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Heavy metal-resistance in fungi isolated from serpentine soils of Andaman

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Eighteen fungal isolates belonging to the genera of *Penicillium, Aspergillus, Mortierella, Trichoderma* and *Rhizopus* were isolated from serpentine soils of north and south Andaman Islands and resistance to Ni^{+2}, Co^{+2} and Cr^{+6} and Cr^{+6} was determined with respect to growth and germination of spores. In general, the serpentine fungi were resistant to Ni^{+2} and Co^{+2} but sensitive to Cr^{+6}. About 88.9 % and 66.7 % of the isolates showed a relative growth > 50 % at 50 mg/ml Ni^{+2} and Co^{+2} respectively while Cr^{+6} at 25 mg/ml inhibited 77.8 % of the isolates. Similarly, germination of spores was adversely affected by Cr^{+6} (22.6 % germination at 25 mg/ml) while Ni^{+2} and Co^{+2} at similar concentrations were well tolerated by the germinating spores. *Penicillium lilacium* F 104 and *Mortierella humilis* F604, the most potent metal-resistant fungi showed a metal resistance profile of Co > Cu > Ni > Zn > Cd > Pb > Hg > Cr and Ni > Co > Zn > Cd > Pb > Cr > Hg respectively.

Key words : Fungi, serpentine soil, Andaman island, metal-resistance

INTRODUCTION

Serpentine soils, derived from weathering of ultramafic rocks, are rich in ferromagnesium minerals but are poor in essential nutrients like N. P. K. and Ca that are required for growth of plants and microorganisms. These soils contain characteristically high levels of heavy metals like nickel, chromium and sometimes cobalt (Brooks, 1987). Microbial density as well as microbial activity in serpentine soils of New Caledonia is reported to be low as compared to agricultural soil (Amir and Pineau, 1998a), but the serpentine microorganisms are well adapted to heavy metals like Ni, Co and Cr (Schlegel *et al.*,1991 ; Stoppel and Schlegel, 1995 ; Mengoni *et al.*, 2001). These metal enriched soils, thus provides information on interaction of microbes with toxic metals and microbial resistance to elevated levels of heavy metals in such naturally metal-percolated ecosystem. Heavy metal-resistant fungi have been previously isolated and extensively studied from soils as well as anthropogenically metal polluted areas (Nakamura, 1962 ; Bewley, 1981 ; Titus and Pfister, 1984 ; Richards *et al.*, 2002). Such strains are able to survive and grow at high concentration of metal ions by variety of intrinsic or induced characters of metal-resistance (Babich *et al.*, 1982a). These included metal ion sequestration, biotransformation of toxic metals or binding of metal ions to cell wall and prevent their translocation into the cytoplasm (Hughes and Poole, 1989).

There are fewer reports on the mycoecological survey of serpentine soils. Mass and Stuntz (1969) collected and characterized 279 fungi from serpentine sites of Cascade Mountains, Washington, USA while Panaccion *et al.* (2001) reported the diversity of ectomycorrhizal fungi from serpentine plots of Soldiers Delight Natural Environment Area, Maryland, USA. Metal-resistance potential of fungi from New Caledonian ultramafic soils were compared with those from non-serpentine soil. Fungal species of serpentine outcrops showed greater metal-resistance ability than bacterial including actinomycetes as well as fungi from agricultural soils (Amir and Pineau, 1998b).

Occurence of serpentine outcorps in north and south Andaman Islands of India is well established. Geological studies of these ultramafic rocks and soils are not uncommon (Roy *et al.*, 1988 ; Halder, 1984 ; Vohra *et al.*, 1989 ; Jafri *et al.*, 2003 ; Pal *et al.*, 2003) but microbiological studies have not been done so far. The present study reports for the first time the isolation and ocurrence of heavy metal-resistant fungi from serpentine soils of Andaman, India.

MATERIALS AND METHODS

Fungal strains and cultivation conditions

A total of 18 fungi isolated from serpentine soils of Andaman, India were obtained from Microbiology Laboratory, Department of Botany, University of Calcutta, The strains were grown on slopes of Czapeck Dox Agar at 28°C for 3-4 days and maintained in the same medium by subculturing at regular intervals.

Screening of fungi for metal resistance

Screening of fungi for resistance to Ni^{+2}, Co^{+2} was determined in 100 ml Erlenmeyer flask containing 20 ml Czapeck Dox broth. The broth was supplemented with 1 ml of sterile metal solution prior to inoculation. Stock metal solutions (1 mg/ml) were prepared by dissolving exact quantities of metal salts in double distilled water and sterilized at 15 p.s.i. for 15 min. For preparation of inculum each isolate was grown on Czapeck Dox Agar plates for 7 days. Circular disces of fungal growth were cut with sterile cock borer (8 mm diameter) from the periphery and transferred to each flask containing 20 ml of sterile broth. The flasks were incubated at 28°C for 5 ays. Mycelial mat was harvested, washed and filtered on pre-weighed filter paper and dry weight of the mycelial mat was determined by drying it to constant weight at 80°C. Relative growth was calculated considering the growth in control set as 100.

