ISSN 0026-2617, Microbiology, 2010, Vol. 79, No. 6, pp. 753–758. © Pleiades Publishing, Ltd., 2010.
Original Russian Text © O.E. Marfenina, G.M. Fomicheva, O.V. Vasilenko, E.M. Naumova, A.B. Kul'ko, 2010, published in Mikrobiologiya, 2010, Vol. 79, No. 6, pp. 767–773.

EXPERIMENTAL ARTICLES

Sporulation in Saprotrophic and Clinical Strains of *Aspergillus sydowii* (Bain. & Sart.) Thom & Church under Various Environmental Conditions

O. E. Marfenina[a, 1], G. M. Fomicheva[a], O. V. Vasilenko[b], E. M. Naumova[a], and A. B. Kul'ko[c]

[a] *Department of Soil Science, Moscow State University, Moscow, Russia*

b *Institute of Medico Biological Problems, Russian Academy of Sciences, Moscow, Russia*

c *Moscow Scientific and Practical Tuberculosis Center, Moscow Health Department, Moscow, Russia*

Received December 22, 2009

Abstract—Molecular genetic characteristics and capacity for sporulation under different levels of temperature and humidity were compared for three saprotrophic and four clinical strains of *A. sydowii*. Analysis of the ITS and D1/D2 loci of these *A. sydowii* strains revealed two clades, each including both the clinical and saprotrophic strains. The differences in sporulation in the saprotrophic and clinical strains of the potentially pathogenic microscopic fungus *A. sydowii* under different environmental conditions were demonstrated. In the clinical *A. sydowii* strains, the level of spore formation was generally higher, especially at humidity levels of 0.90 and 0.95 a_w and 20–25°C. The level of spore formation for the clinical strains inoculated into sterile soil was several times higher than for the saprotrophic ones. On the contrary, nonsterile soils (sod–podzolic and urban soils) exhibited a fungistatic effect against *A. sydowii* populations.

Keywords: opportunistic microscopic fungi, spore formation, saprotrophic and clinical strains, environmental conditions.

DOI: 10.1134/S0026261710060056

A group of micromycetes capable of causing infections in humans has attracted special attention in the last decade. These conditionally pathogenic (opportunistic) fungi usually develop in nature as saprotrophs and may cause diseases in human under certain conditions (usually in the case on immunodeficiency disorders). Members of the genus *Aspergillus* are among the most dangerous opportunistic mycelial fungi, especially *A. fumigatus, A. flavus, A. niger*, and *A. terreus* [1], which are practically always present in the environment. Members of the *Aspergillus versicolor* section, which includes two species, *A. versicolor* and *A. sydowii*, are also mentioned among the opportunistic fungi isolated from humans. *A. sydowii* is widespread in the environment, especially in soil; has been isolated from air and fresh and marine water [2–5]; often occurs in the dust of living areas [2, 6]; etc. Aspergillosis caused by *A. sydowii* has been reported for invertebrates (corals), vertebrates (birds), and humans [1, 3, 7]. Recently, in Russia it was isolated from clinical material in the course of diagnostics of deep mycoses [8, 9].

Infection with opportunistic fungi is known to occur most often by inhaling the fungal spores [10]. The higher the spore concentration in the environment, the higher the risk of infection [11]. However, environmental conditions favoring sporulation in opportunistic fungi are presently poorly studied. No data exist on the possibility for growth and sporulation of the clinical strains of these fungi in soils, the major natural reservoir for opportunistic fungi.

The goal of the present work was comparative investigation of molecular genetic characteristics and sporulation patterns in clinical and saprotrophic *A. sydowii* strains under different conditions of temperature and humidity on nutrient media and in soils.

MATERIALS AND METHODS

The saprotrophic and clinical strains of *Aspergillus sydowii* (Bain. & Sart.) Thom & Church isolated from various environments are listed in the table. Preliminary identification of the strains was carried out using the relevant manuals [12, 13].

