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The influence of ortho- and para-diphenoloxidase substrates on pigment formation in black yeast-like fungi

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Abstract: Dothideaceous black yeast-like fungi (BYF) are known to synthesise DHN-melanin that is inhibited by the systemic fungicide tricyclazole. The final step of the DHN melanin pathway is the conjoining of 1,8-DHN molecules to form the melanin polymer. There are several candidate enzymes for this step, including phenoloxidases such as tyrosinase and laccases, peroxidases, and perhaps also catalases. We analysed the type polyphenoloxidases that are involved in biosynthesis of BYF melanins. For that purpose we used substrates of o-diphenoloxidases (EC 1.10.3.1.): 4-hydroxyphenyl-pyruvic acid, L-β-phenyllactic acid, tyrosine, pyrocatechol, 3,4-dihydroxyphenylalanine and homogentisic acid, as well as substrates of p-diphenoloxidases (EC 1.10.3.2.): syringaldazine, resorcinol, p-phenylenediamine, phloroglucinol, guaiacol and pyrogallic acid. Fourteen strains of black yeasts originating from different natural biotopes were investigated. The tested strains could be divided into four groups based on their ability to produce dark pigments when cultivated on aromatic substrates of o- and on p-diphenoloxidases. It was established that syringaldazine, pyrogallic acid and 4-hydrophenyl-pyruvic acid, β-phenyllactic acid optimally promote melanin biosynthesis. Average intensity of pigmentation of all strains studied was minimal when guaiacol was used as a substrate. The present investigation indicates that the melanisation process may involve more enzymes and more substrates than those commonly recognised. Black yeasts are likely to contain a multipotent polyphenoloxidase.

Key words: Black yeast-like fungi, *Dothideales*, dothideaceous black yeasts, 1,8-dihydroxynaphthalene-melanin, phenoloxidases, o-diphenoloxidases, p-diphenoloxidases.

INTRODUCTION

Black yeast-like fungi (BYF) are either of basidiomycetous or ascomycetous relationship. The basidiomycetes are classified in the genera *Moniliella* and *Trichosporonoides*, of which a precise phylogenetic position has as yet not been established. Most species of these genera are of industrial significance and are rarely seen in clinical practice. In the ascomycete order *Chaetothyriales,* mainly comprising the family *Herpotrichiellaceae,* the genus *Exophiala* is the preponderant yeast-like anamorph (de Hoog *et al*. 2000). The order contains numerous human pathogens, with a wide spectrum of clinical pictures (Vitale & de Hoog 2002, de Hoog *et al*. 2005). The majority of these infections are cutaneous or mild pulmonary, but rarely they may be devastating and fatal. These infections are very difficult to treat because *in vivo* the species are frequently more resistant antimycotics than *in vitro* (Vitale & de Hoog 2002, de Hoog *et al.* 2005). The pathology of these black yeasts and their relatives is poorly understood (de Hoog *et al*. 2000, 2005).

In contrast, the ascomycete order *Dothideales* (anamorph genus *Aureobasidium* and its relatives) mainly comprises saprobic fungi, which are only exceptionally involved in human disease. *Aureobasidium pullulans* is industrially important because of its production of extracellular polysaccharides (EPS), which are applied in biotechnology (Deshpande *et al*. 1992). The EPS concerned comprise pullulan, a poly-α-1,6-maltotriose, and aubasidan, a related glucan with α-1,4-D, β-1,6-D and β-1,3-D-glycosidic bonds. A separate variety, *Aureobasidium pullulans* var. *aubasidani* was described for the strains producing aubasidan-like components (Yurlova & de Hoog 1997).

Dothidealean black yeast-like fungi were found to be predominant in soils highly contaminated with radionuclides emitted during the Chernobyl accident (Zhdanova *et al.* 1994, 2007). They play an important role in blackening of rock and architectural surfaces, in the destruction of marble and limestone (Sterflinger & Krumbein 1995, 1997). The fungi show active growth in extreme ecological niches, surviving low humidity, high temperature, high solar irradiation, presence of long lived radionuclides, and absence of traditional sources of nutrition and energy. The presence of melanin pigments, which possess a wide protective action, provides the dark-coloured fungi a competitive advantage under harsh environmental conditions. The pigments contain stable organic free radicals (Lyakh 1981). Fungal melanins may occur as electron-dense granules located in the fungal cell wall, polymers in the cytoplasm, as extracellular polymers in the medium surrounding the fungus, or in any combination (Butler & Day 1998). In the scientific literature there is information on biological activity of melanins as radioprotectors, antitumor remedies and as growth stimulators of plant seeds (Lyakh 1981). It had been suggested that these pigments might be useful as topical sunscreens and sunlight-protective coatings for plastics. DOPA melanins (of animal and biotechnological origin) have also been recommended for use in cosmetics (Della-Cioppa *et al.* 1990).

Melanins are produced by a variety of higher organisms including humans, but microbes are the melanin producers of choice in biotechnology. Melanin harvest from mammalian tissues sometimes may reach up to 8–10 mg/kg of raw material, while that of fungi can be 100–1000 times higher (Lyakh 1981). Differences were established between the absorption spectra of

Table 1. Strains examined.

Species	Accession no.	Source
Aureobasidium pullulans	CBS 105.22 = ATCC 11942 = VKM F-179	T of *Pullularia fermentans* var. *fusca*
	VKPM F-370	Metallic equipment
	VKPM F-371	Metallic equipment
	VKM F-1125	Fruitbody of *Inonotus obliquus*
	VKM F-2204	Lake water, Latvia
	VKM 2205	Lake water, Yaroslav region, Russia
	SPChPhA 129(11)	Unknown
	SPChPhA 2320	Soil, Chernobyl district, Ukraine
Aureobasidium pullulans var. *aubasidani* T	VKPM F-448 = CBS 100524	Birch sap, *Betula* sp., Russia
Hormonema macrosporum T	VKM F-2452 = CBS 536.94	*Rutilus rutilus*, Vologda region, Russia
Hormonema dematioides	VKM F-2836	Fruit body of *Mycena* sp., Moscow region, Russia
Kabatiella lini T	CBS 125.21	Leaf, *Linum usitatissimum*
Exophiala nigra T	VKM F-2137 = CBS 535.94	T of *Nadsoniella nigra*, seawater
Exophiala prototropha T	CBS 534.94	Unknown

Abbreviation used: T = ex-type strain, CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; SPChPhA = St. Petersburg State Chemical-Pharmaceutical Academy, St. Petersburg, Russia; VKM = All-Russian Collection of Microorganisms, Pushchino, Russia; VKPM = All-Russian Collection of Industrial Microorganisms, Moscow, Russia.

black yeast melanins and commercial Sepia melanin, which have been kept in the dark and photomodified by daylight irradiation. These data indicate perspectives of some black yeast melanins as photoprotectors and stimulators of skin regeneration (Blinova *et al.* 2003, Turkovskij & Yurlova 2002). Some black yeast melanins have higher UV-defensive activity than commercial melanins and they stimulate human skin regeneration significantly (Yurlova 2001, Turkovskij & Yurlova 2002). The photochemical properties of these melanins were found to be dependent on both the producing strain and the condition of its cultivation.

