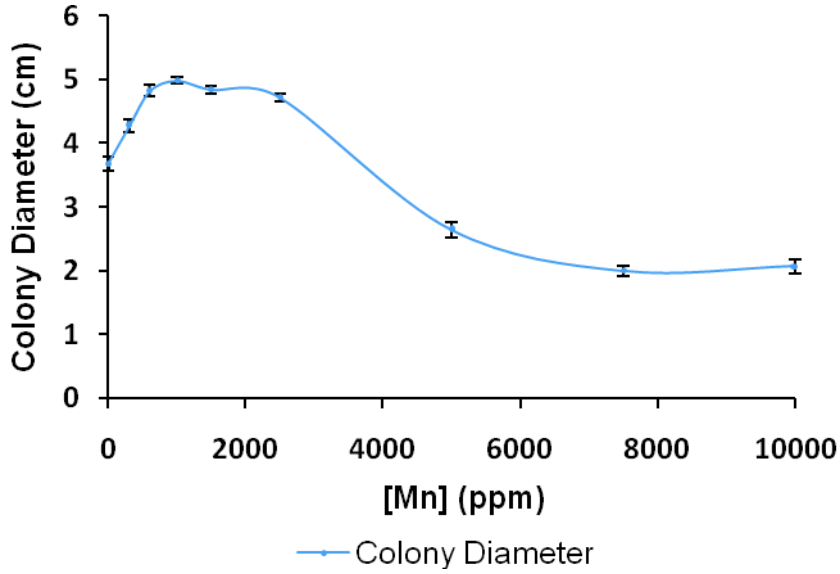


Fig.2.14 Growth response of *Pisolithus tinctorius* PT1 to varying amount of manganese in GMSM

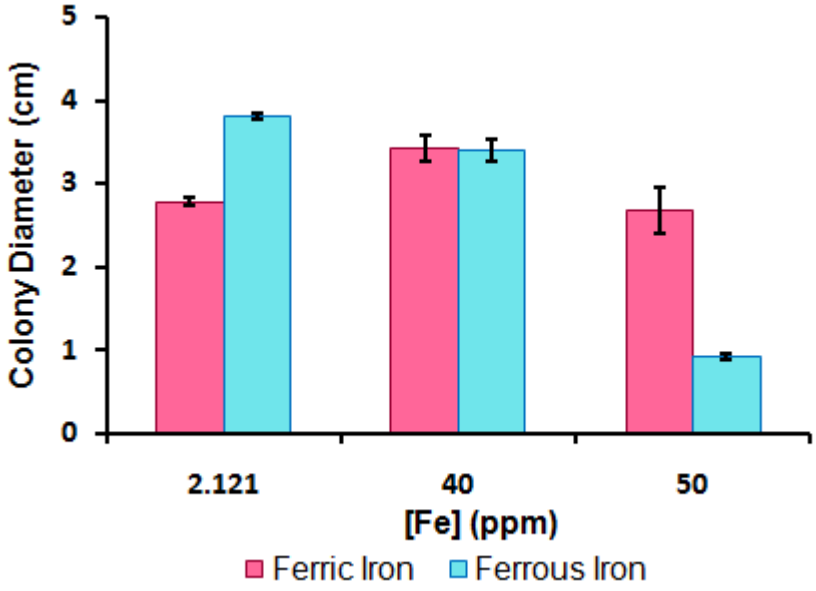


Fig.2.15 Effect of different ionic forms of iron on radial growth of *Pisolithus tinctorius* PT1

The present investigation clearly demonstrated the use of chemically defined medium and identified the actual limits of growth conditions that could be tolerable or inhibitory to *Pisolithus tinctorius* PT1. It identified the optimum physico-chemical parameters for maximum growth and stress region encountered by the isolate in axenic culture conditions (Table 2.1). The table summarizes the optimum and stress values of growth conditions for pH, temperature, NaCl, PEG and manganese. PT1 showed inconsistent response with iron hence the region pertaining to stress was not located.

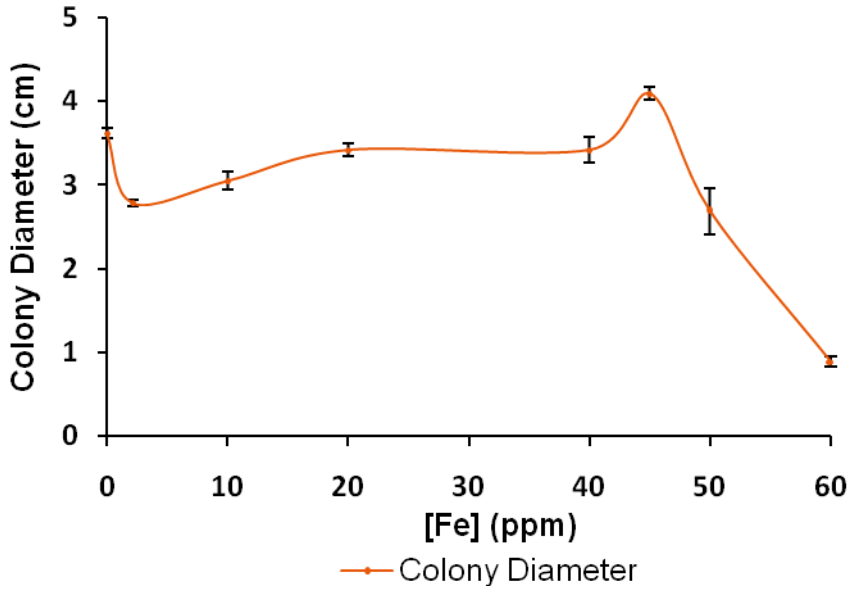


Fig.2.16 Radial growth of *Pisolithus tinctorius* PT1 in GMSM containing varying amount of ferric iron added as ferric chloride

GROWTH FACTOR (TESTED RANGE)	RANGE FOR GROWTH	OPTIMUM GROWTH	STRESS FOR GROWTH	
			LOWER LIMIT	UPPER LIMIT
[PO4] (0.007-0.1M)	0.007 - 0.087M	0.027M	<0.02M	>0.04M
pH (2.2-8.0)	3.0 - 7.0	4.0 - 4.2	≤3.0	≥7.0
TEMPERATURE (10-50^{0}C)	25 - 42^{0}C	25 - 30^{0}C	<25^{0}C	≥37^{0}C
[NaCl] (0-4%)	0 - 3%	0.01 - 1%	ND	≥1.5%
[PEG 6000] (0-35%)	0 - 10%	0%	ND	>10%
[Mn] (0-10000 ppm)	0 - 10000 ppm	500 - 2500 ppm	ND	5000 - 10000 ppm
[Fe] (0-100 ppm)	0 - 50 ppm	VARIABLE RESPONSE	ND	ND

Table 2.1 Growth response of *Pisolithus tinctorius* PT1 indicating optimum and stress values for [Phosphate], pH, temperature, [NaCl], [PEG 6000], [Mn] and [Fe]; ND: Not Determined

2.2.3 Stress response of *Pisolithus tinctorius* PT1

Stress proteins in *Pisolithus* sp. has been mainly studied for thermal stress and heavy metal toxicity (Ferreira *et al.*, 2005, Morselt *et al.*, 1986).The profiles of whole cell soluble protein of *Pisolithus tinctorius* PT1 grown under optimum and stressed growth conditions for pH, temperature, NaCl, PEG and manganese were obtained. Different proteins were expressed during different stress conditions. Protein profiles obtained when fungus grown at pH 4.2 and pH 7.0 that is optimum and stress conditions are placed in Fig.2.17. A 42 KDa protein was newly expressed during pH stress. A 66 KDa protein appeared to be overexpressed and two proteins of 45 and 27 KDa were underexpressed. Protein bands of 40 and 25 KDa were absent during the pH stress.

Organisms in nature are subjected to heat stress due to daily and seasonal temperature fluctuations. Mining region contains non vegetated soil with large variation in temperature. *In vitro* studies showed that the HSPs of high molecular mass (HSP104 and HSP70) act in reactivation of proteins denatured during stress condition (Glover and Lindquist, 1998). Thermotolerance depends on synthesis of one or more heat shock proteins (Sanchez *et al.*, 1992). Two generalizations could be made from the researches on thermal stress were that, HSPs played an important role in acquired thermotolerance and different species used different strategies (different

combinations of HSPs and/or other macromolecules) in acquiring thermotolerance (Trent *et al.*, 1994). Protein profiles of *Pisolithus tinctorius* PT1 grown at 30°C and 37°C that is optimum and stress conditions are displayed in Fig.2.18. Under thermal stress PT1 synthesized three new proteins of molecular size 70, 57 and 55 KDa. Protein bands of 97, 68, 60 and 40 appeared to be upregulated while protein bands of 29 and 25 KDa appeared to be downregulated. Chen and Chen (2004) have shown thermophilic and thermotolerant fungi in Taiwan to produce HSPs with molecular weights ranging between 20-150 kDa in response to 3 h of thermal stress at elevated temperature from 30°C to 50°C. They have observed that heat shock treatments at 40°C induces synthesis of mostly high and medium molecular weight HSPs (40-94 KDa) while at 50°C mostly low molecular weight HSPs (20-35 KDa) are synthesized.

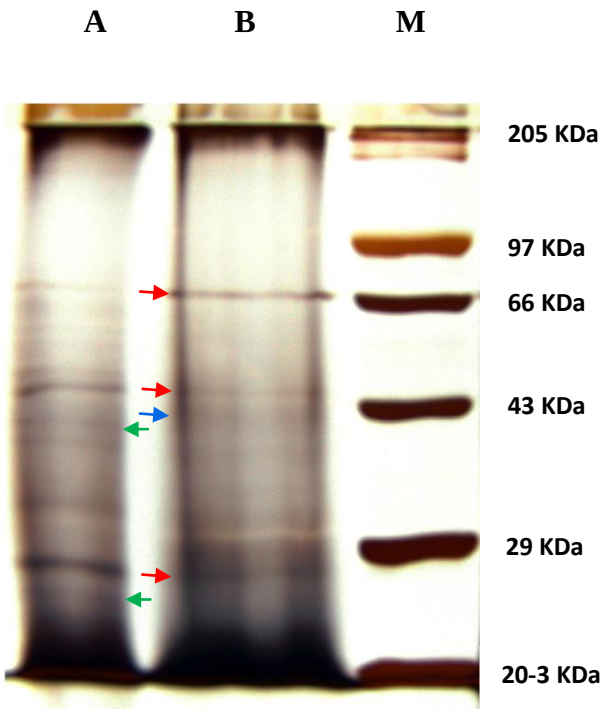


Fig.2.17 Whole cell protein profile of *Pisolithus tinctorius* PT1 grown under pH stress. Lane A - optimum pH 4.2, Lane B - pH stress - pH 7.0, & Lane M - Molecular weight marker. Blue arrow indicates the new protein and red arrow indicate the up- and down-regulated protein under stress condition. Green arrow indicates protein synthesized under optimum condition but absent during stress

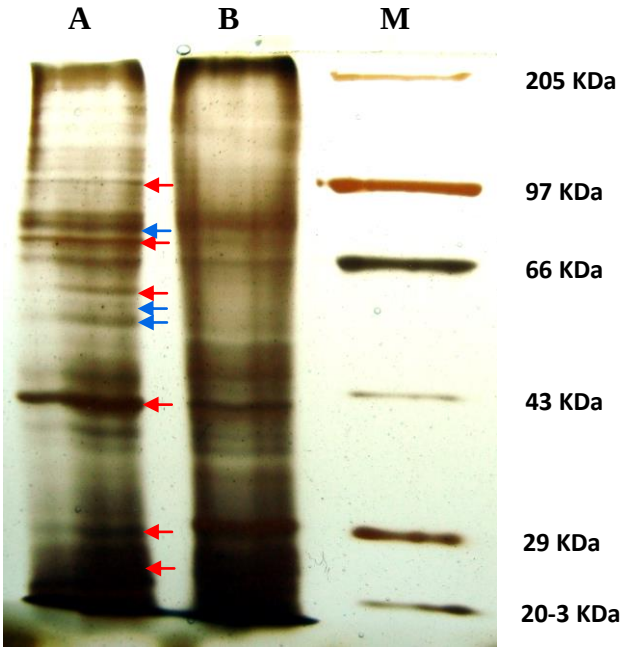


Fig.2.18 Whole cell protein profile of *Pisolithus tinctorius* PT1 grown under thermal stress. Lane A - fungus grown at 37°C, Lane B - fungus grown at 30°C, and Lane M - Molecular weight marker. Blue arrow indicates the new protein and red arrow indicate the up- and down-regulated protein under stress condition

Protein profiles of *Pisolithus tinctorius* PT1 obtained for the isolate when grown under optimum and stress conditions of sodium chloride, i.e., 0.01% and 2% NaCl is shown in Fig.2.19. A 64 KDa protein was newly expressed in PT1 under NaCl stress. Proteins of 55, 45, 40 and 27 KDa were less expressed and a 25 KDa protein was not expressed at all during growth with 2% NaCl. Twenty two proteins related to multiple cellular processes, e.g. metabolisms, energy related processes, DNA repair, cell cycle control, and stress tolerance, were found involved in the stress responses of *Boletus edulis* to salt stress (Liang *et al.*, 2007).

Water availability is one of the most limiting environmental stresses for the life in mining soil as it has low water holding capacity. Protein profiles of *Pisolithus tinctorius* PT1 grown under optimum and stress conditions of water potential, i.e., 0% and 10% PEG is placed in Fig.2.20. During PEG stress the isolate synthesized two new proteins of molecular mass 78 and 38 KDa. A 35 KDa protein was upregulated where as three proteins of molecular mass 37, 33 and 27 KDa were downregulated.

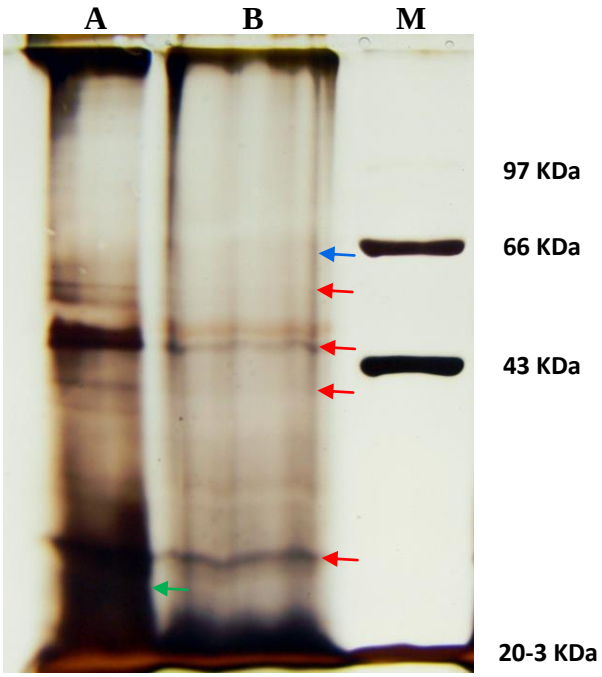


Fig.2.19 Whole cell protein profile of *Pisolithus tinctorius* PT1 grown under NaCl stress. Lane A - isolate grown with 0.01% NaCl, Lane B - isolate grown with 2% NaCl, and Lane M - Molecular marker. Blue arrow - new proteins, red arrow - up- and down-regulated proteins under stress condition. Green arrow - protein synthesized under optimum condition but absent during stress

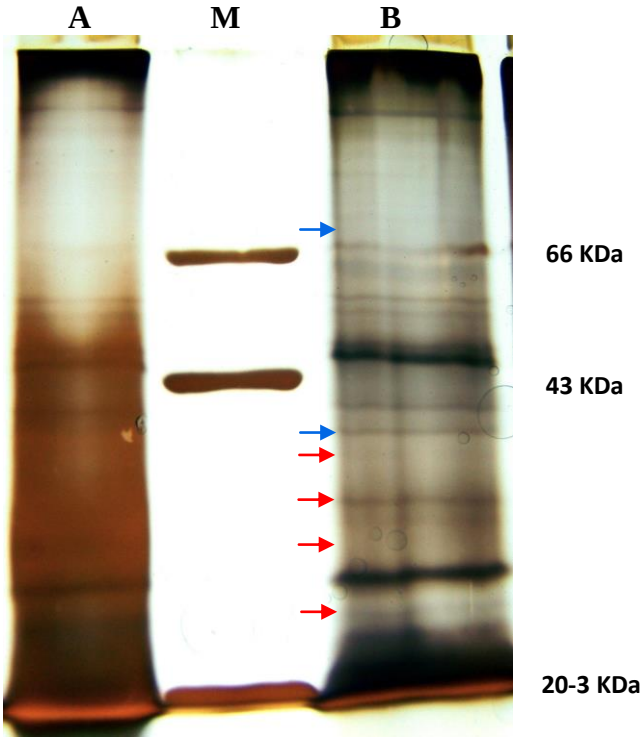


Fig.2.20 Whole cell protein profile of *Pisolithus tinctorius* PT1 grown under drought stress. Lane A - fungus grown with 0% PEG, Lane B - fungus grown with 10% PEG, and Lane M - Molecular marker. Blue arrow - new protein, red arrow - up- and down-regulated protein under stress condition

Howe *et al.* (1996) demonstrated the synthesis of 2.2-2.8 KDa Cu-metallothioneins in ECM fungi like *Laccaria laccata, Paxillus involutus* and *Scleroderma citrinum*. They suggested that tolerance of isolates is not related to their site of origin or isolation. Similarly, tolerance mechanism in ECM fungi may differ for a metal toxicity. Blaudez *et al.*, (2000) have demonstrated cadmium uptake by *Paxillus involutus* and its subcellular compartmentation in cell wall (50%), cytoplasm (30%) and vacuole (20%). An increase in the content of glutathione and γ-glutamyl cysteine was observed when *Paxillus involutus* was exposed to cadmium (Courbot *et al.*, 2004). An additional compound with a 3 KDa molecular mass, probably related to a metallothionein, increased drastically in mycelia exposed to cadmium.

Various mechanisms involved in metal detoxification by Pt includes induction of intracellular metallothionein-like proteins (Morselt *et al.*, 1986), increase in intracellular tyrosinase activity (Gruhn and Miller, 1991), accumulation of polysaccharides and cysteine rich proteins on the outer cell wall (Turnau *et al.*, 1994), accumulation of toxic metal on the outer region of hyphal walls (Turnau *et al.*, 1994), and enhanced accumulation of Ca and Mg that alters the ratio of the divalent cations to toxic metal reducing its binding and absorption (Egerton-Warburton and Griffin, 1995).

Protein bands of 67, 60 and 29 KDa appeared to be overexpressed and approximately 86, 66, 43 and 27 KDa proteins appeared to be underexpressed. Proteins of molecular mass 115 and 26 KDa are synthesized under manganese stress. Protein bands of 40, 37, and 25 KDa were not synthesized during the growth of PT1 with 7500 ppm Mn (Fig.2.21).

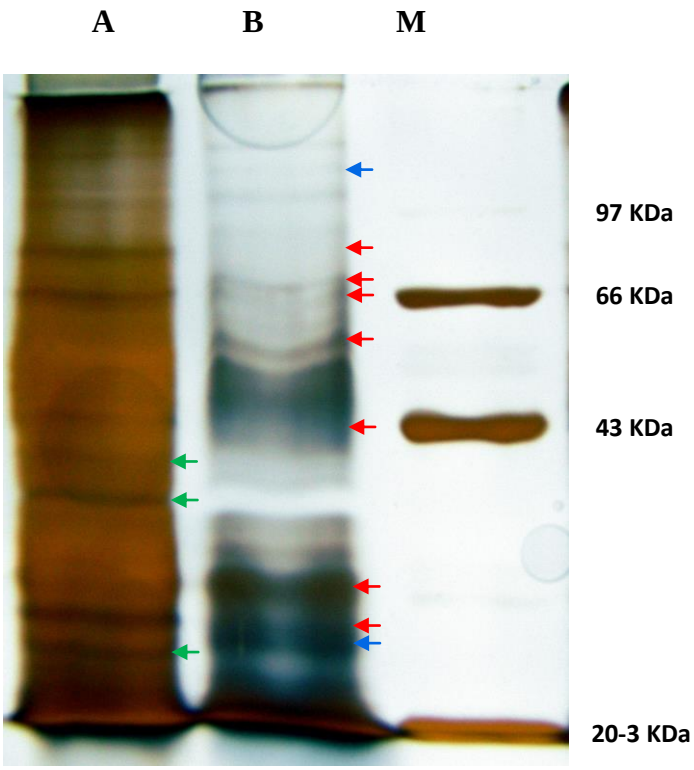


Fig.2.21 Whole cell protein profile of *Pisolithus tinctorius* PT1 grown under manganese stress. Lane A - 1000 ppm Mn, Lane B - 7500 ppm Mn, and Lane M - Molecular weight marker. Blue arrow indicates the new protein and red arrow indicate the up- and down-regulated protein under stress condition. Green arrow indicates protein synthesized under optimum condition but absent during stress

It was observed that 66, 60, 29 and 27 KDa bands appeared to be either overexpressed on underexpressed during various stresses. A 25 KDa band was expressed in optimum growth condition but was absent in the stresses due to pH, NaCl and Mn. Besides the newly synthesized proteins, these proteins probably have some important role in stress tolerance that needs further investigation.

CHAPTER III

Pigments/Phenolics produced by Pisolithus tinctorius PT1

INTRODUCTION

A number of studies have been investigating the different mechanisms of disease resistance in the ECM fungi. Organic compounds liberated by ECM fungi in protection of host plant from pathogen were considered to be the most presumable mechanism (Sylvia and Sinclair, 1983; Duchesne *et al*., 1989; Kope *et al*., 1991; Yamaji *et al.*, 2005). Marx (1969) has reported antibiotic activity of ECM fungi to various root pathogenic fungi and soil bacteria. Antagonistic compounds produced by ECM fungi are phenolics in nature (Sylvia and Sinclair, 1983). Kope and Fortin (1989) have demonstrated inhibition of phytopathogenic fungi by cell free culture media of seven ECM fungi and showed *Pisolithus tinctorius* to have the largest spectrum of activity. Kope *et al*., (1991) have identified two phenolics acids namely Pisolithin A [*p*-hydroxy benzoyl formic acid] and Pisolithin B [R-(-)-*p*-hydroxymendalic acid] from Pt and demonstrated to inhibit spore germination and cause hyphal lysis of phytopathogens and dermatogenic fungi. Thus the phenolic compounds produced by ECM fungi offer important chemical protection to host from pathogenic infections. Suh *et al*. (1991) have attempted fermentative production of antifungal metabolites by Pt and suggested that Pt produces more phenolics when it comes in contact with a pathogen (biotic stress) and co-inoculation could probably increase the production of phenolics. Detailed investigations are required to enhance the production of antagonistic compounds by this fungus.

3.1 MATERIALS AND METHODS

3.1.1 Pigment production by *Pisolithus tinctorius* PT1

In vitro pigment production by *Pisolithus tinctorius* PT1 was checked using GMSM with varying particular medium component or growth condition. Inoculation and incubation was carried out as described in 2.1.5.1.

3.1.1.1A Effect of incubation period

Pisolithus tinctorius PT1 was grown in 100 ml conical flask containing 20 ml GMSM. Medium was inoculated with a single inoculum disc and incubated at RT under stationary condition over a period of 60 days. Fungal biomass and culture broth was harvested on 0, 20, 40 and 60 days. Fungal biomass was determined as dry weight and total phenolics content in the culture broth was estimated.

3.1.1.1B Effect of growth condition

Pisolithus tinctorius PT1 was grown in liquid GMSM and incubated at RT under stationary and submerged (100 rpm) conditions. Fungal biomass and total phenolics content in culture broth were determined after 30 days of incubation.

3.1.1.1C Effect of buffering systems

Effect of buffers on pigment produced by *Pisolithus tinctorius* PT1 was checked on GMSM agar medium. The pH of GMSM was adjusted to pH

6.8 using 0.02 M HEPES buffer, Phosphate-citrate buffer (PCB), and Sodium phosphate buffer (SPB) (Appendix B). Plain GMSM was maintained as control. Visible pigment liberated in the medium was noted.

3.1.1.1D Effect of phosphate

Effect of varying concentration of phosphate on pigment elaboration by Pisolithus tinctorius PT1 was checked on solid GMSM medium. Medium was amended with different concentration of phosphate from 0.007 to 0.107 M using 0.1 M SPB solution (Appendix B). Visible pigment liberated in the medium was noted.

3.1.1.1E Effect of pH

The elaboration of pigment by Pisolithus tinctorius PT1 in the medium with varying pH was checked on GMSM agar plates. The pH of the medium was adjusted to pH 2.2 to 8.0 using 0.04 M PCB.

3.1.1.1F Effect of Temperature

Pisolithus tinctorius PT1 was grown using GMSM broth and incubation at different temperatures from 10 to 50ºC. Culture broth was harvested to determine total phenolics liberated by the isolate.

3.1.1.1G Effect of NaCl

Pisolithus sp. was grown in GMSM liquid medium incorporated with different NaCl concentrations from 0 to 4%.

3.1.1.1H Effect of PEG 6000

Pisolithus sp. was grown in GMSM broth adjusted to different water potential by varying amount of polyethylene glycol (PEG 6000) from 0 to 35%.

3.1.1.1I Effect of Manganese

Effect of manganese on pigment produced by *Pisolithus tinctorius* PT1 was determined on GMSM agar medium. Medium was amended with 0 to 10,000 ppm Mn using 50,000 ppm $MnSO_4$ stock solution (Appendix A).

3.1.1.1K Effect of iron

Effect of iron on pigment produced by *Pisolithus tinctorius* PT1 was determined incorporating 0 to 100 ppm Fe (Appendix A).

3.1.1.3 Analytical procedures

3.1.1.3A Determination of growth

Fungal growth was determined as described in 2.1.5.3A

3.1.1.3C Total phenolics estimation

Total phenolics content in the culture broth was estimated by 4-Aminoantipyrine method (Greenberg *et al*., 1985) (Appendix D).

3.1.1.3B Statistical Analysis

Four replicates were maintained for each experimental condition. Impact of parameters and significant differences between treatments were assessed by analysis of variance (ANOVA) at $P<0.001$ and treatment means were compared by least significant difference ($P<0.05$) using Student-Newman-Keuls Method.

3.1.1.3D Spectral analysis

UV-Visible spectra of culture broth were prepared using UV-Visible Spectrophotometer (model: UV-2450). A ten-fold dilution of culture broth was prepared with de-ionized water. The spectrum of diluted culture broth was prepared against plain GMSM broth (blank) in the UV-visible region from 800 to 190 nm using 1 ml silica cuvettes.

3.1.2 Purification and characterisation of pigment produced by *Pisolithus tinctorius* PT1

3.1.2.1A Pigment extraction from fruiting bodies

Sporocarps were collected from iron ore mines at Codli, Goa. They were cut and dried at 42°C. Dried sporocarps were disintegrated using dry-mixer to form fine powder. Various solvents such as acetone, benzene, chloroform, diethyl ether, ethanol, methanol, and water were tested. 25 ml solvent was added to 0.5g dry sporocarp powder. The mixture was shaken

vigorously and kept standing for 10 h at 4°C. The supernatants were filtered through 0.45µ membrane (HV Millipore) using syringe filter and stored at 4°C.

3.1.2.1B Extraction of pigment liberated in culture broth

About 4 ml of culture broth was dispensed in each 15 ml centrifuge tube. Culture broth was lyophilised for 18 h using ThermoSavant lyophilizer. Acetone, ethanol, methanol, and water were employed to extract pigment from lyophilised culture broth. One ml solvent was added to each centrifuge tube, agitated vigorously and kept standing for 10 h at 4°C. The supernatants were filtered through 0.45µ membrane (HV Millipore) using syringe filter and stored at 4°C.

3.1.2.1C Paper Chromatography

Pigment extracts were chromatographed on paper as described by Odebode, (1996) (Appendix C). Rf of the spots were calculated.

3.1.2.1D Spectral analysis

UV-Visible spectra of pigment extracts were prepared using UV-Visible Spectrophotometer (model: UV-2450). The pigment extracts were diluted with respective solvent. Methanol and water extracts were diluted 100 fold. Acetone and ethanol extracts were diluted 20 and 10 fold, respectively. UV-visible range was corrected for baseline with neat solvents in both the cuvettes to remove the interference in absorption due to solvents. The solvent

in sample cuvette was then replaced with respective diluted pigment extract and read in the UV-visible range from 800 to 190 nm.

3.1.3 Antagonistic activity of *Pisolithus tinctorius* PT1

3.1.3.1A Test pathogens

Fungal phytopathogens used as test organisms were *Fusarium* sp., *Macrophomina phaseolina* and *Sclerotium rolfsii*. The fungal isolates were periodically maintained on PDA plates (HiMedia) (Appendix A). All the isolates were obtained from ICAR Research complex for Goa, Old Goa, Goa.

3.1.3.1B Pigment bioassay

Antibiotic activity of pigments of *Pisolithus tinctorius* PT1 was checked on PDA plates. Water extracted sporocarp pigment (pH 5.0), culture filtrate (pH 3.0) and culture filtrate (pH 5.0) were tested for antibiotic activity against fungal plant pathogens. Culture broth of PT1 grown in GMSM liquid medium at RT was harvested after 30 days and pH was measured. The pH of an aliquot of culture broth was adjusted to 5.0 using KOH solution. The pigment solutions were filtered through 0.45µ membrane-syringe filter assembly and used for assay. An arc was cut on PDA plates using sterile scalpel at about 2.3 cm from the centre. An agar piece (10x10 mm) of test culture previously grown on PDA was placed in the centre of the plate. 300 µl sterile pigment sample was added in the arc. Control plate was maintained with the test culture and arc. Plates were incubated at RT for 10 days and growth of fungal pathogen was noted.

3.2 RESULTS AND DISCUSSION

Fungi are known for their ability to produce secondary metabolites. The fungal extract mostly contains compounds produced from the secondary metabolism. Fungal metabolites include toxins, antibiotics and other compounds (Frisvad *et al*., 2008). These compounds have attracted much attention due to both detrimental (e.g. toxins) and beneficial (e.g. antibiotics) effects on well-being of plants, animals and humans. The regulation of fungal secondary metabolism depends on genes involved in biosynthesis. In addition to pathway-specific regulators, fungal secondary metabolite production is also responsive to general environmental factors, such as carbon and nitrogen sources, temperature, light, pH, etc. (Shwab and Keller, 2008).

3.2.1 Pigment production by *Pisolithus tinctorius* PT1

Pigment production by *Pisolithus tinctorius* PT1 in GMSM is influenced by the parameters that affect growth. Isolate when grown over a period of 60 days, biomass accumulation and total phenolics elaboration increased over the time (Fig.3.1). The steep increase in biomass and phenolics production was observed from 0-20 and 20-40 days, respectively. Maximum pigment was liberated in the stationary phase of growth. UV-Visible spectra of culture broth show the increment of peak at 257 nm with the incubation period (Fig.3.2). Peak at 0 day is of ferrous-EDTA complex in the basal GMSM

medium. UV spectrophotometric assay is satisfactory since each group of phenolics compounds is characterised by one or several UV absorption maxima. Closely related phenolics display wide variations in their molecular absorptive (Pink *et al*., 1994). Simple phenolics have absorption maxima in the region between 220 and 280 nm (Owades *et al*., 1958). *Pisolithus tinctorius* PT1 showed more growth under static condition while total phenolics production was more under submerged condition and vice versa (Fig.3.3). The isolate produced 10% more phenolics under submerged conditions as compared to stationary growth conditions. Biomass accumulation was 16% less than that obtained under stationary conditions. The enhanced pigment liberation under shake condition could be due to shear damage encountered by the isolate.

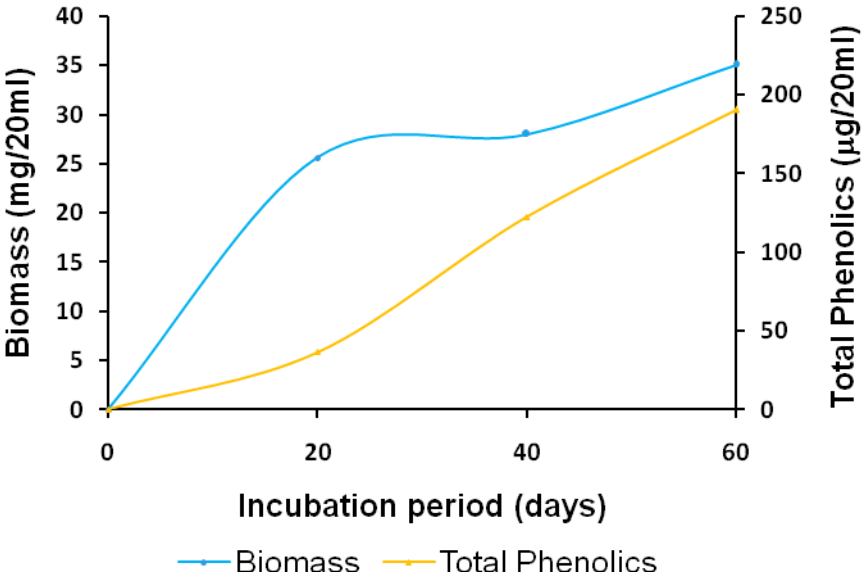


Fig.3.1 Growth and phenolics production by *Pisolithus tinctorius* PT1 in GMSM over a period of 60 days

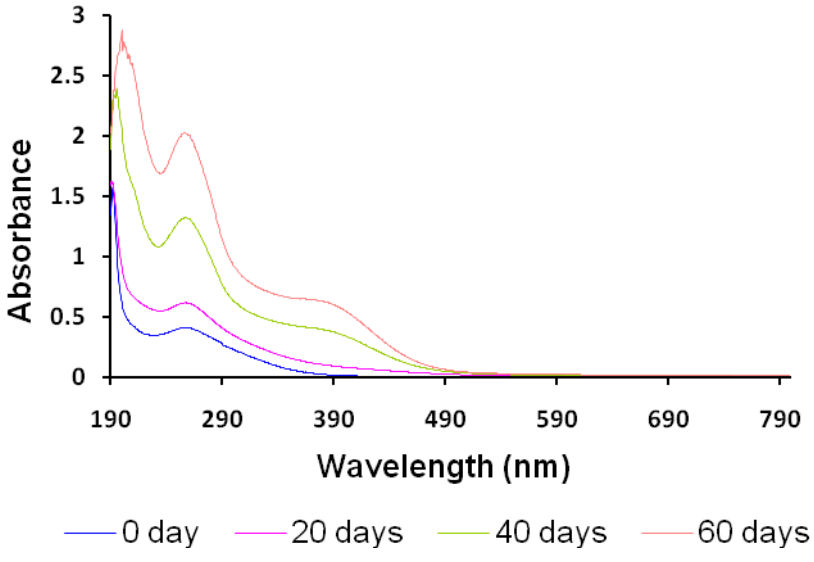


Fig.3.2 UV-visible spectra of culture broth of *Pisolithus tinctorius* PT1 grown in GMSM at different incubation period

High amount of NaCl is known to affect the pigmentation of fungi. Certain strains of *Aspergillus* and *Penicillium* produce brightly coloured pigments. Intensification of pigmentation was frequently seen in many organisms at appropriate NaCl concentration (Tresner and Hayes, 1971). Pigment elaborated by *Pisolithus tinctorius* PT1 was markedly affected by the amount of NaCl in the medium ($P<0.001$) (Fig.3.4) representing a typical bell shaped curve. Visually pigment liberated by PT1 in GMSM with varying amount of NaCl is seen in Fig 3.7. Pigment production increased with increasing NaCl concentration from 0 to 1.5% followed by decrease with increasing NaCl concentration upto 3%. Negligible amount of pigment was seen with 3% NaCl as the growth was meager. Optimum NaCl concentration for maximum phenolics elaboration by the isolate was 1.5%. Maximum yield of phenolics obtained was 149.91 µg/20ml of medium.

