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Evidence for 1,8-dihydroxynaphthalene melanin in three halophilic black yeasts grown under saline and non-saline conditions

Tina Kogej [a,*], Michael H. Wheeler [b], Tea Lanišnik Rižner [c], Nina Gunde-Cimerman [a]

[a] *University of Ljubljana, Biotech. Faculty, Department of Biology, Večna pot 111, SI-1000 Ljubljana, Slovenia*
[b] *Southern Plains Agricultural Research Center, USDA, ARS, 2765 F&B Road, College Station, TX 77845, USA*
[c] *University of Ljubljana, Medical Faculty, Institute of Biochemistry, Vrazov trg 2, SI-1000 Ljubljana, Slovenia*

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Abstract

The ascomycetous black yeasts *Hortaea werneckii*, *Phaeotheca triangularis*, and *Trimmatostroma salinum* are halophilic fungi that inhabit hypersaline water of solar salterns. They are characterized by slow, meristematic growth and very thick, darkly pigmented cell walls. The dark pigment, generally thought to be melanin, is consistently present in their cell walls when they grow under saline and non-saline conditions. We used the inhibitor tricyclazole to test the fungi in this study for the presence of 1,8-dihydroxynaphthalene (DHN)-melanin biosynthesis, since fungal melanins reportedly are derived either from DHN, tyrosine via 3,4-dihydroxyphenylalanine, γ-glutaminyl-3,4-dihydroxybenzene, or catechol. Tricyclazole-treated cultures of the fungi were reddish-brown in color and contained typical intermediates of the DHN-melanin pathway, as demonstrated by high-performance liquid chromatography. This investigation showed that the three fungi synthesized DHN-melanin under saline and non-saline growth conditions.

Keywords: Halophilic black yeast; 1,8-Dihydroxynaphthalene-melanin; *Hortaea werneckii*; *Trimmatostroma salinum*; *Phaeotheca triangularis*

1. Introduction

The black yeasts *Hortaea werneckii*, *Phaeotheca triangularis*, and *Trimmatostroma salinum* are halophilic fungi, and all three fungal species represent indigenous mycobiota present in hypersaline water of solar salterns. These fungi are able to grow in highly changeable saline environments on NaCl concentrations from 0% up to the saturation point [1]. As such, they are rare eukaryotic halophiles and represent highly appropriate model organisms in which to study mechanisms of salt tolerance [2]. Their characteristic stress responses on a morphological level are slow, meristematic growth and very thick, darkly pigmented cell walls. These morphological features are also found in epilithic black yeast species [3] and are therefore typical for black yeasts living in different extreme habitats [1].

Melanins are found in animals, plants, and microorganisms. Melanin is a common term for dark brown to black pigments of high molecular mass formed by oxidative polymerization of phenolic compounds [4], usually complexed with protein and often with carbohydrates as well [5]. Fungal melanins occur in cell walls or as extracellular polymers formed enzymatically or autoxidatively in the medium. The phenolic compounds from which the fungal melanin polymers are derived include tyrosine via 3,4-dihydroxyphenylalanine (DOPA) in various fungi and other microorganisms, γ-glutaminyl-3,4-dihydroxybenzene (GDHB) or catechol in *Basidiomycetes*, and 1,8-dihydroxynaphthalene (DHN) in *Ascomycetes* and related *Deuteromycetes* [4].

Classical chemical and physical tests, used in previous years to confirm that a substance was a melanin [5], usually did not define the type of melanin that was present. However, melanin-deficient mutants and tricyclazole have been used to elucidate details of the DHN-melanin pathway (Fig. 1) in a number of fungi as described previously. Melanins made by the DHN pathway are of particular interest, since they reportedly protect fungi against a number of environmental factors [4,5]. In addition, melanins made by this pathway have been shown to be virulence

* Corresponding author. Tel.: +386 (1) 423 33 88; Fax: +386 (1) 257 33 90.
E-mail address: tina.kogej@uni-lj.si (T. Kogej).

