ISSN 0026-2617, Microbiology, 2012, Vol. 81, No. 2, pp. 244–250. © Pleiades Publishing, Ltd., 2012.
Original Russian Text © A.A. Vasilyeva, L.N. Chekunova, E.N. Bilanenko, A.V. Kachalkin, A.V. Polyakova, 2012, published in Mikrobiologiya, 2012, Vol. 81, No. 2, pp. 266–272.

EXPERIMENTAL ARTICLES

Characterization of the Strain *Monascus floridanus* P. F. Cannon & E. L. Barnard, Isolated from Aviation Fuel

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Received April 4, 2011

Abstract—A fungus was isolated from aviation fuel and identified as *Monascus floridanus* P.F. Cannon & E.L. Barnard (FR827895) according to its morphological and genetic properties. The isolate has some properties that are unusual for the type strain, including a prominent stripe on one of the sides of the ascospores and occurrence, along with the known *Basipetospora*-type thallic conidia, of the *phialophora*-like spore formation. The isolated strain *Monascus floridanus,* like the known kerosene fungus *Hormoconis resinae* (Lindau) Arx & G.A. de Vries, is capable of active growth in aviation fuel.

Keywords: *Monascus floridanus*, *phialophora*-like conidiogenesis, microbiological degradation of aviation fuel, *Hormoconis resinae*.

DOI: 10.1134/S0026261712010158

It is well-known that members many bacterial and fungal species are often detected in aviation fuel, which can cause adverse effects on the aviation equipment. Mycelial fungi are especially hazardous. The acids and enzymes secreted by these fungi can cause corrosion of metal tanks; the rapidly growing biomass may clog valves and filters, resulting in severe accidents. *Hormoconis resinae* (Lindau) Arx & G.A. de Vries, known as the kerosene fungus, is the most active degrader of aviation fuel. However, due to adaptation, new species contaminating fuel may emerge, and the available methods for preventing biodeterioration may lose their efficiency. An investigation of micromycetes growing in aviation fuel is therefore of urgent interest.

In 2007, a dark-colored mycelial fungus, which according to its morphological and cultural properties was classified as a *Monascus* Tiegh species, was isolated as a result of microbiological analysis of the samples of contaminated aviation fuel collected in the filtration system of an airplane. Species of the genus *Monascus*, originally described in 1884, are distinguished by the presence of fruiting bodies, cleistothecia, formed at the ends of specialized hyphae branching from the vegetative mycelium. The cell walls of the cleistothecia consist of two different layers. The internal one is formed by the enlargement of the apical regions of the hyphae, whereas the outer layer consists of interwoven hyphae that form the "sheath" of the fruiting body. Asci in the fruiting bodies are arranged at random and are degraded at early stages of spore formation; ascospores in the asci are hyaline, unicellular, and ellipsoid. A characteristic trait of *Monascus* species is the anamorphous *Basipetospora* stage. The taxonomic position of the genus *Monascus* is constantly under revision; it was recently reclassified into the family *Monascaceae Schroter*, class *Eurotiomycetes* (*Monascaceae, Incertae sedis, Eurotiomycetidae, Eurotiomycetes*, and *Ascomycota*) (http://www.indexfungorum.org/). Species of the genus *Monascus* are often detected in various starch-rich foodstuffs, such as potatoes, rice, and oats, as well as on moldy fruits, feed, silage, and soils. Some representatives of the genus *Monascus* are of great economic importance, since they participate in the fermentation of some foodstuffs and produce antibacterial agents and pigments for food dyes [1–4].

The goal of this work was genetic identification of the strain isolated from the samples of aviation fuel, as well as analysis of its morphological and physiological properties, including its ability to grow due to utilization of the hydrocarbons of aviation fuel.

MATERIALS AND METHODS

Samples of contaminated aviation fuel from which *M. floridanus* was isolated were collected from the filtration system of an airplane making its scheduled flights to China. The samples were dark-colored slimy clots between water–fuel layers. The fungus was isolated on the Czapek medium and malt agar by direct plating and by inoculating serial dilutions [5].