Melanins are high-molecular weight pigments formed by the oxidative polymerisation of phenolic compounds. 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 (Bell & Wheeler 1986). Dothideaceous species that have been found to synthesise DHN-melanin include *Aureobasidium pullulans* (Siehr 1981), *Cladosporium cladosporioides* (Latgé *et al.* 1988), *Hortaea werneckii, Phaeotheca triangularis,* and *Trimmatostroma salinum* (Kogej *et al*. 2004). Chaetothyrialean species include *Cladophialophora carrionii, C. bantiana*, *Exophiala jeanselmei* and *E. mansonii* (Taylor *et al*. 1987). The authors mentioned above used the inhibitor tricyclazole to test the fungi for the presence of 1,8-dihydroxynaphthalene (DHN)-melanin biosynthesis.

The final step in the DHN melanin pathway is the conjoining of 1,8-DHN molecules to form the melanin polymer. There are a number of candidate enzymes for this step, including phenoloxidases such as tyrosinase and laccases, peroxidases, and perhaps also catalases (Butler & Day 1998). DHN appears to be polymerised to melanin via a laccase but not much is know about this enzyme and its function in the melanin pathway (Bell & Wheeler 1986). The aim of the present study was to analyze the influence of ortho- and para-diphenoloxidase substrates on pigment formation in black yeasts and to determine the type polyphenoloxidases that are involved in biosynthesis of black yeast melanins.

MATERIALS AND METHODS

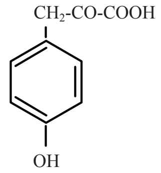
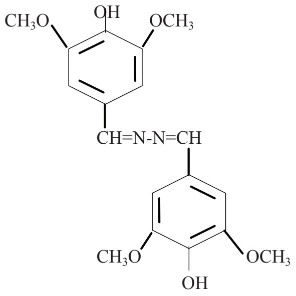
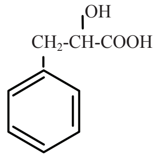
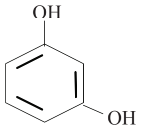
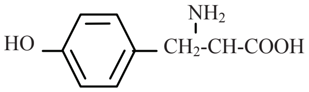
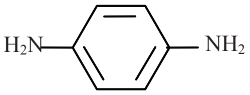
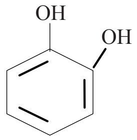
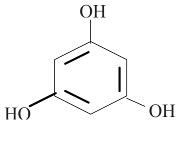
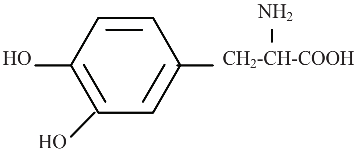
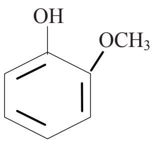
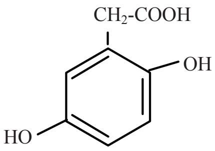
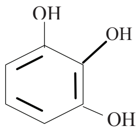
Diphenoloxidase substrates

Stock cultures (Table 1) were maintained on 2 % malt extract agar (MEA) slants. The low molecular weight aromatic compounds tested are listed in Table 2. The formation of melanin from low molecular weight aromatic compounds was determined by a modified auxanographic technique in which plates of Czapek agar (CzA) (in 90 × 15 mm Petri dishes) were divided in half diametrically (Fig. 1). One side of the plate was spread with a suspension of seven-day-old culture cultivated on 2 % MEA at 24 °C. Simultaneously three substrate assay cups were placed on each side. Each cup on each side received 0.1 mL of a solution of aromatic substrate (Table 2) in 0.1 M phosphate buffer (pH 7.0 or 7.2). The other half of the plate served as control for spontaneous oxidation of aromatic compounds. Plates were incubated at 24 °C and observed at intervals for 1 to 7 ds for development of a black-brown colour. The intensity of growth and pigmentation was estimated visually, and the intensity of growth and pigmentation of strain *Aureobasidium pullulans* CBS 105.22 = VKM F-179 (T) cultivated on 4 % MEA was listed as 100 %. The intensity of the pigmentation was represented according to five-grade scale: 100 % (black), 75 % (dark-brown, dark olive-green or dark grey), 50 % (brown or grey), 25 % (light brown or green-brown), and 0 % (yellow or white or pinkish).

Tricyclazole inhibition

Each fungus listed in Table 1 was grown in 90 × 15 mm Petri dishes containing 4 % MEA with tricyclazole, CzA with tricyclazole, and on 4 % MEA (control), and CzA (control). Tricyclazole was first dissolved in 100 % ethanol and then added to cooled medium prior to solidification to produce a concentration of 10–50 μg/mL. The final concentration of ethanol was 1.0 %. Control cultures were established on 4 % MEA and CzA which received only 1.0 % ethanol. All media were adjusted to pH 7.5 prior to dispensing.

Table 2. Substrates of diphenoloxidases tested.

Substrates of o-diphenoloxidases (EC 1.10.3.1.)	Concentration, mM/ml	Substrates of p-diphenoloxidases (EC 1.10.3.2.)	Concentration, mM/ml
4-Hydroxyphenyl-pyruvic acid  	0.02	Syringaldazine  	0.05
L-β-Phenyllactic acid  	0.02	Resorcinol  	0.05
Tyrosine  	0.02	p-Phenylenediamine  	0.05
Pyrocatechol  	0.05	Phloroglucinol  	0.05
3,4-Dihydroxyphenylalanine  	0.005	Guaiacol  	0.05
Homogentisic acid  	0.02	Pyrogallic acid  	0.05

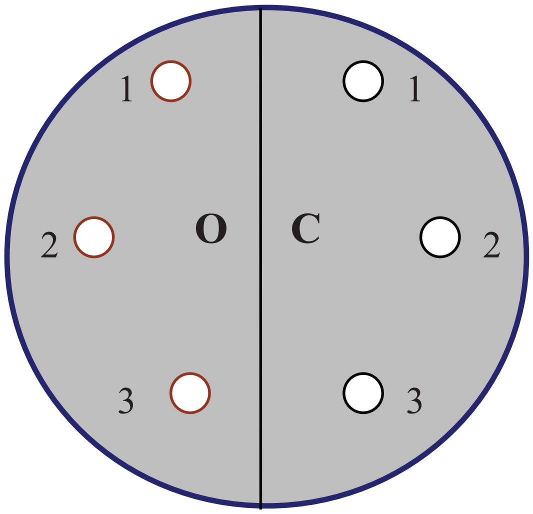


Fig. 1. The effect of the aromatic substrates on pigment production by strains studied. The left half only of the plate was inoculated (O), the right half serving as a control (C) for spontaneous oxidation of aromatic substrates. Cups 1, 2, 3 contained 0.1 mL of a solution of aromatic substrates.