To characterize some molecular genetic properties of the strains, the ITS1, ITS2, and 5.8S loci, as well as the D1/D2 regions of the 28S rDNA, were analyzed as described previously [14]. Analysis of the sequences and assembly of the common contigs were carried out using the Chromas 2.3 software package. Cladistic analysis was carried out by the neighbor-joining method and the confidence parameters were determined by bootstrap analysis for 1000 repeats using the

[1] Corresponding author; e-mail: marfenina@mail.ru

A. sydowii strains used in the present work

Strain	Designation	Source of isolation	GenBank accession nos.
KBP-FC014	C14	Clinical isolate from the bronchopulmonary lavage fluid, Moscow Hematological Scientific Center, Russian Academy of Medical Sciences	AJ937748
KBP-FC095	C95	Clinical isolate from the bronchopulmonary lavage fluid, Moscow Scientific and Practical Tuberculosis Center	AM883157
KBP-FC096	C96	Clinical isolate from the bronchopulmonary lavage fluid, Moscow Scientific and Practical Tuberculosis Center	AM883158
NRRL 254 (Type strain)	C93	Clinical isolate, Waycross, Georgia, United States	AM883159
VKM F-441	E84	Sod–podzolic soil, Moscow oblast	AM883160
VKM F-968	E85	Seawater, Black Sea, Russia	AM883162
VKM F-2488	E86	Ice, Antarctica	AM883163

Designations: VKM, All-Russian Collection of Microorganisms, Moscow; KBP, collection of Department of Soil Biology, Faculty of Soil Science, Moscow State University; NRRL, National Center for Agricultural Research, Agricultural Research Service for Culture Collection, Peoria, IL, United States.

ClustalX 1.83 software package. The TreeView 1.6.6 software package was used to visualize the cladograms. The rDNA sequence of *Emericella nidulans* (strain NHRC-FE064 (E64), accession no. AJ937756) was used as an outgroup. Searching for and ranging of the homologous nucleotide sequences in the international databases (GenBank/EMBL/DDJB, 2009) were carried out using the BLAST software package (NCBI, 2009).

To investigate spore formation by *A. sydowii* strains under different cultivation conditions, 1 ml of spore suspension (10^6 spores/ml) was plated in three repeats on solid Czapek medium in flat-bottom flasks. After 14 days of incubation under specified conditions, the spores were washed off with 50 ml of 0.05% Tween 80. The spores were enumerated in a Goryaev chamber. For this purpose, three aliquots were taken from each flask and the spores were counted three times in each of them under a Carl Zeiss AxioSkop (~800).

The sporulation levels for *A. sydowii* strains were determined at the temperatures of 15, 20, 25, and 30°C and humidity of 0.99, 0.95, 0.9, 0.85, and 0.8 a_w, corresponding to –1.4, –7.0, –14.4, –22.3, and –30.6 MPa, respectively. The specified levels of water activity were achieved by addition of glycerol to the Czapek medium [15]. The intensity of sporulation was calculated from the number of newly formed spores per mother spore.

Development and spore formation of *A. sydowii* in soils were determined in sterile and nonsterile soils (urban soil and sod–podzolic soil) inoculated with spore suspensions (10^6 spores/ml per 1 g soil) of the saprotrophic strain (isolated from sod–podzolic soil) and the clinical strain (type strain NRRL 254). Urban soil (Moscow, Tushino raion) differed from the sod–podzolic soil (Moscow oblast) in its more neutral pH and higher phosphorus content [16]. Soil (20 g) was placed in petri dishes and sterilized by autoclaving at 2 atm for 30 min. Sterile and nonsterile soil samples were moistened to 60% water capacity simultaneously with the introduction of the spore suspension (10^6 spores/ml per 1 g soil). To maintain constant humidity, the dishes with soil were incubated in desiccators with 0.1M K_2SO_4 solution, which provided for 98% air humidity. The desiccators were incubated at 25°C.

