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EXPERIMENTAL ARTICLES

# Growth and Biosynthesis of Rugulovasines in *Penicillium variabile* Sopp 1912

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**Abstract**—Production of clavine alkaloids rugulovasines by *P. variabile* did not depend on the habitat of the producers. During submerged cultivation on a simple synthetic medium in early growth stages, microcyclic conidiation was observed in the tested fungi; its presence or absence, as well as the activity of the cultures as to biosynthesis of rugulovasines, depended on the composition of the culture medium. On a complex medium supplemented with peptone, conidiation occurred but was considerably suppressed. Conidia were completely absent in the medium supplemented with yeast extract. In both cases, no appreciable amounts of rugulovasines were detected.

*Key words*: microscopic fungi, *Penicillium*, microcyclic conidiation, biosynthesis, secondary metabolites, clavine alkaloids, rugulovasines.

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Fungi of the genus *Penicillium* are promising objects in the search for new biologically active compounds [1]. The isolates obtained from unusual, poorly studied habitats are of specific interest. Earlier, out of fungi belonging to the genus *Penicillium* isolated from permafrost, producers of such secondary metabolites as quinoline alkaloids (quinocitrinines) were selected [2] and novel producers of important biologically active compounds (ergot alkaloids and diketopiperazines of the roquefortine group) were found [3, 4]. It was established in the process of those studies that relict fungi of the species *P. variabile* Sopp synthesize clavine alkaloids, atypical of this species: rugulovasines [5]. In the study of growth and development of one of the relic strains, *P. variabile* VKM FW-806, in a medium of defined composition, microcyclic conidiation was detected, which is unusual for producers of the genus *Penicillium*.

This work was designed to study the possible interrelationship between the capability of fungi *P. variabile* for synthesis of rugulovasines and their habitats, as well as to define the factors that determine the character of growth and development of producing strains and the direction and intensity of biosynthesis of secondary metabolites.

## MATERIALS AND METHODS

Strains of *Penicillium variabile* from the All-Russian Collection of Microorganisms (VKM) of the Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences (IBPM RAS) were used: VKM F-2075, FW-806, FW-899, FW-1449, FW-1451, FW-1452, FW-2528, FW-2531, FW-2566, FW-2701, FW-2758, FW-2762, FW-2884, and FW-2886. Growth, development, and biosynthesis of rugulovasines were studied on the medium containing the following (g/l distilled water): mannitol, 50.0; succinic acid, 5.4; $MgSO_4 \cdot 7H_2O$, 0.3; $KH_2PO_4$, 1.0; pH was adjusted to 5.4 with 25% $NH_4OH$ (the control medium). Water suspensions of the spores from 14-day cultures grown on malt extract agar were used as inocula.

The strain VKM FW-806 was additionally cultivated in three media: the control medium supplemented with 4.4 mg/l $ZnSO_4 \cdot 7\ H_2O$ (medium 1) or supplemented with yeast extract or peptone (0.5 g/l; Difco, United States) (media 2 and 3). To study possible effect of rugulovasines on differentiation of fungi, rugulovasines were added to medium 2 either at inoculation (3.3 mg/l) or on the 7th and 11th day of growth.

The strains were cultivated in 150 ml of the medium in 750-ml flasks at 24 ± 1°C on a shaker (220 rpm). To study production of rugulovasines, the cultures were sampled on the 7th and 14th day; to study the possible interrelation of biosynthesis of rugulovasines and conidiation, on the 5th, 6th, 8th, and 13th day. Intensity of conidiation was assessed daily [6]. Growth was evaluated as dry mycelium mass. Metabolites were isolated from the culture liquid filtrate by triple fold extraction with chloroform (vol/vol) according to the previously described procedure [3].

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The extracts were analyzed by TLC on silica gel plates (Silicagel 60 $F_{254}$, Merck, Germany) in the following systems of solvents: chloroform : methanol : 25% $NH_4OH$ 90 : 10 : 0.1 (system I), 90 : 10 : 1 (system II), and 80 : 20 : 0.2 (system III); chloroform : acetone : methanol 93 : 7: 5 (system IV). Rugulovasines were detected by UV-absorbance and after spraying the plates with the Erlich and Dragendorff reagents.

Preparative TLC on silica gel plates was used for further isolation and purification of rugulovasines. Metabolites were identified by co-chromatography in different systems with the standard samples isolated previously in the Laboratory of Secondary Metabolites, Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences using specific Erlich and Dragendorff reagents and also data obtained by other physical and chemical methods of analysis. Quantitative determination of rugulovasines in the samples was carried out by the technique described earlier [5].

