

Halophilic *Aspergillus penicillioides* - a Specialist Group of Fungus and its Morphological Adaptations in Hypersaline Environments

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ABSTRACT

Halophilic fungi undergo various changes that enable them to adapt to osmotic pressure as well as toxicity of salt in the environment. Although several studies have been investigated on osmoadaptations of halophilic fungi to saline conditions, there are few reports on analysis of the fungal mechanisms occurring at micro and macro morphological levels that enable fungi to survive in saline environments. The current work reports the effects of salt on colony growth and micromorphology in obligate *Aspergillus penicillioides* and its mechanism of survival in saline environments.

Keywords: *Aspergillus penicillioides*, Obligate halophile, Colony, Micromorphology, Specialist fungus.

INTRODUCTION

Globally, halophilic fungi have been reported in various saline environments (Gunde-Cimerman *et al.*, 2009; Nazareth, 2014). They are classified into obligate and facultative (Nazareth *et al.*, 2012; Nayak *et al.*, 2012; Gonsalves *et al.*, 2012); furthermore, fungi in extreme environments are classified as generalists and specialists (Gostincar *et al.*, 2010, 2022). The generalists are those that are ubiquitous, polyextremotolerant and can compete with mesophilic species, while the specialists are those that have been less frequently isolated, with narrow ecological amplitudes and cannot compete with mesophilic species.

Among halophilic fungi, extensive studies have been carried out on the extremely halotolerant *Hortaea werneckii*, the obligate halophilic fungus *Wallemia ichthyophaga* and on other halophilic fungi (Gunde-Cimerman *et al.*, 2009; Kuncic *et al.*, 2010; Gunde-Cimerman and Zalar, 2014; Plemenitas, 2021).

High salinity alters the metabolic pattern of organisms, which is reflected as changes in the physiology and morphology (Tresner and Hayes, 1971). Growth of microbes in highly saline environments requires more than one strategy of survival. Unusual morphologies have been recorded in the specialists such as *Wallemia ichthyophaga*, *Exophiala dermatitidis*, and *Hortaea werneckii* (Kuncic *et al.*, 2010; Gostincar *et al.*, 2022). Unusually large phenotypic plasticity has

been reported in polyextremotolerant generalists and this is more than that in specialist fungi (Gostincar *et al.*, 2022).

The present study adds the obligate *Aspergillus penicillioides* to existing list of specialist fungi. Further, this study provides insight into the micromorphological and cultural responses of these specialists at various levels of salt concentrations and discusses similarities in their adaptations in comparison to earlier reported halophilic fungi.

MATERIAL AND METHODS

Isolates for study

Obligate halophilic aspergilli *Aspergillus penicillioides* DSS40 from the athallassohalic, extremely saline Dead Sea (Nazareth *et al.*, 2012), and EM6s137 from the thalassohaline brackish Mandovi estuary, Goa, India (Gonsalves *et al.*, 2012) with GenBank accession number HQ702385 and JQ240645 respectively, were selected for the study.

Radial growth rate

Fungal conidial suspensions were prepared in 10% saline and 0.05% Tween 80. A suspension containing $1.5-1.6 \times 10^3$ conidia was spot inoculated in triplicate on plates of Czapek Dox Agar (CzA) + solar salt. The concentration of salt was 0%, 2%, 5%, 10%, 15%, or 20%. Plates were incubated at room temperature. Growth was recorded daily up to 15 d in terms of diameter of

the colony. Radial growth rate was calculated as given by Reeslev and Kjoller (1995), by regression analysis, from the graph of average diameter plotted against time (mm day^{-1}).

Cultural and morphological changes at various salt concentrations

Fungal conidial suspensions were spot inoculated as above. After 15 days of incubation, changes in the colony such as appearance, surface texture, margin, production of exudate and pigmentation were recorded. Micromorphological changes such as differences in the structure of the mycelia, vesicles and conidia formation were studied using a wet mount of the culture prepared in lactophenol

cotton blue diluted in water (1:2). In instances where growth was not visible, a thin agar plug was used for microscopic examination. The slides were observed at 40x and 100x magnification.

RESULTS

As depicted in **Figure 1**, the two isolates of *A. penicillioides* DSs40 and EM6s137 did not grow in the absence of solar salt and yielded imperceptible growth with addition of 2% solar salt. There was an increase in radial growth rate at 5% salt and was maximum in presence of 10% salt and 15% salt, for isolates DSs40 and EM6s137 respectively. Thereafter, there was a gradual drop in radial growth rate.

