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EXPERIMENTAL ARTICLES

Mycotoxin Production Profiles of *Penicillium vulpinum* (Cooke & Massee) Seifert & Samson Strains

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Abstract—Mycotoxins produced by seven strains of *Penicillium vulpinum* (formerly *Penicillium claviforme*) isolated from different sources were studied. The strains were characterized by specific profiles of secondary metabolites and produced mycotoxins of different structural types. In addition to toxins already known for this fungal species (patulin, roquefortine, 3,12-dihydroroquefortine, oxalin, viridicatin, cyclopenin, and α-cyclopiazonic acid), the strains studied also produced indolyl-3-acetic acid, griseofulvin, meleagrin, and cyclopeptin.

Key words: fungi, *Penicillium vulpinum* (*Penicillium claviforme*), mycotoxins, alkaloids, ecology.

Due to the urbanization of the environment, the previously rare species of the genus *Penicillium,* including the coremium-forming species *Penicillium vulpinum* (formerly *Penicillium claviforme*), became more abundant [1]. However, little is known about the secondary metabolites produced by this fungus.

The aim of the present work was to study the mycotoxin profiles of *P. vulpinum* strains isolated from various habitats.

MATERIALS AND METHODS

The *P. vulpinum* strains used in this study (VKM F-256, VKM F-257, VKM F-258, VKM F-259, VKM F-260, VKM F-1255, and VKM F-2360) were obtained from the All-Russia Collection of Microorganisms (VKM).

The strains were maintained on glucose–potato agar slants inoculated with spore suspensions containing $1–2 \times 10^7$ spores/ml. Fungi were grown at 24 ± 1°C in shaken (220–240 rpm) 750-ml Erlenmeyer flasks containing 150 ml of medium of the following composition (g/l distilled water): mannitol, 50.0; succinic acid, 5.4; $MgSO_4 \cdot 7H_2O$, 0.3; and KH_2PO_4, 1.0. The pH of the medium was adjusted to 5.4 with 25% NH_4OH.

Culture liquid filtrates were analyzed for secondary metabolites after 6, 12, 15, and 20 days of growth. Culture filtrates were alkalinized to pH 8–9 and extracted thrice with chloroform. Extracts were pooled, dehydrated with anhydrous Na_2SO_4, filtered, and completely dried in a vacuum rotary evaporator. After the extraction of neutral and alkaline metabolites, culture filtrates were acidified to pH 2–3 with 10% HCl, and acidic metabolites were extracted from the filtrates with chloroform.

Mycotoxins were analyzed by thin-layer chromatography (TLC) on UV-254 Silufol plates (Czech Republic) developed in the following systems: (I) ethyl acetate–methanol–25% NH_4OH (85 : 15 : 10), (II) chloroform–methanol–25% NH_4OH (80 : 20 : 0.2), (III) chloroform–methanol–25% NH_4OH (90 : 10 : 1), (IV) chloroform–acetone (9 : 1), (V) methylene chloride–acetonitrile (4 : 1), and (VI) toluene–ethyl acetate–formic acid (5 : 4 : 1). Separated compounds were detected by their absorbance or fluorescence under illumination with UV light, as well as by means of spraying the developed plates with the Ehrlich reagent and 1% solution of $Ce(SO_4)_2$ in 6 N H_2SO_4 (detection of indole benzodiazepinic and diketopiperazinic alkaloids) and with a 3% solution of $FeCl_3$ in ethanol (detection of quinoline metabolites).

Individual compounds were obtained by preparative TLC in the aforementioned solvent systems. Separated compounds were eluted from plates with a chloroform–methanol (1 : 1) mixture and evaporated until dry in the vacuum rotary evaporator.

Metabolites were identified by TLC in various solvent systems using reference standards, as well as by UV spectroscopy, and were quantified spectrophotometrically using the known coefficients of molar extinction.

The UV spectra of compounds were recorded on a UV-160A spectrophotometer (Shimadzu, Japan).

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RESULTS AND DISCUSSION

The species *Penicillium vulpinum* (Cooke & Massee) Seifert & Sampson (formerly *Penicillium claviforme* Bain) is placed within the section *Coremigenum* (Biourge) Pitt, subsection *Coremigena* Biourge [2]. Its specific morphological feature is the ability to form spheroid or club-shaped coremia during growth on agar media. The species belongs to coprophiles, can grow under microaerobic conditions, and survives anaerobiosis [3].