The numbers of *A. sydowii* in soils were determined by plating soil suspensions on Czapek medium and by fluorescence microscopy (calcofluor staining) [17]. The samples were analyzed after the time intervals of 0, 7, 14, and 21 days.

Statistical treatment of the data was carried out using the Microsoft Excel 2002 (Microsoft Office XP) and Statistica 6.0 (StatSoft, Inc, 1984–2001) software packages.

The work with clinical strains and molecular genetic analysis were carried out in the Hematological Scientific Center, Russian Academy of Medical Sciences.

RESULTS AND DISCUSSION

Molecular characteristics of the strains confirmed their classification as *A. sydowii*. However, the differences between *A. sydowii* strains were revealed both in the relatively variable rDNA sites (ITS) and in more conservative ones (D1/D2 28S). Comparison of the ITS1-5.8S–ITS2 nucleotide sequences demonstrated that the strains belonged to two groups (Fig. 1). The sequences of these loci in the saprotrophic strains E85 and E76 and the clinical strain C96 were identical. The ITS sequences of the clinical strains C14, C95, and C93, as well as of the strain E84 from sod–podzolic soil, were identical and differed by three nucleotides from the sequences of the first group. The similarity between these groups was 99.4%. The ITS sequences

of the strains belonging to the first group were completely identical to most of the sequences for this species represented in the international databases. According to the data in the GenBank/EMBL/DDJB database, the isolates from mangrove soil, insect guts and excrements, and other habitats belong to the same group as the investigated strains. The ITS nucleotide sequences of strains C14, E84, C95, and C93 form a sister clade with the single identical sequence from GenBank.

Sequence comparison of the D1/D2 sites of the big (28S) subunit rDNA also revealed two groups among the investigated strains. The strains formed the same groups as in the case of ITS sites, with the difference of 1 nucleotide between the clades. All D1/D2 sequences for *A. sydowii* represented in GenBank also belong to either of these two clades.

Thus, analysis of the ITS and D1/D2 rDNA loci revealed two clades among the investigated *A. sydowii* strains. Importantly, the clinical and saprotrophic strains did not form separate clades. Both saprotrophic and clinical strains were present in both clades.

However, we have previously established some differences in the ecophysiological characteristics of *A. sydowii* saprotrophic and clinical strains (rates of colony growth at different temperature and humidity values) [18]. In the present work, a number of differences in the reproductive capacity of these groups were also revealed.

In order to determine the conditions favorable for spore formation by the clinical and saprotrophic *A. sydowii* strains, intensity of sporulation at different values of temperature and humidity was tested (Fig. 2). For all *A. sydowii* strains, the highest levels of spore formation occurred at 20–30°C and water activity of 0.99–0.90 a_w. The highest level of spore formation was 100–420 newly formed spores per mother spore.

At humidity levels 0.95 and 0.90 a_w, most of the clinical strains exhibited significantly higher spore formation than the saprotrophic strains. It was especially pronounced at the temperatures of 20 and 25°C. At these values of temperature and humidity, the intensity of spore formation by saprotrophic strains was reliably lower. The highest level of sporulation in the saprotrophic strains was only 100–150 newly formed spores per mother spore, while for the clinical strains it was several times higher. The highest reproduction level throughout almost all the range of conditions was found in the clinical strain C14. Even at high humidity (0.99 a_w), the intensity of its spore formation was higher than in the saprotrophic strains.

The ecological conditions for active spore formation in *A. sydowii* differ from the conditions favorable for colony growth. Active mycelial growth of this species occurred at higher temperatures than spore formation. At low humidity (0.80–0.95 a_w), the clinical strains had higher growth rates at 25–35°C. For the saprotrophic strains, the highest rates of colony growth under low humidity occurred only at 30°C [18, 19].

