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Role of melanin in the black yeast fungi *Aureobasidium pullulans* and *Zalaria obscura* in promoting tolerance to environmental stresses and to antimicrobial compounds

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ABSTRACT

The role of melanin in *Aureobasidium pullulans* ATCC 15233 and *Zalaria obscura* LS31012019, under simulated osmotic, oxidative, and high temperature stress conditions, on the susceptibility to essential oils (EOs) or antifungals and on the resistance to UV-C radiation was investigated. 93.6% of melanized *A. pullulans* and 92% of *Z. obscura* survived to 40 °C for 1 h compared to 77% and 76% of the non-melanized ones, while both yeasts tolerated a high concentration of NaCl (up to 30%) and H_2O_2 (up to 400 mM) regardless of melanin production. Higher EOs antifungal efficacy was observed in non-melanized cells (growth inhibition zone >30 mm) compared to the melanized ones (25 mm). Similarly, the lowest Minimum Inhibitory Concentrations (MIC) and Minimum Fungicidal Concentration (MFC) values were evidenced for Fluconazole, Clotrimazole, Bifonazole and Amphotericin in the non-melanized fungi. Increasing UV-C intensity (up to 2004.5 J/m^2) caused total death in the non-melanized strains compared to about 30% growth reduction in the melanized ones. The results of this investigation, the first focused on the biological role of melanin in "black-fungi", are novel and encourage a better understanding of the biochemical features of melanin in the environmental adaptive ability of the new species *Z. obscura*.

1. Introduction

Black yeasts include a group of polyextremotolerant fungi able of colonizing a wide range of extreme environments for their ability to survive under acidic, alkaline, and toxic conditions, high temperature, low nutrient availability, and osmotic or mechanical stress. *Aureobasidium pullulans* is a common ubiquitously distributed melanized yeast-like fungus (the so-called black yeast), phylogenetically belonging to *Ascomycota*, order *Dothideales*, family *Dothideaceae* (Schoch et al., 2006; Zalar et al., 2008). This microorganism is well known for its biotechnological potential, particularly for the production of metabolites, such as pullulan, with numerous applications in medicine, pharmacy and the food industry (Leathers 2003; Cheng et al., 2011; Prasongsuk et al., 2018). Furthermore, *A. pullulans* is a commercially available biocontrol species effective against bacteria and fungi pathogens for plants (Johnson and Temple, 2013; Spadaro and Droby, 2016). The ecology of *A. pullulans* is not deeply studied as its biotechnological aspects, but this yeast is widely distributed in many different environments: plants (Andrews et al., 2002; Raspor et al., 2006; Grube et al., 2011; Nováková et al., 2018), coastal hypersaline water (Gunde-Cimerman, 2012), glacial ice as well as arid or topical areas, and also in indoor habitats (Punnapayak et al., 2003; Samson et al., 2010; Zalar et al., 2011). Colonies of *A. pullulans* initially appear as cream, yellow, pink, or light brown (Deshpande et al., 1992), but in the later growth stages, the colonies change colour to dark olivaceous or blackish due to the production of chlamydospores, a typical feature of *A. pullulans*. Moreover, the colonies are initially smooth and later become covered in slime. Hyphae are typically smooth, thin-walled and hyaline with straight conidia. However, these characteristics can be different based on the used culture growth media and among the different strains. The dark colour is due to melanin, a pigment not essential for fungal growth and development, but able to confer important biological functions, such as increased virulence of pathogenic fungi and enhanced survival under environmental stress conditions, including extreme temperatures, high

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salts, desiccation, UV radiation, oxidizing agents and ionizing radiation (Jiang et al., 2016; Revankar and Sutton 2010).

Melanins are negatively charged hydrophobic pigments of high molecular weight composed of polymerized phenolic or/and indolic compounds. Many fungi synthesize melanin via the DHN (1,8-dihydroxynaphthalene) pathway, which starts from the endogenous precursor molecule, acetyl CoA or malonyl CoA, followed by a series of chemical reactions, up to the polymerization of DHN to melanin. Few fungi synthesize melanin via L-3,4-dihydroxyphenylalanine (L-dopa), in a pathway that resembles mammalian melanin biosynthesis (Eisemann and Casadevall, 2012). Melanins are resistant to chemical degradation by acids, insoluble in most substances and can be broken down only by oxidation or dissolution in alkaline solvents. In the literature, it's well reported the protective role of melanins to diverse environmental stress conditions, such as hyperosmotic conditions, heavy metals, desiccation, and ultraviolet radiation (Jiang et al., 2016; Eisemann et al., 2020). Indeed, inhibiting melanin production decreased fungal tolerance to high salts concentration (Kejžar et al., 2013), desiccation or long term-survival (Eisemann et al., 2020).

Recently, a strain belonging to the new species of the genus *Zalaria* (named *Zalaria obscura* LS31012019) was isolated from a damaged wooden sculpture by our research group (Sabatini et al., 2020). The colonies of *Z. obscura* are similar to those of *A. pullulans* and only molecular methods were able to distinguish them. Moreover, both species produce melanin but, in the literature, there is poor information regarding the role of this pigment on biological features of the new species *Z. obscura*.

In this study, we evaluated the production of melanin in *A. pullulans* and *Z. obscura* under nitrogen starvation and the role of melanin in their protection against the osmotic (sodium chloride), oxidative stress (hydrogen peroxide), UV-C radiation, and high temperature. In addition, the susceptibility of melanized and non-melanized cells against natural essential oils (*Rosmarinus officinalis*, *Origanum majorana*, *Ocinum basilicum*, *Thymus vulgaris*, *Origanum vulgare, Melaleuca alternifolia*, *Mentha piperita*, *Cuminum cyminum*, *Cupressus sempervirens*, *Laurus nobilis*, *Cymbopogon citratus*, *Amyris balsaminifera* oil, *Citrus medica*, *Cymbopogon winterianus*), and antifungals (Fluconazole, Clotrimazole, Bifonazole and Amphotericin B) was studied.

2. Material and methods

2.1. Fungal strains and conidial suspension preparation

A. pullulans ATCC 15233 (American Type Culture Collection, Virgina, USA) and *Z. obscura* LS31012019, belonging to our culture collection, were used in this study. The strains were grown on potato dextrose agar (PDA, Liofilchem, Italy) at 25 °C for 5–7 d. Fungal suspensions were prepared according to National Committee for Clinical Laboratory Standards (NCCLS, 2008). For each strain, colonies were harvested from PDA plate and diluted in sterile 0.85 % saline solution with 0.05 % Tween 80. The suspension was vortexed for 15 s and adjusted to an optical density (OD 530 nm) of 0.130, corresponding to about 10^6 cells mL^{-1}. The quantification of each inoculum was verified with the standard plate count agar method on PDA.