To study the morphological properties of the isolate, the Czapek nutrient medium and malt agar were used; the fungus was grown at 25°C. For microscopic

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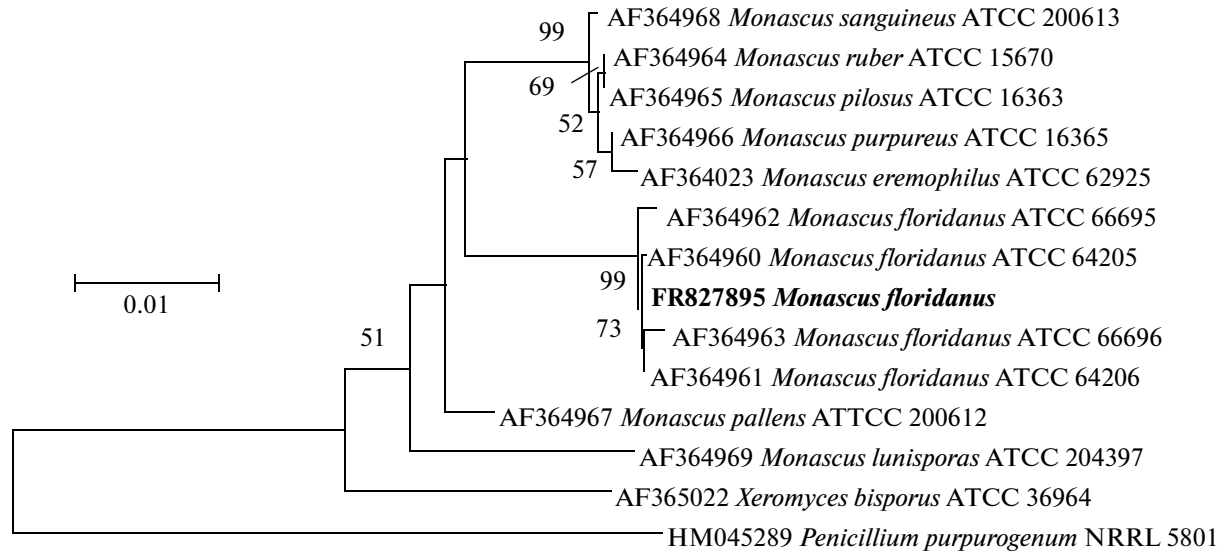

Fig. 1. Phylogenetic position of the isolated *M. floridanus* strain (FR827895) determined using the neighbor-joining method [16]. The numerals at the branching points show the significance of the branching order as determined by bootstrap analysis of 500 alternative trees (only bootstrap values above 50% were considered significant). The bar shows the number of substitutions per each nucleotide. The aligned sequences consisted of 530 bp of the D1/D2 domain of the large subunit (LSU) rDNA. The sequence of *Penicillium purpurogenum* NRRL 5801 was used as the outgroup.

examinations, an Axioskop 40 FL light microscope and a JSM scanning electron microscope were used. The preparations of fungal mycelium for scanning electron microscopy were fixed with glutaraldehyde (2.5%) for 1 h, washed three times with phosphate buffer, dehydrated in ascending concentrations of ethanol (30, 50, 70, 80, 96%) and then in acetone, dried, sputter coated with platinum/palladium alloy, and scanned.

Genetic identification of the isolated fungus was performed according to the results of sequencing of the D1/D2 domain of large subunit (LSU) rDNA. For extraction of total DNA, the mycelium of the 6-day culture was disrupted with glass beads (300–500 μm) and incubated at 65°C for 1 h in lysing buffer (pH 8), containing the following (mM): Tris Base, 50 mM; NaCl, 250; EDTA, 50; and SDS, 0.3%. Amplification of the rDNA domain was carried out with the primers ITS1f (5'-CTTGGTCATTTAGAGGAAGTA) and NL4 (5'-GGTCCGTGTTTCAAGACGG), as well as with the ScreenMix PCR mixture (ZAO Evrogen, Moscow). The PCR product was purified using the BigDye XTerminator Purification Kit (Applied Biosystems, United States). Sequencing of the PCR product was carried out with the primer NL4. Sequencing of the rDNA fragment was performed using a BigDye XTerminator Purification Kit (Applied Biosystems, United States). The reaction products were analyzed on an Applied Biosystems 3130xl Genetic Analyzer by Syntol Co. (Russia). Phylogenetic analysis of the results obtained was carried out using the MAFFT 6 [6] and MEGA4 [7] software packages. The phylogenetic tree was constructed on the basis of the data published in [8]. The obtained nucleotide sequence of the studied strain was deposited in the GenBank database of the National Center for Biotechnology Information (NCBI) under the accession number FR827895. The isolated strain *M. floridanus* was deposited with the All-Russian Collection of Microorganisms (VKM).