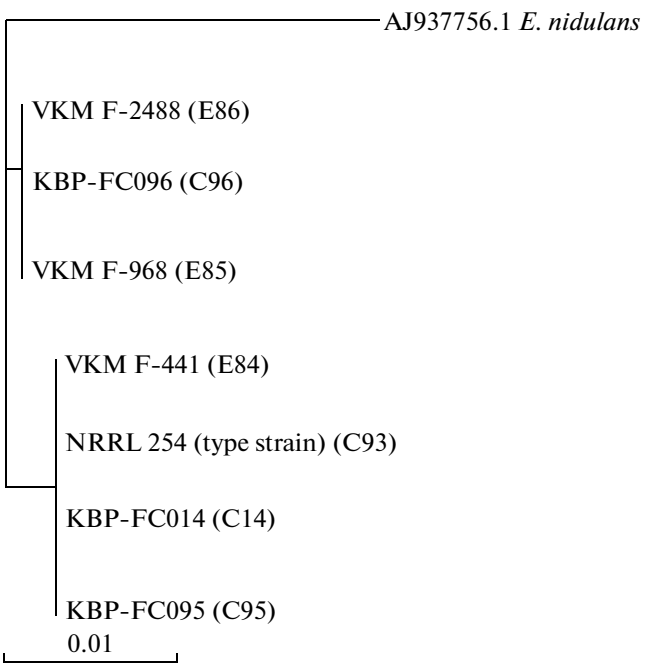

Fig. 1. Phylogenetic tree constructed based on ITS1-5.8S-ITS2 and D1/D2 28S rDNA sequences of *A. sydowii* strains. Scale bar corresponds to 10 nucleotide replacements per 1000 nucleotides.

In order to determine the degree of preservation of capacity for development and sporulation in the clinical strains in the environment, especially soils (the major natural reservoir of microscopic fungi), primary investigation was carried out with some clinical and saprotrophic strains.

For *A. sydowii*, we demonstrated development under soil conditions for the strains isolated as clinical ones. Some differences were revealed in the structure of fungal biomass formed by the populations of the clinical and saprotrophic strains. Two clinical strains inoculated into sterile urban soil produced spores at levels exceeding that of the saprotrophic strain. The content of the spores of the saprotrophic strain was almost stable and insignificantly increase by the end of the experiment. However, active mycelial growth was observed for this strain, while the clinical strains formed practically no new mycelium (Fig. 3).

Fungal spores are considered the primary source for colony development on solid media. More active sporulation in sterile soil revealed for the clinical strain was confirmed by plating the soil suspensions on a nutrient medium. For the clinical strain, the number of CFU in sterile urban soil was twice the level for the saprotrophic strain, especially during the first two weeks of the experiment. The level of stable abundance ($19.2 \times 10^5 \pm 2 \times 10^5$ CFU/g soil) is relatively high and may indicate the ability of the clinical strain (C93) to

