



Unusual thermal resistance of spores of mesophilic fungi

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Abstract

Thermophilic fungi are well-known for their ability to grow at high temperatures of 50–60°C. However, our previous research demonstrated the exceptional thermal resistant capability of the spores of some mesophilic fungi. These fungi, isolated from leaf litters of forests that experience frequent ground fires, were resistant to dry heat at 100–115°C. To determine whether these high thermal resistant spores are unique to fungi in flammable forests, we screened leaf endophytes of trees of a mangrove forest that rarely experience ground fire due to its inundated forest floor. Of the 129 endophyte isolates tested, the spores (conidia/ascospores) of 27 and 13 isolates survived an exposure to dry heat at 100°C for 2 hrs and 4 hrs respectively. The ascospores of *Chaetomium globosum* isolated from the leaves of *Rhizophora stylosa* survived an exposure to 100°C for 10 h. Presence of melanin synthesis inhibitor reduced the thermal resistance in this fungus suggesting that melanin plays a role in the thermal protection.

Key words – Ascospore – Conidia – Heat resistance – Melanin

Introduction

Generally, fungi are mesophilic having temperature optima between 25–35°C for their growth (Cooney & Emerson 1964). A few species are able to grow at temperatures above 50°C and are termed thermophilic fungi (Maheshwari et al. 2000). Although mesophilic fungi cannot grow at temperatures beyond 35°C, Suryanarayanan et al. (2011) reported that the spores of some mesophilic fungi from dry tropical forests which experience episodic forest fires survive exposure to 100–115°C for 2 h. Although the growing mycelia of these fungi are not heat-tolerant, this work reports that their spores represent the most heat-resistant eukaryote cells on record. The authors attributed this thermal resistance of spores to possible adaptations of these fungi to survive fires. To determine if such high thermal resistance is restricted to fungi of flammable habitats, we chose to study fungi from a forest which is not exposed to fire. We screened the spores of 129 isolates of foliar endophytic fungi of mangroves trees of the Andaman Islands for their heat tolerance. Mangrove trees here do not experience forest ground fire as they grow along the coast with permanently submerged roots.

The dark brown melanin pigments of fungi though may not be necessary for their growth and development, play an important role in their survival by protecting them against abiotic stressors including UV irradiation, desiccation, and temperature extremes (Bell & Wheeler 1986). Hence, we

studied the probable role of melanin in the thermal resistance of spores of one of the fungi, which showed extraordinary heat tolerance.

Materials & Methods

We screened fungal endophytes isolated in our earlier study (Rajamani et al. 2018) from the leaves of 20 mangrove species growing in south Andaman Island, in the Bay of Bengal region, India.

Test for thermotolerance

The method of Suryanarayanan et al. (2011) was followed. A sterilized cover glass was used to scrape the surface of a sporulating culture in the agar plate. Such cover glasses bearing the spores (conidia or ascospores) were placed in empty sterile glass Petri dishes without the lid and incubated in a pre-heated hot air oven fitted with a digital thermometer. The oven temperature was set at 100°C and regularly verified with a mercury thermometer. The cover glasses with the spores were incubated for 2 or 4 h. After incubation, the cover glasses were removed from the oven, incubated at 25°C for 30 minutes, and then placed on fresh Potato Dextrose Agar medium contained in Petri dishes. Petri dishes were incubated at 25°C for seven days and examined for growth. Those fungi, which tolerated a 4 hrs treatment, were tested for tolerance to heat by extending the treatment period to 6, 8 or 10 h. All experiments were in triplicates and repeated three times.

Identification of endophytes

The fungi were identified based on their microscopic characteristics (Ellis 1971, 1976, Sutton 1980, Onions et al. 1981, Ellis & Ellis 1988). Fungi whose species could not be confirmed but differed from each other were given species numbers. The *Diaporthe* species had been identified using the molecular method in our earlier study (Rajamani et al. 2018). The ascospores of one species of *Chaetomium*, which exhibited a high thermal resistance, was identified as *C. globosum* based on ITS sequence method in the present study.