To study the ability of strain *M. floridanus* to grow in aviation fuel, tubes with 3 mL of sterile fuel and 3 mL of mineral medium (agar- and sucrose-free Czapek medium) were supplemented with 0.3 mL of the spore suspension and incubated in a thermostat at 28°C for 1 month. Growth was assessed visually.

RESULTS AND DISCUSSION

Initially, investigation into the morphology of the strain isolated from aviation fuel demonstrated that it belonged to the genus *Monascus*, according to the formation of cleistothecia and the *Basipetospora* anamorph typical of the members of this genus. However, its characteristics did not conform to the description of the known species of the genus *Monascus*. Genetic identification of the strain was performed according to the results of sequencing of the D1/D2 domain of large subunit (LSU) rDNA. Phylogenetic analysis showed that the isolated strain belonged to the species *Monascus floridanus* (Fig. 1). The studied strain was conspecific with the type strain *M. floridanus* ATCC 64205 and strain *M. floridanus* ATCC 64206; it differed by 1 bp from *M. floridanus* ATCC 66695 and *M. floridanus* ATCC 66696.

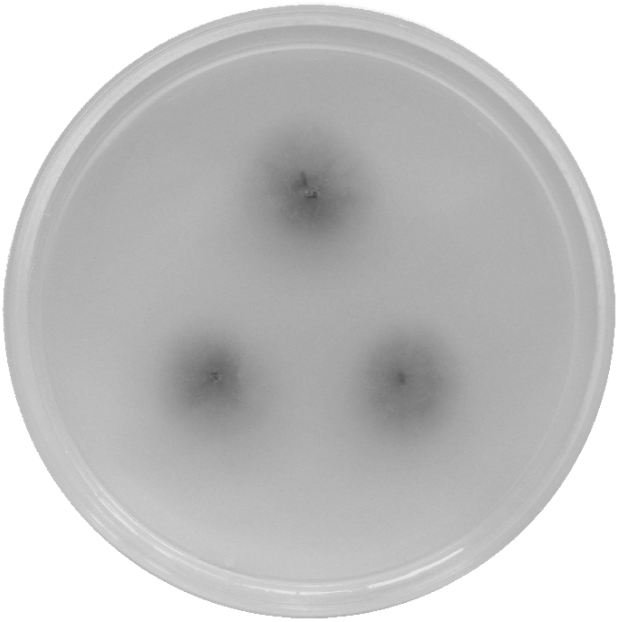

Fig. 2. View of the *Monascus floridanus* colonies grown on the Czapek medium (10 days, 25°C).

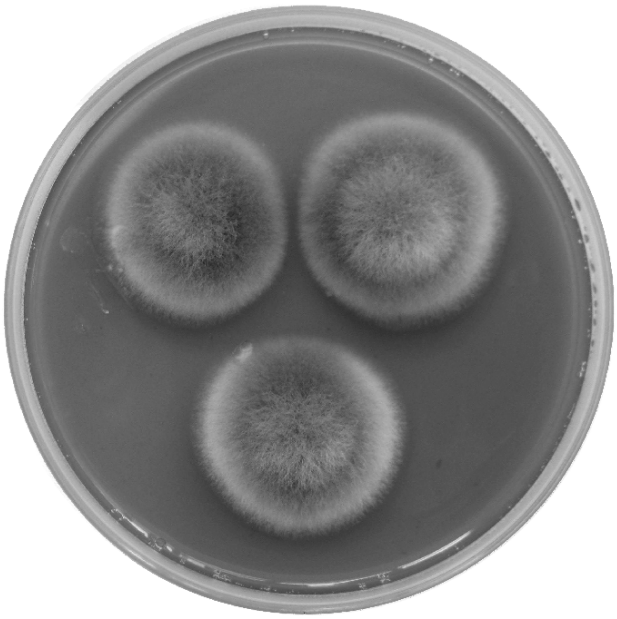

Fig. 3. View of the *Monascus floridanus* colonies grown on wort agar (10 days, 25°C).

The type strain of *M. floridanus*, described in 1987, was isolated in Florida, United States from tarred sites on the trunks and roots of pine trees (*Pinus clausa* (Charm.) Vasey, *P. elliotii* Engelm, and *P. palustris* Mill.) degraded by microorganisms. *M. floridanus* differs from other representatives of this genus by the presence of dark-colored cleistothecia and by the size of its ascospores (3.5–4.5 × 2–3 μm) [1, 8], which is typical of the studied strain as well. However, further studies revealed some interesting aspects of the morphology of the studied *M. floridanus* strain.