2.2. Melanin production under nitrogen starvation

To understand the effect of nitrogen starvation on melanin production, the procedure described by Jiang et al. (2016) was followed with some modifications. Indeed, the presence of $(NH_4)_2SO_4$ in the culture medium affects the production of melanin, thus when the fungi were grown without $(NH_4)_2SO_4$ they produce melanin and when grown with $(NH_4)_2SO_4$ they are not able to produce melanin. For this, *A. pullulans* and *Z. obscura* were grown on PDA, sabouraud dextrose agar (SDA, VWR, Milan, Italy) and yeast potato dextrose (YPD) agar (glucose 20 g L^{-1}, yeast extract 10 g L^{-1}, polypeptone 20 g L^{-1}, agar purified 15 g L^{-1}), with or without 20 g L^{-1} of $(NH_4)_2SO_4$. All the plates were incubated at 25 °C and daily observed for melanin production (presence of dark cultures) for up to 5–7 d.

2.3. Morphological observations

A. pullulans and *Z. obscura* melanized and non-melanized cells were collected from PDA plates and directly observed under normal light with an Axiolab RE 31509 microscope (Zeiss, magnification × 40/0.65).

2.4. Tolerance to osmotic stress

Melanized and non-melanized *A. pullulans* and *Z. obscura* cells were resuspended in sterile 0.85 % saline solution with 0.05 % Tween 80 to about 10^6 cells mL^{-1}. At this point, each suspension was mixed with 200 g L^{-1} or 300 g L^{-1} of NaCl and the mixtures were kept at room temperature by shaking at 180 rpm. After 1 h, NaCl treated cells were serially diluted 10^4 times, 0.1 mL of each dilution was transferred to PDA plate and incubated at 25 °C for 5–7 d. At the end of incubation, the colony-forming units (cfu mL^{-1}) were counted and compared to those of the not treated cell suspensions. The treatments were performed in triplicate and all the data were the average of three independent cultivations.

2.5. Tolerance to oxidative stress

Melanized and non-melanized *A. pullulans* and *Z. obscura* cells were harvested as described above and each suspension was mixed with 200 mM, 300 mM or 400 mM H_2O_2 and left at room temperature by shaking at 180 rpm. After 1 h, H_2O_2 treated cells were serially diluted 10^4 times and 0.1 mL of each dilution was transferred to PDA plate and incubated at 25 °C for 5–7 d. Finally, the colony-forming units (cfu mL^{-1}) were counted and compared to those of the not treated cell suspensions. The treatments were performed in triplicate and all the data were the average of three independent cultivations.

2.6. Heat tolerance

Fungal suspensions, prepared as described above, were incubated in a water bath at 40 °C for 1 h by shaking at 180 rpm. Samples incubated at 25 °C in the same conditions were considered as controls. The heat-treated cells were serially diluted 10^4 times, 0.1 mL of each dilution was transferred to PDA and incubated at 25 °C for 5–7 d. Also, in this case, the colony-forming units (cfu mL^{-1}) were counted and compared to those of the not heat-treated cells. The treatments were performed in triplicate and all the data were the average of three independent cultivations.

2.7. Susceptibility to essential oils

The susceptibility of *A. pullulans* and *Z. obscura* to different EOs was determined by a modified agar diffusion method (Clinical and Laboratory Standards Institute, CLSI 2009). Fourteen commercially available pure EOs were used in this study: *R. officinalis* leaf oil, *O. majorana* leaf oil, *O. basilicum* leaf oil, *T. vulgaris* leaf oil, *O. vulgare* leaf oil, *M. alternifolia* leaf oil (tee tree), *M. piperita* leaf oil, *C. cyminum* seed oil, *C. sempervirens* leaf oil, *L. nobilis* leaf oil, *C. citratus* leaf oil (Lemongrass), *A. balsaminifera* oil, *C. medica* fruit

extracted, *C. winterianus* leaf oil. Carvacrol oil (Sigma, Milan, Italy) was herein considered the positive control for natural substances. Stock solutions (10 % v/v) of each oil were prepared in polyethylene glycol (PEG) and ethanol (1:1, v/v) (Sigma, Milan, Italy). Stock solutions were kept at −20 °C in the dark until use.

Each fungal suspension, prepared as described above, was distributed with a sterile cotton swab on the surface of selected agar plates (PDA, SDA, and YPD with and without $(NH_4)_2SO_4$) for a total of 3 times, with a rotation of approximately 60° each time to ensure a good distribution of the inoculum on the plate. After the plates have been dried for 5 min, 3 blank sterile disks (6 mm diameter) were applied to each plate and covered with 10 μL of the different EOs solutions (10 %). A disk with 10 μL of PEG-Ethanol (1:1, v/v) was used as control. All the plates were incubated at 25 °C for 5–7 d and the growth inhibition diameter (ID) around each disk was daily observed and measured (in mm). In this study, an ID value greater than 10 mm was considered the index of antifungal activity. Each experiment was performed in triplicate.

2.8. Susceptibility to antifungals

The susceptibility of melanized and non-melanized *A. pullulans* and *Z. obscura* to different antifungals was assessed. The Minimum Inhibitory Concentrations (MICs) of Fluconazole (FCZ), Clotrimazole (CTZ), Bifonazole (BFZ) and Amphotericin B (AMP B) (Sigma) were determined following the standard micro-dilution method (NCCLS, 2008), with some modifications. Firstly, concentrated stock solutions of the four antifungals were prepared in pure DMSO (Sigma). Preliminary experiments were performed to exclude possible DMSO antifungal activity; in any case, the volume of DMSO never exceeded 5 % (v/v) of the final volume. Each fungal suspension was prepared as above described (OD 0.1 at 530 nm) and resuspended in Sabouraud dextrose broth (SDB, WVR), as well as in standard RPMI 1640 medium (Sigma). Successively, 100 μL was inoculated into 96-well plates together with the appropriate volumes of each antifungal solution in the range of 512–1 μg mL^{-1} for FCZ, CTZ, BFZ and 32–0.0625 μg mL^{-1} in the case of AMP B. Two rows were left for the positive control (growth control) and negative control (medium only), respectively. Plates were incubated at 25 °C and examined after 48 h of incubation. MIC is defined as the lowest drug concentration that inhibits visible growth in comparison with the untreated control sample. In addition, the turbidity of the 96-wells plate was assessed by spectrophotometer (530 nm) (Multiskan EX, Thermo Scientific, Monza, Italy). Each experiment was performed in triplicate.