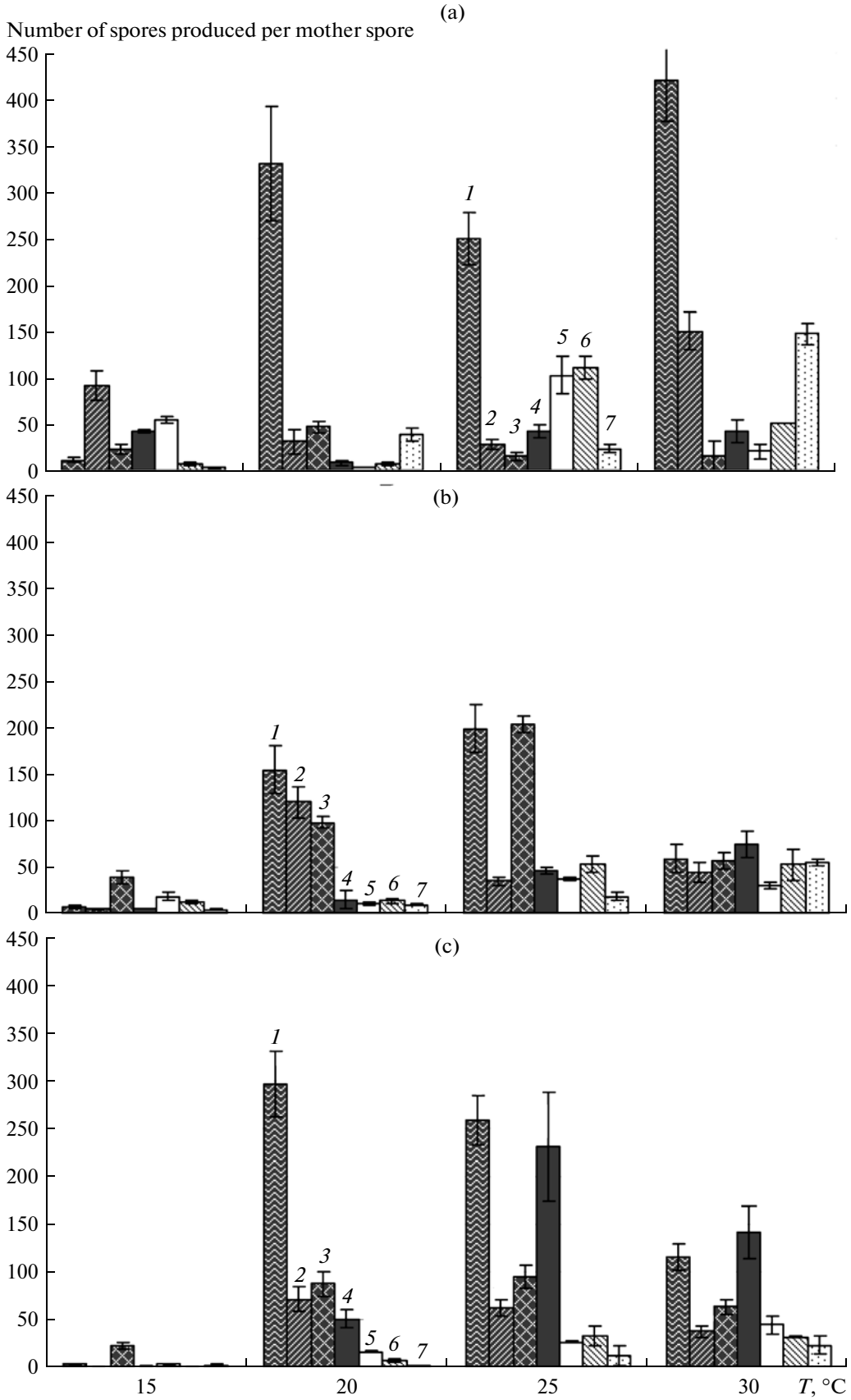

Fig. 2. Sporulation intensity in the saprotrophic and clinical *A. sydowii* strains at different values of temperature and water activity. Water activity: $a_w = 0.99$ (a), $a_w = 0.95$ (b), $a_w = 0.90$ (c). The strains presented in the table: C14 (*1*), C95 (*2*), C96 (*3*), C93 (*4*), E84 (*5*), E85 (*6*), and E87 (*7*).