The appearance of its colonies mostly correlated with the description of the type strain of *M. floridanus* [1]. Growth of the studied *M. floridanus* strain on the Czapek medium was limited; the strain formed colonies 14–16 mm in diameter by day 7 of incubation at 25°C (Fig. 2). The colonies were flat and colorless at the beginning of growth and then turned brownish, mostly in the center; the colony edges remained colorless. The reverse side of the colonies was colorless; exudates were not detected. The fungus exhibited more intense growth on malt agar; the colonies were 18–20 mm in diameter by day 7 of incubation at 25°C (Fig. 3). The colonies were velvety or slightly fluffy and elevated, especially in the center. The strain produced olive-green colonies with brownish centers and white aerial mycelium along the periphery and in the centers; sometimes, weak zonality is observed. With age, the colonies darkened and then turned deep-green. The reverse side of the colonies was dark green or almost black. The centers of the colonies at the reverse site were always light-colored; exudates were not detected.

The fruiting bodies, cleistothecia, in the *M. floridanus* cultures appeared quite early—during 9–11 days of incubation (Fig. 4d)—but matured much later. Cleistothecia were spherical; mature cleistothecia were 25–60 μm in diameter (Figs. 4a–4c). The peridium was brownish-green; the outer sheath consisted of interwoven hyphae, which is typical of species of the genus *Monascus*. The asci were degraded at early stages of spore formation; the ascospores were released from the cleistothecia through disrupted sheaths (Fig. 4b). The ascospores (3.2–3.5 × 2.1–2.4 μm) were unicellular, ellipsoid, hyaline, and smooth (Figs. 4e–4g). Another interesting peculiarity of the ascospores was a prominent stripe (0.4 μm high) running on one of their ventral sides of the ascospore from one pole to another. Another ventral side did not have a stripe and was completely smooth. The description of the sexual stage coincides with that of the type strain of *M. floridanus* [1], except for the presence of the stripe.

In the *M. floridanus* culture, chlamydospores, intercalary and apical, were detected. Unlike single *Basipetospora* conidia, they were large (about 8 μm in length) and surrounded by thick dark capsules.

The *Basipetospora* anamorph of a representative of the genus *Monascus* was described [1, 8] (Fig. 5). Various authors described *Basipetospora* conidia as arthroconidia [9–11], meristem arthroconidia [12], or aleurioconidia [13]. All these types of conidia are produced by thallic conidiogenesis, when a hypha is fragmented into conidia after transverse septation.