UV spectra of compounds in methanol were obtained using a UV-160 spectrophotometer (Shimadzu, Japan).

## RESULTS AND DISCUSSION

The study of low-molecular nitrogen-containing secondary metabolites synthesized by *P. variabile* relict strains VKM FW-655, FW-806, FW-811, FW-816, and FW-818 isolated from permafrost revealed that they all produce an identical set of clavine alkaloids, rugulovasine A and its isomer rugulovasine B [5]. These compounds have been detected in some representatives of the genus *Penicillium* belonging to the two subgenera, *Penicillium* and *Biverticillium* [7, 8]. Rugulovasines were not been previously detected in *P. variabile* Sopp. Strains of this species isolated from modern habitats were earlier known as producers of mycotoxins of the polyketide nature: ochratoxin A and rugulosine [7, 8].

The physiological and biochemical characteristics of fungal strains, including the spectrum of secondary metabolites synthesized by the latter, are known to depend on the habitat from which a specific isolate was obtained. As all the new producers of rugulovasines were isolated from permafrost, it seemed important to determine to what extent the ability to synthesize these secondary metabolites occurs among *P. variabile* strains, especially those isolated from other habitats. Thirteen strains isolated from different habitats in various geographical zones were therefore screened (Table 1). As a result of the study of the spectra of metabolites synthesized by these cultures, it was established that five strains of *P. variabile*, VKM F-2075, FW-2528, FW-2531, FW-2701, and FW-2758, produced an identical set of clavine alkaloids, metabolites 1 and 2. Based on the physical and chemical properties of the obtained metabolites (Table 2), which were almost identical to those described in the literature and obtained from previously known samples [5], they were identified as rugulovasine A (metabolite 1) and rugulovasine B (metabolite 2). All five strains synthesizing rugulovasines were isolated from modern habitats. It is remarkable that both the geography of occurrence of these producers and their habitats were diverse (Table 1). Thus, strains FW-2528, FW-2531, and FW-2701 were recently isolated from extracts of biologically active compounds of natural origin that had arrived from South America. Strain F-2075 deposited in the VKM IBPM RAS for a long time was isolated from liquid fuel in Vietnam, and FW-2758 is a modern isolate from a beeswax phonographic roller which had been stored in the Literature Museum in Moscow for almost one hundred years. Other cultures tested in this work, including the isolates from Arctic and Antarctic permafrost, exhibited no biosynthesis of rugulovasines. Earlier, production of these alkaloids was first established in five *P. variabile* strains from Arctic permafrost [1]. It is remarkable that the level of accumulation of rugulovasines in the strains isolated from modern habitats was three- to sevenfold greater than that of the most active relict strain, *P. variabile* VKM FW-806 (0.6 mg/l) [5].

Thus, the capability for synthesis of rugulovasines is rather widely spread in the moulds fungi *P. variabile*. Since the geography of occurrence and sources of isolation of producer strains are rather varied, one may conclude that the presence or absence of producers of rugulovasines among the cultures of this species does not depend on the isolate's habitat.

The study of morphology of *P. variabile* observed in the process of submerged cultivation on a mineral medium revealed mycelium differentiation with formation of conidiophore structures and conidia in all the strains. The germination of secondary conidia and the development of secondary microcyclic conidiation were also observed in the cultures. By the time of formation of the latter, the strains can be divided in two groups (Table 1). The first group includes the cultures in which (as in the relict strain *P. variabile* VKM FW-806 [5]) conidia at different stages of germination were detected and penicilli with new conidia were formed on the primary hyphae from the fifth day to the end of cultivation. The second group includes the strains in which the germination of secondary conidia and microcyclic conidiation began only in the stationary growth phase. It is noteworthy that in all producing strains mycelium differentiation and microcyclic conidiation occurred in the early growth stages (Table 1).