Pisolithus ticntorius PT1 was monitored for effect of temperature on pigment elaboration (Fig.3.7). There was liberation of phenolics in the broth at 25 to 37^{o}C ($P<0.05$) (Fig.3.5). Further, increasing temperature to 42^{o}C resulted into decreased phenolics production. There is no significant difference in phenolics production at temperatures between 25 to 30^{o}C ($P>0.05$). Significantly, maximum pigment production, 164.07 µg/20ml was observed at 37^{o}C, indicating this temperature as optimum for pigment production.

Unlike growth, total phenolics liberation in the medium was more with the presence of PEG (Fig.3.6 and Fig.3.7). Further, the production was to similar extent in the medium containing 5 and 10% PEG ($P>0.05$), although it was more than that produced in medium having no PEG ($P<0.05$).

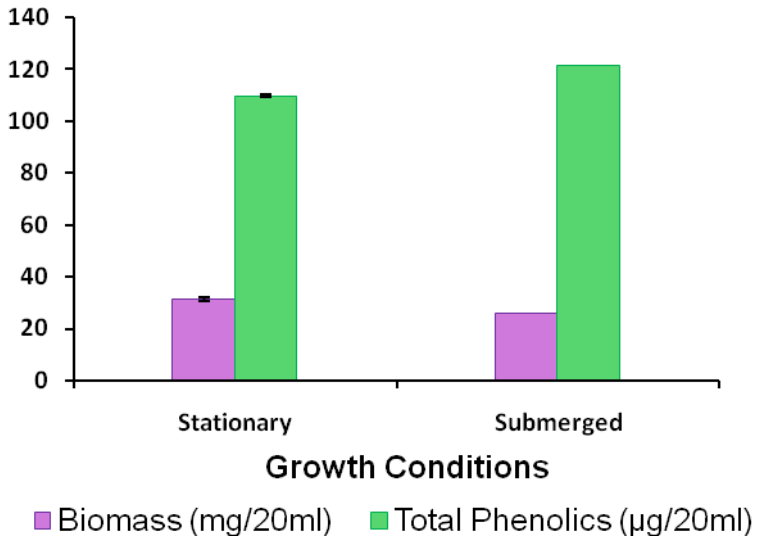


Fig.3.3 Growth of *Pisolithus tinctorius* PT1 and accumulation of phenolics in GMSM under stationary and submerged condition

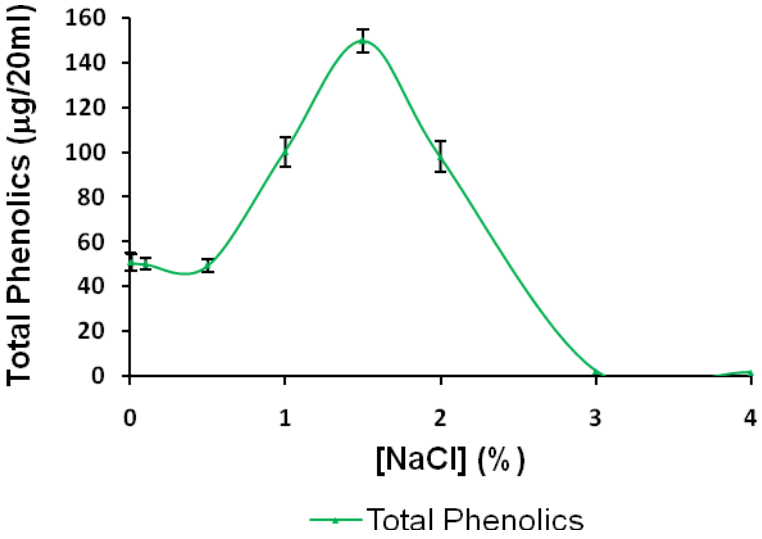


Fig.3.4 Effect of varying amount of NaCl on phenolics production by *Pisolithus tinctorius* PT1 in GMSM

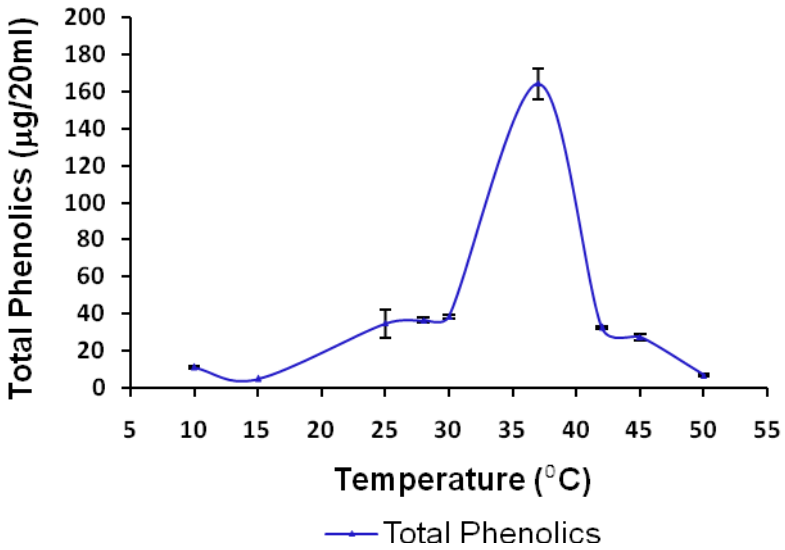


Fig.3.5 Effect of incubation temperature on total phenolics production by *Pisolithus tinctorius* PT1 in GMSM

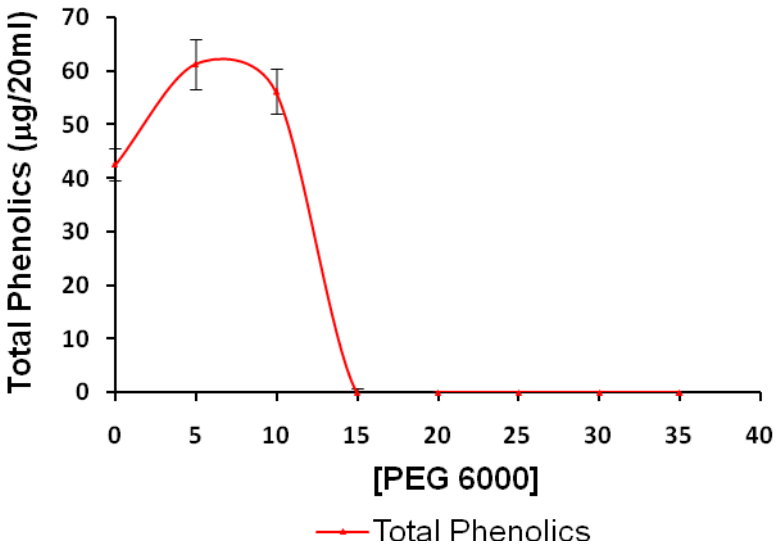


Fig.3.6 Elaboration of total phenolics production by *Pisolithus tinctorius* PT1 in GMSM with varying amount of PEG

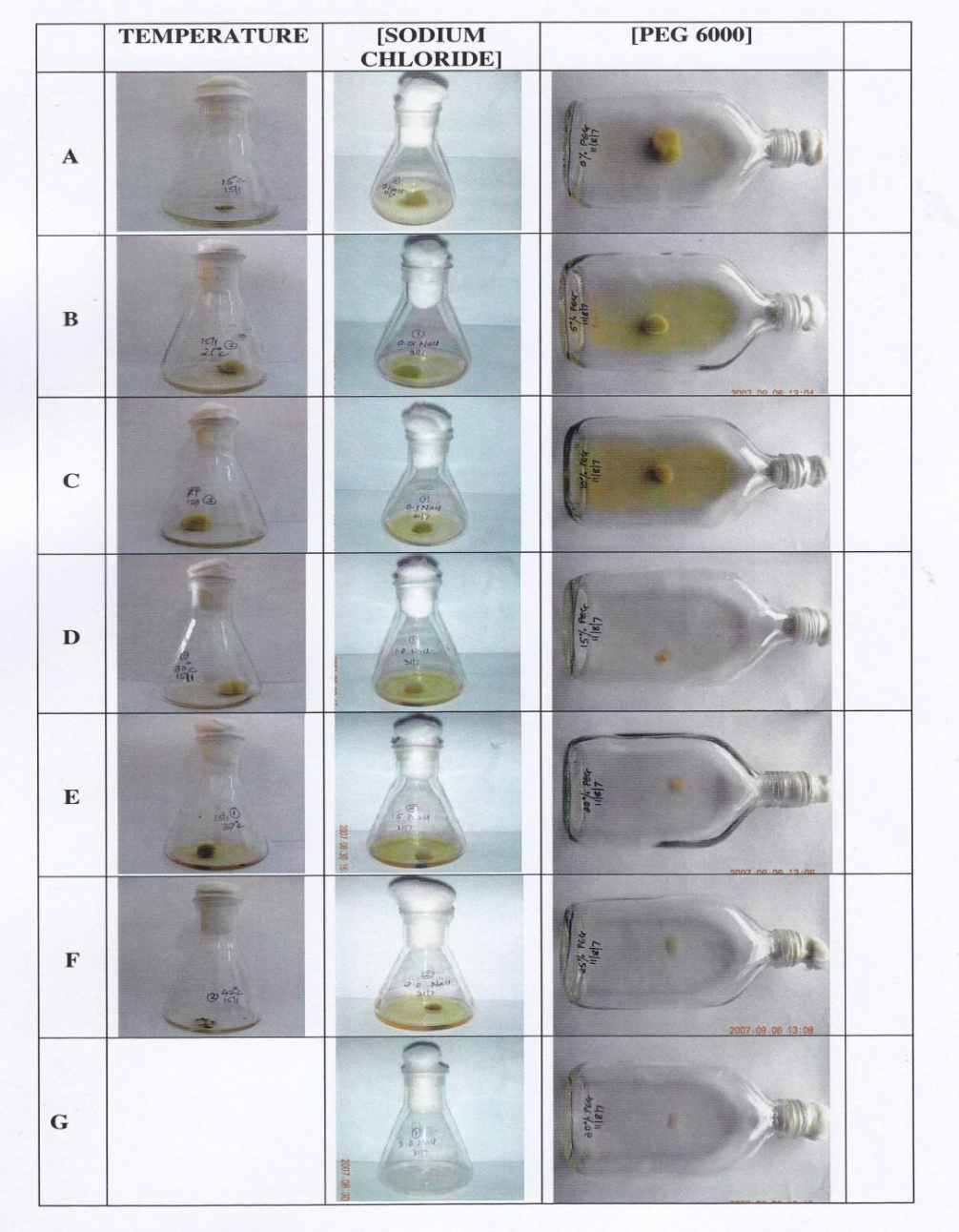


Fig.3.7 Phenolics liberation in GMSM by *Pisolithus tinctorius* PT1 at different incubation temperature, [NaCl], and [PEG]. **Temperature** A-15°C, B-25°C, C-RT, D-30°C, E-37°C, F-42°C; **[NaCl]** A-0%, B-0.01%, C-0.1%, D-1%, E-1.5%, F-2%, G-3%; **[PEG 6000]** A-0%, B-5%, C-10%, D-15%, E-20%, F-25%, G-30%

Pigment liberation by *Pisolithus tinctorius* PT1 was seen to be affected by the buffering systems used to adjust the pH of the medium. Fig.3.8 showed pigment elaborated in media with different buffering systems after 13 days of incubation. Pigmentation was found maximum in GMSM buffered with PCB, followed by SPB, HEPES and plain medium.

Pigmentation was not visible upto 0.027 M phosphate but was more visible in the medium containing phosphate from 0.047 to 0.087M (Fig.3.9). Maximum phenolics were liberated in medium with 0.067 M phosphate. Increasing pH of the media from acidic to neutral showed increase in pigment elaboration by PT1 from pH 4.0 to 7.0 (Fig 3.9). Maximum pigmentation was seen at pH 7.0.

Optimum concentration of manganese for maximum growth was 1000 ppm. Deviations of concentrations of manganese from optimum for growth resulted in elaboration of phenolics by *Pisolithus tinctorius* PT1 (Fig.3.9). This resulted in the two ranges for elaboration of pigment by PT1 in response to Mn in the growth medium. Concentration of Mn at lower range was 0 to 1.1 ppm and towards higher range was 5000 to 10000 ppm for pigment production. Among these, highest amount was observed with 0-1.1 ppm Mn. Similar to variation in growth, elaboration of phenolics also showed variation in response to different amount of $FeCl_3$ in the medium. However, no considerable pigmentation was observed during its growth with iron.

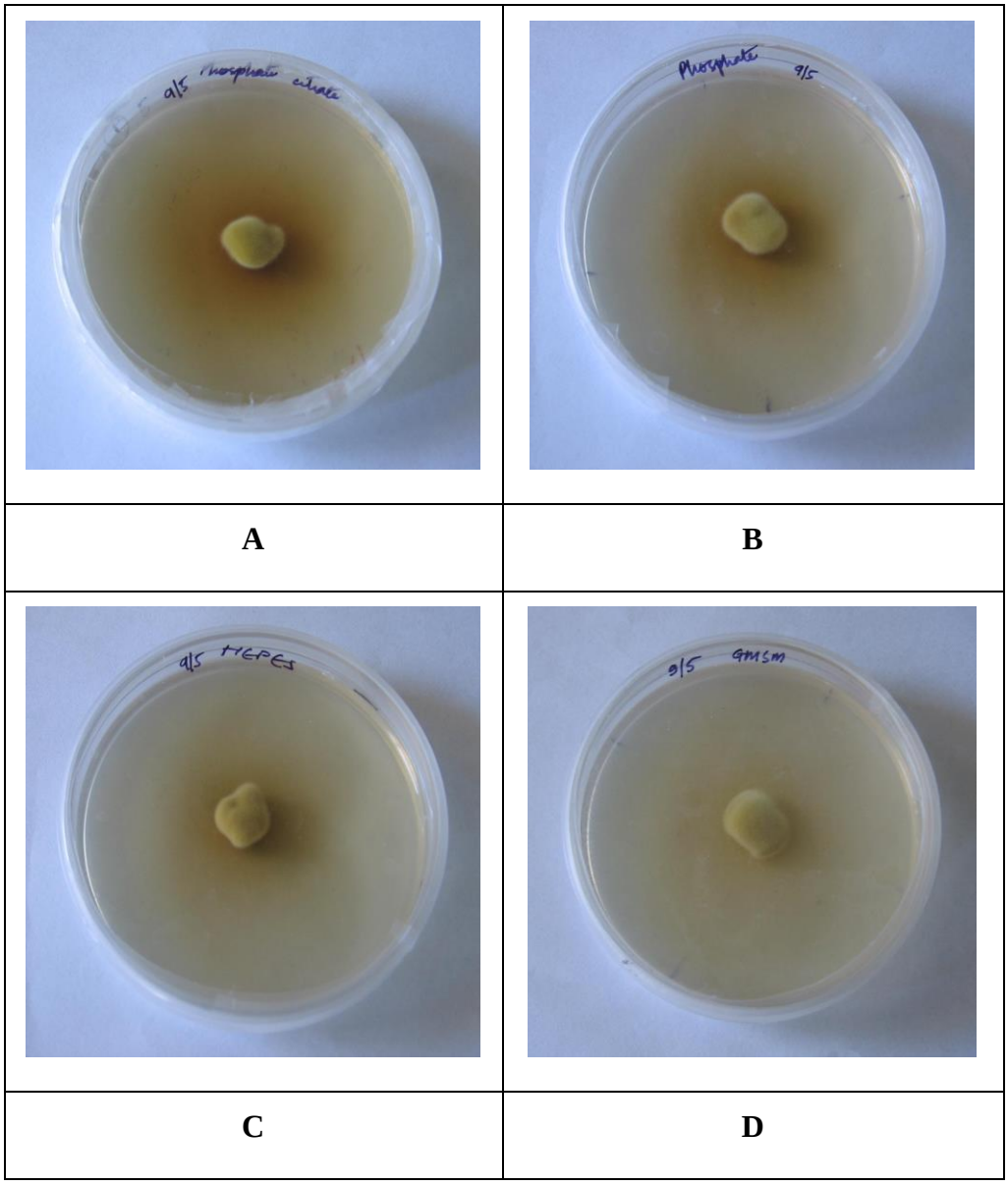


Fig.3.8 Phenolics liberation by *Pisolithus tinctorius* PT1 on GMSM agar medium buffered with (A) PCB, (B) SPB, (C) HEPES, and (D) plain medium without any buffer (control)

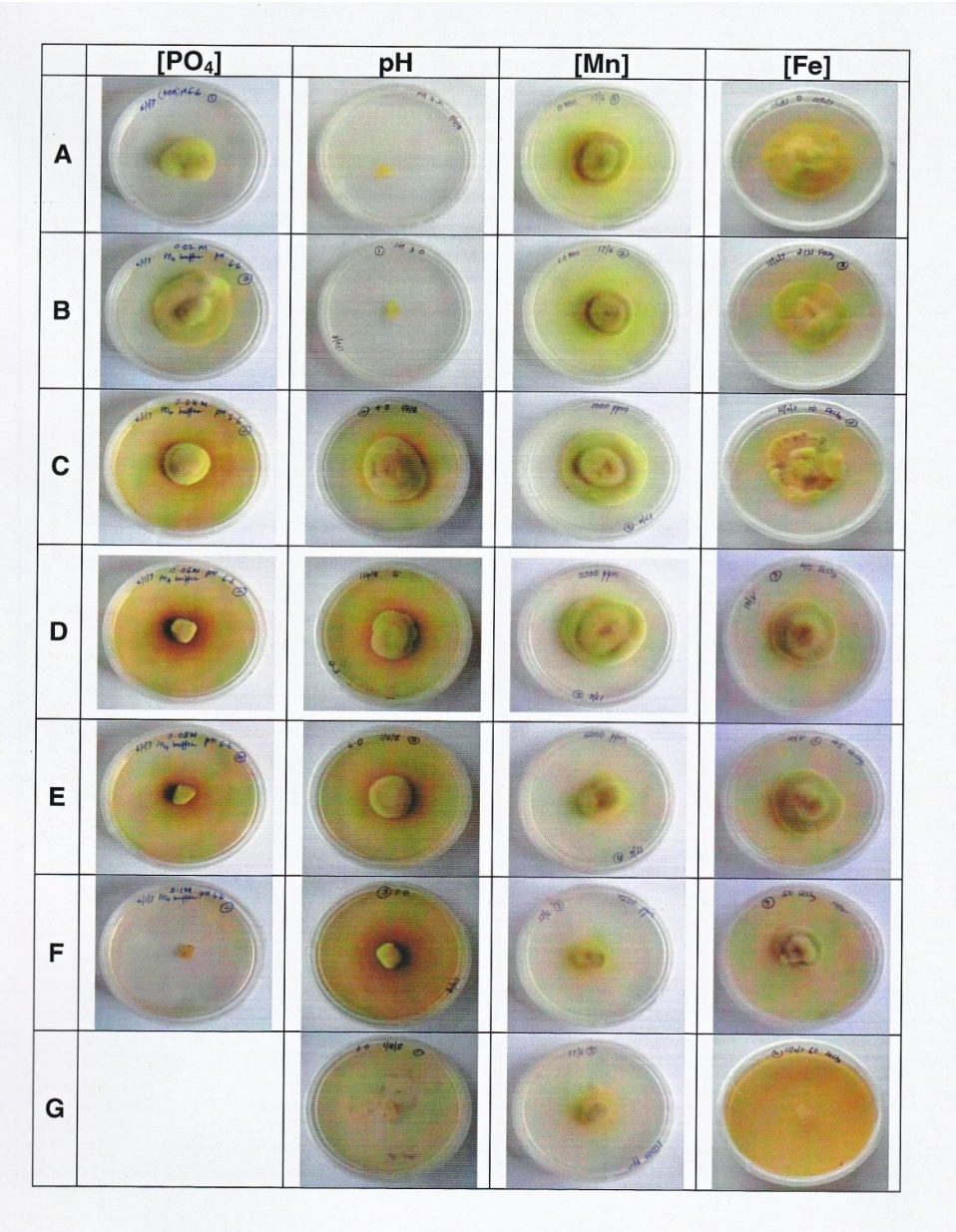


Fig.3.9 Liberation of phenolics on GMSM agar medium by *Pisolithus tinctorius* PT1 with varying [phosphate], pH, [manganese] and [iron]. **[Phosphate]** A-0.007M, B-0.027M, C-0.047M, D-0.067M, E-0.087M, F-0.17M; **pH** A-2.2, B-3.0, C-4.0, D-5.0, E-6.0, F-7.0, G-8.0; **[Manganese]** A-0 ppm, B-1.1 ppm, C-1000 ppm, D-2500 ppm, E-5000 ppm, F-7500 ppm, G-10000 ppm; **[Iron]** A-0 ppm, B-2.121 ppm, C-10 ppm, D-40 ppm, E-45 ppm, F-50 ppm, G-60 ppm

It has been previously suggested that tolerance to metal is associated with the formation of pigments and that the activity of tyrosinase, the melanine biosynthetic complex, is stimulated by metals in ECM fungi. This might probably increase metal sequestration onto cell wall pigments (Gruhn and Miller, 1991).

Present study of phenolics production by *Pisolithus tinctorius* PT1 revealed that the optima of all the variables for elaboration of phenolics were observed at higher values than that required for the maximum growth of isolate (Table 3.1). This indicated that the isolate produced more phenolics while growing under sub optimal conditions where it encountered the stress. Thus, even abiotic stress on the *Pisolithus* sp. will induce phenolics elaboration. Moreover it appeared that pH, NaCl and temperature significantly affected the pigmentation in PT1. Conditions such as pH 7.0, 0.067M phosphate, 1.5% NaCl, 5-10% PEG 6000, 1.1 ppm Mn, 2.121 ppm Fe, incubation temperature of 37°C and submerged cultivation were seen to favour maximum phenolics production in GMSM by *Pisolithus tinctorius* PT1. Further, investigations are needed to identify the impact of interactions of these factors on growth and production of phenolics using statistical methods such as Response Surface Models.

GROWTH FACTOR (TESTED RANGE)	RANGE FOR GROWTH	OPTIMUM GROWTH	STRESS FOR GROWTH		RANGE FOR TOTAL PHENOLICS PRODUCTION	OPTIMUM PHENOLICS PRODUCTION
			LOWER LIMIT	UPPER LIMIT		
[PO4] (0.007-0.1M)	0.007-0.087M	**0.027M**	<0.02M	**>0.04M**	0.027-0.087M	**0.067M**
pH (2.2-8.0)	3.0-7.0	**4.0-4.2**	≤3.0	**≥7.0**	4.0-7.0	**7.0**
TEMPERATURE (10-50^{0}C)	25-42^{0}C	**25-30^{0}C**	<25^{0}C	**≥37^{0}C**	25-42^{0}C	**37^{0}C**
[NaCl] (0-4%)	0-3%	**0.01-1%**	ND	**≥1.5%**	0.05-2%	**1.5%**
[PEG 6000] (0-35%)	0-10%	**0%**	ND	**>10%**	0-10%	**5-10%**
[Mn] (0-10000 ppm)	0-10000 ppm	**500-2500 ppm**	ND	**5000-10000 ppm**	0-1.1ppm & 5000-10000 ppm	**0-1.1 ppm**
[Fe] (0-100 ppm)	0-50 ppm	**VARIABLE RESPONSE**	ND	**ND**	0-45 ppm	**ND**

Table.3.1 Growth response and Total Phenolics elaboration by *Pisolithus tinctorius* PT1 with varying [Phosphate], pH, temperature, [NaCl], [PEG 6000], [Mn] and [Fe]; ND – Not Determined

3.2.2 Purification and characterisation of pigment produced by *Pisolithus tinctorius* PT1

Use of organic solvents is the most routine method for extraction of coloured metabolites or pigments. In this study, attempt has been made to extract pigments of PT1 from fruiting bodies and culture filtrate using polar and non polar organic solvents. Previously, Suh *et al*. (1991) and Kamat (1993) have used different polar solvents such as acetone, ethanol, methanol and non polar solvents such as benzene, chloroform, diethyl ether, ethyl acetate, petroleum ether, toluene, xylene for extraction of pigments from fungi including ECM fungi.

3.2.2a Pigment extraction from sporocarps

Solvents employed to extract sporocarp pigments were acetone, benzene, chloroform, diethyl ether, ethanol, methanol, and water. No pigment was extracted when benzene, chloroform and diethyl ether (non polar solvents) were used for extraction. Orange to brown pigment was extracted in acetone, ethanol, methanol, and water (Fig.3.10). Maximum visible pigment was extracted using water and methanol. UV-visible spectra of pigment extract in acetone show absorption peaks in UV region at 361, 329, 314, 285, 269, 242, 205 & 193 nm, in ethanol at 362, 265, 222 & 203 nm, in methanol at 364, 265 224 & 202 nm, and in water at 316, 223 & 197 nm (Fig.3.11).

A B C D

Fig.3.10 Solvent extraction of sporocarp pigment (A) water, (B) methanol, (C) acetone, and (D) ethanol

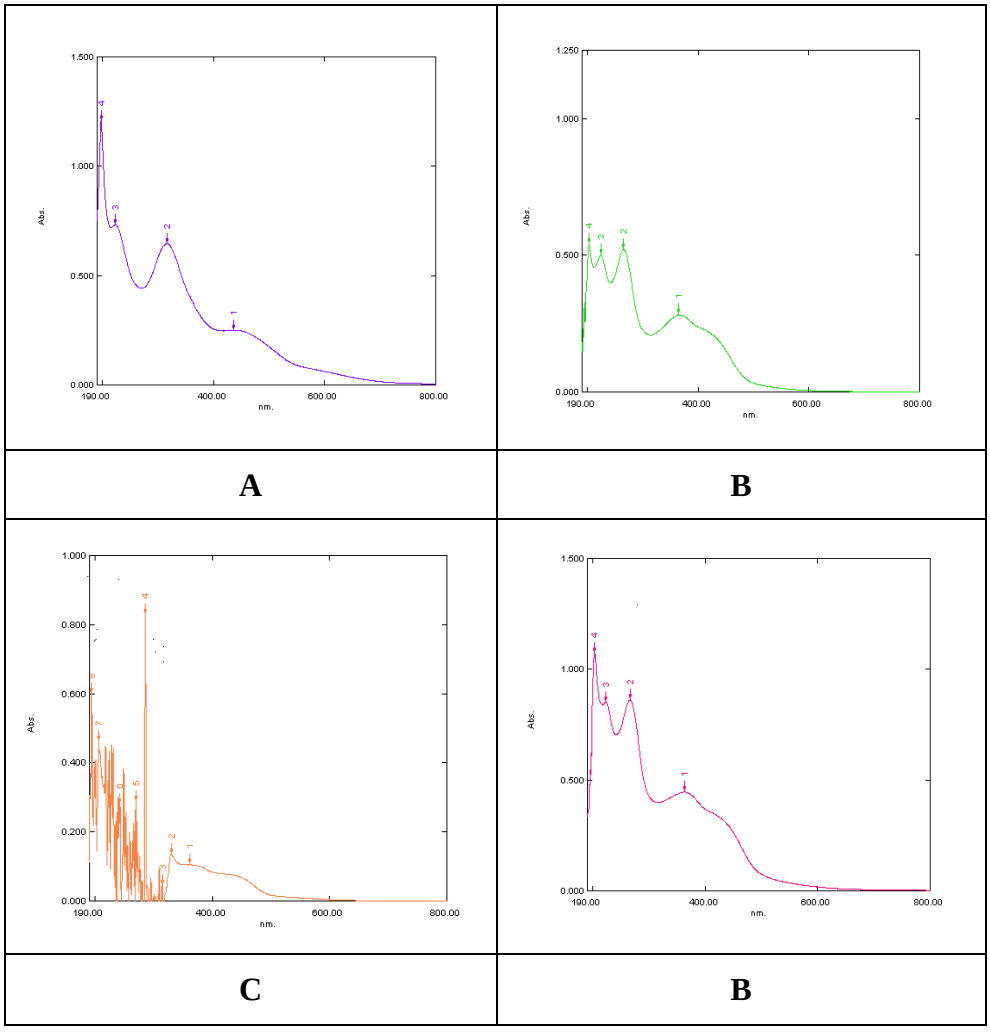


Fig.3.11 UV-Visible spectra of pigment extracted from sporocarp using different solvents (A) water, (B) methanol, (C) acetone, and (D) ethanol

3.2.2b Pigment extraction from culture filtrate

Based on the results of solvent extraction of sporocarp pigments the polar solvents *viz*. Acetone, ethanol, methanol, and water were used for pigment extraction of culture filtrate. Maximum visible pigment was extracted in methanol followed by water, ethanol and acetone (Fig.3.12). UV-visible spectra of acetone extract of culture filtrate pigments show absorption peaks in UV region at 371, 357, 330, 302, 280, 251, 246 & 208 nm (Fig.3.13). Ethanol extract of culture filtrate pigment show absorption peaks at 390, 258 & 204 nm in UV region. Methanol extract of culture filtrate pigment show absorption peaks at 258 & 206 nm in UV region. Culture filtrate pigment extracted with water show absorption peaks at 257 & 193 nm in UV region.

3.2.2a Paper chromatography of sporocarp pigment

Paper chromatography was initially carried out for standard phenolic acids like gallic acid, gentisic acid, mandelic acid and salicylic acid to check the efficiency of resolution of solvent system and developing solution (Fig.3.14). The Rf values were 0.68, 0.89, 0.91 and 0.98 for gallic acid (dark blue), gentisic acid (violet), mandelic acid (yellow) and salicylic acid (purple), respectively. Chromatographic analysis of water extract of sporocarp pigment revealed a single spot with Rf = 0.48 (Fig.3.15). The spot appeared black when observed under UV light and reddish brown when sprayed with

developing solution. Acetone, ethanol and methanol extracts of sporocarp pigment revealed two spots (Fig.3.15). The Rf values of the two spots in acetone extract were 0.38 and 0.45. The Rf values of the two spots in ethanol extract were 0.4 and 0.46. The Rf values of the two spots in methanol extract were 0.42 and 0.51. Under UV light the spot with lower Rf value appeared black and the spot with higher Rf value showed orange fluorescence (Fig.3.15-1). The spot on chromatogram with lower Rf value appeared brown and the spot with higher Rf value appeared yellow when sprayed with developing solution (Fig.3.15-2).

3.2.2b Paper chromatography of pigments from culture filtrate

Chromatographic analysis of pigment extracts of culture filtrate in different solvents revealed a single spot with Rf = 0.6 (Fig.3.16). Under UV light yellow fluorescent spot was observed (Fig.3.16-1). Chromatogram when sprayed with developing solution gave a dark blue spot (Fi.3.16-2).

Thus, chromatographic and spectral analyses clearly revealed that the pigments of *Pisolithus tinctorius* PT1 are phenolic in nature. Moreover, sporocarp pigment and the pigment liberated by PT1 in GMSM appeared to be different compounds.

