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Metal resistance of halotolerant fungi from mangroves and salterns of Goa, India

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ABSTRACT

Filamentous salt-tolerant fungi were isolated from mangroves and salterns of Goa, India; *Aspergillus* and *Penicillium* species were predominant amongst these. The isolates were found to have high levels of tolerance to sodium chloride and showed resistance to salts of heavy metals Pb^{2+}, Cu^{2+} and Cd^{2+}. Species belonging to *Penicillium* showed highest levels of halotolerance as well as resistance to heavy metals, the single isolate of a triverticillate morphotype of *Penicillium* obtained, showing the highest resistance. The aspergilli also displayed fairly high halotolerance as well as metal resistance, with isolates of *A. niger* and *A. flavus* showing higher levels of resistance. The other isolates belonged to the genera *Paecilomyces*, *Fusarium, Alternaria* and *Cladosporium.* Of these, *Paecilomyces* showed a fair amount of halotolerance; the others showed lower tolerance. Resistance to the heavy metals tested was poor in the isolates obtained from these genera. Amongst the isolates obtained, those of *Aspergillus* having a radiate, biseriate spore arrangement, and the single triverticillate morphotype of *Penicillium* displayed a higher degree of resistance to heavy metals. It is suggested that mechanism of resistance in these fungi could be phylogenetically related.

Keywords: Halotolerant; metal resistant; filamentous fungi, mangroves, salterns.

INTRODUCTION

The estuaries in Goa, India, are lined with mangroves and salterns in part, and serve as waterways for barges carrying iron ore, as well as other water traffic. They are, therefore, exposed to metal contamination from metal dust from the barges, run-offs from ore beneficiation plants, as well as from exhaust fumes and /or other industrial or mechanical activities. Since metals are not degradable, they accumulate in the environment. Exposure to metals also leads to the establishment of resistant or tolerant microbial populations, fungi being more tolerant to metals than bacteria or actinomycetes (Gadd, 1993). Such organisms also become important indicators of pollution. These fungi are also subject to selective pressure of the saline environment of the estuary and mangroves and the hypersaline solar salterns, and thus become halotolerant: able to tolerate the presence of high sodium chloride salt concentrations but growing best at concentrations below 0.2M, or halophilic: requiring salt for their optimal growth (Kushner 1978).

Most of the heavy metal salts form aqueous solutions and cannot be easily separated by physical means of separation, nor can they be decomposed by *in situ* biological means (Laws, 1993). Microorganisms that are able to survive in these conditions, are seen as agents of metal pollution control (Wood and Wang, 1983; Vinita and Radhanath, 1992; Vieira and Volesky, 2000; Ezzouhri et al., 2009).

The present work describes the halotolerance, as well as resistance to heavy metal ions such as Pb^{2+}, Cu^{2+} and Cd^{2+}, by fungi from mangroves and salterns of Goa, India, and presents a comparative analysis of halotolerance and metal resistance in the different genera of fungi, and within the different species or morphotypes of a given genus.

MATERIALS AND METHODS

Isolation of microorganisms

Samples of water from mangroves at Ribander (MRw) and salterns near Panjim (SP), were collected in sterile containers and then plated for isolation of organisms on Czapek-Dox Agar (CzA) + 2% NaCl, as given earlier (Marbaniang and Nazareth, 2007). Sediment samples from mangroves (MRs), were suspended in sterile saline (1g in 10 mL) and shaken well; the supernatant was then treated as the water samples. Isolates were picked up based on apparent dissimilarity of cultural characteristics and purified. The purified isolates were identified according to their genera on the basis of cultural characteristics such as nature of growth, spore colour and pigment production when grown on CzA, and on morphological characteristics of mycelia and fruiting bodies (Domsch et al., 1980; Raper and Fennell 1965).

Screening for halotolerance and for metal resistance

The fungal isolates were screened for salt tolerance by spot inoculation on to CzA plates containing 0, 2.0, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5 and 20.0% NaCl concentrations and observed for growth at 30°C up to 7 days. They were then grown in CzB with the above salt

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concentrations and growth was assessed in terms of dry weight of filtered, washed mycelia.