To determine the Minimum Fungicidal Concentration (MFC), from each well showing complete growth inhibition (clear well), the last positive well (growth similar to that of the growth control) and the growth control respectively, 20 μL were removed and streaked on PDA. The plates were incubated at 25 °C for 5–7 d; for each microorganism, MFC is defined as the lowest drug concentration that showed either no growth or fewer than three colonies (approximately 99–99.5 % killing activity).

2.9. Tolerance to UV-C radiation

Firstly, 100 μL of each fungal suspension prepared as described above, was homogeneously distributed, in triplicate, at a concentration of 10^6 cells mL^{-1} on the surface of PDA plates. Inoculated samples were left at room temperature for approximately 15 min to allow the complete absorption of the inoculum. Then, the lids of Petri dishes were removed and the samples were irradiated with a low-pressure mercury vapour lamp UV-C 15 W (G 13, Sankyo Denki G15T8), placed a 50 cm of distance from the samples in a cabinet previously sterilized. The radiation emitted was measured using a radiometer (HD2102.2, LP 471 UV-C) and expressed as J/m^2. The considered times of exposure were 0 (baseline), 60, 180, 300 and 600 s, corresponding to 186.61 J/m^2, 591.6 J/m^2, 1000.7 J/m^2 and 2004.5 J/m^2 respectively. For each strain, two inoculated PDA plates not subjected to UV-C radiation were considered as growth controls. After treatments, all the plates were incubated at 25 °C for 5–7 d and, then, the number of colony-forming units (cfu mL^{-1}) was counted and compared to the related controls. All the experiments were carried out independently at least three times.

2.10. Statistical analysis

Statistical analysis was performed using Prism 5.0 (GraphPad Software, Inc., La Jolla, USA). All the data are

expressed as the mean values obtained in three independent experiments performed in duplicate. The conditions necessary to perform parametric tests were checked before conducting the analysis, otherwise, non-parametric tests were utilized. The level of significance was considered $\alpha = 0.05$.

3. Results

3.1. Melanin production under nitrogen starvation

The results of melanin production in *A. pullulans* and *Z. obscura* under nitrogen starvation were illustrated in Fig. 1. As shown, the melanized yeast strains showed the formation of dark cultures after 7 d of incubation at 25 °C. On the contrary, the cultures grown on PDA and SDA media with 20 g L^{-1} of $(NH_4)_2SO_4$, did not produce any melanin, indicating that melanin biosynthesis was significantly repressed in the presence of $(NH_4)_2SO_4$. No production of melanin was detected in YPD medium with and without $(NH_4)_2SO_4$.

The microscopic observation of *A. pullulans* and *Z. obscura* colonies grown on PDA revealed the presence of strongly melanized cells organized in long hyphae widely distributed (Fig. 2A and B). Interestingly, the same cells grown on PDA with ammonium sulfate resulted not only without the presence of melanin (hyaline) but also without the development of structured hyphae (Fig. 2C and D).

3.2. Tolerance to osmotic stress

Data showing the tolerance of *A. pullulans* and *Z. obscura* to osmotic stress were presented in Table 1. The difference in the viability of both the examined yeast revealed that melanized cells were only a little more tolerant to osmotic stress. In detail, after exposure to 200 g L^{-1} of NaCl, 4.08 ± 0.21 log cfu mL^{-1} were detected for melanized *A. pullulans*, in comparison to 3.95 ± 0.16 log cfu mL^{-1} observed for non-melanized *A. pullulans*. In the case of *Z. obscura*, 4.20 ± 0.12 log cfu mL^{-1} were detected for melanized cells, quite similar to 4.00 ± 0.17 log cfu mL^{-1} observed for *Z. obscura* non-melanized ones. The highest examined concentration of NaCl (300 g L^{-1}) was tolerable for both yeast strains, with the not remarkable difference between melanized and non-melanized cells. Indeed, 3.76 ± 0.30 log cfu mL^{-1} were detected for melanized *A. pullulans* and 3.63 ± 0.25 log cfu mL^{-1} for the non-melanized cells, while 3.79 ± 0.4 log cfu mL^{-1} were detected for melanized *Z. obscura* and 3.58 ± 0.13 log cfu mL^{-1} observed for the non-melanized cells. Otherwise, the differences between controls (untreated cells) and Na Cl 300 g L^{-1} treated cells were statistically significant ($P < 0.05$).

3.3. Tolerance to oxidative stress

When the yeast cells were exposed to 200, 300 or 400 mM H_2O_2 for 1 h, no remarkable differences were observed between

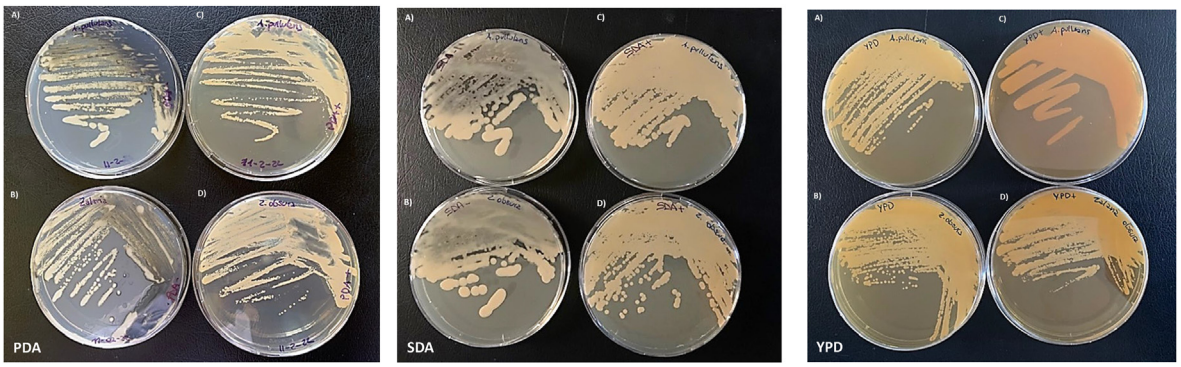

Fig. 1. Growth of *A. pullulans* ATCC 15233 and *Z. obscura* LS31012019 on PDA, SDA and YPD (**A-B**) and in the same media with $(NH_4)_2SO_4$ (**C-D**). The melanin production is well-visible after 7 d of incubation at 25 °C in PDA and SDA without $(NH_4)_2SO_4$.