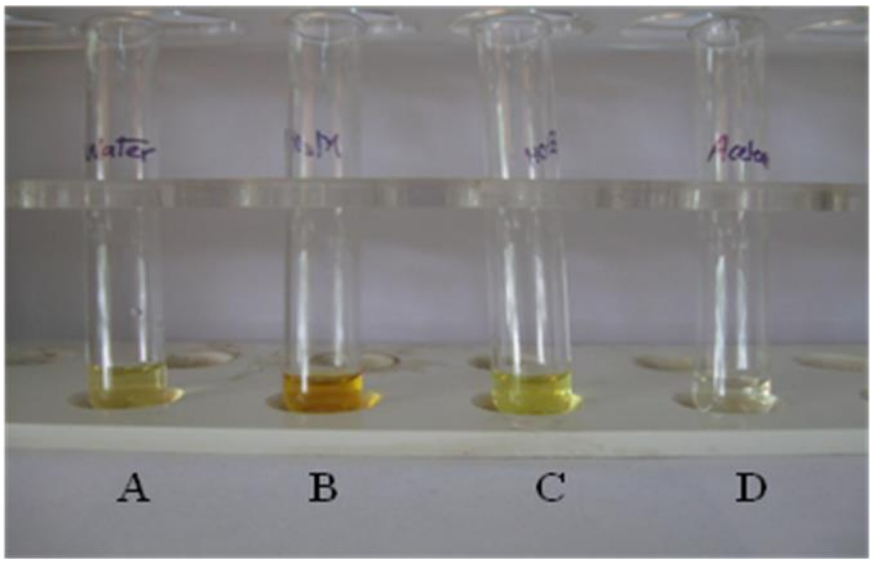


Fig.3.12 Solvent extraction of pigments liberated in culture filtrate. (A) Water, (B) Methanol, (C) Ethanol, and (D) Acetone

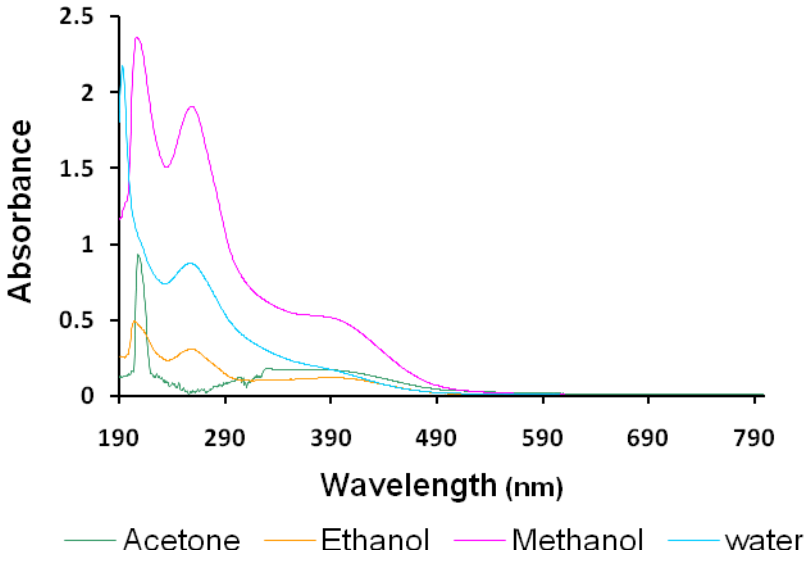


Fig.3.13 UV-Visible spectra of pigment extracts of culture filtrate - (A) water (B) methanol (D) ethanol (C) acetone

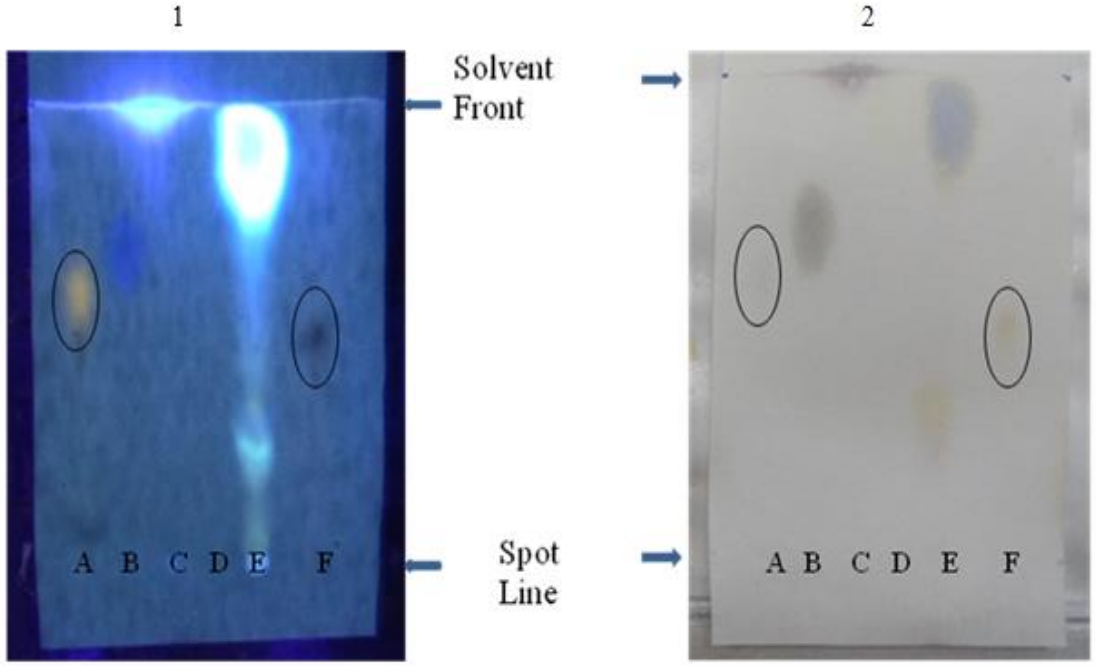


Fig.3.14 Paper chromatogram of standard phenolic acids (1) Chromatogram observed under UV light (2) Chromatogram developed using developing solution. (A) Culture filtrate (B) Gallic acid (C) Salicylic acid (D) Mandelic acid (E) Gentisic acid (F) Water extracted sporocarp pigment

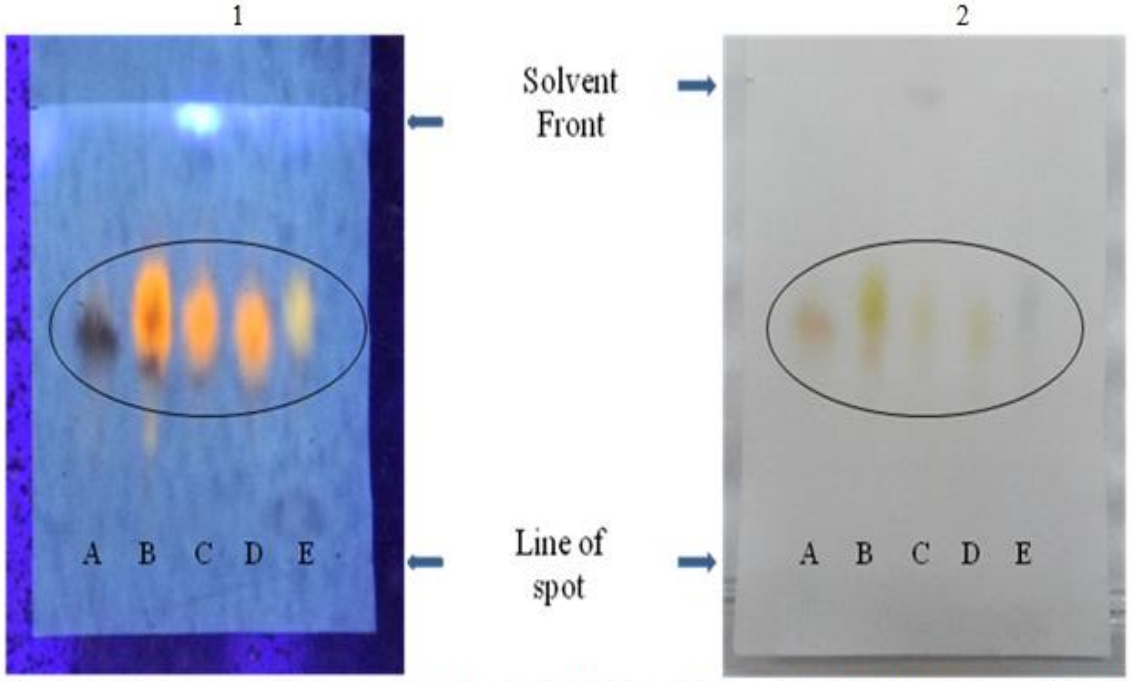


Fig.3.15 Paper chromatogram of sporocarp pigments extracted using different solvents. (A) Water (B) Methanol (C) Ethanol (D) Acetone and (E) Culture filtrate (1) Chromatogram observed under UV light (2) Chromatogram developed using developing solution

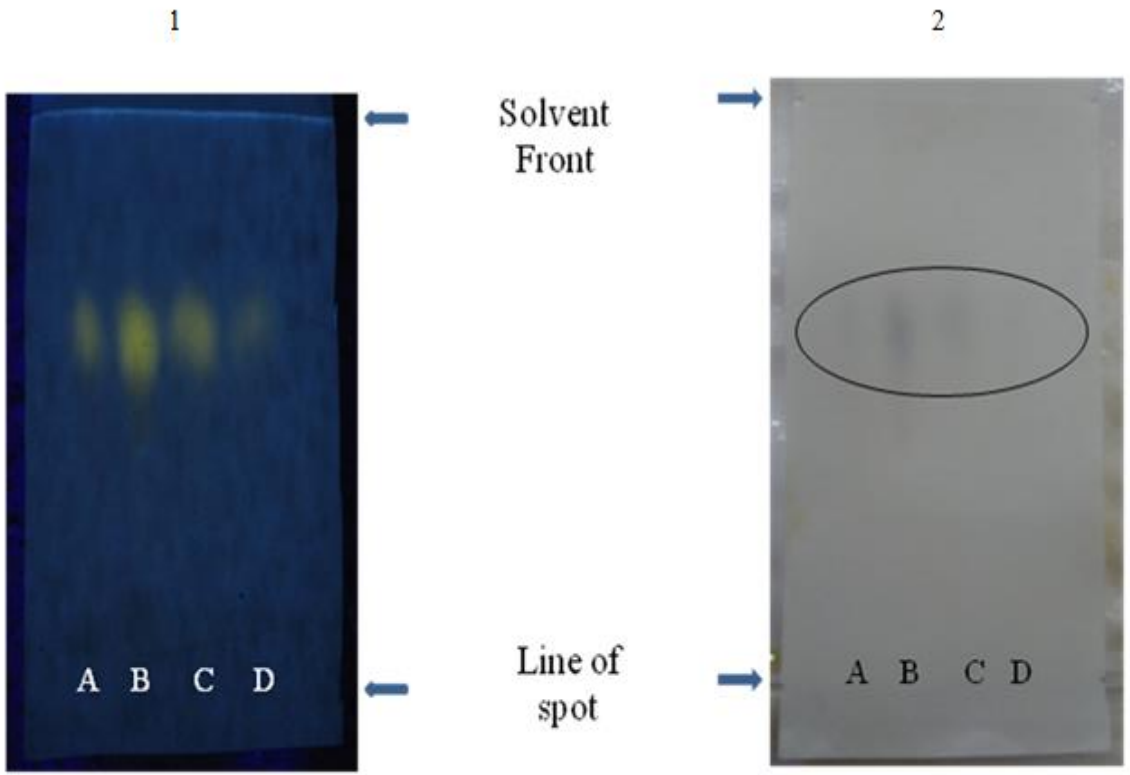


Fig 3.16 Paper-chromatogram of solvent extracted pigments of culture filtrate. (A) Water (B) Methanol (C) Ethanol (D) Acetone (1) Chromatogram under UV light (2) Chromatogram sprayed with developing solution

3.2.3 Antagonistic activity of *Pisolithus tinctorius* PT1

Antagonistic activity of culture filtrate of ECM fungi is known. Various ECM fungi have shown inhibition of growth of *Fusarium* sp.. In this study, an attempt was made to check the antagonistic effect of pigments obtained from *Pisolithus tinctorius* PT1 against *Fusarium* sp., *Macrophomina phaseolina* and *Sclerotium rolfsii*. Besides culture filtrate, pigment extracted from sporocarp was also considered for antagonism studies. Montenegro et al., (2004) demonstrated the cytotoxic potential of pisosterol, a triterpene isolates from sporocarps of Pt against animal cells. Pisosterol strongly inhibited the growth of tumor cell lines, focusing its potential clinical application in cancer chemotherapy.

Water extract of sporocarp pigment arrested the growth of *Fusarium* sp. and showed enhanced sclerotia formation and inhibited aerial growth of *Sclerotium rolfsii*. Sporocarp pigment had no effect on growth of *Macrophomina phaseolina*. Culture fitrate with pH 3.0 significantly arrested the growth of *Fusarium* sp.. *M. phaseolina* and *S. rolfsii* were not affected with culture filtrate having pH 3.0. Culture filtrate with pH adjusted to 5.0 had no effect on growth of *Fusarium* sp. and *M. Phaseolina*, but enhanced sclerotia formation and inhibited aerial growth of *Sclerotium rolfsii*. Thus, pigments of *Pisolithus tinctorius* PT1 showed differential response against the test organisms (Fig.3.17).

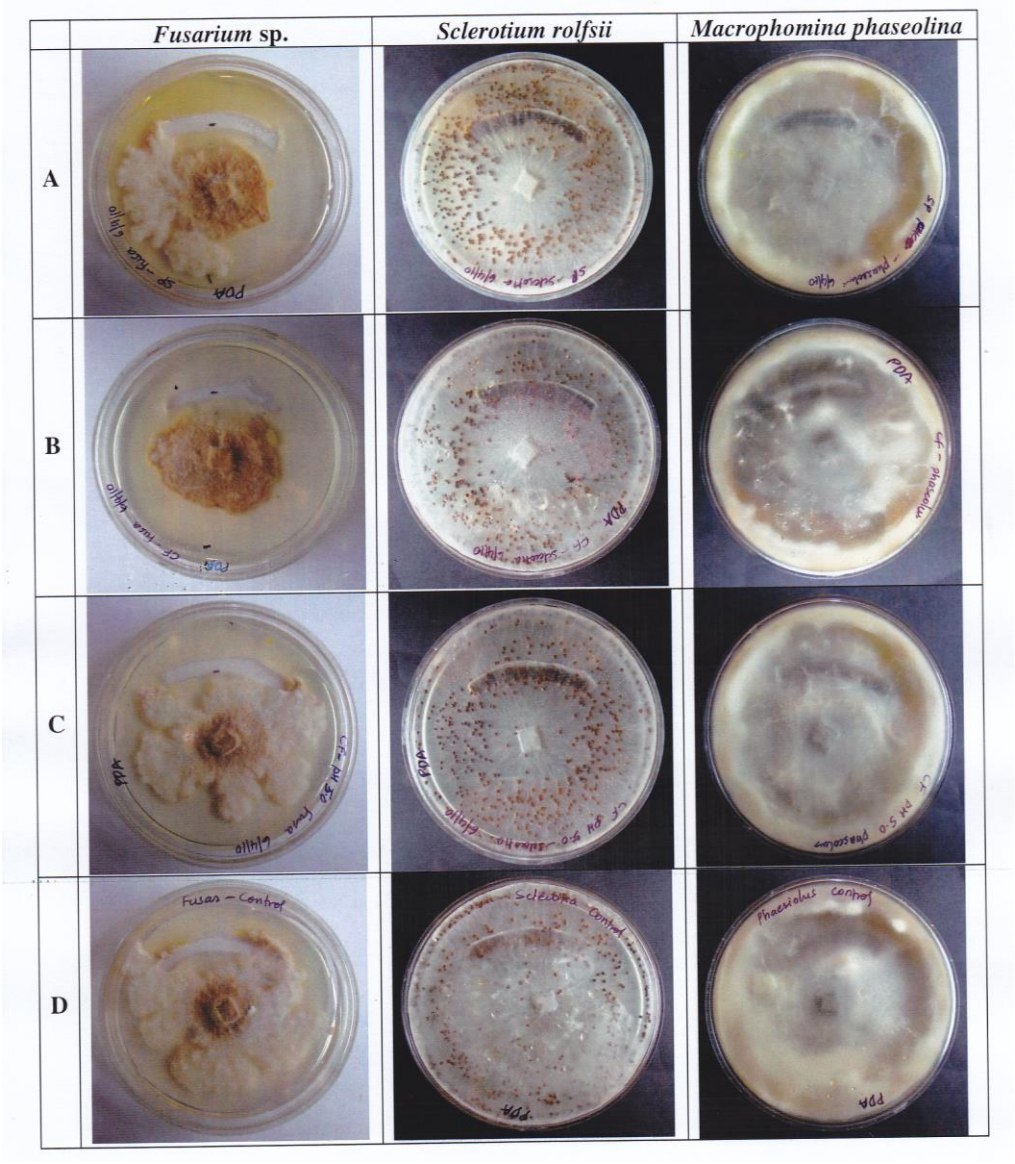


Fig.3.17 Antagonistic effect of *Pisolithus tinctorius* PT1 on *Fusarium* sp., *Sclerotium rolfsii* and *Macrophomina phaseolina*. A - water extracted sporocarp pigment (pH 5.0), B - Culture filtrate (pH 3.0), C - Culture filtrate (pH 5.0), D - Control PDA plate

Among the tested phytopathogens, *Fusarium* sp. was the most affected test organism while *M. phaseolina* was not affected with any of the pigments. Although, *S. rolfsii* showed growth on all the plates, plates treated with water extracted basidiocarp pigment and culture filtrate with pH 5.0 showed enhanced sclerotia formation and inhibited aerial growth of the fungus. Whether these changes in the growth of fungus are due to the effect of pigments, are not clearly explainable. The antagonistic activity of culture filtrate with pH 3.0 is in accordance with that reported by Yamaji *et al*. (2005) that showed antifungal activity of phenolics produced by *Paxillus* sp. 60/92 against *Pythium vexans* under acidic culture condition (pH 3-4). This possibly suggests that bioactivity of phenolics produced by ECM fungi could be pH dependent. Although *M. Phaseolina* and *S. rolfsii* were not inhibited by the pigments of *Pisolithus* sp. PT1, the effect on *S. rolfsii* and *Fusarium* sp. suggests its possible antagonistic potency.

Thus, the results obtained in this investigation suggest the ecological stability of associated host plants in the mining region and possible industrial application of the isolate.

CHAPTER IV

Mycorrhization studies

INTRODUCTION

Mining results in gross disturbance of the existing vegetation and generates a vast amount of reject soil. These rejects have poor nutritive quality, aeration efficiency, high soil temperature and low water holding capacity. Mining soil rejects contain an excess amount of metals that are being mined. Recovery and regeneration of such landscape becomes difficult as survival and establishment rate of young plants is very-very low. ECM is a promising solution to successful and consistent revegetation of such disturbed mine sites. *Pisolithus tinctorius* has been widely investigated for reclamation of mine land with improved tree planting success. Acid coal spoils, kaolin spoils, mineral mine wastes, borrow pits, and other severely disturbed sites have been successfully forested with Pt symbiont tree seedlings (Marx and Artman, 1979; Cordell *et al.*, 2000). Khosla and Reddy (2008) has demonstrated the potential of *Pisolithus albus* with *Eucalyptus* seedlings in reclamation of bauxite mined soil. *Pisolithus tinctorius* offers a practical, economical and effective alternative to intensive mine land reclamation. In southern India, research highlights the diversity of ECM fungi in Western Ghats and efficiency of these fungi on growth and mycorrhizal development with *Pinus*, *Eucalyptus* and some dipterocarps (Natarajan *et al.*, 2005; Reddy and Natarajan, 1997). Extensive mining is being carried out in different parts of Goa. Reclamation of the reject soil using biological tools is need of an hour. In this study we attempted to check the efficiency of inoculation of *Pisolithus tinctorius* on growth and survival of forestry important local tree species in Goa.

4.1 MATERIALS AND METHOD

4.1.1 Inoculum preparation using *Pisolithus tinctorius* PT1

4.1.1.1 Soil based vegetative inoculum

4.1.1.1A Vegetative growth of *Pisolithus tinctorius* PT1

Biomass of *Pisolithus tinctorius* PT1 was multiplied using liquid MMN and GMSM (Appendix A). A shallow layer of liquid medium (10 ml) was inoculated with agar disc, previously grown on MMN agar and incubated in static conditions for 20 days at RT. The biomass was collected and washed with sterile physiological saline solution until no residual sugar was detected in washings. Sugar in the washing was detected using Phenol Sulphuric Acid method (Dubois *et al.*, 1956) (Appendix D).

4.1.1.1B Soil for inoculum preparation

Soil was collected from Goa University campus. Soil was dried overnight in oven at 80°C. The soil was graded using Ro-Tap Sieve shaker (Park, 1997). The fractions, clay and silt (<0.05 mm 'A') and the sand (0.05-1.0 mm, 'B'; 1.0-2.0 mm, 'C') were collected and autoclaved at 121°C for 2 h.

4.1.1.1C Inoculum formulation

Soil fractions A, B and C were mixed in the ratio 1:6:3; soil mixture, 100 g was taken on aluminium foil in a tray. Eight gram wet fungal mycelium was added to the soil. To this mixture 15 g gypsum was added. The contents were thoroughly mixed but gently with sterile distilled water (Appendix E). The mixture was placed in oven at 35°C to dry. The inoculum mixture was dried so as to retain 33% of added water. Inoculum was stored at 4°C, until use.

4.1.1.2 Vermiculite based vegetative inoculum

4.1.1.2A Small scale

Pisolithus tinctorius PT1 was cultivated on solid surface of vermiculite soaked with growth medium. Growth of PT1 was carried out using autoclaved sterilised (121°C/45 min) 10 g vermiculite placed in 250 ml conical flask soaked with 25 ml sterile GMSM. Medium was inoculated with a single inoculum disc and incubated at RT under static condition.

4.1.1.2A Mass scale

Mass multiplication of PT1 was carried out in 3000 ml Haffkine culture bottles with 200 g vermiculite. Sterile vermiculite was soaked with 400 ml GMSM. Inoculum for mass cultivation was prepared in MMN broth. MMN

broth 10 ml with glass pieces was inoculated with a single inoculum disc of PT1. After 22 days of incubation, mycelia were fragmented by shaking the culture bottle vigorously. Suspension containing fragmented culture was transfer to Haffkine culture bottles containing vermiculite soaked with GMSM medium.

4.1.1.3 Soil based spore inoculum

Sporocarps were collected from the dumps of rejects of iron ore mine at Codli, Goa. They were cut and dried at 42°C. Dried sporocarps were disintegrated using dry-mixer to form powder for use in inoculum formulation. The yeast isolate obtained as contaminant during isolation of mycelium of PT1 was maintained on MMN for use in inoculum formulation. 1 Kg sterile soil was taken in a tray. Gypsum (200 g), sporocarp powder (50 g) and yeast cells (10^9 cfu) were added to soil. The contents were thoroughly mixed with distilled water (Appendix E) and dried at 37°C to retain 33% of added water. Inoculum was stored at 4°C.

4.1.2 Inoculation of seedlings with *Pisolithus tinctorius* PT1

4.1.2.1A Inoculation with vegetative inoculum

Seedlings produced in root trainers in the nursery at codli were transferred to vase pots and vegetative inoculum was placed under the roots @ 50 g/ seedling. Plant seedlings used for inoculation were *Acacia mangium*,

Peltophorum pterocarpum, *Pithecellobium dulce*, *Dalbergia sisso* and *Cassia fistula*. Control seedlings were maintained without inoculation with fungal mycelium, i.e., it received all the components of Vegetative Inoculum formulation except fungal mycelium. Seedlings were watered once in 2-3 days and fertilised once a month (Dunabeitia *et al.*, 2004).

4.1.2.1B Inoculation with spore inoculum in containers

Seedlings produced in root trainers in the nursery at Codli were transferred to polyethylene bags and spore inoculum was placed under the roots. Mine reject soil and sand in the ratio 4:1 were used as potting mixture. Plant seedlings used for inoculation were *Albizia lebbeck, Butea monosperma, Lagerstoemia speciosa, Peltophorum pterocarpum, Pithecellobium dulce*, and *Tamarindus indica*. Control seedlings were maintained without inoculation with fungal spores i.e., it received all the components of Spore Inoculum formulation except spores. Seedlings were watered once in 2-3 days and with nutrient solution once a month.

Albizia saman and *Cassia siamea* were inoculated with PT spores by slurry dip method. 5% slurry of sporocarp powder was prepared in physiological normal saline solution. The roots of seedlings were rinsed with deionised water and then dipped in spore slurry.

4.1.2.1B Inoculation of transplanted seedlings with spore inoculum

About 500 transplanted tree seedlings belonging to 22 genera were inoculated with 35 g of spore inoculum (Table 4.1). For inoculation of seedlings, 6" broad and 8-12" deep semi-circle trench at 1.5 ft away from the seedling was prepared. Dimensions of trenches were selected according to the average collar diameter of transplanted seedlings. Each seedling was inoculated with 60g spore inoculum (10^9 spores). Inoculum was spread evenly in the trench made around the seedling, over-layered with soil and 100 g of compost. The trench was covered completely with soil. Control seedlings were treated with only compost. Seedlings were watered once in 2-4 days.

4.1.3 Seedling growth analysis

4.1.3.1A Seedlings in pot culture

Six months after inoculation, the seedlings were uprooted and roots were cleaned gently with water. Roots were examined under stereomicroscope to check mycorrhizal synthesis.

No	Tree species		
	Scientific name	Common name	Family
1	*Albizia lebbeck*	Shiras	Fabaceae-Mimosoideae
2	*Albizia saman*	Raintree	Fabaceae-Mimosoideae
3	*Alstonia scholarias*	Saton	Apocynaceae
4	*Bauhinia racemosa*	Apto	Fabaceae-caesalpinoideae
5	*Butea monosperma*	Palas	Fabaceae-papilionoideae
6	*Cassia fistula*	Bayo	Fabaceae-caesalpinoideae
7	*Cassia seamea*	Cassia	Fabaceae-caesalpinoideae
8	*Dalbergia sisso*	Shisam	Fabaceae-papilionoideae
9	*Delonix regia*	Gulmohar	Fabaceae-caesalpinoideae
10	*Dendrocalamus strictus*	Bamboo	Gramineae
11	*Emblica officinalis*	Awala	Euphorbiaceae
12	*Erythrina indica*	Pongaro	Fabaceae-papilionoideae
13	*Euginea jambolana*	Jamun	Myrtaceae
14	*Gmelina arborea*	Shivan	Verbenaceae
15	*Lagerstoemia speciosa*	Taman	Lythraceae
16	*Melia azadirach*	Mohagany	Meliaceae
17	*Peltophorum pterocarpum*	Peltophorum	Fabaceae-caesalpinoideae
18	*Pithecellobium dulce*	Vilayati chinch	Fabaceae-Mimosoideae
19	*Pongamia pinnata*	Karanj	Fabaceae-papilionoideae
20	*Psidium guajava* Linn.	Guava	Myrtaceae
21	*Pterocarpus marsupium*	Assan	Fabaceae-papilionoideae
22	*Sapindus emarginalis*	Rinte	Sapindaceae
23	*Tamarindus indica*	Tamarind	Fabaceae-caesalpinoideae
24	*Terminalia arjuna*	Arjun	Combretaceae
25	*Terminalia bellerica*	Goting	Combretaceae

Table 4.1 Plant seedlings transplanted on Dump No. 9 inoculated with *Pisolithus*

4.1.3.1B Transplanted seedlings on mine reject dumps

Survived seedlings from each treatment were selected for determination of plant height and collar diameter. Growth parameters were recorded at the time of inoculation (0 month) and 15, 29 and 41 months after inoculation. Growth data of inoculated and un-inoculated control seedlings was indexed using Microsoft Excel. For a single parameter, an average of all the values at 0 month was taken. This mean value was used as denominator to divide each value observed during the entire period of experiment for that parameter for respective tree species. The calculated values were plotted against the time at which the observations were recorded. Linear regression was drawn which was set to intercept Y axis at 1.0. The slope of the linear equation obtained for treated plants was compared with slope of the untreated plants for respective species.

RESULTS AND DISCUSSION

Pisolithus arhizus, Thelephora terrestris, Suillus luteus and *Cenococcum graniforme* (=*Cenococcum geophilum*) have been reported as ectomycorrhizal mycobionts of trees that grow on mine spoils (Medve and Thompson, 1984). Among ECM fungi the value of *Pisolithus tinctorius* to seedlings increased as the quality of site decreased (Marx and Artman, 1979).

Pisolithus sp. therefore offers outstanding potential for use in reclamation of mines and other soils subjected to a variety of edaphic stresses. *Pisolithus* has a global distribution and been used successfully to inoculate plantation stock of several tree taxa including *Acacia*, *Eucalyptus*, *Pinus* and *Oak* (Burgess *et al.*, 1996, Cordell *et al.*, 2000, Jaykumar and Tan, 2005).

Sporocarps of *Pisolithus tinctorius* PT1 were found in association with *Acacia mangium* present in coastal and rain-forest area of Goa including disturbed mining regions. The roots of *A. mangium* were profusely infested with mycelia of Pt. The extensive presence of this combination of host and Pt in the iron ore mining area indicates its possible application in the reclamation of mining soil rejects. These mine rejects consist of porous loose soil with pH 5.5-6.0, phosphorus (Pi) 0-15 mg/Kg, iron 12-50 mg/Kg, manganese 40-70 mg/Kg and negligible fertility. The ECM strain thriving in such nutrient deficient and stressful conditions could be promising in reclamation.

Many important tree species in reforestation programmes are dependent on ECM symbiosis in order to survive and grow, mainly in poor soils and the exploitation to increase plant productivity demands the establishment of inoculum production methods. In the present study, with an objective of using PT1 for reclamation of huge dumps of reject soil generated during mining of iron ore, preparation of various inoculum formulations were carried out. Soil based inoculums were found to be cost efficient and easy to apply. Soil particles less than 2 mm were used in inoculum preparation in order to prevent mechanical damage that would occur to vegetative mycelia during mixing. Gypsum was added as binder in the inoculum. Vermiculite is a

low price, nutritionally inert material that absorbs nutrient solutions well. It also provides a bed structure that enables good aeration of the substrate. Vermiculite soaked with GMSM was used to cultivate PT1 and was found best for mass multiplication of vegetative mycelium (Fig.4.1). The yeast isolate obtained as contaminant during isolation of mycelium of PT1 was included as co-culture in spore inoculum. The isolate was considered important as an activator of spore germination in the inoculum. Earlier researches on spore germination of ECM fungi have revealed the importance of yeast activator (Martin and Gracia, 2000; Wilson and Beneke, 1966). Moreover, Hile and Hennek (1969) observed yeast like organisms appearing on the agar medium during spore germination of Pt and believed the presence of these organisms in the interior of the "peridioles" of the basidiocarp, suggesting their nutritional interdependence. They also suggested that metabolic by-products of the yeast-like organism may enhance growth of Pt.

In containerized conditions, the mycorrhization of plant seedlings inoculated with vegetative inoculum of PT1 is shown in Table 4.2. *Acacia mangium* and *Cassia fistula* have shown mycorrhizae with vegetative inoculum. Association of *Pisolithus* with the roots of the host plant can be easily identified visually because of the characteristics of *Pisolithus* ECM. Typical golden yellowish mycelial growth of *Pisolithus* was observed on fine roots of *A. mangium* and *C. fistula*.

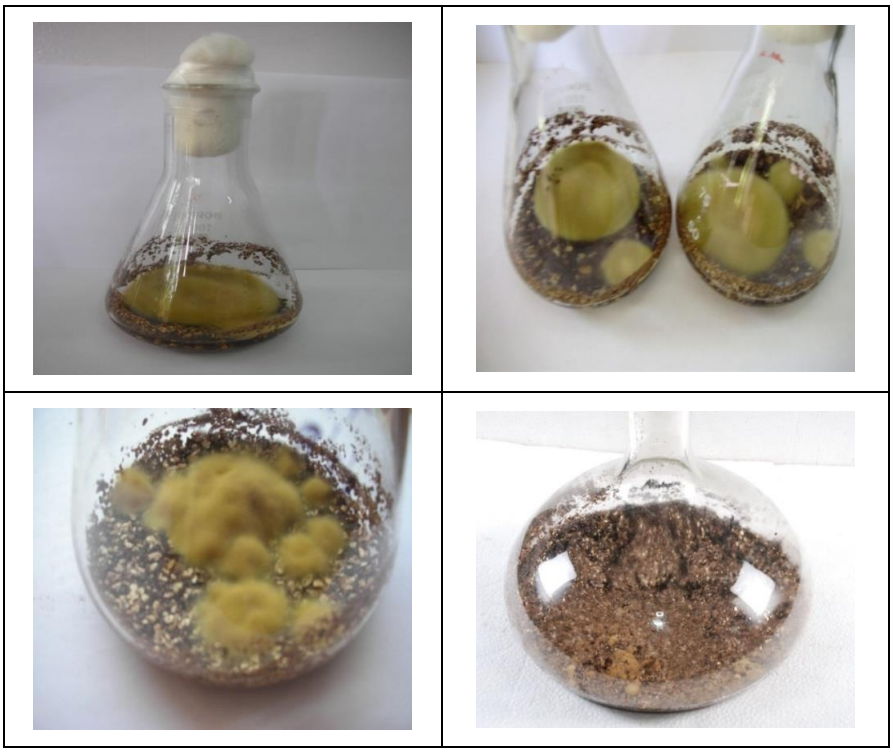


Fig.4.1 Growth of *Pisolithus tinctorius* PT1 on vermiculite soaked with GMSM

Plant species	Mycorrhization
Acacia mangium	+
Cassia fistula	+
Dalbergia sissoo	-
Delonix regia	-
Peltophorum pterocarpum	-
Pithecellobium dulce	-

Table 4.2 Plant seedlings inoculated with vegetative inoculum in vase pots

In laboratory conditions, significant effect of inoculation with spores of PT1, was seen on plant seedlings of *Albizia lebbeck*, *Lagerstoemia speciosa* and *Pithecellobium dulce* (Fig.4.2). Inoculated seedlings showed better growth as compared to un-inoculated control seedlings. Significant increase in plant height was observed in inoculated seedlings. *Albizia saman* and *Cassia siamea* were inoculated with spores of PT1 by dipping the roots of seedling directly in the spore slurry. Inoculated seedlings have shown considerable increase in height, collar diameter and apical shoot diameter (Fig.4.3). The root tips of inoculated seedlings were thicker than the un-inoculated seedlings. Table 4.3 summarizes the growth parameters of inoculated and un-inoculated *Albizia saman* and *Cassia siamea* seedlings, inoculated with spore inoculum by slurry dip method.

Seedling	**Collar diameter (mm)**	**Top diameter (mm)**	**Root tips (mm)**	**Height (cm)**
Albizia saman				
Inoculated	5.6-11.6	1.5-4.4	1.4-3.7	53-86
Un-inoculated	2.3-4.6	1.1-1.5	0.4-0.6	27-49
Cassia siamea				
Inoculated	7.6-8.5	1.9-2.3	0.99-1.5	75-80
Un-inoculated	2.3-4.7	1.3-1.5	0.3-0.7	35-40

Table 4.3 Plant seedlings inoculated with spore inoculum by slurry dip method

Lagerstoemio speciosa	*Albizia lebbeck*	*Pithecellobium dulce*
		
I C	**I C**	**I C**

Fig.4.2 Plant seedlings inoculated with spore inoculum in pot culture (I) Inoculated (C) Un-inoculated control

Albizia saman	*Cassia siamea*
	
I C	**I C**

Fig.4.3 Plant seedlings inoculated with spore inoculum by slurry dip method (I) Inoculated (C) Un-inoculated control

Inoculum formulation with fungal spores contains an additional biological ingredient i.e., yeast culture. Yeast was added as activator for spore germination for inoculums formulation containing *Pisolithus* sp. (Martin and Gracia, 2000; Hile and Hennek, 1969; Wilson and Beneke, 1966). It was added to all the experimental seedlings including control seedlings. Significant improvement was observed in inoculated seedlings as compared to the un-inoculated control plants. This result clearly eliminates the possible effect of yeast on the plant growth. Accordingly, it was decided to omit addition of any components of inoculum in the control plants during the field trial.

Most reports on inoculation techniques with ECM fungi developed in US, Australia, France, Canada and the Philippines involve basidiomycetes on pines, oaks and eucalypts (Marx, 1991). In this study, tree seedlings of forestry important and native of Goa were transplanted on mine reject dumps and inoculated with soil based spore inoculum. Table 4.1 lists the tree species of Goa inoculated with PT1 on dump no 9.

Dump no 9 is the soil rejects of iron ore mines at Codli, Goa. It is nude soil and not received any pre-treatment. Further, it was without any fortification with geotextile and top soil. Subsequent loading of mine rejects was continued on the dump during the experimental period. Seedlings were transplanted in the beginning of the monsoons and inoculated with spore inoculum after monsoons, in the months of January-February. During the

assessment period the dump was highly unstable as it had not been given any pre-treatment. Deep digging on such dump is dangerous. In the interest of the stability of the dump the experimental plants were not assessed for the below-ground parameters including mycorrhizal synthesis. Above ground plant growth parameters were monitored regularly throughout the experimental period. Inoculated tree seedlings exhibited variable response. *Alstonia scholarias* (Fig.4.5), *Dalbergia sisso* (Fig.4.8), *Pongamia pinnata* (Fig.4.16), and *Terminalia bellerica* (Fig.4.19) showed positive effect of inoculation on plant height. Inoculated *Bauhinia racemosa* (Fig.4.6) and *Sapindus emarginalis* (Fig.4.17) showed increase in collar diameter without any effect on the plant height. *Albizia saman* (Fig.4.4), *Delonix regia* (Fig.4.9), *Emblica officinalis* (Fig.4.11), *Lagerstoemia speciosa* (Fig.4.12), *Melia azadirach* (Fig.4.13), and *Pithecellobium dulce* (Fig.4.15) exhibited marked improvement in plant height and collar diameter in inoculated seedlings as compared to un-inoculated control. *Cassia seamea* (Fig.4.7) and *Terminalia arjuna* (Fig.4.18) were not affected by inoculation. *Dendrocalamus strictus* (Fig.4.10) and *Peltophorum pterocarpum* (Fig.4.14) showed negative effect of inoculation on plant height and collar diameter. Growth parameters of *Albizia lebbeck, Butea monosperma, Cassia fistula, Erythrina indica, Euginea jambolana, Gmelina arborea, Pterocarpus marsupium, Psidium guajava,* and *Tamarindus indica* transplanted on mine reject dumps were not determined, either because the control seedlings died or the tree seedlings were washed away along with reject dump soil due to heavy rains. Inoculated plant species *Albizia, Alstonia, Bauhinia, Cassia, Dalbergia, Delonix, Emblica, Lagerstoemia, Melia, Pithecellobium, Pongamia, Sapindus,* and *Terminalia*

showed better growth and survival on mine reject's dumps that needs further confirmation for the mycorrhiza synthesis.

The impact of inoculation on the transplanted seedling with the spores of *Pisolithus tinctorius* at dumps of rejects in Codli is seen in Figure 4.20. Inoculated seedlings (Fig.4.20) have shown tremendous growth as compared to un-inoculated control seedlings (Fig 4.21) over a period of 6 years. The soil on control plot remained loose and unstable. Portion of dump with inoculated plants became stable over the period. These results are very promising and highlight the possibilities of expanding assessment of roots for mycorrhizal colonization and succession studies on dump of mining reject.