The isolates were screened in similar manner for resistance to metals on S-CzA medium containing 0, 2.5, 5.0, 7.5 and 10 mM Pb^{2+} as $Pb(NO_3)_2$, or 0, 1, 2, 3, 4, 5 mM Cu^{2+} as $CuSO_4.5H_2O$, or 0 to 5 mM Cd^{2+} as $3CdSO_4.8H_2O$ or as $Cd(NO_3)_2.4H_2O$. The maximum tolerance concentration (MTC) used in the experiment at which growth was obtained was plotted as a mean of values obtained in triplicate.

Culture and morphology changes in response to heavy metals

Isolates were selected on basis of their high resistance to metals and according to their group subdivision in case of *Aspergillus*, or their morphotype in case of *Penicillium* species, and observed for changes in culture characteristics when grown in presence of heavy metals.

RESULTS

Fungi isolates from mangroves and salterns

Fungi were selected from a total of 55 isolates, based on apparent cultural dissimilarity, with particular reference to colony appearance and spore colour: the mangrove isolates were identified as belonging to the genus *Aspergillus* (7), *Penicillium* (4) and *Paecilomyces* (1); the genera picked from salterns were *Aspergillus* (9), *Penicillium* (12), *Paecilomyces* (3), *Fusarium* (2), *Alternaria* (3) and *Cladosporium* (1).

Halotolerance of the isolates

While all the isolates tested could grow in absence of added NaCl, tolerance curves (Fig 1A) indicated that optimal growth of many were obtained with addition of 2.0 % salt. Most of the isolates could tolerate NaCl concentrations between 8 – 10 % and some up to 15%; some *Penicillium* isolates showed tolerance to 17.5% NaCl; the MTC of NaCl by the isolates, is shown in Fig. 1B.

Resistance to Heavy Metals

The cultures showed a decrease in growth with increasing concentration of heavy metals; the MTC of metals is shown in Fig. 2.

Good resistance was exhibited by all the isolates to a minimum of 5.0 mM Pb^{2+} as $Pb(NO_3)_2$. Most of the *Aspergillus* and *Penicillium* were resistant to 7.5 mM, and some aspergilli were resistant even to 10.0 mM.

Many of the isolates showed resistance to Cu^{2+} in the form of $CuSO_4.5H_2O$, with an MTC of 0.5 – 2.0 mM Cu^{2+}; two of the copper-tolerant aspergilli exhibited an MTC of 3 mM Cu^{2+} and four of the penicilli resisted Cu^{2+} levels in the range of 3 - 5 mM.

Seven of the *Penicillium* isolates could grow in the presence of Cd^{2+} as $Cd(NO_3)_2$ salt, five of these at an MTC of 3-5 mM, and six in the presence of Cd^{2+} as $CdSO_4$ salt, three at an MTC of 3-4 mM; of these, three isolates SP-18, SP-19 and SP-35 showed a resistance to both salts of cadmium. Two *Paecilomyces* isolates MR-15 and SP-51 also grew in the presence of Cd^{2+} as nitrate salt at an MTC of 0.5 mM and *Alternaria* SP-54 at 1 mM of Cd^{2+} as sulphate salt. *Aspergillus, Fusarium, Cladosporium* isolates showed no resistance to Cd^{2+} either as nitrate or as sulphate salt.

A comparative study of the metal resistance of the isolates indicated that only *Penicillium* was resistant to cadmium salt(s), and that the aspergilli and penicilli showed greater resistance to the heavy metals of lead and copper screened, than the other genera, with the isolates from mangroves demonstrating a greater metal resistance than those from salterns. Furthermore, a variation in metal resistance was evidenced between the different species within a given genus and was therefore analyzed with respect to the species of *Aspergillus*, or the morphotypes of *Penicillium*. Amongst the aspergilli, the highest resistance to Cu^{2+} at 3 mM was obtained by isolates of *A. niger* and *A. flavus*, the latter also showing the most resistance to Pb^{2+} at 10mM concentration. Likewise, amongst the penicilli, the greatest metal resistance was demonstrated by the single terverticillate (T) morphotype, followed by that of the biverticillate asymmetric (BA) and the biverticillate symmetric (BS), with the monoverticillate (M) showing a lesser resistance.