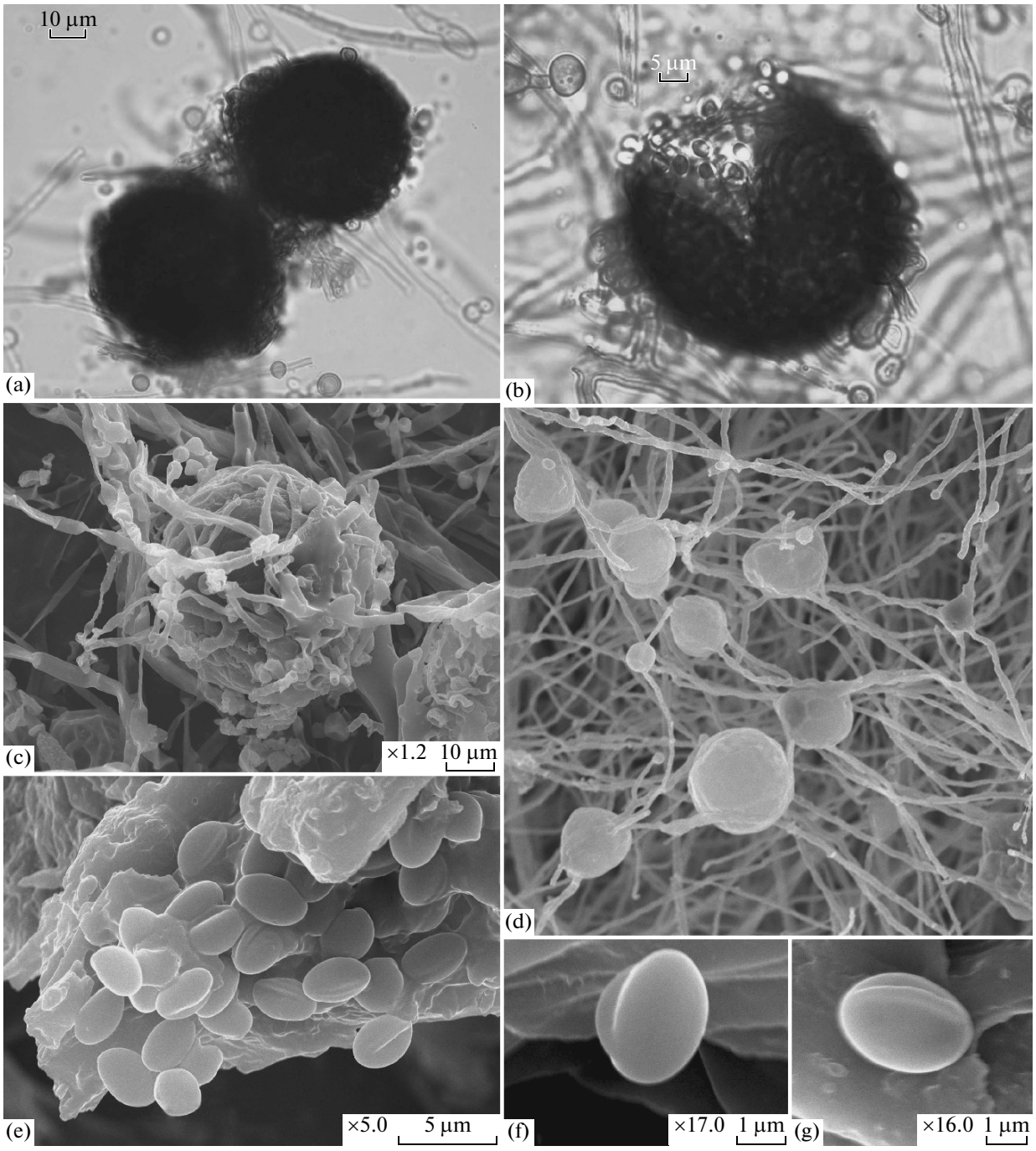

Fig. 4. Sexual stage of *Monascus floridanus*: mature cleistothecia and release of ascospores (light microscopy) (a), (b); mature and young cleistothecia (scanning microscopy) (c), (d); and ascospores (scanning microscopy) (e)–(g).

Conidia-bearing hyphae are simple and similar to the vegetative hyphae; they become shorter as conidia separate from the hypha [2, 9]. Conidia are apical, single, or arranged in chains that can easily split into separate spores. Conidia are unicellular, rounded, truncated, or sometimes pear-shaped. The size of *M. floridanus* conidia ranges between 3.5 and 5.5 μm in diameter; they are usually smooth, but rough forms were detected as well.

The isolated *M. floridanus* strain actively began to produce spores at the first days of growth on the Czapek nutrient medium and malt agar. However, aside from the *Basipetospora*-type spore formation, another type of spore formation was detected. Despite the fact