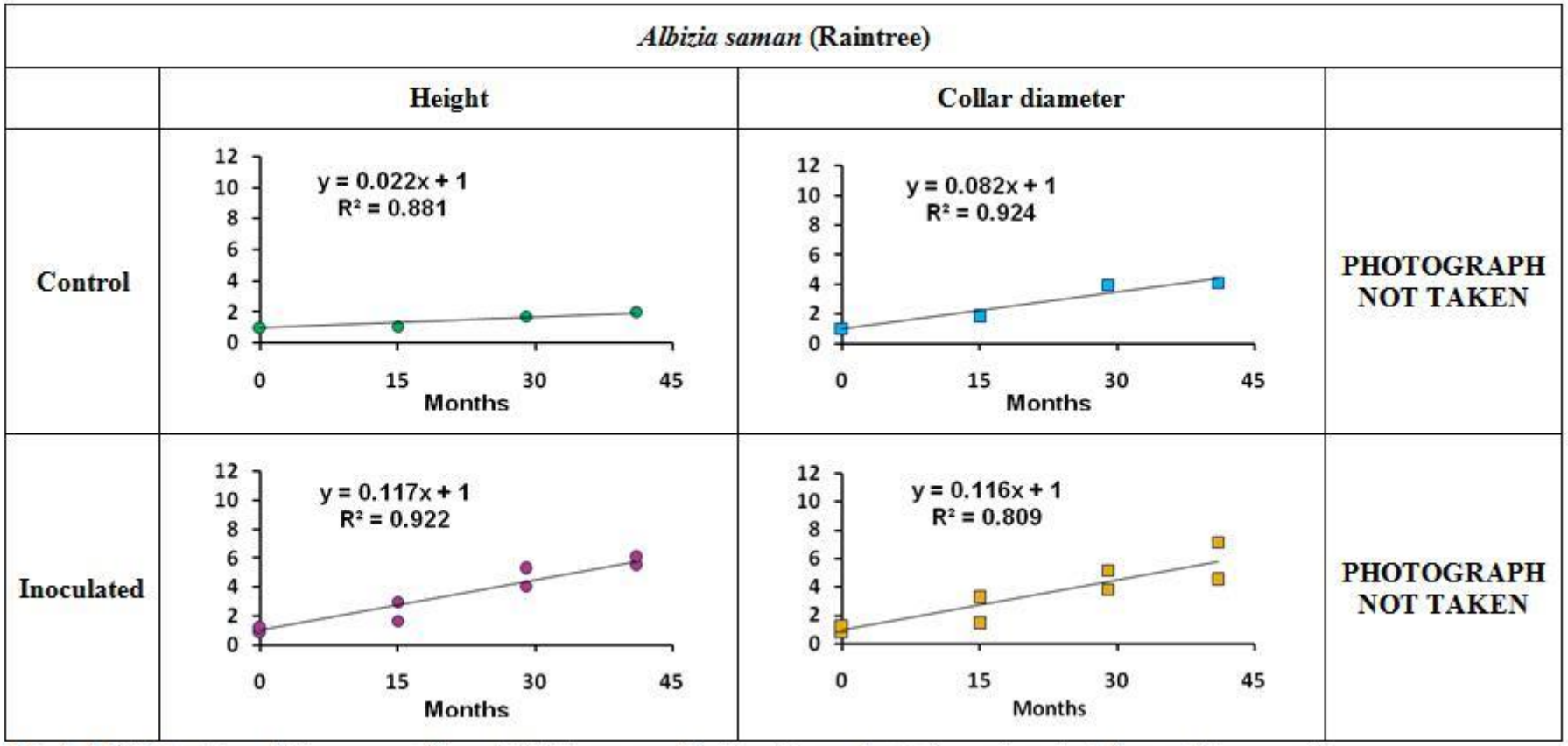


Fig.4. 4 Effect of inoculation on seedling of *Albizia saman* (Raintree) transplanted on mine reject dumps (Dump no 9)

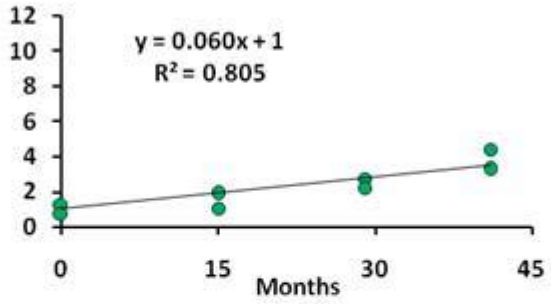
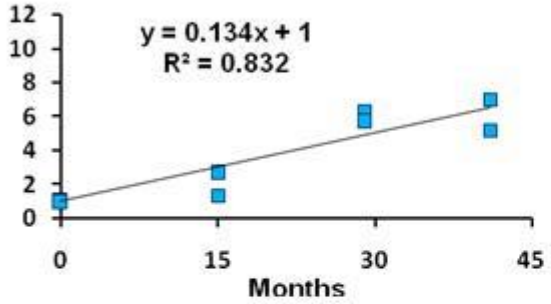
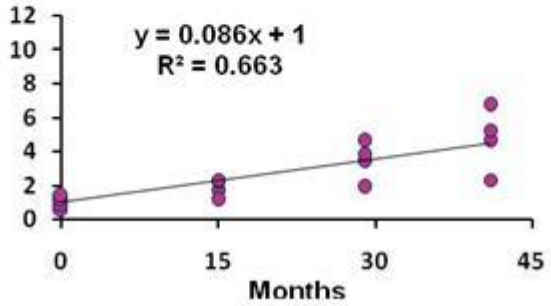
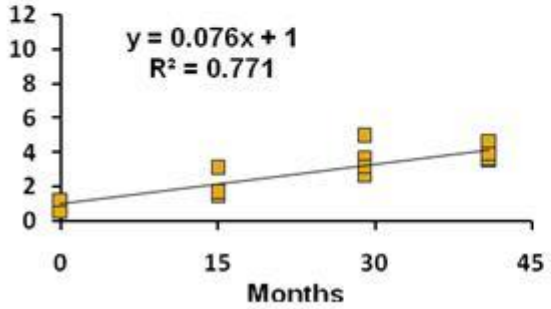
Alstonia scholarias (Saton)			
	Height	**Collar diameter**	
Control			
Inoculated			

Fig.4.5 Effect of inoculation on seedling of *Alstonia scholarias* (Saton) transplanted on mine reject dumps (Dump no 9)

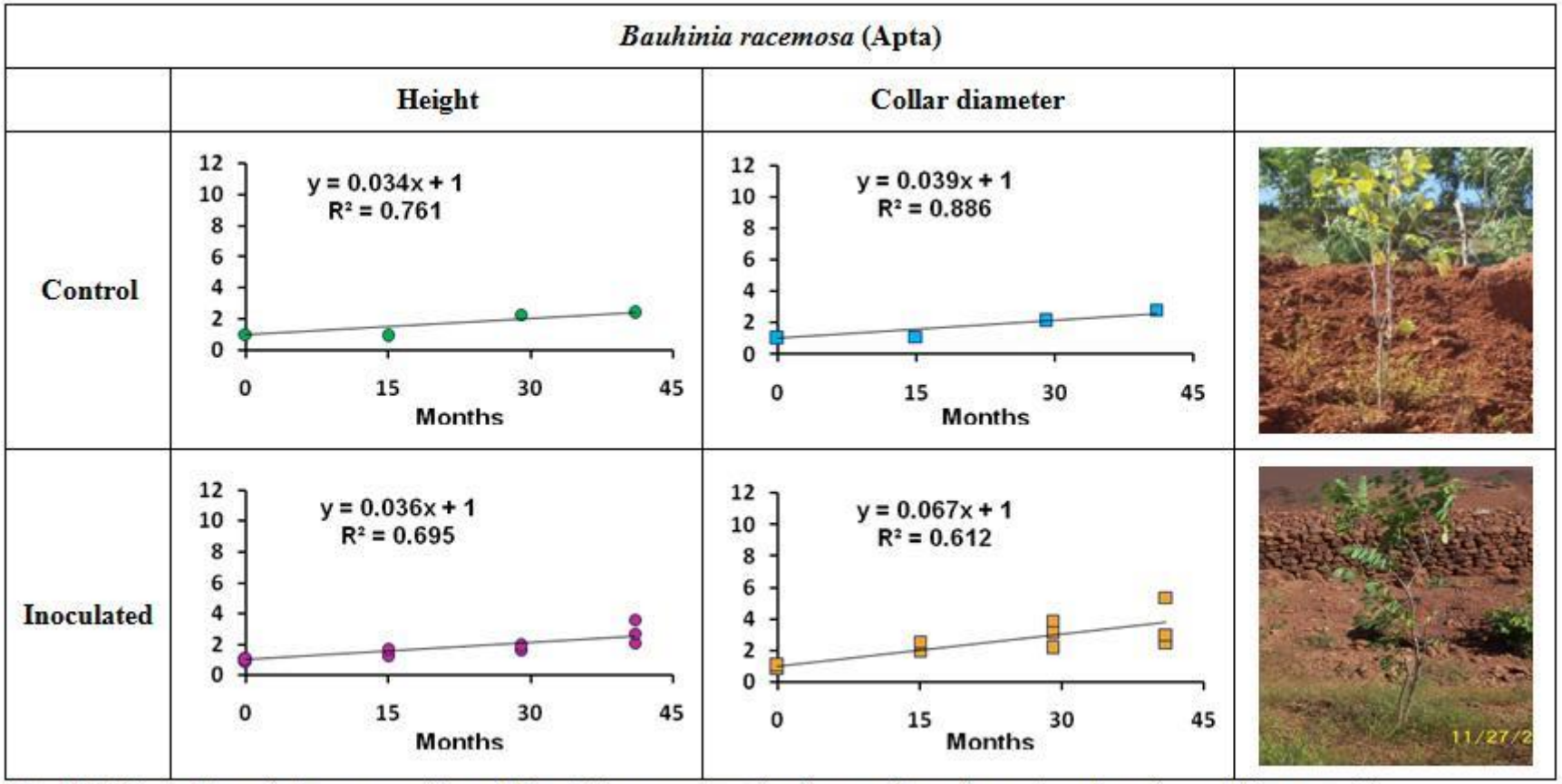


Fig.4.6 Effect of inoculation on seedling of *Bauhinia racemosa* (apta) transplanted on mine reject dumps (Dump no 9)

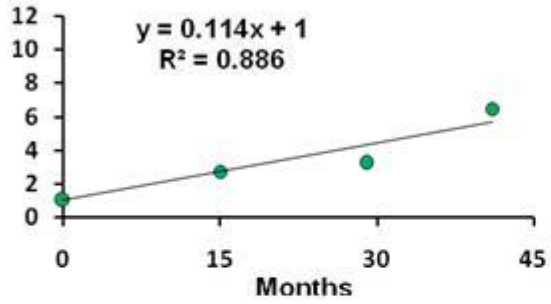
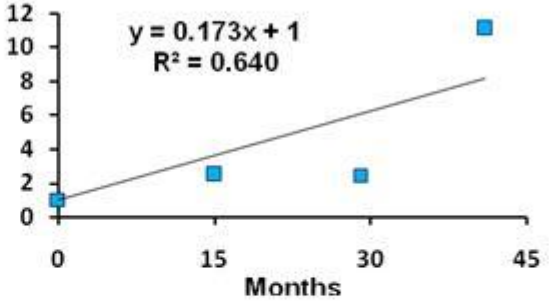
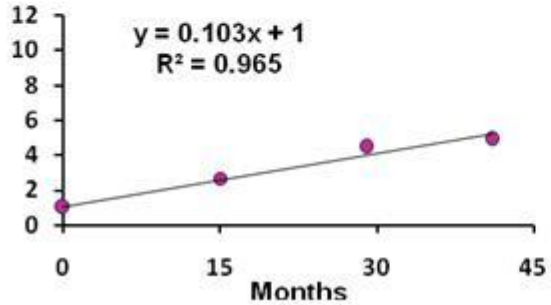
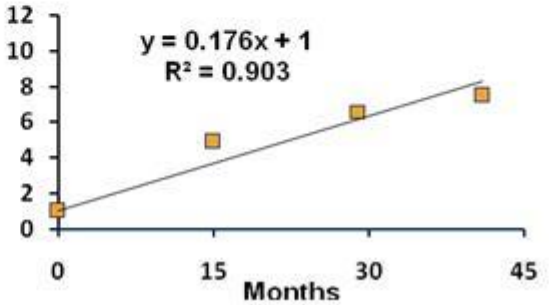
Cassia seamea (Cassia)			
	Height	**Collar diameter**	
Control			**PHOTOGRAPH NOT TAKEN**
Inoculated			**PHOTOGRAPH NOT TAKEN**

Fig.4.7 Effect of inoculation on seedling of *Cassia seamea* (Cassia) transplanted on mine reject dumps (Dump no 9)

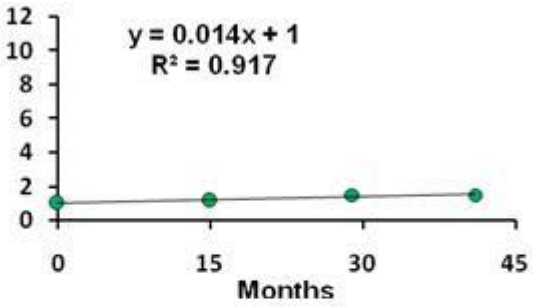
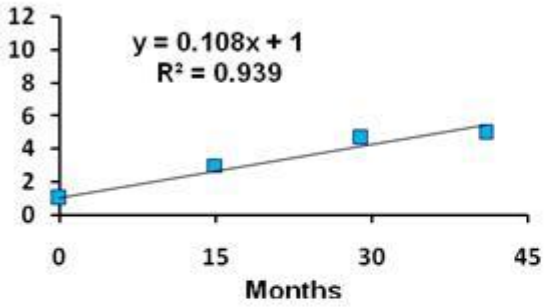
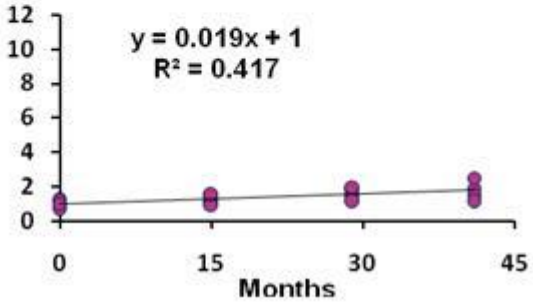
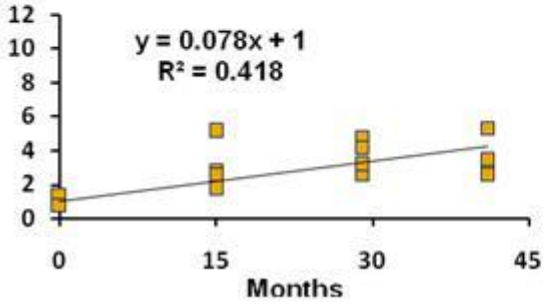
Dalbergia sisso (Shisam)			
	Height	**Collar diameter**	
Control			
Inoculated			

Fig.4.8 Effect of inoculation on seedling of *Dalbergia sisso* (Shisam) transplanted on mine reject dumps (Dump no 9)

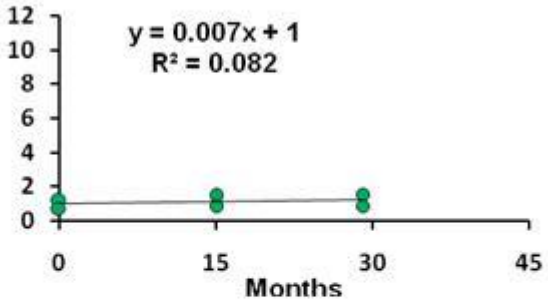
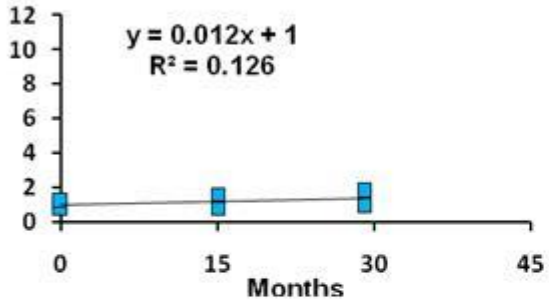
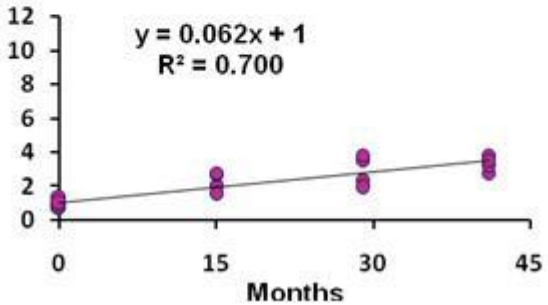
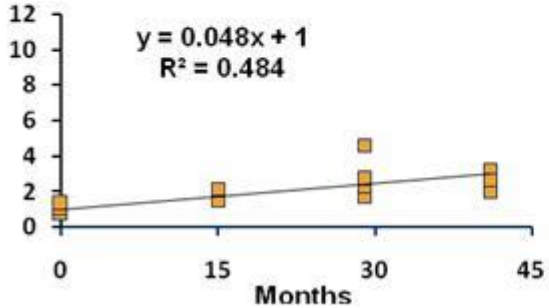
Delonix regia (Gulmohar)			
	Height	**Collar diameter**	
Control			
Inoculated			

Fig.4.9 Effect of inoculation on seedling of *Delonix regia* (Gulmohar) transplanted on mine reject dumps (Dump no 9)

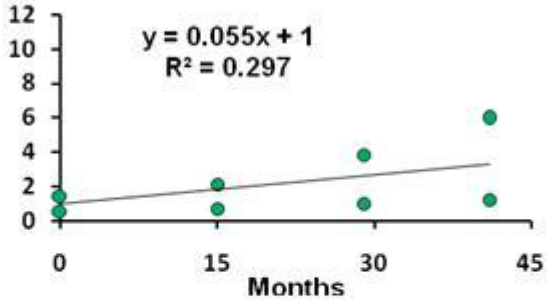
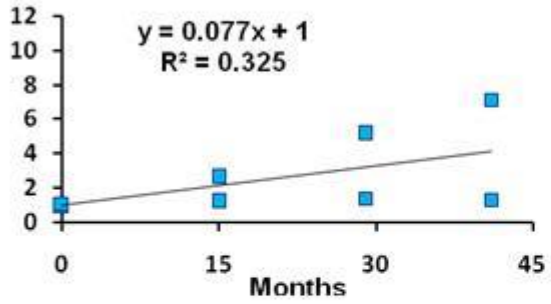
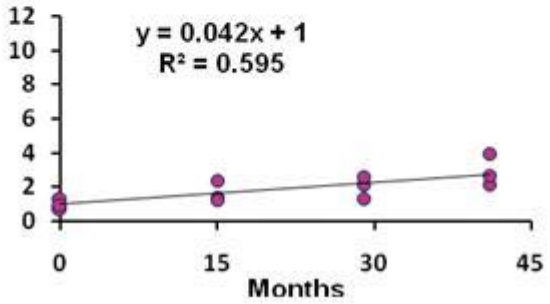
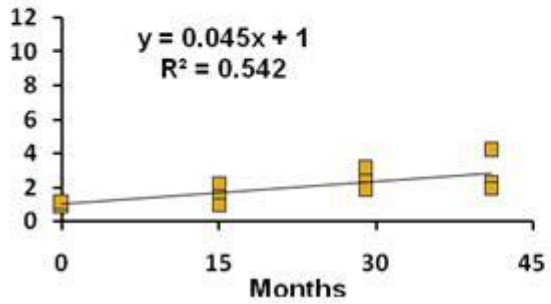
Dendrocalamus strictus (Bamboo)			
	Height	**Collar diameter**	
Control			**PHOTOGRAPH NOT TAKEN**
Inoculated			**PHOTOGRAPH NOT TAKEN**

Fig.4.10 Effect of inoculation on seedling of *Dendrocalamus strictus* (Bamboo) transplanted on mine reject dumps (Dump no 9)

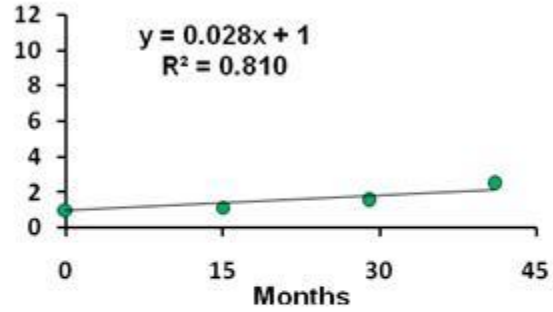
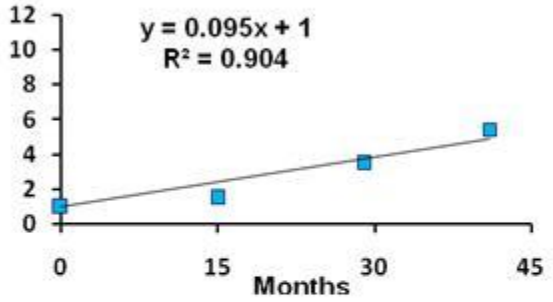
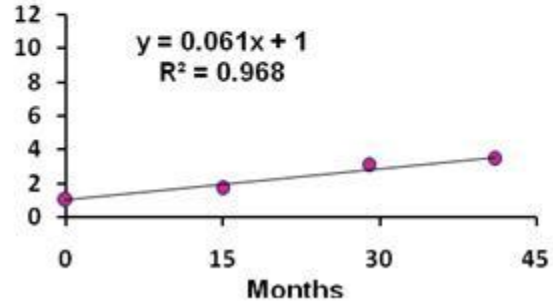
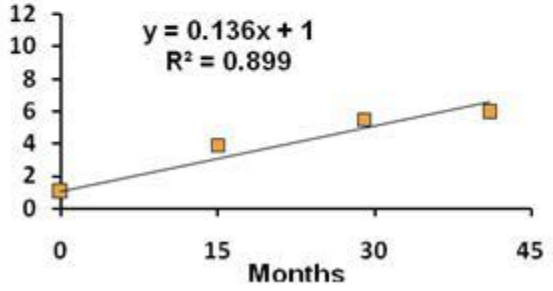
Emblica officinalis (Awala)			
	Height	**Collar diameter**	
Control			
Inoculated			

Fig.4.11 Effect of inoculation on seedling of *Emblica officinalis* (Awala) transplanted on mine reject dumps (Dump no 9)

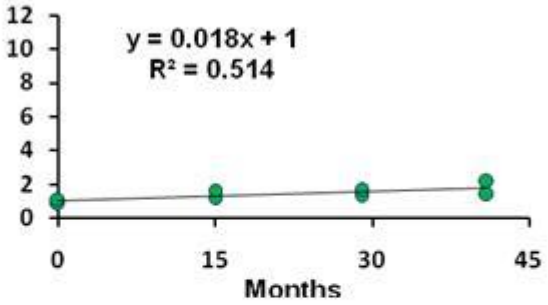
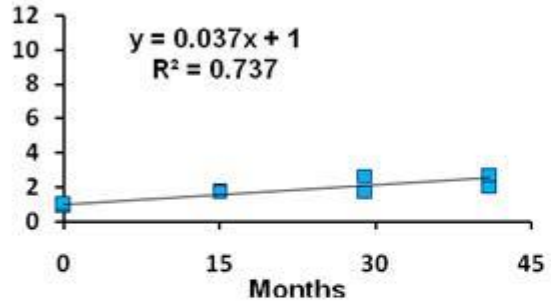
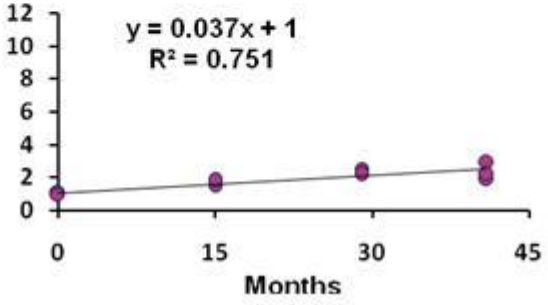
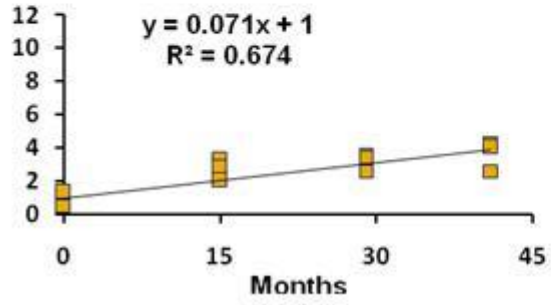
Lagerstoemia speciosa (Taman)			
	Height	**Collar diameter**	
Control			
Inoculated			

Fig.4.12 Effect of inoculation on seedling of *Lagerstoemia speciosa* (Taman) transplanted on mine reject dumps (Dump no 9)

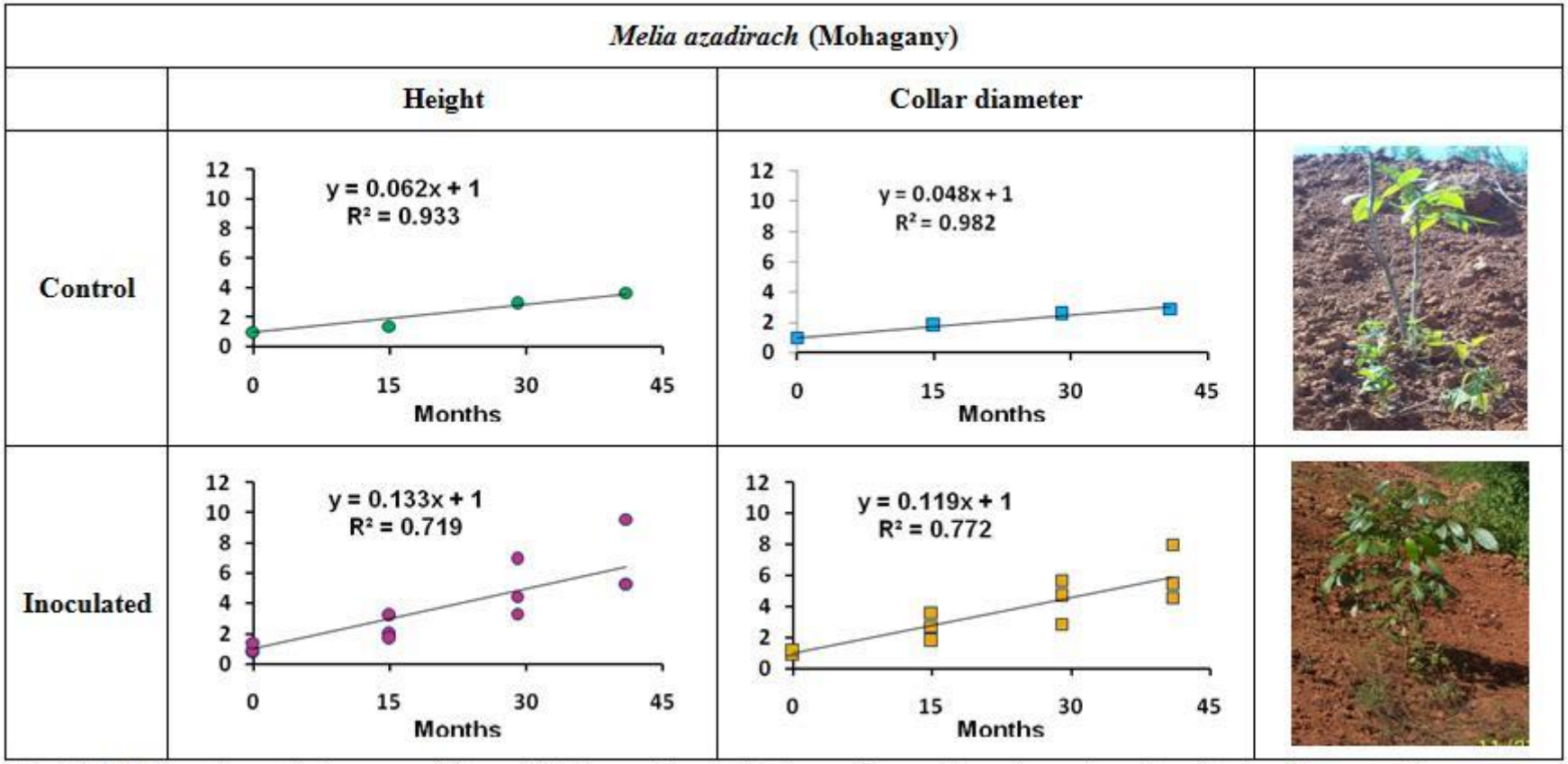


Fig.4.13 Effect of inoculation on seedling of *Melia azadirach* (Mohagany) transplanted on mine reject dumps (Dump no 9)

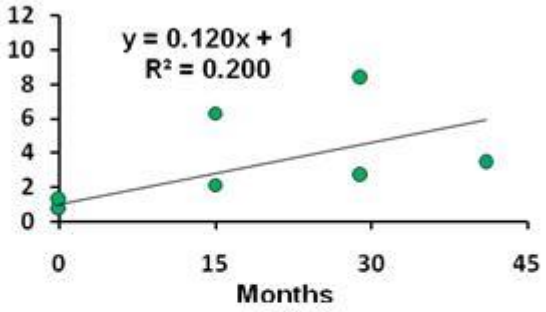
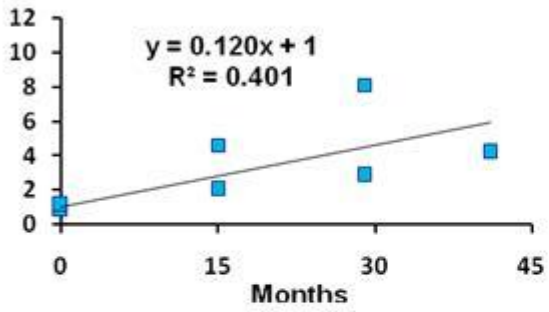
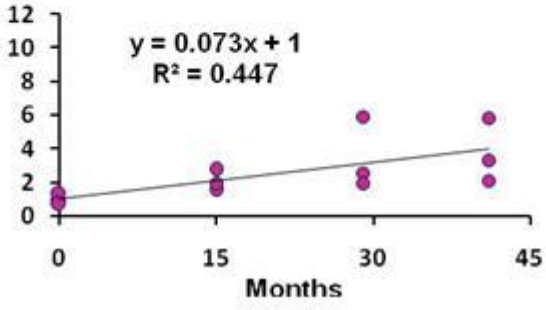
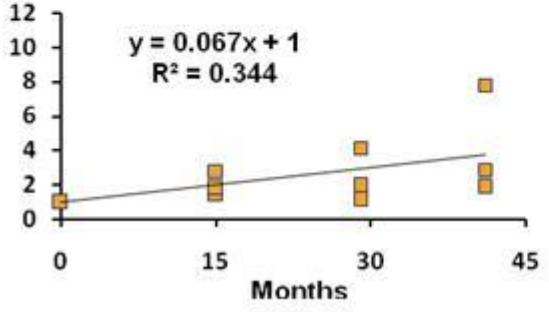
Peltophorum pterocarpum (Peltophorum)			
	Height	**Collar diameter**	
Control			**PHOTOGRAPH NOT TAKEN**
Inoculated			**PHOTOGRAPH NOT TAKEN**

Fig.4.14 Effect of inoculation on seedling of *Peltophorum pterocarpum* (Peltophorum) transplanted on mine reject dumps (Dump no 9)

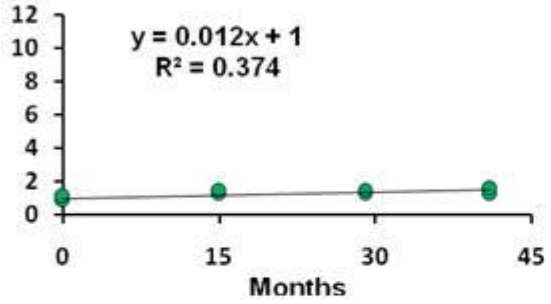
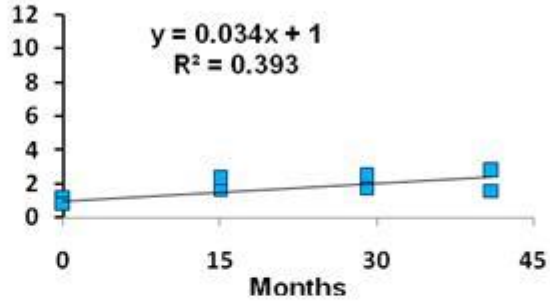
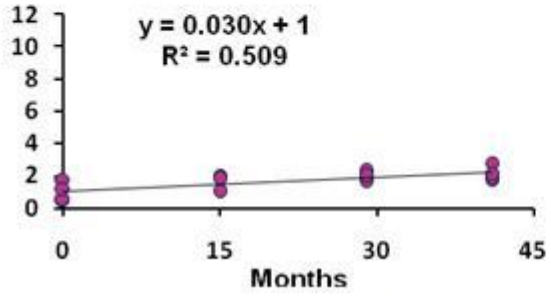
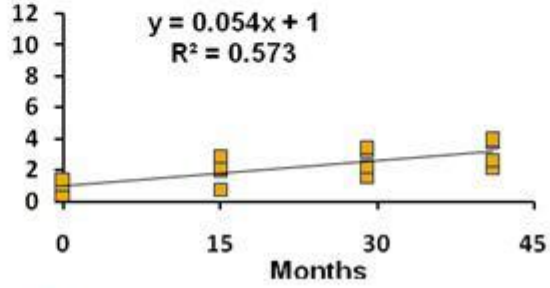
Pithecellobium dulce (Vilayati chinch)			
	Height	**Collar diameter**	
Control			**PHOTOGRAPH NOT TAKEN**
Inoculated			**PHOTOGRAPH NOT TAKEN**

Fig.4.15 Effect of inoculation on seedling of *Pithecellobium dulce* (Vilayati chinch) transplanted on mine reject dumps (Dump no 9)

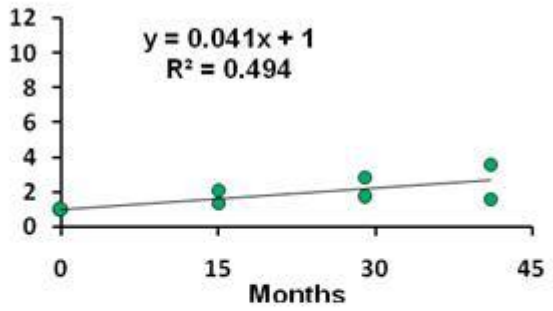
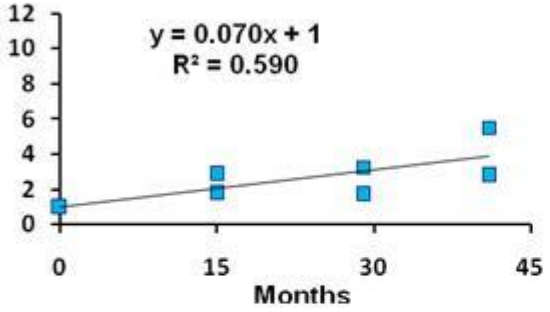
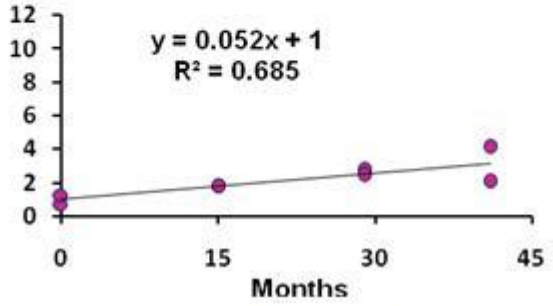
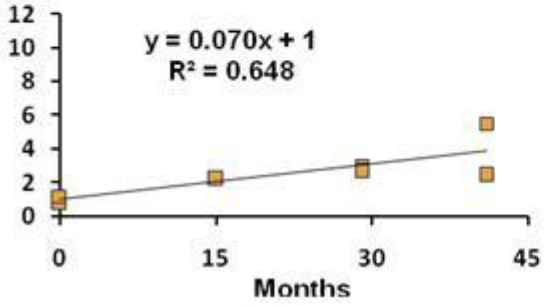
Pongamia pinnata (Karanj)			
	Height	**Collar diameter**	
Control			**PHOTOGRAPH NOT TAKEN**
Inoculated			**PHOTOGRAPH NOT TAKEN**