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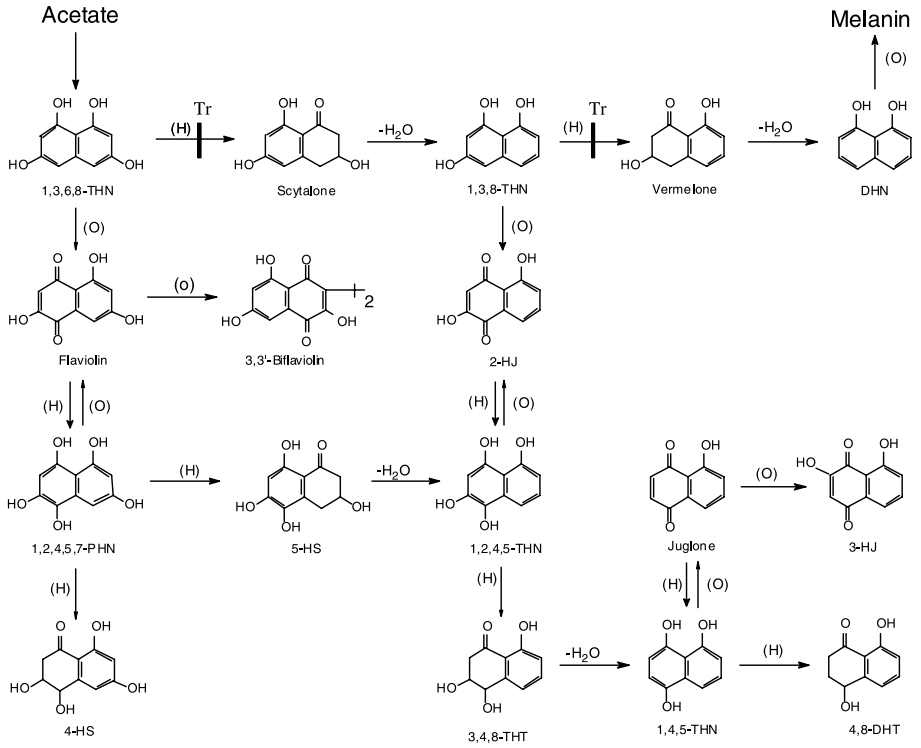

Fig. 1. Biosynthetic pathway of DHN-melanin and related pentaketide metabolites, from the scheme shown by Bell and Wheeler [4]. The first known product of the pathway is 1,3,6,8-THN. This metabolite is reduced to scytalone, which is then dehydrated to 1,3,8-THN. Next, 1,3,8-THN is reduced to vermelone, which is then dehydrated to DHN. The enzyme(s) that catalyze the final polymerization reaction, oxidation of DHN to melanin, have not yet been adequately studied but it appears to be a laccase. Tricyclazole (Tr) inhibits the reduction of 1,3,6,8-THN and 1,3,8-THN to scytalone and vermelone, respectively. Its strongest inhibitory effect is on the reduction of 1,3,8-THN. This results in the accumulation of flaviolin, 2-HJ, and their related shunt products. 1,2,4,5,7-pentahydroxynaphthalene (1,2,4,5,7-PHN), 1,2,4,5-tetrahydroxynaphthalene (1,2,4,5-THN), and 1,4,5-trihydroxynaphthalene (1,4,5-THN) are extremely unstable and have not been isolated from fungi.

factors in both plant and animal pathogens and are known to operate through a variety of mechanisms [4–6].