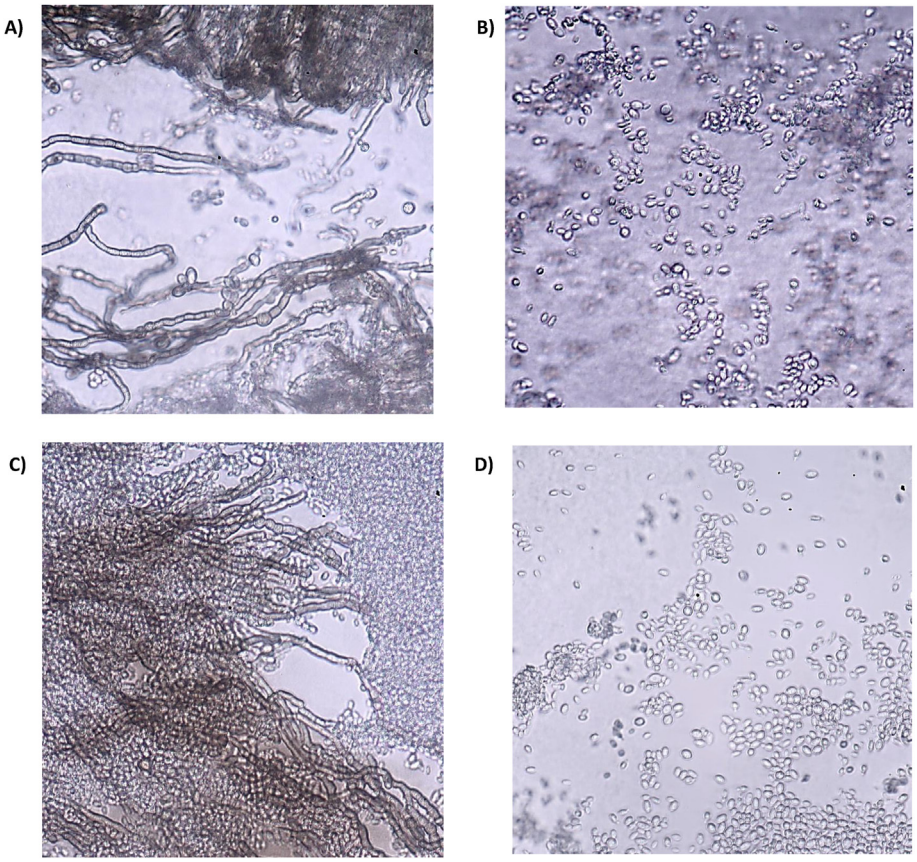

Fig. 2. Microscopic observation of melanized and non-melanized cells of *A. pullulans* ATCC 15233 (**A**, **B**) and melanized and non-melanized cells of *Z. obscura* LS31012019 (**C, D**) grown on PDA. In the melanized yeasts long and black hyphae are well visible, while in the non-melanized ones structured hyphae are absent (magnification × 40/0.65; Axiolab RE 31509, Zeiss).

Table 1
Survival of *A. pullulans* ATCC 15233 and *Z. obscura* LS31012019 melanized and non-melanized cells to osmotic stress. Data are expressed as log cfu mL^{-1} ± sd.

NaCl treatment (1 h)	*A. pullulans* ATCC 15233		*Z. obscura* LS31012019	
	Melanized cells	Non-melanized cells	Melanized cells	Non-melanized cells
Control (untreated cells)	5.49 ± 0.04	5.39 ± 0.12	5.49 ± 0.31	5.29 ± 0.34
NaCl 200 g L^{-1}	4.08 ± 0.21	3.95 ± 0.16	4.20 ± 0.12	4.00 ± 0.17
NaCl 300 g L^{-1}	3.76 ± 0.30*	3.63 ± 0.125*	3.79 ± 0.41*	3.58 ± 0.13*

Asterisks denote values statistically significant compared to the control group (untreated cells) (*P < 0.05).

Table 2
Survival of *A. pullulans* ATCC 15233 and *Z. obscura* LS31012019 melanized and non-melanized cells to oxidative stress. Data are expressed as log cfu mL^{-1} ± sd.

Treatment (1 h)	*A. pullulans* ATCC 15233		*Z. obscura* LS31012019	
	Melanized cells	Non-melanized cells	Melanized cells	Non-melanized cells
Control (untreated cells)	5.64 ± 0.02	5.68 ± 0.17	5.66 ± 0.14	5.63 ± 0.14
H_2O_2 200 mM	4.71 ± 0.15	4.51 ± 0.11	4.64 ± 0.12	4.55 ± 0.24
H_2O_2 300 mM	3.65 ± 0.18*	3.54 ± 0.15*	3.57 ± 0.37*	3.52 ± 0.22*
H_2O_2 400 mM	3.69 ± 0.21*	3.48 ± 0.14*	3.44 ± 0.16*	3.40 ± 0.14*

Asterisks denote values statistically significant compared to the control group (untreated cells) (*P < 0.05).

A. pullulans and *Z. obscura* (Table 2). Specifically, after 1 h of exposure to 200 mM H_2O_2, 4.71 ± 0.15 and 4.51 ± 0.11 log cfu mL^{-1} were detected for melanized and non-melanized *A. pullulans* respectively; similar values were observed for *Z. obscura,* with 4.64 ± 0.12 and 4.55 ± 0.24 log cfu mL^{-1} for the melanized and non-melanized cells respectively. Increased concentration of H_2O_2 (400 mM) induced the progressive reduction of yeast viability, up to 3.48 ± 0.14 log cfu mL^{-1} and 3.40 ± 0.14 log cfu mL^{-1} for *A. pullulans* and *Z. obscura* non-melanized cells respectively. Also in these cases, the differences between controls (untreated cells) and treated cells (300 or 400 mM H_2O_2) were statistically significant (P < 0.05).

3.4. Heat tolerance

Data showing the response to heat treatment of *A. pullulans* and *Z. obscura* were summarized in Table 3. The survival rate was calculated in comparison to the cfu mL^{-1} of the untreated cells (25 °C). The production of melanin affected the viability of both yeasts. In detail, after treatment at 40 °C for 1 h, 93.6 % of the melanized *A. pullulans* survived compared to 77 % of the non-melanized ones; in the case of *Z. obscura*, 92 % of the melanized cells survived to heat treatment in comparison to 76 % of non-melanized ones.

3.5. Susceptibility to essential oils

The antifungal activity of 14 EOs was assessed against *A. pullulans* and *Z. obscura*. To understand the role of melanin production in protecting the yeasts by EOs activity, the test was performed on 3 different solid media (PDA, SDA, YPD with and without $(NH_4)_2SO_4$) (Table 4).