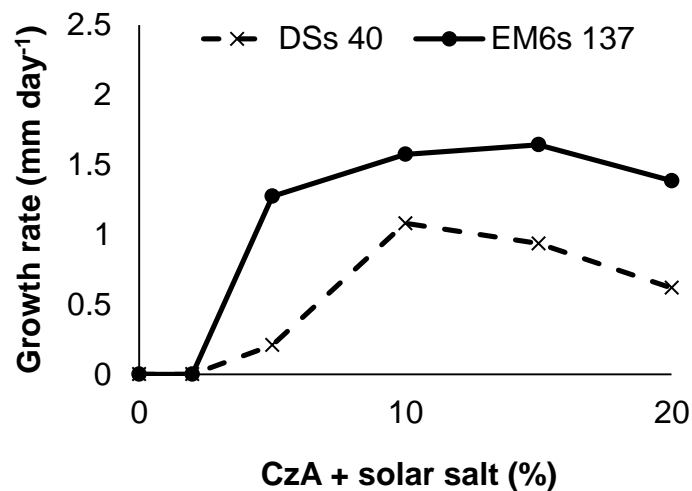


Figure 1: Effect of different salt concentrations on growth rate of *Aspergillus penicillioides* DSs40 and EM6s137.

Aspergillus penicillioides DSs40 and EM6s137 (**Figure 2 and 3**) did not show any colony formation in the absence of salt, and only a slight development at 2% salt. Colony growth was found optimal in the presence of 10% solar salt. On medium with 5% salt, the colonies appeared compact, granular-like and with filamentous margin, while the colonies on 10 – 20% salt appeared undulated, the colony compactness reduced as salt concentration increased in the medium, and the colony surface also changed from velutinous to powdery. Conidiation was not visible on medium with 2 – 5% salt but was seen at 10 – 20% salt. In EM6s137, the reverse of the colony showed pigment production in presence of salt concentrations from 5 to 15%.

Micromorphological examination showed that on media without salt, spores of *Aspergillus penicillioides* DSs40 appeared swollen and

distorted, while those of EM6s137 germinated in 0% salt and then got distorted and no further mycelial growth was seen thereafter in both cultures. In the presence of 2% salt, the mycelia of both cultures appeared distended and deformed, with discontinuous cytoplasm and lysis at a few areas. At 5% salt, the mycelia were only slightly distorted and at 10 – 20% salt the mycelia appeared narrower and normal. Additionally, in EM6s137 at 15% and 20% salt, bulbous growth of the mycelia and frequent septations were seen. The phialides were elongated at a salt concentration of 5%. In DSs40, the number of such elongated phialides decreased at 10% salt and normal vesicles with phialides were seen at salt concentrations of 15% and 20%. In EM6s137, the vesicles appeared normal at $\geq 10\%$ salt, but most of them had long phialides at 10-15% salt and appearing regular at 20% salt.

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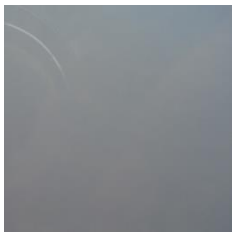
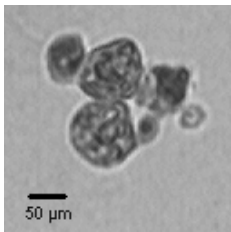
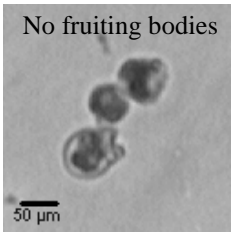
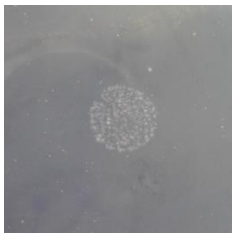
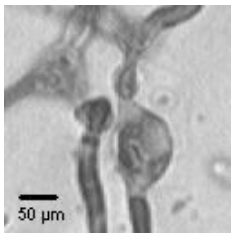
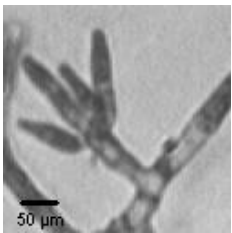
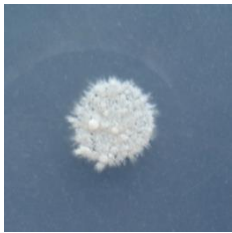
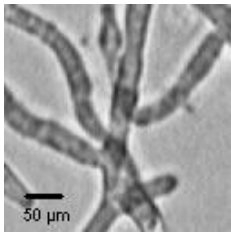
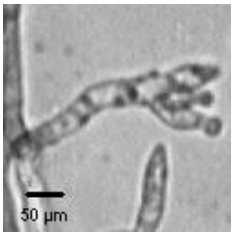

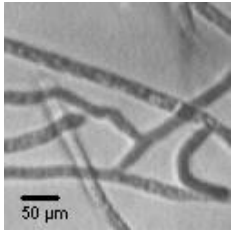
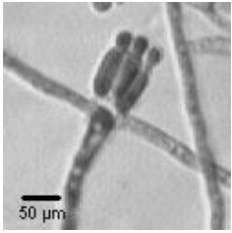