Biosynthesis of secondary metabolites in certain microorganisms is associated with cell differentiation. Thus, in many fungi growth cessation and differentiation of mycelium is accompanied with biosynthesis of secondary metabolites (penicillins, cephalosporins, ergot alkaloids, and other compounds) [9]. It was shown for some producers that both sporogenesis and synthesis of secondary metabolites are induced by defi-

**Table 1.** Biosynthesis of rugulovasines and the time of formation of microcyclic conidiation (MCC) in fungi of the species *P. variabile* Sopp isolated from different habitats

| Strain VKM | Place of isolation | Source of isolation | Rugulovasines, mg/l | MCC, days |
|---|---|---|---|---|
| F-2075 | Vietnam | Liquid fuel | 3.9 | 5 days |
| FW-2528 | South America | Product of processing alkaloid-containing plant material | 4.1 | » |
| FW-2531 | South America | Alkaloid of plant origin (sample 7) | 1.6 | » |
| FW-2701 | South America | Alkaloid of plant origin (sample 47) | 3.7 | » |
| FW-2758 | Moscow, Literature Museum | A fragment of phonographic roller no. 6, light-brown beeswax | 3.0 | » |
| FW-899 | Arctic, Kolyma lowland, borehole 17/99 | Water of a cryopeg with admixture of upper soil layers, depth 17.0–17.3 m, 100–120 thousand years old | 0 | 11 days |
| FW-1449 | Arctic, Kolyma lowland, borehole 2/89 | Permafrost, depth 47.5 m, 1800–3000 thousand years old | 0 | » |
| FW-1451 | Arctic, Kolyma lowland, borehole 3/89 | Permafrost, depth 6.7–7.0 m, 5000–10000 years old | 0 | » |
| FW-1452 | Arctic, Kolyma lowland, borehole 5/90 | Permafrost, depth 1.5 m, 15–40 thousand years old | 0 | » |
| FW-2566 | South America | Alkaloid of plant origin (sample 24) | 0 | » |
| FW-2762 | Moscow, Literature Museum | A cardboard box of the phonographic roller no. 576 | 0 | » |
| FW-2884 | Antarctica, Taylor valley, borehole 2/95 | Permafrost, depth 1.84–1.88 m, 150 thousand years old | 0 | » |
| FW-2886 | Antarctica, Beacon valley, borehole 7/99 | Permafrost, depth 0.6 m, approx. 8000 years old | 0 | » |

ciency of an important nutrient [10]. A physiological correlation exists between biosynthesis of secondary metabolites and sporogenesis. Thus, some inhibitors of sporogenesis suppressed the synthesis of aflatoxins and conidiation in *Aspergfillus flavus*. Synthesis of verrucologen in *P. estinogenum* and of griseofulvin and penitrems in *P. nigricans* was stimulated by the addition of calcium chloride, a known inductor of conidiation in moulds, to the medium [9]. Certain secondary metabolites were proven to participate in the regulation and coordination of cytological differentiation as endogenous signal molecules (triggers) [9–11]. They include peptide antibiotics in bacilli and the A factor that controls morphogenesis and biosynthesis of streptomycin in *Streptomyces griseus* and *S. bikiniensis.* Despite the evident relationship between formation of secondary metabolites and spores, the production of secondary metabolites is not an obligatory condition of sporogenesis. Mutants were obtained that do not produce antibiotics, but retain the ability for sporulation, in particular the producers of bacitracyn (*Bacillus licheniformis*), streptomycin (*S. griseus*), etc. [11]. Furthermore, it was noted that the mutants selected by high productivity of the antibiotic were often incapable of sporulation. Probably, the formation of both secondary metabolites and spores by microorganisms is a consequence, not a cause of transition from vegetative growth to differentiation.

Earlier, in a study of growth and biosynthesis of rugulovasines during submerged cultivation of *P. variabile* VKM FW-806, the synchronicity was observed of the cycles of rugulovasines biosynthesis and conidiation, and a regulatory role of rugulovasines in culture morphogenesis was proposed [5]. However, early differentiation of mycelium and microcyclic conidiation in the tested *P. variabile* strain may also be due to additional requirements of the culture for some compounds, as it is believed that nutrient limitation controls fungal differentiation [9, 10].

The relationships of early mycelium differentiation and the additional requirements of *P. vatriabile* in nutrients were studied in three media. As control, a mineral medium with zinc ions was used, since it had been

shown earlier that the addition of zinc enhanced both the microcyclic conidiation and biosynthesis of rugulovasines [5]. The other two media differed from the control one by the presence of yeast extract or peptone (0.5 g/l), which are often included in the media for microbiological production of different biologically active compounds.