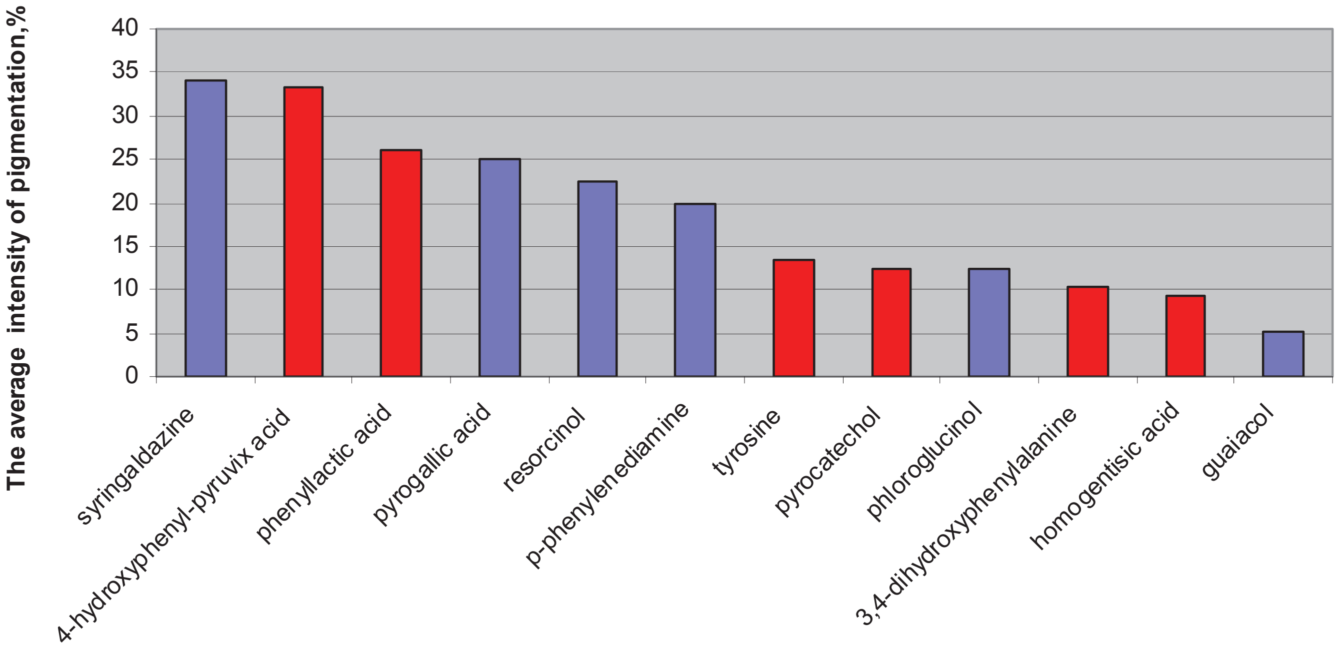


Fig. 2. The average intensity of pigmentation of some strains by presence of different phenolic substracts.
Substrates of o-diphenoloxidases (EC 1.10.3.1.): 4-hydroxyphenyl-pyruvic acid, L-β-phenyllactic acid, tyrosine, pyrocatechol, 3,4-dihydroxyphenylalanine and homogentisic acid are indicated in red colour.

Point inoculation of each fungus was made centrally on the plate in Petri dishes (inoculation was made by a suspension of seven-day-old culture cultivated on 2 % MEA at 24 °C). The cultures were grown in the dark at 24 °C for 21 d. The intensity of growth and pigmentation was estimated visually, and the intensity of growth and pigmentation of strain *Aureobasidium pullulans* CBS 105.22 = VKM F-179 cultivated on 4 % MEA was accepted as 100 %. All tests were performed three times in duplicate.

Thin-layer chromatography (TLC)

Fourteen-day-old Petri dish cultures of *A. pullulans* VKM F-179 = CBS 105.22*, A. pullulans* VKM F-370, *A. pullulans* VKPM F-371*, A. pullulans* var. *aubasidani* VKPM F-448, grown on CzA with (10–50 μg/mL) or without tricyclazole, were cut into small fragments (about 1 cm^3) and extracted in 150 mL acetone for 8 h. The extracts were subsequently filtered, evaporated under reduced pressure and the remaining aqueous solutions extracted twice with equal volumes of ethyl acetate. The ethyl acetate fractions were collected, combined, and residual water was removed over $NaSO_4$. After the ethyl acetate was evaporated under reduced pressure, each sample was reconstituted with 1 mL of ethyl acetate to provide concentrated solutions for chromatographic evaluation (Taylor *et al*. 1987, Kogej *et al*. 2004). The concentrated extracts and the standards of flaviolin, 2-hydroxyjuglone (2-HJ), scytalone, 1,3,8-trihydroxynaphthalene (1,3,8-THN), 1,8-dihydroxynaphthalene were spotted on silica gel-coated TLC plates with fluorescent indicator (Merck) and developed with ether-hexane-formic acid (60:39:1). Once separated, metabolites from the extracts were observed in daylight and under ultraviolet (UV) light at 254 and 365 nm for characteristic colours and R_f values. The plates were then sprayed with an aqueous solution of 1 % $FeCl_3$. Once they were dried, they were again evaluated for colours that appeared in daylight (Taylor *et al*. 1987, Kogej *et al*. 2004).