Change in morphology in response to heavy metals

Isolates of *A. niger* and *A. flavus*, and *Penicillium* monoverticillate, biverticillate and terverticillate morphotypes, showed colony changes such as decrease in growth and/or conidiation, as well as marked changes in micromorphological characteristics: thickened cell walls and bulging mycelia, as also loss of intracellular material, germination of conidia into mycelial strands while still attached to the vesicle; this was seen particularly in response to the presence of copper and cadmium salts, and to a lesser degree in presence of lead. The more prominent changes in morphology of some of the isolates possessing high levels of metal resistance, are indicated in Fig. 3.

DISCUSSION

The isolates obtained from the mangroves and salterns of Goa, India, showed a predominance of *Aspergillus* and *Penicillium* cultures, in keeping with diversity seen in saline environments (Abdel-Hafez, 1981; Radwan et al., 1984; Nayak et al., 2012). The

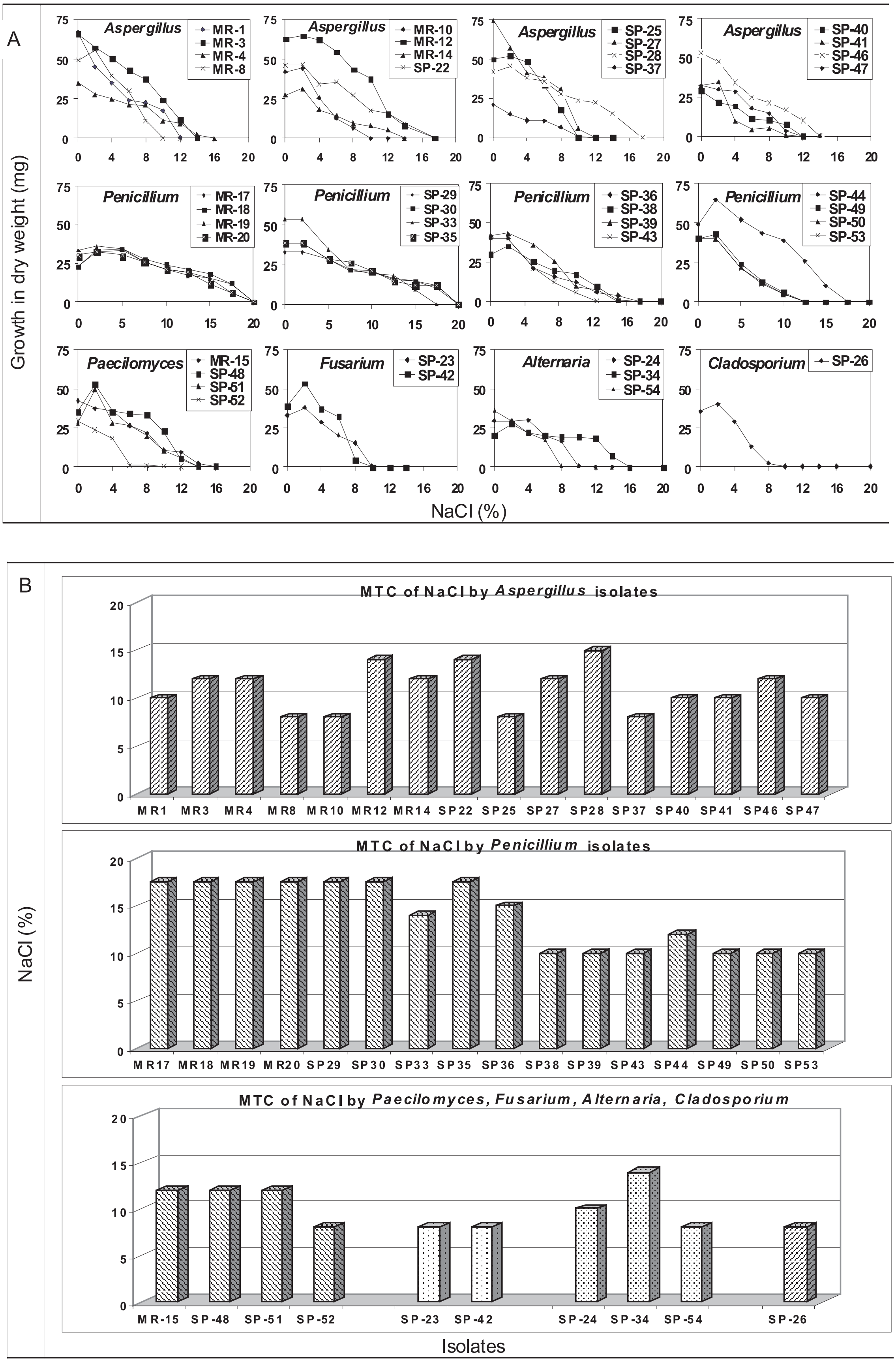


Fig. 1. Salt tolerance of fungal isolates

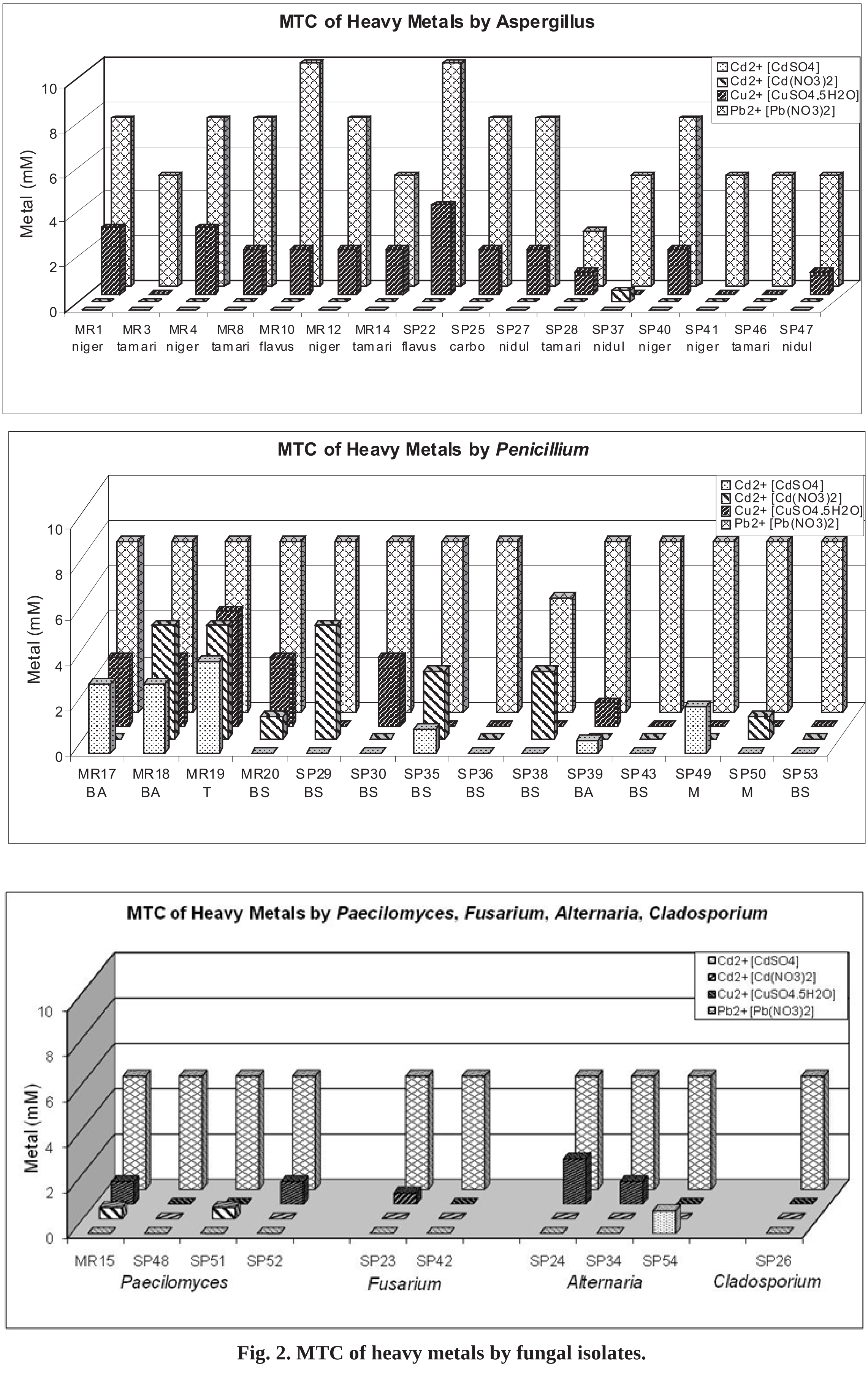
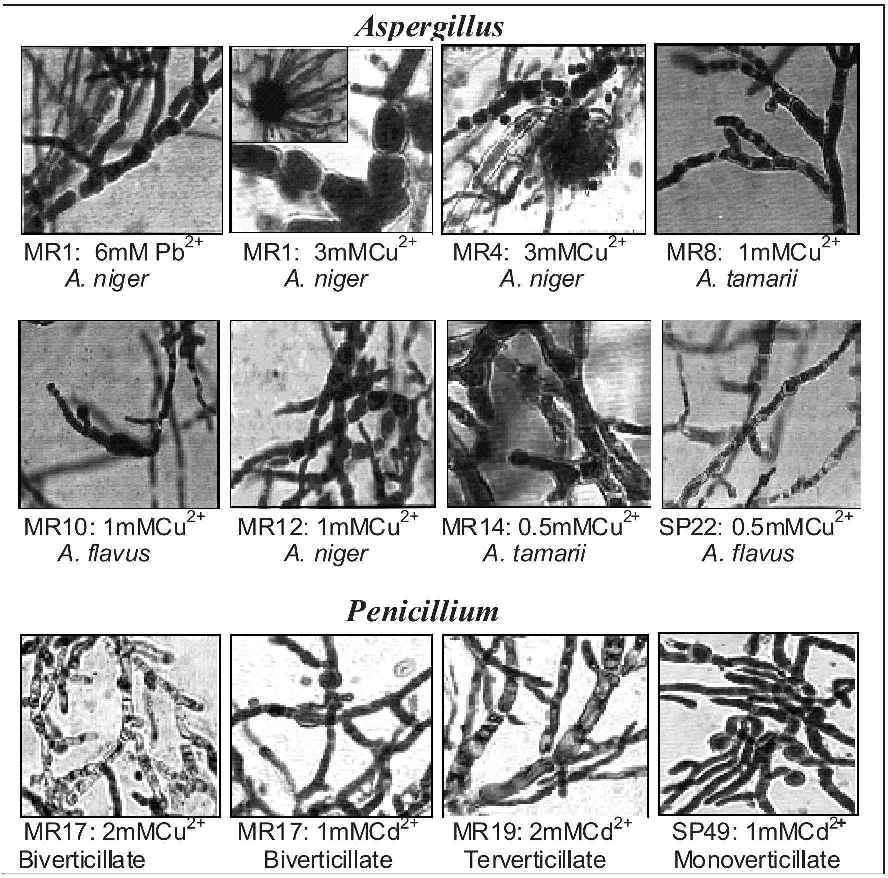


