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Stress tolerance of three opportunistic black yeasts

Toleranca na stres pri treh oportunističnih črnih kvasovkah

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Abstract: Many species of black yeasts can survive extremely harsh conditions and can quickly adapt to novel environments. These traits were proposed to have a role in the ability of some fungal species to colonise indoor habitats inhospitable for majority of microorganisms, and to cause (opportunistic) infections in humans. In order to better understand the stress tolerance of black yeasts and thereby their opportunism, we focused our research on the three model black yeasts: the polyextremotolerant *Aureobasidium melanogenum* and *Exophiala dermatitidis*, and the extremely halotolerant *Hortaea werneckii.* These black yeasts are shown to thrive at temperatures, salinities, pH values and, H_2O_2 concentrations that inhibit growth of mesophilic species. Most importantly, unlike their close relatives they can not only grow, but also synthesize siderophores (*E. dermatitidis*) or degrade proteins (*A. melanogenum*) at 37 °C - traits that are crucial for pathogenesis in humans. These results support the hypothesis that the ability to cope with various environmental stresses is linked to the opportunistic behaviour of fungi. Therefore, better understanding of the connections between the stress-tolerant biology of black fungi and their ability to cause disease is needed, in particular due to their changing interactions with humans and their emerging pathogenicity.

Keywords: melanised fungi, temperature, NaCl, pH tolerance, ROS, oligotrophism, proteolytic activity, capsule

Izvleček: Mnoge vrste črnih kvasovk lahko preživijo ekstremne razmere in se hitro prilagajajo novim okoljem. Te lastnosti imajo vlogo pri sposobnosti nekaterih vrst gliv, da lahko kolonizirajo negostoljubne habitate v notranjosti stavb in povzročijo (oportunistične) okužbe pri ljudeh. Raziskali smo toleranco na stres treh oportunističnih črnih kvasovk: poliekstremotolerantnih kvasovk *Aureobasidium melanogenum* in *Exophiala dermatitidis* ter izjemno halotolerantne kvasovke *Hortaea werneckii*. Vse tri črne kvasovke uspevajo pri temperaturah, koncentracijah NaCl, pH vrednostih in koncentracijah H_2O_2, ki zavrejo rast mezofilnih vrst. Še več, te vrste lahko v nasprotju s svojimi bližnjimi sorodniki ne le rastejo, temveč tudi sintetizirajo siderofore (*E. dermatitidis*) in razgrajujejo proteine (*A. melanogenum*) pri 37 °C, kar sta pomembni

lastnosti za patogenezo pri ljudeh. Ti rezultati se skladajo s hipotezo, da je sposobnost toleriranja različnih okoljskih stresov povezana z oportunističnim značajem gliv. Prav zato moramo bolje razumeti povezavo med biologijo tolerance na stres črnih gliv in njihovo sposobnostjo povzročanja bolezni, zlasti zaradi njihove spreminjajoče se interakcije z ljudmi in njihove porajajoče se patogenosti.

Ključne besede: melanizirane glive, termotoleranca, NaCl, pH toleranca, oksidativni stres, oligotrofizem, proteolitična aktivnost, kapsula

Introduction

Stress tolerance together with great adaptability enable some fungi to inhabit extreme natural environments, which makes them good candidates for colonizing novel habitats, especially those considered as generally hostile to abundant microbial growth (Gostinčar, Grube et al. 2011). Surveys of recently introduced indoor habitats have, for example, uncovered a surprising diversity of polyextremotolerant oligotrophic fungi (Hamada and Abe 2010, Lian and de Hoog 2010, Zalar, Novak et al. 2011). With the advances in technology and by making indoor habitats inhospitable to microbes to prevent their overgrowth, we are exposing microbes to new conditions, such as repeated cycles of thermal stress in dishwashers and novel chemicals such as aromatic pollutants, detergents and, biocides. These conditions appear to select for the most resilient and adaptable species, many of which can cause opportunistic human infections (Gostinčar, Grube et al. 2011, Gostinčar, Gunde-Cimerman et al. 2015).

Black yeasts are a phylogenetically diverse group of fungi with characteristically melanised cell walls that are found in several orders of *Dothideomycetes* and *Eurotiomycetes*. Melanisation and other physiological adaptations enable black yeasts to be highly resistant against different types of environmental stress (Gostinčar *et al.* 2012) and to cause infections in animals (including humans) and plants (Silveira & Nucci 2001, Garcia-Solache & Casadevall 2010, de Hoog *et al.* 2015). Three often neglected opportunistic pathogens belonging to black yeasts have been the subject of our research for several years: the polyextremotolerant *Aureobasidium melanogenum* and *Exophiala dermatitidis*, and the extremely halotolerant *Hortaea werneckii*.

Aureobasidium melanogenum (*Dothideales*, *Dothideomycetes*) is tolerant to various stresses such as low water activity, low and high temperature, fluctuating pH and oligotrophic conditions (Gostinčar, Ohm et al. 2014). It is common in tropical, temperate and polar areas, mainly in natural and anthropogenic aqueous environments, from tap water to household dishwasher interiors (Gostinčar, Ohm et al. 2014, Novak Babič, Zalar et al. 2016). Infections caused by *A. melanogenum* reported in the literature were caused by traumatic inoculation or involved severely immunocompromised patients (reviewed in de Hoog, Guarro et al. (2015)).