It was found that the addition of yeast extract or peptone in the cultivation medium of *P. variabile* resulted in stimulation of vegetative growth. The maximum level of biomass accumulation increased 3- and 1.6-fold, respectively, compared to the control medium (5.5 g/l), which is undoubtedly associated with an increase in the concentration of a nitrogen source in the media. Microscopy revealed significant differences in fungal development depending on the medium. In the medium with yeast extract, neither mycelium differentiation nor conidia formation occurred during the whole period of observation, while in the medium with peptone, as well as in the control medium, early hyphae differentiation, conidia formation, and several cycles of microcyclic conidiation were observed (figure). However, conidia formation decreased sixfold in the presence of peptone compared to the control. Thus, peptone in the medium failed to prevent mycelium differentiation of *P. variabile*, but decreased conidia formation. The results obtained show that in *P. variabile* early mycelium differentiation and microcyclic conidiation are caused by nutrient limitation alleviated by yeast extract. Probably, the *P. variabile* strains with early development of microcyclic conidiation have common features of metabolism, possibly including the absence of de novo biosynthesis of co-factors required for active vegetative growth.

No biosynthesis of rugulovasines was detected in strains grown in the media supplemented with yeast extract or peptone. Thus, a difference in the morphological and genetic development of the culture grown in these media and the absence of biosynthesis of rugulovasines clearly indicate the independence of these two processes.

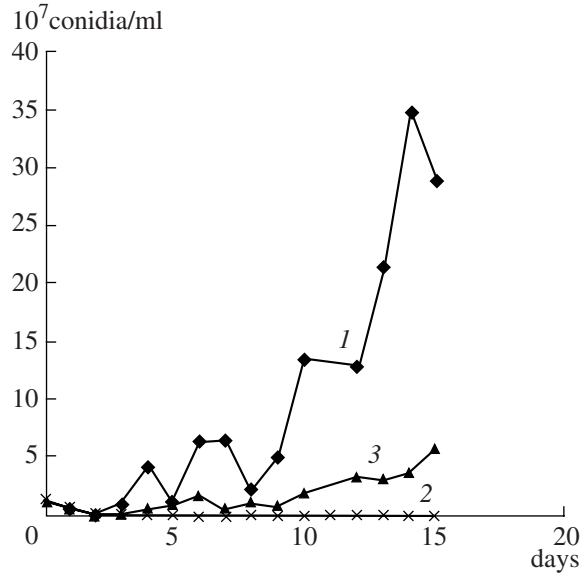
It is known that some secondary metabolites, inductors of sporogenesis, are able to cause conidiation at their addition to the cultivation medium [11]. The addition of a mixture of rugulovasines in the physiological concentration (3.3 mg/l) to the medium with yeast extract inoculated with *P. variabile* on the seventh and then on the eleventh day of growth did not lead to mycelium differentiation and conidiation. Thus, rugulovasines are not endogenous signal molecules (triggers) participating in the regulation and coordination of cytological differentiation in the fungus *P. variabile*.

To date, there are numerous studies devoted to the investigation of adaptive extracellular autoregulators at the genetic level [12]. Autoregulators that adapt microorganisms to "planned" stresses that occur in the course of ontogenesis are the most studied. These stresses include a stress of new habitat occurring at transfer of old or resting cells to a fresh medium favorable for growth and the stresses caused by exhaustion of nutrient sources (starvation stress) or space (critically high cell density) [12]. It is possible that the peculiarity of the fungal species *P. variabile* is the spontaneous removal of the stress pressure of the medium (starvation stress). This is indicated by mass germination of secondary conidia if the fungus cultivation continues.

Thus, the ability of fungi *P. variabile* to synthesize clavine alkaloids, rugulovasines, did not depend on their habitat. At submerged cultivation of producer strains in the mineral medium, microcyclic conidiation

**Table 2.** Physical and chemical properties of metabolites of *P. variabile* Sopp

| Property | Metabolite 1 | Metabolite 2 |
|---|---|---|
| Reaction with Erlich reagent | Violet | Violet |
| $R_f$ TLC (in the systems of solvents): | | |
| I | 0.17 | 0.27 |
| II | 0.14 | 0.25 |
| III | 0.55 | 0.63 |
| IV | 0.15 | 0.05 |
| UV spectrum, $\lambda_{max}$, nm | 224, 277, 286, 295 | 224, 277, 287, 295 |
| Major intensity peaks in the mass spectrum *m/z* (%) | $M^+$268, 225, 197, 171, 149, 130 | $M^+$268, 225, 197, 171, 149, 130 |




Conidiation at submerged cultivation of *P. variabile* FW-806 on the control medium (*1*), the medium with yeast extract (*2*), and with peptone (*3*).

was observed at the early growth stages; its presence or absence as well as the culture activity in relation to biosynthesis of rugulovasines are determined by the composition of the cultivation medium. On addition of peptone to the medium, conidiation took place, but was appreciably suppressed; as for the medium containing yeast extract, conidia were completely absent. In both media no detectable amounts of rugulovasines were detected.

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