Molecular identification of thermotolerant fungus

The identity of a *Chaetomium globosum* isolated from the leaves of *Rhizophora stylosa* that exhibited a high thermotolerance was established using the molecular method as follows. Fungal culture grown in PDA medium for seven days was collected and used for genomic DNA extraction (Sawmya et al. 2013). The obtained DNA was re-suspended in 50 µl of sterile MilliQ water. PCR amplification of the internal transcribed spacer region was carried out using ITS5 and ITS4 primers (White et al. 1990). The PCR conditions were as follows: 95°C for 10 minutes, 30 cycles of 95°C for 60 seconds, 55°C for 60 seconds and 72°C for 90 seconds, and finally at 72°C for 10 minutes. The PCR product was further purified and sequenced using an automated sequencer (ABI 3130 Genetic Analyzer) using ITS4 primer. After manual editing, we checked the sequence for the closest match in the NCBI database using the Blastn algorithm. The sequence was submitted to NCBI GenBank database with the accession number MT125865 and the fungus was deposited in the National Fungal Culture Collection of India (NFCCI), Agharkar Research Institute (ARI), Pune, India (accession number NFCCI 4863).

Phylogenetic analysis

Based on the Blast result, ITS sequences of 21 closely related sequences from type specimens from the NCBI database were downloaded and aligned with our sequence using ClustalW (Thompson et al. 1994) with the default settings. After manual adjustment of the alignment, an evolutionary tree was inferred using the maximum likelihood method following the Kimura 2-parameter model using MEGA version 6 (Kimura 1980, Tamura et al. 2013). A bootstrapping analysis was carried out (1000 replications) to obtain branch support values. For constructing the tree, all positions with less than 95% site coverage were removed, and the tree with the highest log-

likelihood was selected. Similarly, the identity of all the *Diaporthe* isolates from this study was established in our earlier study using a molecular approach (Rajamani et al. 2018).

Test with melanin synthesis inhibitor

Tricyclazole, a melanin synthesis inhibitor, was dissolved in ethanol and added to autoclaved and cooled PDA medium to prepare a series of concentrations at 1, 10, or 100- $\mu\text{g ml}^{-1}$ (Suryanarayanan et al. 2004). This medium was inoculated with a 5 mm diameter plug of mycelium cut from the margin of an actively growing colony and incubated for 20 days at $25 \pm 2^\circ\text{C}$ in the dark. Spores from such colonies were screened for thermotolerance.

Results

Out of the 129 endophyte isolates tested, the spores of 27 and 13 isolates survived exposure to dry heat at 100°C for 2 hrs and 4 hrs, respectively (Table 1). Ascospores of all the seven *Chaetomium* isolates tolerated 4 hrs of exposure. Of the ten *Pestalotiopsis* isolates screened, five survived 4 hrs of exposure; of the two *Curvularia* spp., the conidia of one tolerated 2 hrs treatment while that of the other could withstand 4 hrs of heating. Conidia of *Alternaria* sp.1, *Aspergillus niger*, *Cladosporium cladosporioides*, *Curvularia* spp., and *Trichoderma* spp., and ascospores of *Emericella nidulans* tolerated exposure to 2 hrs of heating. Of the 28 isolates of *Colletotrichum/Glomerella* screened, only one survived exposure to 2 hrs of heating. The conidia of none of the 18 isolates of *Diaporthe* belonging to ten species, 12 isolates of *Phyllosticta capitalensis* and ten isolates of *Nodulisporium* spp. were heat-tolerant (Table 1). The spores of 13 isolates, which survived heating for 4 hrs, were tested with longer durations of heat treatment. While nine and seven of these isolates tolerated exposure to 6 hrs and 8 hrs of heating respectively, the ascospores of *C. globosum* isolated from the leaves of *Rhizophora stylosa* survived exposure to 100°C for 10 hrs (Table 2). In the presence of melanin synthesis inhibitor at a concentration of $100 \mu\text{g ml}^{-1}$, the colonies of *C. globosum* were reddish and its ascospores could not survive exposure to 100°C for 8 and ten hours (Table 3).

The *Chaetomium* isolate, which could grow at 100°C for 10 hrs, was identified using a molecular method. Further, a maximum likelihood tree, constructed by comparing the ITS sequences of closely related sequences obtained from type specimens available in the public database, showed that our sequence clustered with high bootstrap support with sequences belonging to *C. globosum* (Fig. 1).