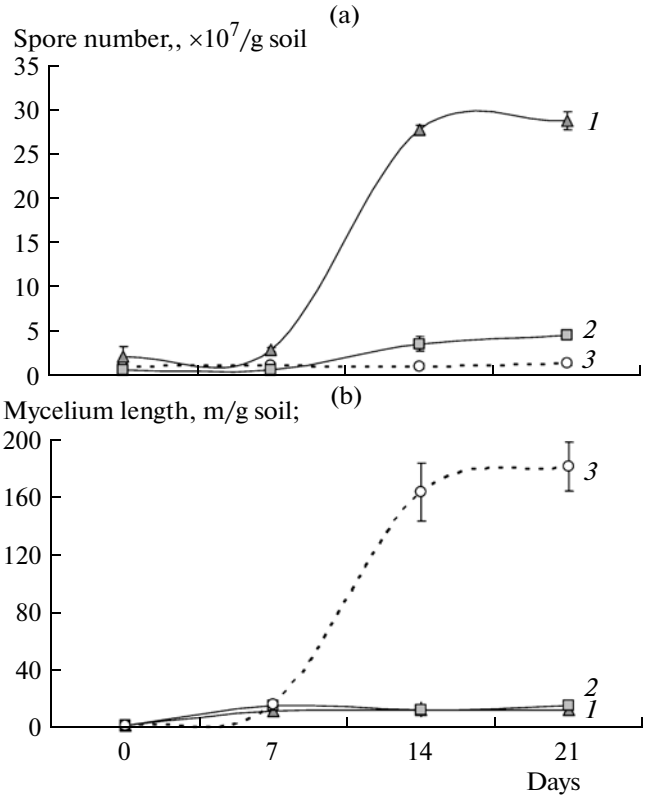

Fig. 3. Number of spores (a) and mycelium length (b) in clinical and saprotrophic *A. sydowii* strains developing in sterile urban soil (fluorescence microscopy). Strain C95 (*1*), strain C93 (*2*), and strain E84 (*3*).

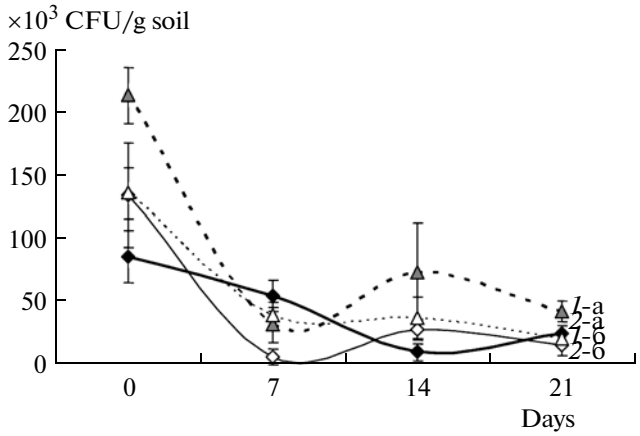

Fig. 4. Dynamics of abundance of the clinical (C93) and saprotrophic (E84) *A. sydowii* strains developing in non-sterile soils (plating on nutrient media). Strain E84 (*1*), strain C93 (*2*), urban soil (a), and sod-podzolic soil (b).

grow actively in sterile soils. For the sod–podzolic soil, the population dynamics for the saprotrophic and clinical isolates was similar to that revealed in urban soil.

In nonsterile soils, however, development of the populations of the clinical and saprotrophic *A. sydowii* strains was inhibited. This occurred both in sod–podzolic and urban soil, and the inhibitory effect was more pronounced for the clinical strain (Fig. 4). Soil microorganisms may therefore play an important part in the regulation of potentially harmful fungi in soils.

Thus, the existence and nature of the differences between environmental and clinical strains of microscopic fungi have not been conclusively established. The main effort has been focused on the possible difference in genetic characteristics of the saprotrophic and clinical strains of conditionally pathogenic fungi. Within the range of genetic characteristics investigated in the present work, the clinical strains (agents of mycoses in humans) had no common properties differentiating them from the saprotrophic strains. Comparison of the nucleotide sequences of D1/D2 and ITS1-5.8S-ITS2 loci revealed that the clinical strains were distributed into two groups together with saprotrophs. These data agree with the results of a similar study for *A. fumigatus* strains [20], which also did not reveal any isolated groups of clinical or saprotrophic strains.

We found, however, that the clinical *A. sydowii* isolates differed from the saprotrophic ones in a number of ecophysiological characteristics. The clinical strains were more resistant to unfavorable humidity conditions than the saprotrophs; we have previously reported their active growth within broader temperature intervals at decreased humidity [18]. The present work demonstrated higher levels of spore formation, compared to the saprotrophic strains, in the clinical strains within a certain range of temperature and humidity. Ecologically speaking, higher resistance to unfavorable environmental factors, together with enhanced capacity for reproduction, indicated an active strategy aimed at population survival.