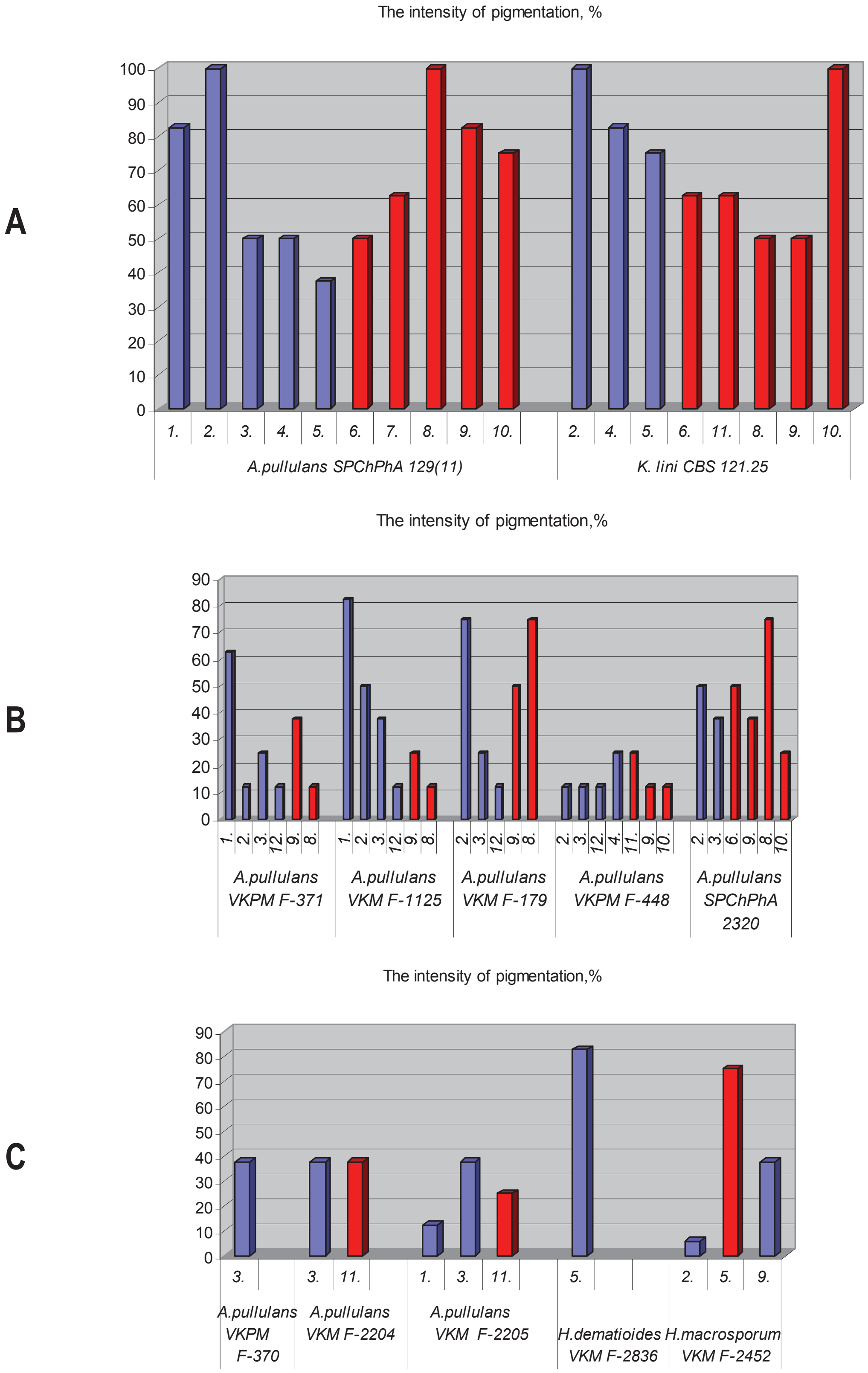


Fig. 3. The intensity of pigmentation of some strains cultivated on media with different aromatic substrates.
Y-axis: intensity of pigmentation, %.
A. Second group; B. Third group; C. Fourth group.
The intensity of pigmentation of strains *Exophiala nigra* F-2137 = CBS 535.94 (T) and *E. prototropha* CBS 534.94 (first group), when cultivated on all 12 aromatic substrates used, was listed as 100 %.
Substrates of o-diphenoloxidases (EC 1.10.3.1.): 4-hydroxyphenyl-pyruvic acid, L- β-phenyllactic acid, tyrosine, pyrocatechol, 3,4-dihydroxyphenylalanine and homogentisic acid are indicated in red colour.

1. phenylenediamine;
2. syringaldazine;
3. pyrogallic acid;
4. phloroglucinol;
5. resorcinol;
6. tyrosine;
7. 3,4-dihydroxyphenylalanine;
8. L-β-phenyllactic acid;
9. 4-hydroxyphenyl-pyruvic acid;
10. homogentisic acid;
11. pyrocatechol;
12. guaiacol.

RESULTS

Fourteen strains of BYF originating from different natural biotopes were investigated (Table 1). It was established that syringaldazine, pyrogallic acid (substrates of p-diphenoloxidases) and 4-hydrophenyl-pyruvic acid, L-β-phenyllactic acid (substrates of o-diphenoloxidases) optimally promoted melanin biosynthesis when compared to other groups of substrates investigated. Average intensities of pigmentation of all strains studied were the lowest when guaiacol (substrate of p-diphenoloxidases) was used as a substrate (Fig. 2).

Diphenoloxidase substrates

Strains investigated were divided into four groups based on their ability to produce dark pigments when they were cultivated on aromatic o- and on p-diphenoloxidase substrates (Fig. 3).

Group 1. *Exophiala nigra* VKM F-2137 and *E. prototropha* CBS 534.94 produced black pigments when they were cultivated on all 12 aromatic substrates used, including o- and on p-diphenoloxidase substrates. The intensity of pigmentation of strains *Exophiala nigra* F-2137 = CBS 535.94 and *E. prototropha* CBS 534.94, cultivated on each of the 12 aromatic substrates, was listed as 100 %.

Group 2. Strains utilising 8−10 aromatic substrates and synthesising dark pigments (Fig. 3A). This group includes two strains. *A. pullulans* SPChPhA 129(11), growing and synthesizing black or dark brown or brown pigments when five substrates of o-diphenoloxidases (4-hydroxyphenyl-pyruvic acid, L-β-phenyllactic acid, tyrosine, 3,4-dihydroxyphenylalanine, homogentisic acid) and five substrates of p-diphenoloxidases (syringaldazine, resorcinol, p-phenylenediamine, phloroglucinol, pyrogallic acid) were used for cultivation. *Kabatiella lini* CBS 125.21 produced black or dark-brown or brown pigment when five substrates of o-diphenoloxidases (4-hydroxyphenyl-pyruvic acid, L-β-phenyllactic acid, tyrosine, pyrocatechol, homogentisic acid) and three substrates of p-diphenoloxidases (syringaldazine, resorcinol, phloroglucinol) were used for cultivation.

Group 3. Strains utilising 5−7 aromatic substrates and synthesizing dark pigments when cultivated on CzA with aromatic substrates (Fig. 3B). This group includes mostly strains of *Aureobasidium pullulans*: VKPM F-371, VKM F-179, VKM F-1125 produced black or dark brown or dark olive-green or dark-grey, brown or grey, light brown or green-brown pigments on two substrates of o-diphenoloxidases (L-β-phenyllactic acid, 4-hydroxyphenyl-pyruvic acid) and on four substrates of p-diphenoloxidases (p-phenylenediamine, syringaldazine, pyrogallic acid, guaiacol). Strain *A. pullulans* var. *aubasidani* VKPM F-448 produced brown or light brown or green brown pigment on three substrates of o-diphenoloxidases (4-hydroxyphenyl-pyruvic acid, pyrocatechol, homogentisic acid) and four substrates of p-diphenoloxidases (syringaldazine, pyrogallic acid, phloroglucinol, guaiacol). Strain *A. pullulans* SPChPhA 2320 formed pigment on four substrates of o-diphenoloxidases (tyrosine, L-β-phenyllactic acid, 4-hydroxyphenyl-pyruvic acid, homogentisic acid) and two substrates of p-diphenoloxidases (syringaldazine, pyrogallic acid).