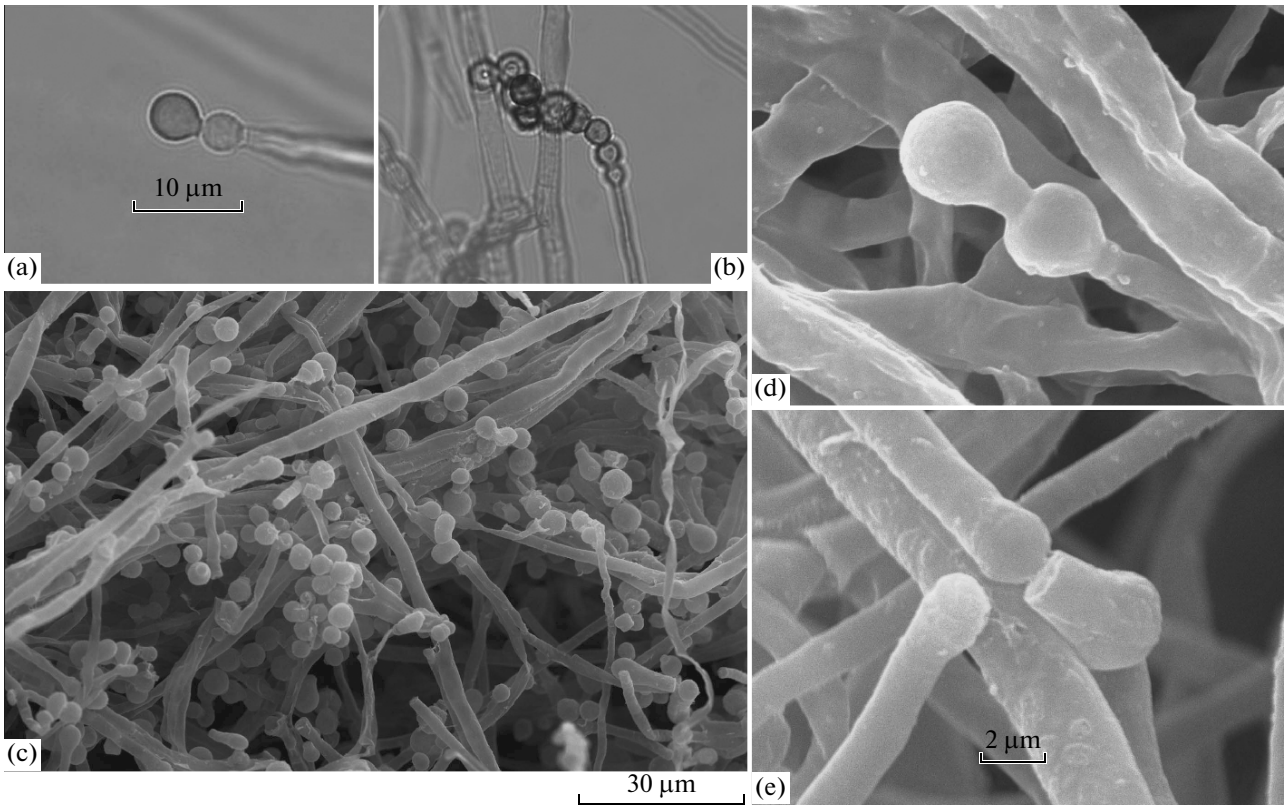

Fig. 5. Conidial *Basipetospora*-type spore formation of *Monascus floridanus*: formation of conidia (light microscopy) (a), (b); formation of spores and conidia (scanning microscopy) (c)–(e).

that the resultant conidia were similar to the above-described ones, the type of conidiogenesis was considerably different. Blastic conidiogenesis was observed. Conidia were formed in specialized cells, phialides, with collarettes at the tips (Fig. 6). This type of spore formation is typical of species of the genus *Phialophora*, which is why it is called *phialophora*-like spore formation [14]. In addition, *phialophora*-like conidiogenesis also occurs in the members of the genus *Cadophora* and other fungal species belonging to at least three orders of ascomycetes [15].

The phialides of *M. floridanus* were formed at the ends of branching vegetative hyphae and grew singly or in bunches. The shapes and sizes of the phialides varied. Elongated, cylindrical phialides, 10–18 μm in length and 1.0–2.5 μm in width, were most often detected. Shorter bottle-shaped phialides, 5.5–10 μm in length and 1.0–3.0 μm in width, tapered at the tip where conidia were formed, were less common. At their apices, all phialides had pronounced, short, cup-shaped collarettes, 0.75–1.0 × 2.0–3.5 μm. Conidia were rounded, 3.5–4.5 μm in diameter, truncated, similar to *Basipetospora* conidia, with a varying degree of roughness, and were arranged in chains that easily split into separate spores. The first conidium was usually smooth and more truncated; the subsequent conidia were rough, with their roughness increasing as the chain grew longer (Fig. 6e). *Phialophora*-like spore formation was first detected in a species of the genus *Monascus*.

The studied *M. floridanus* strain prevailed among the micromycetes isolated from contaminated aviation fuel. Apart from it, the kerosene fungus *Hormoconis resinae* was isolated from the sample, although this species was much rarer in the studied samples. Reinoculation of sterile fuel with the suspension of *M. floridanus* spores demonstrated that the studied strain was capable of active growth utilizing the hydrocarbons of aviation fuel as carbon sources. This was confirmed by the formation of mycelial clots in the test tubes during 7–10 days of incubation. A similar pattern was observed in the case of *H. resinae*, which, until recently, was considered the most active biodegrader of aviation fuel. Data on the ability of *Monascus* species to grow on oil products are lacking in literature; however, we confirmed the capacity of the isolated *M. floridanus* strain for growth in aviation fuel. This strain is of considerable scientific interest and can be used (along with *H. resinae*) in experiments aimed