Among the developmental stages of microscopic fungi (spore germination, mycelial growth, and spore formation), the patterns of sporulation are least known. The level of spore formation in microscopic fungi is considered to be determined by the species genotype and certain environmental factors (temperature, illumination, and nutrient content). Among the environmental factors regulating spore formation, the effect of light is best studied [21]. The possible reasons for the higher reproductive potential in the clinical strains under investigation are not completely clear. It should be noted, however, that the most intensely sporulating strain C14 (initially identified as *A. versicolor*) was isolated from a patient with system mycosis [8].

The strains developing in a human organism will probably acquire new phenotypic characteristics in the course of growth within a macroorganism, so that they may differ from the members of the same species inhabiting the environment. For example, transfer of *Fusarium solani* soil strains in mice was found to increase the pathogenicity of the strains [11]. Apart

from the differences in virulence, the groups of saprotrophic and clinical strains are known to differ in the rates of production of certain enzymes [22]. The differences in sporulation level may result from the so-called microcyclic sporulation in the clinical strains, when the spores germinate directly in a conidiophore with conidia, without the stage of developed mycelium [21]. This suggestion, however, requires further investigation.

Generally, capacity of the clinical *Aspergillus* strains to produce spores under the temperature and humidity common for environmental conditions may be important for assessment of accumulation and distribution of potentially pathogenic species in the environment. Moreover, we demonstrated for the first time that the clinical strains of an opportunistic *Aspergillus* fungus may develop and sporulate in soils. The ecological conditions stimulating and inhibiting their development in soil require further investigation.

ACKNOWLEDGMENTS

The work was supported by the Russian Foundation for Basic Research, project 08-04-00359a.