Fig.4.16 Effect of inoculation on seedling of *Pongamia pinnata* (Karanj) transplanted on mine reject dumps (Dump no 9)

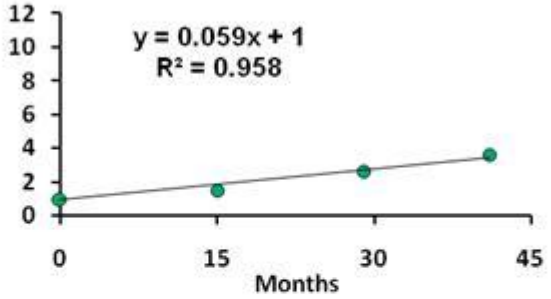
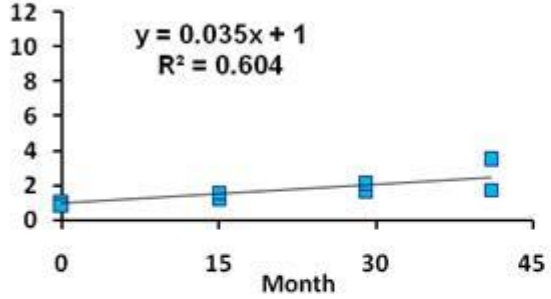
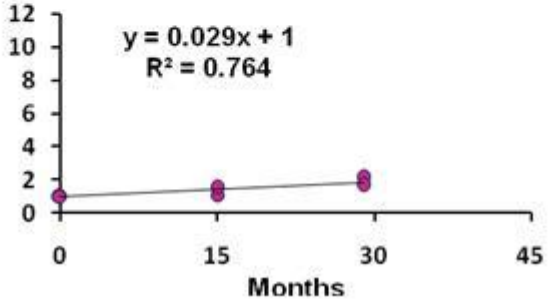
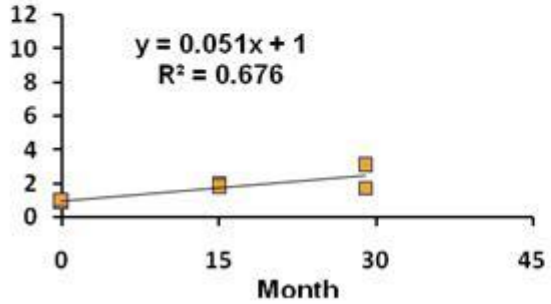
Sapindus emarginalis (Rinte)			
	Height	**Collar diameter**	
Control			**PHOTOGRAPH NOT TAKEN**
Inoculated			**PHOTOGRAPH NOT TAKEN**

Fig.4.17 Effect of inoculation on seedling of *Sapindus emarginalis* (Rinte) transplanted on mine reject dumps (Dump no 9)

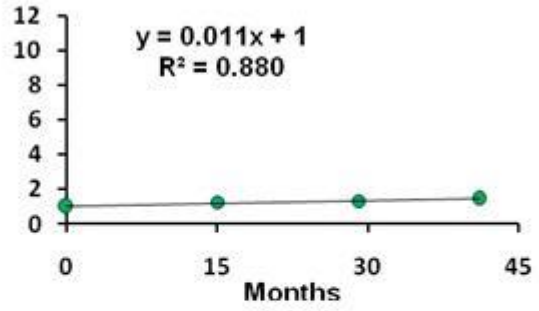
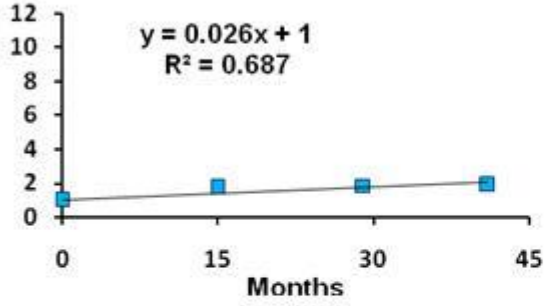
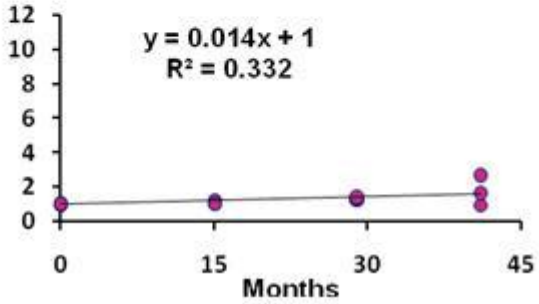
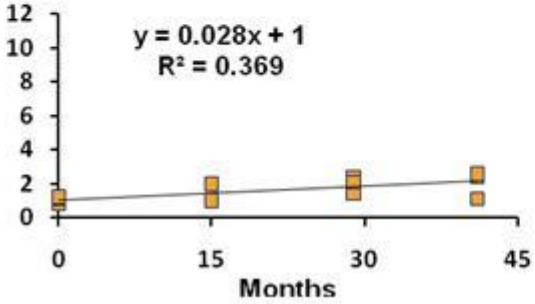
Terminalia arjuna (Arjun)			
	Height	**Collar diameter**	
Control			
Inoculated			

Fig.4.18 Effect of inoculation on seedling of *Terminalia arjuna* (Arjun) transplanted on mine reject dumps (Dump no 9)

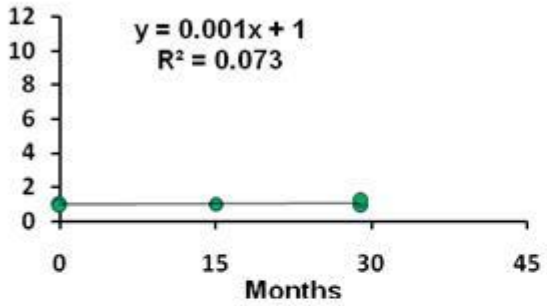
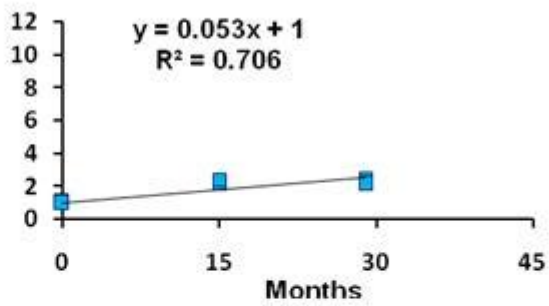
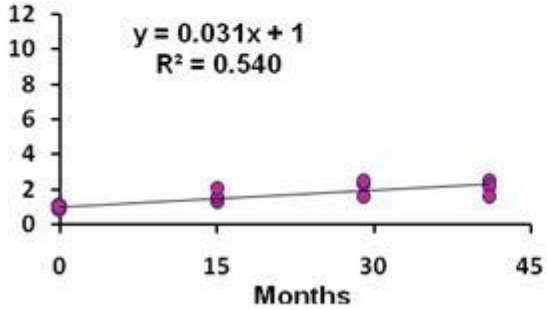
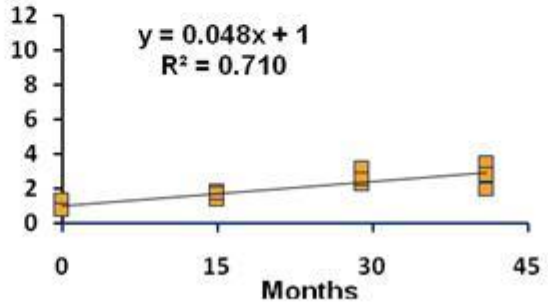
Terminalia bellerica (Goting)			
	Height	**Collar diameter**	
Control			
Inoculated			

Fig.4.19 Effect of inoculation on seedling of *Terminalia bellerica* (Goting) transplanted on mine reject dumps (Dump no 9)

Plot B

Plot Z

Plot B

Figure 4.20 Growth of inoculated seedlings (plot B & Z) on Dump no 9 over a period of 6 years. (Transplantation – June - July 2006; Inoculation – September 2006; Final photos – June 2012)

Plot D

Plot D

Plot D

Figure 4.21 Growth of transplanted seedlings on control plot (plot D) of Dump no 9 over a period of 6 years. (Transplantation period – June - July 2006; Final photos – June 2012)

SUMMARY

&

FUTURE PROSPECTS

SUMMARY

The present investigation was carried out considering the applications of *Pisolithus* sp. The study dealt with characterisation of growth of *Pisolithus tinctorius* PT1 in chemically defined medium. It identified the optimum physicochemical parameters for maximum growth of the isolate PT1. The study characterised the physicochemical region that poses stress to the isolate in axenic culture conditions. The stress response of the isolate to various edaphic factors prevailing on mines was evaluated with respect to whole cell proteins. Further, the effect of growth parameters on the isolate PT1 for enhanced elaboration of pigments/phenolics, having antibiotic activity, was highlighted. The study also investigated the mycorrhization ability of the PT1 and considered the efficiency of inoculation on forestry tree species of Goa. The following points highlights the major outcome of this study

- ECM fungal isolate PT1 obtained from iron ore mines is identified as *Pisolithus tinctorius.*
- *Pisolithus tinctorius* PT1 grew better in chemically defined GMSM medium than the complex medium MMN.
- *In vitro, Pisolithus tinctorius* PT1 tolerated 0.007 to 0.087 M phosphate, pH 3 to 7, 0 to 3% NaCl, 0 to 10% PEG 6000 (≤ -0.1 MPa water potential), upto 50 ppm iron, ≥10000 ppm Mn and temperature of 15-42^{0}C.

- *Pisolithus tinctorius* PT1 grew maximally in GMSM with 0.027 M phosphate, 0.01-1% NaCl, 500-2500 ppm Mn, 0% PEG, at pH 4.0-4.2 and incubation temperature of 25-30^0 C.
- *In vitro, Pisolithus tinctorius* PT1 encountered stress in GMSM with ≥ 0.047 M phosphate, ≥ 1.5% NaCl, ≥ 7500 ppm Mn, ≥10% PEG, pH 7 and above and incubation temperature of 37^0 C and above.
- *Pisolithus tinctorius* PT1 grew even in the presence of 10000 ppm Mn and thus appeared to be Mn-tolerant.
- The tolerance of *Pisolithus tinctorius* PT1 to a wide range of environmental conditions prevailing on dumps of mine's reject suggests its stable existence on such disturbed site.
- In *Pisolithus tinctorius* PT1 profile of whole cell proteins were different with different stress conditions indicating presence of novel proteins.
- PT1 synthesized new protein of 42 KDa under pH stress, proteins of molecular mass 70, 57 and 55 KDa under thermal stress, a 64 KDa protein under NaCl stress, two proteins of 78 and 39 KDa under drought stress and 115 and 26 KDa proteins under Mn stress.
- *Pisolithus tinctorius* PT1 showed more growth under static condition while total phenolics production was more under submerged condition probably due to sheer damage.
- *Pisolithus tinctorius* PT1 produced more phenolics while growing under sub-optimal conditions where it encountered the stress. Thus, even abiotic stress on the *Pisolithus* tinctorius would induce phenolics elaboration.

- Optimum conditions for maximum elaboration of phenolics by PT1 were pH 7.0, 0.067 M phosphate, 1.5% NaCl, 5-10% PEG 6000, 1.1 ppm Mn, 2.121 ppm Fe, incubation temperature of 37^0 C and submerged cultivation.
- Polar organic solvents were found suitable to extract pigments produced by *Pisolithus tinctorius* PT1. Maximum pigments from sporocarps as well as culture filtrate were extracted in water and methanol followed by ethanol and acetone.
- UV-visible spectra and chromatography of the pigment extracted from sporocarps of *Pisolithus tinctorius* PT1 suggests presence of two phenolic compounds.
- UV-visible spectra and chromatography of the solvent extracted pigment in culture filtrate suggests a single phenolic compound liberated by PT1.
- Among the tested phytopathogens, *Fusarium* sp. was the most affected test organism while *M. phaseolina* was not affected with the pigments.
- Pigments of *Pisolithus tinctorius* PT1 showed reduction in aerial growth of *S. rolfsii*, Pigment enhanced the sclerotia formation.
- Vermiculite soaked with GMSM was found suitable for developing vegetative mycelium based inoculum using *Pisolithus tinctorius* PT1.
- *A. mangium* and *C. fistula* showed mycorrhizae with vegetative inoculum of *Pisolithus tinctorius* PT1 in containerized condition.
- Inoculated plant species of *Alstonia, Albizia, Bauhinia, Cassia, Dalbergia, Delonix, Emblica, Lagerstoemia, Melia, Pithecellobium,*

Pongamia, *Sapindus*, and *Terminalia*, showed better growth and survival on mine reject's dumps.

FUTURE PROSPECTS

The present isolate *Pisolithus tinctorius* PT1 affords a promising source of fungal pigments and application for reclamation of mine rejects. The isolate was predominantly found in association with *Acacia mangium* on the iron ore mine and forest along the west coast of India. The abundance of this ECM in the regions rich in iron and manganese suggests the possible applications of this fungus in establishment of vegetation on mining disturbed sites. *In vitro,* the isolate tolerated a wide range of environmental conditions prevailing on dumps of mine's reject and expressed different proteins during various stress condition. This provides a scope of research to further characterize and identify their role with respect to their individual stress. Various growth parameters are seen to affect the pigment production by PT1. This pigment, phenolic in nature, having potential antagonistic activity against soil borne phytopathogens and utility to dye yarns, needs further investigation on fermentative production considering the physicochemical conditions investigated in present study for industrial utilization. PT1 has shown promising results when applied on forestry tree seedlings of Goa, in containerized experiments as well as in field trials. A well designed method

for mass cultivation of PT1 using vermiculite and GMSM is considerable for further studies.

The status of ECM association of forest tree species native to Goa needs thorough investigations. This may lead us in identifying new ECM fungi present in forests of Goa. These new fungi probably are more suitable for inoculation and reforestation practice for mining disturbed sites.

APPENDICES

Appendix A

A.1 Composition of Modified Melin-Norkran's Medium (MMN) (Marx, 1969)

Sucrose	10 g
Malt extract	3 g
$(NH_4)_2PO_4$	0.25 g
KH_2PO_4	0.5 g
$MgSO_4.7H_2O$	0.15 g
$CaCl_2.2H_2O$	0.05 g
NaCl	0.025 g
$FeCl_3$ (1%)	1.2 ml
Thiamine.HCl	100 μg (10 ml working stock solution)
Sreptomycin	100 mg (4 ml streptomycin solution)
Penicillin	18 mg (10 ml penicillin solution)
Distilled water	1 L
Final pH	6.5
Agar	20 g

Sucrose solution (for 250ml medium)

Sucrose	2.5 g

Distilled water	20 ml

Magnesium sulphate solution (1%)

$MgSO_4.7H_2O$	1 g
Distilled water	100 ml

Calcium chloride solution (1%)

$CaCl_2.2H_2O$	1 g
Distilled water	100 ml

Ferric chloride solution (1%)

$FeCl_3$	1 g
Distilled water	100 ml

Thiamine.HCl stock*

Standard stock

Thiamine.HCl	100 mg
Deionized water	100 ml

Working stock

Standard Thiamine.HCl stock	0.5 ml

Deionized water	49.5 ml

Streptomycin sulphate solution*

Streptomycin SO_4	1 g
Deionized water	40 ml

Penicillin solution (3030 units/ml)*

Penicillin benzyl sodium salt (10^6 units)	1 vial
Deionized water	330 ml

A.2 Composition of Glucose Mineral Salts Medium (GMSM) (Garg, 1999)

Glucose	20 g
NH_4Cl	1 g
KH_2PO_4	1 g
NaCl	0.1 g
KCl	0.1 g
$MgCl_2.4H_2O$	0.3 g
$CaCl_2.2H_2O$	0.132 g
$FeSO_4.7H_2O$	10.53 mg
Na_2EDTA	37.52 mg
Thiamine.HCl	100 µg (10 ml working stock solution)
Biotin	40 µg (1 ml biotin solution)
Distilled water	1 L
Final pH	6.0 (adjusted with 0.1N KOH)
Agar	20 g

Micronutrients solution	10 ml

Micronutrients solution 10ml (acidified)

H_3BO_3	2.784 mg
$MnSO_4.H_2O$	3.38 mg
$CuSO_4.5H_2O$	97 µg
$ZnSO_4.7H_2O$	201 µg
$Na_2MoO_4.2H_2O$	338.46 µg
H_2SO_4	240 µl
Distilled water	10 ml

Glucose solution (for 250ml medium) ♦

Glucose	5 g
Distilled water	20 ml

Magnesium chloride-Calcium chloride solution

$MgCl_2.4H_2O$	1 g
$CaCl_2.2H_2O$	0.44 g
Distilled water	100 ml

Ferrous EDTA solution

$FeSO_4.7H_2O$	0.2106 g
Na_2EDTA	0.7504 g
Distilled water	100 ml

Biotin solution*

Biotin	4 mg
Deionized water	100 ml

Micronutrients solution without Mn 10ml (acidified)

H_3BO_3	2.784 mg
$CuSO_4.5H_2O$	97 µg
$ZnSO_4.7H_2O$	201 µg
$Na_2MoO_4.2H_2O$	338.46 µg
H_2SO_4	240 µl
Distilled water	10 ml

Potassium Hydroxide solution (0.1N KOH)

KOH	0.5611 g
Distilled water	100 ml

EDTA solution

Disodium EDTA	37.52 mg
Distilled water	10 ml

$FeSO_4$ -EDTA Stock solution

$FeSO_4.7H_2O$	0.497 g
EDTA	0.038 g
Distilled water	100 ml

1 ml of stock solution in 100 ml medium gives final concentration of 10 ppm Fe in medium. Media with 2.121, 10, 20……90 ppm Fe requires additional 0.98, 0.9, 0.8……0.1 ml EDTA solution.

$FeSO_4$ Stock solution (1000 ppm)

$FeSO_4.7H_2O$	0.4965 g
Distilled water	100 ml

1 ml of stock solution in 100 ml medium gives final concentration of 10 ppm Fe in medium

$FeCl_3$ Stock solution (1000 ppm)

$FeCl_3$	0.2897 g
Distilled water	100 ml

1 ml of stock solution in 100 ml medium gives final concentration of 10 ppm Fe in medium

$MnSO_4$ Stock solution (50,000 ppm)

$MnSO_4.H_2O$	15.366 g
Distilled water	100 ml

2 ml of stock solution in 100 ml medium gives final concentration of 1000 ppm Fe in medium

A.3 Composition of Potato Dextrose Agar (PDA)

Potatoes infusion from	200 g
Dextrose	20 g
Agar	20 g
Final pH	5.1±0.2

Autoclave at 121ºC for 15 mins

♦Autoclave at 121ºC for 10 mins

* Filter sterilize

Appendix B

Sodium phosphate buffer (0.02 M, pH 6.8)

0.02 M NaH_2PO_4

NaH_2PO_4	0.31202 g
Distilled water	100 ml

0.02 M Na_2HPO_4

Na_2HPO_4	0.35598 g
Distilled water	100 ml

The pH of 0.02 M NaH_2PO_4 was adjusted to 6.8 with 0.02 M Na_2HPO_4.

Phosphate citrate buffer (0.02 M, pH 6.8)

0.02 M Citric acid

Citric acid	0.4228 g
Distilled water	100 ml

The pH of 0.02 M Na_2HPO_4 was adjusted to 6.8 with 0.02 M Citric acid.

HEPES buffer (0.02 M)

HEPES buffer	95 mg
Distilled water	20 ml

Sodium phosphate buffer (0.1 M, pH 6.8)

0.1 M NaH_2PO_4

NaH_2PO_4	1.56 g
Distilled water	100 ml

0.1 M Na_2HPO_4

Na_2HPO_4	1.78 g
Distilled water	100 ml

The pH of 0.1 M NaH_2PO_4 was adjusted to 6.8 with 0.1 M Na_2HPO_4.

Sodium phosphate buffer (0.04 M, pH 6.8)

0.04 M NaH_2PO_4

NaH_2PO_4	0.624 g
Distilled water	100 ml

0.04M Na_2HPO_4

Na_2HPO_4	0.712 g
Distilled water	100 ml

The pH of 0.04 M NaH_2PO_4 was adjusted to 6.8 with 0.04 M Na_2HPO_4.

Phosphate citrate buffer (0.04 M, pH 6.8)

0.04M Citric acid

Citric acid	0.846 g
Distilled water	100 ml

The pH of 0.04 M Na_2HPO_4 was adjusted to 6.8 with 0.04 M Citric acid.

Normal saline

NaCl	0.85 g
Distilled water	100 ml

Sodium hypochlorite solution

Sodium hypochlorite (4% active chlorine)	50 ml
Distilled water	50 ml

Lactophenol Cotton Blue solution

Lactophenol cotton blue	50 ml
Distilled water	50 ml

Appendix C

C.1 Scanning Electron Microscopy (SEM) (De Melo and Faull, 2004)

Stock solutions for SEM

Sodium phosphate buffer (0.1M, pH 6.0)

0.1M NaH_2PO_4

NaH_2PO_4	1.56 g
Distilled water	100 ml

0.1M Na_2HPO_4

Na_2HPO_4	1.78 g
Distilled water	100 ml

The pH of 0.1M NaH_2PO_4 was adjusted to 6.0 with 0.1M Na_2HPO_4.

2% glutaraldehyde solution

2 ml glutaraldehyde was dissolved in 0.1M phosphate buffer and final volume was made up to 100ml with 0.1M phosphate buffer.

Acetone solution (30%)

Acetone	30 ml
De-ionized water	70 ml

Acetone solution (50%)

Acetone	50 ml
De-ionized water	50 ml

Acetone solution (70%)

Acetone	70 ml
De-ionized water	30 ml

Acetone solution (90%)

Acetone	90 ml
De-ionized water	10 ml

Procedure for sample preparation:

50 µl of dilute spore suspension in deionized water was spread on 1cm x1cm glass slide. The sample was dried overnight (O/N). The slide was kept immersed in 2% glutaraldehyde in 0.1M sodium phosphate buffer for 2 h (fixation). The slide was then washed with 0.1M sodium phosphate buffer, air dried and passed through a series of increasing acetone concentrations (30, 50, 70, 90, and 100%, v/v) with 10 min per change (dehydration). The slide was dried using critical point dryer. The sample was coated with a thin film of gold using spi-module sputter and observed under x7500 magnification using scanning electron microscope.

C.2 Genomic DNA extraction and PCR amplification (Gardes and Bruns, 1993; Graham *et al*., 1994)

Reagents

Lysis buffer

200 mM Tris-HCl, pH 8.0

100 mM NaCl

25mM EDTA

0.5% SDS

CTAB buffer (5%)

100 mM Tris-HCl, pH 8.0

1.4 M NaCl

5% CTAB

Phenol:chloroform:isoamylalcohol (PCI) (25:24:1, v/v)

Phenol, pH 8.0	25 ml
Chloroform	24 ml
Isoamylalcohol	1 ml

Chloroform:isoamylalcohol (CI) (24:1, v/v)

Chloroform	24 ml
Isoamylalcohol	1 ml

Ethanol (70%)

Absolute ethanol	70 ml
De-ionized water	30 ml

TE buffer

10 mM Tris-HCl, pH 8.0

10 mM EDTA

Procedure

100 mg fresh mycelia was ground in lysis buffer (pH 8.0) using sterile micro pestle and incubated in water-bath at 65°C for 1 hour. 5% CTAB buffer was added to the solution and placed in water-bath at 65°C for 10 minutes. The solution was then extracted at least twice with equal volume of PCI (25:24:1, v/v) and centrifuged at 12,000 rpm for 10 minutes, followed by extraction with CI (24:1, v/v) until the top phase was clear. The upper aqueous phase was transferred to new tube and DNA was precipitated with equal volume of isopropanol. The DNA was washed with 70% ethanol, dried and resuspended in 100 µl TE buffer (pH 8.0). Genomic DNA was visualised in 0.7% agarose gel. The concentration and purity of DNA was determined by measuring absorbance at 260 nm and 280 nm using UV-2450 UV- visible spectrophotometer.

The ITS region of rDNA was amplified using the primer pair ITS 1 and ITS 4 (White *et al*., 1990). The sequences are 5'-TCCGTAGGTGAACCTGCGG-3' for ITS1 and 5'-TCCTCCGCTTATTGATATGC-3' for ITS4. The amplification was carried out using BIOERXP thermal cycler in a 50 µl reaction mixture containing 1 µl genomic DNA, 10X PCR buffer, 1.5 mM $MgCl_2$, 10 mM dNTP mix, 1µM of each primers and 5 units of *Taq* DNA polymerase. The reaction mixture was initially denatured at 94 ºC for 3 minutes and then subjected to 40 cycles of 1 minute each at 94 ºC, 52 ºC and 72 ºC, and final extension step of 5 minutes at 72 ºC. A control reaction without template was run to detect the presence of any contaminated DNA. The amplified products were electrophoresed in 0.8% (w/v) agarose gel, stained with ethidium bromide fluorescing under UV light. Amplified rDNA was sent for sequencing to Bangalore Genei, India.

C.3 Whole cell protein extraction (Burgess et al., 1995; Chen and Chen, 2004)

Reagents

Protein Extraction Buffer (Osherov and May, 1998):

0.1M Tris-HCl. pH 8.0

10mM EDTA

10% glycerol

100mM β-mercaptoethanol

2% SDS

5M Urea

1mM PMSF

Procedure for protein extraction:

The mycelium was ground using mortar and pestle with 10% polyvinyl polypyrrolidone (PVPP) and liquid nitrogen. Minimum amount of protein extraction buffer was added to give a fine paste. The ground sample was distributed in sterile eppendorfs and centrifuged at 12,000 rpm at 4°C for 10 min. Supernatant containing whole cell soluble proteins was collected in fresh tubes. Protein precipitation was carried out using four volume of chilled acetone, mixed and tubes were kept standing at 10-15°C overnight. Samples were centrifuged at 12,000 rpm at 4°C for 10 min. Supernatant was discarded and pellet was stored at -20°C.

C.4 Sodium dodecyl sulphate Polyacrylamide gel electrophoresis (SDS-PAGE) (sambrook *et al.*, 1989)

Stock solutions for SDS-PAGE:

Acrylamide-*bis*-acrylamide solution (Monomer solution):

Acrylamide	29 g
N,N' methylene *bis* acrylamide	1 g
De-ionized water	100 ml

A stock solution containing 29%(w/v) acrylamide and 1%(w/v) N,N' methylene *bis* acrylamide was dissolved in warm de-ionized water. The pH of the solution was ≤ 7.0. The solution was stored in dark bottle at room temperature and used within 3 months.

Resolving gel buffer (1.5M Tris, pH 8.8):

Tris-HCl	18.171 g
De-ionized water	100 ml

18.171g Tris was dissolved in 50ml deionized water. The pH of the solution was adjusted to 8.8 using 6N HCl and the volume was made up to 100ml with deionized water. The solution was stored at 4ºC.

Stacking gel buffer (1.0M Tris, pH 6.8):

Tris-HCl	12.114 g
De-ionized water	100 ml

12.114g Tris was dissolved in 50ml deionized water. The pH of the solution was adjusted to 6.8 using 6N HCl and the volume was made up to 100ml with de-ionized water. The solution was stored at 4ºC.

Ammonium per sulphate (APS, 10% w/v):

Ammonium per sulphate	0.1 g
De-ionized water	1 ml

0.1g APS was dissolved in 1ml deionized water and stored at 4ºC. Fresh solution was prepared weekly.

Sodium dodecyl sulphate (SDS, 10% w/v):

Sodium dodecyl sulphate	10 g
De-ionized water	100 ml

10 g SDS was dissolved in de-ionized water. Final volume was made up to 100ml with deionized water. The solution was stored at room temperature.

Hydrochloric acid (6N):

51ml conc HCl was diluted to 100ml with de-ionized water.

Bromophenol blue (1% w/v):

Bromophenol blue	0.1 g
De-ionized water	10 ml

0.1g bromophenol blue powder was dissolved in 10ml de-ionized water.]

Tris-glycine elecrophoresis buffer (1X):

25mM Tris base

250mM glycine

0.1% SDS

Composition of 5X buffer is as follows:

Tris base	3.02 g
Glycine (pH 8.3)	18.8 g
SDS(10%)	10 ml
De-ionized water	200 ml

To prepare 1X tank buffer, 60 ml of 5X Tris-glycine elecrophoresis buffer was made to 300 ml with de-ionized water.

Gel-loading buffer 1X :

50mM Tris-HCl

200mM β-mercaptoethanol

2% SDS

0.1% bromophenol blue

10% glycerol

Composition of 2X sample buffer (10ml) is as follows:

Tris-HCl (1M, pH 6.8)	1 ml
Glycerol	2 ml
Bromophenol blue (1%)	2 ml
SDS (10%)	4 ml
β-mercaptoethanol	284 µl
Deionized water	716 µl

Preparation of resolving and stacking gel:

Solution	Resolving gel (10ml)			Stacking gel (4ml)
	8%	**10%**	**12%**	**5%**
Monomer	2.7	3.3	4.0	0.67
1.5M Tris, pH 8.8	2.5	2.5	2.5	-
1.0M Tris, pH 6.8	-	-	-	0.5
10% SDS	0.1	0.1	0.1	0.04
10% APS	0.1	0.1	0.1	0.04
Deionized water	4.6	4.0	3.3	2.7
TEMED	0.006	0.004	0.004	0.004

Preparation of sample:

Equal volume of protein sample and 2X sample buffer were mixed and subjected to 100 ºC for 10 min. After cooling, the sample was loaded in the well of the gel.

Procedure:

The SDS-PAGE was carried out in a Banglore Genei apparatus. 20 µl of sample containing 25-50µg of protein along with SDS-PAGE standard molecular weight markers (Genei, Banglore) in a separate well, were loaded in the gel. The electrophoresis was carried out at a constant voltage of 70V till the tracking dye (bromophenol blue) reached the bottom of the gel. The gel was stained with silver stain.

C.5 Silver staining of SDS-PAGE gels (Blum *et al*., 1987):

Reagents

Fixative solution (100 ml)

Methanol	50 ml
Glacial Acetic acid	12 ml
Formaldehyde (37%)	0.05 ml
De-ionized water	37.95 ml

$Na_2S_2O_3$ stock solution (2ml)

$Na_2S_2O_3.5H_2O$	50 mg
De-ionized water	2 ml

Pre-treatment solution (100ml)

$Na_2S_2O_3$ stock solution	0.8 ml
De-ionized water	99.2 ml

Silver solution (100ml)

$AgNO_3$	0.2 g
Formaldehyde (37%)	0.075 ml
De-ionized water	99.92 ml

Developing solution (100ml)

Na_2CO_3	6 g
$Na_2S_2O_3$ stock solution	0.016 ml
Formaldehyde (37%)	0.05 ml
De-ionized water	99.93 ml

Stop solution (100ml)

Methanol	50 ml
Glacial Acetic acid	12 ml
De-ionized water	38 ml

Procedure:

The following specified solutions were added to the polyacrylamide gel in the container. The following steps were performed for specified times exactly on a gel rocker at room temperature.

Step	Solution	Time of Treatment
Fix	Fixing solution	≥ 1 hr.
Wash	50% Ethanol	20 mins.
Wash	30% Ethanol	20 mins.
Pretreat	Pretreatment solution	Exactly 1 min.
Rinse	Deionized water	Exactly 3 x 20 secs.
Impregnate	Silver solution	20 mins.
Rinse	Deionised water	Exactly 3 x 20 secs.
Develop	Developing solution	Until the bands of desired intensity is achieved (<10 mins.)
Stop	Stop solution	10 mins.
Wash	Deionised water	30 secs.
Store	20% Glycerol	Months

C.6 Paper chromatography (Odebode, 1996)

Solvent system

Butanol	40 ml
Glacial acetic acid	10 ml
De-ionized water	50 ml

Developing solution

1% $FeCl_3$

0.1N K_4 $Fe(CN)_6$

Potassium ferrocyanide solution (0.1N)

K_4 $Fe(CN)_6$	10.4 g
De-ionized water	100 ml

To prepare developing solution 1 g FeCl3 was dissolved in 0.1N K_4 $Fe(CN)_6$ solution. Final volume was made upto 100 ml using 0.1N K_4 $Fe(CN)_6$ solution.

Procedure:

Samples were spotted on stripe of Whatman filter paper. After drying the spots, paper was developed in solvent chamber, saturated overnight. Solvent system used was butanol:acetic acid: water, 4:1:5 (upper layer). Solvent was allowed to run up to$3/4^{th}$ of the paper. The chromatogram was removed from the solvent chamber and solvent front was marked. The chromatogram was air-dried, and viewed under ultraviolet light. The spots were developed using 1% ferric chloride in 0.1N potassium ferrocyanide. Rf values were calculated using the formula:

***Rf* = Distance travelled by solute / Distance travelled by solvent**

Appendix D

Methods for Quantitative Estimations

D.1 Folin Lowry's method for proteins (Lowry *et al*. 1951)

Folin Lowry's method for protein estimation uses the Biuret reaction in addition to Folin-Ciocalteau chemistry. The peptide nitrogen[s] reacts with the copper [II] ions under alkaline condition giving dark blue colour and subsequently the Folin-Ciocalteau phosphomolybdic-phosphotungstic acid is reduced to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic amino acids.

Reagents

Reagent A (2% Na_2CO_3 in 0.1N NaOH):

Sodium hydroxide solution (0.1N)

NaOH	0.4 g
De-ionized water	100 ml

Dissolve 2 g Na_2CO_3 in an aliquot of 0.1N NaOH solution and make volume to 100 ml with 0.1N NaOH.

Reagent B (0.5% $CuSO_4$ in 1% sodium potassium tartrate):

Sodium potassium tartrate (1%)

Sodium potassium tartrate	1 g
De-ionized water	100 ml

Dissolve 0.5 g $CuSO_4$ in an aliquot of 1% sodium potassium tartrate solution and make volume to 100 ml with 1% sodium potassium tartrate solution.

Reagent C (Alkaline copper solution):

Reagent A	50 ml
Reagent B	1 ml

Prepare fresh Reagent C solution by mixing Reagent A and Reagent B solutions in the ratio 50:1prior to use.

Reagent D (Folin and Ciocalteau's phenol reagent):

Commercially available reagent diluted with equal volume of distilled water on the day of use. This reagent is a solution of sodium tungstate and sodium molybdate in phosphoric and hydrochloric acids.