Effect of metals on germination of spore

The effect of Ni^{+2}, Co^{+2} and Cr^{+6} on germination of gungal spores was tested in Czapeck Dox broth. Culture tube containing 5ml of sterile media supplemented with Ni^{+2},Co^{+2} and Cr^{+6} (25-50 g/ml) was inoculated with 0.1 ml of homogeneous spore suspension prepared in 0.1 % (w/v) sterile Tween 80 soultion. The tubes were incubated at 28°C for 12-24 h and spore germination was scored microscopically by the visible germ tube formation.

Effect of metals on radial mycelial growth

The effect of Ni^{+2}, Co^{+2}, CD^{+2}, Cu^{+2}, Pb^{+2}, Hg^{+2} and Zn^{+2} on the radial mycelial growth of selected metal-resistant fungi was tested. Fungal isolates were grown on Czapeck Dox Agar medium for 2-4 days and circular plugs of fungal growth were cut from the periphery with sterial crok borer (8 mm) and transferred (with fungal growth uppermost) to the center of petriplates containing the same medium amended with a desired concentration of metal. The plates were incubated at 28°C and the diameter of the colony was measured at an interval of 24 th of 8 days. Ni^{+2}, Co^{+2}, Cu^{+2}, Cu^{+2} and Hg^{+2} were used as chloride salt, while Cr^{+6}, Pb^{+2} and Zn^{+2} were used as K_2CrO_4, $Pb(NO_3)_2$ and $ZnSO_4$ respectively.

All experiments were performed in duplicates and average results were recorded.

RESULTS AND DISCUSSION

During the course of our survey for heavy metal-resistant microoorganisms from sèrpentine soils of Andaman, a total of 18 fungi were isolated from soils of north as well as south Andaman Islands. The metal content of these soils ranged between 396-8033 mg of Ni, 50-533 mg of Co and 571-4436 mg of Cr per kg dry soil. The fungal isolates were subjected to micromorphological studies and identified following the Manual of Soil Fungi (Gilman, 1954) and Manual of Penicillia (Raper and Thom, 1984). The isolates correspond to the most common genera of *Penicillium* spp. (7 isolates), *Aspergillus* spp. (1 isolate). Ultramafic soils of New Caledonia have also been reported to be dominated by fungi belonging to Moniliaceae and the genera *Aspergillus* and *Penicillium* accounted for more than 50% of the microflora (Amir and Pineau, 1998a).

Screening of fungi for metal resistance

Since Ni^{+1}, Co^{+2} and Cr^{+6} are prevalent in serpentine soil, resistance of fungi to these three metals was determined in Czapeck Dox broth at a concentration of 25-50 μg/ml. Results as shown in Fig. 1 have indicated that among the three metals tested, Cr^{+6} appeared to be most toxic and inhibited 77.8 % (14 isolates) of the fungi at a concentration of 25 μg/ml. Similar cases of inhibition of fungi were reported in presence of 10-100 ppm Cr^{+6} (Babich *et al.*, 1982b). *Penicillium vermiculatum* and *Trichoderma viridae* were the exceptions showing resistance to 100 ppm and 1000 ppm Cr^{+6} respectively. In the present study only *Mortierella* F604 showed 25.87 % and 7.54 % relative growth at 25 and 50 μg/ml. Cr^{+6} respectively. As expected, most of the serpentine fungi were resistant to Ni^{+2} and Co^{+2}. About 88.9 % and 66.7 % of the isolates showed a relative growth > 50 % at 50 μg/ml. Ni^{+2} and Co^{+2} respectively. According to Amir and Pineau (1998b) fungi from serpentine outcrops of New Caledonia were resistant to Ni^{+2} as well as Co^{+2} compared to that of bacteria and actinomycetes.

