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

Lakshangy J. Chari, Saroj N. Bhosle and Sandeep Garg*

Department of Microbiology, Goa University, Taliegao Plateau, Goa - 403 206, India.

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***Pisolithus* sp. PT1 was isolated from mushroom found in ectomycorrhizal association with *Acacia mangium* 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

* To whom all correspondence should be addressed.
Tel.: +91-832-6519362/280(Off.) 832-6630717 (Res.)
Fax: +91-832-2451184/2452889
Email: Sandeep@unigoa.ac.in;
Sandeep_garg68@yahoo.co.in

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 phaesiolus* 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 dNTP mix, 1mM of each primers. 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. 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 80ºC. 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 4ºC. 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 $P<0.001$ and treatment means were compared by least significant difference ($P<0.05$) 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 phaesiolus* and *Sclerotia rolsii*. In the present study the pigments of *Pisolithus* sp. PT1showed differential response against the test organisms (Fig. 6). *M.*

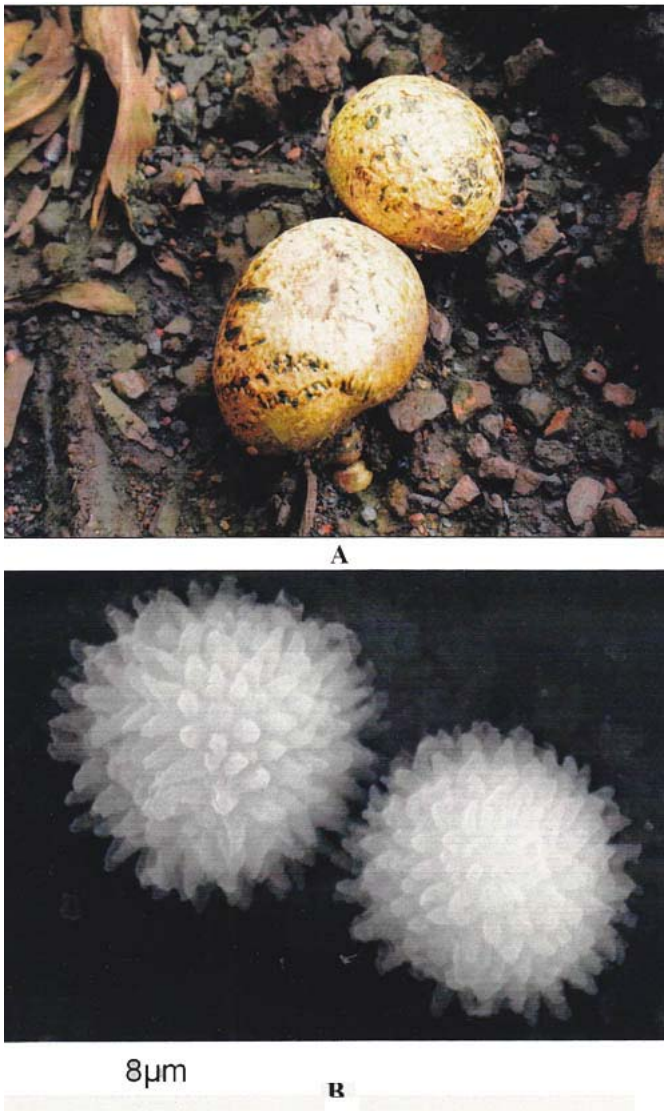

Fig. 1. Basidiocarp (A) and Dry spore of *Pisolithus* sp.PT1 observed under SEM revealing platelet curved spines(B)

phaesiolus was not affected with any of the pigments. Although, *S. rolsii* 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. Phaesiolus* and *S. rolsii* were not inhibited by the pigments of *Pisolithus* sp. PT1, the effect on *Fusarium* sp. suggests its possible antagonistic potency.