In general, melanized *A. pullulans* and *Z. obscura* showed similar sensitivity (50 and 42.8 % respectively) to the examined EOs in all the used solid media. On the contrary, the antifungal efficacy of the tested EOs was higher in the non-melanized yeast (78.6 and 92.8 % for *A. pullulans* and *Z. obscura* respectively), as demonstrated by the larger growth inhibition zone (ID > 30 mm). In detail, melanized *A. pullulans* showed sensitivity to *O. basilicum*, *T. vulgaris*, *O. vulgare*, *M. piperita*, *C. cyminum*, *L. nobilis* and *C. citratus* in all the used media (PDA, SDA and YPD) with ID ranging from 11 ± 0.1 mm to 22 ± 1.2 mm, while the non-melanized cells were sensitive to all the tested EOs, except for *M. alternifolia*, *C. medica* and *C. winterianus*, reaching ID > 30 mm in the case of *T. vulgaris* and *O. vulgare.* In the case of *Z. obscura*, the melanized cells were sensitive to *O. basilicum*, *T. vulgaris*, *O. vulgare*, *M. piperita*, *C. cyminum* and *C. winterianus* in all the used agars, with ID from 11 ± 0.1 mm to 22 ± 1.2 mm. The sensitivity to EOs was higher in the non-melanized *Z. obscura*, resulting sensitive to all the examined compounds (98.2 %), with the only exception of *M. alternifolia.* Also, in this case, the highest IDs were observed for *T. vulgaris* and *O. vulgare* (>30 mm). The activity of Carvacrol was always higher for the non-melanized *A. pullulans* and *Z. obscura* on PDA (IDs 17 ± 0.3 and 22 ± 0.4 mm respectively), compared to the melanized ones (IDs about 15 mm). The activity of Carvacrol on SDA was similar in the case of melanized and non-melanized *A. pullulans* (IDs about 15 mm), while in the case of the non-melanized *Z. obscura* a higher ID was noted (18 ± 0.4 mm), compared to that of the melanized one (16 ± 0.2 mm). The sensitivity to Carvacrol on YPD was quite similar (ID about 16 mm) for both the examined yeasts regardless of the presence of melanin. As expected, the solution PEG-Ethanol showed no antimicrobial activity.

3.6. Susceptibility to antifungals

The susceptibility of melanized and non-melanized *A. pullulans* and *Z. obscura* to different antifungals was summarized in Table 5. As shown, the production of melanin strongly affected the efficacy of the examined antifungals, with a remarkable increase in MIC values in the melanized yeasts.

In detail, MICs of Fluconazole were high for melanized *A. pullulans* in both the media (256 μg mL^{-1} in SBD and 128 μg mL^{-1} in RPMI), while a strong reduction to 4 μg mL^{-1} in SDB and 16 μg mL^{-1} in RPMI were observed in the case of non-melanized *A. pullulans*. Similarly, melanized *Z. obscura* showed MIC of 64 μg mL^{-1} in SDB and 128 μg mL^{-1} in RPMI, compared to 32 μg mL^{-1} in SDB and 16 μg mL^{-1} in SDB of non-melanized *Z. obscura.* MICs of Clotrimazole resulted to be 32 μg mL^{-1} in SDB and 128 μg mL^{-1} in RPMI for melanized *A. pullulans,* reaching 2 μg mL^{-1} in SDB and 8 μg mL^{-1} in RPMI for the non-melanized strain. Low MIC values (16 μg mL^{-1} in SDB and 8 μg mL^{-1} in RPMI) were observed for melanized *Z. obscura*, with a noticeable decrease (2 μg mL^{-1} in SDB and 4 μg mL^{-1} in RPMI) for the non-melanized strain.

Bifonazole showed MICs of 32 μg mL^{-1} in SDB and 8 μg mL^{-1} in RPMI for melanized *A. pullulans*, reaching values of 4 μg mL^{-1} in SDB and 1 μg mL^{-1} in RPMI in the case of the non-melanized *A. pullulans*. For melanized *Z. obscura,* the MICs were 16 μg mL^{-1} in both the media, while in the case non-melanized cells MICs

Table 3
Survival of *A. pullulans* ATCC 15233 and *Z. obscura* LS31012019 melanized and non-melanized cells to heat stress. Data are expressed as log cfu mL^{-1} ± sd.

Heat treatment (1 h)	*A. pullulans* ATCC 15233		*Z. obscura* LS31012019	
	Melanized cells	Non-melanized cells	Melanized cells	Non-melanized cells
25 °C (untreated samples)	5.94 ± 0.12	5.38 ± 0.18	5.93 ± 0.14	5.47 ± 0.20
40 °C	4.75 ± 0.05	4.74 ± 0.04	4.83 ± 0.03	4.75 ± 0.04
Survival (%)	93.6 %	77 %	92. %	76. %

Table 4
Antimicrobial activity of the examined 14 essential oils (EOs) (10 % v/v) against melanized and non-melanized *A. pullulans* ATCC 15233 and *Z. obscura* LS31012019 in PDA, SDA, and YPD. Carvacrol was considered the natural control compound while PEG-Ethanol (1:1, v/v) was tested as a solvent. Data represent the growth inhibition diameter (ID, mm; mean ± sd) of independent experiments performed in triplicate.