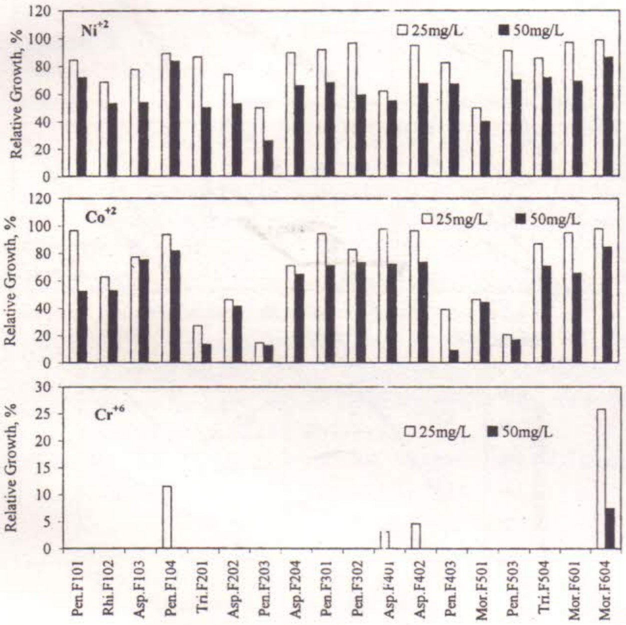

Fig. 1 : Screening of serpentine fungal isolates for resistance against Ni+2, Co+2 and Cr+6. (Pen = *Penicillum*, Asp = *Aspergillus*, Mor = *Mortierella*, Tri = *Trichoderma*, Rhi = *Rhizopus)*

Germination of fungal spores

Germination of spores of serpentine fungi in Czapeck Dox broth with inhibited by the presence of Ni^{+2}, Co^{+6} and Cr^{+6} (Fig 2). Spores apeared to be most sensitive to Cr^{+6} (22.6 % germination at 25 μg/ml). On the other hand, Ni^{+2} and Co^{+2} at similar concentrations were well tolerated by the spores during germination. *Penicillium* F104 and *Mortierella* F604 were resistant to Ni^{+2} and Co^{+2} showing >75 % spore germination efficiency at 50 μg/ml. But their germination percentage diclined significantly in presence of 25 μg/ml of Cr^{+6}. The toxic effects of Cr^{+6} on mycelial growth as well as spore germination are in good agreement with those absorved by Babich *et al.*, (1982b). Spore germination as completely inhibited at 50 ppm Cr^{+6} in *P. vermiculatum* and *Aspergillus gigateus.* According to Amir and Pineau (1998b), Ni^{+2} appeared to inhibit spore germination at concentration of 10 μg/ml and Co^{+2} proved to be the least toxic metal up to 20 μg/ml.

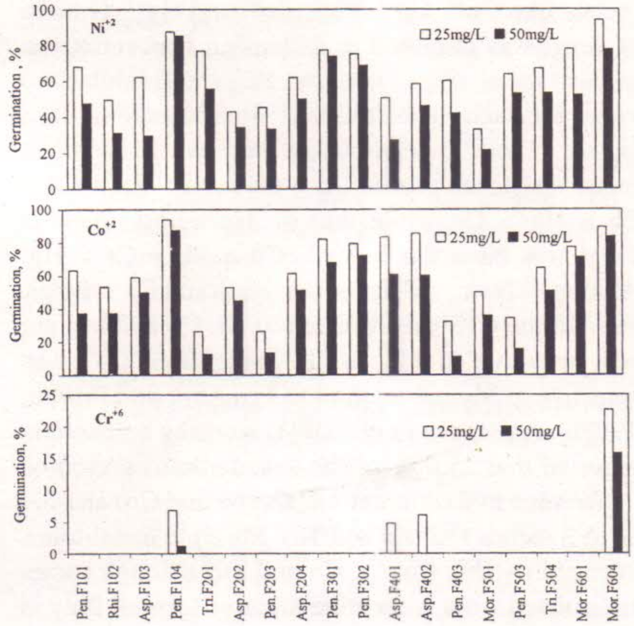

Fig. 2 :Effect of Ni^{+2}, Co^{+6} on the germination of spores of sepentine fungi. (Pen = *Penicillum*, Asp = *Aspergillus*, Mor = *Mortierella*, Tri = *Trichoderma*, Rhi = *Rhizopus)*

Selection of potent metal-resistant fungi

From the results as observed in Figs. 1 and 2 it was evident that fungi from serpentine soils of Andaman were mostly resistant to Ni^{+2} and Co^{+2} but sensitive to Cr^{+6}. Only 4 isolates (22.3 %) were resistant to all 3 metals tested. These observations confirm those of Amir and Pineau (1998b) although they found stimulation of growth of *Aspergillus* C6 and *Aspergillus* C15 in presence of heavy metal ions. Moreover, against a particular metal the response of some fungi with respect to growth and germination of spores were opposite. During the present study growth response of all fungi corresponds to germination of spore against a particular metal ion. *Penicillium* F104 and *Mortierella* F604 were selected as potent metal-resistant fungi and identified as *Penicillium lilacium* F104 and *Mortierella humilis* F604 respectively, according to the Mannual of Penicillia (Raper and Thom, 1984) and the Manual of Soil Fungi (Gilman, 1954).