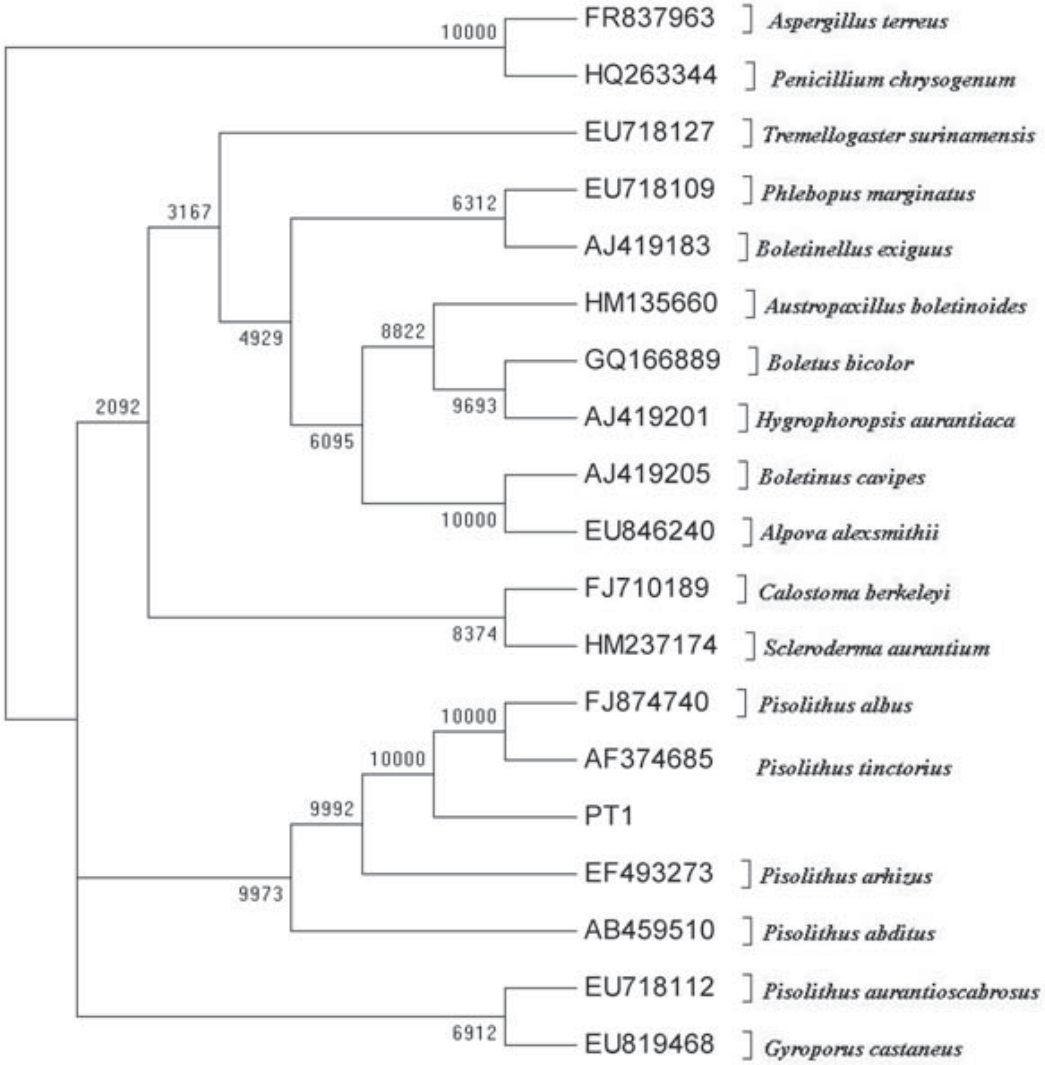

Fig. 2. Phylogenetic position of *Pisolithus* sp. PT1isolate. Tree inferred by maximum likelihood analysis based on rDNA sequences, including ITS1, 5.8S and ITS2 regions *Penicillium chrysogenum* and *Aspergillus terreus* were used as outgroups. The numbers below the branches indicate the percentage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