Group 4. Strains synthesizing dark pigments only on 1−3 aromatic substrates when cultivated on CzA with aromatic substrates (Fig. 3C). Strains included *A. pullulans* VKPM F-370 (light brown pigmentation) and *H. dematioides* VKM F-2836 (dark olive-green pigmentation), producing pigment only when substrates of p-diphenoloxidases (pyrogallic acid, resorcinol) were used. *Aureobasidium pullulans* VKPM F-2204 produced pigment of equal intensity (brown or light brown) on p-diphenoloxidases (pyrogallic acid) and on o-diphenoloxidases (pyrocatechol) substrates. Strain VKPM F-2205 gave light brown pigmentation on two substrates of p-diphenoloxidases (p-phenylenediamine, pyrogallic acid) and on pyrocatechol (substrate of o-diphenoloxidases).

Tricyclazole inhibition

Following the same subdivision:

Group 1. Tricyclazole had no apparent effect on growth of the black yeast strains belonging to the strains of this group, as was observed both on 4 % MEA and on CzA. The strains concerned were blackish or dark brown in colour, when grown on 4 % MEA containing 10–20 µL/mL tricyclazole, and on CzA containing 10–20 µL/mL tricyclazole. We observed reddish pigment only in Group 1 strains (*Exophiala nigra* VKM F-2137 and *E. prototropha* CBS 534.94) when we used higher (40−50 µg/mL) concentrations of tricyclazole. Other groups (Groups 2−4) of strains studied did not form reddish or red-brown pigments even they were cultivated in media with high (30−50 µg/mL) concentrations of tricyclazole.

Group 2. Tricyclazole had no apparent effect on growth and pigmentation of *A. pullulans* SPChPhA 129(11), when grown on 4 % MEA. It slightly inhibited the growth of this strain on CzA and had no effect on pigmentation (Fig. 4). The plant pathogen *K. lini* CBS 125.21 was inhibited by tricyclazole on 4 % MEA and CzA. The intensity of pigmentation was decreased almost in two times, when *K. lini* CBS 125.21 was grown on CzA with tricyclazole (Fig. 4).

Group 3. The growth of the strains belonging to this group was slightly inhibited by tricyclazole both on 4 % MEA and on CzA. Tricyclazole affected intensity of pigmentation of *A. pullulans* VKM F-179, VKM F-1125, SPChPhA 2320, *A. pullulans* var. *aubasidani* VKPM F-448 on CzA. On 4 % MEA an effect was found on pigmentation of only *A. pullulans* var. *aubasidani* VKPM F-448 (Fig. 5).

Group 4. Tricyclazole had no apparent effect on growth of the strains *A. pullulans* VKM F-2204, VKM F-370 of this group, both on 4 % MEA and on CzA (Fig. 6). The intensity of pigmentation of the strains VKM F-2204, VKM F-370 grown on 4 % MEA with tricyclazole was almost the same as on 4 % MEA without tricyclazole. The strains *A. pullulans* VKM F-2204, VKM F-2205, VKM F-370 and *Hormonema macrospora* VKM F-2452 were yellow or light yellow or pinkish, when grown on CzA with or without tricyclazole. *Hormonema dematioides* VKM F-2836 did not grow at all on CzA (Fig. 6). Microscopic comparisons indicated that all strains studied had their normal morphologies in the presence of tricyclazole.

Identification of DHN-melanin intermediates

Metabolites from ethyl acetate extracts of *A. pullulans* VKM F-179 = CBS 105.22, VKM F-370, VKPM F-371 and *A. pullulans* var. *aubasidani* VKPM F-448 were analysed by TLC to determine if DHN-melanin precursors or related metabolites were present. Flaviolin/biflaviolin and 2-HJ were detected in the extracts of 14 ds old cultures of *A. pullulans* VKM F-370 and *A. pullulans* VKPM F-371 when they were grown with tricyclazole; however, they were not found in culture without tricyclazole (Table 3). The TLC results indicated that tricyclazole had blocked the DHN-melanin pathway, causing the accumulation of 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN) and 1,3,8-THN, which were autoxidised to flavolin or 3,3-biflaviolin and 2-HJ, respectively (Table 3). Strains *A. pullulans*

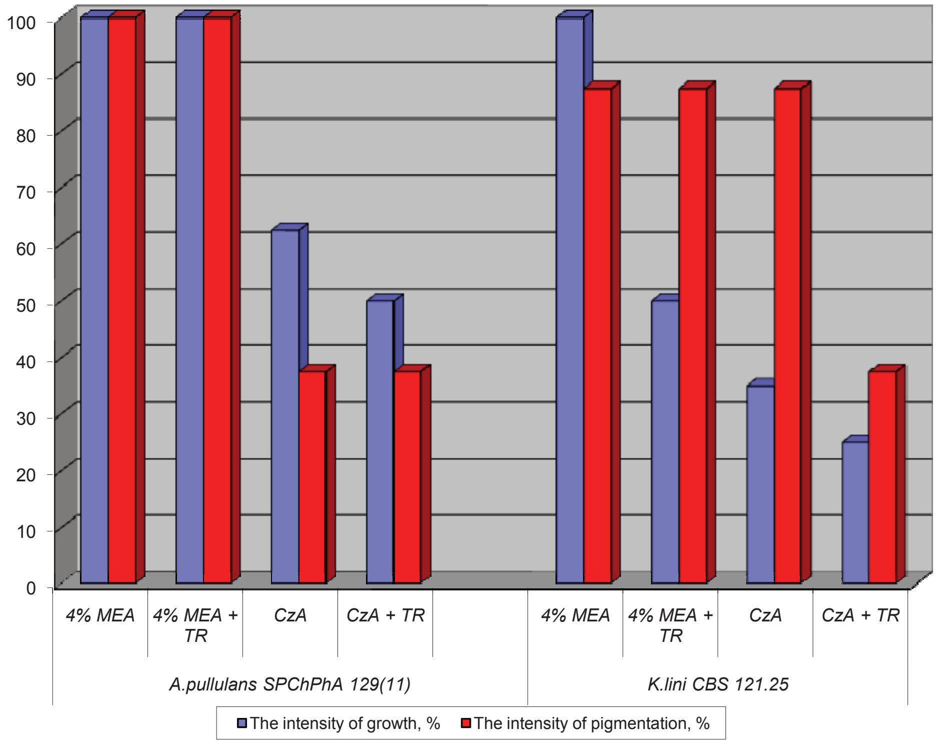


Fig. 4. The influence of tricyclazole on growth and pigmentation of strains belonging to the second group.
Y-axis: intensity of growth, intensity of pigmentation, %.
X-axis: 4 % MEA = 4 % malt extract agar; 4 % MEA +TR = 4 % malt extract agar with 20 µL/mL tricyclazole; CzA = Czapek agar; CzA + TR = Czapek agar with 20 µL/mL tricyclazole.