Effect of metals on redial mycelial growth

Penicillium F104 and *Mortierella* F604 were compared for their redial mycelial growth in Czapeck Dox Agar plates suplemented with increasing concentrations of Ni^{+2}, Co^{+2}, Cr^{+6} as well as other heavy metals like Cu^{+2}, Cd^{+2}, Pb^{+2}, Zn^{+2} and Hg^{+2}. Growth of fungi was inhibited at increasing concentrations of each metal (Fig. 3) but the IC_{50} (50% inhibition) value for each metal differed significantly in each isolate (Table 1). *Penicillium lilacium* F104 has a metal resistance profile Co > Cu > Ni > Zn > Cd > Pb > Hg > Cr, while that of *Mortierella humilis* F604 was Ni > Co > Zn > Cd > Pb > Cr > Hg. Among New Caledonian ultramafic fungi, *Penicillium* C18 was resistant to Ni, Co and Mg but was sensitive to Mn, while *Penicillium* C37 was sensitive to Mg but tolerant to Fe (Amir and Pineau, 1998b). Anisinova *et al.* (1993) working on bacteria reported that 25.6 % of the tested strains presented a tolerance to 2 different metals (Ni and Co) and 6.4 % to 3 metals (Ni, Zn and Te). Multiple metal tolerance ability (Ni, Co, Cr, Cu and Zn) amongh bacteria isolated from serpentine areas of Central Italy is not uncommon (Mengoni *et al.* 2001).

Table 1 : IC_{50} values of some selected heavy metals to metal-resistant *Penicillium* F104 and *Mortierella* F604

Heavy metal	IC50, µg/ml	
	Penicillium F104	*Mortierella* F604
Ni^{+2}	233.2	288.7
Co^{+2}	285.7	255.7
Cr^{+6}	9.0	47.0
Cu^{+2}	277.5	165.5
Cd^{+2}	103.7	183.2
Pb^{+2}	95.0	95.0
Zn^{+2}	135.0	245.0
Hg^{+2}	12.5	26.3

These findings indicate that the metal resistance profile of fungi from Serpentines of Andaman is in good agreement with those reported earlier. Moreover, the data presented here seem to indicate the existance of different combination of genetic determinants for heavy matal-resistance including co-resistance to multiple metals in these fungi. Finally, it may be mentioned that these studies have been carried out under simple laboratory conditions as compared to the actual complexity of ultramafic soils and need further exploration of metal-resistant fungi which can be utilized as possible biotechnological tools for biorecovery of metals from waste waters and soils. Moreover, research on the genetic and molecular basis of heavy metal-resistance could be an interesting perspective towards understanding the physiology of adaption of fungi to metal toxicity in naturally metal percolated ecosystem.

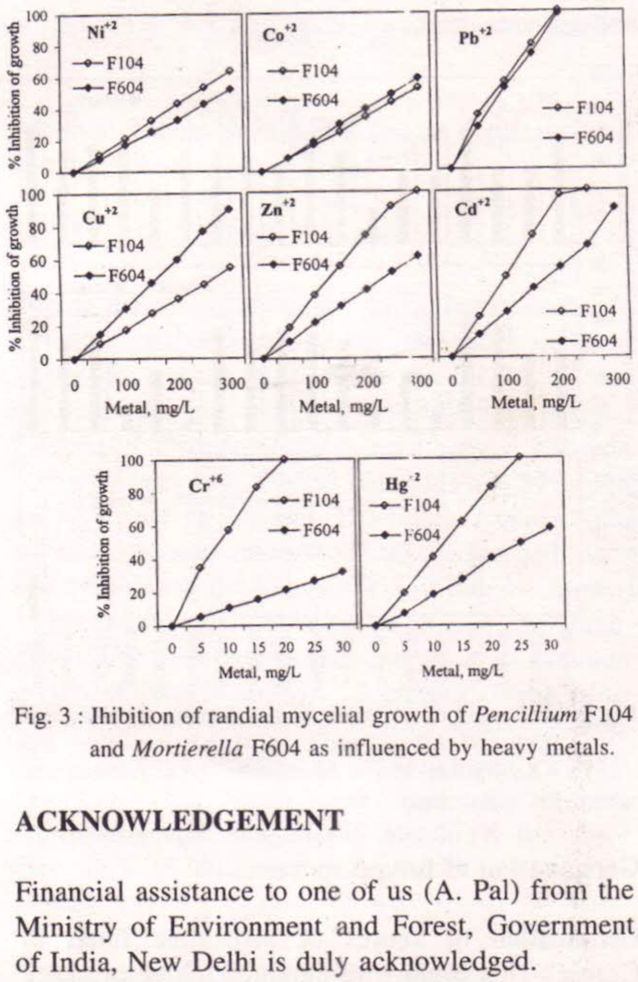

Fig. 3 : Ihibition of randial mycelial growth of *Pencillium* F104 and *Mortierella* F604 as influenced by heavy metals.

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