Fig. 2. MTC of heavy metals by fungal isolates.

Aspergillus and *Penicillium* isolates also displayed a higher level of salt tolerance than the other genera isolated, as also reported by Radwan et al. (1985). The isolates were able to grow in presence of fairly high concentrations of salt and also in absence of salt. Many showed optimal growth in absence of added NaCl and could therefore be regarded as halotolerant; some grew better when 2% salt was added to the growth medium, indicating their slightly halophilic nature (DasSarma, 2002; Nayak et al., 2012; Nazareth et al., 2012) and / or their marine origin (**Mackay et al.,** 1984). A striking observation was that some of the isolates from salterns did not have an ability to grow in presence of very high NaCl concentrations and had an optimal growth at 0-2% salt; these may therefore be of terrestrial/ aerial origin that have survived and/ or adapted to the high brine content.

The halotolerant/mild halophilic isolates were seen to have a good resistance to lead and copper, with resistance to lead being common to all and at a higher level than that of copper ions, similar to other findings (Marbaniang and Nazareth, 2007; Iskander et al., 2011; Gazem and Nazareth, 2012a). The greater resistance by isolates of the genera *Aspergillus* and *Penicillium* to the heavy metals as compared to that of the other genera obtained, corroborated earlier reports (Gadd, 1993; Iqbal et al., 2006). It was also noted that resistance to cadmium was demonstrated only by *Penicillium* species but not by *Aspergillus*, indicating that *Penicillium* could have a wider range of metal-resistance.

The thickening of the cell wall and rounded cells in presence of metals, corroborates earlier findings (Venkateswerlu et al., 1989; Kowshik and Nazareth, 2000; Nazareth and Marbaniang, 2008). Ram et al., (2004) showed that chitin biosynthesis is induced as a response mechanism of the fungal cell of *Aspergillus niger*, *Fusarium oxysporum* and *Penicillium chrysogenum* to stress, thus making it more resistant. When chitin synthesis is affected, growing hyphae form pronounced bulges and tend to lyse (Bago et al., 1996). The involvement of the cell wall in metal sorption (Ezzouhri et al., 2009; Iskander et al., 2011; Gazem and Nazareth, 2012b) might also contribute to the morphological changes seen. Moreover, the unusual phenomenon seen in *A. niger* in presence of copper ions, wherein the conidia showed germination while still attached to the vesicle, could be explained as a response mechanism to stress. Meyer et al. (2007) have shown that stress of antifungals on *A. niger* provoked the establishment of new polarity axes in conidial germination, with a number of germ tubes per conidium.



Aspergillus

MR1: 6mM Pb^{2+} *A. niger*

MR1: 3mMCu^{2+} *A. niger*

MR4: 3mMCu^{2+} *A. niger*

MR8: 1mMCu^{2+} *A. tamarii*

MR10: 1mMCu^{2+} *A. flavus*

MR12: 1mMCu^{2+} *A. niger*

MR14: 0.5mMCu^{2+} *A. tamarii*

SP22: 0.5mMCu^{2+} *A. flavus*

Penicillium

MR17: 2mMCu^{2+} Biverticillate

MR17: 1mMCd^{2+} Biverticillate

MR19: 2mMCd^{2+} Terverticillate

SP49: 1mMCd^{2+} Monoverticillate

Fig. 3. Morphological variations of *Aspergillus* and *Penicillium* species in response to heavy metals.

Isolates belonging to the *A. niger* and *A. flavus* groups were more resistant to the metals used than the other species of *A. tamari* and *A. nidulans*, which is in keeping with earlier reports (Iskander et al., 2011; Gazem and Nazareth, 2012a). Both *A. niger* and *A. flavus* species, which were more resistant, bear a radiate vesicle, with a biseriate, or mainly biseriate, phialide arrangement. In contrast, *A. tamari* are loosely radiate and uniseriate, with only large heads being globose and biseriate, and *A. nidulans* species are columnar with short biseriate phialides, both of which showed less metal resistance.

A similar observation could be found amongst the *Penicillium* isolates, wherein the single triverticillate morphotype with increased branching of the conidial structure, showed greater metal resistance over that of the biverticillate, and more so, over that of the monoverticillate. Developing resistance to environmental stress conditions as a means of survival appears to be an inherent characteristic of this morphotype, the profiles of secondary metabolites such as mycotoxins or antibiotics produced, forming an important feature in their classification (Frisvad and Filtenborg, 1983). Ram et al. (2004) have also indicated the greater resistance of the species of *A. niger* and the terverticillate *P. chrysogenum* to conditions of stress. These observations suggest that metal stress resistance may be phylogenetically related.

The diversity of microorganisms in saline or hypersaline environments is of growing interest and would find application in bioremediation processes. Industrial processes use salts and frequently release brine effluent into the environment. Such halotolerant species, able to grow at high concentrations of salt and possessing a high resistance to heavy metals have a very strong potential as agents for abatement of pollution in saline conditions or in waters of fluctuating salinity, as well as in non-saline environments. The comparative analysis of this study could also serve as an indication of the potential of various fungal genera or species for metal bioremediation.

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