Exophiala dermatitidis (*Chaetothyriales, Eurotiomycetes*) can also resist various extremes, from low to high temperatures (Blasi, Tafer et al. 2015) and broad range of pH (Zalar, Novak et al. 2011). It has the greatest pathogenic potential among the three model black yeasts studied herein (de Hoog, Guarro et al. 2015), causing infections from superficial, cutaneous and subcutaneous, to visceral or systemic (de Hoog, Guarro et al. 2015) and subclinical pulmonary colonisation in patients with cystic fibrosis (Matos, Haase et al. 2003, Kondori, Lindblad et al. 2014, Sood, Vaid et al. 2014, de Hoog, Guarro et al. 2015). Like *A. melanogenum* it is frequently found in tap water (Novak Babič, Zalar et al. 2016), in household dishwashers (Zalar, Novak et al. 2011) and other indoor habitats, but it is rarely recovered from non-anthropogenic habitats (Sudhadham, Prakitsin et al. 2008).

Hortaea werneckii (*Capnodiales*, *Dothideomycetes*) is an extremely halotolerant fungus living in diverse habitats with increased salinity (Gunde-Cimerman, Zalar et al. 2000). It is the cause of *tinea nigra*, a superficial human infection of the palms, and sometimes the soles in tropical and

subtropical regions (Bonifaz, Gomez-Daza et al. 2010, de Hoog, Guarro et al. 2015). It is unable to degrade keratin, but it shows lipolytic activity (de Hoog and Gerrits van den Ende 1992, Göttlich, de Hoog et al. 1995). The systemic cases reported by Ng, Soo-Hoo et al. (2005) in patients with acute myelomonocytic leukaemia are a rare exception.

To better understand the stress-tolerance of black fungi and their pathogenic potential, we focused on the stress tolerance (to a combination of temperature and one additional stress factor) and few selected traits linked to the virulence of the three model black yeasts, such as proteolytic activity, production of siderophores and formation of capsule.

Materials and methods

Strains and culture conditions

Aureobasidium melanogenum (EXF-3378 / CBS 110374), *Exophiala dermatitidis* (EXF-10123 / CBS 525.76 / ATCC 34100) and *Hortaea werneckii* (EXF-2000 / CBS 100457) used in this study were isolated from different extreme environments: a public fountain in Bangkok (Thailand); a human sample (no additional data are available); and hypersaline water of solar salterns (Sečovlje, Slovenia), respectively. The strains are preserved in the Culture Collection Ex (Department of Biology, Biotechnical Faculty, University of Ljubljana, Infrastructural Centre Mycosmo, MRIC UL), at the Westerdijk Fungal Biodiversity Institute (The Netherlands) and American Type Culture Collection (ATTC, USA).

Cultures were maintained on complex malt-extract agar medium MEA (pH 6.0), consisting of (all w/v) 2% malt extract (Biolife, Italy), 2% glucose (Kemika, Croatia), and 0.1% peptone from meat (Merck, Germany); and on defined yeast nitrogen base (YNB) medium (pH 7.0) consisting of (all w/v) 0.17% yeast nitrogen base, 0.08% complete supplement mixture (both Qbiogene), 0.5% ammonium sulphate (Sigma-Aldrich, Germany), 2% glucose (Kemika, Croatia), and 2% agar (Formedium Ltd, UK), in deionised water. The cell suspensions (OD_{600} 0.5) were diluted in sterile deionized water 10^{-2}, 10^{-3} and 10^{-4} and 10 µL was spotted on agar medium for stress tolerance tests. The composition of the specific media for morphological analysis, stress tolerance tests and enzymatic screening are specified below.

Tolerance to acid and alkaline pH

The black yeasts were tested for their tolerance to acid (3, 4) and alkaline pH (8, 9, 10) on MEA. Medium with pH 8 was prepared with 100 mM Sodium Phosphate Buffer (Na_2HPO_4 – NaH_2PO_4) (both Merck, Germany). For the media with pH 9 and pH 10 100 mM glycine - NaOH buffer (both Sigma-Aldrich, Germany) was used. For the media with pH 3 and pH 4 100 mM Citric Acid – Na_2HPO_4 buffer (Carl Roth GmbH+Co, Germany and Merck, Germany) was used. The media were prepared in two parts as follows: (i) malt extract, peptone and glucose were dissolved in appropriate buffer in half the total volume of the medium and filter sterilized (Minisart-Plus 0.20 µm, Sartorius Stedim Biotech GmbH, Germany); (ii) 2 (w/v) % agar was added to water (half of the final medium volume) and autoclaved. The two components were aseptically combined, mixed and poured into petri dishes.

Oligotrophic growth assessment and production of siderophores

The minimal medium consisted of 1% (w/v) glucose (Kemika, Croatia), with the addition of the macroelements (w/v) of 0.6% $NaNO_3$, 0.15% KH_2PO_4, 0.05% $MgSO_4 \times 7H_2O$ and 0.05% KCl, and the microelements (w/v) of 0.01% EDTA, 0.0044% $ZnSO_4$, 0.001% $MnCl_2 \times 4H_2O$, 0.00032% $CoCl_2 \times 6H_2O$, 0.00032% $CuSO_4 \times 5H_2O$, 0.00022% $(NH_4)_6Mo_7O_{24} \times 4H_2O$, 0.00147% $CaCl_2 \times 2H_2O$ and 0.001% $FeSO_4 \times 7H_2O$ (all Sigma-Aldrich, Germany), in ultrapure water. Autoclaved diluted minimal medium was prepared by diluting 1 part of the medium with 9 parts of ultrapure water.

