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).
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 50°C.
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$_2$O 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° 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 80°C. 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 to 11.5 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*. 
**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).
**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. 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 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 tinctorius* 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 37⁰C 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 28-30⁰C. Interestingly, there was no significant difference in the biomass obtained at 25 to 30⁰C (*P*>0.05). PT1 showed growth between 25-37⁰C. These findings are in accordance with that reported by Gupta et al. (1997). Growth at 37 and 42⁰C was significantly lower than at 25 to 30⁰C (*P*<0.05). PT1 tolerated and survived at the temperature of 15 and 42⁰C. The culture was viable at 15 and 42⁰C as the isolate responded when plates were shifted to RT (Fig.2.11). Growth was completely inhibited at 10 and 45⁰C. This isolate can probably tolerate soil temperature higher than 42⁰C 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 46⁰C. Interestingly, isolate recovered its growth after 24-48 h of incubation at 28⁰C.
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, $L$. 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 up to 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 up to 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

<table>
<thead>
<tr>
<th>GROWTH FACTOR (TESTED RANGE)</th>
<th>RANGE FOR GROWTH</th>
<th>OPTIMUM GROWTH</th>
<th>STRESS FOR GROWTH</th>
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<td>[PO₄] (0.007-0.1M)</td>
<td>0.007 - 0.087M</td>
<td>0.027M</td>
<td>&lt;0.02M &gt;0.04M</td>
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<td>pH (2.2-8.0)</td>
<td>3.0 - 7.0</td>
<td>4.0 - 4.2</td>
<td>≤3.0 ≥7.0</td>
</tr>
<tr>
<td>TEMPERATURE (10-50⁰C)</td>
<td>25 - 42⁰C</td>
<td>25 - 30⁰C</td>
<td>&lt;25⁰C ≥37⁰C</td>
</tr>
<tr>
<td>[NaCl] (0-4%)</td>
<td>0 - 3%</td>
<td>0.01 - 1%</td>
<td>ND ≥1.5%</td>
</tr>
<tr>
<td>[PEG 6000] (0-35%)</td>
<td>0 - 10%</td>
<td>0%</td>
<td>ND &gt;10%</td>
</tr>
<tr>
<td>[Mn] (0-10000 ppm)</td>
<td>0 - 10000 ppm</td>
<td>500 - 2500 ppm</td>
<td>ND 5000 - 10000 ppm</td>
</tr>
<tr>
<td>[Fe] (0-100 ppm)</td>
<td>0 - 50 ppm</td>
<td>VARIABLE RESPONSE</td>
<td>ND ND</td>
</tr>
</tbody>
</table>

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 thermitolerance (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-metallothionineins 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 or 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.