The pigment in black yeasts is generally referred to as melanin [3,7], although this has been confirmed only in a few cases [8–10]. Black yeast isolates are continuously obtained from hypersaline water of solar salterns, and a dark pigment is consistently present in the cell walls of the halophilic black yeast species *H. werneckii*, *T. salinum*, and *P. triangularis* [11]. These halophilic black yeasts also are darkly pigmented while growing in the presence of high salt levels. Furthermore, using different media, it has been observed that elevated NaCl concentrations (10% w/v) can affect the color and/or intensity of color of colonies of these fungi, when the colonies grown in the presence of NaCl are compared with those grown on media without NaCl (T. Kogej, unpublished).

In the present study, the systemic fungicide tricyclazole (5-methyl-1,2,4-triazolo(3,4,*b*)-benzothiazole) was used to determine if *H. werneckii*, *T. salinum*, and *P. triangularis* make melanin from DHN via the pentaketide pathway shown in Fig. 1. Melanin biosynthesis in these fungi was investigated under saline and non-saline growth conditions, and tricyclazole was used in an attempt to block their melanin biosynthetic pathway. Metabolites that were produced and accumulated in the presence of tricyclazole were analyzed by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). To our knowledge, melanin biosynthesis has not previously been studied in extremophilic fungi grown in the presence of high salt concentrations.

2. Materials and methods

2.1. Fungal strains, media, and growth conditions

The black yeasts *H. werneckii* MZKI B-736, *P. triangularis* MZKI B-741, and *T. salinum* MZKI B-734 belong to three halophilic species of the ascomycetous order *Dothideales*. The strains were isolated from hypersaline water of a crystallization pond in a solar saltern at the eastern coast of the Adriatic Sea. They were isolated on selective media, containing 17–32% (w/v) NaCl [1]. The isolates are deposited in the Culture Collection of the National Institute of Chemistry (MZKI), Ljubljana, Slovenia.

Asparagine–sucrose agar (ASA) was used to grow the three fungi at pHs usually above 6.0, which is desirable for the formation of DHN-melanin-related metabolites, i.e., flaviolin, 3,3′-biflaviolin, 4-hydroxyscytalone (4-HS), 2-hydroxyjuglone (2-HJ), or 3-hydroxyjuglone (3-HJ), whose

chemical structures are shown in Fig. 1. ASA was first used in DHN-melanin studies with the fungus *Thielaviopsis basicola* and is described elsewhere [12]. Tricyclazole was added to autoclaved ASA as an ethanol solution, so that the final tricyclazole and ethanol concentrations in ASA were 30 μg ml^{-1} and 1%, respectively. For a control, the same amount of ethanol, without tricyclazole, was added to ASA. In addition, the tricyclazole-treated and control cultures of ASA contained 0 and 10% (w/v) NaCl to compare the effects of NaCl on metabolite accumulation.

Inoculations on the four types of ASA media were made by spreading a suspension of 4×10^5 propagules over the surface of 20 ml of the media in 90×15 mm Petri dishes. The cultures were grown in the dark at 25°C for 11–14 days.

2.2. Extraction of intermediates for TLC and HPLC

Four 11–14 days old Petri dish cultures of each of the three isolates were chopped into small fragments, combined, and approximately 80 ml of the agar material extracted twice for 24 h with 160 ml of acetone. This process was repeated for the four types of media. Both extracts were combined (320 ml) and filtered through Whatman No. 1 filter paper (Fisher Scientific, Pittsburgh, PA, USA). The extract was then evaporated under vacuum to remove acetone and an aqueous phase remained. The aqueous phase was adjusted to pH 5 with 1 N H_3PO_4 and saturated with NaCl; it was then extracted twice with equal volumes of ethyl acetate. The ethyl acetate fractions were collected, combined, and residual water was removed over Na_2SO_4. The combined fractions were then filtered through Whatman No. 1 paper, and dried under vacuum [12]. The residues were stored at 2°C until needed for chromatography; then they were dissolved in ethyl acetate for TLC or in methanol for HPLC.

