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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 42°C. 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, 37°C 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 50ºC. 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 60º 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 80ºC. 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.

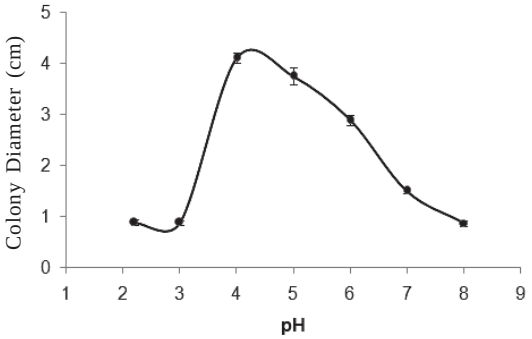

Fig. 1. Response curve of *Pisolithus tinctorius* PT1 in GMSM containing phosphate citrate buffer to maintain the different pH

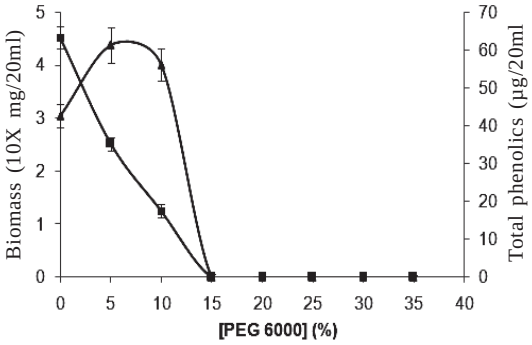

Fig. 2. Effect of varying amount of PEG 6000 in the medium on growth and accumulation of phenolics by *Pisolithus tinctorius* PT1 (■) Dry Biomass 10X mg/20 ml, (♦) Total Phenolics µg/20 ml

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*

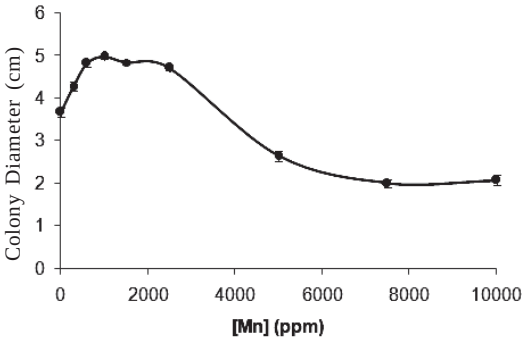

Fig. 3. Response curve of *Pisolithus tinctorius* PT1 to varying amount of manganese in GMSM

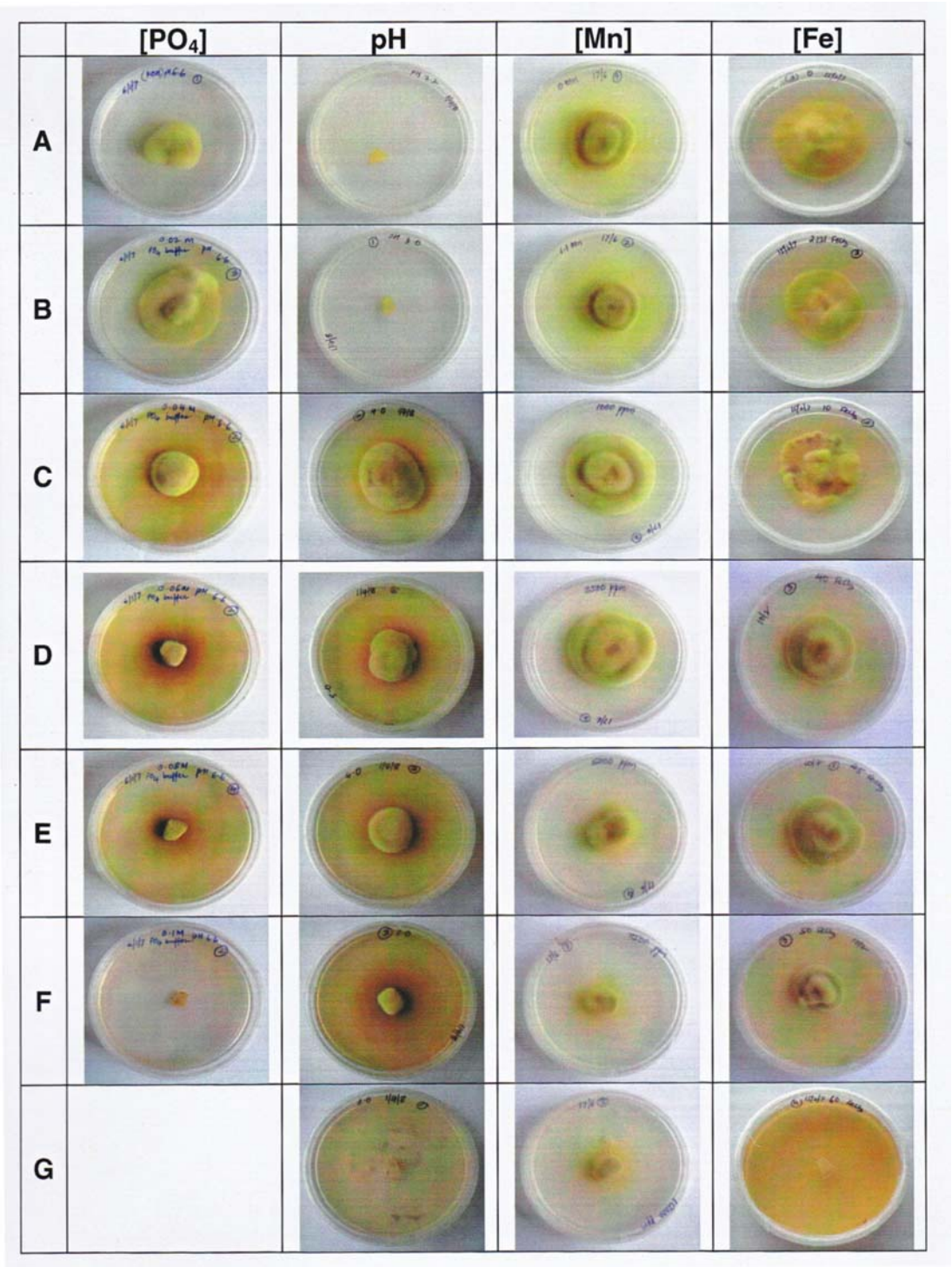

Fig. 4. Growth and phenolic liberation on GMSM agar medium by *Pisolithus tinctorius* PT1 with varying [phosphate], pH, [manganese] and [iron]. [Phosphate] A-0.007M, B-0.027M, C-0.047M, D-0.067M, E-0.087M, F-0.17M; pH A-2.2, B-3.0, C-4.0, D-5.0, E-6.0, F-7.0, G-8.0; [Manganese] A-0 ppm, B-1.1 ppm, C-1000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