The detection of siderophore production was performed on chrome azurole S (CAS) agar based on the modified assay developed by Neilands, Konopka et al. (1987). In short, the siderophore indicator solution (0.12 % (w/v) CAS in deionized water) was mixed with 10 mL of iron (III) Solution (1 mM $FeCl_3$, 10 mM HCl) and hexadecyltrimethylammonium bromide (HDTMA) solution (0.18% (w/v)) (all Sigma-Aldrich, Germany). Buffered

malt extract medium was prepared as follows: 3% (w/v) PIPES, 0.6 % of NaOH (both Sigma-Aldrich, Germany), 2% malt extract (Biolife, Italy), 0.1% pepton (Merck, Germany), 2% glucose (Kemika, Croatia) and 2% agar (Formedium Ltd, UK) was dissolved in 900 mL water. Both solutions were autoclaved separately, cooled to 55 °C, combined carefully to avoid foaming and poured into Petri dishes. After inoculation of the fungi and incubation at 15 °C, 24 °C and 37 °C the presence of siderophores were observed as yellow to orange discoloration of the otherwise blue medium.

Tolerance to salt and oxidative stress

To test the tolerance to salt stress, YNB agar plates with glucose were supplemented with 5% and 10% (w/v) NaCl. Oxidative stress was tested on YNB plates supplemented after with 5 mM and 20 mM of filter sterilized H_2O_2, which were added to the medium after autoclaving and cooling down to 50 °C (Sigma-Aldrich, Germany).

Proteolytic activity

Strains of *A. melanogenum, E. dermatitidis* and *H. werneckii* were tested for degradation of casein, gelatine and keratine without NaCl and with the addition of 5% and 10% (w/v) NaCl. One loop of 7-day-old cultures grown at 24 °C on MEA were resuspended in physiological solution (0.9% [w/v] NaCl) to OD_{600} 0.5, and used for three-point inoculations by dropping 3 µL cell suspensions onto the agar surface, and by inoculation of the media in test tubes with 10 µL cell suspensions. All of the cultures were incubated at 15, 30 and 37 °C for 4 weeks. All of the experiments were carried out in duplicate.

Casein degradation was performed according to Brizzio et al. (2007) and the proteolytic activities of each species was considered positive if a clarification zone around the colonies on otherwise opaque agar was observed (Brizzio, Turchetti et al. 2007, de Garcia, Zalar et al. 2012). Keratinolytic activity was tested on azure dye-impregnated sheep's wool keratin (keratin azure; Sigma-Aldrich, Germany), as previously described by Scott et al. (2004). Briefly, tubes were first filled with 2 mL of agar basal medium without keratin, and then overlaid with 1 mL alkaline (pH 9) keratin azure medium. After incubation, the tubes were examined for the release and diffusion of the azure dye into the lower layer of basal medium (Scott and Untereiner 2004).

The activity of gelatinases was tested in 5 mL nutrient gelatine tubes composed of 12% (all w/v) gelatine (Sigma-Aldrich, Germany), 0.3% beef extract (Becton Dickenson, USA) and 0.5% peptone (Merck, Germany) (Hankin and Anagnostakis 1975), and inoculated with an a loop of the tested black yeasts. After the incubation, the tubes were placed to 4 °C for 30 min to check for liquefaction of the gelatine.

Morphological analysis

Microscopic characteristics were observed on slides of 7-day-old cultures of fungi grown at 30 °C on potato dextrose agar (Biolife, Italy) plates using a light microscope (Olympus BX51) equipped with a digital camera (Olympus DP73). For observation of extracellular polysaccharide (EPS) capsules and layers, negative staining with India ink was used (Becton, Dickinson and Company; Mexico) (Yurlova and de Hoog 2002).

Results

We systematically tested *A. melanogenum*, *E. dermatitidis* and *H. werneckii* for their tolerance to saline (5% and 10% (w/V) NaCl), pH 3-10 and oxidative stress induced by 5 mM and 20 mM H_2O_2 at various temperatures (15, 24, 37 °C) (Figs. 1, 2 and 3 respectively), growth at oligotrophic conditions and the ability to produce siderophores (Fig. 4), all conditions with clinical relevance. Additionally we examined the morphological features and presence of extracellular polysaccharide capsules (Fig. 5) of the *A. melanogenum*, *E. dermatitidis* and *H. werneckii* and tested their proteolytic activity (Tab. 1), which also play important roles in infectivity (Yike 2011, Seyedmousavi, Netea et al. 2014).