EOs (10 % v/v)	*A. pullulans* ATCC 15233						*Z. obscura* LS31012019					
	PDA		SDA		YPD		PDA		SDA		YPD	
	Melanized cells	Non-melanized cells	Melanized cells	Non-melanized cells	Melanized cells	Non-melanized cells	Melanized cells	Non-melanized cells	Melanized cells	Non-melanized cells	Melanized cells	Non-melanized cells
Rosmarinus officinalis	0	12 ± 0.1	0	14 ± 0.4	0	12 ± 0.2	0	13 ± 0.4	0	11 ± 0.4	0	11 ± 0.5
Origanum majorana	0	14 ± 0.1	0	15 ± 0.3	0	13 ± 0.1	0	14 ± 0.3	0	14 ± 0.1	0	11 ± 0.4
Ocinum basilicum	11 ± 0.1	11 ± 0.3	13 ± 0.2	16 ± 1.0	11 ± 0.2	12 ± 0.1	11 ± 0.2	15 ± 0.5	11 ± 0.1	13 ± 0.6	11 ± 0.1	11 ± 0.2
Thymus vulgaris	13 ± 0.2	22 ± 0.5	15 ± 0.5	>30	13 ± 0.2	>30	13 ± 0.4	13 ± 0.5	12 ± 0.2	16 ± 0.1	22 ± 1.2	>30
Origanum vulgare	15 ± 0.1	>30	16 ± 0.3	>30	15 ± 0.3	>30	16 ± 0.8	16 ± 1.0	15 ± 0.9	25 ± 0.3	17 ± 0.4	>30
Melaleuca alternifolia	0	0	0	0	0	0	0	0	0	0	0	0
Mentha piperita	12 ± 1.2	12 ± 0.4	11 ± 0.2	12 ± 0.2	12 ± 0.4	14 ± 0.2	12 ± 0.2	14 ± 1.2	14 ± 0.4	14 ± 1.0	11 ± 1.1	14 ± 0.8
Cuminum cyminum	13 ± 0.7	17 ± 0.5	12 ± 0.1	13 ± 0.2	12 ± 0.1	13 ± 0.2	12 ± 0.5	16 ± 0.8	12 ± 0.6	18 ± 0.2	12 ± 0.6	12 ± 0.4
Cupressus sempervirens	0	11 ± 1.2	0	11 ± 0.1	0	11 ± 0.2	0	12 ± 0.4	0	12 ± 1.2	0	11 ± 0.5
Laurus nobilis	11 ± 0.5	13 ± 0.7	11 ± 0.1	11 ± 0.3	12 ± 0.2	12 ± 0.1	0	11 ± 1.2	0	11 ± 0.8	0	12 ± 0.4
Amyris balsaminifera	0	15 ± 0.4	0	12 ± 0.1	0	14 ± 0.3	0	15 ± 0.8	0	11 ± 0.1	0	11 ± 0.5
Citrus medica	0	0	0	0	0	0	0	14 ± 0.2	0	11 ± 0.4	0	11 ± 0.1
Cymbopogon citratus	15 ± 0.3	15 ± 01.2	11 ± 0.5	22 ± 1.2	11 ± 0.3	12 ± 0.4	0	17 ± 0.4	0	15 ± 0.5	0	12 ± 1.0
Cymbopogon winterianus	0	0	0	0	0	0	11 ± 0.5	11 ± 0.5	11 ± 0.3	14 ± 0.4	11 ± 0.5	11 ± 0.7
Carvacrol oil	15 ± 0.3	22 ± 0.4	20 ± 0.5	20 ± 0.3	16 ± 0.5	16 ± 0.3	15 ± 0.2	17 ± 0.3	16 ± 0.2	18 ± 0.4	16 ± 0.5	16 ± 05
PEG-Ethanol (1:1, v/v)	0	0	0	0	0	0	0	0	0	0	0	0

lowered to 8 μg mL^{-1} in SDB and 2 μg mL^{-1} in RPMI. Amphotericin B resulted the most active examined antifungal, showing the lowest MIC values. Specifically, for melanized *A. pullulans* MICs of 2 μg mL^{-1} in SDB and 4 μg mL^{-1} in RPMI were observed with a remarkable decrease for non-melanized *A. pullulans* (0.5 μg mL^{-1} in SDB and 2 μg mL^{-1} in RPMI). In the case of melanized *Z. obscura,* the MIC values were 2 μg mL^{-1} in SDB and 1 μg mL^{-1} in RPMI, while MICs were lowered to 0.125 μg mL^{-1} in SDB and 0.5 μg mL^{-1} in RPMI for non-melanized *Z. obscura.*

As regards the MFC values, it can be stated that, in most cases, lower MFCs were observed in non-melanized *A. pullulans* and *Z. obscura*, compared to those obtained in the non-melanized strains (Table 5). Fluconazole showed always MFC values > 128 μg mL^{-1} in SDB and 128 μg mL^{-1} in RPMI, regardless of the production of melanin; MFCs of Clotrimazole resulted to be 128 μg mL^{-1} in SDB for melanized *A. pullulans* and *Z. obscura*, while reached 32 μg mL^{-1} in SDB for the non-melanized strains. In the case of RPMI, MFC >128 μg mL^{-1} was noted for melanized *A. pullulans* and 64 μg mL^{-1} for the non-melanized ones. For *Z. obscura*, the MFC of clotrimazole was 32 μg mL^{-1} in both cases (melanized and non-melanized strains). Bifonazole showed MFC values of 128 μg mL^{-1} in SDB for melanized *A. pullulans* and *Z. obscura* and 64 μg mL^{-1} for the non-melanized cells. Similarly, in RPMI MFC values resulted to be 64 μg mL^{-1} for both the melanized yeasts, lowering to 32 μg mL^{-1} for the non-melanized strains. As observed for MICs, Amphotericin B showed the lowest MFC values. Specifically, for melanized *A. pullulans*, MFCs of 4 μg mL^{-1} in SDB and 8 μg mL^{-1} in RPMI were observed with a decrease for non-melanized *A. pullulans* (1 μg mL^{-1} in SDB and 4 μg mL^{-1} in RPMI). In the case of *Z. obscura,* the MFCs were 4 μg mL^{-1} in SDB and 2 μg mL^{-1} in RPMI for the melanized cells, while in the case non-melanized cells MICs were lowered to 1 μg mL^{-1} in both the used media.

3.7. Tolerance to UV-C radiation

In general, a dose-time-dependent effect was observed in both the tested yeasts, more remarkable in the case of non-melanized cells at increasing radiation intensity (Fig. 3). In detail, the exposure to UV-C for 60 s (186.61 J/m^2) resulted in a 15.2 % of growth reduction for *A. pullulans* melanized cells and 15.6 % for the non-melanized ones; the exposure for 180 s (591.6 J/m^2) increased the growth reduction to 43.2 % in the case of non-melanized *A. pullulans* but not in the melanized yeast (15.2 %). After 300 s (1000.7 J/m^2) of UV-C exposure, a 40.9 % growth reduction was observed for melanized *A. pullulans* and 50.5 % for the non-melanized ones. The prolonged exposure to UV-C radiation (600 s, 2004.5 J/m^2) induced the greatest growth reductions, reaching 77.7 % and 100 % for melanized and non-melanized *A. pullulans* respectively.

In the case of *Z. obscura*, the exposure to UV-C for 60 s (186.61 J/m^2) resulted in 7.5 % and 7.4 % of growth reduction for melanized and non-melanized yeast, with a noticeable increase (41.7 %) after exposure for 180 s (591.6 J/m^2) for non-melanized *Z. obscura*. The exposure for 300 s (1000.7 J/m^2) caused a growth reduction of 61.7 % and 65.5 % for melanized and non-melanized *Z. obscura* respectively. The prolonged exposure (600 s, 2004.5 J/m^2) drastically reduced the microbial viability, with 73.1 % and 100 % of growth reduction for *Z. obscura* melanized and non-melanized cells respectively (Fig. 3).