Standard bovine serum albumin solution (200 µg/ml):

BSA standard stock

BSA	0.2 g
De-ionized water	100 ml

BSA working stock

BSA standard stock	1ml
De-ionized water	9 ml

BSA working stock was prepared by mixing 1 ml BSA standard stock solution and 9 ml de-ionized water.

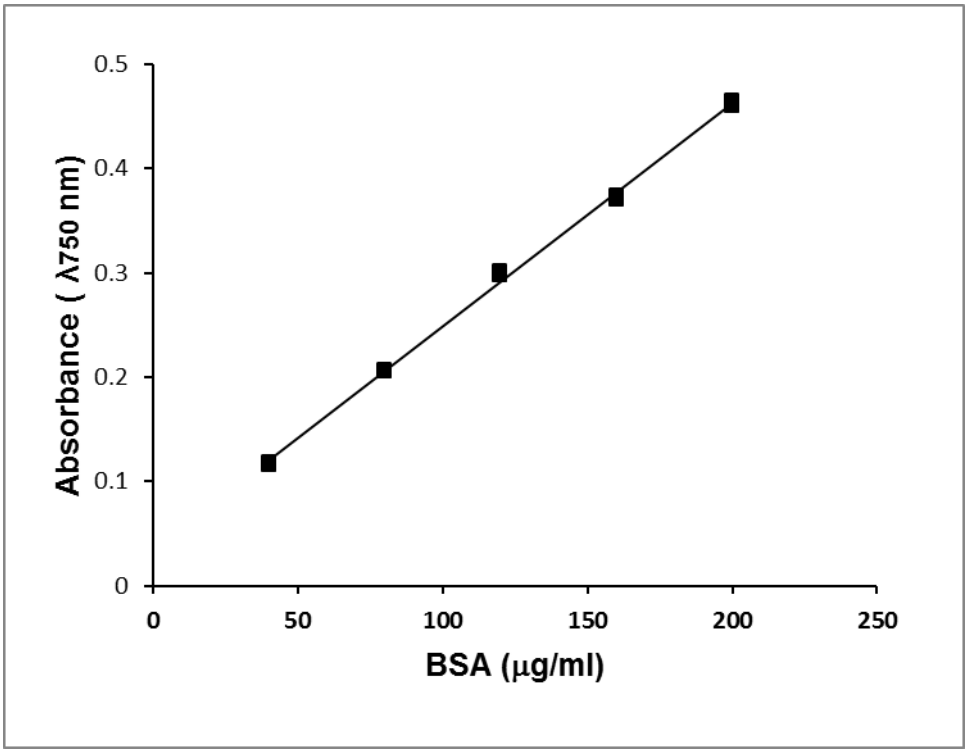
Procedure:

To 1 ml of sample, 5 ml Reagent C was added and mixed. 0.5 ml of Reagent D was then added and tubes were kept standing in dark for 30 minutes. Absorbance was measured at 750 nm against reagent blank. Standard calibration curve was prepared using Bovine serum albumin as standard (50 - 200 µg/ml). Absorption values were converted into protein by using following equation.

$$[\text{Protein}] = (\text{Absorption} - 0.034)/2.142 \qquad R^2 = 0.998$$

This is a linear regression equation of standard calibration curve prepared by using BSA

Standard calibration curve for estimation of Protein:



D.2 4-Aminoantipyrine method for Total Phenolics (Greenberg *et al.* 1958)

The colour reaction is based on the condensation of 4-aminoantipyrine with phenols in the presence of alkaline oxidizing agent, potassium ferricyanide to give antipyrine dyes.

Reagents

Reagent A (Ammonium hydroxide, 0.5N):

NH3	3.743 ml
De-ionized water	96.257ml

To prepare Reagent A 3.743 ml liquid NH_3 (25%) was diluted to 100 ml with de-ionized water.

Reagent B (Phosphate buffer solution, pH 6.8):

K_2HPO_4	10.45 g
KH_2PO_4	7.23 g

To prepare Reagent B 10.45 g K_2HPO_4 and 7.23 g KH_2PO_4 were dissolved in de-ionized water and diluted to 100 ml.

Reagent C (4-aminoantipyrine solution, 2%):

4-amoniantipyrine	2.0 g
De-ionized water	100 ml

To prepare Reagent C 2.0 g 4-amoniantipyrine was dissolved in de-ionized water and diluted to 100 ml. Fresh solution is prepared every time prior to use.

Reagent D (Potassium ferricyanide solution, 8%):

$K_3Fe(CN)_6$	8.0 g
De-ionized water	100 ml

To prepare Reagent D 8.0 g $K_3Fe(CN)_6$ was dissolved in distilled water and diluted to 100 ml. The solution is prepared freshly once in a week and stored in a brown glass bottle.

Standard Phenol solution (0.1 g/100 ml):

Phenol 0.1 g

De-ionized water 100ml

To prepare standard phenol solution 0.1 g crystalline white phenol was dissolved in de-ionized water and diluted to 100 ml.

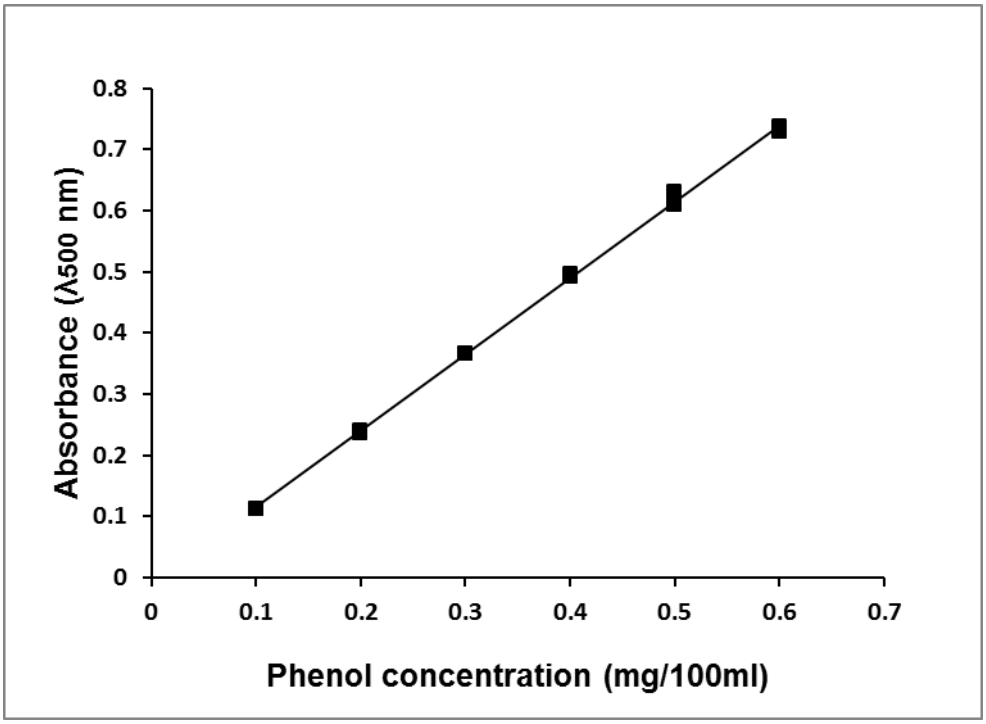
Procedure:

To 10 ml of sample, 0.25 ml Reagent A was added and pH was adjusted to 7.9 ± 0.1 with Reagent B. 0.1 ml Reagent C was then added, mixed, followed by addition of 0.1 ml Reagent D. The reaction mixture was kept standing in dark for 15 minutes. Absorbance was measured at 500nm against reagent blank. Standard calibration curve was prepared using Phenol as standard (0.1 - 0.6 mg/ml). Absorption values were converted into total phenolics by using following equation.

$$[\text{Total Phenolics}] = (\text{Absorption} - 0.01)/1.249 \qquad R^2 = 0.999$$

This is a linear regression equation of standard calibration curve prepared by using Phenol.

Standard calibration curve for estimation of total phenolics:



D.3 Phenol Sulphuric Acid method for sugar (Dubois *et al.*, 1956)

In hot acidic medium carbohydrates are dehydrated to hydroxymethyl furfural. This forms a yellow coloured product with phenol.

Reagents

Reagent A (5% aqueous Phenol solution):

Phenol	5 g
De-ionized water	100 ml

To prepare Reagent A 5 g crystalline white phenol was dissolved in de-ionized water and diluted to 100 ml.

Reagent B (concentrated sulphuric acid):

Standard glucose solution (200 µg/ml):

Glucose standard stock

Glucose	0.2 g
De-ionized water	100 ml

Glucose working stock

Glucose standard stock	1ml
De-ionized water	9 ml

Glucose working stock was prepared by mixing 1 ml glucose standard stock solution and 9 ml de-ionized water.

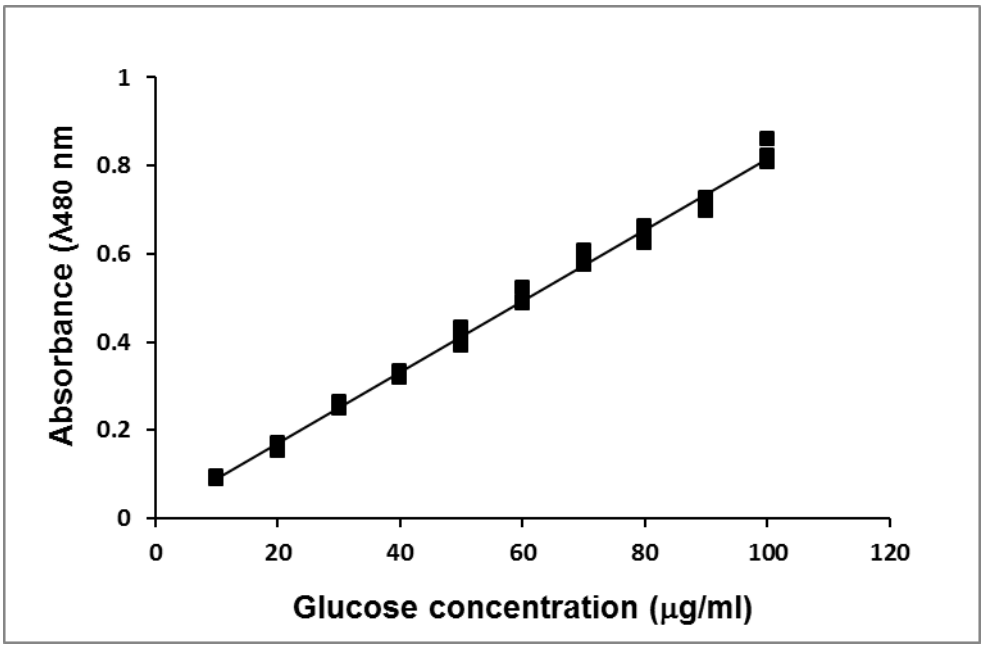
Procedure:

To 1 ml of sample, 1 ml Reagent A was added and mixed. 5 ml of Reagent B was then added and tubes were kept standing on ice bath for 10 minutes. Absorbance was measured at 480 nm against reagent blank. Standard calibration curve was prepared using glucose as standard (10 - 100 μg/ml). Absorption values were converted into sugar by using following equation.

$$[\text{Sugar}] = (\text{Absorption} - 0.009)/0.008 \qquad R^2 = 0.994$$

This is a linear regression equation of standard calibration curve prepared by using glucose.

Standard calibration curve for estimation of sugar:



Appendix E

Composition of soil based inoculum formulations using PT1

E.1 Soil based vegetative inoculum

Constituent	Amount
Soil fraction 'A'*	10 g
Soil fraction 'B'*	60 g
Soil fraction 'C'*	30 g
Gypsum	15 g
Mycelium	8 g (wet weight)
Distilled water**	25-30 ml

E.2 Soil based spore inoculum

Constituent	Amount
Soil	1 Kg
Gypsum	200 g
Powdered sporocarps	50 g
Yeast	10^9
Distilled water**	350 ml

*Sterilized at 121^{O}C for 2 hours and cooled to room temperature.

**Sterilized at 121^{O}C for 15 min and cooled to room temperature.

BIBLIOGRAPHY

1. Alves, J.R., Souza, O., Podlech, P.A.S., Giachini, A.J., Oliveira, V.L. (2001) Effect of ectomycorrhizal inoculants produced by solid state fermentation on growth of *Eucalyptus dunnii* Maiden. Pesq. Agrop. Bras. 36: 349-355.

2. Anderson, I.C., Chambers, S.M., Cairney, J.W.G. (1998) Use of molecular methods to estimate the size and distribution of mycelia individuals of the ectomycorrhizal basidiomycete *Pisolithus tinctorius*. Mycol. Res. 102(3): 295-300.

3. Anderson, I.C., Chambers, S.M., Cairney, J.W.G. (2001) ITS-RFLP and ITS sequence diversity in *Pisolithus* from central and eastern Australia sclerophyll forests. Mycol. Res. 105: 1304-1312.

4. Auge, R.M. (2001) Water relations, drought and vesicular-arbuscular mycorrhizal symbiosis. Mycorrhiza. 11: 3-42.

5. Bevege, D.I. (1968) A rapid technique for clearing tannins and staining intact roots for detection of mycorrhizas caused by *Endogone* spp. and some records of infection in Australasian plants. Trans. of the British Mycol. Soc. 51: 808-810.

6. Bidartondo, M.I., Ek, H., Wallander, H. and Soderstrom, B. (2001) Do nutrient additions alter carbon sink strength of ectomycorrhizal fungi? New Phytol. 151: 543-550.

7. Blaudez, D., Jacob, c., Turnau, K., Colpaert, J.V., Ahonen- J.U., Finlay, R., Botton, B., Chalot, M. (2000) Differential responses of ectomycorrhizal fungi to heavy metals *in vitro*. Mycol. Res. 104: 1366-1371.

8. Blum, H., Beier, H., and Gross, H.J. (1987) Silver staining of proteins in polyacrylamide gels. Electrophoresis. 8: 93-99.

9. Bogeat-Triboulot MB, Bartoli F, Garbaye J, Marmeisse R, Tagu D (2004) Fungal ectomycorrhizal community and drought affect root hydraulic properties and soil adherence to roots of *Pinus pinaster* seedlings. Plant and Soil. 267: 213-223.

10. Bois G, Bertrand AP, Fung M, Khasa DP (2006) Growth, compatible solute and salt accumulation of five mycorrhizal fungal species grown over a range of NaCl concentrations. Mycorrhiza. 16: 99-109.

11. Bolan NS (1991) A critical review on the role of mycorrhizal fungi in the uptake of phosphorus by plants. Plant and Soil. 134: 189-207.

12. Brundrett, M.C., Piche, Y., Peterson, R.L. (1984) A new method for observing the morphology of vesicular-arbuscular mycorrhizae. Can. J. Bot. 62: 2128- 2134.

13. Brundrett, M., Bougher, N., Dell, B., Grove, T., Malajczuk, N. (1995) Examining mycorrhizal associations. In: Working with Mycorrhizas in forestry and agriculture. Australian Centre for international Agricultural Research (ACIAR), Australia, pp. 172-215.

14. Burgess T, Dell B, Malajczuk N (1996) *In vitro* synthesis of *Pisolithus-Eucalyptus* ectomycorrhiza; synchronization of lateral tip emergence and ectomycoeehizal development. Mycorrhiza. 6(3):189-496.

15. Cairney JWG (1999) Intraspecific physiological variation implications for understanding functional diversity in ectomycorrhizal fungi. Mycorrhiza. 9: 125-135.

16. Cairney J.W.G. (2002) *Pisolithus* – death of the pan-global super fungus. New. Phytol. 153: 199-211.

17. Cairney, J.W.G., Chambers, S.M. (1997) Interactions between *Pisolithus tinctorius* and its hosts: a review of current knowledge. Mycorrhiza. 7:117-131.

18. Carlisle, M.J., Watkinson, S.C., Gooday, G.W. (2001) The fungi, 2nd edition. Academic Press, San Diego, pp 394-405.

19. Castellano, M.A. (1990) Outplanting performance of mycorrhizal inoculated seedlings: A review. Proceedings of the 8th North American Conference on Mycorrhizae. Jackson, Wyoming, USA, p 49.

20. Castellano, M.A., Molina, R. (1989) Mycorrhizae. In: The Biological components: Nursery Pests and Mycorrhizae – The Container Tree Nursery Manual, T.D. Landis, R.W Tinus, S.E. McDonald, J.P. Barnett (Eds.) US Department of Agriculture, Forest Service, Washington, DC, USA, pp 101-167.

21. Chen, K-Y., Chen, Z-C. (2004) Heat shock proteins of thermophilic and thermotolerant fungi from Taiwan. Bot. Bull. Acad. Sin. 45: 247-257.

22. Chen DM, Ellul S, Herdman K, Cairney JWG (2001) Influence of salinity on biomass production by Australian *Pisolithus* spp. Isolates. Mycorrhiza. 11: 231-236.

23. Chen, D.M., Khalili, K., Cairney, J.W.G. (2003) Influence of water stress on biomass production by isolates of an ericoid mycorrhizal endophyte of *Woollsia pungens* and *Epacris microphylla* (Ericaceae). Mycorrhiza.13: 173-176.

24. Child JJ, Knapp C, Eveleigh DE (1973) Improved pH control of fungal culture media. Mycologia. 65(5): 1078-1086.

25. Cline ML, France RC, Reid CPP (1987) Intraspecific and interspecific growth variation of ectomycorrhizal fungi at different temperatures. Can. J. Bot. 65: 869-875.

26. Coleman MD, Bledsoe CS, Lopushinsky W (1989) Pure culture response of ectomycorrhizal fungi to imposed water stress. Can. J. Bot. 67: 29-39.

27. Collin-Hansen, C. (2007) First report of phytochelatins in a mushroom: induction of phytochelatins by metal exposure in *Boletus edulis*. Mycologia. 99(2): 161-174.

28. Colpaert JV, Van Assche JA (1987) Heavy metal tolerance in some ectomycorrhizal fungi. Funct. Ecol. 1: 415-421.

29. Colpaert JV, Vandenkoornhuyse, P., Adriaensen, K., Vangronsveld, J. (2000) Genetic variation and heavy metal tolerance in the ectomycorrhizal basidiomycete *Suillus luteus*. New. Phytol. 147: 367-379.

30. Cordell CE, Mans LF, Farley ME, Marx DH (2000) Mycorrhizal fungi and trees – A successful reforestation alternative for mine land reclamation. In: Forest Nursery Proceedings, Kona, HI-2000, US Department of Agriculture Forest Service, Rocky Mountain Research Station, Fort Collins, US, pp 177-187.

31. Cordell, C.E., Marx, D.H, Maul, S.B., Owen J.H. (1988) Production and utilisation of ectomycorrhizal fungal inoculants in the Eastern United States. In: Mycorrhizae in the Next Decade. Practical Applications and Research Priorities, D.M. Sylvia, L.L. Hung, J.H. Grahan (Eds.) University of Florida, Gainesville, USA, pp 287-289.

32. Courbot, M., Diez, L., Ruotolo, R., Chalot, M., Leroy, P. (2004) Cadmium-Responsive Thiols in the Ectomycorrhizal Fungus *Paxillus involutus* App. and Env. Microbiol. 70(12): 7413–7417.

33. Daza, A., Manjon, J.L., Camacho, M., Romero de la Osa, L., Aguilar, A., Santamaria, C. (2006) Effect of carbon and nitrogen sources, pH and temperature on in vitro culture of several isolates of *Amanita caesarea* (Scop.:Fr.) Pers.. Mycorrhiza. 16: 133-136.

34. De la Cruz, R.E. (1990) Current status of nursery and field applications of ectomycorrhizal in the Philippines, Proceedings of the 8th North American Conference on Mycorrhizae, Jackson, Wyoming, USA, p 75.

35. De Mello, I.S., Faull, J.L. (2004) Scanning electron microscopy of conidia of Trichoderma stromaticum, a biocontrol agent of Witches broom disease of Cocoa. Braz. J. Microbiol. 35(4): 330-332.

36. Dixon, R.K., Rao, M.V., Garg, V.K. (1993) Salt stress affects *in vitro* growth and in situ symbioses of ectomycorrhizal fungi. Mycorrhiza. 3: 63-68.

37. Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F. (1956) Colorimetric method for the determination of sugars and related substances. Anal. Chem. 28: 350-356.

38. Duchesne, L.C., Peterson, R.L., Ellis, B.E. (1989) The time-course of disease suppression and antibiosis by ectomycorrhizal fungus *Paxillus involutus*. New. Phytol. 111: 693-698.

39. Dunabeitia, M.K., Hormilla, S., Garcia-Plazaola, J.I., Txarterina, K., Arteche, U., Becerril, J.M. (2004) Differential responses of three fungal species to environmental factors and their role in the mycorrhization of *Pinus radiata* D. Don. Mycorrhiza. 14: 11-18.

40. Durán, N., Teixeira, M.F.S., De Conti, R., Esposito, E. (2002) Ecological-Friendly Pigments From Fungi. Critical Reviews in Food Science and Nutrition. 42(1): 53-66.

41. Dustin (2011) Shades of *Pisolithus*. In: Mushroom dyes. Creative by nature (www.studiobluedesigns.net/blog/?p=845).

42. Egerton-Warburton, L.M., Griffin, B.J. (1995) Differential responses of *Pisolithus tinctorius* isolates to aluminium in vitro. Can. J. Bot. 73: 1229-1233.

43. Ek, H. (1997) The influence of nitrogen fertilization on the carbon economy of *Paxillus involutus* in ectomycorrhizal association with *Betula pendula*. New Phytol. 135: 133-142.

44. Ekblad, A., Wallander, H. and Nasholm, T. (1998) Chitin and ergosterol combined to measure total and living fungal biomass in ectomycorrhizas. New Phytol. 138: 143-149.

45. Ferreira, A.S., Totola, M.R., Borges, A.C. (2007) Physiological implications of trehalose in the ectomycorrhizal fungus Pisolithus sp. under thermal stress. J. Thermal. Biol. 32: 34-41.

46. Ferreira, A.S., Totola, M.R., Kasuya, M.C.M., Araujo, E.F., Borges, A.C. (2005) Small heat shock proteins in the development of thermotolerance in *Pisolithus* sp. J. Thermal. Biol. 30: 595-602.

47. Fillinger, S., Chaveroch, M.K., Dijek, P.V., Vries, R., Euijter, G., Thevelein, J., D'Enfert, C., (2001) Treholose is required for the acquisition of tolerance to a variety of stresses in the filamentous fungus *Aspergillus nidulans*. Microbiology. 147: 1851-1862.

48. Finlay, R.D., Lindahl, B.D., Taylor, A.F.S. (2008) Responses of mycorrhizal fungi to stress. In: Stress in yeasts and filamentous fungi. Avery S, Stratford M, van West P, (eds.) Amsterdam, Elsevier, pp 201–220.

49. Frisvad, J.C., Andersen, B., Thrane. U. (2008) The use of secondary metabolic profiling in chemotaxonomy of filamentous fungi. Mycol. Res. 112(2): 231-240.

50. Gadd, G.M. (1993) Interactions of fungi with toxic metals, Transley review no 47. New. Phytol. 124: 25-60.

51. Galli, U., Schilepp, H., Brunold, C. (1994) Heavy metal binding by mycorrhizal fungi. Physiol. Plant. 92: 364-368.

52. Gardes, M., Bruns, T. D. (1993) ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. Mol. Ecol. 2: 113-118.

53. Garg, S. (1999) Production of ectomycorrhizal fungal inoculum by submerged fermentation, Ph.D. Thesis, University of Delhi, New Delhi.

54. Gill, M., Kiefel, M.J. (2011) Pisoquinone, a new Naphthalenoid Pulvinic Acid from the fungus *Pisolithus arhizus*. Aust. J. Chem. 47(10): 1967 – 1977.

55. Giltrap NJ, Lewis DH (1981) Inhibition of growth of ectomycorrhizal fungi in culture by phosphate. New. Phytol. 87: 669-675.

56. Glover, J.R., Lindquist, S. (1998) Hsp 104, Hsp 70, and Hsp 40: a novel chaperone system that rescues previously aggregated proteins. Cell. 94: 73-82.

57. Graham, G.C., Mayers, P., Henry, R.J. (1994) A simplified method for preparation of fungal genomic DNA for PCR and RAPD analysis. Biotechniques. 16: 48-50.

58. Greenberg, A.E., Trussell, R.R., Clesceri, L.S., Franson, M.A.H. (1985) Standard methods for the examination of water and waste water, 16th edn, American Public Health Association, Washington DC, USA, pp 556-570.

59. Gruhn, C.M., Miller, O.K. (1991) Effect of copper on tyrosinase activity and polyamine content of some ectomycorrhizal fungi. Mycol. Res. 95: 268–272.

60. Gupta V, Garg S, Satyanarayana T (1997) Selection of ectomycorrhizal fungal strains for large scale inoculum production In: Microbial Biotechnology. Scientific Publishers, Jodhpur, India.

61. Han, X.L., Xia, G.X., Niu, Y. (2006) Research of the mechanism of Ectomycorrhizae to drought resistance. Res. Soil Water Conserv. 5: 42-44.

62. Harley, J.L., Smith, S.E. (1983) Mycorrhizal symbiosis. Academic Press, New York.

63. Hile, N., Hennek, J.F. (1969) In vitro culture of *Pisolithus tinctorius* mycelium. Mycologia. 61: 195-198.

64. Hilger, A.B., Thomas, K.I., Krause, H.H. (1986) The effects of several buffers on growth and phosphorus nutrition of selected ectomycorrhizal fungi. Soil Biol. Biochem. 18: 61-67.

65. Ho I (1987) Comparison of eight *Pisolithus tinctorius* isolates for growth rate, enzyme activity and phytohormone production. Can. J. For. Res. 17(1): 31-35.

66. Howe, R., Evans, R.L., Ketteridge, S.W. (1997) Copper-binding proteins in ectomycorrhizal fungi. New. Phytol. 135: 123-131.

67. Hung L-L, Chien CY (1978) Physiological studies on two ectomycorrhizal fungi, *Pisolithus tinctorius* and *Suillus bovines*. Trans. Mycol. Soc. Japan. 19:121-127.

68. Hung L-L, Trappe JM (1983) Growth variation between and within species of ectomycorrizal fungi in response to pH *in vitro*. Mycologia. 75(2):234-241.

69. Jayakumar, P., Tan, T.K. (2005) Phosphorus solubilisation by ectomycorrhizal *Pisolithus tinctorius* in pure culture and in association with *Acacia mangium*. Symbiosis. 39:125-130.

70. Jentschke, G., Godbold, D.L. (2000) Metal toxicity and ectomycorrhizas. Physiologia Plantarum. 109: 107-116.

71. Kamat, N.M. (1993) The wild edible mushrooms (Agaricales) of Goa – Studies on taxonomy, culture and in vitro frutification. Ph.D. Thesis, Goa University, Goa.

72. Kernaghan, G., Hambling, B., Fung, M., Khasa, D. (2002) In vitro selection of Boreal ectomycorrhizal fungi for use in reclamation of saline-alkaline habitats. Restor. Ecol. 10(1): 43-51.

73. Khosla, B., Reddy, M.S. (2008) Response of ectomycorrhizal fungi on the growth and mineral nutrition of *Eucalyptus* seedlings in bauxite mined soil. American-Eurasian J. Agric. Environ. Sci. 3(1): 123-126.

74. Kope, H.H., Fortin, J.A. (1990) Germination and comparative morphology of basidiospores of *Pisolithus arhizus*. Mycologia. 82(3): 350-357.

75. Kope, H.H., Tsantrizos, Y.S., Fortin, J.A., Ogilvie, K.K. (1991) *p*-hydroxy benzoyl formic acid and R-(-)-*p*-hydroxymendalic acid, two

antifungal compounds isolated from the liquid culture of ectomycorrhizal fungus *Pisolithus arhizus*. Can. J. Microbiol. 37(4):258-64.

76. Kope, H.H., Fortin, J.A. (1989) Inhibition of phytopathogenic fungi in vitro by cell free culture media of ectomycorrhizal fungi. New. Phytol. 113: 57-63.

77. Krugner, T.L., Filho, M.T. (1980) Effect of ectomycorrhizal fungi *Pisolithus tinctorius* and *Thelephora terrestris* and mineral fertilisation on growth and survival of *Pinus caribaea* var. *bahamensis* under field conditions in the southern coast of Bahia. IPEF. 21: 41-51.

78. Kuek, C., Tommerup, I.C., Malajczuk, N. (1992) Hydrogel bead inocula for the production of ectomycorrhizal eucalypts for plantations. Mycol. Res. 96: 273-277.

79. Kuek, C. (1996) Shake flask culture of Laccaria laccata an ectomycorrhizal basidiomycete. Appl. Microbiol. Biotechnol. 45: 319-326.

80. Lamhamedi, M.S., Bernier, P.Y., Fortin, J.A. (1991) Growth, nutrition and response to water stress of *Pinus Pinaster* inoculated with ten dikaryotic strains of *Pisolithus* sp.. Tree Physiology. 10:153-167.

81. Landeweert, R., Veenman, C., Kuyper, T.W., Fritze, H., Wernars, K., Smit, E. (2003) Quantification of ectomycorrhizal mycelium in soil by real-time PCR compared to conventional quantification techniques. FEMS Microbiol. Ecol. 45: 283-292.

82. Lapeyrie, F., Ranger, J., Vairelles, D. (1991) Phosphate-solubilizing activity of ectomycorrhizal fungi *in vitro*. Can. J. Bot. 69:342-346.

83. Le Tacon, Jung, G., Mugnier, J., Michelot, P., Mauperin, C. (1985) Efficiency in a forest nursery of an ectomycorrhizal fungus inoculant produced in a liquid medium and entrapped in a calcium alginate gel. Can. J. Bot. 63: 1664-1668.

84. Liang, Y., Chen, H., Tang, M., Shen, S. (2007) Proteome analysis of an ectomycorrhizal fungus *Boletus edulis* under salt shock. Mycol. Res. 111: 939-946.

85. Litchfield, J.H., Lawhon, W.T., (1982) Aerobic submerged fermentation of sporulating ectomycorrhizal fungi. US Patent No. 4327181.

86. Lowry, O.H., Farr, L. and Randell, R.J. (1951) Quantitation of protein using Folin-Ciocalteau reagent. J. Biol. Chem. 93: 265-275.

87. Madsen, T., Mulligan, D. (2007) Effect of salt on the growth of ectomycorrhizal isolates and implications for the coal mining industry in central Queensland. Ecol. Manag. Restor. 8(1): 63-65.

88. Malloch, D., Kuja A.L. (1979) Occurrence of the ectomycorrhizal fungus Pisolithus tinctorius in Ontario. Can. J. Bot. 57: 1848-1849.

89. Martin, F., Delaruelle, C., Ivory, M. (1998) Genetic variability in intergenic spacers of ribosomal DNA in *Pisolithus* isolates associated

with pine, eucalyptus and *Afzelia* in low land Kenyan forests. New. Phytol. 139: 341-352.

90. Martin, F., Diez, J., Dell, B., Delaruelle, C. (2002) Phylogeography of the ectomycorrhizal *Pisolithus* species as inferred from nuclear ribosomal DNA ITS sequences. New. Phytol. 153: 345-357.

91. Martin, M.P., Gracia, E. (2000) Spore germination and development of young mycelia in some *Rhizopogon* species. Acta. Bot. Barc. 46: 31-46.

92. Marx, D.H. (1969) The influence of ectotrophic mycorrhizal fungi on the resistance in pine roots to pathogenic infections I. Antagonisms of mycorrhizal fungi to root pathogenic fungi and soil bacteria. Phytopathology. 59:53-163.

93. Marx, D.H. (1972) Ectomycorrhizae as biological deterrents to pathogenic root infections. Annu. Rev. Phytopathol. 10: 429-454.

94. Marx, D.H. (1975) Mycorrhizas and establishment of trees on stripmined land. Ohio. J. Sci. 75:288-297.

95. Marx, D.H., Cordell, C.E. (1989) The use of specific ectomycorrhizas to improve artificial forestation practices. In: Biotechnology of fungi for improving plant growth. Br Mycol Soc, pp 1-26.

96. Marx, D.H., Zak, B. (1965) Effect of pH on mycorrhizal formation of slash pine in aseptic culture. For. Sci. 11:66-75.

97. Marx, D.H., Artman, J.D. (1979) *Pisolithus tinctorius* ectomycorrhizae improve survival and growth of pine seedlings on acid coal spoils in Kentucky and Virginia. Reclam. Rev. 2:23-31.

98. Marx, D.H. (1975) Mycorrhizae of exotic trees in the Peruvian Andes and synthesis of Ectomycorrhizae on Mexican pines. Forest. Sci. 21: 353-358.

99. Marx, D.H. (1977) Tree host range and world distribution of the ectomycorrhizal fungus *Pisolithus tinctorius.* Can. J. Microbiol. 23: 217-223.

100. Marx, D.H. (1980) Ectomycorrhizal Fungus Inoculation: A Tool for Improving Forest Practices. In: Ectomycorrhiza Research, P. Mikola (Ed.), Clarendon Press, Oxford, UK (1980) pp 13–71.

101. Marx, D.H. (1991) The practical significance of Ectomycorrhizae in forest establishment. In: Ecophysiology of Ectomycorrhizae of forest trees, The Marcus Wallenberg Foundation Symposia Proc. 7. Stockholm, Sweden, pp 54-90.

102. Marx, D.H., Bryan, W.C., Davey, C.B. (1970) Influence of temperature on aseptic synthesis of Ectomycorrhizae by Thelephora terrestris and Pisolithus tinctorius on loblolly pine. For. Sci. 16: 424-431.

103. Marx, D.H., Cordell, C.E. (1989) The use of specific ectomycorrhizas to improve artificial forestation practices. In: Biotechnology of fungi

improving plant growth, J.M. Whipps, R.D. Lumsden (Eds.), New York, USA. pp. 1–25.

104. Marx, D.H., Cordell, C.E., Kenny, D.S., Mexal, J.G. Artman, J.D., Riffle, J.W., Molina, R.J. (1984) Commercial vegetative inoculums of Pisolithus tinctorius and inoculation techniques for development of Ectomycorrhizae on bare-rooted tree seedlings. For. Sci. Monogr. 25: 1-101.

105. Marx, D.H., Hedin, A., Toe, S.F.P. (1985) Field performance of *Pinus caribae* var. *hondurensis* seedlings with specific Ectomycorrhizae and fertiliser after three years on a savanna site in Liberia. For. Ecol. Manag. 13: 1-25.

106. Marx, D.H., Jarl, K., Ruehle, J.L., Bell, W. (1984) Development of *Pisolithus tinctorius* Ectomycorrhizae on pine seedlings using basidiospore encapsulated seeds. For. Sci. 30:897-907.

107. Marx, D.H., Kenney, D.S. (1982) Production of ectomycorrhizal fungus inoculants. In: Methods and Principles of Mycorrhizal research, N.C. Schenk (Ed.), The American Pthytopathological Society, St. Paul, Minnesota, USA, pp. 131-146.

108. Marx, D.H., Marrs, L.F., Cordell, C.E. (2002) Practical use of the mycorrhizal fungal technology in forestry, reclamation, arboriculture, agriculture, and horticulture. Dendrobiology. 47: 27-40.

109. Marx, D.H., Ruehle, J.L., Cordell, C.E (1991) Methods for Studying Nursery and Field Response of Trees to Specific Ectomycorrhiza. In:

Methods in Microbiology, J.R Norris, D.J. Read, A.K. Varma (Eds.), Academic Press, London, UK, pp 383–411.