2.3. TLC analysis

TLC was used for preliminary analysis of the residues. The vacuum-dried residues were dissolved in ethyl acetate [12] and applied to 20×20 cm silica gel-coated TLC plates with fluorescent indicator (Merck Silica gel 60, F_{254}). The plates were then developed in ethyl ether–hexane–formic acid (60:40:1) [8,12]. Once separated, metabolites from the residues were observed in daylight and under ultraviolet (UV) light (254 and 365 nm, respectively) for characteristic colors and R_f values. The plates were then sprayed with an aqueous solution of 1% $FeCl_3$. Once they had dried, they were again evaluated for colors that appeared in daylight. DHN-melanin-related metabolites were identified after comparison with authentic standards of flaviolin, 2-HJ, 3-HJ, 1,3,8-trihydroxynaphthalene (1,3,8-THN), and DHN, which were applied to the plates at the same time as the residues. A detailed protocol for the preliminary TLC procedure was published elsewhere [13].

2.4. HPLC analysis

Analytical HPLC of metabolites was carried out by using a Hewlett Packard 1090 LC system (Agilent Technologies, Palo Alto, CA, USA), equipped with a diode array detector and a Phenomenex Hypersil 5 C18 column (250×4.6 mm). The system was maintained at 40°C. Vacuum-dried residues from the ethyl acetate extractions were dissolved in 1 ml methanol. Authentic standards included 3,3′-biflaviolin, DHN, 4,8-dihydroxytetralone (4,8-DHT), flaviolin, 2-HJ, 3-HJ, 4-HS, scytalone, 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN), 1,3,8-THN, and 3,4,8-trihydroxytetralone (3,4,8-THT); and standard curves were made with 3,3′-biflaviolin, 4,8-DHT, flaviolin, 2-HJ, 3-HJ, 4-HS, so that these six metabolites could be quantified. Table 1 lists typical retention times for all the standards. The injection volume was 10–40 μl. The mobile phase used was a gradient of acetonitrile (MeCN) and water both with 0.7% H_3PO_4 run at 1.25 ml min^{-1}. The gradient was as follows: 10% MeCN (0 min), 40% MeCN (24 min), 40% MeCN (28 min), 100% MeCN (31 min), 100% MeCN (33 min), and 10% MeCN (24 min). The equilibration time between runs was 4 min and the chromatographic signal was obtained at 254 nm (bandwidth 20 nm) with reference to 550 nm (bandwidth 100 nm), while spectra were collected over 210–600 nm.

3. Results

3.1. Normal growth and growth in the presence of tricyclazole

By 11 days, *H. werneckii* formed dark olive-green colonies on ASA medium without NaCl and darker green, almost black colonies on ASA medium with 10% NaCl. The colonies of *P. triangularis* were dark brown on both non-saline and saline media, and colonies of *T. salinum* were black on non-saline medium, and dark brown on saline medium (Fig. 2). The addition of 0.2 ml ethanol

Table 1
HPLC retention times used to detect DHN-melanin metabolites in Fig. 1[a]

Compound	Retention time (min)
4-HS	5.3
3,4,8-THT	7.0
Scytalone	7.8
1,3,6,8-THN	8.9
4,8-DHT	11.6
Flaviolin	13.4
3-HJ	14.5
1,3,8-THN	14.8
2-HJ	17.1
3,3′-Biflaviolin	20.2
DHN	22.2

[a]HPLC procedure is given in Section 2.