Table 5
Minimum Inhibitory Concentration (MIC; μg mL^{-1}) and Minimum Fungicidal Concentration (MFC; μg mL^{-1}) of melanized and non-melanized *A. pullulans* ATCC 15233 and *Z. obscura* LS31012019 to several antifungals. The assay was performed in two different liquid media, SDB and RPMI.

Antifungals and media	*A. pullulans* ATCC 15233		*Z. obscura* LS31012019	
	Melanized cells	Non-melanized cells	Melanized cells	Non-melanized cells
	MIC/MFC		MIC/MFC	
Fluconazole				
SDB	128/>128	4/>128	64/>128	32/>128
RPMI	128/>128	16/128	128/128	16/128
Clotrimazole				
SDB	32/128	2/32	16/128	2/32
RPMI	128/>128	8/64	8/32	4/32
Bifonazole				
SDB	32/128	4/64	16/128	8/64
RPMI	8/64	1/32	16/64	2/32
Amphotericin B				
SDB	2/4	0.5/1	2/4	0.125/1
RPMI	4/8	2/4	1/2	0.5/1

4. Discussion

4.1. Melanin and environmental stress conditions

It's well recognized that yeasts living in harsh environments have developed peculiar mechanisms of stress adaptation, such as the production of exopolysaccharide (EPS), trehalose and melanin (Jiang et al., 2016, 2018**)**. Indeed, melanins may enhance the long-term survival of fungi with the reported spore viability over a 5-y (Nguyen 2018).

In the present study, both of the examined yeasts, *A. pullulans* and *Z. obscura*, were able to produce melanin in standard culture media, such as PDA and SDA, losing the ability to produce the pigmentation under nitrogen starvation conditions, as well as that to develop hyphae, a phenomenon never reported for *A. pullulans* and *Z. obscura*. In old research on *Cryptococcus neoformans* (Nurudeen and Ahearn 1979), was explained that ammonium sulfate repressed the activity of phenyloxidase, the enzyme involved in the early steps of melanin synthesis via DOPA pathway. In the case of *Aureobasidium*, the pathway of melanin biosynthesis follows the one of DHN, catalyzed by polyketide synthase (PKS) (Eisenman and Casadevall, 2012). In the recent literature, Jiang and collaborators (2016) have investigated the behaviour in response to stress environment conditions in two isolates of *Aureobasidium melanogenum*, showing that the strains lose melanin production ability on PDA with ammonium sulfate. In addition, a lower transcription level of PKS gene, encoding the polyketide synthase, was evidenced in yeast grown in a medium with ammonium sulfate. This finding could explain the lost ability to produce melanin in *A. pullulans* ATCC 15233 and *Z. obscura* LS31012019 in presence of ammonium sulfate.

Melanin synthesis is promoted by different types of stress, including high temperature, nutrient-poor growth media, hyper-osmotic pressure (Cordero and Casadevall 2017). However, considering that multiple factors affect melanin biosynthesis, there is no preferable culture media or specific cultivation conditions for growing melanogenic organisms. For these reasons, once determined the ability of *A. pullulans* ATCC and *Z. obscura* to produce melanin, the role of this pigmentation under environmental stressors was investigated. All the experiments were carried out by preparing fungal suspensions of melanized and non-melanized cells. The obtained results showed that both *A. pullulans* and *Z. obscura* survived for 1 h at 20 % and 30 % NaCl concentrations regardless of melanin production, indicating their intrinsic ability to tolerate high salt concentrations (the so-called halophilia). This was not surprising for *A. pullulans* because in the literature was

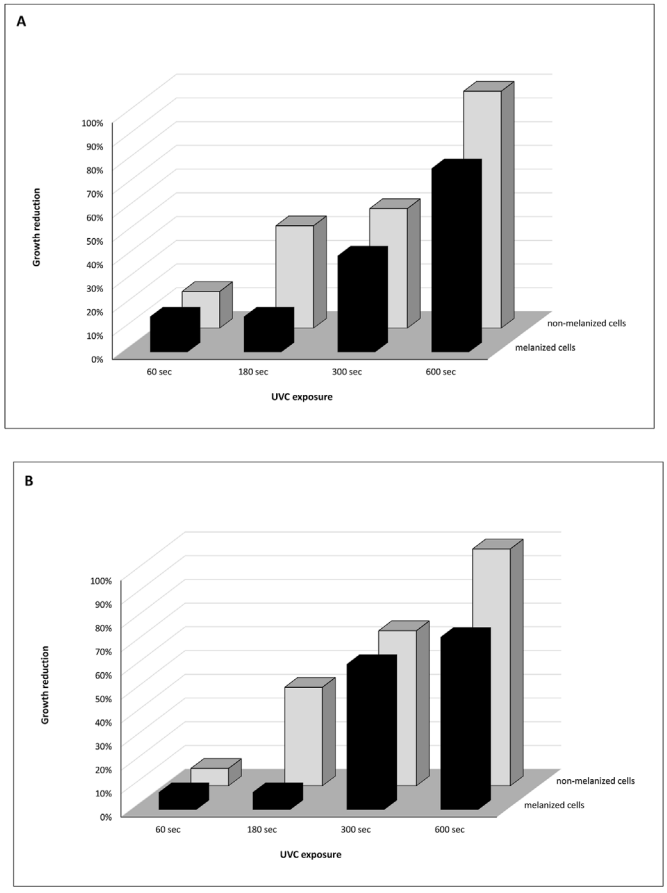

Fig. 3. Resistance to UV-C radiation (from 60 to 600 s) of melanized and non-melanized *A. pullulans* ATCC 15233 **(A)** and *Z. obscura* LS31012019 (**B**).

reported its survival up to 17 % NaCl concentration (Gunde-Cimerman et al., 2000), as well as its ability to grow in different harsh environments (Gostinčar et al., 2019), but gave novel information regarding *Z. obscura*. The treatment of *A. pullulans* and *Z. obscura* with different concentrations of peroxide hydrogen (from 200 to 400 mM) revealed that both were able to survive for 1 h to oxidative stress, regardless of the melanin production. In the literature is reported that yeasts contain effective defence systems for the protection against oxidant damage, including enzymes and antioxidant molecules (Grant, 2001). One of these, glutathione

(GSH), is most present in free form as intracellular (58 %) or extracellular (39 %), but when the cells are exposed to oxidizing agents, such as peroxide hydrogen, the level of extracellular GSH increases and, in parallel, the level of the intracellular one decreases. This constant balance of GSH is consistent with the survival of yeasts to harsh conditions and can explain the observed resistance of *A. pullulans* ATCC 15233 and *Z. obscura* LS31012019 to the high applied concentrations of peroxide hydrogen.