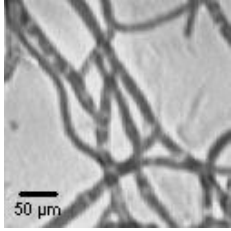
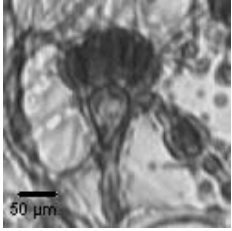
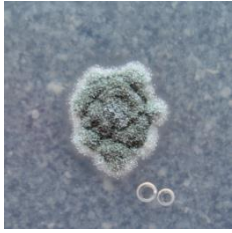
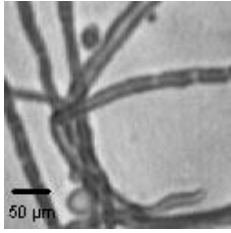
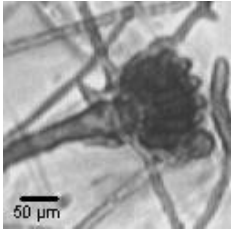
Salt %	Colony	Spores / Mycelia	Fruiting body
0			
2			
5			
10			
15			
20			

Figure 2: Effect of different salt concentrations on colony morphology and micromorphology of obligate halophilic *Aspergillus penicillioides* DSs40.


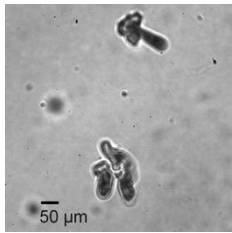
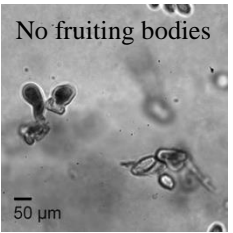

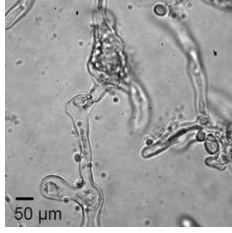
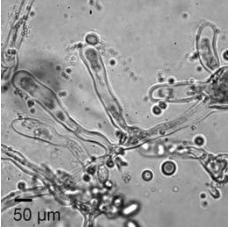

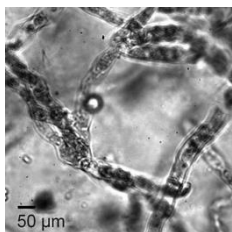
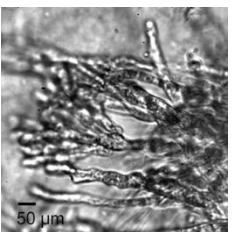

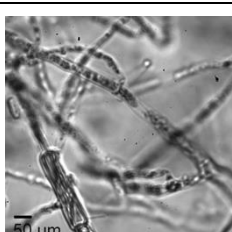
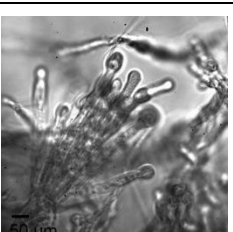

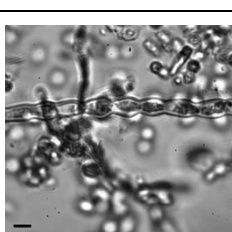


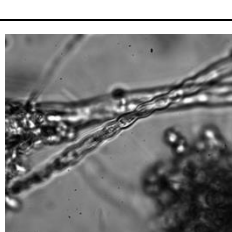
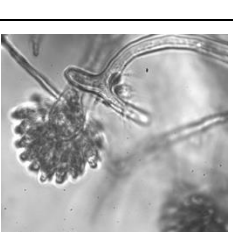
Salt %	Colony	Mycelia	Fruiting body
0		 50 μm	No fruiting bodies  50 μm
2		 50 μm	 50 μm
5		 50 μm	 50 μm
10		 50 μm	 50 μm
15		 50 μm	 50 μm
20		 50 μm	 50 μm

Figure 3: Effect of different salt concentrations on colony morphology and micromorphology of obligate halophilic *Aspergillus penicillioides* EM6s137.

DISCUSSION

The two obligate halophilic *A. penicillioides* DSs40 and EM6s137 were able to grow at a narrow range of salt concentrations and had a slow growth rate. These obligate halophiles grow at a narrow range of salt concentrations and are slow growing, unlike facultative halophiles. Therefore, they are non-competitive in nature against the vast majority of ubiquitous, non-extremophilic, or polyextremotolerant or facultative extremophiles. Thus, they appear to be specialists vis à vis the majority of other generalist fungi, in keeping with the definition of Gostincar *et al.* (2010, 2022).