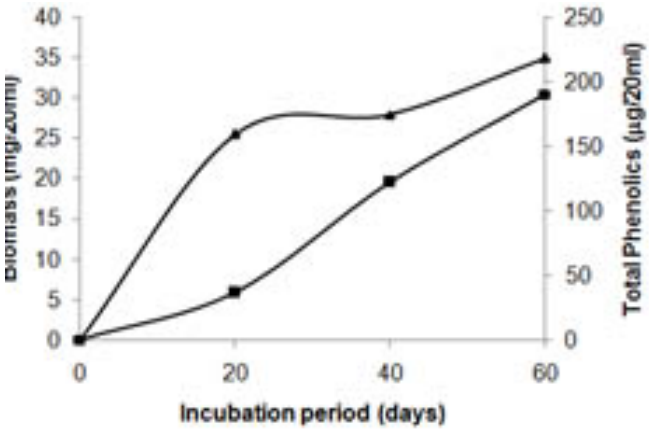

Fig. 3. Growth response of *Pisolithus* sp. PT1and accumulation of phenolics in GMSM over a period of 60 days (²%) Dry Biomass mg/20 ml, (f&) Total Phenolics µg/20 ml

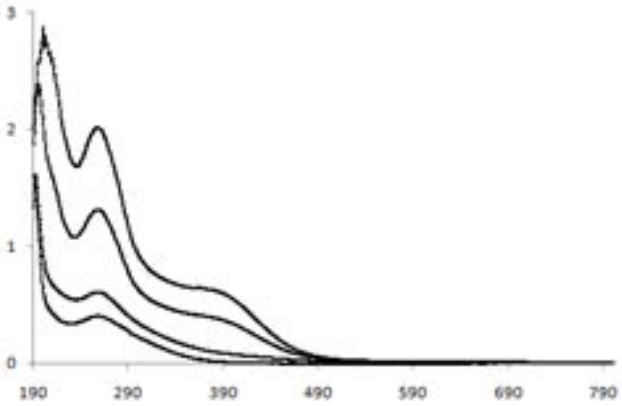

Fig. 4. UV-visible spectra of culture broth of *Pisolithus* sp. PT1 grown in GMSM and harvested at 0, 20, 40 and 60 days

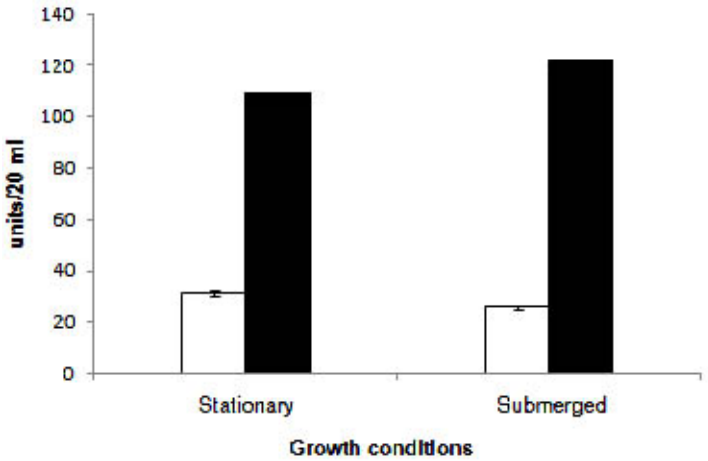

Fig. 5. Growth of *Pisolithus* sp.PT1 and accumulation of phenolics in GMSM under stationary and submerged condition (¡%) Dry Biomass mg/20 ml, (%) Total Phenolics µg/20 ml