110. Marx, D.H., Ruehle, J.L., Kenney, D.S., Cordell, C.E., Riffle, J.W., Molina, R.J., Pawuk, W.H., Navratil, S., Tinus, R.W., Goodwin, O.C. (1982) Commercial vegetative inoculants of *Pisolithus tinctorius* and inoculation techniques for development of Ectomycorrhizae on container-grow tree seedlings. For. Sci. 28: 273-400.

111. Mason, P.A. (1980) Aseptic synthesis of sheathing (ecto-)mycorrhizas. In: Tissue culture methods for plant pathologists, D.S. Ingram, J.P. Hegeson, pp 173-178.

112. Mason, P.A., Last, F.T., Wilson, J. (1986) Effects of different soils on the establishment and influence of sheathing mycorrhizal fungi. In: Physiological and genetical aspects of mycorrhizae. Gianinazzi-Pearson, V. and Gianinazzi, S. (eds.), INRA Press, Paris, pp. 767-772.

113. Matsuda Y, Sugiyama F, Nakanishi S-II (2006) Effects of sodium chloride on growth of ectomycorrhizal fungal isolates in culture. Mycosciences. 47: 212-217.

114. McAfee, B.J., Fortin, J.A. (1986) Competitive interactions of ectomycorrhizal mycobionts under field conditions. Can. J. Bot. 64: 848-852.

115. Medentsev, A.G., Akimenko, V.K. (1998) Naphthoquinone metabolites of the fungi. Phytochemistry. 47: 935-959.

116. Meharg, A.A. (2003) The mechanistic basis of interactions between mycorrhizal associations and toxic metal cations. Mycol. Res. 107(11): 1253-1265.

117. Mexal, J., Reid, C.P.P. (1973) The growth of selected mycorrhizal fungi in response to induced water stress. Can. J. Bot. 51: 1579-1588.

118. Mikola, P. (1973) Application of Mycorrhizal symbiosis in forestry practices. In: Ectomycorrhizae, their ecology and physiology, G.C. Marks, T.T. Kozlowski (Eds.) Acadamic Press, New York, USA, pp. 383-411.

119. Modess, O. (1941) Zur kenntin der mykorrhizabildner von kiefer and Fichte. Symbolae Botanicae Upsaliensis. 5: 1-147.

120. Montenegro, R.C., Jimenez, P.C., Feio Farias, R.A., Andrade-Neto, M., Bezerra, F.S., Moraes, M.E.A., De Moraes, M.O., Pessoa, C., Costa-Lotufo, L.V. (2004) Cytotoxic activity of Pisosterol, a Triterpene isolated from *Pisolithus tinctorius* (Mich.: Pers.) Coker & Couch, 1928. Z. Naturforsch. 59c: 519-522.

121. Morselt, A.F.W., Smits, W.T.M., Limonard, T. (1986) Histochemical demonstration of heavy metal tolerance in ectomycorrhizal fungi. Plant and Soil. 96: 417–420.

122. Mosse, B., XStribley, D.P., Letacon, L. (1981) Ecology of mycorrhizae and mycorrhizal fungi. Adv. Microbiol. Ecol. 5:137-210.

123. Moyersoen, B., Beever, R.E., Martin, F. (2003). Genetic diversity of *Pisolithus* in New Zealand indicates multiple long distance dispersal from Australia. New. Phytol. 160: 569-579.

124. Nagarajan, G., Natarajan, K. (1999) The use of Box-Behnken design of experiments to study *in vitro* salt tolerance by *Pisolithus tinctorius*. World J. Microbiol. Biotechnol. 15: 197-203.

125. Natarajan, K., Senthilarasu, G., Kumaresan, V. And Reviere, T. (2005) Diversity in ectomycorrhizal fungi of a dipterocarp forest in Western Ghats Current Science. 88(12): 1893-1895.

126. Nehls, U., Ecke, M., Manpp, R. (1999) Sugar- and nitrogen-dependent regulation of Amanita muscaria phenylalanine ammonium lyase gene. J. Bact. 181(6): 1931-1933.

127. Odebode, A.C. (1996) Phenolic compounds in the kola nut (*Cola nitida* and *Cola accuminata*) (Sterculiaceae) in Africa. Rev. Biol. Trop. 44(2A): 513-515.

128. Olsson, P.A. (1999) Signature fatty acids provide tools for determination of the distribution and interactions of mycorrhizal fungi in soil. FEMS Microbiol. Ecol. 29: 303-310.

129. Osherov, N., May, G.S. (1998) Optimisation of protein extraction from *Aspergillus nidulans* for gel electrophoresis. Fungal Genetics Newsletter. 45:38-40.

130. Owades, J. L., Rubin, G., Brenner, M. W. (1958) Determination of food tannins by ultraviolet spectrophotometry. J. Agric. Food Chem. 6: 44-47.

131. Park, C. (1997) The environment principles and applications. TR Publications Pvt Ltd.

132. Pink, D., Naczk, M., Baskin, K., Shahidi, F. (1994) Theoretical analysis of Ultraviolet-Visible Spectra of various Phenolic Acid Fractions of Canola J. Agric. Food Chem. 42: 1317-1322.

133. Plesofsky-vig, N., Brambl, R. (1993) Heat shock proteins in fungi. In: Jennings, D.H. (Ed.), Stress tolerance of fungi. Marcel Dekker Inc., New York, pp 45-68.

134. Porcel, R., Ruiz-Lozano, J.M. (2004) Arbuscular mycorrhizal influence on leaf water potential, solute accumulation, and oxidative stress in soybean plants subjected to drought stress. J. Exp. Bot. 55: 1743-1750.

135. Pradella, J.G.C., Zuccolo, M., Lopes, S.A.R., Oliveira, M.S. (1991) *Pisolithus tinctorius* vegetative mycelia production: Effects of nitrogen sources and cultivation in stirred tank fermentor. Rev. Microbiol. 22: 7-11.

136. Rao, C.S., Sharma, G., Shukla, A.K. (1997) Distribution of ectomycorrhizal fungi in pure stands of different age groups of *Pinus kesiya*. Can. J. Microbiol. 43: 85-91.

137. Reddy, M.S., Natarajan, K. (1997) Coinoculation efficacy of ectomycorrhizal fungi on *Pinus patula* seedlings in a nursery. Mycorrhiza. 7: 133-138.

138. Reddy, M.S., Natarajan, K. (1996 *In vitro* ectomycorrhizal formation of *Pinus patula*, *P. pseudostrobus*, *P. oocarpa* and *P. elliotii* grown in Southern India. New. For. 11: 149–153.

139. Reddy, M.S., Singla S., Natarajan, K., Senthilarasu, G. (2005) *Pisolithus indicus*, a new species of ectomycorrhizal fungus associated with Dipetrocarps in India. Mycologia. 97(4): 838-843.

140. Rossi, M.J., Furigo, A., Oliveira, V.L. (2007) Inoculant production of ectomycorrhizal fungi by solid and submerged fermentations. Food Technol. Biotechnol. 45(3): 277-286.

141. Rossi, M.J., Oliveira, V.L. (2011) Growth of the ectomycorrhizal fungus *Pisolithus microcarpus* in different nutritional conditions. Brazilian J. Microbiol. 42: 624-632.

142. Rossi, M.J. (2006) Technology of the production of ectomycorrhizal fungi inoculants employing submerged cultivation in an airlift bioreactor, PhD Thesis, Universidade Federal de Santa Catarina, Florianópolis, Brazil.

143. Rossi, M.J., Souza, J.A.R., Oliveira, V.L. (2002) Inoculant production of the ectomycorrhizal fungus *Pisolithus microcarpus* in an airlift bioreactor. Appl. Microbiol. Biotechnol. 59: 175-181.

144. Saleh-Rastin, N. (1976) Salt tolerance of the mycorrhizal fungus *Cenococcum graniforme* (Sow.) Ferd. Eur. J. For. Path. 6:184-187.

145. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning – A Laboratory Manual, volume 3, second edition, Cold Spring Harbor Laboratory Press, USA.

146. Sanchez, Y. J., Taulien, K. A., Borkovich, and S. Lindquist. (1992) Hspl04 is required for tolerance to many forms of stress. EMBO. J. 11: 2357-2364.

147. Satyanarayana, T., Gupta, V., Garg, S. (1996) Problems and prospects in inoculum production technology of ectomycorrhizal fungi In: Fungi in Biotechnology, CBS Publishers and Distributors, New Delhi, pp 45-50.

148. Schwartz, M.w., Hoeksema, J.D., Gehring, C.A., Johson, N.C., Klironomos, J.N., Abott, L.K., Pringle, A. (2006) The promise and the potential consequences of the global transport of mycorrhizal fungal inoculants. Ecol. Lett. 9:501-515.

149. Selosse, M.A., Bouchard, D., Martin, F., Le Tacon, F. (2000) Effect of *Laccaria bicolor* strains inoculated on Douglas-fir (*Pseudotsuga menziesii*) several years after nursery inoculation. Can. J. Forest Res. 30: 360-371.

150. Shwab, E.K., Keller, N.P. (2008) Regulation of secondary metabolite production in filamentous Ascomycetes. 112(2): 225-230.

151. Siddiqui, Z.A., Kataoka, R. (2011) Mycorrhizal inoculants: Progress in Inoculant Production Technology. In: Microbes and Microbial Technology: Agricultural and Environmental Applications. Ahmad et al., (Eds.) Springer Science + Buisiness media, pp. 489-506.

152. Soil Moist - Mycorrhizal products, JRM Chemical, Inc., 4881 NEO Parkway, Cleveland, Ohio (info@soilmoist.com).

153. Srinivasan, M., Natarajan, k., Nagarajan, G. (2000) Growth optimization of an ectomycorrhizal fungus with respect to pH and temperature *in vitro*, using Designs of Experiments. Bioprocess Eng. 22: 267-273.

154. Suh, H-W., Crawford, D.L., Korus, R.A., Shetty, K. (1991) Production of antifungal metabolites by the ectomycorrhizal fungus *Pisolithus tinctorius* strain SMF. J. Industrial. Microbiol. Biotechnol. 8: 29-36.

155. Sundari, K.S., Adholeya, A. (2003) Growth profile of ectomycorrhizal fungal mycelium: emphasis on substrate pH influence. Antonie van Leeuwenhoek. 83: 209-214.

156. Sylvia, D.M., Fuhrmann, J.J., Hartel, P.G., Zuberer, D.A. (2004) Overview of mycorrhizal symbiosis In. Principles and Applications of soil microbiology, second edition. Prentice Hall Publishers, Upper Saddle River, New Jersey.

157. Sylvia, D.M., Sinclair, W.A. (1983) Phenolic compounds and resistance to fungal pathogens induced in primary roots of Douglas-Fir

seedlings by the ectomycorrhizal fungus *Laccaria laccata*. Phytopathology. 73: 390-397.

158. Tam, P.C.F. (1995) Heavy metal tolerance by ectomycorrhizal fungi and metal amelioration by *Pisolithus tinctorius*. Mycorrhiza. 5:181-187.

159. Theodorou, C. (1971) Introduction of mycorrhizal fungi into soil by spore inoculation of seed. Aust. Forest. 35: 23-26.

160. Theodorou, C., Bowen, G.D. (1973) Inoculation of seeds and soil with basidiospores of mycorrhizal fungi. Soil Biol. Biochem. 5: 765-771.

161. Thompson, G.W., Medve, R.J. (1984) Effects of aluminium and manganese on the growth of ectomycorrhizal fungi. Appl. Environ. Microbiol. 48(3): 556-560.

162. Tibbett, M., Saunders, F.E., Cairney, J.W.G (2002) Low-temperature-induced changes in trehalose, mannitol and arabitol associated with enhanced tolerance to freezing in ectomycorrhizal basidiomycetes (*Hebeloma* spp.). Mycorrhiza. 12: 249-255.

163. Trappe, J.M. (1977) Selection of fungi for ectomycorrhizal inoculation in nurseries. Annu. Rev. Phtopathol. 15: 203-222.

164. Trent, J.D., Gabrielsen, M., Jensen, B., Neuhard, J., Olsen, J. (1994) Acquired thermotolerance and heat shock proteins in thermophiles from three phylogenetic domains. J. Bacteriol. 176(19): 6148-6152.

165. Tresner, H.D., Hayes, J.A. (1971) Sodium chloride tolerance of terrestrial fungi. Appl. Microbiol. 22(2): 210-213.

166. Tsantrizos, Y.S., Kope, H.H., Fortin, J.A., Ogilvie, K.K. (1991) Antifungal antibiotics from *Pisolithus tinctorius*. Phytochemistry. 30(4): 1113-1118.

167. Turnau, K., Kottke, I., Dexheimer, J., Botton, B. (1994) Elemental distribution in mycelium of *Pisolithus arhizus* treated with cadmium dust. Ann. Bot. 74: 137–142.

168. Valdes, M. (1986) Survival and growth of pines with specific Ectomycorrhizae after 3 year on highly eroded site. Can. J. Bot. 64: 885-888.

169. Velišek J., Cejpek K. (2011) Pigments of higher fungi – a review. Czech. J. Food Sci. 29: 87-102.

170. Wallander, H., Nilsson, L.O., Hagerberg, D. and Bafiafith, E. (2001) Estimation of the biomass and seasonal growth of external mycelium of ectomycorrhizal fungi in the field. New Phytol. 151: 753-760.

171. Wallander, H., Massicotte, H.B. and Nylund, J.E. (1997) Seasonal variation in protein, ergosterol and chitin in five morphotypes of *Pinus sylvestris* L. ectomycorrhizae in a mature Swedish forest. Soil Biol. Biochem. 29: 45-53.

172. Walter, J.F., Paau, A.S. (1993) Microbial inoculants production and formulation. In: Soil Microbial Ecology Applications in Agricultural

and Environmental Management, F.B. Metting, Jr. (Ed.), Marcel Dekker, New York, USA, pp 579-594.

173. White, T. J., Bruns, T., Lee, S., Taylor, J.W. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols: A Guide to Methods and Applications, eds. Innis, M. A., D. H. Gelfand, J. J. Sninsky, and T. J. White. Academic Press, Inc., New York. pp 315-322.

174. Whiting, E.C., Rizzo, D.M. (1999) Effect of water potential on radial colony growth of *Armillaria mellea* and *A. gallica* isolates in culture. Mycologia. 91(4): 627-635.

175. Yamaji, K., Ishimoto, H., Usui, N., Mori, S. (2005) Organic acids and water soluble phenolics produced by *Paxillus* sp. 60/92 together show antifungal activity against *Pythium vexans* under acidic culture conditions. Mycorrhiza. 15:17-23.

176. Yamanaka, T. (2003) The effect of pH on the growth of saprotrophic and ectomycorrhizal ammonia fungi *in vitro*. Mycologia. 95(4): 584-589.

177. Zhang, H., Tang, M., Yang, Y. (2011) The response of ectomycorrhizal (ECM) fungi under water stress induced by polyethylene glycol (PEG) 6000. African J. of Microbiol. Res. 5(4): 365-373.

178. Zhou, Z-Y., Liu, J-K. (2010) Pigments of fungi (macromycetes). Nat. Prod. Rep.

179. Zak, B. (1964) Aphids feeding on mycorrhizae of Douglas-fir. For. Sci. 11: 410-411.

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Production of Phenolics Under Abiotic Stress by *Pisolithus tinctorius* PT1 Obtained from Iron Ore Mine

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***Pisolithus* sp. was isolated from ectomycorrhizal association with *Acacia mangium* growing in mining affected forest area along the west coast of India. The *in vitro* growth response and phenolics production by the isolate was checked using mineral medium for various edaphic factors prevailing in rejects of iron ore mines. Phosphate-citrate buffer (0.047 M) was found suitable for maintaining the pH of the medium. The isolate survived at 10 and 42ºC. Interestingly, the isolate showed growth in the presence of 10,000 ppm Mn and appeared to be the most Mn-tolerant. The isolate preferred ferric form of iron over ferrous. The optima of all the variables for phenolics production were found at higher values than that required for the growth of isolate, significantly revealing the enhanced elaboration of phenolics at stress conditions. Maximum yields of phenolics obtained were 149.91, 164.07 and 61.34 µg/20ml with 1.5% NaCl, 37ºC and 5% PEG, respectively. The wide tolerance and elaboration of phenolics by *Pisolithus* sp. to various edaphic factors present in mine rejects could be responsible for its stable existence on such disturbed site. This suggests its possible ecological application and industrial utilization.**

Key words: Metal tolerance; Mine rejects; phenolics; Phosphate inhibition; *Pisolithus tinctorius*.

Pisolithus being ubiquitous organism is subjected to different environment conditions during growth and hence believed to have wider tolerance range. Potential of *Pisolithus* in reclamation of disturbed soils such as mined lands have been widely recognized (Cordell *et al*., 2000; Khosla and Reddy, 2008). Pt is the most frequently used, due to its global geographical distribution, wide host range, greater tolerance to environmental stresses and relatively easy cultivation in laboratory media (Cairney & Chambers, 1997). Thus, *Pisolithus* is a well known ecologically and economically important ECM fungus.

Ectomycorrhizal fungi have been tapped for producing antagonistic phenolic compounds that protects the host plants against root diseases (Sylvia & Sinclair, 1983; Duchesne *et al*., 1989). Two phenolic compounds having antibiotic activity namely Pisolithin A [*p*-hydroxy benzoyl formic acid] and Pisolithin B [R-(-)-*p*-hydroxymendalic acid] have been isolated from *Pisolithus tinctorius* (Pt) (Kope *et al*., 1991). Although earlier reports have suggested the phenolic compounds production under biological stress (Suh *et al*., 1991), it remains to be demonstrated that *Pisolithus* sp. produces phenolics even under abiotic stress. To derive maximum advantage, it is essential to carry out detailed *in vitro* study of the fungus for

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the factors affecting the growth and phenolics production.

The aim of this present work was to study, the *in vitro* growth and survival of *Pisolithus tinctorius* PT1 isolate, obtained from mining region, to various edaphic factors prevailing in mine rejects of iron ore including iron and manganese. Present investigation highlighted the impact of various growth conditions in chemically defined medium giving insight into optimal value of parameter vis-à-vis tolerance of fungus and elaboration of phenolics under abiotic stress.

MATERIAL AND METHODS

Fungal isolate

Pisolithus tinctorius PT1 was isolated from sporocarp associated with *Acacia mangium* on iron ore mine land at Codli, Goa, India. Pure fungal mycelium was maintained by periodical transfer on Modified Melin Norkrans medium, pH 6.5 (MMN) (Marx, 1969) and Glucose Mineral Salt Medium, pH 6.0 (GMSM) (Garg, 1999). Composition of GMSM: basal medium (1L) 20.0 g glucose, 0.1 g KCl, 1.0 g NH_4Cl, 0.1 g NaCl, 1.0 g KH_2PO_4, 0.3 g $MgCl_2.4H_2O$, 0.132 g $CaCl_2.2H_2O$, 10.53 mg $FeSO_4.7H_2O$, 37.52 mg Na_2EDTA, 100 µg Thiamine HCl, 40 µg Biotin, 10 ml micronutrient solution and 20.0 g agar powder. Ten ml micronutrient solution consists of 2.784 mg H_3BO_3, 3.38 mg $MnSO_4.H_2O$, 97 µg $CuSO_4.5H_2O$, 201 µg $ZnSO_4.7H_2O$, 338.46 µg $Na_2MoO_4.2H_2O$ and 240 µl H_2SO_4. Initial pH of the basal medium was adjusted using 0.1 N KOH solution. For all the experiments, single 10x8x5 mm agar piece with fungal mycelia growing on the MMN was used as inoculum.

Growth response of *Pisolithus tinctorius* PT1

The *in vitro* growth response of *Pisolithus tinctorius* PT1 was checked using GMSM with varying particular medium component or growth condition. Effect of varying concentration of phosphate was checked from 0.007 to 0.107 M. The molarity of phosphate in the medium was adjusted using Sorrensen's phosphate buffer (SPB) solution. To check the growth of *Pisolithus tinctorius* PT1 on GMSM (pH 6.8) with various buffer systems, GMSM agar plates containing 0.02 M of HEPES buffer, Phosphate-citrate buffer (PCB), and SPB was inoculated with inoculum disc and colony diameter was recorded after 30 days. In addition, 0.04 M SPB and PCB were also tested. The amount of buffer was in addition to 0.007 M of phosphate present in basal medium. Final pH of the media was taken with pH paper after termination of experiment. The effect of pH was determined by adjusting GMSM medium to pH 2.2 to 8.0 using 0.04 M phosphate-citrate buffer in addition to the phosphate present in basal medium. Growth response of the isolate at different incubation temperature was checked from 10 to 50°C. Increase in colony diameter on GMSM agar and biomass accumulation in liquid medium was monitored. *Pisolithus tinctorius* PT1 was grown using GMSM agar and broth with varying concentration of NaCl from 0.01 to 4%. Fungal isolate was grown on GMSM amended with $FeSO_4.7H_2O$ or $FeCl_3$ salts to give Fe concentration from 0 to 100 ppm. Growth response of *Pisolithus tinctorius* PT1 to manganese (Mn) was determined from 0 to 10,000 ppm by incorporating suitable amount of $MnSO_4$ salt. *Pisolithus tinctorius* PT1 was grown in GMSM broth adjusted to different water potential by varying amount of polyethylene glycol (PEG 6000) from 0 to 35%. Fungal biomass obtained was washed with hot water to remove the residual PEG prior to determination of dry weight.

Four replicates were maintained for each experimental condition. Inoculated plates were incubated at RT. Fungal growth was recorded by measuring colony diameter at 30 days of incubation. The diameter of each colony was measured thrice by rotating the plate at 60° everytime. The values obtained were averaged and reported along with standard errors to account for non-symmetry.

Growth of *Pisolithus tinctorius* PT1 in liquid GMSM broth was carried out by inoculating 20 ml medium in 100ml conical flask. Each flask was inoculated with inoculum disc and incubated for a period of 30 days. Fungal masses were collected and washed with sterile distilled before keeping in pre-weighed aluminium cups at 80°C. Dry weight of fungal was recorded till constant weights of cups were obtained.

Total phenolics content in the culture broth was estimated by 4-Aminoantipyrine method (Greenberg *et al*., 1985). The pH of agar medium and culture broth were determined using pH-paper and pH-electrode, respectively.

Statistical Analysis

Impact of parameters and significant differences between treatments were assessed by analysis of variance (ANOVA) at $P<0.001$ and treatment means were compared by least significant difference ($P<0.05$) using Student-Newman-Keuls Method.

RESULTS AND DISCUSSION

The ECM fungus *Pisolithus tinctorius* PT1 investigated in the present study, was associated with *Acacia mangium* on iron ore mineland at Codli, Goa. This fungus is also present in coastal and rain-forest of Goa. The rejects generated by mining consists of porous loose soil with pH 5.5-6.0, phosphorus (Pi) 0-15 mg/Kg, Fe 12-50 mg/Kg, Mn 40-70 mg/Kg and low fertility. The isolate thriving in such nutrient deficient and stressful conditions have shown wide tolerances to various edaphic factors tested and hence could be promising in reclamation. In containerized studies, fungus formed ectomycorrhizae with *Acacia mangium* and *Cassia fistula*. Moreover, phenolics produced by Pt are potential antimicrobial compounds. Earlier studies demonstrated fermentative production of phenolics by Pt and suggested its enhanced production under biological stress (Suh *et al*., 1991). This is the first report that determines the effect of various growth parameters on phenolics production by *Pisolithus* sp. indicating the enhanced production under abiotic stresses.

The growth response and phenolics production by *Pisolithus tinctorius* PT1 to various growth parameters is summarised in Table 1. The growth of *Pisolithus tinctorius* PT1 under various edaphic factors has revealed some interesting results.

Pisolithus tinctorius PT1 when grown in MMN and GMSM resulted into drastic lowering of pH of growth media to 2.0-3.0. Elaboration of organic acids could be responsible for lowering the pH of the medium (Lapeyrie *et al*., 1991). The available phosphate in the medium was not able to maintain the pH during the growth of *Pisolithus* sp. It is therefore inevitable to have controlled pH of growth medium in order to clearly identify the effect of pH on the growth of ECM fungi (Giltrap and Lewis, 1981; Yamanaka, 2003). However, there is no attempt made earlier to understand the effect of maintained pH on the growth of *Pisolithus* sp.. There are sporadic studies where initial pH of complex MMN medium was adjusted before inoculation (Gupta *et al*., 1997; Sundari and Adholeya, 2003). It is difficult to assess the effect of pH on the fungal growth on conventional complex culture media as they have low buffering capacities (Child *et al*., 1973). Attempts were made to incorporate the inert buffers in the medium to control pH during the growth of Pt and other ECM fungi, but observed that buffers such as ADA,

Table 1. Growth response and Total Phenolics elaboration by *Pisolithus tinctorius* PT1 with varying [Phosphate], pH, temperature, [NaCl], [PEG 6000], [Mn] and [Fe]

Growth Factor (Tested Range)	Range for Growth	Optimum Growth	Stress for Growth		Range for Total Phenolics Production	Optimum Phenolics Production
			Lower Limit	Upper Limit		
[PO4](0.007-0.1M)	0.007-0.087M	0.027M	<0.02M	>0.04M	0.027-0.087M	0.067M
pH(2.2-8.0)	3.0-7.0	4.0-4.2	≤3.0	≥7.0	4.0-7.0	7.0
Temperature(10-50°C)	25-42°C	25-30°C	<25°C	≥37°C	25-42°C	37°C
[NaCl](0-4%)	0-3%	0.01-1%	-	≥1.5%	0.05-2%	1.5%
[PEG 6000](0-35%)	0-10%	0%	-	>10%	0-10%	5-10%
[Mn](0-10000 ppm)	0-10000 ppm	500-2500 ppm	-	5000-10000 ppm	0-1.1ppm & 5000-10000 ppm	0-1.1 ppm
[Fe](0-100 ppm)	0-50 ppm	Variable response	-	≥50 ppm	0-45 ppm	Variable response

ACES, MES and PIPES interferes the metabolism of ECM fungi (Giltrap and Lewis, 1981; Hilger *et al*., 1986).

In vitro studies of *Pisolithus* sp. with reference to growth and pH regulation by phosphate is not yet reported. However, ECM fungi were inhibited at very low concentration of phosphate (Giltrap and Lewis, 1981; Marx and Zak, 1965). *Pisolithus tinctorius* PT1 grew well on buffers containing higher amount of phosphate (SPB and PCB) than on un-buffered control medium. HEPES, an inert buffer was neither able to support maximum growth nor resist the drop in pH, probably because of sub-optimal concentration of buffer (0.02 M) used in the medium. On the contrary, similar molarity of PCB could regulate the pH to 4.5 with stimulating the growth. The isolate showed similar growth on media with SPB and PCB ($P>0.05$). With increase in concentration of SPB and PCB there was no significant difference in growth obtained in the two buffers although there was decrease in growth by increasing molarity, which could be due to combined effect of pH and increasing phosphate molarity. The final pH with PCB was higher than the corresponding phosphate molarity of SPB. This is the first report where Phosphate-citrate buffer (0.047 M) was found suitable for growth without causing any inhibition and maintaining the pH as set initially, throughout the growth of fungus. Maximum phenolics were produced in agar medium containing PCB followed by SPB and HEPES while isolate grown on plain GMSM produced least phenolics.

The final pH of the growth medium with low phosphate concentration was found in acidic region where significant biomass was accumulated. The phosphate concentration of 0.067 M could regulate pH to initial pH 6.6. However, there was quite reduction in growth of the isolate. The growth on 0.087 M phosphate concentration was seen only after 20 days and was as feeble mycelia on inoculum disc.

Best growth of Pt on complex medium spanned over three pH units (Gupta *et al*., 1997). In the current study, *Pisolithus tinctorius* PT1. was monitored for effect of pH on growth using PCB (0.04 M) added to already existing 0.007 M phosphate in basal GMSM. The growth of isolate with varying pH was typically of bell shaped ($P<0.001$) (Fig. 1). Interestingly, the isolate showed growth at pH 3.0 only after 15 days of incubation but was very meager. This isolate although acidophilic in nature could tolerate and survive in the soils with acidic to neutral pH and low phosphorus conditions such as mining soil rejects.

Growth of *Pisolithus tinctorius* PT1 upto 3% NaCl indicated its capacity to tolerate high salt concentration. EC_{50} value of the isolate was found to be approximately 2.3%. *Pisolithus* species could grow above 1.2% NaCl concentration and suggested EC_{50} value for Pt could be well in excess of 1.2% NaCl (Chen *et al*., 2001; Matsuda *et al*., 2006). The growth of isolate with increasing NaCl concentration showed typical bell shaped curve ($P<0.001$). There was very good correlation between colony diameter on GMSM agar and biomass obtained in GMSM broth with varying amount of NaCl. Pt is seen to tolerate more amount of NaCl than other ECM fungi (Dixon *et al*., 1993; Bois *et al*., 2006). Interestingly, a coastal strain of Pt was found to be inhibited during *in vitro* growth with high concentration setting of sodium ions (Nagarajan and Natarajan, 1999). The wide distribution of present isolate of *Pisolithus tinctorius* PT1 along the west coast of India including mining sites and rain-forest could be due to its ability to tolerate the variable salt concentration found in such diverse ecosystems.

Mycorrhizal development is strongly temperature dependent (Mosse *et al*., 1981) and the tolerance in Pt of high temperatures may account for its predominance on mine spoils (Marx, 1975). Darkly pigmented ECM fungi like *Pisolithus* and *Cenococcum* have been found to be more tolerant to high temperatures (Cline *et al*., 1987). The growth patterns and phenolics accumulation were markedly affected by incubation temperature ($P<0.001$) and showed typical bell shaped curves. There was good correlation of colony diameter and biomass produced. Interestingly, the culture was viable at 10 and 15°C and the isolate responded when plates were shifted to incubation temperature to 28°C. Earlier studies reported growth of ECM fungi usually ranges between 25 to 37°C and found Pt as the most tolerant fungus (Gupta *et al*., 1997). This isolate can probably tolerate soil temperature higher than 42°C once in association with the host. Survival of fungus in soils having temperature higher than 50°C in mining sites could be due to

spores as mycelium of *Pisolithus tinctorius* PT1 was found dead at this temperature.

ECM fungi showed variable response to PEG induced water stress (Coleman *et al*., 1989, Zhang *et al*., 2011). *In vitro* growth of *Pisolithus* sp. to water stress is not yet carried out, although response to water stress of *Pinus pinaster* inoculated with dikayotic strains of *Pisolithus* sp. has been investigated and reported that sensitivity of seedlings to water stress depends on associated dikaryons (Lamhamedi *et al*., 1991). In present investigation *Pisolithus tinctorius* PT1 showed growth in mesic zone i.e., water potential ≤ -1.0 MPa created in the medium using PEG ($P<0.001$) (Fig. 2). This coastal isolate requiring high water potential is seen to play a role in drought resistance of trees on the mining site.

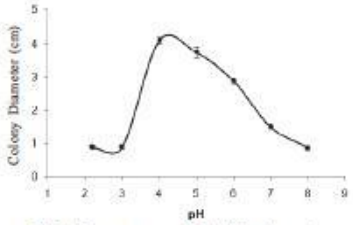

Fig. 1. Response curve of *Pisolithus tinctorius* PT1 in GMSM containing phosphate citrate buffer to maintain the different pH

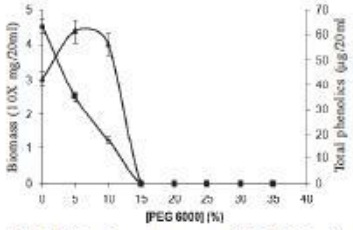

Fig. 2. Effect of varying amount of PEG 6000 in the medium on growth and accumulation of phenolics by *Pisolithus tinctorius* PT1 (■) Dry Biomass 10X mg/20 ml, (♦) Total Phenolics µg/20 ml

Pisolithus tinctorius has been demonstrated to help loblolly and shortleaf pine seedlings establishment in acid coal spoils having high contents of Fe and Mn (Marx and Artman, 1979). Isolate investigated in the current study, showed ectomycorrhizal synthesis with *A. mangium* growing extensively on iron ore mining rejects rich in Fe and Mn. The growth of Pt was not significantly affected at the highest tested Mn concentration of 500 ppm and type of Mn salt (Thompson and Medve, 1984). *Pisolithus tinctorius* PT1 actively responded to Mn concentration *in vitro* and appeared to be most Mn-tolerant (Fig. 3). Interestingly it could survive, tolerate and grow even at 10,000 ppm. This isolate possibly can tolerate even higher Mn concentration as its growth was not inhibited. *Pisolithus tinctorius* PT1 preferred ferric iron over ferrous. Further investigation on this may highlight the preference of ionic form of iron by ectomycorrhizal fungi. The growth pattern of the isolate in present study, under the influence of ferric iron is irregular and similar to that reported earlier (Tam, 1995). Large numbers of fruiting bodies of *Pisolithus* sp. were seen during onset of monsoons on iron ore mining sites of western India. The predominance of this fungus could be explained because of its tolerance to high manganese and ferric iron content.

Total phenolics production by *Pisolithus tinctorius* PT1 was influenced by the parameters that affect growth (Fig. 4). Present detailed study of phenolics production by *Pisolithus tinctorius*

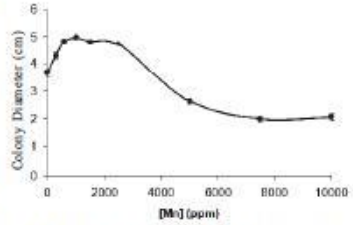

Fig. 3. Response curve of *Pisolithus tinctorius* PT1 to varying amount of manganese in GMSM

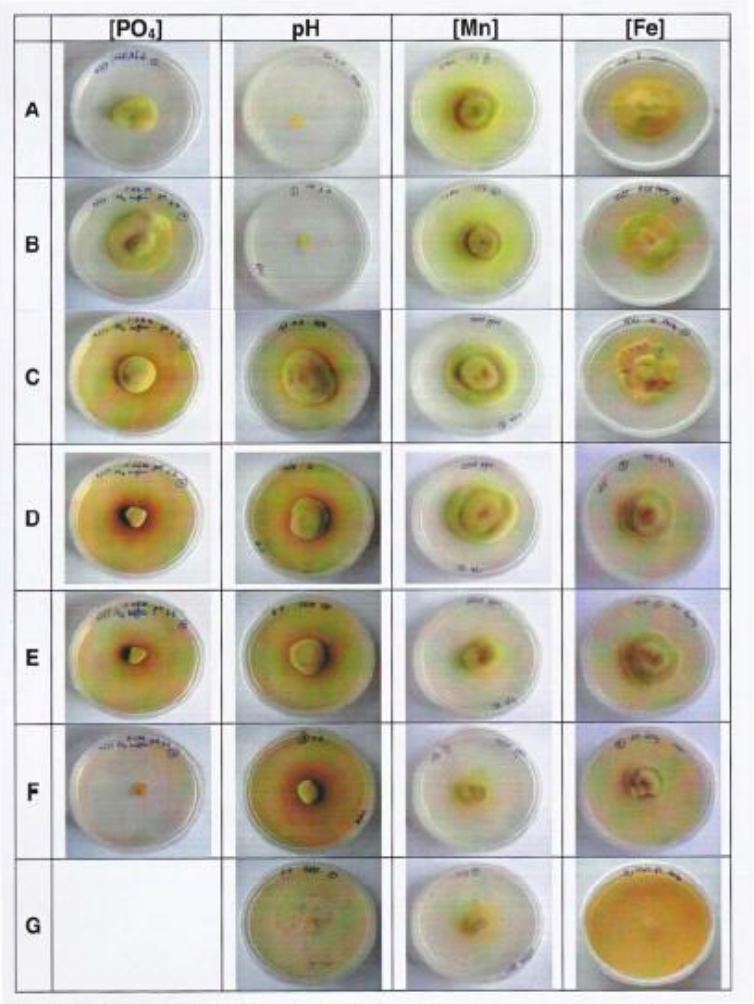

Fig. 4. Growth and phenolic liberation on GMSM agar medium by *Pisolithus tinctorius* PT1 with varying [phosphate], pH, [manganese] and [iron]. [Phosphate] A-0.007M, B-0.027M, C-0.047M, D-0.067M, E-0.087M, F-0.17M; pH A-2.2, B-3.0, C-4.0, D-5.0, E-6.0, F-7.0, G-8.0; [Manganese] A-0 ppm, B-1.1 ppm, C-1000 ppm, D-2500 ppm, E-5000 ppm, F-7500 ppm, G-10000 ppm; [Iron] A-0 ppm, B-2.121 ppm, C-10 ppm, D-40 ppm, E-45 ppm, F-50 ppm, G-60 ppm

PT1 revealed that the optima of all the variables for elaboration of phenolics were observed at higher values than that required for the maximum growth of isolate (Table 1). This indicated that the isolate produced more phenolics while growing under sub optimal conditions where it encountered the stress. Maximum phenolics production was observed at pH 7.0, 0.067 M phosphate, 1.5% sodium chloride, 5% PEG and temperature of 37°C. Thus, even abiotic stress on the *Pisolithus* sp. would induce phenolics elaboration. High amount of NaCl is known to affect the pigmentation of fungi. Certain strains of *Aspergillus* and *Penicillium* produce brightly coloured pigments. Intensification of pigmentation was frequently seen in many organisms at appropriate NaCl concentration (Tresner & Hayes, 1971).