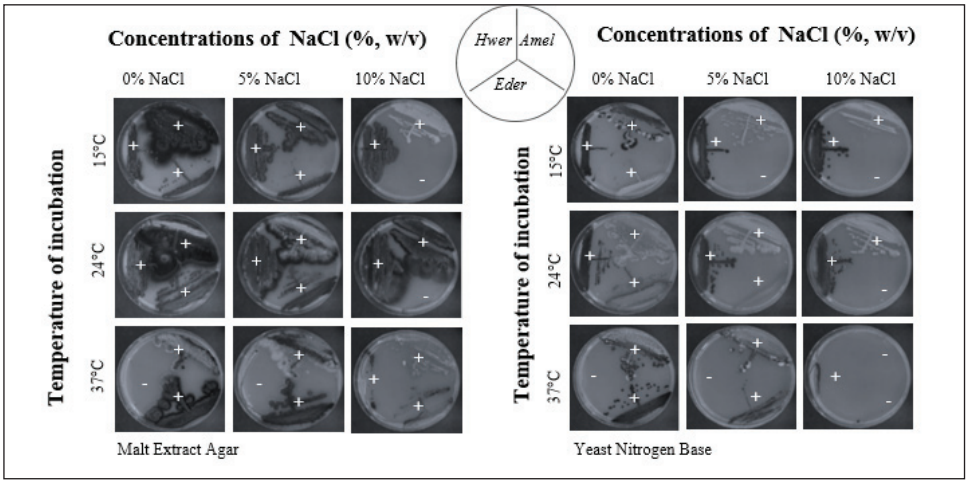

Figure 1: Growth of *Aureobasidium melanogenum* (Amel), *Hortaea werneckii* (Hwer) and *Exophiala dermatitidis* (Eder) at different temperatures and salt concentrations. Left panel -growth on malt extract agar medium (MEA)); right panel - growth on yeast nitrogen base medium with glucose (YNB).

Slika 1: Rast *Aureobasidium melanogenum* (Amel), *Hortaea werneckii* (Hwer) in *Exophiala dermatitidis* (Eder) pri različnih temperaturah in koncentracijah soli. Levo- rast na bogatem gojišču s sladnim ekstraktom (MEA); desno - rast na definiranem gojišču z dušikovo osnovo in glukozo (YNB).

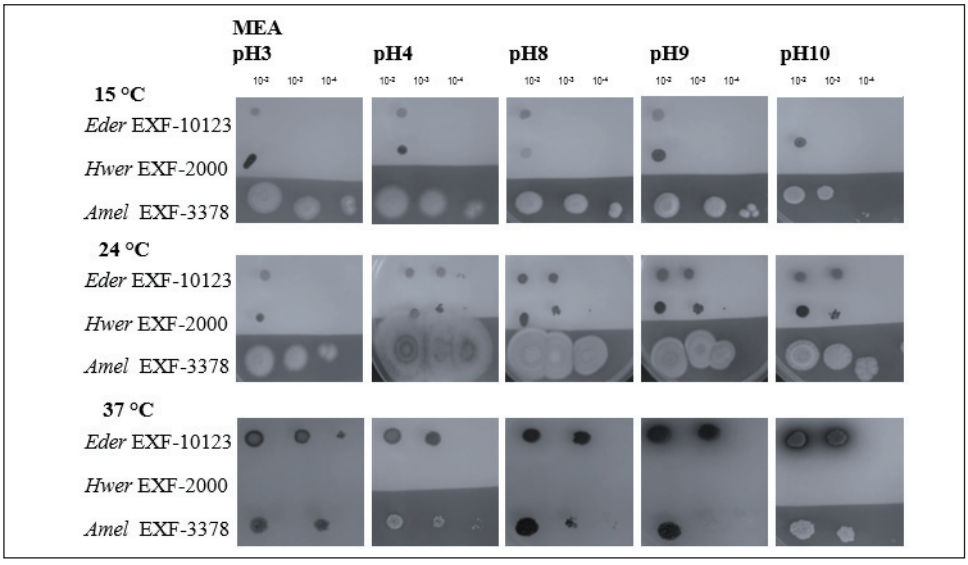

Figure 2: Growth of *Exophiala dermatitidis* (Eder), *Hortaea werneckii* (Hwer) and *Aureobasidium melanogenum* (Amel) at various temperatures and pH values.

Slika 2: Rast *Exophiala dermatitidis* (Eder), *Hortaea werneckii* (Hwer) in *Aureobasidium melanogenum* (Amel) pri različnih temperaturah in pH vrednostih.

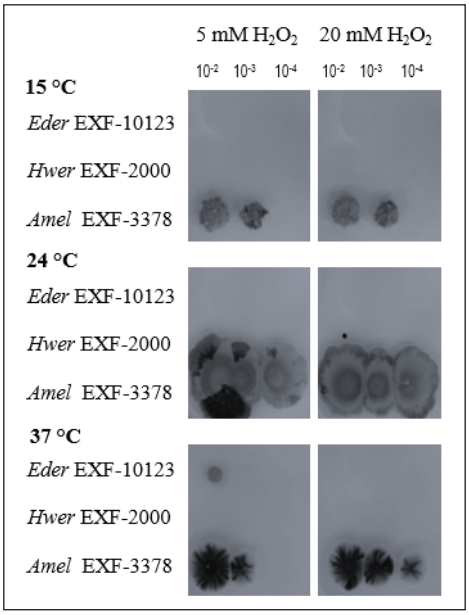

Figure 3: Temperature and H_2O_2 tolerance of *Exophiala dermatitidis* (Eder), *Hortaea werneckii* (Hwer) and *Aureobasidium melanogenum* (Amel).

Slika 3: Temperaturna toleranca in toleranca na H_2O_2 *Exophiala dermatitidis* (Eder), *Hortaea werneckii* (Hwer) in *Aureobasidium melanogenum* (Amel).