The presence of melanin seems to play an important role in response to heat treatment (40 °C for 1 h), as showed by the high percentage of survival of melanized *A. pullulans* and *Z. obscura* (>90 %) compared to the lowest ones observed in the non-melanized yeasts (about the average of 75 %). This difference could be due to the protection ability conferred by melanin, an ability lost when a melanization inhibitor was added to PDA. The use of substances able to limit or completely inhibit melanin production was under investigation since the pigmentation is considered essential for fungal virulence (Nosanchuk et al., 2015), in particular for the pathogenic ones, such as *C. neoformans* (Zimbres et al., 2019). The role of melanin in fungal "thermoregulation" is incomplete, but its role in protection against heat stress was observed for *Wangiella dermatitidis* (Paolo et al., 2006) and *C. neoformans*, with increased tolerance to heat and cold stress by a mechanism probably involving quenching of heat-induced ROS or buffering heat flux (Rosas and Casadevall, 1997).

4.2. Melanin and antimicrobial compounds

In the proposed research, the aspect related to the pharmacological approach was not investigated because *A. pullulans* is mainly considered a biotechnological microorganism (Prasongsuk et al., 2018) and only a few cases reported its role in immunocompromised infection (Hawkes et al., 2005; Mershon-Shier et al., 2011), while *Z. obscura* is not considered pathogen for human. However, the role of melanin production in the resistance of melanized and non-melanized *A. pullulans* and *Z. obscura* to antimicrobials was assessed considering 14 natural essential oils and four antifungals.

As expected, both the melanized yeasts were more resistant to the examined EOs, showing in many cases no inhibition growth diameters (for *R. officinalis*, *O. majorana*, *C. semprevirens* or *A. balsaminifera*), while a remarkable effect was observed in the non-melanized strains. Considering that only a few data are available in the literature on the sensitivity to essential oils of *A. pullulans* (Dawson-Andoh et al., 2000; Domingues et al., 2021) and no information regarding *Z. obscura* is still present, our data are the first reporting findings on this field of investigation. Similar behaviour was evident in response to antifungals, with the lowest MIC or MFC values observed in non-melanized *A. pullulans* and *Z. obscura*. Also for this aspect, a comparison can be reasonable only for *A. pullulans*, referring to data on fluconazole and amphotericin B activity (Mershon-Shier et al., 2011; Najafzadeh et al., 2014), while in the case of *Z. obscura* our data are the first elucidating antifungal sensitivity or resistance of this new species. The concept of melanin as a "shield" protecting fungi from antifungals it's well recognized (Gómez and Nosanchuk 2003) and described for *Penicillum marnaffei* and *Sporothrix brasiliensis* (Eisenman et al., 2020); in this direction, our data add importantly and supporting information on the role of melanin in the so-called "black yeasts".

4.3. Melanin and UV-C treatment

UV light is classified based on wavelength as UV-A (320–400 nm), UV-B (280–320 nm), UV-C (200–280 nm) and UV-V (100–200 nm) (Guerrero- Beltrán and Barbosa-Cánovas, 2004). In terms of germicidal activity, short-wavelength UV-C light (254 nm) is considered active, causing a physical shifting of electrons and breaking of bonds in DNA in most microorganisms (Lopez-Malo and Palou, 2005). UV radiation of 250–260 nm is lethal for fungi, yeast, bacteria, viruses, protozoa and algae and can be used for water disinfection without any change in colour, flavour and pH. Nevertheless, the efficacy of UV-C radiation in liquid can be affected by the type of liquid and UV-C absorption capacity: an increase in the amount of solids, large particles or microbial density could reduce the intensity of the UV-C penetration (Bintsis et al., 2000; Lopez-Malo and Palou, 2005). Fungi showed great resistance to UV-C radiation compared to bacteria. The radiation intensity required to inactivate pathogenic dermatophytes and various species belonging to the genus *Aspergillus* is significantly greater than that required for bacteria, while it's comparable with that of more resistant microorganisms such as protozoan cysts (Hijnen et al., 2006; Sisti et al., 2017). This higher resistance is attributable to the particular structure of the fungal cell and the production of melanin that increases the survival of melanized fungi compared to non-melanized ones (Gómez and Nosanchuk 2003; Revankar and Sutton 2010).

In this study, the tolerance to UV-C radiation of melanized and non-melanized *A. pullulans* and *Z. obscura* was examined. From the data, we can observe a different growth reduction of the two strains during the selected times of UV-C radiation, with complete growth inhibition after 600 s of treatment for the non-melanized cells. This observation stressed the concept that black fungi can survive ionizing radiation levels considered lethal to any other eukaryote. Indeed, it's recognized that melanins can absorb UV–Vis light (Meredith and Sarna 2006) and also dissipate the energy within the structure, which makes these pigments effective photo-protecting agents (Eisenman et al., 2020). In any case, the photo-protection capacity may be related to the different melanin types, the applied radiation frequencies, and the irradiation exposure model (Cordero and Casadevall 2011). The comparison of response to UV-C radiation between *A. pullulans* and *Z. obscura*, showed that at low intensity (60 s corresponding to186.61 J/m^2) the growth reductions of *A. pullulans* were almost double those of *Z. obscura*, regardless of melanin production, indicating that *Z. obscura* was more resistant to the applied UV-C intensity. When the radiation intensity increased, a corresponding increased growth reduction was observed for both the examined yeasts, always higher for the non-melanized ones. Thus, we can state that melanin represents a protective agent affecting survival in different harsh environments and unfavourable conditions, making black yeasts extremophile microorganisms.

5. Conclusions

It's well-known that the presence of melanin confers beneficial effects for fungal survival. This represents the first research specifically focused on this biological aspect in *A. pullulans* and *Z. obscura* and the presented results are interesting and very encouraging. Overall, both the strains can produce melanin in two different culture media, losing this ability in presence of ammonium sulfate. The lack of melanin induces lower resistance to heat treatment and high sensibility to antimicrobial and UV-C treatments, without affecting the tolerance to osmotic stress. Deeper studies are ongoing to better elucidate the biochemical characteristics as well as the adaptive potential of the black yeasts, in particular *Z. obscura*.

CRediT authorship contribution statement

Raffaella Campana: Conceptualization, Methodology, Data analysis, Writing-original draft, Writing-review & editing. **Fabiana**

Fanelli: Methodology. **Maurizio Sisti:** Methodology, Data analysis, Writing-review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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