Heavy metals have shown different effect on phenolics production. Decreasing or increasing the concentrations of Mn from optima for growth results in elaboration of phenolics by *Pisolithus tinctorius* PT1. Similar to variation in growth, elaboration of phenolics also showed variation in response to different amount of $FeCl_3$ in the medium. More secretion of phenolics at higher temperature and amount of metals need further investigation. This could have possible role in growth and survival at the stressed sites. Further, investigations are needed to identify the impact of interactions of these factors on growth and production of phenolics using statistical methods such as Response Surface Models.

In conclusion, the present investigation clearly demonstrated the use of chemically defined medium and identified the actual limits of growth conditions that could be tolerable or inhibitory to *Pisolithus tinctorius* PT1. This study revealed the optimum physico-chemical parameters for maximum growth and phenolics accumulation by *Pisolithus tinctorius* PT1for the first time. Results showed the wide tolerance of the isolate to various edaphic factors prevalent in mining region highlighting its potential in revegetation of disturbed mining sites. This *Pisolithus tinctorius* PT1 isolate appeared to be the most Mn-tolerant (tolerating ≥ 10,000 ppm Mn). This is first report showing any stress on *Pisolithus* sp. would induce phenolics production. Further investigation is required to understand the mechanism of tolerance towards edaphic stresses and optimization for phenolics production.

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REFERENCES

1. Bois, G., Bertrand, A.P., Fung, M., Khasa, D.P. Growth, compatible solute and salt accumulation of five mycorrhizal fungal species grown over a range of NaCl concentrations. *Mycorrhiza*., 2006; **16**: 99-109.
2. Cairney, J.W.G., Chambers, S.M. Interactions between *Pisolithus tinctorius* and its hosts: a review of current knowledge. *Mycorrhiza*., 1997; **7**:117-131.
3. Chen, D.M., Elhul, S., Herdman, K., Cairney, J.W.G. Influence of salinity on biomass production by Australian *Pisolithus* spp. isolates. *Mycorrhiza*., 2001; **11**: 231-236.
4. Child, J.J., Knapp, C., Eveleigh, D.E. Improved pH control of fungal culture media. *Mycologia*., 1973; **65**(5): 1078-1086.
5. Cline, M.L., France, R.C,. Reid, C.P.P. Intraspecific and interspecific growth variation of ectomycorrhizal fungi at different temperatures. *Can J Bot*., 1987; **65**: 869-875.
6. Coleman, M.D., Bledsoe, C.S., Lopushinsky, W. Pure culture response of ectomycorrhizal fungi to imposed water stress. *Can J Bot*., 1989; **67**: 29-39.
7. Cordell, C.E., Mans, L.F., Farley, M.E., Marx, D.H. Mycorrhizal fungi and trees – A successful reforestation alternative for mine land reclamation. In: Forest Nursery Proceedings, Kona, HI-2000, US Department of Agriculture Forest Service, Rocky Mountain Research Station, Fort Collins, US. 2000; pp 177-187.
8. Dixon, R.K., Rao, M.V., Garg, V.K. Salt stress affects *in vitro* growth and in situ symbioses of ectomycorrhizal fungi. *Mycorrhiza*., 1993; **3**: 63-68.
9. Duchesne, L.C., Peterson, R.L., Ellis, B.E. The time-course of disease suppression and

antibiosis by ectomycorrhizal fungus *Paxillus involutus*. *New Phytol*., 1989; **111**: 693-698.
10. Garg, S. Production of ectomycorrhizal fungal inoculum by submerged fermentation. Ph.D. Thesis, University of Delhi, New Delhi.1999.
11. Giltrap, N.J., Lewis, D.H. Inhibition of growth of ectomycorrhizal fungi in culture by phosphate. *New Phytol*., 1981; **87**: 669-675.
12. Greenberg, A.E., Trussell, R.R., Clesceri, L.S., Franson, M.A.H. Standard methods for the examination of water and waste water, 16th edn, American Public Health Association, Washington DC, USA. 1985; pp 556-570.
13. Gupta, V., Garg, S., Satyanarayana, T. Selection of ectomycorrhizal fungal strains for large scale inoculum production In: Microbial Biotechnology. Scientific Publishers, Jodhpur, India. 1997.
14. Hilger, A.B., Thomas, K.I., Krause, H.H. The effects of several buffers on growth and phosphorus nutrition of selected ectomycorrhizal fungi. *Soil Biol Biochem*., 1986; **18**: 61-67.
15. Khosla, B., Reddy, M.S. Response of ectomycorrhizal fungi on the growth and mineral nutrition of *Eucalyptus* seedlings in bauxite mined soil. American-Eurasian *J Agric Environ Sci*., 2008; **3**(1): 123-126.
16. Kope, H.H., Tsantrizos, Y.S., Fortin, J.A., Ogilvie, K.K. *p*-hydroxy benzoyl formic acid and R-(-)-*p*-hydroxymendalic acid, two antifungal compounds isolated from the liquid culture of ectomycorrhizal fungus *Pisolithus arhizus*. *Can J Microbiol*., 1991; **37**(4): 258-64.
17. Lamhamedi, M.S., Bernier, P.Y., Fortin, J.A. Growth, nutrition and response to water stress of *Pinus Pinaster* inoculated with ten dikaryotic strains of *Pisolithus* sp.. *Tree Physiology*., 1991; **10**: 153-167.
18. Lapeyrie, F., Ranger, J., Vairelles, D. Phosphate-solubilizing activity of ectomycorrhizal fungi *in vitro*. *Can J Bot*., 1991; **69**: 342-346.
19. Marx, D.H., Zak, B. Effect of pH on mycorrhizal formation of slash pine in aseptic culture. *For Sci*., 1965; **11**: 66-75.
20. Marx, D.H. The influence of ectotrophic mycorrhizal fungi on the resistance in pine roots to pathogenic infections I. Antagonisms of mycorrhizal fungi to root pathogenic fungi and soil bacteria. *Phytopathology*., 1969; **59**: 53-163.
21. Marx, D.H. Mycorrhizas and establishment of trees on stripmined land. *Ohio J Sci*., 1975; **75**: 288-297.
22. Marx, G.D., Artman, J.D. *Pisolithus tinctorius* ectomycorrhizae improve survival and growth of pine seedlings on acid coal spoils in Kentucky and Virginia. *Reclam Rev*., 1979; **2**: 23-31.
23. Matsuda, Y., Sugiyama, F., Nakanishi, S-II. Effects of sodium chloride on growth of ectomycorrhizal fungal isolates in culture. *Mycosciences*., 2006; **47**: 212-217.
24. Mosse, B., XStribley, D.P., Letacon, L. Ecology of mycorrhizae and mycorrhizal fungi. *Adv Microbiol Ecol*., 1981; **5**: 137-210.
25. Nagarajan, G., Natarajan, K. The use of Box-Behnken design of experiments to study *in vitro* salt tolerance by *Pisolithus tinctorius*. *World J Microbiol Biotechnol*., 1981; **15**: 197-203.
26. Suh, H-W., Crawford, D.L., Korus, R.A., Shetty, K. Production of antifungal metabolites by the ectomycorrhizal fungus *Pisolithus tinctorius* strain SMF. *J Industrial Microbiol Biotechnol*., 1991; **8**: 29-36.
27. Sundari, K.S., Adholeya, A. Growth profile of ectomycorrhizal fungal mycelium: emphasis on substrate pH influence. *Antonie van Leeuwenhoek*., 2003; **83**: 209-214.
28. Sylvia, D.M., Sinclair, W.A. Phenolic compounds and resistance to fungal pathogens induced in primary roots of Douglas-Fir seedlings by the ectomycorrhizal fungus *Laccaria laccata*. *Phytopathology*., 1983; **73**: 390-397.
29. Tam, P.C.F. Heavy metal tolerance by ectomycorrhizal fungi and metal amelioration by *Pisolithus tinctorius*. *Mycorrhiza*., 1995; **5**: 181-187.
30. Thompson, G.W., Medve, R.J. Effects of aluminium and manganese on the growth of ectomycorrhizal fungi. *Appl Environ Microbiol*., 1984; **48**(3): 556-560.
31. Tresner, H.D., Hayes, J.A. Sodium chloride tolerance of terrestrial fungi. *Appl Microbiol*., 1971; **22**(2): 210-213.
32. Yamanaka, T. The effect of pH on the growth of saprotrophic and ectomycorrhizal ammonia fungi *in vitro*. *Mycologia*., 2003; **95**(4): 584-589.
33. Zhang, H., Tang, M., Yang, Y. The response of ectomycorrhizal (ECM) fungi under water stress induced by polyethylene glycol (PEG) 6000. *African J Microbiol Res*., 2011; **5**(4): 365-373.

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Antagonistic Activity of Phenolics Produced by *Pisolithus* sp. PT1

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***Pisolithus* sp. PT1 was isolated from mushroom found in ectomycorrhizal association with *Acccie mengium* growing in mining affected region. The fungal isolate was characterised by morphological and molecular methods. The isolate produced brown phenolics compound in GMSM culture broth. The isolate produced more phenolics in the late growth phase and under submerged growth conditions. Antagonism activity of phenolics was prominent against *Fusarium* sp.. Spore pigment and culture filtrate with pH 3.0, but not at pH 5.0, significantly arrested the growth. *Macrophomina phaesiolus* and *Sclerotia rolsii* were not affected with any of the pigments. This study has given conclusive results that phenolics produced by this *Pisolithus* sp. PT1 isolate are potential antibiotic compounds and maximum production was between 20-60 days and under submerged growth conditions.**

Key words: Antagonism; phenolics; *Pisolithus* sp. PT1.

The ectomycorrhizal (ECM) fungus *Pisolithus tinctorius* (Pt) has wide global distribution and forms association with the roots of numerous hosts in various environments. Pt improves growth and survival of the host plant by not only providing nutrients and tolerating extreme edaphic factors, also protects the host roots against invasion by root-pathogenic soil fungi (Cairney and Chambers, 1997). Many researchers have investigated the antagonistic effect of pigments and other compounds produced by ECM fungi. Marx (1969) has reported antibiotic activity of ECM fungi to various root pathogenic fungi and soil bacteria. The synthesis of antifungal compounds by the ectomycorrhizal (ECM) fungi is demonstrated to protect the ECM plants against the root pathogens (Duchesne *et al*., 1989). Antagonistic compounds produced by ECM fungi are phenolics in nature (Sylvia and Sinclair, 1983). Kope *et al*., (1991) have identified two phenolics acids namely Pisolithin A [*p*-hydroxy benzoyl formic acid] and Pisolithin B [R-(-)-*p*-hydroxymendalic acid] from *Pisolithus tinctorius*. Tsantrizos *et al*., (1991) have demonstrated Pisolithin A and Pisolithin B to inhibit spore germination and cause hyphal lysis of phytopathogens and dermatogenic fungi. Thus the phenolic compounds produced by ECM fungi offer important chemical protection to host from pathogenic infections.

Kope and Fortin (1989) have demonstrated inhibition of phytopathogenic fungi by cell free culture media of seven ectomycorrhizal fungi and showed Pt to have the largest spectrum of activity. On the contrary, Marx (1969) reported Pt being ineffective as an antagonist to root

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pathogenic fungi. Suh *et al*., (1991) have attempted fermentative production of antifungal metabolites by Pt. However the detailed study on growth conditions affecting phenolics production in mineral medium is not yet reported.

The isolate in this present investigation obtained from iron ore mine land produced brown phenolics pigment. To evaluate the antibiotic potential of the phenolic compounds produced by the isolate it is necessary to carry out detailed study on phenolics production. This study is aimed to check the effect of growth conditions on liberation of phenolics by *Pisolithus* sp. PT1in chemically defined medium and its possible bioactivity against common soil borne fungal phytopathogens.

MATERIAL AND METHODS

Culture isolation and maintenance

Basidiocarps that were in mycorrhizal association with *Acacia mangium* were collected from iron ore mine at Codli, Goa. Sporocarps were washed with sterile water. Sporocarp was surface sterilised with 50% sodium hypochlorite solution and cut opened aseptically. Inner tissue of mushroom was placed onto the modified Melin Norkrans medium (MMN, pH 6.5) and incubated at room temperature (RT) (Marx, 1969). Pure fungal mycelia were maintained by periodic transfer on MMN and Glucose Mineral Salt Medium (GMSM, pH 6.0) (Garg, 1999). Composition of GMSM: basal medium (1L) 20.0 g glucose, 0.1 g KCl, 1.0 g NH_4Cl, 0.1 g NaCl, 1.0 g KH_2PO_4, 0.3 g $MgCl_2.4H_2O$, 0.132 g $CaCl_2.2H_2O$, 10.53 mg $FeSO_4.7H_2O$, 37.52 mg Na_2EDTA, 100 µg Thiamine HCl, 40 µg Biotin, 10 ml micronutrient solution and 20.0 g agar powder. Ten ml micronutrient solution consists of 2.784 mg H_3BO_3, 3.38 mg $MnSO_4.H_2O$, 97 µg $CuSO_4.5H_2O$, 201 µg $ZnSO_4.7H_2O$, 338.46 µg $Na_2MoO_4.2H_2O$ and 240 µl H_2SO_4. Initial pH of the basal medium was adjusted using 0.1 N KOH solution.

Test organisms

Fungal phytopathogens used as test organisms were *Fusarium* sp., *Macrophomina phaeasiolus* and *Sclerotia rolsii*. The fungal isolates were periodically maintained on PDA plates. All the isolates were obtained from ICAR Research complex for Goa, Old Goa, Goa.

Culture identification

Morphological characteristics of mushrooms were noted. Transverse sections of the sporocarps were prepared. Mycelial growth and morphology of dried spores were examined microscopically. Basidiospores were sputter coated with gold and examined with a JEOL, 5800LV, Japan, Scanning Electron Microscope (SEM).

Molecular identification of the isolated mycelia was performed using sequence data of the ITS region of the nuclear ribosomal DNA. Total genomic DNA was extracted from 100mg fresh mycelium according to CTAB method as described by Gardes and Bruns (1993) and Graham *et al*. (1994). Samples were first ground in lysis buffer (200 mM Tris- HCl, pH 8.0, 100 mM NaCl, 25mM EDTA, 0.5% SDS) using sterile micro pestle and then incubated in water-bath at 65°C for 1 hour. 5% CTAB buffer (100 mM Tris- HCl, pH 8.0, 1.4 M NaCl, 5% CTAB, w/v) was added to the solution and placed in water-bath at 65°C for 10 minutes. The solution was then extracted at least twice with equal volume of phenol:$CHCl_3$:isoamylalcohol (25:24:1, v/v) and centrifuged at 12,000 rpm for 10 minutes, followed by extraction with $CHCl_3$:isoamylalcohol (24:1, v/v) until the top phase was clear. The upper aqueous phase was transferred to new tube and DNA was precipitated with equal volume of isopropanol. The DNA was washed with 70% ethanol, dried and resuspended in 100 µl TE buffer (10 mM Tris-HCl, pH 8.0, 10 mM EDTA). Genomic DNA was visualised in 0.7% Agarose gel. The concentration and purity of DNA was determined by measuring absorbance at 260 nm and 280 nm using UV-2450 UV- visible spectrophotometer.

The ITS region of rDNA was amplified using the primer pair ITS 1 and ITS 4 (White *et al*., 1990) the amplification was carried out using BIOERXP thermal cycler in a 50 µl reaction mixture containing 1 µl genomic DNA, 10X PCR buffer, 1.5 mM MgCl2, 10 mM dNTPmix, 1mM of each primers. The reaction mixture was initially denatured at 94 °C for 3 minutes and then subjected to 40 cycles of 1 minute each at 94 °C, 52 °C and 72 °C, and final extension step of 5 minutes at 72 °C. A control reaction without template was run to detect the presence of any contaminated DNA. The amplified products were electrophoresed in 0.8% (w/v) agarose gel, stained with ethidium bromide

fluorescing under UV light. The sequencing of amplified rDNA was done by Bangalore Genei, India. Sequence was compared against database using NCBI BLAST search (http://www.ncbi.nlm.nih.gov/entrez). Sequences were aligned and NJ tree was constructed using ClustalX2 programme. Final tree was obtained using MEGA4 software.

Production of phenolics compounds

The total phenolics production by *Pisolithus* sp. was checked using GMSM broth. Each 100 ml conical flask containing 20 ml medium was inoculated with a single inoculum agar piece (10x8x5 mm) with fungal mycelia growing on the MMN and incubated at RT under stationary conditions over a period of 60 days. Fungal biomass and culture broth was harvested on 0, 20, 40 and 60 days. In another set of experiment, inoculated media were incubated at RT under stationary and submerged (100 rpm) conditions. Fungal biomass and culture broth was harvested after 30 days of incubation. Four replicates were maintained for each experimental condition.

Fungal masses were collected and washed with sterile distilled water before keeping in pre-weighed aluminium cups at 80°C. Dry weights were recorded till constant weights of cups were obtained. Total phenolics content in the culture broth was estimated by 4-Aminoantipyrine method (Greenberg *et al*., 1985). UV-Visible spectra of culture broth were prepared using UV-Visible Spectrophotometer.

Antagonism studies

Antibiotic activity of pigments of *Pisolithus* sp. isolate was checked on PDA plates. Water extracted sporocarp pigment (pH 5.0), culture filtrate (pH 3.0) and culture filtrate (pH 5.0) were tested for antibiotic activity against fungal plant pathogens. Water extract of sporocarp pigment was prepared by mixing 0.5 g dried sporocarp powder in 25 ml distilled water. The solution was kept standing overnight at 4°C. The upper clear solution was decanted in another tube and pH of the solution was measured. Similarly culture broth of *Pisolithus* sp. isolate grown in GMSM liquid medium at RT was harvested after 30 days and pH was measured. The pH of an aliquot of culture broth was adjusted to 5.0 using KOH solution. The pigment solutions were filtered through 0.45m syringe filter and used for assay. An arc was cut on PDA plates using sterile scalpel at about 2.3 cm from the centre. An agar piece (10x10 mm) of test culture previously grown on PDA was placed in the centre of the plate. 300 µl sterile pigment sample was added in the arc. Control plate was maintained with the test culture and arc without the pigment. Plates were incubated at RT for 8 days and radial growth was noted.

Statistical Analysis

Impact of parameters and significant differences between treatments were assessed by analysis of variance (ANOVA) at $P \leq 0.001$ and treatment means were compared by least significant difference ($P \leq 0.05$) using Student-Newman-Keuls Method.

RESULTS AND DISCUSSION

Culture characteristics

Sporocarps of *Pisolithus* sp. were of 1.0-11.5 cm in diameter, rounded or club-shaped, yellowish smooth shiny surface, with deeply rooted fibrous base (Fig. 1A). A Transverse Section reveals yellowish brown spore sacs (peridioles) developing in a black gelatinous matrix. Mature dry spores were of 7.5-8.5 µm in diameter, cinnamon brown, globose and bearing triangular shaped flattened curved spines (platelet spines) as seen under SEM (Fig. 1B). These characteristics match the results reported by Kope and Fortin (1990). *Pisolithus* sp. PT1 isolate grew as golden yellowish mycelium on agar medium and showed dense growth on GMSM as compared to conventional MMN. The mycelia were septate with clamp connections. Phylogenetic analyses clearly revealed its evolutionary relatedness with *Pisolithus tinctorius* (Fig. 2).

Production of Phenolic compounds

Studies on production of total phenolics by *Pisolithus* sp isolate *in* the culture broth were checked for two parameters. Isolate when grown over a period of 60 days, biomass accumulation and total phenolics elaboration increased over the time (Fig. 3). UV-Visible spectra of culture broth show the increment of peak at 257 nm with the incubation period (Fig. 4). UV spectrophotometric method is satisfactory as each group of phenolic compounds is characterized by one or several UV absorption maxima (Pink *et al*., 1994). Simple

phenolics have absorption maxima in the region between 220 and 280 nm (Owades *et al.*, 1958). However, closely related phenolics display wide variations in their molecular absorptivities. The steep increase in biomass and phenolics production was observed from 0-20 and 20-40 days, respectively. This indicated that phenolics were produced more when the growth of the isolate was slow. The isolate produced 10% more phenolics under submerged conditions as compared to stationary growth conditions (Fig. 5). Biomass accumulation was 16% less than that obtained under stationary conditions. This could be due to shear stress encountered by the isolate when subjected to shake conditions.

Antagonism studies of *Pisolithus* sp. PT1

Antagonistic activity of culture filtrate of ECM fungi is not new. Similarly various ECM fungi have shown to inhibit *Fusarium* sp., but this study is the first to report the production of extracellular phenolics in chemically defined medium and antagonistic effect on two phytopathogens viz. *Macrophomina phaeziolus* and *Sclerotia rolsii*. In the present study the pigments of *Pisolithus* sp. PT1showed differential response against the test organisms (Fig. 6). *M.*

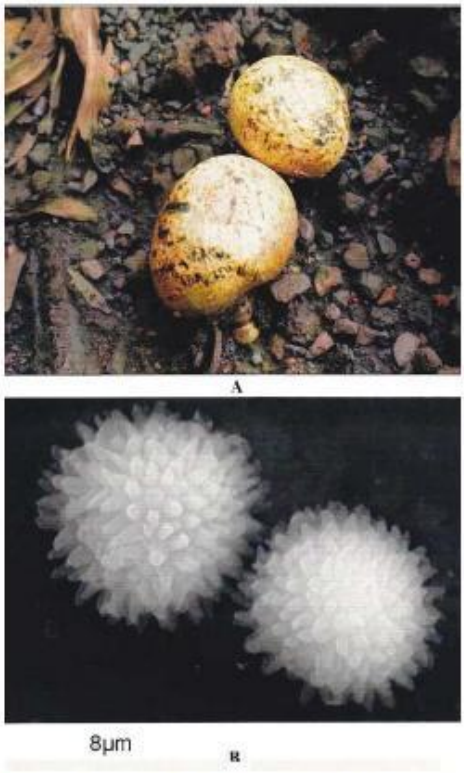

Fig. 1. Basidiocarp (A) and Dry spore of *Pisolithus* sp.PT1 observed under SEM revealing platelet curved spines(B)

phaeziolus was not affected with any of the pigments. Although, *S. rolzii* showed growth on all the plates, plates treated with water extracted basidiocarp pigment and culture filtrate with pH 5.0 showed enhanced sclerotia formation and inhibited aerial growth of the fungus. Whether these changes in the growth of fungus are due to the effect of pigments, are not explainable. *Fusarium* sp. was the most affected test organism. Spore pigment and culture filtrate with pH 3.0 significantly arrested the growth of the fungus. This result is in accordance with that reported by Yamaji *et al*., (2004) that showed antifungal activity of phenolics produced by *Paxillus* sp. 60/92 against *Pythium vexans* under acidic culture condition (pH 3-4). Although *M. Phaeziolus* and *S. rolzii* were not inhibited by the pigments of *Pisolithus* sp. PT1, the effect on *Fusarium* sp. suggests its possible antagonistic potency.

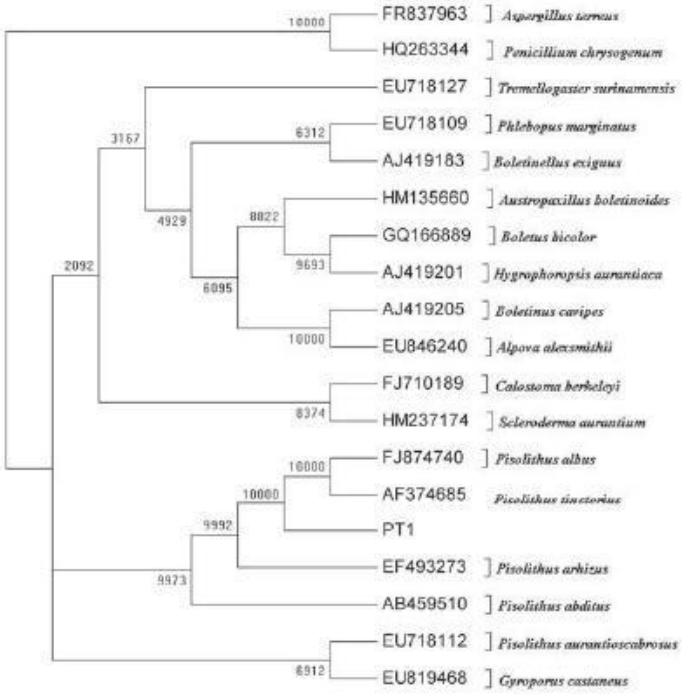

Fig. 2. Phylogenetic position of *Pisolithus* sp. PT1isolate. Tree inferred by maximum likelihood analysis based on rDNA sequences, including ITS1, 5.8S and ITS2 regions *Penicillium chrysogenum* and *Aspergillus terreus* were used as outgroups. The numbers below the branches indicate the percentage at which a given branch was supported in 1000 bootstrap replications

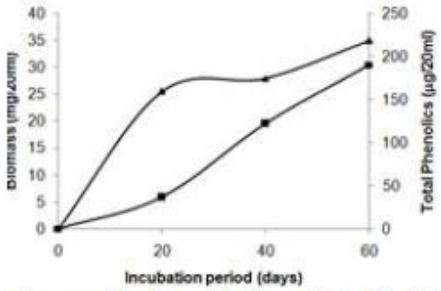

Fig. 3. Growth response of *Pisolithus* sp. PT1and accumulation of phenolics in GMSM over a period of 60 days (*%) Dry Biomass mg/20 ml, (f&) Total Phenolics µg/20 ml

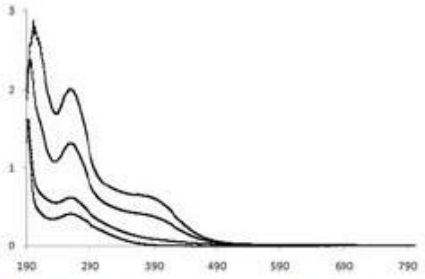

Fig. 4. UV-visible spectra of culture broth of *Pisolithus* sp. PT1 grown in GMSM and harvested at 0, 20, 40 and 60 days

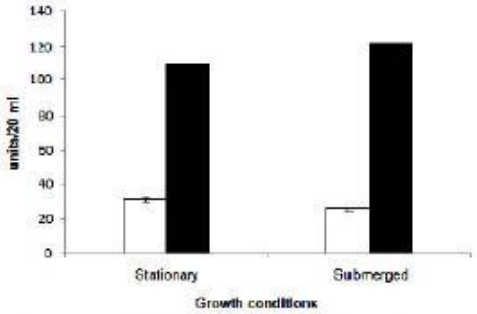

Fig. 5. Growth of *Pisolithus* sp.PT1 and accumulation of phenolics in GMSM under stationary and submerged condition (;%) Dry Biomass mg/20 ml, (%) Total Phenolics µg/20 ml

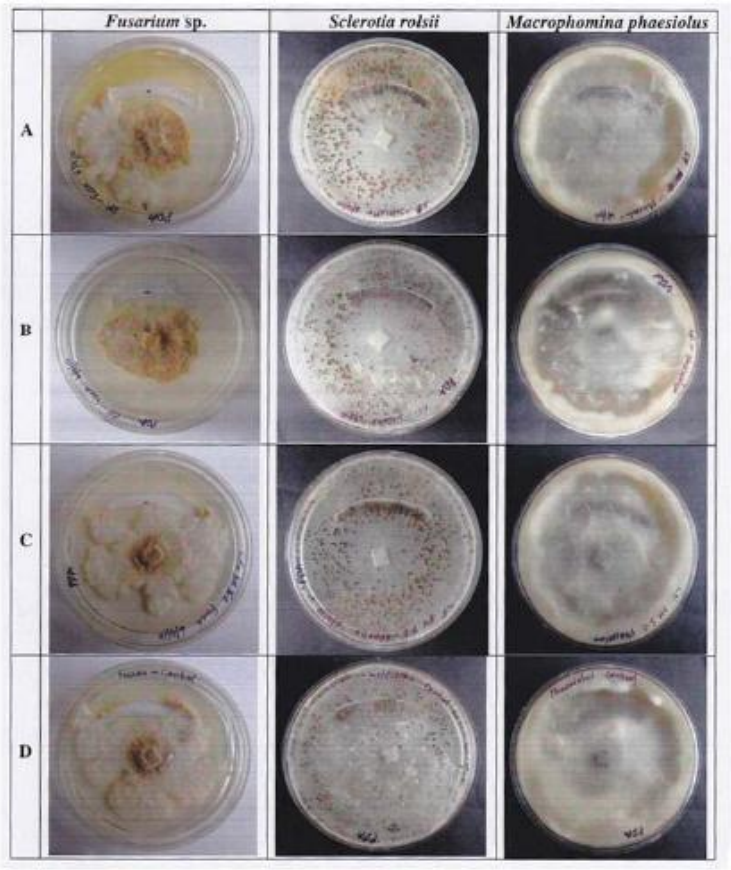

Fig. 6. Antagonistic effect of *Pisolithus* sp. PT1 on *Fusarium* sp., *Sclerotia rolsii* and *Macrophomina phaesiolus*, A-water extracted sporocarp pigment (pH 5.0), B- Culture filtrate (pH 3.0), C- Culture filtrate (pH 5.0), D- Control PDA plate

CONCLUSION

The present work has been taken up with a view to explore the potential of *Pisolithus* sp. PT1 isolate obtained from iron ore mines for phenolics production. The isolate produced significantly more amount of phenolics after 20 days and under submerged growth condition. The water soluble spore pigment and the phenolics produced in the culture broth at pH 3.0 but not at

pH 5.0 significantly arrested the growth of *Fusarium* sp.. This suggests that bioactivity of phenolics produced by ECM fungi could be pH dependent. Thus the results obtained in this investigation suggest the ecological stability of associated host plants in the mining region and possible industrial application of the isolate.

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REFERENCES

1. Cairney, J.W.G., Chambers, S.M. Interactions between *Pisolithus tinctorius* and its hosts: a review of current knowledge. *Mycorrhiza*., 1997; **7**: 117-131.
2. Pink, D., Naczk, M., Baskin, K., Shahidi, F. Theoretical analysis of Ultraviolet-Visible Spectra of various Phenolic Acid Fractions of Canola *J. Agric. Food Chem*., 1994; **42**: 1317-1322.
3. Duchesne, L.C., Peterson, R.L., Ellis, B.E. The time-course of disease suppression and antibiosis by ectomycorrhizal fungus *Paxillus involutus*. *New Phytol*., 1989; **111**: 693-698.
4. Gardes, M., Bruns, T. D. ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. *Mol. Ecol*., 1993; **2**: 113-118.
5. Garg, S. Production of ectomycorrhizal fungal inoculum by submerged fermentation, Ph.D. Thesis, University of Delhi, New Delhi.1999.
6. Graham, G.C., Mayers, P., Henry, R.J. A simplified method for preparation of fungal genomic DNA for PCR and RAPD analysis. *Biotechniques*., 1994; **16**: 48-50.
7. Greenberg, A.E., Trussell, R.R., Clesceri, L.S., Franson, M.A.H. Standard methods for the examination of water and waste water, 16th edn, American Public Health Association, Washington DC, USA. 1985; pp 556-570.
8. Kope, H.H., Tsantrizos, Y.S., Fortin, J.A., Ogilvie, K.K. *p*-hydroxy benzoyl formic acid and R-(-)-*p*-hydroxymendalic acid, two antifungal compounds isolated from the liquid culture of ectomycorrhizal fungus *Pisolithus tinctorius*. *Can J Microbiol*., 1991; **37**(4): 258-64.
9. Kope, H.H., Fortin, J.A. Germination and comparative morphology of basidiospores of *Pisolithus arhizus*. *Mycologia*., 1990; **82**(3): 350-357.
10. Kope, H.H., Fortin, J.A. Inhibition of phytopathogenic fungi in vitro by cell free culture media of ectomycorrhizal fungi. *New Phytol*., 1989; **113**: 57-63.
11. Marx, D.H. The influence of ectotrophic mycorrhizal fungi on the resistance in pine roots to pathogenic infections I. Antagonisms of mycorrhizal fungi to root pathogenic fungi and soil bacteria. *Phytopathology*., 1969; **59**: 53-163
12. Owades, J. L., Rubin, G., Brenner, M. W. Determination of food tannins by ultraviolet spectrophotometry. *J. Agric. Food Chem*., 1958; **6**: 44-47.
13. Suh, H-W., Crawford, D.L., Korus, R.A., Shetty, K. Production of antifungal metabolites by the ectomycorrhizal fungus *Pisolithus tinctorius* strain SMF. *J of Industrial Microbiol Biotechnol*., 1991; **8**: 29-36.
14. Sylvia, D.M., Sinclair, W.A. Phenolic compounds and resistance to fungal pathogens induced in primary roots of Douglas-Fir seedlings by the ectomycorrhizal fungus *Laccaria laccata*. *Phytopathology*., 1983; **73**: 390-397.
15. Tsantrizos, Y.S., Kope, H.H., Fortin, J.A., Ogilvie, K.K. Antifungal antibiotics from *Pisolithus tinctorius*. *Phytochemistry*., 1991; **30**(4): 1113-1118.
16. White, T. J., Bruns, T., Lee, S., Taylor, J.W. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols: A Guide to Methods and Applications, eds. Innis, M. A., D. H. Gelfand, J. J. Sninsky, and T. J. White. Academic Press, Inc., New York. 1990; Pp. 315-322.
17. Yamaji, K., Ishimoto, H., Usui, N., Mori, S. Organic acids and water soluble phenolics produced by *Paxillus* sp. 60/92 together show antifungal activity against *Pythium vexans* under acidic culture conditions. *Mycorrhiza*., 2005; **15**: 17-23.