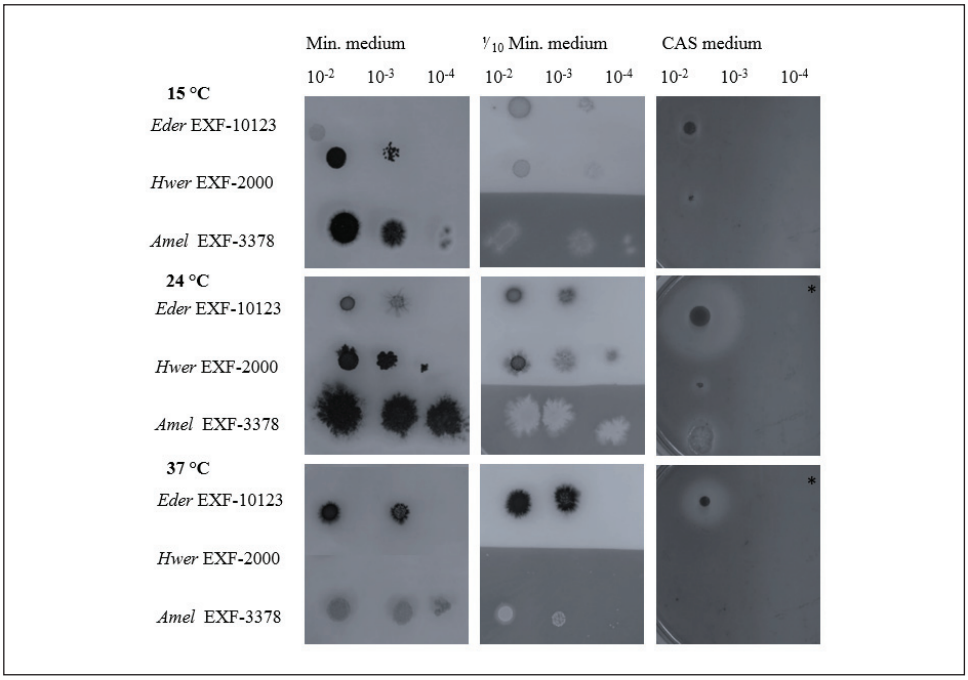

Figure 4: Oligotrophism and siderophore production (on chrome azurole S agar; CAS) of *Exophiala dermatitidis* (Eder), *Hortaea werneckii* (Hwer) and *Aureobasidium melanogenum* (Amel).

Slika 4: Oligotrofizem in proizvodnja sideroforov (na krom azurol S agarju; CAS) *Exophiala dermatitidis* (Eder), *Hortaea werneckii* (Hwer) in *Aureobasidium melanogenum* (Amel).

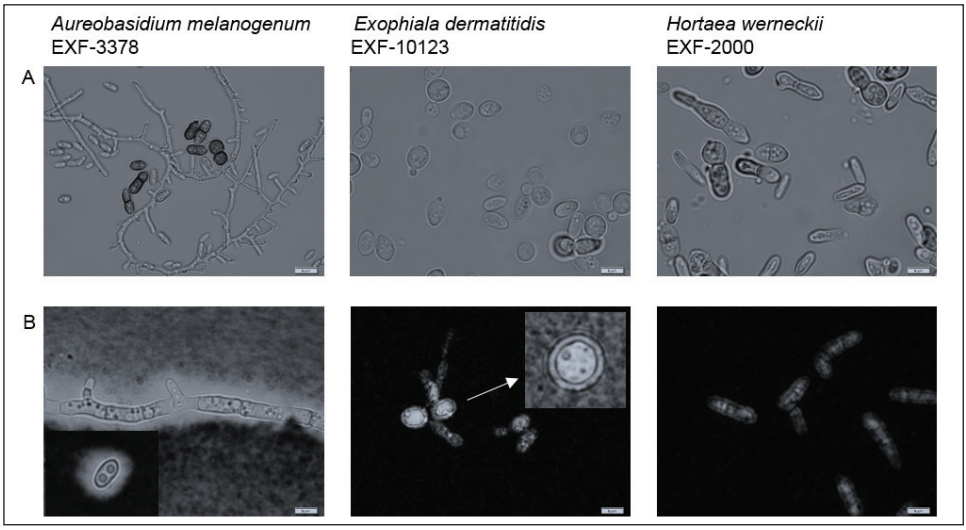

Figure 5: Representative micromorphologies of *Aureobasidium melanogenum*, *Exophiala dermatitidis* and *Hortaea werneckii*. A - Native phase-contrast micrographs. B - Extracellular polysaccharides or capsules negatively stained with India ink. Scale bar: 5 µm.

Slika 5: Reprezentativna mikromorfologija *Aureobasidium melanogenum*, *Exophiala dermatitidis* in *Hortaea werneckii* . A - Nativna fazno-kontrastna mikrografija. B - Zunajcelični polisaharidi ali kapsule, ki so negativno barvani z India črnilom. Merilce: 5 µm.

Table 1: Proteolytic activities of the three black yeasts, *Aureobasidium melanogenum*, *Hortaea werneckii* and *Exophiala dermatitidis* without NaCl and at 5% and 10% NaCl (w/v). Legend: +, activity; -, no activ ity; ng, no growth.

Tabela 1: Proteolitične aktivnosti treh črnih kvasovk, *Aureobasidium melanogenum*, *Hortaea werneckii* in *Exo phiala dermatitidis* brez NaCl in pri 5% in 10% NaCl (m / v). Legenda: +, aktivnost; -, ni aktivnosti; ng, ni rasti.