Discussion

The higher temperature limit for the growth of eukaryotes is 60°C (Tansey & Brock 1972). Thermal tolerance of fungal spores to wet heat is usually studied at temperatures below 60°C since spores of many fungi do not survive exposure to this temperature for several minutes (Wyatt et al. 2013). According to van den Brule et al. (2020), the most heat resistant conidia are produced by a strain of *Paecilomyces variotii*, which could survive exposure to 60°C for 22 minutes. Their work also emphasizes the importance of exposure time to heat since only 1% and 0.1% of the spores were alive after 44 and 66 minutes of treatment. In these and other studies (Bayne & Michener 1979), the test fungi are subjected to heat treatment when suspended in a medium. Our study focuses on dry heat tolerance where spores are subjected to rapid heating by transferring within a few seconds from their fully hydrated state to a heated dry oven (Suryanarayanan et al. 2011). Using this method, Suryanarayanan et al. (2011) showed that spores of mesophilic litter fungi from tropical dry forests experiencing episodic ground fires could tolerate rapid dry heating at 115°C for 2 hours. They opine that such an extraordinary thermotolerance is the outcome of adaptations to prolonged droughts and the prevalence of fire in their dry habitat. The present study using endophytes of trees of mangrove vegetation reveals that this trait is not confined to fungi of dry habitats experiencing sporadic fires but is widespread. This is interesting since even the spores of thermophilic fungi survive exposure to 68°C for 5–60 minutes only (Ogundero & Oso 1980) and

60°C is the upper-temperature limit for these fungi (Maheshwari et al. 2000, Powell et al. 2012). We show here that the resting spores of some mesophilic fungi survive exposure to prolonged periods (2–10 hrs) to 100°C.

Since the fungi studied are from an environment not exposed to fire and the direct transfer from 22°C to 100°C is immediate, it is unlikely that the spores exhibit acquired thermotolerance shown by fungi when transferred from optimal temperature to high temperature in a stepwise manner (Tereshina 2005). The general method of thermal protection in spores adopted by fungi via the synthesis of compatible solutes such as mannitol and trehalose (Hagiwara et al. 2017) and heat shock proteins. This mechanism may not be involved here since dormant fungal spores are very low in their metabolic activities (Thevelein et al. 1984), including respiration (Novodvorska et al. 2016).

Table 1 Thermotolerance of spores of mangrove leaf endophytes.

Endophyte	No. of isolates screened	No. of isolates surviving exposure to 100°C	
		2 Hrs	4 Hrs
<i>Alternaria</i> sp.	4	1	
<i>Aspergillus niger</i>	3	3	
<i>Aureobasidium pullulans</i>	1		
<i>Chaetomium globosum</i>	5	5	5
<i>Chaetomium</i> spp.	2	2	2
<i>Cladosporium cladosporioides</i>	6	1	
<i>Colletotrichum gloeosporioides</i>	11	1	
<i>Colletotrichum</i> spp.	6		
<i>Corynespora</i> sp. 1	3		
<i>Curvularia</i> spp.	2	2	1
<i>Diaporthe discoidispora</i>	3		
<i>Diaporthe eucalyptorum</i>	1		
<i>Diaporthe hongkongensis</i>	2		
<i>Diaporthe kyushuensis</i>	1		
<i>Diaporthe liquidambaris</i>	1		
<i>Diaporthe longicolla</i>	1		
<i>Diaporthe perseae</i>	1		
<i>Diaporthe pseudomangiferae</i>	4		
<i>Diaporthe tectonae</i>	1		
<i>Diaporthe</i> sp. 1	3		
<i>Emericella nidulans</i>	1	1	
<i>Fusarium</i> spp.	9	2	
<i>Glomerella</i> spp.	11		
<i>Lasiodiplodia theobromae</i>	3		
<i>Monilia</i> sp.	1		
<i>Nigrospora</i> sp.	1		
<i>Nodulisporium</i> spp.	10		
<i>Penicillium pedernalense</i>	1		
<i>Penicillium</i> sp. 1	6	1	
<i>Pestalotiopsis</i> sp.	10	6	5
<i>Phomopsis heveicola</i>	1		
<i>Phyllosticta capitalensis</i>	12		
<i>Trichoderma</i> spp.	2	2	
Total Positive Strains	129	27	13

Table 2 Thermotolerance of spores of mangrove leaf endophytes exposed to prolonged heating.