Both the obligate halophilic cultures of *A. penicillioides* used in this study showed similar micromorphological and cultural changes with varying salt concentrations. A general trend was observed in the change of cultural appearance from velutinous to powdery and from compact and umbonate to flat. This finding supports earlier reports on changes in the halophilic *A. sydowii* (Jimenez-Gomez *et al.*, 2020). Fungal colony morphotypes such as in *Aspergillus fumigatus*, *Aspergillus penicillioides* and *Wallemia peruviansis* are affected by several abiotic factors such as oxygen concentration, media composition and salt concentration (Nazareth and Gonsalves, 2014a; Díaz-Valderrama *et al.*, 2017; Liu *et al.*, 2017; Nazareth *et al.*, 2019; Gonsalves and Nazareth, 2020; Kowalski and Cramer, 2020). Kowalski and Cramer (2020) have specified that there are numerous genetic factors that influence these changes. It is possible that the stress, presented as insufficient salt in the medium to support growth, may have caused a change in the colony morphology to a compact umbonate growth.

The colony edges showed a thinning of the fungal mycelia with increase in salt concentration in the medium. This seemed to be reflected also in the decrease in the width of the mycelia, as viewed microscopically. Turgor pressure and/or cell volume is maintained within limits necessary for growth and survival of an organism (Redkar *et al.*, 1996). In the presence of high salt, there is an increase in energy demand to sustain turgor, but cells may be unable to maintain sufficient turgor pressure which depresses cell expansion, resulting in slower volume increase (Redkar *et al.*, 1996; Han and Prade, 2002).

In absence of solar salt, the conidia of the obligate halophiles imbibed water and swelled considerably with distortions, or the conidia germinated with subsequent distortions of the germ tube. Germination occurs at the expense of endogenous resources and the death of a germ tube is inevitable under stressful conditions, when its resources become too depleted to support biosynthetic processes. In the present work, the growth medium provided the nutrition for growth and the high a_w in absence of added solar salt has allowed for transition from dormancy to germination phase. Thus, it appears that neither nutrient limitation nor water availability were causative factors for the inability to grow. It is possible that the endogenous metabolites permit the initial germ tube formation; however, absence of salt in the medium ultimately led to death. Growth of these isolates had an essential requirement for salt (Nazareth and Gonsalves, 2014b). Salt has been shown to induce the expression of hydrophobin genes, the protein of which forms a hydrophobic surface and could play a relevant role in the mycelial growth under halophilic conditions (Perez-Llano *et al.*, 2020). It is apparent therefore that salt plays an essential role for the growth of true halophiles, in the absence of which these fungi are unable to survive.

A few micromorphological changes were evident in the mycelia in response to different concentrations of salt. At lower salt concentrations, the mycelia of obligate halophilic fungi were distended. Jimenez-Gomez *et al.* (2020) have also reported that some hyphal regions show isodiametric growth in absence of salt. Studies have shown that over a wide range of salinities, the halophilic fungus *H. werneckii* has significantly more fluid membranes than the salt-sensitive and halotolerant fungi (Gunde-Cimerman and Zalar, 2014). High membrane fluidity increases permeability of the cell membrane to permeant water molecules. Thus, it may be hypothesized that at higher a_w , increased water permeability may lead to the occurrence of distended cells. Imbibition of water and imbalance in turgor pressure can cause the expansion of the cells. Osmotic stress is balanced by considerable stretching of the cell wall accompanied by isodiametric growth (Cooke and Whipps, 1993; Kuncic *et al.*, 2010). Kuncic *et al.* (2010) have reported that the balance between a rigid and a dynamic structure influences the shape of cells and enables growth and hyphal branching.

The vesicle and phialides appeared normal in the presence of 10 – 20% salt concentrations, which correlated with optimal radial growth. Thus, it may be concluded that salt is essential for formation of normal vesicles in obligate halophilic fungi.

Studies on obligate halophilic *A. penicillioides* bring to light an understanding of the responses of specialist fungi at low water activity and high salinity environment. Notable is that the obligate *A. penicillioides* are from vastly different niches, one from the athalassohalic extremely hypersaline Dead Sea and the other from the thalassohalic brackish estuarine habitats, but both of them had slow growth rates and similar changes in cultural characteristics and micromorphology in response to salt. This less phenotypic plasticity is reported in specialist fungi (Gostincar *et al.*, 2022).

CONCLUSION

The present work presents *A. penicillioides* as a specialist halophilic fungus showing slow growth rate at a narrow range of salt concentrations making these non-competitive in comparison with generalist fungi. Additionally, the specialist halophilic fungi display varied adaptation strategies, both at micromorphological and cultural level at different salt concentrations.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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