		A. melanogenum			*H. werneckii*			*E. dermatitidis*		
Proteolytic activity	Temperature \ Substrate	15°C	24°C	37°C	15°C	24°C	37°C	15°C	24°C	37°C
general	kasein	+	+	+	-	+	ng	-	-	-
	kasein + 5% NaCl	±	-	-	-	+	ng	-	-	-
	kasein + 10% NaCl	-	-	ng	-	+	-	ng	ng	ng
keratinase	keratin	-	-	-	-	-	ng	-	-	-
	keratin + 5% NaCl	-	-	-	-	-	ng	-	-	-
	keratin + 10% NaCl	-	-	ng	-	-	-	ng	ng	ng
gelatinase	gelatine	+	-	-	-	-	ng	-	-	-
	gelatine + 5% NaCl	+	-	-	-	-	ng	-	-	-
	gelatine + 10% NaCl	-	-	-	-	-	-	ng	ng	ng

Our data show that *E. dermatitidis* grows best at 37 °C under all of the tested salinities and pH conditions, whereas *A. melanogenum* and *H. werneckii* grow best at 24 °C (Fig. 2). *E. dermatitidis* grew well with 5% NaCl, while at 10% NaCl it grew only at its optimal growth temperature of 37 °C. The growth of *E. dermatitidis* at 37 °C was documented previously (de Hoog, Guarro et al. 2015), whereas the growth of *H. werneckii* above 35 °C at elevated salinity was observed only recently (Zupančič and Gunde-Cimerman, unpublished data). Here, the growth at 37 °C in the presence of NaCl was confirmed on both of the tested (MEA and YNB) media, although the growth of *H. werneckii* on the defined yeast nitrogen base (YNB) medium was very slow, with little growth even after 40 days of incubation (Fig. 1). The minimum concentration of NaCl that supported growth of *H. werneckii* at 37 °C was 10% (w/v) NaCl. All three black yeasts also grow well across a wide pH range, from pH 3 to pH 10. Our growth tests additionally confirmed that *A. melanogenum* is the most adaptable of the three species tested, not only to the combination of temperatures and salinities (Fig. 1), but also to the combination of temperatures and pH (Fig. 2).

Increasing salinity and/or temperatures reduced the degree of melanisation of *A. melanogenum*, whereas this was not the case for *H. werneckii* and *E. dermatitidis* (Fig. 1). Interestingly, at alkaline pH, a pigmented substance appeared to be leaking from the colonies of *E. dermatitidis*, forming diffuse dark areas around the colonies (Fig. 2).

Tolerance to H_2O_2 in the growth medium (chronic exposure) was greatest in *A. melanogenum*, which grew at both 5 mM H_2O_2 and 20 mM H_2O_2 (Fig. 3). *E. dermatitidis* grew at 5 mM H_2O_2 at optimal temperature (37 °C), while *H. werneckii* did not.

The oligotrophic characters of these three species were assessed on a minimal medium that was diluted to 10% of the original recipe (Fig. 4). All three species grew well on this medium at all tested temperatures (except for *H. werneckii*, which was unable to grow at 37 °C), therefore confirming their oligotrophic nature. The colonies of *H. werneckii* and *A. melanogenum* were smaller and less pigmented on the diluted minimal medium, whereas the colonies of *E. dermatitidis* were comparable in both size and melanisation on the minimal and diluted minimal media. Additionally, the discoloration of otherwise blue CAS agar showed the ability of siderophore production for all three species (Fig. 4). Given the largest halo area around the colony of *E. dermatitidis*, it is considered as the most potent producer of siderophores and importantly it was the only one able to produce siderophores at 37 °C.

Proteolytic activities were tested as the ability to degrade casein, gelatine and keratin supplemented with 0, 5% and 10% NaCl at 15 °C, 24 °C and 37 °C (Tab. 1). The results showed that of these three species, only *A. melanogenum* and *H. werneckii* were able to degrade casein and gelatine at certain conditions, whereas *E. dermatitidis* showed no proteolytic activity. The yeast *A. melanogenum* degraded casein in the absence of NaCl at all three temperatures of incubation and in the presence of 5% NaCl only weakly at 15 °C. *H. werneckii* on the other hand was the only species showing proteolytic activity on casein in the presence of both 5% and 10% NaCl but exclusively at 24 °C (Tab. 1).

We observed extracellular polysaccharides (EPS) covering the cells of *A. melanogenum* and *E. dermatitidis* (Fig. 5) under light microscopy after negative staining with India ink. The EPS layer around all *E. dermatitidis* cells was uniformly thick and well defined, whereas EPS was completely absent in the case of *H. werneckii*. In the case of *A. melanogenum* EPS layer took the form of irregularly thick masses surrounding the individual (or even groups of) chlamydospores and hyphae, whereas no EPS capsules or layers were observed around the non-pigmented yeast cells of *A. melanogenum* (Fig. 5).

Discussion

Species can differ in the depth of their extremotolerance (how extreme a particular type of stress can be before it causes cessation of growth and death), but also in its width (how many different types of stress and their combinations they endure). For the latter type Gostinčar, Grube et al. (2011) introduced the concept ‘polyextremotolerance’. Polyextremotolerant fungi are able to colonise entirely different substrates and endure a variety

of environmental conditions. A typical example of such species is *Aureobasidium melanogenum*, one of the few fungi in its phylogenetic group that is regularly encountered as an agent of human opportunistic infection. *A. melanogenum* is tolerant to high temperature, UV and ionising irradiation, lack of nutrients and other types of stress, resulting in its ability to colonise all kinds of habitats, from the municipal water supply system to window glass or Arctic ice (Gostinčar, Ohm et al. 2014). Nutritional versatility can include the ability to degrade aromatic hydrocarbons, a phenomenon particularly common in the order Chaetothyriales (Prenafeta-Boldu, Summerbell et al. 2006). This is also the order with the highest number of opportunistic species compared to the total number of species known in the order. A similar degree of extremotolerance is seen in *Exophiala dermatitidis*, which is commonly found in anthropogenic habitats such as tap water, dishwashers and creosoted railway sleepers or gasoline-rich environments, while, interestingly, in nature it is found only rarely (Zalar, Novak et al. 2011). On the other side of the spectrum, the monodirectional counterpart of polyextremotolerance might be referred to as 'monoextremotolerance'. *Hortaea werneckii* is a typical representative of this type of ecology, as it is almost invariably selected by presence of high concentrations of NaCl in its natural habitat — despite the fact that *in vitro* it grows well even without added salt (Gunde-Cimerman, Zalar et al. 2000).