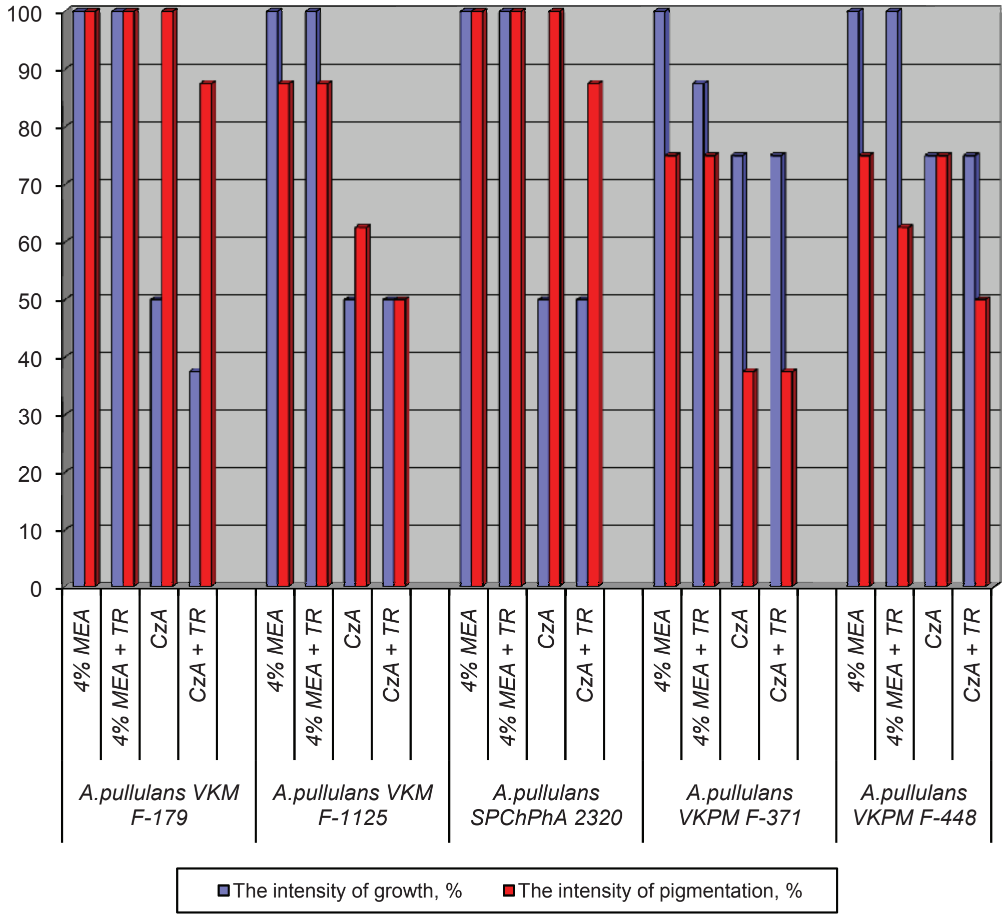


Fig. 5. The influence of tricyclazole on growth and pigmentation of strains belonging to the third group.
Y-axis: intensity of growth, intensity of pigmentation, %.
X-axis: 4 % MEA = 4 % malt extract agar; 4 % MEA +TR = 4 % malt extract agar with 20 µL/mL tricyclazole; CzA = Czapek agar; CzA + TR = Czapek agar with 20 µL/mLtricyclazole.

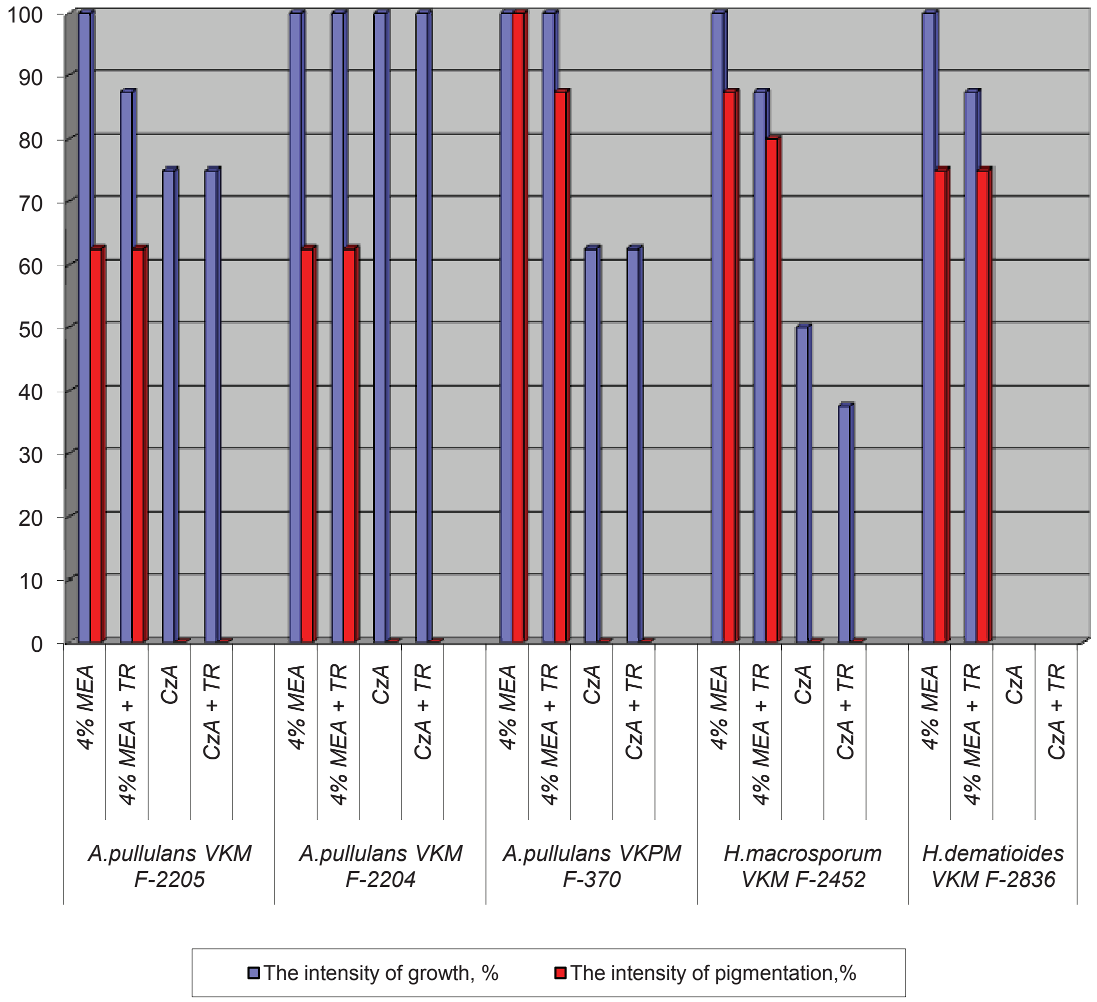


Fig. 6. The influence of tricyclazole on growth and pigmentation of strains belonging to the fourth group.
Y-axis: intensity of growth, intensity of pigmentation, %.
X-axis: 4 % MEA = 4 % malt extract agar; 4 % MEA + TR = 4 % malt extract agar with 20 μL/mL tricyclazole; CzA = Czapek agar; CzA + TR = Czapek agar with 20 μL/mL tricyclazole.