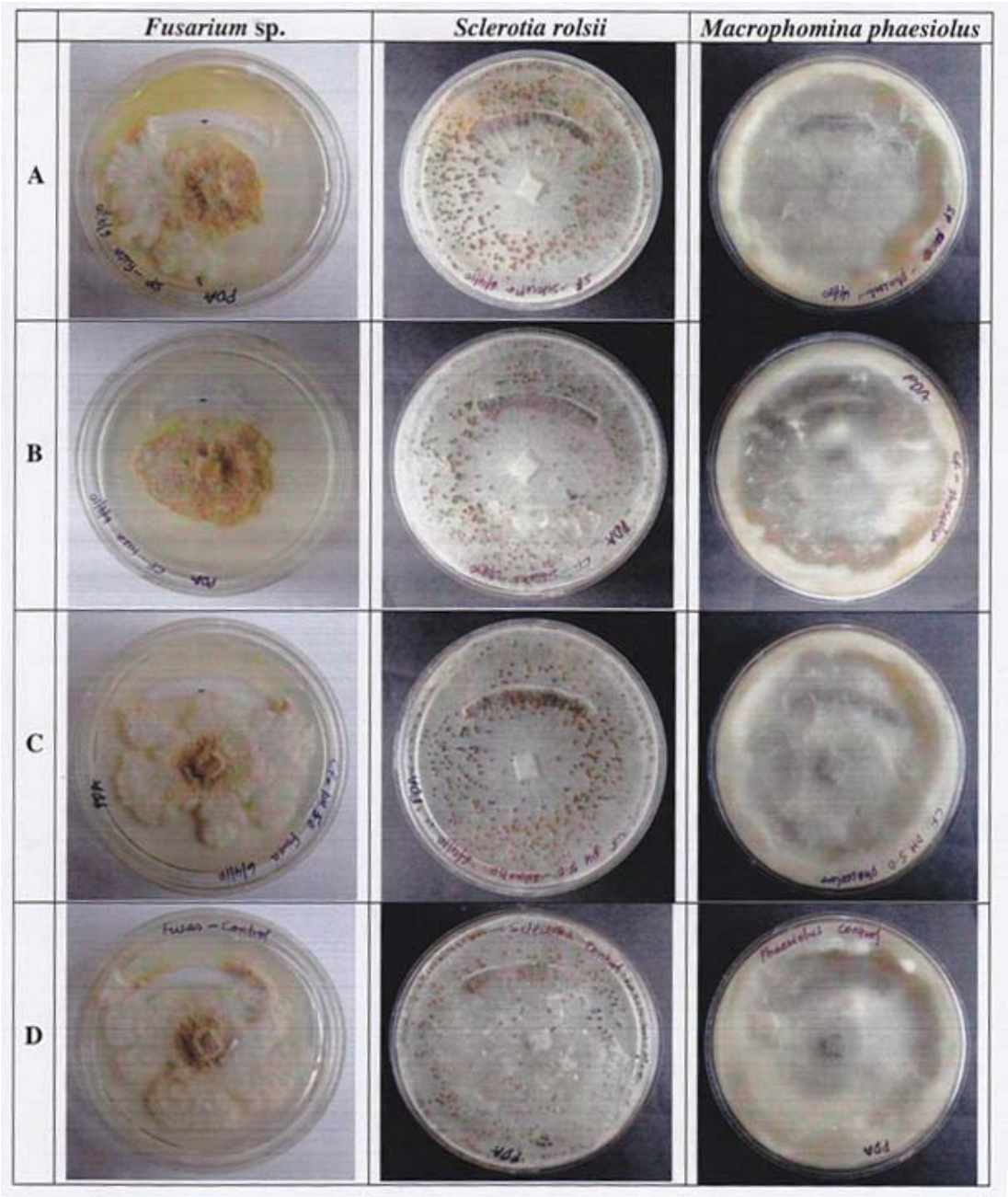

Fig. 6. Antagonistic effect of *Pisolithus* sp. PT1 on *Fusaium* sp., *Sclerotia rolsii* and *Macrophomina phaesiolus*, A-water extracted sporocarp pigment (pH 5.0), B- Culture filtrate (pH 3.0), C- Culture filtrate (pH 5.0), D- Control PDA plate

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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