REFERENCES

1. de Hoog, G.S., Guarro, J., Gene, J., and Figueras, M.J., *Atlas of Clinical Fungi, 2nd ed.*, Boarn: Centraalbureau voor Schimmelcultures, Universitat Rovira I Virgili, 2010.
2. Domsh, K.H., Gams, W., and Anderson, T.-H., *Compendium of Soil Fungi*, Eching: IHW- Verlag, 2007, pp. 91–92.
3. Alker, A., Smith, G.W., and Kim, K., Characterization of *Aspergillus sydowii* (Thom et Church), a Fungal Pathogen of Caribbean Sea Fan Corals, *Hydrobiologia*, 2001, vol. 460, nos. 1–3, pp. 97–104.
4. Douglas, N.L., Mullen, K.M., Talmage, S.C., and Harvell, C.D., Exploring the Role of Chitinolytic Enzymes in the Sea Fan Coral, *Gorgonia ventalina, Mar. Biol.*, 2007, vol. 150, no. 6, pp. 1137–1144.
5. Klich, M.A., Biogeography of *Aspergillus* Species in Soil and Litter, *Mycologia*, 2002, vol. 94, no. 1, pp. 21–27.
6. Antropova, A.B., Bilanenko, E.N., Mokeeva, V.L., Chekunova, L.N., Petrova-Nikitina, A.D., and Zheltikova, T.M., Microbiota of Inhabited Areas in Moscow, *Mikol. Fitopatol.*, 2003, vol. 37, no. 6, pp. 1–11.
7. Gomez-Lopez, A., Garcia-Effron, G., Mellado, E., Monzon, A., and Juan, L., Rodriguez-Tudela, Cuenca-Estrella M. *In vitro* Activities of Three Licensed Antifungal Agents against Spanish Clinical Isolates of *Aspergillus* spp., *Antimicrob. Agents Chemother.*, 2003, vol. 47, no. 10, pp. 3085–3088.
8. Vasilenko, O.V., Serebriiskii, I.I., Bezmel'nitsyn, N.V., Kaplanov, K.D., Pivnik, A.V., Fomicheva, G.M., and Marfenina, O.E., *Aspergillus versisolor* as an Agent of a Systemic Mycosis: Description of a Clinical Case and Molecular Genetic Confirmation of Genetic Heterogeneity of the Species *A. versicolor, Probl. Med. Mikologii*, 2002, vol. 4, no. 2, p. 52.
9. Kul'ko, A.B., Dreval', P.A., Vorob'ev, A.A., and Trusov, V.N., Laboratory Diagnostics of Bronchopulmonary Aspergillesis in Patients with Tuberculosis and Cavernous Formations in the Lungs, *Probl. Med. Mikologii*, 2008, vol. 10, no. 4, pp. 25–28.
10. Latge, J.-P., *Aspergillus fumigatus* and Aspergillosis, *Clin. Microbiol. Rev.,* 1999, vol. 12, no. 2, pp. 310–350.
11. Sugiura, Y., Sugita-Konish, Y., Kumagai, S., and Reiss, E., Experimental Murine Hyalohyphomycosis with Soil-Derived Isolates of *Fusarium solani, Med. Mycol.*, 2003, vol. 41, no. 3, pp. 241–247.
12. Raper, K.B. and Fennell, D.I., *The Genus Aspergillus*, Baltimore: Williams & Wilkins, 1965.
13. Klich, M.A. and Pitt, J.I., A Laboratory Guide to Common *Aspergillus* Species and Their Teleomorphs, North Ryde, N.S.W: Commonwealth Sci. Industr. Res. Org. Div. Food processing, 1992.
14. Fomicheva, G.M., Vasilenko, O.V., and Marfenina, O.E., Comparative Morphological, Ecological, and Molecular Studies of *Aspergillus versicolor* (Vuill.) Tiraboschi Strains Isolated from Different Ecotopes, *Mikrobiologiya*, 2006, vol. 75, no. 2, pp. 228–234 [*Microbiology* (Engl. Transl.), vol. 75, no. 2, pp. 186–191].
15. Pitt, J.I. and Hocking, A.D., Influence of Solute and Hydrogen Ion Concentration on the Water Relations of Some Xerophilic Fungi, *J. Gen. Microbiol.*, 1977, vol. 101, pp. 35–40.
16. *Lesnye ekosistemy i urbanizatsiya* (Forest Ecosystems and Urbanization), Moscow: Tovarishchestvo nauchnykh izdanii KMK, 2008.
17. *Metody pochvennoi mikrobiologii i biokhimii* (Techniques in Soil Microbiology and Biochemistry) Zvyagintsev, D.G., Ed., Moscow: Mosk. Gos. Univ., 1991.
18. Marfenina, O.E., Fomicheva, G.M., and Vasilenko, O.V., Whether There Are Differences between Ecological and Molecular Characteristics of the Environmental and Clinical Strains of Opportunistic Microfungi?, *FEMS 2009, 3rd Congerss of European Microbiologists*, Goethenburg, Sweden, June 28–July, 2009.
19. Marfenina, O.E., Naumova, E.M., and Fomicheva, G.M., Growth of the Clinical and Saprotrophic Strains of *Aspergillus sydowii* at Different Temperature Regimes, *Probl. Med. Mikologii*, 2006, vol. 8, no. 2, pp. 64–65.
20. Warris, A. and Verweij, P.E., Clinical Implications of Environmental Sources for *Aspergillus, Med. Mycol.,* 2005, vol. 43.
21. Carlile, M.J., Watkinson, S.C., and Gooday, G.W., *The Fungi. 2nd ed.*, San Diego: Academic, 2001.
22. Birch, M., Denning, D.W., and Robson, F.D., Comparison of Extracellular Phospholipase Activities in Clinical and Environmental *Aspergillus fumigatus* Isolates, *Med. Mycol.*, 2004, vol. 42, no. 1, pp. 81–86.