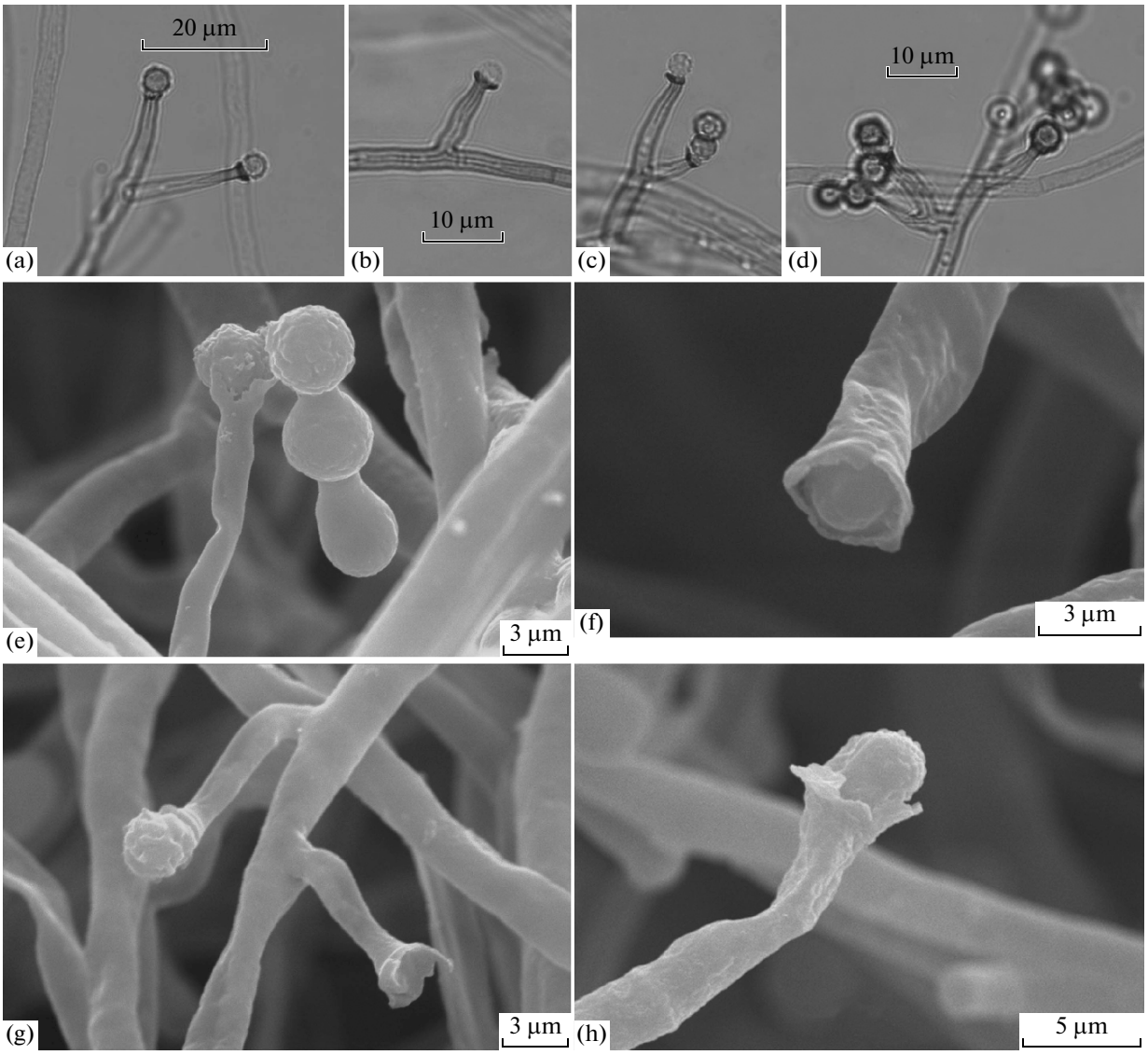

Fig. 6. *Phialophora*-like conidiogenesis of *Monascus floridanus*: formation of conidia on phialides (light microscopy) (a)–(d); formation of conidia on phialides (scanning microscopy) (e)–(h).

at elucidation of the fungal resistance of different fuels and additives.

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