Our results confirm that *A. melanogenum* has the widest ecological amplitude of the three investigated species. This is reflected in a combination of temperatures and salinities (Fig. 1) and of temperatures and pH (Fig. 2) supporting its growth. The fungus is well known to employ extensive stress-combating molecular mechanisms (reviewed in Gostinčar, Ohm et al. (2014)). The highest tolerance to H_2O_2 was seen in *A. melanogenum*, which was previously reported to tolerate short-term exposure to 640 mM H_2O_2 (Castiglia and Kuhar 2015); here we tested chronic exposure, which is more toxic to the cells. The inability of *H. werneckii* to grow in the presence of 5 mM H_2O_2 supports the previous observation (Kejžar, Gobec et al. 2013), while the growth of *E. dermatitidis* at 5 mM H_2O_2 was recently also reported by Song, Laureijssen-van de Sande et al. (2017). *E. dermatitidis* is not exceptionally tolerant to NaCl as it grows poorly at 10% NaCl, but can cope with a wide pH range, from 3-10 at all three temperatures of incubation. Interestingly, at high pH (9 and 10) brown halos appeared around the colonies indication diffusion of the (presumably) melanin pigment from the cell walls. This most probably indicates the effect of alkaline stress on the cell wall as was previously observed in *Saccharomyces cerevisiae* (Serrano, Martin et al. 2006).

Although *E. dermatitidis* does not grow as well in the presence of H_2O_2 as *A. melanogenum* (but exceeds the tolerance to H_2O_2 of *H. werneckii*), it is the most virulent of the three. The fungus shows exceptional temperature tolerance and grows well above its optimal 37 °C (and even at 45-57 °C) (Zalar, Novak et al. 2011, Blasi, Tafer et al. 2015) and is still metabolically active at 1 °C (Blasi, Tafer et al. 2015). It is enriched in habitats that are generally hostile to microbial growth, resulting in a lowered species diversity. This suggests that the fungus has a decreased competitive ability and is pushed to environments where few other microbes survive.

H. werneckii is unable to grow at temperatures above 35 °C (de Hoog and Gerrits van den Ende 1992, Chen, Xing et al. 2012). However, an additional challenge of 10% NaCl (w/v), the salinity that was previously determined as the optimal for *H. werneckii* (Kogej, Ramos et al. 2005), enables the fungus to grow at 37 °C (Zupančič and Gunde-Cimerman, unpublished data; and also confirmed here). The medical relevance of this observation remains to be investigated.

Limitation of nutrients also presents a stressful condition and microorganisms employ various mechanisms to prevent starvation. One of such is the production of siderophores, high affinity iron-chelating organic compounds (Neilands 1993), that have a role both in stress response and in virulence (Johnson 2008). All three here studied black yeasts are oligotrophic, *A. melanogenum* and *E. dermatitidis* also at 37 °C, and able to produce siderophores to overcome iron starvation. Importantly, of the three species *E. dermatitidis* appears to be the most potent siderophores producer and the only one able to produce siderophores at 37 °C. This is of great importance during the establishment of the infection in humans where phagocytes

release mediators that sequester iron and prevent the growth of non-siderophore-producing fungi (Hamad 2008).

Digestion of protein substrates plays an important role in growth and survival of both saprophytic and pathogenic fungi. Extracellular serine, aspartic, and metalloproteases are considered virulence factors of many pathogenic species (Monod, Capoccia et al. 2002, Yike 2011). Secreted proteases are of great importance for the ability of dermatophytes to colonise the surface of skin (*stratum corneum*, nails or hair) and of visceral pathogens, where they are involved in the adherence process and penetration of tissues and in interactions with the immune system of the infected host (Yike 2011). However, in the case of the three black yeasts studied here the proteolytic activities do not appear to be the deciding factor in their pathogenesis. For instance, the most virulent species *E. dermatitidis* showed no proteolytic activity at all, whereas *A. melanogenum* and *H. werneckii* degraded casein and gelatine only under certain conditions. Only *A. melanogenum* showed proteolytic activity at 37 °C, a trait most probably relevant to its pathogenesis.