VKM F-179 and *A. pullulans* var. *aubasidani* VKPM F-448 did not secrete 2-HJ and flaviolin both in the presence of tricyclazole and without tricyclazole (Table 3).

DISCUSSION

Three out of four black yeast genera analysed (*Aureobasidium, Hormonema* and *Kabatiella*) (Table 1) belong to the ascomycetous order *Dothideales*, while *Exophiala* is an anamorph of *Chaetothyriales* (de Hoog *et al*. 1999). Many authors (Siehr 1981, Taylor *et al*. 1987, Butler & Day 1998, Butler *et al.* 2004, Kogej *et al.* 2004) indicated that both types of fungi synthesise a DHN-type melanin. Details of the DHN-type melanin pathway have been elucidated using a number of different fungi. Much of what is known about the pathway and its enzymes has come from the use of melanin-deficient strains and compounds, such as tricyclazole, which inhibit specific enzymes in the pathway (Bell & Wheeler 1986, Butler & Day 1998).

The systemic fungicide tricyclazole [5-methyl-1,2,4-thiazolo(3,4,b)-benzothiazile] (TR) is an inhibitor of biosynthesis of melanins, which form via the pentaketide pathway (Bell & Wheeler 1986). For example, it is known to strongly inhibit the enzymatic reduction (reductase enzymes) of 1,3,8-trihydroxynaphthalene (1,3,8-THN) to vermelone. Tricyclazole has also been shown to weakly inhibit the reduction of 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN) to scytalone (Wheeler & Greenblatt 1988).

Flaviolin and 2-hydroxyjuglone (2-HJ) are known as autoxidative products of 1,3,6,8-THN and 1,3,8-THN, respectively (Fig. 7). The presence of flaviolin and 2-HJ in fungal cultures, treated with tricyclazole, is usually accepted as proof that 1,3,6,8-THN and 1,3,8-THN were involved in the synthesis of DHN-melanin (Butler & Day 1998). Once produced, 1,3,8-DHN is reduced to vermelone, which in turn is dehydrated to 1,8-dihydroxynaphthalene (DHN) (Bell & Wheeler 1986, Taylor *et al*. 1987) (Fig. 7). 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. DHN appears to be polymerised to melanin via a laccase (Butler & Day 1998).

In the present investigation we demonstrated, that the DHN-melanin inhibitor, tricyclazole, inhibited melanin biosynthesis only in some black yeast strains. Four groups were distinguished, differing by their ability to produce pigment with o- and p-diphenoloxidase substrates and to be inhibited by tricyclazole (Table 4).

The effect of tricyclazole on pigment production proved to be more pronounced when strains were grown on CzA. On this medium 53.3 % of the strains were inhibited by tricyclazole, whereas only 26.6 % of the strains decreased their intensity of pigmentation

Table 3. Melanin metabolites analysed by TLC in control cultures and in tricyclazole-inhibited cultures of *A. pullulans* VKM F-179, VKM F-370, VKPM F-371 and *A. pullulans* var. *aubasidani* VKPM F-448.

Accession no.	**Tricyclazole**[1]	**Metabolites**[2]		
		2-HJ	**flaviolin**	**$3,3'$-biflaviolin**
VKM F-179	−	−	−	−
	+	−	−	−
VKM F-370	−	−	−	−
	+	+	+	−
VKPM F-371	−	−	−	−
	+	+	−	+
VKPM F-448	−	−	−	−
	+	−	−	−

[1] Tricyclazole concentration in the medium was 0 µg mL^{-1} (−) or 20 µg mL^{-1} (+).

[2] "+ " = metabolite was observed; "− " = metabolite was not observed.

VKM/VKPM = Russian Collection of microorganisms, Puschuno, Russia.

Table 4. Subdivision of the strains into pigmentation groups.

Strains	**Pigmentation on substrates of**		**Influence of tricyclazole on intensity of**			
	o-diphenoloxidases	**p-diphenoloxidases**	**pigmentation**		**growth**	
			4 % MEA	**CzA**	**4 % MEA**	**CzA**
Group 1 (12/12)[1]						
Exophiala nigra VKM F-2137	+	+	-	+	-	-
Exophiala. prototropha CBS 534.94	+	+	+	+	-	-
Group 2 (8-10/12)[2]						
A. pullulans SPChPhA 129(11)	+	+	-	-	-	+
K. lini CBS 125.21	+	+	-	+	+	+
Group 3 (5-7/12)[3]						
A. pullulans VKPM F-371	+	+	-	-	+	-
A. pullulans CBS 105.22	+	+	-	+	+	-
A. pullulans VKM F-1125	+	+	-	+	-	-
A. pullulans var. *aubasidani* VKPM F-448	+	+	+	+	-	-
A. pullulans SPChPhA 2320	+	+	-	+	-	-
Group 4 (1-3/12)[4]						
A. pullulans VKM F-2204	+	+	-	-	-	-
A. pullulans VKM F-2205	+	+	-	-	+	-
A. pullulans VKM F-370	-	+	+	-	-	-
H. dematioides VKM F-2836	-	+	-	*	+	*
H. macrosporum VKM F-2452	+	+	+	-	+	+

***** *H. dematioides* F-2836 did not grow at all on CzA.

Abbreviations used: "+ " = characteristic was observed; "− " = characteristic was not observed.