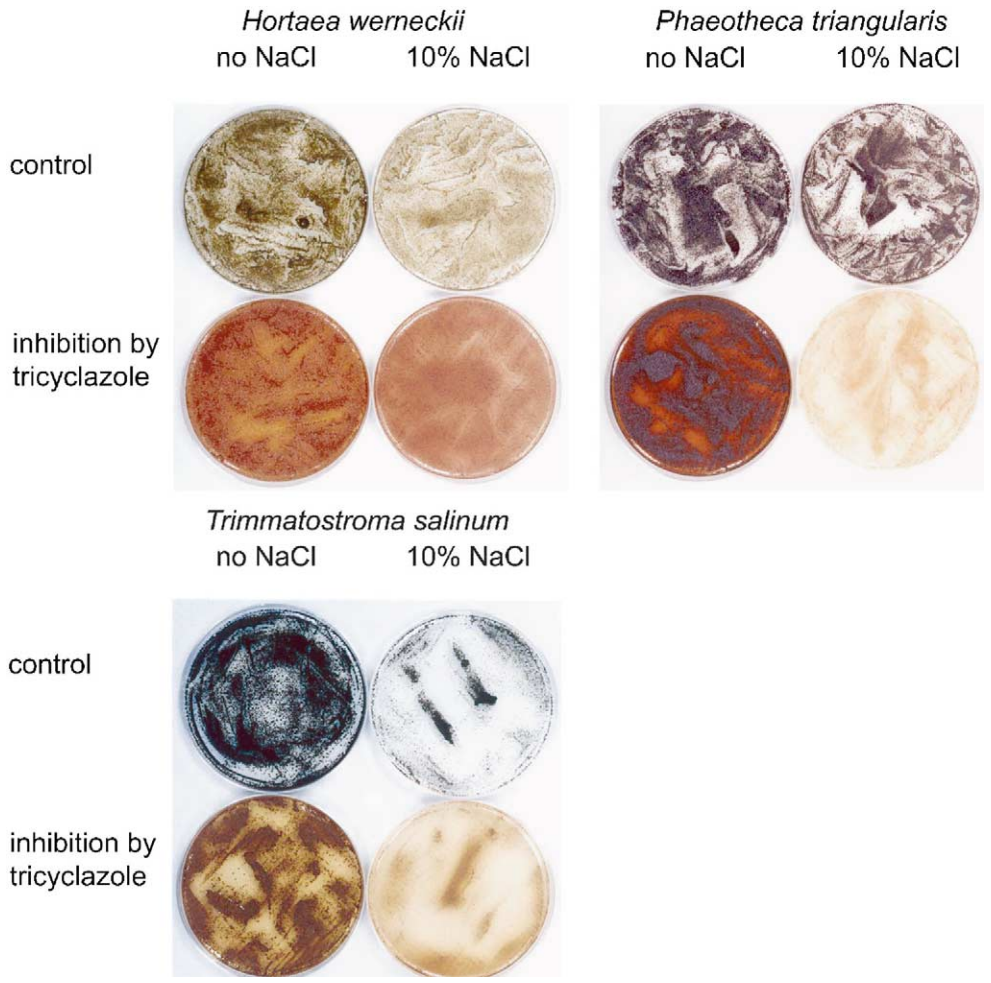

Fig. 2. Control and tricyclazole-inhibited cultures of *H. werneckii*, *P. triangularis* and *T. salinum* on non-saline and saline ASA media. The control cultures show the typical dark brown to black color of the fungi, whereas the tricyclazole-inhibited cultures are reddish-brown in color.

to 19.8 ml of non-saline or saline ASA, prior to hardening and inoculation of the media, did not have an apparent effect on growth rate, color, or morphology of the colonies; and the three fungi still produced large numbers of spores.

The three fungi were reddish-brown in color, when grown on ASA containing 30 μg ml^{-1} tricyclazole, and by 11 days the media of all three fungi contained reddish-brown pigments (Fig. 2). Tricyclazole had no apparent effect on growth, but it affected the color of the colonies, and in *T. salinum* it also influenced the morphology of cells, since one- to two-celled conidia were generally produced instead of the usual mother cells with many conidia (data not shown).