At present, the fungus *P. vulpinum* is encountered in habitats other than feces [1]. Thus, of the seven strains studied, only strain F-256 has been isolated from partridge feces. Strains F-260 and F-1255 have been isolated from soil; F-259, from air; F-258, from a tannin solution; F-2360, from apples; and KBP No. 16, from snow [4].

Upon submerged cultivation in mineral Abe medium, the strains grow in the form of beads 5 to 8 mm in diameter without conidia. Depending on the strain, biomass accumulated by the stationary growth phase (15 days of growth) varied from 3 to 9 g/l. Strains F-2360, F-260, and F-259, which had been isolated from habitats atypical of *P. vulpinum*, accumulated 7–9 g biomass/l. At the same time, the growth of the coprophilic strain F-256 and strain F-257 in mineral medium was poor (their biomass in the stationary phase did not exceed 3 g/l).

To our knowledge, there are only a few publications devoted to the study of secondary metabolites of the fungus *P. vulpinum* [3–5]. The six strains of this species studied by Frisvad exhibited the same profile of mycotoxins produced (patulin, cyclopenin, viridicatin, roquefortine, and oxalin) [3]. At the same time, according to our previous data, fungal metabolites synthesized by various strains of *P. vulpinum* are different. Thus, strain KBP No. 16 produced roquefortine, 3,12-dihydroroquefortine, and auranthioclavin [4], whereas strain KBP No. 113 produced α-cyclopiazonic acid (α-CPA) and its imino derivative [5].

The seven *P. vulpinum* strains studied in the present work also widely differed in the sets of the metabolites produced (Table 1).

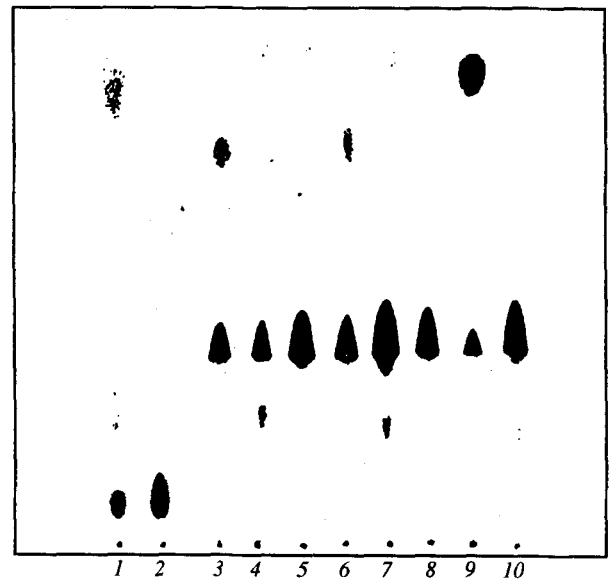
Strains F-256, F-258, F-259, and F-1255 produced diketopiperazine alkaloids of the roquefortine group, which are synthesized via the amino acids tryptophan and histidine. In addition to roquefortine, possessing distinct neurotoxic properties [6], and oxalin, known as an antibiotic, this group of alkaloids also includes meleagrin and 3,12-dihydroroquefortine, whose biological activities can be predicted on the basis of their structure. The maximum production of roquefortine was observed for strains F-259 (up to 15 mg/l) and F-1255 (up to 22 mg/l). The latter strain was also an active producer of meleagrin (up to 18 mg/l), a hitherto unknown metabolite of the species *P. vulpinum*. 3,12-Dihydroroquefortine was produced in the largest amounts (up to 2 mg/l) by strain F-257, whereas other strains synthesized only traces of this metabolite. Oxalin in amounts of up to 9 mg/l was synthesized only by strain F-256 (Table 1).

Table 1. Metabolites produced by *P. vulpinum* strains

Strain	Metabolites
VKM F-256	Oxalin, roquefortine, 3,12-dihydroroquefortine
VKM F-257	Patulin, griseofulvin
VKM F-258	IAA, roquefortine, 3,12-dihydroroquefortine
VKM F-259	Cyclopenin, roquefortine, 3,12-dihydroroquefortine
VKM F-260	α-CPA
VKM F-1255	Patulin, meleagrin, roquefortine, 3,12-dihydroroquefortine
VKM F-2360	α-CPA, viridicatin, cyclopenin, cyclopeptin
KBP No. 16	Roquefortine, 3,12-dihydroroquefortine, auranthioclavin [4]
KBP No. 113	α-CPA, imino α-CPA [5]

Strains F-257, F-260, and F-2360 were unable to synthesize diketopiperazine alkaloids.