Endophyte	Host	Exposed to 100°C		
		6 Hrs	8 Hrs	10 Hrs
<i>Chaetomium globosum</i>	<i>Acanthus ilicifolius</i>	+	+	–
<i>Chaetomium globosum</i>	<i>Avicennia officinalis</i>	+	+	–
<i>Chaetomium globosum</i>	<i>Bruguiera gymnorhiza</i>	–	–	–
<i>Chaetomium globosum</i>	<i>Rhizophora apiculata</i>	–	–	–
<i>Chaetomium globosum</i>	<i>Rhizophora stylosa</i>	+	+	+
<i>Chaetomium</i> sp. 3	<i>Rhizophora mucronata</i>	+	+	–
<i>Chaetomium</i> sp. 4	<i>Rhizophora apiculata</i>	–	–	–
<i>Curvularia</i> sp. 2	<i>Acanthus ebracteatus</i>	–	–	–
<i>Pestalotiopsis</i> sp.	<i>Bruguiera parviflora</i>	+	+	–
<i>Pestalotiopsis</i> sp.	<i>Ceriops tagal</i>	+	+	–
<i>Pestalotiopsis</i> sp.	<i>Nypa fruticans</i>	+	–	–
<i>Pestalotiopsis</i> sp.	<i>Rhizophora mucronata</i>	+	+	–
<i>Pestalotiopsis</i> sp.	<i>Sonneratia alba</i>	+	–	–
Total Positive Strains		9	7	1

– = No growth; + = growth observed.

Table 3 Thermal resistance of ascospores of *C. globosum* in the presence of tricyclazole.

Hours of treatment at 100°C	Inhibitor ($\mu\text{g ml}^{-1}$)			
	0	1	10	100
4	+	+	+	+
6	+	+	+	+
8	+	+	+	–
10	+	+	+	–

– = No growth; + = growth observed.

A few studies show that melanin pigment protects fungi from high temperatures (Butler & Day 1998, Gessler et al. 2014). The mycelia and spores of many of the endophytes of desert plants are pigmented (Suryanarayanan et al. 2005, Sangamesh et al. 2018). The presence of melanin pigment is known to afford protection to fungi against several abiotic stressors including heat (Ravishankar et al. 1995, Money et al. 1998, Paolo et al. 2006, Dadachova & Casadevall 2008, Geib et al. 2016). Spores of melanin deficient *Monilinia fructicola* are more sensitive to heat stress (Rehnstrom & Free 1996). Melanized *Cryptococcus neoformans* cells are less susceptible to heating at 42–47°C than non-melanized cells (Rosas & Casadevall 1997). Melanized *Aureobasidium melanogenum* cells isolated from Taklimakan desert soil tolerated exposure to heat (40°C) better than the non-melanized cells (Jiang et al. 2016). In the present study, ascospores of *C. globosum* showed very high thermotolerance. Since *C. globosum* synthesizes melanin (Hu et al. 2018), we proceeded to see if melanin could be responsible for such high thermotolerance in this fungus. Hu et al. (2012) reported that tricyclazole inhibits melanin synthesis in *Chaetomium globosum*, confirming that it is a 1,8-dihydroxynaphthalene (DHN) type of melanin. We found that the thermotolerance of *C. globosum* spores reduced when melanin synthesis was inhibited in cultures grown in the presence of the inhibitor, suggesting that melanin plays a critical role in the extraordinary thermal resistance of fungal spores. Although fungi like *Phyllosticta capitalensis* reported having DHN melanin (Suryanarayanan et al. 2004), none of the 12 isolates of this fungus

had thermotolerant spores. Similarly, the heat tolerance observed here was not strictly taxon-specific as the same genus or even species isolated from different plants showed different degrees of thermotolerance (Tables 1 & 2). A caveat about the methodology used in the present study should be noted. It involves the immediate transfer of spores from normal temperature to the heated environment under dry condition (without suspending the spores in a medium like water) and hence a spore count could not be made; thus, the number of spores subjected to heating is not known. Furthermore, the possibility of some hyphal bits getting transferred along with the spores cannot be ruled out. It is relevant that heat tolerance in *Saccharomyces cerevisiae* differs with isolates and is a complex polygenic trait (McCusker et al. 1994). Similar studies at the genome level are needed to explain heat resistance trait difference among spores of the same species seen in the present study especially since information on the role of melanin in heat protection in fungi is scarce (Cordero & Casadevall 2017).