3.2. Identification of DHN-melanin intermediates

Metabolites from ethyl acetate extracts of *H. werneckii*, *P. triangularis*, and *T. salinum* were analyzed by TLC to determine if DHN-melanin precursors or related metabolites were present. Flaviolin and 2-HJ were detected in the extracts of 11–14 days old non-saline and saline ASA cultures containing tricyclazole; however, they were not found in cultures without tricyclazole. The TLC results indicated that tricyclazole had blocked the DHN-melanin pathway, causing the accumulation of 1,3,6,8-THN and 1,3,8-THN, which were autoxidized to flaviolin and 2-HJ, respectively (Fig. 1). Neither 1,3,6,8-THN nor 1,3,8-THN were found on the TLC plates, suggesting they rapidly autoxidized to flaviolin and 2-HJ, respectively, before the ethyl acetate extractions. The preliminary experiments with TLC were not used to identify scytalone, 4-HS, 4,8-DHT or other tetralones that may have been present.

HPLC was used in the present studies with the cultures of *H. werneckii*, *T. salinum*, and *P. triangularis*, since it is an excellent technique for identifying melanin precursors and related metabolites. In a typical experiment, for example, only 4,8-DHT was detected in 14 days old saline and non-saline ASA control cultures that were not treated with tricyclazole (Table 2). These controls contained approximately 1% ethanol prior to inoculation, which is equal to the amount of ethanol present in tricyclazole-treated cultures.

In corresponding studies where tricyclazole was used in

Table 2
Melanin metabolites analyzed by HPLC in control cultures and in tricyclazole-inhibited cultures of *H. werneckii*, *P. triangularis*, and *T. salinum* grown on saline and non-saline ASA media[a]

Isolate	NaCl[b]	Tricyclazole[c]	Metabolites[d]					
			4-HS	4,8-DHT	FL	2-HJ	BIF	3-HJ
H. werneckii	−	−	0.00	0.07 ± 0.01	0.00	0.00	0.00	0.00
	+	−	0.00	0.04 ± 0.00	0.00	0.00	0.00	0.00
	−	+	0.98 ± 0.12	0.77 ± 0.01	4.04 ± 0.15	9.99 ± 0.46	8.81 ± 0.31	0.00
	+	+	0.76 ± 0.18	0.16 ± 0.01	3.25 ± 0.56	1.86 ± 0.01	9.57 ± 2.80	0.00
P. triangularis	−	−	0.00	0.05 ± 0.00	0.00	0.00	0.00	0.00
	+	−	0.00	0.03 ± 0.00	0.00	0.00	0.00	0.00
	−	+	0.00	0.00	6.33 ± 0.86	19.87 ± 1.57	7.19 ± 0.30	1.76 ± 0.11
	+	+	0.00	0.00	0.19 ± 0.05	0.15 ± 0.02	3.80 ± 1.83	0.00
T. salinum	−	−	0.00	1.66 ± 0.02	0.00	0.00	0.00	0.00
	+	−	0.00	0.69 ± 0.01	0.00	0.00	0.00	0.00
	−	+	0.00	5.27 ± 0.64	0.39 ± 0.06	3.15 ± 0.19	0.76 ± 0.03	0.00
	+	+	0.00	0.10 ± 0.03	1.60 ± 0.18	4.85 ± 0.45	0.67 ± 0.00	0.00

[a]Values for the different metabolites are in μg ml^{-1} of growth medium; they are the mean and standard deviation of two samples; $n = 2$.
[b]NaCl concentration (w/v) in the medium was 0% (−) or 10% (+).
[c]Tricyclazole concentration in the medium was 0 μg ml^{-1} (−) or 30 μg ml^{-1} (+).
[d]Abbreviations: FL, flaviolin; BIF, 3,3′-biflaviolin.