Among the fungal strains studied, only F-257 and F-1255 were able to synthesize patulin (in amounts of up to 1 mg/l), which is an especially dangerous polyketide mycotoxin that contaminates foods, possesses mutagenic and carcinogenic properties, and produces



Thin-layer chromatograms in solvent system I of extracts of the culture filtrates of *P. vulpinum* strains producing IAA and α-CPA. Lanes: *1*, acid extract, strain F-258; *2*, IAA; *3*, alkali extract, strain No. 113; *4*, acid extract, strain No. 113; *5*, α-CPA; *6*, alkali extract, strain F-260; *7*, acid extract, strain F-260; *8*, α-CPA; *9*, alkali extract, strain F-2360; and *10*, acid extract, strain F-2360.

Table 2. Characteristics of metabolites isolated from *P. vulpinum* strains

Metabolite	Stain with			Chromatographic mobility ($R_f \times 100$) in systems					λ_{max} of UV spectra, nm
	Ehrlich reagent	$Ce(SO_4)_2$	$FeCl_3$	I	II	III	IV	V	
Patulin*	–	–	–					22	275
Griseofulvin**	–	–	–				49	60	326 sh, 291, 254 sh, 236 sh, 207
IAA	Lilac	–	–	11					290, 281, 272, 221
α-CPA	Lilac	–	–	40		11			281, 253, 223
Oxalin	Orange	Dark orange***	–	59	75	63			345, 304 sh, 281, 232 sh, 207
Meleagrin	Orange	Dark orange***	Grayish violet	44	70	50			344, 283, 229, 206
Roquefortine	Dark blue***	Grayish lilac***	–	61	67	49			326, 240, 207
3,12-Dihydro-roquefortine	Dark blue***	Grayish lilac***	–	47	58	33			301, 243, 209
Viridicatin**	–	–	Dirty green***			42		22	330, 318, 280, 222
Cyclopenin	Dirty green***	Yellow***				52	41	21	286
Cyclopeptin	Yellow***	Yellow***				62	36	24	289

*Absorbs UV light.
**Fluoresces under UV illumination.
***Color after 5-min heating at 100°C.

highly toxic effects on the gastrointestinal tract, liver, kidney, and spleen [6]. It should be noted that the UV spectrum of the extract of strain F-257 had a slightly widened and hypsochromically shifted absorption peak at λ_{max} = 273 nm, which was indicative of the possible presence of isopatulin with λ_{max} = 269 nm (Table 2). In addition to patulin, strain F-258 produced another *O*-heterocyclic metabolite, griseofulvin, which exhibits fungicidal activity [7].

The third group of *P. vulpinum* alkaloids involves viridicatin and cyclopenin, whose precursors are anthranilic acid and phenylalanine. As was shown for *P. cyclopium* [8], viridicatin and cyclopenin, as well as other members of this mycotoxin group (cyclopeptin, dehydrocyclopeptin, cyclopenol, and viridicatol) are synthesized in the same metabolic pathway. Among the strains studied in the present work, only strain F-2360 showed the ability to synthesize viridicatin in amounts of up to 1 mg/l. This strain also produced cyclopenin and cyclopeptin (the latter metabolite was also revealed in strain F-259). Viridicatin has been found to possess moderate antibiotic [7] and phytotoxic activities [9]. Generally, alkaloids of the third group are not highly toxic. However, it should be borne in mind that even slightly toxic compounds may become dangerous due to a possible synergistic effect [6].

Analysis of the chromatograms of acid extracts of the culture liquids of *P. vulpinum* strains showed that strain F-258 synthesized up to 8 mg/l of auxin (indolyl-3-acetic acid, IAA), and strains F-260 and F-2360 produced α-CPA in amounts of 12 and 5 mg/l, respectively. Due to the dual acid–base nature of α-CPA, this metabolite was found in both acid and alkali extracts (see the figure). It should be noted that α-CPA is known as a dangerous neurotoxin that affects the immune and nervous systems [6] and as an antimicrobial and mutagenic agent [10].

Clavine and diketopiperazine alkaloids, which differ from the aforementioned fungal metabolites in structure and precursors, were not found in any of the strains studied.

To conclude, all of the *P. vulpinum* strains studied could produce mycotoxins. In addition to known metabolites of *P. vulpinum*, some strains of this species were able to synthesize IAA, griseofulvin, meleagrin, and cyclopeptin. In contrast to Frisvad's data, all the strains studied differed in their secondary metabolite profiles.

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