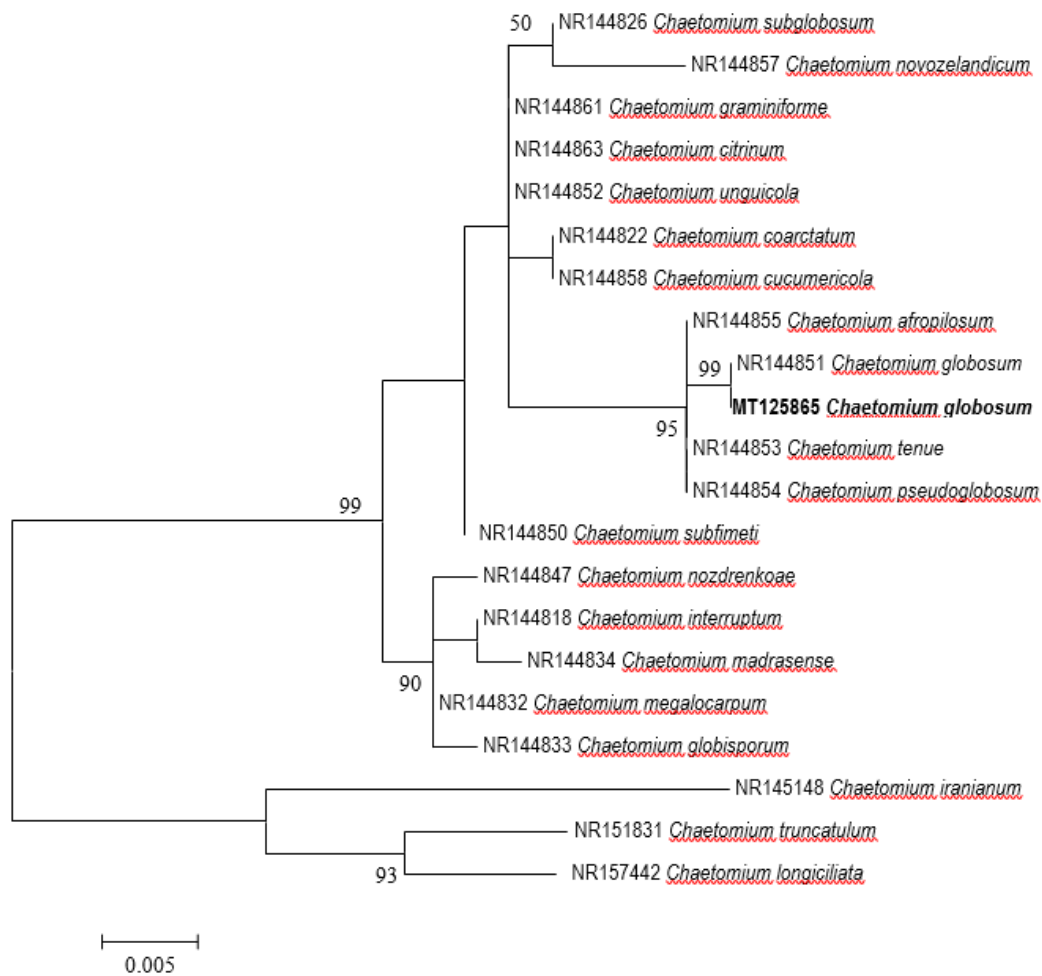


Fig. 1 – Maximum likelihood phylogenetic tree of *C. globosum*

The result that spores of some endophytes like *Alternaria*, *Chaetomium*, *Fusarium* and *Pestalotiopsis* spp. are thermal resistant leads to some speculations. These leaf endophytes have a wide range of plant host and geographic distribution (Suryanarayanan et al. 2018) and are capable of shifting to a saprotrophic litter degrading mode once the leaf falls by elaborating biomass degrading enzymes (Prakash et al. 2015, Reddy et al. 2016). Their ecological amplitude could be enlarged further owing to heat resistant spores especially under the current global warming scenario (Sadyś et al. 2016). Furthermore, thermal resistant spores of plant pathogenic fungi survive longer in soils and could be a major concern since models predict that global warming could increase the relative abundance of plant pathogens worldwide (Delgado-Baquerizo et al. 2020). These

observations, taken together with our study, underscore the importance of studying the performance of crop pathogenic fungi producing melanized resting structures (Velásquez et al. 2018). *Chaetomium* spp. are usually saprotrophic but some including *C. globosum* could cause mycoses including onychomycosis, sinusitis, empyema, pneumonia, and fatal cerebral disease in immunocompromised patients (Barron et al. 2003, Ahmed et al. 2016). Our demonstration that spores of *C. globosum* are extraordinarily heat-tolerant warrants more studies as they may not be inhibited by the natural barrier of higher temperature of the human body (Garcia-Solache & Casadevall 2010). Many species of the order Sordariales, to which *Chaetomium* belongs, are known to be thermophilic (Sandona et al. 2019) and grow better at 45°C (Morgenstern et al. 2012). Though thermophilic fungi, which exhibit growth in higher temperatures, have been studied well, the current study emphasizes the need to study thermal resistance of resting structures of fungi as well.

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