11–14 days old cultures, flaviolin, 3,3′-biflaviolin, 4-HS, 2-HJ, 3-HJ and 4,8-DHT accumulated in saline and non-saline cultures of the three isolates. Tricyclazole-treated 14 days old non-saline cultures of *H. werneckii* accumulated flaviolin, 2-HJ and 3,3′-biflaviolin as primary products, and 4-HS and 4,8-DHT as minor products. In contrast, saline cultures, treated with tricyclazole, accumulated flaviolin and 3,3′-biflaviolin as primary products, and 4-HS, 4,8-DHT and 2-HJ as minor products. The same products accumulated under both saline and non-saline conditions in the tricyclazole-treated cultures of *H. werneckii*, but in different proportions; the main difference was in the relative amount of 2-HJ that was produced (Table 2).

For *P. triangularis*, the results with 14 days old cultures were similar to those with *H. werneckii*. Non-saline cultures of *P. triangularis* with tricyclazole accumulated flaviolin, 2-HJ and 3,3′-biflaviolin as primary products with 2-HJ being present in the largest concentration. 3-HJ was also present but only as a minor component. In saline cultures of *P. triangularis* with tricyclazole, 3,3′-biflaviolin was the primary product, and only small quantities of flaviolin and 2-HJ were present (Table 2).

In the case of tricyclazole-treated *T. salinum*, inhibition of melanin biosynthesis in non-saline cultures resulted mostly in the accumulation of 4,8-DHT; however, the non-saline cultures also accumulated flaviolin, 2-HJ and 3,3′-biflaviolin with 2-HJ present in the greatest concentration. The fungus produced mostly 2-HJ and flaviolin in saline cultures, but also some 3,3′-biflaviolin and a small amount of 4,8-DHT. The four metabolites accumulated under both saline and non-saline conditions, but the major difference was in the amount of 4,8-DHT that was present in the two media (Table 2).

4. Discussion

In the present study, *H. werneckii*, *T. salinum*, and *P. triangularis* were found to make DHN-melanin on non-saline media and media containing 10% NaCl. *H. werneckii* was at first known as the etiologic agent of human tinea nigra [14]; however, it was later discovered to naturally occur in hypersaline waters, such as those in solar salterns, where it was found to be the dominant halophilic fungal species [1,11,14]. *T. salinum* Zalar, de Hoog & Gunde-Cimerman was first isolated from hypersaline water [1] and described as a new halophilic species [15]. *P. triangularis* de Hoog & Beguin was initially isolated from a humidifier of an air-conditioning system [16], but later hypersaline waters were described as its natural niche [1].

It is not surprising that these three fungi have heavily melanized cell walls, since melanins are among the most stable and resistant biochemical materials [17]. A high proportion of melanin-producing microorganisms are known to be associated with environmentally stressed areas such as hot and cold deserts, alpine regions, upper biosphere [4] and saline soil [18]. In addition, melanins are known to confer protection to UV irradiation, temperature extremes and desiccation [4], and they also provide an osmotic role [5].

The dark brown to black melanins in cell walls of *Ascomycetes* and related *Deuteromycetes* are generally synthesized from the pentaketide DHN [4]. Details of the DHN-melanin pathway have been elucidated using a number of different fungi and much of what is known about the pathway and its enzymes has come from the use of melanin-deficient strains and compounds, i.e., tricyclazole, that inhibit specific enzymes in the pathway [4,5]. Tricyclazole, for example, is known to strongly inhibit the en-