Several fungi can produce EPS, either in the form of a well-defined layer surrounding the cell (*i.e.* a capsule), or as a more diffuse EPS covering of the cells (Yurlova & de Hoog 2002). This physical barrier interferes with phagocytosis and immune responses of the host, by inhibiting the production of proinflammatory cytokines and the binding of complement components, and by reducing the migration of leukocytes to the site of inflammation (Kent & Juneann 1998). Furthermore, these EPSs can protect the microorganisms from detrimental conditions by aiding in the formation of a gel-like matrix that promotes their adherence to surfaces and formation of biofilms (Ravella *et al.* 2010). The production of EPS is thus an important virulence factor. This is well-demonstrated for the pathogen *C. neoformans*, for which it was shown that mutants without the EPS capsule are typically avirulent, whereas the capsular strains show (various levels of) virulence (Fromtling *et al.* 1982). The EPS production of our studied strains (Fig. 5) correspond to their pathogenic potential: *E. dermatitidis* had yeast cells enclosed in a rather uniform EPS layer, *A. melanogenum* hyphae and chlamydospores were surrounded by irregular, thick EPS masses, while no EPS were visible around *H. werneckii*. The absence of the EPS capsule or layers for *H. werneckii* corresponds to its non-invasive pathogenic potential reflected in infections resticted to the *stratum corneum*, where immune responses are rarely activated. Without an EPS layer, the cells can retain a high degree of the cell wall hydrophobicity and promote the lipophilic adhesion of the cell wall to the human skin and to the surfaces of plastic medical devices (Göttlich, de Hoog et al. 1995).

If black yeasts are indeed pre-adapted for causing (opportunistic) infections (Gostinčar, Grube et al. 2011, Gostinčar, Muggia et al. 2012), their high stress tolerance might be useful as a marker that indicate species or groups with the potential to thrive in novel anthropogenic habitats and to enter into harmful interactions with humans. Our results clearly demonstrate the (poly)extremotolerance of the three respesentatives of black yeasts and importantly compare their ability of tolerance to a combination of two stress factors at the same time – a condition that is relevant to the pathogenesis of warm-bloded hosts; and uncovered certain traits linked to their pathogenic potential (*e.g.* synthesis of siderophores and degradation of proteins at human body temperature.

Povzetek

Črne kvasovke so filogenetsko raznovrstna skupina gliv iz redov Dothideomycetes in Eurotiomycetes, za katere so značilne melanizirane celične stene, številne med njimi pa so tudi izredno tolerantne na enega ali več različnih stresov. Črne kvasovke lahko tolerirajo različne okoljske strese, kot so visoke in nizke temperature, spremembe v pH, oksidativni stres, pomanjkanje hranil in ionizirajoče sevanje, in sicer do različnih mej. Toleranca na širok spekter ekstremov v okolju se imenuje poliekstremotoleranca, medtem ko lahko toleranco na enega ali nekaj dejavnikov stresa pojmujemo kot monoekstremoleranco.

V raziskavi smo ovrednotili in primerjali toleranco na strese, ki so relevantni za oportuno patogenost pri človeku pri treh črnih kvasovkah, in sicer pri poliekstremotolerantnih vrstah *Aureobasidium melanogenum* in *Exophiala dermatitidis* ter pri monoekstremoterantni *Hortaea werneckii*.

Te tri črne kvasovke so bile izpostavljene kombinaciji različnih temperatur (15, 24 ° C in 37 ° C) in dodatnega stresnega faktorja, kot je NaCl (5 in 10% NaCl), pH 3-10 in prisotnost 5 mM in 20 mM H_2O_2, ki je povzročil kronični oksidativni stres. Poleg tega smo testirali njihov oligotrofen značaj ter proizvodnjo spojin, ki kelirajo železo (siderofori), njihovo proteolitično aktivnost in proizvodnjo zunajceličnih polisaharidnih kapsul. Vse naštete lastnosti so povezane s patogenostjo.

Naši rezultati kažejo, da lahko obravnavane črne kvasovke uspevajo pri nizkih temperaturah (15 °C) in pri temperaturi človeškega telesa (37 °C), pri povišani slanosti, ekstremnih pH vrednosti in H_2O_2 do različnih mej. Vse kažejo oligotrofni značaj, saj lahko rastejo tudi pri zelo omejeni razpoložljivosti hranil in proizvajajo siderofore za vezavo železa. Vrsta *A. melanogenum* je bila najbolj prilagodljiva in edina vrsta, ki je kazala proteolitično aktivnost pri 37 °C, medtem ko je vrsta *E. dermatitidis* edina tvorila siderofore pri 37 °C; dve lastnosti, ki sta ključnega pomena za virulenco pri ljudeh.

Črne kvasovke so se razlikovale tudi glede tvorbe zunajceličnih polisaharidov: pri vrsti *E. dermatitidis* smo okrog celic opazili razločno oblikovane kapsule, pri *A. melanogenum* je bil okoli celic in hif prisoten obilen in nepravilno oblikovan sloj EPS, pri *H. werneckii* pa ni bilo opaznih EPS. To je v skladu s patogenim potencialom teh vrst: *H. werneckii* je namreč neinvaziven patogen na površini kože, kjer je za kolonizacijo potrebna lipofilna adhezija, medtem ko *A. melanogenum* in *E. dermatitidis* lahko povzročita subkutane, visceralne, ter celo sistemske okužbe, pri katerih je vloga EPS pomembna za oteževanje imunskega odziva in fagocitoze gostitelja.

Čeprav je glede na literaturo izrazita proteolitična aktivnost gliv lastnost, ki je tesno povezana s patogenezo pri ljudeh, naši rezultati tega ne podpirajo. Najbolj patogena vrsta *E. dermatitidis* ni pokazala nobene proteolitične aktivnosti ne na kazeinu ne na želatini, vrsta *A. melanogenum* pa je edina razgrajevala kazein pri 37 °C.

Pričujoča študija kaže, da so stopnja tolerance na stres in oligotrofizem, delno pa tudi proteolitična aktivnost, povezane z naraščajočim oportuno patogenim potencialom, vendar pa so za boljše razumevanje te povezave potrebne dodatne raziskave.

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