[1] Strains produced black pigments when they were cultivated on all 12 aromatic substrates used: both on o-diphenoloxidases and on p-diphenoloxidases substrates;

[2] Strains produced dark pigments when they were cultivated on 8–10 aromatic substrates from 12 used: both on o-diphenoloxidases and on p-diphenoloxidases substrates;

[3] Strains produced dark pigments when cultivated on 5–7 aromatic substrates from 12 used: both on o-diphenoloxidase and p-diphenoloxidase substrates;

[4] Strains produced dark pigments when cultivated on 1–3 aromatic substrates from 12 used: both on o-diphenoloxidases and on p-diphenoloxidases substrates.

when they were cultivated on 4 % MEA with tricyclazole (Table 4). Metabolites from ethyl acetate extracts of biomass from *A. pullulans* VKM F-179 = CBS 105.22, VKM F-370, VKPM F-371 and *A. pullulans* var. *aubasidani* VKPM F-448 were analysed by thin-layer chromatography to determine if DHN-melanin precursors or related metabolites were present. Flaviolin and 2-HJ were detected only in the extracts of 14-d-old cultures of *A. pullulans* VKM F-370, *A. pullulans* VKPM F-371, when they were grown on CzA with 10 and 20 µg/mL tricyclazole. However, flaviolin and 2-HJ were not found in acetone extracts of biomasses of these strains, when they were grown in CzA without tricyclazole (Table 3). Strains *A. pullulans* VKM F-179*, A. pullulans* var. *aubasidani* VKPM F-448 did not secrete 2-HJ and flaviolin, neither in the presence of nor without tricyclazole (TR) (Table 3) and even when they were

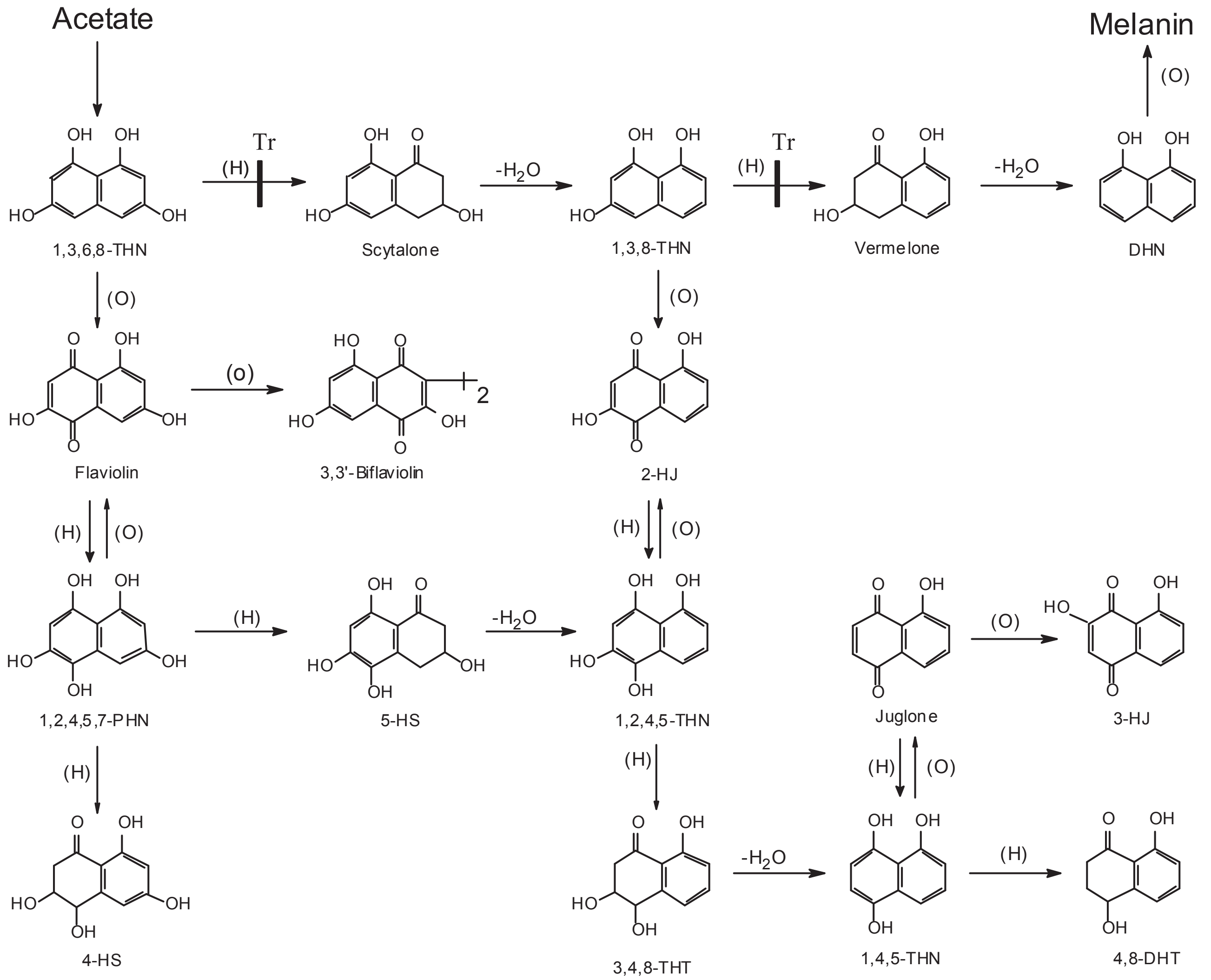


Fig. 7. Biosynthetic pathway of DHN-melanin and related pentaketide metabolites, from the scheme shown by Bell & Wheeler (1986). The first known product of the pathway is I.3.6.8-THN. This metabolite is reduced lo scytalone, which is then dehydrated to 1,3.8-THN. Next, 1.3.8-THN is reduced to vermelone, 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.

cultivated in media with high (30−50 µg/mL) concentrations of tricyclazole. The halophilic ascomycetous black yeasts *Hortaea werneckii, Phaeotheca triangularis* and *Trimmatostroma salinum* accumulated 4,8-dihydroxytetralone (4,8-DHT) in cultures non-inhibited by TR (Kogej *et al*. 2004) (Fig. 7). Small amounts of 4-hydroxyscytalone (4-HS) (Fig. 7) have been reported in wild-type cultures of *Curvularia lunata* non-inhibited by TR (Rižner & Wheeler 2003), as well as of scytalone in *Thielaviopsis basicola* (Wheeler & Stipanovic 1979) and *Sporothrix schenckii* (Romero-Martinez *et al*. 2000). This means that products which are typical for cultures of black yeasts inhibited by tricyclazole (TR) were also found in non-inhibited cultures.

In our earlier investigations (Yurlova & Sindeeva 1996) we proved the presence of intracellular and extracellular laccase activity of 14 above mentioned strains of black yeasts. Tricyclazole decreased laccase activity (Yurlova & Sindeeva 1995). Tyrosinase, which oxidises tyrosine, was not found in any of the strains investigated (Table 1) (Yurlova & Sindeeva 1995). On the basis of the present data we hypothesise that black yeasts contain a multipotent polyphenoloxidase able to oxidise substrates characteristic for o-diphenoloxidases and p-diphenoloxidases. Such kind of multipotent polyphenoloxidase has previously been observed in the marine bacterium *Marinomonas mediterranea* (Fernandez *et al.* 1999). The melanisation process might involve other enzymes and more substrates than those commonly recognised. The mechanism of biosynthesis of black yeast melanins remains to be further elucidated.

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