zymic reduction of 1,3,8-THN to vermelone (Fig. 1), and with some fungi, i.e., *Magnaporthe grisea*, tricyclazole has also been shown to weakly inhibit the reduction of 1,3,6,8-THN to scytalone [19]. The reductase enzymes that produce scytalone and vermelone from 1,3,6,8-THN and 1,3,8-THN, respectively, and the genes that encode for these enzymes have been studied in *Colletotrichum lagenarium* [20], *M. grisea* [21], *Ophiostoma floccosum* [22] and other fungi. Two separate enzymes have been shown to reduce 1,3,6,8-THN in both *M. grisea* [21] and *C. lagenarium* [20]. The results with these two fungi and with *O. floccosum* [22] indicate that multiple reductase enzymes probably reduce 1,3,6,8-THN or 1,3,8-THN in a number of other fungi and may account for differences in tricyclazole sensitivity at different reductase sites. Flaviolin and 2-HJ are known autoxidative products of 1,3,6,8-THN and 1,3,8-THN, respectively, and the presence of flaviolin and 2-HJ in fungal cultures, treated with tricyclazole, is usually accepted as proof 1,3,6,8-THN and 1,3,8-THN were involved in the synthesis of DHN-melanin [5]. Once made, 1,3,8-THN is reduced to vermelone, which in turn is dehydrated to DHN [4,5]. In most cases, these two reactions are carried out by the same reductase and dehydratase enzymes that produce 1,3,8-THN from 1,3,6,8-THN. Finally, DHN appears to be polymerized to melanin via a laccase but not much is known about this enzyme or how it functions in the melanin pathway [5].

In the present investigation, we demonstrated that the DHN-melanin inhibitor, tricyclazole, inhibited melanin biosynthesis in isolates of the halophilic fungi *H. werneckii*, *P. triangularis*, and *T. salinum* on saline and non-saline ASA media. At this time, however, we are unable to explain why different patterns of pentaketide accumulation, as shown in Table 2, were observed under saline and non-saline growth conditions. The three fungi were reddish-brown in color when grown in the presence of tricyclazole under saline and non-saline conditions, and their cultures accumulated flaviolin, 3,3′-biflaviolin, 2-HJ and other DHN-related metabolites. With the three halophilic yeasts, it appears that tricyclazole did not inhibit the reduction of 1,3,6,8-THN as strongly as the reduction of 1,3,8-THN. This would account for the fact that tricyclazole-inhibited cultures of the three fungi accumulated appreciable amounts of flaviolin and 3,3′-biflaviolin at the same time they accumulated 2-HJ. This is consistent with the fact that tricyclazole has been shown to have the same effect on colony color and metabolite accumulation in cultures of other fungi that produce DHN-melanin [4,9,23,24].

The only melanin-related metabolite that accumulated in non-inhibited saline and non-saline cultures of the three halophilic isolates was 4,8-DHT. Although we cannot explain why 4,8-DHT was exclusively found, small amounts of 4-HS have been reported in non-inhibited wild-type cultures of *Curvularia lunata* [24], *T. basicola* [12] and *Monosporascus cannonballus* (M.H. Wheeler, unpublished) as well as small amounts of scytalone in wild-type cultures of *T. basicola* [12] and *Sporothrix schenckii* [23]. The present study with the three halophilic black yeasts and previous studies with other fungi indicate, however, that the melanin pathway is highly efficient in metabolizing melanin precursors, since melanin-related metabolites are seldom found in appreciable amounts in non-inhibited wild-type cultures.

The three halophilic black yeast species *H. werneckii*, *P. triangularis*, and *T. salinum* belong to the ascomycetous order *Dothideales* [1,16]; therefore, it is not surprising that they also synthesize a DHN-type melanin. Other dothideaceous species that have been studied so far and found to synthesize DHN-melanin include *Aureobasidium pullulans* [8], *Cladosporium carrionii*, *Cladosporium bantianum* [9], and *Cladosporium cladosporioides* [10]. Also, a human isolate of *H. werneckii* has been described as making DHN-melanin in non-saline conditions [9]. The type of melanin in *T. salinum* and *P. triangularis* has not been studied until now.

Our results show that tricyclazole inhibited DHN-melanin biosynthesis in the halophilic black yeasts *H. werneckii*, *P. triangularis*, and *T. salinum* under non-saline and hypersaline conditions of growth. The salterns as the natural ecological habitat of these yeasts represent an extreme and highly changeable environment. The most important physicochemical parameters of salterns are high NaCl concentrations, low water activity, and high solar irradiation [25]. The role of melanin in halophilic black yeasts colonizing hypersaline environments remains to be elucidated.

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